Induced Resistance by *Penicillium oxalicum* Against *Fusarium oxysporum* f. sp. *lycopersici*: Histological Studies of Infected and Induced Tomato Stems

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ABSTRACT

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Tomato (*Lycopersicon esculentum*) plants of 'Lorena' were induced with a conidial suspension (10^7 conidia per ml) of *Penicillium oxalicum* before inoculation with *Fusarium oxysporum* f. sp. *lycopersici*, the wilt pathogen. Histological changes occurred in plants under both growth chamber and glasshouse culture conditions and there was a reduction of disease severity. In noninduced plants, the pathogen produced almost a complete loss of cambium (75 to 100% reduction), an increase in the number of bundles, and a decrease in the number of xylem vessels (20% reduction),

in which the diameter also was reduced by 20 to 30% in hypocotyls and epicotyls. The percentage of vessels colonized by *F. oxysporum* f. sp. *lycopersici* was positively correlated to the area under the disease progress curve (AUDPC). However, plants induced with *P. oxalicum* showed less disease, did not lose the cambium, had a lower number of bundles, and had less vascular colonization by *F. oxysporum* f. sp. *lycopersici* (35 to 99%). These effects also were observed in 'Precodor', which is susceptible to races 1 and 2 of *F. oxysporum* f. sp. *lycopersici*, and partially in 'Ramón', which is resistant to both races. Renewed or prolonged cambial activity that led to the formation of additional secondary xylem could be one of the reasons for disease reduction in *P. oxalicum*-induced tomato plants.

Additional keywords: biocontrol, Fusarium wilt.

Fusarium wilt of tomato (*Lycopersicon esculentum* Mill.), caused by *Fusarium oxysporum* Schlechtend.:Fr. f. sp. *lycopersici* (Sacc.) W.C. Snyder & H.N. Hansen, is an economically important disease. The fungus invades plant vascular tissues and induces severe wilting of the foliage by blocking xylem transport and impeding the movement of water (6,27). Vessel walls often are coated in an amorphous electron-opaque material (10). This material occludes xylem parenchyma pit cavities and encrusts intertracheary pit membranes (16). Infection is accompanied by the gradual death of xylem parenchyma cells throughout infected plants (10). Enzymes (19,41), growth-regulating compounds (25), toxins (47,51), and gummosis (26) have all been implicated in symptom development.

As with other vascular plant diseases, chemical control is not effective and sanitation measures are difficult to apply (14). The primary control strategy consists of breeding wilt-resistant cultivars (6). Resistance to Fusarium wilt of tomato is thought to involve a mechanism that restricts the extent of host colonization either physically (by gels, gums, and tyloses [11,18,29,38]) and by the induction of phytoalexins (18,28,36,38), either together or separately. Callose deposition and lignification also restrict lateral penetration from vessels into vascular parenchyma cells (7). However, new races of the pathogen have appeared that overcome resistance in currently grown cultivars (53). 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 (8,9,43). De Cal et al. (22) reported that treatment of tomato plants with

conidia of *Penicillium oxalicum* Durrie & Thom. induced resistance against tomato wilt. *P. oxalicum* and *F. oxysporum* f. sp. *lycopersici* were placed at separate sites on tomato plants or in soil, avoiding a direct interaction between the fungi. *P. oxalicum* induced a reduction in disease severity and colonized the tomato rhizosphere, but the biocontrol fungus was not detected inside stems (22). The possible role of pathogenesis-related (PR) proteins (chitinases Chi3 and Chi9, β -1,3-glucanases Gluc and Glubas, and PR-1) has been studied, but no evidence of involvement of these proteins in the process was found (34). In hydroponic cultures, *P. oxalicum*-treated plants took up more liquid than did nontreated plants when both were infected with *F. oxysporum* f. sp. *lycopersici*, suggesting that *P. oxalicum* partially prevents blocking or collapse of xylem vessels in infected plants (23). Our aim was to identify differences in the structure of the vascular systems of *P. oxalicum*-induced and noninduced tomatoes that might provide some explanation for the resistance shown by induced tomato plants against tomato wilt.

MATERIALS AND METHODS

Cultures. An isolate of *F. oxysporum* f. sp. *lycopersici* race 2 (ATCC number 201829) obtained from a tomato plant in southern Spain was stored at 4°C in tubes containing sterile sand, and the fungus was grown on Czapek Dox agar (CDA) (Difco Laboratories, Detroit) in darkness at 25°C for mycelial production. Microconidial inoculum of *F. oxysporum* f. sp. *lycopersici* was produced in flasks (250 ml) containing 150 ml of sterile Czapek Dox broth (Difco Laboratories), each flask inoculated with three mycelial plugs (1 cm in diameter) of the fungus taken from 7-day-old cultures on CDA (21). The flasks were incubated at 25°C for 5 days in a rotary shaker (model 3527; Lab-Line Instruments, Inc., Melrose Park, IL) at 150 rpm, and the culture was filtered through glass wool and the filtrate used as inoculum. Chlamydosporal inoculum

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of *F. oxysporum* f. sp. *lycopersici* was produced in pots (20 × 20 × 9 cm) containing sterile peat (Gebr. BRILL substrate; GmbH & Co. KG, Georgsdorf, 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 per g of peat). Pots were left in the glasshouse at 20 to 30°C for 30 days (24).

The isolate of *P. oxalicum* was stored on homemade potato dextrose agar (PDA) slants at 4°C. It has been deposited with the American Type Culture Collection (Manassas, VI), awaiting an accession number. Treatments with *P. oxalicum* were made with conidia from PDA plates that were incubated in darkness at 20 to 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 per ml.

