American Journal of

Biochemistry and Molecular Biology

ISSN 2150-4210

Academic
Journals Inc.

www.academicjournals.com

American Journal of Biochemistry and Molecular Biology 1 (2): 121-144, 2011
ISSN 2150-4210 / DOI: 10.3923/ajbmb.2011.121.144
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Biology and Partial Sequencing of an Endophytic *Fusarium oxysporum* and Plant Defense Complex

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ABSTRACT

The capacity of a non-pathogenic strain of *Fusarium oxysporum* (IMI 386351) to control fusarium wilt of a susceptible tomato (*Lycopersicon esculentum* Mill.) cultivar Danish Export caused by *Fusarium oxysporum* f. sp. *lycopersici* was examined along with the biology of that strain, including its phylogenetics. Based on the morphological and the molecular examinations, *F. oxysporum* IMI 386351 appeared to be a member of the *F. oxysporum* complex; phylogenetic trees inferred independently and by combining the two partial sequence genes (a mitochondrial small subunit ribosomal DNA and the nuclear translation elongation factor 1α) suggest that this strain might be a member of *Fusarium oxysporum* f. sp. *melonis*. The results of seed-coating experiments using five doses of *F. oxysporum* IMI 386351 revealed a complex three-organism interaction (host-endophyte-pathogen). Between two of the doses, a dose-dependent shift from one form of systemic induced resistance to a distinctly different form apparently occurred. Corresponding with this shift, the pathogen shifted from being virulent to being benign and disease incidence plummeted to zero. A plant growth promoting effect of *F. oxysporum* IMI 386351, which was probably associated with systemic induced resistance, consistently increased with dose. This study may be the first to demonstrate that, by means of systemic induced resistance, an endophytic fungus (as opposed to a bacterium) can suppress disease symptoms caused by plant pathogenic fungi.

Key words: *Fusarium oxysporum* f. sp. *lycopersici*, endophyte, plant defense and systemic induced resistance, phylogenetic analysis, mtSSU rDNA gene and EF-1α gene

INTRODUCTION

Members of the *Fusarium oxysporum* (*Fo*) species complex are some of the most widely distributed saprophytes. Some Fo strains, which are widespread and highly host-specific, cause vascular diseases (Olivain and Alabouvette, 1997) despite being saprophytic. These pathogenic strains are classified into more than 120 infraspecific taxa (i.e., below species level) (Armstrong and Armstrong, 1981), including some *formae speciales* (f. spp.) and races that infect plants of economic importance, such as *Lycopersicon* sp.

At least some *Fo* strains that are incompatible with a given plant species might be used effectively to protect that plant from its specific pathogens (Matta, 1989). Studies of soils that are

naturally suppressive to *Fusarium* wilts, conducted by Olivain and Alabouvette (1997), for example, show that most suppressive soils support large populations of non-pathogenic *Fo* (Smith and Snyder, 1971; Toussoun, 1975), suggesting that at least some of those populations contributed to soil suppressivness (Rouxel *et al.*, 1979). Studies showing that certain non-pathogenic strains of *Fo* can control fusarium wilts efficiently (Lemanceau and Alabouvette, 1991; Mandeel and Baker, 1991; Postma and Rattink, 1992; Alabouvette *et al.*, 1993; Lemanceau *et al.*, 1992, 1993; Hervas *et al.*, 1995; Minuto *et al.*, 1995; Attitalla *et al.*, 1998; Fuch *et al.*, 1999; Larkin and Fravel, 1999) contributed to our interest in testing whether a non-pathogenic strain of *Fo* could be used to control *Fusarium* wilt of tomato.

Non-pathogenic fungi that are endophytes and reside harmlessly within a plant have the opportunity to participate in the plant's physiological activities, such as storage and secretion of sugars and alcohols (Richardson *et al.*, 1990) and in the plant's modification of its leaf characteristics, which can reduce transpiration losses (Richardson *et al.*, 1992). The possibility that such fungi also facilitate plant photosynthesis and associated processes remains controversial (Monnet *et al.*, 2001).

So far only endophytic bacteria have been found to suppress plant pathogenic fungi, likely by means of Systemic Induced Resistance (SIR), at least in some cases (Chen *et al.*, 1994, 1995; Nejad and Johnson, 2000). Although, many attempts have been made to use fungal strains to control *Fusarium* wilts through induced resistance (Biles and Martyn, 1989), the idea of using non-pathogenic fungal strains that are also endophytic has been largely unexplored. Little is known about the potential of such fungi to control disease and induce plant resistance. *Acremonium kiliense*, an endophytic fungus commonly present in wheat roots (Spiegel, 1990), also colonizes other plant species without causing external symptoms (Bargmann and Schonbeck, 1992). In an earlier study, Attitalla *et al.* (1998) found that as a result of a non-pathogenic *Fo* strain (*Fo* IMI 386351) colonizing tomato plants and existing endophytically within them, disease incidence of *Fusarium* wilt was eliminated, caused by a *Fo* pathogenic strain, *Fusarium oxysporum* f. sp. *lycopersici* (*Fol*). As a follow-up to that study, the primary aim of this study was to examine some of the biological attributes of *Fo* IMI 386351 and to further investigate its potential to deter tomato wilt, in this case, when included in the coating of tomato seeds. To explore the molecular phylogenetics of *Fo* IMI 386351, isolates of *Fusarium* sp. NRRL 22903 and 25184 were used as outgroup species for constructing nuclear and mitocondrial gene genealogies.

MATERIALS AND METHODS

Plant material and growth: The tomato host-plant (*Lycopersicon esculentum* Mill.) cultivar Danish Export (susceptible) was used in all greenhouse experiments. Tomato seedlings (21-day-old, 2-4 leaves) were grown in 10-cm diam. pots containing a non-sterile commercial peat mixture Enhetsjord P (Gerhardson *et al.*, 1985) mixed with sand (80:20). Pots were maintained in a greenhouse with a 12 h photoperiod (light source, Osram HPTI/HQI 400W; intensity and irradiance at plant-top level, 7-10 kLux and 30-35 W m^{-2}; day/night temperatures 26±2°C/22°C, RH 60-70%). Fifty milliliter of water-fertilizer solution Osmocote Plus mikro N-P-K-Mg 5-5-11-1.2 at a concentration of 1.5 kg m^{-3} was applied in the course of watering the tomato plants three-times weekly.

Fungi: *Fusarium oxysporum* f. sp. *lycopersici* (Sacc.) Snyder and Hansen race 2 (*Fol*) was obtained from Centraalbureau Voor Schimmelcultures (CBS 165.85), Baarn, The Netherlands and

maintained on Potato Dextrose Agar (PDA). Conidial suspensions were prepared according to Attitalla *et al.* (2001a) at a concentration of 10^6 condia mL^{-1}. The aggressiveness of *Fol* strain CBS 165.85 race 2 was previously tested and recorded as type 3 (Pineau, 1976). A non-pathogenic strain, *Fusarium oxysporum* IMI 386351 (*Fo* IMI 386351), isolated from roots of broad beans (*Vicia faba*) from the south of Sweden (Attitalla *et al.*, 1998), was maintained on V8-juice agar medium. Ten-day-old cultures prepared the same way as for *Fol* (at concentration 10^5 mL^{-1} conidia) were used as inoculum.

***Fo* IMI 386351 morphology (colony and cell examination):** Cultures of *Fo* IMI 386351 grown on plates of Potato Sucrose Agar (PSA) and on Synthetic Nutrient-poor Agar (SNA) plus filter paper were incubated for 10 to 14 d at 20°C under a 12 h light/dark regime (light near UV). Colony features and microscopic characteristics were observed on PSA and SNA, respectively. To enhance sporulation, 1 to 2 pieces (ca. 1 cm^2) of sterile (autoclaved) filter paper was placed on the agar surface.

In *Fo* IMI 386351 molecular phylogenetics

Biological materials: The fungal strains of the study are listed in Table 1. The fungus *Fo* IMI 386351 (which appears to be endophytic for tomato) was derived from a single microconidium and is being stored cryogenically (-175°C) in the Agricultural Research Service (NRRL) Culture Collection at the National Center for Agricultural Utilization Research (NCAUR), Peoria, Illinois. Genomic DNA of this strain (NRRL 29868 = IMI 386351) was prepared using a hexadecyltrimethylammonium bromide protocol as described previously (O'Donnell *et al.*, 1997).

