SHORT COMMUNICATION

OCCURRENCE IN *PSEUDOMONAS AVELLANAE* STRAINS OF GENE SEQUENCES IDENTICAL TO THE PHASEOLOTOXIN *TOX* GENE CLUSTER, PRESENT IN *P. SYRINGAE* PV. *PHASEOLICOLA* AND *P. S.* PV. *ACTINIDIAE*

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SUMMARY

Field symptoms of hazelnut (*Corylus avellana* L.) decline include the presence of pale foliage as well as rapid wilting of leaves and twigs. Representative strains of *Pseudomonas avellanae*, the causal agent of this disease, were assessed for the possible presence of DNA sequences identical to the phaseolotoxin *argK-tox* gene cluster of *P. syringae* pv. *phaseolicola* and *P. s.* pv. *actinidiae*. Different primers all enabling the detection of PCR products corresponding to different regions of *tox* or *argK* genes were used. Cloning and sequencing of PCR products showed that *P. avellanae* strains have in their genomes DNA sequences identical to the *tox* gene cluster of *P. s.* pv. *phaseolicola* and *P. s.* pv. *actinidiae*: a total of about 1,131 bp out of 6,000 bp. Presence of the *argK* gene was not detected. A possible relationship between presence of the *tox* gene sequences and the wilting and yellowing symptoms is discussed.

Key words: Hazelnut decline, DNA sequencing, phytotoxin, phaseolotoxin, *Pseudomonas avellanae*

Phaseolotoxin [N^6-(N-sulfodiaminophosphinyl)-ornithyl-alanyl-homoarginine] is a non-host specific tripeptide phytotoxin produced by *Pseudomonas syringae* pv. *phaseolicola* (synonym: *P. savastanoi* pv. *phaseolicola*), the causal agent of halo blight of bean (*Phaseolus vulgaris*) (Mitchell, 1978), and by *P. s.* pv. *actinidiae*, the causal agent of canker of kiwifruit (*Actinidia chinensis*) (Tamura *et al*., 2002). In addition, one *P. s.* pv. *syringae* strain, isolated from *Vicia sativa*, showed the 1.9 kb DNA fragment specific to part of the phaseolotoxin gene cluster, currently used for detection of the bean halo blight pathogen (Prosen *et al*., 1993), in addition to inhibitory activity to *Escherichia coli* in the agar diffusion test (Tourte and Manceau, 1995).

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P. avellanae, the causal agent of bacterial canker of hazelnut (*Corylus avellana*) and decline in northern Greece and central Italy, induces rapid wilting of leaves and young branches from spring to early autumn. In addition, characteristic pale foliage over the whole tree canopy can sometimes be observed before tree collapse (Scortichini, 2002). *P. avellanae*, *P. s.* pv. *theae* as well as *P. s.* pv. *actinidiae* can be included in the genomospecies 8 *sensu* Gardan *et al.* (1999) as revealed by amplified rDNA restriction analysis (ARDRA), repetitive-sequence PCR analysis (Scortichini *et al.*, 2002a), and by amplified fragment length polymorphism (AFLP) analysis (Manceau and Brin, 2003). Previously, it has been shown that culture filtrates of *P. avellanae* type-strain BPIC 631, isolated in Greece, and ISPaVe 011, isolated in Italy, obtained from still flasks containing a modified IMM medium, induced a hypersensitivity reaction and chlorosis on tobacco leaves, water-soaking and necrotic lesions on lemon fruits, necrotic lesions on string beans and inhibited the growth of *Rhodotorula pilimanae* and *Bacillus megatherium* [?] (Greco and Scortichini, 2004). In addition, all the *P. avellanae* strains tested did not possess the *syrB* gene coding for the production of cyclic lipodepsinonapeptides (e.g. syringomycin), a phytotoxin produced by many *P. s.* pv. *syringae* strains (Scortichini *et al*., 2002b). In addition, Loreti *et al.* (2003) detected in one *P. avellanae* strain the presence of genes related to open reading frames ORF2 and ORF3 of the pathogenicity island of *P. s.* pv. *phaseolicola.*

These findings prompted us to investigate whether the field symptoms (i.e. the rapid tree collapse and the pale foliage) and the phytotoxic and biocidal activity shown *in vitro* by *P. avellanae* might be connected with the phaseolotoxin gene cluster *argK-tox* that appears to be conserved only in a few pathovars of the *P. syringae* complex (Sawada *et al*., 1999; 2002).

