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RESEARCH PAPER

Role of ethylene in the protection of tomato plants against soil-borne fungal pathogens conferred by an endophytic *Fusarium solani* strain*

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Abstract

An endophytic fungal isolate (Fs-K), identified as a *Fusarium solani* strain, was obtained from root tissues of tomato plants grown on a compost which suppressed soil and foliar pathogens. Strain Fs-K was able to colonize root tissues and subsequently protect plants against the root pathogen *Fusarium oxysporum* f.sp. *radicis-lycopersici* (FORL), and elicit induced systemic resistance against the tomato foliar pathogen *Septoria lycopersici*. Interestingly, attenuated expression of certain *pathogenesis-related* genes, i.e. *PR5* and *PR7*, was detected in tomato roots inoculated with strain Fs-K compared with non-inoculated plants. The expression pattern of *PR* genes was either not affected or aberrant in leaves. A genetic approach, using mutant tomato plant lines, was used to determine the role of ethylene and jasmonic acid in the plant's response to infection by the soil-borne pathogen *F. oxysporum* f.sp. *radicis-lycopersici* (FORL), in the presence or absence of isolate Fs-K. Mutant tomato lines *Never ripe* (*Nr*) and *epinastic* (*epi1*), both impaired in ethylene-mediated plant responses, inoculated with FORL are not protected by isolate Fs-K, indicating that the ethylene signalling pathway is required for the mode of action used by the endophyte to confer resistance. On the contrary, *def1* mutants, affected in jasmonate biosynthesis, show reduced susceptibility to FORL, in the presence Fs-K, which suggests that jasmonic acid is not essential for the mediation of biocontrol activity of isolate Fs-K.

Key words: Biocontrol, endophyte, ethylene, induced systemic resistance, jasmonic acid, *Solanum lycopersicum*.

Introduction

Plants are often challenged by pathogens detrimental to plant health and productivity and capable of triggering an array of local and systemic responses. Beneficial microorganisms that enhance plant resistance against biotic stresses have been widely recognized and described and are regarded as promising means to achieve sustainable, low-input agricultural production. Several types of interaction between biocontrol agents and plant pathogens have been suggested as key determinants of the suppressive activity, for example, competition for nutrients and ecological niches, parasitism and production of cell-wall hydrolytic enzymes and/or of antifungal compounds (Hoitink and Boehm, 1999). Furthermore, treatment with certain pathogens or non-pathogens can result in the induction of local and/or systemic plant resistance to

* Accession numbers: The GenBank accession numbers for the 18S rRNA gene and ITS-5.8S rRNA gene sequences of *Fusarium solani* strain Fs-K are EF621487 and EF621488, respectively.

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subsequent pathogen attack. Induced resistance to pathogens can be subdivided into two broad categories. The first is systemic acquired resistance, known as SAR. This type of resistance develops either locally or systemically and is generally effective against a broad range of pathogens. SAR is mediated via a salicylic acid (SA)-dependent process and is associated with the production of PR proteins (Durrant and Dong, 2004). The second type of induced resistance develops systemically in response to the colonization of plant roots by certain rhizosphere non-pathogenic bacteria, known as plant growth-promoting rhizobacteria (PGPR). This kind of resistance is known as induced systemic resistance or ISR (van Loon *et al*., 1998). For example, ISR in *Arabidopsis* triggered by *Pseudomonas fluorescens* strain WCS417r is effective against different types of pathogens (Pieterse *et al*., 1996). ISR has also been reported in carnation, radish, and tomato plants (Van Peer *et al*., 1991; Leeman *et al*., 1995; Duijff *et al*., 1998). Other ISR-inducing PGPR have also been demonstrated to enhance the plant's defence capacity (Benhamou *et al*., 1996; De Meyer *et al*., 1999; Ahn *et al*., 2002; Kim *et al*., 2004; Tjamos *et al*., 2005).

Although the literature on induced systemic resistance was concentrated mainly on studies with PGPRs, the research has expanded, as the area has matured, to include a number of other micro-organisms. Plant growth-promoting fungi (PGPF) have also been reported to induce systemic resistance in plants. Preinoculation of cucumber plants with the PGPF *Trichoderma asperellum* T203 leads to enhanced defence against the leaf pathogen *Pseudomonas syringae* pv. *lachrymans* (Shoresh *et al*., 2005). Some *Phoma* isolates and *Penicillium simplicissimum* strain GP17-2 are effective in inducing systemic resistance against cucumber anthracnose caused by *Colletotrichum orbiculare* (Meera *et al*., 1994; Koike *et al*., 2001; Chandanie *et al*., 2006). *Piriformospora indica*, a plant root colonizing basidiomycete fungus, has been recently reported to induce systemic resistance in barley against root and leaf pathogens, including the necrotrophic fungus *Fusarium culmorum* and the biotrophic fungus *Blumeria graminis* (Waller *et al*., 2005).

Similarly, arbuscular mycorrhizal fungi have been suggested as bioprotective agents of widespread potential and systemic mechanisms of protection have been implicated in this protective effect. For example, colonization of tomato roots by the fungus *Glomus mosseae* systemically protects the plant against infection by *Phytophthora parasitica* (Cordier *et al*., 1998; Pozo *et al*., 2002). However, although mycorrhizal–plant symbiosis is effective against root diseases (Azcon-Aguilar and Barea, 1997; Borowicz, 2001; Filion *et al*., 2003), mycorrhizal plants have been shown to be either more (Shaul *et al*., 1999; Gernns *et al*., 2001) or less (Liu *et al*., 2007) susceptible to foliage diseases compared with non-mycorrhizal plants.

The molecular basis and the signalling pathways mediating the protective effect of biocontrol agents have been extensively studied and well-described for the interaction by rhizobacteria. The involvement of jasmonic acid (JA)/ethylene (ET) in inducing plant resistance by PGPR against a number of bacterial and fungal pathogens has been shown using the arsenal of *Arabidopsis* signal transduction mutants. This type of induced resistance does not usually involve expression of plant defence genes (Pieterse *et al*., 2002). On the contrary, the mode of action of fungal biocontrol agents is less well studied; most studies on the involvement of the major, interacting signalling pathways in plants, i.e. those mediated by SA, ET, and JA, have focused on fungal pathogens and the basal and induced plant response to their infection (Knoester *et al*., 1998; Kunkel and Brooks, 2002; Geraats *et al*., 2003). Not many studies are available regarding the role of ethylene or JA in the establishment of mutualistic relationships; in general, ethylene is considered to be inhibitory for arbuscular mycorrhiza formation (Geil and Guinel, 2002) or nodulation (Penmetsa and Cook, 1997), and the addition of ethylene decreased bacterial endophytic colonization in *Medicago* spp. (Iniguez *et al*., 2005). On the other hand, in a compatible interaction between grasses and endophytes, JA-inducible stress or defence responses are apparently not important (Miché *et al*., 2006).

