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Repeated applications of *Penicillium oxalicum* prolongs biocontrol of fusarium wilt of tomato plants

Biological control of fusarium wilt in tomato plants

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Abstract

The suppression of fusarium wilt of tomato achieved by *Penicillium oxalicum* (PO) applied one or several times (up to four) was assessed during three glasshouse experiments. The first application of PO (10^6 conidia g^{-1} substrate) to the growing substrate (peat and vermiculite, 1 : 1, v : v) was performed prior to its infestation with *Fusarium oxysporum* f. sp. *lycopersici* (FOL) (10^4–10^6 chlamydospores g^{-1}). Repeated applications of PO prolonged the duration of control of fusarium wilt especially when disease incidence was high. The timing of repeated applications of PO did not affect the efficacy of the control. Disease reduction was not associated with a decrease in density of FOL in the rhizosphere, irrespective of the number of applications of PO. Density of PO in the tomato rhizosphere was higher when repeated applications were made. No relationship was observed between reduction of disease and high densities of PO. Reasons for a longer disease reduction in tomato plants following several applications with PO are discussed.

Introduction

Fusarium wilt in tomato plants (*Lycopersicon esculentum* Mill.) caused by *Fusarium oxysporum* f. sp. *lycopersici* (FOL) (Sacc.) Snyder & H.N. Hansen is an economically important disease. As with other vascular plant diseases, chemical control is not effective and sanitation measures are difficult to apply (Brayford, 1992). The primary control strategy consists of breeding wilt-resistant cultivars (Beckman, 1987). However, new races of the pathogen have appeared which have overcome resistance in the cultivars currently grown (Tello and Lacasa, 1988). This may explain why fusarium wilt of tomato remains a serious and persistent disease, despite intensive efforts to breed for resistance.

In recent years, the process of plant 'immunization' or induced resistance to diseases has received increasing attention (Benhamou et al., 1994, 1996; Kuć, 1987). De Cal et al. (1997a) reported that treatment of tomato plants with conidia of *Penicillium oxalicum* (PO) Thom. induced resistance against tomato wilt. *Penicillium oxalicum* and FOL were placed at separate sites on the tomato plants or in the soil, avoiding a direct interaction between the fungi. *Penicillium oxalicum* induced a reduction in disease severity and colonized the tomato rhizosphere but the biocontrol fungus was not detected inside stems (De Cal et al., 1997a). The application of a conidial suspension of PO by watering the tomato seedlings in seedbeds 7 days before transplanting provided the best control of tomato fusarium wilt (De Cal et al., 1999). Disease suppression was maintained for 60–100 days after inoculation with the pathogen in the glasshouse (De Cal et al., 1999). However, control rates varied from 70% to 20% and diminished with time (De Cal et al., 1995, 1999).

This paper provides information concerning attempts made to improve the biocontrol duration of FOL in glasshouse tomato plants through the application of PO several times after transplanting.

Materials and methods

Isolates and inoculum preparation

An isolate of FOL race 2 (ATCC number 201829) obtained from a tomato plant in southern Spain was stored at 4 °C in tubes containing sterile sand. The fungus was grown on Czapek-Dox agar (CDA) (Difco; Detroit, MI, USA) in darkness at 25 °C for mycelial production. The aggressiveness of FOL was previously tested and recorded as type 3 according to Pineau's scale (Pineau, 1976). Microconidia of FOL were produced in flasks (250 ml) containing 150 ml of sterile Czapek-Dox broth (Difco; Detroit, MI, USA), each inoculated with three mycelial plugs (1 cm diameter) of the fungus taken from 7-day-old cultures on CDA (De Cal et al., 1995). The flasks were incubated for 5 days at 25 °C in a rotary shaker (Lab-Line Instruments, Inc., model 3527, Melrose Park, Illinois, USA) at 150 rpm, and the culture was filtered through glass wool. Chlamydospores of FOL were produced in pots (20 × 20 × 9 cm) containing sterile peat (Gebr. BRILL substrate GmbH & Co. KG, Germany) inoculated with 50 ml of a microconidial suspension of the pathogen in Czapek-Dox broth (giving a final concentration of 4.1×10^5 microconidia g^{-1} peat). Pots were placed in the glasshouse for 30 days at 20–30 °C before transplanting. At this time almost all the microconidia became chlamydospores such as described in De Cal et al. (1997b).