We report on the occurrence in strains of *P. avellanae* of gene sequences partly identical to the phaseolotoxin cluster *argK-tox*. These results were obtained by PCR using four different specific primers enabling amplification of the *argK* gene and the different parts of the *tox* gene cluster of *P. s.* pv. *phaseolicola* and *P. s.* pv. *actinidiae* (Prosen *et al*., 1993; dos Marques *et al*., 2000; Sawada *et al*., 2002; Gonzalez *et al*., 2003) as well as by se-

quencing and comparison of selected PCR products. In addition we were able to induce necrotic lesions and light chlorosis on bean leaves and pods by the causative agent of hazelnut decline, *P. avellanae*. The main properties of the strains used are shown in Table 1. All strains were routinely grown on nutrient agar (Oxoid, Basingstoke, UK) containing 3% sucrose (mNSA).

Total genomic DNA was prepared according to Scortichini *et al.* (2002a). Primers for detection of the *argK-tox* cluster in *P. avellanae* strains are reported in Table 2.

The presence of *argK* was assessed using primers designed by Sawada *et al.* (2002).

The PCR reaction was performed in a 30-µL reaction mixture containing 3 µL of template DNA, 0.2 mM dNTPs, 3 mM $MgCl_2$, 1 mM of each primer, 1.25 U *Taq* polymerase (Promega Corp, Madison, WI, USA) and 1 x PCR buffer. Thermal cycling was: initial denaturation at 94°C for 5 min, 35 cycles of denaturation at 94°C for 1 min, annealing at 60°C for 1 min, extension at 72°C for 1 min, a single final extension step at 72°C for 10 min, and final soak at 4°C.

The presence of a fragment of 480 bp of the *tox* gene cluster of *P. s.* pv. *phaseolicola* (dos Marquez *et al*., 2000) was checked in a 30-µL PCR mixture containing 3 µL of template DNA, 0.2 mM dNTPs, 3 mM $MgCl_2$, 1.2 mM of each primer, 0.25 U of *Taq* polymerase (Promega Corp, Madison, WI, USA). Thermal cycling was: initial denaturation at 94°C for 5 min, 35 cycles of denaturation at 94°C for 1 min, annealing at 58°C for 1 min, extension at 72°C for 1 min, a single final extension step at 72°C for 10 min, and final soak at 4°C.

The presence of a fragment of 1.9 kb of the *tox* gene of *P. s.* pv. *phaseolicola* (Prosen *et al*., 1993) was checked in a 30-µL PCR mixture containing 3 µL of template DNA, 0.2 mM dNTPs, 3 mM $MgCl_2$, 0.4 mM of each primer, 1,25 U of *Taq* polymerase (Promega Corp, Madison, WI, USA). Thermal cycling was: initial denaturation at 94°C for 5 min, 35 cycles of denaturation at

Table 1. *Pseudomonas* strains used in this study.

Strain	Host plant	Origin	Year of isolation
Pseudomonas avellanae BPIC 631^T	*Corylus avellana*	Greece	1976
Pseudomonas avellanae BPIC 632	*Corylus avellana*	Greece	1976
Pseudomonas avellanae BPIC 1077	*Corylus avellana*	Greece	1987
Pseudomonas avellanae ISPaVe011	*Corylus avellana*	Italy	1991
Pseudomonas avellanae ISF 111	*Corylus avellana*	Italy	2004
Pseudomonas avellanae ISF 112	*Corylus avellana*	Italy	2004
P. syringae pv. *actinidiae* NCPPB 3739^T	*Actinidia chinensis*	Japan	1984
P. syringae pv. *theae* CFBP 4097	*Camellia sinensis*	Japan	NK
P. syringae pv. *phaseolicola* 1448A	*Phaseolus vulgaris*	Ethiopia	1985
P. syringae pv. *phaseolicola* 1449B	*Lablab purpureus*	Ethiopia	1985
P. syringae pv. *phaseolicola* 3634	*Phaseolus coccineus*	Italy	1988