A mutualistic relationship is reported here between tomato plants and a fungal endophyte that mediates resistance to root pathogens and systemic resistance to foliar fungal pathogens, namely *Septoria lycopersici*. A genetic approach was used here, with mutant tomato lines, in order to examine closely the plant response in the presence of the two soil-borne micro-organisms, i.e. the pathogen *Fusarium oxysporum* f.sp. *radicis-lycopersici* (FORL) and the endophytic non-pathogenic *Fusarium solani* strain Fs-K. Thus, evidence is provided that the ethylene signalling pathway has a role on the beneficial effects of the endophyte on the host defence.

Materials and methods

Fusarium solani *strain Fs-K isolation and identification*

Seeds of the tomato (*Solanum lycopersicum* formerly *Lycopersicon esculentum*) line ACE55 were surface-sterilized in 2.5% NaOCl and sown directly into pots containing a mixture of peat moss and suppressive compost, derived from grape marc plus extracted olive press cake. The preparation, chemical characteristics, and suppressive properties against plant pathogens of the compost have previously been described (Ntougias *et al*., 2003; Kavroulakis *et al*., 2005). To isolate fungal colonizers, roots from 1-month-old tomato plants were surface-sterilized in 2.5% NaOCl for 10 min and extensively washed with sterilized tap water. The roots were placed in Petri dishes with PDA containing 60 μg cm^{-3} chlorotetracycline, 30 μg cm^{-3} streptomycin, and 30 U penicillin G in order to avoid bacterial growth (Zuberer, 1994). All plates were incubated for

about 4 d at 25 °C. A fungal strain, thereafter referred to as Fs-K, was isolated which was further characterized.

DNA from the fungal strain was extracted as described by Lee and Taylor (1990). A reaction mixture (50 µl) containing 1 µl (50 ng μl^{-1}) genomic DNA, 10× PCR buffer (Finnzymes OY, Finland), 2 mM $MgCl_2$, 200 µM each dATP, dTTP, dCTP, and dGTP, the appropriate set of primers, 0.5 µM each, and 1 U DNA polymerase (Dynazyme EXT-Finnzymes OY, Finland) was prepared. Primers nu-SSU-0817-5' (5′-TTA GCA TGG AAT AAT (AG) (AG)A ATA GGA-3′) and nu-SSU-1536-3' (5′-ATT GCA ATG C(CT)C TAT CCC CA-3′) (Borneman and Hartin, 2000) were used to amplify part of the 18S rRNA gene. Internal Transcribed Spacer 1 (ITS1)–5.8S rRNA gene–Internal Transcribed Spacer 2 (ITS2) DNA region was amplified using primers ITS1 (5′-TCC GTA GGT GAA CCT GCG G-3′) and ITS4 (5′-TCC TCC GCT TAT TGA TAT GC-3′), which derived from the small (SSU) and large (LSU) subunits, respectively (White *et al*., 1990). Partial 18S rRNA gene was amplified using a denaturation step of 2 min at 94 °C, followed by 35 cycles of 30 s denaturation at 94 °C, 30 s primer annealing at 49 °C and 1 min DNA chain extension at 72 °C. A thermocycling amplification, which included an initial denaturation step of 4 min at 94 °C, 35 cycles of 30 s denaturation at 94 °C, 30 s primer annealing at 52 °C, and 1 min DNA chain extension at 72 °C was performed for the amplification of the internal transcribed spacers on 5.8S rRNA gene. All amplifications were completed by 10 min DNA chain extension at 72 °C. Polymerase chain reaction was performed using a PTC-200 thermocycler (MJ Research Inc., USA). PCR products were separated on 1% agarose gels in 1X TAE buffer, extracted using QIAquick gel extraction columns (Qiagen, Hilden, Germany), cloned into the pGEM-T easy vector (Promega, WI, USA). PCR sequencing was performed at the Institute of Molecular Biology and Biotechnology (IMBB), Heraklion, Greece via a Li-Cor Long ReadIR2 4200 automated sequencer (Li-Cor, USA). Similarity searches against the NCBI data base were carried out using the Basic Local Alignment Search Tool (BLAST) programme. Further verification of the taxonomy of strain Fs-K was obtained through the identification service of CABI Bioscience (www.cabi-bioscience.org)

Fungal strains and culture conditions

A *Fusarium solani* isolate (present study) and a strain of *Fusarium oxysporum* f.sp. *radicis-lycopersici* Jarvis & Shoemaker (strain CBS 101587, Centraal Bureau voor Schimmelcultures, Baarn, The Netherlands), were used. The fungal strains were routinely cultured on potato dextrose broth (PDB) at 25 °C for 5 d in the dark. Following removal of mycelium fragments by sieving, conidia were recovered by centrifugation at 4000 *g*, counted using a haemocytometer and suspended in an appropriate volume of 0.85% NaCl in order to achieve the desired inoculum concentration.

The tomato leaf spot pathogen *Septoria lycopersici* strain (NEV) was used to inoculate tomato leaves. Culture conditions and conidia recovery were performed as described in Martin-Hernandez *et al*. (2000).

Plant growth

Tomato (*Solanum lycopersicum*) plant genotypes, ACE 55, Pearson, VFN8, Castlemart *Nr, epi1* and *def1* were used in the disease incidence experiments. Tomato cvs Pearson, VFN8, and Castlemart are parental lines for *Nr, epi1*, and *def1*, respectively and were included as control where appropriate. Seed for Pearson, VFN8, *epi*, and *Nr* genotypes were obtained from the Tomato Genetics Resource Center (University of California, Davis). Seed for the *def1* mutant and the parental line Castlemart were kindly provided by G Howe (Michigan State University).