The isolate of PO was stored on homemade potato dextrose agar (PDA) slants at 4 °C. It has been deposited with the American Type Culture Collection (Manassas, VI, USA). Treatments with PO were made with conidia from PDA plates, which were incubated in darkness at 20–25 °C for 7 days. Spores were removed gently from the surface of each plate culture by adding sterile distilled water, and the suspensions were adjusted to 10^7 conidia ml^{-1}.

Plant material

The tomato cultivar used was Lorena (Novartis), which is susceptible to race 2 of FOL but resistant to race 1. Seventy tomato seeds were sown separately in each tray (1200 cm^2) containing an autoclaved mixture of vermiculite (Termita, Asfaltex, S.A., Barcelona, Spain) and peat (Gebr. BRILL substrate GmbH & Co. KG, Germany) (1 : 1, v : v), and maintained on benches in a growth chamber at 22–28 °C with fluorescent light (100 μE m^{-2} s^{-1}, 16 h photoperiod) and 80–100% humidity for 3 weeks. After that, the trays were placed on benches in a glasshouse at 20–30 °C.

Glasshouse experiments

Three glasshouse experiments were carried out. In all the experiments, tomato plants were treated in seedbeds as described in De Cal et al. (1999). A conidial suspension of PO was watered on seedbeds to give 2×10^6 spores g^{-1} substrate (peat : vermiculite, 1 : 1, v : v) (treatment T3 in Table 1). After 7 days, when the seedlings had two to four leaves, they were carefully transplanted into pots (20 × 20 × 9 cm), containing peat and chlamydospores of FOL. Four plants were cultivated in each pot. These transplants were made on 8 April 1997 (experiment A), on 16 February 1998 (experiment B) and on 23 October 1998 (experiment C). Additional treatments with PO were applied after transplanting (Table 1). Control treatments consisted of: (i) non-protected plants transplanted into sterile peat (autoclaved for 1 h at 1 kg cm^{-2} and 120 °C, three consecutive days) (treatment T1), and (ii) non-protected plants transplanted into FOL-infested peat (treatment T2). Ten pots per treatment were placed in a glasshouse at 20–30 °C in a randomized complete block design. Disease incidence, recorded at 15, 20, 30, 40, 50, 60, 70 and 80 days after transplanting, represented the percentage of plants with fusarium wilt symptoms. To confirm that plants with symptoms were infected with FOL, all plants were transferred to humid chambers at the end of each experiment and the presence or absence of mycelium of the pathogen in the basal stem of the plants was determined after 48 h incubation at 25 °C.

The density of FOL was estimated in the peat just before transplanting (at the beginning of the experiments) as colony forming units per gram of dry soil. At the same time, the density of PO was estimated in the rhizosphere of three plants per treatment, in terms of cfu per gram of fresh root. The densities of both fungi in the rhizosphere of the three plants per treatment were also estimated at the end of the experiments. The soil samples and/or roots were weighed, transferred to 250 ml flasks with 150 ml phosphate buffer (pH = 7), and the flasks shaken for 30 min at 150 rpm; 10- and 100-fold dilutions were made, and aliquots (100 μl) from undiluted and diluted suspensions were spread onto Petri plates containing selective media for the pathogen and the antagonist (De Cal et al., 1997a). Three Petri dishes were used per dilution. Petri dishes were incubated in

Table 1. Treatments of PO applied to tomato plants inoculated with FOL in three glasshouse experiments[a]

Treatment	Treatment no.	Experiment[b]	Description
Controls: no PO application	T1	A, B, C	Non-infested and non-protected plants
	T2	A, B, C	Inoculated and non-protected plants
1 × PO application	T3	A, B, C	PO applied to seedlings 7 days before transplanting to infested peat
2 × PO application	T4	A, B	T3 + PO to plants 7 days after transplanting
	T5	A, B, C	T3 + PO to plants 15 days after transplanting
	T6	C	T3 + PO to plants 20 days after transplanting
	T7	A, B, C	T3 + PO to plants 30 days after transplanting
3 × PO application	T8	A, B	T3 + PO to plants 7 and 15 days after transplanting
	T9	A, B	T3 + PO to plants 7 and 30 days after transplanting
	T10	C	T3 + PO to plants 15 and 20 days after transplanting
	T11	C	T3 + PO to plants 15 and 30 days after transplanting
	T12	C	T3 + PO to plants 20 and 30 days after transplanting
4 × PO application	T13	A, B	T3 + PO to plants 7, 15 and 30 days after transplanting
	T14	C	T3 + PO to plants 15, 20 and 30 days after transplanting