BPIC : Culture Collection of the Benaki Phytopathological Institute, Kiphissia-Athens, Greece
CFBP: Collection Francaise de Bactéries Phytopathogènes, Angers, France
ISF : Culture Collection of C.R.A.-Istituto Sperimentale per la Frutticoltura, Roma, Italy
ISPaVE: : Culture Collection of C.R.A.-Istituto Sperimentale per la Patologia Vegetale, Roma, Italy
NCPPB: National Collection of Plant Pathogenic Bacteria, York, United Kingdom
^{T}: type-strain; NK: not known

Table 2. Primers used in this study to amplify different portions of the *tox-argK* gene cluster.

Primer sequence	Base pair length	Reference
PHA19: 5'-CGTCTGTAACCAGTTGATCC-3' PHA95: 5'-GAATCCTTGAATGCGAAGGC-3'	480	dos Marques *et al*., 2000
HM6: 5'-CGTGTCCGTGGATAAAAGC-3' HM13: 5'-GTTGAATTTCACTACCCG-3'	1.900	Prosen *et al*., 1993
PHTE-F:5'-AATATAGGCTTCAACTTCCTC-3' PHTR: 5'-CCAGGTCAACTCACTTCCG-3'	3.025	Gonzalez *et al.* 2003
OCTF-03: -5'-GACCGTCAAGGAAGAATTCGGGCGC-3' OCTR: 5'-CGACCTTGTTGACCTCCCG-3'	632	Sawada *et al*., 2002

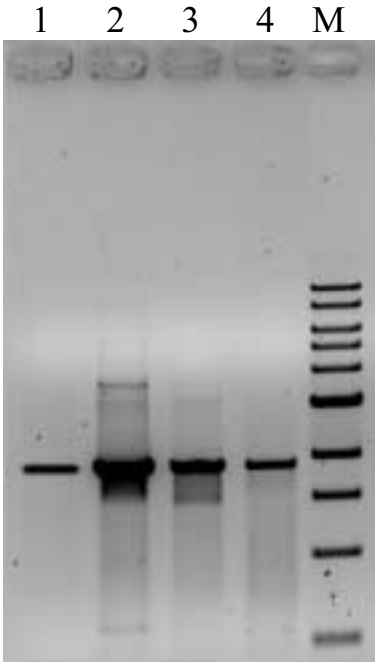

Fig. 1. PCR amplification of a 1,900 bp fragment of the *tox* gene of *Pseudomonas syringae* pv. *phaseolicola* (Prosen *et al*., 1993) in *P. avellanae*, *P. s.* pv. *actinidiae* and *P. s.* pv. *phaseolicola* strains, using the primers shown in Table 2. The expected product is present in *P. s.* pv. *phaseolicola*, *P. s.* pv. *actinidiae* and *P. avellanae*. M: molecular size marker (Kb) 1 kb-ladder (BioLabs, Ipswich, MA, USA). Lane 1, *P. avellanae* ISPaVe 011,; lane 2, *P. s.* pv. *actinidiae* NCPPB 3739; lane 3, *P. s*. pv. *phaseolicola* 1448A; lane 4, *P. avellanae* BPIC 631.

94°C for 1 min, annealing at 60°C for 1 min, extension at 72°C for 1 min, a single final extension step at 72°C for 10 min, and final soak at 4°C.

The presence of a fragment of 3.0 kb of the *phtE* locus of the *tox* gene cluster of *P. s*. pv. *phaseolicola* (Gonzalez *et al*., 2003) was checked in a 30-µL PCR mixture containing 3 µL of template DNA, 0.2 mM dNTPs, 3 mM $MgCl_2$, 1.2 mM of each primer, 1.0 U of *Taq* polymerase (Promega Corp, Madison, WI, USA). Thermal cycling was that of Gonzalez *et al*. (2003).