DNA amplification and sequencing: DNA extraction, PCR amplification and sequencing of a portion of a mitochondrial small subunit (mtSSU) ribosomal DNA (rDNA) was done using reagents and primers described by White *et al.* (1990). A partial fragment of the nuclear translation elongation factor 1α (EF-1α) gene was amplified using primers EF-1 and EF-2, which prime within conserved exons as described by O'Donnell *et al.* (1998) and Baayen *et al.* (2001).

Phylogenetic analysis: Using PAUP (phylogenetic analysis using parsimony) version 4.0b1 for Macintosh (Swofford, 1993), phylogenetic analyses were conducted on DNA sequences of the mtSSU rDNA and the EF-1α gene as a combined dataset for the 37-taxon matrix. Unweighted and weighted parsimony analyses were done on the individual datasets (excluding uninformative characters) using the heuristic search option with 1000 random additional sequences when alignment gaps were tested as missing data. *Fusarium* sp. NRRL 22903 and 25184 were used as outgroups (O'Donnell *et al.*, 1998). The robustness of the internal branches of the trees was estimated by bootstrap analyses using 1000 replications in a heuristic search with random stepwise addition (3 replications) (Vinnere *et al.*, 2002). Bootstrap majority-rule (>50%) consensus trees were obtained.

***Fo* IMI 386351 seed application:** The *in vivo* method described by Hokeberg *et al.* (1997) for seed coating with bacteria was adapted for use in fungi. Seeds of the tomato cv. Danish export were surface sterilized with 1% sodium hypochloride for 30 sec and then rinsed in sterile distilled water and dried for 48 h under an air stream. After dividing the seeds into six groups, *Fo* IMI 386351

Table 1: List of the *Fusarium oxysporum* f. sp. isolates used for the molecular phylogenetic study of Fo IMI 386351

Taxon [C# = clonal lineage][A]	NRRL strain number
Fusarium oxysporum f. sp. *batatas*	26409
Fusarium oxysporum f. sp. *erythroxyli*	26574
Fusarium oxysporum f. sp. *passiflorae*	22549
Fusarium oxysporum f. sp. *radicis-lycopersici*	26033
Fusarium oxysporum f. sp. *radicis-lycopersici*	26379
Fusarium oxysporum f. sp. *radici-lycopersici*	26381
Fusarium oxysporum f. sp. *radicis-lycopersici*	26380
Fusarium oxysporum f. sp. *lycopersici*	26383
Fusarium oxysporum f. sp. *tuberosi*	22555
Fusarium oxysporum f. sp. *lycopersici*	26203
Fusarium oxysporum	26374
Fusarium oxysporum f. sp. *lycopersici*	26034
Fusarium oxysporum f. sp. *lycopersici*	26037
Fusarium oxysporum f. sp. biocontrol (*Fo* IMI 386351)	29868-biocontrol
Fusarium oxysporum f. sp. *lycopersici*	26200
Fusarium oxysporum f. sp. *melonis*	26406
Fusarium oxysporum f. sp. *lycopersici*	26202
Fusarium oxysporum f. sp. *inflexum*	20433
Fusarium oxysporum f. sp. *cubense* [C5]	25609
Fusarium oxysporum f. sp. *cubense* [C5]	25367
Fusarium oxysporum f. sp. *melonis*	26178
Fusarium oxysporum f. sp. *vasinfectum*	25420
Fusarium oxysporum f. sp. *glycines*	25598
Fusarium oxysporum	25369
Fusarium oxysporum	25356
Fusarium oxysporum f. sp. *cubense* [C1]	25607
Fusarium oxysporum f. sp. *cubense* [C3]	26022
Fusarium oxysporum	25357
Fusarium oxysporum f. sp. *cubense* [C5]	25603
Fusarium oxysporum f. sp. *cubense* [C5]	25605
Fusarium oxysporum f. sp. *cubense* [C2]	26038
Fusarium oxysporum f. sp. *perniciosum*	22550
Fusarium oxysporum f. sp. *cubense* [C4]	26024
Fusarium oxysporum f. sp. *cubense* [C4]	26029
Fusarium oxysporum f. sp. *canariensis*	26035
Fusarium sp.	22903
Fusarium sp.	25184

[A]The number in parenthesis following the nine strains identified as: *F. oxysporum* f. sp. *cubense* indicates the clonal lineage

inoculum was applied as a seed coating, 6 doses in all (0, 1, 3, 5, 7 and 10 mL of suspension), each to one of the six groups of seeds. The coated seeds were then dried. Each of the 6 groups of seeds represented a *Fo* IMI 386351 dose, as did the plants grown from those seeds.

To avoid root injury, all 21 day-old seedlings were carefully removed and then their roots were washed in sterile distilled water. The seedlings of each *Fo* IMI 386351 dose were divided into two sets. For one of two sets, a *Fol* suspension was added to the root systems of plants (the B set). For the other set, no *Fol* suspension was added to the root systems of the plants (the A set). Thus, there were 12 treatments, six without *Fol* inoculation, coded A-0, A-1, A-3, A-5, A-7, A-10 and six with *Fol* inoculation, coded B-0, B-1, B-3, B-5, B-7, B-10, as shown in Table 2. Plants of the A-0

Table 2: Code names for treatments. The A-0 plants are the healthy controls, and the B-0 plants, the *Fol* controls. A-3 plants, for example, received a 3 mL dose of Fo IMI 386351, which was not followed by *Fol* inoculation; B-3 plants also received a 3 mL dose of Fo IMI 386351, but *Fol* inoculation did follow

Seed coating	Treatment (*Fo* IMI 386351 dose)	Code name
Without *Fol*	0 mL	A-0 (healthy control)
	1 mL	A-1
	3 mL	A-3
	5 mL	A-5
	7 mL	A-7
	10 mL	A-10
With *Fol*	0 mL	B-0 (*Fol* control)
	1 mL	B-1
	3 mL	B-3
	5 mL	B-5
	7 mL	B-7
	10 mL	B-10

treatment were healthy-controls (no *Fo* IMI 386351 in the seed coating and no *Fol* inoculation) and plants of the B-0 treatment were *Fol*-controls (i.e., diseased controls-*Fol* inoculation but no *Fo* IMI 386351 in the seed coating).

For monitoring *Fo* IMI 386351's ability to grow out from the seed, three seeds from each of the six treatments (*Fo* IMI 386351 doses) were moved to Petri-dishes (one seed per dish) containing PDA or Komada (1975) medium. Other seeds used as healthy controls were placed individually in Petri dishes and treated only with distilled water (after surface sterilization).

Plant colonization by *Fo* IMI 386351 and *Fol*: To monitor (for 1 to 16 weeks) the distribution of *Fo* IMI 386351 and *Fol* inside the stem and petioles of all experimental plants, we adapted the method of Liu *et al.* (1995) as described in Attitalla *et al.* (2001b). This was done after inoculation in the case of *Fol* and after application of *Fo* IMI 386351 in the case of *Fo* IMI 386351. At each monitoring time, the distributions of the two organisms within a plant were determined by re-isolations of *Fo* IMI 386351, *Fol*, or both *Fo* IMI 386351 and *Fol* (at each sampled location) from the first through the fourth petiole and by re-isolations from the stem within the region between the first and fourth internode. Three plants were used for each treatment.

In the same manner, shoots and roots were removed from *Fo* IMI 386351 plants and *Fo* IMI 386351+*Fol* plants for monitoring the distribution of the *Fo* IMI 386351 strain within the root and stem of those plants. The roots were washed lightly under tap water before the stem and roots of each plant were cut into small sections. The stem and root sections of each plant, after immersion in ethanol and distilled sterile water, were placed on PDA and Komada (1975) media, respectively. For each plant (in each treatment), three plates were used to visually observe and evaluate the dispersion of the *Fo* IMI 386351 and/or *Fol* within that plant.

Phytotoxicity: *In vivo* responses of tomato plants to *Fo* IMI 386351 application included phytotoxic signs quantified as the number of lesions appearing on the leaf surfaces of the plant. These lesions were not considered to be disease symptoms because those that occurred were apparently inconsequential to the growth and productivity of plants. Moreover, as pointed out by De Vay (1989), the effects of phytotoxins are likely confined to the immediate vicinity of the

organism that is in contact with the plant and thus, showed no correspondence with the occurrence and severity of wilt disease symptoms in our experiments, other than indicating to some extent the strength of induction of SIR by *Fo* IMI 386351.