Plant material. The tomato cultivars used were Precodor, Lorena, and Ramón. 'Precodor' is susceptible to races 1 and 2 of *F. oxysporum* f. sp. *lycopersici*. 'Lorena' is susceptible to race 2 of *F. oxysporum* f. sp. *lycopersici*, but resistant to race 1. 'Ramón' is resistant to both races of *F. oxysporum* f. sp. *lycopersici*.

Tomato seeds were sown in trays containing an autoclaved mixture of vermiculite (Termita; Asfaltex, S.A., Barcelona, Spain) and peat (Gebr. BRILL substrate; GmbH & Co. KG) (1:1 [vol/vol]) and maintained in a growth chamber at 22 to 28°C with fluorescent light ($100 \mu\text{E m}^{-2} \text{s}^{-1}$, 16-h photoperiod) and 80 to 100% humidity for 4 weeks (growth chamber experiments) or 3 weeks (glasshouse experiments). Seedlings were watered regularly to keep the growth substrate in a wet condition. For glasshouse experiments, trays were then placed in a glasshouse for 7 days before seedlings were transplanted.

Growth chamber experiments. Three types of experiments were carried out. In all of the experiments, tomato seedlings (with two true leaves) were transplanted from seedbeds into 100-ml flasks containing 125 ml of sterile Hoagland solution, allowing the roots to be in contact with the solution as described by De Cal et al. (23). Substrate adhering to the roots was removed gently before placing the plants in the flasks. Seedlings were held by a plastic screen attached to the flask neck. Four seedlings were placed in each flask.

Experiment 1. This experiment was carried out to see if *P. oxalicum* treatments applied at different times induced morphological changes in tomato plants of 'Lorena' infected with *F. oxysporum* f. sp. *lycopersici*. *P. oxalicum* was applied to seedlings at different growth stages as follows: treatment 1, to surface-sterilized seed (in 0.5% sodium hypochlorite for 30 min) before sowing in seedbeds; treatment 2, to seeds immediately after sowing; treatment 3, to seedlings with cotyledons only; treatment 4, to seedlings 7 days before transplanting; treatment 5, to seedlings 4 days before transplanting; and treatment 6, to seedlings just before transplanting. In treatment 1, seeds were immersed for 30 min in a conidial suspension of *P. oxalicum* (10^7 conidia per ml). In treatments 2 to 5, seeds or seedlings in seedbeds were watered with a conidial suspension of *P. oxalicum* (10^7 conidia per ml), giving a concentration of 2×10^6 spores per g of substrate (vermiculite/peat, 1:1 [vol/vol]). In treatment 6, the roots were immersed in the conidial suspension (1×10^7 spores per ml) for 30 min. Inoculation with *F. oxysporum* f. sp. *lycopersici* was made by adding a microconidial suspension of the pathogen in sterile distilled water to the flasks just before transplanting, giving a final concentration in the flasks of 1×10^4 microconidia per ml. Control treatments were treatment 7, flasks not inoculated with *F. oxysporum* f. sp. *lycopersici* for each of the *P. oxalicum* treatments; treatment 8, flasks inoculated with *F. oxysporum* f. sp. *lycopersici* and plants not treated with *P. oxalicum*; and treatment 9, flasks not inoculated with either *F. oxysporum* f. sp. *lycopersici* or *P. oxalicum*. Five replicate flasks, each containing four plants, were used per treatment. The flasks were placed in a randomized complete block design in a growth chamber for about 3 weeks under the conditions described above. The experiment was carried out twice.

Experiment 2. This experiment was carried out to see if *P. oxalicum* induced morphological changes in tomato plants of 'Lorena' infected with *F. oxysporum* f. sp. *lycopersici* at different levels of the stem. Seedlings ('Lorena') in seedbeds were watered 7 days before transplanting (23) with a conidial suspension of *P. oxalicum* (2×10^6 conidia per g of vermiculite/peat, 1:1 [vol/vol]). Inoculation with *F. oxysporum* f. sp. *lycopersici* was made as explained in experiment 1 just before transplanting. Control treatments were plants inoculated with *F. oxysporum* f. sp. *lycopersici* but not treated with *P. oxalicum* or were plants without *F. oxysporum* f. sp. *lycopersici* inoculation and not treated with *P. oxalicum*. Experimental design and plant growth conditions were as in experiment 1. The experiment was carried out twice.

Experiment 3. This experiment was carried out to see if *P. oxalicum* induced morphological changes in cultivars of tomato other than 'Lorena' with other resistance genes. Tomato seedlings of 'Precodor' and 'Ramón' were watered 7 days before transplanting with a conidial suspension of *P. oxalicum* (2×10^6 spores per g of vermiculite/peat, 1:1 [vol/vol]). Inoculation with the pathogen was made as in experiments 1 and 2 just before transplanting. Control treatments were (i) plants inoculated with *F. oxysporum* f. sp. *lycopersici* and not treated with *P. oxalicum*, (ii) plants not inoculated with *F. oxysporum* f. sp. *lycopersici* and treated with *P. oxalicum*, and (iii) plants not inoculated with *F. oxysporum* f. sp. *lycopersici* and not treated with *P. oxalicum*. Five replicate flasks, each containing four plants, were used per treatment and tomato cultivar. Experimental design and plant growth conditions were as in experiments 1 and 2. The experiment was carried out twice.

In all three experiments, when the initial nutrient was consumed in any flask, fresh solution was added to all flasks to give a final volume of 100 ml and the consumption of solution per flask was recorded. Disease caused by *F. oxysporum* f. sp. *lycopersici* was assessed using the disease index described by De Cal et al. (21) each time flasks were replenished. The disease index was as follows: 1 (0% disease severity) = healthy plant, all leaves were green; 1.1 to 1.9 (1 to 24% disease severity) = the first leaf yellow; 2 to 2.9 (25 to 49%) = lower leaves yellow; 3 to 3.9 (50 to 74%) = lower leaves dead and some upper leaves yellow; 4 to 4.9 (75 to 99%) = upper leaves wilted, lower leaves dead; and 5 (100%) = dead plant. The area under the disease progress curve (AUDPC) also was calculated as described by Campbell and Madden (15). Three plants per treatment or cultivar were chosen at random at the end of each experiment for histological studies. Each experiment was carried out twice.

