Production, Survival, and Evaluation of Solid-Substrate Inocula of *Penicillium oxalicum*, a Biocontrol Agent Against Fusarium Wilt of Tomato

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ABSTRACT

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Production of conidia of *Penicillium oxalicum* (ATCC number pending), a biocontrol agent of *Fusarium oxysporum* f. sp. *lycopersici*, was tested in liquid and solid fermentation. *P. oxalicum* produced 250-fold more conidia in solid than in liquid fermentation at 30 days after inoculation of substrate. Solid fermentation was carried out in plastic bags (600 cm³) especially designed for solid fermentation (VALMIC) containing 50 g of peat/vermiculite (PV) (1:1, wt/wt) with 40% moisture, sealed, sterilized, and then inoculated with 1 ml of a conidial suspension of *P. oxalicum* (10⁵ conidia g⁻¹ dry substrate), sealed again, and incubated in darkness at 20 to 25°C for 30 days. Addition of amendments to PV in a proportion of 0.5 (wt/wt) significantly increased conidial production of *P. oxalicum*. The best production was obtained on PV plus meal of cereal

grains (barley) or leguminous seeds (lentil) (100-fold higher). Conidial production obtained after 5 days of inoculation was similar to that obtained at 30 days. However, viability of conidia produced in PV plus lentil meal was 35% higher than that of conidia produced in PV plus barley meal. Changes in proportions (1:1:0.5, wt/wt/wt; 1:1:1, wt/wt/wt; 1:0.5:0.5, wt/wt/wt; 1:1:0.5, vol/vol/vol) of components of the substrate (peat/vermiculite/lentil meal) did not enhance production or viability of conidia. Optimal initial moisture in the substrate was 30 to 40%. At lower moistures, significant reductions of production of conidia were observed, particularly at 10%. There was a general decline in the number of conidia in bags with time of storage at -80, -20, 4, and 25°C, or at room temperature (range from 30 to 15°C), with the highest decline occurring from 60 to 180 days. Conidial viability also was reduced with time, except for conidia stored at -20°C. Fresh conidia produced in solid fermentation system or those conidia stored at -20°C for 180 days reduced Fusarium wilt of tomato by 49 and 61%, respectively.

In agriculture, pests and diseases are predominantly controlled by chemical pesticides (38). New restrictions on application of these chemicals and environmental considerations have led to an increased interest in biocontrol agents (38). In the development of biocontrol agents, it is necessary to find methods for the mass production of inocula (6), and such agents often exhibit inconsistent performance in practical production, resulting in limited commercial use of biocontrol approaches for suppression of plant pathogens (2).

Both solid and liquid fermentation systems have been used for the mass production of biocontrol agents (28). However, some microorganisms, primarily some filamentous fungi, need high oxygen transfer for growth and especially for sporulation (36). Solid fermentation allows some fungi to produce hardy, healthy conidia and mycelia (3). The moisture needed is found in the solid matrix in an absorbed or complex form, with moisture ranging between 12 and 80% (8). Solid-state fermentation mimics the natural environmental conditions and habitat for some microorganisms (25). Solid-state fermentation has been used for the production of some biological agents, such as *Talaromyces flavus* (20), *Coniothyrium minitans* (33), entomopathogenic fungi such as *Metarhizium anisopliae* (18,22), or bioherbicides (11).

Penicillium oxalicum is a fungal biological agent that reduced disease caused by *Fusarium oxysporum* f. sp. *lycopersici* by induction of resistance in tomato plants (16). Induced resistance in tomato plants by *P. oxalicum* was related to renewed or prolonged

cambial activity that led to the formation of additional secondary xylem in *P. oxalicum*-treated plants (13). The application of a conidial suspension of *P. oxalicum* by watering the tomato seedlings in seedbeds 7 days before transplanting provided the best control of tomato Fusarium wilt (12). Disease suppression was maintained for 60 to 100 days after inoculation with the pathogen in the glasshouse (12). In all these experiments, treatments with *P. oxalicum* were made with conidia produced on potato dextrose agar (PDA) plates that were incubated in darkness at 20 to 25°C for 7 days (12,13,15,16). Biocontrol based on *P. oxalicum* conidia is very promising, and an efficient conidia production process should be developed. Production of conidia of *P. oxalicum* in submerged liquid fermentation is feasible but quantities obtained are scarce (43). Furthermore, aerially produced *P. oxalicum* conidia were more effective against Fusarium wilt than submerged conidia or mycelium (41).