Chlorosis: For all plants exposed to *Fo* IMI 386351, *in vivo* response to *Fol* inoculation included chorosis, which was evaluated 30 dfi (days following inoculation) as being present or absent. Chlorosis is an early symptom of wilt (De Vay, 1989; Agrios, 2005) and in our experiments, an indicator of *Fol* inoculation.

Wilt-disease incidence: Disease severity in this experiment included essentially two discrete levels, either health or wilt-death of the plant. Those plants showing wilt disease symptoms died of wilt before the end of the experiment and those showing no symptoms remained healthy. Thus, Disease Incidence (DI) (i.e., the proportion of plants that expressed wilt disease symptoms) was an appropriate assessment of *Fol*'s capacity to cause disease for the B-0, B-1, B-3, B-5, B-7, B-10 plants. Following challenge inoculation with *Fol*, plants were visually examined for disease symptoms during a 120 day period. For treatments, DI ranged from 0 (no diseased plants) to 100 (all plants diseased). Each diseased plant had typical tomato wilt symptoms (Agrios, 2005).

Plant fresh weight: At the end of the 120 day experiment, plant shoots were cut 0.5 cm above the soil surface. The shoot fresh weight of each plant was then measured.

Plant dry weight: After measuring shoot fresh weight, plants were dried overnight at 105°C before measuring shoot dry weight (d.wt.).

Physiological response: For the seedling stage of tomato plants (21 day-old, 2-4 leaves), but not for later stages in development, two indices of physiological response were measured, total phenol and peroxidase activity. The fungal inocula were prepared in sterilized potato dextrose broth (Difco) for 10 d at 28°C in 300 mL Erlenmeyer flasks. Mycelium was harvested and washed twice in sterilized distilled water before being thoroughly shaken in 300 mL Erlenmeyer flasks with 50 mL sterilize distilled water in an electrical shaker for 15 min. The mycelial mats were discarded and the conidial suspensions were centrifuged at 3000 rpm for 1 min. The supernatant was discarded and the conidial concentrates at the bottom were collected (Chakraborty and Sen Gupta, 1995). Spore suspensions (1.5×10^5 mL^{-1}) of the test fungi were mixed in sterilized nutrient solution (Osmocote Plus mikro). After pouring this mixture into small black glass bottles, the seedlings were placed in the bottles in such a way (by loosely plugging with non-absorbent cotton) that the root portion remained immersed in the spore suspension and the upper portion, starting from the collar region, remained outside the bottle. The control plants were grown in nutrient solution without inoculum. Plant tissue (5 g) from each treatment was crushed in a precooled mortar at 0°C with 5 mL of 0.05 M citrate phosphate buffer at pH 5 (with 0.5-1 g neutral sand added). The homogenate was centrifuged at 10 000 rpm for 20 min at 0°C. The supernatant was stored for 24 h before being used for enzyme sampling.

Peroxidase activity was measured following the method of Addy and Goodman (1973). Phenol content was estimated (with Folin-Ciocalteau regent) using the method of Biehn *et al.* (1968). Total phenol, measured as cathechol equivalents relative to a standard curve prepared from pure phenol, was expressed as mg g^{-1} fresh weight of tissue, as described by Meena *et al.* (2001). For comparison, a blank containing water and the reagent was used.

Statistical analysis: Analysis of variance (ANOVA) and tests of significance were carried out using SAS statistical package (SAS Institute, 1988). For all experiments related to *Fo* IMI 386351's capacity for biological control of tomato wilt by means of seed coating, a randomized block design (20 replicates) was used and Duncan's test was used to analyze differences between treatment means ($p = 0.05$). To test the repeatability of results, the complete experiments was repeated twice.

RESULTS

The results of this study derive from (a) examination of cell and colony morphology of both *Fo* IMI 386351 and *Fol*, (b) examination of *Fo* IMI 386351's phylogenetic relatedness to provide an evolutionary perspective for some of the results and (c) examination (by means of *in vivo* experiments) of *Fol*'s ability to cause wilt in tomato plants and of *Fo* IMI 386351's capacity to decrease that ability, that is, to reduce or eliminate DI.

***Fo* IMI 386351 morphology:** Colonies grew rapidly on PSA. Within 10 day their diameters were between 80 and 90 mm (maximum size of our plates). Aerial mycelia were floccose, pale pink, with violet pigment in hyphae (reverse on PSA) (Fig. 1a, b). Cylindrical monophialides 8 to 25 μm long were scattered on the mycelia. Microconidia formed in abundance within 2 to 3 day. The microconidia, which were 0 to 1 septate cylindrical (5-15×2-5 μm), collected in small slimy droplets. Macroconidia (25-55×2.5-6 μm) were 1 to 5 septate. The teleomorph of *Fo* IMI 386351 is unknown. The fungus appeared to be similar to *Fo* Schltdl (Schlechtendahl, 1824; Snyder and Hansen, 1940). These results were confirmed by Dr. John C. David, CABI Bioscience, Egham, UK.

***Fo* IMI 386351 molecular phylogenetics:** The partial sequences of the two genes (mtSSU rDNA and EF-1α) used to examine the phylogenetic relatedness of *Fo* IMI 386351 with GenBank accession number of the nucleotide sequence: AY450432. The tree that is inferred by combining the datasets of those two genes and the trees inferred by the separate independent datasets are given in Fig. 2a-c. In the Fig. 2, *Fo* IMI 386351 is referred to as 29868_biocontrol. The tree inferred by the EF-1α gene is included to show that it is essentially the same as the tree inferred by the mtSSU rDNA gene, except that the former includes greater resolution. Consequently, the tree inferred from the combined datasets is essentially the same as the tree inferred from the EF-1α gene, at least for clarifying the phylogenetics of *Fo* IMI 386351.

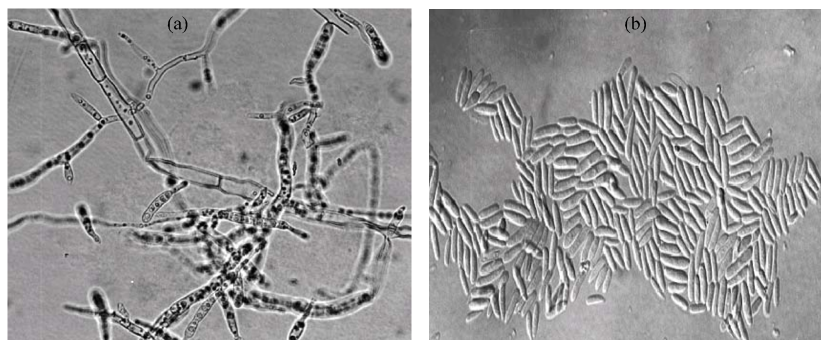
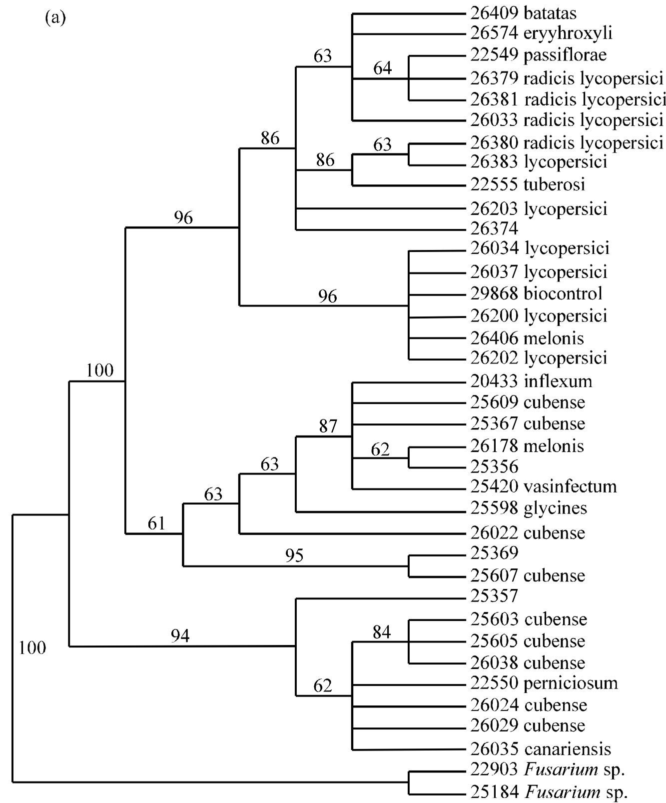