Glasshouse experiment. Tomato plants ('Lorena') in seedbeds placed in a glasshouse were treated 7 days before transplanting by watering with a conidial suspension of *P. oxalicum* to give a concentration of 2×10^6 spores per g of substrate (vermiculite/peat, 1:1 [vol/vol]). When the seedlings had two to four leaves, they were transplanted into pots (four plants per pot) containing peat, and chlamydospores of *F. oxysporum* f. sp. *lycopersici* were produced as described above. Control treatments consisted of (i) non-treated plants transplanted into *F. oxysporum* f. sp. *lycopersici*-inoculated peat, (ii) *P. oxalicum*-treated plants transplanted into sterile peat (autoclaved for 1 h at 1 kg cm^{-2} and 120°C, three consecutive days), and (iii) nontreated plants transplanted into sterile peat. Ten pots per treatment were placed in a glasshouse at 20 to 30°C in a randomized complete block design. Disease severity was recorded at 5, 10, 20, 30, 40, and 50 days after transplanting as described by De Cal et al. (22). The AUDPC also was calculated as described by Campbell and Madden (15). Three plants per treatment were chosen at random at 30, 40, and 50 days after transplanting for histological studies. The complete experiment was carried out twice.

Microscopic study of sections. In all of the experiments, hypocotyls were cut into 1-cm-long segments. In experiment 2 (growth chamber experiment), the first and second internodes above cotyledons of plants also were cut into 1-cm-long pieces.

Segments were transversally cut into 25- to 50- μ m-thick sections with a freezing microtome. Sections were placed in glass petri dishes containing 75% glycerin and heated at 90°C for 45 min, as described by Gao et al. (33). Sections were then placed on slides, stained when required, and viewed with a Zeiss Axioplan microscope (Carl Zeiss, Inc., Oberkochen, Germany) at $\times 25$, $\times 100$, or $\times 400$. Photomicrographs were obtained with a Winder M35 camera (Carl Zeiss, Inc.) using Kodak 160T film (Kodak Limited, Harrow, England). Three pieces per sample, with three sections per piece, were mounted.

Stain for histological studies. Samples from all of the experiments (growth chamber and glasshouse) were used for histological studies. Toluidine blue staining was used to record the number of cell layers in the cambial zone, the number of bundles of xylem elements, the number of vessels of xylem in each bundle, the number of infected vessels (containing the pathogen), and the diameter of vessels. Vascular colonization was determined as the percentage of vessels containing hyphae over the total number of vessels with lumina. Sections were stained with toluidine blue for 5 min, rinsed with sterile water, and mounted with 50% glycerin.

Stains for histochemical studies. Samples from treatment 4 and corresponding controls from growth chamber experiment 1 also were used for histochemical studies (4). The specific stains used were (i) phloroglucinol acid: stains lignin and suberin (red-pink staining reactions) (17); (ii) toluidine blue: stains lignin, tannins, suberin, and phenolics (blue-green) and stains pectic acids (purplish red) (31); (iii) ammoniacal gentian violet: stains suberin deep violet, whereas lignin stains pink (30); (iv) Prussian blue: stains phenolics (green reactions) (30); (v) the acid-Schiff's reaction: stains aldehydes (reddish purple) (45); (vi) ruthenium red: stains pectic acids (purplish red) (39); and (vii) aniline blue (0.5% in 50% ethanol): stains callose (blue) (30).

Fluorescence microscopy. Similar samples described above for the histochemical studies were mounted in a drop of distilled water on a slide, covered with a standard coverslip (55), and observed

under epifluorescence microscopy (UV light: excitation filter B2A [450 to 490 nm], barrier filter 520; dichroic splitter 510; Nikon Labophot; Nikon Corporation, Tokyo). Photomicrographs were obtained with a Nikon AFX-DX camera (Nikon Corporation) using Kodak 160T film.

Data analysis. The percentage of disease severity caused by *F. oxysporum* f. sp. *lycopersici* was converted to $\ln[1/(1 - x)]$, and data of microscopic studies were $x/\Sigma x$ transformed before analyses to improve homogeneity of variances. All data were then analyzed using a factorial analysis of variance (ANOVA), $Y_{ijk} = \mu + A_i + B_j + AB_{ij} + e_{k(ij)}$ (50), in which Y was the variable, A and B were fixed factors (different in each experiment), AB was the interaction effect due to combinations of corresponding factors, μ represented a "grand mean", and e (error) was a random component due to factors not included in the model. In experiments 1 and 3 under growth chamber conditions and in the glasshouse experiments, A represented the treatments applied and B represented the inoculation or noninoculation with *F. oxysporum* f. sp. *lycopersici*. In experiment 2, A represented the treatments applied and B represented the stem internode studied. When the F test was significant ($P = 0.05$), means were compared by the Student-Newman-Keul's multiple range test (50). The correlation coefficient (r) of the percentage of vascular colonization by *F. oxysporum* f. sp. *lycopersici* on the AUDPC was calculated, and its significance was obtained by the t test (50). Data of xylem colonization in the glasshouse experiments were analyzed by a chi-squared test ($P = 0.05$).

