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Pseudomonas sp. strain MF30 suppresses Fusarium wilt of tomato *in vivo*

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Summary. In a search of bacterial biological control agents, 50 bacterial isolates collected from roots of wild plants in northern Sweden were tested *in vivo* for suppression of wilt of tomato caused by *Fusarium oxysporum* f. sp. *lycopersici*. Tomato plants were sown in 10-cm-diam. pots and after 21 d 7 ml of bacterial suspension (ca. $2x10^9$ cfu ml^{-1}) was poured into the soil around each plant. Two days later, 10 ml of pathogen suspension was soil-inoculated (10^6 conidia ml^{-1}) around the same plants. After a further 20 days, disease incidence was measured. One bacterial isolate, MF30, protected plants from Fusarium wilt, even though the fungal pathogen and not MF30 actually colonized the plant. Several mechanisms may have contributed to the suppression of Fusarium wilt, including systemic induced resistance. The MF30 strain is highly similar to members of the RNA group I of the *Pseudomonas fluorescens*, well known for its capacity to induce systemic resistance.

Key words: *Fusarium oxysporum* f. sp. *lycopersici*, biological control.

Introduction

Tomato wilt caused by the fungal pathogen *Fusarium oxysporum* f. sp. *lycopersici* Sacc. Snyder and Hansen (*Fol*) is one of the most prevalent and destructive diseases of this plant, especially where it is grown intensively. It can cause great losses, particularly in susceptible cultivars and under suitable conditions (Agrios, 1997), producing stunting, wilting, and finally death.

Many groups of micro-organisms found in the soil are potential biological control agents (BCAs), including several bacteria that suppress Fusarium wilt, such as *Bacillus subtilis* (Podile *et al*., 1985), *Bacillus* sp. (Kapoor and Kar, 1988), *Streptomyces* sp. (Thirumalachar *et al*., 1970; Turhan, 1981; El-Abyad *et al*., 1993), *Pseudomonas* spp. (Nejad and Johnson, 2000), and *Streptomyces* spp. when combined with herbicides (El-Shansoury *et al*., 1996).

However, the most successful mean of controlling Fusarium wilt is still the use of resistant cultivars (Beckman, 1987), even though the genetically governed disease resistance of such cultivars may be overcome by new pathogenic races (Tello and Lacasa, 1988). Using micro-organisms to control disease is an alternative strategy. However, as Weller (1988) and Schippers (1992) point out, biological control has so far had inconsistent results. Therefore, better BCAs and more knowledge of their disease-suppressive action is needed (Duijff *et al*., 1998).

In this paper, results of screening of bacterial

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isolates to identify effective BCAs against *Fol* are reported. One of the screened isolates, MF30 (*Pseudomons* sp.), eliminated tomato wilt under greenhouse and climate-chamber conditions. To examine constraints on the ability of MF30 to interact directly with *Fol* within the plant by entering and colonizing plant tissue after soil application of *Fol* around the root system, a test was run to monitor the distribution of MF30 and *Fol* within the plant.

Materials and methods

Plant material and greenhouse conditions

The tomato cv. Danish Export (susceptible to race 2 of *Fol*) 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 Wm^{-2}; day/night temperatures 26±2ºC/22ºC, RH 60–70%). Fifty ml 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.

Fungal isolate

Strain ISP218 of *Fol* (race 2) was obtained from the Centraalbureau voor Schimmelcultures, Baarn, The Netherlands, and used in the experiments. *Fol* cultures were stored in potato-dextrose agar (PDA) (Difco Laboratories, Detroit, MI, USA) slants at 5°C and grown in darkness (20–25°C). To obtain the microconidial inoculum, Erlenmeyer flasks each containing 300 ml sterile Czapek-Dox broth (Difco) were each inoculated with five mycelial plugs of *Fol* from a 10-day-old culture on PDA. After incubation in a rotary shaker for 5 d (150 rpm, 20–25°C), the culture was filtered through sterile glass wool, and the suspension was diluted to 10^6 conidia ml^{-1} as described in Attitalla *et al*. (1998). The aggressiveness of the *Fol* isolate was tested according to Pineau (1976).

Bacterial isolates

Bacteria used in the study were isolated from the roots of wild plants in northern Sweden as described in Hökeberg *et al*. (1997). Fifty of the isolates (MF isolates) that grew fastest on agarized (1.2%) tryptic soy (TSA) broth medium (Difco) at 15°C were screened for their biological control of Fusarium wilt.