Seeds of tomato cultivars and mutant lines were surface-sterilized in 2.5% NaOCl and sown directly into 15 cm diameter pots, each containing approximately 300 cm^3 of peat blended with an NPK fertilizer (20-20-20) to a total concentration of 0.8 g l^{-1}. The pots were placed at 20–25 °C with a 16 h photoperiod at 65% RH in controlled-environment growth chambers, and they were watered to the initial weight on alternate days. Once a week the plants were fertilized with a balanced nutrient solution, including micronutrients.

Plant colonization

Application of the inoculum of strain Fs-K was routinely performed 1 week after sowing with 10^4 conidia cm^{-3} of potting mix as water drench. Colonization by Fs-K of all plant genotypes used was assessed by surface-sterilization, plant sectioning (primary and lateral roots; crown tissue; lower, middle, and upper stem; leaves), and plating on PDA medium 5–25 d after inoculation.

Disease scoring

Fusarium oxysporum *f.sp*. radicis-lycopersici *(FORL) bioassay*: Plants, inoculated with FORL were scored either as healthy (no or scarce disease symptoms) or as diseased (severe wilting or dead plants). The routine inoculation process was with 10^4 conidia of Fs-K cm^{-3} of potting mix applied 1 week after sowing and 10^5 conidia of FORL cm^{-3} of potting mix applied at the stage of the first true leaf emergence. In the experiment in which the effect of inoculum concentration was examined, different concentrations (10^2, 10^3, 10^4, 10^5, and 10^6 conidia cm^{-3} of potting mix) of Fs-K was applied to plant potting mixes 1 week after sowing and 10^5 conidia of FORL cm^{-3} of potting mix was added either simultaneously or at the stage of the first true leaf emergence. Control plants received only water. Plants inoculated only with Fs-K conidia (10^4 conidia cm^{-3} of potting mix) never develop any symptom up to the fruit setting stage. Five or eight replications (pots containing five plants each) per treatment were included. The experiments were repeated at least twice with similar results.

Septoria lycopersici *bioassay*: Tomato seeds of the cultivar ACE55 were sown and grown on pots, each containing four plants, as described above. Treatments in the experiments conducted were tomato plants grown (i) in the presence of FORL and strain Fs-K, (ii) in the presence of FORL, (iii) in the presence of strain Fs-K, and (iv) in the absence of any the above biological agents. Three replications (pots) per treatment were used. Plants were inoculated as described above. Detached leaves of the same age from 1-month-old plants were inoculated by brushing the underside with spore suspensions of *S. lycopersici* NEV [1×10^6 spores cm^{-3} in 0.01% (v/v) Tween 80] as described by Martin-Hernandez *et al*. (2000). Mock inoculated leaves were brushed with 0.01% (v/v) Tween 80. Twelve leaves of the same emerging order were used per treatment (four from each replication). Five days after inoculation, infection was evaluated by counting the number of lesions developed on each leaf over background illumination. The experiment was repeated twice with similar results.

Microscopy

Tomato plants (cv. ACE55) were grown and inoculated as described above. Root samples from 1-month-old plant were collected, washed in tap water, and cleared using hot 10% KOH for 10 min. The roots samples were subsequently stained using 0.05% Chlorazol Black E for 15 min. The strain was prepared by mixing with water, glycerine, and lactic acid of 1:1:1 (by vol.). For long-term storage, the stained roots were placed in glass tubes containing

a water–glycerine mix (2:1 v/v). Microscopic observations were carried out using bright-field optics.

RT-PCR of PR *genes*

Tomato seeds of the cultivar ACE55 were sown and grown on pots as described above. Four experimental treatments were used: (i) plants inoculated with strain Fs-K, (ii) plants inoculated with FORL, (iii) plants inoculated with FORL and strain Fs-K, and (iv) plants inoculated with none of the biological agents. Fifteen seedlings (three pots) per treatment were used and the experiment was repeated once more. Each pot was considered as a replicate. Inoculation of the fungal conidia was performed as described. Leaf and root tissues were collected from plants of different treatments separately, 1 week and 2 weeks after inoculation with the pathogen. Samples comprised of all 15 plants and were immediately frozen in liquid nitrogen, and then stored at –80 °C until use. RNA extraction and PCR conditions were performed as described elsewhere (Kavroulakis *et al*., 2006). Host genes assessed include a basic *PR1,* basic and acidic β-1,3-glucanase (*GLUB* and *GLUA-PR2*), basic and acidic chitinase (*CHI3* and *CHI9-PR3*), an osmotin-like *PR5*, and endoproteinase *P69* (*PR7*). The *glyceraldehyde-3-phosphate-dehydrogenase* (*GAPDH*) gene was used as a control. The sequences of gene-specific primers used in RT-PCR analysis are presented in Table 1. All RT-PCR experiments were repeated at least three times.

Ethylene quantification

Ethylene evolution was determined in leaf and root tissues of tomato plants (cv. ACE55) grown either in the presence or absence of strain Fs-K and FORL. The four experimental treatments used are reported in the paragraph concerning the RT-PCR experimental procedure. Leaves and roots of all four treatments were collected 1 week and 2 weeks after inoculation with FORL, weighed and separately placed in 25 cm^3 gas-tight serum flasks, and incubated under climate chamber conditions. At intervals, 1 cm^3 gas samples were withdrawn through the rubber seal. The concentration of ethylene was determined by GS (Perkin-Elmer 8500 GC), equipped with a GS-Q column (3O m×0.32 mm i.d., J & W Scientific, Folsom, CA) and fitted with a flame ionization detector. The oven temperature was 60 °C, and injector and detector temperatures were set at 150 °C. The carrier gas used was helium at a flow rate of 10 $cm^3\ min^{-1}$.

Statistical analysis

All experiments were conducted at least twice and analysed by analysis of variance (ANOVA) followed by Duncan multiple comparison tests (a <0.05). Standard errors were calculated for all mean values and t tests were performed for pairwise comparisons of means at different time points ($P \leqslant 0.05$). All data expressed as percentages were arcsine transformed before analysis. Data from a single representative experiment are presented since repeated tests yielded similar results.

