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INVITED REVIEW

PATTERN RECOGNITION IN PLANT INNATE IMMUNITY

S. Mazzotta and B. Kemmerling

ZMBP - Plant Biochemistry, University of Tübingen, Auf der Morgenstelle 5, 72076 Tübingen, Germany

SUMMARY

All multicellular organisms suffer from microbial pathogen attacks. Recognition of pathogens is the prerequisite and the first step to trigger defence reactions against them. Therefore, plasma membrane-localized receptors play a major role in detecting pathogen-associated molecular patterns (PAMP) or endogenous signals released after attack, so called danger-associated molecular patterns (DAMP). The present review will address perception systems used by plants to perceive pathogen attack and initiate efficient defence responses.

Key words: plant innate immunity, P/M/DAMP, P/M/DAMP-triggered immunity, PTI, receptor-like kinases, FLS2, BAK1.

INTRODUCTION

Plants, as sessile organisms, face the attack of various pathogenic microorganisms they cannot escape. Though plants, in contrast to vertebrates, do not possess an adaptive immune system, their innate immune system effectively protects them from a wide range of different phytopathogenic microorganisms such as bacteria, viruses and fungi. The innate immune system of plants can be divided into two layers of defence responses. The first layer is triggered by pathogen/microbe-associated molecular patterns (P/MAMP) or danger-associated molecular patterns (DAMP), comparable to the innate immune system of animals (Nürnberger *et al.*, 2004). Adopting the P/MAMP-terminology from the animal immunity field had an enormous impact on molecular plant pathology. This was mainly because many of the microbe-associated patterns with immunity-stimulating features were long known as (general) elicitors of cultivar nonspecific defences in many plants. P/MAMPs are unique to microbes, are not produced by

(potential) hosts, and appear to be indispensable for microbial fitness (Medzhitov and Janeway, 1997). Recognition of PAMPs by the corresponding pattern recognition receptors (PRR) in plants leads to PAMP-triggered immunity (PTI), a basal immune status effective against a broad spectrum of pathogens. Pathogens have evolved ways to circumvent this defence by producing effectors to suppress PTI. As a consequence plants learned to specifically recognize some of these effectors. This leads to effector triggered immunity (ETI), the second layer of plant immunity, that was first defined as resistance (R-)/avirulence (AVR-) protein-dependent gene for gene specific resistance (Boller and He, 2009). Plant resistance proteins can directly or indirectly recognize avirulence proteins of invading pathogens. This results in fast, specific, and effective defence responses leading to a hypersensitive response, i.e. localized cell death that restricts further infection by biotrophic microorganisms. Indirect recognition of effectors indicates that some R-proteins might not directly bind avirulence effectors but monitor host targets and observe their perturbation. This phenomenon was described as the guard-hypothesis. Loss or perturbation of the guarder by effectors leads to R-protein dependent HR-based resistance (Jones and Dangl, 2006). In this review we focus on the perception systems that are necessary to initiate basal P/M/DAMP-triggered immune reactions in plants.

PRRs in plants

Pathogen/microbe associated molecular patterns (P/MAMPs) are recognized by plasma membrane-localized pattern recognition receptors (PRRs). P/MAMPs are conserved molecules indispensable for the fitness of the pathogen/microbe and are not present in the host (Medzhitov and Janeway, 1997). The group of microbial P/MAMPs perceived by plants include peptides as, for example, bacterial flagellin or elongation factor Tu or fungal ethylene-inducing xylanase (EIX), oligosaccharides, such as glucans and chitin fragments from fungal cell walls or more complex epitopes, such as glycoproteins from oomycetes or bacterial lipopolysaccharides or peptidoglycan, etc. (for more details see Postel and Kemmerling, 2009; Zhang and Zhou, 2010) (Fig. 1).

The first characterized PRR from plants was FLS2, a

leucine-rich repeat receptor-like kinase (LRR-RLK) that perceives a conserved peptide of bacterial flagellin (flg22). FLS2 consists of an extracellular leucine-rich repeat domain with 28 repeats, a transmembrane domain and a cytoplasmic kinase domain which can initiate phosphorylation-dependent signalling cascades (Boller and Felix, 2009). FLS2 was identified in a forward genetic screen based on flg22-induced root growth inhibition, in order to find flg22-insensitive mutants (Gomez-Gomez and Boller, 2000). This receptor shows structural similarities to animal pattern recognition receptors such as Toll and Toll-like receptors (TLR) from *Drosophila* and mammals, respectively. Together with their associated cytoplasmic kinases the TLR receptors resemble a similar modular structure as the LRR-RLKs (LRRs as recognition domains and cytoplasmic kinases as output domains). In *Drosophila* and mammals these receptors also play important roles in PAMP recognition and initiation of innate immunity. In mammals, for example, TLR5 is involved in sensing bacterial flagellin (Nürnberg *et al.*, 2004; Lehti-Shiu *et al.*, 2009). The plant PRR FLS2 perceives the conserved, N-terminal, 22 amino acid peptide flg22 of the bacterial flagellin protein which is distinct from the epitope perceived by TLR5 in mammals (Nürnberg *et al.*, 2004).

Flg22 recognition leads to several plant defence reactions, such as production of reactive oxygen species (ROS), activation of mitogen-activated protein kinases (MAPK), ethylene production, callose deposition at the cell wall and expression of defence-related genes leading to enhanced immunity as well as growth arrest (Boller and Felix, 2009). Pretreatment of *Arabidopsis* with flg22 induces resistance to the phytopathogenic bacterium *Pseudomonas syringae* pv. *tomato* strain DC3000 (PtoDC3000) and the necrotrophic fungus *Botrytis cinerea* (Zipfel, 2009). Based on homology modelling, the extracellular leucine-rich repeat region forms a predicted horse-shoe-like structure which is involved in direct binding of the peptide flg22 (Chinchilla *et al.*, 2006). Activation of the receptor by binding of its corresponding peptide ligand leads to internalization of FLS2 by endocytosis and further degradation by lysosomal and/or proteasome-related processes (Robatzek *et al.*, 2006).

Another well studied receptor is the elongation factor receptor EFR, which can perceive the N-terminal acetylated peptide elf18 of the bacterial elongation factor Tu (EF-Tu). Activation of EFR leads to activation of similar defence responses as those triggered by flg22 (Zipfel *et al.*, 2006). Activation of both FLS2 and EFR leads to identical calcium-associated plasma membrane anion channel opening as an initial step in the pathogen defence pathway, indicating that both signalling pathways rapidly converge at a very early stage of signalling (Jeworutzki *et al.*, 2010). EFR was identified by a reverse genetic approach in a group of 28 flg22-induced

receptor-like kinases from *Arabidopsis thaliana*. This indicates that PAMP perception leads to an alerted state of the plant represented by the activation of multiple receptors necessary for the perception of additional PAMPs. Proof of EFR function was provided by transient expression of *Arabidopsis* EFR in *Nicotiana benthamiana*, the latter not being responsive towards elf18 because of the lack of an EFR gene (Zipfel *et al.*, 2006). As FLS2, EFR belongs to the LRR-RLK family XII and possesses 21 LRRs. Further analysis of the ligand binding site within the LRR domain was performed with chimeric receptors consisting of different parts of FLS2 and EFR. This led to the discovery of the importance of LRR1-6 and LRR19-21 for binding of elf18 and EFR-dependent signalling (Albert *et al.*, 2010). For FLS2, the binding site for flg22 was narrowed down by mutational analysis to LRR 9 to 15 (Dunning *et al.*, 2007). The exact binding sites for the two PAMPs might be determined in the near future by crystal structures or in-depth structure-function analyses of the receptors. The impact of PAMP recognition on defence is supported by the fact that expression of EFR in solanaceous plants such as tomato and *N. benthamiana* leads to strongly enhanced resistance to a range of phytopathogenic bacteria from different genera (Lacombe *et al.*, 2010). This finding enables crop scientists to engineer resistant plants utilizing new insights from *Arabidopsis* PTI research. EFR-signalling was shown to be dependent on active endoplasmic reticulum quality control (ER-QC) which provide a mechanism for proper folding and glycosylation of membrane proteins, before they are delivered to the membrane (Li *et al.*, 2009; Nekrasov *et al.*, 2009; Saijo *et al.*, 2009) [For more information on plant ER-QC mechanisms refer to Saijo (2010)].

In two independent forward genetic screens a number of *elf18-insensitive-* (*elfins*) and *priority for sweet live-* (*psl*) mutants were identified that show derepression of elf18-induced growth inhibition or of sucrose-induced anthocyanin production, respectively (Li *et al.*, 2009; Nekrasov *et al.*, 2009; Saijo *et al.*, 2009). Several of these mutants are part of ER-QC processes, such as the lectin calreticulin3 (*CRT3*), the folding sensor UDP-glucose glycoprotein glucosyl transferase (*UGGT*) and glucosidase *GIIβ* and *GIIα* enzymes that are involved in the N-glycosylation calnexin/calreticulin pathway. Additional ER-QC components were shown to be necessary for proper EFR production and function involving STT3A, a catalytic subunit of the oligosaccharyltransferase complex involved in co-translational N-glycosylation, ERD2b, an HDEL receptor family member and an ER-residing complex comprised of stromal-derived factor-2 (SDF2) ERdj3b and BiP, necessary for ER-retention of misfolded proteins (Li *et al.*, 2009; Nekrasov *et al.*, 2009). Interestingly, loss of these ER quality control components does not influence FLS2-mediated signalling. This might reflect the exis-

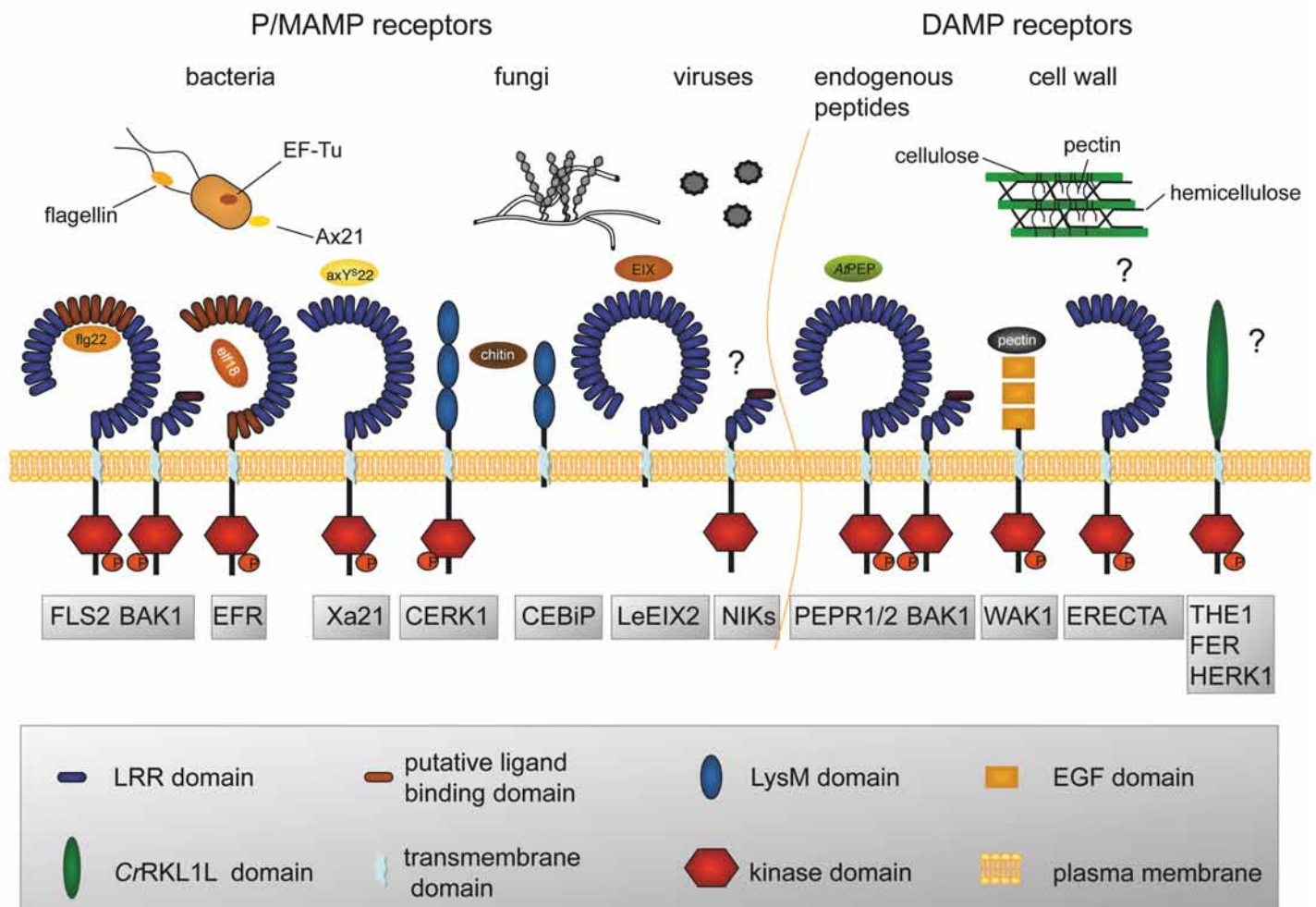


Fig. 1. Membrane-associated pattern recognition receptors can perceive microbial patterns (P/MAMP) from different microbes such as bacteria, fungi, oomycetes or viruses. DAMPs can be released after wounding or pathogen attack. The LRR-RLKs FLS2, EFR and XA21 can perceive bacterial peptides such as flg22 from flagellin, elf18 from EF-Tu and AxY22 from Ax21, respectively. Most likely LRR 1-6 and 19-21 of EFR are necessary for elf18 binding and receptor activation while LRR 9-15 of FLS2 are necessary for flg22 binding. CERK1/LysM-RLK1 is necessary for fungal chitin perception in *Arabidopsis* and acts cooperatively with CEBiP in rice, while tomato EIX2 can perceive the fungal ethylene inducing xylanase protein EIX. NIK1-3 were shown to be involved in virus resistance in tomato and *Arabidopsis*. They belong to the same LRR family II as BAK1, a small LRR-RLK with four and a half LRR-repeats that interacts with several ligand binding receptors such as FLS2, PEPR1/2 and BRI1 (not shown). DAMPs as e.g. the endogenous peptides A1PEPs are perceived by the redundant LRR-receptors PEPR1 and 2. Cell wall fragments can bind to WAK1 and activate oligogalacturonide-dependent defense responses. Other RLKs known to be involved in developmental processes as the LRR-RLK ERECTA and the CrRLK1L proteins FERONIA, HERCULES and THESEUS might be involved in damage associated defence responses.

tence of separate specific pathways for EFR and FLS2 ER-QC (Li *et al.*, 2009). Other RLKs as BRI1 and XA21 were also shown to be under control of these ER-QC cycles. (Jin *et al.*, 2009; Jin *et al.*, 2007; Hong *et al.*, 2008; Park *et al.*, 2010a).

Apart from the two best studied PRRs, FLS2 and EFR, other interesting immune receptors were recently identified. *LeEIX2*, for example, provides sensitivity to the ethylene-inducing xylanase (EIX) of the ascomycete *Trichoderma viride*. The 22 KDa EIX protein induces electrolyte leakage, pathogenesis-related protein expression, and HR in certain varieties of tomato and tobacco. Upon elicitation with EIX, *LeEIX2* becomes internal-

ized by endocytosis as shown for FLS2 (Bar and Avni, 2009). The xylanase enzyme activity of EIX is not needed for the elicitation of defence responses (Ron and Avni, 2004). The receptor *LeEIX2* belongs to the receptor-like protein (RLP) family, and consists of extracellular LRR-repeats, a transmembrane domain and a short cytoplasmic tail with unknown function (Boller and Felix, 2009). Recently, an interaction of the receptors *LeEIX1* (closest homolog of *LeEIX2*) and BAK1 was shown to attenuate EIX responses indicating an inhibitory, potentially competitive effect of *LeEix1* on the receptor *LeEIX2* (Bar *et al.*, 2010).

Tremendous progress has been made on the identifi-

cation of the receptors involved in the perception of fungal chitin or chito-oligosaccharides, the latter responsible for the induction of defence responses in plants. Chitin is a polymer of *N*-acetyl-D-glucosamine found in fungal cell walls, insect exoskeletons, and crustacean shells but not in plants. It was shown by affinity labelling that the chitin elicitor binding protein (CEBiP) is responsible for binding of chito-oligosaccharides in rice (*Oryza sativa*) (Kaku *et al.*, 2006). Knocking down *CEBiP* by RNA interference leads to suppression of ROS production and defence-related gene expression after chitin elicitation (Kaku *et al.*, 2006). This protein also belongs to the family of RLPs and contains an extracellular domain with two LysM domain repeats. Recently, it was shown that CEBiP coordinately operates together with the rice LysM-receptor kinase OsCERK1 (Shimizu *et al.*, 2010). CERK1 (synonymous to LysM-RLK1) was first identified as the chitin receptor in *Arabidopsis* (Wan *et al.*, 2008; Miya *et al.*, 2007). CERK1 consists of 3 extracellular LysM domains, a transmembrane domain and an intracellular kinase domain. Mutations in *CERK1* result in impaired responsiveness towards the elicitor chitin and block the induction of almost all chito-oligosaccharide-responsive genes (CRGs). As a result, resistance to the necrotrophic fungus *Alternaria brassicicola* was lowered in *Arabidopsis cerk1* mutants (Wan *et al.*, 2008; Miya *et al.*, 2007). Surprisingly, its loss leads to a higher susceptibility not only to fungal but also to bacterial pathogens such as *Pseudomonas syringae* (Gimenez-Ibanez *et al.*, 2009). Direct binding of chitin to CERK1 protein was demonstrated *in vitro* (Iizasa *et al.*, 2010; Petutschnig *et al.*, 2010). Furthermore, chitin, chitin oligomers and chitosan, a deacetylated chitin derivative, are able to trigger phosphorylation of CERK1 in *Arabidopsis* cells at several residues in the juxtamembrane and kinase domain (Petutschnig *et al.*, 2010).

Another LRR-RLK-type PRR is XA21 from rice. It confers resistance against the bacterial pathogen *Xanthomonas oryzae* pv. *oryzae* in certain rice cultivars (Lee *et al.*, 2009; Wang *et al.*, 1998). XA21 was initially classified as a R protein because of the narrow spectrum of pathogens it can perceive. Recently it was shown that it perceives the PAMP Ax21 that might be involved in quorum sensing (Han *et al.*, 2011). The minimal active fragment that was identified from Ax21 is the 17 amino acid tyrosine-sulfated peptide axY^S22 (Lee *et al.*, 2009). Ax21 contains all features of a typical PAMP, i.e. it is conserved within a class of microbes, necessary for its life style and not present in the host (Park *et al.*, 2010b).

Besides the detection of dangerous non-self, plants can also sense endogenous signals associated with wounding or weakening of cell walls. These danger signals comprise both non-self representing microbial PAMPs and damaged self-representing DAMPs. In the danger model first described by Matzinger (1998), danger is more generally defined as harmful conditions

which can be sensed by animals (but also by plants) to initiate counteractions for defence. Endogenous molecules which are released after damage by wounding or pathogen attack can function as danger-associated molecular patterns (DAMPs) to induce defence responses as PAMPs (Boller and Felix, 2009). One example of a DAMP is a C-terminal peptide processed from the 92 amino acid precursor protein *AtPROPEP1* that is up-regulated upon wounding or jasmonate/ethylene application. Constitutive overexpression of *AtPROPEP1* leads to resistance against the root pathogen *Pythium irregularare* (Veronese *et al.*, 2006). Photoaffinity labelling and binding assays in tobacco (*Nicotiana tabacum*) cells expressing LRR-RLKs PEPR1 or PEPR2 proved that PEPR1 and PEPR2 are receptors for the damage-associated plant peptide *AtPep1* (and its homologues *AtPep2-6* for PEPR1, or *AtPep1* and 2 for PEPR2). PEPRs again belong to the class of LRR-RLKs. There is clear evidence of differential affinity of the two receptors and their cognate peptides to regulate innate immunity in plants. *AtPep1* action on defence-related gene induction and enhancement of resistance to *Pto* DC3000 were partially reduced in single mutants of *PEPR1* and *PEPR2* and abolished completely in double mutants (Yamaguchi *et al.*, 2010). By root growth inhibition assays and electrophysiological experiments, it was shown that only double mutants in *PEPR1* and its closest homologue *PEPR2* are fully insensitive to *AtPep1* treatment (Krol *et al.*, 2010). As known for other ligand binding LRR-RLKs, PEPR1 and PEPR2 are interacting with the small LRR-RLK BAK1 (BRI1-associated kinase) in yeast two-hybrid experiments. These *in vitro* interaction data are supported by *in vivo* formed *AtPep1*-induced phosphorylation-dependent BAK1 complexes with a protein corresponding to the expected size of PEPR1 or 2 (Postel *et al.*, 2010; Schulze *et al.*, 2010).

Cell wall synthesis and defence responses are closely connected. As shown by Cano-Delgado *et al.* (2003), mutations in the cellulose-synthase subunit *CESA3* in *Arabidopsis* lead to cell wall lignifications and to transcriptional induction of defence-related genes linked to ethylene and jasmonate signalling. A potential PRR involved in sensing cell wall integrity was found by a suppressor screen of another mutant in the cellulose synthase subunit *CESA6*. The *cesa6* mutation leads to short hypocotyls and reduced cellulose content. The suppressor mutant THESEUS (THE1) codes for a CrRLK1L receptor-like kinase (homologous to the first RLK from *Catharanthus roseus*) (Schulze-Muth *et al.*, 1996). The *the1* mutation can partially restore hypocotyl growth defects of *cesa6* mutants grown in the dark without increasing the cellulose content. This hints for a direct inhibitory effect of the receptor on cell elongation rather than the inability of the mutant to produce cellulose *per se* (Hématy *et al.*, 2007). Among the 30 genes upregulated upon THE1 activation, genes related to defence,

oxidative stress, and cell-wall metabolism were largely overrepresented (Hématy *et al.*, 2007). THE1 might directly or indirectly monitor cellulose synthase activity and might function as a mechano-sensor for cell integrity (Hématy and Höfte, 2008). Another indication for an involvement of this class of receptors in plant defence is the fact that a member of the CrRLK1L family is targeted by the bacterial effector AvrPto as shown for FLS2 and EFR (Hématy *et al.*, 2009). For more details on effector targets see heading below.

Quantitative MS analyses of detergent-resistant membranes (DRM) were used to investigate membrane protein dynamics upon flg22 treatment. Several new receptor-like kinases were identified to be enriched in DRMs in an flg22 dependent manner. In addition to FLS2, the RLKs FERONIA (FER) and HERCULES (HERK1) were found (Keinath *et al.*, 2010). These kinases were previously shown to act in concert with THE1 in cell elongation during vegetative growth (Guo *et al.*, 2009). FER and HERK1 also belong to the CrRLK1L family of receptor kinases. FER was first shown to be involved in pollen tube growth (Escobar-Restrepo *et al.*, 2007). *Fer* mutants show deregulation of oxidative burst, MAPK activity and cell death. In addition, they slightly favour bacterial growth (Keinath *et al.*, 2010) but show resistance to powdery mildew infection (Kessler *et al.*, 2010). Recently, it was shown that FER functions in cooperation with NORTIA (allelic to MLO7) in pollen tube reception, and maybe with additional members of the MLO (mildew resistance locus O) family that was previously shown to be involved in powdery mildew defence (Kessler *et al.*, 2010). These examples show that receptors involved in developmental processes might also be utilized as part of the plants defence machinery. Sensing integrity of cells/tissues might be a central part of growth control as well as a tool to sense danger by invading pathogens.

Another group of PRRs is the family of wall associated kinases (WAK). These kinases consist of an epidermal growth factor (EGF)-like motif on the extracellular part that can covalently bind cell wall components as pectin or oligogalacturonides (OGs) *in vitro* (Decreux *et al.*, 2006). OGs are homogalacturonic acids which are activators of plant defence, growth and development (Cervone *et al.*, 1989). WAK genes are upregulated upon pathogen or salicylic acid treatment (Hématy *et al.*, 2009). With chimeric receptors consisting of parts of EFR and WAK1 it was shown that the extracellular WAK1 domain can activate the EFR kinase domain after OG treatment and *vice versa* (Brutus *et al.*, 2010).

Another candidate for sensing cell integrity could be ERECTA. This receptor belongs to the family of LRR-RLKs and is important for regulating developmental processes like inflorescence architecture, lateral organ shape, ovule development, stomatal patterning, and transpiration efficiency (van Zanten *et al.*, 2009) but is also

involved in plant defence. ERECTA was identified in a quantitative trait locus (QTL)-approach searching for the loci responsible for susceptibility to the soil born bacterium *Ralstonia solanacearum* in the *Arabidopsis* accession Landsberg *erecta* (Ler) (Godiard *et al.*, 2003). In another work, encompassing also QTL-mapping, ERECTA was shown to be involved in resistance to the necrotrophic fungus *Plectosphaerella cucumerina* but to none of the other pathogens tested. Callose deposition was impaired in *erecta* mutants which might link ERECTA also to cell wall-related defence responses (Llorente *et al.*, 2005; Sanchez-Rodriguez *et al.*, 2009). In an *erecta* suppressor screen (*ser*), two novel genetic components SER1 and SER2 were found. Though the exact function of these genes is not yet known their mutant phenotypes show a correlation of cell wall composition and susceptibility to *P. cucumerina*. In particular, the content of uronic acids, which are components of the cell wall polysaccharide pectin, was reduced in the suppressor mutants. The amount of uronic acids positively correlated with susceptibility to *P. cucumerina* (Cosgrove, 2005; Sanchez-Rodriguez *et al.*, 2009). The resistance phenotypes of the *ser* mutants can be uncoupled from the developmental effects of *erecta* mutations. The *ser* mutants suppress all immunity-related phenotypes of *erecta* while the developmental *erecta* defects are unaffected indicating that two distinct downstream pathways are initiated by ERECTA (Sanchez-Rodriguez *et al.*, 2009) (Fig. 1).

PRR complexes and downstream signalling

PRR signalling depends on additional partners for activation of the corresponding pathways. One important component involved in the early recognition events is BAK1, a key component in the plant hormone brassinolide (BL) pathway (Nam and Li, 2002; Li *et al.*, 2002), that also has a BL-independent role in cell death control and in PAMP signalling (Chinchilla *et al.*, 2009) (Fig. 2). BAK1 belongs to the family of somatic embryogenesis receptor kinases (SERK) with 5 members. It is a LRR-RLK with a small extracellular domain with only 4 and a half LRR repeats and might therefore be unable to bind ligands by itself. BAK1 (synonymous to SERK3) forms complexes with ligand-binding receptors thereby supporting the respective signalling pathways. In the BL-pathway it interacts with the brassinosteroid receptor BRI1 (Nam and Li, 2002; Li *et al.*, 2002); whereas in PAMP-signalling it interacts with FLS2 in a ligand dependent manner and supports PAMP signalling (Chinchilla *et al.*, 2007; Heese *et al.*, 2007). Further analysis of *bak1* mutants showed enhanced, non-restricted cell death after treatment with necrotrophic fungi and virulent bacteria. This run-away cell death response was uncoupled from the BL-pathway as none of the BL mutants impaired in BL-perception or biosynthesis were affected in their cell death response (Kemmerling *et al.*, 2007).

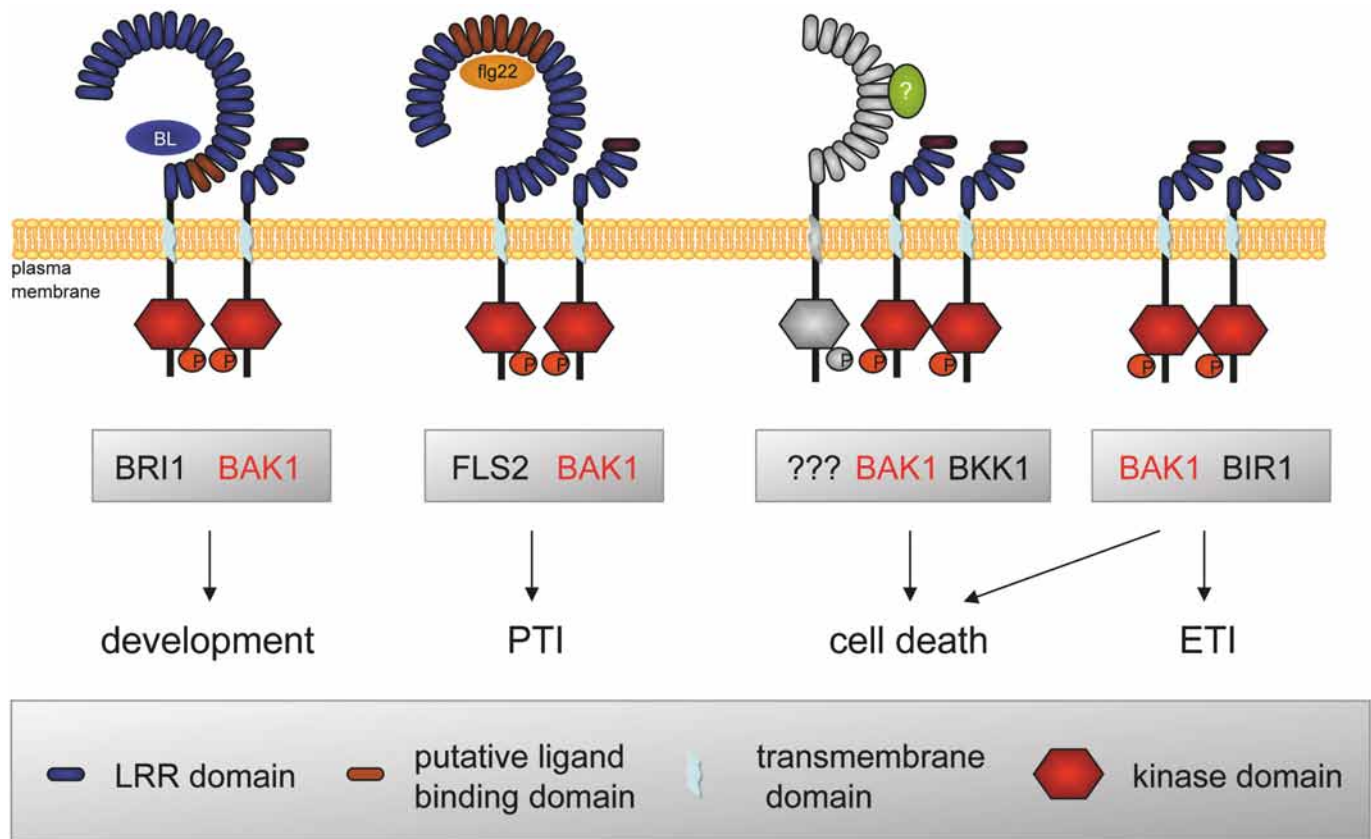


Fig. 2. Interaction of BAK1 with different receptor kinases induces different output. BAK1 interacts with the brassinosteroid receptor BRI1 to enhance hormone induced developmental processes. It forms complexes with FLS2 to support flg22-induced PTI. With its closest homologue BKK1 (SERK4) it is involved in cell death control. Together with BIR1, also a small LRR-RLK, it is necessary for restriction of cell death and the initiation of R-protein specific ETI responses.

In independent studies, an additional role of the closest homologue of BAK1, SERK4 [synonymous to BAK1-like 1 (BKK1)] was unravelled in cell death control. Double mutants of *BAK1* and *BKK1* are seedling-lethal due to constitutive defence-gene expression, callose deposition, ROS accumulation, and spontaneous cell death induction even under sterile growth conditions (He *et al.*, 2007). Additionally, *bak1/bkk1* mutants allow enhanced growth of *Alternaria brassicicola*, an effect that was not observed with any additional member of the SERK-family (Albrecht *et al.*, 2008). More recently found partners of BAK1 are the already discussed receptors PEPR1 and PEPR2 (Postel *et al.*, 2010; Schulze *et al.*, 2010).

The BAK1-interacting receptor like kinase (BIR1), another small LRR-RLK, interacts with BAK1 and shows similar cell death responses as the *bak1/bkk1* double mutants (Gao *et al.*, 2009). The *bir1-1* mutants constitutively activate R-gene specific defence genes involving EDS1 (enhanced disease susceptibility 1), PAD4 (phytoalexin deficient 4) and SAG101 (senescence associated gene 101). These components belong to the TIR-NB-LRR (toll interleukin-1-nucleotide binding-LRR) specific ETI signalling pathway, which was confirmed by

crossing *bir1-1* mutants into *EDS1*- and *PAD4*-defective mutants. Gao *et al.* (2009) discuss the BAK1 and BIR1 complex as a guard. Integrity of the BAK1/BIR1 complex would be guarded by an R-protein and loss of the complex would lead to the activation of R-protein-specific responses and cell death. In *Arabidopsis*, BONZAI1 (BON1) is a regulator of *npr1* (nonexpressor of pathogenesis-related protein 1) suppressor *SNC1* and its expression is induced after PAMP treatment (Denoux *et al.*, 2008). Mutants defective in *BON1* show constitutive defence responses (Yang and Hua, 2004) similar to *bak1/bkk1* double mutants and *bir1* single mutants. BON1 as BIR1 suppresses R-protein dependent cell death reactions. SOBIR (suppressor of BIR1) is a LRR-RLK that was identified in a suppressor screen for loci that can suppress *bir1* mutant cell death phenotypes. SOBIR might be part of an independent cell death suppression pathway (Gao *et al.*, 2009). The molecular mechanism underlying these cell death phenomena is still unclear. The increasing number of RLK mutants causing cell death phenotypes indicates that RLK complexes might be guarded by a control mechanism that senses integrity of these complexes to prevent unintended downstream signalling by unregulated free RLKs.

Since BAK1 is a RD-LRR-RLK with strong auto- and transphosphorylation activity, the regulation of and interaction with its partner is likely an interplay between interaction and transphosphorylation. This was extensively studied in the BRI1/BAK1 interaction which shows a sequential transphosphorylation starting with the activation of BRI1 after BL-binding and transphosphorylation of BAK1. The transphosphorylated BAK1 can transphosphorylate BRI1 again increasing its activity. The expression of inactive BAK1 leads to a strong decrease in BL signalling (Wang *et al.*, 2008). Additionally, BAK1 oligomerizes with FLS2 and becomes phosphorylated after flg22 treatment. Inactive forms of BAK1 can still heterooligomerize with FLS2 upon flg22 treatment, but physiological responses are clearly reduced (Schulze *et al.*, 2010) supporting the importance of kinase activity in activating downstream responses.

The *Arabidopsis* nuclear shuttle protein (NSP)-interacting kinases 1 to 3 (NIK) belong to the LRR-RLK subfamily II, and are closely related to the group of SERKs (Santos *et al.*, 2010). All members of this group have four complete and a fifth incomplete LRR in common (Lu *et al.*, 2010). NIK1 to 3 were shown to have a function in geminivirus defence. In *Arabidopsis* NIK1 to 3 are virulence targets of geminiviruses NSP which inhibits their kinase activities. The loss of activity leads to a higher susceptibility to viral infection (Fontes *et al.*, 2004). In tomato and soybean NIKs are conserved and also targeted by a NSP from the geminivirus *Tomato golden mosaic virus* (TGMV) and *Tomato crinkle leaf yellow virus* (TCrLYV) (Fontes *et al.*, 2004). These RLKs might act as co-receptors of a yet unknown viral PAMP receptor or they might even be able to perceive viral patterns themselves (Lu *et al.*, 2010).

Negative regulation of PRRs can be mediated by phosphatases. Kinase associated protein phosphatase (KAPP) was shown to associate with and dephosphorylate several RLKs, including FLS2 (Gomez-Gomez *et al.*, 2001). Other phosphatases as e.g. XB15, negatively regulate XA21 (Park *et al.*, 2008). Another negative regulator of XA21 is the ATPase XA21 binding protein 24 (XB24). It physically associates with the XA21 juxtamembrane domain, promotes autophosphorylation, and keeps XA21 in an inactive state (Chen *et al.*, 2010). XA21 binding to the XA21 LRR domain induces dissociation of XA21 from XB24 and activates the XA21 non-RD kinase domain (Chen *et al.*, 2010).

Recently, components downstream of the receptor complexes were discovered. Receptor-like cytoplasmic kinases (RLCK) were associated with LRR-receptor mediated innate immune signalling, namely the *Botrytis* induced kinase 1 (BIK1). *Bik1* knockouts show enhanced susceptibility against the necrotrophic fungus *Botrytis cinerea*, but resistance against *Pto* DC3000 (Veronese *et al.*, 2006). In more recent studies BIK1 was discovered to interact and transphosphorylate BAK1 and FLS2 to

mediate early flg22 signalling. Mutants were also impaired in EFR signalling suggesting a more general role in regulating PAMP signalling downstream of the corresponding PRRs (Lu *et al.*, 2010). RLCKs were shown to integrate signals from multiple RLKs and are targets of *P. syringae* effectors (see heading below) (Zhang *et al.*, 2010). These data imply that RLCKs are essential for PTI signalling.

PRR as targets of PTI suppression

As plants succeeded in perceiving pathogens and inducing general defence responses like upregulation of defence-related genes, ROS production and cell wall strengthening by callose deposition, pathogens evolved specific weapons (effectors) to circumvent these PTI reactions. The phytopathogenic bacterium *Pto* DC3000 injects more than 30 effectors into the host cell via the type III secretion system (Zipfel and Rathjen, 2008). The first effector identified to suppress PTI by directly targeting pattern recognition receptors was the avirulence protein AvrPto. It was shown to interact with EFR and FLS2 and to inhibit their kinase activity in a dose dependent manner (Zong *et al.*, 2008). Shan *et al.* (2008) have shown that AvrPto might also target the co-receptor BAK1. Overexpression of AvrPto in *Arabidopsis* results in a dwarf phenotype similar to a weak *bri1* mutant phenotype. BAK1 would be a good target to interrupt several signalling pathways as PAMP signalling, cell death control and also BL-dependent growth at the same time (Shan *et al.*, 2008). Recently, Xiang *et al.* (2011) published contradictory results, indicating that AvrPto interferes with FLS2 but not BAK1. Future studies including *in vivo* data with endogenous proteins will hopefully solve this discrepancy.

Another effector that targets PRRs is AvrPtoB. AvrPtoB has ubiquitin-ligase activity and targets FLS2 for degradation (Göhre *et al.*, 2008). AvrPtoB also interacts with the chitin receptor CERK1 to mediate its degradation. This suppresses all CERK1 responses and leads to higher susceptibility to bacterial infection in *Arabidopsis* (Gimenez-Ibanez *et al.*, 2009). Not all bacteria, not even all strains of *P. syringae*, express AvrPto or AvrPtoB, but might suppress PTI with additional effectors. Of course, PTI can also be targeted at any other component in the signalling cascades and not only at the receptor level as shown, for example, for HOPAL1 which inactivates MAPkinases (Zhang *et al.*, 2007).

The *P. syringae* pv. *phaseolicola* effector AvrPphB targets several members of the RLCK family. AvrPphB is a cysteine protease which proteolytically cleaves PBS1, a member of the subfamily VII of RLCKs, which mediates RPS5- (resistance to *Pseudomonas syringae*) specific disease resistance. Besides this known substrate, also BIK1, the previously described interactor of PRRs and two other RLCKs, PBL1 and PBL2 (PBS1-like 1 and 2), are cleaved by AvrPphB, leading to impaired PTI re-

sponses (Zhang *et al.*, 2010).

Knowledge about common structures of effectors and also access to the increasing number of sequenced genomes will enable scientists to find more effectors and their corresponding targets and most likely also more PRRs.

CONCLUSIONS

Tremendous progress has been made in the last years on the functional analysis of receptor kinases. The first pattern recognition receptor in plants was identified and this led to a paradigm shift in the plant defence field and initiated the acceptance of PTI as a necessary and important part of the plant defence machinery. Since then, multiple new PRR candidate genes have been identified (Fig. 1). Complex networks of receptors and partners as co-receptors, binding proteins and cytoplasmic proteins are formed to specifically initiate different output pathways (Fig. 2). Further analysis is needed to understand the underlying activation mechanisms and the control systems for these complexes. How are these receptor complexes formed and how is the balance between the diverse complexes maintained? Is there crosstalk between pathways that are using the same co-receptors?

Cell death phenomena observed in some RLK mutant analyses might be the consequence of strictly controlled integrity of receptor complexes. Future analyses will disclose the reason for these cell death reactions. The identification of more PRR candidates has also revealed a number of proteins with known functions in developmental processes. These dual or multiple function proteins are interesting links between two different adaptive processes which might shed light on common molecular principles that are used in different adaptive programs. Sensing cell wall integrity is an essential process for developmental growth and tissue control but can equally be used to detect pathogen invasion. Future studies will answer the questions on how receptor complexes are formed and controlled and which signalling pathways are used to efficiently induce plant immunity.

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COMPARISON OF THE EFFECTS OF *FUSARIUM SOLANI* FILTRATES *IN VITRO* AND *IN VIVO* ON THE MORPHOLOGICAL CHARACTERISTICS AND PEROXIDASE ACTIVITY IN PEA CULTIVARS WITH DIFFERENT SUSCEPTIBILITY

L. Svábová¹, A. Lebeda², M. Kitner², M. Sedlářová², M. Petrivalsky³, R. Dostálová¹,
M. Ondřej¹, J. Horáček¹, I. Smykalová¹ and M. Griga¹

¹ Agritec Plant Research Ltd., Zemědělská 2520/16, 787 01 Šumperk, Czech Republic

² Palacky University in Olomouc, Faculty of Science, Department of Botany, Šlechtitelu 11,
783 71 Olomouc – Holice, Czech Republic

³ Palacky University in Olomouc, Faculty of Science, Department of Biochemistry, Šlechtitelu 11,
783 71 Olomouc – Holice, Czech Republic

SUMMARY

Intact plants and *in vitro* cultures of four pea (*Pisum sativum*) cultivars (Adept, Herold, Komet, Menhir) that vary in their degree of susceptibility/resistance to *Fusarium solani* were inoculated with this pathogen or treated with its culture filtrates to compare their reaction patterns at the phenotypic, histological and biochemical level. Changes in activity of three peroxidase forms, cytosolic, membrane- and ion-bound, and in peroxidase isozymes were studied in detail. In addition, the length and weight of roots of regenerating plantlets in explant cultures as well as symptom expression in intact plants were assessed. *In planta*, screening of the four pea cultivars revealed the highest degree of resistance in cv. Adept, and the highest level of susceptibility in cv. Menhir both to *F. solani* and its metabolites. Regarding *in vitro* cultures, the microfiltrated filtrates had stronger inhibitive effect than the autoclaved ones. In terms of peroxidase activity, the only significant difference among cultivars was found in its ionic form, for the most resistant cv. Adept versus the most sensitive cv. Menhir. Minor changes in activity of cytosolic peroxidase were noted *in planta* and *in vitro*, while the membrane- and ion-bound peroxidase significantly decreased in explants. The aim of our study was to compare reaction of pea plants and explants and to verify whether the application of fungal filtrates is able to mimic the *F. solani* pathogenesis. Several responses of explants to filtrates were found to be analogous to the plant reaction to pathogen infection both at morphological and physiological levels. These can be utilized in an early *in vitro* screening for plant tissue resistance to *F. solani*.

Key words: *in planta*, fungal metabolites, pathogenesis, selection for resistance, *Pisum sativum*.

INTRODUCTION

In vitro methods of selection of plant genotypes with enhanced resistance to fungal diseases are primarily based on the utilization of explant cultures and their diversity, conditioned by somaclonal variation. *In vitro* selection proceeds with adapted pathogen-derived selective agents (Svábová and Lebeda, 2005; Lebeda and Svábová, 2010). Fungal culture filtrates or other pathogen-derived selective agents such as chitosan, fusaric acid, FspH DNase, and other elicitors are able to induce defence responses, including oxidative stress in plants (Hadwiger and Beckman, 1980; Hadwiger *et al.*, 1995; Kumar *et al.*, 2008; Kuzniak, 2001; Saikia *et al.*, 2006; Vidhyasekaran *et al.*, 2002). In optimal cases, mixtures of these fungal secondary metabolites elicit in plant tissues *in vitro* a range of physiological and biochemical changes analogous to *in vivo* pathogenesis. Resistant lines of various crops, e.g. banana, barley, grapevine, tobacco, tomato and wheat, were successfully developed via selection *in vitro* (Svábová and Lebeda, 2005; Lebeda and Svábová, 2010). A range of studies was focused on the principles of the interaction between selection agents *in vitro* and pathogenesis *in vivo* (Behnke, 1980; Buiatti *et al.*, 1985; Huang and Hartman, 1998; Hamid and Strange, 2000; Jayasankar *et al.*, 2000; Hollmann *et al.*, 2002), as well as on their biochemical basis, mainly the role of reactive oxygen species (ROS), and pathogenesis-related proteins (PR proteins), in plant-pathogen or explant-selective agent interactions (e.g. Djebali *et al.*, 2007; El-Gendy *et al.*, 2001; Kuzniak, 2001; Morkunas and Gmerek, 2007; Saikia *et al.*, 2006; Singh *et al.*, 2003; Unger *et al.*, 2005).

Expression of plant resistance to pathogens relates to many changes at the physiological, biochemical and molecular levels (Glazebrook, 2005; De Wit *et al.*, 2009). Peroxidases, a complex family of proteins that catalyze oxidation-reduction of various substrates using H₂O₂, are omnipresent in plant tissues. They facilitate cell wall loosening and growth by elongation as well as cross-linking of cell wall components during structural de-

fence (Almagro *et al.*, 2009). The balance between cleavage and cross-linking of cell wall components is regulated by peroxidase (POX) activity and gene expression mainly through H₂O₂ and ascorbate concentrations (Passardi *et al.*, 2004).

Pea defence mechanisms are relatively well known at the intact plant level, but poorly understood in explant cultures *in vitro* (Lebeda *et al.*, 2001; Luhová *et al.*, 2002; Mlícková *et al.*, 2004). The interaction of pea and *Fusarium solani* is only partly understood at the physiological, biochemical and molecular level. The role of several traits of pea resistance to the pathogen has already been explained, e.g. phytoalexin pisatin, cuticle barrier, and activation of the non-host resistance response and PR proteins (Hadwiger, 2008). Several enzymes, mainly those participating in the metabolism of ROS, have been studied in relation to pea resistance against *F. solani* (Luhová *et al.*, 2002, 2003, 2006). However, the involvement of secondary metabolites in *F. solani* pathogenesis and their potential utilisation for resistance screening of pea is not well known (Svábová and Griga, 1997; Svábová *et al.*, 1998; Lebeda and Svábová, 2010; Lebeda *et al.*, 2010).

The major objective of this research was to develop an assay for *in vitro* selection of pea resistance to *Fusarium solani* using fungal culture filtrates. Application of this methodological approach depends primarily on the existence of a positive relationship between *in planta* resistance of pea to *F. solani* and tolerance of pea explants *in vitro* to fungal culture filtrates. These potential relationships were also studied from the viewpoint of phenotypic, histological and physiological (cytosolic, membrane- and ion-bound POX) changes in treated plants grown *in vivo* and *in vitro*.

MATERIAL AND METHODS

Plant material. Four dry seed pea (*Pisum sativum*)

cvs Adept, Herold, Komet, and Menhir with different degrees of susceptibility to various fungal pathogens from the Pea Germplasm Collection of Agritec Plant Research Ltd. (Šumperk, Czech Republic) were used in these experiments (Table 1).

Cultures *in vitro*. Pea seeds were surface-sterilised with 96% ethanol for 30 sec, then in 10% Chloramin B (sodium N-chlorbenzensulphonamide, w/v) (Bochemie Ltd., CZ) for 20 min, and rinsed 3 times in sterile deionized water. Seeds were germinated in 250 ml Erlenmeyer flasks in the dark at 20°C for 3-5 days, on a layer of cellulose wadding covered with filter paper soaked with deionised water. Multiple shoot cultures were initiated from nodal segments of aseptically germinated seeds by stimulation of axillary meristems proliferation and cultured as described by Griga *et al.* (1986). Isolated nodal segments were placed on media containing macro- and microelements, B5 vitamins, 1 µM NAA, 20 µM BAP, 5.5% plant agar (DUCHEFA) and 3% sucrose, and supplemented with autoclaved or microfiltrated fungal filtrates at concentrations according to the particular experiment. The explants were cultured in a growth room at 20-22°C, 16/8h day/night photoperiod and light density 45 µE m⁻² s⁻¹ provided by daylight fluorescent tubes.

For *in vitro* experiments on roots, the shoots were excised from pre-cultured multiple shoots and transferred onto the same media as stated above, omitting 20 µM BAP. The application of filtrates and culture conditions were identical to those described for multiple shoot cultures.

Pathogen culture and preparation of filtrates. The highly virulent isolate FSVG of *Fusarium solani* f. sp. *pisi* (Jones) Snyder et Hansen (syn. = *F. martii* app. et Wu var. *pisi* Jones) (Ondrej *et al.*, 2008) was maintained on Czapek-Dox agar and stored at 4°C. Prior to the production of filtrates, the fungus was grown on pea agar media made from an extract of 200 g pea seeds

Table 1. Passport and description data (according to EVIGEZ) of pea cultivars (*Pisum sativum* L. convar. *sativum*) used in the experiments.

Pea cultivar	Accession No. (EVIGEZ)	Leaf type	Seed colour	Disease resistance to fungal pathogens in the field conditions (1-9 scale)	
				Anthraces	Complex of root diseases
Adept	05L0100762	leaf	yellow	5	7
Herold	05L0100964	afila	yellow	7	6
Komet	05L0100736	leaf	yellow	6	4
Menhir	05L0100768	afila	yellow	5.5	3

EVIGEZ – database of Plant Genetic Resources Documentation in the Czech Republic (http://genbank.vurv.cz/genetic/resources/asp2/default_a.htm).

Disease resistance in field conditions (according to Anonymous, 2000, 2008).

Anthraces (*Ascochyta pisi*, *Mycosphaerella pinodes*, *Phoma medicaginis* var. *pinodella*).

Complex of root diseases (*Pythium* spp., *Aphanomyces euteiches*, *Phoma medicaginis* var. *pinodella*, *Mycosphaerella pinodes*, *Thielaviopsis basicola*, *Fusarium oxysporum* f. sp. *pisi*, *F. solani*, *Rhizoctonia solani*).

Evaluation scale of resistance: 1-highly susceptible; 2,3-susceptible, 4,5-low resistance; 6,7-intermediate resistance; 8,9-resistant.

boiled in 1 litre of tap water, complemented with 15 g D-glucose and 5.5 g agar in Petri dishes (90 mm) and cultured at 20-22°C, 16/8 h day/night photoperiod, and light density 45 $\mu\text{E m}^{-2} \text{s}^{-1}$ provided by daylight fluorescent tubes. Filtrates were prepared by culture of quadrants of agar with well-grown mycelium laid on the surface of liquid media (0.8% w/v KNO_3 , 0.05% w/v KH_2PO_4 , 2% w/v sucrose per 1 l of tap water). After 4 weeks the surface mycelium and spores were removed by straining through sieves and cellulose wadding. Autoclaved filtrate (A) was produced by autoclaving (120°C, 240 kPa, 15 min), whilst a variant of microfiltrated filtrate (F) was prepared by sieving through a 0.22 μm Millipore filter. Both variants were stored in darkness at 4°C before use.

Screening of plants for resistance to *Fusarium solani* and filtrates. A modified test by Ondrej *et al.* (2008) for evaluation of tolerance/sensitivity to *F. solani* and two types of filtrates were utilised in a series of experiments. Seeds were sterilised in a 5% v/v solution of SAVO [5% NaClO (w/v), Bochemie Ltd., CZ], and imbibed in water for 24 h. Seeds were then sown in plastic pots in wet perlite (type EP AGRO, Perlit Ltd. CZ). For the inoculation variant (I) the *F. solani* spore suspension (concentration 10^5 - 10^6ml^{-1}) was poured over the seed (100 ml per row of 20 seeds), whilst for the variants with adjusted selective agents, solutions of A and F at concentrations of 50 and 100% (v/v) were poured over the seed. Plants were grown at 20-22°C, 16/8 h day/night photoperiod and light intensity 220 $\mu\text{E m}^{-2} \text{s}^{-1}$ provided by 400W sodium lamps. Disease symptoms were scored using a 0-4 scale (Lebeda and Buczkowski, 1986): 0 = no visible symptoms; 1 = mild chlorosis (1-25%); 2 = chlorosis and growth depression (26-50%); 3 = severe chlorosis, wilting, root necrosis (51-75%); 4 = complete collapse of the plant (76-100%). Assessments were performed 10 and 20 days post inoculation (dpi) or treatment by filtrates.

Disease index was determined with the following formula:

$$\text{DI} = \Sigma(0 \times A + 1 \times B + 2 \times C + 3 \times D + 4 \times E)/N$$

where: DI = the total degree of infection; A, B, C, D, E = number of plants in each assessed category (0-4); N = total number of assessed plants.

Evaluation of filtrate influence on morphology of *in vitro* explants. The effect of different variants of fungal metabolites on *in vitro* explants was evaluated as percentage of surviving explants, weight (g) and length of roots (mm).

Images of plant samples were captured with a colour digital camera (Nikon 5 Mpi) with controller Nikon DSU-1 (Nikon Instruments, CS-Optoteam, CZ) placed approximately 350 mm above the sample. Images of explants were taken with a macrolens Pentax Cosmocar

(Nikon Instruments, CS-Optoteam, CZ), illumination provided by inferior light box Kaiser Prolite Basic (Kaiser Fototechnik, Germany) and superior tubular light Schott (Schott Glass, Germany). NIS Elements AR 2.30 software (Laboratory Imaging Prague, CZ) was used for subsequent image analysis. Measurement of the root length was based on stamen model.

Rootage of seedlings grown *in vitro* was weighted 20 days following treatment by *F. solani* filtrates (30 and 40% autoclaved, 10 and 20% microfiltrated) using a laboratory scale Mettler PE1600. Data are presented as a mean of cultivars (n = 20-50).

Statistical analyses. Each experimental variant was set up with a minimum of 20 explants or plants. Data were analysed by ANOVA and the means were compared with the Fischer LSD test. Data of POX activity were further processed to calculate correlation coefficients using STATISTICA 8 CZ software (StatSoft, USA).

Enzyme extraction procedures. For determining POX activity a three-step extraction protocol was used (Sedlářová *et al.*, 2007). Cytosolic POX was extracted by 1% polyvinylpyrrolidone (w/v) in 50 mM phosphate buffer (pH 7.0), membrane-bound POX by 1% Triton X-100 in phosphate buffer, and ionic POX by 0.1 M KCl in phosphate buffer. For peroxidase isozyme analysis, ca. 0.5 g of plant tissues (leaves and stems) were homogenized in three volumes of 0.1 M potassium phosphate extraction buffer (pH 7.0) using mortar and pestle. Extracts were centrifuged (12,000 g, 4°C, 15 min) and the supernatant stored in aliquots at -80°C until assayed.

Peroxidase activity. Activity of cytosolic (cPOX), membrane (mPOX) and ionic-bound (iPOX) peroxidases were assayed spectrophotometrically on a microplate reader (Synergy HT, Biotek, USA). POX activity was measured by a modified method with guaiacol (Angelini *et al.*, 1990). The reaction mixture contained plant extract, 0.1 M potassium phosphate buffer (pH 7.0), and 5 mM guaiacol. The reaction was started by pipetting 35 mM hydrogen peroxide (final concentration of 4.4 mM). Time-dependent increases in absorption due to formation of tetraguaiacol at $\lambda = 436 \text{ nm}$ ($\epsilon = 4.5 \text{ nM}^{-1} \text{ cm}^{-1}$) were detected continuously for 1 min at 30°C.

Isozymes and antioxidative enzymes. Electrophoretic separation of POX isoforms was performed by non-denaturing PAGE (30 μl of sample, 4% stacking gel and 8% resolving gel with 0.025 M Tris and 0.19 M glycine buffer pH 8.3, at 4°C). There were no significant differences in protein concentration in sample extracts (data not shown). Specific visualizations of POX isozymes were performed using SigmaFast™ 3,3'-di-

aminobenzidine (DAB) tablets (Sigma-Aldrich, USA). Changes in number, intensity and position of bands were assessed visually.

Histochemical localization of POX. POX activity within plant tissues was visualised by formation of an intense dark brown precipitate due to DAB oxidation. Approximately 5 mm long fragments of pea hypocotyl, adjacent parts of root and stem (i.e. 3 fragments per plant were stained), or from explants were vacuum-infiltrated with DAB-urea hydrogen peroxide solution (SigmaFast™, Sigma-Aldrich, USA). After staining (3 h), the samples were boiled in ethanol for 10 min, rinsed in deionised water and immersed in 70% glycerol until studied. Transverse sections of tissue segments (40-60 µm thick) were cut with a hand slicing microtome and observed with an Olympus BX60 light microscope equipped with a CCD camera DP70 (Olympus Czech Group, CZ).

RESULTS

***In planta* screening of pea cultivars for resistance to *F. solani* and fungal metabolites.** As to the effect of *F. solani* culture filtrates, the only significant differences in disease index (DI) among genotypes were found in cvs Adept and Menhir, both 10 and 20 dpi (Fig. 1). Disease symptoms (Fig. 2) were recorded in all variants of inoculation and filtrate treatment, although the exhibited effects differed with the cultivar and filtrate variants. In both types of filtrates a positive relationship was found between filtrate concentration and intensity of disease symptoms. No substantial differences were found between autoclaved and microfiltrated filtrates applied at identical concentrations. In general, cv. Adept was the most tolerant to both types of filtrates and cv. Menhir the most susceptible. Nevertheless, no direct relationship between the level of pea cultivar susceptibility to fungal infection and filtrate treatment was detected. Tolerant plants of cv. Adept did not show growth depression after inoculation, the roots were attacked only

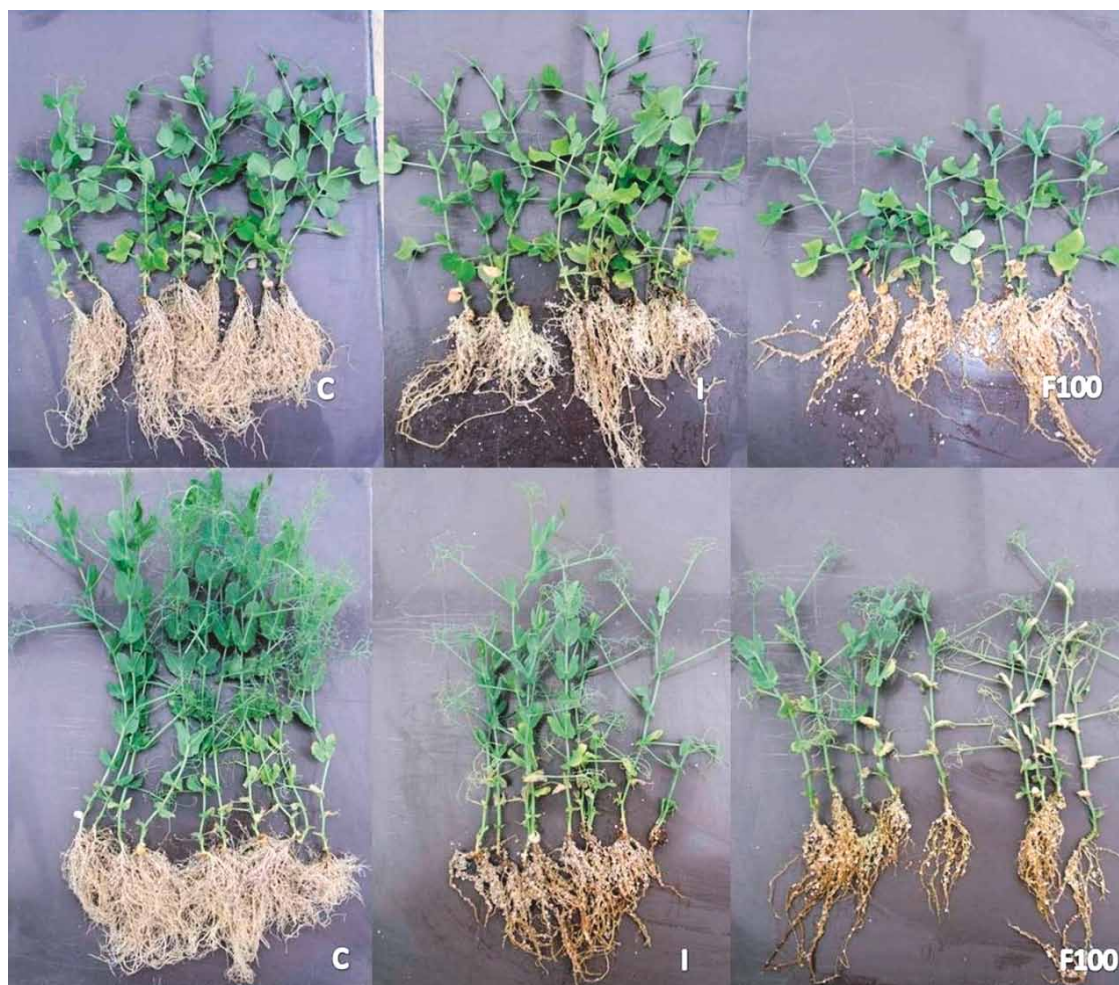


Fig. 1. Symptoms observed in cvs Adept (upper row) and Menhir (lower row), after inoculation by *Fusarium solani* (I), and application of microfiltrated culture filtrate (100% concentration, F100) of *F. solani* after 20 days. C = control.

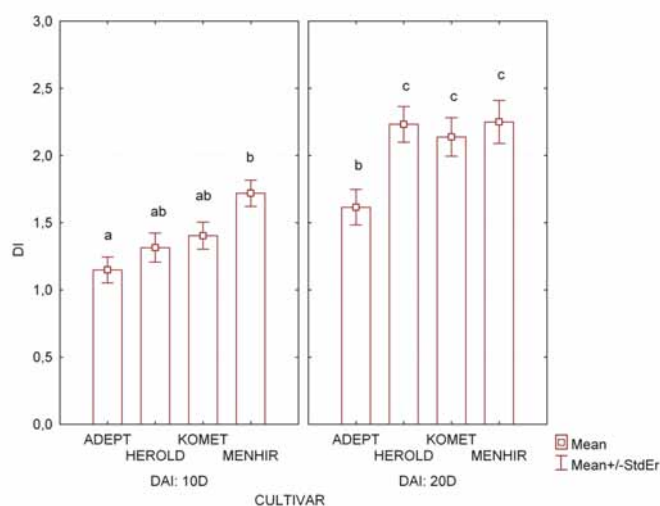


Fig. 2. Differences in susceptibility of four pea cultivars to *Fusarium solani* 10 and 20 days after inoculation, evaluated by disease index (DI); different letters mean statistically significant differences.

slightly, but after treatment with 100% filtrate the growth depression was visible. Plants of cv. Menhir exhibited growth depression, wilting of leaves, and

browning of roots both following inoculation and 100% filtrate treatment.

Effect of autoclaved and microfiltrated filtrates on explants morphology. Four pea cultivars grown in multiple shoot culture were treated with both types of *F. solani* filtrates in concentrations of 25 and 50% (v/v). Generally, the microfiltrated variant of filtrate (F) expressed more inhibitive effect on *in vitro* cultures than the autoclaved (A) variant (Fig. 3). Both 25 and 50% concentrations (v/v) of microfiltrated filtrate had a severe inhibitory effect on pea growth and development *in vitro*, suppressing shoot development four weeks after treatment. The autoclaved filtrate at 25% (v/v) had only a minor effect on *in vitro* explants, but at 50% (v/v) it reduced the development of cvs Herold and Komet but not those of either the most tolerant cv. Adept or the most sensitive cv. Menhir. Moreover, cv. Menhir displayed swelling of the basal parts of explants (Fig. 3). Due to the effect observed in this series of preliminary experiments, the concentrations of fungal filtrates were changed in subsequent experiments aimed at assessing explant survival, root weight and length. The concentrations were decreased to 10 and 20% for

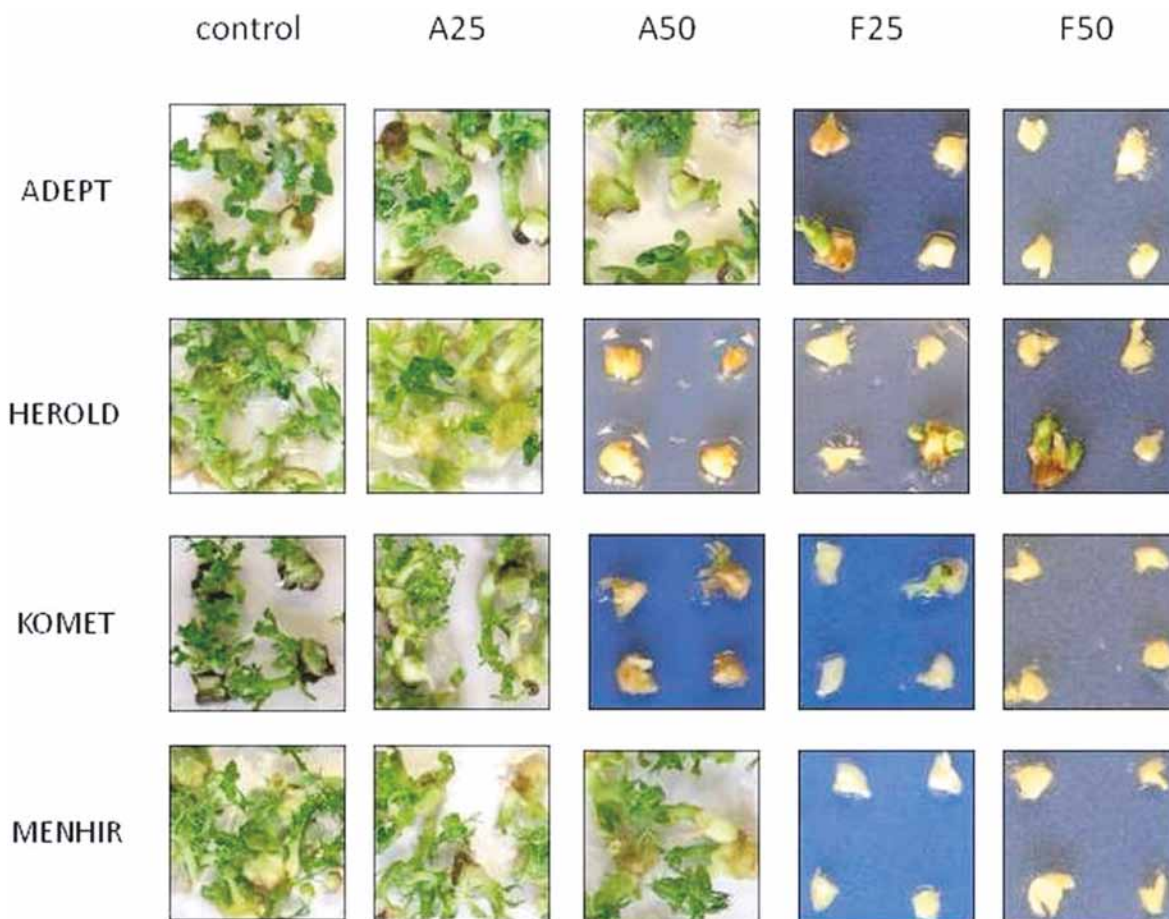


Fig. 3. Effect of treatment by *F. solani* filtrates (A-autoclaved, F-microfiltrated) in two concentrations (25 and 50% v/v) on multiple shoot culture of four pea cultivars, 20 days after treatment compared to untreated control.

microfiltrated variants (10F, 20F), and 30 and 40% for autoclaved ones (30A, 40A).

Effect of filtrates on *in vitro* explant survival, length and weight of roots. When the percentage of surviving explants treated with *F. solani* A and F filtrates was compared, resistant cv. Adept showed a significantly higher percentage of explant survival (84%), whereas the other cultivars did not differ from each other. Similarly, all variants of filtrate treatments differed from the control (mean 91%), but did not differ between each other (Fig. 4).

Length of roots assessed by image analysis was used for evaluation of the relative tolerance/sensitivity of cultivars to fungal filtrates. The mean value of total root length was 37 mm. However, all treatment variants (control, 10F, 30A and 40A), except for 20F, did not differ statistically from one other.

Significant differences in pea cultivar response to application of fungal filtrates were recorded in the weight

of roots. Resistant cv. Adept exhibited the largest and heaviest root system, whereas differences among the remaining pea cultivars were insignificant (Fig. 5). Individual filtrate variants differed in their influence on plant root weight, i.e. F variants significantly impacted plant root weight at 10 and 20% concentration, whereas A variants were not effective at both 30 and 40% concentration.

Isoenzymes of peroxidase. The changes of POX isoenzyme spectra were studied to detect possible biochemical responses of plant tissue to infection and to pathogen-derived selective agents at the level of intact plants and *in vitro* cultures. Electrophoretograms of POX showed no differences in position and intensity of bands (both *in planta* and *in vitro* cultures). The only exception was an increase in the intensity of slow-migrating bands in the upper part of electrophoretograms (samples from 7th and 14th dpi), which might be related to the growth of plants and cultures *in vitro* rather than

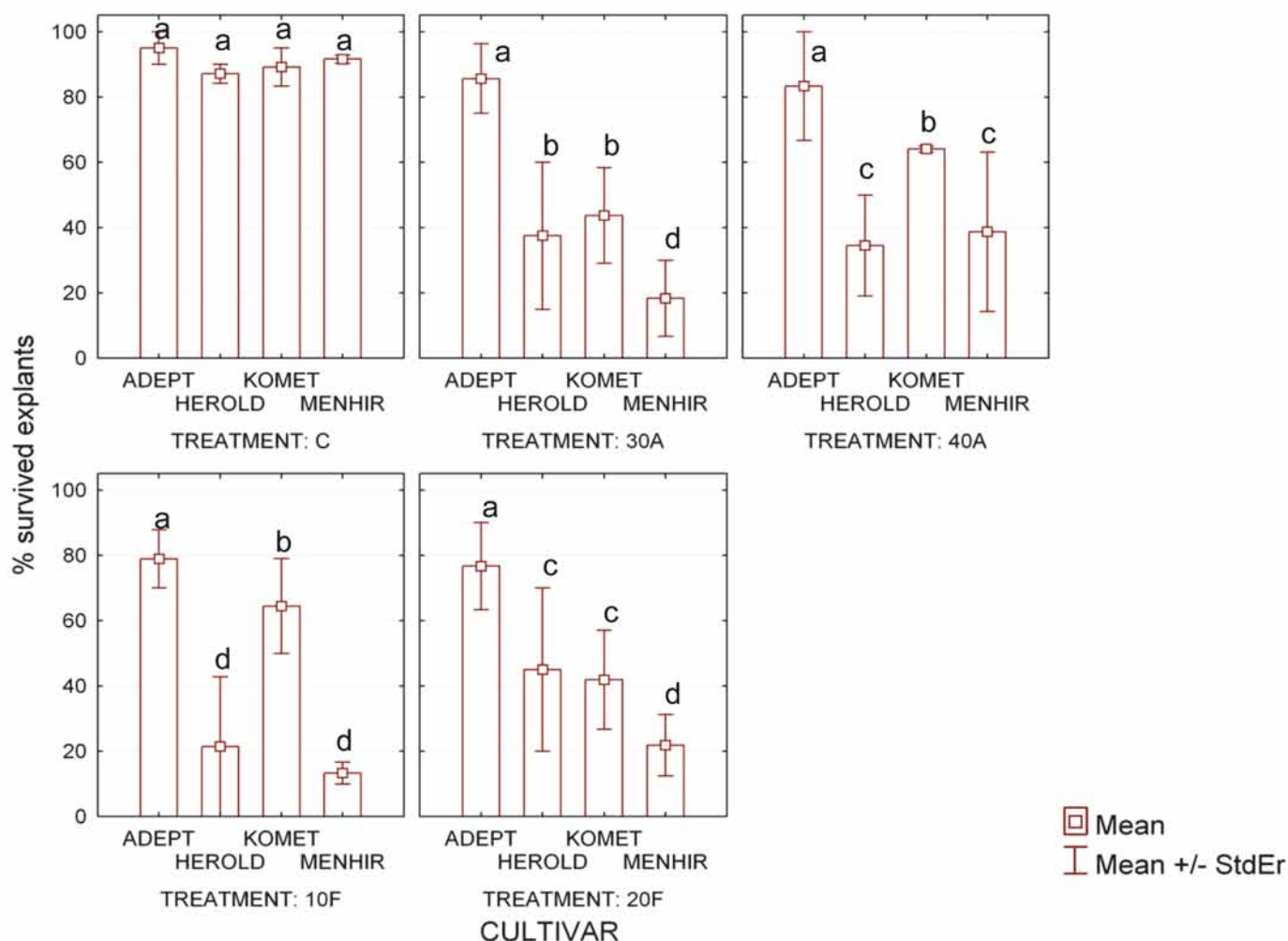


Fig. 4. Effect of *F. solani* filtrates on percentage of survived explants of pea *in vitro* explants (C- control; 30A, 40A-30, 40% v/v autoclaved filtrate; 10F, 20F-10, 20% v/v microfiltrated filtrate); different letters mean statistically significant differences.

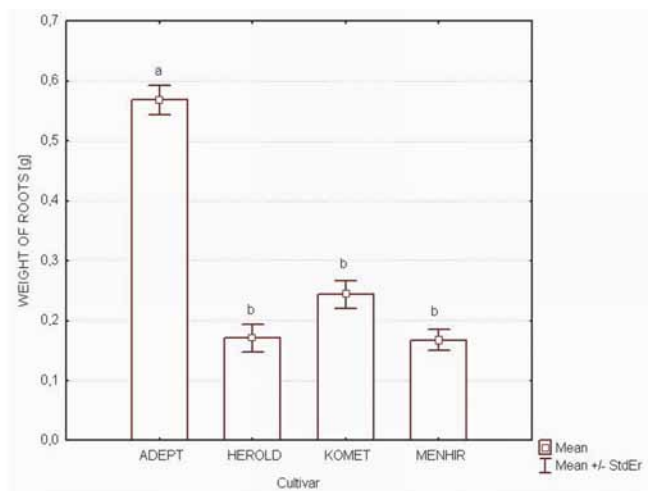


Fig. 5. Weight of roots in *in vitro* cultures after 20 days of treatment by *F. solani* filtrates (C, 30A, 40A, 10F, 20F) expressed as mean of cultivars; different letters mean statistically significant differences.

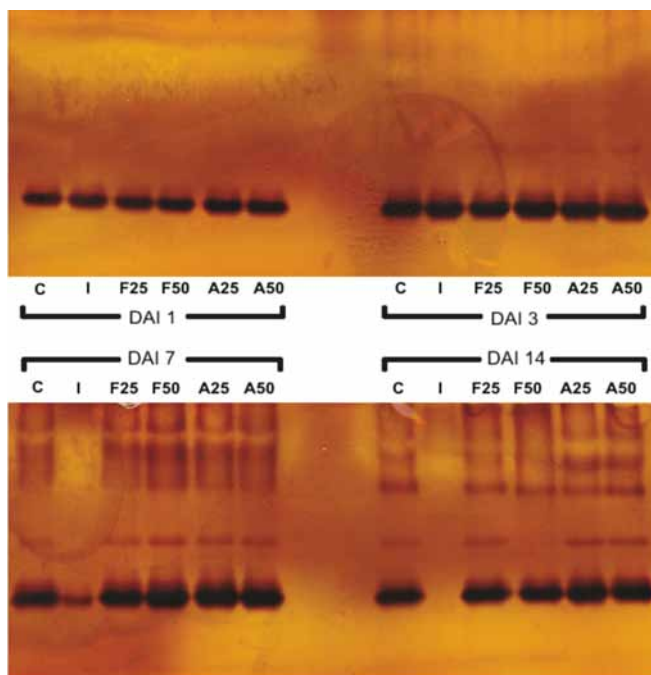


Fig. 6. Changes in peroxidase isoforms in cv. Menhir during 1, 3, 7 and 14 days after treatment (C-untreated control; I-inoculation; A-autoclaved and F-microfiltrated treatment, both 25 and 50% v/v).

to fungal infection. The only significant changes in band intensity were observed in plant samples I (inoculation) as a decrease of intensity at 7 dpi and nearly invisible band at 14 dpi (Fig. 6).

Peroxidase activity. The first experiment with all four pea cultivars was performed 20 dpi, but the inoculated variant had to be removed 10 dpi due to complete necrotization of explants *in vitro*. Before the beginning

of the experiment very low activity of all three POX types, with a mean value of $0.80 \mu\text{kat g}^{-1} \text{FW}$, was recorded in control variants (C1). After 20 days there was a strong increase of POX activity in control samples (C2) to a mean value $12.86 \mu\text{kat g}^{-1} \text{FW}$, but with low activity of mPOX $4.73 \mu\text{kat g}^{-1} \text{FW}$. With the exception of filtrate treatment 50F, all other treatments (25F, 25A, 50A) did not differ significantly from the control (C2). All cultivars, except for cv. Komet, possessed the expected activity values of the three POX types, with respect to their tolerance or sensitivity to *F. solani*. Cvs Adept, Herold and Menhir were characterized by mean values over all three POXes of 9.84 , 7.21 , and $5.42 \mu\text{kat g}^{-1} \text{FW}$, respectively, i.e. POX (especially iPOX) activity decreased with cultivar sensitivity to *F. solani*. However, the highest average value of POX activity, $10.49 \mu\text{kat g}^{-1} \text{FW}$, was recorded in the moderately sensitive cv. Komet.

Detailed experiments with the most susceptible cv. Menhir focused on differences between the two variants *in planta* and *in vitro*. The activities of all three forms of peroxidases were assayed 1, 3, 7 and 14 dpi in *in vitro* and *in planta* samples treated with *F. solani* filtrates. The mean values of activity for all POX forms increased with time interval both within *in vitro* and *in planta* samples. Two main differences between *in vitro* and *in planta* samples were observed: (i) increased activity of cytosolic POX in *in vitro* conditions versus gradual increase of ionic POX in *in planta* samples during the experimental period (Fig. 7, 8); (ii) in general, 9- to 10-fold higher POX activity was detected in *in planta* samples. Mean of cytosolic POX activity in plants was slightly higher ($0.95 \mu\text{kat g}^{-1} \text{FW}$) than in samples from *in vitro* cultures ($0.67 \mu\text{kat g}^{-1} \text{FW}$). The membrane-bound POX was 30-fold higher in plants than *in vitro* ($2.13 \mu\text{kat g}^{-1} \text{FW}$ vs. $0.07 \mu\text{kat g}^{-1} \text{FW}$), and ionic POX was 33-fold higher in plants compared to *in vitro* cultures ($5.27 \mu\text{kat g}^{-1} \text{FW}$ vs. $0.16 \mu\text{kat g}^{-1} \text{FW}$).

In planta mPOX activity slightly correlated with mPOX activity *in vitro* ($r = 0.56671$). In contrast, iPOX activities did not correlate ($r = 0.09314$), and there was a negative correlation ($r = -0.5361$) between *in planta* and *in vitro* response of cPOX activities (data not shown).

Localization of POX within plant tissues. Histochemical detection of POX in intact plants and explants of pea treated with *F. solani* or with individual variants of filtrates (25F, 50F, 25A, 50A) showed no significant differences in the effects of filtrate concentration on POX accumulation (data not shown). The only difference in POX accumulation was found between untreated controls and samples co-cultivated with the pathogen, where growing mycelium induced necrosis of host tissues (Fig. 9). A strong signal for POX activity was detected both in pathogen's hyphae and in plant cells in intimate contact with *F. solani*. No variation in

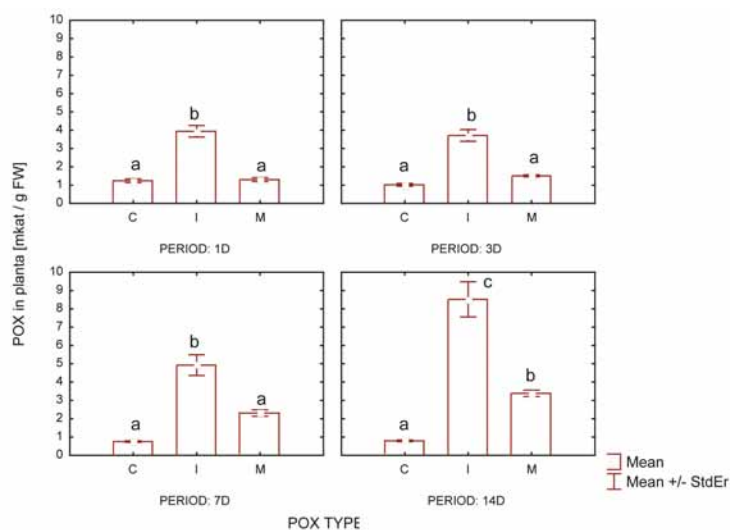


Fig. 7. POX activity during 14 days in cv. Menhir, differences in activities of cytosolic (C), ionic (I), membrane-bound (M) POXes between *in planta*.

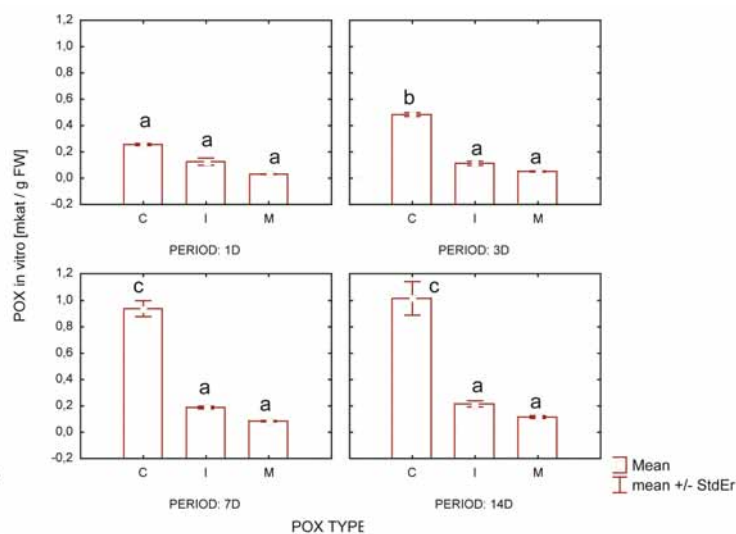


Fig. 8. POX activity during 14 days in cv. Menhir, differences in activities of cytosolic (C), ionic (I), membrane-bound (M) POXes between *in vitro* cultures.

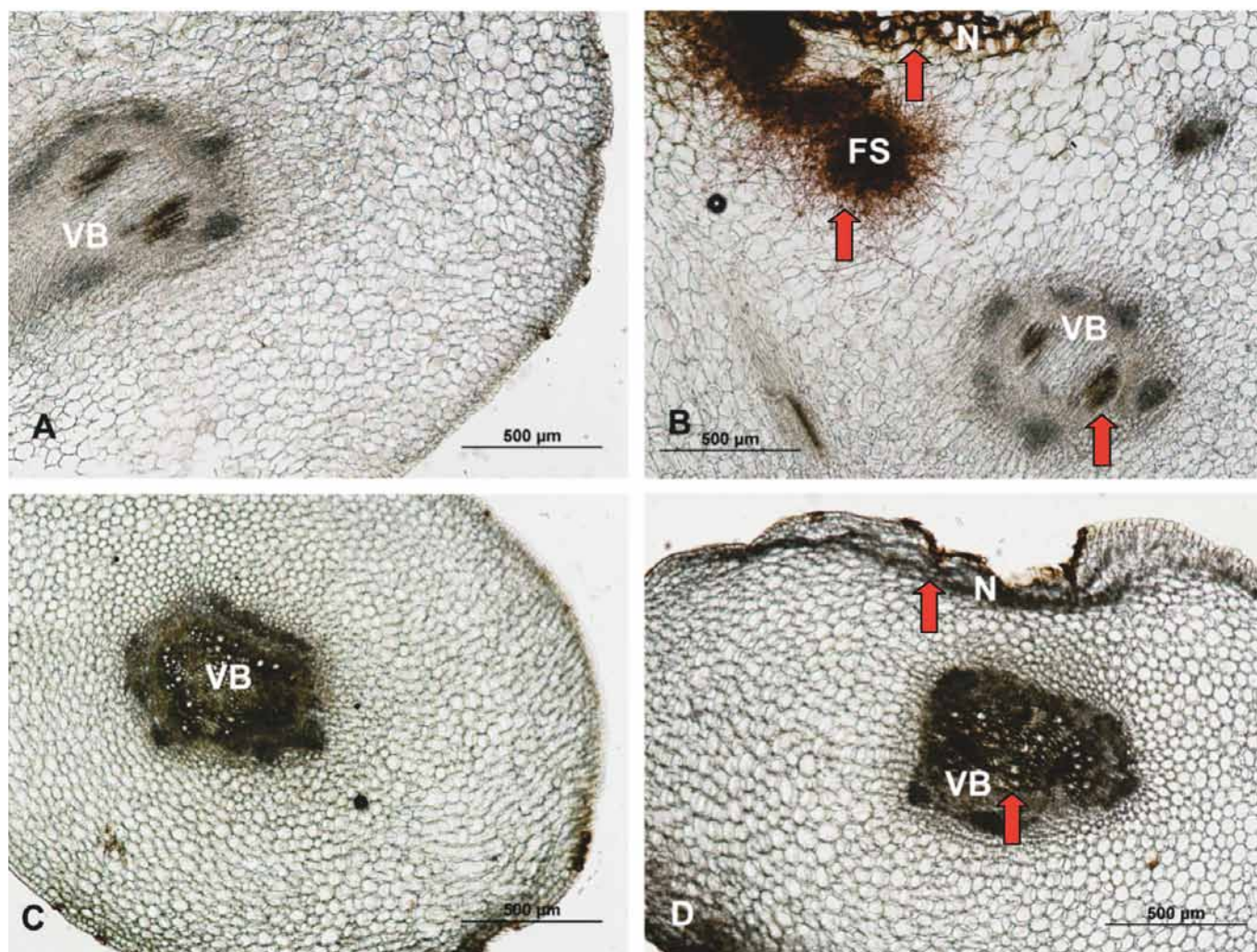


Fig. 9. Localization of POX in tissues of explants (A,B) and stems closely above hypocotyl (C, D) in *Pisum sativum* cv. Adept without treatment (A,C) and 7 dai by *Fusarium solani* (B,D). Dark staining for POX was localized in vascular bundles (VB), hyphae of *F. solani* (FS) and necrotizing cells (N).

POX presence among roots, hypocotyls and stems was revealed in intact plants. Localization of POX changes in explants was influenced by a strong accumulation in tissues due to previous mechanical injury of plant tissues during preparation of explants.

DISCUSSION

Culture filtrates of some plant pathogenic fungi are able to produce disease symptoms and thus can be used for resistance selection (Svábová and Lebeda, 2005; Lebeda and Svábová, 2010). This paper expands our previous studies focused on defence mechanisms of *P. sativum* against *Fusarium* spp. (Svábová and Griga, 1997; Svábová *et al.*, 1998; Luhová *et al.*, 2002, 2003, 2006) and contributes to the general understanding of disease resistance in this pathosystem (Hadwiger, 2008), and to the possibility of using fungal culture filtrates for resistance selection *in vitro* (Lebeda and Svábová, 2010).

The host-pathogen interaction between pea and *F. solani* is very variable. Previous data on the variation in resistance/susceptibility of peas to *Fusarium* spp. have been reviewed (Hagedorn, 1984; Jacobsen, 1992; Lebeda *et al.*, 2010) and Ali *et al.* (1994) have summarised the available information on valuable sources of resistance to soil-borne root diseases of peas. It is evident that sources of resistance in peas are rather limited. Our *in planta* screening of four pea cultivars with *F. solani* showed genotypic differences in resistance. Twenty days after inoculation or treatment with filtrates, cv. Adept expressed a higher level of resistance compared with cvs Herold, Komet and Menhir (Fig. 2). Treatment of the same set of intact pea plants by inoculation and with filtrates resulted in a similar reaction pattern. However, significant differences were recorded between two tested concentrations (50 and 100%) of filtrates. In general, the most similar reaction pattern to *in planta* inoculation was recorded with the 100% microfiltrated fungal suspension. These data disclose a relationship between pathogenicity of *F. solani* and symptoms expression after treatment of pea plants with the fungus and fungal filtrates in the studied pathosystem. A similar relationship was reported between plant response to inoculation by pathogen and treatment by filtrates of *F. oxysporum* in chickpea (Hamid and Strange, 2000) and pineapple with *F. subglutinans* (Borrás *et al.*, 2001). Moreover, a positive correlation between the area under disease progress curve of inoculated plants and the cut seedling test was observed in soybean using culture filtrates of *F. solani* (Huang and Hartman, 1998).

Inactivated, i.e. autoclaved or microfiltrated, fungal cultures represent complex mixtures of substances derived from the pathogen isolate. Fungal culture filtrates may contain a spectrum of secondary metabolites like

polysaccharides, oligosaccharides, proteins, glycoproteins, unsaturated fatty acids, growth regulators as auxin, cytokinins and gibberellic acid, along with toxins that may play a role as co-determinants of pathogenicity during disease development (Svábová and Lebeda, 2005). The application of filtrates to plant cultures *in vitro* can trigger the elicitation of various defence responses, including the induction of enzymes such as peroxidases, β 1,3-glucanase, and chitinase, and the synthesis and accumulation of phytoalexins, phenols and phenolic acids (Lebeda and Svábová, 2010).

A phenotypic response of plants to filtrates, similar to that elicited by fungal inoculation, is one of the basic pre-requisites for the possibility to substitute fungus treatment with filtrates for *in vitro* experiments. Based on the phenotypic responses of pea cultivars in our experiments, it can be concluded that fungal filtrates are able to mimic symptoms induced by *F. solani* inoculation. Our results show that the microfiltrated variant of *F. solani* filtrate was more efficient and have a higher inhibitive effect on *in vitro* pea explants compared with the autoclaved filtrate (Fig. 3). The process of autoclaving is responsible for inactivation or decomposition of some compounds as thermo-labile proteins which are involved in the pathogenicity and the expression of symptoms. The responses of pea explants showed significant genotypic differences in parameters such as percentage of surviving explants, length and weight of roots. Cv. Adept expressed a significantly higher tolerance to the negative effect of filtrate, whereas other cultivars were more sensitive and did not differ in tolerance. This relationship is in agreement with the expression of resistance degree *in planta* (Fig. 2). From the viewpoint of selectivity, the microfiltrated variant (20F) was the most efficient. A comparable phenomenon, inhibition of callus growth, was reported for chickpea treated with *F. oxysporum* filtrates (Singh *et al.*, 2003) and in cell culture of soybean treated with *F. solani* f. sp. *glycines*, where a decrease of viability of mesophyll cells was observed and correlated with foliar symptom severity (Li *et al.*, 1999).

Oxidative stress, including ROS production, was reported in different plant species after inoculation or natural fungal attack by *Fusarium* spp. or other pathogens, e.g. in the pathosystems bean-*Botrytis cinerea* (Unger *et al.*, 2005), carnation-*F. oxysporum* (Van Pelt-Heerschap and Smit-Bakker, 1999), chickpea-*F. oxysporum* (Singh *et al.*, 2003), potato-*F. sambucinum* (Ray and Hammerschmidt, 1998), and yellow lupine-*F. oxysporum* (Morkunas and Gmerek, 2007).

Plants are protected against ROS damage by an antioxidant system which includes enzymes of ROS catabolism such as superoxide dismutase and peroxidases (Lebeda *et al.*, 2001). Rapid production of ROS in the apoplast in response to pathogen attack has been proposed to orchestrate different defensive barriers against

the pathogens (Torres *et al.*, 2006). This trend was observed in resistant lines of *Medicago truncatula* which revealed rapid increase of iPOX after inoculation with *Phoma medicaginis* (Djebali *et al.*, 2007), and in mungbean (*Vigna radiata*) after treatment with an elicitor from *Macrophomina phaseolina* (Vidhyasekaran *et al.*, 2002).

Cell wall-bound peroxidases also belong to potential sources of ROS in plants (Able, 2003; Polkowska-Kowalczyk and Maciejewska, 2001). Generally, the response of resistant genotypes is characterised by an increase of activities of extracellular POXs, i.e. membrane-bound and ionic, during the first 24 h and then a stable increased level or gradual decrease. The *in vitro* response of POX in cv. Komet is more similar to the trend recorded by the more resistant cv. Adept. There are two possible explanations for this reaction: cv. Komet is either more resistant than previously reported, or it may have a very good adaptability to *in vitro* conditions which may also cause the increased activity of POX. In our experiments, susceptible cv. Menhir showed delayed increase of mPOX and iPOX as a result of late pathogen recognition, possibly following a weak strengthening of the cell wall as well as a late start of cell signalling (Fig. 7, 8). Low values of cPOX activities may be a result of the lack of resistance gene expression by sensitive cv. Menhir. Similar results were recorded in *Solanum* cell culture of a sensitive genotype after treatment with culture filtrate of *Phytophthora infestans*, where the activity of intracellular peroxidases did not change, whereas extracellular POX activity increased by 30% (Polkowska-Kowalczyk and Maciejewska, 2001).

Several papers dealing with the study of POX activities do not differentiate the forms of POX and record only total POX activity (Djebali *et al.*, 2007; Singh *et al.*, 2003; Vidhyasekaran, 2002), whilst other studies differentiate POX types (Cachinero *et al.*, 2002; Morkunas and Gmerek, 2007; Polkowska-Kowalczyk and Maciejewska, 2001; Vance *et al.*, 1976). POX influences not only plant-pathogen interactions, but also the growth and ageing of cells (Passardi *et al.*, 2004), and morphogenesis, i.e. somatic embryogenesis (Vranová *et al.*, 2002). The differences in the amount of POX types are probably also strongly influenced by specific *in vitro* conditions, such as hermetic sealing, limited space, limited exchange of gases and metabolic waste products and the content of nutritive substances and phytohormones in the media.

Our results represent one of the first studies focused on the *P. sativum*-*F. solani* interaction *in vivo* and *in vitro*, from the viewpoint of morphological changes as well as changes in the activity of peroxidases in plants and explant cultures exposed to the fungus and fungal filtrates. Results suggest that responses to *F. solani* filtrates mimic the physiological response *in planta* as well as *in vitro*. The activity of three POX types, cytosolic, mem-

brane-bound and ionic, is suitable for the assessment of the adaptability to stress, including biotic stress caused by *F. solani* and its filtrates. The resistant response is characterised by the immediate increase of all three types of POX. After an immediate increase of POX activity, a successive decrease comes as a result of plant adaptation to the infection. Conversely, the sensitive response is specific in later start of POX increase, as a result of late pathogen recognition, and the decrease of POX activity is parallel to plant cell death.

Recent studies have shown that there is some positive relationships between the pathogenicity of *F. solani* and pathogenesis processes on pea plants grown *in planta* and *in vitro*. It is evident that culture filtrates of *F. solani* could mimic the activity of inoculation by the pathogen, i.e. they could be used for *in vitro* selection. However, a detailed understanding of the physiological and biochemical processes responsible for this interaction remains unclear.

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TEMPORAL PROGRESS AND INTEGRATED MANAGEMENT OF FROSTY POD ROT (*Moniliophthora roreri*) OF COCOA IN TABASCO, MEXICO

M. Torres de la Cruz¹, C.F. Ortiz García², D. Téliz Ortiz¹, A. Mora Aguilera¹ and C. Nava Díaz¹

¹ Colegio de Postgraduados, Instituto de Fitosanidad, Km 36.5 Carretera México-Texcoco, 56230 Texcoco, México

² Colegio de Postgraduados, Campus Tabasco, Km. 3.5 Carretera Cárdenas-Huimanguillo, 86500 H. Cárdenas, Tabasco, Mexico

SUMMARY

Frosty pod rot of cocoa, induced by *Moniliophthora roreri*, has become the principal parasitic limitation for cocoa production in Tabasco (Mexico), which has prompted studies on its temporal progress and management. The temporal progress was desumed from estimation of the percentage of diseased fruits in three fruit flushes in the production cycle of 2008-09, whereas a regional management was compared with an Integrated Crop Management (ICM) in two systems of shade (50 and 70%). Three epidemics occurred from May 2008 to April 2009 with crop losses of over 70%. The epidemics occurred during 11 consecutive months associated with cool temperature (20-26.9°C) and relative humidity (higher than 90%) 49 days prior to the appearance of the symptoms. The ICM treatment with 50% shade showed a protection effectiveness of 90%. The reduction of shade to 50% alone reduced the incidence of frosty pod rot by 20%. The results suggest the convenience of independent management of the epidemics and the advantage of the following integrated control measures in each of the three fruit flush periods: weekly removal of diseased fruits, pruning of lateral and internal branches, reduction of tree height to 4 m, total elimination of fruits in low production period (purge), treatment of eliminated fruits and harvested litter with 15% urea, a single application with Azoxystrobin (250 g a.i./ha) to fruits under two months of age, and after it, three monthly sprays with copper hydroxide (1500 g a.i./ha). Additionally, weed control and improvement of soil drainage were implemented in both ICM and regional treatments.

Key words: epidemiology, moniliasis, integrated crop management.

INTRODUCTION

Cocoa (*Theobroma cacao* L.) is one of the main agricultural resources of Tabasco, Mexico. This crop is

grown on 41,086 ha distributed in ten municipalities, and nearly 30,000 families depend economically on it (OEIDRUS, 2008). In Tabasco, the most important disease of cocoa is black pod caused by *Phytophthora capsici* (Ortiz, 1996). However, in April 2005, frosty pod rot (FP), also known as moniliasis, caused by the fungus *Moniliophthora roreri* [(Cif and Par.) Evans *et al.*] was reported for the first time in Tabasco (Phillips-Mora *et al.*, 2006). By 2007, this disease had invaded all of the cocoa producing zones of the state, becoming its major limiting factor.

M. roreri attacks only fruits where it causes internal and external necrosis (chocolate spot), and the total loss of the pod. It lives in mummified fruits that remain on cocoa trees after harvest, thus supplying high levels of inoculum throughout the next fructification period (Phillips-Mora, 2003; Evans, 1981). This fungus was reported to reduce production dramatically by as much as 80% in Costa Rica (Hidalgo *et al.*, 2003; Phillips-Mora *et al.*, 2005), Ecuador and Colombia (Ampuero, 1967).

Isolated control measures for *M. roreri* have included: weekly removal of diseased fruits (González *et al.*, 1983; Soberanis *et al.*, 1999; Krauss *et al.*, 2003), total removal of fruits in periods of low production or at the end of the principal production stage (González *et al.*, 1983; Bateman *et al.*, 2005), pruning, weed control, improvement of drainage (Barros, 1966), opportune harvest of fruits (Bateman *et al.*, 2005), pruning of shade trees (Galindo, 1984), biological control (Krauss and Soberanis, 2002), chemical control (González *et al.*, 1983; Murillo and González, 1984; Bateman *et al.*, 2005) and genetic resistance (Phillips-Mora *et al.*, 2005).

There are no epidemiological studies of FP in Mexico for effective control strategies. Hence, the objective of the present study was to determine the temporal progress of FP and to develop an integrated management program for the cocoa-growing zones of Tabasco.

MATERIALS AND METHODS

Site and design. A cocoa plantation was selected in the municipality of Jalpa de Méndez (Tabasco) located at 13 m asl, 18°10'33.5" latitude north and 93°08'45"

longitude west. The area was planted with a mixture of susceptible 20-year-old hybrids, planted at 3.5×3.5 m. The trees were not fertilized during the tests.

Temporal progress. A plot with regional management was selected, without any measure against the disease. A rectangular area of 10×18 trees was selected from which the central 4×12 trees were used to follow the disease temporal progress. Disease incidence was evaluated in the three fruit flush periods that occurred during a complete production cycle (May 2008 to April 2009). Fruit formation was registered in each of the three fruit flushes. A population of young fruits, 1.5 to 2 months of age (80 to 100 mm length) was selected from each fruit flush (Phillips-Mora *et al.*, 2005). Dead fruits smaller than 80 mm were attributed to “Cherelle wilt”, a physiological disorder that affects young fruits and impedes the accurate identification of the disease in this stage (Bateman *et al.*, 2005). Incidence of fruits with chocolate spot and external sporulation (Sánchez and González, 1989) were quantified weekly. During evaluation, local agronomic practices in the regional plot included the elimination of shoots and weeds, control of *Phytophthora capsici* with CuSO₄ (2 kg/ha) and partial elimination of diseased fruits every 15 days during each of the three harvest periods. Discarded fruits were left on the soil. The accumulated progress of the disease was graphed for each of the three fruit flush periods.

Meteorological monitoring. The temperature was registered daily at 2 h intervals, with a meteorological station equipped with a Weather Wizard III (Davis Instruments, USA) console installed 100 m from the regional plot. Relative humidity was registered with a data logger (Hobo H8, Onset Instruments, USA) every 2 h. The meteorological information of the console and data logger were transferred to a calculation sheet (Excel of Microsoft) for analysis.

Temporal analysis. The epidemic for each production period was characterized by means of the Weibull distribution model (Pennypacker *et al.*, 1980) simplified to two parameters (b and c) (Thal *et al.*, 1984). The apparent infection rate was estimated by the inverse of the b parameter (b^{-1}) and the form of the curve by the c parameter. The intensity of each epidemic was estimated through the calculation of the area under the disease progress curve (AUDPC) from the trapezoidal integration method (Campbell and Madden, 1990) and the values of the initial incidence (Y_0) and final accumulated incidence (Y_f) of each epidemic.

Correlations. The incidence of the disease was related, through the estimation of the Pearson coefficient of correlation (r), with environmental variables that considered its duration in h/week/month, in the following

intervals: temperature (<20, 20-26.9, 27-29.9, 30-34.9 and >35°C), and relative humidity (<60, 60-90 and >90%).

Comparison of management. A regional management (RM) was compared against an Integrated Crop Management (ICM) in two successive fruit flush periods, in the same productive year and in a neighboring plot to the one where the temporal progress was studied. Each experimental plot consisted of 180 trees in a rectangle of 10×18 trees. The treatments were applied to the totality of trees of the plot, from which the experimental unit of 4×12 = 48 central trees were evaluated. A total of eight plots were established with a factorial design, completely randomized, with two replicates. The treatments were: two regimes of shade (70 and 50%) and two systems of management. For each shade regime, four plots were established, two of which were subjected to an ICM strategy and two were maintained with RM.

Two years prior to the evaluation, pruning and thinning were carried out in the shade trees of the plantation. To verify the conditions and stability of radiation during the evaluations, the photosynthetically active radiation (PAR) was measured using a PAR meter model BQM-SUN-5133 (Spectrum Technologies, USA). Ten readings were registered monthly following two cross lines within each experimental unit, above and below the cocoa tree canopy (4 and 1.5 m, respectively), as well as under full sunlight at the start and the end of each measurement to obtain the incident PAR.

During the two periods of highest production (July 2008 to March 2009), fruit formation was registered, distinguishing by production period a population of young fruits (80 to 100 mm). Dead fruits smaller than 80 mm were attributed to “Cherelle wilt” because plating of necrotic tissue in potato dextrose agar and incubation in a moist chamber excluded that any of these fruits was infected by *M. roreri*. A weekly quantification was made of the number of fruits that showed chocolate spot and sporulation as external symptoms of FP. The accumulated progress curve was graphed with the incidence of diseased fruits (Phillips-Mora *et al.*, 2005) in each production period.

Regional management. The RM consisted of manual weed control, conservation of drainage ditches, manual elimination of root shoots every year, chemical control of *Phytophthora capsici* with CuSO₄ (2 kg/ha) and elimination every 15 days of diseased fruits only during the harvest period (Table 1).

Integrated crop management. The ICM, besides weed control, improvement of drainage and elimination of shoots, differed in that the removal of diseased fruits was carried out weekly, canopy was managed by pruning of lateral and internal branches, tree height was kept

Table 1. Annual comparative agronomic calendar of integrated (ICM) and traditional (RM) management measures.