Biocontrol experiments

Experiment 1 (greenhouse)

Each of the 50 MF isolates, which had been stored freeze-dried, was streaked on a TSA medium plate. One to 3 colonies from each TSA plate were used to inoculate 250-ml half-strength TS broth in a 1-l Erlenmeyer flask. The flasks were incubated at 15°C for three days, and at 18–20°C on a rotary shaker (120 rpm) in darkness for 48 h. The resulting bacterial suspension was adjusted to a concentration of about $2x10^9$ cfu ml^{-1} and immediately used in the experiments. Seven ml of suspension was poured on the soil surface around the root system of 21-day-old potted tomato seedlings (1 seedling/pot; 10 pots/isolate).

For the MF+*Fol* treatment, application of MF isolates was followed 48 h later by *Fol* inoculation. For this, 10 ml of *Fol* suspension (10^6 conidia ml^{-1}) was added to the soil around each plant. For 50 days after inoculation with *Fol*, the disease incidence (DI) (percentage of diseased plants) was calculated by visual inspection. The following served as control plants: (1) plants treated with only TSB (TSB-ctrl); (2) plants treated with only tap water (H-ctrl); (3) plants inoculated with only bacteria (MF-ctrl) and, (4) plants inoculated with only *Fol* (*Fol*-ctrl).

Experiment 2 (growth chamber)

One of the 50 MF isolates, MF30, which completely eliminated Fusarium wilt (DI=0) under greenhouse conditions, was further tested in a climate chamber (day/night temperatures 24±2/20ºC, RH 60%). Each potted plant received 20 ml of the water+fertilizer (Osmocote Plus mikro) solution twice weekly. At the end of this 60-d experiment, shoots of plants inoculated with MF30 or only *Fol* were cut 0.5 cm above the soil surface, and shoot d wt was measured after the shoots were dried overnight at 105ºC.

To determine the colonization and distribution of MF30 and/or *Fol* in the MF30-ctrl, *Fol*-ctrl and MF30+*Fol* plants, the microscopic method of Bargmann and Schönbeck (1992) with modifications was

used. Three times during the inoculation period, at plant heights of 7, 20 and 60 cm, one plant per treatment was sampled. For sampling, the root and stem was washed lightly under tap water, and then cut into 1-cm long sections. The sections were immersed in 70% ethanol for 1–2 min, rinsed in sterile distilled water, and then placed on plates containing PDA to test for the bacterium and plates containing TSA to test for the fungus. Any colonization by MF30 or *Fol* in each MF30+*Fol* plant was measured as the percentage of root and shoot sections colonized by the bacterium and/or fungus.

In vitro experiments

MF30 and, for comparison, 7 other MF isolates (MF7, MF9, MF11, MF20, MF27, MF29, MF44), all of which reduced the DI of Fusarium wilt by at least 50%, were tested for their ability to antagonize mycelial growth of *Fol* in three culture media (TSA, PDA, and King's B agar), using the method of Cartwright and Benson (1995). Because the MF30 strain was taken from a plant growing in the far north of Sweden, the capacity of this isolate to grow at warmer temperatures was also determined. Cells of the MF30 strain were streaked in Petri dishes on the same three media as those used for the antagonism studies and incubated at 25°C and 28±2°C.

Characterization of strain MF30

The MF30 bacterial strain was identified with the API NE 20 test (BioMerieux, Marcy l'Etoile, Marcy, France). Further characterization was performed at Deutsche Sammlung von Mikro-organismen aus Zellkulturen GmbH, Braunschweig, Germany (DSMZ).

Statistical analysis

A randomized complete block design (10 blocks) was used to test the 50 MF isolates for their effectiveness as BCAs (biocontrol experiment 1). The experiment was done twice to check for repeatability. Two-way ANOVA with 10 replications was used to analyze the data, and Duncan's test (P=0.05) was used to compare differences between means. The same experimental design and statistical analysis was used to determine the biocontrol capacity of the MF30 strain (biocontrol experiment 2).

Results

Biocontrol experiments

Experiment 1 (greenhouse)

In the effectiveness test of the 50 MF isolates, 8 isolates showed a reduction in Fusarium wilt (P=0.05) of at least 50%. These isolates were tested *in vitro*. One, MF30, was particularly effective, and completely eliminated Fusarium wilt (DI=0). This isolate was further tested for its biocontrol capacity under climate chamber conditions in experiment 2.