RESULTS

In all of the ANOVAs, both factors A and B were significant ($P = 0.05$). Since factor interactions (AB) were significant in each analysis, mean comparisons between factor A were made for each factor B (uppercase letters [superscripts] in tables and figure); similarly, the effect of factor B was analyzed for each factor A (lowercase letters [subscripts] in tables and figure).

TABLE 1. Effect of different treatments of *Penicillium oxalicum* on disease development caused by *Fusarium oxysporum* f. sp. *lycopersici* and on stem microscopic morphology in tomato plants of 'Lorena' grown in a growth chamber (experiment 1) at 21 days after inoculation

<i>F. oxysporum</i> f. sp. <i>lycopersici</i> ^w	<i>P. oxalicum</i> treatments ^x	AUDPC ^y	Stem microscopic morphology ^z				
			Xylem colonization by <i>F. oxysporum</i> f. sp. <i>lycopersici</i> (%)	Cambial layers	Vessels per bundle	Bundles per section	Diameter of vessels (μ m)
+	1	358 ^B _b	71.4 ^C _b	0.0 ^A _a	10.0 ^A _a	4.0 ^A _a	21.7 ^A _a
	2	389 ^B _b	64.4 ^{BC} _b	0.0 ^A _a	12.0 ^{AB} _a	4.0 ^A _a	25.6 ^A _a
	3	344 ^B _b	54.1 ^{ABC} _b	1.8 ^C _a	13.6 ^B _a	7.8 ^D _b	31.7 ^B _a
	4	197 ^A _b	41.4 ^A _b	1.6 ^{BC} _a	12.6 ^{AB} _a	5.6 ^B _b	37.1 ^B _a
	5	345 ^B _b	56.3 ^{ABC} _b	0.0 ^A _a	12.8 ^B _a	4.0 ^A _a	33.7 ^B _a
	6	183 ^A _b	51.7 ^{AB} _b	1.0 ^B _a	13.2 ^B _a	6.2 ^C _a	36.8 ^B _a
	7 (control)	361 ^B _b	64.4 ^{BC} _b	0.2 ^A _a	11.0 ^{AB} _a	6.5 ^C _b	31.4 ^B _a
-	1	0 ^A _a	0.0 ^A _a	1.4 ^B _b	13.8 ^B _b	4.0 ^A _a	30.8 ^A _b
	2	0 ^A _a	0.0 ^A _a	2.0 ^B _b	13.2 ^B _a	4.7 ^B _b	45.3 ^B _b
	3	0 ^A _a	0.0 ^A _a	4.6 ^D _b	17.6 ^C _b	5.0 ^B _a	36.9 ^A _a
	4	0 ^A _a	0.0 ^A _a	4.2 ^D _b	23.2 ^D _b	4.0 ^A _a	39.0 ^A _a
	5	0 ^A _a	0.0 ^A _a	4.3 ^D _b	19.3 ^C _b	4.0 ^A _a	36.7 ^A _a
	6	0 ^A _a	0.0 ^A _a	0.5 ^A _a	10.0 ^A _a	6.0 ^C _a	36.7 ^A _a
	7 (control)	0 ^A _a	0.0 ^A _a	3.4 ^C _b	18.6 ^C _b	4.2 ^A _a	47.1 ^B _b
MSE		5004.6	1043.6	0.18	2.7	0.17	8.8

^w Inoculation of tomato plants with *F. oxysporum* f. sp. *lycopersici*.

^x *P. oxalicum* treatments applied to tomato: treatment 1, to disinfected seeds before sowing in seedbeds; treatment 2, to seeds immediately after sowing; treatment 3, to seedlings with cotyledons only; treatment 4, to seedlings 7 days before transplanting; treatment 5, to seedlings 4 days before transplanting; treatment 6, to seedlings just before transplanting; and treatment 7, not treated (control).

^y AUDPC = area under the disease progress curve. Data are the mean of five replicates, with four tomato plants per replicate. Data were analyzed using analysis of variance. Means were compared by Student-Newman-Keul's multiple range test. Means within an inoculum with no common uppercase letter (superscript) differ significantly at $P = 0.05$. Means within a treatment with no common lowercase letter (subscript) differ significantly at $P = 0.05$.

^z Microscopic study of the percentage of vascular colonization by *F. oxysporum* f. sp. *lycopersici*, the number of cell layers in the cambial zone, the number of vessels of xylem in each bundle, and the diameter of vessels. Data are the mean of three replicates, with three sections per replicate. Data were analyzed using analysis of variance. Data were transformed to $x/\Sigma x$ before analysis to improve homogeneity of variances. Means were compared by Student-Newman-Keul's multiple range test for each column. Means within an inoculum with no common uppercase letter (superscript) differ significantly at $P = 0.05$. Means within a treatment with no common lowercase letter (subscript) differ significantly at $P = 0.05$. MSE was the mean square error of analysis with nontransformed data.

Experiment 1. A significant reduction of the AUDPC was recorded only in treatment 4 (antagonist applied 7 days before transplanting) and in treatment 6 (antagonist applied just before transplanting) (Table 1). No disease was recorded in noninoculated plants (Table 1). *P. oxalicum* was never observed inside the plant stem and did not produce any disease symptoms. Xylem tissues of plants inoculated with *F. oxysporum* f. sp. *lycopersici* commonly contained mycelium of the pathogen (Fig. 1A). Mycelium stained deep red or purple with toluidine blue. Mycelium growth was restricted mostly to vascular tissues and the neighboring parenchyma cells. Sixty-four percent colonization of xylem vessels was recorded in plants inoculated with *F. oxysporum* f. sp. *lycopersici* and not treated with *P. oxalicum* (Table 1). This colonization was reduced in plants of treatment 4 (antagonist applied 7 days before transplanting) (Table 1; Fig. 1B). The percentage of vascular colonization was positively correlated with the AUDPC ($r = 0.97$, $P = 0.1\%$).