Production period	Management measures	RM	ICM
End of harvest of fruit flush 3 (April-May)	Weed control (April).	YES	YES
	Pruning of ventilation and height of cocoa trees (May).		YES
	Elimination of shoots (May).	YES	YES
	Total elimination of fruits (purge) (May).		YES
Fruit flush 1 (June-September)	Spraying of Azoxystrobin, (250 g a.i./ha ⁻¹), only to fruits under two months of age (June).		YES
	Weekly elimination of diseased fruits (June-September).		YES
	Elimination every 15 days of diseased fruits (only at harvest time).	YES	
	Treatment of eliminated fruits and harvest litter with solution of urea at 15%.		YES
Fruit flush 2 (August-January)	Spraying of Azoxystrobin (250 g a.i./ha ⁻¹) only to fruits under two months of age (August).		YES
	Monthly spraying of Copper Hydroxide (1500 g a.i./ha ⁻¹) (September-November).		YES
	Weekly elimination of diseased fruits (August-January).		YES
	Elimination every 15 days of diseased fruits (only at harvest time).	YES	
	Treatment of eliminated fruits and harvest litter solution of urea at 15%.		YES
	Improvement of drainage (August).	YES	YES
	Weed control (September).	YES	YES
Fruit flush 3 (December-April)	Control of black pod (<i>Phytophthora capsici</i>) (November).	YES	
	Spraying of Azoxystrobin (250 g a.i./ha ⁻¹) only to fruits under two months of age (December).		YES
	Monthly spraying of Copper Hydroxide (1500 g a.i./ha ⁻¹) (January-March).		YES
	Weekly elimination of diseased fruits (December-March).		YES
	Elimination every 15 days of diseased fruits (only at harvest time).	YES	
	Treatment of eliminated fruits and harvest litter with solution of urea at 15%.		YES

at 4 m; total elimination of fruits in low production period (purge), treatment of eliminated fruits and harvest litter with 15% urea; chemical control of FP with a single spray of Azoxystrobin (250 g a.i./ha), whose biological effectiveness was previously verified *in vitro*, applied to fruits under two months of age, and after it, three monthly sprays with copper hydroxide (1500 g a.i./ha) (Bateman *et al.*, 2005). The fungicides were applied with a back pump spray (Table 1).

Effect of treatments. To measure and compare the development of the disease between the two shade regimens (70 and 50%) and two management systems (ICM and RM), an analysis of variance was made under a factorial design with the procedure PROC GLM of SAS (SAS, 1988) for AUDPC, parameter b^{-1} and final accu-

mulated incidence (Y_p) of each epidemic/plot. The test of separation of means (Tukey $P=0.05\%$) was applied in the treatments in which the test of F was significant (Little and Hills, 1987).

RESULTS AND DISCUSSION

Temporal progress. Three epidemics were registered in concordance with an equal number of fruit flush periods that occurred during the year of evaluation (2008-09) (Fig. 1). The epidemics coincided with production periods regionally known as: “Venturero loco” (15 June-15 September), “Alegrón-invernada” (16 September-15 February) and “Cosecha” (February-June), previously described by Yanes (1994).

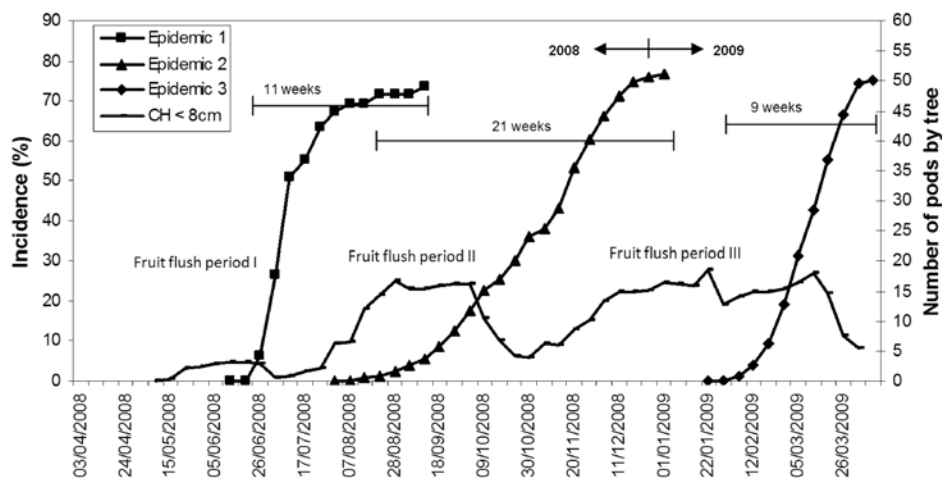


Fig. 1. Curves of temporal progress of frosty pod rot (*Moniliophthora roreri*) epidemics of cocoa in a grove with traditional management in Tabasco, during a year of production. CH = Chilillos (young fruits) (2008-2009).

In epidemic one (fruit flush period I), the start of the disease (Y_0) was observed at the end of June and the final incidence (Y_f) was reached in the middle of September. The Y_0 in epidemic two (fruit flush period II) appeared at the beginning of August and the Y_f in the first week of January. In epidemic three (fruit flush period III), the Y_0 started in the first week of February and the Y_f in the second week of April. The marked separation of the epidemics suggests the possibility of independently managing each epidemic with integrated actions that reduce the incidence of the FP in the reproductive stage of highest economic importance. The FP occurred during 11 consecutive months, which indicates favorable conditions for the continuous infection throughout the productive year in Tabasco.

FP epidemics were adequately described by the Weibull model (Table 2). Epidemic one had an $r^2=0.94$ and epidemics two and three had an r^2 of 0.99. The Y_f in the three epidemics affected an average of 75% of the fruits (Table 2) of each fruit flush period. The apparent infection rates (b^{-1}) fluctuated between 0.0079-0.0306, with epidemic one showing the highest rate (0.0306). The values of intensity of epidemics (AUDPC) fluctuated between 514.3-536.2, where the second epidemic was the most intense. Incidences of FP higher than 70% and under natural field conditions were

found in Costa Rica by Hidalgo *et al.* (2003) and Phillips-Mora *et al.* (2005).

Correlations. The incidence of the disease was positively associated with cool temperature (20-26.9°C) ($r=0.76$) and relative humidity higher than 90% ($r=0.76$), which appeared 49 days before the appearance of the symptoms and reflect environmental conditions favorable for critical stages of infection for Tabasco. Merchan (1981) found that there is a positive correlation between the incidence of FP and relative humidity, 60 days before the appearance of symptoms. Evans (1981) mentioned that one of the characteristics of FP is the long period of incubation from penetration until the appearance of symptoms. Desrosiers and Suárez (1974) found that the appearance of symptoms were related to the age of fruits, since symptoms occurred in 40 days when the fruits were infected at 20, 30 and 60 days of age, whereas in fruits inoculated at 80 days of age, the symptoms appeared in 60 days.

Management of FP. Significant differences were found in the AUDPC ($P=0.0004$), b^{-1} ($P=0.0003$) and Y_f ($P=0.0004$) between the RM and the ICM (Table 3, Fig. 2). The RM with 70% of shade registered the highest values of the disease (AUDPC, b^{-1} , and Y_f) in the three

Table 2. Epidemiological parameters of frosty pod rot of cocoa.

Epidemic (fruit flush)	AUDPC absolute	Y_f	b^{-1}
1	514.3	73.5	0.0306 ^a ($r^2=0.950$)
2	536.2	76.7	0.0079 ($r^2=0.993$)
3	522.3	74.9	0.0159 ($r^2=0.991$)

^aInfection rate was obtained through regression analysis of the Weibull model. AUDPC= area under the disease progress curve. Y_f = final accumulated incidence. b^{-1} = rate of apparent infection.

Table 3. Effect of treatments on the parameters of temporal progress of frosty pod rot of cocoa epidemics.

Epidemiological Parameters				
Treatments	AUDPC ^z absolute	Y_f^z	b^{-1z}	
Evaluation 1				
RM-70% shade	536.2 a	76.3 a	0.0079 ($r^2=0.993$)	a
RM-50% shade	360.6 b	52.3 b	0.0059 ($r^2=0.980$)	b
ICM-70% shade	141.7 c	20.2 c	0.0029 ($r^2=0.987$)	c
ICM-50% shade	54.0 c	7.7 d	0.0021 ($r^2=0.973$)	c
Evaluation 2				
RM-70% shade	522.3 a	74.9 a	0.0159 ($r^2=0.991$)	a
RM-50% shade	415.1 b	59.5 b	0.0135 ($r^2=0.971$)	b
ICM-70% shade	100.8 c	15.1 c	0.0062 ($r^2=0.968$)	c
ICM-50% shade	27.2 d	4.2 d	0.0052 ($r^2=0.962$)	c

AUDPC = area under the disease progress curve. Y_f = final accumulated incidence. b^{-1} = rate of apparent infection of the disease. RM = regional management. ICM = Plot with integrated management. ^z Means followed by the same letter do not differ statistically (Tukey, 0.05%).

treatments evaluated (Table 3, Fig. 2). The ICM treatments resulted in lower AUDPC, b^{-1} and Y_f in the two fruit flush periods. The lowest values occurred in the ICM with 50% of shade (54.0-27.2; 0.0021-0.0052 and 7.7-4.2, respectively) (Table 3, Fig. 2).

Significant differences were found among shade regimens in each single parameters evaluated: AUDPC ($P=0.0076$), b^{-1} ($P=0.0100$) and Y_f ($P=0.0071$) (Table 3, Fig. 2); observing that in the plots with 50% shade, independently of the agronomic management, RM or ICM, presented the lowest values in AUDPC, b^{-1} and Y_f , which coincided with the theoretical proposition of Fowler *et al.* (1956) and Díaz (1957).

The incidence of the disease was reduced by 68.64% in the ICM treatment with 50% shade in the first evaluation and by 70.77% in the second, with respect to the RM-70% of shade, which shows the consistency of the

effect of ICM in both evaluations. The ICM-50% of shade represented a protection effectiveness of 90% compared with the absolute total incidence observed in RM-70% of shade (Table 3), and the shade, regardless of the management, contributed to reducing the incidence of FP by 20%. Our results confirm the advantage of the integrated management approach compared with the implementation of isolated measures followed by Bateman *et al.* (2005), Krauss *et al.* (2003) and Krauss and Soberanis (2002).

In conclusion, our results suggest the convenience of applying ICM to each of the three fruit flush periods to improve the FP control efficiency, while differing from those of other studies based on the annual evaluation (Bateman *et al.*, 2005; Krauss and Soberanis, 2002), without a specific analysis of each one of the fruit flush periods that determines crop profitability.

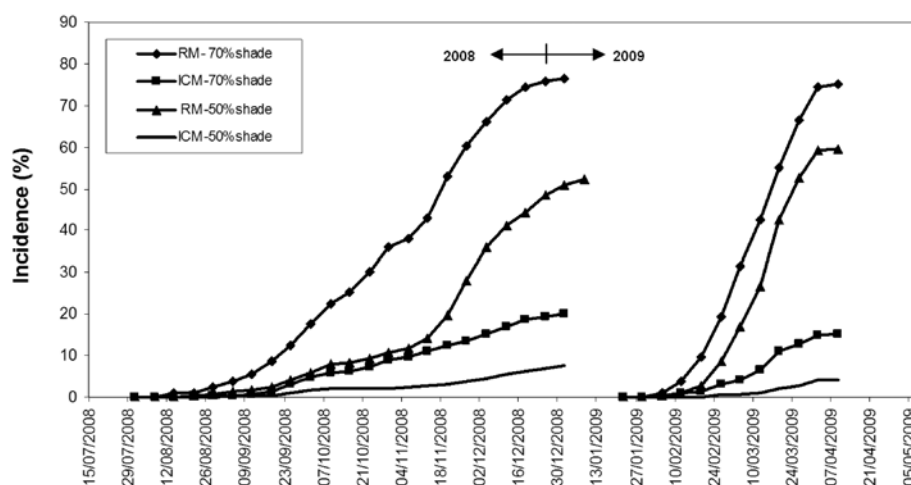


Fig. 2. Curves of temporal progress of frosty pod rot (*Moniliophthora roreri*) epidemics of cocoa in Tabasco. RM = Plot with regional management. ICM = Plot with integrated management (2008-2009).

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EFFECT OF SOME ENDEMIC PLANTS ESSENTIAL OILS ON BACTERIAL SPOT OF TOMATO

S. Altundag¹ and B. Aslim²

¹ Plant Protection Central Research Institute, PK 49, 06172 Ankara, Turkey

² Gazi University, Molecular Biology Research Center, 06830 Ankara, Turkey

SUMMARY

Essential oils of *Origanum minutiflorum*, *Sideritis erythrantha* subsp. *erythrantha*, *Satureja wiedemanniana*, *Salvia tchibatcheffii* and *Thymus sipyleus* subsp. *sipyleus* were tested against *Xanthomonas vesicatoria*, the agent of bacterial spot of tomato. Essential oil yields of these endemic plants were between 0.35-4.40% v/w per 100 g plant material. Essential oils inhibition zones, measured with calipers, were in the range of 4-43 mm. The lowest minimum inhibitory concentration (MIC) value against *X. vesicatoria* of essential oil was that of *O. minutiflorum* (200 µg/ml). The major constituents of the oils determined by GC-MS technique were carvacrol (C₁₀H₁₄O), γ-terpinene (C₁₀H₁₆), p-cymene (C₁₀H₁₄) and 1,8-cineole (C₁₀H₁₈O). The essential oil of *O. minutiflorum* reduced bacterial spot severity significantly (81.25%).

Key words: antimicrobial effect, bacterial spot, essential oil, *Origanum minutiflorum*, *Xanthomonas vesicatoria*.

INTRODUCTION

Turkey is as an important diversification center for the plant family Lamiaceae. The leafy part of members of this family such as oregano, thyme and savory (*Origanum*, *Thymus*, *Satureja*) are since long used in Mediterranean cookery. The genera *Thymus*, *Origanum*, *Sideritis*, *Salvia* and *Satureja* are represented by 241 species in the flora of Turkey and the East Aegean Islands, 114 of them being endemic (Davis *et al.*, 1984, 1988). Essential oils of these species, those of *Origanum* in particular, due to their antioxidant and antimicrobial activities, have recently become of interest as potential natural additives to foodstuff to replace synthetic products. Chemical control remains the main measure to reduce the incidence of diseases in various fruits and vegetables but their application may cause serious problems to the

environment. The demand for a reduced use of chemical pesticides has increased the interest for the possible application of essential oils to control plant pathogens (Daferera *et al.*, 2003). The acceptance of traditional medicine as an alternative form for health care and the development of microbial resistance to antibiotics have favoured investigations on the antimicrobial activity of medicinal plants. Although there are several studies on the antimicrobial activity and the essential oil composition of *Origanum* spp., the antimicrobial activity of the essential oil of *Origanum minutiflorum* against plant pathogen bacteria has never been studied before.

Bacterial spot of tomato, a serious disease effecting both foliage and fruit, is caused by *Xanthomonas vesicatoria* whose control with copper and streptomycin sprays has been attempted. However, chemical applications have disadvantages such as residues on fruits, cost, and development of resistant bacterial strains (Stall, 1993; El-Hendawy *et al.*, 2005; Al-Dahmani *et al.*, 2003). In recent years, there has been an increasing interest for biological control of bacterial diseases using natural products such as plant essential oils, which prompted the present investigations on the effectiveness of these products against *X. vesicatoria*. Therefore, the objective of this study was to compare the bactericidal activity of five plant essential oils against *X. vesicatoria* and to relate it to chemical composition of essential oils extracted from plants of the family Lamiaceae.

MATERIALS AND METHODS

Plant material. Plants of *Origanum minutiflorum* and *Sideritis erythrantha* subsp. *erythrantha* were collected in Isparta (1684 m a.s.l.) in 2004, *Satureja wiedemanniana* in Amasya (415 m a.s.l.) in 2005, *Salvia tchibatcheffii* and *Thymus sipyleus* subsp. *sipyleus* in Cankiri (735 and 644 m a.s.l.) in 2005, during the flowering stage, and their identification was confirmed by taxonomist of Gazi University, Ankara.

Extraction of essential oils. Samples from leaves, flowers and stems of all the above species were dried in the shade, ground in a grinder with a 2 mm diameter

Table 1. Three major components (%) of essential oils and essential oil yields.

Plant species	Yield (% v/w)	Major component	Composition (%)
<i>O. minutiflorum</i>	4.40	Carvacrol	73.93
		<i>p</i> -cymene	7.20
		γ -terpinene	3.99
<i>S. erythraea</i> subsp. <i>erythraea</i>	0.45	γ -terpinene	17.75
		β -caryophyllene	11.81
		Sabinene	9.96
		<i>p</i> -cymene	11.55
<i>S. wiedemanniana</i>	0.35	Menthadiene	9.45
		Spathulenol	7.22
		1.8-cineole	44.45
<i>S. tchibatche</i>	0.70	Camphor	12.19
		Thujene	8.80
		1.8-cineole	44.98
<i>T. sipyleus</i> subsp. <i>sipyleus</i>	0.60	Camphor	8.69
		Camphene	8.51

mesh and submitted for 3 h to steam distillation, using a Clevenger apparatus to extract the essential oil. Oil yield was 2.2% (v/w) based on the dry weight of the samples. Oils were stored in the dark at 4°C for no more than a week, until used (Oke *et al.*, 2009; Yaltirak *et al.*, 2009).

Test microorganism and inoculum preparation. *X. vesicatoria* NCPPB 422 used in this study was provided by the Bacteriology Laboratory of the Plant Protection Central Research Institute Ankara, and was routinely grown on nutrient agar (NA) at 28°C. The strain was stored at -20°C in an appropriate medium (nutrient broth containing 10% glycerol) and regenerated twice before use in the experiments. Bacterial cultures were suspended in sterile distilled water and turbidity was adjusted to an OD_{630nm} 0.100 using a spectrophotometer (Hitachi U-1800), corresponding to a cell concentration of ca. 10⁸ CFU/ml.

Inhibitory effect determination. The inhibitory effect of essential oils on *X. vesicatoria* was determined by the agar diffusion method (Kalemba *et al.*, 2003). Plates 90x20 mm with 20 ml of NA were inoculated with 200 µl of the bacterial culture suspension. The essential oils

were diluted in ethanol to a concentration of 1:5 (20 µl) and placed in four 7 mm diameter wells punched in the agar. The negative control consisted of straight ethanol, whereas a 1:5 copper sulfate solution in water was used as positive reference standard. The inoculated plates were incubated at 28°C for 48-72 h, then the inhibition zone diameters were measured with a caliper.

Determinations of minimum inhibitory concentration (MIC). To this aim, the microdilution broth susceptibility assay was used (Koneman *et al.*, 1997; Oke and Aslim, 2010). A stock solution of essential oil was prepared in 10% dimethylsulfoxide (DMSO), serial dilutions were then made in a concentration range from 7.8 to 500 µg/ml. An aliquot of 95 µl of nutrient broth (NB), 100 µl of essential oil and 5 µl of bacterial inoculum was dispensed in each well of a 96-well plate. A positive control (inoculum with NB but no essential oil) and negative control (essential oil but no inoculum) were included on each microtitre plate. The content of the wells was mixed by pipetting and the microplates, placed in their own sterile bags, were incubated at 28°C for 24 h. MIC was defined as the lowest concentration of the compounds able to inhibit bacterial growth.

Table 2. Inhibition zones and MIC values of different essential oils and copper sulfate against *Xanthomonas vesicatoria*.

Plant species	Essential oil (1/5)	
	Inhibition zone (mm)	MIC (µg ml ⁻¹)
<i>O. minutiflorum</i>	43 ± 0.7	200
<i>S. erythraea</i> subsp. <i>erythraea</i>	ND	>500
<i>S. wiedemanniana</i>	5 ± 0.3	>500
<i>S. tchibatcheffii</i>	ND	>500
<i>T. sipyleus</i>	4 ± 0.4	>500
Copper sulfate	18 ± 0.3	15.6

ND: No inhibition zone.

Gas chromatography/mass spectrometry analysis.

The chemical composition of essential oils was analyzed by gas chromatography/mass spectrometry. The mass spectrometer was Agilent 6890N GC/5973MSD-SCAN in the electron impact (EI) ionization mode (70eV) and HP-5MS capillary column (bonded and cross-linked 5% phenyl-methylpolysiloxane, 30 mm x 0.25 mm, coating thickness 0.25 µm). Injector and detector temperatures were set at 220°C. The oven temperature was held at 50°C for 30 min, then programmed to 240°C at a rate of 3°C/min. Helium (99.99%) was the carrier gas at a flow rate of 1 ml/min. Diluted samples (1/100 in hexan, v/v) of 1.0 µl were injected manually (Tabanca *et al.*, 2001; Aliannis *et al.*, 2001). Identification of the components was based on the comparison of their mass spectra with those of Wiley7N, Nist 2002 and Flavor libraries and comparison of their retention times.

Growth of test plants and inoculation of bacteria.

Tomato seeds Selin F1 were used as test plants. The soil was sterilized at 121°C and 1 atm air pressure for 15 min. Plants were grown in a growth chamber on sterile soil at 25°C with a 16 h light-8 h dark photoperiod and 70-75% relative humidity. Tomato seedlings at the three-leaf stage were transplanted in pots (14 cm diameter; 13 cm height). *X. vesicatoria* NCPPB 422 at concentration of *ca.* 10⁸ CFU/ml was spray-inoculated onto the abaxial and adaxial leaf surface of 20-day-old seedlings 15-20 cm in height. There were 5 theses in this experiment: (i) 200 µg/ml (MIC value) of the essential oil were sprayed on the seedlings followed after 48 h by bacterial inoculation; (ii) the bacterial suspension was first delivered to seedlings, followed after 48 h, by a spray with essential oil; (iii) treatment with essential oils only; (iv) inoculation with bacterial suspension only; (v) no treat-

ments (negative control). After the treatment, tomato seedlings were covered with plastic bags for 48 h. The experiment was carried out in four replicates.

Disease severity and disease rate were calculated according to formula by Townsend and Heuberger (1943):

Disease severity (%):

$$\frac{[\sum(\text{disease plant number of each scale} \times \text{scale value})]}{4 \times \text{total plant number}} \times 100$$

Disease rate (%):

$$\text{Disease plant number} / \text{total plant number} \times 100$$

For disease severity (magnitude of symptom shown by inoculated plants) the empirical scale: 0 = No symptoms; 1 = 1/4 of the plants show symptoms; 2 = half of the plants show symptoms; 3 = 3/4 of the plants show symptoms; 4 = all plants show symptoms

Statistical analysis. Inhibitory effects estimated by the agar-well diffusion, minimum inhibitory concentration and growth of tomato seedlings, and bacterial inoculation assays were performed in four, three and four replicates, respectively. Statistical analysis was by Duncan's multiple range tests using SPSS 10.0.

RESULTS AND DISCUSSION

Yields of *O. minutiflorum*, *S. tchibatcheffii*, *T. sipyleus* subsp. *sipyleus* *S. erythraea* subsp. *erythraea* and *S. wiedemanniana* essential oils were found to be 4.40, 0.70, 0.60, 0.45 and 0.35% (v/w), respectively. The three major components of five essential oils are reported in Table 1. The main components of *S. erythraea* subsp. *erythraea*, *S. wiedemanniana*, *S. tchibatcheffii* and *T. sipyleus* subsp. *sipyleus*, *O. minutiflorum* essential

Table 3. Effect of *Origanum minutiflorum* essential oil in the control of *X. vesicatoria* infection to tomato.

Experiment	Replicate number	Disease rate (%)	Diseases severity (%)
<i>X. vesicatoria</i> suspension only	4	100 a	64.06±2.99 a (56.25–68.75)
<i>X. vesicatoria</i> inoculation + essential oil application after 48 h	4	100 a	64.06±4.69 a (56.25–75.00)
Essential oil application + <i>X. vesicatoria</i> inoculation after 48 h	4	18.75±6.25 b (0.00-25.00)	4.69±1.56 b (0.00-6.25)
Essential oil only	4	0 c	0 c
Negative control (no treatment)	4	0 c	0 c

Values followed by different letters within each column are significantly different based on Duncan's multiple range test.

oil were γ -terpinene ($C_{10}H_{16}$), p-cymene ($C_{10}H_{14}$), 1,8 cineole ($C_{10}H_{18}O$), 1,8 cineole ($C_{10}H_{18}O$), carvacrol ($C_{10}H_{14}O$), respectively. *O. minutiflorum* essential oil (Table 2) had the highest inhibitory effect against *X. vesicatoria* (43 mm), i.e. higher than that of copper sulphate (18 mm) and oils from *S. wiedemanniana* (5 mm) and *T. sipyleus* subsp. *sipyleus* (4 mm). The negative control did not show any inhibitory effect.

The MIC value of *O. minutiflorum* was the lowest ($200 \mu\text{g ml}^{-1}$), while MIC values of the other oils were higher than $500 \mu\text{g ml}^{-1}$ (Table 2). Therefore, the essential oil of *O. minutiflorum* was selected for testing its effect against *X. vesicatoria* in tomato plants. As reported in Table 3, *X. vesicatoria* infection was inhibited at the rate of 81.25% after the application of *O. minutiflorum* essential oil.

In terms of essential oil yield, our results are in accordance with those from the literature (Sarer, 1996; Tabanca *et al.*, 2001; Demirci *et al.*, 2002). Most of the antimicrobial activity of these essential oils seems to be associated with phenolic compounds. In fact, compounds purified from essential oils such as carvacrol, eugenol, linalool and thymol inhibit the growth of a variety of microorganism (Bagamboula *et al.*, 2004).

However, no antimicrobial activity has been reported for γ -terpinene (synonyms: Crithmene, Moslene 1-Isopropyl-4-methyl-1,4-cyclohexadiene) and p-cymene (synonyms: Dolcymene, 1-Isopropyl-4-methylbenzene, 4-Isopropyltoluene) (Aliğianis *et al.*, 2001; Sivropoulou *et al.*, 1996) which are the main components of the oils of *S. erythraea* subsp. *erythraea* (17.75%) and of *S. wiedemanniana* (11.55%), respectively.

The main component of *S. tchihatcheffii* (44.45%) and *T. sipyleus* subsp. *sipyleus* (44.98%) oils is 1,8 cineole (synonyms: eucalyptol, limonene oxide, 1,8-epoxy-p-menthane), whereas carvacrol (synonyms: 2-Hydroxy-4-cymene, Isothymol 5-isopropyl-2-methylphenol) (73.93%) prevails in *O. minutiflorum* oil, in agreement with Sarer (1996) who reported carvacrol to be the major component (92.95%) of the essential oil from the same plant species. Interestingly, the essential oil of *Origanum scabrum*, which contains 75% of carvacrol, was reported to have a very high antibacterial effect against *Streptomyces aureus* and *Escherichia coli* (Kalemba and Kunicka, 2003), a finding is in line with our results that indicate carvacrol as the most effective inhibitor of *X. vesicatoria* (81.25% inhibition rate).

At the application rate used in our experiments the essential oil of *O. minutiflorum* did not cause any apparent phytotoxicity to tomato plants. This increases the potential of this compound for use in IPM programmes for the control of *X. vesicatoria*, provided that outdoor field experiments will confirm the results obtained under growth chamber conditions.

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DIFFERENTIAL EFFECTS OF ORGANIC COMPOUNDS ON CUCUMBER DAMPING-OFF AND BIOCONTROL ACTIVITY OF ANTAGONISTIC BACTERIA

B. Li^{1,2}, S. Ravnskov², G. Xie¹ and J. Larsen^{2*}

¹ State Key Laboratory of Rice Biology, Key Laboratory of Molecular Biology of Crop Pathogens and Insects, Ministry of Agriculture, Institute of Biotechnology, Zhejiang University, Hangzhou, China

² Department of Integrated Pest Management, Faculty of Agricultural Science, University of Aarhus, Slagelse, Denmark

*Present address: Centro de Investigaciones en Ecosistemas, Universidad Nacional Autónoma de México, Apartado Postal 27-3 Santa María de Guido, 58090 Morelia, Michoacán, México

SUMMARY

The influence of the organic compounds tryptic soy broth, cellulose, glucose and chitosan on cucumber damping-off caused by *Pythium aphanidermatum* and biocontrol efficacy of the biocontrol agents (BCAs) *Paenibacillus macerans* and *P. polymyxa* were examined in a seedling emergence bioassay. Results showed that the organic compounds differentially affected both pathogen and BCAs. Tryptic soy broth, glucose and chitosan increased *Pythium* damping-off of cucumber, compared to the control treatment without organic compounds, whereas cellulose had no effect. Both *Paenibacillus* species had biocontrol effects against *Pythium* damping-off compared with the corresponding treatments with *P. aphanidermatum* alone, but the biocontrol efficacy depended on the type of organic compounds added. Both BCAs counteracted damping-off in treatments with TSB and chitosan. However, *P. polymyxa* counteracted damping-off in combination with either glucose amendment or without organic compound amendment, while *P. macerans* increased damping-off in combination with either cellulose amendment or without organic compound amendment. Damping-off did not correlate with *Pythium* biomass estimated by ELISA, nor with background microbial communities as examined using biomarker fatty acids.

Key words: biocontrol agents, *Paenibacillus*, plant growth promoting rhizobacteria, *Pythium*, resource availability.

INTRODUCTION

Pre- and post-emergence damping-off in cucumbers, mainly caused by *Pythium aphanidermatum* (Wulff *et al.*, 1998; Abbasi and Lazarovits, 2006), causes serious economic losses under conditions favorable for its development (Georgakopoulos *et al.*, 2002; Abbasi and

Lazarovits, 2006). Although fungicides are available, they are not always sufficiently effective for disease control, and their use may also cause environmental problems (Abbasi *et al.*, 2004). Several antagonists against *Pythium*-induced damping-off of cucumber have been identified (Larsen *et al.*, 2003; Folman *et al.*, 2004). Two bacterial strains, *Paenibacillus polymyxa* MB02-226 and *P. macerans* MB02-429, which were isolated from the cucumber rhizosphere (Mansfeld-Giese *et al.*, 2002), have potential to inhibit pre- and post-emergence damping-off of cucumber seedlings caused by *P. aphanidermatum* (Li *et al.*, 2007).

The use of organic compounds has also been proposed, for both conventional and biological agriculture systems, to decrease the incidence of plant diseases caused by soil-borne pathogens (Siddiqui, 2004). Potassium silicate amendments to nutrient solutions were found to be effective in controlling *Pythium* infections in hydroponic culture systems (Chérif and Bélanger, 1991). Furthermore, fish emulsion amendment was shown to biologically activate peat and soil suppressing *Pythium* damping-off of cucumber seedlings (Abbasi *et al.*, 2004). In addition, chitosan effectively inhibited the growth of *P. aphanidermatum* in both potato dextrose agar (PDA) plates and nutrient solutions (EI Ghaouth *et al.*, 1994).

Recent studies indicated that highly effective biological control of soil-borne plant pathogens can be attained only with the combined application of organic amendments and microbial biocontrol agents (Siddiqui, 2004; Chang *et al.*, 2007; Singh *et al.*, 2007; Siddiqui and Akhtar, 2008). The addition of chitosan to a formulation of *Bacillus pumilus* increased the effectiveness of this bacterium in controlling Fusarium wilt of tomato (Benhamou *et al.*, 1998). Furthermore, chitosan and isolates of *Pseudomonas* spp. effectively reduced the severity of Fusarium seedling blight of wheat and barley (Khan, 2006). In addition, glucose amendment improved the biocontrol activity of *Pseudomonas fluorescens* strain CHA0 against *Macrophomina phaseolina* both under laboratory and glasshouse conditions (Shaukat and Siddiqui, 2003). Therefore, the use of organic compounds to increase plant protection, in combination with antagonistic bacteria, seems to be a prom-

ising approach for the control of *Pythium* damping-off.

However, relationships between the populations of soil microorganisms, the soil environment, organic amendments and plant health are obviously complex and make it difficult to assess activities occurring in soil. Some researchers have indicated that the interaction between soil-borne plant pathogens and BCAs could be influenced by a number of factors, such as the nature of the organic amendments, the microorganisms present, the properties of the soil, and environmental conditions (Shaukat and Siddiqui, 2003; Singh *et al.*, 2007; Siddiqui and Akhtar, 2008). Interestingly, the biocontrol ability of bacteria from the genus *Paenibacillus* against *Pythium*-caused damping-off of cucumber has been well documented in six-well microtiter plates with sterilised sand as growth substrate (Li *et al.*, 2007), which exclude the influence of other factors. Therefore, this sand system may be suitable to examine the influence of organic compounds with different nutritional components on the interaction between soil-borne plant pathogen and BCAs.

The purpose of this study was to investigate the role of organic compounds in the rhizosphere on the development of *Pythium* damping-off of cucumber and the biocontrol ability of antagonistic bacteria against this disease. Our main hypothesis was that organic compounds differentially affect the interaction between *P. aphanidermatum* and BCAs.

MATERIALS AND METHODS

Microbial inoculants. The pathogen and both antagonistic bacteria were obtained from the culture collection at the Department of Integrated Pest Management, University of Aarhus, Denmark. The virulent strain of *P. aphanidermatum* FC42 was grown on corn meal agar (CMA; Becton Dickinson and Company, USA) at 28°C for 48 h. The antagonistic bacteria *P. macerans* MB02-429 and *P. polymyxa* MB02-226 were grown for 24 h in tryptic soy broth (TSB; Becton Dickinson and Company, USA) at 30°C on a rotary shaker (200 rpm) and harvested by centrifugation for 5 min at 3,000 rpm at 20°C. Following two washes in phosphate buffer, the bacteria were re-suspended in sterile deionized water before seed application. The population density of bacteria were determined after plating of serial dilutions on TSB agar plates.

Experimental design. The experiment had a fully randomized 5 x 4 factorial design with organic compounds and microbial inoculation as the main factors. Five different levels of organic compounds were used: (i) control with water; (ii) TSB; (iii) cellulose; (iv) glucose; and (v) chitosan; and four different levels of microbial inoculation were employed: (i) without microbial inoculation; (ii) inoculation with *P. aphanidermatum*; (iii) inoculation with

P. aphanidermatum in combination with *P. macerans* and (iv) inoculation with *P. aphanidermatum* in combination with *P. polymyxa*, giving a total of 20 treatments, each with five replicates.

Cucumber seedling emergence assay. A cucumber seed emergence bioassay was developed using six-well microtiter plates with sterilized sand as described by Li *et al.* (2007). The sand in the microtiter was left untreated or uniformly mixed with one of the following substrates (0.5 mg/g sand): glucose, cellulose, chitosan, or 1/10 strength TSB (0.7 ml/g sand). Chitosan (85% deacetylated) was powdered in liquid nitrogen before application. Sterile deionized water was included as control. All chemicals were obtained from Sigma Chemical (USA). Thiram-coated cucumber seeds of cv. Mystica (F1 hybrid) were washed in distilled water for 3 min to get rid of the fungicide, then one seed was placed in each well. Each replicate consisted of one plate with six wells.

***Pythium* inoculation and bacterial application.** *P. aphanidermatum* FC42, whose pathogenicity to cucumber was confirmed in a previous study (Li *et al.*, 2007), was grown on CMA at 28°C for 48 h. Agar plugs (5 mm in diameter) covered with actively growing mycelium of were used as inoculum and one agar plug was applied directly to each seed. The respective controls without *Pythium* received sterile agar plugs. In the combined treatment with bacteria, 1 ml of a bacterial suspension (approximately 3×10^8 CFU/ml) was applied at sowing to each well. The control treatment without bacteria received 1 ml sterile deionized water.

Growth conditions and disease assessment. After sowing, the microtiter plates were incubated for 7 days in a growth chamber with 16 h photoperiod (16 h day at 21°C and 8 h night at 19°C). Each experimental unit was scored for seedling emergence and damping-off. Lids of the plastic plates were removed after seedling emergence (four days after sowing) and sterile deionized water was added regularly to full water holding capacity.

Harvest and plant analyses. Seven days after sowing, emerged seedlings from each microtiter plates were counted and pooled. Dry weights of shoots and root of the pooled seedlings were determined after drying at 80°C for 24 h. Sand samples from each microtiter plate were pooled, mixed, freeze-dried and ground for whole cell fatty acid (WCFA) analysis and ELISA detection of *Pythium*.

Biomarker fatty acids. A 30 g sample of freeze-dried sand was powdered in a steel mill, and 1.0 g was subjected to lipid extraction and further analysed for the content of whole cell fatty acids (WCFAs), as described by Thygesen *et al.* (2004). Analyses of fatty acid methyl

esters were performed using the Sherlock 3.1 software package (MIDI Inc., USA) with an HP Chemstation (Hewlett Packard, USA) and an HP5890 GC fitted with a 25-m fused silica capillary column (HP part no. 19091B-102) and hydrogen as the carrier gas. The injector and detector temperatures were set to 250 and 300°C, respectively. The column temperature was programmed so that it increased from an initial 170°C to an ultimate 270°C at a rate of 5°C/min.

ELISA detection of *Pythium*. *Pythium* biomass was estimated by ELISA with the Agriscreen Kit (Adgen, UK) in extracts from different sand samples. A 10.0 g sample of freeze-dried sand was powdered in a steel mill, and 1.0 g was mixed with 4.0 ml extraction solution provided with a 96 well ELISA detection kit and the suspension was vortexed for 30 sec before the assay. One hundred microliters of positive control, negative control and each of the prepared samples were placed into the appropriate micro wells. The ELISA protocol for estimation of *P. aphanidermatum* FC42 biomass in sand followed the manufacturer's instructions.

Statistical analyses. The software STATGRAPHICS Plus, version 5.1 (Copyright Manugistics Inc., USA) was used to perform statistical analyses. Levels of significance of the main factors and their interactions were calculated by two-way analyses of variance after testing for normality and variance homogeneity. Treatment means were compared using LS means analyses. All percentage values were arc-sin transformed before analyses of variance.

RESULTS

Plant parameters. Seedling emergence. The interaction between organic compounds and microbial inoculation was significant for seedling emergence (Table 1).

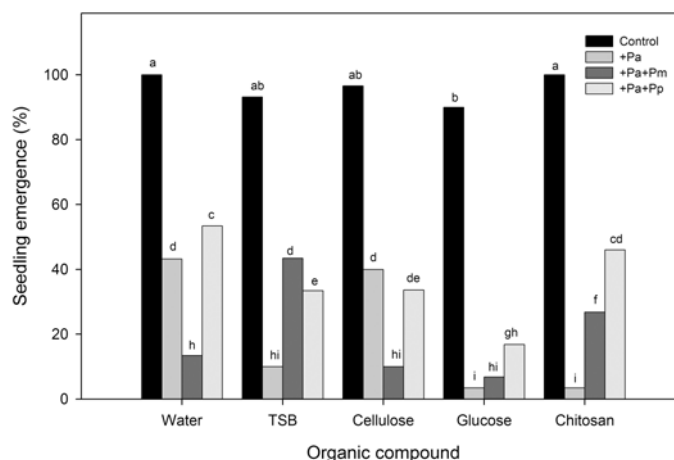


Fig. 1. Effect of organic compound amendments on the virulence of *Pythium* alone and biocontrol activity of antagonistic bacteria against *Pythium* damping-off of cucumber seedlings in a sterile sand system. Columns with the same letters are not significantly different ($n=5$). Pa, *Pythium aphanidermatum*; Pm, *Paenibacillus macerans*; Pp, *Paenibacillus polymyxa*.

Without microbial inoculations, cucumber seedling emergence was 100% in the treatment without organic compounds (Fig. 1). Amendment of TSB, cellulose and chitosan did not reduce the seedling emergence in the treatment without microbial inoculation, however glucose caused a 10% reduction in seedling emergence 7 days after sowing, compared to the treatment with water (Fig. 1). In general, in the presence of *P. aphanidermatum*, cucumber emergence percentage decreased by 73.3% compared with the treatment without *P. aphanidermatum* that had an emergence percentage of 96%. However, the suppressive effect of *Pythium* on cucumber seedling emergence was increased by more than 76.9% in treatments with TSB, glucose and chitosan, but was unaffected by cellulose compared with the control treatment with water that had an emergence percentage of 43.2% (Fig. 1). Inoculation with *P. macerans*

Table 1. Probability (P) values from all measured parameters from two way analyses of variance for substrates and microorganisms as the main factors, and their interactions ($n=5$).

Parameters	Organic compound (O)	Microorganisms (M)	O x M
<i>Plant parameters</i>			
Seedling emergence	***	**	**
Healthy seedlings	*	***	*
Shoot dry weight	***	*	*
Root dry weight	***	***	*
<i>Microbial parameters</i>			
Pythium ELISA	***	0.697	0.269
G- bacteria fatty acids	***	**	0.082
G+ bacteria fatty acids	***	**	0.162
Fungal fatty acids	***	*	***
Total microbial fatty acids	***	0.146	*

*, $P<0.05$; **, $P<0.01$; ***, $P<0.001$.

counteracted damping-off in treatments with TSB and chitosan, which had an emergence percentage of 43.4% and 26.8%, respectively. However, *P. macerans* increased damping-off by more than 50% in combination with either cellulose amendment or without organic compound amendment, which had emergence percentages of 10.0% and 13.4%, respectively (Fig. 1). Inoculation with *P. polymyxa* counteracted damping-off in all treatments by more than 23.6%, except in combination with cellulose that had an emergence percentage of 33.6% (Fig. 1). The emerged seedlings remained healthy seven days after sowing.

Shoot and root dry weight. The interaction between organic compounds and microbial inoculation was significant for shoot and root dry weight (Table 1). Without microbial inoculations shoot and root dry weight did not differ between treatments with diverse organic compound amendment (Table 2). Inoculation with *P. aphanidermatum* FC42 alone, had no effect on shoot dry weight, but increased the root dry weight by 23.1% in the treatment without organic compounds (Table 2). Combination of *P. aphanidermatum* inoculation and amendment with all organic compounds reduced both shoot and root dry weight at different levels, except for cellulose which only slightly reduced shoot dry weight and had no effect on root dry weight (Table 2). Dual inoculation with *P. aphanidermatum* and *P. macerans* resulted in a 40 to 66.9% decrease in shoot and root dry

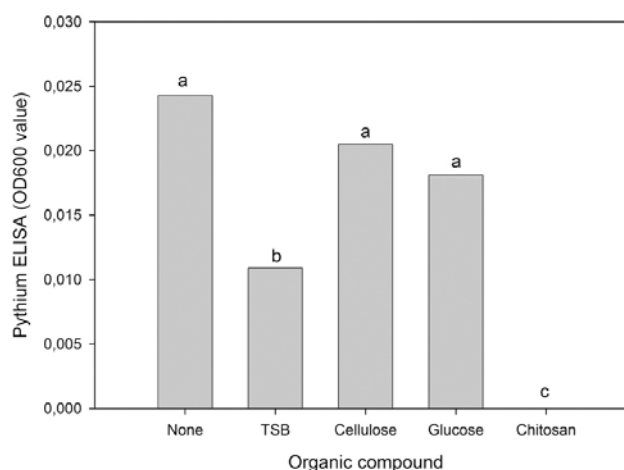


Fig. 2. Effect of organic compound amendments on *Pythium* ELISA (OD600 value) in a sterile sand system. Columns with the same letters are not significantly different (n=5).

weight in combination without organic compound amendment, and with cellulose, whereas TSB, glucose and chitosan resulted in more than 78.9% increase in shoot and root dry weight compared to the corresponding treatments with *P. aphanidermatum* alone (Table 2). Dual inoculation with *P. aphanidermatum* and *P. polymyxa* resulted in more than 24.0% increase in shoot and root dry weight in combination with all organic

Table 2. Shoot and root dry weight of cucumber plants in treatments with different combinations of organic compound amendments and microbial inoculations (Pa, *Pythium aphanidermatum*; Pm, *Paenibacillus macerans*; Pp, *Paenibacillus polymyxa*). Treatments with different letter within each column are significantly different (n=5).

Treatments		Plant biomass	
Organic compound	Microbial inoculation	Shoot dry weight (mg)	Root dry weight (mg)
None	None	24.7 f	5.2 e
	+Pa	26.6 f	6.4 f
	+Pa+Pm	8.8 b	2.4 bc
	+Pa+Pp	25.1 f	5.1 e
TSB	None	25.9 f	5.7 ef
	+Pa	10.9 c	2.7 c
	+Pa+Pm	19.5 e	4.9 de
	+Pa+Pp	19.2 e	5.1 e
Cellulose	None	24.5 f	5.3 e
	+Pa	20.4 e	5.0 de
	+Pa+Pm	10.4 c	3.0 c
	+Pa+Pp	26.7 f	6.2 f
Glucose	None	23.6 f	4.4 de
	+Pa	4.0 a	0.5 a
	+Pa+Pm	8.8 b	1.7 b
	+Pa+Pp	19.2 d	2.5 bc
Chitosan	None	23.7 f	4.8 de
	+Pa	0 a	0 a
	+Pa+Pm	14.2 c	3.5 c
	+Pa+Pp	18.4 de	4.0 cd

compounds, compared with inoculation with *P. aphanidermatum* alone (Table 2).

Microbial parameters. *Pythium* ELISA. Organic compounds significantly affected *Pythium* ELISA, whereas microbial inoculation had no effect and the two main factors did not interact (Table 1). Tryptic soy broth and chitosan reduced *Pythium* ELISA by 55% and 100%, respectively, compared to the treatment without organic compound amendment, whereas glucose and cellulose had no effect (Fig. 2). The background *Pythium* ELISA level in the treatment without *Pythium* inoculation was high corresponding to 78% of the total amount of *Pythium* ELISA value in the treatments with *Pythium* alone. However, when comparing the *Pythium* ELISA values in treatments with and without *Pythium* inoculation, the *Pythium* ELISA value was 27% higher in the treatments with *Pythium* than in the treatments without *Pythium* ($P < 0.001$, data not shown).

Biomarker fatty acids. Both organic compounds and microbial inoculation significantly affected the amount of Gram-negative and Gram-positive bacterial biomarker fatty acids, but the two factors did not interact (Table 1). The amount of Gram-negative and Gram-positive biomarker fatty acids was higher in treatments with microbial inoculation than without microbial inoculation (Table 3). The microbial inoculations with *P. aphanidermatum* alone or in combination with either *P. macerans* or *P. polymyxa* were similar concerning Gram-negative and Gram-positive bacterial biomarker fatty acids (Table 3). Glucose decreased the level of Gram-negative bacterial biomarker fatty acids by 62.9% compared with the treatment without organic compound amendment (Table 3). The other organic compounds, TSB, cellulose and chitosan, had no effects on the amount of Gram-negative bacterial biomarker fatty acids (Table 3). Tryptic soy broth increased the level of Gram-positive bacterial fatty acid biomarkers by 46.8% compared to the treatment without organic compound amendment (Table 3), whereas the other organic compounds had no effect.

Concerning the fungal biomarker fatty acid 18:2 ω 6,9 the two main factors interacted significantly (Table 1). In general, the amount of 18:2 ω 6,9 in the cucumber rhizosphere sand was higher in treatments with microbial inoculation and did not differ between treatments with *P. aphanidermatum* alone and in combination with *P. macerans* or *P. polymyxa* (Table 4). However, in the absence of organic compounds, the amount of 18:2 ω 6,9 was higher in the treatment with dual inoculation with *P. aphanidermatum* and *P. macerans* than in the two other microbial inoculation treatments (Table 4).

A significant interaction between organic compounds and microbial inoculation occurred concerning total microbial biomarker fatty acids (Table 1). In general, the amount of total microbial biomarker fatty acids in sand samples was higher in treatments with microbial inocu-

Table 3. Means of factor treatments of bacterial biomarker fatty acids in the rhizosphere of cucumber seedlings representing Gram-negative (sum of 10:0 2OH, 10:0 3OH, 12:0 3OH and 17:0 cyclic) and Gram-positive bacteria (sum of 15:0 anteiso and 17:0 anteiso) relative to the internal standard (%). Individual factor treatments within separate columns are significantly different ($n=5$).

Factors	Gram-negative bacteria	Gram-positive bacteria
<i>Microbial inoculation</i>		
None	0.04 a	0.29 a
+Pa	0.39 b	1.43 b
+Pa+Pm	0.43 b	1.69 b
+Pa+Pp	0.31 b	1.67 b
<i>Organic compound</i>		
None	0.35 bc	1.09 ab
Tryptic soy broth	0.25 ab	1.60 c
Cellulose	0.45 c	0.92 a
Glucose	0.13 a	1.47 bc
Chitosan	0.31 bc	1.26 abc

Pa, *Pythium aphanidermatum*.

Pm, *Panabacillus macerans*.

Pp, *Paenibacillus polymyxa*.

lation than without microbial inoculation (Table 4). The organic compounds had differential effects on the impact of the different types of microbial inoculations on the amount of total microbial biomarker fatty acids (Table 4). In the treatments without organic compounds, dual inoculation with *P. aphanidermatum* and *P. macerans*, increased the level of microbial biomarker fatty acids more than 1.3 fold compared to that of the two other microbial inoculations (Table 4). In the treatments with TSB, the amount of microbial biomarker fatty acids did not differ between the three different treatments with microbial inoculation (Table 4). In treatments with cellulose and glucose the level of microbial biomarker fatty acids was higher and lower, respectively, in the treatment with dual inoculation with *P. aphanidermatum* and *P. macerans* compared with the two other treatments with microbial inoculation (Table 4). In the treatments with chitosan the amount of microbial biomarker fatty acids was lower in the treatment with dual inoculation (*P. aphanidermatum* and *P. polymyxa*), compared with that of the two other treatments with microbial inoculation (Table 4).

DISCUSSION

The present results confirm our main hypothesis that organic compounds can affect both *Pythium* damping-off and the efficacy of bacterial biocontrol agents, and that the interaction between organic compounds and microbial inoculation is affected by the type of organic compounds and the type of biocontrol agents.

The fact that organic compounds affect *P. aphaniderma-*

Table 4. Amount of fungal and total microbial biomarker fatty acids (relative to internal standard) in the rhizosphere of cucumber seedlings in treatments with different combinations of organic compound amendments and microbial inoculations (Pa, *Pythium aphanidermatum*; Pm, *Paenibacillus macerans*; Pp, *Paenibacillus polymyxa*). Treatments within each group of microbiota with different letters are significantly different (n=5).

Treatments		Biomarker fatty acids (relative to internal standard)	
Organic compound	Microbial inoculation	True fungi (18:2 ω 6,9)	Total microbial fatty acids*
None	None	0 a	0.62 a
	+Pa	1.48 bc	12.79 b
	+Pa+Pm	11.69 d	32.53 e
	+Pa+Pp	2.24 bc	14.14 b
Tryptic soy broth	None	0 a	0.55 a
	+Pa	1.81 bc	14.57 b
	+Pa+Pm	1.20 b	12.12 b
	+Pa+Pp	1.57 bc	14.11 b
Cellulose	None	0.45 ab	0.40 a
	+Pa	3.54 c	19.45 d
	+Pa+Pm	3.29 c	16.32 c
	+Pa+Pp	3.45 c	18.15 d
Glucose	None	0.77 ab	0.66 a
	+Pa	2.00 bc	12.39 b
	+Pa+Pm	3.60 c	19.74 d
	+Pa+Pp	1.57 bc	12.15 b
Chitosan	None	0 a	0.36 a
	+Pa	3.34 c	15.66 c
	+Pa+Pm	2.25 bc	15.36 c
	+Pa+Pp	2.02 bc	11.72 b

* Total microbial fatty acids represent the sum of bacterial and fungal fatty acids and the general microbial fatty acids 16:1 ω 7, 18:1 ω 7 and 18:1 ω 9.

tum pathogenicity is in agreement with our previous result showing that TSB increase *Pythium* damping-off, and emphasize the importance of nutrient availability in terms of pathogen inoculum potential (Shah *et al.*, 2005; Li *et al.*, 2007). However, our finding that chitosan increases *Pythium* damping-off in cucumber is in contrast with the report by El Ghaouth *et al.* (1994) who observed an inhibitory effect of chitosan on *Pythium* damping-off when applied to the nutrient solution. The reason for these conflicting results may be due to differences in the experimental set-up, such as the growth matrix and/or nutritional factors.

In treatments with TSB and chitosan, *Pythium* damping-off was suppressed by bacterial biocontrol agents regardless of the bacterial species, which is in agreement with the result of Postma *et al.* (2009), who found that addition of chitosan enhanced the biocontrol efficacy of *Lysobacter enzymogenes* against *P. aphanidermatum* in hydroponically-grown cucumbers. However, *P. macerans* and *P. polymyxa* showed differential effects against *Pythium* damping-off in the present study. Recently, Larsen *et al.* (2009) also found that *P. polymyxa* markedly suppressed root colonization of an arbuscular mycorrhizal fungus, *Glomus intraradices*, irrespective of wheat

bran amendment, whereas *P. macerans* suppressed root colonization of *G. intraradices* only in combination with wheat bran amendment. These results suggest that the influence of organic compounds also depends on the specific biocontrol agent, even in the case of closely related biocontrol agents.

Signature fatty acids have been used to estimate biomass of specific organisms (Larsen *et al.*, 1998, 2000; Larsen and Bødker, 2001). As we anticipated, the amount of Gram-negative and Gram-positive bacterial as well as fungal and total fatty acids was very low in treatments without microbial inoculation. However, inoculation with *P. aphanidermatum* alone caused a significant increase in the amount of Gram-negative and Gram-positive bacterial as well as fungal and total fatty acids. These results suggest that the observed shift in microbial community structure may have been associated with a progressive change from oligotrophic to more copiotrophic conditions that are suitable for saprophytic bacteria and fungi due to nutrients available from dead cucumber plants.

Larsen *et al.* (2009) used the biomarker fatty acid 15:0 anteiso to estimate the biomass of *P. polymyxa* and *P. macerans* in cucumber rhizosphere, but in our case, the

amount of this biomarker fatty acid or other Gram-positive biomarker fatty acids did not differ between treatments, most likely because of a high background value of Gram-positive bacteria (data not shown). In addition, the total numbers of Gram-positive bacteria also depends on the fate of the introduced Gram-positive bacteria when applied with the different organic compounds.

It was recently reported that the population of *L. enzymogenes* gradually decreased from 10^6 to 10^4 CFU/ml in a cucumber hydroculture (Postma *et al.*, 2009). Likewise, in the present study only TSB increased the amount of Gram-positive bacterial fatty acid, whereas glucose reduced the amount of Gram-negative bacterial fatty acids compared to the treatment without organic compound amendment.

Changes in the amount of total microbial fatty acids also revealed that the interaction between *Pythium* and biocontrol agents depends on both organic compounds and *Paenibacillus* species. However, fungal fatty acids (18:2 ω 6,9) were unaffected by *Paenibacillus*, irrespective of the bacterial species in treatments with different organic compounds. Interestingly, Aye *et al.* (2008) found that isolates of *Pythium* spp. showed large variations and components of fatty acids compared to that of true fungi. Indeed, the fatty acids of 16:0, 18:1 ω 9c and 18:2 ω 6c were significantly detected in isolates of *Pythium* spp., and especially 14:0 and 16:1 ω 7c were specific in this oomycete genus (Aye *et al.*, 2008; Timmusk *et al.*, 2009).

Fatty acid biomarkers such as 14:0, 16:1 ω 7c, 20:4 ω 6c and 20:5 ω 3c were recently proposed for quantifying the extent of *Pythium* infection in plant tissues (Aye *et al.*, 2008). However, fatty acids of 14:0, 20:4 ω 6c and 20:5 ω 3c were not detected in our sand samples, which may be due to differences in tested samples. Although an increase in the relative amount of 16:1 ω 7c was found in our sand samples with *Pythium* (data not shown), this fatty acid was reported to occur in measurable amounts in many bacteria. Hence the application of biomarker fatty acids for quantification of *Pythium* may be limited due to lack of specificity.

In general, ELISA and the fatty acid data from this study showed low specificity and sensitivity, which may have resulted from the sampling protocol. Indeed, our ELISA and the fatty acid data reflect the average amount of microbial biomass in sand samples from each microtiter plate. However, since damping-off may be more related with microbial biomass in cucumber rhizosphere, it could be suggested that sand samples from cucumber rhizosphere should be collected for fatty acid analysis and ELISA detection of *Pythium*. In addition, the low specificity and sensitivity may be also partially attributed to the specific plant growth system, in which the sand is very poor in nutrients, thus limiting microbial growth.

Bacteria from the genus *Paenibacillus* are able to inhibit a range of plant pathogenic fungi by producing a variety of antibiotics (Raza *et al.*, 2008; Kim *et al.*, 2009;

Son *et al.*, 2009). However, our results indicate that competition for nutrients seems to play a more important role in the interaction between the biocontrol bacteria and *Pythium*. Obviously, TSB and chitosan improved the nutritional status of the sand substrate for bacterial biocontrol agents. Interestingly, Chun *et al.* (2003) investigated carbon utilizations of *Bacillus* and *Pythium* species using a Biolog (R) microplate assay, which made it possible to determine the differences in the carbon utilizations by pathogens and antagonistic bacteria (Sim *et al.*, 2008). In addition, the antifungal and antioomycete activity of chitosan itself should also be considered. Indeed, chitosan showed strong antioomycete activity on hyphal growth of *P. aphanidermatum* in our *in vitro* experiment (data not shown).

In conclusion, our results clearly show that organic compounds differentially affect the development of *Pythium* damping-off of cucumber and the biocontrol ability of antagonistic bacteria against this *Pythium* disease. However, a close correlation was not observed between microbial parameters and *Pythium* damping-off of cucumber plants, which may be due to the complex interaction between organic compounds and *Pythium* as well as the bacterial biocontrol agents.

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DETECTION AND CHARACTERIZATION OF PHYTOPLASMAS ASSOCIATED WITH DISEASES OF *RUBUS* spp. IN POLAND

M. Cieslinska

Department of Plant Protection, Research Institute of Pomology and Floriculture,
96-100 Skierniewice, Pomologiczna 18, Poland

SUMMARY

Rubus spp. plants with severe symptoms of stunting, short and thin shoots were observed in central Poland in both production fields (raspberry and blackberry) and their natural environments. Nested PCR of DNA extracted from symptomatic plants and healthy raspberry, conducted using phytoplasma universal and group-specific primer pairs, showed the presence of phytoplasmas in all symptom-showing plants selected for this study. RFLP with *Hpa*II, *Rsa*I, *Hha*I and *Bfa*I and sequence analysis of the 16S rDNA fragment amplified with universal primers R16F2n/R16R2 revealed that assayed plants were infected by phytoplasmas belonging to three different groups. This is the first report of the natural occurrence of 'Candidatus Phytoplasma asteris' and X disease phytoplasma in *Rubus* spp. in Poland.

Key words: aster yellows, X disease, elm yellows, PCR/RFLP, sequencing.

INTRODUCTION

Phytoplasma diseases affect wild and cultivated red raspberry (*Rubus idaeus* L.), black raspberry (*Rubus occidentalis* L.), blackberry (*Rubus fruticosus* L., *R. laciniatus* Willd., *R. caesius* L.), loganberry (*Rubus* x *loganobaccus*) and crosses of these species throughout Europe, north-eastern USA and Turkey (Mäurer and Seemüller, 1994; Converse *et al.*, 1982; Davis *et al.*, 2001; Sertkaya *et al.*, 2004). Infected plants may show a variety of symptoms such as stunting, shoot proliferation, small leaves, short internodes, enlarged sepals, phyllody, flower proliferation and fruit malformations (van der Meer, 1987; Mäurer and Seemüller, 1994). These diseases are associated with infection by phytoplasmas belonging to the following groups: elm yellows (Marani *et al.*, 1977; Schneider *et al.*, 1993; Mäurer and Seemüller, 1994; Bertaccini *et al.*, 1995; Lee *et al.*, 1995;

Marcone *et al.*, 1997; Davies, 2000; Vindimian *et al.*, 2004), X disease (Davies, 2000; Davis *et al.*, 2001), aster yellows (Borroto Fernández *et al.*, 2007; Fahmeed *et al.*, 2009; Reeder *et al.*, 2010), and stolbur (Borroto Fernández *et al.*, 2007).

Symptoms of rubus stunt were observed in Polish raspberry fields in the 1970's (Dobrowolska-Wilsz, 1973), whose agent was later identified by Cieslinska (2001). This paper reports the results of a more extensive study on detection and molecular properties of phytoplasmas present in naturally infected *Rubus* spp. in Poland.

MATERIALS AND METHODS

Plant material. Symptomatic raspberries (*Rubus idaeus*) of cvs Canby, Polka and Vetén, loganberry (*Rubus loganobaccus*), and tayberry (*R. loganobaccus* x *R. idaeus*), grown in commercial and experimental fields, as well as the wild blackberry WB8 (*Rubus fruticosus* L.) were investigated for the presence of phytoplasmas. *Rubus* spp. plants showed stunting (Fig. 1A), yellowing, premature reddening and epinasty of the leaves, shortening and proliferation of shoots, phyllody and virescence of flowers and abnormal fruits (Fig. 1B). Loganberry and wild blackberry (WB8) plants showed phyllody and virescence (Fig. 1C).

Nucleic acid extraction, PCR/RFLP analysis of 16S rDNA. To verify phytoplasma association with diseases of *Rubus* spp. plants, molecular identification of the agents was carried out using PCR/RFLP and sequencing analyses. Samples of 0.3 g of fresh leaf midribs collected from cvs Canby, Polka and Vetén, tayberry, loganberry and wild blackberry WB8 plants were ground in liquid nitrogen. Total DNA was extracted from all samples using the commercial DNeasy Plant Mini Kit (Qiagen, Germany) according to the manufacturer's instructions. Phytoplasmas detection was by nested PCR. The phytoplasma-universal primer pair P1/P7 (Deng and Hiruki, 1991; Schneider *et al.*, 1995) was used for amplification of a 1.8 kb product representing the almost the complete 16S ribosomal RNA (rRNA) gene,



Fig. 1. A. Stunting of naturally infected blackberry WB8 (healthy blackberry on the left). B. Malformed fruits of cv. Veten. C. Proliferation, phyllody and virescence of loganberry flowers.

the 16-23S rRNA spacer region, and the 5'-end of the 23S rRNA gene. Products obtained after P1/P7 amplifications were diluted 1:39 with sterile deionized water and used as templates in nested PCRs primed by universal primers R16F2n/R16R2 (Gundersen and Lee, 1996) and primer pairs R16(I)F1/R1, R16(III)F2/R1, R16(V)F1/R1 (Lee *et al.*, 1994) specific for 16SrI, 16SrIII and 16SrV phytoplasma groups, respectively.

PCRs were performed with thermocycler PTC-200 (MJ Research, USA) and the amplification products (10 µl) were separated in 1% agarose gel followed by staining in ethidium bromide and visualization of DNA bands using an UVi-Tec transilluminator (Syngen,

USA). The molecular weight of PCR products was estimated by comparison with a 100 bp DNA ladder (Fermentas, Lithuania). Two symptomless (healthy) raspberry plants and sterile deionized water were included in the PCRs as negative controls. *C. roseus* infected with aster yellows phytoplasma (16SrI-B) from strawberry, and DNA of phytoplasmas: OAY ('*Candidatus* Phytoplasma asteris', subgroup 16SrI-B), CX (X disease phytoplasma, subgroup 16SrIII-A) and RS ('*Candidatus* Phytoplasma ulmi', subgroup 16SrV-E) kindly supplied by Prof. A. Bertaccini (University of Bologna, Italy) were included in this study as positive controls.

Nested PCR products primed by R16F2n/R16R2

were digested singly with restriction endonucleases *Hpa*II, *Rsa*I, *Hha*I and *Bfa*I according to the manufacturer's instructions (Fermentas, Lithuania). Restriction patterns were analyzed by electrophoresis in 8% polyacrylamide gels in 1% TBE buffer. The resulting RFLP patterns, after staining in ethidium bromide and visualization, were compared with profiles of positive controls and the restriction patterns of the reference strains of phytoplasmas (Lee *et al.*, 1998, 2004b).

Nucleotide sequencing of 16S rRNA gene fragments

Specific DNA fragments amplified with primer pair R16F2n/R16R2 from six symptomatic *Rubus* spp. plants, were excised from the gel, eluted using QIAquick gel extraction kit (Qiagen, Germany) and sequenced in both directions in the Oncology Center of Maria Skłodowska-Curie Institute in Warsaw, using ABI Prism 3100 Genetic Analyzer (Perkin Elmer, USA). Nucleotide sequences of partial 16S rDNA (1.2 kb nested PCR products amplified with primer pairs R16F2n/R16R2) were compared with phytoplasmal sequences from GenBank using the BLAST algorithm (<http://ncbi.nlm.nih.gov/BLAST/>). Multiple alignment of these sequences was made using CLUSTALW of the DNASTAR's Lasergene software (DNASTAR Inc., USA). Phylogenetic and molecular evolutionary analyses were carried out using the neighbor-joining method implemented in CLUSTALW of the genetic analysis software Molecular Evolutionary Genetic Analysis (MEGA), version 4.02 (Tamura *et al.*, 2007). The data were replicated 1000 times and the

bootstrap percentage values are given at the nodes of the phylogenetic tree.

RESULTS AND DISCUSSION

PCR/RFLP analysis of 16S rDNA. No PCR products were obtained after amplification 16S rDNA using P1/P7 universal primers. Nested PCR primed by R16F2n/R16R2 yielded products of the expected size (1245 bp) from all six symptomatic *Rubus* spp. plants and from the infected *C. roseus* (positive control). It was possible to amplify products from cvs Vetén, Canby, Polka' and tayberry using R16(V)F1/R1 primers specific for the elm yellows phytoplasma group. Nested PCR with the primer pair R16(I)F1/R1 specific for the aster yellows phytoplasma group gave positive result only with sample isolated from wild blackberry WB8. R16(III)F2/R1 primers specific for the X disease phytoplasma group, amplified products of expected size only from loganberry. No products were amplified from DNAs of healthy plants.

RFLP patterns obtained after digestion of PCR products using the restriction enzymes *Hpa*II, *Rsa*I, *Hha*I and *Bfa*I showed that *Rubus* spp. plants were infected by phytoplasmas belonging to three different groups (Fig. 4). RFLP analysis of 1.2 kb of 16S rDNA fragment from cvs Vetén, Canby, Polka and tayberry, yielded patterns indistinguishable from those of strains RuSR19, RuS400, RuS971 and RUS belonging in subgroup E of

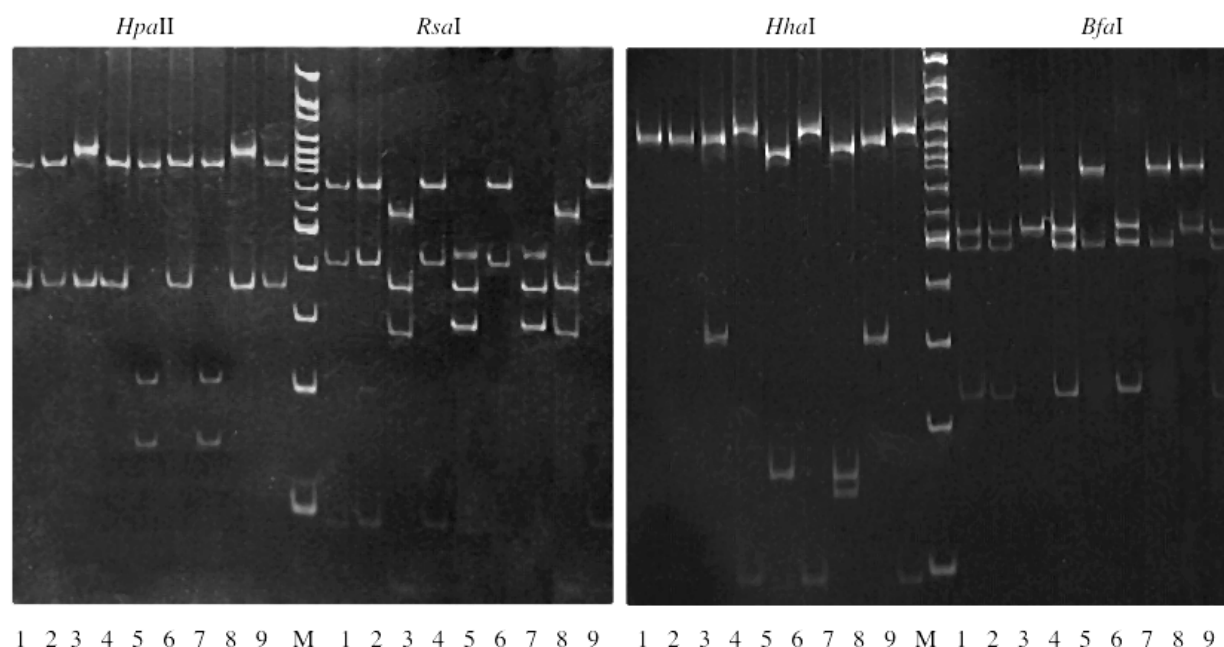


Fig. 2. RFLP profiles of 1.2 kb nested PCR amplified with primers R16F2n/R16R2 from six samples of *Rubus* spp. plants and the phytoplasma control strains. *Hpa*II, *Rsa*I, *Hha*I and *Bfa*I endonucleases were used for restriction analyses. Lanes M, 100 bp DNA Ladder (Fermentas, Lithuania). Lane 1, cv. Vetén; lane 2, cv. Canby; lane 3, loganberry; lane 4, Polka; lane 5, WB8; lane 6, tayberry; lane 7, OAY; lane 8, CX; lane 9, EY.

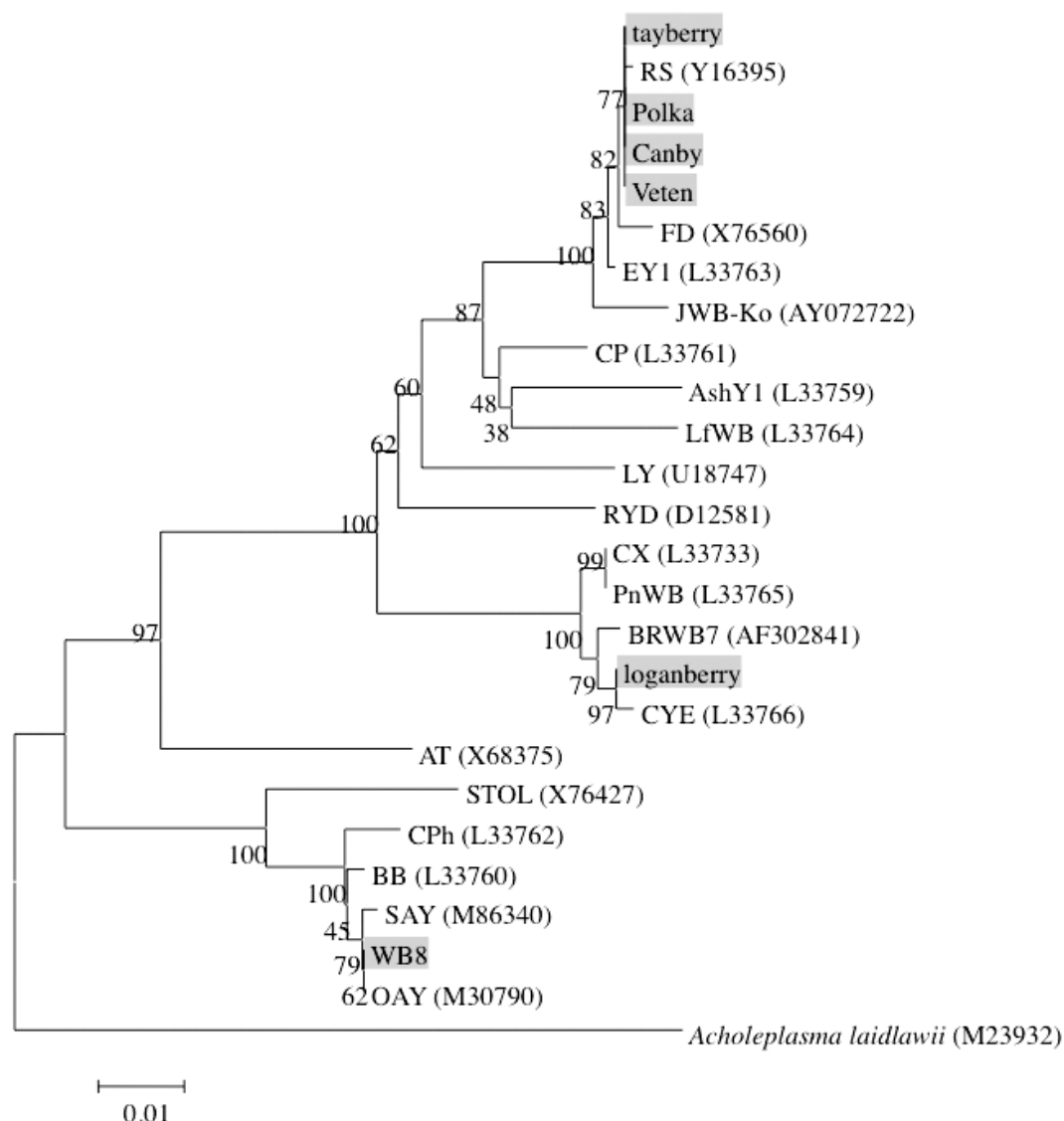


Fig. 3. Phylogenetic tree constructed with partial nucleotide sequences of the 16S rDNA from phytoplasmas infecting *Rubus* plants and phytoplasma reference strains of the aster yellows, X disease and elm yellows groups available in GenBank. *Achleplasma laidlawii* (M23932) is included as an outgroup.

the elm yellows group, designed as the novel taxon '*Candidatus* Phytoplasma ulmi' (Lee *et al.*, 2004b). R16F2n/R16R2 product from loganberry plant digested singly with four endonucleases gave profiles identical to those of CX strain classified in subgroup A of the X-diseases phytoplasma group (16SrIII). Restriction profiles allowed also the preliminary identification of the phytoplasma infecting wild blackberry WB8, as belonging to subgroup B of the aster yellows phytoplasma group denoted '*Candidatus* Phytoplasma asteris' (Lee *et al.*, 2004a).

Nucleotide sequences and phylogenetic analyses. Sequence analysis of the rRNA gene fragment amplified with primers R16F2n/R16R2 confirmed the presence of a phytoplasma of the group 16SrV, '*Ca. P. ulmi*' in cvs Vetten, Canby, Polka and tayberry and showed a 99.7-

99.9% sequence similarity to each other. This phytoplasma was closely related to the rubus stunt isolate (AC: Y16395) and the FD isolate (AC: X76560, probably 16SrV-C). A phytoplasma belonging to group 16SrV (Rubus stunt phytoplasma subgroup) was detected and described in wild and cultivated *Rubus* spp. in Italy (Mäurer and Seemüller, 1994; Bertaccini *et al.*, 1995; Lee *et al.*, 1995; Marcone *et al.*, 1997; Vindimian *et al.*, 2004), the UK (Davies, 2000) and Poland (Cieslinska, 2001). Multiple alignments revealed that strain WB8 shared high 16S rDNA nucleotide sequence similarity (99.9% and 99.5%, respectively) with strains OAY (AC: M30790) and SAY (AC: M86340). Both of these strains belong to subgroup B of 16SrI group (Aster yellows phytoplasma group, '*Candidatus* Phytoplasma asteris'). Occurrence of aster yellows phytoplasma (16SrI-B) has also been reported from wild raspberry and blackberry

grown in Austrian forests (Borroto Fernández *et al.*, 2007), from blackberry in Pakistan (Fahmeed *et al.*, 2009) and in the UK (Reeder *et al.*, 2010). The 1148 bp rDNA fragment of the phytoplasma strain from loganberry shared 99.8% and 99.6% sequence similarity, with that of strains CYE (AC: L33766) and BRWB (AC: AF302841), respectively, both belonging in the 16SrIII group (X disease phytoplasma group). X disease phytoplasma was previously identified in loganberry in the UK (Davies, 2000) and in black raspberry (*Rubus occidentalis*) with Witches' broom symptoms in Oregon (Davis *et al.*, 2001).

Phylogenetic analysis grouped isolates from four samples (Veten, Canby, Polka, tayberry) together with RS strain and close to the other members of the Elm yellows phytoplasma group, '*Ca. P. ulmi*' (Fig. 5). In the phylogenetic tree the WB8 isolate clustered in one group with strains OAY and SAY, the members of subgroup B of aster yellows phytoplasma group, '*Ca. P. asteris*'. The loganberry isolate grouped in the same cluster together with phytoplasma strains CYE and BRWB of the X disease phytoplasma group.

The sequences obtained during the study were deposited in GenBank under accession Nos GU125723 (Canby), GU125724 (Polka), GU125725 (Veten), GU125726 (loganberry) and GU125727 (*Rubus fruticosus* clone WB8).

Observations and testing of *Rubus* spp. plants conducted randomly for several years, indicated that phytoplasma diseases do not seem to be widespread in Polish commercial raspberry and blackberry fields (data not shown). The presence of these agents is mostly connected with their transmission with infected plant material during vegetative propagation. However, wild raspberries and blackberries can become a phytoplasma reservoir as these pathogens were detected in many *Rubus* spp. plants growing in a natural environment. After detecting phytoplasmas from aster yellows and stolbur groups in wild raspberries and blackberries, Borroto Fernández *et al.* (2007) suggested that infected plants growing in a natural environment may play an important role in their spreading.

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DECOMPOSITION OF *MEDICAGO SATIVA* RESIDUES AFFECTS PHYTOTOXICITY, FUNGAL GROWTH AND SOIL-BORNE PATHOGEN DISEASES

G. Bonanomi¹, V. Antignani¹, E. Barile¹, V. Lanzotti² and F. Scala¹

¹ *Dipartimento di Arboricoltura, Botanica e Patologia Vegetale, Università degli Studi di Napoli "Federico II", Via Università 100, 80055 Portici (NA), Italy*

² *Dipartimento di Scienze degli Alimenti, Università degli Studi di Napoli, Via Università 100, 80055 Portici (NA), Italy*

SUMMARY

Disease problems resulting from replanting alfalfa (*Medicago sativa*, MS) have been associated both to autotoxicity of undecomposed plant residues and activity of soil-borne fungal pathogens such as *Pythium* spp. and *Rhizoctonia solani*. However, the interaction between MS residues and soil-borne pathogens has not been previously investigated. In this work we studied the effects of the decomposition process of MS residues, in aerobic and anaerobic conditions on: (i) the growth of MS seedlings; (ii) the growth of 17 fungal species selected among saprophytic, biological control agents, foliar and soil-borne pathogens and (iii) the outcome of the plant-pathogen interactions. MS chemical changes during decomposition were characterized by ¹³C-CPMAS-NMR spectroscopy. Autotoxicity and phytotoxicity of MS extracts rapidly decreased during decomposition, in both laboratory and litterbag in aerobic conditions, but increased sharply in anaerobic conditions. Undecomposed MS extracts positively affected fungal growth, while extracts from MS residues decomposed both in aerobic and anaerobic conditions showed an inhibitory effect. MS seedling damping-off caused by *Pythium ultimum* and *R. solani* increased when soil was amended with MS residues, suggesting that availability of more nutrients and energy sources may be important in increasing disease severity. ¹³C-CPMAS-NMR analysis revealed that dramatic chemical changes occurred during decomposition. O-bearing carbon compounds, mainly associated with sugars, decreased steadily, while aliphatic compounds increased during decomposition. It was concluded that there is a consistent relationship among MS chemical changes, its autotoxicity and the effects on fungal growth.

Key words: autotoxicity, allelopathy, ¹³C-CPMAS-NMR, organic amendment, *Pythium ultimum*, *Rhizoctonia solani*.

INTRODUCTION

Disease problems resulting from replanting are common with many woody (Mazzola, 2004) and vegetable species (Singh *et al.*, 1999). Replanting of the perennial legume forage crop alfalfa (*Medicago sativa*, MS) in monosuccession is often unsuccessful because of the low germination and poor seedling growth due to damping-off and reduced vigour of survived seedlings (Jennings and Nelson, 2002; Chon *et al.*, 2006). MS replant-induced disease has mainly been associated with autotoxicity of plant residues (Miller, 1996), although it has been recognized that the activity of associated soil-borne fungal pathogens such as *Pythium* spp. and *Rhizoctonia solani* can aggravate the incidence of the phenomenon (Handelsman *et al.*, 1990; Jennings and Nelson, 2002).

Autotoxicity is a special case of allelopathy in which plant species release chemical compounds that inhibit seed germination and growth of the same species (Singh *et al.*, 1999). MS autotoxicity has been often reported and ascribed to release of several phytotoxic compounds including chlorogenic, vanillic, ferulic and salicylic acid, medicarpins, saponins, coumarins, trans-cinnamic and caffeic acid following leaf, seed coat, stem and root degradation (Miller, 1992). However, none of these compounds has conclusively been identified as the main autotoxic factor (Chung and Miller, 1995). Commonly, disease problems resulting from replanting are avoided by allowing a "rotation" interval between the end of an existing MS crop and the establishment of a new crop in the same field. However, data from field studies on the required time-lag are not consistent, as it was reported to range from few weeks (Miller, 1992) to several months or years (Webster *et al.*, 1967; Jennings and Nelson, 2002). Furthermore, MS autotoxicity tends to disappear sooner from sandy than clayey soils (Jennings and Nelson, 1998). The inconsistency of these field results make it difficult to predict the success of a MS re-establishment.

Leaching of autotoxic water-soluble compounds (Jennings and Nelson, 1998) and their decomposition driven by microbial soil communities (Xuan *et al.*, 2005) were proposed as the main processes involved in the

elimination of MS autotoxicity. In more general terms, the release has often been reported of allelochemical compounds during organic matter (OM) decomposition (reviewed by Rice, 1984). However, although the allelopathic effects of plant residues have been extensively studied (Putnam, 1994; Miller, 1996), little attention has been given to the dynamics of phytotoxicity during decomposition processes (Bonanomi *et al.*, 2006a). Organic compounds produced by plant residues during decomposition undergo several physical, chemical, and biological processes in the soil, such as adsorption and polymerisation by soil organic matter and clay minerals and transformation by microorganisms (Blum *et al.*, 1999). Changes over time of both composition and quantity of allelochemicals can either increase or decrease the effects of decomposing plant materials (An *et al.*, 2001).

Phytotoxicity dynamics has been documented for several crop species in aerobic conditions (Rice, 1984; Putnam, 1994). In this case, severe inhibition observed in early stages of decomposition are followed by phytotoxicity decrease (Xuan *et al.*, 2005). In contrast, anaerobic conditions have been reported to produce stronger and more durable phytotoxic levels (Patrick, 1971; Bonanomi *et al.*, 2006a).

In addition to the direct effect on plants, decomposing crop residues affect the activity of soil-borne pathogens (Blok and Bollen, 1993; Bonanomi *et al.*, 2007) and biocontrol agents (Hoitink and Boehm, 1999). However, despite the large body of available literature (Mazzola, 2004; Bonanomi *et al.*, 2010), the mechanisms underlying this phenomenon are not yet completely elucidated. It is noteworthy that many studies have reported contrasting results, with OM having either positive effect on soil-borne pathogens, by providing the substrate for their saprophytic growth (Croteau and Zibilske, 1998; Bonanomi *et al.*, 2006b), or negative effects, by causing fungistasis or producing fungitoxic compounds (Lockwood, 1977). Specifically, soil amendment with MS residues has been reported to both increase (Snyder *et al.*, 1959; Papavizas, 1968; Gilpatrick, 1969) or decrease (Zentmyer, 1963; Gilpatrick, 1969; Adams, 1971) the incidence of soil-borne pathogens. In the latter cases, the observed effect has partially been related with the presence of fungitoxic compounds, especially saponins, present in undecomposed MS residues (Oleszek *et al.*, 1992; Avato *et al.*, 2006).

This work investigated the influence of the decomposition process of MS residues on plant and microbe development. Specifically, the effects of decomposition on the growth of MS plants and associated soil-borne fungi, and disease incidence of residues were studied under contrasting conditions (aerobic vs. anaerobic). MS residues were characterized by ^{13}C -CPMAS nuclear magnetic resonance spectroscopic analysis which allows a detailed characterization of the chemical changes that

take place during the decomposition process (Kögel-Knabner, 2002).

Based on previous work on other plant-pathogen systems (Bonanomi *et al.*, 2006b), the hypotheses were: (i) undecomposed MS residues are autotoxic, but during decomposition their toxicity can either decrease or increase, depending on the presence and on the duration of aerobic and anaerobic conditions, respectively; (ii) fresh MS residues support the saprophytic growth of several soil-borne fungi, including pathogens, but during decomposition their effects become progressively negative; (iii) soil amendment with fresh MS residues increases the incidence of MS seedling damping-off.

MATERIALS AND METHODS

Plant material collection. Green leaves were collected in Campania (southern Italy) from field-grown MS plants of cv. Classe during blooming in spring 2006. Immediately after collection materials were slowly dried ($+30^{\circ}\text{C}$ for 10 days), chopped by scissors (size <1 cm), and stored at room temperature. Only leaves were used because a previous study had shown that this material has the highest autotoxicity (Chung and Miller, 1995) compared to other MS parts (e.g. roots, flowers, crowns and stems).

Laboratory and litterbag decomposition experiments. Decomposition experiments were carried out in two different ways: under laboratory conditions and in litterbags. In the first case, dry MS leaves were wetted by distilled water (5% dry weight, i.e. 50 g l^{-1}). Decomposition processes were carried out in 2 litre beakers containing 1 litre of water at $25\pm 2^{\circ}\text{C}$ for 30 days. The decomposition process was replicated using three different 2 litre beakers. Saturated aerobic conditions were obtained by pumping air in the suspension, whereas anaerobic conditions were generated by keeping the beakers sealed. Distilled and autoclaved water was added to the beakers to compensate for evaporation losses. Anaerobic decomposition was carried out to simulate waterlogged soil, a common condition during winter and early spring in many areas of MS cultivation. A microbial inoculum consisting of a mixture obtained from 1 g soil collected in the field (top 20 cm) and 9 g of water, was added to facilitate onset of the decomposition process. This method of decomposition was used because it is rapid, easily reproducible and allows standardization that avoids the effect of soil type (Bonanomi *et al.*, 2006a). Furthermore, the experimental conditions of decomposition in water proved to be comparable to those occurring in the field with the litterbag methods (Bonanomi *et al.*, 2006a), because the soil microbial community always operates in thin water films either surrounding solid particles or inside aggregates

Table 1. Synthetic results of two three-way ANOVA of root growth inhibition of *Lepidium sativum* and *Medicago sativa* in the laboratory experiments.

Effect	<i>Lepidium sativum</i>			<i>Medicago sativa</i>		
	df	F	P-value	df	F	P-value
Condition of decomposition	1	698.9	<0.001	1	450.2	<0.001
Time of decomposition	4	9.2	<0.01	4	6.1	<0.01
Extract concentration	2	391.6	<0.001	2	76.6	<0.001
Condition x time	4	172.5	<0.001	4	70.4	<0.001
Condition x concentration	2	24.7	<0.001	2	9.9	<0.01
Time x concentration	8	14.4	<0.001	8	8.6	<0.01
Condition x time x concentration	8	19.7	<0.01	8	1.22	0.29

Condition of decomposition (aerobic and anaerobic), time of decomposition (0, 5, 10, 20, 30 days) and extract concentration (50, 16.6 and 5 g l⁻¹) are the main factors of the two analyses. P-values < 0.05 in bold type.

(Stotzky, 1997). Samples of the aqueous suspensions were collected at different stages of the decomposition process. Sampling was done after 5 h from the start of the experiment and after 5, 10, 20 and 30 days. Samples were centrifuged (2,395 g for 10 min), sterilised (with PES 0.22 µm pore filters), diluted by distilled water at three levels (50, 16.6 and 5 g l⁻¹) and stored at -20 °C.

Phytotoxicity dynamics of MS leaves was also studied using the litterbag method (Berg and McClaugherty, 2003) to simulate field decomposition conditions. Plastic litterbags (mesh size 1 mm) were filled with 5 g of dry MS intact leaves. Litterbags were placed in trays (30x20x5 cm), kept in a growth chamber under controlled temperature (25±2°C), moisture and aerobic (litterbags were daily watered to water holding capacity) conditions. We used the same microbial inoculum of the laboratory decomposition experiment, but in this case it was sprayed over the litterbags. Litterbags (n=8) were sampled after 10, 30, 60 and 90 days of decomposition for a total of 32 samples. The bags were dried in the laboratory (40°C for 5 days), weighted and the material was then mixed with distilled water in beakers at 5% of dry weight (50 g l⁻¹) and shaken for 5 h. Suspensions were centrifuged (2,395 g for 10 min), filter-sterilised (with PES 0.22 µm pore), diluted by distilled water at three levels (50, 16.6 and 5 g l⁻¹) and stored at -20 °C until bioassay.

Autotoxicity and phytotoxicity of MS extracts. Phytotoxicity was assessed using a root elongation test on *Lepidium sativum* L., a species known as particularly sensitive to phytotoxic compounds (Bonanomi *et al.*, 2006a). The same test was carried out on MS to assay extract autotoxicity. Root elongation was measured because of its higher sensitivity to aqueous extracts compared to seed germination (Chung and Miller, 1995). Experiments were done in a growth chamber at constant temperature (25°C) and in the dark. Ten seeds

were placed in a 9 cm Petri dish over a sterile filter paper with 4 ml of test solution. Three different concentrations of aqueous extracts (50, 16.6 and 5 g l⁻¹) were used for each of five decomposition times (0, 5, 10, 20, and 30 days). For each solution ten replications were made, plus the control with distilled water. Bioassays were carried out with MS aqueous suspensions from both laboratory and litterbag decomposition experiments.

Petri dishes were placed in a growth room according to a totally randomised design and the length of seedling roots was measured after 36 h from germination of *L. sativum* and after 5 days of MS. Data were always expressed as percent of inhibition of root growth compared to the control. Three-way ANOVA was performed to test the main effects and interactions of the conditions of decomposition (aerobic vs. anaerobic), decomposition time and extract concentration level on the inhibition of *L. sativum*. The same test was performed for MS root elongation. Two separate three-way ANOVA were done for *L. sativum* and MS because root measurements were recorded at different times (36 h versus 5 days). Data of the litterbag decomposition experiment were analyzed by two-way ANOVA to test the main effects and interaction of decomposition time and extract concentration level on the inhibition of *L. sativum*. The same test was performed for MS root elongation.

Two independent root elongation tests were done to test the effects of different pH (4.4, 5.5, 6.5, 7.5, 8.5 and 9.5) and electrolytic conductivity (0.1, 0.7, 1.6, 4.3, 6.2, 7.9 and 12 mS/cm) values on MS growth. The effects of pH and EC on the growth of *L. sativum* are reported in Bonanomi *et al.* (2006). Different pHs were obtained using a MES buffer in distilled water (Sigma-Aldrich, Germany) adjusted with 1 M NaOH. The EC values were obtained using 10, 20, 50, 80, 100 and 150 mmol l⁻¹ of NaCl solution. One-way ANOVA was applied two

times to test the effects of pH and EC on MS root elongation. Each experiment was repeated twice.

Effects of MS extracts on fungal growth. In these experiments the effect of MS extracts from the laboratory decomposition experiment was tested on the saprophytic growth of *Pythium ultimum* (PU) and *Rhizoctonia solani* (RS) as potential agents of MS damping-off. Furthermore, the effect of MS extract was tested on 15 additional fungal species selected among saprophytic, foliar and soil-borne pathogens and biological control agents (Table 2). Media were prepared by mixing water agar (WA) and sterile MS residue extracts to obtain two dilution levels (50 and 5 g l⁻¹). Since the aim of this experiment was to test the ability of fungi to use MS residues as a single source of nutrient, fungal growth started from a nutrient-poor medium (WA). Six extracts obtained at three decomposition times (5 h, 10 and 30 days) and two conditions of decomposition (aerobic and anaerobic) were used in these experiments. As control, we used WA medium for all fungi. Five replications were used for each treatment. Ten millilitres of each dilution were placed in a 9 cm Petri dish. Fungal inoculum was prepared from colonies growing on WA at

24°C. After seven days of culture, a 4 mm diameter plug was collected from the edge of the growing colony and placed in the centre of the Petri dish. After 72 h, hyphal density and radial growth of each colony were measured on five randomly chosen points. Hyphal density was measured by counting the number of hyphae crossing a 1 mm line at 250X amplification under microscope. The growth index was calculated as the product of the area of fungal colony, calculated from the measured colony radius, and the hyphal density (Tuitert *et al.*, 1998). A second experiment was done according to the same procedures to test the effects of different pH values (4.5, 5.0, 6.0, 7.0, 8.0 and 9.0) in Czapek medium 0.1 strength and V8 for *Phytophthora* species. The different pHs were obtained using NaOH (5 M) and HCl (0.1 M). For each fungal species, a three-way ANOVA was performed to test the main effects and interactions of conditions of decomposition (aerobic vs. anaerobic), decomposition time (0, 10 and 30 days) and extract concentration levels on the mycelial growth expressed as growth index. One-way ANOVA was applied to test the effects of pH on growth of each fungal species. Each experiment was repeated twice.

Table 2. Synthetic results of 19 three-way ANOVA of growth response of 17 fungal species on *Medicago sativa* extracts obtained from residues decomposed in aerobic or anaerobic conditions.

Species	Condition		Time		Concentration		Condition x time		Condition x concentration		Time x concentration		Condition x time x concentration	
	F	P	F	P	F	P	F	P	F	P	F	P	F	P
FA	9.2	<0.01	89	<0.01	4.6	0.03	5.1	0.02	2.4	0.12	25	<0.01	1.4	0.25
FOL	4.6	0.03	6.6	<0.01	4.2	0.04	6.1	<0.01	16	<0.01	4.0	0.04	5.6	<0.01
FORL	9.9	<0.01	4.3	0.03	22	<0.01	44	<0.01	48	<0.01	25	<0.01	1.8	0.17
FS	4.1	0.04	26	<0.01	43	<0.01	1.6	0.22	24	<0.01	0.9	0.42	6.8	<0.01
PC	4.4	0.03	193	<0.01	130	<0.01	4.8	0.03	4.3	0.03	153	<0.01	0.2	0.81
PCAP	6.0	<0.01	22	<0.01	29	<0.01	4.1	0.04	4.2	0.04	5.6	<0.01	5.6	0.01
PCIN	4.7	0.03	43	<0.01	4.1	0.04	5.2	0.02	5.7	0.01	7.7	<0.01	1.2	0.33
PN	4.0	0.04	209	<0.01	9.0	<0.01	5.3	0.02	5.2	0.02	33	<0.01	1.6	0.21
PU	9.4	<0.01	232	<0.01	185	<0.01	4.0	0.04	24	<0.01	179	<0.01	0.3	0.73
RS	9.1	<0.01	47	<0.01	8.1	<0.01	0.15	0.85	2.2	0.14	1.2	0.28	0.1	0.85
SR	27	<0.01	31	<0.01	162	<0.01	6.8	<0.01	27	<0.01	6.7	<0.01	6.8	<0.01
TB	4.3	0.03	16	<0.01	18	<0.01	4.0	0.04	15	<0.01	16	<0.01	3.9	0.04
AA	6.1	<0.01	98	<0.01	4.6	0.03	4.5	0.03	4.9	0.02	17	<0.01	1.2	0.29
BC	6.5	<0.01	77	<0.01	5.2	0.02	1.5	0.21	6.5	<0.01	52	<0.01	1.5	0.22
CG	11	<0.01	232	<0.01	38	<0.01	2.0	0.13	2.2	0.14	90	<0.01	7.4	<0.01
AN	12	<0.01	170	<0.01	14	<0.01	3.9	0.04	12	<0.01	32	<0.01	3.8	0.04
M sp.	4.2	0.04	15	<0.01	8.6	<0.01	1.2	0.31	4.0	0.04	34	<0.01	1.1	0.38
TH (P1)	11	<0.01	61	<0.01	4.3	0.03	0.1	0.91	45	<0.01	111	<0.01	9.6	<0.01
TH (T22)	14	<0.01	168	<0.01	76	<0.01	5.2	0.02	14	<0.01	22	<0.01	5.3	0.01

Condition of decomposition (aerobic and anaerobic), time of decomposition (0, 10, 30 days) and extract concentration (50 and 5 g l⁻¹) are the main factors of the 19 analyses. *P*-values < 0.05 in bold type.

Species names: FA = *Fusarium avenacearum*; FOL = *Fusarium oxysporum* f.sp. *lycopersici*; FORL = *Fusarium oxysporum* f.sp. *radicis lycopersici*; FS = *Fusarium solani*; PC = *Phytophthora cactorum*; PCAP = *Phytophthora capsici*; PCIN = *Phytophthora cinnamomi*; PN = *Phytophthora nicotianae*; PU = *Pythium ultimum*; RS = *Rhizoctonia solani*; SR = *Sclerotium rolfsii*; TB = *Thielaviopsis basicola*; AA = *Alternaria alternata*; BC = *Botrytis cinerea*; CG = *Colletotrichum gloeosporioides*; AN = *Aspergillus niger*; M = *Mucor* sp.; TH strain P1 = *Trichoderma barzianum*; TH strain T22 = *Trichoderma barzianum*. Microbial strains were supplied by the Department of Arboricoltura, Botanica e Patologia Vegetale, University of Naples Federico II.

Effects of MS extracts on *P. ultimum* and *R. solani* damping-off. The incidence of PU and RS damping-off on MS seeds was evaluated in a system consisting of quartz sand amended with MS extracts at different decomposition stages and concentrations. In this experiment, fine (0.45-1 mm diameter) sand was washed with tap water, dried at 80°C for 24 h in the oven and sterilized twice by autoclaving (120°C for 22 min). Each 9 cm Petri dish was filled with 50 g of sand and was subsequently wetted with 15 ml of sterile MS extract at three concentrations (0, 5 and 50 g l⁻¹), equivalent to 0,0.1% and 1% MS residues in dry weight (w/w), respectively. Twenty surface-sterilized MS seeds (disinfection was done by shaking seeds in a 1% hypochlorite solution for 1 min followed by a thorough rinsing with sterile water) were placed in the dish on the amended quartz sand. Each treatment was replicated five times for a total of 105 plates. Inoculum was prepared by placing at the bottom of sand in the centre of the Petri

dish a 4 mm plug collected from the edge of a PU and RS growing colony on WA. Plates were incubated at 24°C and disease incidence and severity were monitored after 24, 48 and 72 h after inoculation by checking fungal colonization of seeds by microscopic observation (250X magnifications). After 72 h, seedlings were harvested and root length measured. For each fungus, three-way ANOVA was performed to test the main effects and interactions of conditions of decomposition (aerobic vs. anaerobic), decomposition time (0 and 30 days) and extract concentration level on the seedling root length and the percentage of MS seeds colonized by PU or RS. Each experiment was repeated twice.

Chemical analyses and ¹³C-CPMAS NMR characterization of MS extracts. Redox potential (E_h), pH and electrolytic conductivity (EC) of aqueous extracts were measured to assess the conditions of the decomposition processes.

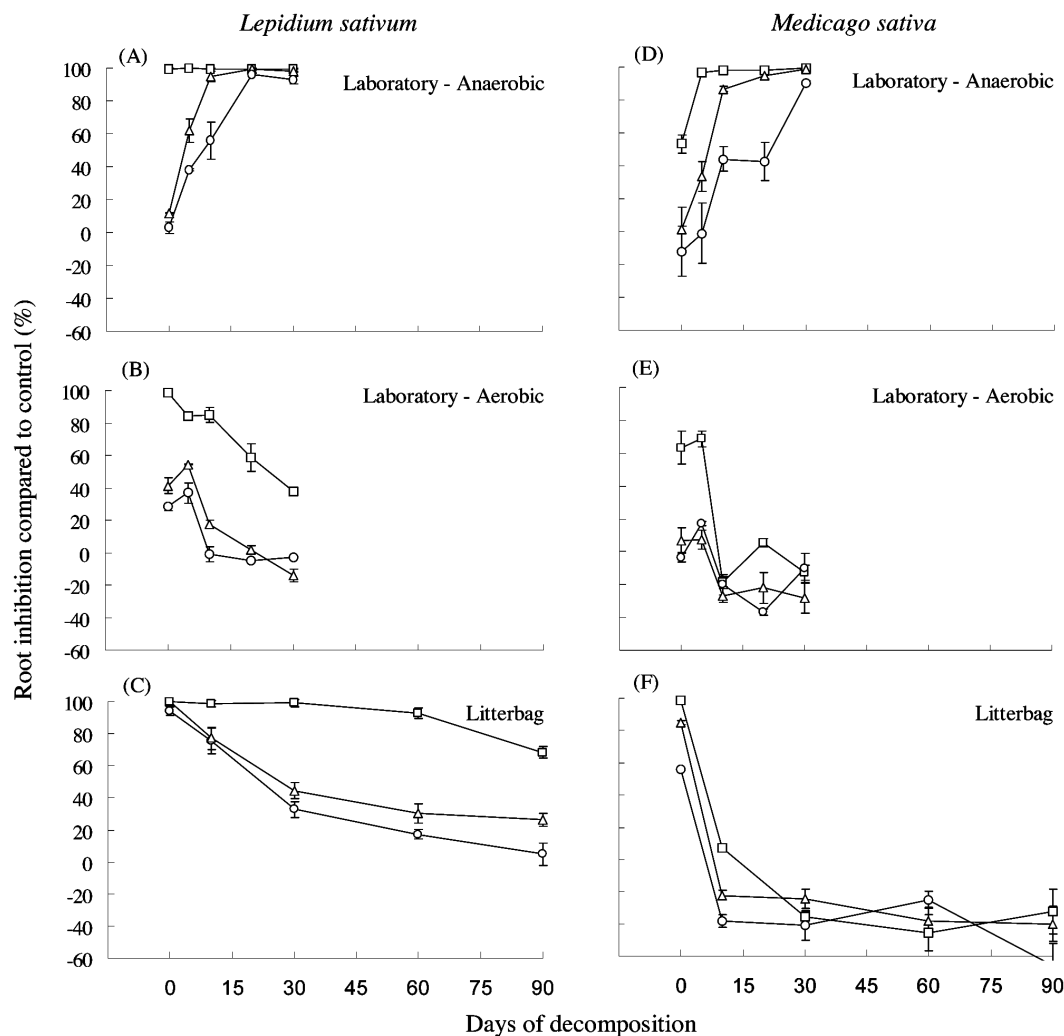


Fig. 1. Inhibition of root length compared to control (=0%) of *Lepidium sativum* (left) and *Medicago sativa* (right) by aqueous extracts at three concentrations (50, 16.6 and 5 g l⁻¹, squares, triangles and circles, respectively) obtained during anaerobic (A,D) and aerobic (B,E) decomposition in laboratory conditions and in litterbags (C,F) of *Medicago sativa* residues. Data are averages of ten replicates, bars indicate ±1SE.

MS materials from the litterbag experiment were characterized by ^{13}C -CPMAS NMR obtained in solid state under the same conditions for quantitative comparison among spectra. Samples were analyzed at five different times: undecomposed leaves and decomposed for 10, 30, 60 and 90 days. The spectrometer used was a Bruker AV-300 equipped with a 4 mm wide-bore MAS probe. NMR spectra were obtained by using 13000 Hz of rotor spin, 1 s of recycle time, 1 ms of contact time, 20 ms of acquisition time, 2000 scans. Samples were packed in 4 mm zirconium rotors with Kel-F caps. The pulse sequence was applied with a ^1H ramp to account for non-homogeneity of the Hartmann-Hahn condition at high spin rotor rates.

Each ^{13}C -CPMAS NMR spectrum was automatically integrated to calculate the area of the peaks which appeared in the chosen region. Signals of the spectra were assigned using information from previous studies on soil organic matter and plant litter (Spaccini *et al.*, 2000; Kögel-Knabner, 2002). In detail, the following resonance intervals, associated with different C types, were considered: 0-45 p.p.m. = aliphatic-alkyl C; 46-110 p.p.m. = C-N and C-O in polypeptide and carbohydrate compounds (O-alkyl C); 111-140 p.p.m. = aromatic C; 141-160 p.p.m. aromatic, phenolic related C; 161-190 p.p.m. carboxylic C. The degree of hydrophobicity (HB/HI) of MS residues was calculated according to Spaccini *et al.* (2000).

