Dispersal Improvement of a Powder Formulation of *Penicillium oxalicum*, a Biocontrol Agent of Tomato Wilt

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ABSTRACT

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Sugars, polyalcohols, inorganic salts, and detergents were added to conidia of *Penicillium oxalicum* at three different points of the production-formulation process to improve water dispersal. Effects also were tested on conidial germination and production. Conidial germination without additives ranged from 51 to 79%. Additives did not reduce conidial germination except for 50% polyethylene glycol (PEG) 300 and 10% CaCl_2 . Sunflower oil and sodium alginate, sucrose (0.5, 15, 30, and 60%), D-sorbitol (30 and 60%), glycerol (2, 5, 20, and 30%), 30% PEG 300, CaCl_2 (0.01 to 1%), Tween 20 (0.01, 0.02, 0.5, and 1%), and Tween 80 (0.01 to 1%) enhanced conidial germination. Production without additives ranged from 0.57 to 4.58 conidia $\times 10^8 \text{ g}^{-1}$ substrate. Additives did not affect conidial production except for reduction by 60% D-sorbitol, 60% fructose, and 10% CaCl_2 . Conidial dispersal in water improved when 1.5% sodium alginate was added to substrate in bags before production, and when 1.5% sodium alginate, 60% sucrose, 60% D-sorbitol, 60% fructose, 5 to 20% PEG 8000, or 20% glycerol were added to conidia before drying. Dispersal of dried conidia was enhanced with 1% Tween 20, 1% Tween 80, 1% Triton X-100, 10% Agral, and 1.5% sunflower oil. Two *P. oxalicum* formulations (conidial suspensions maintained with 60% sucrose or 1.5% sodium alginate for 10 min before drying) significantly reduced tomato wilt caused by *Fusarium* spp. under greenhouse conditions and, in a preliminary trial, by *Verticillium* spp. in a field assay.

Additional keywords: biofungicide, formulation

Penicillium oxalicum Currie & Thom is a promising fungal agent for biological control of soilborne diseases of tomato (8,10). Application of a conidial suspension of *P. oxalicum* by watering the tomato seedlings in seedbeds 7 days before transplanting usually results in a significant reduction of *Fusarium* and *Verticillium* wilts of tomato plants (7,21). Induction of resistance in tomato plants was demonstrated as the main mode of action of *P. oxalicum* against *Fusarium oxysporum* f. sp. *lycopersici* (Sacc.) W. C. Snyder & H. N. Hans. (11). Conidia of *P. oxalicum* must contact the tomato roots (6,11) and a range of 10^6 to 10^7 conidia g^{-1} in seedbed substrate and rhizosphere before transplanting is required for effective control of wilts of tomato (21).

The main drawback to commercial biocontrol products is the necessity of a shelf-

stable formulated product retaining biocontrol activity comparable to fresh cells of the agent (16). Biocontrol of tomato wilt is based on application of *P. oxalicum* conidia (24). A method for mass production of conidia has been developed and conidial applications have reduced wilts of tomato. Unfortunately, viability of these conidia declined within 30 days at room temperature (19). Conidia of *P. oxalicum* can be dried by fluid bed drying, retaining 100% viability at the end of the process and 40 to 50% viability after 180 days of storage at room temperature (20). *P. oxalicum* conidia dried by fluid bed drying reduced the incidence of *Fusarium* wilt of tomato under glasshouse and field conditions (20).

An important problem associated with applications of *P. oxalicum* is the hydrophobic nature of conidia, especially of dry conidia (24). For commercial use of *P. oxalicum*, it is essential to enhance conidial dispersal in water, decrease dust hazard (14), reduce settling of conidia and aggregate formation, and thereby improve efficiency of root drenches (15).

The objective of the present study was to evaluate the effect of different additives for dispersal of dried *P. oxalicum* conidia in water, in order to improve the application of the product. Disease control efficacy of the formulated product was tested in glasshouse trials and in a preliminary field trial.

MATERIALS AND METHODS

Cultures. *P. oxalicum* (American Type Culture Collection [ATCC] number 201888) was stored on potato dextrose agar (PDA) (Difco Laboratories, Detroit) slants at 4°C and grown on PDA in petri dishes in darkness at 20 to 25°C for 7 days for inoculum production. Conidia of *P. oxalicum* were produced on peat/vermiculite/lentil meal (1/1/0.5; wt/wt/wt) in plastic bags (19). Mixture (50 g) enclosed in 600-cm³ plastic bags was inoculated with 40 ml of a distilled water conidial suspension of *P. oxalicum* (10^5 conidia g^{-1} of dry substrate), sealed again, and incubated in darkness at 20 to 25°C for 5 days. Fresh conidia obtained after incubation were concentrated and resuspended in sterile distilled water for 10 min, and numbers of conidia were estimated with a hemacytometer. Conidial suspensions then were filtered through a 1- μm filter paper using a Büchner funnel. Conidial paste was dried in a fluid bed dryer 350s (Burkard Manufacturing Co Ltd., Hertfordshire, UK) as previously described (20). Conidial moisture content was measured using a humidity analyzer (BOE-CKEL, GmbH +Co, Hamburg, Germany). Conidia were dried until moisture contents decreased below 10%.