Results

A non-pathogenic Fusarium solani *(strain Fs-K) colonizes root tissues and protects tomato plants against fungal pathogens*

Previously, the suppressive capability of a compost derived from grape marc plus extracted olive press cake (GM-EPC) against soil and foliar fungal pathogens of

Table 1. *Sequences of gene-specific primers used in RT-PCR analysis*

Gene family	Specific class	Accession number	5′ primer	3′ primer	References
PR1	PR1b, Basic PR1	AJ011520	5′-CCAAGACTATCTTGCGGTTC-3′	5′-GAACCTAAGCCACGATACCA-3′	Van Kan *et al*., 1992
PR2	GLUA, acidic glucanase	M80604	5′- TATAGCCGTTGGAAACGAAG-3′	5′-TGATACTTTGGCCTCTGGTC-3′	Van Kan *et al*., 1992
	GLUB, basic glucanase	M80608	5′-CAACTTGCCATCACATTCTG-3′	5′-CCAAAATGCTTCTCAAGCTC-3′	Van Kan *et al*., 1992
PR3	Chitinase 3, acidic	Z15141	5′-CAATTCGTTTCCAGGTTTTG-3′	5′-ACTTTCCGCTGCAGTATTTG-3′	Danhash *et al*., 1993
	Chitinase 9, basic	Z15140	5′-AATTGTCAGAGCCAGTGTCC-3′	5′-TCCAAAAGACCTCTGATTGC-3′	Danhash *et al*., 1993
PR5	Osmotin-like PR5	AY093593	5′-AATTGCAATTTTAATGGTGC-3′	5′-TAGCAGACCGTTTAAGATGC-3′	Rep *et al*., 2002
PR7	P69G	DQ157774	5′-AACTGCAGAACAAGTGAAGG-3′	5′-AAC GTGATTGTAGCAACAGG-3′	Kavroulakis *et al*., 2006
GAPDH	Glyceraldehyde-3-phosphate-dehydrogenase	M64114	5′-GAAATGCATCTTGCACTACCA-ACTGTCTTGC-3′	5′CTGTGAGTAACCCCATTCATTAT-CATACCAAGC-3′	Shih *et al*., 1991

tomato plants has been reported (Kavroulakis *et al*., 2005). In an attempt to determine biotic factors that may cause or contribute to the compost suppressiveness, a fungal strain (named Fs-K) was isolated using semi-selective media. Based on 18S rRNA and ITS sequence analysis, the strain was identified as *Fusarium solani* and its taxonomic position was further verified by classical taxonomic characters (CABI-Bioscience).

Inoculation of tomato plants (cultivar ACE55) with fungal conidia resulted in colonization of the root tissues and endophytic growth of the isolated fungus. Figure 1 demonstrates that strain Fs-K is capable of penetrating the root and growing in the root cortex at 15 d post-inoculation. The formation of round bodies was observed (Fig. 1A). Interestingly, the fungus proliferates even in the vascular system of the plants as shown by Chlorazol Black staining and light microscopy (Fig. 1B). Colonization, as determined by surface-sterilization of sectioned plant tissues and microscopic analysis, extends to the crown, but not to the stem and leaf tissues (data not shown).

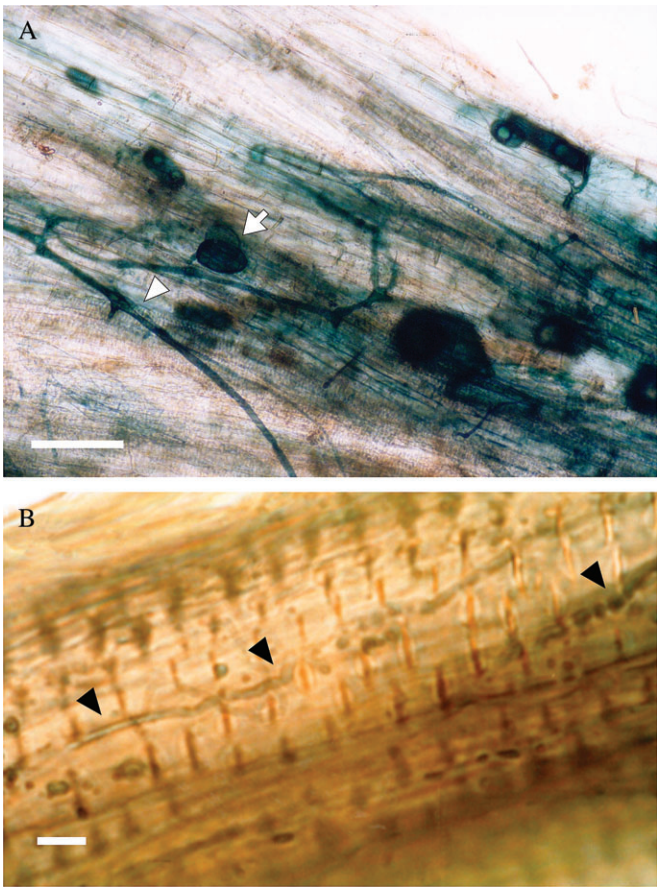

Fig. 1. Light micrographs of tomato root tissue at 15 d post-inoculation with strain Fs-K using Chlorazol Black staining. (A) The fungus is capable of penetrating the root cells (arrowheads). The formation of round bodies was observed (arrows) and (B) colonization of the vascular bundle by strain Fs-K is detectable (arrowheads). Bars represent 50 μm.

Tomato plants grown on peat amended with a conidial suspension of strain Fs-K were significantly more resistant to crown and root rot caused by *F. oxysporum* f. sp. *radicis-lycopersici* (FORL) when compared with tomato plants grown on peat alone. This phenomenon has been repeatedly observed over a period of three years and a typical graph depicting the disease process in plants inoculated or non-inoculated with strain Fs-K is shown in Fig. 2A. Different tomato lines were used for these experiments, all of which were colonized by the fungus and exhibited increased disease resistance, although the response pattern differed as regards the time-course and