Instead of analysing single chemical compounds, this method allows a broad characterization of the decaying OM by assessing the changes of different classes of compounds: phenols and organic acids that are important for their allelopathic effects (Rice, 1984), and carbohydrates that are a source of energy for microbial growth (Boehm *et al.*, 1997).

RESULTS

Autotoxicity and phytotoxicity of MS extracts. Inhibition of root growth of *L. sativum* and MS was significantly affected by the decomposition conditions, decomposition time and extract concentration in the experiments carried out under laboratory conditions (Table 1). Statistical interactions, with the exception of the three factor interaction for MS, were all significant (ANOVA, $P < 0.01$ in all cases). The effect of decomposition time, extract concentration and their interaction were also significant for the decomposition carried out in litterbag for both *L. sativum* and MS (Two-way ANOVA, $P < 0.01$ in all cases).

In both species, the increase of the MS extract concentration caused a proportional increase of root growth inhibition (Fig. 1A-F). Aerobic and anaerobic decomposition of MS residues produced divergent dynamics in phytotoxicity. At the initial stage of decompo-

sition, phytotoxicity was high for both *L. sativum* and MS. During decomposition carried out in anaerobic conditions, toxicity progressively increased for both *L. sativum* and MS (Fig. 1A, D). Differently, extracts obtained under laboratory aerobic conditions reduced their phytotoxicity during the decomposition (Fig. 1B, E). MS autotoxicity completely disappeared after 10 days of decomposition under the same conditions (Fig. 1E). The phytotoxic trends during aerobic decomposition in litterbag and laboratory conditions were similar (Fig. 1B, C, E, F), but while phytotoxicity (toward *L. sativum*) declined more rapidly in laboratory conditions, autotoxicity toward MS disappeared after 10 days in both decaying conditions. MS extracts obtained after more than 10 days of aerobic decomposition significantly stimulated MS root growth (Fig. 1E, F).

MS root length was not affected by pH values within the tested range (ANOVA, $F = 1.08$, $P = 0.41$), while it was significantly inhibited by EC with values above 6.2 mS/cm (ANOVA, $F = 44.02$, $P < 0.001$).

Effects of MS extracts on fungal growth. Mycelial growth of all tested fungi were significantly affected by decomposition conditions, decomposition time, and extract concentrations of MS (Table 2). In addition, several statistical interactions resulted highly significant (Table 2). Mycelial growth showed a similar behaviour for the majority of the tested fungi in relation to the main variables studied. The highest fungal growth was recorded in the presence of the undecomposed extract, with the exception of FOL, FORL, FS and TB (Table 3). Mycelial growth decreased on extracts from both aerobic and anaerobic decomposition, with a more evident reduction in the second case (Table 3). Ten species grew very slowly on the concentrated extracts obtained after 30 days of decomposition in aerobiosis, while no species developed on the 30 day anaerobic extracts (Table 3). The fungal response to the extract concentrations was strictly dependent on the timing of decomposition. On undecomposed extracts, 11 fungi grew more at the highest concentration, and 1 (SR) at the lowest concentration. After 30 days of aerobic and anaerobic decomposition, 9 and 13 fungi, respectively, grew significantly more at the lowest extract concentration (Table 3). The highest mycelial growth was recorded for PU and TH strain P1 on the undecomposed material. On extracts obtained during decomposition, the growth of these two species was dramatically reduced, while that of *Fusarium* species (FOL, FORL and FS) decreased but was the less affected (Table 3). Fungal growth was not affected by pH values within the tested range with the exception of BC, PU, SR, RS and TH strain T22.

Effects of MS extracts on *P. ultimum* and *R. solani* damping-off. MS seedling root length and percentage of infected seedlings by both PU and RS were significantly

Table 3. Growth response of 17 fungal species after 72 hours at two concentrations (high = H; low = L; 50 and 5 g l⁻¹, respectively) of *M. sativa* extracts obtained from residues undecomposed or decomposed for 10 and 30 days in aerobic or anaerobic conditions. Control is water agar (WA). Growth index values are average \pm standard error of 5 replicates.

Fungal group	Species	Concentration	Undecomposed	Aerobic		Anaerobic		Control
			0	10	30	10	30	WA
Soilborne pathogens	FA	H	4.1±0.6	0.9±0.3	1.4±0.7	0.0±0.0	0.0±0.0	0.0±0.0
		L	2.3±0.8	1.4±0.7	1.1±0.9	1.8±0.4	0.0±0.0	
	FOL	H	2.5±1.0	2.1±0.6	3.1±1.2	0.0±0.0	0.0±0.0	0.8±0.8
		L	2.1±1.5	1.4±0.3	2.4±1.2	4.0±1.1	1.5±0.4	
	FORL	H	11.9±5.0	5.1±0.7	1.0±0.1	0.0±0.0	0.0±0.0	0.0±0.0
		L	6.1±0.4	5.2±2.0	2.6±1.0	7.9±2.4	12.9±3.7	
	FS	H	5.6±1.6	4.2±1.0	2.9±0.7	3.6±1.3	0.0±0.0	0.0±0.0
		L	7.5±1.3	4.6±1.5	2.3±0.1	8.6±2.8	6.7±0.5	
	PC	H	4.0±0.8	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.1±0.01
		L	0.3±0.1	0.3±0.1	0.1±0.04	0.0±0.0	0.0±0.0	
	PCAP	H	1.8±0.6	2.3±1.7	1.4±0.3	0.0±0.0	0.0±0.0	0.6±0.1
		L	4.4±1.4	2.0±1.1	1.9±0.9	3.2±1.0	0.3±0.4	
	PCIN	H	1.8±0.5	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.07±0.1
		L	1.8±1.2	0.1±0.01	0.2±0.1	1.6±0.2	1.2±0.6	
	PN	H	8.3±0.9	0.0±0.0	0.2±0.2	0.0±0.0	0.0±0.0	0.8±0.6
		L	3.9±1.1	1.5±0.8	0.5±0.3	0.6±0.3	0.7±0.3	
	PU	H	62.5±14	10.7±2.5	5.0±2.3	0.0±0.0	0.0±0.0	0.06±0.02
		L	6.8±2.6	3.2±0.3	2.3±1.7	2.0±0.4	8.5±3.5	
	RS	H	4.4±0.4	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.6±0.3
		L	6.4±4.5	1.3±0.53	1.7±1.1	1.2±0.3	0.0±0.0	
	SR	H	1.4±0.7	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
		L	6.8±1.6	6.1±1.4	5.1±2.4	1.6±0.2	0.0±0.0	
	TB	H	2.3±0.7	0.4±0.3	1.2±0.2	0.0±0.0	0.0±0.0	0.8±0.1
		L	1.7±0.8	1.3±0.4	1.0±0.6	2.5±0.6	2.0±0.6	
Airborne pathogens	AA	H	4.3±1.2	0.1±0.1	0.0±0.0	0.0±0.0	0.0±0.0	0.4±0.1
		L	2.5±1.1	1.4±0.6	1.5±0.3	0.0±0.0	0.0±0.0	
	BC	H	14.8±5.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.8±0.4
		L	4.8±2.6	1.0±0.4	1.1±0.5	5.4±0.9	6.0±2.0	
	CG	H	23.6±5.0	6.4±0.8	1.4±0.4	0.0±0.0	0.0±0.0	2.4±0.4
		L	8.7±1.8	4.9±1.7	4.6±1.6	5.9±0.5	0.3±0.1	
Saprophytes and antagonists	AN	H	2.4±0.7	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.7±0.18
		L	1.8±0.5	0.6±0.3	0.1±0.1	1.8±0.4	0.6±0.3	
	Msp.	H	9.6±5.5	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
		L	4.7±1.2	5.0±0.6	2.7±0.7	0.0±0.0	0.0±0.0	
	TH (P1)	H	70.9±9.3	8.7±1.6	20.4±1.7	0.0±0.0	0.0±0.0	5.6±2.7
		L	16.8±2.1	20.7±8.2	10.2±5.8	48.0±1.3	45.2±1.5	
	TH (T22)	H	11.1±2.4	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	1.9±0.7
		L	11.2±2.1	3.8±1.1	2.6±0.6	10.6±1.1	5.5±1.9	

Species names: FA = *Fusarium avenaceum*; FOL = *Fusarium oxysporum* f.sp. *lycopersici*; FORL = *Fusarium oxysporum* f.sp. *radicis lycopersici*; FS = *Fusarium solani*; PC = *Phytophthora cactorum*; PCAP = *Phytophthora capsici*; PCIN = *Phytophthora cinnamomi*; PN = *Phytophthora nicotianae*; PU = *Pythium ultimum*; RS = *Rhizoctonia solani*; SR = *Sclerotium rolfsii*; TB = *Thielaviopsis basicola*; AA = *Alternaria alternata*; BC = *Botrytis cinerea*; CG = *Colletotrichum gloeosporioides*; AN = *Aspergillus niger*; M = *Mucor* sp.; TH strain P1 = *Trichoderma barzianum*; TH strain T22 = *Trichoderma barzianum*. Microbial strains were supplied by the Department of Arboricoltura, Botanica e Patologia Vegetale, University of Naples Federico II.

affected by the decomposition conditions, decomposition time and extract concentration (ANOVA, $P < 0.05$). MS seedling root growth was reduced both by amendment with MS residues and pathogen inoculum (Table 4). Percentage of infected seeds by PU and RS significantly increased, compared to the control, where sand was amended with all types of MS extracts at the highest concentration (Table 4). On undecomposed extracts all MS seeds were infected by PU after 24 hours from inoculum. RS infection was higher, compared to control, with undecomposed extracts at both concentra-

tions and decreased with both aerobic and anaerobic decomposed materials.

Chemical analyses of MS extracts and ¹³C-CPMAS NMR characterization of MS litter. The pH of extracts increased during decomposition in aerobic conditions and decreased in anaerobiosis (Fig. 2A). In both conditions, the EC values of the extracts that ranged between 3 and 5.5 mS/cm, slightly increased in the early phase of the decomposition and decreased after 30 days (Fig. 2B). Redox potential values of aqueous extracts were al-

ways higher in aerobic than in anaerobic conditions (Fig. 2C). However, these values decreased at 5 days of decomposition in both conditions, but, as decomposition proceeded, increased in aerobiosis while remained fairly stable and very negative in anaerobiosis.

The ^{13}C -CPMAS NMR spectra revealed changes of the main fractions of chemical compounds during decomposition (Fig. 3), with the largest differences occurring in the first 10 days of decomposition (Table 5). The aliphatic alkyl-C region (0-45 ppm characteristic of lipid waxes and cutins) sharply increased in the first 10 days of decomposition, but in the later stages of decomposition (30-90 days) progressively decreased. The O-alkyl-C region (46-110 ppm), that is associated mainly with poly-

saccharides, decreased in the first 10 days of decomposition and then remained almost unchanged (Fig. 3; Table 5). The aromatic fractions (111-140 and 141-160 ppm) progressively increased during decomposition with the largest increase for phenolic C (Table 5). The carboxylic

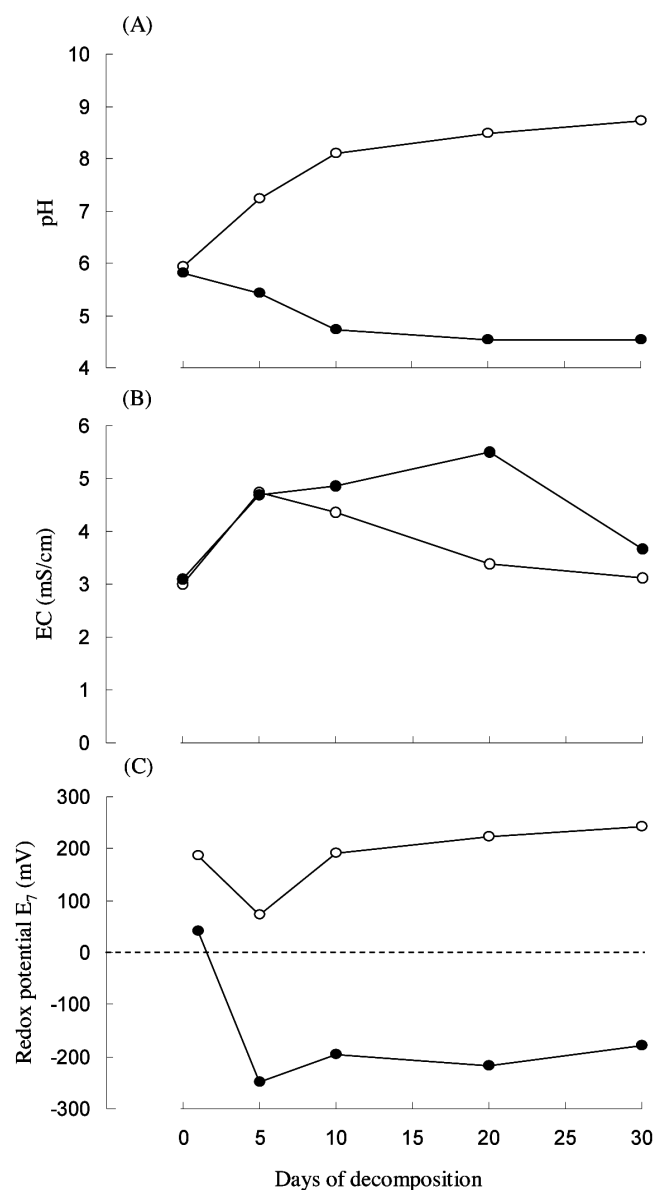


Fig. 2. Variation of pH (A), EC (B) and redox potential (C) of aqueous extracts at the highest concentration (50 g l^{-1}), during aerobic (open circles) and anaerobic (closed circles) decomposition of *Medicago sativa* residues.

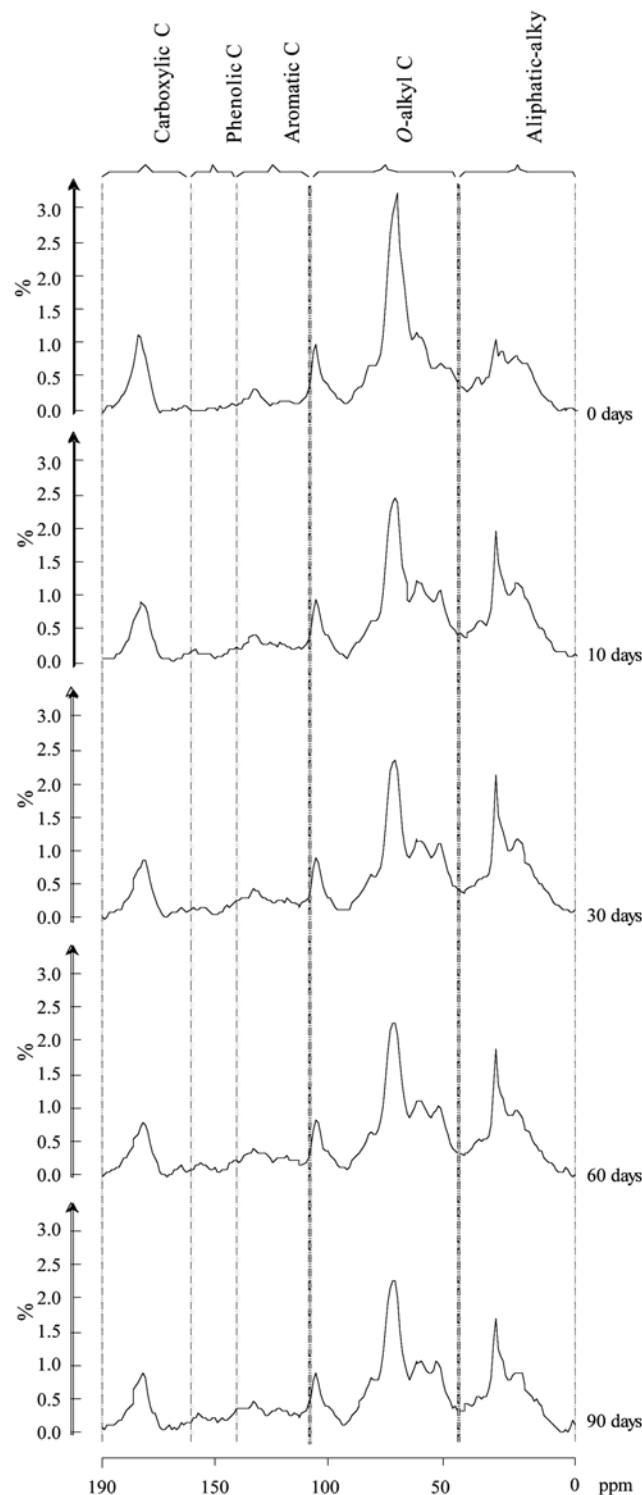


Fig. 3. ^{13}C -CPMAS NMR spectra of undecomposed *Medicago sativa* leaves and at four level of decomposition in litterbags under controlled environmental conditions. Vertical lines delimitate the different spectral regions.

fraction showed a decrease in the first ten days and a limited increase in the later stages of decomposition (Table 5). The degree of hydrophobicity (HB/HI) and the ratio between alkyl-C and O-alkyl-C showed a strong increase in the early phases of decomposition followed by a limited decrease in the later stages (30-90 days). Finally, the aromaticity index and the ratio between aromatic-C and O-alkyl-C rapidly increased in the first 10 days and then showed a further progressive increase (Table 5). MS leaves showed a rapid decomposition with a total weight loss of 67.6% in 90 days. However, the decay rate was rapid in the early phases of decomposition, then progressively decreased and almost stopped in the later stage (60-90 days) (Table 5).

Comparative effects of MS extracts on plants and fungi. A contrasting response of fungi (average of 17

species) and plants (average of two species) to decaying MS residues was observed (Fig. 4). Undecomposed material was strongly phytotoxic but supported well the saprophytic growth of almost all fungi. During aerobic decomposition two opposite trends were observed for plants and fungi with an increasing and a decreasing trend, respectively. Differently, MS residues decomposed in anaerobiosis generated detrimental conditions for growth of both plants and fungi (Fig. 4).

DISCUSSION

This study analyzed the dynamical patterns of MS residue phytotoxicity and the effects on soil-borne fungi during the decomposition process. The results show that decomposition conditions are the major determi-

Table 4. Percentage of infected *M. sativa* seedlings and seedling root length in sterile sand, amended with sterile *M. sativa* extracts at two concentrations (high = H; low = L; 50 and 5 g l⁻¹, respectively) and decomposed in aerobic and anaerobic conditions for 30 days, after *P. ultimum* or *R. solani* inoculation. In the control, no pathogens were inoculated. Values are average \pm standard error of 5 replicates.

Sand amendment	Concentration	Infected seedlings (%)			Seedling root length (mm)		
		Control	<i>Pythium</i>	<i>Rhizoctonia</i>	Control	<i>Pythium</i>	<i>Rhizoctonia</i>
Water		0 \pm 0.0	8.3 \pm 0.9	5.2 \pm 2.4	7.5 \pm 1.1	4.2 \pm 0.6	7.8 \pm 0.8
Extract 0 days	H	0 \pm 0.0	100 \pm 0.0	81.5 \pm 7.6	3.1 \pm 0.4	0.12 \pm 0.09	2.5 \pm 0.2
	L	0 \pm 0.0	73.4 \pm 7.3	76.7 \pm 16.0	6.3 \pm 0.5	1.6 \pm 0.5	3.2 \pm 0.4
Extract 30 days – Aerobic	H	0 \pm 0.0	100 \pm 0.0	21.5 \pm 7.6	4.1 \pm 0.4	3.0 \pm 0.4	3.1 \pm 0.8
	L	0 \pm 0.0	46.7 \pm 4.2	0 \pm 0.0	7.2 \pm 0.6	4.1 \pm 0.7	6.9 \pm 1.1
Extract 30 days – Anaerobic	H	0 \pm 0.0	100 \pm 0.0	25.1 \pm 5.1	1.5 \pm 0.2	0.15 \pm 0.1	3.0 \pm 0.3
	L	0 \pm 0.0	78.3 \pm 2.8	8.3 \pm 3.5	4.9 \pm 0.3	1.0 \pm 0.3	4.1 \pm 0.3

Table 5. Litter weight loss and relative changes of carbon types assessed by ¹³C-CPMAS NMR during decomposition of *M. sativa* leaves in litterbags. Values are changes in percentage during the indicated period of decomposition (days).

Parameter	0-10 days	10-30 days	30-60 days	60-90 days	0-90 days
Aliphatic-alkyl C (0-45 ppm)	33	0	-11	-7	10
O-alkyl C (46-110 ppm)	-12	-1	2	0	-11
Aromatic C (111-140 ppm)	24	4	11	10	58
Phenolic C (141-160 ppm)	21	4	30	28	108
Carboxylic C (161-190 ppm)	-23	0	7	2	-16
Hydrophobicity (HB/HI)	50	2	-9	-4	32
(Aromatic + Phenolic C)/O-alkyl C	40	5	13	15	92
Aromaticity	23	4	15	15	70
Alkyl C / O-alkyl C	51	1	-12	-7	24
Litter weight loss	48.5	12.7	5.8	0.64	67.6

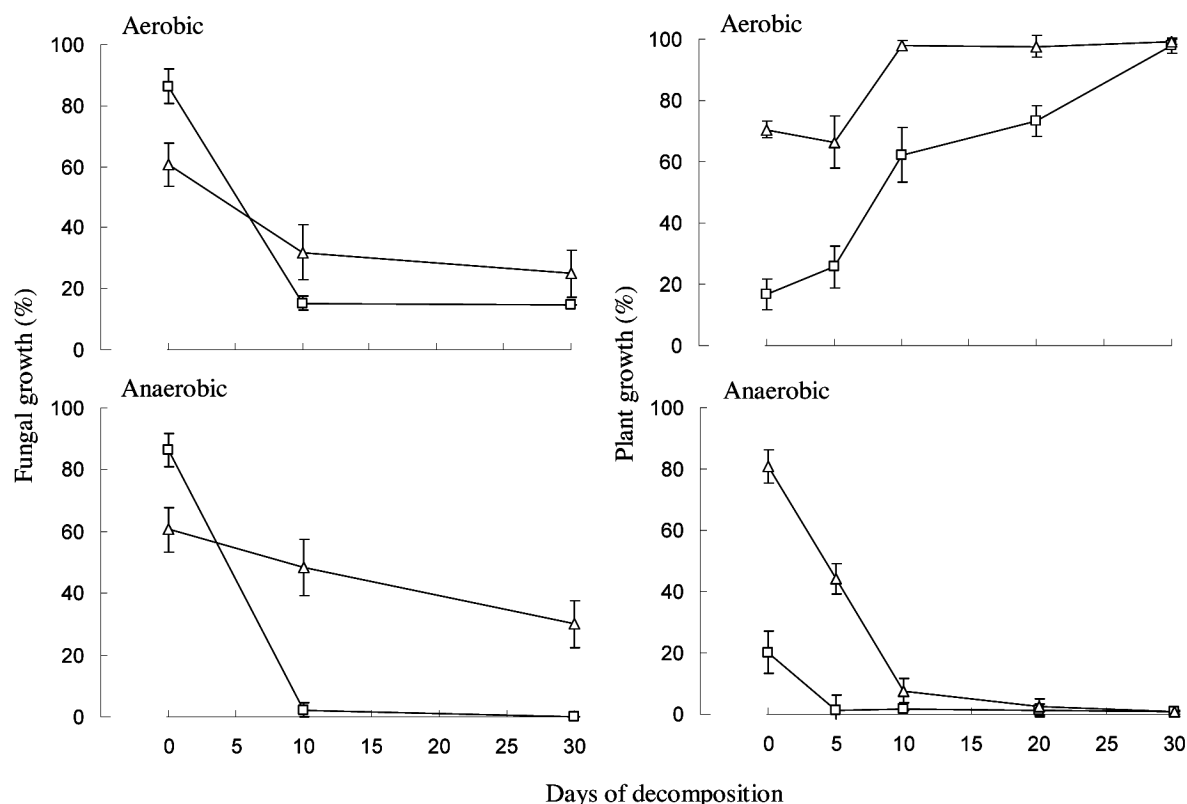


Fig. 4. Effect of *M. sativa* extracts at two concentrations (squares = high; triangles = low; 50 and 5 g l⁻¹, respectively) on fungal and plant growth during the decomposition process. Values are average \pm standard of all tested fungi (n=19) and plants (n=2) expressed in percentage compared to the highest growth value within each species.

nants of the OM impact on plants, soil microbes and their interactions. Furthermore ¹³C-CPMAS NMR spectroscopic analysis revealed changes in the chemical composition of the MS residues decomposed in aerobic conditions.

It is known that during decomposition of decaying plant residues, phytotoxic compounds can be produced, released, transformed and destroyed simultaneously by microbial activities (Blum *et al.*, 1999), thus affecting both plants and microorganisms. Many studies investigated the chemical nature of MS autotoxic compounds. However, most of the work has been done on undecomposed extracts (Read and Jense, 1989; Miller, 1996; Chung *et al.*, 2000), whereas only little attention has been paid to the MS chemical transformation and its ecological impacts during decomposition (Xuan *et al.*, 2005). The initial phase of decomposition fundamentally consists of plant tissue breakdown and the subsequent release of cell contents. Almost all fungi cultured in presence of MS extracts at this stage showed a positive response with a remarkably high growth rate for PU and TH strain P1. These results are surprising because of the high MS content of saponins and other antifungal compounds (e.g. medicagenic acid and derivatives, hederagenin and bayogenin) (Oleszek *et al.*, 1992; Avato *et al.*, 2006). Probably, the activity of these

antifungal compounds is reduced or overbalanced by the availability of nutrients and carbon sources, prevalently carbohydrates that may attenuate the deleterious effect of toxic compounds. Fungal growth decreased for all species on extracts obtained both in aerobic and anaerobic conditions, with a stronger effect in the second case. Hypotheses to explain these observations may be: (i) progressive reduction of energy and nutrients during decomposition; (ii) progressive accumulation of fungitoxic compounds, and (iii) both processes together. A partial support to the second hypothesis comes from the observation of a higher growth on the most concentrated undecomposed extracts, and a growth reduction on concentrated extracts decomposed for 10 and 30 days. This suggests that MS undecomposed extracts mainly act as substrate (i.e. fungal growth increases with concentration), whereas decomposed material has a fungitoxic effect (i.e. fungal growth decreases with concentration). ¹³C-CPMAS NMR data, although obtained only from the litterbag experiment, show a dramatic carbohydrate reduction during the MS aerobic decomposition, which indirectly supports the hypothesis that an energy reduction might cause the fungal growth decrease. Changes in pH and EC of the extracts cannot explain these results because their values fall within the range of tolerance of many fungi. Fungal re-

sponses to decomposition depend on the species: PC, PCAP, PCIN, PN, RS, SR, AA, AN, BC, M, and TH strain T22 showed a dramatic growth decrease, while *Fusarium* species (FA, FOL, FORL) were less affected. *Fusarium* species have been often described as aggressive saprophytes, able to colonize different crop residues (Gordon and Martyn, 1997).

MS extracts appeared detrimental for all tested fungi when decomposed in anaerobic conditions, with increasing effects during the decomposition processes. The role of different mechanisms such as the oxygen reduction or the production of fungitoxic compounds are not yet fully clarified (Blok *et al.*, 2000). It has been reported that the survival of several soil-borne pathogens can be strongly reduced by anaerobic soil conditions, especially when coupled with plant residue amendments (Blok *et al.*, 2000; Bonanomi *et al.*, 2007). Our results seem to confirm that the accumulation of fungitoxic products could be one of the key factors, in agreement with the findings of Blok *et al.* (2000) obtained with broccoli and grass residues. The fungitoxic effect, observed with different plant materials, indicates that the microbial anaerobic decomposition produces non-specific rather than crop-specific fungitoxic compounds (Blok *et al.*, 2000). Fungitoxic compounds such as methane, short-chain organic acids, aldehydes, alcohols and sulphur-containing compounds are among the compounds produced in anaerobiosis (Armstrong and Armstrong, 1996). The tested fungi tolerated the relatively low pHs and the changes of EC in the anaerobic extracts. However, at the lowest pHs observed during the MS residue decomposition in anaerobiosis, organic acids were present in undissociated form, which is more fungitoxic (Armstrong and Armstrong, 1999). Further studies are needed to clarify the involvement of these chemical changes in the fungitoxic activity of plant residues.

On plants, MS undecomposed extracts showed high phytotoxic and autotoxic effects, as reported in previous studies (Miller, 1992; Xuan *et al.*, 2005). This is coherent with the evidence that nitrogen-fixing species, such as MS, produce highly phytotoxic residues (Bonanomi *et al.*, 2006a). Under aerobic conditions, in both laboratory and litterbag experiments, as decomposition advanced, phytotoxicity steadily decreased and autotoxicity disappeared after 10 days. By contrast, in anaerobic conditions plant responses were similar to that of fungi. The phytotoxic effect of extracts on root growth steadily increased during the decomposition process. The observed dynamics of phytotoxicity and autotoxicity could not be explained by the pH and EC values observed during residue decomposition: both *L. sativum* (Bonanomi *et al.*, 2006a) and MS root growth are not directly affected by the pHs and ECs within the observed ranges. However, pH affects the dissociation of compounds such as organic acids, thus increasing phy-

totoxicity in acidic conditions (Armstrong and Armstrong, 1999). This is consistent with our results, since a higher phytotoxicity was observed at lower pHs produced in anaerobic conditions. ^{13}C -CPMAS NMR data may explain some changes in phytotoxicity, occurred during decomposition: phytotoxicity decline in aerobic conditions could be related with the marked reduction of O-alkyl C and carboxylic compounds in the early stages of decomposition, but also with the simultaneous increase of hydrophobicity. The increase of hydrophobicity and aromaticity of MS residues, which indicates a progressive stabilization of organic matter during decomposition, is consistent with the findings of previous studies (Spaccini *et al.*, 2000; Alarcón-Gutiérrez *et al.*, 2008).

One major criticism to the allelopathic theory is that although most plant species produce phytotoxic compounds, these are rapidly degraded into non-toxic molecules by soil microbes, thus reducing the impact on plant growth. MS autotoxicity in the field may last from few weeks (Miller, 1992) to months and, in some cases, several years (Webster *et al.*, 1967; Jennings and Nelson, 2002). Our results clearly showed that in laboratory anaerobic conditions of decomposition, autotoxicity remains high for at least 30 days. Anaerobic or partially anaerobic conditions may be present in poorly drained fields in relation to soil micro-topography and climatic conditions (Patrick, 1971). The long-lasting autotoxic effect can likely interact with other stresses of anoxic environments, such as oxygen deficit and the presence of inorganic toxic compounds. The higher autotoxicity of extracts from anaerobic compared to those from aerobic decomposition, are consistent with the findings that autotoxicity disappears from sandy soils sooner than from clayey soils (Jennings and Nelson, 1998), because the latter are more susceptible to water logging. Recently, Xuan *et al.* (2005) showed that the phytotoxic effect towards weed species of MS leaves and stems decomposing in aerated soil lasts from 10 to 20 days. However, both these experiments and ours have been carried-out at optimal temperatures for decomposition and without water limitations. Further studies are needed to investigate the dynamics of autotoxicity also during decomposition in sub-optimal environmental conditions, with special attention to the effect of temperature and water availability.

Pathogenicity assays showed that the addition of MS residues, both fresh and decomposed, increased the incidence of PU and RS damping-off. To explain these results, three hypotheses may be proposed: (i) residue autotoxicity could produce root lesions [browning of root apex was observed (data not shown)] that may facilitate fungal attacks, (ii) autotoxicity may reduce growth and weaken seedlings which become more susceptible to the pathogens, (iii) the presence of sterile MS residues may improve pathogen growth causing a build-up of a larger

inoculum. Previous studies found that diseases caused by *Pythium* spp. are primarily related to their saprophytic growth before the attack, but not to the primary inoculum density (Grünwald *et al.*, 2000; Knudsen *et al.*, 2002). The very fast saprophytic growth of PU and RS over undecomposed MS residues, exceeding 2 cm day⁻¹ for PU, supports the hypothesis that the presence of available nutrient and energy sources is a major factor for the severity of MS damping-off. However, our bioassay was carried out in the absence of competing microbes, and consequently, of conditions suppressive to the development of the soil-borne pathogens. Although, it is well known that soil amendment with MS residues affects disease incidence, the results obtained are erratic and often contradictory (Bonanomi *et al.*, 2007). For example, the incorporation of MS residues in soil may increase the incidence of damping-off caused by *Fusarium solani* f.sp. *phaseoli* on bean (Snyder *et al.*, 1959), but decreases the incidence of root rot caused by *Phytophthora cinnamomi* on avocado (Zentmyer, 1963). It is difficult to explain all these results because of the poor knowledge about the saprophytic ability of the soil-borne pathogens on MS residues (Bonanomi *et al.*, 2010). Our results suggest that the suppressive effect of undecomposed MS on soil-borne pathogens (Zentmyer, 1963; Gilpatrick, 1969; Adams, 1971) is not due to a direct fungitoxic effect, but more likely to the stimulation of other microorganisms that are antagonists to the pathogens. Further studies about this issue would improve the capability to predict the effect of MS and other crop residues on soilborne pathogens.

From this study the evidence emerges that there are contrasting responses of fungi (17 species) and plants (two species) to decaying MS residues. In particular, decomposition in anaerobic conditions generated detrimental conditions for both plants and fungi. Utilization of crop residues followed by anaerobic decomposition has been proposed for soil-borne pathogen control (Blok *et al.*, 2000). In this view, although MS in anaerobiosis has a fungitoxic effect, we do not recommend this application because of the simultaneous high phytotoxicity. These results emphasize the importance of studying both plant and pathogen responses to OM during decomposition. An agronomic management that takes into account the removal of MS above-ground residues from the field could reduce autotoxicity. Appropriate aerobic decomposition processes before replanting could avoid autotoxic effects, whereas conditions like water-logging producing anaerobiosis would increase autotoxicity. Additionally, to reduce the risk of fungal damping-off, MS residues must be fully colonized by saprophyte microorganisms (Boehm *et al.*, 1997), thus avoiding the direct stimulation of plant pathogens. Further studies are needed to define managing guidelines to reduce the risk of MS autotoxicity and of fungal damping-off after MS stand re-establishment in field conditions.

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VIRULENCE DIVERSITY IN NORTH INDIAN ISOLATES OF *SCLEROSPORA GRAMINICOLA*, THE PEARL MILLET DOWNY MILDEW PATHOGEN

R. Sharma¹, V.P. Rao¹, S. Senthilvel¹, S.C. Rajput² and R.P. Thakur¹

¹ International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Patancheru, 502324 Hyderabad, Andhra Pradesh, India

² Regional Research Station, Chandra Shekhar Azad University of Agriculture and Technology, 202125 Kalai, Aligarh, Uttar Pradesh, India

SUMMARY

On-farm surveys were conducted in the Uttar Pradesh (India) during the two rainy seasons 2007 and 2008 to monitor pearl millet (*Pennisetum glaucum*) downy mildew incidence. Twenty-one isolates of *Sclerospora graminicola*, the pearl millet downy mildew pathogen, were collected from different hybrid cultivars. These isolates were established on seedlings of the highly susceptible line 7042S grown in the greenhouse and were characterized for their virulence diversity using a set of seven host differential lines. Quantitative differences in virulence among pathogen isolates were determined by calculating virulence index (percent disease incidence \times latent period⁻¹). Results were submitted to cluster analysis using the Average Linkage method to determine similarity among pathogen isolates. The two highly virulent isolates, Sg 492 from Aligarh and Sg 510 from Badaun, representing geographically diverse locations were selected for use in greenhouse screening of pearl millet breeding lines.

Key words: *Sclerospora graminicola*, downy mildew, pearl millet, virulence, pathotype, breeding.

INTRODUCTION

Downy mildew (DM) caused by *Sclerospora graminicola* is an important disease of pearl millet [*Pennisetum glaucum* (L.) R. Br.]. The disease is highly destructive and widespread in the major pearl millet-growing areas of the world (Williams, 1984; Jeger *et al.*, 1998). In India, the disease is quite severe on single-cross F₁ hybrids and causes substantial yield losses. *S. graminicola* is largely heterothallic and has a rapid asexual generation cycle, thus it can produce millions of spores in a short time (Idris and Ball, 1984; Michelmores *et al.*, 1982). Existence of mating types and their frequency greatly contributes towards the development of new recombinants in the pathogen populations (Pushpavathi *et al.*, 2006a).

These characteristics help the pathogen to produce new variants with changed virulence that can be selected on resistant cultivars. Pathogen populations with new virulence genes could arise either because of sexual recombination/mutation or because of gene flow. Evolution of host-specific virulences in pearl millet downy mildew is well documented (Thakur *et al.*, 1992; Sastry *et al.*, 2001; Pushpavathi *et al.*, 2006b), as a result of which resistant genotypes lose their effective resistance within a short period, leading to the development of new pathotypes/races in the pathogen populations (Kolmer *et al.*, 2006).

Pathogenic variability in *S. graminicola* studied through a collaborative International Pearl Millet Downy Mildew Virulence Nursery has revealed differences in *S. graminicola* populations at different locations, including those within India (Thakur *et al.*, 2004, 2006; Rao *et al.*, 2005). The on-farm DM surveys during 1994-2004 in the hybrid-intensive states of Maharashtra, Rajasthan, Gujarat, and Haryana revealed increased DM susceptibility of a hybrid when grown in the same field for more than three consecutive crop seasons, indicating the emergence of a pathotype with new/changed virulence (Thakur *et al.*, 2003; Rao *et al.*, 2005). Therefore, pathogen populations in the major crop-growing areas need to be periodically monitored and characterized to identify new pathotypes in the target area. Virulence change in *S. graminicola* populations is monitored through on-farm surveys for downy mildew incidence and by characterizing pathogen isolates for virulence diversity. Studies done at ICRISAT and elsewhere have shown large pathogenic variability of *S. graminicola* populations from India and other countries (Shivaramakrishna *et al.*, 2003; Thakur *et al.*, 2004; Sharma *et al.*, 2010).

S. graminicola isolates from the major pearl millet-growing areas of India have been characterized and the most virulent pathotypes from different regions are being maintained at ICRISAT for greenhouse screening of breeding material. However, pathogen populations from Uttar Pradesh, the fourth largest pearl millet producing state (about 0.9 million ha annually) in India, have not yet been characterized.

This study aimed at determining the variability in pathogenicity (ability of a pathogen isolate to cause

>10% DM incidence) and virulence (high DM incidence and low latent period in the differentials) among *S. graminicola* populations by means of reaction of pathogen isolates on host differentials and identification of virulent pathotypes of the fungus for use in the screening of pearl millet hybrid parent lines targeted for development of hybrids suited for cultivation in the northern plains in India.

MATERIALS AND METHODS

DM survey and collection of isolates. The surveys were conducted to monitor DM severity in fields of eight districts of Uttar Pradesh during the rainy seasons 2007 and 2008 when the crop was at the preboot to flowering stages. During the survey, one pearl millet field was sampled at approximately every 10 km along the roadside, depending on crop intensity and hybrid diversity. In each field (≈ 0.4 ha), 5 random subplots (4 subplots at the corners and one in the center) were selected and within each subplot a minimum of 50 plants were visually checked in 2-3 rows for DM symptoms. Percent disease incidence was calculated for each field as total diseased plants/total observed plants $\times 100$.

Twenty-one samples, collected from DM-infected leaves as sporangial isolates, came from six highly susceptible hybrids, hybrids of unknown identity, and local cultivars from seven Uttar Pradesh districts (Aligarh, Badaun, Bulandshahr, Etah, Hathras, Mathura and

Moradabad) (Table 1, Fig. 1). Two isolates Sg 298 (from New Delhi) and Sg 334 (from Bhiwani, Haryana), the two neighboring states of Uttar Pradesh, collected during 1999 and 2001 surveys, respectively, and being presently used at ICRISAT for greenhouse screening of pearl millet breeding lines targeted for northern plains, were included in the study as reference isolates. These isolates were maintained through asexual generations in isolation chambers in the greenhouse on ICMP 451, 841 B, W 504-1 and 7042S seedlings.

Virulence diversity. Host differentials. Seven pearl millet genotypes P 7-4, P 310-17, 700651, 7042R, IP 18292, IP 18293 and 852B exhibiting differential reaction in the International Pearl Millet Downy Mildew Virulence Nursery (Thakur *et al.*, 2004), and two known DM susceptible lines (ICMP 451 and 7042S) were selected as host differentials for variability studies.

Inoculum preparation and inoculation. Sporangial inocula of the 23 isolates were raised on seedlings of the highly susceptible genotype 7042S in isolation chambers in a greenhouse at ICRISAT. Sporangia from sporulating leaves were harvested in ice-cold water and the spore concentration was adjusted to 1×10^6 ml⁻¹. Pot-grown seedlings of the differential lines were spray-inoculated at the coleoptile stage using an atomizer. Inoculated seedlings were incubated at 20°C and >90% relative humidity (RH) for 20 h, then transferred to greenhouse benches maintained at $25 \pm 2^\circ\text{C}$ and >95% RH for disease development for the next 2 weeks. The experiment

Table 1. *Sclerospora graminicola* isolates collected in Uttar Pradesh.

Isolate	Year	Cultivar	Location
Sg 482	2007	Pioneer 86M32	Bedai Sadabad, Hathras
Sg 483	2007	Kaveri 456	Chandola sujampur, Aligarh
Sg 489	2007	GK 1044	Bahidpur Kasgunj, Etah
Sg 490	2007	Pioneer 86M32	Bhudia, Aligarh
Sg 491	2007	Pusa 383	Kalai, Aligarh
Sg 492	2007	Pioneer 86M32	Iglas, Aligarh
Sg 493	2007	Pioneer 86M32	Sathini Aligarh
Sg 494	2007	Pioneer 86M32	Perusuva Mott, Mathura
Sg 506	2008	Pioneer 86M32	Koyal Mat, Mathura
Sg 507	2008	Krishna	Ira, Mathura
Sg 508	2008	Hybrid	Jamunanagar, Bulandshahr
Sg 509	2008	Local	Beechpuri, Badaun
Sg 510	2008	Hybrid	Gannur, Badaun
Sg 511	2008	Hybrid	Duvari, Badaun
Sg 512	2008	Local	Berpur, Badaun
Sg 513	2008	Hybrid	Ganeshpur, Badaun
Sg 514	2008	Hybrid	Baagwala, Badaun
Sg 515	2008	Hybrid	Baburata, Moradabad
Sg 516	2008	Local	Narora, Buladshahr
Sg 517	2008	Hybrid	Salari, Bulandshahr
Sg 518	2008	Pioneer 86M32	Nanglababu, Hathras
Sg 298	1999	W 504-1	New Delhi
Sg 334	2001	Hybrid	Neemriwali, Bhiwani, Haryana

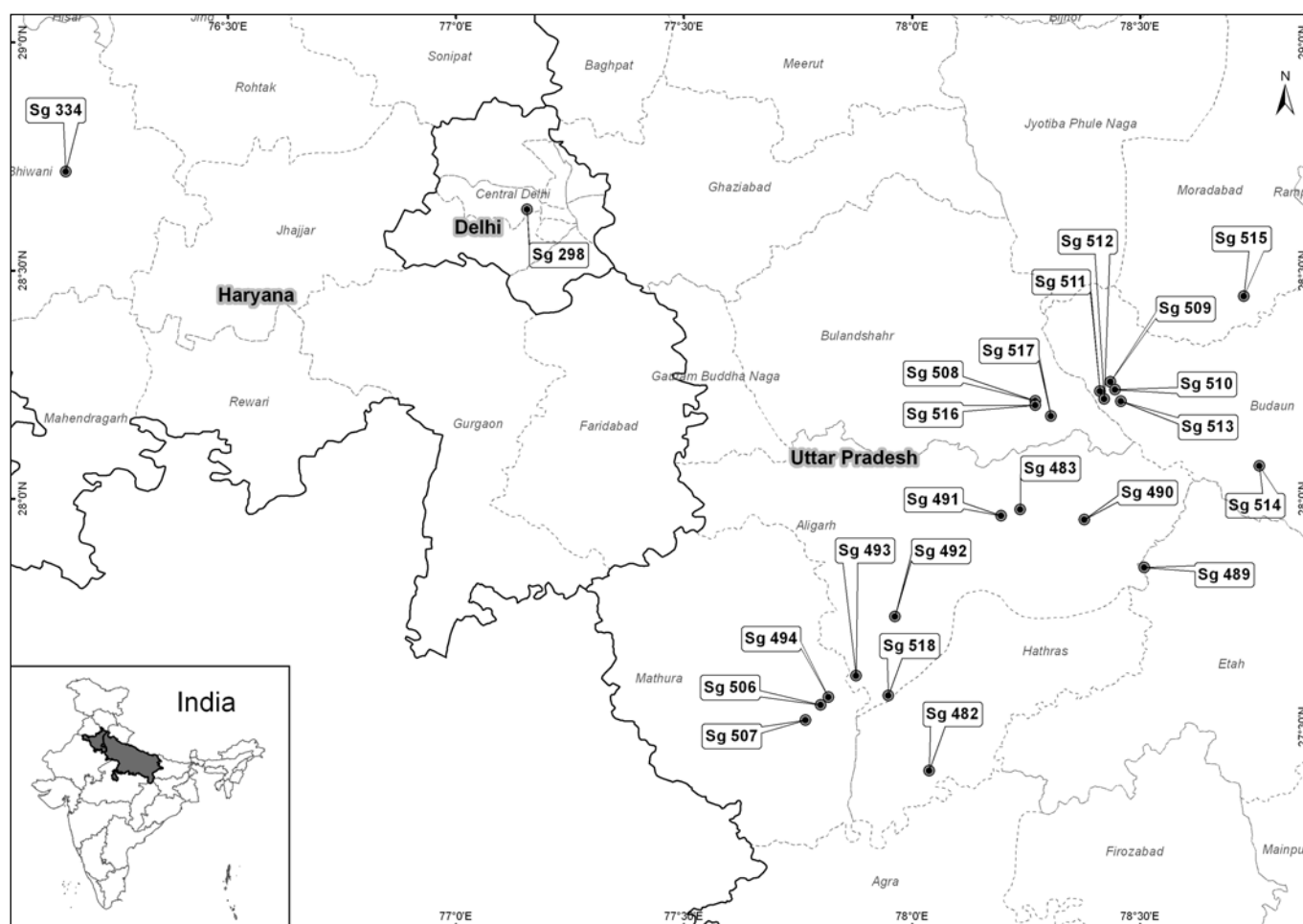


Fig. 1. Origin of *Sclerospora graminicola* isolates collected from Uttar Pradesh (India).

was conducted in a complete randomized design with three replications, and 35-40 seedlings per replication.

Data recording and analysis. Latent period_{50%} (number of days after inoculation for typical symptom appearance on 50% of the infected seedlings) was recorded from 4th day onwards post inoculation. DM incidence was recorded 14 days post inoculation as percent of infected plants. Quantitative differences in the virulence levels of the isolates were determined by calculating virulence index (percent disease incidence \times latent period⁻¹) (Thakur and Rao, 1997).

Data sets were subjected to analysis of variance (ANOVA) to determine significant differences among isolates for latent period, DM incidence and virulence index. Based on the proximity matrix, calculated by using Euclidean distance measure, cluster analysis was done using the Average Linkage method (GENSTAT 9.1, Lawes Agricultural Trust, UK) to determine the similarity among isolates and to classify isolates into different virulence groups based on virulence index.

RESULTS

On farm DM survey. DM was quite severe in all the eight districts of Uttar Pradesh surveyed. A total of 97 fields were surveyed and DM was found in 86 fields (89%) with incidence ranging from 2 to 100% with a mean incidence of 52% across infected fields (Table 2). DM incidence was very high in the Hathras district with 100% incidence. None of the fields surveyed in Badaun, Bulandshahr, Mathura and Moradabad districts were free from DM and the mean DM incidence in these districts ranged from 45 to 83%.

Fifteen hybrid cultivars (Alankar, GK 1044, HS 68, JKBH 26, Kanchan, Kaveri 456, Krishna, Nirmal 1651, PAC 931, SBH 768, Pusa 383, Pioneer 86M32, -86M52, -86M64 and Proagro 9444) were observed on farmers' fields (Table 3). Pioneer 86M32 was observed in 29 of the 97 fields surveyed (36% area surveyed) and DM was quite severe (up to 100% incidence) on this hybrid. Eighteen hybrids of unknown identity were also observed, all of them being susceptible to DM with a range of 44-94% disease incidence. Twenty local cultivars were also observed with relatively low DM inci-

Table 2. Prevalence of downy mildew (DM) in eight Uttar Pradesh districts surveyed during the 2007-08 rainy seasons.

District	Villages (No.)	Fields surveyed		Area (ha) surveyed		DM incidence (%) ^a	
		Total	DM-infected	Total	DM-infected	Mean	Range
Agra	5	21	15	10.0	7.4	28	3-100
Aligarh	13	22	21	14.1	13.7	49	10-93
Badaun	7	19	19	11.8	11.8	49	11-86
Bulandshihar	3	5	5	3.4	3.4	45	12-94
Etah	2	4	3	1.0	0.8	11	2-25
Hathras	2	8	5	3.6	2.4	100	100
Mathura	3	9	9	7.0	7.0	83	61-93
Moradabad	2	9	9	6.0	6.0	51	10-94
Total	37	97	86	56.9	52.5	52	2-100

^aBased on DM-infected fields.

dence (2 to 29%). Of the 15 hybrids monitored in the field, six (Kanchan, Nirmal 1651, PAC 931, Pioneer 86M52, -86M64 and Proagro 9444) were free from DM, two (Pusa 383 and JKBH 26) were moderately resistant (10-20% incidence) and seven highly susceptible (>50% incidence).

Virulence diversity. Analysis of variance showed significant differences ($P < 0.001$) between pathogen isolates, host genotypes and their interactions for DM incidence, latent period and virulence index indicating pathogenic variability among test isolates (Table 4). Percent disease incidence varied from 1 to 100%. Mean DM incidence for the test isolates across differentials ranged from 41% (Sg 508) to 97% (Sg 517) (Table 5). Mean DM incidence in the old isolates Sg 298 (from

New Delhi) and Sg 334 (from Bhiwani, Haryana) was 52 and 35%, respectively. Eighteen of the 21 isolates collected from Uttar Pradesh had more than 60% DM incidence across differentials, indicating high levels of virulence in the pathogen populations in farmers' fields. Based on the pathogenicity factor (number of differentials with >10% DM incidence), 23 isolates formed 5 pathogenic groups (Table 6). Group 1, comprising 10 isolates (Sg 483, -489, -490, -492, -493, -494, -507, -511, -513, and -517), was the most virulent and the isolates belonging to this group were virulent on all the seven differentials (pathogenicity factor 7). Group 2, comprising 8 isolates (Sg 491, -509, -510, -512, -514, -515, -516, -518), and isolate Sg 298 from New Delhi was virulent on all differentials except IP 18292 (pathogenicity factor 6). Group 3, represented by Sg 482 was also virulent

Table 3. Prevalence of downy mildew (DM) in different pearl millet cultivars in Uttar Pradesh surveyed during the 2007-08 rainy seasons.

Cultivars	Fields surveyed		Area (ha) surveyed		DM incidence (%) ^a	
	Total	DM-infected	Total	DM-infected	Mean	Range
Alankar	2	2	0.6	0.6	61	60-62
GK 1004	1	1	0.2	0.2	25	-
HS 68	1	1	0.4	0.4	68	-
Unknown hybrids	18	18	12.4	12.4	67	44-94
JKBH 26	5	2	2.3	1.0	11	6-16
Kanchan	1	0	0.2	0.0	0	-
Kaveri 456	4	4	1.6	1.6	54	36-68
Krishna	3	3	2.2	2.2	52	10-93
Local cultivars	20	20	11.3	11.3	13	2-29
Nirmal 1651	1	0	0.4	0.0	0	-
PAC 931	1	0	0.4	0.0	0	-
Pioneer 85M32	29	29	20.7	20.7	52	3-100
Pioneer 86M52	3	0	1.3	0.0	0	-
Pioneer 86M64	1	0	0.4	0.0	0	-
Proagro 9444	1	0	0.4	0.0	0	-
Pusa 383	1	1	0.1	0.1	19	-
SBH 768	5	5	2.0	2.0	51	10-92
Total	97	86 (89%)	56.9	52.5 (92.3%)	28	-

^aBased on DM-infected fields.

Table 4. Analyses of variance for downy mildew (DM) incidence, latent period and virulence index for 21 *Sclerospora graminicola* isolates collected from Uttar Pradesh.

Source of variation	df	MS		
		DM incidence (%)	Latent period (days)	Virulence index
Replications	2	8.72	3.58	6.02
Isolates (I)	20	7814.81***	38.15***	205.50***
Genotypes (G)	8	21941.79***	58.82***	865.48***
I × G	160	1399.54***	6.23***	40.31***
Residual	376	24.91	0.57	1.72
Total	566			

***Significant at $P < 0.001$.**Table 5.** Downy mildew incidence of *Sclerospora graminicola* isolates from Uttar Pradesh on pearl millet host differential lines.

Isolate	Downy mildew incidence (%) ^a									
	P 7-4	P 310-17	700651	7042R	852B	IP 18292	IP 18293	ICMP 451	7042S	Mean
Sg 482	23	19	24	39	36	47	10	100	100	44
Sg 483	49	43	62	88	79	21	19	98	100	62
Sg 489	75	81	85	100	97	29	82	96	100	83
Sg 490	73	80	77	67	97	95	45	100	100	82
Sg 491	85	83	90	100	98	10	85	100	100	83
Sg 492	90	70	54	97	99	100	72	100	100	87
Sg 493	91	91	90	94	97	100	68	100	100	92
Sg 494	60	81	43	48	96	100	59	100	100	76
Sg 506	38	25	38	84	0	3	21	100	100	45
Sg 507	100	100	99	69	100	100	100	100	100	96
Sg 508	30	24	45	51	1	5	17	100	100	41
Sg 509	96	100	99	100	100	7	77	100	100	87
Sg 510	100	100	100	100	100	4	73	100	100	86
Sg 511	90	98	63	100	100	14	69	99	100	81
Sg 512	100	90	59	99	100	10	41	99	100	78
Sg 513	91	88	52	66	100	100	51	100	100	83
Sg 514	100	83	46	35	100	3	39	100	100	67
Sg 515	98	83	93	100	100	0	76	99	100	83
Sg 516	100	95	92	100	100	10	91	100	100	88
Sg 517	100	100	98	97	100	100	74	100	99	96
Sg 518	98	100	98	100	100	10	82	99	100	87
Sg 298	29	25	46	54	82	7	26	99	100	52
Sg 334	16	19	17	29	14	10	9	98	100	35
Trial mean	75	73	68	79	82	38	56	99	100	

^aMean of 2 runs, 3 replications/entry, 30-35 seedlings/replication.LSD ($P < 0.05$) for isolate means = 1.78; for genotype means = 1.11; for isolate × genotype means = 5.33.

on 6 differentials (pathogenicity factor 6), but avirulent on IP 18293. Isolates Sg 506 and Sg 508 formed group 5 and were the least virulent among the isolates collected from Uttar Pradesh. Bhiwani isolate (Sg 334) represented group 4 and was less virulent (pathogenicity factor 5) and pathogenically different from isolates collected in Uttar Pradesh, whereas the Delhi isolate Sg 298 was similar to 8 of the 21 Uttar Pradesh isolates (pathogenic group 2, Table 6).

A dendrogram generated by the average linkage cluster analysis of virulence index clustered the 23 isolates into 5 major groups (Fig. 2). Based on virulence index, group V appeared as the most virulent group comprising 4 isolates (Sg 490, -492, -493, and -494) with >13 mean virulence index across differentials. Groups II (9 isolates) and III (3 isolates) had moderate to high levels of virulence. Isolate Sg 514 (group IV) with 8.6 virulence index appeared separately in the dendrogram. Isolates

Table 6. Pathogenic groups/pathotypes of *Sclerospora graminicola* isolates based on their reaction on host differentials.

Pathogenic group	Representative isolates	Reaction type ^a							Pathogenicity factor
		700651	7042R	852B	IP18292	IP18293	P310-17	P7-4	
1	Sg 483, -489, -490, -492, -493, -494, -507, -511, -513, -517	+	+	+	+	+	+	+	7
2	Sg 491, -509, -510, -512, -514, -515, -516, -518, -298	+	+	+	-	+	+	+	6
3	Sg 482	+	+	+	+	-	+	+	6
4	Sg 334	+	+	+	-	-	+	+	5
5	Sg 506, -508	+	+	-	-	+	+	+	5

^aMean of 3 replications, 35-40 seedlings/replication.

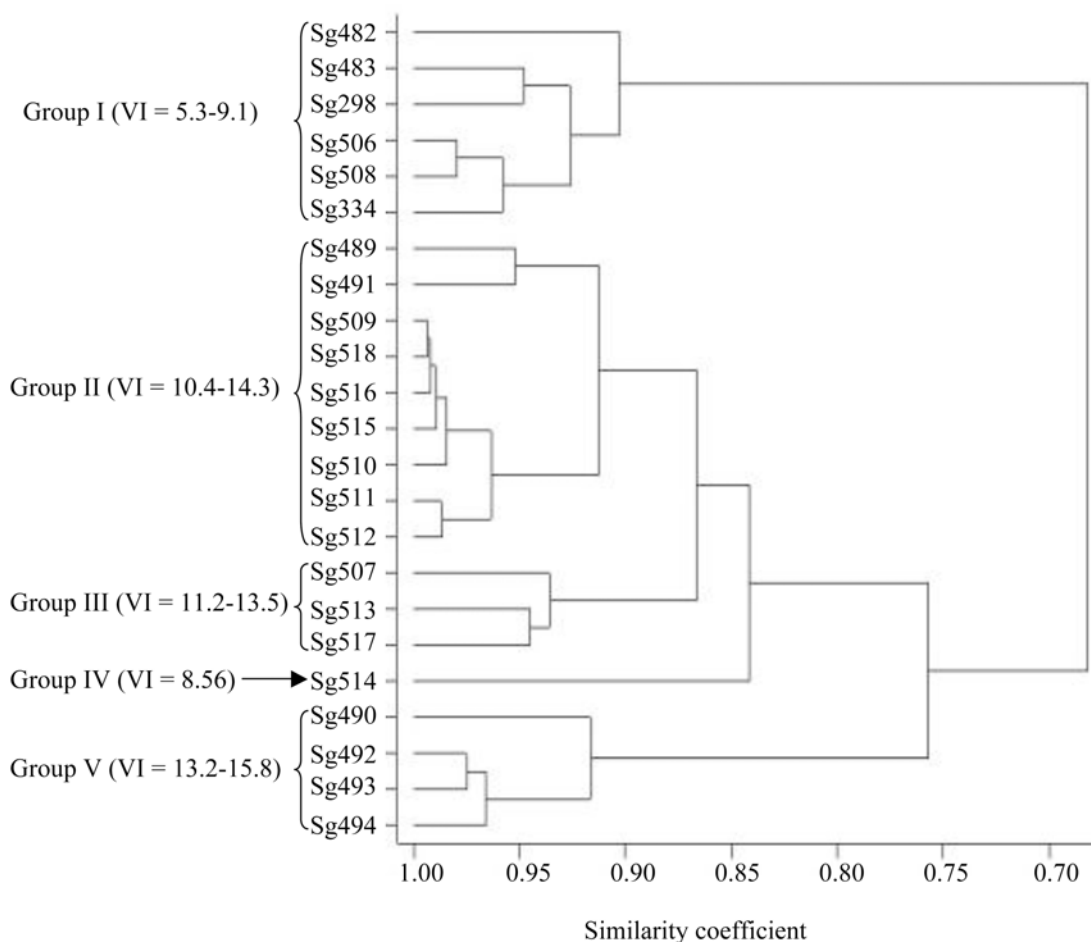
+ = Susceptible (S) (>10% downy mildew incidence); - = Resistant (R) (≤10% downy mildew incidence).

Sg 482, -483, -506 and -508 clustered in the least virulent group I along with old isolates Sg 298 and Sg 334.

DISCUSSION

ICRISAT has a major research focus on development of pearl millet hybrid parent lines, especially diversifying the genetic base of A-lines, which are disseminated to public organizations and private seed companies for

use in developing F₁ hybrid cultivars. Breeding lines are screened against different pathotypes in the greenhouse under high disease pressure (>85% disease incidence in the susceptible check) and those found resistant (≤10% disease incidence) to at least two pathotypes are designated and disseminated as seed parents. The isolates collected from Uttar Pradesh were more virulent than the isolates Sg 298 (from New Delhi) and Sg 334 (from Haryana) being used at ICRISAT for greenhouse screening of hybrid parent lines targeted for the north-

**Fig. 2.** Grouping of *Sclerospora graminicola* isolates based on virulence index.

ern plains in India. Therefore, these old less virulent pathotypes need to be replaced with new more virulent pathotypes for the greenhouse screening of pearl millet lines targeted for hybrid development for Uttar Pradesh.

In the pearl millet downy mildew pathosystem, disease incidence levels indicate quantitative differences for virulence in the pathogen and resistance in the host. Quantitative variation in *S. graminicola* isolates was studied by calculating the virulence index from two independent measures of pathogenicity, disease incidence and latent period (Thakur and Rao, 1997). Significant differences were observed in the virulence index of the test isolates indicating virulence diversity in isolates from Uttar Pradesh. Differential line IP 18292 recorded minimum mean virulence index (6.4) across 23 isolates tested and <2.5 virulence index against 15 isolates, indicating that resistance in this genotype is comparatively stable and might be governed by several QTLs for downy mildew resistance. The number of QTLs for host plant resistance has been reported in pearl millet against different pathotypes of *S. graminicola* (Hash and Witcombe, 2001; Jones *et al.*, 2002).

There was good correspondence between pathogenic and virulence groups. Isolates Sg 490, -492, -493 and -494 from the pathogenic group 1, virulent on all the differentials were clustered in one virulence group (V) with highest virulence index. Similarly, isolates Sg 491, -509, -510, -512, -514, -515, -516, and -518 comprising pathogenic group 2, virulent on 6 differentials were clustered in virulence group II with moderate virulence levels. Isolate Sg 298 (from Delhi) had low virulence index and was grouped in less virulent group I along with other less virulent isolates Sg 334 (from Bhiwani). The overlap between pathogenic and virulence groups may be due to the same genes for pathogenicity and virulence, however, in addition to major genes for pathogenicity, some minor genes may be responsible for quantitative differences in the virulence of these isolates.

The main objective of this study was to characterize pathogen populations from Uttar Pradesh state and to select highly virulent and geographically diverse isolates for greenhouse screening of pearl millet lines targeted for this Indian region. This is the first report on pathogenic diversity study in the *S. graminicola* populations from the northern Indian plains. From this study, we could select two highly virulent isolates Sg 492 from Aligarh and Sg 510 from Badaun two locations more than 100 km apart. Thus, these two isolates from Uttar Pradesh in addition to one each from New Delhi and Bhiwani will be used for greenhouse screening of pearl millet breeding lines targeted for hybrid development best suited for northern India.

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OCCURRENCE AND PATHOGENICITY OF *PSEUDOMONAS FLUORESCENS* AND *P. PUTIDA* ON TOMATO PLANTS IN ITALY

M. Dimartino¹, S. Panebianco¹, A. Vitale¹, I. Castello¹, C. Leonardi², G. Cirvilleri¹ and G. Polizzi¹

¹ *Dipartimento di Scienze e Tecnologie Fitosanitarie, Sezione di Patologia Vegetale, Università degli Studi, Via S. Sofia 100, 95123 Catania, Italy*

² *Dipartimento di Orto-Floro-Arbicoltura e Tecnologie Agro-Alimentari, Sezione di Ortofloricoltura, Università degli Studi, Via Valdisavoia 5, 95123 Catania, Italy*

SUMMARY

During 2006-2008, a survey conducted in soilless and soil-grown tomato crops in eastern and southeastern Sicily (Italy) disclosed the presence of widespread leaf chlorosis and necrosis associated with yellow, pink or brown discolouration of vascular and pith tissues. Bacteria were always isolated from symptomatic tissues on KB and NA media. A total of 158 isolates showed the LOPAT characters of group Va (-+-+) and group Vb (+++-) and 21 of these strains were identified as different biovars of *Pseudomonas fluorescens* and *P. putida*. Koch's postulates showed that 42 fluorescent *Pseudomonas* isolates are able to induce vascular discolouration of tomato plants but not leaf chlorosis and necrosis. The interactions of vascular infections and abiotic stresses were studied in growth chamber experiments. Differences in virulence were found among *P. fluorescens* biovar I and *P. putida* bv. A strains on tomato plants grown under saline stress conditions. Both bacteria were able to induce leaf chlorosis, necrosis or death of tomato plants under saline stress conditions.

Key words: fluorescent *Pseudomonas* species, characterisation, pathogenicity, soilless system, stress.

INTRODUCTION

In Italy greenhouse fresh market tomato (*Lycopersicon esculentum* L.) is a major vegetable crop grown on almost 7,280 ha with a total production of 516,260 tons (ISTAT, 2008). Recently, following the elimination of methyl bromide some substantial changes occurred in cultural practices and more interest was focused on tomato cultivation in soilless system for its advantages on productivity and environmental concerns. These changes could have negative repercussions on phytopathological issues.

Observations of recent years revealed a widespread leaf chlorosis and necrosis associated to discolouration of pith and vascular tissues caused by *Pseudomonas fluorescens* and *P. putida* on soilless tomatoes in greenhouse grown in eastern Sicily (Polizzi *et al.*, 2007). Occurrence and severity of leaf chlorosis and necrosis symptoms were hypothesised to be related to abiotic stresses conditions (e.g. high salinity or extreme temperature in the rhizosphere) which can easily occur in soilless cultivations.

Pseudomonads are opportunistic pathogens (Lo Cantore and Iacobellis, 2007), widely distributed in the natural environment and can be found in soil and plant rhizosphere (Palleroni, 1984; Bradbury, 1986). In detail, under specific environmental conditions (high relative humidity, temperature changes, high N fertilization), *P. fluorescens* may result in significant damage to agricultural crops (Dhavantari and Dirks, 1987; Lo Cantore and Iacobellis, 2002).

In relation to the widespread diffusion of the infections, the objectives of this study were: (i) to evaluate the occurrence of bacterial infections in soil-grown and soilless tomato cultivations; (ii) to characterize species involved in the syndrome; (iii) to assess the pathogenicity of isolates; (iv) to assess interactions between vascular *pseudomonads* and abiotic stresses with particular reference to salinity stress.

MATERIALS AND METHODS

Survey and pathogen detection. During 2006-2008, a survey was conducted on several tomato crops located in different agricultural areas of eastern and southeastern Sicily (36.7÷37.2 N). Specifically, 21 farms of the provinces of Caltanissetta, Catania, Ragusa and Syracuse were inspected: 11 carried out in soilless systems using perlite bags, the remaining were carried out on soil. Symptomatic plant samples were collected from each crop and farm investigated. Pieces of symptomatic tissues were dipped for 1 min in 0.6% sodium hypochlorite, rinsed in sterile distilled water (SDW), then blotted on sterile paper towels. They were placed in plates of potato dextrose agar (PDA, Oxoid, UK)

and acidified PDA (APDA) (lactic acid; pH=3.6) and incubated at 25°C for 4-5 days. At the same time, loopfuls of plant tissue suspension in SDW were streaked onto plates of nutrient agar (NA, Oxoid, UK) and King's medium B (KB) (King *et al.*, 1954). The plates were incubated at 25°C and examined after 48 h.

Characterisation. The identity of bacterial isolates was determined by morphological, physiological and biochemical tests according to the methods described by Lelliot and Stead (1987) and by Schaad *et al.* (2001). Bacterial isolates were tested for Gram reaction and production of fluorescent pigment on KB medium. Fluorescent strains were grouped on the basis of levan-type colonies formation on NA with 5% sucrose (SNA), oxidase test, pectolytic and arginine dihydrolase activity, hypersensitivity reaction on tobacco leaves (LOPAT scheme) according to Lelliot and Stead (1987). A total of 21 bacterial strains was tested for gelatin liquefaction and nitrate reduction using commercial API20NE strips (BioMérieux, France) and identified on the basis of their different ability to utilize 95 carbon sources by using Biolog analysis (MicroLog TM System Release 4.2; Biolog Inc., USA). These strains were selected on the basis of source, isolation frequency and LOPAT scheme. *P. fluorescens* biovar I 2102 (CFBP, INRA Angers, France) was used as reference.

Fatty acids profiles of 3 bacterial isolates belonging to *P. putida* were analysed using the Sherlock (Version 4.5) Microbial Identification System (MIDI) by Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ) Identification Service.

Pathogenicity tests. The pathogenicity of 42 representative fluorescent strains, including 21 identified by API and Biolog analysis, was tested on 4 week-old

tomato plants cvs Tyty, Piccolo and Shiren. The isolates were selected on the basis of source, isolation frequency and LOPAT scheme (Table 1). Bacterial suspensions (50 µl, at concentrations of 10⁵ and 10⁸ CFU ml⁻¹) in SDW, prepared by growing strains for 24 h at 25°C on NA, were injected into the pith at the first true leaves, using a sterile hypodermic syringe. Six tomato plants were used for each isolates. *P. fluorescens* bv. I (CFBP 2102) and SDW was used as a positive and negative control respectively. Inoculated plants and SDW controls were covered with plastic bags for 48 h and maintained in a growth chamber at 25°C and 70% relative humidity with a photoperiod of 16 h. After 14 days, four plants from each treatment were sectioned to determine vascular and pith discolouration. The remaining two plants were maintained in a growth chamber for five months. Bacterial re-isolates were characterized on the basis of morphological, physiological and biochemical tests.

Interactions between fluorescent pseudomonads and abiotic stresses. Two trials were carried out on tomato plants grown in a phytotrone to assess the interactions between stress and manifestation of leaf chlorosis and necrosis symptoms. Both experiments were performed on potted 4-week-old tomato plants cv. Shiren, grown on peat.

In the preliminary trial (first trial) 9 treatments were included, each consisting of 8 plants. Plants were inoculated by injecting 50 µl of bacterial suspension (10⁸ CFU ml⁻¹) of either *P. fluorescens* bv. I (DISTEF-4.4) or *P. putida* bv. A (DISTEF-21.11) as previously described. SDW injected into the stem of tomato plants served as a control. Specifically, the treatments were: water-stressed plants inoculated with either *P. fluorescens* or *P. putida*; salinity-stressed plants inoculated with either pathogen; adequately watered plants inoculated with either

Table 1. Location, source and number of fluorescent *Pseudomonas* isolates obtained from soilless and soil-grown tomato employed for pathogenicity tests.

Location	Cultivar	Symptoms ^a	Growing system	Isolates (No.)
Caltanissetta	Esperanza	SD	Soil	1
Catania	Shiren	SD, LCN	Soilless	2
Ragusa	Jessica	SD, LCN	Soilless	9
Ragusa	Piccolo	SD, LCN	Soilless	1
Ragusa	Flavorino	SD	Soil	1
Ragusa	Shiren	SD	Soil	1
Ragusa	Desiderio	SD, LCN	Soilless	1
Ragusa	Shiren	SD	Soil	1
Ragusa	Tyty	SD	Soil	1
Ragusa	Panarea	SD	Soil	1
Syracuse	Clave	SD, LCN	Soilless	18
Syracuse	Shiren	SD	Soil	3
Syracuse	CLX	SD	Soil	1
Syracuse	Marinda	SD	Soil	1

^aSD = stem discolouration, LCN = leaf chlorosis and necrosis.

pathogen; water-stressed control plants; salinity-stressed control plants; adequately watered control plants.

Irrigation was done daily with nutrient solution volumes suitable to ensure adequate leaching fraction. The nutrient solution had an EC (electrical conductivity)= 1.80 mS cm⁻¹, a pH= 6 and the following composition (meq l⁻¹): 9.5 of NO₃⁻, 0.5 of NH₄⁺, 1.5 of H₂PO₄⁻, 6.2 of K⁺, 4.2 of Ca⁺⁺, 1.5 of Mg⁺⁺, 1.7 of SO₄⁻, 31 of Na⁺, 31 of Cl⁻ (plus micronutrients).

To determine salt stress, sodium chloride was added to the standard solution in order to reach an EC = 4.50 mS cm⁻¹. To determine water stress, plants were irrigated only when they withered. The pH and EC values were measured with a GroChek pH/EC/TDS meter (Hanna Instruments, model HI 9813). Plants were maintained in the phytotrone at 27°C/22°C day/night cycle under lamps set for 14 h/10 h day/night photoperiods.

Table 2. Discriminating characters of tomato fluorescent *Pseudomonas* isolates.

Characteristic	<i>P. putida</i> bv. A	<i>P. putida</i> bv. B	<i>P. fluorescens</i> bv. I	<i>P. fluorescens</i> bv. III	<i>P. fluorescens</i> bv. IV	<i>P. fluorescens</i> bv. V	<i>P. fluorescens</i>
Levan production	- ^a	-	+ ^b	-	+	-	+
Oxidase	+	+	+	+	+	+	+
Pectolytic activity	-	-	-	-	-	-	-
Arginine dihydrolase	+	+	+	+	+	+	+
Tobacco hypersensitivity	-	-	-	-	V ^c	-	-
Gelatin liquefaction	-	-	+	+	+	-	+
Nitrate reduction	-	-	-	+	+	-	+
Tween 80	V	+	-	+	+	+	+
Adonitol	-	-	+	+	-	-	+
L-Arabinose	-	+	+	-	V	+	+
D-Arabitol	-	V	+	+	V	+	+
D-Cellobiose	-	-	-	-	-	-	-
D-Fructose	V	+	+	+	+	+	+
D-Galactose	-	+	+	+	+	+	+
α-D-Glucose	+	+	+	+	+	+	+
m-Inositol	-	-	+	+	+	-	+
D-Mannitol	-	+	+	+	+	+	+
D-Mannose	-	+	+	+	V	+	+
D-Sorbitol	-	-	+	-	+	-	+
Sucrose	-	-	+	-	+	-	+
D-Trehalose	-	-	+	+	V	+	+
Pyruvic acid methyl ester	+	+	+	+	+	+	+
Cis-Aconitic acid	+	+	+	+	+	+	+
Citric acid	+	+	+	+	+	+	+
D-Galacturonic acid	+	+	+	-	+	+	+
D-Gluconic acid	+	+	+	+	+	+	+
D-Glucuronic acid	+	+	+	-	+	+	+
Malonic acid	-	+	+	+	+	+	+
Propionic acid	+	+	+	+	+	+	+
D-Saccharyd acid	+	+	+	-	+	+	+
L-Asparagine	+	+	+	+	+	+	+
L-Aspartic acid	+	+	+	+	+	+	+
L-Leucine	+	+	+	+	+	+	+
L-Phenylalanine	-	-	-	-	-	-	-
D-Serine	V	-	-	+	-	-	-
Urocanic acid	-	+	+	+	+	+	+
Inosine	-	-	+	+	+	+	+
Putrescine	+	+	-	-	-	+	-

^a - negative; ^b + positive; ^c V between 50-80% of strains negative.

After 15 weeks, incidence and severity of disease expressed as leaf chlorosis and necrosis were evaluated. The following empirical scale was used to determine disease severity: 0= no disease symptoms; 1= light leaf chlorosis or leaf necrosis; 2= leaf chlorosis or leaf necrosis up to 25%; 3= 26-50% of chlorotic or necrotic leaves; 4= 51-90% of chlorotic or necrotic leaves; 5= plant death. Tomato plants were also sectioned to evaluate vascular and pith discolouration symptoms. The re-isolated bacteria were characterized on the basis of LOPAT scheme and Biolog analysis.

The second trial was conducted to assess the role of salt stress in the symptomatic expression of the disease. A randomized block design included 6 treatments with three repetitions per treatment and 9 plants per repetition. Specifically, the treatments were: salinity-stressed plants inoculated with either *P. fluorescens* bv. I (DISTEF-4.4) or *P. putida* bv. A (DISTEF-21.11); adequately watered plants inoculated with either *P. fluorescens* bv. I (DISTEF-4.4) or *P. putida* bv. A (DISTEF-21.11); salinity-stressed control plants; adequately watered control plants.

The management of nutrient solution both for salt stressed and non stressed plants, was carried out as described above.

Incidence and severity of disease were evaluated 15 weeks after inoculation by the empirical scale as previously described. At the end of the experiment plants were removed from the phytotrone and sectioned to determine vascular and pith discolouration. The etiology of infections was assessed on four plants for each repeti-

tion of single treatment by isolation in culture on agar media (PDA and KB). Then re-isolated bacteria were subjected to the biochemical assays for the identification (LOPAT tests and Biolog analysis).

In both trials statistical analyses were conducted using Duncan's multiple range test at 5% significance level.

RESULTS

Survey and pathogen detection. A widespread leaf chlorosis and necrosis was observed on most soilless tomato crops (80%) surveyed. Severe symptoms of disease were found on tomato cvs Piccolo, Jessica, Clave, Shiren and Desiderio grown on new and recycled perlite bags (Fig. 1). These symptoms usually appeared during winter and spring on tomatoes mainly at ripening time. Transverse sections of stems revealed, especially at the crown level, a yellow, pink or brown discolouration of vascular and pith tissues. Sometimes, wilting of plant was observed. These symptoms, similar to infections caused by *Fusarium oxysporum*, were found on most cultivated plants (50-100%). Brown spots on tomato stem surface were sporadically detected only in a plastic house located in Ragusa province. Occasionally the plants (about 5%) showed symptoms of swelling at the crown, internal decay of the lower stem, and root rot. Sometimes on these plants typical fungal sporulation resembling that of *F. oxysporum* f. sp. *radicis-lycopersici* (FORL) Jarvis and Shoemaker was detected.



Fig. 1. Widespread leaf chlorosis and necrosis in a soilless tomato plastic-house as a consequence of stem infections caused by fluorescent pseudomonads.

Vascular and pith discolourations were also detected on soil-grown tomato at the end of the cropping season. Disease symptoms were detected on cvs Shiren, Tyty, Panarea, Aranca, Flavorino, Esperanza, Clx, Marinda, Ikram, Piccadilly, Lancilot in 10 farms located in the provinces of Ragusa, Caltanissetta and Syracuse. The percentage of symptomatic plants ranged between 33% and 100%. No leaf chlorosis and necrosis were found associated to vascular and pith discolourations.

Bacteria were always isolated from the internal stem tissue of tomato plants on NA and KB media. All bacterial isolates were maintained in 15% glycerol at -80°C for long-term storage. *Fusarium* spp. was detected only occasionally.

Characterisation. A total of 158 bacterial isolates were collected from different locations. All isolates were gram negative and fluorescent on KB medium. They showed the LOPAT characters of Va or Vb group according to Lelliott and Stead (1987). Strains belonging to group Va were negative for levan production, pectolytic activity and tobacco hypersensitivity and positive for oxidase and arginine dihydrolase. Instead, those belonging to group Vb were oxidase and arginine dihydrolase positive, negative for tobacco hypersensitivity, they produce levan-type colonies on SNA and none caused soft rot on potato slices. Using API20NE system and Biolog analysis, 21 isolates were identified at the biovar and species level on the basis of their metabolic fingerprints: 5 strains as *P. putida* bv. A [similarity index (s.i.) ranging from 0.70 to 0.79]; 4 strains as *P. putida* bv. B (s.i. = from 0.59 to 0.69); 1 strain as *P. fluorescens* bv. I (s.i. = 0.69); 1 strain as *P. fluorescens* bv. III (s.i. = 0.69); 2 strains as *P. fluorescens* bv. IV (s.i. = from 0.53 to 0.77); 5 strains as *P. fluorescens* bv. V (s.i. = from 0.61 to 0.78); 3 strains as *P. fluorescens* (s.i. = from 0.56 to 0.62). The most discriminating characters are shown in Table 2. The isolates of *P. putida* bv. B differed from those of *P. putida* bv. A for the utilization of L-arabinose, D-galactose, D-mannitol, D-mannose, malonic and urocanic acid and both biovars were gelatin liquefaction, nitrate reduction and D-trehalose negative. All biovars of *P. fluorescens* were able to use D-galactose, D-mannitol, malonic and urocanic acid, while they differed for gelatin liquefaction, nitrate reduction, L-arabinose, D-mannose and D-trehalose utilization.

Fatty acid analysis confirmed the identity of 3 bacterial isolates which have been characterized as *P. putida* bv. A. The similarity indices for these bacterial isolates ranged between 84% and 86%. The *P. putida* bv. A isolates had 17 fatty acids that included C8:0 3OH, C10:0 3OH, C12:0, C12:0 2OH, C12:1 3OH, C12:0 3OH, C14:0, C15:0, Sum in Feature 2 (C14:0 3OH/C16:1 ISO I), Sum in Feature 3 (C16:1 w7c/15 iso 2OH-C15:0 ISO 2OH/C16:1 w7c), C16:0, C17:0 CYCLO, C17:0, C16:0 3OH, C18:1 w7c, C18:0.

Pathogenicity tests. All bacterial isolates tested at two different concentrations (10^5 and 10^8 CFU ml^{-1}), including *P. fluorescens* bv. I reference strain (CF2102), caused vascular and pith discolouration symptoms on tomato plants cvs Tyty, Piccolo and Shiren 14 days after inoculation. All bacterial isolates caused more extensive vascular discolouration, but leaf chlorosis and necrosis or wilting symptoms were not observed on inoculated tomato plants after five months. No disease symptoms appeared on control plants. No difference related to the pathogenicity between the isolates and to bacterial concentrations. Re-isolated bacteria on KB medium were recognized as identical to the original strains by LOPAT scheme and Biolog analysis.

Interactions between fluorescent pseudomonads and abiotic stresses. In the first trial, symptoms of leaf chlorosis and necrosis appeared 15 weeks after pathogen inoculation on plants irrigated with saline solution. These symptoms were identical to those observed in naturally infected tomato crops and appeared on all salinity-stressed plants (100%) inoculated with *P. fluorescens* bv. I and *P. putida* bv. A strains. No leaf chlorosis and necrosis symptoms were detected on both inoculated water stressed and non stressed plants and in all control plants (stressed and non stressed).

Under saline stress conditions the severity of disease expressed as leaf chlorosis and necrosis was significantly higher on plants inoculated with *P. fluorescens* bv. I DISTEF-4.4 (severity index= 3.7) than in those inoculated with *P. putida* bv. A DISTEF-21.11 (severity index= 2.0). Under these conditions both strains led the plant to death (Table 3).

Examination of stem sections revealed discolouration symptoms in all plants inoculated with either pathogen. In detail, under conditions of saline stress average discolouration of 24.9 cm and 28.1 cm were found on plants inoculated with DISTEF-4.4 and DISTEF-21.11, respectively. Extension of symptoms on non stressed plants inoculated with either DISTEF-4.4 (12.3 cm) or DISTEF-21.11 (16 cm) was lower than salinity-stressed and inoculated plants. Light discolourations (2.9 cm) detected on all water-stressed and inoculated plants were not significantly different from controls. No stem discolouration symptoms were detected in control plants (Table 4). Re-isolated bacteria were identified as identical to the two inoculated species.

In the second trial leaf chlorosis and necrosis symptoms as a consequence of stem infections were detected after 15 weeks on plants stressed and inoculated with either DISTEF-4.4 (*P. fluorescens* bv. I) or DISTEF-21.11 (*P. putida* bv. A) (Fig. 2A). The disease severity was significantly higher on salinity-stressed plants artificially infected by DISTEF-4.4 (severity index= 4.4) than those inoculated with DISTEF-21.11 (severity index= 1.3). Under these conditions both pathogens led the plants to

Table 3. Pathogenicity of *Pseudomonas fluorescens* bv. I (DISTEF-4.4) and *P. putida* bv. A (DISTEF-21.11) expressed as leaf chlorosis and necrosis under salt stress conditions in the first trial.

	Incidence (%)	Severity index
<i>P. fluorescens</i> bv. I (4.4)	100	3.7 b
<i>P. putida</i> bv. A (21.11)	100	2.0 a

Data are the mean of eight values. Values marked with different letters are significantly different according to Duncan's multiple range test ($P \leq 0.05$). Disease assessment was based on a 0-to-5 severity scale reported in the text.

death. In particular, 25% of the plants died when inoculated with *P. fluorescens* bv. I, only 5% with *P. putida* bv. A, while none of the inoculated plants died. No leaf chlorosis and necrosis were observed on non stressed inoculated plants and in all control plants (saline-stressed and non stressed).

Stem discolouration was detected on all plants inoculated with either DISTEF-4.4 or DISTEF-21.11 (Figs. 2B and 2C). Discolourations caused by *P. fluorescens* bv. I (DISTEF-4.4) were significantly more extensive on salinity-stressed plants than those detected on non

stressed plants. No significant difference on discolouration extent was found between stressed and non stressed tomato plants inoculated with *P. putida* bv. A (DISTEF-21.11). No vascular infections were detected on control plants (Table 5).

The bacteria were re-isolated from pieces of symptomatic stem tissue and identified as *P. putida* bv. A and *P. fluorescens* bv. I by LOPAT scheme and Biolog analysis.

DISCUSSION

Widespread symptoms of vascular and pith discolouration were detected on most of the surveyed tomato crops. In detail, the research conducted in soil-less tomatoes allowed to detect leaf chlorosis and necrosis associated to discolouration of internal stem tissues. Gram negative bacteria belonging to Va and Vb groups of fluorescent pseudomonads were always isolates from the internal stem tissues of tomato plants. Within these groups *P. fluorescens* bv. I, III, IV, V and *P. putida* bv. A and B were detected.

Koch's postulates showed that 42 fluorescent *Pseudomonas* isolates were able to induce a stem discolouration

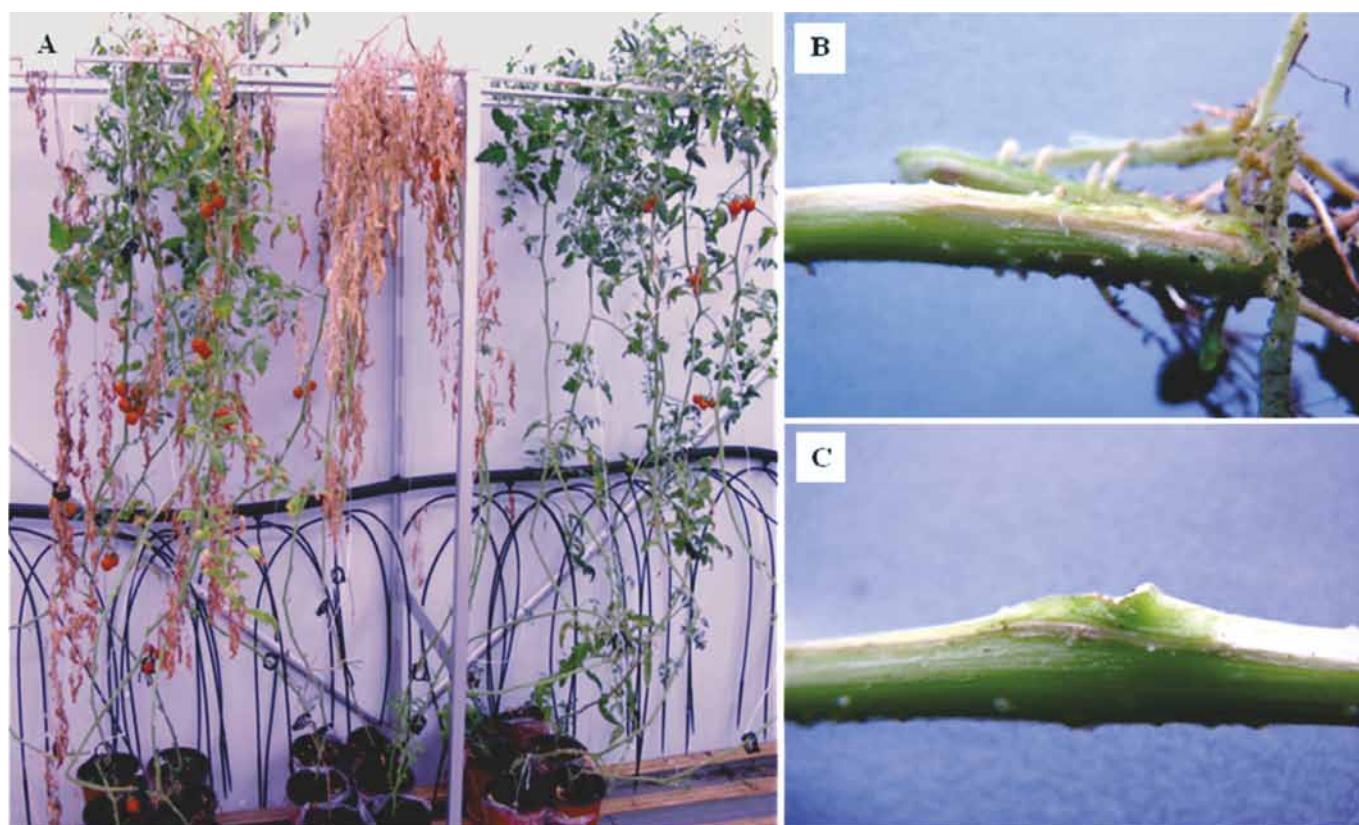


Fig. 2. (A) Leaf chlorosis and necrosis on tomato plants under saline stress condition induced by inoculations with *Pseudomonas putida* bv. A DISTEF-21.11 (on the left) and *P. fluorescens* bv. I DISTEF-4.4 (in the centre) compared with stressed uninoculated control (on the right) in phytotrone. (B-C) Stem discolouration symptoms caused by *P. fluorescens* bv. I DISTEF-4.4 (B) and *P. putida* bv. A DISTEF-21.11 (C) on inoculated plants.

Table 4. Pathogenicity of *Pseudomonas fluorescens* bv. I (DISTEF-4.4) and *P. putida* bv. A (DISTEF-21.11) expressed as vascular and pith discolouration under different growth conditions in the first trial.

Treatments	<i>P. fluorescens</i> bv. I		<i>P. putida</i> bv. A	
	Incidence (%)	Severity (cm)	Incidence (%)	Severity (cm)
Salinity-stressed and inoculated	100	24.9 c	100	28.1 c
Water-stressed and inoculated	100	2.9 a	100	2.9 a
Non stressed and inoculated	100	12.3 b	100	16.0 b
Salinity-stressed control	0	0.0 a	0	0.0 a
Water-stressed control	0	0.0 a	0	0.0 a
Uninoculated control	0	0.0 a	0	0.0 a

Data are the mean of eight values. Values marked with different letters are significantly different according to Duncan's multiple range test ($P \leq 0.05$).

Table 5. Pathogenicity of *Pseudomonas fluorescens* bv. I (DISTEF-4.4) and *P. putida* bv. A (DISTEF-21.11) expressed as vascular and pith discolouration under different growth conditions in the second trial.

Treatments	<i>P. fluorescens</i> bv. I		<i>P. putida</i> bv. A	
	Incidence (%)	Severity (cm)	Incidence (%)	Severity (cm)
Salinity-stressed inoculated	100	27.6 c	100	16.3 b
Non stressed inoculated	100	18.6 b	100	15.7 b
Salinity-stressed control	0	0.0 a	0	0.0 a
Uninoculated control	0	0.0 a	0	0.0 a

Data are the mean of twenty four values. Values marked with different letters are significantly different according to Duncan's multiple range test ($P \leq 0.05$).

tion within 14 days, but did not demonstrate their ability to induce leaf chlorosis and necrosis symptoms on tomato plants five-months after inoculation.

Our data on pathogenicity tests under saline stress conditions provide evidence that *P. fluorescens* bv. I and *P. putida* bv. A are responsible for leaf chlorosis, leaf necrosis and tomato plants death. These symptoms are identical to those observed in the soilless tomato cultivation and can be considered as a consequence of stem infections caused by fluorescent *Pseudomonas* species. Salinity stress plays a key role in the expression of leaf chlorosis and necrosis symptoms. Under salinity conditions more severe symptoms of disease expressed as leaf chlorosis and necrosis were observed in plants inoculated with *P. fluorescens* bv. I showing a greater virulence of this pathogen than *P. putida* bv. A. According to these results, irrigation with saline water induces stress in crops and increases disease symptoms with severe damaging consequences for tomato production. A previous work showed the increase of FORL incidence in soil-grown tomatoes irrigated with salinized water (Triky-Dotan *et al.*, 2005). This aspect is more evident in soilless cultivation systems where plants are particularly susceptible to diseases under stress conditions because

of high temperatures and/or low levels of dissolved oxygen in the nutrient solution and, especially, the limited buffer capacity of the substrate (Gold and Stanghellini, 1985; Jarvis, 1991; Menzies *et al.*, 1996; Cherif *et al.*, 1997; Stanghellini and Kim, 1998; Utkhede *et al.*, 2000; Khan *et al.*, 2003).

In southeastern Sicily tomato crops are frequently irrigated with saline water. Although tomato crop is considered to be moderately sensitive to salinity (Maas, 1986), saline water is widely used in greenhouse cultivations (Leonardi and Martorana, 2005). Indeed, increasing salt concentrations determine sugar accumulation in the fruits (Cuartero and Fernandez-Munoz, 1999).

High salinity may affect plant physiology via morphological, anatomical, metabolic, and biochemical changes (Campbell and Pitman, 1971; Kylin and Quatrano, 1975; Poljakoff-Mayber, 1975; Hasegawa *et al.*, 2000; Bernstein and Kafkafi, 2002; Parida and Das, 2005), and in addition may impose on the plant osmotic stress, lowered water potential and water deficit (Bernstein and Kafkafi, 2002; Munns, 2002).

In a preliminary study *P. fluorescens* bv. I and *P. putida* bv. A have been reported as vascular pathogens on tomato plants (Polizzi *et al.*, 2007) and the role of abiot-

ic stresses was suspected. In addition, a similar disease has also been observed on tomato plants in Turkey (Saygili *et al.*, 2004) and ascribed to *P. fluorescens* bv. I. Several authors have reported *P. fluorescens*, alone and in combination with other bacterial species, as causal agent of tomato pith necrosis (Malathrakakis and Goumas, 1987; Lo Cantore and Iacobellis, 2002; Molan and Ibrahim, 2007; Pekhtereva *et al.*, 2009).

It is well known that *P. putida* is a soil microorganism that normally does not cause plant disease, but our data have shown the ability of this bacterium to induce infections. This pathogen could represent a serious threat for tomato crops grown under salinity stress conditions.

However, the symptoms described herein could be confused with those induced by *F. oxysporum* although they were associated in the most surveyed farms with vascular infections caused by fluorescent *Pseudomonas* species.

Comprehensively, these findings confirm that the recent changes in tomato growing systems and in soil disinfection techniques could determine new phytopathological issues.

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ARTIFICIAL EVOLUTION CORRECTS A REPULSIVE AMINO ACID IN POLYGALACTURONASE INHIBITING PROTEINS (PGIPs)

M. Benedetti¹, E. Bastianelli¹, G. Salvi¹, G. De Lorenzo¹ and C. Caprari²

¹ Dipartimento di Biologia e Biotechnologie "C. Darwin", Università La Sapienza, Rome, Italy

² Dipartimento di Scienze e Tecnologie per l'Ambiente e il Territorio (S.T.A.T.),
Università degli Studi del Molise, Pesche (IS), Italy

SUMMARY

Polygalacturonase-inhibiting proteins (PGIPs), extracellular proteins that specifically inhibit fungal endopolygalacturonases (PGs), play a critical role in plant protection by favouring the accumulation of oligogalacturonides (OGs), which are elicitors of plant defence responses. The genes encoding PGIP2 of *P. vulgaris* and the variant PGIP2.Q224K were subjected to error prone PCR (epPCR) to generate mutated inhibitors with novel and improved recognition capabilities. Using a *Pichia pastoris* expression library and a high-throughput screening method, two mutated PvPGIP2.Q224K-derived inhibitors active against the PG produced by the phytopathogenic fungus *F. phyllophilum* (FpPG) were isolated. Both variants were better inhibitors than PGIP2.Q224K and were characterized by the replacement of the lysine in position 224, supporting the view that the absence of this positively charged amino acid at position 224 is a primary requirement for gaining the inhibition capability against FpPG.

Key words: artificial evolution, PGIPs, LRR, polygalacturonase, *Pichia pastoris*, Error prone PCR.

INTRODUCTION

Molecular recognition events play a key role in plant defence against pathogens. For example, microbe-associated molecular patterns (MAMPs) are recognized by specific plant pattern recognition receptors (PRR) to trigger defence responses. On the other hand microbial effectors, secreted to help the pathogenicity process, may act as avirulence products upon recognition by specific receptors (resistance proteins) and elicit resistance responses (Boller and Felix, 2009). Furthermore, several enzymes produced by microbes or insects are specifically recognized by plant proteins for inhibition of their

enzymatic activity (De Lorenzo and Ferrari, 2002) or as non-self molecules for activation of the immune response (Boller and Felix, 2009), in order to limit colonization and disease.

The majority of plant recognition proteins share domains characterized by leucine-rich repeats (LRR). This structure is also found in many receptors involved in hormone response and development (Becraft, 2002; Szekeres, 2003; Boller and Felix, 2009). A prototypical plant LRR protein involved in defence is polygalacturonase-inhibiting protein (PGIP). PGIP exhibits 10 imperfect LRRs of the extracellular type [consensus motif: xxLxLxxNxLt/sGxIPxxLxxLxx] and acts as a specific inhibitor of endopolygalacturonases [poly (1,4- α -D-galacturonide) glycanohydrolase, EC 3.2.1.15; PGs] from fungi and insects, but not of plant-derived PGs (De Lorenzo *et al.*, 2001). The degradation of the mechanical barrier represented by the plant cell wall is a critical event during pathogenesis caused by phytopathogenic fungi that produce a variety of cell wall degrading enzymes both to facilitate the invasion of plant tissues and to release nutrients to be used as carbon source. PGs are among the first enzymes produced during infections and degrade homogalacturonan, a component of pectin that acts as a cohesive element for the entire cell wall structure (Lionetti *et al.*, 2010). To accommodate pathogenesis in a variety of conditions and on various hosts, many PG isoenzymes, which often exhibit polymorphism in different isolates or races, are produced by pathogens (Caprari *et al.*, 1993; De Lorenzo *et al.*, 2001; Daroda *et al.*, 2001; Poinssot *et al.*, 2003; Mariotti *et al.*, 2009). Against these PGs, plants have evolved many PGIPs, often encoded by small gene families of clustered paralogs, with different specificities and regulation (Frediani *et al.*, 1993; D'Ovidio *et al.*, 2004). For instance, all four members of the *Phaseolus vulgaris* PGIP family inhibit, with different efficiencies, PGs from *Botrytis cinerea* and *Colletotrichum lupini*, but only PvPGIP2 inhibits PG from *Fusarium moniliforme* FC-10 strain [now reclassified as *F. phyllophilum* strain FC912 (Mariotti *et al.*, 2008)] (Leckie *et al.*, 1999; D'Ovidio *et al.*, 2004). By interacting with PGs, PGIPs favour the formation of oligogalacturonides (OGs) capable of inducing plant defense responses (De Lorenzo

and Ferrari, 2002; Brutus *et al.*, 2010). PGIPs hamper the invasion process by limiting host tissue colonization and can therefore be exploited to obtain transgenic plants with increased resistance (Powell *et al.*, 2000; Ferrari *et al.*, 2003; Aguero *et al.*, 2005; Manfredini *et al.*, 2005; Joubert *et al.*, 2007; Janni *et al.*, 2008).

Computational analysis of the PvPGIP2 structure predicts that the xxLxLxx region within each LRR has a strong propensity to be involved in protein-protein interactions (Sicilia *et al.*, 2005; Casasoli *et al.*, 2009) and a biochemical analysis has shown that this region contains residues influencing the specificity of PvPGIP2 (Leckie *et al.*, 1999; D'Ovidio *et al.*, 2004; Sicilia *et al.*, 2005). The highly variable xxLxLxx motifs in the LRR are responsible for ligand binding. In the crystal structure of PGIP, which is so far the only available structure of a plant LRR protein, these motifs form a concave beta-sheet surface. Single residues located in this concave surface differentially affect the inhibition of fungal PGs. For example, the glutamine residue 224 is crucial for the inhibition of PG of *F. phyllophilum*, hereon indicated as FpPG (Leckie *et al.*, 1999). The combination of evolutionary analyses with structural, biochemical, and physiological data allowed the identification of residues in the third LRR (H104A, Y105A, and Y107A) and in the seventh LRR module (F201) of PvPGIP2 as "hotspots" for the interaction, i.e., essential residues that if mutated impede or severely affect protein-protein interactions (Casasoli *et al.*, 2009).

In this work, an *in vitro* evolution approach has been used in the attempt to obtain inhibitors with novel and improved recognition capabilities against a fungal PG that is not inhibited by any known PGIP such as that from *Fusarium verticillioides* (Raiola *et al.*, 2008). Approaches of artificial evolution based on random mutagenesis by error-prone PCR (epPCR) have been already applied to tobacco LRR resistance protein Rx to generate variations in disease resistance specificities (Farnham and Baulcombe, 2006), indicating directed evolution as a common laboratory tool for altering and optimizing the functions of proteins characterized by a high evolvability (Romero and Arnold, 2009). Random mutagenesis is a suitable method to generate *in vitro* variability of LRR proteins, because leucine, the key aminoacid for the maintenance of the stability of the protein scaffold, is coded by six different codons and characterized by the lowest mutation frequency. Here we present the results obtained by screening two different libraries of PGIP variants expressed in *Pichia pastoris*. The first library was generated using, as a starting protein, PGIP2Q224K, which is impaired in recognition of FpPG and was mainly aimed at recovering gain-of-function variants and demonstrating the feasibility of the approach. The second library was generated using the wild-type PvPGIP2 and aimed at isolating variants with novel and improved recognition specificities.

MATERIALS AND METHODS

Construction of the expression vector containing the PvPGIP2 gene. The wild-type PvPGIP2 gene was cloned in pGAPZ α A (Invitrogen BV, The Netherlands) using the *EcoRI* and *XbaI* restriction sites introduced by the following primers: EcoFw 5'-ATCGATGAATTC-GAGCTATGCAACCCACAA-3' and *Xba*Rvtag 5'-CTTGTCTCTAGAGAAGTGCAGGCAGGA-3' (underlined sequence indicates the restriction site introduced), to generate a fusion protein where the signal sequence of PvPGIP2 was replaced by the yeast alpha factor signal sequence for secretion and a 6 histidine tag was added at the C-terminus. pGAPZ α A allows the constitutive expression of heterologous proteins under the control of the GAP promoter. The construct was amplified by transforming *E. coli* DH5 α competent cells [genotype: F- Φ 80lacZ Δ M15 Δ (lacZYA-argF)U169 *deoR* *recA1* *endA1* *hsdR17*(rk⁻, mk⁺) *phoA* *supE44* *thi-1* *gyrA96* *relA1* λ -provided by Invitrogen BV, The Netherlands]. Transformants were selected on low-salt Luria-Bertani (LB) plates containing 25 μ g/ml zeocin (Duchefa Biochemie, Italy) and analysed by direct PCR amplification using specific primers for the alpha-factor signal peptide and the 3'-AOX terminator sequences according to the manufacturer's instruction. One PCR-positive colony was picked with a sterile tip and used to inoculate 5 ml of low-salt LB medium [1% tryptone (Duchefa Biochemie, Italy), 0.5% yeast extract (Duchefa Biochemie, Italy), 0.5% NaCl (Carlo Erba Reagenti, Italy), pH 7.4] containing 25 μ g/ml zeocin. The culture was grown overnight at 37°C at 250 rpm. Plasmid DNA was extracted from the cells using a plasmid mini prep kit (Macherey and Nagel, Germany) and analyzed by digestion with *EcoRI* and *XbaI* restriction enzymes, followed by 1% agarose gel analysis.

Site-directed mutagenesis of PvPGIP2.Q224K and PvPGIP2.Q224E. Site-directed mutagenesis of the PvPGIP2 gene was performed using the Quick Change site-directed mutagenesis kit (Stratagene, Canada). PCR was carried out directly on pGAPZ α A/ PGIP2 construct. The internal overlap primers that hybridize at the site of the desired mutation, containing the relevant mismatched bases (underlined bases), were the following: PGIP2.Q224K Fw: 5'-CGGATCAGATAAGAACACG AAGAAGATACATCTGGCGAAG-3'; PGIP2.Q224K Rv 5'-CTTCGCCAGA TGTATCTTCTTCGTGTTC TT ATCTGATCCG-3'; PGIP2.Q224E Fw 5'-CGGATCAGATAAGAACACG GAGAAGATACATCTGGCGAAG-3'; PGIP2.Q224E Rv 5'-CTTCGCCAGA TGTATCTTCTCCGTGTTC TT ATCTGATCCG-3'.