Three cambium layers were common in the hypocotyls of 'Lorena', which was not induced with *P. oxalicum* and not inoculated with *F. oxysporum* f. sp. *lycopersici*; vascular tissue was differentiated into four distinct xylem bundles, each bundle contained 15 to 25 xylem vessels with a diameter ranging between 40 to 50 μm (Fig. 1C). The number of cambium layers, the number of xylem vessels per bundle, and the vessel diameter decreased in plants inoculated with *F. oxysporum* f. sp. *lycopersici* and not treated with *P. oxalicum*, while the number of bundles per cross section increased (Table 1).

Changes in variables studied by microscopy were observed in infected plants treated with *P. oxalicum*, although these changes were most apparent in plants of treatment 4 (Table 1). Treatments 3, 4, and 6 induced an increase of the number of cambium layers in plants inoculated with *F. oxysporum* f. sp. *lycopersici*. The number of vessels per bundle and the diameter of vessels were the same in infected plants either treated or nontreated, except for treatments 1 and 2, with regard to the diameter of vessels. Treatments 1, 2, 4, and 5 reduced the number of bundles per section in infected plants, while treatment 3 increased this variable.

Changes of the different variables also occurred in plants treated with *P. oxalicum* and not inoculated with *F. oxysporum* f. sp. *lycopersici* in relation to nontreated and noninoculated plants (Table 1). Treatments 3, 4, and 5 increased the number of cambium layers; treatment 4 increased the number of vessels per bundle; treatments 2, 3, and 6 increased the number of bundles per section; and all of the treatments, except treatment 2, reduced the diameter of vessels.

Cross sections of stem tissues induced by *P. oxalicum* showed sporadic gumlike deposits on the walls of xylem elements such as in noninduced plants after infection with *F. oxysporum* f. sp. *lycopersici*.

Samples from treatment 4 (application of *P. oxalicum* to seedlings 7 days before transplanting) and the corresponding controls also were studied histochemically. No differences in staining patterns of the xylem vessels and cambium were observed when comparing induced and noninduced plants, when they were also both not inoculated with *F. oxysporum* f. sp. *lycopersici*: (i) positive reaction on all cells with phloroglucinol acid, toluidine blue, ammoniacal gentian violet, acid-Schiff's, and aniline blue; (ii) positive reaction on some cells with ruthenium red; and (iii) no reaction with Prussian blue.

Tomato plants inoculated with *F. oxysporum* f. sp. *lycopersici* and induced by *P. oxalicum* also presented the same staining pattern in xylem vessels and cambium as those described above. Plants inoculated with *F. oxysporum* f. sp. *lycopersici* and not induced with *P. oxalicum* showed abnormalities of cambial cells, as was recorded previously in the histological studies. No positive reaction was observed in the cambial zone with any of the stains used.

The examination of samples from both noninduced and induced tomato plants infected with *F. oxysporum* f. sp. *lycopersici* using the epifluorescent method revealed that all xylem bundles had yellow-green fluorescent material within the xylem cells. The fluores-

cent cells appeared to be stained dark brown when observed by white light. Tomato plants not infected with *F. oxysporum* f. sp. *lycopersici* did not present fluorescence in the xylem.

Experiment 2. A significant reduction of the AUDPC was recorded in *P. oxalicum*-treated plants (AUDPC = 208.7) in relation to nontreated plants (AUDPC = 510.6), both inoculated with *F.*

Fig. 1. Cross sections of tomato stems of 'Lorena' showing vascular tissue forming a cylinder with an external phloem separated from xylem by the vascular cambium ($\times 100$). **A**, Infected by *Fusarium oxysporum* f. sp. *lycopersici*; **B**, infected with *F. oxysporum* f. sp. *lycopersici* and treated with *Penicillium oxalicum*; and **C**, not infected with *F. oxysporum* f. sp. *lycopersici* and not treated with *P. oxalicum*. Abbreviations: x = xylem; c = cambium; p = phloem; and m = mycelium of *F. oxysporum* f. sp. *lycopersici*.

oxysporum f. sp. *lycopersici*. No disease was recorded in plants nontreated and noninoculated.

In each of the stem internodes studied, xylem colonization by the pathogen was lower in infected plants treated with *P. oxalicum* than in nontreated plants (Fig. 2A). When comparing each treatment between stem internodes, an increase of xylem colonization was observed in the second internode of the epicotyl in treated and inoculated plants, while this increase was observed in the first and second internodes in nontreated and inoculated plants (Fig. 2A). The percentage of vascular colonization was positively correlated with the AUDPC ($r = 0.78$, $P = 0.1\%$).

In each of the stem internodes studied, treated and inoculated plants showed a higher number of cambial layers than did nontreated plants, both those inoculated or noninoculated (Fig. 2B). The highest number of cambial layers was recorded in the hypocotyl in treated and inoculated plants. The number of cambial cells was the same in all stem internodes studied in nontreated and inoculated plants, while it was lower in the epicotyl internodes than in the hypocotyl, both in treated and inoculated plants as well as those nontreated and noninoculated. (Fig. 2B).

