SHORT COMMUNICATION

OCCURRENCE OF AN ENDOPHYTIC, POTENTIALLY PATHOGENIC STRAIN OF *PSEUDOMONAS SYRINGAE* IN SYMPTOMLESS WILD TREES OF *CORYLUS AVELLANA* L.

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SUMMARY

Fluorescent colonies were obtained from surface-sterilized twigs of healthy wild trees of *Corylus avellana* L. growing in central Italy. LOPAT, biochemical and pathogenicity tests, and 16S rDNA gene sequencing indicated that one endophytic isolate belonged to the *Pseudomonas syringae* species complex. This isolate incited hazelnut twig dieback only when inoculated at 1-2 $\times 10^5$ CFU/ml per leaf scar. Lower doses did not induce symptoms. The isolate also caused necrosis in pear and lilac twigs, lemon fruits and bean pods. To our knowledge, this is the first report of an endophytic *P. syringae* strain obtained from wild *C. avellana* trees.

Key words: Hazelnut, pseudomonads, endophytic bacteria, 16S rDNA, gene sequencing

Endophytic bacteria are generally considered as harmless to the host plant and can be isolated from surface-sterilized plant tissue or from the inner parts of the plant (Hallman *et al.*, 1997). Studies aimed at identifying endophytic bacteria from crops and wild plants have shown that apart from saprophytic, mutualistic or beneficial species, plant pathogens were also found in some cases. Examples are *Pseudomonas viridiflava* in pea stems (Elvira-Recuenco and Van Vuurde, 2000), *Curtobacterium flaccumfaciens* and *Clavibacter michiganensis* in prairie plants (Zinniel *et al.*, 2002), *Curtobacterium flaccumfaciens* and *Xanthomonas campestris* in orange and tangerine (Araujo *et al.*, 2002), *Burkholderia gladioli*, and *Curtobacterium flaccumfaciens* pathovars *flaccumfaciens*, *oort* and *poinsettiae* in coffee seeds (Vega *et al.*, 2005). However, such studies did not assess the pathogenicity, and therefore the exact identity, of the endophytic strains towards the host plants from which they were originally isolated.

Corylus avellana L. (hazelnut), is widely cultivated in temperate areas of Asia, North America, South America and in Europe, where pathogenic pseudomonads, namely *P. avellanae*, *P. syringae* pv. *coryli* and *P. s.* pv. *syringae,* can cause severe damage to crops (Scortichini *et al.*, 2005). We describe here some *P. syringae* strains obtained from wild *C. avellana* trees in central Ital, one of which appeared to be potentially virulent to some *C. avellana* cultivars when experimentally inoculated.

Healthy twigs of wild *C. avellana* trees located in the province of Frosinone (central Italy) were collected in summer 2003, stored at 4-5°C, but used within 24 h. The twigs were cut into 10 cm pieces, washed in running tap water and surface-sterilized according to Araujo *et al.* (2002). The pieces were washed in 70% ethanol for 5 min, followed by washings in 1% sodium hypochlorite for another 5 min, then in 70% ethanol for 30 sec, and finally rinsed three times in sterile distilled water. The bark was removed with a sterile razor blade and the pieces were cut into lengths of 1 cm. These were crushed in sterile mortars containing sterile saline (0.85% NaCl in distilled water). Ten-fold serial dilutions were prepared and aliquots of 0.1 ml were spread on plates containing medium B of King *et al.* (1954). The plates were incubated at 25-27°C for 48 h.

With fluorescent colonies, the LOPAT tests as well as nitrate reduction, glucose metabolism, arbutin and esculin hydrolysis and gelatin liquefaction tests were done following the techniques described by Lelliott and Stead (1987). For pathogenicity tests, potted hazelnut plants of the cvs Tonda Gentile delle Langhe and Nocchione were inoculated either in autumn or spring 2005. In autumn (early October) inoculations were done by placing 10 µl of bacterial suspension (1-2 $\times 10^5$ and 1-2 $\times 10^7$ CFU/ml) onto a leaf scar immediately after leaf removal. In spring (early May), the young shoots were gently wounded with a sterile scalpel and drops of inoculum were placed on the wounds. Shoots of potted plants of pear (*Pyrus communis* L.) and lilac (*Syringa vulgaris* L.) were wounded and inoculated in spring as described for hazelnut. After inoculation, the plants were covered with plastic bags for 48 h and subsequently kept outdoors for observation. Control plants were wounded in the same way and treated with sterile phosphate buffer. Re-isolations were made after the appearance of symptoms using the technique described by