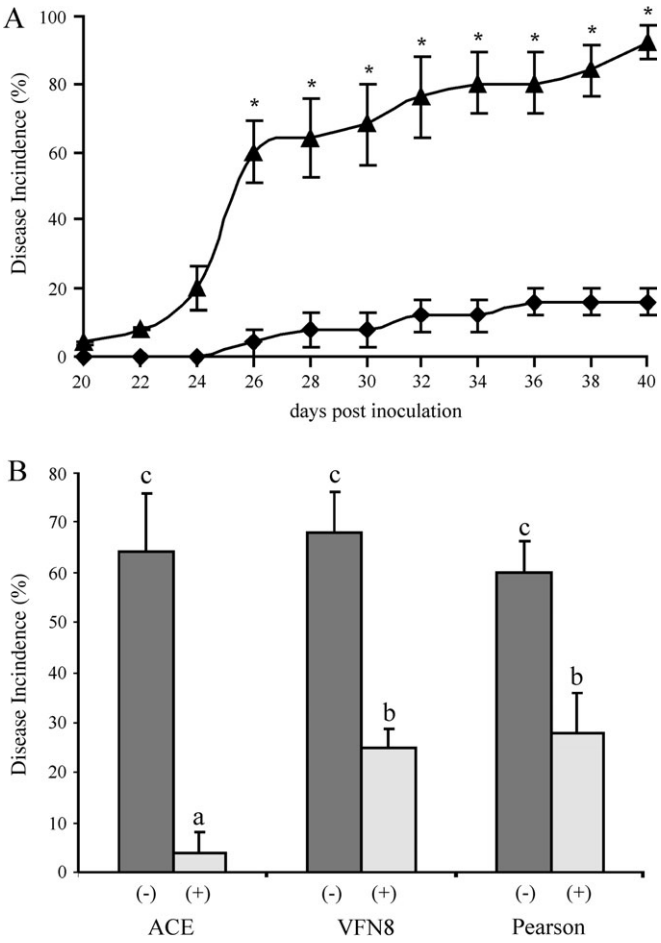

Fig. 2. Disease incidence in tomato plants caused by inoculation with 10^5 conidia cm^{-3} of *Fusarium oxysporum* f.sp. *radicis-lycopersici* (FORL). (A) Disease progress curves in tomato plants (cultivar ACE) grown on peat in the absence (triangles) or presence (diamonds) of 10^4 conidia cm^{-3} of Fs-K. Disease incidence was assessed as percentage of dead plants (five pots, each containing five plants per treatment, n=5). Mean values that were statistically different at each time point (t test $P \leq 0.01$) are indicated by an asterisk. Bars indicate standard errors of means. (B) Disease incidence caused by FORL in three different tomato cultivars in the absence (–) or presence (+) of strain Fs-K. Disease incidence at 30 d post-inoculation is depicted, assessed as above. Statistical comparisons within all cultivars were performed by Duncan tests (a <0.05). Indicator letters in common denote a lack of significant difference. Bars indicate standard errors of means.

the level of protection conferred by the endophyte (Fig. 2B). An apparent growth-promoting effect was not observed, although we have not specifically addressed this case. To investigate the effect of inoculum concentration on disease incidence caused by FORL, tomato plants were inoculated with strain Fs-K prior to or at the same time with FORL inoculation. Ratios of inoculum density needed for a 40–100% reduction in disease incidence are 1:1000 or 10:1 (endophyte:pathogen) for the inoculation with strain Fs-K prior to or at the same time with FORL inoculation, respectively (Table 2), indicating that strain Fs-K represents a very efficient antagonist for FORL.

Strain Fs-K systemically protects tomato plants against foliar pathogens

Induction of systemic resistance by the fungal isolate Fs-K was examined in a different pathosystem using the foliar fungal pathogen *Septoria lycopersici*. Significantly fewer disease lesions were observed on the leaves when plants were grown in the presence of strain Fs-K (Fig. 3). The presence of the root pathogen was also expected to induce resistance to the foliar pathogen, through a SAR mechanism. Indeed, infection by FORL also resulted in a reduction in lesions caused by *S. lycopersici* but notably this effect was not as strong as the protection conferred by Fs-K (Fig. 3). The induction of systemic resistance due to the simultaneous presence of the antagonist *Fusarium solani* strain Fs-K and the pathogen was also investigated. In the latter case, no higher levels of protection were achieved and a similar extent of lesion development was observed as in the presence of strain Fs-K alone.

Strain Fs-K down-regulates expression of PR *genes in root tissue*

The expression of certain pathogenesis-related (*PR*) genes (*PR1, PR5, P69*/*PR7*) was previously shown to be induced in the root tissues of tomato plants grown on the GM-EPC compost (Kavroulakis *et al*., 2006). To examine whether this phenomenon could be attributed to the presence of strain Fs-K in the root tissues, RT-PCR analysis of *PR1, PR5, P69*/*PR7, PR2, PR3* transcripts was performed in the root and leaf tissues of plants following the inoculation with the fungus. The experimental set-up also included plants inoculated with the root pathogen FORL and with both fungal strains. No significant or consistent differences were observed in leaf tissues (data not shown). On the contrary, a reduction in the level of *PR5* and *P69*/*PR7* gene expression was observed in the root (Fig. 4). Interestingly, in tomato root tissues, the reduction in the transcript levels of *PR5* and *PR7* genes, mediated by Fs-K, was evident regardless of the presence or absence of the root pathogen FORL. Moreover, the same reduction was observed in the levels of *PR1*, whose expression was induced, as expected by the presence of the pathogen (Fig. 4). No difference was observed in the expression levels of genes encoding for chitinases or glucanases (basic or acidic expression levels of the basic forms are presented in Fig. 4).

Role of ethylene and jasmonic acid in biocontrol activity of strain Fs-K

To examine the role of ethylene and jasmonate in induced plant resistance conferred by strain Fs-K, mutant plants defective in ethylene- and jasmonate-signalling pathways were used. Initially, the wild-type and mutant lines were inoculated with Fs-K alone to examine any effect on the plant growth but it was not possible to detect any difference in the presence or absence of Fs-K for all lines. Furthermore, Fs-K was recovered from surface-sterilized root sections of all tomato lines.

There is no report, at least to our knowledge, on the effect of either ethylene or jasmonate and their respective signalling pathways on the plant response to infection by the root pathogen used in this study, FORL. Thus,

Table 2. *Effect of inoculum concentration of endophytic strain Fs-K on the control of disease incidence caused by* F. oxysporum *f.sp.* radicis-lycopersici *(FORL) to tomato plants*

Inocula of both fungi were added as a water drench. Statistical comparisons between treatments were performed by Duncan's tests (a <0.05). Letters in common in parenthesis indicate a lack of significant difference (comparisons are valid within each row).