Treated and inoculated plants and those nontreated and noninoculated showed a higher number of vessels per bundle than

Fig. 2. Effects of *Penicillium oxalicum* on stem ultrastructure of tomato plants of 'Lorena' grown in a growth chamber (experiment 2) at 21 days after inoculation with *Fusarium oxysporum* f. sp. *lycopersici*. *P. oxalicum* was applied 7 days before transplanting. **A**, Percentage of colonization by the pathogen; **B**, number of cambial layers; **C**, number of vessels per bundle; and **D**, number of bundles per section were recorded in hypocotyls (H) of tomato stems and in the first and second internodes (1°E and 2°E, respectively) above cotyledons of treated and inoculated plants, nontreated and inoculated plants, and nontreated and noninoculated plants. Data are the mean of three replicates, with three sections per replicate. Data were transformed to $\ln(\Sigma x)$ before analysis of variance. Means were compared by Student-Newman-Keul's multiple range test. Means within an internode with no common uppercase letter (superscript) differ significantly at $P = 0.05$. Means within a treatment with no common lowercase letter (subscript) differ significantly at $P = 0.05$. MSE was the mean square error of analysis with nontransformed data.

did nontreated and inoculated plants in all of the stem internodes (Fig. 2C). The highest number of vessels per bundle was recorded in the hypocotyl in treated and inoculated plants. A reduction in the number of vessels per bundle was not shown in the internodes studied in nontreated and noninoculated plants (Fig. 2C).

In both internodes of the epicotyl, the number of bundles per section was lower in treated and inoculated plants than in those nontreated and inoculated (Fig. 2D). An increase of the number of bundles per section was observed with the height of the plant in all plants (Fig. 2D). In nontreated and noninoculated plants, this increase of the number of bundles per section was observed in the second internode of the epicotyl, while it was observed in the first and second internodes of the epicotyl in plants inoculated and treated as well as in plants inoculated and nontreated.

Experiment 3. A significant reduction of disease and vascular colonization caused by *F. oxysporum* f. sp. *lycopersici* was obtained in tomato plants of 'Precodor' (susceptible to races 1 and 2 of *F. oxysporum* f. sp. *lycopersici*) treated with *P. oxalicum*. No infection was obtained in 'Ramón' (resistant to both races) (Table 2). An increase in the percentage of vascular colonization by *F. oxysporum* f. sp. *lycopersici* resulted in an increase in the AUDPC ($r = 0.89$, $P = 0.1\%$).

The number of cambial layers and the number of bundles per cross section were increased and reduced, respectively, in plants infected with *F. oxysporum* f. sp. *lycopersici* and treated with *P. oxalicum* in relation to infected plants not treated with *P. oxalicum* in both cultivars (Table 2).

Glasshouse experiment. Results were observed in 'Lorena' at 50 days after the inoculation with *F. oxysporum* f. sp. *lycopersici*. A significant reduction of disease severity and colonization of xylem vessels caused by *F. oxysporum* f. sp. *lycopersici* was obtained in tomato plants induced by *P. oxalicum* (Table 3). An increase in the percentage of vascular colonization by *F. oxysporum* f. sp. *lycopersici* resulted in an increase in the AUDPC ($r = 0.98$, $P = 0.01\%$).

In plants inoculated with *F. oxysporum* f. sp. *lycopersici* and treated with *P. oxalicum*, the number of cambium layers increased and the number of bundles per sections decreased in relation to plants inoculated with *F. oxysporum* f. sp. *lycopersici* and not induced with *P. oxalicum* (Table 3).

DISCUSSION

P. oxalicum induced systemic morphological changes in tomato plants infected by *F. oxysporum* f. sp. *lycopersici* under both growth chamber and glasshouse conditions. In these plants, disease and vascular colonization by the pathogen were also reduced.

The percentage of colonization by *F. oxysporum* f. sp. *lycopersici* was correlated to the AUDPC in all of the experiments made and tomato cultivars studied. Gao et al. (32) observed that the extent of *F. oxysporum* f. sp. *lycopersici* colonization determined the degree of symptom development in eight tomato cultivars with

TABLE 3. Effect of *Penicillium oxalicum* on disease development caused by *Fusarium oxysporum* f. sp. *lycopersici* and on stem ultrastructure of tomato plants of 'Lorena' grown in the glasshouse at 50 days after inoculation

<i>F. oxysporum</i> f. sp. <i>lycopersici</i> ^w	<i>P.</i> <i>oxalicum</i> ^x	AUDPC ^y	Microscopic study ^z		
			Colonization by <i>F.</i> <i>oxysporum</i> f. sp. <i>lycopersici</i> (%)	Cambial layers	Bundles per section
+	+	807 ^A _b	9.87*	4.6 ^B _a	4.5 ^A _a
	–	1,187 ^B _b	28.48	0.8 ^A _a	5.2 ^B _b
–	+	0 ^A _a	–	4.8 ^B _a	4.7 ^A _b
	–	0 ^A _a	–	3.5 ^A _b	4.7 ^A _a
MSE		34,616		0.4	0.6

^w Inoculation of tomato plants with *F. oxysporum* f. sp. *lycopersici*.

^x Treatments of tomato plants with *P. oxalicum* 7 days before transplanting.

^y AUDPC = area under the disease progress curve. Data are the mean of 40 replications. Data were analyzed using analysis of variance. Means were compared by Student-Newman-Keul's multiple range test. Means within an inoculum with no common uppercase letter (superscript) differ significantly at $P = 0.05$. Means within a treatment with no common lowercase letter (subscript) differ significantly at $P = 0.05$.

^z Microscopic study recorded the number of cell layers in the cambial zone, the number of bundles per cross section, and the percentage of vascular colonization by *F. oxysporum* f. sp. *lycopersici*. Data are the mean of three replicates, with three sections per replicate. Data were analyzed using analysis of variance. Data were transformed to $\sqrt{x}$ before analysis to improve homogeneity of variances. Means were compared by Student-Newman-Keul's multiple range test for each column. Means within an inoculum with no common uppercase letter (superscript) differ significantly at $P = 0.05$. Means within a treatment with no common lowercase letter (subscript) differ significantly at $P = 0.05$. MSE was the mean square error of analysis with nontransformed data.