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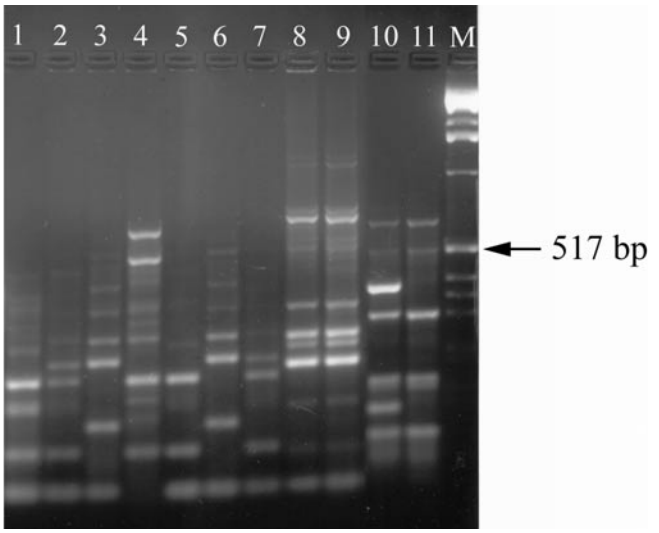

Fig. 1. Repetitive-sequence PCR fingerprinting using ERIC primer sets of endophytic pseudomonads isolated from healthy, wild *Corylus avellana* L. trees belonging to LOPAT group Ia (lanes 1 to 7) compared with *Pseudomonas syringae* pv. *coryli* NCPPB 4273 and ISF 598 (lanes 8 and 9 respectively), and *P. avellanae* BPIC 631 and ISPaVe 2059 (lanes 10 and 11, respectively). M: molecular size marker, 1-kb ladder (Gibco-BRL). *P. syringae* ISF FR1 pattern is shown in lane 4.

Scortichini *et al.* (2002). Lemon fruits and bean pods were also inoculated with the same isolates and doses. Lemon [(*Citrus limon* (L.) Burn.] fruits and bean (*Phaseolus vulgaris* L.) pods were surface-sterilised with 0.5% sodium hypochlorite, rinsed with sterile distilled water and inoculated by wounding the epidermis with a sterile syringe and placing 10-20 µl of the bacterial suspension at the same dose as above. After inoculation, the fruits and pods were placed in a humid chamber for 48 h at room temperature. Lemon fruits inoculated with *P. syringae* pv. *syringae* NCPPB 3869 were used as positive control. Fruits and pods treated with sterile distilled water were used as negative control.

The repetitive-sequence PCR genomic fingerprinting method of Louws *et al.* (1994) was used with ERIC primer sets (Primm, Milano, Italy) used to compare the endophytic pseudomonads with *P. avellanae* BPIC 631 and ISPaVe 2059 and with *P. s.* pv. *coryli* NCPPB 4273 and ISPaVe 598. DNA preparations were obtained as described by Scortichini *et al.* (2002). The thermal cycling procedure was that of Louws *et al.* (1994). PCR products were separated by gel electrophoresis on 15% agarose gels, stained with ethidium bromide, visualized with a Spectroline UV transilluminator (Spectronic Corporation, Westburg, NY, USA) and photographed with Polaroid type 55 film. PCR was done in duplicate.

The entire 16S rDNA gene of isolate ISF FR1 was sequenced. A loopful of pure culture, grown on NSA at 25-27°C for 24 h, was suspended in 0.5 ml of sterile reagent-grade water (Millipore Corp., Billerica, MS, USA) and placed in boiling water for 10 min to obtain a lysed cell suspension for use as PCR template.

To amplify the 16S rDNA, the primers P0 (5'-GAGAGTTTGATCCTGGCTCAG-3') and P6 (5'-CTACGGCTACCTTGTTACGA-3') (Grifoni *et al.*, 1995) were used. PCR conditions, purification of amplicons and sequencing were done as described by Scortichini *et al.* (2005).