Fig. 1: (a) Aerial mycelia and cylindrical mono-phialides of *Fo* IMI 386351, about 8 to 25 μm, were scattered on the mycelia. (b) Microconidia (smaller-5-15×2-5 μm) and macroconidia (larger-25-55×2.5-6 μm) are illustrated



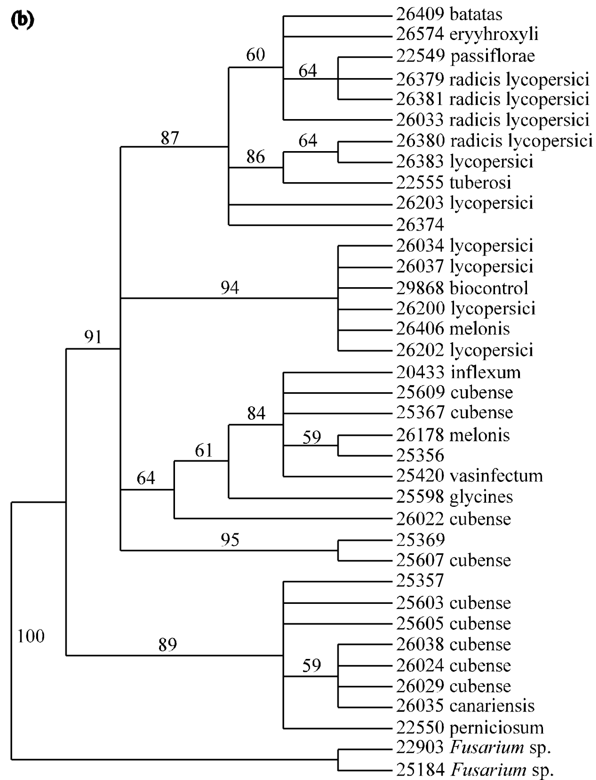


Fig. 2: Continued

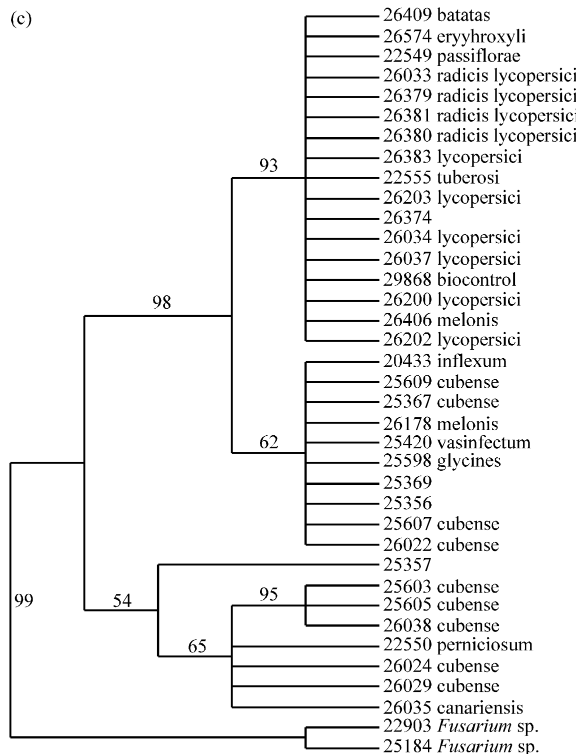


Fig. 2: Phylogenetic trees of all the examined f. sp. of *Fusarium oxysporum* based on the mitochondrial small subunit (mtSSU) ribosomal DNA (rDNA) and elongation factor 1α (EF-1α) genes. The numbers on each branch correspond to bootstrap values. (a) The tree inferred by combining the data of the two genes; (b) the tree inferred by the EF-1α gene; (c) the tree inferred by the mtSSU rDNA gene. The GenBank accession number of the nucleotide sequence: AY450432

Within the tree inferred by the combined data set, *Fo* IMI 386351 is nested in the terminal clade that also includes two f. sp., *Fol* and *Fusarium oxysporum* f. sp. *melonis* (*Fom*). *Fusarium oxysporum* f. sp. *radicis-lycopersici* (*Forl*), the other f. sp. of interest, although morphologically indistinguishable from *Fol* (Boyer and Charest, 1989), occurs in a different terminal clade. *Fo* IMI 386351 is indistinguishable from f. sp. *Fol* and by our results, cannot be a member of *Forl*. Based on the tree, *Fo* IMI 386351 might be a member of *Fom*, but note that *Fol* and *Fom* have polyphyletic evolutionary origins.

RFLP analysis of mtDNA by digestion of total genomic DNA (Cooley, 1992) was used to compare *Fo* IMI 386351 to *Fol* strains collected from several geographical locations in Europe and the Middle East. The analysis showed that *Fo* IMI 386351 has the typical mtDNA profile for *Fol* strains (unpublished data). In addition, the inferred phylogenetic trees indicate that *Fo* IMI 386351 is genetically similar to two American *Fol* strains (NNRL), with about 96% similarity. Every f. sp. that is represented by more than one strain (see the tree inferred by the combined data) is polyphyletic.

Assuming that *Fo* IMI 386351 has the typical mtDNA profile for *Fol* strains, evolution through natural selection may have established a distinct relation between *Fo* IMI 386351 and *Fol* that

Table 3: Colonization of the stem and petioles of tomato plants by Fo IMI 386351 and *Fol*

Treatment[A] *Fo* IMI 386351 (dose)	Presence or absence of *Fo* IMI 386351 after application; and of *Fol*, after challenge inoculation[B]							
	Stem				Petiole			
	4 week	8 week	12 week	16 week	4 week	8 week	12 week	16 week
Seed coating (no *Fol*)								
A-0	–	–	–	–	–	–	–	–
A-1	C	C	C	C	C	C	C	C
A-3	C	C	C	C	C	C	C	C
A-5	C	C	C	C	C	C	C	C
A-7	–	–	C	C	C	C	C	C
A-10	C	C	C	C	C	C	C	C
Seed coating (*Fol*)								
B-0	F	F	F	F	–	F	F	F
B-1	C	F	C,F	C,F	–	C	C,F	C,F
B-3	C	C	C	C	C	C	C,F	C,F
B-5	C	C	C	C	C	C	C,F	C,F
B-7	–	F	F	F	C,F	C,F	C,F	
B-10	C	F	F	F	C	C	C,F	C,F

[A]Stem or petiole tissues incubated on potato dextrose agar (PDA) and Komada's selective medium. Three plants were used per treatment. C, Presence of *Fo* IMI 386351 as a disease control agent. F, Presence of *Fol* as the pathogen causing tomato wilt. C,F, Presence of *Fol* and *Fo* IMI 386351 on the stem and petiole

takes into account their high similarity with distinct differences, particularly concerning their effects and behaviors when they meet in the same host plant, which in some places or circumstances may be frequent.

Fo IMI 386351 seed application

Plant colonization by *Fo* IMI 386351 and *Fol*: *Fo* IMI 386351 was detected in the stem and petioles of those *Fo* IMI 386351 plants receiving the three lowest *Fo* IMI 386351 doses (A-1, A-3 and A-5) for all weeks monitored (from the 4th week through the 16th week after planting) as shown in Table 3. Yet, for the next higher dose (A-7), *Fo* IMI 386351 was undetected until the 12th week and for the next higher dose (A-10), *Fo* IMI 386351 was detected (again) for all weeks monitored. This qualitative shift in plant-endophyte response as the dose proceeded from 5 to 7 mL (A-5 to A-7 plants) was apparently not an experimental artifact or consequence of sampling error. Several other results, to be presented, confirm this interpretation of a sudden shift in the nature of plant defense in proceeding from A-5 to A7 plants, including the DI results.