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 (13). The fungus was grown on Czapek-Dox agar (CDA; Difco Laboratories) in darkness at 25°C for mycelial production. Microconidia of *F. oxysporum* f. sp. *lycopersici* were produced in Czapek-Dox broth (Difco Laboratories) for 5 days at 25°C on a rotary shaker at 150 rpm (10). Chlamydospores of *F. oxysporum* f. sp. *lycopersici* were produced in bags (80 by 40 by 20 cm³) containing sterile peat. Bags were inoculated with a microconidial suspension in Czapek-Dox broth giving a final density of 10^5 microconidia g^{-1} of peat, and were left in the glasshouse for 30 days at 20 to 30°C. At this time, almost all the microconidia became chlamydospores (12). The population of *F. oxysporum* f. sp. *lycopersici* in the peat was estimated as number of CFU per gram of dry peat employing methods previously described (7,10,12,21).

Plant material. Tomato cv. San Pedro, susceptible to races 1 and 2 of *F. oxysporum* f. sp. *lycopersici* and to *Verticillium dahliae*, were used in all experiments. Tomato seed for glasshouse experiments

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were sown in trays (27 by 42 by 7 cm³) containing an autoclaved mixture of one part vermiculite and one part peat. The trays were maintained in a growth chamber at 22 to 28°C with fluorescent light (100 µE m⁻² s⁻¹, 16-h photoperiod) and 80 to 100% humidity for 3 weeks, then placed in a glasshouse for 7 days before seedlings were transplanted to bags previously inoculated with *F. oxysporum* f. sp. *lycopersici* as described above.

Tomato seed for field experiments were sown in trays with 40 individual cells (4 by 4 by 6 cm/cell) containing an autoclaved mixture of vermiculite and peat as described above. Trays were maintained in a growth chamber for 3 weeks, and then placed in a glasshouse at 20 to 30°C for 7 days. Before transplanting into the field, plants were maintained in the trays on benches close to the field for 2 weeks. Manure was applied to soil approximately 3 months before transplanting in fallowed fields.

Additives. Several compounds such as sugars (sucrose, D-sorbitol, and fructose), polyalcohols (glycerol, polyethylene glycol 300 [PEG 300], PEG 8000, and nonilphenyl-PEG ether [Agral]), inorganic salts (NaCl, KCl, and CaCl₂), detergents (PEG sorbitan monolaurate [Tween 20], PEG sorbitan monooleate [Tween 80], and mono[p-(1,1,3,3-tetramethylbutyl) phenyl]-PEG ether [Triton X-100]) were used due to their features as dispersants,

binders, humectants, emulsifiers, and wetters (1). Additives were dissolved in distilled water wt:vol (solids) or vol:vol (liquids) and then autoclaved at 1.0 kg cm⁻² at 120°C for 20 min.

Dispersal assays. Before additives were selected for dispersal assays, the different compounds described above were tested for toxicity (effect on conidial germination and production) against *P. oxalicum* at the dose range previously described (2,17) (Tables 1 to 5). Agral (10%) was tested as recommended by its manufacturer (Zeneca Agro, Madrid). Three experiments were performed to evaluate the effect of additives on germination of dried conidia of *P. oxalicum* (9). A conidial suspension of 2 × 10⁶ conidia ml⁻¹ was prepared in sterile Czapek broth (Difco Laboratories). Additive solutions were prepared as described above at 2× the dose of those shown in Tables 1 to 5, except for doses of 60%. Droplets (15 µl) of conidial suspensions were mixed on a slide with 15 µl of each additive solution. In the case of 60% doses (Table 2), each sugar was added to Czapek broth and, after autoclaving at 1.0 kg cm⁻² at 120°C for 20 min, dry conidia of *P. oxalicum* were added to these solutions to give a concentration of 10⁶ conidia ml⁻¹. Aliquots of 30 µl of each mixture were placed on sterile glass slides. All slides were placed in 15-cm-diameter glass petri dishes lined with moist paper and incubated for 16 h at 20 to 25°C in darkness.

Germination of 50 conidia was assessed in each replicate and three replicates were made for each sample. A spore was considered germinated when the germ tube was longer than the length of the spore (more than 7 µm).

Two experiments were performed to test the effect of additives on *P. oxalicum* conidial production. Conidia were produced as described above. Substrate contained in plastic bags (50 g) was inoculated with 40 ml of a suspension of *P. oxalicum* conidia (10⁵ conidia g⁻¹ of dry substrate) in each additive solution (Tables 1 to 5).

To determine the effect of additives on dispersal of *P. oxalicum* conidia, additives were added to conidia at three different steps of the production-drying process (Table 6): (i) to substrate contained in bags before production, (ii) to conidial paste obtained after production before drying, and (iii) to conidia after drying by fluid bed drying. Within each group, the dose tested was the highest nontoxic one, according to the results obtained in toxicity assays (germination and production). Conidia without additives were used as control and three replicates were made per additive and step. For methods i and ii, conidia were produced and dried as described above and suspended in water; for method iii, an additive solution was used and each was rotated at 150 rpm for 30 min. Dispersal of conidial suspensions or additive solutions was estimated by measuring absorbance at 420 nm in a spectrophotometer. Three readings were made for each sample and the complete experiment was conducted three times. In each experiment, the percentage of increase of conidial dispersal was calculated by the formula [(dispersal of conidia with additive – dispersal of conidia without additive)/dispersal of conidia without additive] × 100.