Transformation and growth of *E. coli* as well as selection and analysis of transformants were performed as reported in the previous paragraph. The mutations were checked by sequencing analysis (PRIMM, Italy).

Error-prone PCR. The conditions for random mutagenesis were optimized on the basis of the method described by Leung *et al.* (1989). The reaction mixture (50 µl final volume) contained: 10 mM Tris-HCl pH 8.3 (Carlo Erba Reagenti, Italy), 50 mM KCl (Carlo Erba Reagenti, Italy), 7 mM MgCl₂ (Carlo Erba Reagenti, Italy), 0.35 mM MnCl₂ (Carlo Erba Reagenti, Italy), 25 pmol of each primers, unbalanced dNTPs mix (1mM dCTP and dTTP, 0.2 mM dATP and dGTP) (Bioline, England) and 2.5 U Taq polymerase (Bioline, England). The primers used were EcoFw and XbaRvtg described above. We used 22 ng of pBS23-Pvpgip2Q224K (Leckie *et al.*, 1999) for each reaction tube for a total of 50 tubes. This was the program utilized: 1x2 min 94°C, 26-30 x (1 min 94°C, 1 min 56°C, 1 min 72°C), 1 x 7 min 72°C.

Electroporation. The bacterial and yeast libraries were obtained by electroporation using the Gene-Pulser Xcell Electroporation System (Bio-Rad Laboratories, USA). Preparation and electroporation of DH5α was carried out according with the Gene-Pulser Xcell Electroporation System manual. Preparation and electroporation of *Pichia pastoris* X33 (genotype: wild type, provided by Invitrogen BV, The Netherlands) was carried out as described (Wu and Letchworth, 2004).

Preparation of PGs. A homogeneous PG of *Colletotrichum lupini* (strain SHK788 var *setosum*) was prepared as described (Bonivento *et al.*, 2008). A homogeneous PGII of *Aspergillus niger* (strain N400) was prepared according to D'Ovidio *et al.* (2004). *Botrytis cinerea* (strain BO5-10), *Fusarium phyllophilum* (strain FC912), *Fusarium graminearum* (strain XM391184) and *Fusarium verticillioides* [strain PD; (Raiola *et al.*, 2008)] were grown for 20 days on potato dextrose agar (Oxoid, Italy) at 22°C under constant light. Mycelium of *Botrytis cinerea*, *F. phyllophilum*, *F. graminearum* and *F. verticillioides* (1 cm²) were harvested, used to inoculate Czapek-Dox medium (2 g l⁻¹ NaNO₃, 1 g l⁻¹ K₂HPO₄, 0.5 g l⁻¹ MgSO₄, 0.5 g l⁻¹ KCl, 10 mg l⁻¹ FeSO₄, pH 7.0; all chemicals were from Carlo Erba Reagenti, Italy), and supplemented with 1% citrus pectin (Sigma Aldrich, Italy). Cultures were incubated in a rotary shaker at 180 rpm and 21°C for 5 days, and filtrates were used for the PG activity assay.

Screening of the libraries. *Pichia* colonies obtained by electroporation were replicated by replica plating using a sterile filter paper and transferred to a plate containing 40 ml of YTGA medium [25 mM citrate buffer pH 4.5, 1.3% glycerol, 0.5% yeast extract (Duchefa Biochemie, Italy), 1% tryptone (Duchefa Biochemie, Italy), 0.8% polygalacturonic acid from citrus fruit (Sigma Aldrich, Italy), 1.2% agarose (Jena Bioscience, Germany), 100 µg/ml zeocin (Duchefa Biochemie, Italy)]. After replica plating, plates were incubated at 28°C for

4 days. Plates were covered by a solution containing the polygalacturonase of *Fusarium phyllophilum* (1.5 ng/ml of FpPG in 25 mM citrate buffer pH 4.5) and incubated overnight at 28°C. After incubation, the development of the assay was acted adding 6 M HCl for 1 min.

PG-PGIP assay. PG-PGIP assay was performed as previously published (Taylor and Secor, 1988; Ferrari *et al.*, 2003). The sample containing purified PGIP and purified PG (for AnPGII and CIPG) or the fungal crude preparation (for FpPG, FgPG, FvPG and BcPG) was added to 0.9 cm wells on plates containing 100 mM sodium acetate (Carlo Erba Reagenti, Italy), pH 4.6, 0.5% PGA from citrus fruit (Sigma Aldrich, Italy) and 0.8% agarose (Jena Bioscience, Germany). Plates were incubated for 16 h at 30°C, and the halo caused by enzyme activity was visualized after 1 min of treatment with 6 M HCl. PG activity was expressed as agarose diffusion units, with 1 agarose diffusion unit defined as the amount of enzyme that produced a halo of 0.5 cm radius (external to the inoculation well) after 16 h at 30°C.

Production and purification of PGIP. The medium used for production of PGIP2, PGIPA and PGIPB contained 1% yeast extract (Duchefa Biochemie, Italy), 1% tryptone (Duchefa Biochemie, Italy) and 2% glucose (Carlo Erba Reagenti, Italy). Purification included the following steps: cultural filtrates were concentrated using a Vivaflow 200 (Sartorius Stedim, France) and dialyzed against 20 mM Na acetate pH 4.6 (Carlo Erba Reagenti, Italy). Dialyzed proteins were mixed with a suspension of diethylaminoethyl (DEAE) cellulose (DE52, Whatman, UK) pre-equilibrated with 20 mM Na acetate pH 4.6. The not-absorbed proteins were passed on a column HiTrap SP-Sepharose (GE Healthcare, USA) pre-equilibrated with 20 mM Na acetate pH 4.6 (Carlo Erba Reagenti, Italy). Elution were acted using a linear gradient of NaCl (0 to 1 M in 10 min) (Carlo Erba Reagenti, Italy) in 20 mM Na acetate pH 4.6 (Carlo Erba Reagenti, Italy). Fractions that showed the highest inhibitory activity were assayed by SDS gel 10% acrylamide (Sigma Aldrich, Italy), quantified by Blue Coomassie staining and immunoblotting.

SDS-PAGE and Western blotting. Purified proteins were analyzed by SDS-PAGE acrylamide gel 10% (Sigma Aldrich, Italy) and stained using a solution of Coomassie brilliant blue R-250 (Sigma Aldrich, Italy). Western blotting was done utilizing the antibody His-probe (Santa Cruz Biotechnology, USA) at the concentration suggested by the manual. Protein bands were detected by chemiluminescence by ECL reagent (Amersham, USA) and quantified using the program Quantity One (Bio-Rad Laboratories, USA).

Statistical analysis. Each experiment (PG- PGIP assay) was performed at least five times. Data are reported as means. Statistical significance between samples was calculated using the Student's t-test. $P < 0.003$ was considered significant.

RESULTS AND DISCUSSION

In a previous paper, *P. vulgaris* PvPGIP2, in which glutamine 224 had been substituted by a lysine (Q224K), was reported to be affected in its inhibitory activity against FpPG (Leckie *et al.*, 1999). In order to assess whether PGIP is amenable to random mutagenesis to obtain improved variants, the PvPGIP2.Q224K mutated gene was chosen as a starting point for epPCR to generate a library of PGIP variants for the isolation of function-gaining inhibitors of FpPG. The signal peptide sequence and the stop codon of the *Pvpgip2* gene were removed and the ORF was fused in frame between the sequence encoding the yeast α -factor sequence for translocation in the ER and the sequence encoding the histidine tag (6His) present in the expression vector pGAPZ α A. The resulting plasmid was subjected to site-directed mutagenesis to obtain the plasmid pGAPpgip2Q224K. This was introduced into *P. pastoris* and the inhibitory activity of the expressed protein was tested by the agar diffusion

assay against FpPG as well as against PGs from *Aspergillus niger* (AnPGII), *Colletotrichum lupini* (CLPG), *Botrytis cinerea* (BcPG) *Fusarium graminearum* (FgPG) and *Fusarium verticillioides* (FvPG), in parallel with the wild type PGIP2 (PGIP2wt) (Table 1).

The gene *pgip2*Q224K was then used as a template for epPCR and the amplified DNA was used to obtain an *E. coli* library (about 2×10^6 independent clones) in pGAPZ α A. The average mutation frequency of the *pgip2*Q224K gene was 5.4 ± 3.6 mutations/kb of DNA as determined by sequencing 40 independent transformants. Substitutions were randomly distributed along the gene without apparent preferential sites; in some cases (8%) single-base deletions were observed. It was decided to introduce a low number of mutations because preliminary experiments had shown that a high number of mutations causes a large proportion of transformed *P. pastoris* colonies with no expression of PvPGIP2Q224K, as determined by Western blot analysis using an anti-His antibody (data not shown). This is likely due to the instability and degradation of highly mutated PvPGIP2 and is in agreement with the notion that the mutation rate of *in vitro* evolutionary approaches should be decided on the basis of the evolvability degree of the specific protein, i.e. the ability of a protein to adapt in response to mutation and to selective pressure (Romero and Arnold, 2009).

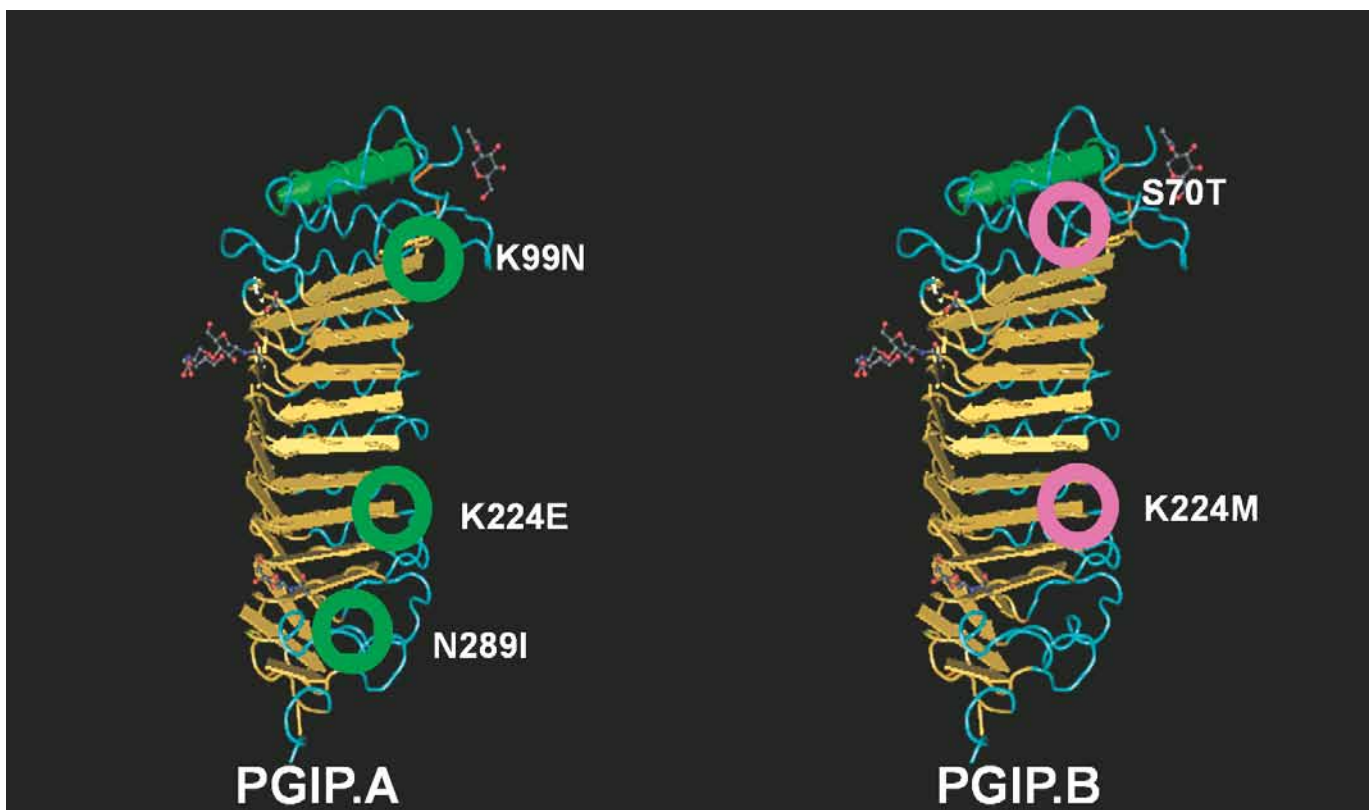


Fig. 1. Tridimensional structure of PGIP. Green and purple circles indicate the mutated sites found in PGIP.A and PGIP.B, respectively.

Table 1. Inhibitory activities^a of native and mutated PGIP2 variants.

	PGIP2wt	PGIP2.Q224K	PGIP.A	PGIP.B	PGIP2.Q224E
AnPG	0.4	15*	0.4	0.9*	0.4
FpPG	1.3 ^b	∞*	1.5	7.5*	1.5
CIPG	2.1 ^b	2.5	1.3*	3*	1.1*
BcPG	1.2 ^b	4.0*	1.1	2.5*	1.0
FgPG	1.2	3.4*	0.9	3*	0.8
FvPG	∞	∞	∞	∞	∞

^aValues indicate the amount (in ng) of PGIP that determines 50% inhibition of 1 agarose plate unit of PG. The symbol ∞ indicates >600 ng. The asterisk indicates cases where inhibitory activities of PvPGIP2 and the mutated variant are significantly different ($P < 0.003$).

^bThe apparent discrepancy between these values and those reported in previous articles (D'Ovidio *et al.*, 2004) is due to the different enzyme preparations used in this work (for details see Materials and Methods).

The plasmid DNA purified from the bacterial library was linearized with *AvrII* and used to transform *P. pastoris* by electroporation (Wu and Letchworth, 2004). An *in vitro* high-throughput screening method was developed, based on the detection of the inhibitory activity secreted by single transformed *P. pastoris* colonies. The colonies were grown for 4 days on YTGA medium containing polygalacturonic acid. A PG solution was poured into the YTGA Petri dish and the enzyme incubated overnight at 28°C. HCl 6 M was used as a detector of enzyme activity; a typical dark spot, due to the absence of substrate degradation, appeared around the colonies producing an active PGIP. Upon screening 3×10^3 clones, two colonies showing FpPG inhibition were isolated. Sequencing of the insert showed that the proteins secreted by both colonies had a missense mutation on residue 224, with two different amino acids introduced, i.e. glutamic acid and methionine. The mutant proteins, named PGIP.A and PGIP.B, had two (K99N and N289I) and one (S70T) additional substitutions, respectively (Fig. 1). Each mutated protein was purified and used for further characterization. Although PGIP.B showed activity against FpPG, it was less active than PvPGIP2. On the contrary, PGIP.A had fully recovered activity against FpPG and an improved inhibition capability against CIPG (Table 1).

Next, we investigated by site-directed mutagenesis whether the amino acid substitution at site 224 (Q224E) was the main responsible for the improved PGIP inhibition features. The inhibitor carrying glutamic acid at position 224 (PGIP2.Q224E) had recovered activity against FpPG (Table 1). This result, together with the observation that a variant carrying an alanine at site 224 shows nearly wild type activity, including the ability to inhibit FpPG (Casasoli *et al.*, 2009), supports the view that the requirement for the interaction with FpPG might be the absence of a lysine at position 224, rather than the presence of a glutamine. Position 224, rather than representing a contact point, is likely to negatively

affect the interaction when occupied by a lysine, because of charge repulsion with the nearby lysine at position 225 and possibly with positive charges in the partner PG. Notably, a natural PGIP2 variant of genotype BAT93 that carries a 3-amino acid deletion comprising both 224 and 225 sites shows no inhibition against FpPG (Farina *et al.*, 2009). Interestingly, both PGIP.A and PGIP2.Q224E had a better recognition capability against CIPG, indicating site 224 as a primary variability spot for improving PGIP inhibition. However, none of the natural PGIP variants so far analysed shows a glutamic acid or a methionine at site 224 although this site is characterized by a high variability (De Lorenzo and Ferrari, 2002; Casasoli *et al.*, 2009).

A second library of variants was generated starting from the wild-type *PvPpgip2* gene (2×10^6 independent clones and a frequency of 5 mutations/fragment). Although a total of 6×10^4 transformants were screened for gain of inhibition of FvPG; no positive colonies were found. This suggests that a single nucleotide mutation is not sufficient for PGIP2 to acquire the capability to inhibit FvPG, probably due to the complexity of the interaction.

In other PG-PGIP combinations this complexity is clearly reflected by the different mechanism of inhibition (competitive, non-competitive, mixed), the different areas of the enzyme recognized by the inhibitor and the different sets of contacts required to stabilize the complex (Casasoli *et al.*, 2009). It is therefore difficult to generate gain of function inhibitors unless, like in the case of lysine 224, a repulsive action is targeted. Preventing an interaction is easier than generating one. A valid alternative to improve the recognition capabilities of PGIPs may be represented by DNA shuffling. This technology allows to exchange large functional domains of sequences, thus mimicking and accelerating the process of sexual recombination during natural evolution (Stemmer, 1994). To perform a successful DNA shuffling, genes encoding the bean *prip* family (D'O-

vidio *et al.*, 2006) or members belonging to different gene families may be mixed, fragmented into random size pieces and reassembled into full-length genes *via* self-priming PCR and extension. This process may yield crossovers carrying different PGIP contact points that can be analyzed in the *P. pastoris* expression system.

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INFLUENCE OF THE QUANTITY AND VARIABILITY OF *CITRUS TRISTEZA VIRUS* ON TRANSMISSIBILITY BY SINGLE *TOXOPTERA CITRICIDA*

Y. Zhou¹, C.Y. Zhou¹, X.F. Wang¹, Y.Q. Liu¹, K.H. Liu¹, Q. Zou¹, Y. Xiang² and Z.A. Li¹

¹ National Citrus Engineering Research Center, Citrus Research Institute, Southwest University, 400712 Chongqing, China

² Division of Plant Quarantine, National Agriculture Technology Extension and Service Center, Ministry of Agriculture, 100026 Beijing, China

SUMMARY

Citrus tristeza virus (CTV) is transmitted in a semi-persistent manner by several aphid species, among which *Toxoptera citricida* is most efficient. After 24 h acquisition access period, eight CTV isolates were detected in single *T. citricida* with frequency of 63.3-91.1% and 71.1-91.1% by nested RT-PCR and real-time RT-PCR, respectively. More copies of CTV-targets were detected in single viruliferous *T. citricida* individuals that acquired high transmissibility CTV isolates. Analysis by single strand conformation polymorphism (SSCP) of viruliferous *T. citricida* individuals that had acquired CTV isolates ST9, GS14, CT11, HH12, and of CTV-infected receptor plants showed that most of the CTV-infected receptor plants and individual of viruliferous aphids carried one CTV haplotype, and that CTV in the receptor plants showed less haplotype diversity than that in aphids.

Key words: CTV, aphid transmissibility, quantity and variability of CTV, epidemiology, SSCP.

INTRODUCTION

Citrus tristeza virus (CTV), the agent of one of the most destructive citrus diseases in the world, induces a variety of symptoms among which stem pitting, decline and small fruits (Bar-Joseph *et al.*, 1989). Economic losses caused by CTV have been recorded in some areas of China (Zhao *et al.*, 1979; Zhou, 1997), and the virus has become more harmful to Chinese citrus production in recent years (Xu *et al.*, 2006; Zhou *et al.*, 2007). Tolerant rootstocks are extensively used to control tristeza decline syndrome, and mild strain cross protection (MSCP) has played an important role in controlling stem pitting in grapefruit and sweet orange (Lin *et al.*, 2002; Powell *et al.*, 2003).

CTV is disseminated by grafting and some aphid species in a semi-persistent manner. *Toxoptera citricida* (Kirkaldy) is the most efficient vector (Bar-Joseph *et al.*, 1989). It can spread different CTV strains, including those occasionally transmitted by other aphid species (Rocha-Pena *et al.*, 1995). *T. citricida* used to be widely distributed in southern China, southeast Asia, South America, and Africa (Bar-Joseph *et al.*, 1989), but has recently spread to Caribbean, Central America and Florida, breaking down cross protection in grapefruit and sweet orange (Powell *et al.*, 2003).

As to molecular interactions between CTV and aphid vectors, Cambra *et al.* (2000) found that there was a correlation between RT-PCR detection of CTV in single *Aphis gossypii* individuals and transmission rate to Mexican lime seedling. Thereafter, Satyanarayana *et al.* (2001) suggested that CTV quantification in the aphids is important to assess its epidemiology. Moreno *et al.* (2009) found that the amount of *Plum pox virus* (PPV) plays a role in aphid transmissibility. The availability of more sensitive virus detection techniques in plant and vectors, now facilitates the study of relationships between virus titre in the aphids and transmission efficiency.

In this study, real-time RT-PCR was used to estimate if the number of CTV targets in single *T. citricida* individuals had a bearing on transmission efficiency, and the level of CTV variability during single aphid transmission was analyzed by single strand conformation polymorphism (SSCP).

MATERIALS AND METHODS

Maintenance of CTV. CTV isolates used in this investigation are part of a collection kept at the Citrus Research Institute in Chongqing. Isolates SS7, HH12, GS14 and HB1 are severe and cause stem pitting in Symons sweet orange (*Citrus sinensis*), Feng-huang pummelo (*C. grandis*) and Duncan grapefruit (*C. paradisi*), whereas CT11, ST9, HH3 and LJ1 are mild isolates that do not induce apparent symptoms in the above hosts. All these isolates were graft-inoculated onto Jincheng sweet orange (*C. sinensis*) seedlings in the greenhouse at 15 to 27°C. Virus infection was con-

Corresponding author: Z.A. Li
Fax: +86.23.68349592
E-mail: zhongan369@yahoo.com.cn

firmed 90 days post inoculation by direct tissue blot immunoassay (DTBIA) (Garnsey *et al.*, 1993).

Single aphid transmission. CTV-free *T. citricida* colonies were established as reported by Broadbent *et al.* (1996) and maintained on young flushes of healthy Jincheng sweet orange seedlings in insectaries. CTV-infected Jincheng seedlings were used as donor plants to feed 100 to 200 CTV-free apterae adult aphids. After 24 h acquisition period, the aphids were placed singly on young flushes of CTV-free Jincheng seedlings for 24 h. Receptor plants were then transferred into an insect-proof greenhouse at 18 to 25°C.

Thirty apterae adult aphids were used to transmit each CTV isolate in single aphid transmission tests. Transmissions were repeated three times to insure consistency in transmission rate. Four months later, the receptor plants were tested by DTBIA (Garnsey *et al.*, 1993).

RNA extraction. Total RNA was extracted from donor plants and individual aphids which had fed on CTV-infected donor plants for 24 h by Trizol reagent (Invitrogen, USA). RNA extracts were resuspended in 25 µl of RNase-free water and treated with RNase-free DNase (TaKaRa, Japan). After single aphid transmission of isolates ST9, GS14, HH12 and CT11, total RNAs from infected receptor plants were extracted as reported by Zhou *et al.* (2001). All RNA extracts were stored at -80°C.

Detection and characterization of a conserved 3' UTR fragment from CTV isolates in aphids and receptor plants. Nested PR-PCR amplification was done according to Olmos *et al.* (1999). Nested PR-PCR products of CTV RNA from single aphids that had acquired isolates ST9, GS14, HH12, CT11 and from CTV-infected receptor plants were examined by SSCP analysis in 8% polyacrylamide gel at 4°C and 200 V for 3 h, as

described (D'Urso *et al.*, 2000).

Sequence analysis. Twenty nested RT-PCR products of each particular SSCP pattern were transformed into the vector PMD-18T (TaKaRa, Japan) and cloned into *E. coli* JM-109. Five clones of each RT-PCR products were custom sequenced (Shengong, China)

Standard curves. The cDNA used as template for *in vitro* transcription was obtained by RT-PCR with primer PM198R and PM261F that includes the T7 promoter sequence at its 5' terminus (Ruiz-Ruiz *et al.*, 2007). RT-PCR products were transcribed *in vitro* with T7 RNA polymerase (Promega, USA), and the transcripts were purified with Transcript RNA Clean Up Kit (TaKaRa, Japan). The concentration of transcripts was estimated twice with a NanoDrop ND-1000 UV Spectrophotometer (Thermo Scientific, USA). Ten-fold serial dilutions were prepared using RNA extracts (10 ng/µl) from healthy citrus, and stored at -80°C. Dilutions from 10⁹ to 10² were employed to generate standard curves.

CTV detection by real-time RT-PCR. Real-time RT-PCR with and without reverse transcriptase were run in parallel, to ensure the absence of DNA template in transcript preparations. The RNA extracts from *T. citricida* and CTV donor plants were tested in the iCycler iQ platform (Bio-Rad, USA) with primers PM261F/PM198R targeting conserved sequence in CTV ORFs 1b (Ruiz-Ruiz *et al.*, 2007).

Statistic analysis. The percentages of viruliferous *T. citricida* that acquired different CTV isolates were statistically analyzed using the generalized linear model (McCullagh *et al.*, 1989). Differences among quantitative CTV levels in single *T. citricida* individuals were calculated with the one-way ANOVA method of the SPSS13.0 package (Bertolini *et al.*, 2008).

Table 1. Detection of CTV in the receptor plants after single aphid transmission.

Isolates	Pathogenicity	Transmissibility	Mean transmissibility (%)	Isolates	Pathogenicity	Transmissibility	Mean transmissibility (%)
SS7	Severe	7/30 ^a	22.2	CT11	Mild	11/30	37.8
		8/30				11/30	
		5/30				12/30	
HH12		15/30	47.8	ST9		2/30	8.89
		15/30				3/30	
		13/30				3/30	
HB1		9/30	28.9	HH3		7/30	27.8
		10/30				9/30	
		7/30				9/30	
GS14		0/30	2.22	LJ1		0/30	1.11
		1/30				0/30	
		1/30				1/30	

^aNumerator = number of plants infected; denominator = number of test plants used.

Table 2. Detection of CTV in the donor plants by real-time RT-PCR.

Transmissibility	High					Intermediate	Low	
CTV isolate	HH12	CT11	HB1	HH3	SS7	ST9	GS14	LJ1
Ct \pm S.D ^a	20.6 \pm 0.8	21.3 \pm 0.6	22.1 \pm 0.8	21.8 \pm 0.5	21.9 \pm 0.4	21.5 \pm 0.9	19.9 \pm 0.3	21.7 \pm 1.0
Mean number of CTV targets	621,420,766	542,414,268	474,226,829	497,625,047	487,255,109	520,862,377	692,576,873	497,825,509

^a Average threshold cycle and standard deviation.

RESULTS

Single aphid transmission. The single aphid transmission rate of different CTV isolates ranged from 1.1 to 47.7% (Table 1). The average transmission rate of severe and mild CTV isolates was 25.3% and 18.9%, respectively, but this difference was not statistically significant ($P=0.892$). Based on the transmissibility by *T. citricida*, CTV isolates were classified into three groups with low (0-5%), intermediate (6-15%) and high (over 16%) transmission rate (Yokomi *et al.*, 2010). Most of our CTV isolates fell in group three (high transmissibility).

CTV detection in aphids by nested RT-PCR. After 24 h acquisition period, CTV targets were detected in 533 out of 720 aphids (74.0%). No significant difference was found between transmissibility rate by *T. citricida* and percentage of viruliferous *T. citricida* that acquired the different isolates ($P=0.127$). Isolate GS14 had highest transmission rate (91.1% of the aphids were viruliferous), whereas with 63.3% of viruliferous aphids, isolate HH12 proved to be the least transmissible (Table 2).

Detection and quantification of CTV in donor plants and single aphid. To estimate the amount of CTV in donor plants, these were analyzed by real time RT-PCR, and the results showed the estimate number of CTV targets in donor plants ranged from 474,226,829 to 682,576,873 (Table 2). Detection rates obtained by real-time RT-PCR ranged from 71.1% to 91.1% (Table 3). When CTV isolates with high, intermediate and low transmissibility were acquired by *T. citricida*, the mean value was 2,984,953, 993,593 and 1,207,566 copies of CTV-targets in a single viruliferous aphid, respectively (Table 3, Fig. 1). According to the mean number of CTV-targets quantified, significant differences were observed when CTV isolates with high transmissibility were compared with those with intermediate ($P=0.0257$) and low transmissibility ($P=0.0412$). However, when low and intermediate transmissibility groups were compared, no significant difference was observed in the number of acquired CTV targets ($P = 0.783$).

SSCP analysis. Sequence analysis of five clones from each nested RT-PCR product showed that no more than four nucleotide difference was found among the same

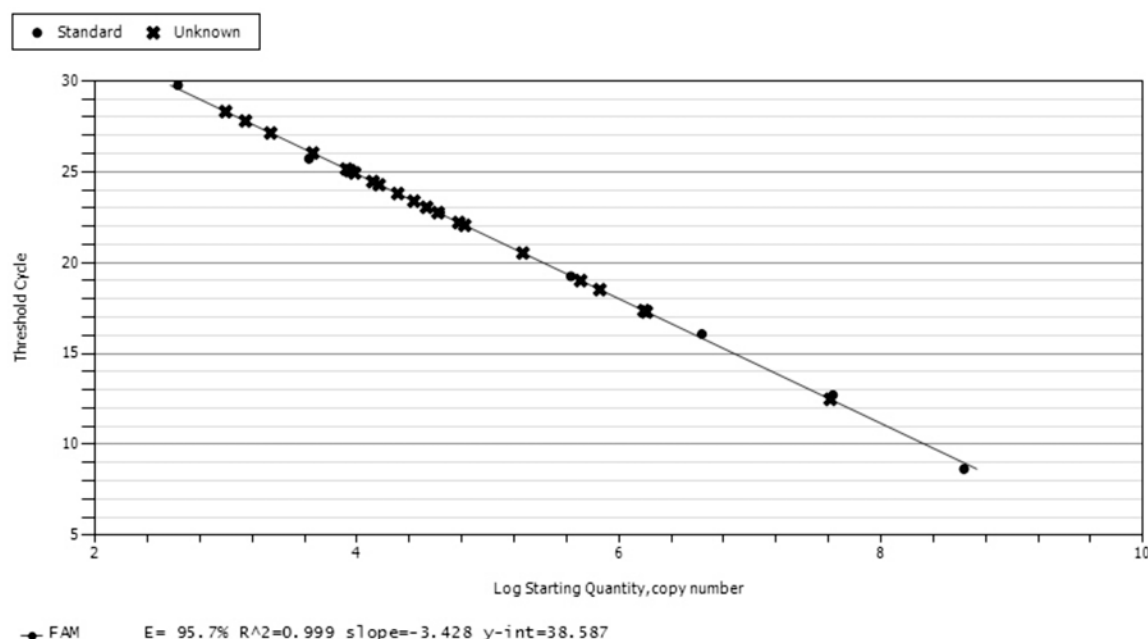


Fig. 1. Quantification range of the real-time assay based on the standard curve obtained with eight repetitions of 10-fold serial dilutions of CTV control transcripts.

Table 3. Detection and quantitation of CTV in *T. citricida* by nested RT-PCR and real-time RT-PCR.

Transmissibility	Isolate	Detection by nested RT-PCR		Detection by real-time RT-PCR		
		Number of positive aphids ^a	Detection rate (Mean \pm SE ^b)	Number of positive aphids ^a	Ct \pm S.D ^c	Number of CTV targets (mean \pm SE ^d)
High	HH12	18/30	0.7471 \pm 0.1415a	20/30	25.4 \pm 1.1	2,984,953 \pm 1,019,170a
		17/30		21/30		
		22/30		23/30		
	CT11	27/30		25.5 \pm 0.7		
		15/30			22/30	
		25/30			25/30	
	HB1	26/30		26.3 \pm 0.3		
		22/30			23/30	
		25/30			25/30	
	HH3	25/30		25.7 \pm 0.8		
		18/30			24/30	
		22/30			22/30	
SS7	17/30	26.8 \pm 0.5				
	22/30		23/30			
	26/30		25/30			
Intermediate	ST9	11/30	0.6567 \pm 0.2483a	22/30	28.5 \pm 1.1	993,593 \pm 278,637b
		24/30		21/30		
		24/30		25/30		
Low	GS14	28/30	0.8283 \pm 0.0998a	28/30	27.9 \pm 0.9	1,207,566 \pm 468,849b
		27/30		27/30		
		27/30		27/30		
	LJ1	24/30		28.5 \pm 0.7		
		20/30			22/30	
		23/30			25/30	
				24/30		

^a Numerator: number of viruliferous aphids; denominator: number of test aphids used.

^b Means followed by different letters are significantly different using the generalized linear model statistical analysis.

^c Average threshold cycle and standard deviation.

^d Means followed by different letters are significantly different using a one-way ANOVA after transforming the response variable by the natural logarithm statistical analysis.

Table 4. SSCP analysis of CTV isolates.

Haplotypes of CTV isolates	ST9 Aphids ^a	Plants ^b	GS14 Aphids	Plants	HH12 Aphids	Plants	CT11 Aphids	Plants
ST9I	20	8						
ST9II	3	0						
ST9III	29	0						
ST9I+III	7	0						
GS14I			11	1				
GS14II			14	0				
GS14III			13	0				
GS14I+II			21	1				
GS14I+III			23	0				
HH12I					34	30		
HH12II					5	3		
HH12III					3	0		
HH12I+II					10	8		
HH12I+III					5	2		
CT11I							45	34
CT11II							22	0

^a Number of CTV haplotype in a single viruliferous aphid.

^b Number of CTV haplotype in the receptor plants after single aphid transmission.

SSCP pattern, suggesting that the CTV isolates with the same SSCP patterns belong to the same haplotype. CTV from receptor plants showed less haplotype diversity than CTV from aphids, since most of the receptor plants and single viruliferous aphid contained one haplotype.

Compared with the severe isolates, mild isolates ST9 and CT11I could be more easily separated. When high transmissibility CTV isolates were moved by *T. citricida*, aplo-types HH12I and CT11I were more readily transmitted to the receptor plants than others (Table 4, Fig. 2).

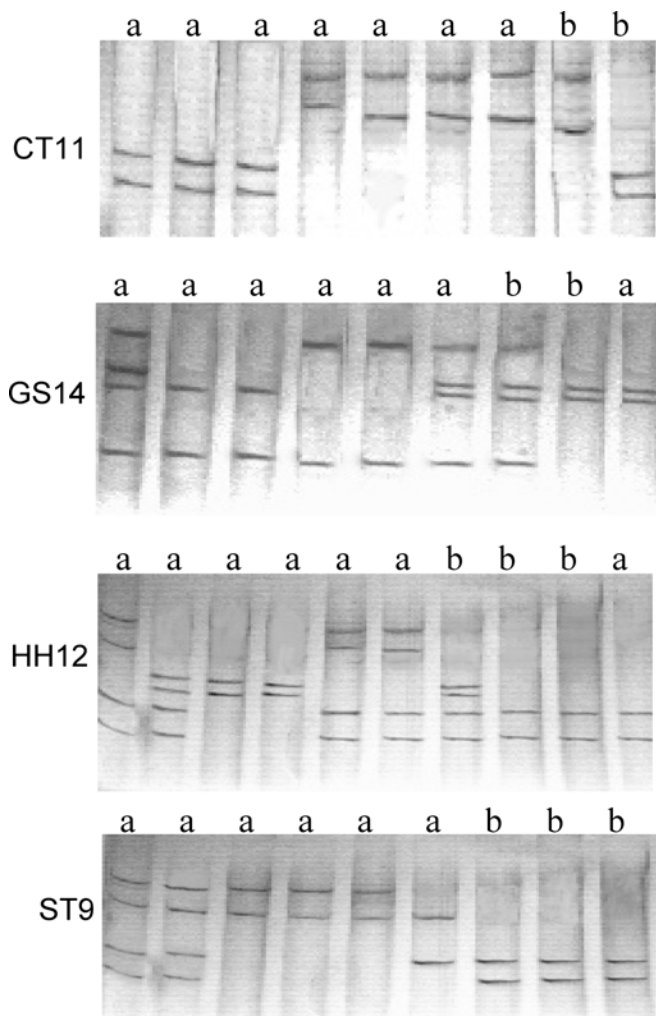


Fig. 2. SSCP patterns of virus variants of CTV isolates CT11, GS14, HH12 and ST9. a: SSCP patterns of virus variants carried by single *T. citricida*; b: SSCP patterns of CTV aphid-transmitted sub-isolates in the receptor plants.

DISCUSSION

In this study, although there was a clear trend towards a higher transmissibility of severe CTV isolates, differences between transmission rates of severe and mild isolates were not statistically significant. However, severe CTV isolates could spread quickly in the field (Sharma, 1989), thus sub-isolates of aphid transmission need to be further studied by indexing.

Previous studies on CTV, *Potato leaf roll virus* (PLRV) and *Barley yellow dwarf virus* (BYDV) indicated that the percentage of viruliferous migrant aphids is one of the major factors influencing virus epidemiology (Singh *et al.*, 1995; Cambra *et al.*, 2000; Fabre *et al.*, 2003).

In this study, all aphids were analyzed by techniques, such as nested and real-time RT-PCR, that allow CTV detection also in samples testing negative by other detection methods (Olmos *et al.*, 1999; Bertolini *et al.*, 2008). The results showed that more than 71.1% of *T.*

citricida individuals were viruliferous after 24 h acquisition access period and that there was no significant difference between the transmission rate by *T. citricida* and the percentage of viruliferous aphids that had acquired different CTV isolates. Cambra *et al.* (2000) reported that, although there was a correlation between CTV detection in *A. gossypii* individuals by PCR and its transmission to Mexican lime seedling, CTV isolates were still detected consistently in *T. citricida*, *A. spiraeicola*, *T. aurantii*, *A. nerii* and *Hyalopterus pruni*, regardless of their transmissibility. Furthermore, Moreno *et al.* (2009) showed that 88.5% of tested aphids inoculated *Plum pox virus* (PPV) to receptor plants, whereas PPV infection rate was only 20%. These results indicate that it is the amount of virions involved in aphid transmission rather than the percentage of viruliferous aphids that has a bearing on transmission efficiency.

Escriu *et al.* (2000) reported that the efficiency of transmission of *Cauliflower mosaic virus* (CMV) depended on the virus titre in the plants, and a correlation between virus accumulation and transmissibility with a possible plateau was positive when virus concentration reached 500 µg/g of leaf or higher. Previous studies indicated that an aphid could transmit more efficiently when it fed on CTV-infected Mexican lime, because the CTV titre in this host is higher than that in other citrus plants (Marroquin *et al.*, 2004). In this study, the possible interference from the abundant subgenomic RNAs and defective RNAs in assessing CTV titre was excluded by targeting ORF1b (Ruiz-Ruiz *et al.*, 2007). However, no obvious relationship between transmissibility and CTV accumulation in the donor plant was found and the amount of CTV in different donor plants was similar.

Moreno *et al.* (2009) reported that the amount of PPV inoculated in a plant by a single aphid could influence the frequency of infection. For CTV, however, little information is available on the influence of the number of virus particles acquired by a single aphid on the efficiency of transmission (Cambra *et al.*, 1981; Saponari *et al.*, 2008). In our study, CTV isolates with high transmissibility were present with a much higher number of particles in single *T. citricida* individuals than isolates with a lower transmissibility, suggesting that the quantity of CTV copies acquired by the aphid determines transmission efficiency. This finding may have potential value for explaining the epidemiological behaviour of CTV in regions where annual epidemics are caused by migrant viruliferous aphids. Furthermore, in other studies, the number of virions required for effective infection and the aphid behaviour were both involved in virus acquisition and inoculation processes as previously reported (Soosaar *et al.*, 2005; Moreno *et al.*, 2009).

Nolasco *et al.* (2008) found that almost all CTV haplotypes from a donor plants were present in the aphids, some of which carried more than one haplotype, although 54% of the aphids carried similar haplotypes

with very low nucleotide diversity. Because of its conservation, the CTV 3' UTR region is useful for assessing changes in viral populations. So in this study, nested RT-PCR amplicons of the 3' UTR region were used by SSCP and sequencing for further analysis of the genetic structures of CTV populations during aphid transmission. Results indicated that some aphids carried a viral content distinct from that of other aphids, and that the majority of aphids acquired one haplotype of CTV.

We were unable to detect some CTV haplotypes in receptor plants. This may taken as an indication that CTV isolates contain some non-transmissible components, or that the transmission rate of some components is too low to be detected with the number of replications used in our study (Bar-Joseph *et al.*, 1989; Rocha-Pena *et al.*, 1995). A further step toward improving real-time RT-PCR with specific primers or probes may be used to detect the different CTV haplotypes, so as to provide a better understanding of the relationship between viral genotype and its transmission.

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GENETIC CHARACTERIZATION OF SYRIAN *ERWINIA AMYLOVORA* STRAINS BY AMPLIFIED FRAGMENT LENGTH POLYMORPHISM TECHNIQUE

H. Ammounh, M.I.E. Arabi, A. Shoaib, M. Rajeh and A. Al-Daoude

Department of Molecular Biology and Biotechnology, AECS, PO Box 6091, Damascus, Syria

SUMMARY

Thirty *Erwinia amylovora* strains, collected from the main rosaceous crop-growing regions of Syria, were chosen as representatives of all major pathogenicity groups and were genetically studied by AFLP. Eight primer combinations were utilized and approximately 300 scorable bands in total were generated. Based on similarity coefficient, *E. amylovora* strains were placed into a main cluster containing two sub-clusters, indicating very low genetic variations among the studied pathogen. The existence of two plasmids, pEA29 (present in nearly all *E. amylovora* isolates) and pEL60 (present mainly in Lebanese strains), was confirmed using multiplex PCR in all tested Syrian *E. amylovora* strains, indicating that Lebanese and Syrian isolates may share a common origin.

Key words: fire blight, AFLP, genetic characterization, plasmid, survey.

INTRODUCTION

Fire blight caused by *Erwinia amylovora* (Burrill Winslow *et al.* is the most destructive disease of many rosaceous plants including apple (*Malus domestica*), pear (*Pyrus communis*) and quince (*Cydonia oblonga*) (Van der Zwet and Keil, 1979). Susceptible plants can be severely damaged or killed in nurseries, commercial orchards and landscape plantings (Vanneste, 2000). A survey of all major Syrian pome fruit-growing regions conducted in 2005 and 2006 allowed the establishment of a collection of *E. amylovora* strains from different hosts (quince and pear) and geographic locations (Ammounh *et al.*, 2008).

E. amylovora is a genetically homogenous species, as established by biochemical, serological and host range studies with a few exceptions (Vantomme *et al.*, 1986;

Beer *et al.*, 1996; Momol and Aldwinckle, 2000). Several molecular marker techniques currently available allow a more precise assessment of genetic diversity than morphological and biochemical properties (Rademaker *et al.*, 2000; Ruppitsch *et al.*, 2006; Donat *et al.*, 2007). Among these, AFLP analysis is a high resolution technique used for determining the taxonomic diversity and phylogenetic structure of bacterial population including plant pathogens (Restrepo *et al.*, 1999; Savelkoul *et al.*, 1999; Rico *et al.*, 2004). AFLP markers do not require a priori knowledge of DNA sequences and can detect all types of polymorphism. It performs better than a series of other molecular marker techniques (Vos *et al.*, 1995; Barionovi *et al.*, 2006).

Nearly all naturally occurring strains of *E. amylovora* contain the non-transmissible plasmid pEA29 or pEA28 (Falkenstein *et al.*, 1989; Vanneste, 2000; Barionovi *et al.*, 2006). pEA29 has received considerable attention because it confers some traits advantageous to the bacterium and its loss leads to reduced virulence (Falkenstein *et al.*, 1989). PCR-based detection of pEA29 is the most commonly used method for the detection and identification of *E. amylovora* and is based on amplification using primers within the 1.1Kb *Pst*I fragment (MacManus and Jones, 1995; Llop *et al.*, 2000; Ammounh *et al.*, 2008). In contrast, knowledge of other less widely occurring plasmids in *E. amylovora* is quite limited. Accordingly, the aims of this study was to deploy AFLP technique to determine the relatedness of 30 *E. amylovora* isolates representing the major pathogenicity groups found in Syria and to examine the plasmid content of these tested isolates.

MATERIALS AND METHODS

Bacterial isolates. It is noteworthy that in Syria, fire blight disease prevail in only three isolated foci in regions with warm and humid climates (Al-Zabadani area, Zarzar Lake, Kafer Hor and Zaher AlJabal). Consequently, the thirty *E. amylovora* isolates chosen for this study were obtained from all these locations that are in a close vicinity to each other and less than 10 Km from the Lebanese border (Ammounh *et al.*, 2008). All iso-

Table 1. Sequences of oligonucleotide adapters and primers used in the pre-amplification step and the selective AFLP primer combinations.

Name	Reaction	Code	Sequence
<i>Eco</i> RI adapter	Ligation		5'-AATTGGTACGCAGTCTAC-3'
			3'-CCATGCGTCAGATGCTC-5'
<i>Mse</i> I adapter			5'-TACTCAGGACTCAT-3'
			3'-GAGTCCTGAGTAGCAG-5'
<i>Eco</i> RI	Pre-amplification	E	5'-GACTGCGTACCAATTC3'
<i>Mse</i> I		M	5'-GATGAGTCCTGAGTAA3'
<i>Eco</i> RI + A	Selective amplification	E-A	5'-GACTGCGTACCAATTC A-3'
<i>Eco</i> RI + G		E-G	5'-GACTGCGTACCAATTC G-3'
<i>Eco</i> RI + C		E-C	5'-GACTGCGTACCAATTC C-3'
<i>Eco</i> RI + T		E-T	5'-GACTGCGTACCAATTC T-3'
<i>Mse</i> I + C		M-C	5'-GATGAGTCCTGAGTAA C-3'
<i>Mse</i> I + T		M-T	5'-GATGAGTCCTGAGTAA T-3'
<i>Mse</i> I + A		M-A	5'-GATGAGTCCTGAGTAA A-3'
<i>Mse</i> I + G		M-G	5'-GATGAGTCCTGAGTAA G-3'

lates were grown on sucrose nutrient agar (SNA) plates (Billing *et al.*, 1961) and incubated at 26±1°C until bacterial colonies developed.

Isolation of total DNA. Bacterial strains were grown overnight at 26±1°C in 10 ml LB, centrifuged and the resulting pellets lysed with lysozyme and SDS. DNA was purified by repeated extraction with phenol and chloroform-isoamylalcohol (24:1), and precipitated with ethanol. Concentrations were measured using Gene Quant (Pharmacia Biotech, Sweden) and working solutions of 10 ng/μl were made by dissolving DNA in sterile double distilled water.

PCR analysis. Multiplex PCR was performed using three primer pairs: (i) A (CGGTTTTTAACGCTGGG) and B (GGGCAAATACTCGGATT) for the amplification of a ~900 bp band [*Pst*I fragment of the plasmid pEA29, Bereswill *et al.*, (1992)]; (ii) AJ889 (GC-CGGGGCGTGGAACAGAAG) and AJ890 (TCAT-GCCGGAAGAGTCAAACC) for the amplification of a 483 bp band [*virB10* gene for the detection of pEU30, Foster *et al.*, (2004)]; (iii) AJ901 (GCCGGCCGCTGT-TACCGGGTTTG) and AJ902 (TCTGTGGTGC-CGCGTTGATGAAGC) for the amplification of 402 bp band [*parB* and *nuc* regions for the detection of pEL60, Foster *et al.* (2004)]. PCR reactions were carried out in a volume of 25 μl containing 100 ng of template DNA, 1% Tween 20, 0.2 mM each of dATP, dCTP, dGTP and dTTP (Promega, USA), 15-25 pmol of each primer, 2 mM MgSO₄, 3% dimethyl sulfoxide (DMSO) and 0.7 U of Taq polymerase (Promega, USA). A Bio-Rad T gradient thermocycler was programmed for 1 cycle of 5 min at 94°C followed by 37 cycles, each consisting of 1 min at 94°C, 2 min at 52°C, and 2 min at 72°C. The last cycle was followed by 15 min incubation period at 72°C. PCR products were separated on a

2% agarose gel, stained by ethidium bromide and photographed under UV light.

AFLP analysis. The AFLP protocol was as described by Vos *et al.* (1995) with a few modifications. Pre-amplification reaction was performed using E and M primers (*Eco*RI and *Mse*I) with no selective nucleotide at the 3' end to increase the number of amplified fragment. Oligonucleotide adapters and primers sequences used in this study are presented in Table 1. The pre-amplification was carried out in 8.5 μl volume containing 125 ng of DNA using the following cycling parameters: 20 cycles of 30 sec at 94°C and 40 sec at 56°C and 50 sec at 72°C. The pre-amplified DNA was diluted 10 times by adding double distilled H₂O, using 2.5 μl as a template for the consequent selective amplification in which *Eco*RI and *Mse*I primers with one selective nucleotide were employed. DNA was amplified as follows: 12 cycles with annealing temperatures from 68°C to 59.6°C (decreasing by 0.7°C in each cycle), then 23 cycles at 59°C annealing temperature. In each cycle, the denaturation and elongation steps were the same: 94°C for 30 sec and 72°C for 1 min, respectively. To assure reproducibility, AFLP analysis was repeated twice starting from restriction. PCR products were mixed with 4 μl loading buffer (98% formamide, 10 mM EDTA, 0.025% xylene cyanol, and 0.025% bromophenol blue) and denatured for 3 min at 95°C. AFLP fragments were separated on 6% polyacrylamide gel with 7 M urea, and 1x TBE buffer (12.1 g Tris, 5.11 g boric acid, 0.37 g EDTA). Gels were run at constant power (1500 V, 80 W, 100 mA, 3 h and 20 min), and visualized with silver staining (Silver Sequence kit, Promega, Cat. Q4132, USA) following the manufacturer's instructions. Glass plates were treated as described in the instructions replacing SigmaCote solution with Repeal-Silane (Pharmacia Biotech, Sweden).

Data analysis. For each primer pair, the number of polymorphic and monomorphic bands was determined. Bands clearly visible in at least one strain were scored (1 for the presence and 0 for the absence) and entered into a data matrix. Percentage of polymorphism was calculated as the proportion of polymorphic bands over the total number of bands. Allelic polymorphic information content (PIC) was calculated using the following formula:

$$\text{PIC} = 1 - \sum (\text{P}_{ij})$$

where P_{ij} is the frequency of the 1st pattern revealed by the 1st primer summed across all patterns revealed by the primers (Botstein *et al.*, 1980). Data for all eight different primer combinations were used to estimate the similarity among strains on the basis of the number of shared amplification products according to Nei and Li (1979) coefficient. Principal coordinate analysis (PCA) based on genetic distance matrix was performed using the DCENTER and EIGEN algorithms of the NTSYS-pc software package (Rohlf, 1993).

RESULTS

Eight AFLP primer combinations with one selective nucleotide were sufficient to produce 285 bands in total among which 236 bands were polymorphic (Table 2). PCA analysis with the entire AFLP data separated *E. amylovora* strains from *E. carotovora* strains and *E. coli* placing them in three distinct groups. The PIC values ranging from 0.10 to 0.15 between the 30 *E. amylovora* strains indicate very low dissimilarity. Such a low genetic variation at the DNA level indicates a possibly common origin of the tested Syrian strains as with the strains from Lebanon where fire blight was detected much earlier (Saad *et al.*, 1999). AFLP analysis could not differentiate isolates according to their host and location and were grouped in one main cluster (not shown). However, the

technique successfully revealed substantial genetic variability among *E. amylovora* strains. In contrast, Donat *et al.* (2007) reported that AFLP technique was able to group Spanish isolates of *E. amylovora* according to their geographical origin which might be due to multiple introductions of the pathogen in Spain from different European countries (Rico *et al.*, 2004).

As to the presence of plasmids, all tested isolates including the reference strain, *E. amylovora* L 4778 (positive control) but not other bacteria (*E. coli*, negative control) produced PCR products of the expected size (~0.9 and 402 bp) when primers A-B and AJ901 -AJ902 were used. However, all isolates failed to produce a pEU30-specific product (483 bp) when primers AJ889 and AJ890 were used, indicating the absence of this plasmid in the tested isolates.

DISCUSSION

Results presented in this study suggest that the population of *E. amylovora* in Syria is homogenous with PIC values ranging from 0.1 to 0.15 which are in general agreement with the findings of Manulis *et al.* (1998) who studied the genetic diversity of this pathogen in Israel. Contrary to expectations, isolates with different characteristics [host, sensitivity to streptomycin and virulence (Table 3)] were found to be genetically similar and thus grouped in the same cluster (not shown). Homogeneity of *E. amylovora* strains isolated from fruit trees was reported also in other works (McManus and Jones, 1995).

The presence of the pEL60 plasmid, known to be present mainly in Lebanese *E. amylovora* isolates, in all tested Syrian isolates was confirmed by multiplex PCR utilizing specific oligonucleotides (AJ901-AJ902) that amplify sequences comprising the 3' end of parB of this plasmid (Saad *et al.*, 1999; Foster *et al.*, 2004). Most of the *E. amylovora* strains studied were mainly obtained

Table 2. AFLP bands numbers, frequency and PIC^a revealed by eight primer pairs.

Primer pair	Total No. of scored AFLP bands	Mean frequency of scored bands	PIC
E-A x M-T	44	0.919	0.128
E-G x M-C	37	0.920	0.126
E-G x M-A	49	0.885	0.152
E-A x M-G	20	0.942	0.100
E-C x M-T	30	0.906	0.141
E-G x M-G	31	0.934	0.108
E-T x M-C	47	0.934	0.105
E-T x M-A	27	0.937	0.108
ALL	285	0.922	0.121

^aPIC = Polymorphism information content calculated according to Anderson *et al.* (1993).

from a Syrian region that neighbours the Lebanese village Maarabounne in which all studied isolates are streptomycin sensitive and harbour the pEL60 plasmid (Saad *et al.*, 1999). Thus, it is likely to suggest that *E. amylovora* isolates found in Syria and harbour the pEL60 plasmid came from neighboring countries, possibly Lebanon where the disease was detected much earlier

and isolates share common characteristics (Saad *et al.*, 1999; Foster *et al.*, 2004). Nonetheless, Foster *et al.* (2004) reported the presence of pEL60 in *E. amylovora* strains only from Lebanon, Canada, USA, France and New Zealand. Consequently, the validity of using this plasmid as a traceable marker for strains of the fire blight pathogen from possibly Lebanon to Syria would

Table 3. Codes, host, location, year of collection, virulence level, streptomycin resistance and copper sulfate tolerance of *E. amylovora* strains used in this study.

Strain	Host, place and year of isolation	Virulence level	Streptomycin resistance	Copper sulphate tolerance
EaSy11	Quince, Al-Zabadani, 2005	virulent	Sensitive	Tolerant
EaSy112	Quince, Zarzar lake, 2006	virulent	Sensitive	Susceptible
EaSy113	Quince, Zarzar lake, 2006	virulent	Sensitive	Susceptible
EaSy114	Pear, Zaher Al Jabal, 2006	virulent	Sensitive	Susceptible
EaSy14	Quince, Al-Zabadani, 2005	virulent	Highly resistant	Susceptible
EaSy16	Quince, Al-Zabadani, 2005	virulent	Resistant	Susceptible
EaSy17	Quince, Al-Zabadani, 2005	Weakly virulent	Sensitive	Highly tolerant
EaSy19	Quince, Al-Zabadani, 2005	virulent	Sensitive	Tolerant
EaSy2	Quince, Al-Zabadani, 2005	Weakly virulent	Sensitive	Highly tolerant
EaSy20	Quince, Al-Zabadani, 2005	virulent	Resistant	Susceptible
EaSy21	Quince, Al-Zabadani, 2005	Weakly virulent	Highly resistant	Susceptible
EaSy22	Quince, Al-Zabadani, 2005	Weakly virulent	Sensitive	Tolerant
EaSy23	Quince, Al-Zabadani, 2005	virulent	Resistant	Susceptible
EaSy24	Quince, Al-Zabadani, 2005	virulent	Sensitive	Tolerant
EaSy26	Quince, Al-Zabadani, 2005	Weakly virulent	Sensitive	Highly tolerant
EaSy27	Quince, Al-Zabadani, 2005	virulent	Sensitive	Susceptible
EaSy29	Quince, Al-Zabadani, 2005	Weakly virulent	Sensitive	Susceptible
EaSy30	Quince, Al-Zabadani, 2005	virulent	Sensitive	Susceptible
EaSy4	Quince, Al-Zabadani, 2005	virulent	Sensitive	Highly tolerant
EaSy47	Quince, Al-Zabadani, 2006	Highly virulent	Sensitive	Susceptible
EaSy50	Pear, Kafer Hor, 2006	virulent	Sensitive	Susceptible
EaSy51	Pear, Kafer Hor, 2006	virulent	Sensitive	Susceptible
EaSy52	Quince, Al-Zabadani, 2006	Highly virulent	Sensitive	Susceptible
EaSy59	Quince, Al-Zabadani, 2006	Highly virulent	Sensitive	Susceptible
EaSy66	Pear, Al-Zabadani, 2006	virulent	Sensitive	Susceptible
EaSy6	Quince, Al-Zabadani, 2005	Weakly virulent	Sensitive	Susceptible
EaSy8	Quince, Al-Zabadani, 2005	virulent	Highly resistant	Susceptible
EaSy80	Quince, Al-Zabadani, 2006	Highly virulent	Sensitive	Susceptible
EaSy85	Quince, Al-Zabadani, 2006	Highly virulent	Sensitive	Susceptible
EaSy9	Quince, Al-Zabadani, 2005	Weakly virulent	Sensitive	Highly tolerant
<i>Erwinia carotovora</i> (Ec)	Potato			
<i>Erwinia carotovora</i> (Ec)	Potato			
<i>E.coli</i> DH5 α	Commercial			

Al-Zabadani: A region 40 Km west of Damascus.

Kafer Hor: A region 40 Km south of Damascus.

Zarzar lake: A region 30 Km west of Damascus.

Zaher Al-Jabal: AlSewdaa province, 200 km south of Damascus.

be better supported if strains of *E. amylovora* from other countries with a long history fireblight, such as Turkey, Cyprus, Israel, Egypt and Jordan were also tested. Moreover, the existence of streptomycin resistant *E. amylovora* isolates that harbour the pEL60 plasmid in Syrian orchards could be explained by the illegal importation of infected plant materials and rootstocks from countries with a long history of streptomycin use such as Israel, possibly through Lebanon. This hypothesis has to be further assessed and evaluated using other approaches.

Although streptomycin could be used to control fire blight which is still sporadic in Syria, cautious plant management and quarantine measures, together with reliable disease detection and monitoring, may help minimizing its spread (Zhang and Geider, 1997).

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IMPLICATIONS OF PLANT MATERIAL ORIGIN, LAND USE HISTORY AND SOIL PROPERTIES IN THE INCIDENCE OF VERTICILLIUM WILT IN OLIVE GROVES

E. Rodríguez¹, J.M. García-Garrido¹, P. A. García² and M. Campos¹

¹Departments of Agroecology-Plant Protection and Soil Microbiology and Symbiotic Systems, Estación Experimental del Zaidín (CSIC), Profesor Albareda 1, 18008 Granada, Spain

²Department of Statistics and O.R. University of Granada, Campus de Fuente Nueva s/n, 18071 Granada, Spain

SUMMARY

The increased presence of Verticillium wilt (*Verticillium dahliae* Kleb.) in olive groves is often related to the use of infected propagation material and to the planting of new olive trees in contaminated soils. This study assessed the implications of plant propagation, land-use history and soil properties on disease prevalence in southern Spain, the most important olive-growing area worldwide. To this purpose, a large-scale sampling survey was carried out in this area, *V. dahliae* pathotypes were identified by PCR, and GIS was used to analyze soil properties and cropland-use history. Finally, multiple correspondence analysis was performed to show the statistical association between the variables taken into account. Results strongly indicated the potential risk of planting olive in valleys with irrigated cropland history, especially those that had hosted herbaceous crops, highlighted the importance of using pathogen-free certified planting material as a key component for a successful disease management, and confirmed the role played by saline, alkaline, and steep-slope soils in enhancing *V. dahliae* prevalence.

Key words: defoliating *Verticillium* pathotypes, land-use, nondefoliating pathotypes, *Olea europaea*, soil, disease management.

INTRODUCTION

Verticillium wilt (VW), caused by the soil-borne fungus *Verticillium dahliae* Kleb., is a disease affecting a wide variety of host plants (Harris, 1998), and is considered the most important olive disease worldwide (Jiménez-Díaz *et al.*, 1998).

Soils pathogen-contaminated by previous crops or inter-cropped with susceptible hosts are a major factor for *V. dahliae* occurrence (Cirulli, 1981; Tjamos, 1993;

Blanco-López and Jiménez-Díaz, 1995; Serrhini and Zerroual, 1995; Naser and Al-Raddab, 1998). In these soils the pathogen can survive for long time (in excess of 10 years) thanks to the microsclerotia produced in dying tissues of the host plant, which represent the main inoculum source for VW development (Wilhelm, 1955; Schnathorst, 1981). Therefore, the knowledge of land-use history can help estimating the risk of disease outbreaks in any given plot to be planted with olive. Since the use of infested plant propagation material determines the appearance of VW in pathogen-free soils, nurseries may have been instrumental in spreading accidentally the highly virulent *V. dahliae* pathotype (Jiménez-Díaz *et al.*, 1998; Nigro *et al.*, 2005).

According to their virulence to cotton and olive, *V. dahliae* isolates can be classified as highly virulent defoliating (D) or non-defoliating (ND) pathotypes, depending on their ability or not to induce leaf shedding (Schnathorst and Sibbet, 1971; Schnathorst, 1973). The D pathotype was first reported in south-western Spain in an area of intensive cotton cropping (Bejarano-Alcázar *et al.*, 2001), and in the last 5-10 years has spread to distant pathogen-free olive-growing areas in south-eastern Spain. Currently, spreading of the D pathotype is a very serious threat to olive crops since no resistant cultivars are available, and control measures are not efficient (Bejarano-Alcázar *et al.*, 1996; López-Escudero and Blanco-López, 2001; Navas-Cortés *et al.*, 2001; Rodríguez *et al.*, 2009). Knowing the origin of plant propagation material would allow a better understanding of the spread and spatial patterns of the highly virulent pathotype for improved management and protection programs of olive groves.

Soil properties have a pronounced effect on disease spread and development. Tenuta and Lazarovits (2004) found that effectiveness of nitrogenous amendments (meat and bone meal) is related to soil properties such as organic carbon content and pH. Addition of meat and bone meal to a sandy soil killed microsclerotia but had no effect in loamy soil. Overall, VW is more of a problem in neutral or alkaline soils, rather than acid soils (Pegg and Brady, 2002), but there is no information on a soil-specific effect on VW in olive groves. Such knowledge would be vital to identify the factors

involved in wilt development and to predict which soils facilitate VW occurrence.

In this study, a large-scale sampling survey was carried out to assess the implications of the origin of plant propagation materials, land-use history and soil properties for VW prevalence in olive groves. GIS was employed to identify agricultural land-use history and soil properties impacting on disease risk. PCR assays were used to identify *V. dahliae* pathotypes and multiple correspondence analysis was done to analyze the patterns of relationships of categorical variables with pathogen prevalence.

MATERIAL AND METHODS

Study area. The study was carried out in Granada province (Andalusia, south-east Spain) where olive is the most extensive crop, covering a surface of 175,000 ha (Fig. 1). Olive is characterized by a high degree of heterogeneity, production regions differing greatly from one another for physical characteristics of the groves, management practices, socioeconomic situations and environmental factors (Rodríguez *et al.*, 2008, 2009).

Many studies blame irrigation as a cause of VW spread (Cirulli, 1981; Blanco-López *et al.*, 1984; Al-Ahmad and Mosli, 1993; Serrhini and Zeroual, 1995; Rodríguez *et al.*, 2009). Due to the heterogeneity of different olive regions and use of irrigation, we designed a stratified double-sampling technique to determine the number of olive groves needed to make an accurate estimation of the disease (Rodríguez *et al.*, 2009). Surveys were conducted in 873 olive groves, covering an area of 4,087.2 ha and comprising 527,903 olive trees with a standard error $\leq 3.3\%$ (95% confidence interval).

From 2002 to 2005 a questionnaire was distributed to randomly chosen farmers with queries on the agro-nomic features (Rodríguez *et al.*, 2008), origin of plant propagation materials, previous cropping history and symptoms present in their plantations. To reduce bias from farmer's subjectivity, all groves with VW symptoms were visited directly to validate survey results.

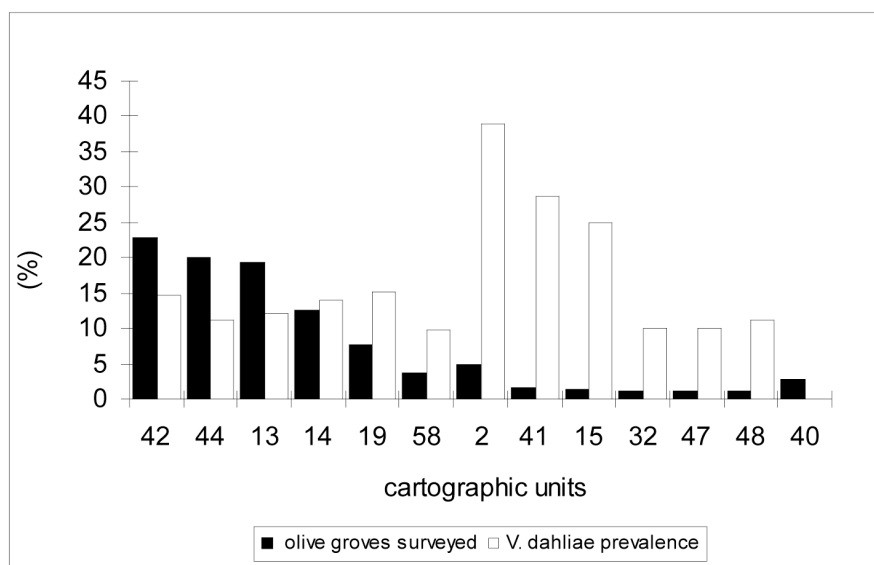
Isolation of *V. dahliae* from olive trees. Samples were collected from March to June and from September to November, the most appropriate time for detecting *V. dahliae* presence within plants (Levin *et al.*, 2003). Olive trees with wilt symptoms were inspected, counted and sampled. Branches and stems from at least 10 affected trees were collected, and analysed in the laboratory. Twenty-four small internal fragments (~ 1 cm²) of vascular tissue from different affected branches of each tree were surface-sterilized with NaClO (10%) for 1 min, rinsed with sterile water for 1 min, and placed in Petri plates in water agar with chlortetracycline (30 mg/l). Fungal colonies were periodically examined for

V. dahliae identification (Rodríguez *et al.*, 2009).

Characterization of *V. dahliae* pathotypes. Fungal isolates were PCR-assayed using specific primers for *V. dahliae* pathotype identification. For DNA extraction, isolates were cultured on Potato Dextrose Broth (PDB) in 100 ml Erlenmeyer flasks, agitated on an orbital shaker (120 rpm) for 7 days at 24°C in the dark. Mycelia mats were harvested by filtration, frozen at -20°C, lyophilized and ground as in Pérez Artes *et al.* (2000). DNA was extracted from ground mycelia using the DNeasy Plant Kit (Qiagen, Germany) and its concentration was determined using Nanodrop and by agarose gel electrophoresis, according to standard procedures. DNA preparations were stored at -20°C for future use. Primers DB19 and DB22 amplify *V. dahliae*-specific products 523 or 539 bp in size (Carder *et al.*, 1994). *V. dahliae* population was screened for D or ND pathotypes using the primers DB19/DB22/espedf01, which, in a single-reaction, yield one of the 523 or 539 bp amplicons together with an additional amplicon of 334 bp in the case of D isolates (Mercado Blanco *et al.*, 2003).

GIS and data analysis. The very center of each olive orchard was identified using the "Sistema Integrado de Gestión" (SIG) (integrated olive-management system) of the Spanish Ministry of Agriculture and Fisheries (http://w3.mapya.es/dinatierra_v3/). All information from questionnaires and georeferenced olive groves were transferred to a GIS database. ArcGIS (version 9.1) was used to display data and analyze the correlation between prevalence of *V. dahliae* and agricultural properties (i.e. soils and land-use history). The layers used were the 1:400 000 soil map of Andalusia (IARA-CSIC, 1998) to analyze soil properties and 1995-96 land-crop/land-use map (Consejería de Agricultura y Pesca, Junta de Andalucía, Province of Granada) to analyze land-use history. The 1:400 000 soil map of Andalusia consists of 64 cartographic units (c.u). Four basic variables are included on the soil map. The soils themselves are divided into major groups and further subdivided to give individual soil units. The map also specifies categories of texture and slope. Finally, information is provided on different soil phases, such as stoniness and salinity, i.e. land attributes that are particularly important to land management. The map is composed of soil associations, each of which may include up to eight soil units. In each association, the dominant soil is indicated by a capital letter and a lower case letter; associated soils occupy at least 20% and inclusions at least 10% percent of the area (FAO, 1974; European soil map, Commission of the European Communities, 1985).

Statistics. "Pathogen prevalence", the term used for disease assessment, describes the proportion (or percentage) of olive groves where the pathogen was detect-



† Types of soils were characterized by cartographic units (c.u.). Source: 1:400 000 soil map of Andalusia (IARA-CSIC, 1998).

Main characteristic of the cartographic units (FAO 1974) taxonomy:

- 42: Bk (Rc Jc Lk). Medium and fine-textured material. Terraces, undulating or hilly terrain. Calcaric cambisols with moderate to deep soil profiles are very productive soils and intensively used. On seep slope are covered with almond and olive.
- 44: Bk Rc I (E). A weakly developed soil found on unconsolidated materials. They are typical of the mountainous regions. High percentage of limestone. Land use vary widely. Suitable for olive and dry land farming.
- 13: Rc Bk (I Jc E). Hilly landscape with white and loamy soils called “albarizas” that provide an excellent soils for vineyards, olive, sunflower and cereal. High percentage of limestone depleted of N and organic matter.
- 14: Rc Bk (Lc Jc). Hilly terrain with unconsolidated material. Young and poorly developed soils with continuous threat of erosion. Vegetation includes *Retama sp.*, shrubs, olive and cereal.
- 19: I Lc E (Bk). Steep slope (more than 30%) with shallow and stony soils. Limited potential for tree crop production. Only suitable for forest and livestock.
- 58: Lk Bk Lc (Rc). Luvisols calcic are a clayey fertile soils suitable for a wide range of agricultural uses. These soils are commonly used for cereals and for sugar beet while the upper slopes are best suited for fruit trees, vineyards, olives and grazing.
- 2: Jc. Clay, deep and rich soils, with great agricultural capacity located in valleys (< 2% slope). Soils particularly suitable for intensive irrigated crops.
- 41: Bk (Rc). Strongly sloping and loamy soils with high salt concentrations in solum. Xerophytic and halophytic vegetation.
- 15: I Re Lc (Be). Various kinds of rock and unconsolidated materials over shallow soils. Stony and steep slope (>25% slope) only suitable for livestock and forest.
- 32: Be Re Lc (I). Mountainous terrain with loamy-sandy soils. Soils with seep slope and with low water-holding capacity. Xerophytic vegetation, forest, vineyards, olive and fig.
- 47: Bk Lk Lc (I Jc). Hilly soils in which amount of calcium carbonate are found. Soils with a strong summer drought. The land use of these soils is highly variable including orange tree, olive, horticultural crops and cereals.
- 48: Bv Rc Vc (Bk). Deep, rich and dark clay soils called “bujeo” that are excellent for dry land farming.
- 40: Bk (Rc). Loamy soils on nearly level to undulating terrain with gypsum concentrations. Land-use include *Quercus coccifera*, *Rhamnus lycioides*, *Ephedra fragilis* and dry land farming.

Fig. 1. Percentage of olive groves surveyed and *V. dahliae* prevalence related to types of soils. Chi-square test was performed $\chi^2=30.883$ at the 0.05 probability level.

Table 1. *Verticillium dahliae* prevalence in olive groves surveyed in southern Spain in relation to the previous land-use.

Land use history [†]	Prevalence <i>V. dahliae</i>	Surface surveyed (ha)	Prevalence of pathotypes	
			ND	D
Agricultural land				
Irrigated cropland				
Orchards and mixed crops	27.1	60	91	9
Herbaceous crops	56.4	212	86	14
Non irrigated cropland				
Orchards and crops	10.6	887	73	27
Olive groves	7.5	1915	54	46
Herbaceous crops	15.6	611	70	30
Heterogeneous agricultural land				
Irrigated and non-irrigated mixed cropland	33.3	161	100	0
Mixed crops with natural vegetation	4.2	51	100	0
Irrigated surfaces	8.3	101	100	0
Forest land				
Shrub land	0.0	19	-	-
Pasture and shrubland with or without oak	30.0	38	100	0
woodland				
Natural areas with scarce vegetation	0.0	22	-	-
Mixed forest land	0.0	14	-	-

[†] Source: 1995-96 land-use digital maps. Province of Granada. Consejería de Agricultura y Pesca, Junta de Andalucía

ed, divided by the total number of olive groves that were inspected (Nutter *et al.*, 2006). Chi-square tests were used to assess the statistical significance of associations between categories. Statistical analysis excluded soil-type with less than five observations.

Multiple correspondence analyses (MCA) was used to analyze associations among qualitative variables and their influence on *V. dahliae* prevalence in olive groves (Savary *et al.*, 1995). MCA is an extension of correspondence analysis which allows one to analyze on the same graph the pattern of statistical relationships of several categorical dependent variables (Abdi and Valentine, 2007). In this study, the nominal variables and levels considered were: (i) origin of propagation material; (ii) land-use history and (iii) cartographic units. Prevalence of the pathogen was described as one of the two unique categorical variables: high risk or low risk.

The interpretation in MCA is often based upon proximity between points in a low-dimensional map (i.e., two or three dimensions). Proximity was meaningful only between points from the same set (i.e., rows with rows, columns with columns). When two row points were close to each other they tended to select the same level of nominal variables. For the proximity between variables, two cases were needed for discrimination. First, the proximity between levels of different nominal variables meant that these levels tended to appear together in the observations. Second, because the levels of the same nominal variable could not occur together, a different type of interpretation was needed in this case. Here the proximity between levels meant that the

groups of observations associated with these two levels were themselves similar (Savary *et al.*, 1995; Abdi and Valentine, 2007).

RESULTS

Implications of plant propagation material. The most traditional technique of olive tree reproduction in Spain is by cuttings from the annual pruning which are planted directly in the field. The majority of the olive groves surveyed (52.7%) were established in this way. Development of modern olive growing has increased the establishment of intensive olive groves with plants from nurseries. In fact, 30% of the groves surveyed originated from nursery productions. Conversion of traditional olive groves into more productive plantations requires inter-planting with young trees from nurseries so as to double tree density. This type of orchards represented about 5.3% of the total. Finally, 12% of the farmers interviewed did not know the origin of their trees.

Olive groves planted with nursery material registered the highest percentage of *V. dahliae* prevalence (24.5%), followed by groves with double density (17.4%) and those with unknown tree origin (13.2%). Conversely, groves planted with cuttings from old trees had the lowest *V. dahliae* prevalence since infection rate was only 8.5% ($\chi^2 = 105.42$; $p < 0.05$) (Table 1). However, no significant differences were detected between the prevalence of *V. dahliae* pathotypes in relation to the differ-

ent origin of plant propagation material ($\chi^2 = 6.51$ $p > 0.05$). In all cases, prevalence was around 76% for the ND pathotype and around 24% for the D pathotype.

Implications of land-use. *V. dahliae* prevalence in olive groves varied according to previous land-use. Prevalence was higher in olive groves established in lands classified as irrigated herbaceous crops (56.4%), followed by irrigated and non-irrigated mixed cropland (33.3%) (Table 1). Questionnaires indicated that the top 10 crops grown in the irrigated herbaceous category were potato, beet, bean, pepper, tomato, garlic, tobacco, alfalfa, maize and asparagus. Likewise, sunflower, vegetables, cereals, and legumes (chick-pea, lentil and bean) accounted for the largest crops in the irrigated and non-irrigated mixed cropland category. Typically, olive groves established in certain forest land had the lowest *V. dahliae* incidence (Table 1).

Prevalence of the ND pathotype appeared to increase with irrigated land-use, whereas the prevalence of the D pathotype was higher in olive groves established in non irrigated than irrigated cropland (Table 1). Although areas classified as olive groves made up most of the surface surveyed, the prevalence of the disease was low (7.5%). An unexpectedly high prevalence of pathotype D was registered in olive grove lands (Table 1).

Implications of soil properties. Olive groves surveyed were mainly located in c.u. 42, 44 and 13, followed by 14 and 19 (Fig. 1). The five c.u. (42, 44, 13,

14, 19) have similar soils and climatic patterns. The most important soils in c.u. 42 were calcic cambisols. C.u. 42 is usually located on hilly to mountainous terrain with high potential for cereals, almond and olive, but with strong limitations due to summer drought. Calcic cambisols and calcic regosols, the typical soils in c.u. 44, are suitable for non irrigated crops, olive and ilex. Their main limitations are summer drought and the high percentage of active limestone. Calcic regosols, the most abundant soils in c.u. 13, are fairly poor in organic matter (less than 2%) and have a calcium carbonate content ranging from 30% to 70%. Regosol/cambisol is the association mainly found in unit 14. These soils are typical of mountainous regions. Regosols are shallow soils found on loosely arranged materials but when deep soil occurs, cambisols support scrubs, *Retama* spp. and can be used for dry land farming. The continuous threat of erosion is the main limitation of this c.u. The most important soils in c.u.19 are leptosols, i.e. shallow soils lying directly over a calcareous bank, or soils having less than 20% (by volume) fine earth material, or extremely stony deeper soils. Slope are often more than 30%.

VW was significantly more prevalent in olive groves located in c.u. 2, 41, and 15, with pathogen prevalence of 39% for c.u. 2, 29% for c.u. 41 and 25% for c.u. 15 ($\chi^2 = 30.883$; $p < 0.05$) (Fig. 1). C.u. 2 included eutric fluvisols, i.e. deep soils derived from recent alluvial sediments, slightly basic at the surface (pH nearly 9) and having a sandy-clay to clayey-loam constitution. C.u. 2 was located in fertile valleys of Andalusia on flat terrain

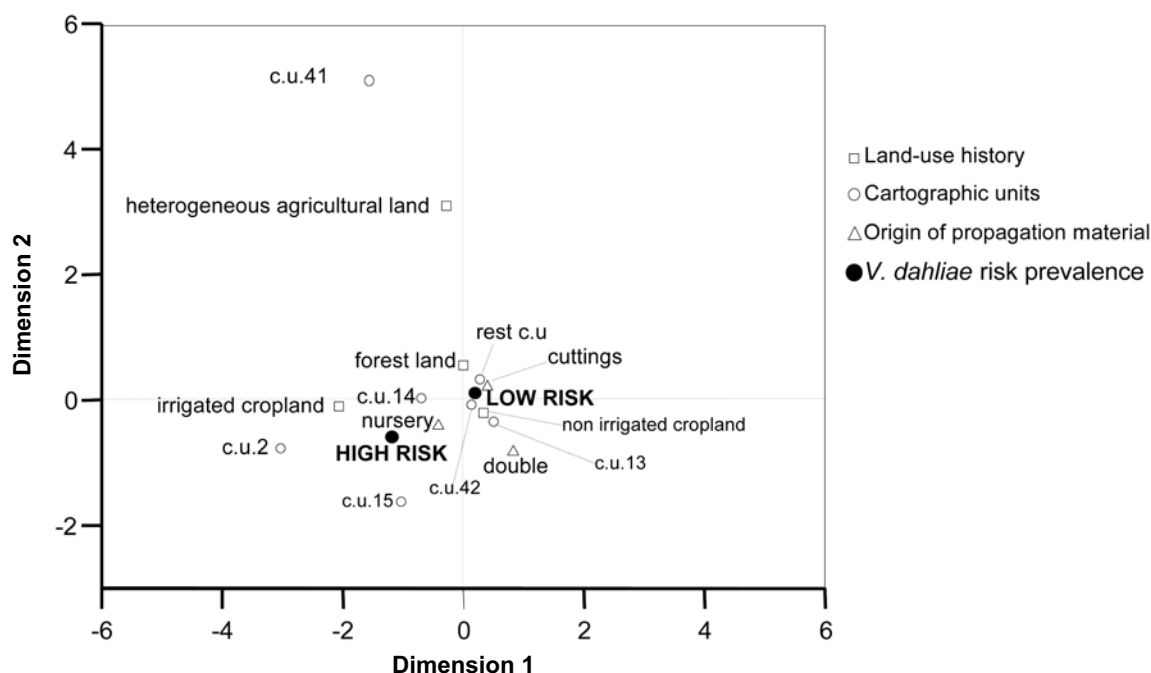


Fig. 2. Multiple correspondence analysis showing the statistical relationships between categorical variables taken into account in this study and *Veticiillium dahliae* prevalence.

around 400 m a.s.l. and with a slope of less than 2%. The cover and land-use is calcicole plants, mainly grass. Natural vegetation was scarce due to intensive cropping. C.u 15 included thin rocky soils located on mountainsides with steep slopes (more than 25%). Leptosols predominated in craggy and highly eroded areas whereas eutric regosols with chromic luvisols prevailed in levelled areas. These soils were distributed from 200 to 1600 m a.s.l. The principal land-use was forest (*Quercus rotundifolia*, *Retama sphaerocarpa*) as well as extensive livestock rearing. Calcic cambisols, the dominant soils in c.u. 41, are located from 750 to 900 m a.s.l. and are characterized by a loamy texture and high salt concentrations. Their natural vegetation consists of saline plants and xerophytes typical of a semi-arid climate. The principal land-use is low-productive dry land farming as well as extensive livestock rearing.

Multiple correspondence analysis (MCA). Relationship among the origin of plant propagation material, land-use history, c.u. and prevalence of VW risk in olive groves is shown in Fig. 3. There were two clusters of risk prevalence, i.e. high risk in the left half, and low risk in the right half. High risk prevalence was graphically associated with propagation material from nurseries (intensive olive groves), irrigated herbaceous crops as previous land-use and soil types corresponding to c.u. 2, 14, 44. Low risk was associated with propagation material from cuttings, and with olive groves with double density (called in the graph “double”). Furthermore, low risk were associated with forest, non-irrigated cropland as previous land-use and with soil types corresponding to c.u. 42, 13 and the rest of the soil units grouped and called “rest”. This indicates that c.u. 2, nurseries and irrigated cropland are strongly correlated with *V. dahliae* prevalence in olive groves ($r = +0.734$), and negatively correlated with the rest of the variables ($r = -0.734$).

DISCUSSION

Implications of plant propagation. There was a statistical relationship between VW and intensive olive cropping, consequent to the origin of plants from nurseries, whereas olive groves established with the traditional propagataion system had the lowest level of infection. In previous reports, *V. dahliae* spreading via greenhouse and nursery stock had been reported from Greece (Thanassouloupoulos, 1993) and Italy (Nigro *et al.*, 2005). In our case, traditional olive groves that doubled the density by inter-planting old with young trees from nurseries, had an intermediate level of disease. Therefore, these “mixed olive groves” disclosed the role played by nurseries in *V. dahliae* dissemination, with serious implications for the spread of the highly virulent pathotype.

In Spain, the D pathotype was restricted to cotton crops in the south-west in the mid-1980s (Bejarano-Alcázar *et al.*, 2001) but now it is well established in the olive area surveyed in the south-east of the country (Rodríguez *et al.*, 2009). As fungal isolates from cotton and olive show cross-virulence in olive (Schnathorst and Sibbett, 1971; Schnathorst, 1973), the spread of pathotype D in olive groves has been related with previous cotton growing in areas where new olive groves have been established (Collins *et al.*, 2005). However, cotton was not grown in the area we have surveyed or in surrounding fields, suggesting that the introduction of D pathotype could have occurred through infected planting stocks. Thus, nurseries had a central role in D pathotype spreading in pathogen-free areas of eastern Spain.

Implications of agricultural land-use history. *V. dahliae* prevalence differed considerably depending on previous land-use. For instance, soils previously cropped with irrigated vegetable crops (potato, beet, bean, pepper, tomato), tobacco or alfalfa displayed the highest level of disease, in agreement with literature reports (Zachos, 1963; Wilhelm and Taylor, 1965; Cirulli and Montemurro, 1976; Thanassouloupoulos *et al.*, 1979; Blanco-López *et al.*, 1984; Bejarano-Alcázar *et al.*, 2001; Serrhini and Zeroual, 1995).

Pathogen prevalence increased with irrigation and intensification of the previous land-use, i.e. from olives grown in previous forest land showing the lowest disease level, to olive cultivated in irrigated cropland, that exhibited the highest disease level. However, VW was found with a prevalence of 33.3% in olives grown in soils classified as forest land category (“pasture and shrub land with or without oak woodland”), which were assumed to be pathogen-free, thus highlighting again the role of propagating material in disease spread. Prevalence of ND isolates increased also in irrigated cropland, whereas the prevalence of the highly virulent pathotype increased in non-irrigated cropland, especially in olive groves. This is consistent with previous results (Rodríguez *et al.*, 2009) that showed the ND pathotype to prevail in irrigated olive groves, whereas the D pathotype was prevalent in non-irrigated olive stands.

Implications of soil properties. VW prevailed more in olive groves established in saline, alkaline, and poor soils (steep slope soils) with low levels of organic matter. In cotton, the disease was favoured by neutral to alkaline soils (pH 6-9) and natural soil salinity or salinity induced by recycling of salt-rich irrigation water (Pegg and Brady, 2002). Saline irrigation can exacerbate the problem in olive groves (Levin *et al.*, 2003). The highest pathogen prevalence was found in orchards established in soils corresponding to c.u. 2, i.e. soils from fertile valleys with a long history of intensive cultivation with irrigated crops. Therefore, a high incidence of VW was ex-

pected in olive groves established in these kind of soils naturally located in fertile valleys and characterized by a long land-use with crops susceptible to *V. dahliae* (Zachos, 1963; Wilhelm and Taylor, 1965; Cirulli and Montemurro, 1976; Thanassouloupoulos *et al.*, 1979; Blanco-López *et al.*, 1984; Al-Ahmad and Mosli, 1993; Serrhini and Zeroual, 1995).

In conclusion, the application of multiple correspondence analyses of data relative to soil properties, land-use history and origin of propagation material of the groves surveyed, made it possible to acquire a wider knowledge of certain epidemiological aspects of VW in olive groves. The role of saline, alkaline and steep slope soils favouring a higher *V. dahliae* incidence was confirmed. Furthermore, the noteworthy potential pathway for introduction of the pathogen into olive orchards from infected nursery plants was shown. It is clear from these analyses that it is best not to plant olive in soils previously cropped with irrigated crops, especially herbaceous crops, without making use of preventive measures such as soil disinfection. On the other hand, establishing olives in soils with non-irrigated, olive or forest land-use history seems to allow prolonged olive culture without an excessive risk of *V. dahliae* prevalence.

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MULTILOCUS SEQUENCE ANALYSIS OF *FUSARIUM POAE*

T. Kulik and A. Pszczółkowska

*Department of Diagnostics and Plant Pathophysiology, University of Warmia and Mazury in Olsztyn,
Plac Łódzki 5, 10-957 Olsztyn, Poland*

SUMMARY

Emerging prevalence of *F. poae* in fungal biota of small-grain cereals has been documented in many diverse geographic areas. We studied the phylogenetic relationships among 40 *F. poae* isolates from different geographic origins and hosts based on partial sequences of elongation factor-1 alpha (*ef1*), enniatin synthase (*esyn1*), intergenic spacer of rDNA (*igs*), RNA polymerase II (*rpb2*) and trichodiene synthase (*tri5*) data sets. Sequence alignments of five individual data sets showed low genetic variability within *F. poae* with limited development of clonal lineages. Evolutionary divergence varied between genes, with high phylogenetic incongruence between *ef1*, *igs*, *rpb2* and genes involved in secondary metabolism (*esyn1* and *tri5*). MLS (multilocus sequence analysis) bootstrap consensus tree inferred from the MP separated all 40 *F. poae* isolates into four well-supported subgroups, although there was no clear geographic or host type basis on this separation. Lack of correlation between subgroups of any individual data set and geographic origin of the isolates and host (wheat) suggests absence of geographic isolation between local populations of *F. poae* and high adaptive potential of this species to changing environmental conditions, hosts or substrates. The results of the present study may also suggest that recently reported emerging prevalence of *F. poae* in grain mycobiota is not associated with dramatic changes in the population structure of *F. poae*.

Key words: *Fusarium poae*, multilocus sequence analysis, genetic variability, population structure.

INTRODUCTION

Fusarium poae is a cosmopolitan pathogen of numerous plant species widespread in temperate zones (Leslie and Summerell, 2006). Emerging prevalence of *F. poae*

in fungal biota of small-grain cereals has been documented in many diverse geographic areas including Argentina, Canada, Japan and eastern, western and northern European countries (Stenglein, 2009). Isolates of *F. poae* can synthesize mycotoxins including enniatins (ENN) and trichothecenes. *In vitro* experiments revealed that toxin productivity by particular strains of *F. poae* differs (Torp and Langseth, 1999; Thrane *et al.*, 2004; Jestoi *et al.*, 2008; Vogelgsang *et al.*, 2008a) and two studies revealed the positive correlation between *F. poae* DNA and levels of ENNs and nivalenol (NIV) in infected grain (Yli-Mattila *et al.*, 2008; Vogelgsang *et al.*, 2008b). Several studies aimed to study intraspecific variability within *F. poae* utilizing vegetative compatibility tests (VCG), random amplified polymorphic analysis (RAPD) (Kerényi *et al.*, 1997) and sequences of different DNA targets such as elongation factor-1 alpha (*ef1*) (Knutsen *et al.*, 2004; Kristensen *et al.*, 2005; Stenglein *et al.*, 2009), trichodiene synthase (*tri5*) (Niessen *et al.*, 2004), β -tubulin, internal transcribed spacer (*its*) and intergenic spacer of rDNA (*igs*) (Yli-Mattila *et al.*, 2004) and mitochondrial small subunit rDNA (*mtSSU*) (Stenglein *et al.*, 2009). Kerényi *et al.* (1997) and Stenglein *et al.* (2009) showed moderate to low genetic variability of *F. poae* comprised of subgroups with no clear correlation to geographical origin and type of host. Results of phylogenetic analysis revealed that *F. poae* is a monophyletic species with its closest genetic relationship to *F. kyushuense*, *F. venenatum* and *Gibberella pulicaris* (anamorph: *F. sambucinum*) (Kristensen *et al.*, 2005), whereas the morphology of *F. poae* is similar to *F. langsethiae* (Torp and Langseth, 1999; Yli-Mattila *et al.*, 2004).

The sexual stage of this species is unknown although, according to Kerényi *et al.* (1997), fungal cultures of *F. poae* often form sectors that differ from the original colony in morphological and toxicological characteristics. Further studies of Kerényi *et al.* (2004) revealed mating potential of the fungus by identification of conserved mating type (MAT) sequences in genome of *F. poae*. RT-PCR, together with northern blot analysis, revealed that both *MAT-1-1* and *MAT-2* genes are functional, however, evidence for meiotic recombination of *F. poae* has not been confirmed to date (Kerényi *et al.*, 2004). The aim of this study was to determine: (i) the

Table 1. List of *F. poae* isolates used for phylogenetic analyses.

CBS/DDPP/IBT code ¹	Geographical origin, host/habitat of origin	<i>F. poae</i> haplotypes ²					
		<i>Ef1</i>	<i>Esyn1</i>	<i>Rpb2</i>	<i>Igs</i>	<i>Tri5</i>	MLS
DDPP 07602	Poland, wheat kernel	1	1	1	1	1	I
DDPP 06404		1	1	1	1	1	I
IBT 9928	Austria, corn	1	1	1	1	1	I
CBS 177.64	Norway, wheat kernel	1	1	1	1	1	I
TPU2A	Argentina, wheat kernel	1	2	1	1	1	I
CBS 185.96	Norway, barley	1	1	2	1	1	I
DDPP 071201	Poland, wheat kernel	1	1	2	1	1	I
DDPP 03107		1	1	2	1	1	I
CBS 186.96	Poland, wheat	1	1	1	2	1	I
DDPP 07501	Poland, wheat kernel	1	1	1	2	1	I
DDPP 07314		1	1	1	3	1	I
DDPP 0607e	England, wheat kernel	1	1	1	3	1	I
DDPP 06605	Poland, wheat kernel	1	1	1	4	1	I
DDPP 07001		1	1	1	4	1	I
CBS 623.87	Denmark, barley	1	1	1	6	1	I
IBT 9982	Italy, wheat kernel	1	1	1	10	1	III
CBS 317.73	Australia, bud of <i>Dianthus</i> sp.	1	1	1	11	1	II
DDPP 072019		1	1	1	11	1	II
DDPP 060903	Poland, wheat kernel	1	1	1	7	2	I
DDPP 03171		1	1	1	7	2	I
TPUAA	Argentina, wheat	1	2	1	9	1	IV
DDPP 0373	Poland, wheat kernel	1	2	1	10	1	III
CBS 178.96	Norway, wheat	1	3	1	9	1	IV
CBS 181.96	Norway, barley	1	4	2	1	1	I
DDPP 04357	Poland, wheat kernel	1	5	1	9	1	IV
CBS 184.96	Norway, barley	1	5	1	9	1	IV
IBT 9929	Norway, oats	1	5	1	9	1	IV
DDPP 0601	Poland, wheat kernel	1	6	1	10	3	III
IBT 8452	Denmark, barley	1	6	1	10	3	III
IBT 9999	USA, durum wheat	1	7	1	10	1	III
DDPP 079305		2	1	1	7	2	I
DDPP 079822	Poland, wheat kernel	2	1	1	7	2	I
DDPP 06303		2	1	1	7	2	I
DDPP 08g5	Germany, wheat kernel	2	1	1	7	2	I
CBS 183.96	Norway, barley	3	1	1	6	1	I
CBS 175.96	Norway, barley	3	2	1	11	1	II
CBS 446.67	Germany, <i>Anthoxanthum odoratum</i> , inflorescence	4	1	1	3	1	I
DDPP 07410	Poland, wheat kernel	4	1	1	3	1	I
DDPP 08g4	Germany, wheat kernel	4	1	1	5	2	I
DDPP 06504	Poland, wheat kernel	4	2	1	8	1	I

¹ (CBS) Fungal Biodiversity Centre, Utrecht, The Netherlands; (DDPP) Department of Diagnostics & Plant Pathophysiology, University of Warmia and Mazury in Olsztyn, Poland; (IBT) Center for Microbial Biotechnology; (CMB) Department of Systems Biology, Technical University of Denmark.

² (*ef1*) elongation factor-1 alpha, (*esyn1*) enniatin synthase, (*igs*) intergenic spacer of rDNA, (*rpb2*) RNA polymerase II, (*tri5*) trichodiene synthase, (MLS) multilocus sequence analysis.

phylogenetic relationships among 40 *F. poae* isolates with respect to geographic origin and host (wheat); (ii) the separation of the putative lineages within *F. poae*; (iii) determine recombination events within *F. poae*. MLS (multilocus sequence analysis) used in this study incorporated data sets widely used in fungal phylogenetics (*efl*, *igs*, and *rpb2* (RNA polymerase II)) as well as two genes responsible for trichothecene and enniatin synthesis, *tri5* and *esyn1* (enniatin synthase), respectively.

MATERIALS AND METHODS

Collection of *F. poae* isolates. All fungal isolates analyzed in this study are listed in Table 1. Isolates of *Fusarium* tested are held in the CBS (CBS Fungal Biodiversity Centre, Utrecht, The Netherlands) fungal collection. IBT isolates analyzed are held in IBT culture collection [Center for Microbial Biotechnology (CMB), Department of Systems Biology, Technical University of Denmark]. TPUAA and TPU2A isolates were kindly provided by Dr S.A. Stenglein [Laboratorio de Biología Funcional y Biotecnología (BIOLAB-CEBB), Facultad de Agronomía (UNCPBA), Buenos Aires, Argentina]. Nineteen Polish, 2 German and 1 English field isolates (DDPP, Department of Diagnostics and Plant Pathophysiology, University of Warmia and Mazury in Olsztyn, Poland) were obtained from wheat seed samples collected during 2003-2008 (data not shown). Fungal

isolates are stored in 15% glycerol at -80°C in the fungal collection of the DDPP and are available upon request. The *Fusarium* isolates were cultured on PDA (potato dextrose agar) (Booth, 1971) at 25°C prior to DNA extraction. Species identity of all *F. poae* isolates were confirmed by BLAST searches using *efl* gene sequence.

DNA extraction, PCR and DNA sequencing. DNA extraction, PCR analyses and DNA sequencing were carried out as previously described (Kulik *et al.*, 2007). Primers used for amplification of partial *efl*, *esyn1*, *igs*, *rpb2* and *tri5* are listed in Table 2. Several sets of primers (data not shown) were designed for the amplification and sequencing of partial *igs*. However only primer sets (Table 2) designed to the *igs* end flanking 18S rDNA allowed to generate PCR products from all the isolates analyzed. All sequences were deposited in NCBI database: *efl* (GU270944-GU270984), *esyn1* (GU139965-GU140004), *igs* (GU270985-GU271024), *rpb2* (GU271025-GU271065) and *tri5* (GU271066-GU271105). The sequence identity of each gene was confirmed by BLAST searches (Altschul *et al.*, 1997) (<http://www.ncbi.nlm.nih.gov/blast/index.shtml>).

Phylogenetic analyses. Sequence data were edited and aligned using Clustal W algorithm (Thompson *et al.*, 1994) implemented in Geneious Pro 4.0.4 with the default settings (Drummond *et al.*, 2009). Data for each gene were analyzed both separately and together as a

Table 2. List of primers used for amplification and sequencing of *efl*, *esyn1*, *igs*, *rpb2* and *tri5* data sets.

DNA target	Primer sequence	Annealing temperature	Reference
<i>Efl</i>	Ef1	58°C	O'Donnell <i>et al.</i> , 1998
	ATGGGTAAGGA(A/G)GACAAGAC		
	Ef2 GGA(G/A)GTACCAGT(G/C)ATCATGTT		
<i>Esyn1</i>	Esy1	58°C	This study
	TTCAAGGGCTGGACGTCTATG		
	Esypoae2 CAGCATATCGATACGCGCTGAG		
<i>Igs</i>	Igspoae1	60°C	This study
	GCAGTAGTATAGGGTAAGTGAAA		
	Igspoae2		
	AACCAGTGAATCAGCACGCAGT		
	Igsspo11 AGCCCGGTCTGGCGTGCGACC		
<i>Rpb2</i>	Igsspo12 CCCCTCTAGATTCCCCAGGGC	58°C	This study
	Rp1		
	ACGCCCATTGGACGTGACGG		
	Rp2 CTCACAATGTGTGTACATGTG		
<i>Tri5</i>	Tox5-1	58°C	Niessen and Vogel, 1998
	GCT GCT CAT CAC TTT GCT CAG		
	Tox5-2 CTG ATC TGG TCA CGC TCA TC		

combined multilocus sequence (MLS). Variable substitution sites and parsimony informative sites were calculated using MEGA v3.1 (Kumar *et al.*, 2004). Maximum parsimony (MP) and maximum likelihood analyses (ML) were conducted using PAUP* v4.0b10 (Swofford, 2001) implemented in Geneious Pro 4.0.4 using heuristic search option and in case of ML with starting trees obtained by random stepwise addition. Furthermore, Modeltest version 3.06 (Posada and Crandall, 1998) with Akaike Information Criterion (AIC) (Akaike, 1973) model selection was used to determine the nucleotide substitution model best suited to the data set. Sequences of *G. avenacea* [anamorph: *F. avenaceum* (FJ939659, EF026103)], *F. armeniacum* (GQ505494), *F. langsethiae* (AF449793), *F. sporotrichioides* (EF521145, AJ854656, GU271047, EU128232), *G. pulicaris* [anamorph: *F. sambucinum* (Z48743)] served as outgroups of individual data sets. Stability of clades was assessed by 1000 bootstrap replications.

Partition homogeneity test. Congruence between individual gene data sets was tested using the partition homogeneity test (Farris *et al.*, 1995) implemented in PAUP* v4.0b10 (Swofford, 2001). One thousand replicates were analyzed in a heuristic search. Max trees was set to 1000. Invariant characters were deleted prior to analysis and 0.01 was used as a significance threshold (Cunningham, 1997).

RESULTS

Sequence characterization. Aligned sequence lengths of *F. poae* isolates of *ef1*, *esyn1*, *igs*, *rpb2* and *tri5* genes were 513, 501, 844-845, 564 and 471 nucleotides, re-

spectively. Among the 40 isolates, 4 *ef1*, 7 *esyn1*, 11 *igs*, 2 *rpb2* and 3 *tri5* haplotypes were identified (Table 1). The number of variable characters in individual data sets varied. Among data sets analyzed, *igs* region had 37 (4.4%) variable sites. *Esyn1* gene had 25 (5%) variable sites. Both *ef1* and *tri5* data sets had 4 (0.8%) variable sites. *Rpb2* had only one (0.2%) variable site. The number of parsimony informative sites also varied between data sets analyzed. *Igs* region had 16 (1.9%) parsimony informative sites. *Esyn1* had 6 (1.2%), while *ef1* gene had only one (0.2%) parsimony informative site. Parsimony informative sites within *rpb2* and *tri5* were not revealed.

Phylogenetic analysis of individual and combined gene sequences. ML and MP analysis of the *ef1*, *esyn1*, *igs*, *rpb2* and *tri5* genes differentiated all *F. poae* isolates from other *Fusarium* species (Fig. 1-5). Bootstrap consensus trees inferred from the MP analysis of all loci had identical topologies to the trees produced by ML (data not shown). Monophyly of the ingroups within *ef1*, *esyn1*, *igs*, *rpb2* and *tri5* data set was strongly supported by bootstrap value (100%). Within *F. poae* one, four, one and two well-supported groups were observed at the *ef1*, *esyn1*, *rpb2* and *tri5* genes, respectively. However, in the case of *igs*, the three subgroups revealed were not supported with strong bootstrap values (Fig. 1-5). MLS nucleotide alignment within *F. poae* consisted of 2,895 characters. Bootstrap consensus tree inferred from the MP analysis revealed four well-supported subgroups within *F. poae* (Fig. 6).

Partition homogeneity test. The partition homogeneity test showed significant incongruence in phylogenetic signal between the combined all five data sets ($P = 0.001$). However, excluding of both *tri5* and *esyn1* genes

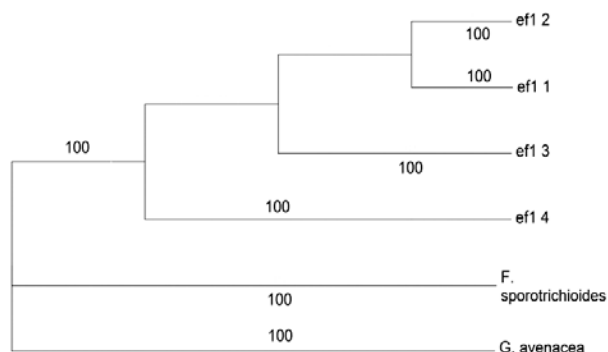


Fig. 1. Bootstrap consensus tree inferred from the MP analysis based on *ef1* sequence data set. Where species is not indicated, isolates belong to *F. poae* *ef1* haplotypes. Values at branches indicate branch support with bootstrapping percentages based on maximum parsimony analysis. Bootstrap values $\geq 70\%$ are shown. Tree was rooted with *G. avenacea* (anamorph: *F. avenaceum*) and *F. sporotrichioides*.