	Endophyte:pathogen (conidia cm^{-3} of peat)							
	0:0	10^4:0	0:10^5	10^2:10^5	10^3:10^5	10^4:10^5	10^5:10^5	10^6:10^5
	Endophyte PRIOR to pathogen[b]							
% Disease incidence[a]	0	0	88 (c)	52 (b) (40.9)	40 (b) (54.5)	22.6 (a) (74.2)	18.6 (a) (78.8)	
	Endophyte WITH pathogen[c]							
% Disease incidence[a]	0	0	97.6 (b)	97.6 (b) (0)	94.3 (b) (3.38)	100 (b) (0)	95.3 (b) (2.35)	62 (a) (36.4)

[a] Disease incidence was assessed as percentage of dead plants (n=5) 30 d post-inoculation with FORL. Numbers in parenthesis indicate % disease reduction compared to FORL inoculation alone.
[b] Strain Fs-K was applied 1 week after sowing, FORL was applied at the stage of first-true-leaf.
[c] Strain Fs-K and FORL were applied 1 week after sowing.

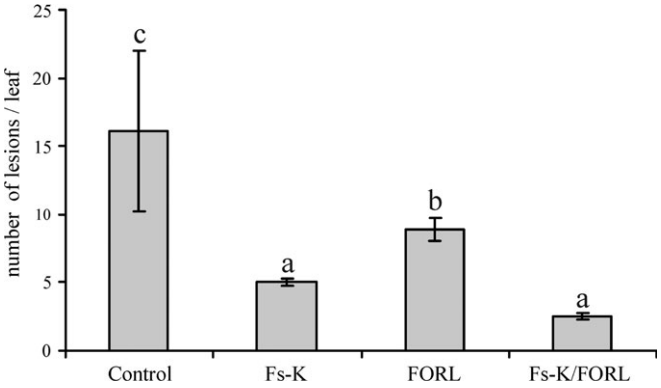

Fig. 3. Effect of strain Fs-K presence on the development of symptoms caused by *Septoria lycopersici* strain NEV on tomato plants grown for 1 month on peat (control) or peat amended with 10^5 conidia cm^{-3} of FORL or 10^4 conidia cm^{-3} of strain Fs-K or with conidia from both fungal strains. Number of lesions was counted per leaf 1 week after inoculation with 10^6 conidia cm^{-3} of *S. lycopersici* spore suspension. Bars indicate standard deviations of means (n=12). Statistical comparisons were performed by Duncan's tests (a <0.05). Letters in common indicate a lack of significant difference.

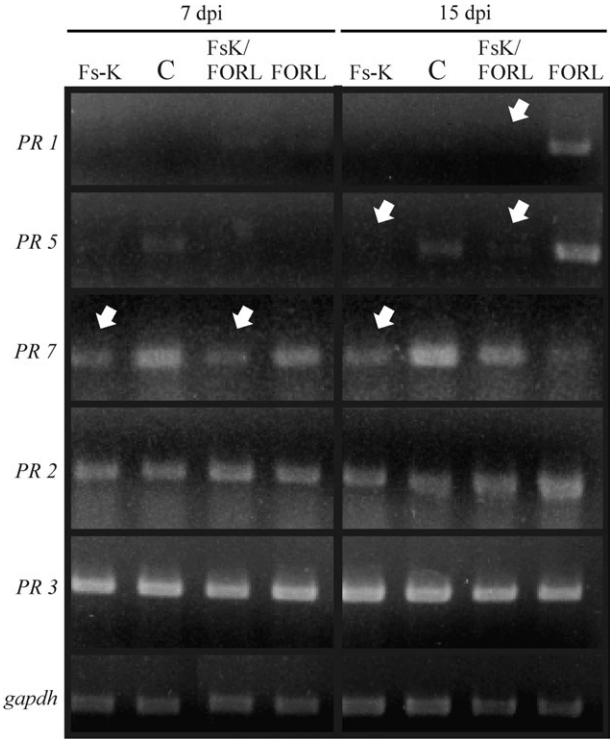

Fig. 4. Expression of *PR* genes as determined by RT-PCR analysis of plant samples in the presence or absence of strain Fs-K (10^4 conidia cm^{-3}) with or without prior inoculation with *Fusarium oxysporum* f.sp. *radicis-lycopersici* (10^5 conidia cm^{-3}). Host genes assessed include a basic *PR1,* an osmotin-like *PR5*, endoproteinase *P69* (*PR7*), basic β-1,3-glucanase (*PR2*), and basic chitinase (*PR3*). Tthe *GAPDH* gene was used as a control. Plants were grown on peat. Root tissues were collected from plants at 7 d and 15 d post-inoculation with the pathogen. Arrows indicate the reduced levels of transcripts observed in the presence of strain Fs-K. Repeated RT-PCR assessment of gene expression was performed. The experiment was conducted twice with similar results.

experiments with all plant genotypes were conducted that allowed us to conclude in parallel on the role of the two phytohormones when the plant is affected by the pathogen alone and when both the pathogen and the endophytic Fs-K are present (Fig. 5).

As regards the role of ethylene, two mutant lines, that is the ethylene-insensitive *Never ripe* (*Nr*) (Lanahan *et al*., 1994) and the *epinastic* (*epi*) genotype, demonstrated to be constitutively activated in a subset of ethylene responses (Barry *et al*., 2001), were used (Fig. 5A, B). It must be noted here that *Nr* plants are marginally less susceptible than the wild-type plants and occasionally exhibited increased resistance to FORL. Interestingly, ethylene-deficient mutant lines (*Nr, epi1*) are not protected against the root pathogen (Fig. 5A, B). Therefore, a functional ethylene pathway seems to be a necessary component of the mechanism used by the endophyte to induce resistance.

The jasmonate-deficient mutant *def1* was tested for susceptibility to FORL by being compared to the wild-type progenitor cultivar Castlemart (CastM); as it was expected and since the *defenceless-1* (*def1*) mutant is deficient in the biosynthesis of jasmonic acid (Howe *et al*., 1996; Li *et al*., 2002), the JA-biosynthesis mutant (*def1*) proved to be highly susceptible to the pathogen. Nevertheless, the presence of Fs-K clearly protected the plants against the pathogen, inducing a 40–70% reduction in the disease incidence observed in the absence of the endophyte (Fig. 5C).

Discussion

A *Fusarium solani* strain (Fs-K), capable of entering the host tissue and residing as an endophyte in tomato plants, reducing, subsequently, root pathogen infection and disease development by FORL and inducing systemic protection against foliar pathogens, is reported here.