Glasshouse experiment. Tomato cv. San Pedro plants were used to evaluate the bio-control efficacy of *P. oxalicum* containing various dispersal additives against *F. oxysporum* f. sp. *lycopersici* in April 2003. The following formulations were applied to seedlings in seedbeds, 7 days before transplanting: (i) a conidial suspension of *P. oxalicum* maintained with 60% sucrose for 10 min before the drying process (FOR1) and (ii) a conidial suspension of *P. oxalicum* maintained with 1.5% sodium alginate for 10 min before the drying process (FOR2). FOR1 and FOR2 were selected in the glasshouse because of good dispersal and germination results obtained previously in preliminary studies. All treatments were made to give 6 × 10⁶ spores g⁻¹ of seedbed substrate. Seedlings were transplanted into sterile peat bags (80 by 40 by 20 cm³) which previously were inoculated with *F. oxysporum* f. sp. *lycopersici* as described before. Ten tomato plant seedlings were cultivated per bag. Control treatments consisted of plants transplanted into peat with *F. oxysporum* f. sp. *lycopersici* added without *P. oxalicum* (C1) and plants

Table 1. Effect of hydrophobic compounds on germinability of dry conidia and on conidial production of *Penicillium oxalicum*^v

Hydrophobic compounds, doses (%) ^w	Conidia germinated (%) ^x	Germination enhancement (%) ^y	Production (conidia × 10 ⁸ g ⁻¹ of substrate) ^z
Sunflower oil			
0	71 a	–	0.57 a
0.1	88 bc	24	NT
2	88 bc	24	NT
5	95 bc	34	NT
10	96 c	36	0.81 a
20	94 bc	33	NT
50	85 b	20	NT
MS _{within}	58	...	0.27
Sodium alginate			
0	61 (38) a	–	0.83 (0.23) a
0.1	88 (64) c	45	NT
0.5	88 (63) c	45	NT
1	88 (63) c	46	1.37 (0.31) a
1.5	77 (51) b	27	2.15 (0.49) a
MS _{within}	(94)	...	(0.03)
Methyl cellulose			
0	71 a	–	0.65 a
0.1	67 a	–	NT
0.5	83 a	–	NT
1	77 a	–	NT
1.5	72 a	–	0.76 a
MS _{within}	82	...	0.32

^v Means for each additive followed by the same letter in each column are not significantly different (*P* = 0.05) by Duncan's multiple range test.

^w MS_{within} = error mean square.

^x Data are the mean of three replications; 50 conidia were counted per replication. Data in parentheses were subjected to arcsin transformation before analysis.

^y All values shown were significantly different from control (*P* = 0.05); – indicates no germination enhancement.

^z Data are the mean of three fermentation bags. Data in parentheses were subjected to log₁₀(*x* + 1) before analysis. NT = not tested.

transplanted into clean peat (C2) with no *P. oxalicum* or *F. oxysporum* added. Five bags per treatment were placed in a glasshouse at 20 to 30°C in a randomized block design. Plants were fertilized with Nitrophoska Stabil 14+7+17 (BASF, Germany) at 100 g m⁻² 40 days after transplanting. Disease incidence on 50 plants per treatment was recorded at 15, 20, 30, 40, 50, 60, 70, and 80 days after transplanting based on the plants with Fusarium wilt symptoms. The area under the disease progress curve (AUDPC) was calculated (3). The crowns of plants were cut lengthwise and transferred to humid chambers and the presence or absence of *F. oxysporum* f. sp. *lycopersici* on xylem tissues was determined after 5 days of incubation at 20 to 25°C. Fungal structures were taken from diseased tissues and were placed on slides, stained with methylene blue, squashed, and examined microscopically. When only mycelia were observed on the diseased tissues, isolations of mycelia on PDA amended with streptomycin sulfate at 0.5 g liter⁻¹ were made to identify *F. oxysporum* f. sp. *lycopersici*. Identification was based on presence of chlamydospores formed singly or in pairs, single-cell, oval to kidney-shaped microconidia borne in false heads on short monophialides, and slightly sickle-shaped, thin-walled macroconidia with an attenuated apical cell and a foot-shaped basal cell (23).

Prior to transplanting (day 7 after treatment), three plants per treatment were selected at random from seedbeds to estimate the population of *P. oxalicum* in the rhizosphere. Population of the fungus also was estimated as described below at the end of the experiment in three plants chosen at random from the 50 plants of each treatment. The roots of each plant were taken from peat, shaken to remove most of the peat, weighed, and added into 250-ml flasks with 150 ml of phosphate buffer (pH = 7, 0.01M). Flasks then were rotated for 30 min at 150 rpm, and 10 to 1,000-fold dilutions were made. Aliquots of 100 µl from undiluted and diluted suspensions were spread onto petri dishes containing selective media for *P. oxalicum* (11). Petri dishes were incubated in the dark at 25°C for 5 to 7 days, after which the colonies were counted (18). The CFU per plant were calculated per gram of fresh root, plus the peat adhering to the roots, considered to be part of the rhizosphere. Three petri dishes were used per dilution.