Experiment 2 (growth chamber)

In the growth chamber isolate MF30 demonstrated its ability to suppress *Fol* wilt (Fig. 1). There was an absence of wilt also in the MF30-ctrl group, consisting of plants inoculated only with MF30, in the TSB-ctrl group, consisting of plants inoculated only with TSB, and in the H-ctrl group,

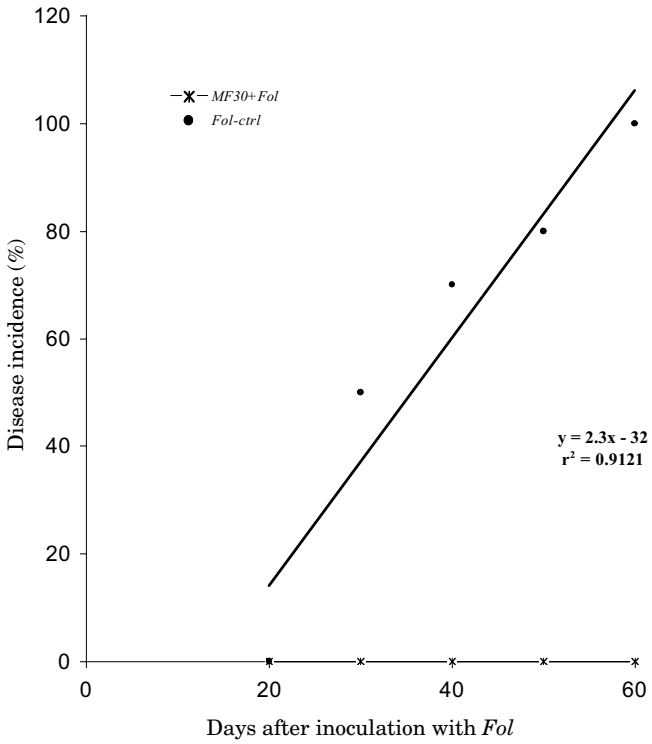

Fig. 1. Effect of MF30 soil bacterization under climate chamber conditions on disease incidence caused by *Fusarium oxysporum* f. sp. *lycopersici (Fol)*. Both MF30+*Fol* plants and *Fol*-control (*Fol*-ctrl) plants were inoculated with *Fol*, but in the MF30+*Fol* plants *Fol* inoculation was preceded by MF30 bacterization.

consisting of plants inoculated only with water. In the *Fol*-ctrl group, on the other hand, which were inoculated with only *Fol*, about 50% of plants showed wilting and chlorosis of the leaves 30 d after inoculation, and by the end of the trial, after 60 d, the DI had increased to 98%.

Soil inoculation with strain MF30 caused a slight reduction (4%) in the d wt of tomato stems (Table 1). This reduction reached 22% in the *Fol*-ctrl group but was only 12% in the MF30+*Fol* group. Inoculation with only MF30 did not cause any changes in tomato (e.g., necrotic spots on the leaves, chlorosis, lower stem length).

Reisolation tests with MF30 and *Fol* on tomato plants in the MF30-ctrl group, the *Fol*-ctrl group and the MF30+*Fol* group, showed that only the fungus penetrated into the roots from where it invaded the stem and eventually reached the plant apex. Nevertheless, it seemed that in the MF30+*Fol* group soil inoculation with MF30 reduced the extent to which *Fol* spread into the host. In the *Fol*+ctrl group 65–60% of tomato stem sections were colonized with *Fol* in 60-cm-high plants, and 80–100% in 7 and 20-cm-high plants; but in the MF30+*Fol* group the colonization percentages were much lower: 10–65% in 7-cm-high plants and 0–10% in 20 and 60-cm-high plants. *Fol* was also abundantly reisolated from the roots of tomato plants from the *Fol*+ctrl group (data not shown).

In vitro experiments

None of the eight strains that most reduced the DI in the greenhouse inhibited radial growth of *Fol* when grown in dual culture in any of the three culture media. MF30, although acclimated to the relatively low temperatures of northern Sweden, also grew well *in vitro* at the higher temperatures 25°C and 28±2°C (data not shown).

Characterization of strain MF30

On the basis of the characteristics revealed by the API NE 20 test (Table 3), the bacterial strain MF30 was assigned to the genus *Pseudomonas*. Partial sequences of 16 SrDNA showed a 99.6% similarity to *Pseudomonas veronii* and a high similarity to other members of RNA group I of the *Pseudomonas*. Strain testing was done by DSMZ which also found that the profile of the cellular fatty acids was typical for that RNA-group; and that, although physiological tests were unable to distinguish between the species in this group, yet they were all closely related to biovars of *Pseudomonas fluorescens*.