The strain's biocontrol ability against root pathogens was examined in further detail and it was demonstrated that different tomato genotypes were all protected against FORL. The discrepancies among tomato lines regarding the level of protection conferred by strain Fs-K against FORL cannot easily be attributed. Similarly, though, differences among tomato lines have been reported before, regarding either their resistance to FORL (Brayford, 1996) or their mechanism of resistance against *Botrytis cinerea* (Diaz *et al*., 2002). Furthermore, it has been shown in a previous study using a GUS transformed FORL isolate, that the plant genotype appears to affect the GUS activity measurements obtained for a given level of disease progression caused by FORL (Papadopoulou *et al*., 2005). Thus, most probably a number of genetic factors determine the resistance level of each plant genotype and, in a comparable manner, the level of protection conferred by biocontrol agents, like strain Fs-K.

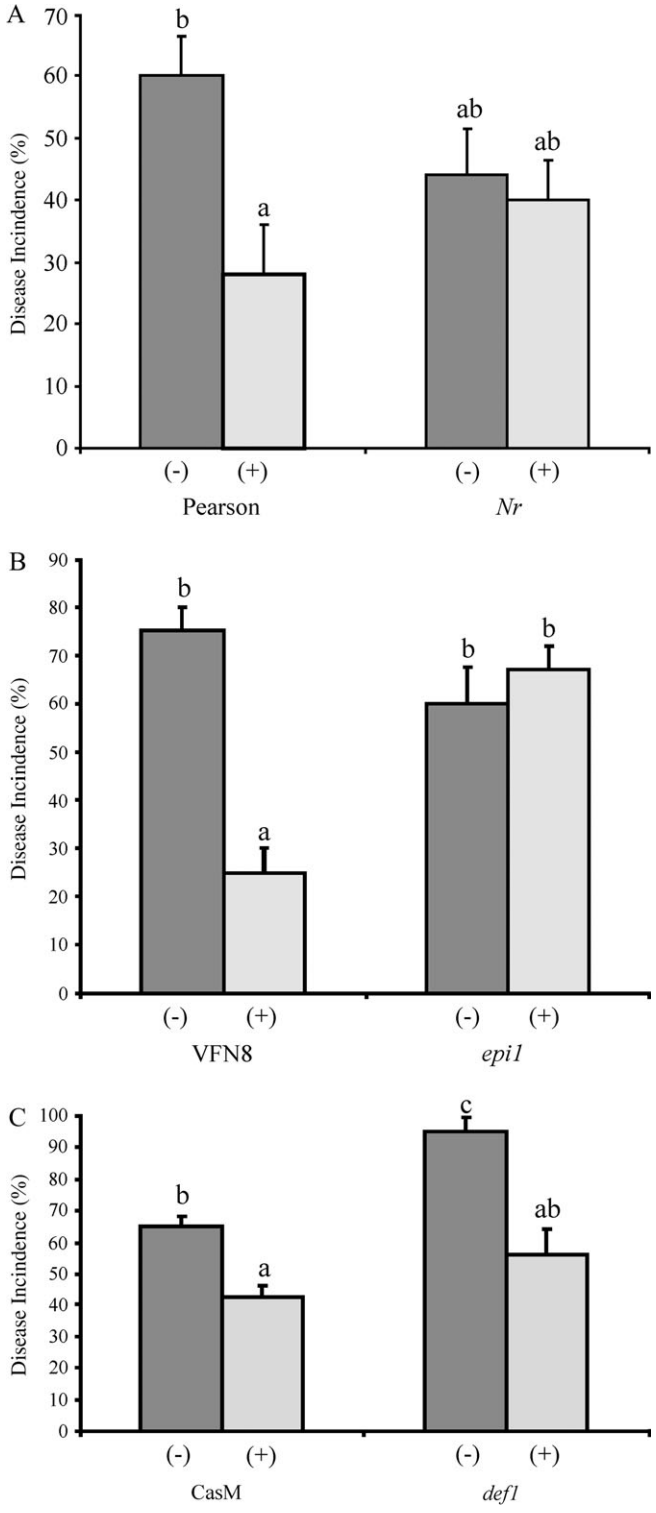

Fig. 5. Disease incidence caused by FORL in wild-type and mutant tomato plants in the presence (+) or absence (–) of strain Fs-K at 50–54 d post-inoculation with the pathogen. Inoculation was performed as described in Fig. 2. Disease incidence was assessed as percentage of dead plants (eight pots, each containing five plants per line, n=8). Bars indicate standard deviations of means. Statistical comparisons were performed by Duncan's tests (a <0.05). Letters in common indicate a lack of significant difference. Plant genotypes tested: Pearson, *Never ripe* (*Nr*), VFN8, *epinastic1* (*epi1*), Castlemart (CasM), *defenceless* (*def1*).

A higher inoculum of strain Fs-K is needed to confer resistance against the root pathogen FORL when the endophyte is applied to the plant at the same time as the pathogen as compared to its application prior to pathogen inoculation. This indicates that strain Fs-K could represent a strain that outcompetes the pathogen by competition for niche colonization. Nevertheless, other mechanisms of action (e.g. an effect of Fs-K on the survival of the pathogen and on its spore germination efficacy due to the production of antifungal compounds), which have not been studied conclusively as yet, can not be excluded.

Many other fungal species have been reported to act as biocontrol agents against plant pathogenic fungi (Punja and Utkhede, 2003), amongst them *F. solani* strains (Larkin and Fravel, 1998) and the well-described non-pathogenic *Fusarium oxysporum* strain Fo47. The mode of action of the latter was attributed to colonization and competition for nutrients, although systemic induction accompanied by a SAR-like mechnanism and induction of PR1, chitinase, and PR2 has also been reported (Fuchs *et al*., 1997; Duijff *et al*., 1998). Other well-known biocontrol fungi are *Trichoderma* spp., initially studied for their mycoparasitic mode of action and the production of antifungal compounds (Harman *et al*., 2004; Djonovic *et al*., 2006). Lately, though, it is becoming evident that induction of systemic resistance is also involved in their antagonistic effect against bacterial pathogens in cucumber plants, by modulating the expression of genes implicated in the jasmonate/ethylene signalling defence pathways (Shoresh *et al*., 2005).