Field assay. A field trial was established in a randomized complete block design with five replications (plots) per treatment during the 2003 growing season. The field had a loam soil, pH = 7.0, and was located in Aranjuez, Madrid, Spain, where Verticillium wilt disease had been prevalent in previous years (21). Fifteen days after addition of *P. oxalicum*, when the seedlings had four to six leaves, they were carefully transplanted into the field. Ten tomato seedlings of cv. San Pedro per rep-

lication or 50 plants per treatment were transplanted on 20 May in 24-m² plots spaced 1 m within rows with 1.2 m between rows. The field received a manure treatment about 3 months before planting. During the growing season, tomato plants were flood irrigated as necessary. Tomato plants received two applications of insecticide (Omatoato 50% p/v [SL], FOLIMAT 50LS; Bayer Hispania S.A., Barcelona, Spain) on 18 June and 2 July. Plots were mechanically cultivated as needed to remove weeds. Tomato plants were treated in seedbeds with the same formulations of *P. oxalicum* described for glasshouse experiment (FOR1 and FOR2). The control treatment had no *P. oxalicum* applied (C1). Disease incidence was recorded every 15 days for 120 days and symptoms characteristic of wilts of tomato were recorded. The AUDPC was calculated (3). To determine the cause of wilting, aerial parts of all wilted plants were transferred to humidity chambers in the laboratory and the presence or absence of fungal pathogens on them was determined after 5 days of incubation at 20 to 25°C. Fungal structures were taken from the vegetative tissues to be observed under the microscope as described above for the glasshouse experiment. When

only mycelia were observed, fungi were isolated in PDA amended with streptomycin sulfate at 0.5 g liter⁻¹.

Three plants per treatment were selected at random from seedbeds to estimate *P. oxalicum* population in the rhizosphere, just before transplanting into the field, as described above.

Data analysis. Data were analyzed by analysis of variance (ANOVA) with the Statgraphics Plus 4.1 program. Prior to analysis, some data were subjected to various transformations to improve homogeneity of variances: arcsin for data of conidial germination with sodium alginate, sucrose, fructose, D-sorbitol, glycerol, PEG 300, PEG 8000, calcium chloride, and Triton TX-100; cos 10⁻¹ for data of conidial germination with Tween 20; (log₁₀ + 1) for data of conidial production with fructose, D-sorbitol, PEG 300, and calcium chloride, and for data of percentage of increase of conidial dispersal with all the additives; and cos for data of CFU of *P. oxalicum* g⁻¹ of fresh root in the field experiment. Germination and production of conidia experiments were repeated with similar results. Dispersal experiments were repeated three times. These three experiments showed similar results. An ANOVA was

Table 2. Effect of sugars on germinability of dry conidia and conidial production of *Penicillium oxalicum*^v

Sugars, doses (%) ^w	Conidia germinated (%) ^x	Germination enhancement (%) ^y	Production (conidia × 10 ⁸ g ⁻¹ of substrate) ^z
Sucrose			
0	70 (45) a	–	0.70 a
0.1	69 (44) a	–	NT
0.5	82 (57) b	17	NT
1	80 (55) ab	–	NT
2	71 (47) ab	–	NT
15	83 (58) b	20	NT
30	95 (75) c	37	NT
60	93 (70) c	33	0.61 a
MS _{within}	(197)	...	0.31
D-sorbitol			
0	70 a	–	2.52 (0.27) a
0.1	74 ab	–	NT
0.5	71 a	–	NT
1	78 abc	–	NT
2	72 ab	–	NT
15	81 abc	–	NT
30	85 bc	22	NT
60	90 c	29	0.13 (–0.69) b
MS _{within}	256	...	(0.14)
Fructose			
0	67 (45) ab	–	4.58 (0.73) a
5	78 (54) b	–	NT
10	78 (54) b	–	NT
20	78 (54) b	–	NT
30	72 (48) b	–	NT
60	44 (29) a	–	0.28 (0.10) b
MS _{within}	(223)	...	(0.009)

^v Means for each additive followed by the same letter in each column are not significantly different (*P* = 0.05) by Duncan's multiple range test.

^w MS_{within} = error mean square.

^x Data are the mean of three replications; 50 conidia were counted per replication. Data in parentheses were subjected to arcsin transformation before analysis.

^y All values shown were significantly different from control (*P* = 0.05); – indicates no germination enhancement.

^z Data are the mean of three fermentation bags. Data in parentheses were subjected to log₁₀(*x* + 1) before analysis. NT = not tested.

made with all three assays and, because the factor assay was not significant ($P = 0.05$), a pool of data from each assay was made and further analyzed by ANOVA with nine replications (three experiments with three replications each). When the F test was significant at $P = 0.05$ or $P = 0.01$, means were compared by Duncan's multiple range test (25).