For *Fo* IMI 386351+*Fol* plants, as dose increased for the three lower doses (B-1, B-3 and B-5 plants), *Fol* ceased to be found in the stem and was only found in the petioles after the 8th week. On the other hand, *Fo* IMI 386351 was found more frequently in the petioles for B-3 and B-5 plants than for B-1 plants, as one might expect from higher doses. Then, consistent with the pattern for *Fo* IMI 386351 plants (unchallenged by *Fol*), in proceeding from B-5 to B-7, a sudden reversal occurred. That is, in B-3 and B-5 plants, *Fo* IMI 386351 was found in the stem at all four weeks, but no *Fol* was found; yet for B-7 and B-10 plants, *Fo* IMI 386351 was undetected in the stem for all four weeks and instead, *Fol* was detected for the last three weeks of monitoring. Yet, as will be shown later, *Fo* IMI 386351 application resulted in elimination of DI for B7 and B-10

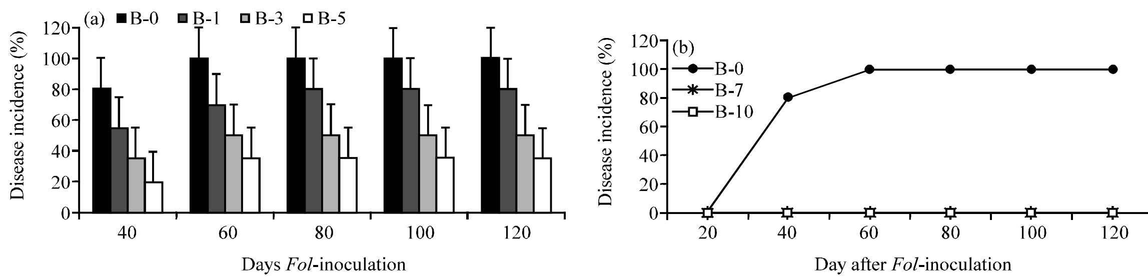


Fig. 3: Disease incidence (DI) response to *Fo* IMI 386351 seed-coating dosage. (a) The DI for *Fo* IMI 386351 plants (B-0, B-1, B-3, B-5) showing the progression of incidence over the 5 sampling times given as days after *Fol* inoculation. Note that only *Fo* IMI 386351+*Fol* plants expressed observable wilt symptoms and that of those plants, B-7 and B-10 plants did not express observable symptoms (DI = 0). In each case, *Fol* was inoculated into the soil of 21-day-old tomato seedlings. Bars indicate 99% confidence intervals ($p \leq 0.001$). (b) The rapid increase in DI for B-0 plants (*Fol*-control), which quickly went to completion (100%). In contrast, the B-0, B-7, B-10 plants did not express wilt symptoms, so their DI trajectories lie along the X-axis. Note that after *Fol*-inoculation no typical wilt disease symptoms could be detected for the first 40 days of observation and therefore DI = 0

plants, indicating that during the 5 to 7 mL dose shift in plant response, the plant-endophyte defense changed qualitatively. That shift involved, among many other things mentioned later, a transformation of *Fol*, whose presence changed from being virulent to being benign. Moreover, as *Fo* IMI 386351 dose was increased further (B-7 to B-10 plants), *Fo* IMI 386351 was present in both the stem and the petiole by the fourth week and continued to be present in the petioles, but not the stem. In other words, one form, pattern, mechanism, or at least expression of plant-endophyte defense seems to have shifted to another.

For *Fo* IMI 386351+*Fol* plants, although *Fol* was undetected in B-3 and B-5 plants, yet *Fol* was apparently able to cause wilt in those plants (i.e., in about 50% of B-3 plants and in about 35% of B-5 plants) (Fig. 3a). At the same time, as mentioned above, *Fo* IMI 386351 plants showed no wilt symptoms. Hence, to assume that wilt in *Fo* IMI 386351+*Fol* plants was caused by the presence of *Fol* and not by the presence of *Fo* IMI 386351 seems reasonable, even though *Fol* cells were not detected in the petioles of *Fo* IMI 386351+*Fol* plants until after the 8th week (56 day). More than 25% of B-3 plants and about 20% of B-5 plants bore wilt symptoms within 40 d (Fig. 3a) (16 day before *Fol* was detected in the petioles of plants) (Table 3). Since *Fol* appeared in the petioles, at least some *Fol* (apparently undetected) had to have colonized the stem in order for *Fol* cells to have reached the petioles. Although the density of *Fol* cells (in the stem) remained too low to be detected by the experimental procedure, the density was presumably enough to cause some of the B-3 and B-5 plants to express wilt symptoms and die.

The fact that *Fol* cells presumably existed in the stem at a density too low for detection may have been partly an experimental artifact, resulting from *Fo* IMI 386351's ability to propagate. *Fo* IMI 386351 quickly grew over *Fol in vitro* (Attitalla *et al*., 1998). Hence, *Fo* IMI 386351 may have quickly established itself at most sites within the stem, leaving very few sites for *Fol*, but apparently enough for *Fol* to cause wilt in 25% of B-3 plants (Fig. 3a). Thus, direct interaction between *Fo* IMI 386351 and *Fol* may have dominated plant protection in the experiments and may have determined DI for B-1, B-3 and B-5 plants; or alternatively, as discussed later, a *Fo* IMI

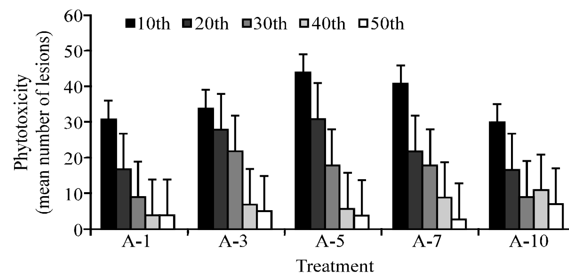


Fig. 4: Phytotoxicity resulting from seed treatment with *Fo* IMI 386351 (no. *Fol* inoculation). Plants were examined 10, 20, 30, 40 and 50 days after planting. No phytotoxicity was observed for the A-0 plants (healthy controls). Bars indicate 99% confidence intervals ($p \le 0.001$)

386351-induced SIR may have been largely responsible, with the level or effectiveness of that SIR being *Fo* IMI 386351-dose-dependent (i.e., dose-dependent SIR-induction strengths).

Phytotoxicity: Although, *Fo* IMI 386351 plants showed no wilt symptoms (DI = 0) and appeared to be healthy, nevertheless, they did bear phytotoxic lesions as early as 10 day after planting. However, the number of lesions soon began decreasing and by 50 day after planting, the number of lesions was reduced by more than 85% for those plants, whose old lower leaves fell off in due course. For A-10 plants reduction was somewhat less (about 80%) (Fig. 4). For all plants, no new lesions appeared 50 day after planting. Similarly, when applying *Fo* IMI 386351 to the soil around the root systems of tomato plants (Attitalla *et al.*, 1998), the plants recovered. In a study by Chakraborty and Sen Gupta (2001), non-pathogen inoculated pigeonpea (*Cajanus cajan* (L.) Millsp.) seedlings showed some initial symptoms of wilt in its early stages, but the seedlings gradually recovered and fresh leaves started appearing after a few days.

Overall, phytotoxicity (at 10 day) appears to have increased with dose for A-1, A-3 and A-5 plants by about 50% and then it decreased with dose in going from A-7 to A-10 plants, reflecting the 5 to 7 mL dose shift in plant-response to *Fo* IMI 386351 and suggesting that, independent of any pathogen, the plant-response to *Fo* IMI 386351 was dose-dependent. Moreover, because the plant's exposure to *Fo* IMI 386351 was through seed treatment, the dose-dependent plant response included dose-dependent systemic physiological response.

For *Fo* IMI 386351+*Fol* plants, phytotoxicity also existed, apparently due to *Fo* IMI 386351 application insofar as *Fol*-control plants (B-0) experienced no observable phytotoxicity. This fact also indicates that phytoxicity was probably not a symptom of *Fusarium* wilt of tomato, in agreement with Duniway (1971). That is, the phytotoxicity for the B-3, B-5 and B-7 plants (Fig. 5) probably did not contribute to the observed wilt of some of those plants, corroborating the opinion of De Vay (1989) that toxins are not actively or directly involved as causes of vascular wilt. The dose-dependent phytotoxicity levels presented in Fig. 5 are again congruent with the 5 to 7 mL dose shift in the defense response of the tomato-*Fo* IMI 386351 complex.

Chlorosis: Only the presence of *Fol* and not the presence of *Fo* IMI 386351, in the root or shoot of a plant caused chlorosis (Table 4), except for B-7 and B-10 plants, in which case chlorosis was absent regardless of the presence of *Fol* in the root and shoot. This again supports the idea that a threshold shift (between the 5 and 7 mL dose) in plant response occurred in the experiments.