TABLE 2. Effect of *Penicillium oxalicum* on disease development caused by *Fusarium oxysporum* f. sp. *lycopersici* and on stem ultrastructure of tomato plants of 'Ramón' and 'Precodor' grown in a growth chamber (experiment 3) at 21 days after inoculation

Cultivars	<i>F. oxysporum</i> f. sp. <i>lycopersici</i> ^w	<i>P. oxalicum</i> ^x	AUDPC ^y	Microscopic study ^z		
				Colonization by <i>F. oxysporum</i> f. sp. <i>lycopersici</i> (%)	Cambial layers	Bundles per section
Precodor	+	+	32 ^A _a	0.9 ^A _a	2.3 ^B _a	4.0 ^A _a
		−	240 ^B _b	98.9 ^B _b	0.0 ^A _a	6.0 ^B _b
	−	+	0 ^A _a	0.0 ^A _a	2.3 ^A _a	4.0 ^A _a
		−	0 ^A _a	0.0 ^A _a	2.3 ^A _b	5.0 ^B _a
MSE			863.8	2.9	0.2	1.1 × 10 ^{−23}
Ramón	+	+	0	5.7 ^A _a	1.0 ^B _a	4.0 ^A _a
		−	0	0.0 ^A _a	0.1 ^A _a	7.0 ^B _b
	−	+	0	0.0 ^A _a	3.2 ^A _b	6.0 ^B _b
		−	0	0.0 ^A _a	4.2 ^B _b	4.0 ^A _a
MSE			NS	22.0	0.1	2.6 × 10 ^{−23}

^w Inoculation of tomato plants with *F. oxysporum* f. sp. *lycopersici*.

^x Treatment of tomato plants with *P. oxalicum* 7 days before transplanting.

^y AUDPC = area under the disease progress curve. Data are the mean of five replicates, with four tomato plants per replicate. Data were analyzed using analysis of variance. Means were compared by Student-Newman-Keul's multiple range test for each cultivar. Means within an inoculum with no common uppercase letter (superscript) differ significantly at $P = 0.05$. Means within a treatment with no common lowercase letter (subscript) differ significantly at $P = 0.05$. NS = no significant data.

^z Microscopic study recorded the number of cell layers in the cambial zone, the number of bundles per cross section, and the percentage of vascular colonization by *F. oxysporum* f. sp. *lycopersici*. Data are the mean of three replicates, with three sections per replicate. Data were analyzed using analysis of variance. Data was transformed to $\sqrt{x}$ before analysis to improve homogeneity of variances. Means were compared by Student-Newman-Keul's multiple range test for each column and cultivar. Means within an inoculum with no common uppercase letter (superscript) differ significantly at $P = 0.05$. Means within a treatment with no common lowercase letter (subscript) differ significantly at $P = 0.05$. MSE was the mean square error of analysis with nontransformed data.

different degrees of resistance/susceptibility. Vascular colonization was markedly higher in susceptible than in resistant tomato plants (32). Different mechanisms have been described that may be responsible for the reduced colonization in resistant cultivars, including formation of barriers preventing the progress of the pathogen (8), confinement of the pathogen to primary xylem (8,47,48), proliferation of pathogen-free vessels (48), and inhibition of pathogen growth (18,29,32,38). Vascular colonization by *F. oxysporum* f. sp. *lycopersici* was less extensive in *P. oxalicum*-treated than in nontreated tomato plants. De Cal et al. (23) reported that under growth chamber conditions tomato plants infected by *F. oxysporum* f. sp. *lycopersici* consumed 45% less nutrient solution than did uninfected plants, while *P. oxalicum*-treated and *F. oxysporum* f. sp. *lycopersici*-infected tomato plants consumed 25% more nutrient solution than did infected, nontreated plants. Consumption of nutrient solution by tomato plants was correlated with other wilt-related parameters such as AUDPC, weight of aerial parts, and stunting (23). Bargmann and Schönbeck (5) also reported induction of resistance by *Acremonium kiliense* to wilt diseases of tomato by reducing the spread of the pathogens within the plant and increasing the number of xylem vessels.

In infected and noninduced plants, *F. oxysporum* f. sp. *lycopersici* caused almost a complete loss of cambium (Fig. 1A). A decrease both in the number and the diameter of xylem vessels and an increase in the number of bundles were also observed. Cambium is a lateral meristem in charge of secondary growth (54). Cambium regression or its total differentiation has a negative effect on vascular development with inefficient water transport in the plant and subsequent stunting (25,27). Several authors have described the reduction in the number of xylem vessels as an effect of vascular pathogens (3,35) including *F. oxysporum* f. sp. *lycopersici* (5). However, only Peterson and Pound (44) observed several instances of cambial disorganization and deterioration in radish plants in response to the presence of *F. oxysporum* f. sp. *conglutinans*. Effects on the cambial zone in *Ulmus americana* (12) and *Prunus pensylvanica* (46) also have been described with other wilt pathogens such as *Ophiostoma ulmi*.

P. oxalicum prevented the morphological changes caused by *F. oxysporum* f. sp. *lycopersici* (Fig. 1B). Tomato plants induced with *P. oxalicum* did not lose the cambium and had a lower number of bundles than did the noninduced and infected plants. Preservation or regeneration of the cambium is extremely important for the survival of the infected plant (2). The cambial activity in *P. oxalicum*-treated plants improved secondary growth and subsequently arranged the water movement through the xylem of infected tomato plants and could be the reason for disease reduction in *P. oxalicum*-treated tomato plants. Production of new xylem vessels for the continued activity of the vascular cambium was observed in wilt diseases of hop (52) and oak (49), although it was also shown by regeneration from parenchyma cells in wilted carnations (3,4,37).