RESULTS

Dispersal assays. Toxicity of additives to *P. oxalicum* conidia was tested by effects on germination and production (Tables 1 to 5). Germination of conidia without additives ranged from 51 to 79% (Tables 1 to 5). Additives assayed did not significantly ($P = 0.05$) reduce conidial germination except for 50% PEG 300 and 10% CaCl_2 (Tables 1 to 5). Several additives significantly ($P = 0.05$) enhanced germination of *P. oxalicum* conidia. These included all doses of sunflower oil and sodium alginate (Table 1); 0.5, 15, 30, and 60% sucrose; 30 and 60% D-sorbitol (Table 2); 2, 5, 20, and 30% glycerol; 30% PEG 300 (Table 3); 0.01 to 1% CaCl_2 (Table 4);

0.01, 0.02, 0.5, and 1% Tween 20; and 0.01 to 1% Tween 80 (Table 5). No effect was observed on conidial germination with methylcellulose, fructose, PEG 8000, Agral, NaCl, KCl, or Triton TX-100 (Tables 1 to 5).

Production of *P. oxalicum* conidia without additives ranged from 0.57 to 4.58 conidia $\times 10^8 \text{ g}^{-1}$ of substrate (Tables 1 to 5). Additives did not have an effect on conidial production of *P. oxalicum*, except for reductions observed for 60% D-sorbitol, 60% fructose, and 10% CaCl_2 (Tables 2 and 4). Conidial production was not significantly enhanced for the additives tested.

Additives with no effects on germination or production of *P. oxalicum* conidia were further tested to improve conidial dispersal. In the first step, additives were added to substrate in bags before production. Sodium alginate at 1.5% provided higher percentages of conidial dispersal but was not significantly different from 12 other additives (Table 6). Other additives tested did not significantly enhance the dispersal of conidia in water.

Five additives significantly ($P = 0.05$) increased the dispersal of conidial suspensions when they were added to conidia before drying. These included 1.5% sodium alginate, 60% sucrose, 60% D-sorbitol, 60% fructose, and 20% PEG 8000 (Table 6).

Once conidia were dried, their dispersal in water was only enhanced when suspended in detergent solutions such as 1% Triton X-100 and 10% Agral (Table 6).

Glasshouse experiment. Tomato plants without *P. oxalicum* added showed symptoms of Fusarium wilt 15 days after transplanting, and 55% of the plants were diseased 80 days after transplanting. At this time, old leaves drooped and curved downward, and some were yellow. The plants had fewer leaves, frequently wilted, and were stunted. At the end of the assay, only *F. oxysporum* f. sp. *lycopersici* were observed and isolated from the vascular system of plants. Initial population of *F. oxysporum* f. sp. *lycopersici* in the soil just before transplanting was $1.37 \times 10^6 \text{ CFU g}^{-1}$ dry weight. After 80 days, control plants (C2) were healthy and vigorous, although 4% were diseased. When infection was detected in control plants, the assay was concluded (80 days after transplanting).

Use of FOR2 reduced tomato wilt incidence and AUDPC at the end of the experiment (Table 7). FOR1 only reduced tomato wilt incidence but did not significantly reduce AUDPC.

The population of *P. oxalicum* in the rhizosphere of seedlings 7 days after treatment with formulations was higher than 10^6 CFU g^{-1} of fresh root in all cases (Table 7). The highest population was obtained in rhizosphere of plants treated with FOR2, 20 times more than with FOR1. In the case of control plots (C1 and C2, nontreated with *P. oxalicum*), some populations of *P. oxalicum* were isolated, in both cases less than 10^3 CFU g^{-1} of fresh root. The population of *P. oxalicum* decreased to levels close to 10^3 CFU g^{-1} of fresh root at the end of assay. For the controls, C1 and C2, a population of *P. oxalicum* was not detected at the end of assay (Table 7).

Field assay. In the field experiment at 57 days after treatment, initial symptoms included mature leaves that were drooping, curved downward, and chlorotic. A total of 26 plants wilted after 120 days of transplanting (51% incidence; Table 8) in the control treatment; *Verticillium* spp. were isolated from all of these plants. *F. oxysporum* f. sp. *lycopersici* also was isolated from four plants. The treatments FOR1 and FOR2 reduced tomato wilt incidence 22 and 14%, respectively, and AUDPC at the end of the experiment (Table 8).