[a]Each application with PO was made by watering the substrate with a conidial suspension of the fungus to give 2×10^6 spores g^{-1} substrate. Inoculation with FOL was made by producing chlamydospores of the fungus in peat pots before transplanting tomato plants as described in Materials and methods.
[b]Tomato seedlings were transplanted to pots on 8 April 1997 (experiment A), on 16 February 1998 (experiment B) and on 23 October 1998 (experiment C).

the dark at 25 °C for 5–7 days, after which the colonies were counted (Lacey et al., 1980).

Data analysis

Data of densities were log transformed before being analysed by variance analysis (Snedecor and Cochran, 1980). When the F-test was significant at $P = 0.05$, treatment means were compared, using the Student–Newman–Keuls test. Incidence data from each of the glasshouse experiments were analysed independently by variance analysis and by contrast analysis with F-test at $P < 0.05$ (Snedecor and Cochran, 1980). After analysis of variance, when the F-test was significant ($P = 0.05$), treatment means were compared using the Least Significant Difference test at $P < 0.05$ (Snedecor and Cochran, 1980). Treatment 1 (non-infested and non-protected control) was excluded from analyses. Before analysis, disease incidence data were subjected to arcsin (experiments A and C) or sqrt (experiment B) transformation to achieve homogenization of variances.

Results

Tomato plants infected with FOL showed symptoms of fusarium wilt manifested as leaf and stem wilting (De Cal et al., 1995, 1997a, 1999) in all experiments. Disease incidence for treatment 2 (infested and non-protected plants) at 80 days after transplanting was higher in experiments A and C (100% for both experiments) than in experiment B (62%) (Table 2). Non-protected and non-infested plants (T1) did not show disease symptoms, except for experiment C at 80 days after transplanting.

The reference treatment T3 (one application of PO to seedlings 7 days before transplanting) resulted in a significant reduction of disease incidence that decreased with time (Table 2). Tomato plants treated with T3 showed a control of disease incidence until 60, 70 and 20 days after transplanting in experiments A, B and C, respectively (Table 2). At these dates, disease was reduced at similar levels with all the treatments (1 × PO, 2 × PO, 3 × PO or 4 × PO), except in experiment B, where T13–T14 (4 × PO) reduced more fusarium wilt than T8–T12 (3 × PO) (Tables 2 and 3). After these dates, new applications of PO were necessary to control disease: T3 (1 × PO) did not control disease (Tables 2 and 3). When disease incidence was high (100% in experiments A and C, Table 2), PO should be applied three or four times (T8-14) to control fusarium wilt, while only twice (T4, T5 and T7) with lower disease pressure (62% in experiment B). No significant differences were observed in any of the three experiments when comparing the control of disease incidence

Table 2. Disease incidence of tomato plants (cv. Lorena) infested with FOL and treated with PO in three glasshouse experiments at different days after transplanting[a]

Treatments[b]	Experiment A		Experiment B		Experiment C	
	60 days	80 days	70 days	80 days	20 days	80 days
T2	96.0 (82.60)	100.0 (90.0)	50.0 (6.54)	62.0 (7.56)	96.0	100.0 (90.0)
T3 (1 × PO)	76.0 (56.00)	94.0 (81.0)	24.0 (3.60)	34.0 (5.01)	62.0	96.0 (84.7)
T4 (2 × PO)	78.0 (62.80)	90.0 (75.9)	14.0 (1.97)	32.0 (4.31)	—	—
T5 (2 × PO)	68.0 (45.70)	98.0 (86.3)	14.5 (2.65)	12.0 (2.42)	76.0	86.0 (68.6)
T6 (2 × PO)	—	—	—	—	72.0	98.0 (86.3)
T7 (2 × PO)	82.0 (63.00)	100.0 (90.0)	34.0 (4.65)	38.0 (4.89)	80.0	100.0 (90.0)
T8 (3 × PO)	54.0 (41.70)	86.0 (68.3)	32.0 (4.60)	36.0 (5.23)	—	—
T9 (3 × PO)	68.0 (48.50)	91.5 (74.7)	28.0 (4.90)	32.0 (5.23)	—	—
T10 (3 × PO)	—	—	—	—	72.0	90.0 (75.6)
T11(3 × PO)	—	—	—	—	70.0	84.0 (66.6)
T12 (3 × PO)	—	—	—	—	76.0	96.0 (82.6)
T13 (4 × PO)	64.6 (43.20)	82.0 (65.1)	8.0 (1.78)	16.0 (3.05)	—	—
T14 (4 × PO)	—	—	—	—	78.0	84.0 (72.0)
LSD	(15.79)	(11.65)	(1.92)	(1.92)	15.18	(12.3)