Discussion

The genus *Pseudomonas* is well known for its biocontrol capacities. The biovars of *Pseudomonas fluorescens* in particular, to which MF30 appears closely related, naturally suppress Fusarium wilts (Alabouvette and Lemanceau, 1996). These biovars are known to employ several biocontrol mechanisms: production of anti-fungal compounds (e.g., hydrogen cyanide, antibiotics, or extracellular enzymes antagonistic to the wilt fungus), siderophore production, nutrient competition, niche exclusion,

Table 1. Effect of soil inoculation of *Pseudomonas* strain MF30 alone or in combination with *Fusarium oxysporum* f. sp. *lycopersici* (*Fol*) on the growth and occurrence of leaf chlorosis of tomato plants.

Treatment	MF 30	*Fol*	Shoot d wt (%)	Chlorosis
H-ctrl	NI	NI	100	-
MF30-ctrl	I	NI	96	-
Fol-ctrl	NI	I	78	+
MF30+*Fol*	I	I	88	-

H, tap water treatment; ctrl, control.
I, inoculated (with MF30 or *Fol*); NI, not inoculated.
+, presence of chlorosis on leaves.
-, absence of chlorosis on leaves.

Table 2. Percentage of sections of tomato stems colonized by *Fusarium oxysporum* f. sp. *lycopersici* (*Fol*) after the plants were inoculated with *Fol*, or with *Fol* + *Pseudomonas* strain MF30.

Treatment	Plant height (cm)		
	7	20	60
Fol-ctrl	++++	++++	+++
MF30+*Fol*	++	+	+

+, 0–10% of stem sections colonized by *Fol*; ++, 10–65%; +++, 65–80%; ++++, 80–100%.

Table 3. Phenotypic characteristics of *Pseudomonas* strain MF30.

Characteristic	Result
Shape of cells	rods
width µm	0.6–0.8
length µm	2.0–3.5
Gram reaction	-
Lysis by 3% KOH	+
Aminopeptidase (Cerny)	+
Oxidase	+
Catalase	+
Motility	+
Flagella	polar>1
Urease	-
Hydrolysis of gelatin	+
" " esculin	-
Utilization of	
glucose	+
phenylacetate	-
citrate	+
malate	+
arabinose	+
mannose	+
mannitol	+
adipate	-
caparte	+
gluconate	+
maltose	-
trehalose	+
sorbitol	+
citraconate	+
erythrite	+
m-inosite	+
L-lysin	+
iso-butyrate	-
α-amino-butyrate	-
butyrate	-
Growth at 41ºC	-
Denitrification	-
Lecithinase	+
Hydrolysis of tween 80	-
Levan from sucrose	-
Flurorescence	+
Pyocyanin	-

and induction of systemic resistance (Kloepper and Schroth, 1978; Cook and Baker, 1983; Thomashow and Weller, 1988; Weller, 1988).

Van Loon *et al*. (1998) state that the clearest evidence for systemic induced resistance mediated by a plant growth-promoting rhizobacterium (PGPR) is obtained when the rhizobacterium does not actually antagonize the pathogen in culture. The experimental results of Wei *et al*. (1991) support this statement. In our *in vitro* experiments too, MF30, which is probably a strain of *Pseudomonas fluorescens*, had no antagonistic effect on *Fol* grown in dual culture on any of the media, suggesting that systemic induced resistance was involved in the *in vivo* disease suppression. Another possibility is that MF30 produced a siderophore which lowered the growth of *Fol* in the soil and thus reduced or removed its virulence (Lucas, 1998).

In conclusion, three properties make MF30 a promising candidate for field tests and for practical application against Fusarium wilt caused by *Fol*: (1) It eliminates Fusarium wilt when inoculated in the soil around tomato plants in the greenhouse and the climate chamber; (2) it may do so by triggering systemic resistance in the plant; and (3) the biological process of eliminating Fusarium wilt does not appear to have any damaging side effects: no necrotic spots or other signs of toxicity were noted in any of the plants inoculated with MF30, or with MF30+*Fol*.

Experiments with MF30 are now being run to determine the dose response. Further experiments will explore the biochemical and physiological mechanisms involved in Fusarium wilt suppression by application of MF30.

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