No differences were observed between just before transplanting and 15 days after treatment in the populations of *P. oxalicum*

Table 3. Effect of polyalcohols on germinability of dry conidia and on conidial production of *Penicillium oxalicum*^a

Polyalcohols, doses (%) ^w	Conidia germinated (%) ^x	Germination enhancement (%) ^y	Production (conidia $\times 10^8 \text{ g}^{-1}$ of substrate) ^z
Glycerol			
0	68 (43) a	—	0.76 a
0.1	82 (56) ab	—	NT
0.5	81 (55) ab	—	NT
2	97 (78) c	38	NT
5	93 (73) c	37	1.34 a
20	98 (80) c	43	0.86 a
30	90 (65) bc	33	NT
50	78 (53) ab	—	NT
MS _{within}	(111)	...	0.34
PEG 300			
0	76 (53) b	—	2.73 (0.51) a
5	84 (60) b	—	2.47 (0.45) a
10	77 (53) b	—	NT
20	84 (61) bc	—	1.95 (0.38) a
30	92 (72) c	25	NT
40	88 (65) bc	—	NT
50	56 (36) a	—	NT
MS _{within}	(131)	...	(0.07)
PEG8000			
0	79 (55) a	—	1.32 a
5	79 (55) a	—	1.47 a
10	82 (56) a	—	NT
20	92 (70) a	—	2.02 a
30	94 (73) a	—	NT
40	94 (71) a	—	NT
50	89 (67) a	—	NT
MS _{within}	(176)	...	0.30
Agral			
0	51.3 a	—	4.58 a
10	32.2 a	—	5.03 a
MS _{within}	161.1	...	2.19

^a Means for each additive followed by the same letter in each column are not significantly different ($P = 0.05$) by Duncan's multiple range test.

^w MS_{within} = error mean square.

^x Data are the mean of three replications; 50 conidia were counted per replication. Data in parentheses were subjected to arcsin transformation before analysis.

^y All values shown were significantly different from control ($P = 0.05$); — indicates no germination enhancement.

^z Data are the mean of three fermentation bags. Data in parentheses were subjected to $\log_{10}(x + 1)$ before analysis. NT = not tested.

estimated on tomato rhizosphere in any treatment (Table 8).

DISCUSSION

P. oxalicum has shown a good potential for development as a commercial biocontrol product (21). Nondusty, highly miscible, wettable-powder formulations of *P. oxalicum* conidia were developed by adding compounds at one of three different steps of the production-formulation process.

Problems with conidial suspension in water of *P. oxalicum*, particularly after the drying process, were addressed (20,24). Dusty powders, such as conidia that form dry aggregates, result in mixtures with aggregates that settle when added to water and produce irregular distribution in applications with erratic control (2). These aggregates also obstruct nozzles in spray tanks (2). Several additives were tested in this study to overcome these drawbacks.

Hydrophobic compounds, such as sugars, polyalcohols, inorganic salts, and detergents, were used as dispersal additives. Detergents added in this study to suspend dried conidia in water just before application enhanced dispersal of conidia. Detergents helped to reduce surface tension at the liquid-solid interface and facilitated air distribution around particles (17).

Additives should not affect production and germination of conidia. Dispersal assays were carried out with additives at doses that had no effect on conidial production or germinability. In these assays, conidia were germinated in Czapek-Dox broth, a complete medium conducive to germination of conidia, as observed in the controls without additives. Several doses of some additives (Tables 1 to 5) enhanced germination of *P. oxalicum* conidia in these conditions; therefore, these additives may improve water and nutrient absorption of *P. oxalicum* conidia. Water and nutrients are essential, together with gas ratios and favorable temperature, in rehydration of conidia to activate certain enzyme systems, to initiate other internal chemical changes, and to increase the volume of germinating spores (5,22,26). However, there could be a negative effect of additives. If all spores are induced to germinate by the additive, there will be no spore reserves in the mix, because spores that are induced to germinate and are not in the root niche will die.

The best results on dispersal of conidia were obtained when additives (glycerol, PEG 8000, sodium alginate, and sugars) were added to conidia before drying. These compounds form hydrogen bonds with functional groups of protein molecules on dry conidia surfaces (4,24,27) and allow rehydration and improve dispersal by reducing the hydrophobicity of conidial surfaces of *P. oxalicum*. Conidial dispersal also was enhanced when sodium alginate was added to the substrate. These studies demonstrate that adding additives to the

Table 4. Effect of inorganic salts on germinability of dry conidia and on conidial production of *Penicillium oxalicum*^v

Inorganic salts, doses (%) ^w	Conidia germinated (%) ^x	Germination enhancement (%) ^y	Production (conidia × 10 ⁸ g ⁻¹ of substrate) ^z
Sodium chloride			
0	74 a	—	0.98 a
0.5	82 a	—	NT
1	74 a	—	NT
5	77 a	—	NT
10	77 a	—	1.20 a
MS _{within}	311	...	0.29
Potassium chloride			
0	74 a	—	0.98 a
0.5	75 a	—	NT
1	76 a	—	NT
5	76 a	—	NT
10	76 a	—	1.06 a
MS _{within}	274	...	0.17
Calcium chloride			
0	64 (42) b	—	2.99 (0.57) a
0.01	88 (68) c	39	NT
0.05	86 (62) c	35	NT
0.1	88 (62) c	38	NT
0.5	93 (69) c	46	NT
1	88 (63) c	37	NT
10	26 (16) a	—	1.40 (0.37) b
MS _{within}	(186)	...	(0.021)

^v Means for each additive followed by the same letter in each column are not significantly different ($P = 0.05$) by Duncan's multiple range test.

^w MS_{within} = error mean square.