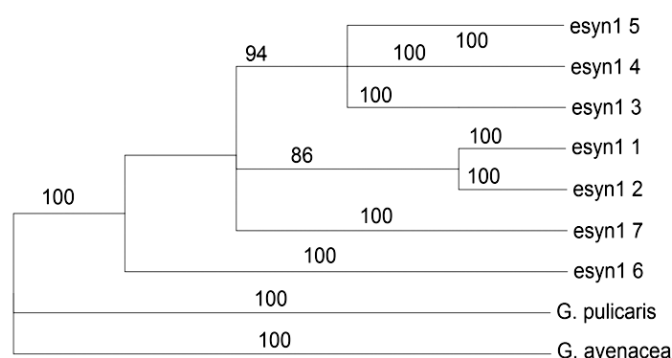


Fig. 2. Bootstrap consensus tree inferred from the MP analysis based on *esyn1* sequence data set. Where species is not indicated, isolates belong to *F. poae* *esyn1* haplotypes. Values at branches indicate branch support with bootstrapping percentages based on maximum parsimony analysis. Bootstrap values $\geq 70\%$ are shown. Tree was rooted with *G. avenacea* (anamorph: *F. avenaceum*) and *G. pulicaris*.

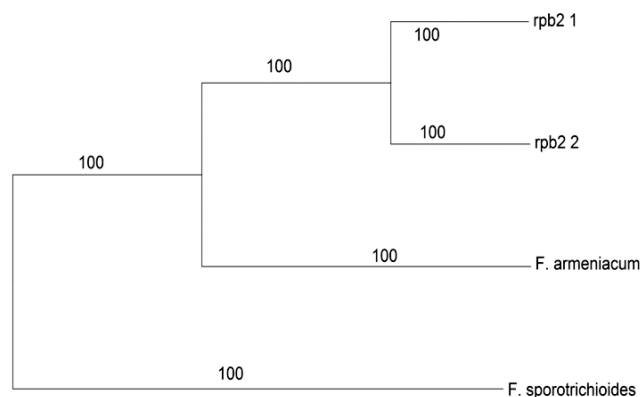


Fig. 4. Bootstrap consensus tree inferred from the MP analysis based on *rpb2* sequence data set. Where species is not indicated, isolates belong to *F. poae rpb2* haplotypes. Values at branches indicate branch support, with bootstrapping percentages based on maximum parsimony analysis. Bootstrap values $\geq 70\%$ are shown. Tree was rooted with *F. armeniacum* and *F. sporotrichioides*.

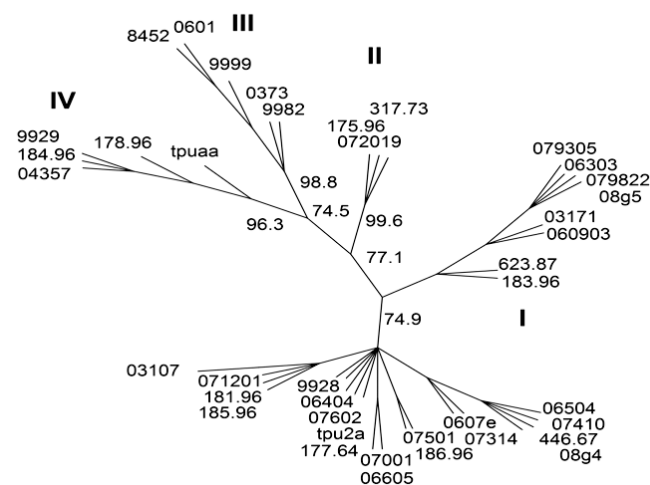


Fig. 6. Bootstrap consensus tree inferred from the MP analysis based on multilocus sequence data sets of *ef1*, *esyn1*, *igs*, *rpb2* and *trr5*. Values at the main internal branches are only shown and indicate branch support, with bootstrapping percentages based on maximum parsimony analysis. Bootstrap values $\geq 70\%$ are shown.

tri5) was determined. Among the genes analyzed, *esyn1* and *rpb2* were used to study intraspecific variability of this species for the first time. Sequence alignment of all five data sets revealed low variability within *F. poae* (total pairwise identity 99.4%), although it was moderately varied among data sets analyzed. Additionally, relatively high number of clonal strains was found. Of the 40 individuals tested in this study, 28 shared identical nucleotide sequence within 11 subgroups of clonal lineages. These results are in agreement with previous phylogenetic studies of Knutsen *et al.* (2004), Niessen *et al.* (2004), Yli-Mattila *et al.* (2004), Kristensen *et al.* (2005)

DISCUSSION

In this study, phylogenetic relationship within *F. poae* based on five nuclear data sets (*ef1*, *esyn1*, *igs*, *rpb2* and

and Stenglein *et al.* (2009) that showed very low intraspecific variability of *F. poae*. Other methods such as VCG, RAPD (Kerényi *et al.*, 1997), IGS-RFLP (Konstantinova *et al.*, 2004) and AFLP (Schmidt *et al.*, 2004) also underlined moderate intraspecific variability within this species. Surprisingly, most recent studies of Somma *et al.* (2010) showed high level of variability of Italian population of *F. poae* by using AFLP analyses. Similarly, inter-simple sequence repeats (ISSR) analysis of Argentinean and English populations of *F. poae* indicated high intraspecific variability within *F. poae* (Dinolfo *et al.*, 2010). Such contradictory results may be explained by the type of molecular technique used. Both AFLP and ISSR survey entire genome and are extremely effective in detecting genetic variation (Meng and Chen, 2001) but their dominant nature makes them generally unusable as phylogenetic markers. It seems that “additional” sequence variation within *F. poae* may be revealed by surveying entire genome although previous studies of Kerényi *et al.* (1997) and Schmidt *et al.* (2004) do not support it. VCG together with RAPD revealed subgroups within *F. poae* with no clear correlation to geographical origin and host plant (Kerényi *et al.*, 1997). Similar results were obtained by Stenglein *et al.* (2009) and Dinolfo *et al.* (2010).

The current results did not reveal clear correlation between *F. poae* subgroups on the basis of individual data sets and geographical origin of the isolates, although weak correlation might be observed. For example, *ef1* 2, *ef1* 4, *igs* 4, *igs* 7 and *tri5* 2 haplotypes were only revealed in Poland and Germany. *Esyn1* 3 haplotype was only revealed in Norway. Kerényi *et al.* (1997) suggested that the lack of correlation between *F. poae* subgroups and origin of the isolates may be due to long-distance transport of infested plant materials resulting in the movement of pathogen genotypes and nullifying the effects of geographic isolation on evolution. If so, *F. poae* isolates should have high adaptative potential to changing environmental conditions, hosts or substrates. The present study aimed at finding potential host speciation by including high number of isolates associated with head blight of wheat, however, no clear correlation was revealed between subgroups of any individual data sets and wheat. Among the haplotypes analyzed, *ef1* 2, *igs* 2, *igs* 4, *igs* 7 and *tri5* 2 haplotypes were associated with wheat only, however more isolates are required to confirm the correlation of these haplotypes to wheat. Lack of host speciation may be most likely explained by lack or little effect of host selective pressure on *F. poae* (Kerényi *et al.*, 1997). Sequences of all five data sets were combined into a MLS for analysis (Fig. 6). It should be noted that 69.6% of phylogenetically informative sites within the MLS of *F. poae* isolates were contributed by the *igs* data set and, thus, the structure of the MLS phylogeny obtained is heavily weighted towards the evolutionary history of that target. MLS boot-

strap consensus tree (Fig. 6) inferred from the MP separated *F. poae* isolates into four well-supported subgroups, although there was no clear geographic or host type basis on this separation. Subgroup I supported by bootstrap value of 77.1% represented 27 isolates. Notably, 21 isolates from this subgroup were associated to wheat. Subgroup II supported by bootstrap value of 99.6% represented 3 isolates from Australia, Poland and Norway that shared identical *igs*, *rpb2* and *tri5* sequences. Subgroup III supported by bootstrap value of 98.8% contained isolates from Denmark, Italy, Poland and the USA that shared identical *ef1*, *igs* and *rpb2* sequences. Subgroup IV supported by bootstrap value of 96.3% contained 5 isolates from Argentina, Poland and Norway sharing identical *ef1*, *igs*, *rpb2* and *tri5* data sets. It should be noted that excluding of *esyn1* and *tri5* data sets from MLS bootstrap consensus tree had no effect on the tree topology (data not shown).

Significant incongruence ($P < 0.001$) was evident between combined all five data sets analyzed in this study. However, excluding of both *tri5* and *esyn1* genes from analysis indicated that the phylogenetic signal of the combined *ef1*, *rpb2* and *igs* was not significantly incongruent ($P = 0.026$). Incongruence in phylogenetic signal between *ef1*, *igs*, *rpb2* and genes responsible for toxin synthesis, suggests different evolutionary histories or evolutionary origins for *esyn1* and *tri5* genes. Several processes, including incomplete lineage sorting, variable evolutionary rates, parasexual recombination and sexual recombination, could generate differences in tree topologies (Scott and Chakraborty, 2006).

On the other hand, congruence between *ef1*, *rpb2* and *igs* might be due to the low sequence variation within *ef1* and *rpb2* genes. Evidence that trichothecene metabolite profiles are not well correlated with evolutionary relationships within the *F. graminearum* clade has been previously reported. It has been demonstrated that polymorphism within *tri* cluster is transpecific and appears to originate in the common ancestor of type B trichothecene producers (Ward *et al.*, 2002). Some clusters involved in secondary metabolism appear to have moved into fungal genomes by horizontal gene transfer from either prokaryotes or other fungi (Khaldi *et al.*, 2008). Further studies are now required in order to examine the discord between *ef1*, *rpb2*, *igs* and *esyn1* and *tri5* in *F. poae*.

In conclusion, the results of the present study provide new insights into population biology, reproductive mode and the degree of clonality within *F. poae*. These results may also indicate that emerging prevalence of *F. poae* in grain mycobiota is rather not associated with dramatic changes in the population structure of *F. poae*. Given their importance to agriculture and food safety, information gained from these studies should promote more informed disease control and plant breeding strategies.

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This paper is dedicated in the memory of my father Jan Kulik.

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DEVELOPMENT OF PCR-BASED TECHNIQUES FOR THE DETECTION OF IMMOBILISED *POTATO VIRUS Y* VIRIONS

S.J. Gawande¹, A. Shukla², V.P. Chimote³, N. Kaushal², P. Kaundal², I.D. Garg² and K.P. Chimote¹

¹Division of Plant Pathology, Directorate of Onion and Garlic Research, Rajgurunagar, 410505 Pune, India

²Division of Plant Protection, Central Potato Research Institute, 171001 Shimla, India

³State Level Biotechnology Laboratory, Mahatma Phule Krishi Vidyapeeth, 413722 Rahuri, India

SUMMARY

Detection of *Potato virus Y* (PVY), one of the important potato viruses, was undertaken using various PCR-based techniques, i.e. immunocapture reverse transcription polymerase chain reaction (IC-RT-PCR), direct binding RT-PCR (DB-RT-PCR) and print capture RT-PCR (PC-RT-PCR). These techniques were evaluated for detection of PVY virions immobilized on solid supports. The 5'UTR and P1 region (969 bp) of the PVY genome were amplified using these techniques, which were compared with DAS-ELISA and nucleic acid spot hybridization (NASH) using fluorescein labeled cDNA probes. The detection limit of purified PVY in serially diluted healthy potato leaf sap was 10 ng for DAS-ELISA, 1 ng for NASH and DB-RT-PCR, 1 pg for PC-RT-PCR, IC-RT-PCR and standard RT-PCR. Of all techniques, PC-RT-PCR proved to be the simplest, economical and reliable. It does not require antibodies for the capture of virions nor lengthy RNA extraction processes.

Key words: *Potato virus Y*, *Solanum tuberosum*, diagnosis, RT-PCR, IC-RT-PCR, PC-RT-PCR.

INTRODUCTION

Potato virus Y (PVY) is the type species of the genus *Potyvirus*, family *Potyviridae*, i.e. the largest and economically important group of plant viruses, transmitted non-persistently by about 40 aphid species (Sigvald, 1984). The predicted crop losses due to PVY infections range from 10 to 80% (De Bokx and Huttinga, 1981). PVY detection is very important not only for the production of healthy planting material, but also for screening in resistance breeding programs. Therefore rapid detection methods with high degree of accuracy and sensitivity are necessary. ELISA has been used widely in plant virus diagnosis (Clark and Adams, 1977; Carlebach *et al.*, 1982). However, ELISA has relatively low

sensitivity which is not suitable for detection of trace amounts of the virus in single viruliferous aphid vectors and dormant tubers. By contrast, PCR is an effective and efficient tool for *in vitro* amplification of DNA templates and has been extensively used for the diagnosis of viral and subviral pathogens with DNA and/or RNA genomes. There are various methods to detect RNA viruses by RT-PCR technique (Barker *et al.*, 1993; Singh and Singh, 1996; Singh, 1999; Singh *et al.*, 1996, 2002). In recent years several variants of PCR and nucleic acid-based detection techniques, such as immunocapture RT-PCR (IC-RT-PCR) (Jansen *et al.*, 1990; Wetzel *et al.*, 1992), radioactive nucleic acid spot hybridization (R-NASH) (Singh and Singh, 1996), digoxigenin (DIG)/fluorescein labeled cDNA probes (Singh and Singh, 1995; Verma *et al.*, 2003), and real time PCR (Cockerill and Smith, 2002; Mackey *et al.*, 2002) have been evaluated for the detection of viral RNAs. Although these methods are sensitive and specific, they are complex and time-consuming as they require multiple steps in sample preparation.

Cumbersome nucleic acid extraction procedures, non-specific amplification and poor detection efficiency for low-titer viruses are some of the factors limiting the application of PCR technology in virus diagnosis. To overcome these constraints, immunocapture (IC) PCR was developed for the detection of *Hepatitis A virus* (HAV; Jansen *et al.*, 1990) and a similar procedure (IC-RT-PCR) was applied to plant viruses (Wetzel *et al.*, 1992). However, this method needs production of virus-specific IgGs which is expensive and labor-intensive. Thus, direct binding (DB)-PCR on polypropylene tubes was developed for detection of some apple, *Prunus* and grapevine viruses (Rowhani *et al.*, 1995).

There is a scarce literature on sensitive detection of non-persistent viruses in a single aphid vector (Singh *et al.*, 1996; Varveri, 2000). Protocols for print capture on nylon membranes and filter paper were developed for *Plum pox virus* (PPV) and PVY (Olmos *et al.*, 1996; Varveri, 2000), but they require virus immunocapture in tissue and aphid-printed spots prior to PCR analysis. In the present study, we describe a direct print capture technique for the detection of immobilized PVY virions. The technique is cost-effective as it does not re-

Table 1. Comparison of detection limits of different PCR-based methods with fluorescein-labeled cDNA probe and ELISA.

Method	Detection limit	Sensitivity	Sample volume
RT-PCR	1pg	10 ⁻⁴	10 µl
IC-RT-PCR	1pg	10 ⁻⁵	100 µl
DB-RT-PCR	1ng	10 ⁻²	100 µl
PC-RT-PCR	1pg	10 ⁻⁴	10 µl
Fluorescein -labeled cDNA probe	1ng	10 ⁻²	10 µl
DAS-ELISA	10 ng	10 ⁻²	100 µl

quire either immunocapture of the virus or viral RNA, and is suitable for the detection of very low amounts of virus even in single viruliferous aphids or dormant tubers.

MATERIALS AND METHODS

Virus source. An Indian PVY^o strain (Khurana *et al.*, 1975) isolated from field-grown potato plants was maintained in *Nicotiana glutinosa* in an insect-free greenhouse. To study the suitability of reported protocols, potato leaf samples with PVY symptoms were collected from eight different potato varieties (Table 2). These samples and the negative control (samples of healthy *Solanum chacoense*) were obtained from the Central Potato Research Institute (CPRI), Shimla, India. Aphids (*Myzus persicae* Sulz.) were collected from potato fields and reared on cabbage plants (*Brassica oleracea* var. *capitata*). The second generation of aphids at the nymph stage was used for PC-RT-PCR detection.

Virus purification and antiserum production. PVY was purified according to Khurana *et al.* (1987) from infected *N. glutinosa* leaves harvested 20-30 days post in-

oculation. Antiserum was raised following weekly intramuscular injections of 1 mg of purified virus into a rabbit for six weeks. Polyclonal IgGs were isolated and an alkaline phosphatase conjugate was prepared as described (Khurana *et al.*, 1987). Purified PVY was used to estimate spectrophotometrically the protein content, for determining the detection limits of the protocols used in our experiments.

DAS-ELISA. Anti PVY IgGs (2 µg/ml) were used to coat microtiter plates and the conjugate was used at 1:800 dilution. Potato leaf samples and PVY-infected *N. glutinosa* leaf tissues were extracted in 1x PBS-T buffer (140 mM NaCl, 1.5mM KH₂PO₄, 20mM Na₂HPO₄, 2.5mM KCl, 0.05% Tween-20, 2% PVP and 1% egg albumin) at 1:10 tissue to buffer ratio. Absorbance values were read at 405 nm. Positive reactions were deduced from detection threshold equal to twice the mean value of healthy control.

RNA extraction. The coat protein of purified virus was removed by proteinase K (5 µg/ml) treatment in the presence of SDS (0.5%) at 37°C for 30 min, RNA was extracted with phenol-chloroform and was precipitated with 2.5 vol of ethanol and 1/10th of 3 M sodium ac-

Table 2. Detection of PVY in leaf sap of field potato samples by RT-PCR and PC-RT-PCR as compared with fluorescein-labeled cDNA probe and ELISA.

Potato cultivar	RT-PCR	PC-RT-PCR	Flourescein-labeled cDNA probes	DAS-ELISA
Kufri Badshah	+++	+++	++	++
K. Jyoti	+++	+++	+++	+++
K. Surya	+	+	-	-
K. Pukhraj	++	+++	+	-
K. Chandramukhi	+	++	-	-
K. Chipsona I	-	-	-	-
K. Chipsona II	-	-	-	-
K. Bahar	++	++	+	-

etate pH 5.2. RNA concentration was estimated spectrophotometrically. Potato RNA was isolated from PVY symptomatic leaf samples using RNeasy Plant Mini Kit (Qiagen, Germany) following the manufacturer's protocol.

RT-PCR. Reverse transcription was carried out in a 50 µl vol containing 5 µl of 10x reaction buffer (250 mM Tris HCl, pH 8.3, 375 mM KCl, 15 mM MgCl₂), 20 µl of 10 mM dNTP mix, 2 µl of M-MuLV (100 U/µl) reverse transcriptase (Fermentas, USA), 10 µl viral RNA, and random hexanucleotide primers (200 ng). This reaction mixture was incubated for 1 h at 37°C and the reaction was stopped by heating at 95°C for 15 min. PCR was performed on a reaction mixture consisting of 10 µl of 10x PCR buffer (100 mM Tris HCl, 500 mM KCl, 15 mM MgCl₂, 0.01% w/v gelatin), 5 µl dNTP mix (2 mM each), 200 ng each upstream 5' and downstream primers (Tordo *et al.*, 1995), 5 U DNA polymerase (Applied Biosystems, USA), template cDNA and distilled water to 100 µl total reaction volume. Cycling conditions were optimized to an initial denaturation at 94°C for 5 min and 40 subsequent cycles of 1 min at 94°C, 1 min at 55°C, 1 min at 72°C and a final extension at 72°C for 10 min. For virus purification, artificially inoculated *N. glutinosa* leaves were harvested 20-30 days post inoculation. Amplified products were analyzed on ethidium bromide stained 1% agarose gel.

IC-RT-PCR. Anti-PVY IgGs (2 µg/ml) diluted in carbonate buffer (15 mM Na₂CO₃, 35 mM NaHCO₃, pH 9.6) were used to pre-coat sterile 0.2 ml PCR tubes (Axygen, USA) and polystyrene ELISA plates by incubating them at 37°C for 2 h. Pre-coated tubes were washed thrice with 100 µl PBS-T wash buffer. PVY-infected *N. glutinosa* leaf tissues were extracted in PBS-T buffer, pH 7.4, at 1:10 tissue to buffer ratio containing 2% PVP and 1% egg albumin. The extract was added to the anti-PVY IgG pre-coated tubes and further incubated for 2 h at 37°C. After washing three times with sterile PBS-T, cDNA synthesis was carried out in the same PCR tubes and polystyrene ELISA plates as described above for RT-PCR. Instead of RNA template, sterile distilled water was added to make the reaction mixture to 20 µl. PCR reactions were performed as described above.

DB-RT-PCR. Sterile 0.2 ml PCR tubes were loaded with 100 µl aliquots of PVY-infected *N. glutinosa* leaf tissues extracted in PBS-T buffer (pH 7.4) at 1:10 ratio containing 2% PVP and 1% egg albumin. These tubes were incubated for 2 h at 37°C. After washing three times with sterile PBS-T, cDNA synthesis was carried out in the same tubes as described in the RT-PCR. Here too, instead of RNA template, sterile distilled water was added to make the reaction mixture to 20 µl. After-

wards, PCR was performed as described above.

PC-RT-PCR. Potato leaf samples and PVY-infected *N. glutinosa* leaf tissues were cut with sharp razors and cut edges were printed onto nylon membranes (Hybond N⁺, Pharmacia Biotech, USA) and 3MM Whatman Filter paper. Similarly, each single aphid (aphids were starved for 2 h prior to feeding on PVY-infected *N. glutinosa* leaves in Petri plates for 30 min) was pressed with the help of a glass rod on the membrane or filter paper. Purified virus was serially diluted (ten-fold dilutions) in healthy potato leaf sap and 10 µl of each dilution was spotted on the membrane to determine sensitivity and detection limit. These spots were dried at 37°C for 1 h. The printed spots were cut into pieces and transferred to 0.2 ml PCR tubes and 20 µl of sterile distilled water was added to each tube. To elute viral RNA, tubes were heated at 95°C for 10 min. Ten µl aliquots from the tubes were used for cDNA synthesis and PCR was performed as described earlier.

Preparation of non-radioactive cDNA probe, sample preparation and hybridization. The 5'UTR and P1 region of PVY genome was amplified by PCR and cloned into pGEM-T easy vector. The recombinant plasmid was digested with *Eco*RI. The released fragment was gel eluted and labeled using Gene Images Random Prime Labeling and Detection kit (Pharmacia Biotech, USA). DNA (1 µg) to be labeled was diluted in 30 µl nuclease-free water, denatured by heating in boiling water for 5 min, then chilled on ice. The reaction mix [composed of DNA (30 µl), fluorescein-11-dUTP nucleotide mix (10 µl), random primers (5 µl), DNA polymerase (Klenow) 5 U/µl and distilled water (3 µl)] was incubated overnight at 37°C and the labeling reaction was stopped by addition of 2 µl of 0.5 M EDTA.

Purified virus mixed with healthy potato sap in ten-fold serial dilutions was spotted onto the nylon membrane followed by baking at 80°C for 1 h. The baked membrane was prehybridized in hybridization buffer [5x SSC, 1/20 vol of blocking buffer, 0.1% SDS, 5% dextran sulfate and distilled water] at 60°C for 30 min. The probe was denatured by boiling for 5 min and immediately chilled on ice. The membrane was hybridized with denatured probe at 60°C overnight with gentle agitation. It was then subsequently washed in 1x SSC+0.1% SDS for 10 min at 60°C followed by 0.5x SSC+0.1% SDS for 5 min. The spotted membrane was then incubated for 1 h in blocking buffer diluted in buffer A (100 mM Tris, 300 mM NaCl, pH 9.5) and antibody conjugate (1:5000) in 0.5% BSA in buffer A (0.3 ml/cm²) at room temperature. The blot was placed on Saran wrap, 30-40 µl/cm² of detection reagent (CDP-Star) were added onto it and incubated for 5 min. The membrane was then exposed to X-ray film (Kodak-X-Omat AR Film) for 3 h and results were then analyzed.

RESULTS

Comparative detection limits of different PCR-based methods. Amplified product of the expected size (969 bp) was obtained from PVY-infected plant material with all four PCR methods viz. RT-PCR, IC-RT-PCR, PC-RT-PCR and DB-RT-PCR (Fig. 1). PVY in single viruliferous aphids was detected by PC-RT-PCR (Fig. 1). Amplification was also obtained with single aphids printed either on nylon membrane or 3MM filter paper, whereas there was no amplification from non-viruliferous controls. The results of PC-PCR using Whatman 3MM filter paper and nylon membranes were very similar. For obvious cost and convenience reasons the Whatman 3MM filter paper was used throughout the study. The detection limit of ELISA, IC-RT-PCR, DB-RT-PCR, PC-RT-PCR and fluorescein labeled cDNA probes were determined using tenfold serial dilutions of purified virus samples and the results are summarized in Table 1.

Gel electrophoresis analysis (Fig. 2 and 3) of PCR products revealed that an identical detection level per assay was obtained for standard RT-PCR, IC-RT-PCR and PC-RT-PCR. With these three techniques up to 1 pg of virus per assay was detected. With DB-RT-PCR the detection threshold obtained was down to 1 ng of virus.

To determine the sensitivity of IC-RT-PCR, PC-RT-PCR and DB-RT-PCR for virus detection in their hosts, serially diluted purified virus was mixed with healthy tissue extracts to investigate the applicability of these methods for testing bulk samples from fields (Fig. 2 and 3). Comparison of DB-RT-PCR with ELISA showed 10-fold improvement of the former method in detection sensitivity over ELISA. Whereas the detection sensitivity of fluorescein-labeled cDNA probes and DB-RT-PCR was the same, IC-RT-PCR was 1,000 and 10,000-fold more sensitive than the fluorescein-labeled probes and ELISA, respectively. The detection sensitivity of RT-PCR and PC-RT-PCR was very similar. Comparison of fluorescein-labeled cDNA probes and ELISA with PC-PCR and RT-PCR showed 100 and 1,000-fold improvement in detection sensitivity, respectively.

Detection of PVY in potato field samples. To study the applicability of PC-RT-PCR to detect PVY infection in symptomatic potato field samples, the results obtained with this method were compared with those of standard RT-PCR, fluorescein labeled NASH and DAS-ELISA (Table 2). Of the eight samples tested, 2 were PVY-negative (Kufri Chipsona1 and Kufri Chipsona2) with all tests performed. PC-RT-PCR and RT-PCR detected PVY in six samples, non-radioactive NASH in four samples, and ELISA in only two samples.

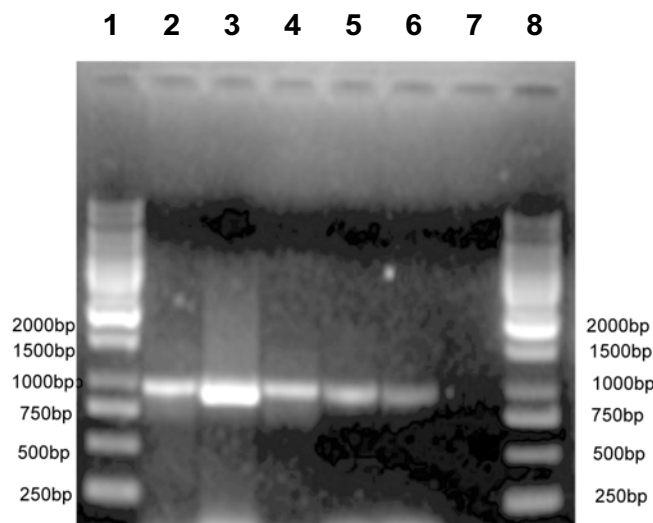


Fig. 1. Gel analysis of amplified 5'UTR and P1 gene (969 bp) from infected leaf sap and single aphid. Lanes 1 and 8, 1 kb DNA marker; lane 2, RT-PCR; lane 3, IC-RT-PCR; lane 4, PC-RT-PCR from crude sap; lane 5, PC-RT-PCR from a single aphid; lane 6, DB-RT-PCR; lane 7, healthy control.

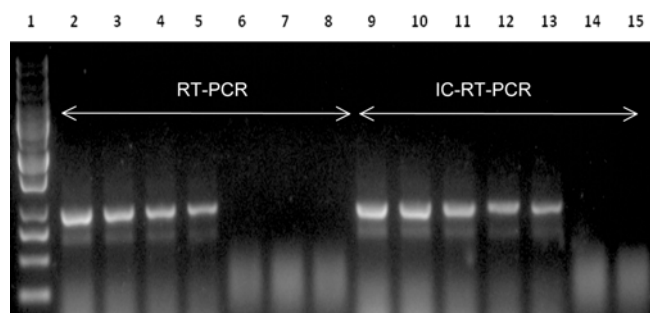


Fig. 2. Gel analysis of 5'UTR and P1 gene (969 bp), 10-fold serially diluted from infected leaf sap (left to right). Lane 1, 1 kb DNA marker (Fermentas, USA); lanes 2-7, RT-PCR; lane 8, healthy control for RT-PCR; lanes 9-14, IC-RT-PCR; lane 15, healthy control for IC-RT-PCR.

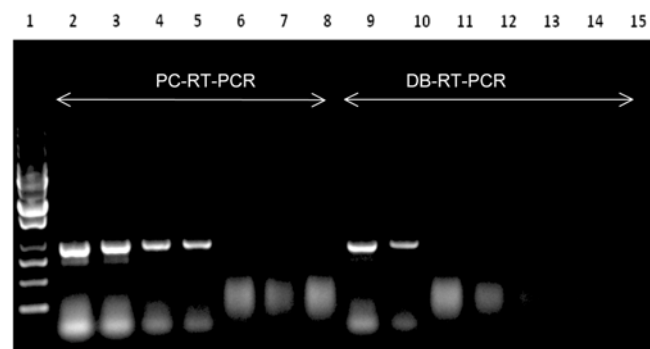


Fig. 3. Gel analysis of 5'UTR and P1 gene (969 bp), 10-fold serially diluted from infected leaf sap (left to right). Lane 1, 1 kb DNA marker (Fermentas, USA); lanes 2-7, PC-RT-PCR; lane 8, healthy control for PC-RT-PCR; lanes 9-14, DB-RT-PCR; lane 15, healthy control for DB-RT-PCR.

DISCUSSION

The protocols used in the present investigation detected PVY with varying degrees of sensitivity. IC-RT-PCR was ten-fold more sensitive than conventional RT-PCR. This was due to greater specific binding of the virions on the IgG-coated surface and higher sample volume (Jelkmann and Keim-Konald, 1997; Koenig *et al.*, 1995; Nolasco *et al.*, 1993; Wetzel *et al.*, 1992). The much lower sensitivity of DB-RT-PCR was due to the competition between virions and host cell components for binding sites (Rowhani *et al.*, 1995; Demeke and Adams, 1992). DB-RT-PCR is attractively simple and economical, but it needs further refinement and optimization to improve its sensitivity and specificity to be used in routine detection programs.

Non-persistent viruses are difficult to detect in their aphid vectors. With the development of molecular techniques, few reports have appeared on this subject. The first such study was the detection of PVY in aphids by RT-PCR, which involved laborious RNA extraction (Singh *et al.*, 1996). A PC-PCR technique involving prior virus immunocapture on membrane before PCR reaction was described, which was used for the detection of PVY in *N. tabacum* cv. Xanthi (Olmos *et al.*, 1996; Varveri, 2000).

The most significant achievement in the present study is that we have standardized a protocol for simple, sensitive and cost-effective PC-RT-PCR protocol that does not require any specific antiserum. This protocol also does not involve the requirement for chemical disruptors such as Triton X-100. In this procedure, we simply heated the printed membrane at 95°C for 10 min in distilled water to elute viral RNA followed by RT-PCR. This is a very simple procedure to detect virus in crude sap and even in a single aphid and, most importantly, the sensitivity of PC-RT-PCR is comparable to that of standard RT-PCR. This method is suitable for replacing the present methods involving RNA extraction such as the conventional RT-PCR and IC-PCR using virus specific IgGs, widely used in routine virus detection. In addition, this method not only allows the long-term preservation of spotted membranes but facilitates convenient exchange with other laboratories involved in PCR-based virus diagnosis. PC-RT-PCR is successful even on Whatman 3MM filter paper. Although in principle this method might be used for detection of viroids which lack protein coat, no experiments were done using purified viroid or viroid-infected samples.

To assess the applicability of the described protocol to field potato samples, the results of PC-RT-PCR detection were compared with those of commonly used viral detection techniques viz. RT-PCR, DAS-ELISA and non-radioactive NASH. From this comparison it is clear that PC-RT-PCR detected PVY with a high level of sensitivity and is comparable with standard RT-PCR. These

results establish the advantages of PC-RT-PCR over the current methods for PVY detection in potato, and recommend this technique for its simplicity, general applicability, specificity and sensitivity as required in routine virus diagnosis.

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EFFECTIVENESS OF PLANT ESSENTIAL OILS AGAINST *ERWINIA AMYLOVORA*, *PSEUDOMONAS SYRINGAE* pv. *SYRINGAE* AND ASSOCIATED SAPROPHYTIC BACTERIA ON/IN HOST PLANTS

B. Kokoskova¹, D. Pouvova² and R. Pavela³

¹Laboratory of Diagnostics and Epidemiology of Microorganisms, Crop Research Institute, 161 06 Prague 6, Czech Republic

²Faculty of Agrobiological Sciences, Food and Natural Resources, Czech University of Life Sciences, 165 21 Prague 6, Czech Republic

³Department of Entomology, Plant Medicine Division, Crop Research Institute, 161 06 Prague 6, Czech Republic

SUMMARY

Plant essential oils of five aromatic herb species of the family Lamiaceae (*Origanum* sp., *Thymus* sp., *Melissa* sp., *Mentha* sp. and *Nepeta* sp.) were investigated for antimicrobial effect against plant pathogenic (*Erwinia amylovora* and *Pseudomonas syringae* pv. *syringae*) and saprophytic (*Pseudomonas fluorescens*, *Pantoea dispersa* and *P. agglomerans*) bacteria commonly associated with both pathogens in plant tissue of healthy and/or diseased fruit trees in orchards. The screening was carried out *in vitro* on agar plates seeded with the target organism. All screened essential oils exhibited a higher level of antibacterial activity than streptomycin used as a standard in all tests. Plant essential oils from *Origanum compactum*, *O. vulgare* and *Thymus vulgaris* were significantly more effective against all tested bacteria than essential oils from *Melissa officinalis*, *Mentha arvensis* and *Nepeta cataria*. The main compounds of essential oils from *O. compactum*, *O. vulgare* and *T. vulgaris* were carvacrol and thymol. Apart from the three above most effective oils, those from *Nepeta cataria* and *Mentha arvensis* were also effective against *E. amylovora*, and *M. officinalis* and *M. arvensis* against *P. syringae* pv. *syringae*, however only *M. officinalis* was effective against *P. fluorescens*. All tested herb essential oils exhibited the highest antimicrobial activity against *P. agglomerans* and the lowest inhibitory activity against *P. dispersa*.

Key words: anti-microbial activity, essential oils, *Erwinia amylovora*, *Pseudomonas syringae* pv. *syringae*, *Pseudomonas fluorescens*, *Pantoea dispersa*, *Pantoea agglomerans*.

INTRODUCTION

Fire blight caused by *Erwinia amylovora* [(Burrill) Winslow *et al.*, 1920], is the most serious bacterial disease of apple, pear, hawthorn, cotoneaster and other

plant species in the family Rosaceae (Sobiczewski *et al.*, 1997; Vanneste, 2000). The pathogen is included among quarantine organisms in many countries around the world and very strict quarantine measures are enforced (Smith *et al.*, 1997). Since sanitation methods could not stop the spreading of the disease, fire blight management using appropriate chemicals and bio-control agents is the focus of ongoing efforts. Effective control can be achieved through streptomycin treatments (Johnson and Stockwell, 1998). However, its use has been prohibited in many countries due to the risk of resistance development in the population of the fire blight agent and non-target bacteria (Iacobellis *et al.*, 2005).

Pseudomonas syringae pv. *syringae* van Hall 1902 is polyphagous plant pathogenic bacterium, which survives usually as an epiphyte on host plants to become pathogenic under appropriate environmental conditions. This bacterium causes serious losses to stone fruits, in which it elicits a variety of symptoms, i.e. blossom blast, spur dieback, leaf necroses, bark cankers and gummosis of woody tissues (Renick *et al.*, 2008). As *Pss* is an active ice nucleation bacterium, it is harmful to plants particularly at the time of spring and autumn frosts (Renick *et al.*, 2008). The only bactericide registered for bacterial canker management is copper, which is of limited use because of its potential phytotoxicity (Kennelly *et al.*, 2007). The efficacy of copper has also been limited by the development of copper-resistant strains of *Pss* (Sundin *et al.*, 1989; Vanneste *et al.*, 2005).

Commonly occurring saprophytic bacteria such as *Pseudomonas fluorescens*, *Pantoea dispersa* and *Pantoea agglomerans* survive in the phylloplane of fruit trees together with plant pathogenic bacteria. These bacteria occurring on/in plant tissue do not cause harm to woody plants and some strains can even be useful as antagonists against plant pathogenic bacteria (Johnson and Stockwell, 1998; Elkins *et al.*, 2005).

Control of plant bacterial diseases remains difficult due to the limited availability of bactericides. Preparations based on copper compounds, which are applied most frequently, are not sufficiently effective in disease management for apple and stone fruit orchards and could have unfavourable effects either on the environment or on human and animal health (Iacobellis *et al.*,

2005; Vanneste *et al.*, 2005). Search of an environmentally friendly biological alternative is a permanent task of present research (Chen *et al.*, 2009).

In plant protection, the use of herb essential oils looks promising against plant bacterial pathogens because some of them have a strong antimicrobial activity. The potential effectiveness of herb essential oils against different plant pathogenic bacteria has been verified by many authors (Dorman and Deans, 2000; Iacobellis *et al.*, 2005; Vasinauskiene *et al.*, 2006; Kokoskova and Pavela, 2007; Rhouma *et al.*, 2009).

Some essential oils proved useful also against fungi, as shown by those from oregano, thyme, dictamnus and majoram that were effective against *Clavibacter michiganensis* subsp. *michiganensis* and *Botrytis cinerea* and *Fusarium* sp. (Daferera *et al.*, 2003). Moreover, extracts from *Pistacia* and *Schinus* spp. were effective against *Agrobacterium* sp. and *Pseudomonas* sp., and also against *Fusarium* sp. and *Rhizoctonia* sp. (Rhouma *et al.*, 2009). Combination of plant extracts or etheric oils from plants with copper and other chemical compounds can increase their effectiveness (Zeller, 2005).

Mosch *et al.* (1989, 1993) reported that extracts from *Rhus typhina*, *Juglans nigra*, *Berberis vulgaris*, *Mahonia aquifolium*, *Alium sativum*, *Hedera helix* and *Viscum album* inhibited the *in vitro* and *in vivo* growth of *E. amylovora*. Extracts from *H. helix* and *V. album* showed a remarkable inhibitory effect of bacterial infections to *Cotoneaster* sp. probably connected with resistance induction (Mosch *et al.*, 1996). Scortichini and Rossi (1989, 1991) observed that the terpenoids geraniol and citrolleol decreased the growth of *E. amylovora* most effectively out of 20 terpenoidal compounds tested.

Many control agents have been used in the management of fire blight and tested against blossom blight infections. Some compounds used against *E. amylovora* did not have any bactericidal activity, but either triggered the plant defence mechanism leading to systemic acquired resistance (SAR) (acibenzolar-S-methyl, trade name Bion) or suppressed shoot growth (prohexadione-

calcium, trade name Regalis), thus lowering shoot susceptibility to infection (Psallidas and Tsianthos, 2000; Kennelly *et al.*, 2007).

Promising tools for fire blight control are also yeast preparations such as BPASc and Blossom-Protect. They contain blastospores of *Aureobasidium pullulans*. Blossom-Protect reduces pH to approximately 4 and has two mechanisms to prevent neutralisation, i.e. a strong buffer (component A) which decreases the pH on the plant surface immediately after application, which is then kept low by *A. pullulans* during growth on blossoms surfaces. Their disadvantage is a potential fruit russet (Kunz *et al.*, 2006).

Recently, two toxins from *Bacillus amyloliquefaciens* FZB42T with strong antagonistic effects against *E. amylovora*, proved promising for fire blight control (Chen *et al.*, 2009).

This study is a further development of a previous work on the *in vitro* efficacy of 34 essential oils from different aromatic herbs against *E. amylovora* (Kokoskova and Pavela, 2007). The most effective essential oils were selected for detailed tests against *P. syringae* pv. *syringae*, *E. amylovora* and associated saprophytic bacteria.

MATERIALS AND METHODS

Bacteria. Three reference strains of *E. amylovora* (IVIA 1525-6, NCPPB 1114, GER 270/97) and two reference strains from other species were used in these tests, i.e. *P. s.* pv. *syringae* (CCM 4073, LMG 1247), *P. fluorescens* (CCM 2115, CRI 22), *P. dispersa* (CCM 4414, CCM 4341) and *P. agglomerans* (CCM 2406, CCM 3490). Bacterial cultures of *E. amylovora*, *P. dispersa* and *P. agglomerans* were grown on nutrient beef peptone agar (Schaad *et al.*, 1991) and *Pss* on King B medium and kept at 25°C (King *et al.*, 1954).

Plant material and chemicals. All plant essential oils (EOs) used in this study, extracted by steam or hy-

Table 1. Main compounds of selected essential oils tested for potential inhibitory effect against plant pathogenic and saprophytic bacteria.

Plants	Origin	Plant organ	Main compounds (relative area %) ^a
<i>Melissa officinalis</i>	Spain	Flowers/leaves	citronellal (12.9), citronellol (6.3), neral (24.5), geraniol (31.3), β -caryophyllene (3.9)
<i>Mentha arvensis</i>	India	Aerial part	menthol (74.5), menthone (9.2), methyl acetate (3.1)
<i>Nepeta cataria</i>	Canada	Flowering tops	nepetalactone (81.1), β -caryophyllene (10.8)
<i>Origanum compactum</i>	Morocco	Aerial part	carvacrol (36.2), p-cymene (22.3), thymol (18.6), γ -terpinene (5.2)
<i>Origanum vulgare</i>	Greece	Aerial part	thymol (28.5), thymyl methyl ether (5.7), carvacrol (19.5), β -bisabolene (12.6)
<i>Thymus vulgaris</i>	Spain	Aerial part	p-cymene (16.3), γ -terpinene (5.6), geraniol (8.3), thymol (6.8), carvacrol (7.9)

^a According to the data of the gas chromatography analysis of essential oils provided by the manufacturer.

drodistillation (Table 1), were purchased from Essential Oil University, Charlestown, USA. Streptomycin (Sigma, Germany) in concentration 0.02% was used as control.

Experiments. Antimicrobial activity tests were carried out *in vitro* on agar plates seeded by the target organism. ENA II medium (6.6 g nutrient agar no. 2, 6.6 g glucose, 0.7 g yeast extract, 15.0 g agar, 1 liter sterile water, pH 6.6) was used for the screening of EOs (Kokoskova, 1992). *E. amylovora* was used as 24 h culture but the other bacteria were used as 48 h cultures in a concentration corresponding to $OD_{620} \approx 0.5$ in all tests. EOs were delivered on the bacteria-seeded agar surface at a dose of 1 μ l after its preparation. Each EO was assayed in six replicates. Agar plates without bacteria or with bacteria but no essential oils served as negative and positive controls, respectively.

After treatment, agar plates were covered with Parafilm and incubated at $25 \pm 1^\circ\text{C}$ for three days, prior to the measurement of the inhibition zones. The antimicrobial efficacy index (IAE) of each EO was calculated for each bacterium. EO effectiveness was directly proportional to the size of the inhibition zone (Kokoskova and Pavela, 2007) and was evaluated according to four levels of effect: (i) weaker than the standard (by more than 10%); (ii) equal to the standard ($\pm 10\%$); (iii) up to 50 % higher than the standard (by 10-50%); (iv) more than 50% higher than the standard.

Statistical analysis. The antimicrobial efficacy index (IAE) was calculated with the formula:

$$\text{IAE (\%)} = \{-1 \times [(C-T)/(C+T)]\} \times 100$$

where C is the average inhibitory zone (cm) on the standard dish (streptomycin 0.02%) and T is the aver-

age inhibitory zone on the treated dish, to which the EO was applied (Table 2a, 2b). The IAE (%) indicates whether the efficacy of EOs is lower and/or higher than the streptomycin standard (Kokoskova and Pavela, 2007) (Fig 1).

A one-way analysis of variance (ANOVA test) was performed to compare the areas of effectiveness (inhibitory zones) of EO with streptomycin, followed by a ranking of their averages using Tukey's test. Differences between means were considered significant when $P \leq 0.05$ (Table 2a, 2b).

RESULTS

Erwinia amylovora. EOs from *Thymus vulgaris* and *Origanum compactum* showed antimicrobial activity significantly (more than 50%) higher ($P \leq 0.05$) than streptomycin. The antimicrobial effect of essential oils from *Origanum vulgare*, *Nepeta cataria* and *Mentha arvensis* was also higher (up to 50%), whereas the essential oil from *Mellissa officinalis* showed approximately the same biological effectiveness as streptomycin (Table 2a, Fig. 1).

***Pseudomonas syringae* pv. *syringae*.** The antimicrobial activity of EOs from *O. compactum*, *O. vulgare* and *T. vulgaris* was significantly (more than 50%) higher ($P \leq 0.05$) than that of streptomycin. EOs from *M. officinalis* and *M. arvensis* showed also a higher effectiveness (up to 50%) than streptomycin, but the EO from *N. cataria* was as effective as streptomycin (Table 2a, Fig. 1).

Pseudomonas fluorescens. The antimicrobial activity of EOs from *T. vulgaris* and *O. vulgare* was significantly

Table 2a. Effectiveness of plant essential oils against *Erwinia amylovora* and *Pseudomonas syringae* pv. *syringae*

Essential oil/Chemical	Growth reduction expressed as average of inhibition zone diameters (cm)						
	<i>Erwinia amylovora</i>				<i>Pseudomonas syringae</i> pv. <i>syringae</i>		
	IVIA 1525-6	NCPPB 1114	GE 270/97	Average	CCM 4073	LMG 1247	Average
<i>Mellissa officinalis</i>	6.17 \pm 1.33	7.0 \pm 0.89	8.7 \pm 2.23	7.11 \pm 1.71	5.8 \pm 0.75	7.7 \pm 0.82	6.7 \pm 1.22
<i>Mentha arvensis</i>	7.67 \pm 2.5	12.5 \pm 2.26	12.7 \pm 0.82	10.94 \pm 3.04	4.5 \pm 0.55	5.5 \pm 1.22	5 \pm 1.04
<i>Nepeta cataria</i>	24.00 \pm 0.89	23.7 \pm 1.51	12.1 \pm 0.75	19.94 \pm 5.75 *	3.5 \pm 0.55	4.2 \pm 0.75	3.8 \pm 0.72
<i>Origanum compactum</i>	21.33 \pm 4.5	29.3 \pm 3.33	24.8 \pm 4.31	25.17 \pm 5.10 *	15.5 \pm 1.22	26.3 \pm 3.83	20.9 \pm 6.27 *
<i>Origanum vulgare</i>	14.50 \pm 1.38	24.2 \pm 1.47	25.5 \pm 1.22	21.39 \pm 5.20 *	14.5 \pm 0.55	25.7 \pm 1.37	20 \pm 5.92 *
<i>Thymus vulgaris</i>	14.33 \pm 2.5	37.0 \pm 3.52	31.3 \pm 4.47	28.11 \pm 10.71 *	16.0 \pm 0.89	24.0 \pm 1.55	20 \pm 4.35 *
Streptomycin 0.02 %	12 \pm 3.41	5.3 \pm 0.52	6 \pm 0.63	7.78 \pm 3.62	3.7 \pm 0.52	3.7 \pm 0.82	3.7 \pm 0.65

Values represent means of six replicates

*The diameter (cm) of inhibitory zones (mean \pm standard error); Asterisks indicate means that are significantly different from control ($P \leq 0.05$)

IVIA - Collection of Instituto Valenciano de Investigaciones Agrarias, Moncada, Valencia, Spain

NCPPB - National Collection of Plant Pathogenic Bacteria, York, UK

GE 270/97 - strain provided by Dr. K. Richter, Institute of Epidemiology and Resistance, Federal Centre for Breeding Research on Cultivated Plants, Aschersleben, Germany

CCM - Czech Collection of Microorganisms, Brno, Czech Republic

LMG - BCCM/LMG - Laboratory of Microbiology Gent Bacteria Collection, Gent, Belgium

Table 2b. Effectiveness of plant essential oils against *Pseudomonas fluorescens*, *Pantoea dispersa* and *Pantoea agglomerans*.

Essential oil/Chemical	Growth reduction expressed in average of diameter inhibitory zones (cm)									
	<i>Pseudomonas fluorescens</i>			<i>Pantoea dispersa</i>			<i>Pantoea agglomerans</i>			
	CCM 2115	CRI 22	Average	CCM 4114	CCM 4341	Average	CCM 2406	CCM 3490	Average	
<i>Melissa officinalis</i>	7 ± 1.41	6.5 ± 0.55	6.7 ± 1.06	6.2 ± 0.75	4.3 ± 0.52	5.2 ± 1.14 *	9.33 ± 1.03	19.2 ± 1.33	14.2 ± 5.26 *	
<i>Mentha arvensis</i>	4.8 ± 0.75	5.5 ± 0.55	5.2 ± 0.72	10.3 ± 1.03	7.3 ± 0.52	8.8 ± 1.75	11 ± 1.10	11.2 ± 0.75	11.1 ± 0.90 *	
<i>Nepeta cataria</i>	3.5 ± 0.55	3.33 ± 0.52	3.4 ± 0.51	8 ± 0.00	3.8 ± 0.75	5.92 ± 2.23 *	11.3 ± 1.21	7.5 ± 0.55	9.4 ± 2.19	
<i>Origanum compactum</i>	15.2 ± 0.75	11.3 ± 1.03	13.2 ± 2.18 *	15.7 ± 0.52	12.7 ± 0.52	14.2 ± 1.64 *	40.0 ± 0	33.7 ± 4.97	36.8 ± 4.71 *	
<i>Origanum vulgare</i>	10.5 ± 1.05	17.7 ± 2.25	14.1 ± 4.1 *	15.3 ± 0.52	9.7 ± 0.52	12.5 ± 3 *	40.0 ± 0	30.8 ± 2.04	35.4 ± 4.98 *	
<i>Thymus vulgaris</i>	16.2 ± 1.83	17 ± 2.28	16.6 ± 2.02 *	17.33 ± 0.52	11.8 ± 0.52	14.6 ± 2.94 *	40.0 ± 0	35.5 ± 5.43	37.7 ± 4.35 *	
Streptomycin 0.02 %	3.7 ± 0.52	5.5 ± 1.05	4.6 ± 1.24	9.7 ± 0.52	8.7 ± 0.52	9.2 ± 0.72	5.0 ± 0.63	4.8 ± 1.17	4.9 ± 0.9	

Values represent means of six replications

* The diameter (cm) of inhibitory zones (mean ± standard error); Asterisks indicate means that are significantly different from control ($P \leq 0.05$).

CCM - Czech Collection of Microorganisms, Brno, the Czech Republic

CRI - Collection of Plant Pathogenic Bacteria, Dept. of Bacteriology, Crop Research Institute, Prague, Czech Republic

(slightly more than 50%) higher ($P \leq 0.05$) than that of streptomycin. EOs from *O. compactum* and *M. officinalis* were also more effective (up to 50%) than streptomycin, whereas EO from *M. arvensis* showed the same efficacy of streptomycin and *N. cataria* EO was less effective than streptomycin (Table 2b, Fig. 1).

Pantoea dispersa. EOs from *T. vulgaris*, *O. compactum* and *O. vulgare* had antimicrobial activity significantly (up to 50%) higher ($P \leq 0.05$) than that of streptomycin. *M. arvensis* EO showed the same biological effectiveness as streptomycin, but essential oils from *Nepeta cataria* and *M. officinalis* were less effective than streptomycin (Table 2b, Fig. 1).

Pantoea agglomerans. EOs from *T. vulgaris*, *O. compactum* and *O. vulgare* had antimicrobial activity significantly (more than 50%) higher ($P \leq 0.05$) than that of streptomycin, the same as EOs from *M. officinalis*, *M. arvensis* and *N. cataria* that showed higher biological effectiveness (up to 50%) than streptomycin (Table 2b, Fig. 1).

DISCUSSION

Based on previous results (Kokoskova and Pavela, 2007), where thirty-four plant EOs had been screened for potential *in vitro* effectiveness against *E. amylovora*, the six most effective oils, i.e. those from *M. officinalis*, *M. arvensis*, *N. cataria*, *O. compactum*, *O. vulgare* and *T. vulgaris* were chosen for further experiments. In the present study, the efficacy of these EOs was verified against at least two strains of one species of plant pathogenic and saprophytic bacteria to obtain more reliable results.

We wanted to know whether the tested EOs were able or not to decrease the growth of saprophytic bacteria, because some strains of these species have been used as antagonists or as biocontrol agents against fire blight and other diseases (Johnson and Stockwell, 1998; Elkins *et al.*, 2005). Streptomycin was used as standard, because of the unsatisfactory efficacy of oxychloride-Cu (Kokoskova and Pavela, 2007).

The plant pathogenic bacteria *P. syringae* pv. *syringae* and *E. amylovora* and the saprophytic bacteria *P. fluorescens*, *P. dispersa* and *P. agglomerans* showed sensitivity to all plant EOs presently tested, although differences in the antibacterial activity of some oils were found (Table 2a, 2b). Streptomycin was more effective against Gram-negative anaerobic bacteria (*E. amylovora*, *P. dispersa* and *P. agglomerans*) than against Gram-negative aerobic bacteria (*Pss*, *P. fluorescens*) as is clear from Table 2a and 2b.

The variability of efficacy of plant EOs compared to streptomycin (axis X) is shown in Fig. 1. In general, the

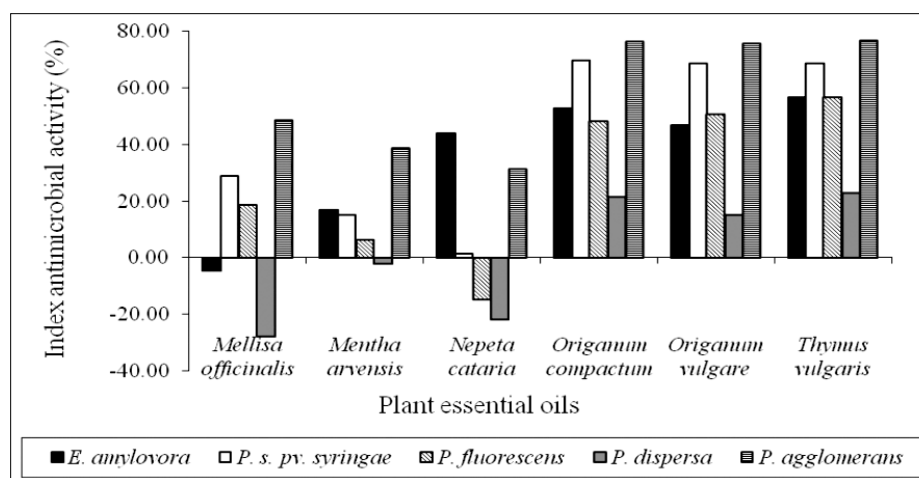


Fig. 1. Effectiveness of plant essential oils against *Erwinia amylovora*, *Pseudomonas syringae* pv. *syringae* and bacteria associated with them on/in plant tissue compared to 0.02% streptomycin. Effectiveness: >> control (more than 50%); > control (10 - 50%); ~ control ($\pm 10\%$); < control (less - 10%)

majority of EOs showed a higher effectiveness than streptomycin. EOs from *O. compactum*, *O. vulgare* and *T. vulgaris* were significantly more effective than those from *M. officinalis*, *M. arvensis* and *N. cataria*. All EOs were most effective against *P. agglomerans* and least effective against *P. dispersa*. Since a higher efficacy of EOs against phytopathogenic than saprophytic bacteria was expected, the high sensitivity of *P. agglomerans* came as a surprise, which, however, may depend on the specific strain tested. As to *Pseudomonas* spp., all EOs were more effective against *Pss* than *P. fluorescens*. This is not surprising because *P. fluorescens*, like *P. aeruginosa*, is among the most resistant organisms, representing a problem particularly in human medicine (Papadopoulos *et al.*, 2006; Bouhdid *et al.*, 2008).

Regarding plant pathogenic bacteria, EOs from *O. compactum*, *O. vulgare*, *T. vulgaris* and *M. arvensis* were more effective against *P. syringae* pv. *syringae* than *E. amylovora* while *M. officinalis* EO showed a good efficacy against *P. syringae* pv. *syringae*, but not *E. amylovora*, and the *N. cataria* EO was more effective against *E. amylovora*, than *P. syringae* pv. *syringae* (Fig. 1).

Our results show that the screened EOs are potentially highly effective against Gram-negative bacteria in general, in agreement with literature records relative to oils from *Origanum* sp. and *Thymus* (Chaira *et al.*, 1996; Soyulu *et al.*, 2005; Iacobellis, 2005; Kokoskova *et al.*, 2006; Bouhdid *et al.*, 2008). EOs from *Origanum* sp. and *Thymus* sp. are rich in antioxidative phenolic compounds, which are believed to be responsible for their marked antimicrobial activity (Zaika and Kissinger, 1981; Chizzola *et al.*, 2008). The studies of Dorman and Deans (2000), which found the volatile oils thymol from *T. vulgaris* and carvacrol from *O. vulgare* subspecies *hirtum* to have a wide spectrum of antimicrobial activity,

are in agreement with our results. In addition, Vanneste (1996) reported that some plant extracts and some EOs could inhibit *E. amylovora* *in vitro*, thyme oil in particular, which again tallies with our results.

Vasinauskiene *et al.* (2006) found a significant inhibitory effect *in vitro* of all nine chemo-types of *O. vulgare* EO on the growth of *P. syringae* pv. *syringae* and other plant pathogenic bacteria. EOs from *O. compactum* and *T. vulgaris* have shown a high level of antimicrobial activity against *P. putida* (Oussalah *et al.*, 2006). Interestingly, we found that the same oils were effective against *P. fluorescens*. In our study, essential oil from mint showed a lower level of inhibitory activity than EOs from oregano and thyme, as reported by others (Sivropoulou *et al.*, 1996; Mazzanti *et al.*, 1998).

Chemical analysis of the six EOs tested (Table 1) showed that their major constituents are phenolic monoterpenes (carvacrol and thymol), monoterpene hydrocarbons (p-cymene or γ -terpinene) and aldehydes (geranial or citronellol) (Table 1). The components with phenolic structures, such as carvacrol and thymol, were highly active against all the screened microorganisms, in agreement with previous reports (Sivropoulou *et al.*, 1996; Dorman and Deans, 2000). Many authors have suggested that polyphenols inhibit the growth of microorganisms by forming complexes with their enzymes and proteins (Rhouma *et al.*, 2009). Phenolic compounds can dissolve the bacterial membrane, thus penetrating the cell, where they interact with cell metabolism (Judis, 1963; Juven *et al.*, 1972; Oussalah *et al.*, 2006). Carvacrol and thymol disrupt the plasma membrane, which increases its permeability and depolarizes its potential (Xu *et al.*, 2008).

Comparison of our data with previously published results is based on the fact that the composition of plant

essential oils and extracts is influenced by the geographical origin of the hosts from which they are extracted, the environmental conditions, and the host species or subspecies (Faleiro *et al.*, 2003; Sivropoulou *et al.*, 1996; Sarac and Ugur, 2008). EO composition varies also depending on the host variety, time and the way of harvesting, type of storage, extraction method, etc. All these factors influence the chemical composition and the relative proportion of individual constituents of EOs (Oussalah *et al.*, 2006), so that EOs form the same plant species collected from different locations can show different chemical composition and different levels of antimicrobial activity (Vasinauskiene *et al.*, 2006; Sarac and Ugur, 2008, Chizolla *et al.*, 2008). Moreover, the biological activity of EOs can be influenced by the different percentage of inhibitory active compounds and by their antagonistic and/or synergistic effect (Hummelbrunner and Isman, 2001).

In any case, the essential oils screened in our study seem to have a promising potential as new pesticide products or as templates for new, more effective compounds.

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TRANSCRIPT ANALYSIS OF THE BEAN POLYGALACTURONASE INHIBITING PROTEIN GENE FAMILY REVEALS THAT *PVPGIP2* IS EXPRESSED IN THE WHOLE PLANT AND IS STRONGLY INDUCED BY PATHOGEN INFECTION

R.M. Kalunke¹, M. Janni¹, L. Sella², P. David³, V. Geffroy^{3,4}, F. Favaron² and R. D'Ovidio¹

¹ Dipartimento di Agrobiologia e Agrochimica, Università della Tuscia, 01100 Viterbo, Italy

² Dipartimento Territorio e Sistemi Agro-Forestali, Sezione Patologia Vegetale, Università degli Studi di Padova, 35020 Legnaro (PD), Italy

³ Institut de Biologie des Plantes, UMR-CNRS 8618, bât. 630, Université Paris-Sud, 91405 Orsay, France

⁴ Unité Mixte de Recherche de Génétique Végétale, Institut National de la Recherche Agronomique, 91190 Gif-sur-Yvette, France

SUMMARY

The expression analysis of the four polygalacturonase-inhibiting protein (*Pgip*) genes composing the bean (*Phaseolus vulgaris* L.) *Pgip* family showed a pattern of transcriptional variation in young leaves, hypocotyls, roots and pods with *Pvpgip1* not expressed, *Pvpgip2* expressed in all organs, *Pvpgip3* and *Pvpgip4* poorly expressed in roots. We compared also transcript accumulation of the four *Pvpgip* genes during infection of bean plants with the fungal pathogens *Botrytis cinerea*, *Sclerotinia sclerotiorum* and *Colletotrichum lindemuthianum*. qRT-PCR analyses showed that the transcript level of *Pvpgip1*, *Pvpgip2* and *Pvpgip3* increases significantly following fungal infection, whereas *Pvpgip4* remains unchanged. The level of induction was different between the three genes, *Pvpgip2* exhibiting the strongest transcript accumulation. The induction pattern was similar in the pathosystems bean-*S. sclerotiorum*, bean-*B. cinerea*, and in the compatible interaction bean-*C. lindemuthianum*, with a maximum of transcript accumulation in the late stage of infection. Instead, in the incompatible interaction bean-*C. lindemuthianum*, *Pvpgip1*, *Pvpgip2* and *Pvpgip3* showed an early and transient transcript accumulation, with *Pvpgip2* exhibiting an earlier and higher induction. These results extend previous analyses of the whole *Pvpgip* transcript and provide additional evidences of the relevant role of PvPGIP2 in plant defence.

Key words: polygalacturonase-inhibiting protein (PGIP), gene family, quantitative real-time polymerase chain reaction, *Phaseolus vulgaris*, fungal pathogens.

INTRODUCTION

During infection and colonization of the plant tissue, pathogens produce a number of molecules to surmount

the host barriers. Among these, cell wall degrading enzymes (CWDEs) represent important pathogenicity factors for several pathogens. Endopolygalacturonases (PGs; EC 3.2.1.15) are some of the first CWDEs secreted during the infection process. They cleave the α -(1→4) linkages between D-galacturonic acid residues in homogalacturonan causing cell separation and maceration of host tissue. The importance of PG in pathogenesis has been demonstrated for some fungal pathogens, including *Botrytis cinerea* (ten Have *et al.*, 1998), *Alternaria citri* (Ishiki *et al.*, 2001) and *Claviceps purpurea* (Oeser *et al.*, 2002). The activity of this enzyme is inhibited by the polygalacturonase-inhibiting protein (PGIP) and the overexpression of this inhibitor in transgenic plants showed a significant reduction of disease symptom development caused by several fungal pathogens (Powell *et al.*, 2000; Ferrari *et al.*, 2003; Aguero *et al.*, 2005; Manfredini *et al.*, 2006; Joubert *et al.*, 2006; Janni *et al.*, 2008).

PGIPs are plant cell wall glycoproteins that belong to the superfamily of leucine-rich repeat proteins (LRRs) of the extracytoplasmic type (Jones and Jones, 1997). The genes encoding these proteins have been characterized in a number of plants, including both monocot and dicot species, and in all these species the deduced encoded product is composed by 9-10 imperfect LRRs of 24 residues each (D'Ovidio *et al.*, 2004a). These analyses showed also inter-specific variation in the number of *Pgip* genes per genome, ranging from 2 in *Arabidopsis thaliana* (Ferrari *et al.*, 2003) to 16 in *Brassica napus* (Hegedus *et al.*, 2008). Furthermore, the *Pgip* genes showed inter- and intra-specific variation in the transcriptional regulation and inhibition properties against fungal and insect PGs (De Lorenzo *et al.*, 2001; D'Ovidio *et al.*, 2004a; Federici *et al.*, 2006; Protsenko *et al.*, 2008).

The *Pgip* family of common bean (*Phaseolus vulgaris*) is one of the best studied such families. The full complement comprises four clustered genes of about 1 kbp each (*Pvpgip1*, *Pvpgip2*, *Pvpgip3*, *Pvpgip4*) oriented in the same direction and spanning a 50 kbp region (D'Ovidio *et al.*, 2004b) on the linkage group B2 (Geffroy *et al.*, 2000). Genomic arrangement and sequence similarities (>80% at nucleotide level) between the four bean *Pgip* genes suggested that they derive from a common ancestor through a sequence of duplication-divergence-

duplication events (D'Ovidio *et al.*, 2004b). Sequence comparison between the bean *Pgip* genes and those from the close relative soybean (*Glycine max*) indicates that *Pvpgip1* and *Pvpgip2* are the most closely related to the ancestral gene, since their sequence is much more similar to *Gmpgip3* (>85% nucleotide identity), one of the four characterized soybean *Pgip* genes, than to *Pvpgip3* or *Pvpgip4* (D'Ovidio *et al.*, 2006).

Inhibition assays with the four heterologous expressed bean *Pgip* genes revealed both partial redundancy and subfunctionalization against PGs, with PvPGIP2 being the most effective and wide spectrum PG inhibitor of fungal PGs (Manfredini *et al.*, 2006; D'Ovidio *et al.*, 2004b, 2006). The sequence and inhibition activity of this inhibitor is also strongly conserved in the bean germplasm (>99% nucleotide identity) and in the related *Phaseolus* species (>97% nucleotide identity), suggesting its adaptive significance (Farina *et al.*, 2009). Further analyses at the protein level to determine the contribution of each single PvPGIP in plant defence are hampered by their high level of sequence similarity (>80%). Consequently, their occurrence and accumulation during normal growth or following stress conditions has been detected by Western blot only as whole PvPGIP accumulation (Bergmann *et al.*, 1994). These analyses showed that wounding or salicylic acid treatment of bean tissues can induce PvPGIP accumulation following a transient accumulation of the whole *Pvpgip* transcript (Bergmann *et al.*, 1994).

Evidence for the specific contribution of each *Pvpgip* member in plant defence has been detected at the transcript level. Gene-specific transcript analysis of the four *Pvpgip* genes showed that these genes can differentially respond to elicitors, salicylic acid or mechanical injury (D'Ovidio *et al.*, 2004b). These results prompted us to investigate the patterns of transcriptional variation of the four *Pvpgip* members during normal growth and following fungal pathogen infection with the specific aim of verifying whether the transcript accumulation of some of them correlates better with the host defence response. We investigated the kinetics of transcript accumulation in three different bean-pathogen interactions that included the necrotrophic fungal pathogens *Sclerotinia sclerotiorum* (Lib. De Bary) and *Botrytis cinerea* Pers. ex Fr., the agents of white and grey mold of bean, respectively, and the hemibiotrophic pathogen *Colletotrichum lindemuthianum* (teleomorph *Glomerella lindemuthiana*), the causal agent of anthracnose, one of the main diseases of common bean. In parallel, we verified also the inhibiting activity of the four PvPGIPs against the PG activity produced by these three fungal pathogens.

MATERIAL AND METHODS

Biological materials and plant treatments. Common

bean seeds of cv. Etna were surface-sterilized in sodium hypochlorite (0.5% v/v) for 10 min, then rinsed thoroughly in sterile water. Plants were grown at maturity or for 7 days with a 14 h photoperiod at 21°C in sterilized moist vermiculite. Uniformly growing seedlings were selected for tissue preparations or for infection experiments. For tissue preparation, 20 seedlings were sectioned into young leaves, hypocotyls and roots; hypocotyls and roots were further sectioned in three equal parts (apical, middle and basal) and two parts (apical and basal), respectively. Young pods were collected 12 days post anthesis (DPA). The material was frozen in liquid nitrogen and stored at -80°C until used for total RNA isolation.

The B-24 isolate of *S. sclerotiorum* and the PM-10 isolate of *B. cinerea* were grown for 3 days at 25°C on potato dextrose agar (PDA) to obtain mycelium for inoculation of bean seedlings.

Bean seedlings were artificially infected with *S. sclerotiorum* or *B. cinerea* by inoculating the hypocotyls with a plug of mycelium-colonized agar (10×2 mm) as reported (Sella *et al.*, 2005). Infected seedlings were incubated at 25°C in the dark at high humidity. Hypocotyls of control plants were treated similarly using a plug of agar without the fungal mycelium. Hypocotyl segments (about 10 mm) from six different infected or control plants were cut transversally exactly below the agar plugs at 0, 8, 16, 24 and 48 h post inoculation (hpi). Samples were frozen in liquid nitrogen and stored at -80°C until used for RNA extraction.

For infection experiments with *C. lindemuthianum*, seeds of *P. vulgaris* genotype BAT93 were germinated and grown for 8 days as described previously (Geffroy *et al.*, 1999). The two cotyledonary leaves of BAT93 seedlings were sprayed, 8 days after germination, with M126 (virulent) or C531 (avirulent) *C. lindemuthianum* conidia according to Geffroy *et al.* (1999). Control plants were sprayed with water and incubated in a control growth chamber under the same temperature (19°C) and humidity conditions (90% relative humidity) as the infected plants. The cotyledonary leaves from three different plants were harvested at 10, 24, 48, 72 and 96 hpi, frozen in liquid nitrogen and stored at -80°C until used for RNA extraction. All infection experiments were repeated at least twice.

RNA extraction, RT-PCR and quantitative RT-PCR.

Total RNA was extracted using the RNeasy Plant Mini Kit (Qiagen, Italy) or the Nucleospin RNA plant Kit (Macherey Nagel, Germany) according to manufacturer's instructions. Contaminant DNA was removed with DNA-free TM (Applied Biosystems/Ambion, Italy) and its absence was verified by the absence of the unprocessed *Ubiquitin* gene amplification product in PCR assays performed by using total RNA as template. RNA concentration was determined spectrophotometrically.

RT-PCR was carried out on total RNA using “Ready To Go RT-PCR Beads” (Amersham Biosciences Europe GmbH, Germany), according to manufacturer’s instructions in a MyCycler thermal cycler (Bio-Rad, Italy). Amplification conditions and primer combinations specific for the four *Pvpgip*s and the house-keeping *Ubiquitin* gene (*Pvubq*) were those described in D’Ovidio *et al.* (2004b). Each RT-PCR reaction was made in triplicate. RT-PCR analysis were repeated in at least two different biological replicas.

qRT-PCR experiments were performed using the iCycler (Bio-Rad, Italy) and the master mix iQTMSYBER Green Supermix (BioRad, Italy) containing the flurogenic SYBER Green I DNA binding dye. Each reaction was made in triplicate. Oligonucleotide primers were designed on the basis of the four *Pvpgip* genes (EMBL/GenBank data libraries accession Nos AJ786408, AJ786409, AJ786410, AJ786411) using Primer 3 [http://fokker.wi.mit.edu/primer3/ (Rozen *et al.*, 2000)] and have the following sequences (sense and antisense, respectively): 5'-CCTCACCGGGAAGATTC-CA and 5'-TTAGCTGCGCTAGTCCCTGA for *Pvpgip1*, 5'-CCTCACCGGGAAGATTCCG and 5'-TTAGCTGCGTCAGTCCCTGC for *Pvpgip2*, 5'-TCTCACCGGCAATATACCGG and 5'-CTAAGTCCTTC-GACTTCTGA for *Pvpgip3*, 5'-CTCACCGGCAATATTC-CGA and 5'-CTAAGTTCTTCGACCTCAAA for *Pvpgip4* and 5'-CAGCTGGAGGATGGAAGGA and 5'-TCCGAAGTCTCCACCTCAAGA for *Pvubq*.

Total reaction volume was 20 µl and included 10 µl (2X) Mastermix, 100 ng of cDNA, 0.5 µl of 10 µM of each forward and reverse primers and volume adjusted with water. Reaction conditions were as follows: one cycle at 50°C for 2 min, 94°C for 5 min, then 40 cycles at 94°C for 30 sec, 60°C for 40 sec and 72°C for 30 sec.

The specificity of the primers was verified in separate PCR experiments using, as template, recombinant plasmid DNA containing the appropriate *Pvpgip* genes. Each specific amplicon obtained was also verified by nucleotide sequence.

The CT values of target genes and house-keeping genes were used for further analysis and samples with more than 0.6 value difference within triplicates were not considered. The relative expression analysis was determined by using the $2^{-\Delta\Delta CT}$ method (Livak *et al.*, 2001), (Applied Biosystems User Bulletin No. 2-P/N 4303859). Calculation and statistical analyses were performed by Gene Expression Macro™ Version 1•1 (Bio-Rad, Italy). The qRT-PCR experiments were repeated at least three times for each sample in two different biological replicas.

Nucleotide sequence analysis and *in silico* search of putative regulatory elements. Amplicons were subjected to sequencing reactions using the ABI PRISM dye terminator cycle sequencing ready reaction kit and

DNA sequences were determined with the semiautomatic ABI PRISM 310 sequencer (Applied Biosystem, Italy). Nucleotide sequences were also determined through the MWG-BIOTECH AG (Germany). Sequence analyses were performed using the DNAMAN software (Lynnon Biosoft, Canada).

The 5' flanking region of all four *Pvpgip* genes were scanned for the presence of *cis*-elements identical with or similar to the motifs deposited in two different plant *cis*-acting regulatory elements databases, PlantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) and PLACE (http://www.dna.affrc.go.jp/htdocs/PLACE/signalscan.html), using the database associated search tools.

RESULTS

***Pgip* gene expression in bean seedlings.** RT-PCR assays were performed to verify the expression of each member of the *Pgip* family during the normal growth and development of bean seedlings. As mentioned, expression of *Pvpgip* genes was analyzed in different light-grown tissues, including young leaves, hypocotyls and roots of 10-day-old seedlings and young pods of adult plants. The hypocotyls and roots were further divided in three (apical, middle and basal) and two (apical and basal) regions, respectively. RNA transcripts corresponding to at least one *Pgip* gene were detected in all organs and sections analyzed. *Pvpgip1* transcript was at undetectable level in all tissues analyzed. However, when a higher number of amplification cycles was used (45 cycles), a faint *Pvpgip1* amplicon was obtained (data not shown). In contrast, *Pvpgip2* was present in all tissues analyzed. *Pvpgip3* and *Pvpgip4* were present in young pods, in the hypocotyl regions and in the basal root region. Conversely, no amplification product was clearly detected in the apical root region (Fig. 1). However, when a higher number of cycles (45 cycles) was used, a small amount of amplicon was also visible in the apical root region (data not shown).

qRT-PCR of *Pgip* genes during fungal infection. In order to define the regulation of each single *Pgip* member during infection, we determined the transcript accumulation of these genes following infection with *C. lindemuthianum*, *B. cinerea* and *S. sclerotiorum*. Since the host-pathogen system bean-*C. lindemuthianum* is regulated by a gene-for-gene interaction, transcript accumulation was analysed in the bean genotypes BAT93 following infection with isolates M126 (virulent strain) or C531 (avirulent strain) which produce in this genotype a compatible or incompatible interaction, respectively.

For *B. cinerea* or *S. sclerotiorum*, inoculations were performed in the middle region of 10-day-old bean hypocotyls using actively growing mycelia. In infected

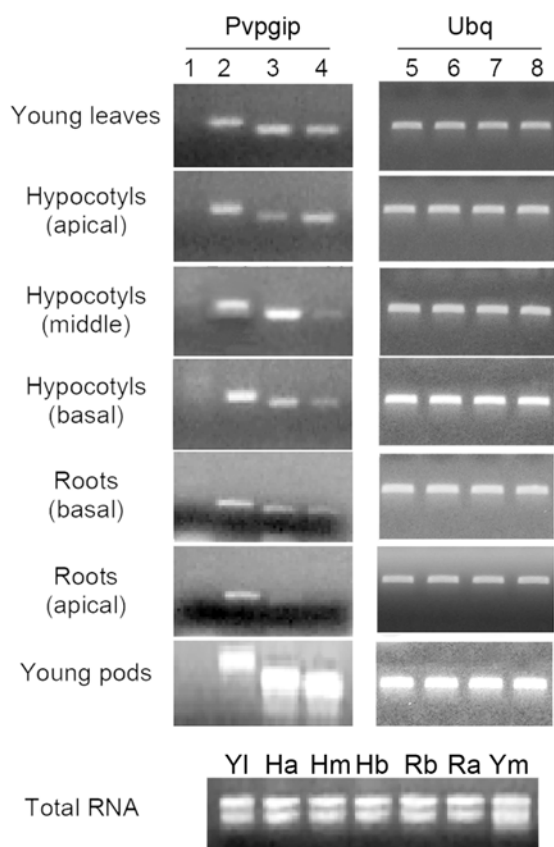


Fig. 1. Expression of the bean *Pvpgrp* genes in seven different organs during normal growth. The specific amplicons were obtained by RT-PCR on total RNA (300 ng) extracted from hypocotyls, roots, leaves of 7-day-old seedlings and flower/pod of adult plants and fractionated on 1.5% agarose gel. Ubiquitin (*Pvubq*) gene was used as internal control. Each experiment was repeated at least three times with similar results. Lane 1, *Pvpgrp1* (493 bp); lane 2, *Pvpgrp2* (493 bp); lane 3, *Pvpgrp3* (451 bp); lane 4, *Pvpgrp4* (445 bp). Yl, Young leaves; Ha, hypocotyls apical; Hm, hypocotyls middle; Hb, hypocotyls basal; Rb, Root basal; Ra, Root apical; Ym, Young pods.

hypocotyls, symptoms were faintly detectable until 8 hpi; at 16 hpi, a necrotic zone appeared below the agar plug and was clearly detectable after 24 hpi, still below the agar plug. Finally, at 48 hpi tissue maceration was clearly visible below the agar plug with the symptoms expanding along the hypocotyls outside the area of contact with the inoculum.

Transcript accumulation of each single *Pvpgrp* gene was followed by qRT-PCR in time-course experiments encompassing 8, 16, 24 and 48 hpi. In the bean-*B. cinerea* interaction, *Pvpgrp1* transcripts are present at 8 hpi and accumulate gradually until 48 hpi, when a significant 5-fold increase was detected. By contrast, *Pvpgrp2* transcripts underwent a stronger accumulation, i.e. at 8 hpi *Pvpgrp2* was slightly induced, then increased gradually until 24 hpi and reached a significant 19-fold increase at 48 hpi. *Pvpgrp3* transcripts were slightly induced only at 24 hpi and increased until 48 hpi (7-fold) whereas

Pvpgrp4 transcripts were not induced (Fig. 2A).

The qRT-PCR analysis performed on the bean-*S. sclerotiorum* interaction showed a kinetic of *Pvpgrp* accumulation similar to that observed in the bean-*B. cinerea* interaction. Transcript of *Pvpgrp1* and *Pvpgrp2* accumulated progressively, with *Pvpgrp2* reaching a significant 17-fold increase at 48 hpi. *Pvpgrp3* was slightly induced (4-fold at 48 hpi), whereas *Pvpgrp4* was not induced (Fig. 2B).

For the bean-*C. lindemuthianum* interaction, inoculations were performed with conidia of M126 or C531 isolates on the two cotyledonary leaves of BAT93 seedlings. Anthracnose symptoms appeared at 96 hpi with the M126 virulent strain while small necrotic hypersensitive-like symptoms appeared at 44 hpi with the C531 avirulent strain. Transcript accumulation of each single *Pvpgrp* genes was determined by qRT-PCR at 10, 24, 48, 72 and 96 hpi. The kinetic of *Pvpgrp* transcript accumulation was different between compatible or incompatible interaction. In the compatible interaction (BAT93 bean genotype-M126 fungal virulent strain) the kinetics of transcript accumulation was similar to that observed for *B. cinerea* or *S. sclerotiorum* infection. *Pvpgrp1*, *Pvpgrp2* and *Pvpgrp3* genes were induced and reached a maximum of transcript accumulation in the late stage of infection (between 72 and 96 hpi). However, only *Pvpgrp2* showed a notable 8-fold induction at 96 hpi, whereas the other *Pvpgrp* genes showed less than 3-fold induction (Fig. 3A). *Pvpgrp4* was not induced.

In the incompatible interaction (BAT93 bean genotype-C531 fungal avirulent strain) *Pvpgrp* genes showed an earlier and transient induction, with *Pvpgrp2* showing a significant 4-fold induction at 24 hpi and a sustained 3-fold induction at the following time points. *Pvpgrp1* showed a maximum 3.5-fold induction at 72 hpi, *Pvpgrp3* reached a 2.5-fold induction at 24 hpi and remained at this level up to 72 hpi and, finally, *Pvpgrp4*, similarly to what observed in *B. cinerea* and *S. sclerotiorum* infection, was not induced.

***In silico* analysis of the 5' flanking region of bean *Pgip* genes.** In a previous analysis of the 5' flanking region of all four bean *Pgip* genes we identified a number of sequences with significant similarity to *cis*-acting elements known to mediate transcriptional responses to pathogen-derived elicitors (D'Ovidio *et al.*, 2004b). Among these, we identified W box elements, that are recognized by the WRKY transcription factors which, in *Arabidopsis*, were shown to play a major role in regulating plant defence transcriptome (Eulgem *et al.*, 2007). These elements have a conserved core sequence TGAC plus a few additional flanking nucleotides. Recently, the sequence 5'-TTGACC/T-3' have been defined as a minimal W-box element (Ciolkowski *et al.*, 2008). Consequently, we have refined and extended our analysis within the first two kilobases of all four *Pvpgrps*. We identified eight and three 5'-TTGACC/T-3' W box

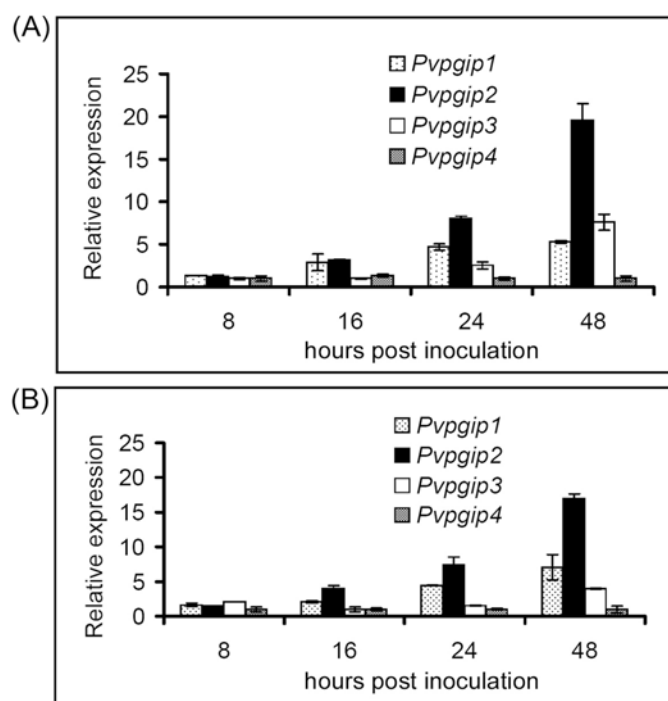


Fig. 2. Expression analysis by qRT-PCR of the four *pqip* genes during hypocotyl infection of common bean. (A) *B. cinerea*; (B) *S. sclerotiorum*. Quantification of gene expression was performed using the comparative Ct method (Livak and Schmittgen, 2001). Relative expression of each gene is reported as the number of fold increase of the transcript level in infection experiments relative to each corresponding control sample. Amplicon size were: *Pvpqip1* (247bp); *Pvpqip2* (238bp); *Pvpqip3* (193bp); *Pvpqip4* (186bp). Bars represent standard errors.

sequences in *Pvpqip1*, *Pvpqip2*, respectively, and none in *Pvpqip3* and *Pvpqip4* (Table 1). Four and three W boxes of *Pvpqip1* and *Pvpqip2*, respectively, were localized within the first 800 bp (Table 1).

Because *Pvpqip* genes showed also expression variation in different tissues we extended this search to all *cis*-acting regulatory DNA elements present in the databases Plant Care and Place with the aim of identifying *cis*-acting elements implicated in tissue specificity. *Cis*-acting elements that control tissue specificity were not

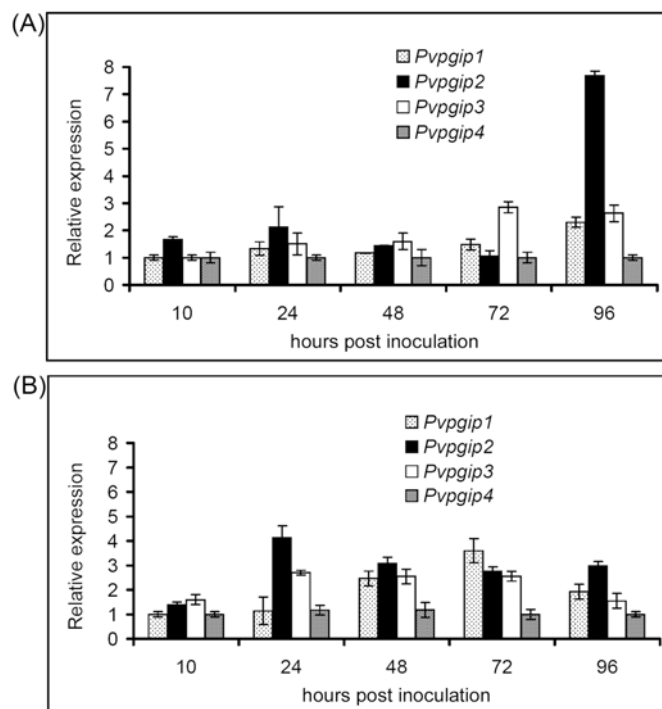


Fig. 3. Expression analysis by qRT-PCR of the four *pqip* genes during hypocotyl infection of common bean. (A) Compatible interaction with *C. lindemuthianum* isolate M126; (B) Incompatible interaction with *C. lindemuthianum* isolate C531. Quantification of gene expression was performed using the comparative Ct method (Livak and Schmittgen, 2001). Relative expression of each gene is reported as the number of fold increase of the transcript level in infection experiments relative to each corresponding control sample. Amplicon size were: *Pvpqip1* (247bp); *Pvpqip2* (238bp); *Pvpqip3* (193bp); *Pvpqip4* (186bp). Bars represent standard errors.

identified within the 2-Kb 5' flanking region of the four *Pvpqip* genes, however, searching the Plant Care database, we identified a sequence within the 5' flanking region of *Pvpqip3* and *Pvpqip4* that showed a similarity with the *as-2 box*, a *cis*-element involved in leaf specific expression and, in particular, in the vascular tissues of the leaf (Lam and Chua, 1989). This sequence is located within a 500-bp proximal region upstream from the start of the coding region of both *Pvpqip3* and *Pvpqip4*, which suggests that it could be functional (Table 1).

Table 1. Sequences in the 5' flanking region of the four *Pvpqip* genes sharing similarity with known *cis*-acting elements. *In silico* analysis was performed on Place and Plant Care databases.

<i>Cis</i> -element	<i>Pvpqip1</i>	<i>Pvpqip2</i>	<i>Pvpqip3</i>	<i>Pvpqip4</i>
	Sequence ^c	Sequence ^c	Sequence ^c	Sequence ^c
W box element ^a	ttgacc (-1958, -1920, -1356)	ttgact (-75, -534, 732)	---	---
ttgac(c/t)	ttgact (-1128, -482, -328, -97, -80)			
As-2 box ^b			gataaggatg (-478)	gataaggatg (-474)
(gtggattgatgtgatatctcc)				

^a Minimal W-box element as defined in Ciolkowski *et al.*, 2008.

^b as-2 tetramer sequence as reported in Lam and Chu, 1989.

^c In parenthesis the position in base pair (bp) of the *cis*-element with respect to the translation start (+1).

DISCUSSION

We analyzed the expression of the complete set of the four bean *Pgip* genes in seven different organs and in response to the infection of three different fungal pathogens. These data extend our previous analysis that included the response of these genes following wounding and treatment with elicitors and salicylic acid (D'Ovidio *et al.*, 2004b).

The expression pattern of the four *Pvpgip* genes during normal growth of bean seedlings showed a wide variation in the different organs analyzed. Only *Pvpgip2* is expressed in all organs analyzed, whereas *Pvpgip1* transcript is not detected at the standard experimental conditions used, and *Pvpgip3* and *Pvpgip4* are mainly expressed in the aerial parts of the plant. The expression of *Pvpgip3* and *Pvpgip4* mainly in the epigeous part of bean seedlings is supported by the presence in their promoter region of a sequence sharing similarity with the as-2 box that directs the expression in leaf tissue and, in particular, in the vascular tissues of the leaf (Lam and Chua, 1989). We do not know whether the expression of *Pvpgip3* and *Pvpgip4* is directed especially in the vascular tissue of the green tissue, however, this type of tissue specificity has been already reported for the bean *Pgip* genes. Transgenic tobacco plants expressing the *gusA* gene under control of the *Pvpgip1* promoter showed most of the β -glucuronidase (GUS) activity to be localized in the vascular tissues at the level of the differentiating node in the internal and external phloem (Devoto *et al.*, 1998).

The expression of *Pvpgip3* and *Pvpgip4* in the green tissue could be related to the effectiveness of the product of these genes against pathogens attacking these tissues. In this context, it is worth noting that PvPGIP3 and PvPGIP4, although weak inhibitors of fungal PGs, are the only bean PGIPs that inhibit PGs from phytophagous insects (D'Ovidio *et al.*, 2004b) that damage the apical meristem (Conti and Bin, 2006). The possible involvement of these inhibitors in insect defence do not rule out, however, the possibility that they might contribute also to physiological aspects related with the development of aerial tissues.

qRT-PCR analysis showed that the basal transcript level of *Pvpgip1*, *Pvpgip2* and *Pvpgip3* increased significantly upon fungal infection, whereas that of *Pvpgip4* remained unchanged. The level of induction of the three genes was different and *Pvpgip2* underwent the stronger transcript accumulation in all three pathosystems analyzed. Moreover, the level and kinetics of induction of the three bean *Pgip* genes (*Pvpgip1*, *Pvpgip2* and *Pvpgip3*) was similar in the bean-*S. sclerotiorum* and bean-*B. cinerea* pathosystems, whereas clear differences were detected in the bean-*C. lindemuthianum* interaction. In this pathosystem, the level of induction of all three *Pgip* genes was lower than in the other two pathosystems. This aspect could be related to the hemibiotrophic lifestyle of this pathogen

(O'Connell *et al.*, 2000), whereas *S. sclerotiorum* and *B. cinerea* are necrotrophs and cause a more rapid and extensive tissue rot.

The kinetics of transcript accumulation of *Pvpgip1*, *Pvpgip2* and *Pvpgip3* in the compatible bean-*C. lindemuthianum* interaction was similar, although less pronounced, to that observed in the other two pathosystems, with an increased transcript accumulation in the late stage of infection. On the contrary, an earlier and transient induction of these genes was observed in the incompatible interaction. These results are consistent with those obtained in the same pathosystem by northern blot and *in situ* hybridization analyses for the whole *Pgip* transcript. In these analyses a delayed or an early and spatially localised accumulation of the whole *Pgip* transcripts have been detected in the compatible or incompatible interaction, respectively (Devoto *et al.*, 1997; Nuss *et al.*, 1996). Our gene-specific transcript analyses extended these findings showing that *Pvpgip4* does not contribute to the accumulation of the whole *Pgip* transcript and that *Pvpgip2* undergoes an earlier and stronger induction. In particular, *Pvpgip2* reached the highest accumulation in the earlier stage of infection (24 hpi) during the incompatible interaction and in the late stage of infection (96 hpi) during the compatible interaction.

The differential regulation of members belonging to the same *Pgip* family in response to stress-related stimuli has been reported for different species including *Arabidopsis* (Ferrari *et al.*, 2003), soybean (D'Ovidio *et al.*, 2006), *Medicago truncatula* (Song *et al.*, 2005) and *Brassica napus* (Hegedus *et al.*, 2008). The genome of this last species contains the largest *Pgip* family so far reported and the 16 *Bnpgip* genes analyzed showed a wide range of transcriptional response and only a few of them respond to all the stresses analyzed (Hegedus *et al.*, 2008). The finding that *Pvpgip2* is the only bean member clearly expressed in all tissues analyzed and the one that undergoes the earlier and stronger transcript accumulation following *S. sclerotiorum*, *B. cinerea* or *C. lindemuthianum* infections reinforces the notion of a primary role of *Pvpgip2* in host defence against fungal pathogens. *Pvpgip2* responds promptly to several stress signals such as wounding, salicylic acid, glucan and oligogalacturonides (D'Ovidio *et al.*, 2004b) and its encoded product showed *in vitro* stronger inhibitor activity against fungal PGs than the other PvPGIPs (Manfredini *et al.*, 2006; D'Ovidio *et al.*, 2004b). In particular, PvPGIP2 is the stronger inhibitor of the PG activity of *B. cinerea* (Manfredini *et al.*, 2006; D'Ovidio *et al.*, 2004b) and inhibits efficiently the two PGs secreted by *S. sclerotiorum* during host infection (Sella *et al.*, 2005; Farina *et al.*, 2009). Moreover, a number of reports showed the ability of the bulk or partially purified bean PGIP, that contains PvPGIP2 (Desiderio *et al.*, 1997), to inhibit completely the PGs secreted by virulent or avirulent races of *C. lindemuthianum* (Albersheim and

Anderson, 1971; Anderson and Albersheim, 1972; Lafitte *et al.*, 1984; De Lorenzo *et al.*, 1990).

These findings suggests that the stronger induction of *Pvpgip2* transcript during fungal infection could be regarded as a strategy of the host plant to counteract the colonization of the tissue with a higher amount of a particularly effective PGIP. This possibility is further supported by the reduction of disease symptoms caused by *B. cinerea* or *Bipolaris sorokiniana* in transgenic *Arabidopsis*, tobacco and wheat plants ectopically expressing *Pvpgip2* (Manfredini *et al.*, 2006; Janni *et al.*, 2008). In particular, Janni *et al.* (2008) reported that the transgenic wheat tissue expressing PvPGIP2 showed a reduced degradation when treated with a fungal PG that is specifically recognized by PvPGIP2.

Differently from *Pvpgip2*, the contribution of the other *Pvpgip* members is not clear. *Pvpgip4* is not induced during any of the fungal-pathogen interactions here analyzed. This gene is also not induced by stress signals such as wounding, salicylic acid, glucan and oligogalacturonides (D'Ovidio *et al.*, 2004b), consistently with the lack of W boxes in its 5' flanking region. *Pvpgip1* and *Pvpgip3* are induced following pathogen infection but their transcript accumulation does not exceed 7-fold induction during necrotrophic infection. Moreover, the overexpression of PvPGIP1 does not enhance disease resistance of transgenic tomato plants against the pathogenic fungi *Fusarium oxysporum* f. sp. *lycopersici*, *B. cinerea*, and *Alternaria solani*, most probably because of its limited capacity to inhibit PGs from these fungal pathogens (Desiderio *et al.*, 1997).

In conclusion, the pattern of transcriptional variation of the four bean *Pgip* genes provided additional information to assess the significance of specific members during normal growth and following fungal infections. In particular, the finding that *Pvpgip2* is the only member of the bean *Pgip* family that is expressed in all tissue analyzed and undergoes the stronger transcript accumulation following pathogen infection further reinforces the notion of a primary role of PvPGIP2 over the other three PvPGIP members, PvPGIP1, PvPGIP3 and PvPGIP4, to counteract the colonization of the host tissue by fungal pathogens.

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INCIDENCE OF POTATO VIRUSES AND BACTERIAL WILT DISEASE IN THE WEST AMHARA SUB-REGION OF ETHIOPIA

B. Bekele¹, E. Abate², A. Asefa² and M. Dickinson³

¹ Ethiopian Institute of Agricultural Research, Plant Protection Research Centre, P.O. Box 37, Ambo, Ethiopia

² Amhara Region Agricultural Research Institute, P.O. Box 527, Bahar Dar, Ethiopia

³ School of Biosciences, University of Nottingham, Sutton Bonington Campus, Loughborough LE12 5RD, UK

SUMMARY

A survey of virus diseases and bacterial wilt was carried out in four major potato growing administrative zones in the west Amhara sub-region of Ethiopia in 2008. Leaf samples with symptoms suggestive of virus infection were collected from 38 randomly selected fields in 16 locations, whilst for bacterial wilt detection tuber and stem samples were collected from 23 and 12 fields in 15 and 12 locations, respectively. Disease incidences were visually assessed in the field and the identities of the pathogens were confirmed by laboratory testing using double antibody sandwich (DAS)-ELISA for viruses, and nitrocellulose membrane (NCM)-ELISA kits for *Ralstonia solanacearum*. In addition, an enrichment procedure was used to determine latent infection by *R. solanacearum*. Virus disease incidence varied from zero to 100% in different potato growing systems, whilst bacterial wilt incidence as high as 25% was recorded in farms in the west Gojam and north Gonder zones when assessed based on visual field symptoms. Results of laboratory testing for viruses confirmed the occurrence of at least five viruses, with *Potato virus S* (PVS) being the most widely distributed. Other viruses identified included *Potato virus X* (PVX), *Potato virus M* (PVM), *Potato leaf roll virus* (PLRV) and *Potato virus Y* (PVY), in order of importance. Mixed infections with two or more viruses were also detected. *Potato virus A* (PVA) was not detected in any of the samples tested. Latent infection by *R. solanacearum* was found in various potato fields, including experimental plots, farmers' seed potato production fields, suggesting the need to consider strict quarantine measure and restrict the free movement of seed tubers.

Key words: potato, Ethiopia, viruses, bacterial wilt, survey.

INTRODUCTION

Ethiopia is among the top potato (*Solanum tuberosum* L.) producers in Africa, with 70% of its arable land in the high altitude areas above 1500 m being suitable for potato production (FAOSTAT, 2008). Currently, potato is produced mainly in the north western, central and eastern highlands of Ethiopia. The north western part of the country which mainly includes the highlands of the west Amhara sub-region (Fig. 1) is the major production area, and this region makes up over one third of the total area allotted to potato nationally. About 600,000 rural households are involved in potato production in the region, and according to CACC (2003) the area covered by potatoes in the region may be as high as 70,000 ha. The highlanders produce potato as a food security crop because of the limited crop choice that they have, whilst in the mid altitude areas potato is considered as an emergency crop as it is usually ready for consumption when the grain crops are not.

The national average productivity of potato in Ethiopia is 8 tons/ha, which is below the African continent average (10.8 tons/ha) (FAOSTAT, 2008). Diseases caused by viruses, bacteria and fungi are considered among the major biotic production constraints of potato. Symptoms suggestive of viral diseases are widely observed and distributed in the major production areas in the west Amhara sub-region. Evidence of the occurrence of potato viruses in Ethiopia was first reported in studies conducted in central, south and southeast Ethiopia during the 1984 and 1985 crop seasons (Agranovsky and Bedasso, 1985, 1986). The results of these consecutive studies indicated the presence of *Potato virus X* (PVX), *Potato virus S* (PVS), *Potato leafroll virus* (PLRV), *Potato virus Y* (PVY), *Potato virus A* (PVA) and *Potato virus M* (PVM). These studies, however, did not include the main production areas in the country such as the west Amhara sub-region, and the identity, distribution and status of viruses attacking potato have not been systematically studied using specific diagnostic methods. Studies made elsewhere indicate that yield losses as high as 90% can be incurred by viral diseases that can cause varietal degeneration (Cyperus and Bokx, 2005). In addition, symptoms of some potato viruses are often not apparent when in association with

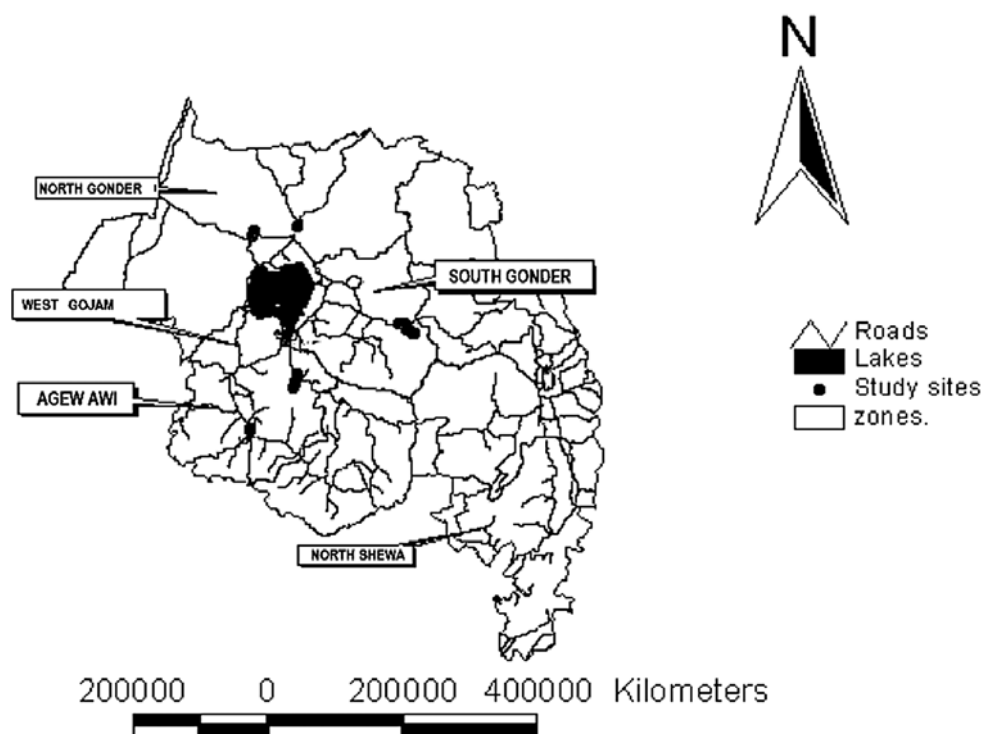


Fig. 1. Map of the west Amhara sub-region of Ethiopia from which samples were collected during the main season 2008. The four sampling zones of west Gojam, Agew Awi, north Gonder and south Gonder is shown, with the sampling sites marked.

the mosaics caused by PVX, PVY and PVS, so identification by visual observation of symptoms alone is not reliable (Fletcher *et al.*, 1996; Burrows and Zitter, 2005).

Bacterial wilt of potato (*Ralstonia solanacearum*) can also cause significant yield loss to potato (Ajanga, 1993). Because this pathogen stays in the soil for several years it prohibits subsequent production of potato in the same field. Moreover, this pathogen may stay latent without showing any symptoms in the field with the consequence of high impact on tuber yield in the upcoming season. Detection of latent infections by *R. solanacearum* requires sensitive diagnostic methods but, as yet, such methods have not been adopted in Ethiopia to inspect potato seed tubers and monitor the status of latent infection and its consequences. As the crop is vegetatively propagated, the diseases can easily be transmitted through tubers and cause very high economic losses across wide geographic areas.

This study was carried out to fill the current knowledge gap on identity, incidence and distribution of viral diseases and bacterial wilt of potato in the major production areas of the west Amhara sub-region with particular emphasis on emerging seed production schemes and germplasm evaluation work.

MATERIALS AND METHODS

Field visits and sample collection. An intensive sur-

vey and sample collection was conducted during the main rainy season of 2008 (from July 31–August 15, 2008) in selected representative major potato growing areas in four administrative zones of the west Amhara sub-region (Fig. 1). Samples for virus and bacterial wilt assay were collected from three systems: potatoes grown for seed, ware potato, and research plots in the west Gojam and north Gonder zones (Table 1). Sampling was done to meet three main objectives: typing of potato viruses occurring in the sub region, confirmatory test for bacterial wilt, and assessment of virus and bacterial wilt disease incidence and status.