Specific staining for defense substances did not indicate any difference in the composition of the cambial zone of tomato plants induced or not induced with *P. oxalicum*, suggesting that compounds such as lignin, suberin, callose, polysaccharides, or pectin acids were not involved in the induced resistance or that they could not be stained. In some cases, differences in the accumulation of phenolics, lignin, suberin (13), and callose (8) have been described as characteristics of some resistant tomato cultivars. At the intensity used, autofluorescence was only observed in the xylem of tomato plants infected by *F. oxysporum* f. sp. *lycopersici*, similar to the situation in sugarcane phloem infected by luteovirus (55). The fluorescence is interpreted as evidence of the accumulation of phenolic compounds (55).

The timing and method of application of *P. oxalicum* affected the suppression of morphological effects caused by *F. oxysporum* f. sp. *lycopersici* in tomato plants. Plants of 'Lorena' treated with *P. oxalicum* in seedbeds 7 days before transplanting and inoculated

with *F. oxysporum* f. sp. *lycopersici* showed the most dramatic morphological changes: the most cambium layers, the least vascular colonization by the pathogen, and a low number of bundles per cross section of stem. These effects were higher in hypocotyls than in studied epicotyls, suggesting that the application of *P. oxalicum* on tomato root induced systemic changes along the stem. Application to tomato seedlings in seedbeds 7 days before transplanting also provided the most effective disease suppression, although some morphological changes also were recorded in *P. oxalicum*-treated plants, in which disease reduction was not observed, or in treated and noninfected plants. The suppression of disease caused by *F. oxysporum* f. sp. *lycopersici* due to application of *P. oxalicum* was demonstrated previously to be related to mechanisms of induced resistance in tomato plants (22). Morphological changes observed in plants treated with *P. oxalicum* in seedbeds 7 days before transplanting should have a role in resistance to Fusarium tomato wilt, and they are systemic in the tomato plants.

F. oxysporum f. sp. *lycopersici* caused morphological changes both in cultivars resistant ('Ramón') or susceptible ('Precodor' and 'Lorena') to race 2 of *F. oxysporum* f. sp. *lycopersici*. Thus, resistance to *F. oxysporum* f. sp. *lycopersici* in 'Ramón' should be due to factors other than these morphological changes studied. The experiments carried out with plants of 'Ramón' did not show either disease symptoms or vascular colonization by the pathogen. However, in previous experiments carried out in glasshouse conditions, De Cal et al. (20) showed that when *F. oxysporum* f. sp. *lycopersici* was artificially inoculated in the stem of tomato plants of 'Ramón' it caused the same disease severity as in another susceptible cultivar, Novy. Other failures of commercial cultivars considered "resistant" against Fusarium wilt have already been described (40). As *P. oxalicum* induced morphological changes in all cultivars, independent of their resistance/susceptibility to *F. oxysporum* f. sp. *lycopersici*, and these changes led to decreased vascular damage caused by *F. oxysporum* f. sp. *lycopersici*, treatment with *P. oxalicum* could improve control of vascular wilt even in these cases of resistance "failure." This is important, since the use of resistant cultivars is the most important means of control of Fusarium wilt of tomato.

We may surmise that a sequential two-component resistance process was operating to restrict pathogen invasion in *P. oxalicum*-treated plants. First, treatment with *P. oxalicum* limited the secondary distribution of *F. oxysporum* f. sp. *lycopersici* within the vascular tissues. Secondly, treatment with *P. oxalicum* prevented the distortion of cambial cells and the total differentiation of cambium. Plants inoculated with *F. oxysporum* f. sp. *lycopersici* and not treated with *P. oxalicum* would differentiate all the cambium into more bundles of vessels with a lower number and smaller diameter of vessels. In addition, the differentiation and degradation of cambium led to the loss of this meristem and, consequently, no more vessels could even be formed. The histological changes observed in *P. oxalicum*-induced tomato plants could be related to the production of growth regulators (e.g., hormones) causing division, differentiation, and growth of cells. Phytohormones are a major factor controlling vascular differentiation in plants (1,42). The polar movement of auxin from leaves to roots induces continuous vascular tissues along the flow of auxin and controls the size and density of vascular elements along the plant axis. The highest number of bundles and the least number of cambial layers in healthy tomato plants of 'Lorena' were in the second internode of epicotyl. High auxin levels near the young leaves in healthy plants induce numerous vessels that remain small because of their rapid differentiation, while low auxin concentrations further down result in slow differentiation and, therefore, in fewer and larger vessels (1). Cytokinin, which is the major developmental signal of the root, also is involved in the differentiation of vessels. Cytokinin, in the presence of auxin, stimulates early stages of vascular differentiation, at a time when many cell divisions occur in the differentiating tissue (1). Some of morphological ef-

fects observed in tomato plants inoculated with *F. oxysporum* f. sp. *lycopersici* were similar to those described above for high stem internodes in healthy tomato plants. *P. oxalicum* prevented the morphological changes caused by *F. oxysporum* f. sp. *lycopersici*. Infected tomato plants induced with *P. oxalicum* did not lose the cambium and had a lower number of bundles than did the non-induced and infected plants. Tomato plants induced with *P. oxalicum* and not infected by the pathogen showed an increase of the number of cambium layers and a reduction of the number of vessels per bundle. Although, in some cases, an increase of the number of bundles per section and a decrease of the diameter of vessels were recorded too. Moreover, *P. oxalicum* promotes growth of tomato plants (R. García-Lepe, A. De Cal, S. Pascual, and P. Melgarejo, unpublished data) in different growth conditions without a pathogen, which supports the hypothesis that growth regulators are at least one part in the mechanism of action of *P. oxalicum* as an inducer of resistance. The possibility of some hormonal process inducing differentiation and division in cells of *P. oxalicum*-induced tomato plants merits further investigation.

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