^x Data are the mean of three replications; 50 conidia were counted per replication. Data in parentheses were subjected to arcsin transformation before analysis.

^y All values shown were significantly different from control ($P = 0.05$); — indicates no germination enhancement.

^z Data are the mean of three fermentation bags. Data in parentheses were subjected to $\log_{10}(x + 1)$ before analysis. NT = not tested.

Table 5. Effect of non-ionic surfactants on germinability of dry conidia and on conidial production of *Penicillium oxalicum*^v

Detergents, doses (%) ^w	Conidia germinated (%) ^x	Germination enhancement (%) ^y	Production (conidia × 10 ⁸ g ⁻¹ of substrate) ^z
Tween 20			
0	70 (0.992) a	—	0.87 a
0.01	81 (0.989) b	16	NT
0.02	84 (0.989) b	21	NT
0.3	80 (0.990) ab	—	NT
0.5	82 (0.989) b	18	NT
1	85 (0.988) b	21	0.97 a
MS _{within}	(8 × 10 ⁻⁶)	...	0.13
Tween 80			
0	71 a	—	0.97 a
0.01	87 b	23	NT
0.02	89 bc	25	NT
0.3	95 c	34	NT
0.5	94 bc	32	NT
1	94 c	33	1.21 a
MS _{within}	39	...	0.24
Triton TX-100			
0	74 (50) a	—	0.80 a
0.01	77 (54) a	—	NT
0.02	85 (60) a	—	NT
0.3	86 (61) a	—	NT
0.5	86 (61) a	—	NT
1	84 (59) a	—	0.83 a
MS _{within}	(184)	...	0.14

^v Means for each additive followed by the same letter in each column are not significantly different ($P = 0.05$) by Duncan's multiple range test.

^w MS_{within} = error mean square.

^x Data are the mean of three replications; 50 conidia were counted per replication. Data were subjected to cos/10 and arcsin for Tween 20 and Triton TX-100, respectively, before analysis.

^y All values shown were significantly different from control ($P = 0.05$); — indicates no germination enhancement.

^z Data are the mean of three fermentation bags. NT = not tested.

Table 6. Effect of different additives added before production, before drying process, and to dry conidia, on conidial dispersal of *Penicillium oxalicum*^y

Additives ^z	Before production	Before drying	After drying
1.5% Sunflower oil	1.20 ab	0.54 abc	1.20 cde
1.5% Sodium alginate	1.44 b	1.98 c	0.53 abcd
1.5% Methyl cellulose	1.08 ab	1.08 abc	0.21 ab
60% Sucrose	0.50 ab	1.82 c	0.00 a
60% D-sorbitol	NT	1.80 c	0.00 a
60% Fructose	NT	1.90 c	0.00 a
5% Glycerol	0.51 ab	0.82 abc	0.00 a
20% Glycerol	0.73 ab	1.74 bc	0.00 a
5% PEG300	0.13 a	0.00 a	0.00 a
20% PEG300	0.00 a	0.98 abc	0.00 a
5% PEG8000	1.18 ab	1.62 bc	0.35 abc
20% PEG8000	1.17 ab	1.84 c	0.00 a
10% Agral	1.15 ab	0.20 ab	1.46 e
10% NaCl	0.47 ab	0.00 a	0.69 abcde
10% KCl	0.75 ab	0.00 a	0.00 a
1% Tween 20	0.79 ab	0.47 abc	0.96 bcde
1% Tween 80	0.00 a	1.18 abc	1.32 de
1% Triton X-100	0.62 ab	0.61 abc	1.47 e
MS _{within}	0.39	0.25	0.20

^y Data are $\log_{10}(x + 1)$ percentage of increase of conidial dispersal and were calculated by the formula $\log_{10}(x + 1)$ where $x = ([\text{dispersal of conidia with additive} - \text{dispersal of conidia without additive}]/[\text{dispersal of conidia without additive}]) \times 100$. Dispersal of conidia suspensions was measured as absorbance at 420 nm in a spectrophotometer. Data are the mean of three experiments with three replications in each experiment (nine replications). Means for each additive followed by the same letter in each column are not significantly different ($P = 0.05$) by Duncan's multiple range test; NT = treatments not tested.

^z Additives were added to (i) substrate contained in bags before production, (ii) conidial paste obtained after production before drying, or (iii) conidia after drying by fluid bed drying. MS_{within} = error mean square.

Table 7. Disease incidence (percent with *Fusarium* spp.) on tomato plants treated with *Penicillium oxalicum* and populations of *P. oxalicum* in the rhizosphere^y

Treatments ^z	Disease incidence	AUDPC	Populations of <i>P. oxalicum</i> in rhizosphere ($\times 10^3$ CFU g ⁻¹ of fresh root)	
			Before transplanting	End of assay
FOR1	22.0 c	970 ab	3,697 a	11.0 a
FOR2	26.9 bc	855 b	85,528 b	5.0 a
C1	55.4 a	1,569 a	<10 ³	ND
C2	4.0 c	130 c	<10 ³	ND
MS _{within}	260.1	240,663	5.3×10^{10}	1.96×10^4

^y Means followed by the same letter in each column are not significantly different by Duncan's multiple range test at $P = 0.05$ (incidence and area under the disease progress curve [AUDPC]), or $P = 0.01$ (*P. oxalicum* population). Data of incidence and AUDPC are the mean of five replicates with 10 plants per replicate (50 plants). Data of CFU are the mean of three replicates (three randomly chosen plants per treatment). ND = not detected.