Virus diseases. For virus typing, leaf samples with symptoms suggestive of virus infection were collected from about 1,460 plants in the surveyed areas. From each field, one to three composite samples were taken depending on the type and diversity of symptoms encountered, with each composite sample being a mixture of 10 individual plant samples. A total of 146 composite samples were collected from 38 fields in 16 locations, including experimental plots at the Adet Agricultural Research Centre (AARC) in west Gojam and the Gonder Agricultural Research Centre (GARC) in north Gonder. Leaflets were collected from the upper, middle and lower parts of at least 10 individual plants per field. Sampling was done at a constant interval depending on the distribution of crop in the respective administrative zones/locations surveyed following simple random sampling techniques and by moving diagonally across each

field. At the time of sampling crops were at the flowering-tuber setting stage. Disease incidence in the field was recorded visually as percent infection. Both symptomatic and non symptomatic samples were collected, but as the main aim of this survey was to type viruses occurring in the sub-region, emphasis was given to symptomatic plants, with plants showing different symptoms collected separately. Testing for six viruses was conducted in the tissue culture and serology laboratory of the Amhara Region Agricultural Research Institute (ARARI), Bahar Dar.

Bacterial wilt disease. Sampling for bacterial wilt in the field was performed following the simple random sampling strategy outlined for the viral diseases. Instead of leaves, in this case, tuber and stem samples were collected. In addition to random samples for testing latent infection, symptomatic plants with bacterial wilt-like symptoms were collected for confirmatory tests of *R. solanacearum* infection. Stem and tuber samples were mainly collected from released varieties in seed and ware potato producers' fields and in variety evaluation experimental plots at research stations. A total of 62 tu-

ber and 29 stem samples were collected for laboratory testing in 23 and 12 fields from 15 and 12 locations, respectively. Emphasis was given to tuber sampling in all production systems to determine the level of infection, as tubers are the major source of inoculum as well as means by which the bacterium spreads from field to field and location to location.

Collected leaf, tuber and stem samples were labelled, put in plastic bags, taken to the laboratory and processed immediately or kept at 4-6°C in the refrigerator until processed for testing by DAS-ELISA and NCM-ELISA, respectively, for detection of viruses and bacterial wilt pathogens. During potato field inspections, data related to crop variables such as growth stage and variety, disease symptoms, disease incidences (%), purpose of production (ware, seed or research), altitude of each location and their corresponding geographical position using the geographical positioning system (GPS) were collected.

Laboratory tests. *Virus diseases.* All composite leaf samples were tested by DAS-ELISA (Clark and Adams,

Table 1. Virus disease incidence in potato as determined from symptomatic samples in growers fields and experimental plots during a survey in the west Amhara sub-region, Ethiopia, in 2008.

Zone	Farm type*	No. of Fields	No of Samples	*No. of fields / exp. Plots with incidences (%) of:							
				<5	5-15	16-25	26-35	36-45	46-55	56-65	>65
West Gojam	EP (VT-1)	1	18	4	7	0	0	0	5	0	2
	EP (VT-2)	1	8	0	0	1	4	0	1	0	2
	EP(VT-3)	1	11	2	3	1	1	0	0	0	4
	EP(VT-4)	1	14	1	5	4	3	1	0	0	0
	EP (VT-5)	1	20	15	2	2	1	0	0	0	0
	OSSI	1	15	4	3	6	1	0	0	0	1
	GH	1	5	1	0	0	0	0	0	0	0
	FF	3	3	0	1	2	0	0	0	0	0
	OFSI	6	6	3	0	1	2	0	0	0	0
	Sub total	16	100								
Agew Awi	OFSI	5	5	0	2	0	0	0	3	0	0
	Sub total	5	5								
North Gonder	EP (OFT)	1	3	0	3	0	0	0	0	0	0
	EP (VT-1)	1	10	4	3	1	1	0	0	0	1
	EP(VT-2)	1	14	5	4	4	0	0	0	0	1
	FF	4	4	1	3	0	0	0	0	0	0
	Sub total	7	31								
South Gonder	FF	8	8	5	3	0	0	0	0	0	0
	OFSI	2	2	1	1	0	0	0	0	0	0
	Sub total	10	10								
	Total	38	146								

*EP (VT 1-5) = Experimental plots sampled in different on-station variety trials at AARC and GARC; EP (OFT) = on-farm trial; OSSI = On-station seed increase; OFSI = Farmers potato seed production cooperative farm; FF = Farmers ware potato producers field; GH = Greenhouse samples.

*For experimental plots and OSSI, each sample represents a single plot of an experiment or seed increase plot and incidence was recorded per plot, whilst for other samples incidence was calculated per field.

1977) following standard protocols described in the International Potato Centre (CIP) DAS-ELISA kit instruction manual. DAS-ELISA kits were provided by the CIP Serology Laboratory at Lima (Peru). Each sample was tested for 6 viruses, namely *Potato leafroll virus* (PLRV), *Potato virus A* (PVA), *Potato virus M* (PVM), *Potato virus S* (PVS), *Potato virus X* (PVX) and *Potato virus Y* (PVY).

Bacterial wilt. Stem and tuber samples were tested for *R. solanacearum* by NCM-ELISA. Sample preparation and serological tests were performed according to procedures outlined in the CIP NCM-ELISA kit instruction manual for detection of *R. solanacearum* in potato (Priou, 2001). Also this kit was supplied by the CIP Serology Laboratory. Tuber and stem samples with bacterial wilt symptoms were directly processed and tested by NCM-ELISA without enrichment. In addition, to increase the sensitivity of the test and detect latently infected plants, an enrichment procedure was carried out for non symptomatic plants in semi selective broth (modified SMSA) before performing NCM-ELISA. The enrichment was done by incubating the tuber and stem extracts prepared from non symptomatic samples in M-SMSA for 48 h at 30°C (Priou, 2001). In each of the tests, CIP positive (at concentrations of 10^6 , 10^7 , 10^8 bacteria/ml) and negative controls were used.

RESULTS

Viral and bacterial wilt diseases incidence in the field based on symptoms. The altitudes and geographical positions of surveyed areas were between 1,800 and 3,260 metres above sea level, and N10° 46.743'-N12° 34.249' and E037° 027.690'-E038° 30.823'. The most commonly observed virus-like symptoms in potato were leaf curling, interveinal mosaic, mottling, reduced leaf size, deepening of leaf veins, narrow leaves and stunting. At the time of sampling, most plants were at the flowering-tuber setting stage, which is a good stage for symptom expression. Virus disease incidence assessed visually varied from zero to 100% in different farms and variety evaluation experimental plots (Table 1). The highest incidences for virus-like symptoms were commonly recorded in experimental plots. For instance, in variety trial-1 (VT-1) assessed at AARC, virus incidence as high as 100% was recorded on cv. Guassa planted as a standard check, whilst an incidence of 50% was recorded in two other varieties, Gudenie and Key Abeba (used as a local check). An incidence of 85% was also recorded in one of the test clones included in this variety trial. In variety verification trial (VT-2), disease incidence ranged from 25-70%, whereas in other variety development experiments (VT-3) evaluated, incidences of 100%, 80% and 80% were recorded, respectively, on three test clones. Low incidence (2.5%) was recorded on the standard check (cv. Jalene), while a test clone

'CIP 392640.539' was apparently free of any virus-like symptoms at the time of survey. When on-station seed increase (OSSI) plots of released potato varieties were visually evaluated, highest (>90%) incidence for virus-like symptoms was recorded in cv. Degemegn, and lowest incidence (2%) in cv. Tolcha. The other varieties had incidence of virus-like symptoms ranging from 15-30%.

On farmers' fields, disease incidence was less when compared to experimental plots. The highest incidence recorded was in the range of 50-60% in three out of five fields assessed around the Injibara area in the Tilili district of the Agew Awi zone on cv. Jalene. Farmers' fields inspected around Adet in the Yilmana Densa district (west Gojam) had highest virus disease incidences in the range of 20-30% (Table 1).

In north Gonder, disease incidence was between 10 and 15% on cv. Jalene planted at an on-farm fertilizer trial field at Chiliga. In the same location, 100% incidence was recorded on one of the clones included in the variety trial. Other test clones had incidences ranging between 2 and 30%. In another variety trial, the highest and lowest incidences were 75% and 5%, respectively. On the other hand, low disease incidences of 5-10% were recorded on farmers' fields planted with improved varieties in the north Gonder administrative zone. Low levels of virus-like symptoms were recorded in fields inspected in the south Gonder zone with common symptoms being deepening of veins and interveinal mosaic. The latter was particularly widely distributed in fields planted with local potato varieties.

With respect to bacterial wilt, plant wilting, browning of vascular tissue when cut and oozing of milky fluid from the vascular ring of cross-sectioned tubers were the most commonly encountered disease symptoms. Incidences as high as 25% were observed in localities around Adet Zuria in the Yilmana Densa district (west Gojam zone) and some farms in the Chiliga districts of the north Gonder administrative zone (Table 3).

Viral disease identity and incidence as determined by DAS-ELISA. DAS-ELISA testing of symptomatic plants indicated that out of the six viruses for which antibodies were provided, the occurrence of five viruses was confirmed in the west Amhara sub-region. PVA was not detected in any of the samples tested. Among the five viruses, PVS was the most widely distributed in the sub-region followed by PVX, PVM, PLRV and PVY (Table 2). In addition, the first two viruses (PVS and PVX) had high incidences compared to the other viruses identified. In most cases, simultaneous detection of two or more viruses was common, particularly in samples collected from experimental plots (Table 2). In addition, some uncommon symptoms such as narrow leaves were observed in some fields, which were assumed to be viral, but no virus could be detected by the antibodies used in this study.

Table 2. Detection of potato viruses by DAS-ELISA in samples collected from the west Amhara sub-region during the main rainy season, 2008.

Zone	District	Field types	No. of fields	No of samples	Viruses detected out of the samples collected and (percent)*					
					PLRV	PVA	PVM	PVS	PVX	PVY
West Gojam	Adet	EP	5	71	43 (60.6)	0	38 (53.5)	60 (84.5)	40 (56.0)	20 (28.0)
		OSSI	1	15	2 (13.3)	0	5 (33.3)	6 (40.0)	2 (13.3)	2 (13.3)
		FF	3	3	1 (33.3)	0	0	2 (66.6)	0	0
		OFSI	6	6	2 (33.3)	0	1 (16.6)	4 (66.6)	4 (66.6)	1 (16.6)
	Bahir Dar	GH	1	5	0	0	3 (60.0)	4 (80.0)	1 (20.0)	1 (20.0)
Agew Awi	Tilili/Kosober	OFSI	5	5	0	0	0	5 (100)	2 (40.0)	0
North Gonder	Chilga	EP	3	27	12 (44.4)	0	16 (59.0)	16 (59.0)	6 (22.0)	0
		FF	4	4	0	0	1 (25.0)	4 (100)	0	0
South Gonder	Tach Gaint	OFSI	2	2	0	0	0	1 (50.0)	2 (100)	0
		FF	5	5	0	0	0	3 (60.0)	5 (100)	0
	Lai Gaint	FF	3	3	0	0	0	2 (66.6)	3 (100)	0
Total			38	146	60 (41.0)	0	64 (43.8)	117 (80.1)	65 (44.5)	24 (16.4)

EP- Experimental plots; OFSI-farmers seed potato production field; OSSI- On station seed increase; FF- Farmers ware potato field planted with local cultivars, GH = green house samples.

*Figures in parenthesis indicate percent infection.

Table 3. Detection of bacterial wilt in symptomatic tuber and stem potato samples from the west Amhara sub-region in Ethiopia by direct NCM-ELISA without enrichment.

Symptomatic samples								
Zone	District/Locality	Altitude (m)	Variety	Field type sampled	Plant part sampled	Field incidence (%)	No of samples	NCM-ELISA Positive samples
West Gojam	Y/Densa-Goshiye	2626	Gera	OFSI	tuber	25	9	6 (66.7%)
	Y/Densa-Goshiye	2626	Gera	OFSI	Stem	25	15	13 (86.7%)
	Y/Densa -Adet	2205	Jalene	OFSI	Tuber	15	2	2 (100%)
	Y/Densa -Adet	2205	Local	FF	Tuber	5	1	0
North Gonder	Chilga	2254	Guassa	OFT	Stem	20	4	4 (100%)
Sub total					Tuber		12	8 (66.7%)
					Stem		19	17 (89.5%)
Total symptomatic samples							31	25 (80.7%)

OFSI -farmers' seed potato production field; FF- Farmers ware potato field planted with local cultivars; GH-Samples from green house potato; OFT – on-farm trial.

All the five viruses were detected in samples collected from experimental and on-station seed increase plots at AARC, and farmers' seed production fields in the vicinity of Adet in the west Gojam zone. The highest incidence was among samples collected from experimental plots (Table 2). In all the variety trials inspected at AARC, mixed infections of all the five viruses (PLRV, PVM,

PVS, PVX and PVY) were recorded. In the north Gonder zone, four of the five viruses were recovered in samples collected from experimental plots at Chilga. In this area, PVS and PVM were equally identified in 59% of the samples. PLRV and PVX were identified from respectively, 48% and 22% of the samples collected in Chilga at on-farm experimental plots. Mixed infection of

one to four viruses were recorded in samples collected from experimental plots and farmers field in north Gonder. The most common virus combination was PLRV, PVM and PVS in four samples, while mixed infections of PVM, PVS and PVX; PLRV and PVS; PVM and PVS were each recorded in two samples. Combinations of PLRV, PVM, PVS and PVX; PLRV and PVM; PVS and PVX; PLRV and PVX were each detected in one sample. Two viruses (PVS and PVX) were detected in five samples collected from farmers' seed potato production cooperative fields at Enjibara and Tilili areas (Agew Awi zone). PVS was identified in all samples, while PVX was recovered in mixed infection with PVS from only 2 (40%) of the samples (Table 2).

In the south Gonder zone, leaf samples with symptoms suggestive of virus infection were collected from 10 fields (seven in Tach Gaint and three in Lai Gaint locations). The result showed that two (PVS and PVX) of the six viruses tested were detected, and all the tested samples were positive for PVX, with or without PVS. Of the total samples, 6 of 10 were PVS-infected. Mixed infections of PVS and PVX were detected in six out of 10 samples. Most of the samples tested from south Gonder were collected from farmers' fields planted with local varieties indicating wider distribution of PVS and PVX in local potato production systems as well (Table 2).

Bacterial wilt incidence and distribution as determined by NCM-ELISA. A total of 31 symptomatic plant samples (19 stem and 12 tuber samples) were collected for confirmatory testing of *R. solanacearum* infection (Table 3). All tuber samples collected from farmers' seed potato production cooperative farms planted with cvs Jalene at Adet gave positive reactions to the pathogen. Similarly, at the same location, 86.7% of the stem samples and 66.7% of the tuber samples collected from farmers' seed increase plots planted with cv. Gera tested positive. All the stem samples collected from symptomatic plants at the Chilga on-farm experimental plots were found positive when tested by NCM-ELISA.

In addition to symptomatic samples, a post enrichment NCM-ELISA test was carried out for non-symptomatic (apparently healthy looking) potato plants to detect latent infection by *R. solanacearum* (Table 4). Of the total of 50 tuber and 10 stem samples tested, *R. solanacearum* was detected in 11 (18.3%) out of 60 samples. As shown in Table 4, of the five administrative zones sampled, the bacterium was detected in all except south Gonder. Latent infection by *R. solanacearum* was recovered from both the local and improved varieties that were tested.

Table 4. Detection of bacterial wilt in random asymptomatic tuber and stem potato samples from the west Amhara sub-region in Ethiopia by enrichment NCM-ELISA.

Random samples							
Zone	District/Locality	Altitude (m)	Variety	Field type sampled	Plant Part Sampled	No. of samples	Positive samples
West Gojam	Adet	2400	Square(Local)	FF	tuber	2	0
		2626	Jalene	OFSI	tuber	2	0
		2400	Sisay (Local)	FF	tuber	1	0
		2205	Zengena	OSSI	tuber	1	0
		2626	Gera	OFSI	stem	5	2
		2626	Gera	OFSI	tuber	30	6
	Bahir Dar-ARARI	1800	Zengena	GH	tuber	1	0
		1800	Guassa	GH	tuber	1	0
Agew Awi	Enjibara	2503	Jalene	OFSI	tuber	2	0
		2503	Deme (Local)	FF	tuber	1	0
		2503	Samuni (Local)	FF	tuber	1	1
North Gonder	Chilga	2254	Chilga local	FF	tuber	1	0
		2254	Guassa	EF	stem	2	1
		2254	Guassa	EF	tuber	2	1
South Gonder	Tach Gaint	2892	Kara (Local)	FF	tuber	1	0
		3260	Jalene	OFSI	tuber	3	0
		3260	Jalene	OFSI	stem	3	0
	Lai Gaint- Gob gob	3054	Local	FF	tuber	1	0
		Sub total				Tuber	50
				Stem	10	3 (30%)	
Total random samples						60	11 (18.3%)

OFSI -farmers' seed potato production field; OSSI- On station seed increase; FF- Farmers ware potato field planted with local cultivars; GH-Samples from green house potato.

DISCUSSION

From this survey in the west Amhara sub-region of Ethiopia, PVS was the most frequently identified and distributed among six potato viruses tested in respective zones and across zones studied, followed by PVX, PVM, PLRV and PVY. Mixed infections with two or more viruses were also commonly detected, among which PVS and PVX combination was recorded in 12 samples. This finding correspond with the survey results conducted in central, south and southeast Ethiopia during the 1984/85 seasons (Agranovsky and Bedasso, 1985, 1986) that reported PVS and PVX as the most common viruses identified with PLRV, PVY, PVM and PVA being less widely distributed in the regions surveyed. However, PVA was not identified in any of our samples collected in the west Amhara sub-region. Causing a mild mosaic, PVS is the most frequently found virus in potato worldwide and is very contagious (Cyperus and Bokx, 2005). Infection rates of 100% have been reported from many countries (Cyperus and Bokx, 2005), which agrees with the present results. It is also known that infection by PVS may result in yield losses of up to 20%, but higher losses can be incurred if infection is combined with PVX. Being the second and third most widely distributed viruses next to PVS, PVX and PVM cause mild symptoms and bring about low yield loss, however, they are reported to cause significant impact on potato yield when in combination with PVS and other viruses. PLRV was the fourth most widely distributed virus, and Cyperus and Bokx (2005) indicated that yields of plants with secondary infection of PLRV are often reduced by more than half; while in highly sensitive varieties yield loss can be as much as 90%. As mixed infections of two or more of these viruses were recorded in many of the locations surveyed, one can assume that potato farmers are facing heavy yield losses every year, and evidence for this comes from the high disease severity and incidence observed in some farmers' fields and experimental plots at research stations.

Some solanaceous plant hosts, weeds (*Chenopodium amaranticolor*, *Datura metel*, and *Datura stramonium*) and plants belonging to other families such as *Nicotina* spp. and *Phaseolus vulgaris* are commonly grown in the study area, and are alternate hosts for some of the viruses detected (Kook-Hyung, 2001), contributing to the widespread occurrence of these viruses along with the presence of aphid and biting insect vectors. Another possible factors to account for the widespread and high virus disease incidences may be the overlapping potato growing seasons and lack of a seed potato health testing program.

The presence of different viruses at a higher incidence rate on variety trials under research managed experimental plots than farmer's fields and on-farm seed increase plots seems paradoxical as far as the high level

of management in research stations is concerned vis-à-vis farmers' poor management. However, this result seems to have been associated with the presence of susceptible clones in the test materials and/or because of high inoculum build up in the experimental stations over years without break. Rarely encountered symptoms in some potato experimental fields sampled such as narrow leaves, which were suspected to be caused by viral infection, did not give positive reaction when tested against antibodies for the six viruses. This may indicate the presence of other viruses or virus-like organisms that could not be detected by the reagents used in these tests, which calls for further investigation.

One of the objectives of using NCM-ELISA was a confirmatory test whether symptomatic potato plants are caused by bacterial wilt or not. In the direct NCM-ELISA test (Table 3), of the total of 12 tuber and 19 stem composite samples collected from symptomatic plants, *R. solanacearum* was recovered from 8 (66.7%) tuber and 17 (89.5%) stem samples, respectively. Overall, *R. solanacearum* was detected in 25 (80.7%) of the 31 tuber and stem symptomatic samples. From this result it is evident that there is high chance that most of the potato plants with wilting symptoms are infected by *R. solanacearum*. Only six (19.4%) out of the 31 samples tested were negative. Priou (2001) reported that post enrichment NCM-ELISA can detect as few as 10 bacteria per ml of extract instead of 10^6 - 10^7 bacteria/ml without enrichment. In this study, the negative reaction of some symptomatic samples when tested by NCM-ELISA without the enrichment procedure may be attributed to low bacterium concentrations in the extracts, or due to infection of plants by other soil-borne pathogens and/or insects that could cause wilting symptoms similar to *R. solanacearum*, and this probably resulted in false negatives. However, had it not been for shortage of reagents during this study, it would have been imperative re-testing and confirming all symptomatic samples that were negative by using post enrichment ELISA. This therefore suggests the need for paying particular attention while collecting and rating bacterial wilt disease incidence in the field and emphasizes the importance of using efficient detection methods such as post enrichment NCM-ELISA.

When 60 random samples (50 tuber and 10 stem samples) collected from apparently healthy looking plants were tested by post enrichment NCM-ELISA for latent infection, *R. solanacearum* was recovered from 11 (18.3%) of the samples, both from improved and local varieties in different farms as well as in tuber and stem samples at higher altitudes over 2,500 m above sea level. This result supports findings by Ciampi *et al.* (1980) and Hayward (1991) who have confirmed latent infection of *R. solanacearum* in tropical cool conditions at altitudes above 2,500 m, and that of Janse (1996) who has indicated that bacterial wilt has become a serious threat

to potato seed production in cool, temperate countries of northern Europe. This level of latent infection is high, particularly considering that bacterial wilt is a quarantine disease of zero tolerance level in seed tuber production (Priou *et al.*, 1999b).

Interestingly, assuming that bacterial wilt is a quarantine disease of zero tolerance level in seed tuber production, higher levels of latent infection (20%) were recorded in samples collected from potato seed tuber production fields at altitudes of 2,626 m above sea level (Table 3). These potato seed tubers were meant to be distributed to growers for use as a planting material in the subsequent growing season, indicating the potential danger of using potato seed tubers from such infected fields as planting materials in the upcoming season. As suggested by Nortje (1997) and Kakuhenze *et al.* (2000), this is a consequence of a lack of rigorous seed health testing and a certification programme, which is also one of the major drawbacks within the potato seed production system in the area. This drawback may in part be associated with the lack of technical capacity, facilities and availability of affordable and efficient detection methods. The classical detection method of tuber infection is time-consuming (requiring tuber incubation for three to four weeks at 30°C) and space-consuming and may not reveal low infection rates, whereas post enrichment NCM-ELISA can have the combined advantages of low-cost (about \$0.30/sample for supplies), ease, and speed (6 h after enrichment of the extracts), and does not require extensive laboratory equipment (Priou *et al.*, 1999a). Therefore, the use of NCM-ELISA with the post enrichment procedure can be recommended as a powerful tool for efficient and economical detection of latent infection by *R. solanacearum* for routine use in quarantine procedures, seed certification and quality testing, as well as in assessing susceptibility of breeding lines to bacterial wilt in experimental stations in the Amhara region and other potato growing regions in the country.

Results of this study have shown comparably high levels of latent infection of *R. solanacearum* in both stem and tuber samples tested, indicating the potential for using stem sampling as an alternative to tubers, since tuber sampling has economic implications. Mwangi *et al.* (2008) observed similar results and found a positive correlation between stem and tuber testing. We recommend use of stems as an alternative sampling to tubers in seed health testing programmes.

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TRANSGENIC PLUMS EXPRESSING *PLUM POX VIRUS* COAT PROTEIN GENE DO NOT ASSIST THE DEVELOPMENT OF VIRUS RECOMBINANTS UNDER FIELD CONDITIONS

I. Zagrai¹, M. Ravelonandro², I. Gaboreanu³, B. Ferencz⁴, R. Scorza⁵, L. Zagrai¹, B. Kelemen⁴, D. Pamfil³ and O. Popescu⁴

¹ Fruit Research and Development Station Bistrita, Drumul Dumitrei Nou 3, 420127 Bistrita, Romania

² INRA-1090 Unité Mixte de Recherches Génomique, Diversité, Pouvoir Pathogène, 33883 Villenave d'Ornon, France

³ University of Agricultural Sciences and Veterinary Medicine Cluj-Napoca, Romania

⁴ Institute for Interdisciplinary Research in Nano-Bio-Sciences, Molecular Biology Center, Cluj-Napoca, Romania

⁵ USDA-ARS Appalachian Fruit Research Station Kearneysville, WV, USA

SUMMARY

The serological and genetic variability of *Plum pox virus* (PPV) isolates from transgenic plum trees expressing the PPV coat protein gene and conventional plums was analyzed. PPV isolates were characterized serologically by TAS-ELISA using PPV-D and PPV-M specific monoclonal antibodies and by molecular typing across three genomic regions as well as RFLP analysis of the 3' terminus of the CP gene. PCR products spanning the (Cter)CP and (Cter)NIb-(Nter)CP regions were sequenced. Sequence information revealed no significant difference between serological and molecular features of PPV isolates from transgenic and conventional plums that were growing together in experimental orchards for 6-8 years. These results show that transgenic plums do not promote the emergence of new PPV variants under field conditions.

Key words: pathogen derived resistance, GMO, CP gene, safety issue, recombinant viruses.

INTRODUCTION

Pathogen derived resistance (PDR) (Sanford and Johnston, 1985) can provide an alternative to conventional breeding for obtaining virus-resistant plants. This technology holds great promise for perennial crops because conventional breeding is time-consuming and expensive (Dolgov and Hanke, 2006). In many cases, sources of virus resistance are unknown, multigenic, or exist only in primary germplasm, requiring many generations of selection to produce resistant commercial cultivars (Hartmann and Petruschke, 2002). In the case of *Plum pox virus* (PPV), the most devastating virus of stone fruits (Cambra *et al.*, 2006) PDR has been used to produce plums resistant to PPV (Ravelonandro *et al.*, 1997; Malinowski *et al.*, 2006). The mechanism of resistance in PPV-resistant plums has been shown to be post-

transcriptional gene silencing (PTGS) in which both transgene mRNA and homologous viral RNA are degraded (Scorza *et al.*, 2001; Hily *et al.*, 2004).

Several environmental safety issues have been raised with the application of PDR, particularly when viral transgene transcripts are expressed. The issues of concern include complementation, transcapsidation, synergism, and recombination (Thomas *et al.*, 1998; Tepfer, 2002). Recombination is of particular concern since it has been suggested that a virus transgene inserted in a plant genome may generate recombinant viruses with new biological properties (Wintermantel and Schoelz, 1996). Most studies of recombination have been carried out under greenhouse conditions and under high to moderate selection pressure to favor the development of recombinants (Tepfer, 2002; Turturo *et al.*, 2008), including PPV (Varrelmann *et al.*, 2000). Perennial crops are grown in the field for many years and are continually exposed to virus infection. Therefore, perennial crops engineered for virus resistance may have increased potential for recombination compared to annual crops. Yet few long-term studies have been undertaken with perennial crops to address the emergence of recombinant viruses (Vigne *et al.*, 2004; Fuchs and Gonsalves, 2007; Capote *et al.*, 2008).

Recombination has been assessed in PPV CP transgenic and conventional plums grown in the field under Mediterranean conditions. PPV-D strain was used as inoculum but no recombination between transgene transcript and viral RNA was detected, nor was the genetic diversity of virus populations affected (Capote *et al.*, 2008). Here, we expanded on this work and evaluated the occurrence of recombination in PPV CP transgenic plum trees that were exposed to infection by the D and Rec strains of PPV for 6-8 years in the field under continental European conditions.

MATERIALS AND METHODS

Plum trees and field plots. The transgenic plum clones C2, C3, C4 and PT3 planted for these trials express the CP gene (Scorza *et al.*, 1994). The transgenic clone C6 contains one copy of the PPV CP gene and

Corresponding author: I. Zagrai
Fax: +40263214752
E-mail: izagrai@yahoo.com

does not accumulate viral CP (Scorza *et al.*, 1994). All these transgenic clones are susceptible to PPV (Ravelo-nandro *et al.*, 1997; Hily *et al.*, 2004; Malinowski *et al.*, 2006). Transgenic clones were grafted onto the GF-8.1 rootstock (*Prunus mariana*) at INRA in Bordeaux (France), and subsequently established in experimental orchards at the Fruit Research and Development Station Bistrita, Romania under Ministry of Agriculture import authorization no. 1166/02/1996. Following EU recommendations, the experimental plots were surrounded by a buffer of apple trees. The first plot was set up in 1996 with 55 transgenic plum trees belonging to five clones (C2, C3, C4, C6 and PT3). Ten trees each of transgenic clones C2, C3, C4, and C6, and five of transgenic clone PT3 were planted in the spring. Plum trees were planted within a 6-year-old PPV-infected plum orchard in a single row, at a spacing of 4 m between trees and 5 m between rows. In this plot, a high number of transgenic plants died after planting, probably due to the poor adaptation of the GF-8.1 rootstock to the heavy soil and environmental conditions in the Carpathian region in Romania. In order to improve tree survival, a new experimental plot was established in 1998 with 11 plum trees belonging to four transgenic clones (C2- three trees, C4- three trees, C6- two trees, and PT3- three trees) grafted onto myroblan rootstock. Tree spacing was identical as in 1996 but the transgenic plums were randomly dispersed within the orchard.

PPV infection and sampling. Leaf samples were collected from transgenic trees showing typical PPV symptoms and symptomatic conventional trees surrounding transgenic trees in June 2004 and/or 2005. Leaf samples were collected at random throughout the canopy. If symptoms were limited to particular branches, leaves were only sampled from symptomatic branches. Virus infection was confirmed by serological and molecular detection.

Serological and molecular detection of PPV. Serological tests were performed by DAS-ELISA (Clark and Adams, 1977) using a commercial polyclonal antiserum to PPV according to the manufacturer's instructions (Bioreba, Switzerland). Absorbance values were measured at 405 nm after 1 h substrate hydrolysis. Samples were considered positive if their absorbance values were more than twice those of the negative control. Molecular PPV detection was performed by IC-RT-PCR using primer pair P1/P2 that amplifies a 243 bp fragment corresponding to the 3'-terminus of the PPV CP gene (Wetzel *et al.*, 1991). For immunocapture, PPV was trapped with the above PPV polyclonal antiserum adsorbed on an Eppendorf microtube. The enhanced Avian kit (Sigma, USA) was used for RT-PCR. The thermal cycling scheme consisted of 30 min at 50°C followed by 2 min at 94°C and 35 cycles of 30 sec at 94°C,

45 sec at 61°C and 60 sec at 72°C and a final elongation step of 10 min at 72°C. An aliquot of the amplified products (10 µl) was fractionated in 1.5% agarose gel electrophoresis in Tris-borate EDTA (TBE) buffer. DNA bands were visualized by ethidium bromide staining under UV light.

Strain characterization. In order to identify the serotype of the PPV isolates, TAS - ELISA was run with specific monoclonal antibodies raised to PPV-D (Dideron or chlorotic strain) (Durviz, Spain) (Cambra *et al.*, 2004) and PPV-M (Marcus or necrotic strain) (Agritest, Italy) (Boscia *et al.*, 1997).

Molecular strain typing was done by IC-RT-PCR targeting three genomic regions corresponding to: (i) (Cter) CP using specific primers PD and PM that distinguish the two major PPV strains D and M (Olmos *et al.*, 1997); (ii) (Cter) N1b - (Nter)CP using the primer pair mD5/mM3 (Subr *et al.*, 2004) that detects recombinant PPV strain (PPV-Rec) between D and M (Glasa *et al.*, 2002, 2004); (iii) CI using C1f/ C1D or C1M primer sets (Glasa *et al.*, 2002) to confirm the presence of PPV-Rec. Aliquots of PCR products corresponding to (Cter) CP were subjected to RFLP analysis in order to distinguish the D and M strains based on *Rsa* I polymorphism. Digested products were fractionated in 8% polyacrylamide gel electrophoresis (PAGE) in TBE buffer and photographed under UV light after staining with ethidium bromide.

Sequencing. To confirm the molecular variability of PPV isolates, amplified DNA products were purified by Wizard SV Gel and PCR Clean-Up System (Promega, USA) and sequenced by using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA) on the ABI Prism 310 Genetic Analyzer (Applied Biosystems, USA). Alignment of nucleotide sequences from 44 PCR products corresponding to the (Cter)CP gene (15 PPV isolates from transgenic plums and 29 PPV isolates from conventional plums) and seven amplified fragments spanning the (Cter) N1b - (Nter)CP region (four isolates from transgenic plums and three isolates from conventional plums) was done using the program package BioEdit version 5.0.9 (Hall, 1999). Subsequently, the newly sequenced fragments were submitted to GenBank and compared with other PPV sequences available in this database. A phylogenetic tree was constructed with the Mega 3.1 program using Minimum Evolution method Jukes-Cantor model (Bootstrap value 10,000) for sequences corresponding to the (Cter)CP gene (Felsenstein, 2004).

RESULTS

Variability of PPV populations. PPV isolates D and

Rec are endemic in Bistrita (Romania) and natural aphid populations readily transmit PPV isolates among plum trees in experimental orchards (Zagrai *et al.*, 2008). The first trial with transgenic plums was established in 1996 in an experimental orchard for which 23% (49 of 211) of the trees were infected with PPV. In that plot, 10 transgenic and 37 conventional plums showing typical PPV symptoms were sampled in 2004 and 2005. The second trial with transgenic plums was established in 1998 in another experimental orchard for which 4.5% (27 of 598) of the trees were infected with PPV. In the second plot, five transgenic and 17 symptomatic conventional plums were sampled.

TAS-ELISA indicated that the 15 isolates from transgenic plums were PPV-D (eight isolates) and PPV-M (seven isolates). These results were confirmed by IC-RT-PCR using PD and PM specific primers, respectively. Similarly, the two PPV strains were identified by RFLP analysis and the presence of *Rsa* I sites in PPV-D strain (Table 1). All PPV isolates selected from conventional plums surrounding the transgenic plums also reacted positively to at least one of the two monoclonal antibodies as well as to PPV-D or/and PPV-M specific primers (Table 2). A slight discrepancy between serological and molecular test results was observed. Indeed, 28 of 54 isolates were identified as PPV-D by TAS-ELISA, 24 of

Table 1. Serological and molecular differentiation of PPV isolates from transgenic plums.

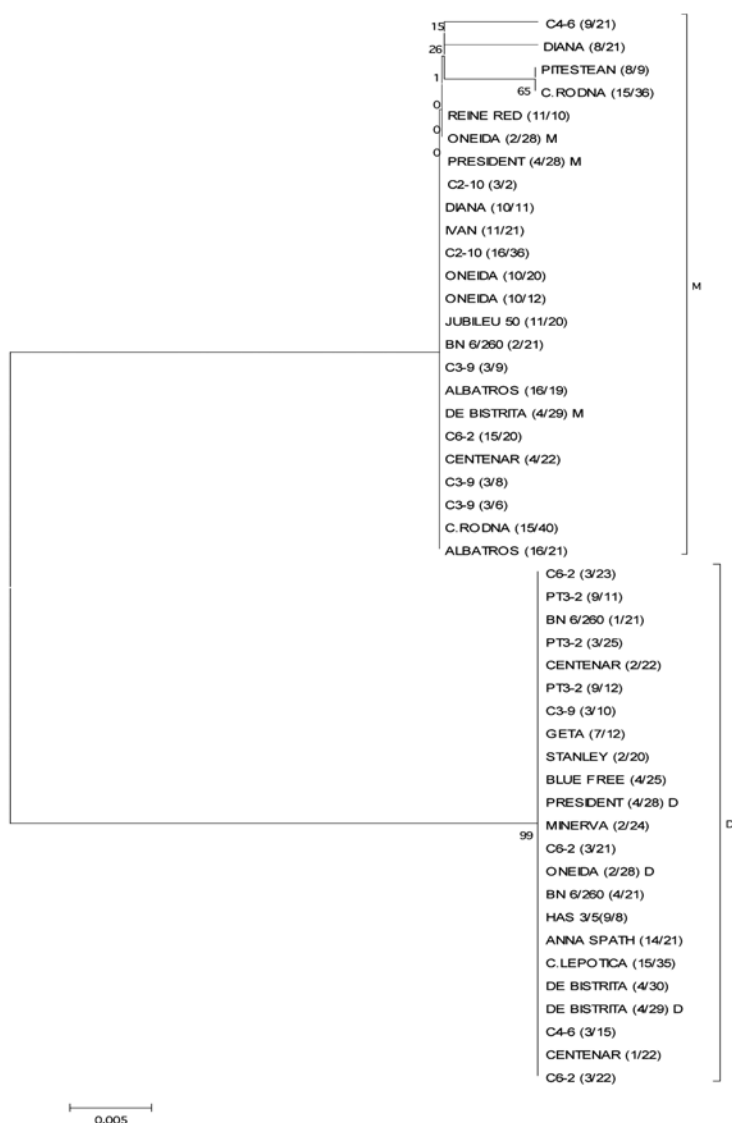
Isolate (row/tree)	DAS / TAS-ELISA				IC-RT-PCR				RFLP <i>Rsa</i> I		
	PPV poly	PPV- D	PPV- M	PPV- D+M	PPV poly	PPV- D	PPV- M	PPV- D+M	PPV- D	PPV- M	PPV- D+M
<i>Plot no. 1</i>											
C6-2 (3/21)	+	+	-	-	+	+	-	-	+	-	-
C6-2 (3/23)	+	+	-	-	+	+	-	-	+	-	-
PT3-2 (3/25)	+	+	-	-	+	+	-	-	+	-	-
C6-2 (3/22)	+	+	-	-	+	+	-	-	+	-	-
C3-9 (3/6)	+	-	+	-	+	-	+	-	-	+	-
C3-9 (3/8)	+	-	+	-	+	-	+	-	-	+	-
C3-9 (3/9)	+	-	+	-	+	-	+	-	-	+	-
C3-9 (3/10)	+	+	-	-	+	+	-	-	+	-	-
C4-6 (3/15)	+	+	-	-	+	+	-	-	+	-	-
C2-10 (3/2)	+	-	+	-	+	-	+	-	-	+	-
<i>Plot no. 2</i>											
PT3-2 (9/11)	+	+	-	-	+	+	-	-	+	-	-
PT3-2 (9/12)	+	+	-	-	+	+	-	-	+	-	-
C4-6 (9/21)	+	-	+	-	+	-	+	-	-	+	-
C6-2 (15/20)	+	-	+	-	+	-	+	-	-	+	-
C2-10 (16/36)	+	-	+	-	+	-	+	-	-	+	-
TOTAL (%)	15 (100)	8 (53.3)	7 (46.7)	0 (0)	15 (100,0)	8 (53.3)	7 (46.7)	0 (0)	8 (53.3)	7 (46.7)	0 (0)

Table 2. Serological and molecular differentiation of PPV isolates from conventional plums surrounding transgenic plums.

No. of isolates	DAS / TAS-ELISA				IC-RT-PCR				RFLP <i>Rsa</i> I		
	PPV poly	PPV- D	PPV- M	PPV- D+M	PPV poly	PPV- D	PPV- M	PPV- D+M	PPV- D	PPV- M	PPV- D+M
<i>Plot no. 1</i>											
24	+	+	-	-	+	+	-	-	+	-	-
10	+	-	+	-	+	-	+	-	-	+	-
1	+	+	-	-	+	-	-	+	-	-	+
1	+	-	+	-	+	-	-	+	-	-	+
1	+	-	-	+	+	-	-	+	-	-	+
<i>Plot no. 2</i>											
3	+	+	-	-	+	+	-	-	+	-	-
13	+	-	+	-	+	-	+	-	-	+	-
1	+	-	-	+	+	+	-	-	+	-	-
TOTAL (%)	54 (100)	28 (51.9)	24 (44.4)	2 (3.7)	54 (100)	28 (51.9)	23 (42.6)	3 (5.5)	28 (51.9)	23 (42.6)	3 (5.5)

Table 3. Molecular typing of PPV isolates from transgenic and conventional plums in different viral genomic regions.

Plot no.	Number of isolates		Target region		
	Transgenic	Conventional	(C-ter) CP	C-ter (NIB)–(N ter) CP	CI
1	6	24	D	-	D
	4	10	M	Rec	D
	0	3	D+M	Rec	D
2	2	4	D	-	D
	3	13	M	Rec	D
	0	0	D+M	Rec	D
Total	8	28	D	-	D
	7	23	M	Rec	D
	0	3	D+M	Rec	D

**Fig. 1.** Phylogenetic grouping of PPV isolates based on nucleotide sequences corresponding to the 3'-terminus of the PPV coat protein gene. Isolates President 4/28, Oneida 2/28 and De Bistrita 4/29 represent mixed infection of PPV D and M strains.

54 as PPV-M and two as a mixed infection of PPV-D and PPV-M. IC-RT-PCR analysis revealed the presence of PPV-D in 28 isolates, PPV-M in 23 isolates, and a mixed infection of D and M strains in three isolates. These results were confirmed by RFLP analysis using *RsaI* digestion.

Occurrence of natural PPV-Rec. All PPV isolates from transgenic and conventional plums initially typed as PPV-M in the (Cter)CP region were identified as PPV-Rec in the (Cter) NIB - (Nter)CP region by IC-RT-PCR using primer pair mD5/mM3 (Subr *et al.*, 2004) (Table 3). As expected no serological differentiation was observed between PPV-M and PPV-Rec. In addition, the use of specific primers to distinguish strains D and M in the CI region detected only fragments of PPV-D, confirming their status as PPV-Rec.

The distribution of PPV strains in transgenic (PPV-D: 53% and PPV-Rec: 47%) and conventional (PPV-D: 52% and PPV-Rec: 43%) plums surrounding the transgenic plums was similar, except for mixed infections (PPV-D + PPV-Rec: 5.5%) that were detected only in conventional plums. Mixed PPV-D and -Rec infections in conventional plums may have been the result of older trees exposed over longer periods of time to aphids carrying both strains.

No emergence of new PPV variants in transgenic plums. The phylogenetic grouping of PPV isolates based on nucleotide sequences corresponding to the 3'-terminus of the PPV CP gene confirmed two major groups and a similarity of PPV isolates from transgenic and conventional plums (Fig. 1). No recombination was found in this genomic region. Our PPV-D and -Rec sequences were 100% and 98-99% identical to the sequences from the NCBI database, respectively. Four nucleotide substitutions were detected in PPV-Rec, one in an isolate from a transgenic plum (C4-6, row 9/tree 21) and three in isolates from conventional plums [Pitesteian (8/9), C. Rodna (15/36), Diana (8/21)]. The PPV sequences determined in this study are available in Gen-

Bank as accessions numbers HQ218871 to HQ218917.

To check if the recombination breakpoint position suspected to occur in the (C-ter)NIB - (N-ter)CP region corresponded with those of PPV-Rec previously reported in conventional plums (Glasa *et al.*, 2002, 2004), seven PCR products spanning this genomic region [four from transgenic plums (accession Nos HQ218918-HQ218921) and three from conventional plums (accession Nos HQ218922-HQ218924)], were sequenced (Fig. 2). The multiple nucleotide sequence alignment indicated that the recombination breakpoint is located at the same nucleotide position 8450 as other PPV-Rec. These results were consistent with the notion that the genetic variability of PPV-Rec isolates is similar in transgenic and conventional plums. In addition, a high similarity (98-99%) with different sequences of PPV-Rec previously reported [BNE-10 (accession No. AF450311), LOZ-3 (accession No. AF450312), BOR-3 (accession No. AY028309)] was found. This genetic similarity confirms that PPV Rec is widespread in Ro-

mania and likely belongs to the same ancestral group with a common evolutionary origin (Glasa *et al.*, 2004; Zagrai *et al.*, 2008).

DISCUSSION

In this report we show that transgenic plum-trees expressing a PPV CP gene do not assist the emergence of PPV recombinants in the field under continental European conditions. The transgenic plums that are PPV susceptible and produce PPV-CP mRNA (except for the C-6 clone) were grown for 6-8 years in experimental orchards, and no significant differences in serological and genetic variability of PPV isolates sampled in transgenic plums and surrounding conventional plum trees was detected. As previously observed by Capote *et al.* (2008), there are no apparent differences in the genetic make up of PPV isolates from transgenic and conventional plum trees. The similarity in the ratio between

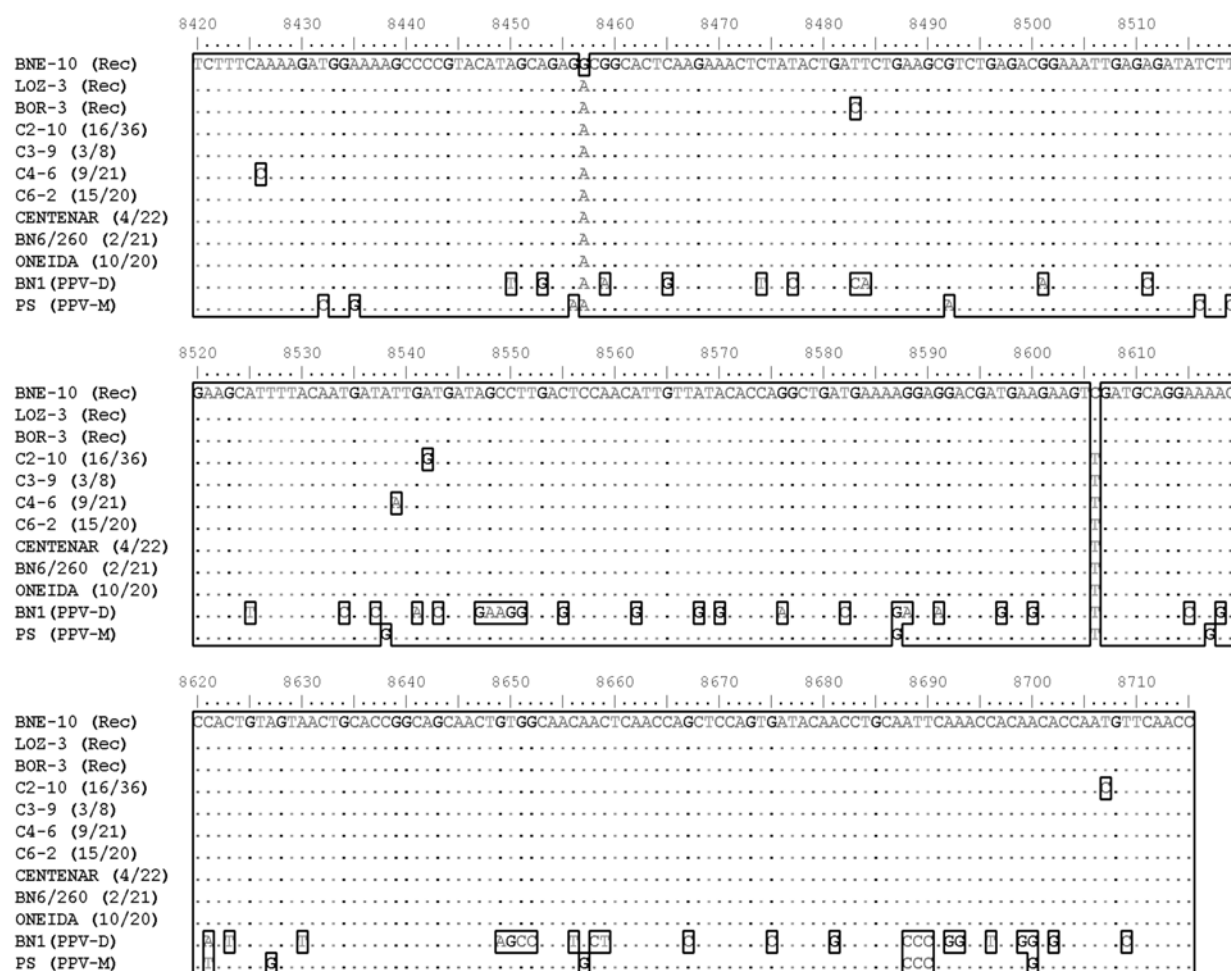


Fig. 2. Multiple alignment of recombinant sequences (NIB/CP) of seven Romanian PPV isolates from transgenic [C2-10 (16/36), C3-9 (3/8), C4-6 (9/21), C6-2 (15/20)] and conventional plums [Centenar (4/22), BN 6/260 (2/21), Oneida (10/20)] and three isolates [BNE-10 (accession number AF450311), LOZ-3 (accession number AF450312), BOR-3 (accession number AY028309)] previously reported.

PPV strains in transgenic and conventional trees suggests that aphid vectors do not differentiate between the two types of plums. The occurrence of a few nucleotide mutations (substitutions and silent mutations) was identified in the CP gene of a few isolates. These results were consistent with the investigation of PPV D populations in Spain (Capote *et al.*, 2008). The major difference between our study and the work of Capote *et al.* (2008) is the diversity of PPV populations. Trees were exposed to PPV D and Rec strains in Romania but only to D strains in Spain. The fact that identical transgenic plum trees were evaluated under distinct environmental conditions for which diverse aphid populations and climatic factors occur in continental Romania and Mediterranean Spain strengthen our collective conclusions on a lack of effect of transgenic trees on the serological and genetic variability of natural PPV populations.

The fact that new PPV variants were not detected in transgenic plums is in contrast with observations in annual species where the emergence of recombinants is well documented (Tepfer, 2002; Turturo *et al.*, 2008). Although PPV Rec isolates were identified in our study, the recombination breakpoint was identical in the (C-ter)N1b-(N-ter)CP region of isolates from transgenic and conventional plums, indicating that recombination did not involve transgene PPV CP transcripts. Instead, the presence of PPV Rec isolates in transgenic plums resulted from aphid-mediated transmission from adjacent naturally infected nontransgenic plum trees.

Our results and those of Capote *et al.* (2008) indicate that transgene transcripts in plum trees expressing a PPV CP gene do not contribute to the creation of PPV recombinants with PPV D and Rec strains. Further, since PPV is the only known potyvirus transmitted in a non-persistent manner in *Prunus*, the risk of heterocapsidation is limited unlike in annual crops for which multiple potyviruses can coexist and interact with aphid vectors (Fuchs and Gonsalves, 2007).

Together with results reported from work with transgenic grapevines (Vigne *et al.*, 2004), our findings provide strong evidence that transgenic perennial fruit trees expressing a virus CP gene do not assist the development of recombinants under field conditions. Field studies in woody perennial hosts do not support recombination reported from greenhouse studies (Tepfer, 2002; Turturo *et al.*, 2008).

A high level and a durable resistance to PPV was achieved in transgenic plums through PTGS (Scorza *et al.*, 2001; Hily *et al.*, 2004) and the production of siRNA (Hily *et al.*, 2005; Scorza *et al.*, 2007; Kundu *et al.*, 2008). The resistant transgenic clone C-5 does not produce detectable PPV-CP mRNA (Scorza *et al.*, 1994) but accumulates the siRNA doublet that has been analyzed extensively by Hily *et al.* (2005) and Kundu *et al.* (2008). C-5 plum trees were not evaluated in this study

since this clone is not infected following natural aphid-mediated transmission of PPV (Malinowski *et al.*, 2006).

In summary, our study shows that recombination was not detected in PPV-susceptible transgenic plums expressing the PPV CP gene. Also the genetic diversity of PPV strains in transgenic and conventional plums was similar. Therefore, we conclude that transgenic plums expressing the PPV CP gene do not present a greater risk in terms of virus recombination and diversity than conventional plums.

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IDENTIFICATION OF *CIRCULIFER HAEMATOCEPS* (HEMIPTERA:CICADELLIDAE) AS VECTOR OF *SPIROPLASMA CITRI* IN THE KERMAN PROVINCE OF IRAN

M. Omid¹, A. Hosseini-Pour¹, H. Rahimian², H. Massumi¹ and C. Saillard^{3,4}

¹ Department of Plant Protection, College of Agriculture, Shahid Bahonar University, Kerman, Iran

² Department of Plant Protection, College of Agriculture, Mazandaran University, Sari, Iran

³ Université de Bordeaux 2, UMR 1090 Génomique Diversité et Pouvoir Pathogène, F-33883 Villenave d'Ornon, France

⁴ INRA, UMR 1090 Génomique Diversité et Pouvoir Pathogène, F-33883 Villenave d'Ornon, France

SUMMARY

A study of leafhoppers collected in citrus-growing areas of Kerman province as vectors of *Spiroplasma citri*, the causal agent of citrus stubborn disease, was carried out by transmission tests to liquid medium and/or to healthy periwinkle plants. From seven field-collected leafhopper species only *Orosius albicinctus* and *Circulifer haematoceps* delivered *S. citri* into LD10 broth medium by feeding through parafilm membrane and *C. haematoceps* was the sole leafhopper species capable of transmitting *S. citri* to healthy periwinkle plants. *C. haematoceps*, *O. albicinctus* and *Austroagallia sinuata* collected from sesame fields in citrus-growing areas, were raised on garden stock and sesame plants in the greenhouse. For *S. citri* acquisition, each progeny leafhopper colony was caged on *S. citri* infected periwinkles. After the acquisition access period leafhoppers were transferred to healthy periwinkle plants. Detection of spiroplasmas in leafhoppers was done by PCR performed separately on insect bodies and heads. Spiroplasmas were detected in the bodies of all three leafhopper species but in the head of *C. haematoceps* only. Symptoms typical of *S. citri* infection were observed only on periwinkle plants fed on by *C. haematoceps*. Results indicate that among the leafhoppers species collected in Kerman province, only *C. haematoceps* was capable of transmitting *S. citri*, and the other two species while capable of acquiring *S. citri*, were unable to transmit it to healthy plants.

Key words: sesame, PCR, transmission, mollicutes, citrus stubborn disease, epidemiology.

INTRODUCTION

Members of the class *Mollicutes*, phylogenetically related to Gram-positive bacteria are the smallest free-living organisms known. They are characterized by a small

genome size (580 to 2200 kbp) and low G+C contents (Barre *et al.*, 2004). In the *Mollicutes*, the genus *Spiroplasma* consists of a group of motile helical procaryotes that are associated primarily with arthropods, mainly insects (Gasparich *et al.*, 2004). Only three species, *Spiroplasma citri* (Saglio *et al.*, 1973), *S. kunkelii* (Whitcomb *et al.*, 1986), and *S. phoeniceum* (Saillard *et al.*, 1987) are pathogenic to plants. *S. citri*, the first plant pathogenic mollicute to be cultured and characterized (Saglio, *et al.*, 1971, 1973), is an important pathogen, causing citrus stubborn disease in the Mediterranean area and California (Calavan and Bové, 1989; Bové, 1986) as well as horseradish brittle root (Fletcher *et al.*, 1981) and carrot purple leaf (Lee *et al.*, 2006) diseases in the United States. Plant pathogenic spiroplasmas are transmitted from plant to plant by phloem-feeding leafhoppers in a persistent propagative manner (Purcell, 1983). *Circulifer tenellus* (Baker) was identified as the major vector of *S. citri* in California (Oldfield *et al.*, 1976), whereas in Turkey and Syria, *C. haematoceps* is regarded as the main vector of this pathogen (Bové *et al.*, 1988). Cochran and Samadi (1976) reported the incidence of citrus stubborn disease in Iran based on symptom observation in the groves and graft transmission tests. *S. citri* was cultured from an infected citrus tree in southwestern Iran in 1974 (Bové, 1995) and since then, has been isolated from citrus trees in many areas of southern and southeastern Iran (Rahimian, 1982; Hosseini-Pour, 2000). Stubborn disease is the major problem in the citrus production areas of Kerman province, one of the main citrus-growing regions of Iran. *S. citri* has been previously detected in field collected leafhoppers in southern Kerman province by ELISA and isolation of the spiroplasma in culture (Lori *et al.*, 2007). However, the transmission capabilities of this leafhopper species have not been verified.

The purpose of the present study was to evaluate the ability of leafhopper species collected in citrus orchards of Kerman province, to transmit *S. citri* to periwinkle (*Catharanthus roseus* (L.) G. Don) plants or to inoculate the spiroplasma into liquid media through Parafilm membrane.

Corresponding author: A. Hosseini-Pour

Fax: +98.3413220067

E-mail: Hosseini@mail.uk.ac.ir

MATERIALS AND METHODS

Insect source and rearing. Leafhoppers collected from sesame (*Sesamum indicum* L.) fields in the citrus growing-areas of southeastern and southern Kerman province using a sweep net during early autumn 2004, were sorted out and identified to the species in the laboratory. For mass rearing of *C. haematoceps*, *O. albicinctus* and *A. sinuata*, adult insects were captured in the sesame fields in Jiroft (southern Kerman province), transferred to a greenhouse and caged on garden stock (*Matthiola incana* L.) and sesame plants in plexiglass cylinders (15×50 cm), with four 10×10 cm ventilation apertures made of gauze. Transmission experiments were carried out with individuals from the third generation of the reared leafhoppers. Samples of adults from each leafhopper species, as well as samples from stock and sesame plants used in rearing trials, were tested regularly by PCR and culturing in liquid media to check their freedom from *S. citri* contamination. Material tested for the absence of spiroplasmas by both methods were used for rearing.

Transmission of *S. citri* by field-collected leafhoppers to periwinkle. Disease-free periwinkle plants were grown from seeds in leafhopper-proof screenhouses. Plants at the 6 to 8 leaf stage (15-20 cm in height) were used for transmission assays. Collected leafhoppers (Table 1) were caged on healthy periwinkle plants in groups of 10 adults per plant (except *Empoasca decipiens* with 15) for an inoculation access period of two weeks. At the end of the period, the leafhoppers were killed with an insecticide and the plants were kept in a leafhopper-proof screen house at 30±5°C and checked daily for the appearance of symptoms. Spiroplasma infection of plants used in transmission experiments was determined by culture assay. Periwinkle plants with severe symptoms of *S. citri* infection and from which *S. citri* was isolated in culture were propagated and pre-

served by grafting as *S. citri*-infected periwinkles for further experiments. They were regularly tested by PCR for the presence of *S. citri*.

Acquisition and transmission assays with laboratory-reared insects. Adults of each *S. citri*-free leafhopper species were caged on *S. citri*-infected periwinkle plants for an acquisition-access period of three days. They were then transferred to young healthy stock plants for an incubation period of two weeks. Leafhoppers were finally caged, in batches of 10 individuals, on young healthy periwinkle plants until they died (ca.10-15 days). Periwinkles exposed to insects were kept in the greenhouse at 30±5°C, checked regularly for symptom expression and tested for infection with *S. citri*. At the end of the incubation period, leafhopper heads bearing the salivary glands were cut off the thoraxes as described (Kwon *et al.*, 1999). DNA was extracted separately from heads and bodies and used in PCR assay for detection of *S. citri*.

Transmission of *S. citri* through membranes. Field-collected leafhoppers were transferred in groups of four to conical microfuge tubes on which a Parafilm membrane separating the leafhoppers from the LD10 culture medium (Lee and Davis, 1984) which was poured into the inner chamber of the lid, and the insects were allowed to feed through the membranes (Rana *et al.*, 1975; Foissac *et al.*, 1995). Infected leafhoppers were expected to inject *S. citri* into the culture medium while feeding through the membrane. After leaving the assembly for 24 h at room temperature, the culture medium was collected and kept at 32°C. Growth of spiroplasmas was checked after 4 days by the change of color of the medium from pink to yellow and by dark field microscopy.

Culture of *S. citri* from periwinkle plants. Old and young leaves of symptomatic periwinkle plants were col-

Table 1. Transmission of *Spiroplasma citri* to periwinkle plants by field-collected leafhoppers in Kerman province.

Leafhopper species ^a	No. of insects used for transmission trials	No. of <i>S. citri</i> -infected plants/ no. of plants exposed to insects
<i>Anaceratagallia laevis</i>	150	0/15
<i>Austroagallia sinuata</i>	200	0/20
<i>Circulifer haematoceps</i>	200	2/20
<i>Empoasca decipiens</i>	150	0/10
<i>Orosius albicinctus</i>	150	0/15
<i>Psammotettix alienus</i>	100	0/10
<i>Psammotettix striatus</i>	100	0/10

^aOne thousand fifty field leafhoppers were collected from sesame fields in Kerman province during October 2004. For each leafhopper species 10 insects were caged on each periwinkle plant, but *E. decipiens* with 15 insects. A total of 100 periwinkle plants were used in the transmission assays. Plants inoculated with *C. haematoceps* showed symptoms after three weeks and *S. citri* was isolated from them.

lected randomly. Midribs (0.2 to 0.5 g) were minced with a razor blade in 2 ml of liquid LD10 medium. After 30 min of incubation at room temperature, the macerates were filtered through 0.45- μ m-pore-size filters. The filtered extracts were serially diluted in the culture medium and incubated at 32°C.

Extraction of DNA from leafhoppers and plants.

DNA was extracted from insects by the method of Maixner (1994). Briefly, samples from whole insects, heads, and the headless bodies were ground in an Eppendorf tube (1.5 ml) with 400 μ l of extraction buffer (100 mM Tris-HCl, 1.4 M NaCl, 1% PVP, 2% CTAB, 20 mM EDTA, pH 8.0), incubated at 65°C for 30 min, and centrifuged at 1,200 g for 5 min. An equal volume of chloroform-isoamyl alcohol (24:1, v/v) was added to the supernatant, mixed by repeated inversions and the emulsion was centrifuged again. A 0.7 vol of isopropyl alcohol was added to the supernatant, mixed well and the tube was kept at -20°C overnight. Following centrifugation the pellet was dissolved in 50 μ l of sterile distilled water, and used as a template DNA for direct PCR amplification. DNA from midribs of healthy and infected plants used in different experiments was extracted by as described by Maixner *et al.* (1995).

PCR amplification. Detection of *S. citri* in insects and plants was done by PCR amplification of a 336 bp fragment of the spiralin gene using primers designed by Najjar *et al.* (1998). Each PCR reaction in a total volume of 25 μ l contained 1 μ l of DNA template from plants or insects, 1 μ M of each primer, 1 unit of *Taq* DNA polymerase (Cinagen, Iran), 0.2 mM each dNTPs, 2.5 μ l of 10 \times PCR buffer, and 1.5 mM MgCl₂. Samples lacking template DNA and those containing 1 μ l of a liquid culture of *S. citri* (as DNA template) were run as negative and positive controls in each PCR reaction, respectively. The reaction mixture was overlaid with 25 μ l of mineral oil. After an initial 3 min incubation at 94°C, amplification was carried out for 40 cycles of 94°C for 45 sec, 62°C for 45 sec, and 72°C for 30 sec. Final extension

step was at 72°C for 7 min. Five μ l of each reaction mixture was analyzed on 1% agarose gel in Tris-borate buffer (0.045 M Tris-borate, pH 8.0, 1 mM EDTA), stained with ethidium bromide, visualized at 312 nm on a UV transilluminator and photographed.

RESULTS

Transmission of *S. citri* to periwinkle plants and to culture medium by field-collected leafhoppers. Eight hundred forty leafhoppers, belonging to the seven species, collected from sesame fields were allowed to feed for 24 h on LD10 medium through Parafilm membrane. *C. haematoceps* and *O. albicinctus*, were the only species that inoculated *S. citri* into the culture medium (Table 2) as evidenced by the change of the colour of the medium. The growth of *S. citri* was further confirmed by observing spiroplasma cells in culture medium under a dark-field microscope and the amplification of a 336 bp DNA fragment in PCR assay of the cultures.

PCR detection of *S. citri* in field-collected leafhoppers. PCR assays with spiralin-specific primers were performed with DNA extracted from leafhoppers collected in sesame fields. The 336 bp fragment amplified from DNA extracted from *C. haematoceps* and *O. albicinctus* corresponded to that of *S. citri* positive control. No amplification occurred with DNA extracted from the other leafhopper species (Fig. 1).

Transmission of *S. citri* to periwinkles following acquisition by leafhoppers from spiroplasma-infected plants. All of eight periwinkle plants on which *C. haematoceps* individuals that had acquired *S. citri* through feeding on infected plants were caged, developed leaf yellowing and malformation symptoms (Table 3). *S. citri* was isolated from symptomatic leaves in LD10 medium and was also cultured from 40% of the *C. haematoceps* adults that had fed on infected periwinkle plants following the inoculation access period.

Table 2. Inoculation of *Spiroplasma citri* by field-collected leafhoppers into culture medium through membrane feeding in Kerman province.

Leafhopper species ^a	No. of insects used for <i>S. citri</i> -inoculation assays through membrane feeding	Presence of spiroplasma cells in culture medium after four days (No. of positive assays/No. of assays)
<i>Anaceratagallia laevis</i>	120	0/30
<i>Austroagallia sinuata</i>	120	0/30
<i>Circulifer haematoceps</i>	120	4/30
<i>Empoasca decipiens</i>	120	0/30
<i>Orosius albicinctus</i>	120	1/30
<i>Psammotettix alienus</i>	120	0/30
<i>Psammotettix striatus</i>	120	0/30

^aEight hundred forty leafhoppers were collected from sesame fields in Kerman province in October 2004. Four insects of each leafhopper species were caged in each membrane feeding apparatus. Thirty feeding apparatus per leafhopper species were used.

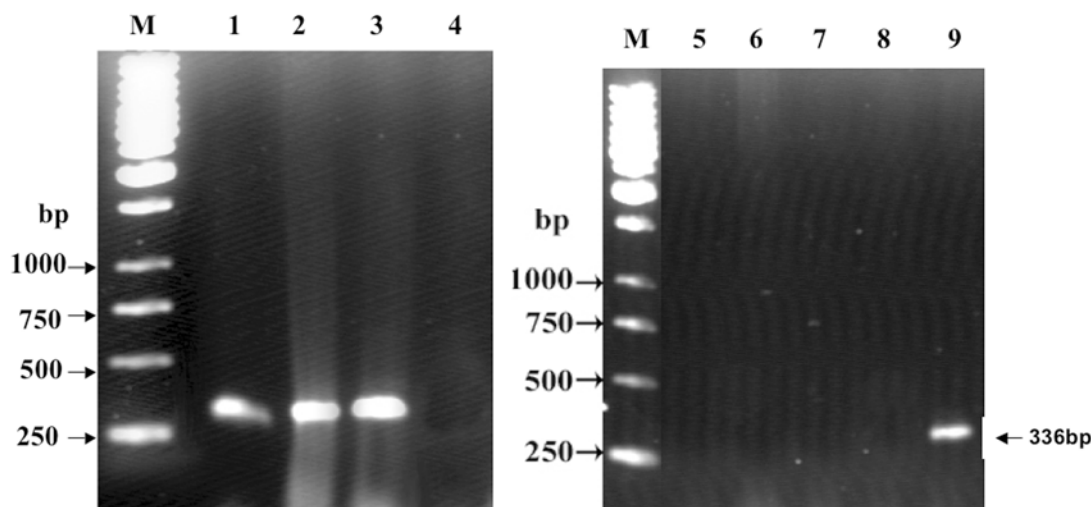


Fig. 1. PCR detection of *S. citri* in total DNA extracted from adults of *Circulifer haematoceps* (lane 1), *Orosius albicinctus* (lane 2), *Psammotettix alienus* (lane 5), *Austroagallia sinuata* (lane 6), *Anaceratagallia laevis* (lane 7), and *Empoasca decipiens* (lane 8) collected in sesame fields from Kerman province. DNA extracted from a *S. citri* culture as positive control (lanes 3 and 9) and water control (lane 4). M: 1 kb DNA ladder (MBI, Fermentas).

Table 3. Experimental transmission of *Spiroplasma citri* to periwinkle plants by laboratory reared-leafhoppers following their feeding on *S. citri*-infected periwinkle plants.

Leafhopper species ^a	No. of insects used for transmission trials	No. of <i>S. citri</i> -infected plants/no. of plants exposed to insects
<i>Austroagallia sinuata</i>	60	0/6
<i>Circulifer haematoceps</i>	80	8/8
<i>Orosius albicinctus</i>	40	0/4

^aPlants inoculated with *C. haematoceps* showed symptoms after two weeks and *S. citri* was isolated from them. Despite three months of keeping, none of the plants inoculated with the other two species showed any symptoms and isolation of *S. citri* from such plants was unsuccessful.

PCR detection of *S. citri* in leafhoppers after acquisition from infected periwinkles. After feeding on infected periwinkles and completion of the 2-week incubation period on healthy stock plants, PCR assays for detection of *S. citri* were performed on DNAs of *C. haematoceps*, *O. albicinctus*, and *A. sinuata*. DNA was extracted from the head and body of each leafhopper species and each was used, separately, as template in PCR. A PCR product with a size corresponding to that of positive control DNA (data not shown) was obtained with DNAs extracted from the body of all leafhopper species used (Fig. 2, lanes 2, 4, 6). However, amplification was obtained with DNA extracted only from the head of *C. haematoceps* (Fig. 2, lane 5). PCR amplification were repeated at least three times from each insect species. No amplicons were obtained from DNAs ex-

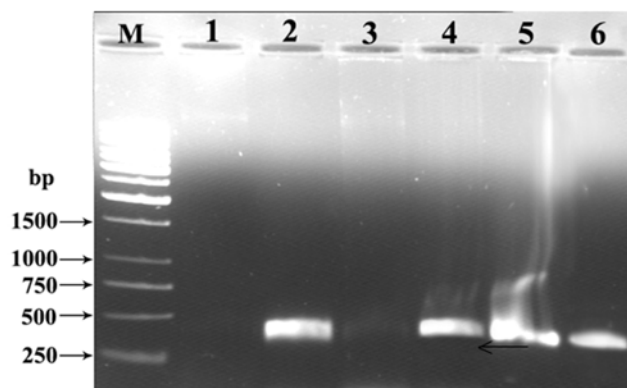


Fig. 2. PCR detection of *S. citri* in total DNA extracted from heads (lanes 1, 3, 5) and bodies (lanes 2, 4, 6) of leafhoppers *Orosius albicinctus*, *Austroagallia sinuata* and *Circulifer haematoceps*, respectively. The reared healthy leafhoppers were fed on *S. citri*-infected periwinkle plants for an acquisition-access period of 3 days and then on young healthy stock plants for an incubation period of two-weeks. M: 1 kb DNA ladder (MBI, Fermentas).

tracted from the head or decapitated bodies of healthy leafhopper species reared only on garden stock, a non-host plant for *S. citri* (data not shown).

DISCUSSION

Seven leafhopper species were collected from sesame fields in citrus-growing areas (Jiroft, Shahdad, and Bam) of Kerman province. In transmission experiments only *C. haematoceps* was able to transmit *S. citri* to periwinkle plants. *S. citri* had been previously detected by

ELISA in *C. haematoceps*, *O. albicinctus*, *P. striatus*, *A. sinuata* and *P. alienus*, in southern Kerman province (Lori *et al.*, 2007). However, the capability of these leafhopper species to transmit the pathogen to healthy plants through direct tests has remained undetermined. In the present work, field collected *C. haematoceps* transmitted *S. citri* to 10% of the exposed periwinkle plants. In Turkey *S. citri* was cultured from field-collected leafhopper species *Baclutha hebe*, *Cicadulina bipunctella*, *Exitianus capicola*, *P. striatus*, *O. orientalis*, and *C. haematoceps*. However, in transmission experiments, only *C. haematoceps* leafhoppers collected from corn (*Zea mays* L.) and sesame plants and not the ones collected from *Salsola kali* L. could transmit *S. citri* to periwinkle plants with a transmission rate of 25% (Kersting and Sengonca, 1992). In California, the two field-collected leafhopper species *C. tenellus*, and *Scaphytopius acutus delongi* transmitted *S. citri* to periwinkle plants with infection rates of about 8% and 0.7%, respectively (Oldfield *et al.*, 1984). Isolation of spiroplasma in culture or a positive PCR assay would merely indicate that the insect is capable of acquiring *S. citri* and allowing its multiplication in digestive tract but would not imply that the insect could act as a vector. Whereas both *O. albicinctus* and *C. haematoceps* injected *S. citri* into culture medium through Parafilm membrane, the former species was unable to transmit spiroplasma to periwinkle plants under the conditions of this study. Caglayan (1987) using *E. capicola* succeeded in transmitting *S. citri* to culture medium but not to periwinkle plants. Spiroplasmas ingested via phloem-sap feeding leafhoppers traverse the insect gut wall and reach the hemolymph, where they multiply and circulate. They eventually invade the salivary glands, where they multiply further and are introduced, with the saliva, into the sieve tubes of plants while the leafhoppers are feeding on their host plants (Liu *et al.*, 1983; Kwon *et al.*, 1999). The *S. citri* spiralin primers allowed amplification of a specific DNA fragment from the headless bodies of *A. sinuata* and *O. albicinctus* fed on *S. citri*-infected periwinkle following a 3 and 14 days of acquisition access and incubation periods, respectively, but not from their heads. However, the fragment was amplified from both the heads and headless bodies of *C. haematoceps*. Oldfield *et al.* (1984) reported that *Ollarianus strictus* occasionally harbors spiroplasmas and readily acquires *S. citri* from the experimentally infected periwinkle plants but has never been able to transmit this pathogen to healthy plants. *O. albicinctus* probably behaves like *O. strictus*. It is possible that spiroplasma cells acquired by *O. albicinctus*, do multiply in the hemolymph, but cannot cross the salivary gland barriers efficiently; thence the insects are not able to introduce sufficient number of spiroplasma cells with the saliva, into the plant phloem tissue. However, the quantity of spiroplasmas injected by this leafhopper species into the medium

seems to be enough for getting a culture. Culture medium may act as a better nutritive environment than the phloem sap for *S. citri* growth. The results obtained with the laboratory reared insects which fed on *S. citri*-infected periwinkle plants, support this proposition. The relationships between leafhoppers and mollicutes are quite specific. One type of specificity operates when a leafhopper has the ability to acquire a mollicute through feeding on an infected host plant, but lacks the ability to transmit it to another plant. Although these interactions are clearly crucial to the biology and epidemiology of the pathogen and the disease, little is known about the specific events at the microbe-vector interface that determine the nature of transmission specificity (Fletcher *et al.*, 1998). Some authors (Fletcher *et al.*, 1998; Mowry, 1986) speculate that the mechanisms of spiroplasma penetration and traversal of the insect intestinal lining are probably different from those leading to salivary cell penetration. The physical and physiological factors that determine whether phytopathogenic mollicutes are transmissible by a certain insect may also determine whether an insect can harbor a particular mollicute and whether that microorganism is able to penetrate the intestine wall and colonize the hemolymph and subsequently get passed the salivary gland barriers. Considering that *O. albicinctus* can acquire *S. citri* that multiplies in its body, but cannot cross efficiently the salivary glands, this leafhopper is a good candidate for studying the specific interaction between *S. citri* and its leafhopper vectors.

Results of the present study show that, like in Mediterranean countries, *C. haematoceps* is the main vector of *S. citri* that causes citrus stubborn disease in Iran. Information from the present study would help to minimize the damage caused by *S. citri* in Iran by focusing on control measures targeted against *C. haematoceps*.

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CHARACTERIZATION AND VARIABILITY OF SOFT ROT-CAUSING BACTERIA IN CHINESE CABBAGE IN NORTH EASTERN BRAZIL

I.C.M. Alvarado¹, S.J. Michereff¹, R.L.R. Mariano¹, E.B. Souza¹, A.M. Quezado-Duval²,
L.V. Resende¹, E. Cardoso¹ and E.S.G. Mizubuti³

¹ Universidade Federal Rural de Pernambuco, Departamento de Agronomia, 52171-900 Recife, Pernambuco, Brazil

² Embrapa Hortaliças, 70359-970 Brasília, Distrito Federal, Brazil

³ Universidade Federal de Viçosa, Departamento de Fitopatologia, 36571-000 Viçosa, Minas Gerais, Brazil

SUMMARY

Yield of Chinese cabbage (*Brassica pekinensis*) may be limited by the occurrence of the soft rot caused by pectinolytic bacteria. Thirty-nine bacterial isolates associated with soft rot in Chinese cabbage, obtained from north-eastern Brazil, were identified as *Pectobacterium carotovorum* subsp. *carotovorum* (*Pcc*) based on biochemical tests and URP-PCR. The variability of all isolates was assessed with reference to disease components, i.e. incubation period (IP), initial severity (ISEV), final severity (FSEV) and area under the disease progress curve (AUDPC), sensitivity to 12 antibiotics and the banding pattern of REP, ERIC and BOX in Rep-PCR. Based on IP, ISEV, FSEV and AUDPC, the isolates were distributed in six similarity groups after cluster analysis. There was significant correlation ($P \leq 0.05$) between IP and ISEV. Based on the sensitivity to antibiotics, *Pcc* isolates were distributed in 14 groups. Significant correlations between sensitivity to gentamicin and IP ($r = -0.41$), as well as between sensitivity to clindamycin and FSEV ($r = -0.45$) were detected. There was high genetic variability among the 39 isolates based on the molecular markers. A total of 32 similarity groups were formed. No significant correlations were found between the linkage distances of molecular markers and either the disease components or antibiotic sensitivity. Overall, there was high variability in populations of *Pcc* affecting Chinese cabbage in north-eastern Brazil.

Key words: *Pectobacterium carotovorum* subsp. *carotovorum*, *Brassica pekinensis*, disease components, resistance to antibiotics, molecular markers.

INTRODUCTION

Pernambuco state (north eastern Brazil), is one of the main producers of Chinese cabbage (*Brassica pekinensis* L.), the average yield between 2001 and 2005 being estimated to be 75.6 t/year. Camocim de São Félix is the

most important producing county, and accounts for 45% of the total crop (Ceasa-PE, 2007).

Yield of Chinese cabbage can be limited by diseases among which soft rots, caused by pectinolytic bacteria, are the most destructive worldwide (Mew *et al.*, 1976; Kikumoto, 1980; Ren *et al.*, 2001). Soft rots are frequent in Pernambuco state. For instance, in a survey conducted in 2004 the disease was recorded in all fields of Chinese cabbage sampled in the Agreste and in the Zona da Mata regions. Disease prevalence was 100%, and an incidence of up to 67% was recorded in a sampled field (Silva *et al.*, 2007). The initial symptom of soft rot in Chinese cabbage is the maceration of leaf base tissues that are in contact with soil. The disease can progress quickly and symptoms can be observed in the main stem. The whole plant can collapse after a few days (Kikumoto, 1980).

The main bacterial species associated with soft rots in Chinese cabbage is *Pectobacterium carotovorum* (Jones) Hauben *et al.*, predominantly *P. carotovorum* subsp. *carotovorum* (Jones) Hauben *et al.* (Mew *et al.*, 1976; Ren *et al.*, 2001). However, *P. carotovorum* subsp. *odoriferum* (Gallois *et al.*) Hauben *et al.* (Seo *et al.*, 2004) has also been reported to cause soft rot in this crop. Classification of the *Pectobacterium* spp. is mainly based on biochemical and phenotypic characteristics (Seo *et al.*, 2004). One important characteristic is the capacity to grow and form a depression in crystal-violet pectate medium (CVP) (Hyman *et al.*, 2002). According to De Boer and Kelman (2001), *P. carotovorum* can be differentiated from other species of the genus based on the ability to grow at 37°C, production of acid from α -methyl glucoside, indol production, phosphatase activity, and sucrose reduction. Nevertheless, phenotypic identification of the bacteria is time-consuming and reliability is questionable (Toth *et al.*, 2001). The high genetic and phenotypic diversity of *Pectobacterium* spp. makes identification a challenging issue (Seo *et al.*, 2000, 2002).

Molecular methods are rapid, sensitive, cost effective, and widely used in diagnostic bacteriology (Janse, 2005). They also can quantify pathogen variability or polymorphism (Hu *et al.*, 2007).

The genetic variability of *P. carotovorum* subsp. *caro-*

Corresponding author: S.J. Michereff
Fax: +55.81.33206205
E-mail: sami@depa.ufrpe.br

toovorum (*Pcc*) has been investigated by restriction fragment length polymorphism (RFLP), rep-PCR (Seo *et al.*, 2000) using BOX, ERIC, and REP primers that correspond to the conserved repetitive elements of the bacterial genome (Louws *et al.*, 1999). *Pcc* isolates have also been studied using DNA sequences of the 16S and 23S of rDNA (Fessehaie *et al.*, 2002) and the intergenic region 16S-23S (ITS) (Toth *et al.*, 2001).

However, for practical purposes (see below), the association of genetic variability assessed with molecular markers must be complemented with the analysis of variation regarding variables directly related to disease development and management strategies, such as disease components and sensitivity to chemical compounds.

Bacterial isolates can differ in severity of the disease they cause and sensitivity to antibiotics. Quantification of disease components related to the infection cycle is important to infer for determining pathogenic variations. Adoption of this approach makes it possible to assess differences among isolates regarding the speed of host tissue colonization, thus the possible severity of disease in the field (Costa *et al.*, 2001; Silveira *et al.*, 2003). Sensitivity of bacterial isolates to antibiotics can also vary. The effectiveness of these antibiotics can be affected by differential responses among isolates.

Knowledge about the diversity of soft rot-causing bacteria in Chinese cabbage can potentially contribute to design more effective disease management strategies (Seo *et al.*, 2000). Despite the importance of both crop and disease in the Agreste region (Pernambuco state), no thorough investigation of the population of these plant pathogenic bacteria has been conducted. The objective of this study was to characterize soft rot-causing bacteria that affect Chinese cabbage in the main producing areas of Pernambuco state and to quantify the amount of variability among isolates of *Pcc* based on disease components, sensitivity to antibiotics and molecular makers.

MATERIALS AND METHODS

Sampling and characterization of bacterial isolates.

Chinese cabbages with typical soft rot symptoms collected from different producing areas in Camocim de São Félix during 2004 and 2005, were taken to the laboratory and selective isolation of *P. carotovorum* was carried out in sweet pepper as described (Takatsu *et al.*, 1981). Preliminary identification of the isolates was done using Casamino acid-peptone-glucose culture medium (CPG). Young *Pectobacterium* colonies (36-48 h) observed under a stereoscope with oblique illumination, had a "broken glass" appearance (Kelman and Dickey, 1995). Thirty nine isolates with these characteristics were subjected to the following tests: Gram coloration, oxidation/fermentation, oxidase, catalase, soft rot in potato tuber

(Hyman *et al.*, 2002) and pathogenicity in detached leaves of Chinese cabbage (cv. Komachi). Isolate identification at the subspecies level was done based on the following tests: growth at 37°C; reducing substances from sucrose; utilization of α -methyl glucoside; acid production from sorbitol; melibiose and lactose; sensitivity to erythromycin; growth in 5% NaCl, and production of phosphatase and lecithinase (Hyman *et al.*, 2002). Additional tests such as hydrolysis of casein (Dye, 1969) and colony color on nutrient agar-glycerol-MnCl₂ medium (NGM) were done to differentiate *Pcc* from *Dickeya chrysanthemi* (Burkholder *et al.*) Samson *et al.* (Lee and Yu, 2005). Pure cultures were preserved in sterilized distilled water (SDW) and stored in the culture collection of the Laboratório de Fitobacteriologia of the Universidade Federal Rural de Pernambuco.

DNA extraction. Total genomic DNA of each isolate was extracted from 36 to 48 h old cultures grown in 5 ml of nutrient broth and incubated at 25±4°C. The concentration of the bacterial suspension was adjusted to $A_{600} = 0.3$ using a photocolormeter. DNA was extracted using the CTAB method (Wilson, 1999). An aliquot of 1.5 ml of bacterial suspension was transferred to microtubes, centrifuged twice at 14,000 *g* for 2 min, and the supernatant was discarded. The pellet was resuspended in SDW and centrifuged at 14,000 *g* for 3 min. The pellet was resuspended with 567 μ l of TE buffer, pH 8. A volume of 30 μ l of 10% SDS and 3 μ l proteinase K (20 mg ml⁻¹) were added to the tubes and vortexed. Tubes were kept at a 37°C for 1.5 h. After incubation, 100 μ l of 5 M NaCl were added to each tube and tubes were shaken. CTAB/NaCl (4.1 g of NaCl and 10 g of CTAB in 100 ml of water) was added and tubes were kept in a water bath at 65°C for 10 min. After incubation, 780 μ l of chlorophorm-isoamyl alcohol (24:1) were pipetted, tubes were manually agitated for 10 min, and centrifuged at 14,000 *g* for 5 min. Supernatant was transferred to a new tube, phenol-chlorophorm-isoamyl alcohol (25:24:1) was added, tubes were manually agitated for 10 min, and centrifuged at 14,000 *g* for 5 min. Supernatant was transferred to new tubes and 360 μ l of isopropanol were added to precipitate DNA by gently agitation. After precipitation, tubes were kept at -70°C for 10 min and centrifuged at 14,000 *g* for 20 min. Supernatant was discarded and DNA was washed with 70% ethanol followed by a centrifugation at 14,000 *g* for 10 min. Ethanol was discarded and tubes were placed in Speed-Vac for 5 min to dry. DNA was resuspended with 50 μ l TE buffer pH 8 with RNase (10 μ g ml⁻¹). The amount of DNA was estimated by electrophoresis in agarose gel (0.9%) by adding 2 μ l of *Sybr* Gold (Invitrogen, USA) to each sample which were visualized in an image analysis system (Vilber Lourmat, France). DNA samples were stored at 4°C.

Primers EXPCCR (5'-GCCGTAATTGCCTACCT-GCTTAAG-3') and EXPCCF (5'-GAACTTCGCAC-CGCCGACCTTCTA-3') were used in the URP (universal rice primer)-PCR protocol (Kang *et al.*, 2003). Nested-PCR was carried out with the URP-PCR product using primers INPCCR (5'-GGCCAAGCAGTGC-CTGTATATCC-3') and INPCCF (5'-TTCGATCAGC-CAACCTGCATTACT-3') (Kang *et al.*, 2003). In both PCR protocols gelatin and KCl were not used since gelatin improves reaction efficacy which was not necessary and KCl was already in the Taq polymerase buffer (Invitrogen, Brazil). PCR products were separated in 2% agarose gel in TBE 0.5% buffer at 100 V for 1 h and documentation was secured in the Vilber Lourmat image system. *P. carotovorum* subsp. *carotovorum* Pcc 867 was used as positive control and *P. betavascularum* Pb 787 as negative control (IBSBF Phytobacteria Culture Collection, Laboratório de Bacteriologia Vegetal, Instituto Biológico - CEIB, P.O. Box 70, Campinas, SP, Brazil).

Variability of isolates. Variability of the 39 isolates of *Pcc* obtained from Camocim de São Félix was assessed based on pathogenic (disease components), phenotypic (sensitivity to antibiotics), and molecular markers (REP-PCR).

Disease components. Isolates were grown in nutrient agar-yeast extract-dextrose medium (NYDA) for 36-48 h and kept at 28±2°C. A cell suspension was obtained by flooding Petri plates with SDW. Concentration of each isolate suspension was adjusted with a photocolormeter (M3, Metronic) at $A_{570} = 0.36$, which corresponds to approximately 10^9 CFU ml⁻¹. Tests were conducted in 43-day-old Chinese cabbage cv. Komachi plants grown in a greenhouse (25-30°C and RH = 71.7±18.2%). The second fully developed leaf of each plant was inoculated in the basal portion of the petiole with the deposition of a 5 µl droplet of bacterial suspension after wounding with a sterilized toothpick. After inoculation plants were kept in a moist chamber (plastic bags with the inner part moistened with distilled water) at 25-30°C for 6 h.

Disease assessments started 1 h after inoculation and were carried out at hourly intervals up to 6 h after inoculation. Afterwards, disease assessments were conducted at 6 h intervals up to 48 h after inoculation. The following variables were recorded: (i) incubation period (IP), defined as the number of hours between inoculation and symptom appearance; (ii) initial soft rot severity (ISEV) value, 6 h after inoculation, rated with an arbitrary scale ranging from 1 to 9 (Ren *et al.*, 2001), where: 1 = no lesion at the inoculation site; 2 = lesions smaller than 5 mm; 3 = lesions between 5 and 10 mm; 4 = lesions larger than 10 mm, but not reaching the leaf blade; 5 = lesions in the leaf blade and in the main stem;

6 = stem infected, but inoculated leaf is not affected; 7 = stem and uninoculated leaves are infected; 8 = whole plant almost dead; 9 = dead plant; (iii) final soft rot severity (FSEV), 48 h after inoculation, rated with the above scale; (iv) area under the disease progress curve (AUDPC), which was calculated as

$$[\Sigma (y_i + y_{i+1})/2 \cdot d_{ti}]/n,$$

where y_i and y_{i+1} are severity values recorded in two consecutive time intervals, d_{ti} is the time interval between assessments, and n is the duration of the assessment period (Fry, 1978).

The experiment was set in a completely randomized design with 5 replicates. Each pot with a plant was considered an experimental unit.

Sensitivity to antibiotics. Sensitivity of isolates to 12 antibiotics was determined by antibiogram analysis based on agar diffusion. Cell suspensions in SDW of each isolate were prepared from 36 to 48 h-old cultures developed in test tubes containing NYDA. Aliquots of 3 ml of the cell suspension were transferred to Erlenmeyers containing 100 ml of molten semi-solid NYDA medium, homogenized by vigorous shaking and the mixture was poured into Petri plates. After solidification of the culture medium, paper discs containing amoxicillin (AMO) 10 µg, cefoxitin (CFO) 30 µg, clindamycin (CLI) 2 µg, erythromycin (ERI) 15 µg, gentamicin (GEN) 10 µg, nalidixic acid (NAL) 30 µg, oxacillin (OXA) 1 µg, rifampicin (RIF) 5 µg, trimetoprim (TRI) 5 µg, teicoplanin (TEC) 30 µg, tetracycline (TET) 30 µg, and vancomycin (VAN) 30 µg were placed in four equidistant points in a Petri plate. Plates were kept in an incubator at 28±2°C for 24 h. After incubation, the diameter of the inhibition halo was measured in two perpendicular directions. The average value of the two measurements was subjected to statistical analysis. The experiment was set in a completely randomized design with 5 replicates. Each plate containing one disc of an antibiotic was considered an experimental unit.

Rep-PCR analysis. DNA of each isolate was extracted as described above. The amount of DNA was quantified by electrophoresis in a 0.8% agarose gel, stained with ethidium bromide and visualized with the Eagle Eye II documentation system (Stratagene, USA). Rep-PCR analysis was carried out with the ERIC, BOX, and REP primers (Louws *et al.*, 1994). PCR products were resolved by electrophoresis in a 1.5% agarose gel running in TBE 0.5% buffer at 80V for 2 h and visualized with the Eagle Eye II system.

Data analysis. Disease components and sensitivity to antibiotics data were subjected to multivariate analysis of variance (MANOVA) to test variation among isolates. One MANOVA analysis was conducted for the disease components data set and a separate analysis was con-

ducted for the sensitivity to antibiotics data set. After testing for variation among isolates, cluster analysis was carried out aimed at finding groups of isolates with similar properties. Isolates were grouped based on the Euclidean distance and the unweighted pair-group method with arithmetic average (UPGMA). Additionally, correlation (Pearson) analyses were conducted among disease components (IP, ISEV, FSEV, and AUDPC) and between these variables and sensitivity to antibiotics and the linkage distance calculated for the cluster analysis with the molecular marker. All statistical analyses were conducted with the STATISTICA for Windows program (StatSoft Inc., Tulsa, USA, 2000).

For the molecular marker data a haplotype of each isolate was constructed based on the banding pattern for each primer. The presence or absence of a band in a locus was assumed to represent allelic state 1 or 0, respectively. The simple match coefficient was used to estimate similarity (Kosman and Leonard, 2005). Finally, the correlation between pathogenic and molecular variation was assessed by analyzing the two distance matrices (Euclidean distance based on disease components and the genetic distance for the REP-PCR) with the Mantel test (Mantel, 1967). The null hypothesis of no correlation ($H_0: \rho=0$) was tested after constructing a simulated data set with 1000 permutations.

RESULTS AND DISCUSSION

Characterization of isolates. All bacterial colonies growing on CPG medium had the typical “broken glass” aspect (Kelman and Dickey, 1995). Isolates were Gram-negative, oxidase negative, catalase positive, had fermentative-oxidative metabolism, caused soft rot in potato tubers, and were pathogenic to detached leaves of Chinese cabbage. These isolates were classified as *Pectobacterium*. Identification at the species and subspecies level resulted in the classification of all isolates as *Pcc*. Isolates grew at 37°C, did not utilize α -methyl glucoside, did not produce acid from sorbitol, produced acid from lactose, were resistant to erythromycin, grew in 5% NaCl, and did not produce phosphatase and lecithinase. Sucrose reduction was observed for 10.2% of the isolates and 12.8% did not produce acid from melibiose. The results regarding sucrose and melibiose metabolism are not uncommon, possibly due to higher genotypic and phenotypic diversity of *Pcc* in relation to other subspecies of the *P. carotovorum* complex (Avrova *et al.*, 2002). Growth capacity at 36°C and sucrose reduction are not suitable to differentiate *P. carotovorum* from *P. atrosepticum* (Seo *et al.*, 2002). Thus, other methods should be employed to identify the subspecies (Yap *et al.*, 2004). The casein hydrolysis test (Dye, 1969) and the colony color on NGM medium (cream color) confirmed the subspecies as *Pcc* (Lee and Yu, 2005).

Furthermore, identity of isolates was confirmed by typical banding pattern with PCR tests, i.e. one 555 bp band from URP-PCR and a 380 bp band from nested-PCR that are characteristic of *Pcc* (Kang *et al.*, 2003).

Disease components. Based on several tests (Wilks' Lambda, Pillai's Trace, Hotelling-Lawley's Trace and Roy's Greatest Root), there was a highly significant effect of isolates ($P < 0.0001$). Therefore, isolates differed among each other regarding the assessed disease variables. According to the 50% total linkage distance criterion, six groups of isolates were formed based on ISEV, FSEV, and AUDPC (Fig. 1). The large number of cluster supports the high variability among isolates detected with the MANOVA analysis.

No report of utilization of disease components to assess variability of *Pcc* was found. However, from the epidemiological standpoint, pathogenic variability has direct implications on disease management (McDonald and Linde, 2002). In most studies dealing with pathogenicity, assessments are completed in 2 to 3 days after inoculation (Seo *et al.*, 2001, 2002) and final severity is the variable commonly recorded. Most investigations are not concerned with symptom appearance (IP) and their progress (AUDPC). In one study, the IP of soft rot of tomato fruits was an informative variable and differences in IP among isolates ranged from less than 24 h to 3 weeks (Bartz, 1981). In the present study, the short values of IPs (from 4.0 to 5.4 h) indicate the rapid colonization of host tissues by bacteria. There was rapid progress from the base of the petiole to the stem, which led to plant collapse. Variation in isolate virulence was evident when soft rot severity was assessed: ISEV varied from 0.6 to 2.0 and FSEV from 3.0 to 6.2. As a consequence, there was also variation for the AUDPC values (2.01 to 4.16). AUDPC can reflect isolate characteristics that affect disease development (Fry, 1978). Thus, pathogenic variation among isolates was most likely revealed when this parameter was calculated. However, because no effective chemical control is available or employed by growers in Pernambuco state, preventive control measures, mainly those related to seed quality, crop rotation, soil tillage, planting density, and irrigation management should be carefully planned in fields where highly virulent isolates are prevalent.

The intensity of soft rot was inversely related to the time of symptoms appearance. Significant correlation were detected between IP and ISEV ($r = -0.57$) (Table 1). Given that soft rot is a monocyclic disease (Kikumoto, 1980), lack of correlation between IP and FSEV or AUDPC is not unusual because disease progress is not influenced by IP (Hau and Vallavielle-Pope, 1998). The lack of correlation among ISEV, FSEV, and AUDPC suggest that these variables are independent of each other. Thus, variation in soft rot severity are more likely to be determined by the pathogen isolate, amount of inocu-

Table 1. Correlation matrix between incubation period (IP), initial severity (ISEV), final severity (FSEV), and area under the disease progress curve (AUDPC), sensitivity to antibiotics and linkage distance (LD) for molecular markers, estimated for 39 isolates of *Pectobacterium carotovorum* subsp. *carotovorum* from different Chinese cabbage growing areas of Camocim de São Félix, Pernambuco state, Brazil.

Variable	Pearson's correlation coefficient			
	IP	ISEV	FSEV	AUDPC
SEVI	-0.57*	-	-	-
SEVF	-0.24	0.30	-	-
AACPD	-0.15	-0.01	0.34	-
AMO ¹	0.01	-0.04	-0.08	-0.36
CFO	0.18	-0.05	0.03	-0.12
CLI	0.07	-0.14	-0.45*	-0.18
ERI	0.03	0.19	0.13	-0.02
GEN	-0.41*	0.25	0.18	0.10
NAL	-0.16	0.18	0.18	0.01
OXA	0.05	-0.14	-0.32	-0.17
RIF	0.06	0.12	0.06	-0.15
TRI	-0.10	0.16	0.11	-0.21
TEC	-0.09	0.03	-0.10	-0.27
TET	0.09	-0.04	0.04	-0.29
VAN	0.13	-0.15	-0.22	0.20
LD at: 0.05	0.13	-0.04	0.21	-0.12

* Significant at $\alpha = 0.05$.

¹ Amoxicillin (AMO), cefoxitin (CFO), clindamycin (CLI), erithromycin (ERI), gentamicin, (GEN), nalidixic acid (NAL), oxacillin (OXA), rifampicin (RIF), trimetoprim (TRI), teicoplanin (TEC), tetracycline (TET), and vancomycin (VAN).

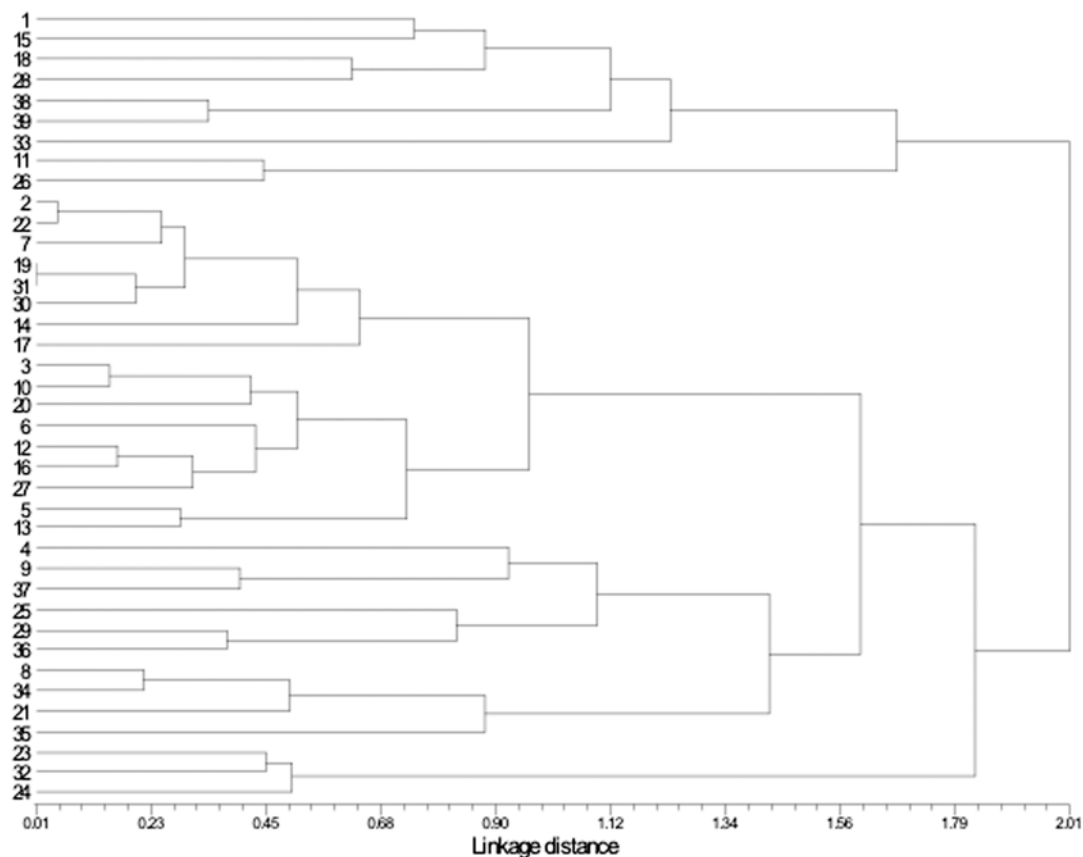


Fig. 1. Cluster analysis, based on UPGMA, of 39 isolates of *Pectobacterium carotovorum* subsp. *carotovorum* from different Chinese cabbage growing areas in Camocim de São Félix, based on the disease components: incubation period (IP), initial severity (ISEV), final severity (FSEV), and area under the disease progress curve (AUDPC). Numbers at the tip of the branches correspond to the isolate code.

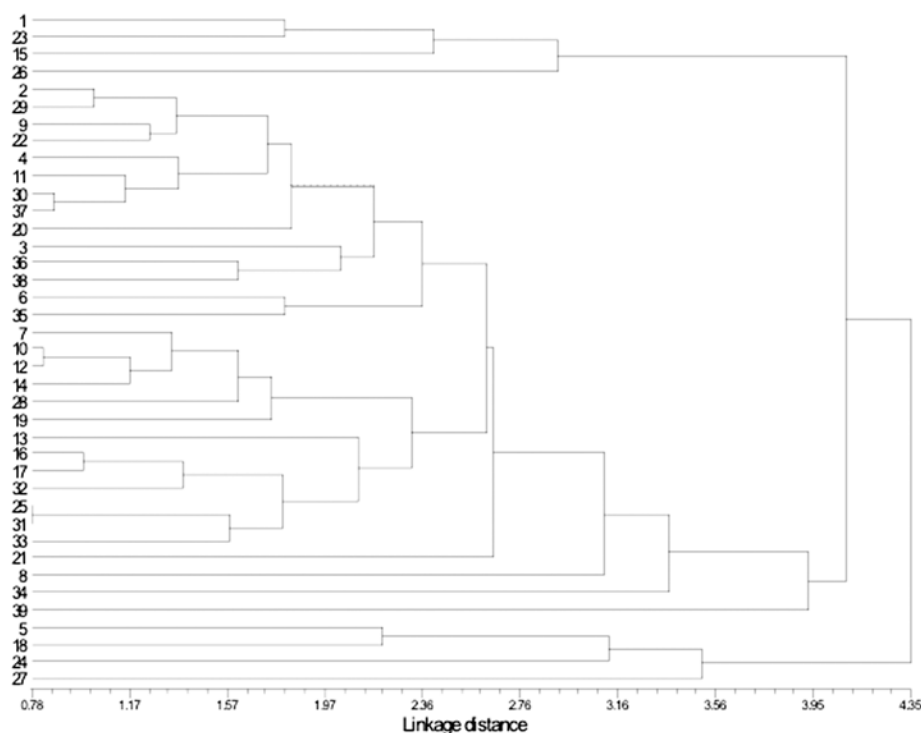


Fig. 2. Cluster analysis, based on UPGMA, of 39 isolates of *Pectobacterium carotovorum* subsp. *carotovorum* from different Chinese cabbage growing areas in Camocim de São Félix, based on the sensitivity to the antibiotics amoxicillin, cefoxitin, clindamycin, erythromycin, gentamicin, nalidixic acid, oxacillin, rifampicin, trimetoprim, teicoplanin, tetracycline, and vancomycin. Numbers at the tip of the branches correspond to the isolate code.