PCR products of *P. s.* pv. *phaseolicola* 1448A and those of *P. avellanae* BPIC 631 showing a fragment of the size expected for the the phaseolotoxin *argK-tox* gene cluster, were analyzed by electrophoresis in 1% agarose gels. In particular, the 480 bp and 1,900 bp fragments of the *tox* gene cluster were sequenced. The bands of expected size were isolated with the Wizard SV Gel and PCR clean up system (Promega Corp, Madison, WI, USA), and cloned into pGEM-T easy vector (Promega Corp, Madison, WI, USA). Ligated plasmids were transformed into competent *E. coli* strain TOP10 F' cells (Invitrogen, Milano, Italy) and the bacteria spread onto LB agar plates containing 50 µg.ml^{-1} ampicillin, 0.5 mM IPTG, and 80 µg.ml^{-1} X-gal. Plasmid DNA isolated from several white colonies was purified and digested according to Sambrook *et al.* (1989), and digested with *EcoR I* to verify the presence of the inserts. These clones were sequenced with primers T7: 5'-TAATACGACTCACTATAGGG-3' and SP6: 5'-GATTTAGGTGACACTATAG:3' by Primm (Milano, Italy).

P. avellanae as well as *P. s*. pv. *phaseolicola*, *P. s.* pv. *actinidiae* and *P. s.* pv. *theae* strains were inoculated to bean (*Phaseolus vulgaris* L.) cv. Sofia leaves and cv. Corallo pods to verify possible disease development. For inoculation of bean pods, the strains listed in Table 1 were grown for three days on mNSA, at 18°C. Bacterial suspensions of 1 to 2. 10^8 cfu. ml^{-1} were then prepared in sterile 0.85% NaCl (physiological saline, PS). Bean pods were inoculated by placing 10 µl of the bacterial suspension on the pod and, immediately pricking the pod through the drop. Ten drops for each strain were used. Sterile PS, inoculated in the same way, served as negative control. Similarly, fully expanded leaves of pot-grown bean plants cv. Sofia were inoculated with the same strains. The leaves were infiltrated using a sterile syringe until they appeared water-soaked. For each strain, six leaflets were inoculated. Sterile PS

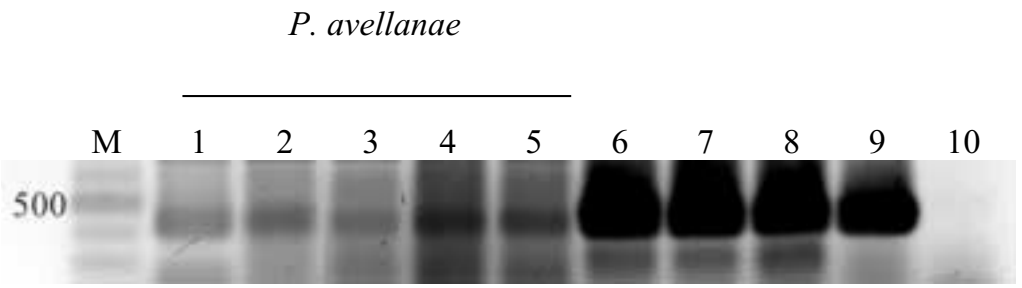

Fig. 2. PCR amplification of a 480 bp fragment of the *tox* region gene of *Pseudomonas syringae* pv. *phaseolicola* (dos Marques *et al*., 2000) in *P. avellanae*, *P. s* pv. *actinidiae*, *P. s.* pv. *theae* and *P. s.* pv. *phaseolicola* strains, using the primers shown in Table 2. The expected product is present in *P. s.* pv. *phaseolicola*, *P. s.* pv. *actinidiae* and in *P. avellanae*, and absent in *P. s.* pv. *theae*. M: molecular size marker (bp) (100 bp DNA ladder; Promega). Lane 1, *P. avellanae* ISF 111; lane 2, *P. avellanae* ISF 112; lane 3, *P. avellanae* BPIC 1077; lane 4, *P. avellanae* BPIC 631; lane 5, *P. avellanae* BPIC 632; lane 6, *P. s*. pv. *phaseolicola* 3634; lane 7, *P. s*. pv. *phaseolicola* 1449B; lane 8, *P. s*. pv. *phaseolicola* 1448A; lane 9, *P. s.* pv. *actinidiae* NCPPB 3737; lane 10, *P. s.* pv. *theae* CFBP 4097.

was infiltrated as negative control. The pods and the plants were placed in a glasshouse where the temperature never exceeded 18°C, to incite the appearance of the halo blight symptom. The symptoms were checked for up to ten days after the inoculation.