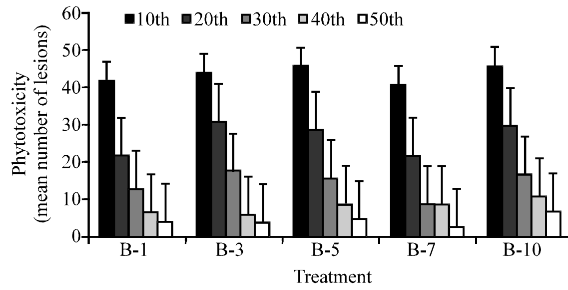


Fig. 5: Phytotoxicity resulting from seed treatment with *Fo* IMI 386351 (*Fol* inoculation). Plants were examined 10, 20, 30, 40 and 50 days after planting. No phytotoxicity was observed for the B-0 plants (*Fol*-control), which presented typical wilt symptoms, as described by Agrios (2005). Bars indicate 99% confidence intervals ($p \leq 0.001$)

Table 4.: Chlorosis after *Fol* inoculation (20 to 30 days), along with presence and absence at that time of *Fo* IMI 386351 and *Fol* in the shoots and/or roots of tomato plants

		Presence in shoot		Presence in root	
Treatment	Chlorosis	*Fo* IMI 386351	*Fol*	*Fo* IMI 386351	*Fol*
A-0	-	-	-	-	-
A-1	-	+	-	-	-
A-3	-	+	-	-	-
A-5	-	+	-	-	-
A-7	-	+	-	-	-
A-10	-	+	-	-	-
B-0	+	-	+	-	+
B-1	+	+	+	-	+
B-3	+	+	+	-	+
B-5	+	+	-	-	+
B-7	-	+	-	-	-
B-10	-	+	+	-	+

+: Presence, -: Absence

Wilt-disease incidence: Any *Fo* IMI 386351-*Fol* plant that expressed wilt symptoms (beyond chlorosis) within 80 dfi was wilt-dead by the end of the experiment. After 60 dfi, which was about 40 day after the first appearance of wilt symptoms, there was no change in DI for B-0 plants (Fig. 3b), since all plants were diseased. For B-1 plants, after 80 dfi (about 8.5 week), no increase in DI occurred.

If the mechanism of plant-endophyte defense had been the same for all *Fo* IMI 386351 doses, then, by inspection of the dose-dependent reduction in DI as the *Fo* IMI 386351 dose increases (Fig. 3a), the bar in the graph for the DI of B-7 plants, instead of being absent (i.e., no DI), would be expected to have been in the neighborhood of about 20%. This observation corroborates the proposed switch from a lower-dose form or degree of plant protection (B-1, B-3, B-5 plants) to a higher-dose form or degree (B-7, B-10 plants). This idea is further corroborated by another observation. For two of the three lower doses of *Fol* (B-3, B-5 plants), the density of *Fol* cells in the stem was too low to be detected. Yet a substantial proportion of those plants developed wilt

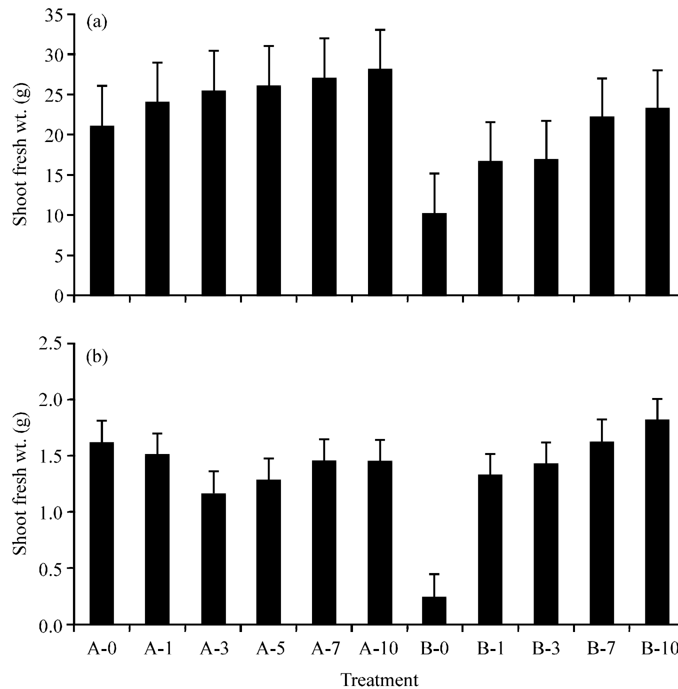


Fig. 6: Plant shoot weights, (a) fresh and (b) dry. Bars indicate 99% confidence intervals ($p \leq 0.001$)

symptoms and paradoxically, stems of the B-7 and B-10 plants contained a detectable density of *Fol* cells and no *Fo* IMI 386351 cells after the 8th week, yet those plants developed no detectable wilt symptoms.

Plant fresh weight: For *Fo* IMI 386351 plants, plant-growth consistently increased with dose (Fig. 6a). Indeed, even A-1 plants (having the smallest *Fo* IMI 386351 dose) had greater fresh weight than the A-0 plants (healthy controls having the benchmark dose of 0). This plant-growth promoting effect of *Fo* IMI 386351 held also for *Fo* IMI 386351+*Fol* plants, but the shoot fresh weight for the B-0 plants (dose of 0) was less than half that of the corresponding *Fo* IMI 386351 plants (A-0 plants). In addition, *Fol* inoculation consistently lowered fresh weight significantly for all doses (Fig. 6a), indicating that both *Fo* IMI 386351 and *Fo* IMI 386351+*Fol* plants underwent a substantial systemic response to *Fo* IMI 386351 application, a response that increased with induction strength. This distinct dose-dependent systemic response to *Fo* IMI 386351 application, which apparently entailed the occurrence of phytotoxic lesions caused by *Fo* IMI 386351, may well have also entailed SIR, with levels being dose-dependent and with A-1 and B-1 plants representing the lowest levels of SIR in our experiments.

Plant dry weight: For *Fo* IMI 386351 plants, shoot dry weight seems to have been dose-dependent (Fig. 6b), such that the first two lower doses (A-1 and A-3 plants) reduced dry weight somewhat and then the next three higher doses increased that weight, but below that of the healthy plants (A-0). This dose-dependent reversal may well have been related to the 5 to 7 mL dose shift in plant-response pattern is shown in Table 5.

Although, the dry weight of B-0 plants was only about 15% of the dry weight of A-0 plants (Fig. 6b), even the lowest dose of *Fo* IMI 386351 (B-1 plants) increased the dry weight of *Fo* IMI

Table 5: Colonization of plant stems by *Fo* IMI 386351 and *Fol* at three plant heights. The times of measurement (given as days after planting) occurred within the indicated intervals

	Plant height (cm)		
Fo IMI 386351 dose	15 (25–35 day)	55 (65–75 day)	105 (90–105 day)
Seed coating (no *Fol*)			
A-0	-	-	-
A-1	++	++	+++
A-3	++	++	+++
A-5	++	+++	+++
A-7	++++	++++	++++
A-10	++++	++++	++++
Seed coating (*Fol*)			
B-0	+	+++	++++
B-1	++	+++	+++
B-3	++	+++	+++
B-5	+++	++++	++++
B-7	+++	++++	++++
B-10	++++	++++	++++

+ = 0–10% of stem sections colonized by *Fo* IMI 386351 or *Fol*; ++ = 10–65%;+++ = 65–80%; ++++ = 80–100%. *Fol* and *Fo* IMI 386351 re-isolated from the plant were tested again using the method described in Attitalla *et al.* (1998). For identifying *Fol* and *Fo* IMI 386351, each fungus produced typical pigments when grown on PDA. Colonies of *Fo* IMI 386351 produced a dark purple color, whereas those of *Fol* produced a pink color

386351+*Fol* plants over that of *Fol* control plants (B-0 plants) by more than 300%. With further increase in *Fo* IMI 386351-dose, the dry weight of *Fo* IMI 386351+*Fol* plants consistently increased. In fact, the dry weight of B-10 plants (i.e., having the highest *Fo* IMI 386351 dose) was even greater than that of the healthy control plants (A-0 plants).

Fol mycelia within the xylem vessels of a tomato plant's stem causes those cells to collapse, become distorted and filled with mycelia and gum (lignin, etc.), which plug the vessels and thereby break down the water economy of the plant. The result is wilted leaves. Note that the two highest *Fo* IMI 386351 doses (the B-7 and B-10 plants) resulted in elimination of DI and that one of the defense pathways provided by induction of SIR is that of lignification of pathogen-exposed plant cells (Pearce and Ride, 1978; Ride, 1975, 1980; Vance *et al.*, 1980; Kuc, 1982; Pan *et al.*, 1992; Kessmann *et al.*, 1994; Terras *et al.*, 1995; Epple *et al.*, 1997). Hence, because lignification of a cell increases its dry weight by adding carbon, the high dry weight of B-10 plants (Fig. 6b) may have been at least partly the result of lignification, an expected effect of SIR. Hammerschmidt and Kuć (1982) found that SIR in cucumber against the fungus *Colletotrichum lagenarium* appeared to involve plant cell lignification, which may have explained the enhanced protection they observed for resistant cultivars.