Our aim was to develop a method to produce conidia in masses that could be used in industrial processes for a filamentous fungus such as *P. oxalicum*. These conidia should be efficient and consistent for the biological control of tomato disease, especially Fusarium wilt of tomato.

MATERIALS AND METHODS

Cultures. The isolate of *P. oxalicum* Currie & Thom was stored on homemade (PDA) slants as described in Pascual et al. (42) at 4°C and grown in darkness at 20 to 25°C for 7 days on PDA on petri plates for conidial inoculum production. It has been deposited with the American Type Culture Collection (Manassas, VA), awaiting an accession number. An isolate of *Fusarium oxysporum* Schlechtend.:Fr. f. sp. *lycopersici* (Sacc.) W. C. Snyder & H. N. Hans. race 2 (ATCC number 201829), obtained from a tomato

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plant in southern Spain, was stored at 4°C in tubes containing sterile sand. Sand culture of the fungus was sprinkled on Czapek-Dox agar (CDA) (Difco Laboratories, Detroit) with mycelial production completed after 7 days in darkness at 25°C. Microconidial inoculum of *F. oxysporum* f. sp. *lycopersici* was produced in Erlenmeyer flasks (250 ml) containing 150 ml of sterile Czapek-Dox broth (Difco Laboratories). Each flask was inoculated with three mycelial plugs (1 cm in diameter) of the fungus taken from 7-day-old cultures on CDA (15). The flasks were incubated for 5 days at 25°C in a rotary shaker (Model 3527; Lab-Line Instruments, Inc., Melrose Park, IL) at 150 rpm (three-quarter-inch orbital path). The culture was filtered through glass wool, concentrated by centrifugation at 14,040 × g for 10 min, and diluted in water prior to use as inoculum.

Plant material. The tomato cultivar used, Precodor, is susceptible to races 1 and 2 of *F. oxysporum* f. sp. *lycopersici*. Tomato seed were sown in trays containing an autoclaved mixture of vermiculite (Termita, Asfaltex, S.A., Barcelona, Spain) and peat (Gebr. BRILL substrate GmbH & Co. KG, Germany) (1:1, vol/vol), and 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% relative humidity for 4 weeks. Seedlings were watered regularly to keep the growth substrate in a wet condition.

Conidia production in liquid fermentation versus solid fermentation. *P. oxalicum* was produced in liquid fermentation such as described Pascual et al. (41). *P. oxalicum* was grown in 250-ml Erlenmeyer flasks with 50 ml of liquid medium adjusted to pH 6 and containing, per liter, 50 g of glucose, 1 g of KH₂PO₄, 0.5 g of MgSO₄·7H₂O, 1 g of KNO₃, 2.94 g of Cl₂Ca (20 mM), and 1 ml of a trace element solution containing, per liter, 225 mg of ZnSO₄·7H₂O, 220 mg of FeSO₄·7H₂O, 40 mg of (NH₄)₆Mo₇O₂₄·4H₂O, 38 mg of CuSO₄·5H₂O, and 24 mg of MnSO₄. Flasks were inoculated with 1 ml of a spore suspension (1 × 10⁷ conidia ml⁻¹) from PDA plates and incubated at 20 to 25°C and 150 rpm for 30 days.