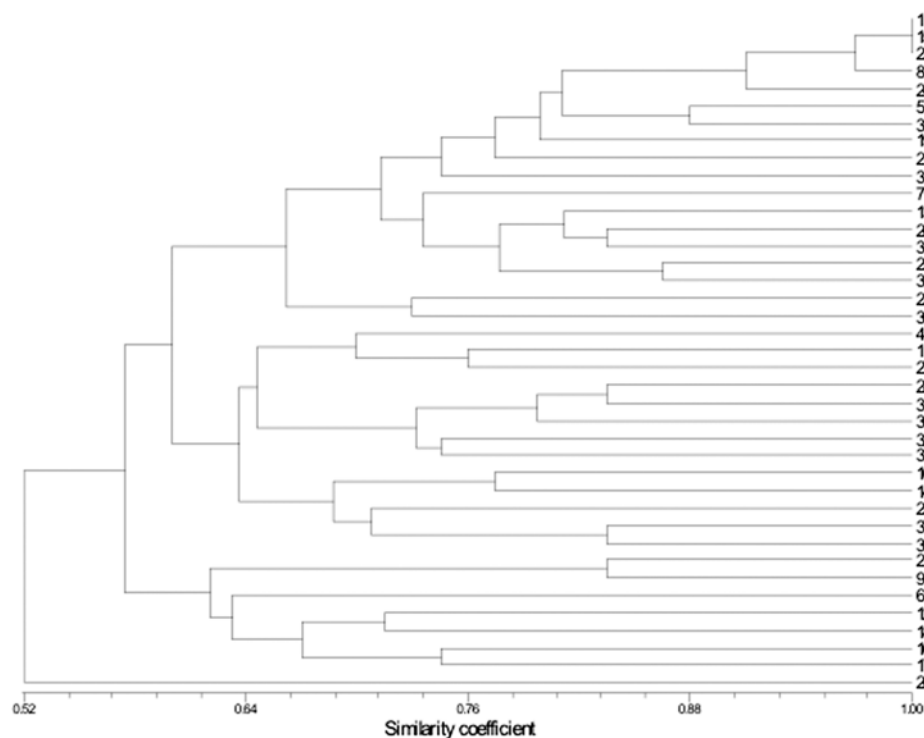


Fig. 3. Cluster analysis, based on UPGMA, of 39 isolates of *Pectobacterium carotovorum* subsp. *carotovorum* from different Chinese cabbage growing areas in Camocim de São Félix, based on the Simple Match similarity coefficient for the banding pattern generated by the REP, ERIC, and BOX primers used for Rep-PCR. Numbers at the tip of the branches correspond to the isolate code.

lum, the degree of wounding during inoculation, the genotype and phenological state of the host, and also the prevailing environmental conditions (Kikumoto, 1980).

Sensitivity to antibiotics. Isolates also varied according to the sensitivity to different antibiotics ($P < 0.0001$) and could be grouped into 14 clusters according to the 50% total linkage distance criterion (Fig. 2). No isolate was sensitive or insensitive to all antibiotics analyzed. The majority of antibiotics inhibited to some degree the growth of several isolates. Resistance of bacteria to antibiotics is regulated by genes located either in the chromosome or in the plasmid (Madigan *et al.*, 1997). Cases of constitutive multiple resistance of whole populations of plant pathogenic bacteria to one or several antibiotics have been documented (Romeiro *et al.*, 1998). However, this was not evaluated in the present study. Significant correlations ($P \leq 0.01$) were found between sensitivity to gentamicin and IP ($r = -0.41$), as between sensitivity to clindamycin and FSEV ($r = -0.45$) (Table 1), however this correlation is not commonly detected (Costa *et al.*, 2001).

Molecular analysis. REP, ERIC, and BOX PCR markers were polymorphic and revealed high variation among isolates of *Pcc*. A total of 32 groups of similarity were formed according to the 50% linkage distance criterion (Fig. 3). These markers are based on repetitive and relatively conserved sequences of the bacterial genome (Louws *et al.*, 1999) and are commonly used to assess variation of Gram-negative species (Versalovic *et al.*, 1991). Therefore, there is strong evidence of high genetic variability among *Pcc* isolates collected from Chinese cabbage in Pernambuco state. High genetic variability in populations of *Pcc* was also reported in other studies that used similar (Seo *et al.*, 2000, 2001, 2002) or distinct molecular markers such as AFLP (Avrova *et al.*, 2002), and PCR-RFLP (Darrasse *et al.*, 1994; Helias *et al.*, 1998). High genetic variability even among isolates collected from a single host such as potato (Mäki-Valkama and Karjalainen, 1994) or from a region within a given crop season (Yap *et al.*, 2004) has also been reported. Most likely, several genetic and environmental factors may have acted upon the population of *Pcc* associated with Chinese cabbage in north-east Brazil.

High genetic variability within populations of *Pcc* can be the result of mutation, differential selection exerted by the host plants and population subdivision (Avrova *et al.*, 2002; Seo *et al.*, 2002). High mutation rates are reported in genes that affect pathogenesis, mainly those encoding cell-wall degrading enzymes (Andresen *et al.*, 2007; Fan *et al.*, 2007). DNA sequence analysis should be conducted to properly address this hypothesis regarding the Brazilian isolates of *Pcc*. Another potential evolutionary mechanism that can contribute to diversity is the wide range of horticultural crops that are grown in most areas where Chinese cabbage is produced. More than one

species of Brassicaceae are commonly planted in the same farm, as well as plants from other families that are host of *Pcc*. Host diversity can contribute to variation in the bacterial population. From a population biology perspective, the genetic variability determines the evolutionary potential of the population (McDonald and Linde, 2002).

High genetic variability was also reported for other soil-inhabiting plant pathogenic bacteria, such as *Burkholderia cepacia* associated with corn rhizosphere (Dalmastri *et al.*, 1999) and *Ralstonia solanacearum* race 1, biovar III isolated from infected potato, tomato and *Solanum chacoense* in a single field (Grover *et al.*, 2006).

The disease components were not correlated to either the molecular makers or to sensitivity to antibiotics. Probably, the markers used in the present study were not closely associated with the distinct regions of the genome related to pathogenicity. Like the results presented here, no correlation between markers was detected using ERIC-PCR (Seo *et al.*, 2001) and RFLP for rDNA 16S, ISRs and the *pel* gene (Seo *et al.*, 2002). Additionally, no correlation between sensitivity to antibiotics and pathogenicity can be drawn. Disease components can be considered a polygenic trait and direct correlation with antibiotic resistance, not uncommonly reported to be a single-gene trait, would not be easily observed unless a strong clonal population structure of the pathogen is present in the field. However, based on the molecular marker data, this was not our case.

The high variability of *Pcc* from several Chinese cabbage growing areas in Pernambuco state has important implications for disease management since this might be a reason for the wide host range of this bacterium and for quick breakdown of disease resistance in host plants.

Due to the difficulty of designing specific diagnostic protocols for this pathogen careful studies aiming at detection and correct identification need to be developed. The epidemiology and control of soft rot should be analyzed on a case-specific basis, since subpopulations of a habitat can differ genetically from others in close proximity. The evolutionary mechanisms driving this variability remain largely unknown and need to be investigated in properly designed field and controlled condition experiments.

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SHORT COMMUNICATION

SAMPLE SIZE FOR QUANTIFICATION OF CERCOSPORA LEAF SPOT IN SWEET PEPPER

S.J. Michereff¹, R.B. Martins², M.A. Noronha³ and L.P. Machado¹

¹ Universidade Federal Rural de Pernambuco, Departamento de Agronomia, 52171-900 Recife, Pernambuco, Brazil

² Universidade Federal de Alagoas, Campus de Arapiraca, 57309-005 Arapiraca, Alagoas, Brazil

³ Embrapa Meio Norte, Laboratório de Fitopatologia, 64006-220 Teresina, Piauí, Brazil

SUMMARY

Cercospora leaf spot of sweet pepper (*Capsicum annuum*) caused by *Cercospora capsici* is an important disease occurring in the tropics. Due to the lack of standard methods to quantify this disease in field conditions, the objective of this study was to determine the ideal sample size for Cercospora leaf spot quantification in the field. Disease severity was determined in eight commercial sweet pepper (cv. All Big) fields located in the Agreste region of Pernambuco state (northeast Brazil). In each field, two diagonals (X) were established in a 0.5 ha area. Twenty-five plants were evaluated along each diagonal with the aid of an empiric scale to consider different sampling rates (3, 6, 9, 12 leaves/plant). The spatial pattern of the disease was estimated using Lloyd's Index of Patchiness (LIP) and ideal sample sizes were calculated based on degrees of acceptable error (5, 10 and 20%). The spatial pattern was predominantly random and there was no correlation ($P = 0.44$) between the LIP values and sample size. Considering a sample of 3 leaves/plant with 10 and 20% rate of acceptable error, the mean ideal sample size was 103 and 26 plants, respectively, for each 0.5 ha of cultivated field.

Key words: *Cercospora capsici*, *Capsicum annuum*, sampling, epidemiology.

Sweet pepper (*Capsicum annuum* L.), one of the most valuable vegetables grown in Brazil, is grown throughout the country. Cercospora leaf spot, an important disease of sweet pepper, is caused by the fungus *Cercospora capsici* Heald et Wolf, that affects the entire plant canopy, especially the leaves. The main symptoms of Cercospora leaf spot are circular, brown, and necrotic lesions exceeding 1 cm in diameter, with a light gray central portion. Similar lesions, but of smaller size, are found on the stalk, branches and leaf stems (Lopes and

Ávila, 2003). The occurrence and development of leaf spot is favored at temperatures above 25°C and at a relative humidity greater than 90%, which are common growing conditions in warm regions or during summer. Leaf spot causes a considerable reduction in the photosynthetic area due to the loss of tissue caused either by the increase in the size and/or number of lesions or by leaf shedding during the development of the disease. Under these conditions, pepper fruits are small, twisted, sun-burned and unfit for marketing (Monteiro *et al.*, 2000).

Surveys are fundamental for drafting, planning, implementing and evaluating plant disease management strategies (Campbell and Madden, 1990; Holderness, 2002). Through such surveys, it is possible to determine the relative importance of diseases, monitor fluctuations in intensity throughout the growing season and determine the efficiency and acceptance of recommended control measures (Holderness, 2002). However, surveys are only reliable when the methods employed in the quantification of disease severity and sampling are standardized. In other words, methods should be previously established for assessing severity, number of samples and collection method (Campbell and Madden, 1990; Holderness, 2002). For the quantification of Cercospora leaf spot in the sweet pepper, a diagrammatic scale was designed that offers a good degree of accuracy in estimating disease severity (Michereff *et al.*, 2006). However, there is not an established method for field sampling.

Sample size should optimize the balance between the number of samples and reliability of the data. Although faster and easier to perform, small sample sizes can provide unreliable data, whereas a very high number of samples may contribute little to reliability when compared with the confidence level and precision of an intermediate number of samples (Campbell and Madden, 1990).

There are at least three methods to estimate sample size, which depend on the operational definition of the reliability and on the costs of sample collection: (i) reliability is defined by the coefficient of variation of the mean or standard error; (ii) equations of probability define reliability; (iii) components of variance and cost functions are used to optimize sample number, consid-

Table 1. Number of sweet pepper plants to be sampled in 0.5 ha surface for quantification of severity of *Cercospora* leaf spot (*Cercospora capsici*) infections based on four numbers of leaves sampled per plant and reliability defined by the degree of acceptable error.

Field	Localization (City) ¹	Number of leaves/plant	Severity (%) ²	LIP ³	Sample size ⁴ Error/Number of plants		
					5%	10%	20%
A	BEZ	3	2.5 ± 7.3	1.8	467	117	29
		6	2.4 ± 4.4	1.3	306	77	19
		9	2.2 ± 2.3	1.0	186	46	12
		12	2.5 ± 2.0	0.9	125	31	8
B	CGR	3	3.1 ± 13.0	2.0*	539	135	34
		6	2.9 ± 6.8	1.5	322	80	20
		9	2.2 ± 3.6	1.3	298	75	19
		12	2.7 ± 4.8	1.3	266	66	17
C	BEZ	3	5.4 ± 38.4	2.1*	527	132	33
		6	5.9 ± 32.5	1.8	373	93	23
		9	5.6 ± 16.0	1.3	204	51	13
		12	6.1 ± 18.5	1.3	199	50	12
D	CSF	3	7.3 ± 57.8	1.9*	434	108	27
		6	8.2 ± 70.6	1.9*	420	105	26
		9	7.1 ± 41.0	1.7	325	81	20
		12	7.9 ± 34.8	1.4	223	56	14
E	CGR	3	10.1 ± 81.0	1.7	318	79	20
		6	10.5 ± 84.6	1.7	307	77	19
		9	8.9 ± 49.0	1.5	247	62	15
		12	9.5 ± 53.3	1.5	236	59	15
F	BEZ	3	9.5 ± 96.0	2.0*	426	106	27
		6	10.2 ± 90.3	1.7	347	87	22
		9	9.7 ± 65.6	1.6	279	70	17
		12	10.3 ± 51.8	1.4	195	49	12
G	CSF	3	11.1 ± 94.1	1.7	305	76	19
		6	11.8 ± 90.3	1.6	259	65	16
		9	12.4 ± 77.4	1.4	201	50	13
		12	12.6 ± 65.6	1.3	165	41	10
H	CSF	3	13.5 ± 130.0	1.6	285	71	18
		6	13.9 ± 125.4	1.6	260	65	16
		9	14.7 ± 96.0	1.4	178	44	11
		12	13.1 ± 59.3	1.3	138	35	9
Mean		3	7.8 ± 64.7	-	413 a ⁵	103 a	26 a
		6	8.2 ± 63.1	-	324 ab	81 ab	20 ab
		9	7.9 ± 43.9	-	240 bc	60 bc	15 bc
		12	8.1 ± 36.3	-	193 c	48 c	12 c

¹BEZ = Bezerros, CGR = Chã Grande, CSF = Camocim de São Félix.²Estimated with empirical scale (Michereff *et al.*, 2006). Mean ± variance of 50 plants evaluated per field.³Lloyd's Index of Patchiness. Values marked with an asterisk are significantly greater than 1.0 ($P=0.05$) and indicate a patchy spatial pattern (Campbell and Madden, 1990).⁴Calculated using a random pattern of diseased plants based on mean severity, variance and level of acceptable error (Campbell and Madden, 1990).⁵Means followed by the same letter in a column do not differ from each other, according to Kruskal-Wallis test ($P=0.05$).

ering that each sample type has an associated cost (Campbell and Madden, 1990). Considering that the reliability of disease estimate is directly related to sample size and spatial heterogeneity of disease, all above methods can be associated to distributions, which represent different models of spatial distribution of the disease in the field (Perry, 1994).

Despite the importance of *Cercospora* leaf spot in sweet pepper, no studies have been made to establish the number of plants to be sampled for disease severity quantification. Thus, the aim of the present study was to determine the ideal sample size for the quantification of leaf spot in different growing areas with varying levels of disease severity.

Pilot samplings were carried out to assess the severity of leaf spot in eight commercial fields of cv. All Big located in the Agreste region of the state of Pernambuco (Brazil) (Table 1). The minimal distance between fields was 3.5 km. The trench system was employed in all fields, with flood irrigation and a spacing of 1.0x0.80 m between rows and plants, respectively. All plants were vegetating when evaluated.

On each field, a 0.5 ha area was chosen, consisting of 50 rows and 125 plants/row, for a total of 6,250 plants. Two diagonals (X) were established in each area and 25 plants were evaluated along each diagonal, with the first plant located approximately 5 m from the beginning of the diagonal and the remaining plants spaced at distances of approximately 5 m. With the aid of the empiric scale, which includes levels from 0 to 50% of damaged leaf area (Michereff *et al.*, 2006), the leaf damage by *Cercospora* leaf spot was estimated using 50 selected plants, with further selection of 3, 6, 9 and 12 leaves/plant. In the three-leaf sample, one leaf from each third of the plant (lower, middle and upper) was evaluated. In the 6-, 9- and 12-leaf samples, two, three and four leaves were evaluated in each third of the plant, respectively.

The *Cercospora* leaf spot data obtained from the pilot samplings were used in the determination of the ideal sample sizes based on the spatial pattern of the diseased plants. The spatial pattern was evaluated for each field area using Lloyd's Index of Patchiness (LIP) through the equation:

$$LIP = [(\bar{x} + (S^2/\bar{x}) - 1)]/\bar{x}$$

in which \bar{x} is the mean severity of the disease from 50 plants, using 3, 6, 9 and 12 leaves/plant, and S^2 is the sample variance. Values of LIP lower than, equal to or greater than 1.0 indicate regular, random or patchy spatial patterns, respectively (Campbell and Madden, 1990). The significance ($P = 0.05$) of the values was tested using the chi-square test (X^2) with (n-1) degrees of freedom.

Ideal sample sizes (n) were estimated for each crop area based on the coefficient of variation of the mean ($CV_{\bar{x}}$) and random pattern of diseased plants, consider-

ing sample of 3, 6, 9 and 12 leaves/plant by the equation:

$$n = S^2/(x^2 \cdot CV_{\bar{x}}^2)$$

with pre-established acceptable errors of 5, 10 and 20% ($CV_{\bar{x}} = 0.05, 0.1$ and 0.2) (Campbell and Madden, 1990). Using the data obtained from each field, the mean ideal sample size was calculated when 3, 6, 9 and 12 leaves/plant were assessed. Mean sample sizes within each category of acceptable error were compared with the Kruskal-Wallis test ($P = 0.05$). Pearson's correlation analysis ($P = 0.05$) was used to determine a possible influence from the degree of disease severity on the intensity of the patchiness (LIP) among the diseased plants in the different sample sizes.

The severity of *Cercospora* leaf spot on sweet pepper from the different fields ranged from 2.2 to 13.9% (Table 1). Because the ideal sample size can vary depending on the spatial pattern of the disease in the field (Kranz, 1988; Perry, 1994), the *Cercospora* leaf spot pattern was estimated using LIP. The pattern did not differ from 1 ($P > 0.05$) in 84% of the situations evaluated (Table 1), indicating that the *Cercospora* leaf spot pattern in the field is predominantly random. This type of pattern was expected for this disease, as wind is the principal dispersion agent (Monteiro *et al.*, 2000). Therefore, diseased plants that are relatively distant from a set of healthy plants can serve as a source of inoculum (Burdon, 1987). The different samplings of 3, 6, 9 and 12 leaves/plant did not affect the LIP estimates, a result that was confirmed by the lack of a significant correlation ($r = 0.14$; $P = 0.44$) between the degree of disease severity and intensity of patchiness within samplings with different numbers of leaves. This conclusion supports the hypothesis that the origin of the inoculum is an exogenous source.

Sample size for quantification of *Cercospora* leaf spot on sweet pepper was estimated based on a random pattern of diseased plants. No significant correlation ($r = -0.42$; $P = 0.28$) was found between the degree of disease severity and the number of plants sampled.

In terms of mean among fields, there was no significant difference in sample size for the quantification of leaf spot when 3 or 6 leaves per plant were assessed; however, with 12 leaves there was a significantly different sample size (Table 1). The number of plants to be sampled (regardless of the number of leaves evaluated) was reduced when the degree of acceptable error was increased (Table 1). The choice of the degree of acceptable error depends on the purpose of the sampling (Kranz, 1988). For regional phytopathological surveys, which are often faced with financial restrictions, a 20% error can be used (sampling with 80% accuracy). Considering this degree of error and the mean value obtained, sampling 26 plants with 3 leaves/plant is recommended for each 0.5 ha of cultivated field (Table 1) in surveys for assessment of *Cercospora* leaf spot severity

in sweet pepper. If there are no limitations to the survey execution, a 10% error is considered ideal for field surveys (Southwood, 1978), in which case, the sampling would require 103 plants with 3 leaves/plant for each 0.5 ha of cultivated field (Table 1). Either way, 3-leaf samples were chosen with a greater number of plants, to increase the precision of sampling.

When using different methods for estimating sample size, the data from the locations analyzed should be representative of what could occur in other fields, an assumption whose validity varies between pathosystems (Campbell and Madden, 1990). Thus, sample size for quantification of *Cercospora* leaf spot severity on sweet pepper derived from this study are applicable across areas where this crop is grown, because the data came from fields with different conditions and were estimated based on increasing need for precision.

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SHORT COMMUNICATION

HOST RANGE AND PHYLOGENETIC ANALYSIS OF IRANIAN ISOLATES OF ZUCCHINI YELLOW MOSAIC VIRUS

H. Massumi¹, M. Shaabani¹, J. Heydarnejad¹, A. Hosseini Pour¹ and H. Rahimian²¹ Department of Plant Protection, College of Agriculture, Shahid Babonar University, Kerman, Iran² Department of Plant Protection, College of Agronomy Sciences, University of Agricultural Sciences and Natural Resources, Sari, Iran

SUMMARY

The host range and molecular variation of several Iranian isolates of *Zucchini yellow mosaic virus* (ZYMV) were investigated. Of 1,119 symptomatic cucurbit samples and one symptomatic weed species (*Colocynthis*; *Citrullus colocynthis*) collected from 13 Iranian provinces, 430 samples were ZYMV-infected as indicated by DAS-ELISA. Twenty one viral isolates were chosen for biological and molecular characterizations based on their host range and geographical distribution. Based on biological properties, the isolates clustered in three groups. Isolates of group I and II, induced similar symptoms on watermelon, *Chenopodium quinoa* and *C. amaranticolor*, whereas isolates of group III produced different symptoms. The complete coat protein (CP) gene, the N-terminal coding region of the CP gene and the C-terminal part of the polymerase gene (NIb) of the 21 isolates were amplified by PCR, cloned and sequenced. Phylogenetic analysis using the 836 bp fragment comprising the complete CP gene and the 250 bp of NIb-CP fragment showed that ZYMV isolates clustered in three groups. Iranian isolates belonged to group I which was divided into two subgroups, I_A and I_B.

Key words: ZYMV, sequencing, Cucurbitaceae, host range, DAS-ELISA, PCR.

Zucchini yellow mosaic virus (ZYMV), genus *Potyvirus*, family *Potyviridae*, first reported from Italy in 1973 (Lisa *et al.*, 1981), was subsequently found to cause devastating epidemics in commercial cucurbits worldwide (Lisa and Lecoq, 1984). Sequence data for the 3' terminus of the ZYMV genome, including the region coding for the coat protein (CP), were obtained for many isolates (Gal-On *et al.*, 1990; Lin *et al.*, 2000; Desbiez *et al.*, 2002). Amino acid sequence identity in the CP of distinct ZYMV isolates is over 90%, except for the Singapore and Reunion isolates, which are particularly variable in the N-terminus of the CP-coding region

(Zhao *et al.*, 2003). Isolates of ZYMV may differ also in serological properties, symptomatology, host range, capacity to overcome resistance genes (Lecoq and Pitrat, 1984; Paris *et al.*, 1988) and aphid transmissibility (Lecoq *et al.*, 1991).

In Iran, viral diseases caused by ZYMV, *Watermelon mosaic virus* (WMV) and *Cucumber mosaic virus* (CMV) are among the major limiting factors for cucurbit production (Rahimian and Izadpanah, 1978; Massumi *et al.*, 2007; Sharifi *et al.*, 2008). Cucumber plants infected by ZYMV show an array of symptoms such as severe stunting, mosaic, and malformation of leaves and fruits, and produce few unmarketable fruits. A 489-nucleotide sequence of the C-terminal part of the NIb and N-terminal part of CP of 12 Iranian isolates infecting squash and melon has recently been determined and compared with that of isolates from other countries (Bananej *et al.*, 2008). We now report the partial biological properties of 21 ZYMV isolates collected from 13 Iranian provinces, the complete nucleotide sequence of their CP and NIb-CP coding regions, and their phylogenetic relationships.

Wintersquash (*Cucurbita maxima* Duch. E Lam.), cucumber (*Cucumis sativus* L.), pumpkin (*Cucurbita muschata* Duch.), watermelon (*Citrullus vulgaris* Schard.), melon (*Cucurbita melo* L.) and the weed species colocynthis [*Citrullus colocynthis* (L.) Schrad.] were surveyed for the presence of ZYMV from 2004 to 2007 in 19 Iranian regions, covering cucurbit-producing areas with different climatic conditions (Sharifi *et al.*, 2008) where cucurbits are grown under field and/or greenhouse conditions (Table 1). Samples from total of 1,119 cucurbit plants and the symptomatic colocynthis were collected, consisting of the two youngest fully expanded symptomatic leaves from each plant. All samples were tested by DAS-ELISA (Clark and Adams, 1977) using specific polyclonal antibodies (Bioreba, Switzerland) against ZYMV, CMV, WMV, *Squash mosaic virus* (SqMV) and *Papaya ringspot virus-W* (PRSV-W).

Twenty one ZYMV isolates were selected based on distinct geographic origin and host plant and maintained on *C. pepo* cv. Maragheh in a temperature-regulated insect-proof greenhouse for subsequent studies. These isolates were numbered according to the abbrevi-

ated names of the province, region and the host plant (Table 1) and were partially characterized biologically based on the reaction to inoculation of a range of test plants. Leaf tissues were ground in 1% (w/v) solution of K_2HPO_4 pH 7.5 containing 0.01% Na_2SO_3 , 2% polyvinylpyrrolidone (PVP) and 0.05% ethylenediaminetetraacetic acid (EDTA) and the extracts were mechanically inoculated on 24 different hosts from 19 species belonging to five families (Table 2). A minimum of five plants of every experimental host were inoculated for each virus isolate in two independent experiments. ELISA and symptomology were used to check the infection and the reaction of inoculated plants to virus isolates.

Of the 1,119 samples collected from winter squash, cucumber, pumpkin, watermelon and melon and tested by ELISA, 403, 240, 65 and 4 were infected by ZYMV, WMV, CMV and SQMV, respectively, whereas none was infected by PRSV-W. ZYMV was also detected in *C. colocynthis*. Double infection (WMV+ZYMV, CMV+ZYMV or WMV+ CMV) and triple infection (WMV+ CMV+ ZYMV) were found in 92 and 3 samples, respectively.

Table 2 shows that the 21 ZYMV representative isolates selected for further studies could be classified into three groups based on the type and severity of symptoms induced on test plants. Isolates in group I infected systemically the seven varieties of squash and melon causing mottling, vein clearing and vein banding and in some varieties mild to severe narrowing of leaf lamina, which became filiform. Group II isolates produced leaf mottling occasionally accompanied by blistering on melon and squash varieties. Isolates of both groups induced mosaic and yellowing on inoculated watermelon plants whereas *Chenopodium quinoa* and *C. amaranticolor* reacted with chlorotic local lesions. Group III isolates caused chlorotic local lesions and systemic symptoms (vein clearing) like ZYMV isolates from northern Italy (Lisa *et al.*, 1981). Finally, groups II and III isolates induced necrotic lesions on inoculated leaves of *Datura stramonium*. Except for these, none of the other isolates infected any member of family Solanaceae, Fabiaceae and the single species of the family Ranunculaceae tested.

Total RNA was isolated from *C. pepo* L. cv. Maragheh experimentally infected with each of the ZYMV isolates using High Pure Viral Nucleic Acid Kit (Roche Biochemical, Germany). RT-PCR was done as described (Sharifi *et al.*, 2008). Following alignment of published nucleotide (nt) sequences of ZYMV strains, two conserved regions were identified for amplification of the CP gene. The sense primer, ZYMV-F (5' ATC AGG CAC TCA GCC AAC TGC 3') is located in a region from nt 8541 to nt 8562 of the complete gene of TW-TN3 strain (accession No. AF343979); the antisense primer ZYMV-R (5' ACA CTA AAG CTT CCG ACA GGA C 3') corresponds to a region spanning from

nt 9524 to nt 9546. The amplified fragment was expected to be 1005 bp. Amplification of the CP gene was done using the first-strand cDNAs procedure (Sharifi *et al.*, 2008) with the antisense primer ZYMV-R. PCR, carried out in a Techne TC 312 thermal cycler (Techne, UK), consisted of an initial denaturation step of 5 min at 95°C, followed by 40 cycles of 30 sec at 94°C for denaturation, 30 sec at 56°C for primer annealing and 45 sec at 72°C for elongation. The reaction was terminated by a final elongation step of 5 min at 72°C. Each PCR product (3 µl aliquots) was electrophoresed on 1% agarose gel, stained with ethidium bromide, and visualized with an UV transillumination.

The primer pairs R-DAG/F-DAG (Desbiez *et al.*, 2002) and ZYMV-F/ZYMV-R were designed for amplification of NIB-CP and whole CP genes, respectively. InsT/Aclone™ PCR Product Cloning Kit (Fermentas, Lithuania) was used for cloning PCR products according to manufacturer's instruction. Purified products were ligated into the pTZ57R/T vector and transferred into competent *Escherichia coli* DH5α cells. Recombinant clones were screened, plasmids DNAs were extracted using High Pure Plasmid Extraction Kit (Roche, Germany) and custom sequenced (Macrogen, South Korea). At least three clones of each isolate were sequenced. Sequences were aligned and compared with the NIB-CP coding region and the whole CP gene sequences of other ZYMV isolates available in databases using the BLAST (NCBI) program (Altschul *et al.*, 1990).

Phylogenetic analysis of the 21 Iranian ZYMV isolates was conducted by comparing separately the 836 nucleotides (nt) of the whole CP gene and the 250 nts that included the 132 nts of the variable N-terminal part of the CP-coding region with the comparable sequences of other ZYMV isolates from GenBank. This comparison extended to deduced amino acid sequences. Multiple sequence alignment was carried out using the DNAMAN software package (Lynnon, Biosoft, Quebec, Canada). Phylogenetic trees were constructed with the DNAMAN software using similarity matrix and the neighbor-joining method and maximum parsimony (MEGA version 2.1) (Kumar *et al.*, 2001). Tree branches were bootstrapped with 1000 replications. The validity of trees was also evaluated by estimation of the cophenetic coefficient based on the mantel test (Mohammadi and Prasanna, 2003).

As mentioned, PCR amplification of CP and NIB-CP segments of the 21 viral isolates yielded fragments of 459 bp and 1005 bp, respectively. The two PCR products contained an overlapping of 208 bp to give a contiguous fragment of 1,257 bp encompassing NIB, CP and the 3'UTR. The nucleotide sequence identity of the NIB-CP gene of the Iranian ZYMV isolates ranged from 92.8 to 99.6%, whereas the whole CP gene had sequence identities ranging from 93.7 to 99.8%. The pres-

ent ZYMV isolates showed 94.8 to 99.1% identity in the nucleotide sequences of the NIb-CP gene with the two isolates Iran-8 and Iran-12 of ZYMV, previously reported from Iran (Bananej *et al.*, 2008).

The maximum and minimum nucleotide sequence identities of the NIb-CP gene between the Iranian isolates and those deposited in GenBank were between isolates Ham.Asd.C and FI96-1 (99.6%) and isolates Ker.Ker.S and HA117 (75.4%), respectively. When the sequences of the whole CP gene were compared, Azr.Mak.W and Hor.Haj.W isolates showed 99.6 and 81.8% sequence identity with isolates Berlin1 and SJP, respectively. At the deduced amino acid sequence level of the NIb-CP, the Iranian isolates Sis.Zab.W, Ham.Mal.W, Ham.Asa.C, Far.Mar.M, Hor.Haj.W,

Azr.Tab.S, Gil.Ras.C, Hor.Min.S, Azr.Sha.C and Teh.Kar.S were 100% identical. The Iranian isolates Sis.Zab.W, Far.Mar.M, Azr.Tab.S and Ker.Jir.W showed 100% identity at the amino acid level of the whole CP gene (data not shown).

Comparison of the ZYMV CP and NIb-CP sequences of the Iranian isolates with those from GenBank showed that the former are members of Zhao *et al.* (2003) group I, which could be divided into two subgroups (I_A and I_B) (Fig. 1). Both trees had essentially the same topology with the Iranian isolates placed in subgroups (I_A and I_B). The only exceptions were isolates Azr.Mah.W and Ker.Ker.S that belonged either to subgroup I_A when NIb-CP sequences were compared, or to subgroup I_B when the whole CP sequences were compared. Nonetheless, iso-

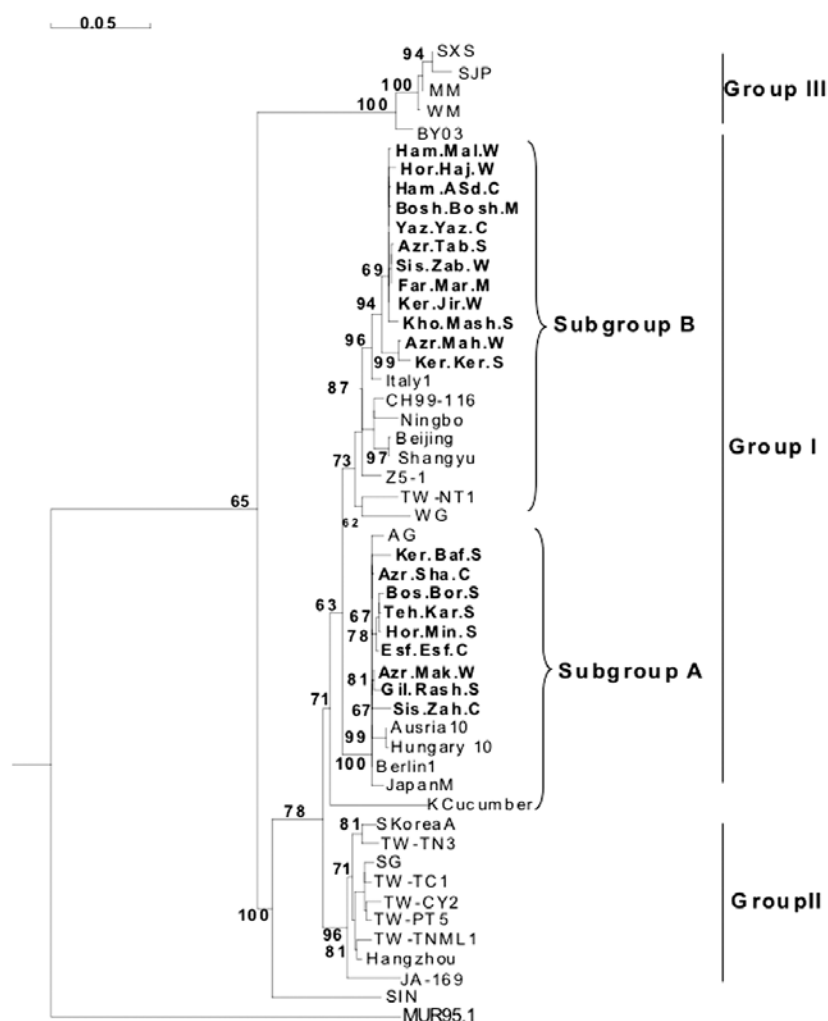


Fig. 1. Neighbor-joining tree constructed with whole CP sequences, showing the phylogenetic relationships between Iranian and other ZYMV isolates. Multiple sequence alignments of the nucleotide sequences were generated with DNAMAN program (Version 5.2.2; Lynnon Biosoft, Canada). Bootstrap values are shown on the nodes. The Iranian isolates are in bold. MUR95.1 is *Watermelon mosaic virus* included as outgroup. Accession numbers: SXS (AJ515907), SJP (AJ515909), MM (AJ515911), WM (AJ515908), BY03 (AY611025), Italy1 (AJ420020), CH99-116 (AY611021), Ningbo (AY074810), Beijing (AY074809), Shangyu (AF513550), Z5-1 (AB188115), TW-NT1 (AF127933), WG (AJ316229), AG (EF062583), Ausria10 (AJ420015), Hungary10 (AJ251527), Berlin1 (AJ420019), JapanM (AB004641), KCucumber (AF062518), SKoreaA (AJ429071), TW-TN3 (AF127929), SG (AJ316228), TW-TC1 (AF127931), TW-CY2 (AF127930), TW-PT5 (AF127934), TW-TNML1 (AF127932), Hangzhou (AF435425), JA-169 (AB004640), SIN (AF014811).

lates Bos.Bor.S, Hor.Min.S, Teh.Kar.S, Esf.Esf.C, Sis.Zah.C, Azr.Sha.C, Ker.Baf. S, Azr.Mak.W and Gil.Ras.C appeared to be less than 84.7% similar in the nucleotide sequence of NIB-CP to isolates from Israel, Turkey, Jordan, Poland, Taiwan, Germany, Syria, Slovakia and Czech Republic clustered in subgroup I_A. Isolates Hor.Haj.W, Yaz.Yaz.C, Bos.Bos.M, Ham.Aas.C, Ham.Mal.W, Azr.Tab.S, Sis.Zab.W, Far.Mar.M, Ker.Jir.W and Kho.Mash.S, that were 93.2 to 99.2% similar to isolates from Taiwan, China, Nepal, Spain, USA, France and Italy belonged to subgroup I_B.

Nine of the Iranian isolates recovered from edible cucurbits and the one from *C. colocynthis* shared >98% nucleotide sequence similarity in the CP and NIB-CP fragments with those reported from south east Asia, USA and the Mediterranean region (Spain, Italy, France). Eleven other isolates were more similar (93.2-99.2%) to those from the Middle East, south east Asia and some European countries (Germany, Poland, Slovakia, Austria, Hungary).

Phylogenetic trees were constructed using neighbor-joining (DNAMAN) and maximum parsimony methods for the sequence of both the NIB-CP fragment and the whole CP gene of ZYMV isolates. The estimated cophenetic coefficient of the dendrograms (0.96 and 0.98 for

NIB-CP and whole CP genomes, respectively) confirmed the reliability of the groupings.

The CP gene is often used to determine the phylogenetic relationship among potyviral isolates (Tobias and Palkovics, 2003). As said above, our phylogenetic tree classified ZYMV isolates into three major groups [previously designated as groups I, II, III by Zhao *et al.* (2003)] based on the nucleotide sequences of whole CP gene and the NIB-CP gene fragment. According to our results and those of Desbiez *et al.* (2002) and Bananej *et al.* (2008) isolates of group I (denoted as group A by the latter authors) are the most widespread. Group I of this study however, was composed of two subgroups (I_A and I_B). The Iranian isolates in subgroup I_A based on NIB-CP gene were from cucumber, winter squash (*C. maxima*) and watermelon and the those in subgroup I_B were from melon, cucumber, watermelon, pumpkin, colocynth and winter squash (Table 1).

Host range and molecular properties have been used for the identification and differentiation of strains and pathotypes of viruses (Shukla *et al.*, 1994; Xiao *et al.*, 1993; Zhao *et al.*, 2003). In this study, ZYMV isolates have been grouped on the basis of their biological properties and phylogenetic relationships using NIB-CP and CP fragments.

Table 1. Names and origins of ZYMV isolates from different parts of Iran used in this study and accession numbers of gene sequences determined.

Isolate name	Province-region	Original host	Accession No. for NIB/CP	Accession No. for CP
Ker.Ker.S ^a	Kerman-Kerman	<i>Cucurbita maxima</i>	FJ752261	FJ705266
Esf.Esf.C	Esfahan- Esfahan	<i>Cucumis sativus</i>	FJ752252	FJ705257
Teh.Kar.S	Tehran-Karaj	<i>C. maxima</i>	FJ752265	FJ705270
Gil.Ras.C	Gilan-Rasht	<i>C. sativus</i>	FJ752254	FJ705259
Ker.Jir.W	Kerman-Jiroft	<i>Citrullus colocynthis</i>	FJ752260	FJ705265
Yaz.Yaz.C	Yazd-Yazd	<i>C. sativus</i>	FJ752245	FJ705271
Hor.Min.S	Hormozgan-Minab	<i>C. maxima</i>	FJ752258	FJ705263
Sis.Zah.C	Sistan and Baloochestan-Zahedan	<i>C sativus</i>	FJ752264	FJ705269
Ham.Asa.C	Hamadan-Asadabad	<i>C. sativus</i>	FJ752255	FJ705260
Azr.Tab.S	East Azarbayjan-Tabriz	<i>Cucurbita muschata</i>	FJ752249	FJ705254
Ham.Mal.W	Hamadan-Malayer	<i>Citrullus vulgaris</i>	FJ752256	FJ705261
Bos.Bos.M	Booshehr-Booshehr	<i>Curcubita melo</i>	FJ752251	FJ705256
Aza.Mah.W	West Azarbayjan-Mahabad	<i>Citrullus vulgaris</i>	FJ752246	FJ705252
Bos.Bor.S	Booshehr-Booshehr	<i>C. maxima</i>	FJ752250	FJ705255
Azr.Mak.W	West Azarbayjan-Makoo	<i>Citrullus vulgaris</i>	FJ752247	FJ705272
Ker.Baf. S	Kerman-Baft	<i>C. maxima</i>	FJ752259	FJ705264
Hor.Haj.W	Hormozgan-Hajibade	<i>Citrullus vulgaris</i>	FJ752257	FJ705262
Sis.Zab.W	Sistan and Baloochestan-Zabole	<i>Citrullus vulgaris</i>	FJ752263	FJ705268
Far.Mar.M	Faras-Marvdasht	<i>Cucurbita melo</i>	FJ752253	FJ705258
Kho.Mash.S	Khorasan-Mashahad	<i>C. maxima</i>	FJ752262	FJ705267
Azr.Sha.C	East Azarbayjan-Shabstar	<i>C. sativus</i>	FJ752248	FJ705253

^a Each isolate was identified by location including province, regions, and host plant.

Table 2. Symptoms induced by Iranian isolates of ZYMV on selected plant species.

Families, species and cultivars	Symptoms		
	Group I ^a	Group II ^b	Group III ^c
Cucurbitaceae			
<i>Cucurbita pepo</i> L.cv. Khoy (local cultivar)	FL, Mo ^d	Mo, Bl	CLL, Mo
<i>C. pepo</i> var. <i>pepo</i>	FL, Mo, Vb	Mo, Bl	CLL, Mo
<i>C. pepo</i> L.cv. Maragheh (local cultivar)	FL, Mo, Vb	Mo, Bl	CLL, Mo
<i>Cucumis melo</i> L. var. <i>conomon</i>	Vc, Mo	M, Mo	CLL, Mo, Vc
<i>C. melo</i> L. var. <i>inodorus</i>	Vc, Mo	Mo, Bl	CLL, Mo, Vc
<i>C. melo</i> L. var. <i>flexuosus</i>	Vc, Mo	M, Mo	CLL, Mo
<i>C. melo</i> L. var. <i>cantalupensis</i>	Vc, Mo	Mo	Mo, Bl
<i>Citrullus lantanus</i> cv. Crimson Sweet	Y, Mo	Y, Mo	-
Chenopodiaceae			
<i>Chenopodium quinoa</i>	CLL	CLL	NLL
<i>C. amaranticolor</i>	CLL	CLL	NLL
<i>C. murale</i>	-	-	-
Solanaceae			
<i>Datura stramonium</i>	-	NLL	NLL
<i>D. metel</i>	-	-	-
<i>D. maxima</i>	-	-	-
<i>Nicotiana clevelandii</i>	-	-	-
<i>N. tabacum</i> . cv. Samsun NN.	-	-	-
<i>N. glutinosa</i>	-	-	-
<i>N. debneyi</i>	-	-	-
<i>N. bentamiana</i>	-	-	-
<i>Solanum lycopersicum</i> cv. Gavrish	-	-	-
<i>Gompherena globosa</i> .	-	-	-
Fabaceae			
<i>Pisum sativum</i>	-	-	-
<i>Phaseolus vulgaris</i> cv. Pinto	-	-	-
Ranunculaceae			
<i>Rannunculus sardous</i>	-	-	-

^aGroup I includes the Iranian isolates Esf.Esf.C, Teh.Kar.S, Gil.Ras.C, Ker.Jir.W, Hor.Min.S, Sis.Zah.C, Aza.Mah.W, Bos.Bor.S, Ker.Baf. S, Azr.Sha.C.

^bGroup II includes the Iranian isolates Ker.Ker.S, Azr.Mak.W.

^cGroup III includes the Iranian isolates Yaz.Yaz.C, Ham.Asd.C, Azr.Tab.S, Ham.Mal.W, Bos.Bos.M, Hor.Haj.W, Far.Mar.M, Kho.Mash.S, Sis.Zab.W.

^dExplanation of symbols: Mo=mottling, M=mosaic, Bl=blistering, Y=yellowing, Vb=vein banding, Vc=vein clearing, CLL=clorotic local lesion, NLL=necrotic local lesion, FL= filiform leaves, -= no symptoms and no virus recovered.

Based on biological properties, the Iranian isolates falling in groups I and II showed similar symptoms on watermelon, *C. quinoa* and *C. amaranticolor*. Whereas only isolates in group III elicited different symptoms in these hosts. ZYMV isolates of biological groups I and II were all comprised in subgroup I_A of the tree constructed with the whole CP gene sequence. The only exception was isolate Ker.Jir.W from colocynth, which fell in subgroup I_B. In contrast, all nine Iranian isolates of biological group III were classified in subgroup I_B (Fig. 1). Similar results, with the exception of isolates Azr.Mah.W and Ker.Ker.S, were also obtained when phylogenetic trees were generated with nucleotide sequences of NIB-CP gene fragment (data not shown).

ZYMV isolates belonging to groups I and II induced

in squash and melon varieties varying degrees of mosaic, mottling, vein clearing, blistering and filiform leaves, whereas isolates of group III caused clorotic local lesion followed by mottling. These differences in symptomatological reactions could be indicative of a possible relationships between biological and molecular properties, so as to suggest that each subgroup may share a common origin or has spread from a unique infection focus.

Frenkel *et al.* (1991) suggested that potyviral CP may play a role in determining host specificity and induction of symptoms. One amino acid (aa) variation was observed in the putative CP sequence of Iranian isolates that clustered in subgroups I_A and I_B. The difference was due to the substitution of aa V at position 39 of the CP sequence of the Iranian ZYMV isolates in subgroup

I_A with aa A in those of subgroup I_B. The only exception was isolate Ker.Ker.S that has an Isoleucine (I) instead of an alanine (A) at the CP N-terminus. Thus, this aa variation between Iranian ZYMV isolates that clustered in subgroups I_A and I_B and their relationships with biological properties raise the question that some aa substitutions might be related with symptoms production.

All Iranian ZYMV isolates contained in the CP a pair of amino acids DK at positions 16 and 17, conserved in almost all molecularly characterized isolates of this virus with rare exceptions (Glasa and Pittnerova, 2006). Likewise, all isolates had the DAG amino acid triplet required for virus transmission by aphids (Atreya *et al.*, 1990, 1995).

Based on the result of this study ZYMV appears to be the most prevalent and damaging virus in several cucurbit-growing areas of Iran where is perpetuated in weed species, such as colocynth, that may serve as overwintering host. The variability potential of this virus could lead to the emergence of resistance-breaking strains, which makes this kind of investigations a prerequisite for planning and enforcing appropriate control measures, especially via genetic engineering (Garcia-Arenal *et al.*, 2000). Furthermore, control programs must integrate several strategies to remain effective (Desbiez and Lecoq, 1977).

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SHORT COMMUNICATION

EVALUATION OF BIOCONTROL EFFICIENCY OF *PSEUDOMONAS FLUORESCENS* UTPF61 IN DIFFERENT NITROGEN SOURCES

F. Heidari-Tajabadi, M. Ahmadzadeh and R. Sharifi

*Laboratory of Biological Control of Plant Disease, Department of Plant Protection,
College of Agriculture, University of Tehran, Karaj, Iran*

SUMMARY

Understanding the environmental factors and their impediments to commercialization of microbial biocontrol agents is an essential step for improving biocontrol efficiency. One of these impediments is the lack of liquid culture technology for cost-effective mass production of biocontrol agents and their insufficient longevity during storage. *Pseudomonas fluorescens* UTPF61 is a biocontrol agent against sclerotinia wilt of sunflower, an important disease caused by *Sclerotinia sclerotiorum*. This strain was originally isolated from rice rhizosphere and was selected out of 47 strains. In this work liquid culture assays were made to identify nitrogen sources that had a differential influence on the biocontrol activity of UTPF61. Rapid growth in flask was observed in media containing M1+peptone, M1+2-ammonium hydrogen phosphate, M1+ammonium sulfate and M1+urea+ammonium sulfate, respectively. Medium containing M1+urea or M1+ammonium phosphate had the best effect on the antagonistic efficiency. The best effect on plant growth was observed in treatments with M1+2-ammonium hydrogen phosphate under greenhouse conditions since, compared to the control, it significantly increased the length of roots and stems by 124.18% and 168.25%, respectively, as well as the wet weight (by 222.3%) and dry weight (by 246.15%) of the plants.

Key words: antagonistic efficiency, antifungal activity, growth rate, biological control.

Sclerotinia sclerotiorum (Lib.) de Bary is a pathogen that causes destructive diseases of numerous plants (Agrios, 2005), among which sunflower (*Helianthus annuus* L.). There has been no progress in breeding a type of sunflower resistant to sclerotinia wilt and no effective chemical control of this disease is known (Expert and Digat, 1995). Therefore interest in biological control has

recently increased (Duffy and Defago, 1999). Although some strains of *Pseudomonas fluorescens* are known as biocontrol agents of various soil-borne fungi (Weller, 1988; Defago *et al.*, 1990; Expert and Digat, 1995; Ahmadzadeh and Sharifi-Tehrani, 2009) they may perform in a dissimilar way under different conditions. Understanding which environmental factors are important and how they influence disease suppression is recognized as a key to improving the level and reliability of biocontrol (Duffy and Defago, 1999).

This study aimed at (i) finding nitrogen sources other than by-products from food industries (Costa *et al.*, 2001) that would allow maximum biomass production of *Ps. fluorescens* UTPF61; (ii) improving its biocontrol potentiality against *S. sclerotiorum*, and (iii) increasing plant growth promotion activity.

UTPF61 was selected out of 40 strains of fluorescent pseudomonads based on dual culture assays, growth promotion activity of sunflower, production of HCN and protease (Heidari-Tajabadi, 2008). This strain was originally isolated from the rhizosphere of rice from Ghazvin province (Iran). Stock cultures were prepared for storage at -80°C in 1.5 ml vials by mixing equal volumes of 50% glycerol and 24 h broth from single-colony inoculum grown in Luria Bertani medium. The basal medium (M1) we used contained 1 g/l yeast extract, 10 g/l sucrose, 0.4 g/l CaCO₃, 0.4 g/l MgSO₄ and 0.98 g/l K₂HPO₄·3H₂O, to which the following nitrogen sources (at a 4 g l⁻¹ concentration) were added separately: urea, ammonium sulfate, 2-ammonium hydrogen phosphate, ammonium nitrate, ammonium phosphate, corn steep, urea+ammonium sulphate and peptone. All media were autoclaved at 121°C for 15 min. Flasks containing 40 ml of medium were inoculated using 1 ml of a 24 h UTPF61 culture adjusted to 10⁶ CFU ml⁻¹ in sterile distilled water. After 48 h incubation at 25°C in a rotary shaker (120 rpm), cell density was measured at 600 nm (T70 Spectrophotometer. PG Instruments, UK) and cultures were used for dual-culture assays and greenhouse experiments.

The *in vitro* inhibition of mycelial growth of *S. sclerotiorum* by strain UTPF61 was determined as described by Keel *et al.* (1996). Three 10 µl drops from different flask cultures were equidistantly placed at the margins

of potato dextrose agar (PDA) plates and incubated at 20°C for 24 h. A 7 mm agar disc from fresh PDA cultures of *S. sclerotiorum* was then placed at the centre of the PDA plate and incubated at 20°C for five days. Inhibition of fungal growth was assessed five days later by measuring the size of the inhibition zone.

Greenhouse trials for plant growth promotion (PGPR) were performed in 7.5×8 cm plastic pots containing a sterilized 3:1:1 compost of sand, clay and humus (HB-Zarin, Iran). Surface-sterilized (5% sodium hypochlorite for 3 min and rinsed five times with sterile distilled water) sunflower seeds bacterized by soaking in UTPF61 suspension (1×10^8 CFU ml⁻¹) for 30 min with a mild shaking were then sown in the pots. Bacterial suspension was prepared in a 1% carboxyl methyl cellulose (CMC) solution in sterile water. Control seeds were rinsed only in sterile 1% CMC solution for 30 min.

S. sclerotiorum inoculum consisted of millet seeds sterilized twice at 121°C for 20 min, inoculated with mycelial plugs, and incubated at 20°C for 20 days. One gram of 24 h millet seed inoculum was mixed in the upper part of sterile soil, prior to seed planting. Sterile millet seeds mixed with the compost served as control. In PGPR test, bacterized seeds were planted in sterile perlite and irrigated by half-strength Hoagland solution (Hoagland and Arnon, 1950). Pots were kept in a greenhouse at 22±3°C, 60% relative humidity and a photoperiod of 14 h light/10 h darkness. Pots were fully randomized and treatments were replicated three times.

In the antagonistic trial, the percentage of healthy plants was determined 30 days after planting, according to Expert and Digat (1995) and in the PGPR trial, the length of root and stem, the wet and dry weight of roots and stem were calculated 30 days after sowing. Data were analyzed by Duncan's multiple range test ($P < 0.05$), with the SAS general linear model procedure (SAS institute, Cary, NC). Normal distribution and homogeneity of variances were checked beforehand. A complete randomized design (CRD) was used in all experiments and each treatment was replicated four times. Correlation analysis was performed with the SPSS 16 statistical software package.

Media containing peptone, 2-ammonium hydrogen phosphate, ammonium sulphate and urea+ammonium sulphate increased bacterial growth by 131.32%, 127.92%, 124.97% and 125%, respectively, compared to M1 control (Table 1). *S. sclerotiorum* was more sensitive by 153.3% and 156.7% to strain UTPF61 when grown in media which contained urea, ammonium nitrate or ammonium phosphate, respectively, than in media supplemented with ammonium sulphate or 2-ammonium hydrogen phosphate (Table 1). Media containing urea or ammonium phosphate showed more antifungal activity than the other media by 93.3% (Fig. 1).

In greenhouse PGPR, ammonium sulphate was more effective in increasing the length of root by 148.45% than

the other media, whereas 2-ammonium hydrogen phosphate was more effective in stimulating stem length, and also the wet and dry weight of stem and root by 168.25%, 222.3% and 246.15%, respectively (Table 2).

Since the commercial production of *P. fluorescens* to be used as a biocontrol agent requires both low cost and high cell density, the optimization of nutritional and environmental conditions to produce this bacterium are based on minimizing cost and maximizing cell density. The results obtained in this study showed that the nature of nitrogen source in culture media influenced the rate of bacterial growth, but it is worth mentioning that media showing the highest effect on the rate of bacterial growth did not necessarily have the same effect on the antagonistic efficiency. In other words, no correlation was found between rate of bacterial growth and antagonistic efficiency, which agrees with previous results (Duffy and Defago, 2000; Costa *et al.*, 2001).

Conditions under which biocontrol agents are grown may have a significant influence on the production of antimicrobial substances (Duffy and Defago, 1999), survival in soil, and biocontrol ability (Fuchs *et al.*, 2000; Gu and Mazzola, 2001). We have now shown that also cultural media can influence the biocontrol ability of the *P. fluorescens* strain used in our study.

Media containing ammonium phosphate, ammonium nitrate and urea with 11.179, 11.132 and 11.068 log CFU/ml respectively, support moderate cell yields of *P. fluorescens* strain UTPF61 but not as much as ammonium sulphate (11.916), peptone (12.587), 2-ammonium hydrogen phosphate (12.245) and urea+ ammonium sulphate (11.808 log CFU/ml).

Corn steep liquor provided the least bacterial growth but it had a significant positive effect (13.33 mm) on antagonistic efficiency and a moderate effect on plant

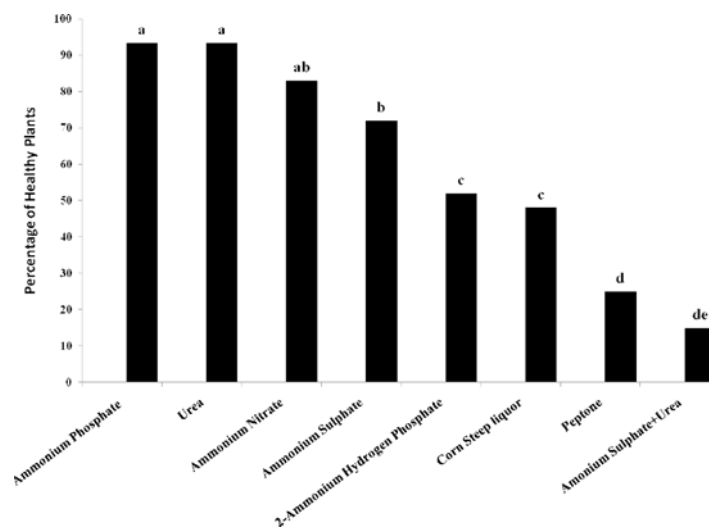


Fig. 1. Percentage of healthy plant which treated with strain UTPF61 grown in different nitrogen sources.

Table 1. Effect of some nitrogen sources on the growth rate and antagonistic efficiency of *P. fluorescens* strain UTPF61 at 25°C and after 48 h.

Media	Inhibition zone (mm)	Log CFU/ml
M1+ peptone	12.67 bc*	12.587 a
M1+ 2-ammonium hydrogen phosphate	10 d	12.245 a
M1+ ammonium sulphate	10 d	11.916 a
M1+ urea	15.33 a	11.068 ab
M1+ ammonium nitrate	15.33 a	11.132 ab
M1+ ammonium phosphate	15.67 a	11.179 ab
M1+ corn steep	13.33 ab	10.124 b
M1+ urea+ ammonium sulphate	10.67 dc	11.808 a

*statistical significance was judged at the $P < 0.01$ level.

Table 2. Effect of different nitrogen sources on the ability of bacteria to improve growth of sunflower.

Content of the media on which strain UTPF61 was grown	Root length (cm)	Stem length (cm)	Wet weight (g)	Dry weight (g)
Ammonium sulfate	16.33 a	17.67 bc	2.68 ab	0.22 bc
2-ammonium hydrogen phosphate	13.66 ab	23 a	3.29 a	0.32 a
Peptone	14.33 ab	21.33 ab	2.61 ab	0.25 ab
Ammonium nitrate	13.67 ab	16.33 cd	2.19 b	0.22 bc
Ammonium phosphate	12.33 b	16.33 cd	1.98 b	0.16 c
Urea	15 ab	15.33 cd	2.29 b	0.15 c
Ammonium sulfate+ urea	13 b	17cd	2.54 ab	0.25 bc
Corn steep liquor	13.33 ab	13 d	2.03 b	0.2 bc
Control	11 c	13.67 d	1.48 bc	0.13 d

Figures followed by the same letter are not significant at the $P < 0.01$ level.

growth and antifungal activity (Table 2). By contrast, 2-ammonium hydrogen phosphate was the best inorganic source as it showed a significant effect both on bacterial (12.245 log CFU/ml) and plant growth (Table 2), but it had little effect on antagonistic efficiency (Fig. 1). This result agrees with those by Dharani-Aiyer (2004) and Musial *et al.* (2004).

In conclusion, the laboratory conditions used to prepare the inocula need to be carefully considered when optimizing the production of a biocontrol pseudomonad. An improvement in biocontrol efficiency may be achieved when rich laboratory media are replaced with less rich media, which lowers also the manufacturing costs of biocontrol products. However, the encouraging results of the present study, obtained under experimental conditions (laboratory and greenhouse) need to be verified in the field.

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SHORT COMMUNICATION

**CHARACTERISATION OF THE COAT PROTEIN GENE
OF *DASHEEN MOSAIC VIRUS* INFECTING ELEPHANT FOOT YAM**

B. Babu, V. Hegde, T. Makeshkumar and M.L. Jeeva

*Division of Crop Protection, Central Tuber Crops Research Institute,
Thiruvananthapuram Kerala 695017, India*

SUMMARY

A strain of *Dasheen mosaic virus* (DsMV) from *Amorphophallus paeoniifolius* (Elephant foot yam) plants showing leaf mosaic, puckering and shoestringing was characterized by RT-PCR and amplification of a 1.2 kbp fragment encoding the coat protein (CP) gene and the 3' untranslated region (UTR). BLAST analysis of the cloned DNA amplicon of DsMV-Amp1 revealed at least 80% nucleotide sequence identity with other DsMV isolates from different hosts and geographic origins. The CP gene and the 3' UTR of DsMV-Amp1 consisted of 942 and 260 nucleotides, respectively. The highest nucleotide sequence identity (89%) was with DsMV-ch from *Caladium* from the USA, and the deduced CP amino acid sequence had 92-98% identity with other DsMV isolates. Nucleotide sequence identity of 82-85% was also found with *Vanilla mosaic virus* (VMoV), whereas the identity with *Bean common mosaic virus* (BCMV), *Hardenbergia mosaic virus* (HaMV) and *Watermelon mosaic virus* (WMV) ranged from 74 to 79%. Several potential threonine- and asparagine-rich N-glycosylation motifs and a DVG motif potentially involved in aphid transmission were found in the CP of DsMV-Amp1. This is the first characterization of DsMV from *A. paeoniifolius* plants in India.

Key words: coat protein, *Dasheen mosaic virus*, Elephant foot yam, potyvirus, RT-PCR, sequencing.

Amorphophallus paeoniifolius or Elephant foot yam (Whitespot giant arum), family Araceae, is an important tuber crop, grown widely in the Philippines, Malaysia, Indonesia and other south-east Asian countries. Its tubers are used as vegetables and in preparation of indigenous ayurvedic medicines (Misra *et al.*, 2002). It has become a cash crop and is gaining popularity due to its high production potential (50-60 t/ha), nutritional and

medicinal values, and high economic returns (Srinivas and Ramanathan, 2005). Occurrence of mosaic disease of *A. paeoniifolius* (previously known as *A. campanulatus*) was reported from India (Capoor and Rao, 1969) and 24 to 88% mosaic incidence with yield losses of 3.5 to 38% were recorded in Uttar Pradesh (Nehalkhan *et al.*, 2006). Disease incidence of 5 to 10% was observed in other major Elephant foot yam-growing states of India (Kerala, Andhra Pradesh and Orissa) (B. Babu *et al.*, unpublished information). Immunoelectron microscopy observations have shown the association of a potyvirus with Elephant foot yam mosaic disease (Ahlawat *et al.*, 2003).

Among the species of the genus *Potyvirus*, *Dasheen mosaic virus* (DsMV) infects a wide variety of aroid plants worldwide, including at least 16 genera of the family Araceae (Zettler and Hartman, 1986, 1987; Zettler *et al.*, 1978). It causes serious damage to ornamentals like *Caladium*, *Dieffenbachia*, and *Zantedeschia*, and is ubiquitous in commercial plantings of tropical root crops of the genera *Colocasia*, *Xanthosoma* and *Amorphophallus*. The virus is experimentally transmitted by infected sap and, in nature, by several widely distributed aphid species, including *Myzus persicae* and *Aphis gossypii*. Since most of the commercially cultivated hosts, including Elephant foot yam, are vegetatively propagated, the infection primarily spreads by infected planting material. Pandit *et al.* (2001) reported DsMV as the causal agent of mosaic disease of Elephant foot yam in India based on serology and virus identity was later confirmed by sequencing of the core region of coat protein (CP) gene (B. Babu *et al.*, unpublished information).

Partial genome sequences of DsMV isolates from *Colocasia* (Pappu *et al.*, 1994), *Xanthosoma* (Reyes *et al.*, 2009) and *Zantedeschia* (Chen *et al.*, 2001) and the complete genome sequence of a *Zantedeschia* isolate have been determined (Chen *et al.*, 2001). However no sequence information of DsMV from India or from Elephant foot yam plants has so far been reported. Therefore, the objective of the present study was to characterise the sequences of the CP gene and 3' untranslated region of a DsMV isolate infecting Elephant foot yam in India, and to analyse the phylogenetic relationship of the virus with known DsMV isolates.

Table 1. Percentage identity of nucleotide (nt) and amino acid (aa) sequences of the coat protein gene of *Dasheen mosaic virus*-Amp1 with other viral isolates.

No.	Accession No.	Isolate	Percentage identity	
			nt	aa
1	AF045065	<i>Bean common mosaic virus</i> , strain GGSUS- USA	78	89
2	AJ305434	<i>Dasheen mosaic virus</i> - DeSLK2-UK	79	92
3	DQ925464	<i>Dasheen mosaic virus</i> - VN/Cel-Vietnam	87	94
4	AF511485	<i>Dasheen mosaic virus</i> - S-Taiwan	85	95
5	AJ298035	<i>Dasheen mosaic virus</i> - DK-China	87	95
6	AJ298036	<i>Dasheen mosaic virus</i> - TW- Taiwan	87	96
7	AJ298034	<i>Dasheen mosaic virus</i> - ND-China	85	96
8	AF048981	<i>Dasheen mosaic virus</i> - USA	89	98
9	AY994105	<i>Dasheen mosaic virus</i> - DsMV2- New Zealand	83	94
10	DMU00122	<i>Dasheen mosaic virus</i> - USA	88	96
11	EF199550	<i>Dasheen mosaic virus</i> - YN80- China	87	96
12	DQ925466	<i>Dasheen mosaic virus</i> - VN/Tt1- Vietnam	87	91
13	AY994104	<i>Dasheen mosaic virus</i> - Taro- New Zealand	84	94
14	DQ925465	<i>Dasheen mosaic virus</i> - VN/Ce2- Vietnam	86	91
15	AF169832	<i>Dasheen mosaic virus</i> -Taiwan	86	96
16	AJ298033	<i>Dasheen mosaic virus</i> - M13	86	96
17	U08124	<i>Dasheen mosaic virus</i> - USA	85	95
18	EU420058	<i>Dasheen mosaic virus</i> - China	84	88
19	AM910401	<i>Dasheen mosaic virus</i> -NiNG67-Nicaragua	88	96
20	AM910407	<i>Dasheen mosaic virus</i> -NiNG78-Nicaragua	88	96
21	AM910398	<i>Dasheen mosaic virus</i> -NiNG1-Nicaragua	88	96
22	DQ898206	<i>Hardenbergia mosaic virus</i> - WHP-1 - Australia	76	87
23	DQ898197	<i>Hardenbergia mosaic virus</i> - MU-3A- Australia	78	88
24	DQ898188	<i>Hardenbergia mosaic virus</i> -BB-6- Australia	79	89
25	AJ616721	<i>Vanilla mosaic virus</i> - CI-NAT- New Zealand	82	93
26	AJ616720	<i>Vanilla mosaic virus</i> - CI-AT- New Zealand	82	92
27	AJ616719	<i>Vanilla mosaic virus</i> - FP- New Zealand	84	94
28	AJ429525	<i>Vanilla mosaic virus</i> - CI-AT- New Zealand	88	93
29	EU660586	<i>Watermelon mosaic virus</i> - FBR04-37- France	76	86
30	AB369278	<i>Watermelon mosaic virus</i> - South Korea	77	87

nt = nucleotide; aa = amino acid

A. paeoniifolius plants showing mottling, chlorotic feathering, puckering leaf deformation and shoestringing (Fig. 1) were collected from Kerala, Andhra Pradesh and Orissa, and the representative tubers were also collected and maintained in a glasshouse. Total RNA was extracted from 100 mg infected leaves using the Qiagen RNeasy Plant mini kit (Qiagen, USA) according to the manufacturer's protocol and resuspended in 50 µl nuclease free water.

Extracted total RNA was subjected to RT-PCR using a one step kit (Finnzymes, Finland) in a reaction mixture (20 µl) containing 5 µl RNA, 1 µl of 20 pmol oligo (dT)₁₆, 1 µl of 20 pmol each of DsMV(I)-F and DsMV(I)-R, 2.0 µl of 10x/5x reaction buffer, 0.5 µl of 10 mM dNTPs, 0.5 µl of 50 mM magnesium chloride (MgCl₂), 0.25 µl of AMV reverse transcriptase and 1.0 µl of Dynazyme DNA polymerase in Eppendorf Mastercycler Gradient ES with the following profile: 48°C for 45 min, 94°C for 2 min, followed by 35 cycles of 94°C for 30 sec, 58°C for 1 min, 72°C for 1 min and a final extension at 72°C for 10 min.

Degenerate primers DsMV (I)F and DsMV (I)R were designed 21 nucleotides upstream of the 5' end of the CP gene comprising the NIb region [DsMV (I)F 5'-GATGCCCTTTRMCTTTGARNTGWKSKGTG-3'] and in the 3' UTR [DsMV (I)R 5'-ACCGTGCAC-DAAGCAYCTCGC-3'], respectively, using the Primer Premier Software version 5 and five DsMV sequences from GenBank (accession Nos AAJ298033, AJ628756, DQ925464, DQ925465 and DQ925466). Amplified products 1.2 kb in size, which potentially encoded the complete CP gene and the 3'UTR region of the virus, were analyzed in 1% agarose gel, stained with ethidium bromide and photographed with a UV-gel doc system (Alpha Imager, USA). Amplicons were then purified using the Gel extraction kit (Qiagen, USA), cloned into the pGEM-T Easy vector (Promega, USA) and transferred into *E. coli* strain DH5α (Sambrook and Russell, 2001). Thirteen clones carrying the insert were sequenced using T7 and SP6 primers, and a representative sequence was deposited in GenBank (accession No. FJ160764).

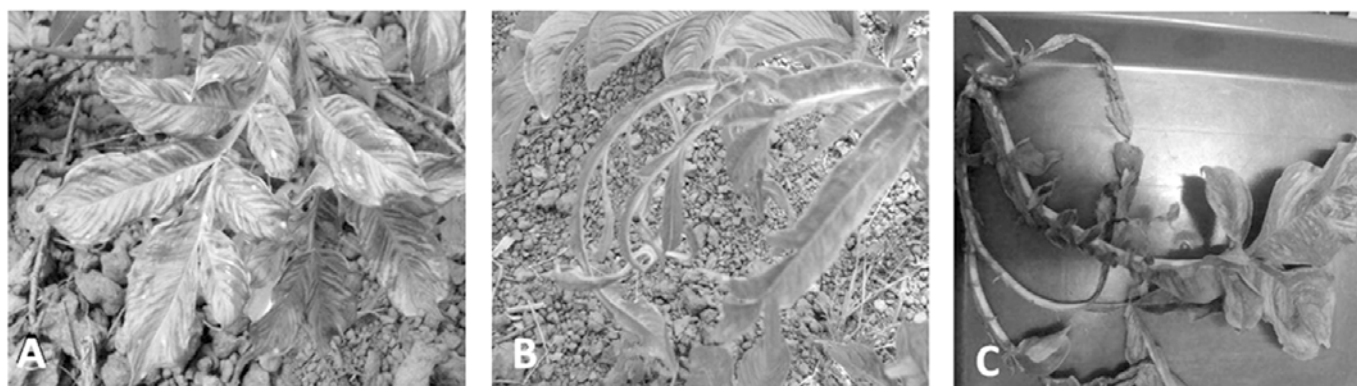


Fig. 1. Symptoms shown by *Amorphophallus paeoniifolius* leaves infected with *Dasheen mosaic virus*. A. Mosaic and chlorotic feathering. B. Shoestringing (severe narrowing of leaf lamina). C. Puckering and leaf distortion.

Sequences were edited using the BIOEDIT Software (Hall, 1999). Nucleotide (nt) and the deduced amino acid (aa) sequences were compared with those in the NCBI database using the Basic Local Alignment Search Tool (BLAST; <http://www.ncbi.nlm.nih.gov/blast/>), with Blastn and Blastp, respectively. Deduced amino acid sequences were analysed using the GCG programme MOTIFS (Devereux *et al.*, 1984). Multiple sequence alignment of the virus isolates and the percent identity between aligned sequences were calculated using Clustal W (Thompson *et al.*, 1994). Phylogenetic analysis was done between DsMV and other selected

members of the genus *Potyvirus*. From the aligned sequences, a phylogenetic tree was constructed using the neighbor-joining method (Tajima and Nei, 1984). The data sets were subjected to 100 bootstrap replicates and the tree was constructed using the TREECON software.

The 1.2 kb amplicon from DsMV-Amp1 contained 21 bp of Nib gene, the full-length CP cistron consisting of 942 nts, which potentially encoded a 313 aa protein, and the 3' UTR region made up of 260 nts. Multiple sequence alignment of DsMV-Amp1 CP with that of other DsMV isolates revealed that although the N-terminal of the CP region varies considerably, two thirds of the

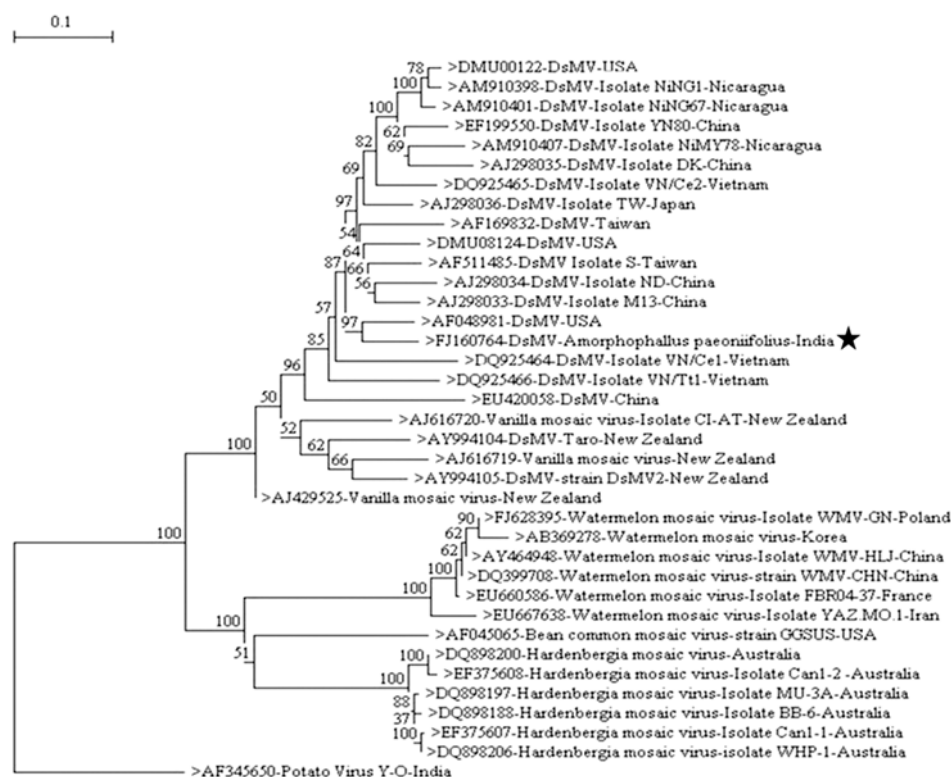


Fig. 2. Phylogenetic tree constructed with CP nucleotide sequences of *Dasheen mosaic virus*-Amp1 and other viral isolates using the neighbor-joining method with 100 bootstrap replicates. *Potato virus Y* was used as outgroup.

C-terminal was highly conserved. A nucleotide sequence identity ranging from 83 to 89% was found between DsMV-Amp1 and DsMV sequences available in GenBank. The highest nt sequence identity (89%) was with DsMV-ch from *Caladium* in the USA (AF048981) and the lowest (79%) with DsMV-DeSLK2 (AJ305434) (Table 1). Furthermore, the DsMV-Amp1 CP nt sequence showed 82-85% identity with that of *Vanilla mosaic virus* (VMV) and 74-79% identity with *Hardenbergia mosaic virus* (HaMV), *Bean common mosaic virus* (BCMV) and *Watermelon mosaic virus* (WMV) (Table 1). In the phylogenetic tree, DsMV isolates clustered with VMV, confirming that these two viruses are closely related (Fig. 2). Incidentally, VMV has been reported to be a DsMV strain that exclusively infects vanilla (Farreyrol *et al.*, 2006).

Analysis of the 5' end region of the CP revealed that the DsMV-Amp1 differs from three other DsMV isolates (AY994104, AY994105 and AJ616719) as it lacks 66 nts, whereas it lacks 58 nt compared with 10 other viral isolates (AM910398, DMU00122, AF169832, EF199550, AJ298035, DQ925464, AM910407, AM910401, AM994104 and AY994105) (data not shown).

Protease cleavage sites in potyvirus polyprotein sequences for the production of the CP are Q/A, Q/S or Q/G residues (Pappu *et al.*, 1994). DsMV-Amp1, had two potential protease cleavage sites within the 313 residue polypeptide. The first site occurs at position 7 and 8, between Q/A to produce a CP of 313 amino acids (Adams *et al.*, 2005). Another possible cleavage site is suspected to occur 62 amino acids downstream of the first protease cleavage site at a Q/V site. The possibility of two protease cleavage sites in the CP of DsMV was also previously reported (Pappu *et al.*, 1994). The presence of the sequence DEVVL in the Nib region, upstream of the glutamine residue of the Q/A site at position 6, suggests that this is the protease digestion site. This region also correlates with the protease sites of other potyviruses (Yu *et al.*, 1989). The presence of the sequence ADDTV following the first putative cleavage site indicates that this site is likely active, as reported for *Tobacco etch virus* (TEV) and *Tobacco vein mottling virus* (TVMV) (Pappu *et al.*, 1994; Yu *et al.*, 1989).

DsMV-Amp1 possessed a DVG motif (residues 13 to 15) instead of the aphid transmission motif DAG of potyviruses (Atreya *et al.*, 1991; Pappu *et al.*, 1994). Following the DVG motif an unusual and unique stretch of 36 aa was found, which is repetitive and rich in threonine and asparagine. A 3-residue sequence (NNT) was repeated three times within this stretch and was followed by six proline residues. The CP of DsMV-Amp1 has several threonine- and asparagine-rich (NGT, NNT, NVT and NVS) potential N-glycosylation motifs in the central region, including some that cluster among the repeat sequences. Similar motifs were also found in the CP of other DsMV isolates (Pappu *et al.*, 1994).

In conclusion, the CP nucleotide and amino acid sequence of DsMV analysed in this study may be useful for the development of both virus resistance strategies and diagnostic tools for DsMV infecting Elephant foot yam.

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SHORT COMMUNICATION

PHOMOPSIS sp. ASSOCIATED WITH POST-HARVEST FRUIT ROT OF KIWIFRUIT IN ITALY

L. Luongo, A. Santori, L. Riccioni and A. Belisario

CRA-Centro di Ricerca per la Patologia Vegetale (CRA-PAV), Via C.G. Bertero 22, 00156 Roma, Italy

SUMMARY

Kiwifruit is a very important commercial crop in Italy, the most important producer of the fruit worldwide, followed by China and New Zealand. The relatively rapid expansion of the area given over to kiwifruit has resulted in the spread of various diseases including those developing post-harvest. Stored kiwifruit showing post-harvest symptoms of inner rot with disorganized, soft and lighter green tissues, known as stem end rot, were investigated. *Phomopsis* isolates recovered from symptomatic tissues were examined morphologically, by DNA sequencing and pathogenicity tests, but could not be identified at the species level. Nevertheless, as reported for *Diaporthe actinidiae*, they were able to induce a soft rot decay that was artificially reproduced only on wound-inoculated fruit. ITS sequences had 98 to 100% identity with *Phomopsis* spp. from grapevine (*N6*), *Rosa* sp., blueberry (*P. vaccinii*) and Norway spruce (*Diaporthe conorum*), but were clearly distinct from *D. actinidiae*. These results call for extreme caution in fruit handling, as they suggest that only wounded fruits are susceptible to infection by this *Phomopsis* sp.

Key words: *Actinidia deliciosa*, fruit diseases, fungal diseases, *Diaporthe*.

Kiwifruit (*Actinidia deliciosa*) is a recently domesticated plant whose fruits are produced mostly by one female cultivar, Hayward, following pollination by the male cultivars Tomuri or Matua (Palombi and Damiano, 2002). This crop is grown on 26,700 ha in Italy, the leading kiwifruit producer in the world, followed by China and New Zealand (Testolin and Ferguson, 2009). The relatively rapid expansion of the area given over to kiwifruit has resulted in the spread of various diseases including those causing post-harvest decay. Kiwifruit can be stored for more than four months if storage conditions are optimal. However, post-harvest diseases

cause severe losses during storage, transportation, marketing, and in retail stores during shelf-life. Postharvest losses have often been underestimated since a considerable number of fruits that look apparently healthy, turn out to be decayed after peeling off their skin.

Several fungi have been reported to be associated with post-harvest rots of kiwifruit, e.g. *Botryosphaera* spp., *Phomopsis* sp., *Alternaria* spp., *Phoma* sp., *Colletotrichum* spp. (Hawthorne *et al.*, 1982; Pennycook, 1985; Koh *et al.*, 2005; Garibaldi *et al.*, 2010), *Botrytis cinerea* and *Phialophora* sp. (Tonini, 2001), *Diaporthe actinidiae* (Koh *et al.*, 2005; Lee *et al.*, 2001). These fungi are all agents of important diseases of stored fruits (Zuccherelli, 1979; Scapin *et al.*, 1983). *Phomopsis* rot, caused by *Phomopsis* sp., has been reported as one of the most damaging among the most common postharvest diseases of kiwifruit, besides Botrytis, Phoma and Sclerotinia rots (Sommer and Beraha, 1975; Koh *et al.*, 2005). The fruit disease caused by *Phomopsis* sp. occurs at the stem-end of the fruit as it ripens, and so it is known as stem-end rot. The brown pubescent skin at the affected end of the fruits becomes soft and lighter in colour than the adjacent firm healthy tissues. When the skin is peeled, the affected flesh appears water-soaked, disorganized, soft and light green. A fermentation scent is often associated with rotted fruit. These symptoms have also been reported as caused by *D. actinidiae*, the *Phomopsis* sp. teleomorph (Sommer and Beraha, 1975; Lee *et al.*, 2001; Kho *et al.*, 2005).

During 2006-2007, kiwifruit produced on a farm located at Colonna (Rome) showing post-harvest symptoms of inner rot were investigated with the aim of identifying the disease agent and searching for *D. actinidiae*, which has never been reported from Italy. Although *Phomopsis* sp. infections of kiwifruit have recently been observed in Italy (Garibaldi *et al.*, 2010), and Greece (Elena, 2009), the symptoms described resembled those induced by *D. actinidiae*, a pathogen occurring in Spain and Portugal (Pintos Varela *et al.*, 2000). Since the difficulties of distinguishing *Phomopsis* species are well known, due to the wide host range of some species and their morphological plasticity (van Niekerk *et al.*, 2005), isolates from kiwifruit were analyzed morphologically and by DNA sequencing and were subjected to pathogenicity tests.

Table 1. *Phomopsis* isolates used in this study.

Isolate	Host	Geographic origin	Accession numbers
<i>Phomopsis</i> sp. ISPaVe1968	Kiwifruit (fruit)	Italy	FN597584
<i>Phomopsis</i> sp. ISPaVe1947	English Walnut	Italy	FN597587
<i>Phomopsis</i> sp. ISPaVe ERK310	Kiwifruit (wood)	Italy	FN597586
<i>Phomopsis</i> sp. ISPaVe ERK27	Kiwifruit (wood)	Italy	FN597585
<i>Phomopsis</i> sp. 6 ATCC 56789	Blueberry	USA	AF317580
<i>Phomopsis</i> sp. 6 STE-U2680	<i>Rosa</i> sp.	South Africa	AF230766
<i>Phomopsis</i> sp. 6 STE-U5160	Grapevine	South Africa	AY485737
<i>Phomopsis</i> sp. 6 STE-U5133	Grapevine	South Africa	AY485741
<i>Phomopsis</i> sp. 6 STE-U5166	Grapevine	South Africa	AY485734
<i>Phomopsis</i> sp. 6 STE-U5161	Grapevine	South Africa	AY485732
<i>Phomopsis</i> sp. 6 STE-U5167	Grapevine	South Africa	AY485733
<i>Phomopsis</i> sp. MAFF 665005	Kiwifruit (fruit)	Japan	AB247175
<i>Phomopsis</i> sp. MAFF 665007	Kiwifruit (fruit)	Japan	AB247176
<i>Phomopsis</i> sp. MAFF 665006	Kiwifruit (fruit)	Japan	AB107890
<i>Phomopsis</i> sp. MAFF 237229	Kiwifruit (fruit)	Japan	AB105145
<i>Phomopsis</i> sp. MAFF 237221	Kiwifruit (fruit)	Japan	AB247165
<i>Phomopsis</i> sp. MAFF 237220	Kiwifruit (fruit)	Japan	AB107884
<i>Phomopsis asparagi</i> MAFF 237556	Kiwifruit (fruit)	Japan	AB107885
<i>Phomopsis theicola</i> STE-U2668	Grapevine	Portugal	AF230762
<i>Phomopsis vaccinii</i> BI-PH02B	Blueberry	Lithuania	EU571096
<i>Phomopsis viticola</i> STE-U2671	Grapevine	Italy	AF230747
<i>Diaporthe</i> sp. G1B	Garlic mustard	USA	EF432278
<i>Diaporthe actinidiae</i> DAR 55786	Kiwifruit (fruit)	Australia	FN668392
<i>Diaporthe conorum</i> CBS 186/37	Norway spruce	Norway	DQ116551
<i>Diaporthe medusaea</i> MAFF 410599	Satsuma mandarin	Japan	AB245073
<i>Diaporthe medusaea</i> MAFF 410311	Poplar	Japan	AB201444
<i>Diaporthe phaseolorum</i> ISPaVe 473-4	Soybean	Italy	AF001021
<i>Diaporthe viticola</i> STE-U2677	Grapevine	Portugal	AF230765

Isolations were made from 30 kiwifruits surface-disinfected with sodium hypochlorite (1%) for 1 min. Skins were peeled and small pieces of flesh (3-5 mm) were excised at the margin of affected tissue and plated onto potato dextrose agar (PDA) (Oxoid, UK). Subcultures were incubated 15 days at 25°C, after which single alfa conidial cultures were made from sporulating colonies. Six such cultures were considered, which produced white aerial mycelial mats with concentric rings, and black spherical or bluntly conical picnidia bearing alpha and beta conidia. Conidia were extruded from ostioles in yellowish masses. Alpha conidia were hyaline, biguttulate, fusoid-ellipsoidal (3-7x1.2-2 µm); beta conidia were hyaline, filiform and curved at one end (16-29x1-1.5 µm). No perithecia were formed on PDA

plates incubated for more than 8 weeks at 22°C, following the protocol described for *D. actinidiae* (Lee *et al.*, 2001; Koh *et al.*, 2005). Colony and conidial morphology were consistent with those of a *Phomopsis* sp. Notwithstanding the great variation in morphological and cultural characteristics between isolates reported for *Phomopsis* spp. (Rehener and Uecker, 1994), all colonies obtained from kiwifruit displayed the same morphological traits.

To confirm morphological identification, sequence analysis of the ITS region of nuclear ribosomal DNA (nrDNA) was performed. For DNA extraction, six fungal strains were grown on PDA medium for 7 days at 25°C. Mycelium was scraped directly from the plates, freeze-dried in liquid nitrogen and homogenized with a

pestle following the protocol of the Puregene for Genomic DNA purification Kit (Gentra Systems, USA). The ITS region was amplified using the universal primers ITS4 and ITS5 (White *et al.*, 1990). PCR reaction was performed in a total volume of 25 µl with approximately 4 ng of DNA template, 0.25 µM of each primer, 0.25 mM of each dNTP, 2.5 mM MgCl₂, and 0.02 U/µl of *Taq* DNA polymerase in 1x *Taq* PCR buffer II (Bioline, USA). Cycling parameters were 95°C for 3 min, followed by 35 cycles of 30 sec at 94°C, 30 sec at 55°C, 1 min at 72°C, and a final extension for 5 min at 72°C. PCR products were visualized in 1% agarose gels after staining with ethidium bromide, and purified using NucleoSpin Extract II (Macherey-Nagel, Germany) according to the manufacturer's instructions. Both strands were custom sequenced (Bio-Fab Research, Italy). Clustal W (Thompson *et al.*, 1994) was used to generate consensus sequences (based on 5' and 3' sequence data). Since all the 550 bp ITS sequences of the *Phomopsis* sp. isolates from kiwifruit were 100% identical, the sequence of a single isolate (ISPaVe1968) was used for comparisons and deposited in GenBank (accession No. FN597584).

Homologous sequences were identified by BLAST analysis (Altschul *et al.*, 1997) against GenBank sequences using NCBI nucleotide BLAST tool (<http://www.ncbi.nlm.nih.gov/blast/>). ITS analysis confirmed the morphological identification.

For phylogenetic analysis, the kiwifruit isolate ISPaVe1968 sequence was compared with those of other *Phomopsis* isolates present in the CRA-PAV fungal collection, a *D. actinidiae* isolate kindly provided by M. Priest of NSW Department of Primary Industries (Australia) and *Phomopsis/Diaporthe* sequences retrieved from GenBank. (Table 1).

Distance-based phylogenetic analysis by neighbor-joining (NJ) method and a tree topology was constructed with the MEGA 4.0 program (Tamura *et al.*, 2007). The robustness of the tree was tested with 1,000 bootstrap trials. Topology resolved ISPaVe1968 isolate in a well supported branch with a bootstrap value of 96. This isolate grouped with sequences belonging to the well characterized *Phomopsis* sp. N6 from grapevine and *Rosa* sp. (van Niekerk *et al.*, 2005) and with *Phomopsis* sp. and *P. vaccinii* from blueberry, and *Diaporthe conorum* from Norway spruce. The identity ranged from 98 to 100% (Fig. 1).

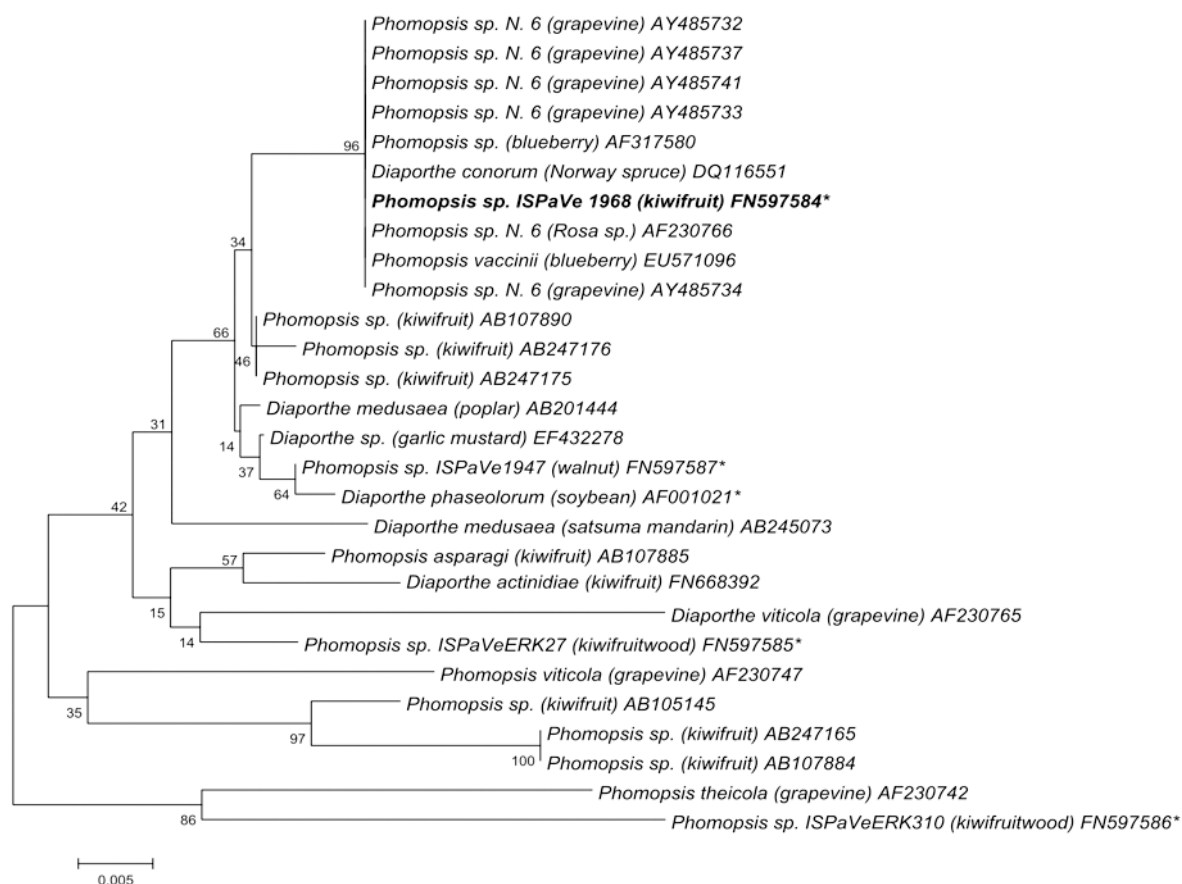


Fig. 1. Neighbor-Joining (NJ) phylogenetic tree showing the taxonomic relationship of *Phomopsis* sp. ISPaVe1968 isolate (accession No. FN597584) with *Phomopsis/Diaporthe* retrieved from GenBank. Numbers at the nodes indicate the level of bootstrap support based on NJ analysis of 1,000 replicates. GenBank accession numbers are on the right of species names. Scale bar unit: number of nucleotide substitutions per site. *Sequences of the CRA-PAV isolate collection.

ISPaVe1968 was clearly separated from Japanese isolates of *Phomopsis* sp. from kiwifruit, and from Italian *Phomopsis* spp. obtained from woody tissues of kiwifruit trees. No significant variation was observed either associated with host or geographic origin, confirming the results of Rehner and Uecker (1994). Our *Phomopsis* sp. from rotted kiwifruit was rather distant genetically from *D. actinidiae* (95% identity) (Fig. 1) and from similar fungi pathogenic to grapevine, e.g. *Diaporthe viticola*, *P. viticola* (van Niekerk *et al.*, 2005) and *P. theicola* (van Rensburg *et al.*, 2006).

For pathogenicity tests, isolate ISPaVe1968 was compared with four other *Phomopsis* sp. isolates from different sources namely, ISPaVeER473-4 (soybean), ISPaVe1947 (walnut) and ISPaVeERK310 and ISPaVeERK27, both from woody kiwifruit tissues. Single-spore cultures of the selected isolates were subcultured on PDA and incubated at 25°C for 2 weeks to obtain α -conidia. Inoculations were made on healthy, untreated, near ripe kiwifruit cv. Hayward. For each isolate twenty fruit were surface-disinfected with sodium hypochlorite (1%) for 1 min, rinsed in sterile water and air-dried. They were inoculated either by injecting 100 μ l of a conidial suspension (10^6 α -conidia/ml) at a depth of 2-3 mm or with a 6-mm mycelium plug cut from the margin of an actively growing culture, following the methods described by Lee *et al.* (2001) and Hawthorne *et al.* (1982). Controls were inoculated either with sterile water or agar plugs. Inoculation points were wrapped with parafilm. Fruits were placed in a



Fig. 3. Symptoms of disorganized soft decay tissue in kiwifruit inoculated with a conidial suspension of *Phomopsis* sp. ISPaVe1968 7 days post inoculation.

sealed plastic bag at room temperature (20-25°C). Seven days post inoculation, symptoms on the fruits were recorded and the length of lesions measured. Inoculations were repeated twice. Isolates morphologically identical to those used for inoculation were recovered. No lesions developed on control fruits. In both experiments, an analysis of variance (ANOVA) followed by a Duncan's multiple range test showed no significant differences ($P \leq 0.01$) in the length of lesions between ISPaVe1968 and the other isolates used in the comparison (Fig. 2). However, only ISPaVe1968 induced symptoms like those observed on naturally infected fruits. These symptoms consisted of a disorganized soft decay clearly visible after peeling the skin (Fig. 3), which worsened as the disease progressed and was accompanied by a sour or fermented scent, thus resembling the whole picture of the disease caused by *D. actinidiae*.

Isolates used for pathogenicity tests were also assessed for colony growth at eight different temperatures (5, 10, 15, 25, 28, 30, 32, 38°C) on PDA in the dark. Three replicate dishes were used. Measurements were made after 6 days incubation averaging two orthogonal diameters per colony. The optimum temperature range for mycelial growth of ISPaVe1968 was 25 to 28°C, being the same at both temperatures. There was no growth under 10°C or over 32°C. These results, agree with those reported for *D. actinidiae* by Koh *et al.* (2005) in Korea.

The genus *Phomopsis* (Sacc.) Bubák contains more than 800 plant pathogenic or saprophyte species (Uecker, 1988). This high number of species, mainly based on host affiliation, is due to the plasticity of morphological and cultural characteristics of *Phomopsis* species. Recent studies have shown, however, that several *Phomopsis* species can infect a wide variety of hosts and that host association is no longer sufficient for their identification (Rehner and Uecker, 1994; Mostert *et al.*, 2001). Genetic data suggest

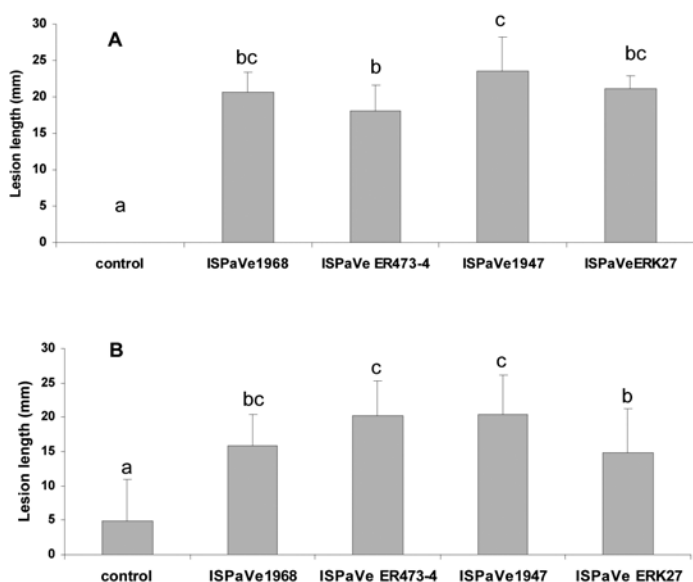


Fig. 2. Lesion length (mm) on kiwifruit inoculated with 100 μ l of a conidial suspension (10^6 α -conidia/ml) (A) and with 6-mm mycelium plug (B). Lesions were measured 7 days post inoculation. Each value represents the mean of twenty replicates and bars indicate the standard deviation (SD). Values with the same letter are not significantly different by Duncan's multiple range test ($P \leq 0.01$).

that apparent morphological and cultural variation may not correlate well with genetic variation and the relationships between *Phomopsis* isolates from different hosts remain a “blackbox” (Johnston *et al.*, 2005).

Our results exclude *D. actinidiae* as the agent of the kiwifruit rot investigated in the present study. Conversely, a *Phomopsis* sp. from rotted kiwifruit induced the typical symptoms of disorganized tissue and soft rot decay of kiwifruit described for *D. actinidiae*. Furthermore, symptoms were obtained only on wound-inoculated fruits (Lee *et al.*, 2001), whereas mycelial plugs and droplets of a conidial suspension did not induce any symptoms on non-wounded fruit. These results suggest that *Phomopsis* sp. cannot readily infect sound fruits, which become susceptible when wounded. Since kiwifruit have numerous opportunities to be wounded during harvest, selection, storage, packing, transportation and marketing (Lee *et al.*, 2001), it is conceivable that postharvest fruit rots occur directly after harvest, cool storage, or a cool storage followed by a period of shelf-life (Pennycook, 1985). The conclusion is that kiwifruit must be handled carefully to prevent wounds that are conducive to *Phomopsis* rotting

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SHORT COMMUNICATION

DIVERSITY OF THE *OMP* GENE IN *CANDIDATUS* LIBERIBACTER ASIATICUS IN CHINAW.Z. Hu^{1,3}, X.F. Wang^{1,2}, Y. Zhou^{1,2}, Z.A. Li^{1,2}, K.Z. Tang^{1,2} and C.Y. Zhou^{1,2}¹ National Citrus Engineering Research Center, Southwest University, Chongqing 400712, China² Key Laboratory of Horticulture Science for Southern Mountainous Regions, Ministry of Education, Chongqing 400715, China³ College of Plant Protection, Southwest University, Chongqing 400716, China

SUMMARY

Huanglongbing (HLB), is a destructive quarantine disease of citrus transmitted by psyllids and grafting, with which three species of *Candidatus* Liberibacter (*Ca. Liberibacter asiaticus*, *Ca. Liberibacter africanus* and *Ca. Liberibacter americanus*) are associated. To investigate the diversity of the *Ca. Liberibacter asiaticus*, the *omp* genes of 23 *Ca. Liberibacter asiaticus* isolates with different symptoms from seven provinces in China were assayed by restriction endonucleases *Taq*I, *Bcl*II, *Apo*I, *Ssp*I and *Eco*RI. Sequence analysis of *omp* genes showed that the HLB isolates under study shared 99% identity with *Ca. Liberibacter asiaticus* (AY642159) and 72% identity with *Ca. Liberibacter africanus* (AY642158). In a phylogenetic tree, *Ca. Liberibacter* isolates grouped in two major clusters. The 23 isolates under study grouped with the Asian strains. The groups of *Ca. Liberibacter asiaticus* was distributed in three subgroups depending on their geographical origin. No genetic evidence for a host determinant was found in *Ca. Liberibacter asiaticus* isolates except the genetic diversity of the geographical origins.

Key words: Huanglongbing (HLB), *Ca. Liberibacter asiaticus*, polymorphism, genetic diversity.

Citrus Huanglongbing (HLB), previously known as Citrus greening, is one of the most destructive citrus diseases in Asia, Africa, North and South America (de Graça, 1991; Bové, 2006). HLB infects almost all citrus cultivars and causes substantial economic losses by reducing fruit production, shortening the lifespan of the trees (Bové, 2006), and even killing trees. More than 60 million trees have already been killed worldwide by this disease (Das *et al.*, 2007; Halbert and Manjunath, 2004). The causal agent of HLB is a non-culturable Gram negative, phloem-limited bacterium (Garnier *et al.*,

1984). Based on the sequence of 16S rDNA, the 16S/23S ribosomal intergenic region and the *rp*/KAJL-*rpo*BC gene cluster, this bacterium proved to belong to *Candidatus* Liberibacter, a new genus in the alpha sub-division of the Proteobacteria (Murray and Schleifer, 1994) comprising three species, i.e. *Ca. Liberibacter asiaticus* in Asia and America, *Ca. Liberibacter africanus* in Africa (Jagoueix *et al.*, 1994), and *Ca. Liberibacter americanus* in Brazil (Coletta-Filho *et al.*, 2005). The pathogens are transmitted by both contaminated budwoods in nurseries and the psyllids *Trioza erytreae* in Africa (McClellan and Oberholzer, 1965) and *Diaphorina citri* in Asia, North and South America (Halbert *et al.*, 2004).

In previous studies, monoclonal antibodies directed against *Ca. Liberibacter* isolates from different geographical areas were shown to react with one or several isolates, but none of the antibodies reacted with all isolates (Garnier *et al.*, 1991; Gao *et al.*, 1993). Gao *et al.* (1993) classified 11 *Ca. Liberibacter asiaticus* isolates from different geographical regions into six distinct serotypes, suggesting that there is genomic variation among isolates (Garnier *et al.*, 1987, 1991). In further studies, molecular techniques provided useful complementary tools for the identification and genetic characterization of *Ca. Liberibacter*. Several molecular targets such as 16S rRNA gene, *rp*/KAJL-*rpo*BC cluster and the intergenic 16S/23S rRNA gene spacer region were used to characterize different isolates of *Ca. Liberibacter*. No significant discrepancy between sequences of these genes amplified from different isolates of *Ca. Liberibacter asiaticus* was found (Garnier *et al.*, 2000; Jagoueix *et al.*, 1994, 1997; Planet *et al.*, 1995; Villechanoux *et al.*, 1993; Okuda *et al.*, 2005; Hocquellet *et al.*, 1999). More recently, the outer membrane protein (*omp*) gene was found to be the most promising for studying inter- and intraspecies variability of '*Ca. Liberibacter asiaticus*' (Bastianel *et al.*, 2005).

Although HLB is prevalent in the citrus-growing areas of southern China, except for Chongqing, information on the genetic diversity of Chinese strains of *Ca. Liberibacter asiaticus* is still very limited (Deng *et al.*, 2008; Ding *et al.*, 2009; Zhou *et al.*, 2009). Thus, the *omp* gene was used to study the diversity of *Ca. Liberibacter*

Table 1. RFLP profiles of isolates of *Ca. Liberibacter asiaticus* in China.