[a]Data are the means of ten replicates, with four plants per replicate. Data in brackets are subjected to arcsin (experiments A and C) or sqrt (experiment B) transformation before analysis to achieve homogenization of variances.
[b]See Table 1 for details of treatments. Inoculation with FOL was made by producing chlamydospores of the fungus in peat pots before transplanting tomato plants as described in Materials and methods.

Table 3. Contrast analysis of disease incidence of tomato plants (cv. Lorena) infested with FOL and treated with PO in three glasshouse experiments at different days after transplanting[a]

Contrasts[a]	Experiment A		Experiment B		Experiment C	
	60 days	80 days	70 days	80 days	20 days	80 days
T2 (non-protected) vs T3 (1 × PO)	*	ns	*	ns	*	ns
T2 (non-protected) vs T4–T7 (2 × PO)	*	ns	*	*	*	ns
T2 (non-protected) vs T8–T12 (3 × PO)	*	*	*	*	*	*
T2 (non-protected) vs T13–T14 (4 × PO)	*	*	*	*	*	*
T4–T7 (2 × PO) vs T8–T12 (3 × PO)	ns	*	ns	ns	ns	*
T4–T7 (2 × PO) vs T13–T14 (4 × PO)	ns	*	ns	ns	ns	*
T8–T12 (3 × PO) vs T13–T14 (4 × PO)	ns	ns	*	ns	ns	ns
T8 vs T9	ns	ns	ns	ns	—	—
T10 vs T11	—	—	—	—	ns	ns
T10 vs T12	—	—	—	—	ns	ns
T11 vs T12	—	—	—	—	ns	ns

* = F-test significant at $P < 0.05$; ns = not significant; —not tested.
[a]See Table 1 for details of treatments. Inoculation with FOL was made by producing chlamydospores of the fungus in peat pots before transplanting tomato plants as described in Materials and methods.

observed on tomato plants treated with three or four PO applications 80 days after transplanting. In addition, the timing of repeated applications of PO is not related to the control of disease.

The densities of FOL were $\log_{10}5.5$–6 cfu g^{-1} dry peat in peat before transplanting. Treatments with PO did not affect rhizosphere densities of FOL (Table 4) which were approximately $\log_{10}4$ cfu g^{-1} fresh root in experiments A and C, and approximately $\log_{10}3$ cfu g^{-1} fresh root in experiment B at the end of experiments.

Density of PO in the rhizosphere of tomato seedlings prior to transplanting was $\log_{10}7$ cfu g^{-1} fresh root in experiment A, and $\log_{10}6$ cfu g^{-1} fresh root in

Table 4. Densities of FOL and PO ($\log_{10}$ cfu g^{-1} fresh root) in the rhizosphere of tomato plants cv. Lorena treated with a conidial suspension of PO in three glasshouse experiments 80 days after transplanting[a]

Treatment[b]	Experiment A		Experiment B		Experiment C	
	FOL	PO	FOL	PO	FOL	PO
T2	4.01 a	0.0 a	3.24 a	0.0 a	4.01 a	0.0 a
T3	4.27 a	4.61 b	2.83 a	3.89 b	4.12 a	4.88 b
T4	4.07 a	5.50 c	2.97 a	4.33 bc	—	—
T5	4.17 a	5.39 c	3.18 a	4.69 bc	4.17 a	5.06 bc
T6	—	—	—	—	4.40 a	5.31 bc
T7	4.52 a	5.51 c	3.15 a	4.35 bc	4.03 a	5.56 c
T8	4.42 a	5.57 c	3.39 a	4.42 bc	—	—
T9	4.49 a	6.0 c	3.05 a	5.08 c	—	—
T10	—	—	—	—	4.25 a	5.64 c
T11	—	—	—	—	4.15 a	5.72 c
T12	—	—	—	—	4.59 a	5.66 c
T13	4.14 a	6.01 c	2.91 a	5.16 c	—	—
T14	—	—	—	—	3.89 a	5.43 bc