P. oxalicum also was produced in a solid fermentation system. The fungus was grown on a mixture of peat (Gebr. BRILL substrate GmbH & Co. KG, Germany) and vermiculite (Termita, Asfaltex, S.A., Barcelona, Spain) (1:1, wt/wt) as basal substrate

(PV). Fifty grams of PV with 20 ml of distilled water (≈40% moisture) were placed in a plastic bag (600 cm³) especially designed for solid fermentation (VALMIC), sealed, and sterilized by autoclaving at 1.0 kg cm⁻² and 120°C for 1 h, on each of three consecutive days. Bags then were inoculated with 1 ml of a conidial suspension of *P. oxalicum* (10⁵ conidia g⁻¹ of dry substrate) produced on PDA plates, sealed again, shaken vigorously to evenly distribute conidial inoculum, and incubated in darkness at 20 to 25°C for 30 days.

Estimation of conidial production was made at 5, 10, 20, and 30 days after inoculation in three replicates (flasks or bags) both in solid and liquid fermentation. Conidia were obtained from solid fermentation bags by taking 1-g samples of PV from each bag and suspending each in 10 ml of sterile water. Suspensions of conidia from liquid and solid fermentation then were vigorously shaken in a laboratory mixer (MS 2 Minishaker IKA-Works, Inc., Wilmington, NC) operated at full speed for 2 min, filtered through glass wool, washed in 100 ml of sterile distilled water, concentrated by centrifugation at 14,040 × g for 10 min, and resuspended in 5 ml of sterile distilled water. Appropriate dilutions were made to estimate conidia concentrations in a hemacytometer. The complete experiment was repeated twice.

Determination of optimal conditions for conidia production in solid fermentation. Four types of experiments were carried out to improve production and viability of *P. oxalicum* conidia in PV by solid fermentation.

The first experiment was carried out to see the effect of different sources of nutrient amendments (Table 1) on conidial production of *P. oxalicum* when they were added to PV in proportion peat/vermiculite/amendment, 1:1:0.5, wt/wt/wt, respectively. All the amendments were ground in a mixer (Osterizer, cyclotroll, Milwaukee, WI) and then passed through a 1-mm-pore-size sieve. PV alone (1:1:0; wt/wt/wt) was used in the controls. Fifty grams of the mixture (40% moisture) was placed in a plastic bag following a procedure similar to that described above. Three replicate bags of each solid-substrate were inoculated with 1 ml of a conidial suspension of *P. oxalicum* (10⁵ conidia g⁻¹ of dry substrate) in sterile distilled water. Bags were incubated in darkness at 20 to 25°C for 30 days and shaken periodically. After selection of the best amendments, the following combinations were tested: barley meal plus lentil meal, barley meal plus orange peel meal, lentil meal plus orange peel meal, and barley meal plus lentil meal plus orange peel meal.

The effect of amendments on sporulation of *P. oxalicum* was determined as described above at 5, 10, 20, and 30 days after inoculation.

The second experiment was carried out to see the effect of barley meal or lentil meal on the viability of *P. oxalicum* conidia when amendments were added to PV in proportion peat/vermiculite/amendment, 1:1:0.5, wt/wt/wt, respectively. The experiment was carried out as described above except PV only was amended with barley meal or lentil meal. Germination of conidia produced at 5, 10, 20, and 30 days after inoculation was tested in sterile Czapek broth (Difco Laboratories) by the bioassay described in De Cal et al. (14) in three bags per substrate. Sterile glass slides were placed on glass 15-mm-diameter petri dishes lined with moist paper. A 15-µl droplet

TABLE 1. Nutrient contain of the amendments added to peat and vermiculite in proportion peat/vermiculite/amendment, 1:1:0.5, wt/wt/wt, respectively^z

Amendment	Nutrient containing		
	Proteins	Fats	Carbohydrates
Cereal grains			
Oat meal	17.3	7	66.2
Barley meal	12.5	2.3	73.5
Rice meal	13.4	20.9	49.7
Leguminous seeds			
Lentil meal	28.1	1	57.1
Chickpea meal	19.3	6	60.7
Vetch meal	26.51	1.7	56
Fruit peels			
Apple peel meal	Traces	3.05	15.29
Orange peel meal	1.5	0.2	25

^z Data are the mean values per 100 g.