Physiological response: In this study, tomato plants clearly responded physiologically to seed coating with *Fo* IMI 386351, based on several observations mentioned above and on the observed full recovery of *Fo* IMI 386351 plants from early phytotoxicity. Indeed, compared to healthy-control plants, *Fo* IMI 386351 plants (A-1 to A-10, B-1 to B-10) had significantly higher shoot fresh weight, which consistently increased with increase in *Fo* IMI 386351 dosage. Hence, the physiological response to seed coating with *Fo* IMI 386351 was apparently substantial and systemic.

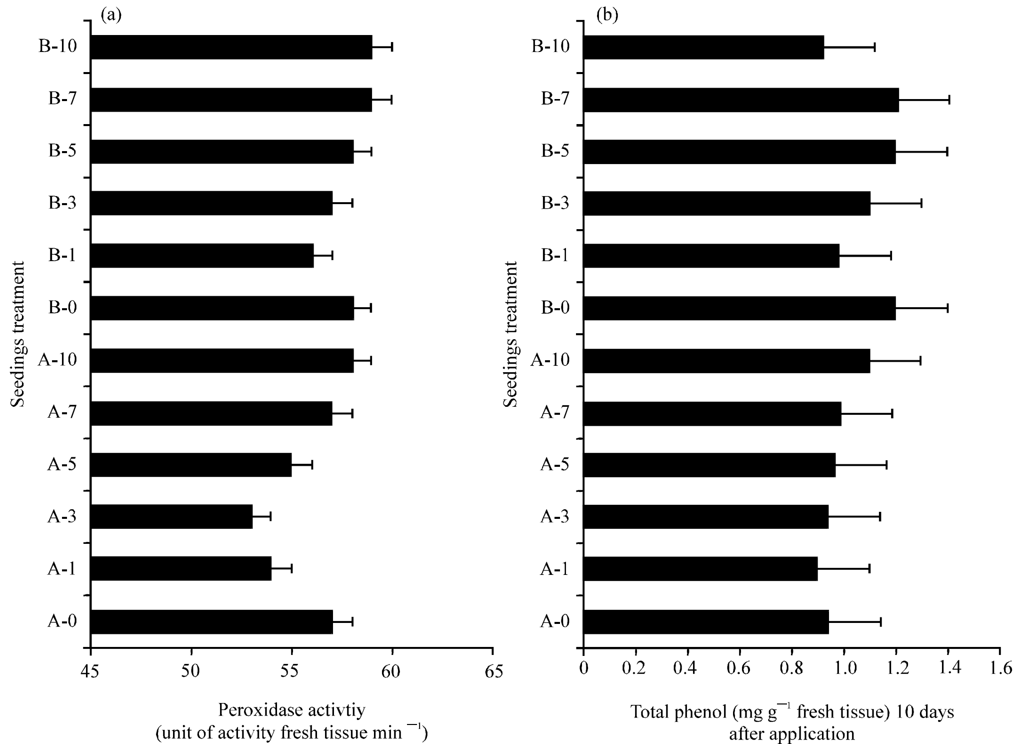


Fig. 7: Physiological responses as indicated by plant-produced compounds. (a) Peroxidase (21 day old seedlings) and (b) Phenol (10 dfi). Bars indicate 99% confidence intervals ($p \leq 0.001$)

Although, in this study we focused on the plant pathological consequences of this systemic physiological response, we did observe dose-dependent enhanced peroxidase activity (Fig. 7a), indicating that change in peroxidase activity was a systemic physiological response of the plants to *Fo* IMI 386351. For *Fo* IMI 386351 plants, the lowest dose of *Fo* IMI 386351 (A-1 plants) significantly reduced peroxidase activity relative to healthy control plants (A-0), but then activity rapidly and consistently increased with higher *Fo* IMI 386351 doses (A-3, A-5 and A-7 plants) and for A-10 plants, reached the same level as reached by *Fol*-control plants (B-0 plants). The increase in peroxidase activity with *Fo* IMI 386351 dose was even more apparent for *Fo* IMI 386351+*Fol* plants (B-1 to B-10 plants), a result that is consistent with enhanced peroxidase activity observed and linked with synthesis of lignin (Ride, 1975). Matta and Gentile (1970) and Matta and Gentile (1973) observed enhanced peroxidase activity following infection of a non-susceptible (resistant) and a susceptible cultivar.

Fo IMI 386351 application also resulted in increase in total phenol, as observed by 10 dfi. Levels of total phenol increase with increase in *Fo* IMI 386351 dose for *Fo* IMI 386351 plants (A-1 to A-10, B-1 to B-10 plants) (Fig. 7b), indicating that change in total phenol content was directly or indirectly involved in the systemic physiological response of tomato plants to seed application with *Fo* IMI 386351. For *Fo* IMI 386351+*Fol* plants, B-1, B-3 and B-5 plants (having lower *Fo* IMI 386351 doses) experienced increase in total phenol with increase in dose, with a maximum occurring, it seems, somewhere between the 5 and 7 mL doses. This observation is again consistent with the proposed shift in plant-physiological response in going from the 5 to 7 mL *Fo* IMI 386351 dose.

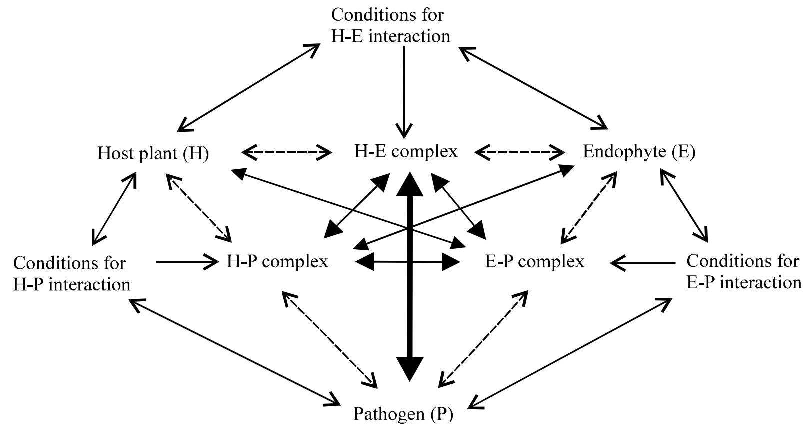


Fig. 8: A graphical illustration of the complexity of interaction that can occur as a consequence of evolution when two fungi (an endophyte and a pathogen) inhabit a plant. Solid arrows represent forms of interaction and each dashed arrow represents the interaction between a complex (being formed) and one of the organisms participating in forming that complex. In the diagram, systemic induced resistance can be viewed as being a critical part of the thickest arrow in the figure

Overall, present results suggest that the relationship between the tomato plant as host, the fungus *Fo* IMI 386351 as endophyte and the fungus *Fol* as pathogen is complex. The evolutionary potential for such complexity seems to be considerable (Fig. 8).

DISCUSSION

When isolates are morphologically identical but attack different hosts, they are classified by host range into f. sp., as in the case of rust fungi (Agrios, 2005). Present results support the idea that such categorization of strains may or may not correspond with phylogenetic clustering, or with natural subdivisions within a pathogenic fungal species, as pointed out by Kistler (1997).Our phylogenetic analysis confirms that *Fo* IMI 386351 is a member of the *Fo* complex. *Fo* IMI 386351 is non-pathogenic toward tomato, at least under the conditions of our experiments, which include the form of inoculation of the fungus. The results of actual inoculations are the essential test of pathogenicity to a given plant cultivar (Olivain and Alabouvette, 1997).

Our observation that *Fo* IMI 386351 lived within the tissues of the tomato plants of this study, without doing substantive harm and without gaining benefit (as far as we know) other than residency, indicated that *Fo* IMI 386351 existed endophytically within the tomato plants., But *Fo* IMI 386351 is probably an exogenous endophyte for tomato because it may well encounter those plants only occasionally.