Of the four primer pairs tested, only two (PHA19 and PHA95 and HMA6 and HMA13), allowed amplification of PCR products of the same size as the phaseolotoxin *tox* gene cluster (Figs 1 and 2). Sequencing of these products revealed complete identity between bp 435bp and 696bp of *P. avellanae* BPIC 631 and the corresponding sequences of *P. s.* pv. *phaseolicola* 1448A, indicating that c. 19% of the *tox* gene cluster is also present in *P. avellanae*. The sequences have been deposited in the GenBank nucleotide sequence database (Accession numbers: AM283540 and AM283541).

The pathogenicity tests performed with on bean pods confirmed that only *P. s.* pv. *phaseolicola* induces the water-soaked symptoms four-five days after inoculation. All the other pathogens tested, including *P. avellanae*, incited only a necrotic lesion around the inoculation site. Inoculation with the *P. avellanae* strains on bean leaves gave a water-soaked area that lasted for two-three days. However, afterwards no enlargement of the symptom or the typical halo blight was observed. *P. s.* pv. *actinidiae* and *P. s.* pv. *theae* incited a hypersensitivity reaction only. No reaction was observed upon the infiltration with sterile PS.

This study shows that sequences of the *tox* gene cluster coding for the production of phaseolotoxin by *P. s.* pv. *phaseolicola* and *P. s.* pv. *actinidiae* are also present in the genome of *P. avellanae*, the causal agent of hazelnut decline. In fact, primers enabling the amplification of the region corresponding to ORF5 and ORF6 of about 1.9 kb of the *tox* gene (Gonzalez *et al*., 2003), allowed detection of a 696 bp sequence 100% identical to that of *P. s.* pv. *phaseolicola*. This region codes for a fatty acid desaturase belonging to a NAD-dependent epimerase/ dehydratase family. In addition, we also found in *P. avellanae* a sequence of 435 bp identical to a putative lysine amidinotranseferase of *P. s.* pv. *phaseolicola*. However, the other fundamental part of the cluster, the phaseolotoxin-resistant OCTase gene, *argK*, as well as the remaining part of 3.0 kb of the *tox* gene, seem not to be present or are markedly different in *P. avellanae*. These results provide some evidence that the phytotoxic and biocidal compounds produced by *P. avellanae* (Greco and Scortichini, 2004; Lops, 2005) do not belong to phaseolotoxin, even though they would seem related to it in some part. Previous studies (Scortichini *et al*., 2002a) also indicated the absence in *P. avellanae* of the *syrB* gene coding for lipodepsinonapeptides, which are involved in production of *P. syringae* pv. *syringae* syringomycin.

Some interaction between *P. avellanae* and bean leaves is present, since water-soaked lesions were observed three days after inoculation. Afterwards, however, there was no enlargement of the lesion or development of halo blight By contrast, no water-soaking was observed upon inoculation of bean pods, suggesting that different types of plant-microbe interaction may occur in bean leaves and pods when challenged by *P. avellanae*.

REFERENCES

Dos Marques A.S., Corbière R., Gardan L., Tourte C., Manceau C., Taylor J.D. 2000. Multiphasic approach for the identification of the different classification levels of *Pseudomonas savastanoi* pv. *phaseolicola*. *European Journal of Plant Pathology* **106**: 715-734.

Gardan L., Shafik H., Belouin S., Broch R., Grimont F., Grimont P.A.D. 1999. DNA relatedness among the pathovars of *Pseudomonas syringae* and description of *Pseudomonas tremae*, sp. nov., and *Pseudomonas cannabina*, sp. nov. (*ex* Sutic and Dowson 1950). *International Journal of Systematic Bacteriology* **49**: 469-478.