Multiple alignment of 16S rDNA sequences were performed using the ClustalW algorithm (Higgins and Sharp, 1998). Cluster analysis was conducted using MEGA, version 3.1 (Kumar *et al.*, 2004) software and computed using the Kimura 2-parameter model (Kimura, 1980) and the neighbour-joining (NJ) clustering algorithm (Saitou and Nei, 1987). Bootstrap analysis was performed to estimate the significance level of the NJ tree internal branches (Hedges, 1992). The 16S rDNA gene sequence of isolate ISF FR1 was compared with those of other *Pseudomonas* spp. and *P. syringae* pathovars using GenBank accessions.

Medium B of King *et al.* (1954) gave fluorescent colonies from the surface-sterilized hazelnut twigs. Fifteen isolates were recovered and LOPAT tests showed that seven of them belonged to group Ia: levan-positve, oxidase-negative, potato soft rot-negative, arginine dehydrolase-negative, tobacco hypersensitivity-positive. In addition, these isolates oxidatively metabolised glucose, hydrolized arbutin and esculin and liquefied gelatin but did not reduce nitrates. Based on these results, the seven pseudomonads could provisioonally be identified as *P. syringae*. The pathogenicity tests carried out on hazelnut showed that, among the seven LOPAT group Ia isolates, only ISF FR1 was able to induce wilting of the twigs (autumn inoculation) and necrosis around the inoculation site (spring inoculation). However, twig wilting and necrosis were observed only when the highest dose of inoculum (i.e. $1\text{-}2 \times 10^5$ CFU/ml per leaf scar) was used. Re-isolations yielded colonies showing the same biochemical and rep-PCR patterns as ISF FR1. None of the other isolates incited symptoms. The control plants showed no sign of infection. ISF FR1 was the sole isolate able to incite enlarging necrotic lesion at a dose of 10^7 CFU/ml on lilac and pear twigs as well as on lemon fruits and bean pods.

Repetitive-sequence PCR using ERIC primers distinguished the isolates obtained from wild hazelnut from both *P. avellanae* and *P. s.* pv. *coryli.* Moreover, each of the seven isolates showed different band patterns (Fig. 1). The 16S rDNA gene sequence of ISF FR1 was deposited in GenBank with accession n. AM495723. Cluster analysis performed with 16S rDNA gene sequences and the NJ algorithm showed that the endophytic, potentially pathogenic isolate is similar to some phytopathogenic pseudomonads. In fact, it was similar to some *P. syringae* pathovars, namely, *syringae*, *coryli*, *mori*, *glycinea*, *broussonetiae* and *P. ficuserectae* (Fig. 2). The data obtained