In the experiments, clear systemic physiological responses of tomato seedlings to inoculation by seed-coating with the non-pathogenic *Fusarium* strain *Fo* IMI 386351 included measured quantitative changes in amounts of peroxidase activity and total phenol (phenols and/or phenol oxidizing enzymes). However, even though these systemic quantitative changes were *Fo* IMI 386351-dose dependent, this does not mean that those particular physiological responses were directly involved in reduction of DI or in SIR, although they might have been.

On the other hand, our study does confirm that phenols might be one of the disease inhibitors and can accumulate as a consequence of infection with non-pathogenic forms of *Fo* (Matta, 1989),

such as *Fo* IMI 386351. Present study also confirms that increase in peroxidase activity can accompany such accumulation of soluble phenols in tomato plants (Ferraris *et al.*, 1987; Gentile *et al.*, 1988). When disease symptoms appeared for susceptible tomato plants, Retig (1974) found a marked increase in peroxidase activity in the stems. He presumed the activity was probably a result of host-senescence acceleration caused by pathogen invasion. He found, however, no such changes in the stems of resistant plants.

The role of phenols in SIR induced by a tomato-nonpathogenic form of *Fo* seems to be indirect (Ferraris *et al.*, 1987), with transitory accumulation of phenols being part of a complex physiological reaction directed to repair stress-induced injuries. The reaction includes increased ethylene evolution (Gentile and Bovio, 1986; Bovio *et al.*, 1987) and the activation of peroxidase, polyphenoloxidase and glycosidases (Gentile *et al.*, 1988; Matta, 1989).

Although, present results for total phenol are consistent with the possibility that general resistance for tomato plants is related to a prompt increase in total phenol (i.e., within 10 dfi), they provide no convincing evidence for that possibility, consistent with the results obtained by Gentile *et al.* (1982) for pepper plants. Based on their data, they assumed that general resistance against the majority of *Verticillium* isolates, coming from hosts other than pepper plants, was related to a prompt increase within pepper plants of phenols and/or phenol oxidizing enzymes.

Fo IMI 386351 caused phytotoxic lesions on plants, but the plants fully recovered (i.e., lesions ceased to appear) and compared to healthy-control plants, plants exposed to *Fol* had significantly higher shoot fresh weight, which consistently increased with increase in *Fo* IMI 386351 dosage. Hence, plants exposed to *Fo* IMI 386351 may well have increased not only in growth, but in health and vigor. Under natural conditions, the fitness of wild tomato plants might well generally be improved by exposure to a non-pathogenic endophytic fungus such as *Fo* IMI 386351. Critically, *Fo* IMI 386351 caused clear systemic physiological responses, which included physiological change underlying plant growth promotion and which caused dose-dependent systemic changes in peroxidase activity and in total phenol.

The clear dose-dependent physiological response to *Fo* IMI 386351, independent of plant exposure to *Fol*, but yet modified by such exposure, is in itself a compelling reason to suspect that SIR was induced by *Fo* IMI 386351. In addition, DI was reduced as a result of *Fo* IMI 386351 exposure; the reduction was *Fo* IMI 386351-dose dependent; and there was a discrete shift in the systemic physiological response of *Fo* IMI 386351 plants to *Fo* IMI 386351. That shift, occurring between two *Fo* IMI 386351 doses, resulted in a change in plant response to *Fo* IMI 386351 and *Fol*, a change that appeared to be qualitative. The shift in plant response resulted in elimination of DI (based on DI observed for previous lower doses of *Fo* IMI 386351), but also in a shift in (a) the distribution of *Fo* IMI 386351 and *Fol* in *Fo* IMI 386351+*Fol* plants, (b) the response of *Fo* IMI 386351+*Fol* plants to phytotoxicity (apparently caused by *Fo* IMI 386351) and (c) the amount of total phenol for *Fo* IMI 386351+*Fol* plants. In addition, *Fol* shifted from a virulent to an apparently benign presence in the plant.

Collectively, these observations strongly suggest that SIR was induced by exposure to *Fo* IMI 386351 (in the manner of seed-coating), at least for the two highest doses of *Fo* IMI 386351 and likely for the three lower doses as well.

At the same time, even if seed application of *Fo* IMI 386351 did trigger SIR, this does not rule out contributions of other mechanisms in the observed tomato-*Fo* IMI 386351 reduction of *Fusarium*-wilt DI (especially at the three lower *Fo* IMI 386351 doses). Site competition, niche exclusion, nutrient competition, siderophore production, or production of antifungal compounds might well have contributed (Cook and Baker, 1983).

Natural selection has the possibility of designingall three of the pairwise relations (complexes) that can determine the fate of a plant colonized by an endophyte and a pathogen, namely, the host-endophyte complex, the host-pathogen complex and the endophyte-pathogen complex. These three pairwise complexes can be integrally interrelated, enough that a modification by natural selection of any one will involve modifications or adjustments in the other two. The close phylogenetic relation between *Fo* IMI 386351 and members of the f. sp. *Fol*, as shown above, suggests that between them a distinct endophyte-pathogen relation may well exist, implemented by natural selection. We presumably observed aspects of such a relation in our experiments when cells of *Fol* and *Fo* IMI 386351 colonized the same plants.

A plant genotype can enjoy a selection advantage by having a superior capacity to provide non-pathogenic fungi such a *Fo* IMI 386351 with residency (whenever such fungi encounter individuals of the plant genotype) and a capacity to take advantage of such residency. Advantages can include induced growth promotion, induced increase in vigor and SIR (i.e., an induced standby capacity for disease resistance and inhibition). Because of such selection advantages, plants in general may well have, by virtue of natural selection, mechanisms for physiological adjustments so as to take full advantage of the residency of non-pathogenic and endophytic fungal genotypes that provide their host plants with means of timely induction of systemic standby states that prepare the plant for appropriate response to environmental change, such as exposure to pathogens. Thus, in at least some cases, by allowing the appropriate plant responses to be dependent on the type or amount of contact with the endophytic fungus, a plant genotype might well gain a selective edge over other genotypes in the population.

Although, such adjustments may be difficult to detect or demonstrate, Krokene *et al*. (2001), for example, observed adjustment in SIR level for Norway spruce trees, based on strength of induction related to the extent of host damage caused by pretreatment. It seems plausible that most plants may have means for making graduated adjustments in induced systemic states, including adjustments in SIR. Such adjustments might be based upon continuous physiological monitoring of circumstances that occur inside the plant and at or near the plant's surface. Quintanilla and Brishammar (1998) observed that induction of resistance in three potato cultivars (susceptible, moderately resistance and resistant) was cultivar-sensitive and within each cultivar, sensitive to the type of inducer, such as an organism or chemical compound.

Plants in general are also likely to have means for making discrete qualitative shifts in induced systemic states, including shifts in SIR level and quality, which occur when certain threshold physiological conditions (local or systemic) are passed. Such shifts can be stepwise, where one standby state includes a capacity for triggering another. In this study of the tomato-*Fo* IMI 386351-*Fol* complex, *Fo* IMI 386351 dose was varied from 0 to 10 mL and within that interval, a threshold dose (somewhere between the 5 mL dose and 7 mL dose) was crossed, which resulted in a qualitative shift in what may well have been a standby state induced by exposure to *Fo* IMI 386351. That qualitative shift included a discrete change in the behavior of *Fol*, which went from a virulent to an apparently benign presence in the plant.

When applied to seeds of the tomato plants, this study shows that *Fo* IMI 386351 can eliminate DI *in vivo* by establishing endophytic residence within the plant and then assisting the plant with protection from pathogens, probably by triggering an induced systemic standby state that includes SIR. Such a form of protection has similarities with cross-protection (Chen *et al*., 1994, 1995). *Fo* IMI 386351's potential for biological control of tomato wilt by means of seed application deserves further study and consideration, as does its biology.

ACKNOWLEDGMENTS

We gratefully acknowledge Professor Lennart Johnsson, MASE lab, Uppsala, Sweden for help with the statistical analysis; Professor Siv Andersson Department of Organism Biology, Upssala University, Uppsala, Sweden for helpful comments and discussion; Professor Mark Bailey at CEH-Oxford, Virology and Environmental Microbiology, UK for reading the paper and useful suggestions; Professor Paul Johnson for assistance in interpreting the data and for critical reading and refining of the manuscript; Universiti Sains Malaysia (Malaysia) for a visiting scientist opportunity and Omar Al-Mukhtar University, Al-Bayda, (Libya) and Uppsala University (Sweden) for the financial support.

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