Gonzalez A.I., de la Vega M.P., Ruiz M.L., Polanco C. 2003. Analysis of the *argK-tox* gene cluster in nontoxigenic strains of *Pseudomonas syringae* pv. *phaseolicola*. *Applied and Environmental Microbiology* **69**: 4979-4982.

Greco M., Scortichini M., 2004. Phytotoxic and biocidal metabolites produced in culture by *Pseudomonas avellanae*. *Journal of Plant Pathology* **86**: 296.

Lops R., 2005. Antimicrobial activity of secondary metabolites produced by different pathovars of *Pseudomonas syringae* and by strains of *P. avellanae*. *Phytopathologia Mediterranea* **44**: 56-70.

Loreti S., Gallelli A., Jackson R., Butcher D., Arnold D., Vivian A., 2003. Chracterisation of effector genes of pseudomonads causing disease on hazelnut. In: Iacobellis N.S., Collmer A., Hutcheson S.W., Mansfield J.W., Morris C.E., Murillo J., Schaad N.W., Stead D.E., Surico G., Ullrich M.S. (eds.), *Pseudomonas syringae* and Related Pathogens, pp. 393-398. Kluwer Academic, Dordrecht, The Netherlands.

Manceau C., Brin C. 2003. Pathovars of *Pseudomonas syringae* are structured in genetic populations allowing the selection of specific markers for their detection in plant samples. In: Iacobellis N.S., Collmer A., Hutcheson S.W., Mansfield J.W., Morris C.E., Murillo J., Schaad N.W., Stead D.E., Surico G., Ullrich M.S. (eds.), *Pseudomonas syringae* and Related Pathogens, pp. 503-512. Kluwer Academic, Dordrecht, The Netherlands.

Mitchell R.E. 1976. Bean halo-blight toxin. *Nature* **260**: 75-76.

Prosen D., Hatziloukas E., Schaad N.W., Panopoulos N.J. 1993. Specific detection of *Pseudomonas syringae* pv. *phaseolicola* DNA in bean seed by polymerase chain reaction-based amplification of a phaseolotoxin gene region. *Phytopathology* **83**: 965-970.

Sambrook J., Fritsch E.F., Maniatis T. 1989. Molecular cloning: a laboratory manual. 2nd edition. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, U.S.A.

Sawada H., Suzuki F., Matsuda I., Saitou N. 1999. Phylogenetic analysis of *Pseudomonas syringae* pathovars suggests the horizontal gene transfer of *argK* and the evolutionary stability of *hrp* gene cluster. *Journal Molecular Evolution* **49**: 627-644.

Sawada H., Kanaya S., Tsuda M., Suzuki F., Azegami K., Saitou N. 2002. A phylogenetic study of the OCTase genes in *Pseudomonas syringae* pathovars: the horizontal transfer of the *argK-tox* cluster and the evolutionary history of OCTase genes on their genomes. *Journal of Molecular Evolution* **54**: 437-457.

Scortichini M., Marchesi U., Rossi M.P., Di Prospero P., 2002a. Bacteria associated with hazelnut (*Corylus avellana* L.) decline are of two groups: *Pseudomonas avellanae* and strains resembling *P. syringae* pv. *syringae*. *Applied and Environmental Microbiology* **68**: 476-484.

Scortichini M., Marchesi U., Di Prospero P. 2002b. Genetic relatedness among *Pseudomonas avellanae*, *P. syringae* pv. *theae* and *P. s.* pv. *actinidia*e, and their identification. *European Journal of Plant Pathology* **108**: 269-278.

Tamura K., Imamura M., Yoneyama K., Kohno Y., Takikawa Y., Yamaguchi I., Takahashi H. 2002. Role of phaseolotoxin production by *Pseudomonas syringae* pv. *actinidiae* in the formation of halo lesions of kiwifruit canker disease. *Physiological and Molecular Plant Pathology* **60**: 207-214.

Tourte C., Manceau C. 1995. A strain of *Pseudomonas syringae* which do not belong to pathovar *phaseolicola* produce phaseolotoxin. *European Journal of Plant Pathology* **101**: 483-490.

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