Symptoms	Geographic origin	Host	HLB isolates	Profile				
				<i>ApoI</i>	<i>TaqI</i>	<i>BclI</i>	<i>SspI</i>	<i>EcoRI</i>
Nutrition deficiency	Guangxi	Shatang ju (<i>Citrus reticulata</i>)	GX-stj	1	1	1	1	1
Nutrition deficiency	Guangdong	Luogang sweet orange (<i>C. sinensis</i>)	GD-ltc	1	1	1	1	1
Nutrition deficiency	Sichuan	navel orange (<i>C. sinensis</i>)	SC-qc	3	3	1	1	1
Nutrition deficiency	Jiangxi	Moroge Sanguine orange (<i>C. sinensis</i>)	JX-qxc	2	1	1	1	1
Nutrition deficiency	Yunnan	Satsuma (<i>C. unshiu</i>)	YN-wzm	1	1	1	1	1
Nutrition deficiency	Fujian	Ponkan (<i>C. reticulata</i>)	FJ-lg	1	1	1	1	1
Blotchy mottle	Fujian	Newhall navel (<i>C. sinensis</i>)	FJ-nhe	1	1	1	1	1
Blotchy mottle	Guangdong	Newhall navel (<i>C. sinensis</i>)	GD-tc	1	2	1	1	1
Blotchy mottle	Guangdong	Shatang mandarin (<i>C. microcarpa</i>)	GD-stj	1	1	1	1	1
Blotchy mottle	Guangxi	Nanfeng mandarin (<i>C. reticulata</i>)	GX-nfmj	1	1	1	1	1
Blotchy mottle	Hunan	Shatian pummelo (<i>C. grandis</i>)	HN-sty	1	1	1	1	1
Blotchy mottle	Hunan	Valencia orange (<i>C. sinensis</i>)	HN-xc	1	1	2	1	1
Blotchy mottle	Jiangxi	Nanfeng mandarin (<i>C. reticulata</i>)	JX-nfmj	1	1	1	1	1
Blotchy mottle	Yunnan	Ponkan (<i>C. reticulata</i>)	YN-pg	4	4	3	2	2
Blotchy mottle	Yunnan	Eureka (<i>C. limon</i>)	YN-ylk	1	1	1	1	1
Blotchy mottle	Yunnan	Bintangcheng orange (<i>C. sinensis</i>)	YN-tc	1	1	1	1	1
Blotchy mottle	Yunnan	Shatang mandarin (<i>C. microcarpa</i>)	YN-stj	1	1	1	1	1
Blotchy mottle	Sichuan	Ponkan (<i>C. reticulata</i>)	SC-pg	3	3	1	1	1
Yellowing	Guangxi	Kumquat (<i>Fortunella japonica</i>)	GX-jg	1	1	1	1	1
Yellowing	Guangxi	Ponkan (<i>C. reticulata</i>)	GX-pg	1	1	1	1	1
Yellowing	Hunan	Ehime Kashi No. 14 mandarin (<i>C. unshiu</i>)	HN-ay	1	1	1	1	1
Yellowing	Jiangxi	Newhall navel orange (<i>C. sinensis</i>)	JX-qc	1	1	1	1	1
Yellowing	Yunnan	Bintangcheng orange (<i>C. sinensis</i>)	YN-btc	1	1	1	1	1

acter asiaticus in China, and to evaluate the relationship between genetic diversity and geographic origin.

HLB isolates, collected from different citrus species (mandarin, sweet orange, pummelo, mandarin hybrids, lemon and kumquat) that showed blotchy mottle, yellowing and nutrition deficiency-like symptoms (Table 1) were graft-inoculated onto sweet orange seedlings in an insect-proof greenhouse and maintained at 25 to 32°C.

Total nucleic acids were extracted as described by Zhou *et al.* (2001) and PCR was performed using primers HP1asinv (5'-GATGATAGGTGCATAAAAGTACAGAAG-3') and Lp1c (5'-AATACCCTTATGGGATACAAA-3'), designed on the *omp* gene sequence (Bastianel *et al.*, 2005).

For RFLP analysis, each PCR product was digested with restriction endonuclease *TaqI*, *BclI*, *ApoI*, *SspI* and *EcoRI* following the manufacturer's instruction (TakaRa, Japan).

PCR products of the *omp* gene were purified using Biospin Gel Extraction Kit (Bioer, China) and ligated into pGEM-T easy vector (Promega, USA). Recombinant plasmids were subjected to PCR and restriction analysis to verify the presence of an insert of the expected size. Three randomly selected clones from each isolates were sequenced with an ABIPRISM DNA sequencer (Perkin-Elmer, USA). The results were analyzed by the Neighbor-joining method.

A PCR product of 2.4 kb, expected from *Ca. Liberibacter asiaticus* DNA, was obtained from the 23 HLB-infected plants and the positive control, regardless of their origin or the symptoms shown, but there was no

amplification signal from healthy citrus plants and the water control (Fig. 1).

Following digestion of PCR amplicons from the *omp* gene by restriction endonucleases (*ApoI*, *TaqI*, *BclI*, *SspI* and *EcoRI*) the 23 *Ca. Liberibacter asiaticus* isolates clustered into 6 groups, one of which comprised all *Ca. Liberibacter asiaticus* isolates. *ApoI* and *TaqI* resulted in more polymorphism than others (Table 1, Fig. 2). The polymorphism of isolates from Fujian and Guangxi and Sichuan, and those causing yellowing was very similar, whereas isolates from Yunnan showed a wider polymorphism.

To further investigate the diversity of Chinese *Ca. Liberibacter asiaticus* isolates, their *omp* gene sequences were compared with those present in GenBank database and were used to construct a phylogenetic tree. BLAST analysis showed that the nucleotide sequences of the 23 Chinese *Ca. Liberibacter asiaticus* isolates had

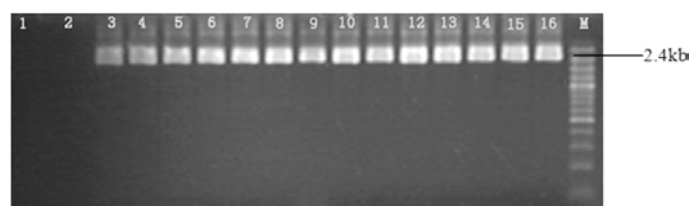


Fig. 1. Agarose gel electrophoresis of the amplicons. Lane 1, water control; lane 2, healthy citrus plant; lane 3, positive control; lane 4-16, HLB samples; lane M, 100 bp DNA marker, the most intense band is 1000 bp.

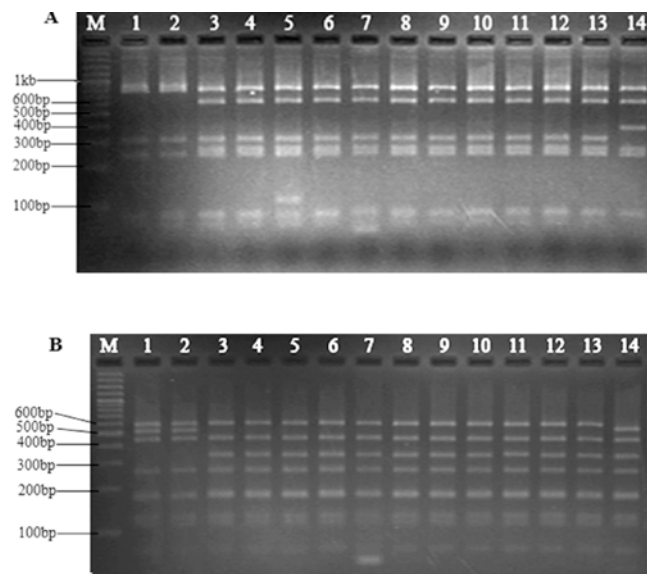


Fig. 2. *ApoI* (A) and *TaqI* (B) restriction profile of the *omp* gene from various isolates of *Ca. Liberibacter asiaticus*. Lane M, 100 bp DNA size markers; lane 1, SC-qc; lane 2, SC-pg; lane 3, HN-xc; lane 4, HN-sty; lane 5, JX-qxc; lane 6, JX-qc; lane 7, GD-tc; lane 8, GX-nfmj; lane 9, GX-jg; lane 10, FJ-lg; lane 11, YN-tc; lane 12, GD-stj; lane 13, YN-btc; lane 14, YN-pg.

a high BLASTn matching with GenBank *Ca. Liberibacter asiaticus* DNA sequences. Furthermore, a 99.3% to 99.9% identity at the nucleotide level was found in the *omp* gene of the 23 Chinese isolates although they were collected from different areas, hosts and showed different symptoms. However, isolates FJ-lg, SC-pg, HN-xc, JX-qxc, GD-tc and YN-pg had 26 nucleotide substitution positions, in agreement with the apparent difference in their polymorphism (Table 2).

As shown by the phylogenetic tree, the isolates clustered into two major groups one of which comprised *Ca. Liberibacter africanus* (AY642158) and the other the *Liberibacter asiaticus* isolates including those from China. However, *Liberibacter asiaticus* isolates separated into three subgroups depending on their geographical origins (Fig. 3). HN-ay and GX-nfmj came together in the first subgroup, isolates from Indonesia (accession No. AB480132), Vietnam (AB480111), Nepal (AY842430), India (AY642159) and SC-qc in the second subgroup, and the others in the third subgroup.

Table 2. *Omp* gene sequence difference among the six *Ca. Liberibacter asiaticus* isolates.

Isolate	Nucleotide positions								
	59	387	395	574	601	926	1090	1250	2252
FJ-lg	A	C	T	A	T	A	A	C	T
SC-pg	A	C	T	A	A	A	A	T	T
HN-xc	A	T	C	A	T	A	A	T	T
JX-qxc	A	C	T	T	T	A	A	T	T
GD-tc	A	C	T	A	T	C	A	T	T
YN-pg	G	C	T	A	T	A	G	T	C

The genetic diversity of *Ca. Liberibacter asiaticus* isolates revealed by PCR-RFLP analysis of the *omp* gene tallies with previous serological data that disclosed the existence of several distinct serotypes within this *Liberibacter* species (Gao *et al.*, 1993). Nevertheless, PCR-RFLP analysis of the *omp* gene may be more manageable and useful than other techniques for isolate discrimination, as supported by the clear-cut separation of Chinese isolates of *Ca. Liberibacter asiaticus* into six distinct groups. Interestingly, although the *omp* gene sequence was highly conserved, still the isolates associated with blotchy mottle showed more polymorphism than those associated with yellowing.

Further investigations of HLB-infected samples showing blotchy mottle seem desirable to confirm this



Fig. 3. Neighbor-joining phylogenetic tree generated from alignment of *omp* sequences from *Ca. Liberibacter* and other α -proteobacteria. The phylogenetic tree indicated that all the *Ca. Liberibacter* isolates were separated into two major groups. The *Ca. Liberibacter asiaticus* isolates group was separated into three subgroups depending on their geographical origins (according to the bootstrap values). HN-ay and GX-nfmj came together as the first subgroup, the isolate from Indonesia, Vietnam, Nepal, India and SC-qc belonged to the second subgroup, and the others as the third subgroup. Bootstrap analysis was conducted using 1000 replicates to assess the reliability of inferred tree topologies.

finding and for exploring other *Ca. Liberibacter asiaticus* genes (Duan *et al.*, 2009) for the presence of additional discriminating polymorphisms.

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SHORT COMMUNICATION

EFFECT OF 1,3;1,6- β -D-GLUCAN ON THE FORMATION OF ABNORMAL *TOBACCO MOSAIC VIRUS* PARTICLES IN THE LEAVES OF *NICOTIANA TABACUM* cv. SAMSUN

A.V. Reunov, L.A. Lapshina, V.P. Nagorskaya and L.A. Elyakova

*Pacific Institute of Bioorganic Chemistry, Far East Branch of Russian Academy of Sciences,
Vladivostok 690022, Russia***SUMMARY**

The carbohydrate-containing polymer 1,3;1,6- β -D-glucan was obtained by transformation of laminaran from the alga *Laminaria cichorioides* with endo- β -1,3-glucanase from marine mollusks. In electron microscope observations of phosphotungstic acid-stained preparations from *Nicotiana tabacum* cv. Samsun leaves inoculated with a mixture of *Tobacco mosaic virus* (TMV) (1 μ g/ml) and glucan (1 mg/ml) or with TMV alone, we found that such preparations contained, along with virus particles of normal diameter (about 18 nm), abnormal (swollen and thinner) particles. The highest number of thin viral particles was found in dips from leaves inoculated with TMV together with glucan. It is suggested that this may be caused by a glucan-mediated increase of TMV particle proteolysis in infected leaves.

Key words: antiviral activity, 1,3;1,6- β -D-glucan, *Laminaria cichorioides*, *Nicotiana tabacum*, TMV infection.

In developing methods for protecting plants from pathogen infection, viruses in particular, investigators are increasingly attracted by induction of natural plant resistance with ecologically safe and biologically active substances (Lyon *et al.*, 1995; Reunov, 1999), among which carbohydrate-containing polymers. It was shown that antiviral activity in plants can be elicited by different substances with a carbohydrate nature, such as: 1,3;1,6- β -D-glucans from fungi (Kopp *et al.*, 1989) and brown algae (Reunov *et al.*, 1996); 1,3;1,4- β -D-glucan from the lichen *Cetraria islandica* (Stübler and Buchenauer, 1996); xyloglucan- (Subíková *et al.*, 1994) and galactoglucomannan-derived oligosaccharides (Slováková *et al.*, 2000), chitosan (Iriti *et al.*, 2006), κ/β -carrageenan from the red alga *Tichocarpus crinitus* (Reunov *et al.*, 2004); and fucoidan from the brown alga

Fucus evanescens (Lapshina *et al.*, 2006). However, the mechanisms underlying the antiviral action of these polysaccharides remain to be elucidated.

It is known that plants respond to pathogen infection by activation of defense-related genes that leads to a large range of responses, including the production of reactive oxygen intermediates, accumulation of pathogenesis-related (PR) proteins, and synthesis of enzymes of the phenylpropanoid pathway (Hammond-Kosack and Jones, 1996). Some of defense genes can be activated in both resistant and susceptible plants in response to pathogen invasion. However, they are expressed more rapidly and to a greater extent in resistant plants challenged with avirulent pathogens (Dong *et al.*, 1991). Certain defense plant genes can be elicited by compounds referred to as elicitors (Shinya *et al.*, 2007).

There is evidence that an important role in cell defense against pathogens involves hydrolases such as chitinases and 1,3- β -glucanases (van Loon *et al.*, 2006), proteases (Van der Hoorn, 2008; Shindo and van der Hoorn, 2008) and RNases (Hugot *et al.*, 2002). Chitinase and 1,3- β -glucanase activity are two major functions of some PR proteins causing degradation of fungal and bacterial cell walls (van Loon *et al.*, 2006). Their antiviral role, however, remains to be established.

Plant proteases and RNases were shown to be involved in a range of processes, including defense responses. The endoproteinase P-69 induced by *Citrus exocortis viroid* (CEVd) in tomato plants is one of the major PR proteins of this host (Vera and Conejero, 1988). The vacuolar processing enzyme identified by Hatsugai *et al.* (2004) in *Tobacco mosaic virus* (TMV)-infected *Nicotiana* plants carrying the *N* resistant gene is believed to play a key role in induction of virus-mediated hypersensitive response, whereas RNases may be involved in antiviral plant resistance caused by different factors (by action of gibberellin, steroid glycosides, vaccination with mild viral strains, etc.) (for review see Reunov and Reunov, 2008). The PR-10 protein from hot pepper was shown to have RNase and antiviral activity (Park *et al.*, 2004) and antiviral proteins from some plants proved to possess strong RNase activity (Begam *et al.*, 2006; Choudhary *et al.*, 2008).

According to our concept (Reunov, 1999; Reunov

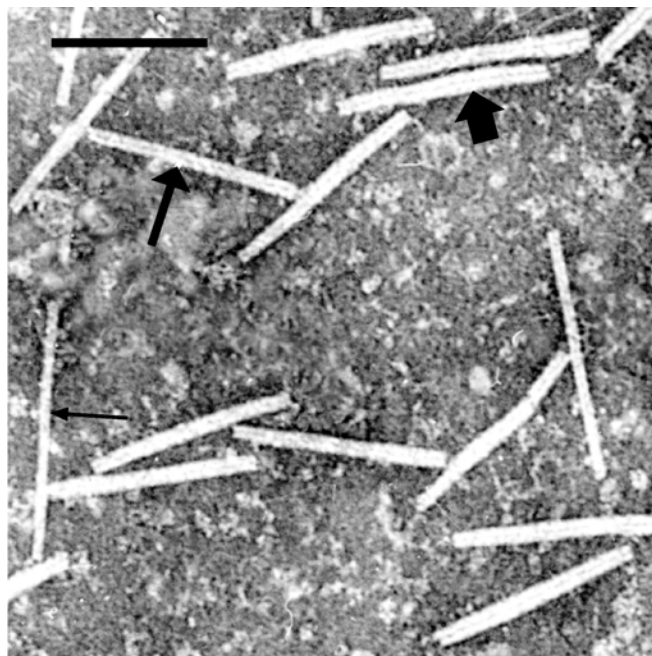


Fig. 1. PTA-stained TMV particles from *N. tabacum* leaves 4 days after inoculation with a mixture of TMV and glucan. The intermediate, thick, and thin arrows indicate normal, swollen, and thin virions, respectively. Bar = 200 nm.

and Reunov, 2008), an important role in antiviral cell defense is played by the lytic compartment, i.e. destruction of viral particles by hydrolases, such as proteases and RNases.

Recently, we showed (L.A. Lapshina *et al.*, unpublished information) that glucan preparations, whose anti-TMV activity was reported previously (Reunov *et al.*, 1996), cause an increase in protease and RNase activity in *N. tabacum* cv. Samsun leaves infected or not by TMV. Taking into account the possibility of destruction of viral particles by such hydrolases, we examined the glucan action on the morphology of TMV particles in Samsun tobacco leaves.

Assays were carried out on leaves of 4-week-old Samsun tobacco plants grown in a greenhouse, using the TMV U1 strain which was purified from systemically infected tobacco leaves according to Otsuki *et al.* (1977).

The glucan preparation was obtained by transformation of laminaran from the brown seaweed *Laminaria chorioides* with endo- β -1,3-glucanase from marine mollusks as described by Zvyagintseva *et al.* (1995). This preparation was composed of a highly branched β -D-glucan with terminal, 3-, 6-, and 3,6-linked glucosyl residues.

Leaves from the middle plant tier were detached, cut in half along the midrib and dusted with carborundum. Test leaf halves were inoculated with a mixture of the virus (1 μ g/ml) and glucan (1 mg/ml) whereas control leaf halves were inoculated with the virus alone. The processed leaf halves were washed with water and

placed in a moist chamber that was then transferred to a constant environment room (24°C, 16 h photoperiod, 16,000 lux, 70% relative humidity).

For electron microscope observations, tissue fragments were cut 4 days post inoculation with a razor blade in a drop of distilled water, which was placed on Formvar-coated grids, desiccated and negatively stained for 5 min with 2% phosphotungstic acid (PTA), pH 7.0. In separate experiments, virus preparations were obtained from leaf tissue pieces fixed for 3 h in 6.5% glutaraldehyde in phosphate buffer pH 7.4. Measurement of the diameter of PTA-stained particles (400 virions from both test and control preparations) was carried out in 20 randomly selected viewing fields at a 50,000 X magnification.

For immuno-electron microscope (IEM) tests the grids coated with virus from leaf dips were placed onto a drop of TMV U1 antiserum and kept in a moist chamber for 15 min at 37°C. A normal serum and an antiserum to *Potato virus X* (PVX) were used as controls. Samples were carefully rinsed with distilled water, desiccated and stained with 2% PTA, pH 7.0.

Negatively stained dips from leaves inoculated with a mixture of TMV and glucan or with TMV alone, contained virions with a normal diameter (about 18 nm) as well as swollen (thick) and thinner particles (Fig. 1). PTA-stained dips from infected leaves previously fixed with glutaraldehyde, also contained normal, swollen and thin virions, approximately in the same proportions as in preparations from unfixed leaves.

IEM assays showed that the TMV antiserum completely decorated virions with a normal diameter (Fig. 2 A-C) and the swollen ones (Fig. 2 B), but not the thinner particles (Fig. 2 C). In virions with variable diameter, decoration was preferably seen on the wider parts of the particle but not on the thinner areas (Fig. 2 A). There was no decoration with control antisera.

Analysis of the distribution of TMV particle diameters showed that dips from infected leaves inoculated with a mixture of TMV and glucan contained substantially more abnormal virions than those from leaves inoculated with TMV alone. Thus, in preparations from leaves inoculated with a mixture of TMV and glucan, 42% of particles had a normal diameter, 13% were swollen and 45% were thinner (Fig. 3). In dips from leaves inoculated with TMV only, 59% of the particles had a normal diameter, 11% were swollen and 30% were thinner.

The present observations have revealed that in negatively stained dips from infected leaves treated or not with glucan, TMV particles undergo significant changes resulting in the formation of abnormal (swollen and thin) virions. It should be emphasized, however, that normal, swollen and thin virions were seen in approximately the same proportions in PTA-stained preparations from infected leaves, regardless of whether they

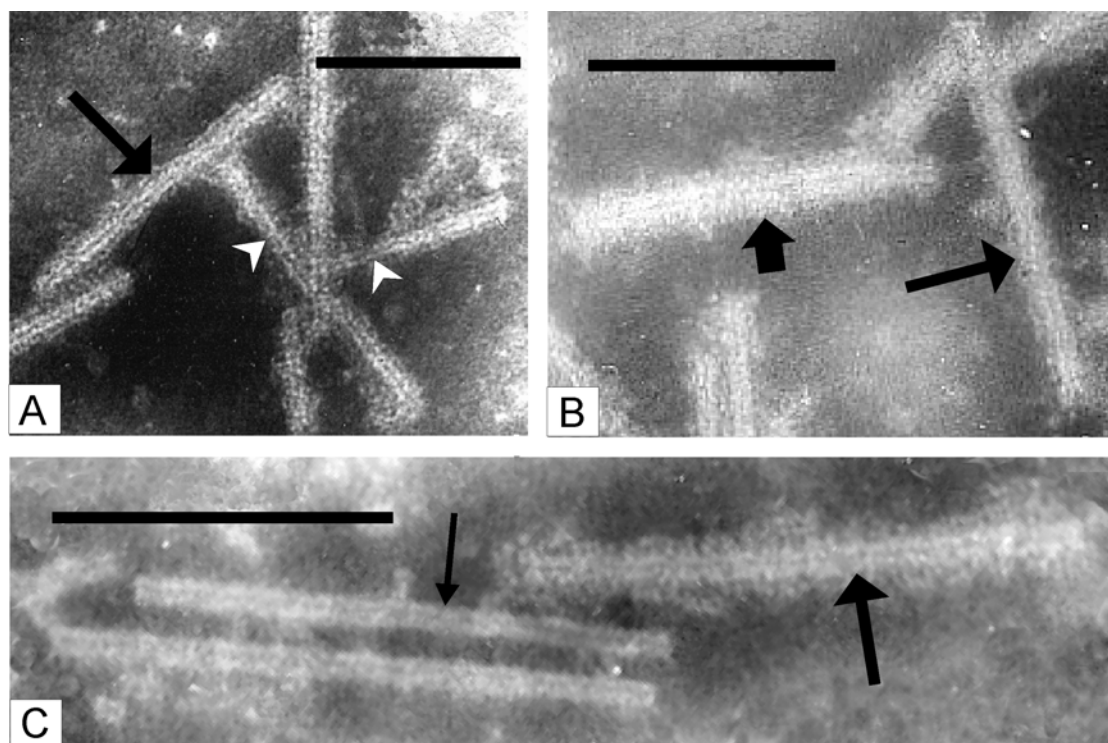


Fig. 2. Preparation of TMV particles from *N. tabacum* leaves four days post inoculation with a mixture of TMV and glucan. The preparation was exposed to a TMV-specific antiserum and stained with PTA. The middle, thick, and thin arrows indicate normal, swollen, and thin virions, respectively. Arrowheads (A) indicate thin virion areas uncoated by the antiserum; the areas of these virions with a normal diameter are decorated. Bars = 200 nm.

were fixed or not. Therefore, the presence of abnormal viral particles in the preparations analyzed does not seem to be artificial. The fact that thin TMV particles, unlike those of normal diameter, lost the ability to bind specific antibodies is likely caused by partial proteolysis of the capsid subunits leading to elimination of the determinants responsible for antibody binding.

Appearance of abnormal TMV particles in infected cells was reported earlier (Reunov, 1999) and attributed to the onset of virus-induced lytic processes leading to destructive intracellular changes affecting the virions. The generation of abnormal particles appears to be a biphasic process. For instance, due to an infection-induced change of the environmental conditions (e.g. pH), TMV protein subunits seem to undergo conformational alterations resulting in partial loosening and untwining. As a result of subunit loosening, the virions become swollen whereas during untwining of the capsid subunit polypeptides, the peptide bonds may be cleaved by proteases. This leads to the appearance of thin virions. Earlier, Everitts *et al.* (1988) described the partial proteolysis of hexons of *Adeno-associated virus 2* (AAV-2) by proteases in a similar fashion.

A glucan-mediated increase of protease activity in tobacco leaves, both infected or not with TMV, has recently been observed (L.A. Lapshina *et al.*, unpublished

information). We suggest that protease activation in cells under glucan action promotes TMV particle destruction leading to an increase in the number of thin viral particles and, thus, it may be one of the protective antiviral mechanisms.

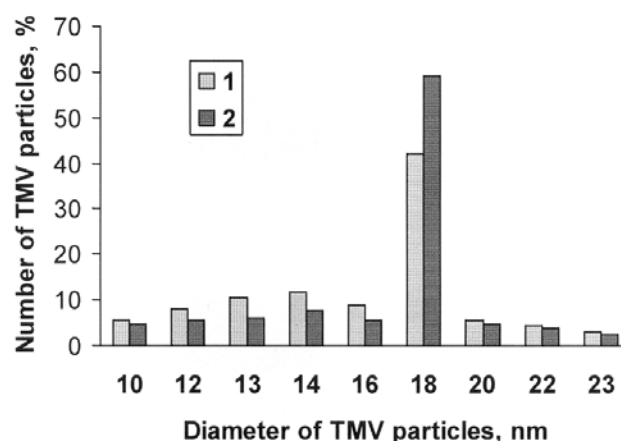


Fig. 3. Diameter distribution of 400 TMV particles in dips from *N. tabacum* leaves four days post inoculation with a mixture of TMV and glucan (1) and with TMV alone (2).

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SHORT COMMUNICATION

DEVELOPMENT AND VALIDATION OF A DIAGRAMMATIC SCALE FOR ESTIMATION OF ANTHRACNOSE ON SWEET PEPPER FRUITS FOR EPIDEMIOLOGICAL STUDIES

C. Pedroso¹, D.A.C. Lage¹, G.P. Henz² and A.C. Café-Filho¹¹ Departamento de Fitopatologia, Universidade de Brasília, 70910-900 Brasília, DF, Brazil² Embrapa Hortaliças, 70359-970 Brasília, DF, Brazil

SUMMARY

A diagrammatic scale for the evaluation of anthracnose severity on sweet-pepper fruits, intended for epidemiological field studies was developed. One hundred symptomatic fruits were collected, photographed, and the images were digitally processed to quantify the percentage diseased area of one half of the fruit surface. Anthracnose symptoms ranged from apparently sound, healthy fruits, to fruits displaying more than 90% diseased area. Fruits were separated into nine classes, from class 0 (0% diseased area, sound fruits) to class 8 (81% or more diseased area), roughly following Horsfall and Barratt (1945) midpoint values. Validation was done with additional images of naturally-infected fruits, collected from a grower's field, presented to 12 raters, half of them experienced and the other half relatively inexperienced. Evaluators' subjective ratings, with and without use of the scale, were compared statistically to the actual severity values (calculated by Quant^R software) by linear regression analysis, and the variance of absolute errors. The t-test was applied to check the null hypotheses of linear coefficient = 0 and angular coefficient $b = 1$. Use of the scale consistently improved accuracy and precision of all raters, bringing scores close to the real severity values. In addition, the scale proved easy to use in field experiments, allowing sequential evaluations in the same plots and fruits, as needed in temporal dynamic studies, and avoiding destructive sampling of infected fruit.

Key words: *Capsicum annuum*, *Colletotrichum acutatum*, *Colletotrichum gloeosporioides*.

Anthracnose is a very destructive disease of solanaceous crops such as sweet pepper (*Capsicum annuum*), hot peppers (*Capsicum* spp.), eggplant (*Solanum melongena*), 'jiló' (*Solanum gilo*) and tomato (*Solanum lycopersicum*) (Hadden and Black, 1989; Pernezny *et al.*, 2003).

Anthracnose in Solanaceae manifests itself mainly by direct fruit infection which causes severe losses in the field and during the postharvest period.

Although poorly documented, losses caused by anthracnose to sweet peppers and hot peppers are high in Brazil, reaching up to 100% in individual fields. Conducive environmental conditions for the disease prevail in the most important pepper production areas, which are located in tropical and subtropical regions. Disease is favored by moderate to high temperatures, high relative humidity and the frequent incidence of rains (Pernezny *et al.*, 2003). In all areas of pepper production, farmers complain of severe outbreaks of anthracnose, especially during the summer (rainy) season. In Turuçú county (southern Brazil), a traditional pepper production area for dried-and-ground market, anthracnose has been responsible for losses of up to 50% almost every year (Silva *et al.*, 2005). Occasionally, sweet pepper anthracnose is also a problem at the seedling stage.

Studies on the *Colletotrichum* x *Capsicum* pathosystem in Brazil focused mainly on host resistance, species identification, and pathogen variability (Fernandes *et al.*, 2002). Three *Colletotrichum* species have been identified as the cause of anthracnose of solanaceous vegetable crops in Brazil: *C. gloeosporioides*, *C. acutatum* and *C. coccodes*. Of these, *C. gloeosporioides* and *C. acutatum* are probably the most important *Capsicum* pathogens. Although most reports of *Capsicum* anthracnose have so far been associated with *C. gloeosporioides* (Manandhar and Hartmann, 1995), *C. acutatum* has been recorded on *Capsicum* in central Brazil (Azevedo, 2006) and elsewhere (Ivey *et al.*, 2004). In addition, Tozze-Junior *et al.* (2006) reported *C. acutatum* as the predominant sweet pepper pathogen in Brazil.

Repeated measurements of disease severity are routinely made for the estimation of treatment effects, for the construction of disease progress curves, and to establish crop loss estimates. Such epidemiological studies must rely on precise, accurate and preferably non-destructive assessment methods. Accurate disease estimates are also important for estimating effects of control measures or to evaluate host resistance. Many diagrammatic scales have been proposed, of which the se-

Table 1. Performance of experienced and inexperienced disease evaluators with and without the use of the pepper anthracnose diagrammatic scale. Intercept (a), angular coefficient (b) and coefficient of determination (R^2) of linear regressions of estimated and real severity values of anthracnose -infected sweet-pepper fruit.

Rater	Without scale			With scale		
	a	b	R^2	a	b	R^2
Inexperienced						
A	0.57	1.22**	0.92	5.60*	1.03	0.91
B	5.77**	0.98	0.96	-1.44	1.00	0.95
C	6.63**	0.94	0.94	-1.27	1.00	0.97
D	7.77**	1.09*	0.93	-0.01	1.03	0.97
E	1.63	1.07*	0.97	1.25	0.98	0.97
F	3.42*	1.06*	0.96	1.04	0.98	0.97
Mean	4.30	1.06	0.95	0.86	1.00	0.96
Experienced						
G	4.81**	1.06*	0.97	1.98	0.98	0.96
H	8.35**	1.02	0.94	1.92	0.99	0.96
I	-4.45*	1.14**	0.95	5.05*	1.01	0.95
J	2.17	0.94	0.94	-0.20	0.96	0.95
K	-0.30	0.96	0.95	-1.81	0.97	0.97
L	2.98*	1.03	0.98	-0.17	1.02	0.96
Mean	2.26	1.03	0.96	1.13	0.99	0.96

*Null hypothesis ($a=0$ or $b=1$) rejected by t-test ($p<0.05$)

**Null hypothesis ($a=0$ or $b=1$) rejected by t-test ($p<0.01$)

ries described by James (1971) are still widely used. Indeed, the use of direct measurement of diseased plant parts has been shown to be superior to more sophisticated risk analysis indicators for disease control (Makowski *et al.*, 2005). Several other scales have recently been created for the estimation of foliar diseases (Michereff *et al.*, 2006). Others have adapted to new pathogens scales previously described for use in other pathosystems, such as the scale to evaluate bean anthracnose (*C. lindemuthianum*) used by Ferraz and Café-Filho (2005). However, no specific or adapted scale has yet been proposed for the study of the dynamics of sweet pepper anthracnose, and the usefulness of such a tool for the study of the temporal progress of the disease for epidemiological studies was discussed recently among pepper pathologists (Pedroso *et al.*, 2007).

This paper describes the development and validation of a new diagrammatic scale created to evaluate anthracnose severity on sweet-pepper fruits under field conditions, intended for epidemiological studies. No other scale is available for disease progress studies of pepper anthracnose.

Diseased fruits for the development of the diagrammatic scale were collected from an experiment at the University of Brasília Biology Field Station, designed to

study the effects of fungicides and soil cover on epidemics of sweet pepper anthracnose. Sweet pepper hybrid 'Maximos F1' (Clause Seeds) was selected for the trial because it is one of the most locally popular genotypes. The experiment followed a randomized complete block design, with four treatments (two fungicides over plastic or straw mulch) and three replicates. Experimental units consisted of 20 plants spaced 0.5 m apart with 0.6 m between rows. Plots were irrigated by overhead irrigation twice a day for 15 min each time in order to stimulate anthracnose development. Plants had no symptoms of anthracnose up to 80 days after transplanting. At this point in time, plots were infested by random distribution of sweet pepper fruit parts that had been infected with virulent isolates of *C. gloeosporioides* and showed abundant anthracnose lesions.

One hundred sweet pepper fruits, at various developmental stages and displaying a wide variety of anthracnose severity, were collected randomly, starting seven days after field infestation. For estimation of the diseased fruit area, each fruit was photographed, and the images processed with the aid of Quant^R v. 1.0.2 software (Vale *et al.*, 2003). The total area and the diseased area of every fruit were estimated. According to the percentage diseased area, a scale was proposed con-

sisting of 9 classes, approximately coincident to Horsfall and Barratt's (1945) midpoint values (Madden *et al.*, 2007; Kranz, 1988): 0%, 1%, 2%, 5%, 10%, 20%, 40%, 60%, 80% (classes 0 to 8, respectively). To facilitate field evaluations, drawings from actual diseased fruits representative of each class were reproduced in black ink (Fig. 1). For some severity classes, two fruits with similar diseased percentage areas were chosen to reproduce the shape, size and distribution pattern of the lesions.

Scale validation was based on an additional set of 40 images of naturally-infected sweet-pepper fruits, collected in a grower's field, displaying a wide range of anthracnose severity levels. Actual severity (% area with anthracnose symptoms) of every digital fruit image was obtained by use of the Quant^R v. 1.0.2 image processing software. The images were then presented to 12 evaluators, divided into two groups, according to their recognized experience as plant disease raters. Six members were assigned to the inexperienced group, composed of undergraduate agriculture students that had never rated disease severity values, while the six members of the experienced group were graduate students or young post-doctoral graduates with more familiarity with plant diseases. Evaluators estimated anthracnose severity of the images twice on computer screens. First, they estimated severity of all 40 images without the aid of the scale. Subsequently, they estimated disease severity for the same images, in a different order of display, with the aid

of the scale. Estimated and actual severities were compared for each subject by linear regression and graphic analysis of absolute errors with and without use of the scale.

Linear coefficient *a* (intercept) and angular coefficient *b* were used to verify the accuracy of the each rater. Intercepts not different from zero and angular coefficients not different from 1.0 are usual indicators of accuracy (Kranz, 1988). The t-test was applied to check the null hypotheses of $H_0: a = 0$ and $H_0: b = 1.0$. According to Nutter Junior *et al.* (1993) intercept values significantly different from zero indicate constant deviation by the rater while angular values significantly different from 1.0 indicate systematic deviations in rating. Evaluator precision was based on the coefficient of determination of the linear regression (R^2) and in the graphic analysis of absolute errors between real and estimated severities (Madden *et al.*, 2007; Nutter Junior and Shults, 1995).

The naturally-infected fruits used in the validation studies had severity values ranging from 1 to 95%. The scale was a useful tool for both experienced and inexperienced raters, improving accuracy and precision of almost all subjects (Table 1; Fig. 2, 3 and 4). Accuracy has been defined as the closeness of an estimate to the truest value and is often measured by linear coefficients *a* and *b*. With aid of the scale, mean *a* and *b* values of inexperienced raters were reduced from 4.30 to 0.86, and from 1.06 to 1.00, respectively (Table 1). Without the

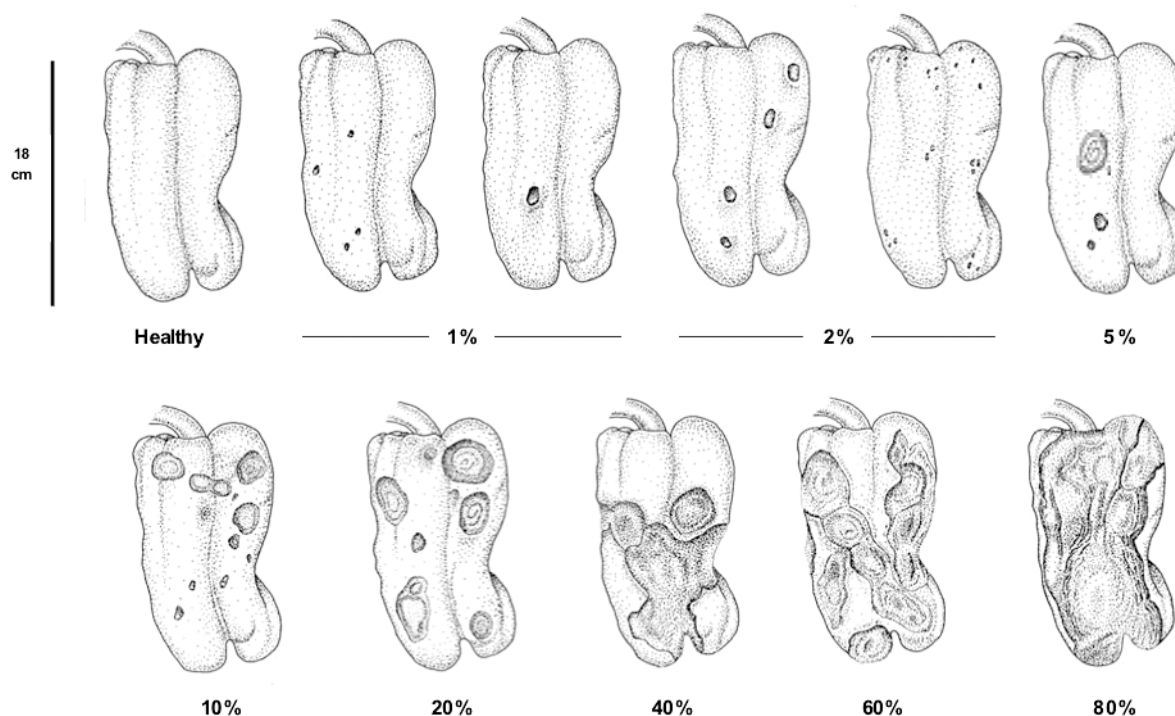


Fig. 1. Diagrammatic scale for the field evaluation of anthracnose severity on sweet-pepper fruits.

aid of the scale, the null hypothesis was rejected for eight and six of the subjects for the intercept and angular coefficient, respectively. With use of the scale, only two intercept values and no angular values were significantly different from zero or 1.0, respectively (subjects A and I, Table 1). Some experienced evaluators (subjects J and K) did not significantly improve their performances with the aid of the scale, because they were already quite accurate originally (Table 1). Without the aid of the scale, a trend towards superestimation was observed, as revealed by the skew of the estimated

severity line to the left of the real severity line. This trend was considerably reduced with the aid of the scale (Fig. 2 and 3).

Precision refers to the variation associated with a sample estimate (Kranz, 1988; Madden *et al.*, 2007). When measured by the values of the coefficient of determination between the estimated and real severity values, raters' precision was originally high ($R^2 > 90\%$) and was not improved by use of the scale (Table 1). However, when the pooled distribution of absolute errors of all evaluators was examined, a clear trend towards greater

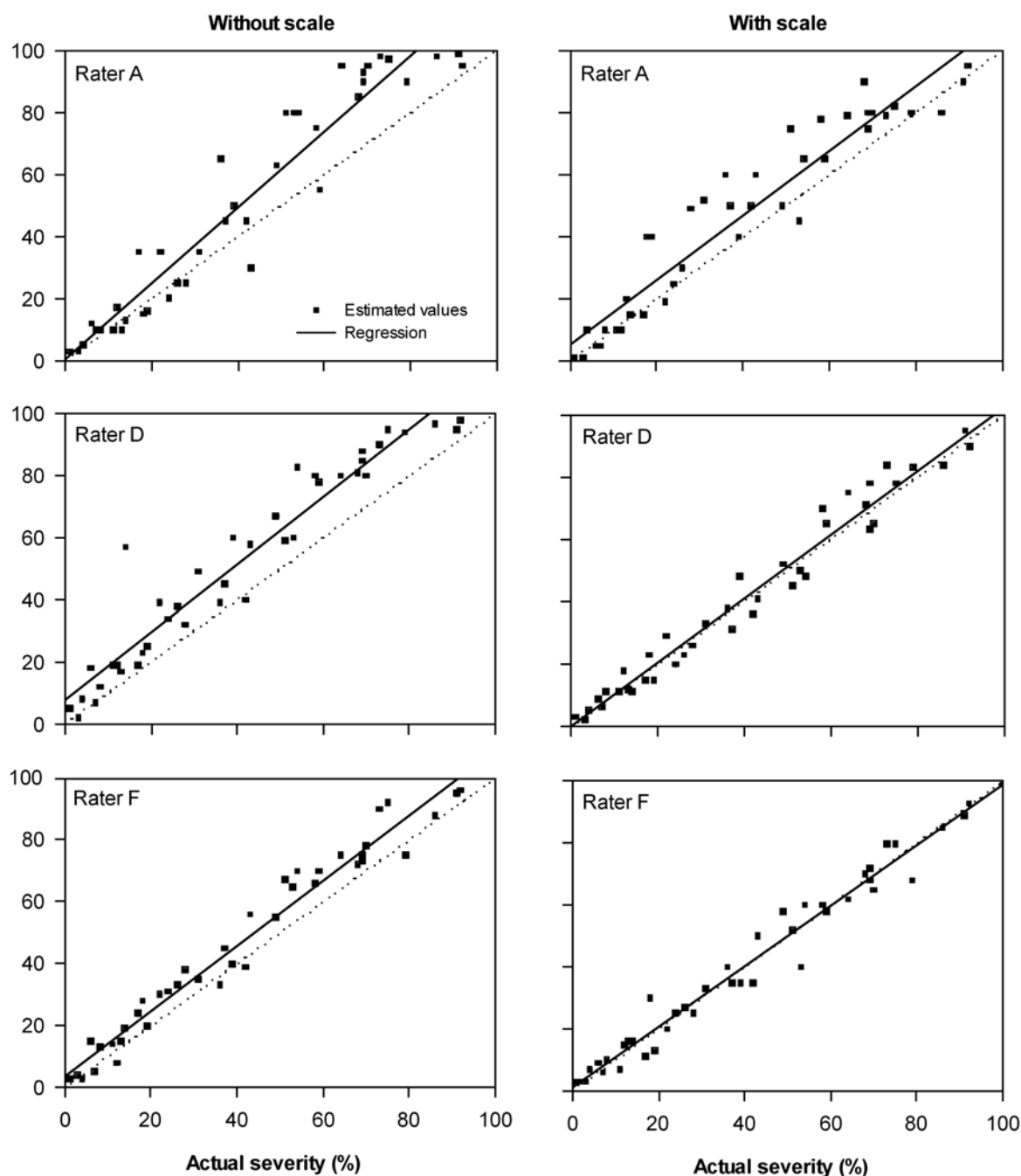


Fig. 2. Estimation of sweet-pepper anthracnose by inexperienced raters, with and without the aid of the diagrammatic scale. The dotted lines represent the ideal response, where estimated severity scores are equal to the real severity values.

precision became apparent, by the greater proximity of absolute errors to the zero axis (Fig. 4).

In conclusion, using naturally-infected fruits collected in a grower's field, the scale consistently improved both accuracy and precision of experienced and inexperienced raters. Furthermore, the proposed diagrammatic scale also proved to be instrumental in facilitating precise measurements of disease and separation of treatment effects in other field experiments, conducted in the dry and rainy seasons (not shown).

Presently, one of the main constraints of evaluating

the progress of anthracnose epidemics of sweet pepper is determining the disease incidence and rating the severity accurately at several points in time, while avoiding destructive sampling. Removal of diseased fruits in epidemiological studies requires destructive sampling, which is a major drawback in the estimation of treatment effects, especially during the early stages of the epidemics, when removal of inoculum sources may greatly impact disease progress, hampering the secondary spread of disease.

Despite its importance, no diagrammatic scale had

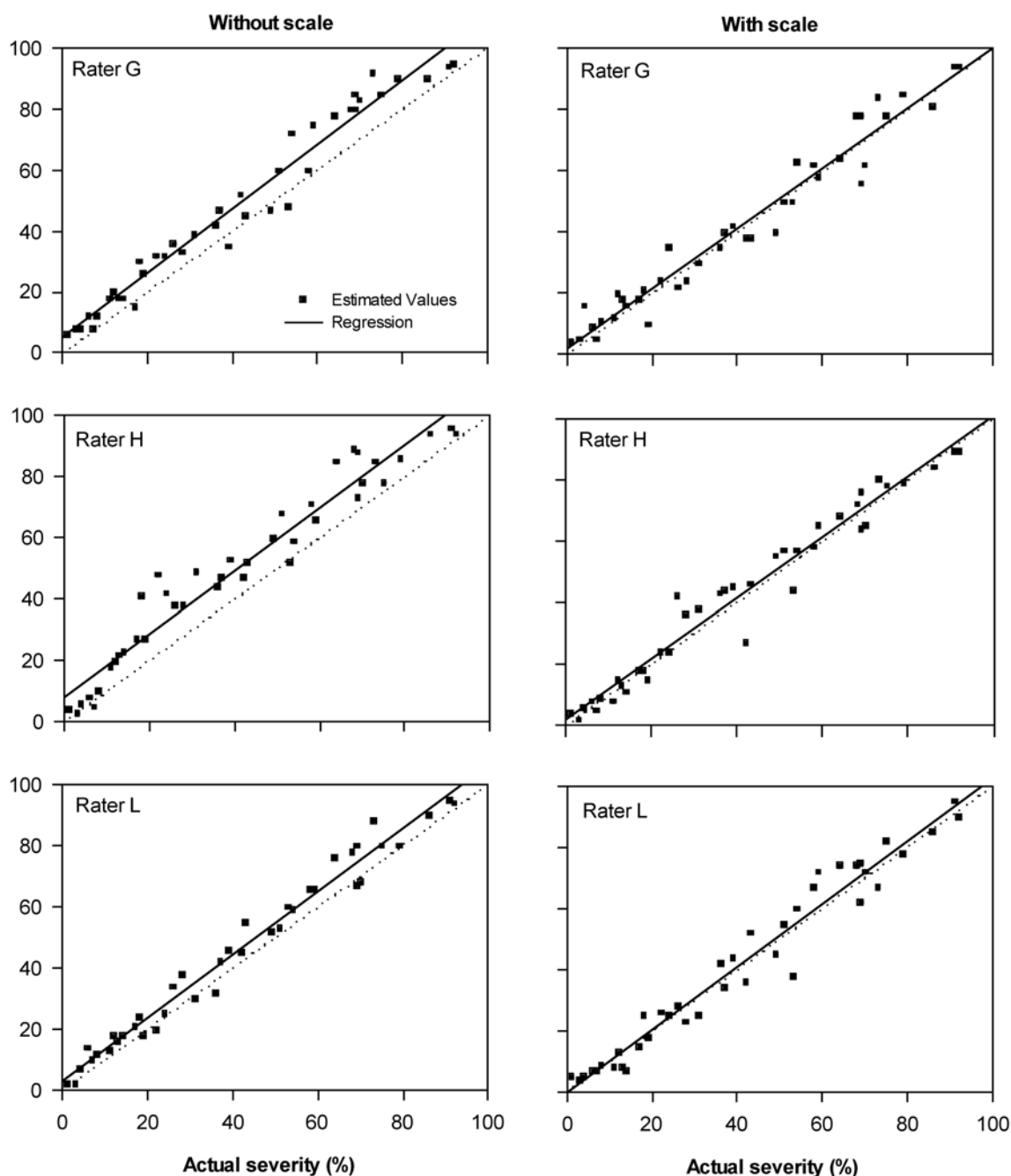


Fig. 3. Estimation of sweet-pepper anthracnose by experienced raters, with and without the aid of the diagrammatic scale. The dotted lines represent the ideal response, where estimated severity scores are equal to the real severity values.

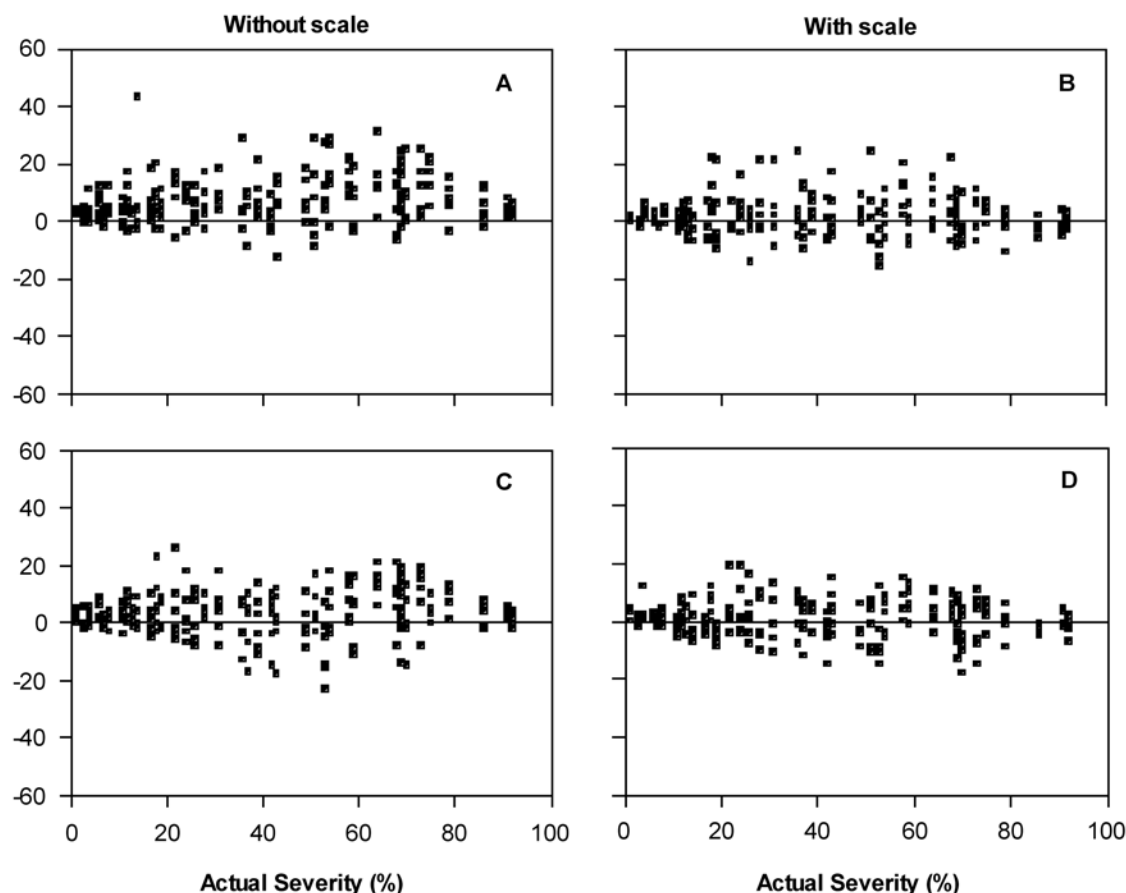


Fig. 4. Absolute errors (difference between real and estimated severities) for the pool of inexperienced raters (A, B) and experienced raters (C, D). Dots represent 6_40 scores given by each group with and without the aid of the diagrammatic scale.

yet been proposed for sweet pepper anthracnose for epidemiological or disease resistance studies. We stress that the main goal of the scale is to support non-destructive, repeated measurement studies, without hindering disease progress. Moreover, detailing the damage realistically is extremely useful for epidemiological studies: the scale was created based on field-infected fruits, and validated using real grower's field samples. Therefore it approximates authentic field situations very closely. Basic recommendations for the preparation of disease scales (Madden *et al.*, 2007), such as the consideration of the maximum limit of disease severity in the field, accurate representation of real disease under field conditions, and visual accuracy in setting the subdivisions, were all considered in the construction of this anthracnose scale.

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SHORT COMMUNICATION

CHARACTERIZATION OF LATENT VIRAL INFECTION OF OLIVE TREES
IN THE NATIONAL CLONAL GERMPLASM REPOSITORY IN CALIFORNIAM. Al Rwahnih¹, Y. Guo², S. Daubert¹, D. Golino¹ and A. Rowhani¹¹Department of Plant Pathology, University of California, One Shields Avenue, 95616 Davis, CA, USA²Foundation Plant Services, 455 Hopkins Road, 95616 Davis, CA, USA

SUMMARY

The oil- and table olive industry in California is growing rapidly. Propagation material is being requested in ever-larger quantities from the USDA National Clonal Germplasm Repository at the University of California, Davis. This collection is recognized as one of the richest sources of olive material in the U.S. The repository maintains 107 different olive varieties imported from 19 different countries. However, the collection's viral infection status has never been systematically analyzed. We have now completed the first comprehensive virus testing of the collection using molecular diagnostic tools. A total of 49 trees from 36 different cultivars were sampled. Though these trees were asymptomatic, the samples from 97.9% of them showed dsRNA profiles indicating viral infection. The 93.8% of these trees tested positive for Olive leaf yellowing-associated virus (OLYaV; an unassigned virus in the *Closteroviridae*) by RT-PCR analysis, while 34.7% were positive for *Cucumber mosaic virus* (CMV). PCR amplicons of the OLYaV heat shock 70 homologue (HSP70h) gene were cloned and sequenced to analyze diversity of isolates from trees originating from different geographical regions. Sequence analysis showed a maximum of 32% divergence between amplicons obtained from these selections.

Key words: California, olive germplasm, olive viruses, OLYaV, CMV, HSP70h gene.

The olive oil industry in California is growing rapidly, and olive tree (*Olea europaea*) cultivation is increasing at an average of 4000 acres per year. High density culture of cvs Arbequina, Arbosana and Koroneiki is prevalent, and the possibility of viral infection and spread through orchards is a growing concern. Spread via infected propagating material likely represents a major means of

virus dissemination through olive acreage (Martelli, 1999). Vector-borne viral spread is a further consideration. Assessment of the infection status of the crop is central to management of the industry.

To date, 15 different viruses belonging to eight different genera have been identified in olive (Martelli, 1999; Cardoso *et al.*, 2004; Fernandes *et al.*, 2006; Alabdullah *et al.*, 2010). Prevalent among them are *Arabis mosaic virus* (ArMV), *Strawberry latent ringspot virus* (SLRSV), *Cherry leaf roll virus* (CLRV), *Olive latent ringspot virus* (OLRSV), *Tobacco necrosis virus* (TNV), *Cucumber mosaic virus* (CMV), *Olive latent virus* -1 and -2 (OLV-1, OLV-2) and *Tobacco mosaic virus* (TMV). Another prevalent olive virus, Olive leaf yellowing associated virus (OLYaV) is the only virus thus far reported in U.S. olive orchards (Essakhi *et al.*, 2006).

The occurrence of double-stranded RNAs (dsRNAs) in a majority of olive stocks tested suggests that the extent of viral infection in olive may be underestimated (Martelli *et al.*, 2002). Most viral infection in olive is symptomless, but can be detected by dsRNA analysis or PCR. dsRNA profiles are indicative of viral infection, but the banding patterns are not definitive indicators of individual virus species. Specific RT-PCR tests are required to verify the identity of the viruses in infected trees. RT-PCR tests are now available for eight olive viruses (Faggioli *et al.*, 2005).

The USDA National Clonal Germplasm Repository (NCGR) at the University of California Davis contains 107 olive varieties from 19 countries. Since the collection is becoming widely used as source material by California growers, we undertook a systematic study using dsRNA and RT-PCR analyses to update the sanitary status record of the repository. We sampled 49 trees by collecting 5-6 cuttings about 12-15 inches in length from 1- to 2-year-old twigs from all four quadrants of each tree. dsRNA was extracted from 30 g of cortical scrapings as described by Grieco *et al.* (2000). Ten µl aliquots of dsRNA from each sample were profiled by acrylamide gel analysis. Seven µl of the remaining dsRNA were used as a template for the synthesis of cDNA primed with random hexamers. The cDNA was used in PCR for screening for ArMV, CLRV, CMV, OLRSV, OLV-1, OLV-2, SLRSV, and OLYaV using the

Table 1. List of olive cultivars in the USDA National Clonal Germplasm Repository which were sampled for dsRNA and RT-PCR tested for *Olive leaf yellow associated virus* (OLYaV) and *Cucumber mosaic virus* (CMV). Cultivars were RT-PCR tested for eight viruses (see text), but were only positive for two. Products from cultivars shown in bold were used for sequencing.

Number	Variety	Origin	dsRNA	OLYaV	CMV	Number	Variety	Origin	dsRNA	OLYaV	CMV
1	Arbequina	Spain	+	+	-	26	Midx-elbasan	Unknown	+	+	+
2	Ascolana tenera 1	Italy	+	+	+	27	Mission	Algeria	+	+	+
3	Ascolana tenera 2	Italy	-	-	-	28	Mission 2	Algeria	+	+	-
4	Ascolana dura	Cyprus	+	+	+	29	Mission 3	Italy	+	+	-
5	Azapa	Chile	+	+	+	30	Mission 4	France	+	+	-
6	Barnea	Israel	+	+	+	31	Mission 5	Unknown	+	+	-
7	Bidh El Hammam	Tunisia	+	+	-	32	Mission 6	Italy	+	+	-
8	Bouquetier	Australia	+	+	+	33	Mission 7	Algeria	+	+	-
9	Chalkidiki	Greece	+	+	-	34	Mission 8	Spain	+	+	-
10	Columello	Unknown	+	+	-	35	Mission 9	Unknown	+	+	-
11	Conservolia	Greece	+	+	-	36	Mission Leiva	Unknown	+	+	-
12	Cypress 31	Unknown	+	-	+	37	Mostazal	Unknown	+	+	-
13	Franklin	Unknown	+	+	-	38	Nevadillo	Spain	+	+	-
14	Frantoio	Italy	+	+	-	39	Oblonga	France	+	+	-
15	Gaidourelia	Greece	+	+	-	40	Ogliarola	Italy	+	+	-
16	Grossa di Spagna 1	Italy	+	+	+	41	Rouget	France	+	+	+
17	Grossa di Spagna 2	Italy	+	-	-	42	San Francesco	Italy	+	+	-
18	Grossane	France	+	+	+	43	Sevillano	Spain	+	+	-
19	Kadesh	Israel	+	+	+	44	Sevillano2	Spain	+	+	-
20	Koroneiki	Greece	+	+	-	45	Sevillano3	Spain	+	+	+
21	Late Blanquette	Australia	+	+	-	46	Sevillano-lovisone	Unknown	+	+	+
22	Leccio	Italy	+	+	-	47	Souri	Palestine	+	+	-
23	Leccino	Italy	+	+	+	48	Tragolea	Greece	+	+	-
24	Lucca	Australia	+	+	-	49	Zitoum	Morocco	+	+	+
25	Manzanilla	Spain	+	+	+						

primers described by Faggioli *et al.* (2005). Positive controls from known infections in well characterized olive material served to confirm the reliability of the tests for all virus analyses. Specificity of the virus assays was confirmed by direct sequencing of PCR products.

The survey revealed an extensive presence of two viruses. Of the sampled trees, 46 (93.8%) were positive for OLYaV and 17 (34.7%) were positive for CMV (Table 1). From the 49 samples examined for the presence of dsRNA, 48 (97.9%) contained bands visualized

electrophoretically. One plant (cv. Grossa di Spagna 2-Italy) was positive for dsRNAs, but was negative for all the viruses specifically tested in the RT-PCR analysis. Another tree (cv. Ascolana tenera 2-Italy) was negative for all specifically tested viruses and also showed no dsRNAs.

Sequences of OLYaV amplicons, generated using primers that amplify 383 nt from the HSP70h gene (Sabanadzovic *et al.*, 1999), revealed a broad range of OLYaV strains infecting the trees. PCR products for sequencing were eluted from agarose gels using the Zymo-Clean Gel DNA Recovery Kit (ZymoResearch, USA), ligated directly into pGEM-T Easy (Promega, USA) according to the manufacturer's instructions, and used to transform *E. coli* DH5 α cells. Clones were sequenced, and the sequences analyzed using Sequence Analysis and Molecular Biology Data Management software Vector NTI Advance™ 11 (Invitrogen, USA). Sequences were subjected to BLAST analysis (Altschul *et al.*, 1997) through the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov>). Clustal W (Thompson *et al.*, 1994) and the neighbor-joining method (Saitou and Nei, 1987) were used to align the multiple OLYaV sequences and determine their relationships. Amplicon sequences were submitted to GenBank under accession numbers from HQ288836 to HQ288849, and from HQ316956 to HQ316961.

These amplicons were sequenced from each of the 34 OLYaV-infected olive cultivars. In each case, the number of infecting viral strains in each tree was estimated from inspection of the sequences of 3-5 clones. Most trees were found to carry only a single viral strain. Trees from three cultivars were found to carry two distinct OLYaV sequences: two OLYaV sequences from a cv. Sevillano-Spain tree were only 88% identical; two OLYaV sequences from cv. Oblonga-France were only 79% identical, as were two OLYaV sequences from a Mostazal-unknown tree.

The genomic variability of OLYaV isolates appeared to be at least as high as that reported for European strains of the virus (Saponari *et al.*, 2002; Fadel *et al.*, 2005; Essakhi *et al.*, 2006). The RNA sequences were found to vary from 98 to 68% in identity with the GenBank reference OLYaV sequence. Of the 37 different OLYaV sequences in this study, 27 differed from the GenBank reference sequence by 15% or more.

A phylogenetic tree was assembled from the 31 OLYaV sequences from trees with single strain infections, plus the 6 sequences from the three trees that were infected with two strains of the virus. In the 37 member phylogenetic tree, the OLYaV diversity in the NCGR was resolved into six groups (Fig. 1). In none of the three cases of trees supporting mixed strain infection did the sequences from the two co-infecting viral strains fall into the same group.

The most divergent isolate represented in Fig. 1, re-

covered from olive tree cv. Nevadillo-Spain, shared only 68% nucleotide sequence homology with the GenBank reference (Fig. 2A). At the amino acid level the identity was 60% (Fig. 2B). The divergence of this OLYaV strain was of such a magnitude that in the phylogenetic tree it sorted into its own single member group (Fig. 1).

The wide variation shown in the OLYaV sequences from the NCGR suggests that further analysis may show OLYaV to be a complex, made up of different viral species. Amino acid variation in the HSP70h gene of

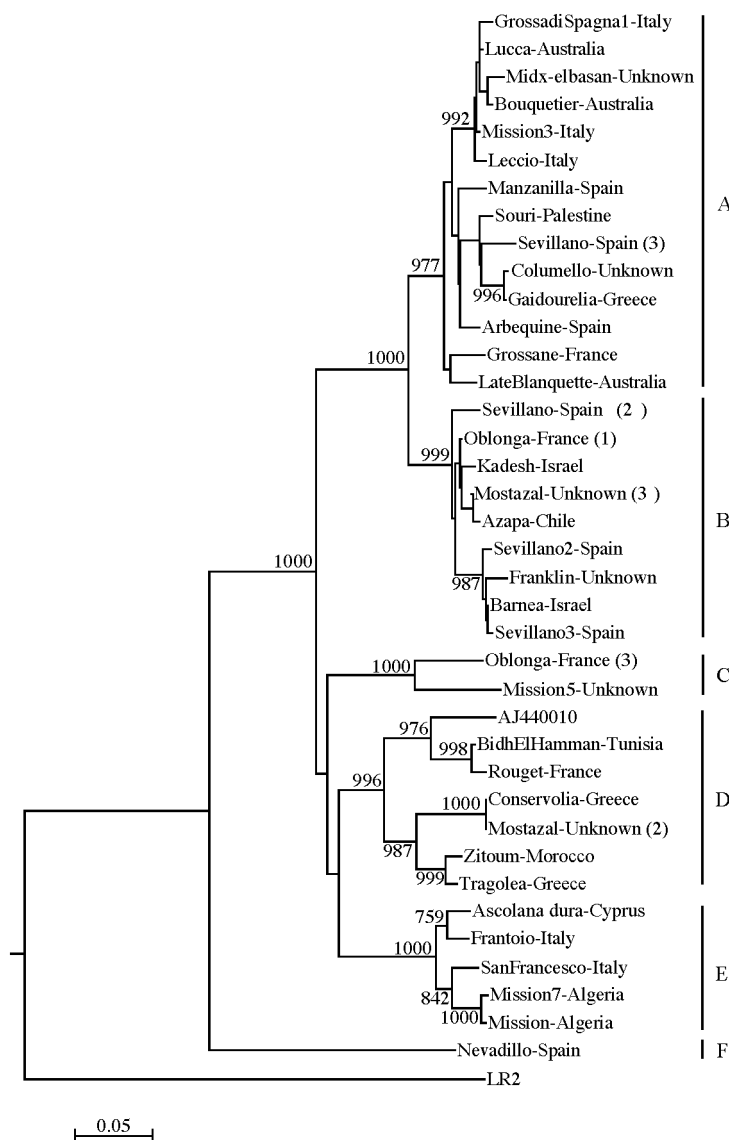


Fig. 1. Dendrogram constructed from genetic distances by the neighbor-joining method using Arithmetic Means with PHYLIP and 1,000 bootstrap replicates. The numbers above the branches are the percent support for the group. Groups were named A-F. Sequences are designated by variety-country of origin of the source trees. AJ440010 is the OLYaV reference sequence. A partial HSP70h sequence of *Grapevine leafroll-associated virus 2* (accession No. AY881628) was used as the outgroup. The three trees infected by two strains of the virus (Sevillano, Oblonga, and Mostazal) are parenthetically numbered.

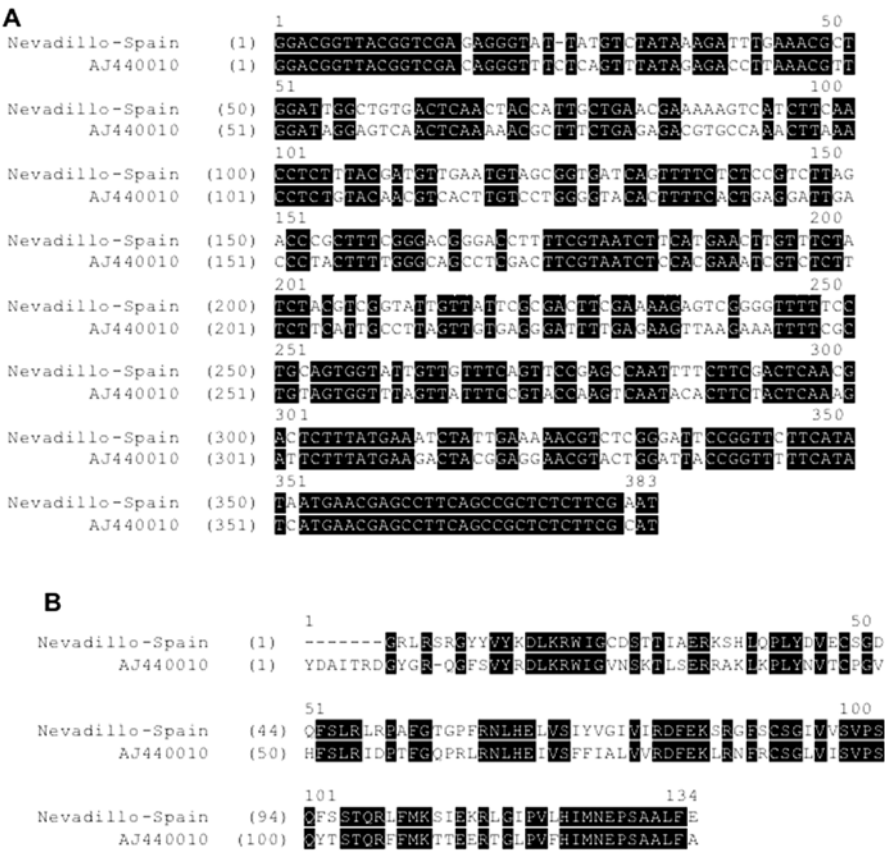


Fig. 2. Alignment of the HSP70h amplicon sequence of the very divergent viral isolate from cv. Nevadillo with the reference sequence in the GenBank. A: nucleotide alignment; B: amino acid alignment. AJ440010 is the OLYaV reference sequence from the GenBank.

10% or more is one of the generally accepted criterion for separate species in the *Closteroviridae* (Fauquet *et al.*, 2005). Further sequence analysis of these genomes will be needed to confirm the taxonomic relationships of the viruses in this complex group.

CMV was detected by PCR in 17 trees. Five amplicons from separate trees were sequenced. Contrary to OLYaV, the CMV sequences were all 100% identical to each other. They differed from the reference sequence in the GenBank at only 2 positions out of 275.

The general sanitary status inspection of the olive collection in the NCGR in Davis revealed no symptoms related to virus infection. The number of infecting virus species detected was low. However, when compared to the status of European olive orchards as reported by Saponari *et al.* (2002) and Fadel *et al.* (2005) the prevalence of latent infection as revealed by the presence of dsRNA species was just as high in the NCGR as it is in Europe. This extensive latent infection was attributable to a pervasive presence of OLYaV.

The source of this pervasive infection is currently unknown. A high percentage of OLYaV infection in southern Italy has been explained by possible viral transmission by olive psylla *Euphyllura olivina* (Costa) and by unidentified mealybugs of genus *Pseudococcus* (Sa-

banadzovic *et al.*, 1999). As a consequence of the findings from this study, a cleanup program using both heat therapy and *in vitro* propagation has been initiated in the NCGR. The cleaned material will be used for the establishment of a new Olive foundation block at Foundation Plant Services at U.C. Davis.

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SHORT COMMUNICATION

COLEUS, A NEW HOST FOR *RALSTONIA SOLANACEARUM* RACE 1 BIOVAR 3 IN INDIAK.N. Chandrashekara¹, M.K. Prasannakumar², M. Deepa³ and A. Vani¹¹ Division of Biotechnology, Indian Institute of Horticultural Research, Hessaraghatta Lake Post, Bangalore 560 089, India² Department of Plant Pathology, University of Agricultural Science, GKVK, Bangalore 560 064, India³ Division of Soil Science and Agricultural Chemistry, Indian Institute of Horticultural Research, Hessaraghatta Lake Post, Bangalore 560 089, India

SUMMARY

Ralstonia solanacearum was isolated from wilting coleus plants (*Coleus forskohlii*) in six commercial nurseries around Bangalore (south India). Colonies of all isolates were irregular with smooth margins, slimy dull white with pink to red centre on SMSA medium, Gram-negative, non-capsulated and non-spore forming. Bacterial identity was confirmed by PCR with species-specific 16S rDNA-based primers OLI1+Y2 and DAS-ELISA. The isolates could infect tomato, potato, and ginger, but failed to infect mulberry and banana. Six isolates were identified as race 1, based on pathogenicity tests, and as biovar 3, based on carbohydrate utilization.

Key words: coleus, PCR, *Ralstonia solanacearum*, race-1, biovar-3.

The medicinal plant coleus (*Coleus forskohlii* Brig.) is grown on 2,500 ha in India (Farooqi and Sreeramu, 2001), but in several parts of southern India its cultivation is limited by a bacterial wilt caused by *Ralstonia solanacearum*, a pathogen recently recorded from this area (Chandrashekara and Prasannakumar, 2010). Affected plants show yellow discolouration, loss of turgidity and drooping of the leaves, wilting and brownish vascular bundles (Fig. 1). A tentative identification of the causal agent as a bacterium was made by the ooze test (Danks and Barker, 2000), following which bacteria were recovered from symptomatic plants, characterized and identified as detailed below.

Six bacterial isolates (GCR-1, GCR-2, DCR-1, KCR-1, RCR-1 and BCR-1) were obtained from wilt-affected coleus plants collected in commercial nurseries in 5 locations around Bangalore (south India) [two isolates from Gouribidanur (GCR-1 and GCR-2), one each from Doddaballapur (DCR-1), Kolar (KCR-1), Rajankunte (RCR-1), and Bangalore BCR-1]]. Bacteria

were isolated on modified SMSA medium (Elphinstone *et al.*, 1998), purified and stored in sterile water (Kelman, 1954; Schaad, 1988) for morphological, physiological, cultural, biochemical and pathogenicity studies along with three reference strains of race-1 biovar-3 (GSC-26, 134, JGS 3/118) provided by the Central Potato Research Institute, Shimla, Himachal Pradesh. Tests for starch hydrolysis, nitrate reduction, oxidase, indole production, esculin hydrolysis, arginine dihydrolase, curdling of skimmed milk were carried out (Anonymous, 1957) and morphological characteristics of colonies were matched following Schaad (1988). Bacterial cells were observed with an electron microscope (JEOL-100S).

The six bacterial isolates from coleus (5×10^8 CFU/ml), suspected to be *R. solanacearum* and three reference cultures (*R. solanacearum*) were differentiated into biovars using carbohydrate fermentation discs (Hayward, 1964). Observations were recorded at 24 h and 48 h for change in colour from light red to white and then to yellow with whitish creamy growth around the disc.

The differential hosts tomato (*Lycopersicon esculentum*) cv. Avinash-II, banana (*Musa accuminata*) cv. Yalla-ki bale, ginger (*Zingiber officinale*), mulberry (*Morus alba*) cv. M-5 and potato (*Solanum tuberosum*) cv. Kufri jyothi were grown in a greenhouse and inoculated by pouring 20 ml of bacterial suspension (5×10^8 CFU/ml) on the root zone which was then covered with soil. Plants similarly inoculated with sterile water served as control.

The hypersensitivity (HR) test was done as described by Granada and Sequeira (1975). Bacterial suspensions (5×10^8 CFU/ml) were infiltrated into fully expanded tobacco leaves (*Nicotiana tabacum* cv. Samsun) and the reaction recorded after 24, 48, and 72 h. Purified bacterial cultures were inoculated and incubated overnight in 100 ml casein peptone glucose (casamino acid 1g/l, peptone 10g/l, glucose 10g/l, pH 7.2) at room temperature.

A newly developed technique was used for DNA extraction, i.e. bacterial cultures (100 ml) were centrifuged (7,000 rpm for 10 min) and pellets were frozen at -20°C for 2 h and thawed for 3 min at 37°C. The suspension in 10 ml lysis buffer (0.15 M NaCl, 0.05 M sodium citrate



Fig. 1. Natural infection of coleus in field condition.

buffer) was incubated with 200 μ l of lysozyme (10 mg/ml) at 37°C for 60 min. The cells were further lysed with 500 μ l of 20% SDS by gentle shaking for 5 min. Thereafter 15 ml extraction buffer was added [2.5 ml of 5 M sodium perchlorate and 12.5 ml of chloroform: isoamylalcohol mixture (24:1)]. Preparations were incubated at -20°C for 2 h and allowed to thaw at room temperature by gentle shaking for 30 min and subsequently centrifuged at 5,000 rpm for 10 min. Supernatant was precipitated with an equal volume of isopropanol, washed with 70% ethanol. DNA was air dried and dissolved in 1 ml $T_{10}E_1$ buffer. After spectrophotometric quantification, the DNA was further analysed on 0.7% agarose gel.

Isolates were PCR-amplified using primers (OLI1 and Y2) corresponding to 16S rDNA (Seal *et al.*, 1993). Further, serological confirmation was carried out by DAS-ELISA (Priou *et al.*, 1999).

Bacterial colonies were highly fluid, irregularly shaped, convex, dark reddish with red center and whitish margin, i.e. morphologically similar to those of *R. solanacearum* (Elphinstone *et al.*, 1998). Bacteria were Gram-negative, rod shaped, non-capsulated and non-spore forming. Biochemical tests were positive for oxidase and negative for indole production, were able to utilize various sucrose, glucose, rhamnose, maltose, mannose, cellobiose, trehalose and mannitol as carbon source, were negative for indole and gelatin lignifications, positive for oxidase, levan production, nitrate reduction and did not produce fluorescent pigments on Kings-B medium. In the electron microscope bacterial cells appeared rod shaped, lophotrichously flagellated with one to three flagella at one end (Fig. 2).

Biovars of *R. solanacearum* are differentiated according to their ability to oxidize disaccharides *viz.*, cellobiose, lactose and maltose and utilize sugar alcohols such as dulcitol, mannitol and sorbitol (Hayward,

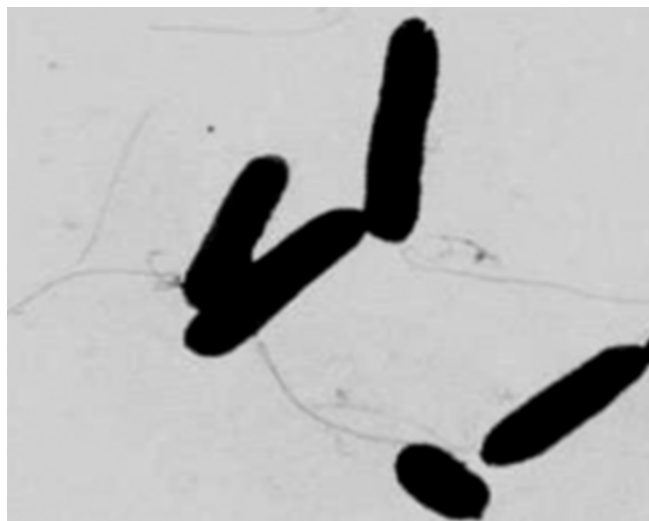


Fig. 2. Electron micrograph of *R. solanacearum* from coleus.

1964). Since our isolates were able to utilize both disaccharides and sugar alcohols they could be assigned to biovar-3.

All isolates produced typical wilt symptoms on their respective host (6 on coleus and 3 reference cultures on potato) (Wang and Berk, 1997) and wilting within 4 to 5 weeks in tomato, potato and ginger, but not in mulberry and banana. These results support the identification of all bacterial isolates from coleus as *R. solanacearum* race-1, which is further supported by the hypersensitive reaction on tobacco leaves within 24 h (Granada and Sequeira, 1975).

Partial sequences of 16S rDNA genes are excellent targets for identification of bacteria at the species level and have been used for PCR amplification (Woese, 1987). The PCR-based diagnostic test using OLI1+Y2 primers specific to 16S rDNA (Seal *et al.*, 1993) yielded 292 bp product expected for *R. solanacearum* (not shown).

Detection of *R. solanacearum* by CIP's post-enrichment DAS-ELISA kit originally developed for identifying latent infection in potato seed tubers was adopted to authenticate the isolates. Coleus isolates that produced positive reaction with bright purple colour at a concentration of 10^4 CFU/ml on nitrocellulose membrane were positively compared with control strips provided with the kit (Priou *et al.*, 1999).

In conclusion, the present study on bacterial wilt of coleus in south India constitutes the first record of *R. solanacearum* race-1 biovar-3 as the casual agent of the disease.

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SHORT COMMUNICATION

THE ARBUSCULAR MYCORRHIZAL SYMBIOSIS REDUCES DISEASE SEVERITY IN TOMATO PLANTS INFECTED BY *BOTRYTIS CINEREA*V. Fiorilli¹, M. Catoni², D. Francia³, F. Cardinale³ and L. Lanfranco¹¹Dipartimento di Biologia Vegetale, Università degli Studi, Viale Mattioli 25, 10125 Torino, Italy²Istituto di Virologia Vegetale del CNR, Strada delle Cacce 73, 10135 Torino, Italy³Dipartimento Colture Arboree – Fisiologia Vegetale, Università degli Studi di Torino, Via Leonardo da Vinci 44, 10095 Grugliasco (TO), Italy

SUMMARY

The majority of plants establish symbiotic associations with arbuscular mycorrhizal (AM) fungi. The symbiosis provides the plants with an improved mineral nutrition and, to some extent, higher tolerance to biotic and abiotic stresses. In this work we have evaluated whether AM symbiosis modifies the response of tomato plants to the attack of the necrotrophic pathogen *Botrytis cinerea*. Leaves of tomato plants, colonized or not by the AM fungus *Glomus mosseae*, were infected with *B. cinerea*. A higher disease index in control plants (60.3%) compared to mycorrhizal plants (37.5%) was observed. To assess the potential involvement of salicylic acid (SA), jasmonic acid (JA) and abscisic acid (ABA) in this response, the levels of these hormones were also measured in the leaves of mycorrhizal and non mycorrhizal plants. While JA was not detected and no differences were observed in the SA content between the two biological conditions, a statistically significant lower content of ABA was detected in mycorrhizal *vs* control plants. Our results show that AM symbiosis reduces disease severity in tomato plants infected by *B. cinerea* and suggest that ABA is one component of the AM-induced lower susceptibility to *B. cinerea*.

Key words: arbuscular mycorrhizal symbiosis, tomato, *Botrytis cinerea*, abscisic acid.

The arbuscular mycorrhizal (AM) symbiosis is the association between soil fungi, belonging to the phylum Glomeromycota, and most land plants (Parniske, 2008). AM fungi colonize the root cortex, developing intercellular hyphae and extensively branched intracellular hyphae called arbuscules (Bonfante and Genre, 2010). Arbuscules are thought to be the site of nutrient exchanges, i.e. the fungus provides the plant with mineral

nutrients (i.e. phosphorus, nitrogen) (Javot *et al.*, 2007; Guether *et al.*, 2009) while, in return, it receives carbon compounds that are essential for the completion of its life cycle. This symbiosis has a multifunctional nature because AM fungi provide other significant benefits to the host plants, including higher tolerance to biotic and abiotic stresses (Pozo and Azcón-Aguilar, 2007; Aroca *et al.*, 2008). In particular, the AM symbiosis can have an important impact on plant interactions with other microbes: mycorrhizal plants often show a reduced susceptibility to pathogens.

Most studies on protection by AM symbiosis deal with the reduction of incidence and/or severity of soil-borne diseases caused by fungi and by oomycetes (for a review see Whipps, 2004). Reports about the effect of mycorrhizal colonization on aboveground diseases are scarcer and less conclusive. It seems that the effect greatly depends on the life-style of the attacker and on the pathogen-host plant combination (Pozo and Azcón-Aguilar, 2007). A higher susceptibility to fungal pathogens (Lindermann, 1994; Dugassa *et al.*, 1996; Gernns *et al.*, 2001; Shaul *et al.*, 1999), aphids (Gange and West, 1994) and viruses (Daft and Okusanya, 1973; Dehne, 1982; Shaul *et al.*, 1999) has been reported for different mycorrhizal *vs* non-mycorrhizal host plant species. However, mycorrhization reduced symptoms caused by a phytoplasma (Lingua *et al.*, 2002), the necrotroph *Alternaria solani* (Fritz *et al.*, 2006) and the bacterial pathogen *Xanthomonas campestris* (Liu *et al.*, 2007) in some host plants. The mechanisms at the basis of these responses are largely unidentified (Pozo and Azcón-Aguilar, 2007). As far as the regulation of plant genes involved in defense response to biotic stress is concerned, contrasting results were obtained in *Nicotiana tabacum* and *Medicago truncatula* shoots (Shaul *et al.*, 1999; Liu *et al.*, 2007).

Many plant reactions to pathogen infection are regulated by the coordinated activity of an elaborate matrix of signal transduction pathways, in which the plant hormones salicylic acid (SA), jasmonic acid (JA), and ethylene (ET) act as key signaling molecules (Grant and Lamb, 2006; Adie *et al.*, 2007). It is commonly accepted that SA promotes resistance against pathogens with a biotrophic life-style, whereas JA and ET act as positive

signals in the activation of defenses against necrotrophic pathogens and herbivorous insects (Thomma *et al.*, 2001; Rojo *et al.*, 2003; Glazebrook, 2005). However, several exceptions to this model are reported, especially for plants other than *Arabidopsis thaliana* and for pathogens whose lifestyle is more difficult to fit into either category (see for example Thaler *et al.*, 2004; Francia *et al.*, 2007). More recently, also auxin (Kazan and Manners, 2009) and abscisic acid (ABA) were shown to play a role in plant-pathogen interactions (Mauch-Mani and Mauch, 2005 and references therein; Fan *et al.*, 2009). Dealing with ABA, ABA-deficient tomato plants (*sitiens*) were shown to be less susceptible to infection by *Botrytis cinerea* (Audenaert *et al.*, 2002; Asselbergh *et al.*, 2007; Asselbergh and Höfte, 2007), *Oidium neolycopersici* (Achuó *et al.*, 2006), *Pseudomonas syringae* (Thaler and Bostock, 2004), and *Erwinia chrysanthemi* (Asselbergh *et al.*, 2008). In addition, exogenous application of this hormone was reported to increase the susceptibility of various plant species to bacterial and fungal pathogens (Mohr and Cahill, 2007; Thaler *et al.*, 2004; Achuo *et al.*, 2006). Altered phytohormone balance has been observed in mycorrhizal plants, although whether this shift has a role in the interaction with the AM fungus is still unknown (Ludwig-Müller, 2000; Hause *et al.*, 2007; Lopez-Raez *et al.*, 2010; Pozo and Azcon-Aguilar, 2007).

In the present study, we evaluated whether the AM symbiosis modifies the response of tomato plants to the attack of the necrotrophic pathogen *B. cinerea*. In addition, the levels of SA, JA and ABA were measured in the leaves of mycorrhizal and non-mycorrhizal plants to assess their potential involvement in this response.

Solanum lycopersicum (cv. Moneymaker) seedlings

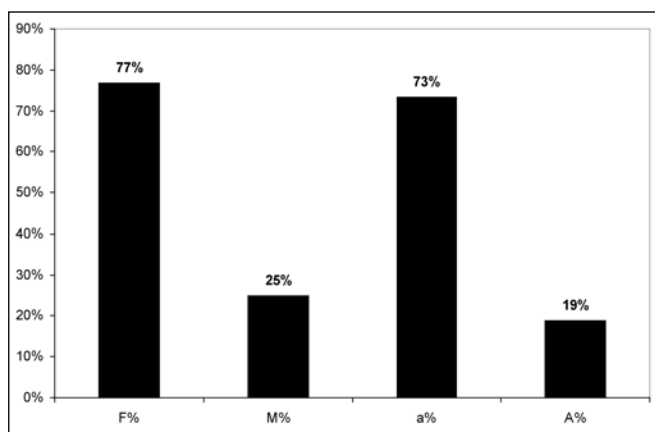


Fig. 1. The mycorrhization level was assessed according to Trouvelot *et al.* (1986) at plant harvest, at the end of each experiment. The different parameters considered were: F% (frequency of mycorrhization); M% (intensity of mycorrhization); a% (percentage of arbuscules within infected areas); A% (percentage of arbuscules in the root system). Similar results were obtained in a second independent experiment.

were inoculated with *Glomus mosseae* Gerd. et Trappe BEG12 (Biorize) as described by Fiorilli *et al.* (2009). The plants were grown in a growth chamber under 14 h light (24°C)/10 h dark (20°C) regime, and watered with 125 ml/plant twice a week, and once a week with a modified Long-Ashton solution with low phosphorus concentration (3.2 μM $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$) (Hewitt, 1966). The plants were harvested 44 days post inoculation.

To verify and evaluate the extent of AM colonization, the mycorrhization level was assessed according to Trouvelot *et al.* (1986) at plant harvest, at the end of each experiment. In the representative example reported in Fig. 1, mycorrhizal plants showed a colonization intensity of 25% and a high percentage of arbuscules within colonized areas (77%). Similar values were obtained for all other experiments.

Forty-two days after AM fungal inoculation, plants were inoculated with *B. cinerea* as follows: agar plugs from V8 medium (5 mm in diameter) with freshly grown mycelium were placed face down on fully expanded leaflets. Before inoculation, micro-wounding was made with a needle to facilitate fungal penetration. For each condition, 50 plants and a minimum of four leaflets per plant were inoculated. Plants were covered for 24 h with plastic bags to guarantee a high relative humidity (95 to 100%). Disease severity was evaluated 48 h after pathogen inoculation by scoring individual leaves for each plant, and assigning a percentage value based on the severity of disease symptoms. In particular, values of 0, 20, 40, 60, 80 or 100%, refer to increasing necrotized or collapsed leaf surface. Average values were then calculated for each plant as means of the percentages assigned to the leaves analyzed for a given plant, thus obtaining the percentage representing the plant disease index. Finally, disease index values for every plant of each treatment were averaged to obtain the disease index for that condition. Data were analyzed statistically by mean comparison by the least significant difference (LSD) test.

Two days after *B. cinerea* infection, the disease index showed the higher susceptibility of control plants (60.3%) compared with the mycorrhizal ones (37.5%) (Fig. 2). Data showed a significant difference between treatments in accordance to the LSD statistical test ($P = 0.00004$). Similar results were obtained from two independent experiments. In addition, based on a qualitative phenotypic analysis, older leaves of mycorrhizal and control plants, exhibited higher disease severity than younger leaves.

Different results were obtained by Shaul *et al.* (1999) who showed that *Nicotiana tabacum* plants colonized by the AM fungus *Glomus intraradices* were more susceptible to *B. cinerea*. This discrepancy might be due to the fact that a different plant-fungus combination was used. Differences in colonization rate, hormonal and tran-

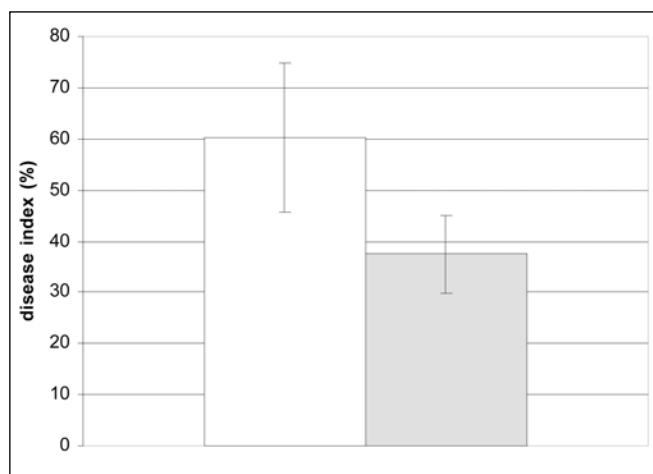


Fig. 2. Mean disease index of control (white bar) and mycorrhizal (hatched bar) plants inoculated with *Botrytis cinerea*. Standard deviations are shown for each bar. A significant difference was found using the LSD statistical test ($P = 0.00004$). Similar results were obtained in a second independent experiment.

scriptional responses to AM colonization and to salt stress between plants infected by *G. intraradices* or *G. mosseae* were previously reported for several plant species (Pozo *et al.*, 2002; Feddermann *et al.*, 2008; Lopez-Raez *et al.*, 2010; Peng *et al.*, 2011) suggesting that these differential responses could be related to the AM fungal genotype. In addition, *G. mosseae* was shown to provide more protection to tomato plants towards a soil-borne pathogen compared to *G. intraradices* (Pozo *et al.*, 2002).

To evaluate whether the response of tomato plants to *B. cinerea* infection was mediated by changes in hormone levels, the amount of JA, ABA and SA was deter-

mined in shoots of control and mycorrhizal plants grown in the same conditions used for the pathogenicity test. To exclude variations of the ABA content due to water stress, all plants were abundantly watered 16 h before harvesting. Shoot samples from six plants for each condition were collected and analyzed by liquid chromatography coupled to mass spectrometry that allowed simultaneous measuring of free JA, ABA and SA from each sample. Samples of mycorrhizal and control plants were placed in liquid nitrogen and freeze-dried. For each sample two aliquots of 10 mg of powdered tissue were extracted according to Forcat *et al.* (2008) with two re-extractions instead of one. Before starting the extraction process internal standards (10 ng of ABA, 12 ng of JA and 200 ng SA) were added to one of the two aliquots to calculate the recovery percentage of each hormone with the method described by Zadra *et al.* (2006).

Samples (50 μ l) were then analyzed by LC-MS/MS (ACQUITY UPLC system, Waters, UK) equipped with a 2.1 mm \times 50 mm \times 1.7 mm ACQUITY UPLC BEH C18 column (Waters) using the binary solvent system described by Saika *et al.* (2007). A flow rate of 0.6 ml min⁻¹ was used to increase the speed of analysis. Solvent gradients and the MS/MS conditions were optimized to obtain the best peaks.

A similar percentage of recovery was observed for SA and ABA (70%) while the recovery percentage was lower for JA (40%).

The ABA content was lower to a statistically significant extent in leaves of mycorrhizal plants compared to control plants (Fig. 3). This condition was already suggested by the expression profiles of ABA-related genes in a microarray study of tomato mycorrhizal and non-mycorrhizal plants (Fiorilli *et al.*, 2009).

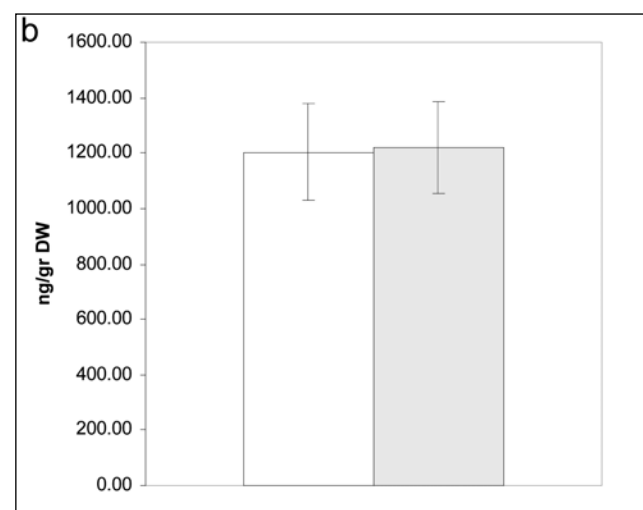
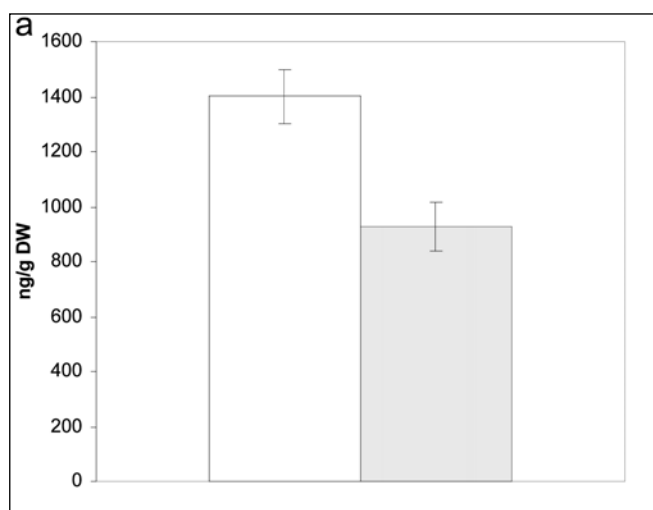


Fig. 3. ABA (a) and SA (b) content analyzed by LC-MS/MS in the leaves of control (white bar) and mycorrhizal (hatched bar) plants. Error bars show the standard errors. A significant difference was found using the program SYSTAT 10 ANOVA with Tukey's test as a post-hoc test.

Interestingly, it has been shown that an accumulation of ABA and low SA levels are probably responsible for an enhanced susceptibility of tomato to *B. cinerea* (Audenaert *et al.*, 2002; Asselbergh *et al.*, 2007). Transcriptional analyses showed an up-regulation of defense-related transcripts prior to infection in the ABA-deficient mutant *sitiens* compared to wild type. In addition, accumulation of hydrogen peroxide following *B. cinerea* infection occurred earlier and more strongly in *sitiens* epidermal cells compared to wild-type cells (Asselbergh *et al.*, 2007). The lower ABA content in leaves of mycorrhizal plants might therefore explain the increased resistance to *B. cinerea* in our experiments (Fig. 2, 3). This is in line with what reported by Audenaert *et al.* (2002), Asselbergh *et al.* (2007) and Curvers *et al.* (2010). The effect of ABA may be indirect, since it is known that there is a complex cross-talk among different plant hormones regulating defense gene expression and disease resistance (de Torres-Zabala *et al.*, 2009; Ton *et al.*, 2009; De Vleeschauwer *et al.*, 2010).

The key role of SA in defense response mechanisms against biotrophic pathogens is well established. In tomato, however, SA is reported to contribute also to defense against *B. cinerea* (Achuo *et al.*, 2002). In spite of this, no statistically significant differences in SA content were observed between the two biological conditions tested in our experiments (Fig. 3). As far as we know, there are no data in the literature concerning SA content in the leaves of mycorrhizal plants (Hause *et al.*, 2007). In contrast, no JA was detected, probably due to the low amount of this hormone in the analyzed samples.

In conclusion, our results show that AM symbiosis reduces disease severity in tomato plants infected by *B. cinerea* and suggest that ABA is one component of the response to *B. cinerea* mediated by AM symbiosis.

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DISEASE NOTE

FIRST REPORT OF THE BREAKDOWN OF THE Yr17 RESISTANCE GENE TO WHEAT STRIPE RUST IN THE GRAND-DUCHY OF LUXEMBOURG

M. El Jarroudi¹, F. Giraud^{2*}, B. Tychon¹,
L. Hoffmann² and P. Delfosse²

¹ Université de Liège, Campus d'Arlon, 185 Avenue de Longwy,
6700 Arlon, Belgium,

² Centre de Recherche Public - Gabriel Lippmann, Département
Environnement et Agro-biotechnologies (EVA), 41 Rue du Brill,
4422 Belvaux, Luxembourg

* Present address: Staphyt/BIORIZON, Rue Magendie/Bordeaux
Montesquieu, 33650 Martillac, France

Yr17 resistance gene (Yr17⁺ cultivars) induces yellow rust resistance in wheat at the seedling stage and operates throughout the plant life. A new Yr17 pathotype that overcomes resistance to wheat stripe rust in Yr17⁺ cultivars was first detected in 1997 in countries neighboring Luxembourg (Bayles *et al.*, 2000). In 1999, stripe rust was suspected to occur also in this country (GDL) and, in 2003, *Puccinia striiformis* f. sp. *tritici* was identified. Disease severity was recorded in the 1999-2009 period in fungicide untreated experiments with various cultivars grown in Everlange, Christnach, Burmerange and Reuler (El Jarroudi *et al.*, 2009). Over this 11-year period, the difference between Yr17⁺ (Achat, Akteur, Astron, Flair) and Yr17⁻ (Aron, Batis, Busard, Drifter, Ritmo, Vivant) cultivars was highly significant, the highest severity being observed in Yr17⁺ cultivars (mean severity of 13%) compared to Yr17⁻ cultivars (0.2%). In 1999, Yr17⁺ cultivars showed the highest disease severity (19%) whereas in 2000 and 2001, the highest severity was observed in cv. Flair (Yr 17⁺) with 30% and 22%, respectively at Everlange, and 50% and 11%, respectively at Christnach. In both years symptoms on Yr17⁻ cultivars were rare (severity <1%) or absent. During 2002-2009 yellow rust occurred with relatively low severity (average 6%) on Yr17⁺ cultivars (cv. Achat at Everlange in 2002, 2008 and 2009, cv. Akteur at Everlange and Reuler in 2007). These observations support the hypothesis of the presence in GDL of the Yr17 pathotype that overcomes the Yr17 gene. This new virulent pathotype appears more aggressive on Yr17⁺ than Yr17⁻ cultivars. Since most resistant European cultivars are Yr17⁺, this renders control strategies based on this monogenic resistance to wheat stripe rust vain.

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Corresponding author: M. El Jarroudi
Fax: +32.63230800
E-mail: meljarroudi@ulg.ac.be

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DISEASE NOTE

FIRST REPORT OF BACTERIAL BLIGHT CAUSED BY AN UNKNOWN ERWINIA sp. ON VERBENA × HYBRIDA IN ITALY

A. Garibaldi¹, D. Bertetti¹, C. Moretti²,
R. Buonauro² and M.L. Gullino¹

¹ Centre for Agro-Environmental Innovation
(AGROINNOVA), Università degli Studi di Torino,
Via Leonardo da Vinci 44, 10095 Grugliasco, Italy

² Department of Agricultural and Environmental Sciences,
Università degli Studi di Perugia, Via Borgo XX Giugno 74,
06121 Perugia, Italy

In June 2008, brown necrotic spots were observed on leaves and stems of *Verbena x hybrida* grown in Piedmont. The incidence of the disease was approximately 50%. Pale yellow, circular bacterial colonies developed on LB agar. Pathogenicity was determined by growing three strains (Ve1, Ve2, Ve3) in LB broth, diluting the suspensions to 10⁷ CFU ml⁻¹, and spraying the foliage of healthy *Verbena x hybrida* plants. Control plants were sprayed with sterile nutrient broth. After 5 days of incubation at 20±1°C, lesions identical to those observed in the field developed only on plants inoculated with strain Ve3. From these a bacterium identical to the inoculated strain was readily recovered. Strain Ve3 was subjected to API 20E system (bio-Mérieux, France) generating the 7digit code 1205373, identical to that reported by Mergaert *et al.* (1984) for the type strain (NCPBP 1578^T) of *Erwinia rhapontici*. Growth occurred at 37°C. When the 16S rDNA gene sequence of strain Ve3 was compared by BLASTn with nucleotide sequences from GenBank, it showed 97% identity with the comparable sequence of *E. rhapontici* ATCC 29283. However, Ve3 strain did not produce the typical *E. rhapontici* pink bacterial colonies and a pink pigment on potato dextrose agar (Wise *et al.*, 2008). Based on cultural, biochemical, nutritional and pathogenicity tests as well as on 16S rDNA sequence, the bacterium isolated from *Verbena x hybrida* seems to belong to an unknown *Erwinia* sp. This is apparently the first report of bacterial blight of *Verbena x hybrida* caused by an *Erwinia* sp. in Italy and in the world.

Mergaert J., Verdonk L., Kersters K., Swings J., Boeufgras J.M., De Ley J., 1984. Numerical taxonomy of *Erwinia* species using API systems. *Journal of General Microbiology* 130: 1893-1910.

Wise K.A., Zhao Y.F., Bradley C.A., 2008. First report of pink seed of pea caused by *Erwinia rhapontici* in North Dakota. *Plant Disease* 92: 315.

Corresponding author: M.L. Gullino
Fax: +39.011.6709307
E-mail: marialodovica.gullino@unito.it

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Journal

Elad I., Volpin H., 1991. Heat treatment for the control of rose and carnation grey mould. *Plant Pathology* **40**: 278-286.

Book

Abel F.B., Morgan P.W., Saltveit M.F., 1992. Ethylene in Plant Biology. 2nd Ed. Academic Press Inc., San Diego, USA.

Book chapter

Alleweldt G., 1987. The contribution of grapevine breeding to integrated pest control. In: Cavalloro R. (ed.). Integrated Pest Control in Viticulture, pp. 369-377. A.A. Balkema, Rotterdam, The Netherlands.

Thesis or Dissertation

Hammer P., 1992. Mechanisms of resistance to infection by *Botrytis cinerea* in rose flowers. Ph.D. Thesis. Pennsylvania State University, University Park, USA.

Proceedings

Mortensen K., Makowsky R.M.D., 1989. Field efficacy of different concentrations of *Colletotrichum gloeosporioides* f.sp. *malvae* as a herbicide for round-leaved mallow (*Malva pusilla*). In: Delfosse E.S. (ed.). *Proceedings of the 7th International Symposium on Biological Control of Weeds, Rome 1988*: 523-530.

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