^z FOR1 = a conidial suspension of *P. oxalicum* maintained with 60% sucrose for 10 min before drying process, FOR2 = a conidial suspension of *P. oxalicum* maintained with 1.5% sodium alginate for 10 min before drying process, C1 = no *P. oxalicum* and no additive, all inoculated with *F. oxysporum* f. sp. *lycopersici*, C2 = no *P. oxalicum*, no additive and not inoculated, and MS_{within} = error mean square.

Table 8. Incidence of *Verticillium* spp. on tomato cv. San Pedro plants in a field experiment at 120 days after transplanting, and population of *Penicillium oxalicum* in the rhizosphere just before transplanting^y

Treatments ^z	Incidence of <i>Verticillium</i> spp. (%)	AUDPC	<i>P. oxalicum</i> CFU g ⁻¹ of fresh root ($\times 10^3$)
FOR1	22 b	204 b	27,000 (0.15) a
FOR2	14 b	335 b	11,700 (0.22) a
C1	51 a	551 a	<10 ³
MS _{within}	305	16,682	(0.27)

^y Data of incidence are the mean of five replicates with 10 plants per replicate (50 plants). Data of CFU are the mean of three replicates (three randomly chosen plants per treatment), and they were subjected to cos transformation before analysis (data in parentheses). Means followed by the same letter in each column are not significantly different by Duncan's multiple range test ($P = 0.05$) (incidence and area under the disease progress curve [AUDPC]), or $P = 0.01$ (*P. oxalicum* population).

^z FOR1 = a conidial suspension of *P. oxalicum* maintained with 60% sucrose for 10 min before drying process, FOR2 = a conidial suspension of *P. oxalicum* maintained with 1.5% sodium alginate for 10 min before drying process, C1 = no *P. oxalicum* and no additive, and MS_{within} = error mean square.

growth substrate significantly improved the ability of conidia of *P. oxalicum* to disperse in water. This approach can have wide and useful applications.

Based on dispersal results, two formulations were tested in glasshouse and field trials. *P. oxalicum* formulations with 60% sucrose (FOR1) or 1.5% sodium alginate (FOR2) added to conidia before drying enhanced both dispersal and germination of FOR1 and FOR2, reduced wilts caused by *Fusarium* spp. in the greenhouse, and, in a preliminary trial, reduced wilts caused by *Verticillium* spp. in a field assay. Enhancement of dispersal of conidia increased when suspensions of formulations were made before application. Conidia were suspended in the tank of the sprayer without difficulties because conidial settling was avoided. In addition, without aggregate formation, nozzles were not obstructed.

All the plants from the glasshouse experiment were picked up at the end of the experiment to isolate the fungal pathogens causing wilts. *F. oxysporum* f. sp. *lycopersici* was the only fungus isolated from the vascular system of these plants. In the field experiment, *Verticillium* spp. were isolated from all diseased plants and *F. oxysporum* f. sp. *lycopersici* was isolated from a few plants.

Population levels of *P. oxalicum* in rhizospheres of tomato plants treated with FOR1 and FOR2 before transplanting were in the range of 10^6 to 10^7 CFU g⁻¹ of fresh root for both formulations and were similar to previous greenhouse and field experiments (7,8,21) in which disease reduction of *Fusarium* and *Verticillium* wilts of tomato were demonstrated. Disease reduction was obtained with FOR1 and FOR2. The presence of nutrients included in FOR1 (60% sucrose) or FOR2 (1.5% sodium alginate) may contribute to enhance biocontrol. Previously, we (7,8,21) have demonstrated that conidia applied together with nutrients enhanced consistency of control. We demonstrated here that 60% sucrose or 1.5% sodium alginate, among others, enhanced germination of *P. oxalicum* conidia. *Typhula phacorriza* formulated as sodium alginate pellets was effective at low rates (20 g m⁻²) to control gray snow mold of turf grass and cereals caused by *T. incarnata* and *T. ishikariensis* in other studies (28). Nutrient bases may enable propagules of biocontrol agents to germinate and colonize the rhizosphere or may be a nutrient base for the antagonist during the early stages of proliferation after application to the soil.

Interplot interference could occur due to flood irrigation in field assays because conidia of *P. oxalicum* may have been moved by water between plots. To reduce this problem, a distance of 1 m² was established between plants, and plots were established in a randomized block design. In addition, although some conidia from a

plot were moved to the rhizosphere of plants of another plot, the levels were low, and less than the 10^6 to 10^7 conidia per grams of fresh root necessary to reduce disease (21).

Our results provide an effective method to add additives to the conidial production process or during formulation as a dry product to improve *P. oxalicum* dispersal, increasing disease reduction from wilt of tomato caused by *Fusarium* and *Verticillium* spp.

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