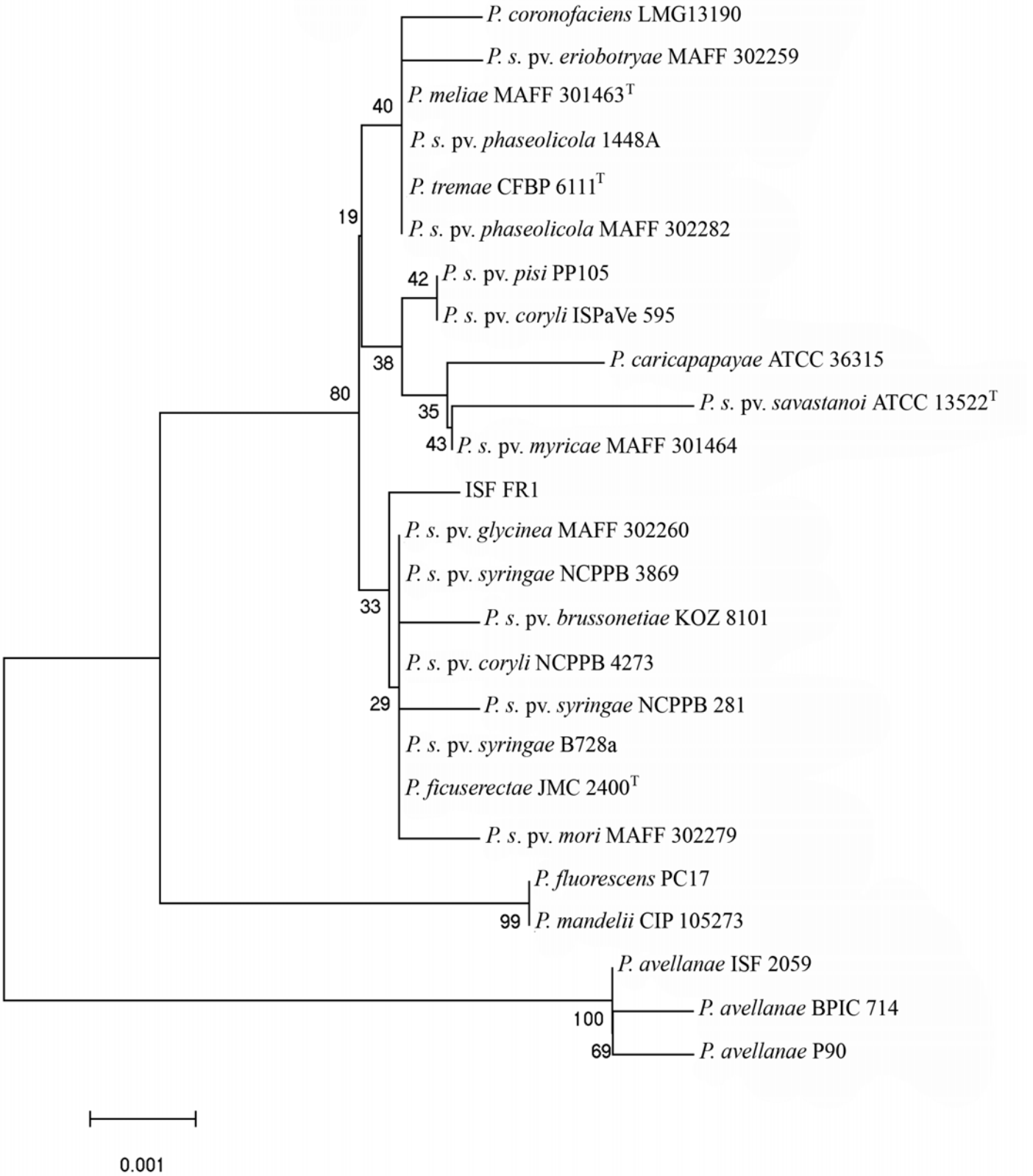

Fig. 2. Dendrogram based on 16S rDNA gene sequences of endophytic *Pseudomonas syringae* ISF FR1, *P. syringae* pathovars and *Pseudomonas* spp. obtained with neighbor-joining algorithm. Estimated distances were based on Kimura's two parameters. Horizontal distances are proportioned to phylogenetic distances expressed in substitutions per 100 sites. The scale bar represents the number of substitutions in each sequence. Bootstrap values (1,000 replicates) are also shown.

from biochemical and pathogenicity tests and from repetitive-sequence PCR and 16S rDNA gene sequences indicate that ISF FR1 belongs to the *P. syringae* species complex. The other endophytic *P. syringae* strains isolated from wild hazelnut trees did not incite any symptom when experimentally inoculated.

This study has shown for the first time the occurrence of endophytic pseudomonads within twigs of healthy wild *C. avellana* in central Italy one of which, belonging to the *P. syringae* complex, was able to incite twig dieback of hazelnut cultivars when artificially inoculated at 1-2 $\times$ 10^5 CFU/ml per leaf scar. The behaviour of this potentially pathogenic bacterial strain inside the plant is unknown. Likewise, nothing is known on colonization and movement of natural endophytic bacteria within *C. avellana*. However, it was earlier shown that the hazelnut pathogen *P. avellana* can colonize the twigs and roots of *C. avellana* cultivars after its release in early autumn onto leaf scars and that host colonization was more effective at the onset of spring vegetation (Scortichini and Lazzari, 1996).

We have also shown strain ISF FR1 elicits dieback of hazelnut twigs and necrosis of other plant organs only when a high inoculum is used. However, the conditions promoting active multiplication of pseudomonads within *C. avellana* tissues in nature are still unknown.

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