Most cases reporting induced resistance by biocontrol fungal strains refer to systemic resistance, where disease control occurs at a site distant from the location of the antagonist. It is difficult to examine the role of induced resistance to pathogens that cause root diseases because of the putative direct effects of the soil-borne biocontrol organism on the pathogen. Most such studies use the split-root system in order to examine the inductive and systemic nature of protection. An alternative approach was used by using mutant plant lines, defective in the pathways of induced resistance mediated by ethylene and JA. By this approach, it was possible to examine the induced nature of resistance conferred by Fs-K to the soil-borne pathogen under normal conditions, that is when the pathogen and the non-pathogen reside at their physiological niches. Our results show that, the protective ability of strain Fs-K prerequisites plant responses mediated by well-known ethylene signalling pathways. Thus, *Never ripe* mutant plants, unable to respond to ethylene, are not protected against root pathogens by the mechanism used by strain Fs-K to confer resistance in the plant. On the contrary, highly effective protective action is exerted by isolate Fs-K on the wild-type plants. The lack of ethylene perception does not appear to play a crucial role on the infection capability of the pathogen and on disease

development, since *Nr* plants are not significantly different in susceptibility to FORL than the wild-type plants. Similarly, *epi* mutants are also not less susceptible to FORL, contrary to reports on the susceptibility of foliar pathogens like *Botrytis* (Diaz *et al*., 2002). Furthermore, *epi* mutants are not protected by strain Fs-K. Taking into account that the two mutant lines are affected differentially in the ethylene signalling pathway, i.e. *Nr* is an ethylene receptor mutant (Wilkinson *et al*., 1995) while *epi* is constitutively activated in a subset of ethylene responses and also displays elevated ethylene synthesis (Barry *et al*., 2001), either lack of ethylene perception or differences in the endogenous ethylene levels among the plant genotypes apparently intervene in the protective mechanism used by strain Fs-K. In any case, the results of the present study indicate that the ethylene signalling pathway has an effect on the endophyte Fs-K itself, either on its initial interaction with the plant or with the pathogen and the subsequent entrance and establishment in the root tissues or during the mounting of induction of plant resistance.

Root colonization by strain Fs-K does not induce elevated ethylene production and ethylene levels produced in the root and aerial parts of tomato plants were not significantly different to levels in tomato plants grown in the absence of the endophyte. When plants were also inoculated with the root pathogen FORL, no increase in ethylene level was detected (data not shown). In a similar manner, rhizobacteria-mediated induced resistance is not based on the local or systemic induction of changes in the biosynthesis of ethylene (Pieterse *et al*., 2000) although colonization of *Arabidopsis* roots by *P. fluorescens* primes the plant to produce higher levels of ethylene in the leaves upon pathogen infection (Hase *et al*., 2003).

Interestingly, a functional JA biosynthesis pathway is not necessary for Fs-K-mediated resistance against FORL since *def1* mutant plants are protected. The dependence of the protective action on ethylene and its independence from JA seems to deviate from the generally recognized concerted action of JA and ET in activating both basal and induced disease resistance in plants. More specifically, JA-dependent defence responses are essential for rhizobacteria-mediated ISR and an enhancement of extant JA- and/or ET-dependent defence responses seems to be the basis of ISR (Pozo *et al*., 2004). The discrepancy could be attributed to the fact that both organisms in the present study are soil-borne and thus the induced resistance observed is not systemic.

On the other hand, wild-type tomato plants need JA to combat FORL and *def1* mutants are extremely susceptible to FORL. These results, demonstrating that JA-deficient mutant plants are compromised in resistance against a root rot pathogen, further reinforces previous work reported by Thaler *et al.* (2004), which showed that JA-mediated resistance in tomato is effective against a wide range of pathogens. Remarkably, the level of protection conferred by Fs-K in the *def1* mutant plants is much higher than the protection exhibited in the wild-type plants. It could be hypothesized that JA also acts as a defence line against the endophyte, too. Therefore, in the absence of JA, a more efficient colonization by Fs-K leads to higher levels of protection and the reduced disease incidence observed.

Whether Fs-K root colonization *per se* triggers, as most probably expected, a host recognition system or, if strain Fs-K is able to manipulate the plant's defence system, is not yet clear. However, our results point out that the endophyte definitely has the ability to alter plant response and, in particular, SA-mediated responses. Thus, it is clearly evident that even baseline expression of certain *PR* genes, known to act as means of the plant's defence line (van Loon *et al*., 2006), are down-regulated by Fs-K. Attenuation of SA-dependent defence responses in host plants such as suppression of expression of *PR* genes has been reported for certain plant pathogens. For example, susceptibility of tomato to *Pst*DC3000 is associated with repression of *PR* genes in *Arabidopsis* (Kloek *et al*., 2001) and in tomato (Zhao *et al*., 2003). We have previously reported on an inducible by compost, root-specific orthologue of the PR7/P69 subtilase family in tomato roots and have shown its sporadic expression on cell 'islands' of the root cortex of plants grown on GM-EPC compost as compared to basal levels detected in all parenchyma cells of plants grown on peat (Kavroulakis *et al*., 2006). Our initial hypothesis that the colonization by the fungal endophyte may be responsible for this localized expression of the gene was not confirmed. On the contrary, a reduction on the gene's expression levels was detected. This was further verified in *in situ* studies, where, in the presence of strain Fs-K, the expression of P69G was below detection levels in almost all cells of the root parenchyma (data not shown). Recently, it has been reported that a different member of *P69* (*P69B*) may be inhibited in the leaves of tomato by the extracellular protease inhibitor EPI1 from *Phytophthora infestans* as a mechanism of defence response suppression of this biotrophic pathogen, leading to colonization of the host apoplast (Tian *et al*., 2004). Therefore, it may be suggested that Fs-K achieves colonization by attenuating the defence reaction through the excretion of metabolites locally in the root tissues.

In most cases, fungal biocontrol agents have been reported to colonize root tissues internally, mainly the epidermal and cortical cell layers (Bolwerk *et al*., 2005; Deshmukh *et al*., 2006). Extremes ranging from obligate biotrophy, exemplified by arbuscular mycorrhiza fungi, to the host cell death-inducing *Piriformospora indica* (Deshmukh *et al*., 2006) have been described. To our knowledge, though, the ingress in the vascular bundle is a characteristic of root pathogens and therefore, the

capability of strain Fs-K to grow abundantly and without causing any disease symptoms to the plant, denotes a rather unique plant–microbe interaction. The pattern of colonization and mechanisms underlying this unusual symbiotic relationship will be pursued further.

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