[a]Data are the means of three replicates. Means followed by the same letter in each column are not significantly different ($P = 0.05$) by Student–Newman–Keuls range test.
[b]See Table 1 for details of treatments. Inoculation with FOL was made by producing chlamydospores of the fungus in peat pots before transplanting tomato plants as described in Materials and methods.

experiments B and C. All of the treatments with more than one application with PO showed higher rhizosphere densities of PO at 80 days after transplanting in experiment A (Table 4). However, a significantly higher density of PO was only observed in the tomato plant rhizosphere treated with T9 and T13 in experiment B or with T7, T10, T11 and T12 in experiment C (Table 4).

Discussion

Repeated application of PO improved control of fusarium wilt in tomato plants in glasshouse. The control of fusarium wilt obtained after application of PO to tomato seedlings in seedbeds 7 days before transplanting (T3), which is the reference treatment used in other works (De Cal et al., 1997a, 1999), was effective for a variable period time depending on disease severity. A relationship has been described between the inoculum level of the pathogen and the control level of a microorganism (Meera et al., 1994). De Cal et al. (1997c) reported this relationship between inoculum concentration of FOL and disease control by PO. When PO was applied more than once (two or three times in cases of low or high disease severity, respectively), reduction of fusarium wilt was prolonged in time. Repeated applications of the antagonist have been used for improving the efficacy of biocontrol in other diseases. Cartwright and Benson (1995) obtained more effective control of Rhizoctonia stem rot with three spray applications of *Pseudomonas cepacia* to Poinsettia cuttings during a 2 week period than either one or two bacterial sprays. Weekly spray applications of *Trichoderma harzianum* were necessary for highly effective control of turf grass diseases under severe disease situations (Lo et al., 1997). Three applications of *Tilletiopsis pallescens* reduced re-infection of rose powdery mildew (Ng et al., 1997). However, multiple applications of hypovirulent isolate of *Sclerotinia homoeocarpa* did not result in higher disease suppression of dollar spot (Zhou and Boland, 1998).

Lo et al. (1997) showed that monthly applications of conidial suspensions of *T. harzianum* to turf grass significantly reduced disease severity caused by several fungi, but were less effective than weekly spray applications under severe disease pressure. However, reduction percentages of fusarium wilt obtained with one or with several applications of PO were similar. In addition, the timing of repeated application was not an important factor affecting control. Control obtained

with two or more applications of PO was similar when the applications were repeated each 7, 15, 20 or 30 days after transplanting.

The disease suppression in tomato plants by application of PO to seedlings was demonstrated (De Cal et al., 1997a). In all the experiments reported previously, disease reduction was never apparently associated with a decrease in density of FOL in the rhizosphere. This has also occurred in the experiments reported here, independently of the number of applications of PO.

Mechanisms for inducing resistance in the plant may be too slow to protect them against primary infections and are best suited to limiting disease development after infection (Cook, 1993). In our system, as demonstrated previously in De Cal et al. (2000), plants induced with PO showed less disease due to an induced resistance mechanism related to renewed or prolonged cambial activity that led to the formation of additional secondary xylem in PO-treated plants. However, infection of tomato plants by FOL usually still occurs despite the induced resistance.

Competition for space, nutrients or oxygen between PO and FOL in the rhizosphere of tomato plants leading to a better spatial coverage and improved contact, thus avoiding new infections by FOL, could also explain the improved control obtained after repeated application of PO. Treatments with more than one application with PO showed 10-fold higher rhizosphere densities of PO at 80 days after transplanting. The density of PO with only one antagonist application was 10^4 cfu g^{-1} fresh root at the end of experiments. In previous works (De Cal et al., 1995; 1997c; 1999) where fusarium disease control was reported, densities of PO at the end of the assay were similar to those obtained in this study corresponding to one application with PO. A relationship between the higher levels obtained with repeated antagonist applications and the degree of disease reduction does not seem to be evident. Levels of density of PO lower than 10^2 cfu g^{-1} fresh root have been observed when fusarium control was not reported (De Cal et al., 1999).

These results indicate that repeated PO applications may be effective for improved duration of biological control of fusarium wilt of tomato, especially under severe disease situations.

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