TABLE 2. Conidial production of *Penicillium oxalicum* in liquid fermentation (conidia × 10⁶ ml⁻¹) or in solid fermentation (conidia × 10⁶ g⁻¹)^y

Fermentation method	Days after inoculation			
	5	10	20	30
Liquid	0.027 (4.14) a	0.034 (4.49) a	0.083 (4.89) a	0.068 (4.89) a
Solid	2.12 (6.23) b	3.25 (6.09) b	5.70 (6.71) b	16.97 (7.20) b
MS _{within} ^z	0.26	0.31	0.045	0.018

^y Data are the means of three samples. Data in parentheses were subjected to log₁₀ transformation. Means followed by the same letter in each column are not significantly different (*P* = 0.05) by Student-Newman-Keul's range test.

^z MS_{within} = error mean square.

of conidia suspension of *P. oxalicum* (1×10^6 conidia ml⁻¹) from each substrate was mixed on a slide with a 30-μl droplet of sterile Czapek broth and incubated for 16 h at 20 to 25°C in darkness. The percentage of germination of 50 conidia was counted in each replicate. Three replicates were made for each sample. A spore was considered germinated when a germ tube was longer than the length of the spore.

The third experiment was carried out (as in the first two experiments) to see if the amount and viability of *P. oxalicum* conidia produced in solid fermentation were improved in proportions of peat/vermiculite/lentil meal of 1:1:1, wt/wt/wt; 1:0.5:0.5, wt/wt/wt; and 1:1:0.5, wt/wt/wt. The proportion of peat/vermiculite/meal of 1:1:0.5, wt/wt/wt, was used as control.

The fourth experiment was carried out (as in experiments 1 and 2) to see the effect of different percentages of initial moisture of substrates (10, 20, 30, and 40%) on production and viability of *P. oxalicum* conidia. The moisture of bags was measured after autoclaving before inoculating the bags as (wet weight – dry weight)/wet weight. All the experiments were repeated twice.

Shelf life of conidia of *P. oxalicum* produced in solid-substrate fermentation. To monitor shelf life of conidia, plastic fermentation bags containing 50 g of peat/vermiculite/lentil meal (1:1:0.5, wt/wt/wt) with 40% moisture were inoculated with *P. oxalicum* as described above and incubated for 5 days in the darkness at 20 to 25°C. Then, bags were stored at –80, –20, 4, or 25°C or at room temperature (ambient) (three replicates for each temperature). After 30, 60, 90, 120, 150, and 180 days, 1-g samples were taken from each replicate bag, suspended in sterile distilled water, and conidial production and viability were estimated as described in experiments 1 and 2. Moisture of samples also was determined as described in experiment 4. The complete experiment was repeated twice.

Biocontrol efficacy of conidia obtained in solid fermentation. Two types of experiments were carried out in growth chamber conditions to study the biocontrol efficacy of conidia obtained in solid fermentation.

The first experiment was carried out to see the biocontrol efficacy of conidia produced in fermentation bags containing vermiculite/peat/lentil meal (1:1:0.5, wt/wt/wt, with 40% moisture) that were incubated for 5 days at 20 to 25°C in the darkness. Tomato seedlings with two true leaves were transplanted from seedbeds into 100-ml flasks containing 125 ml of sterile Hoagland's solution, allowing the roots to be in contact with the solution as described by De Cal et al. (17). Substrate adhering to the roots was removed gently before placing the plants in the flasks. A plastic screen attached to the flask

TABLE 3. Conidial production (conidia $\times 10^8$ g⁻¹ of substrate) of *Penicillium oxalicum* in solid fermentation in plastic bags containing vermiculite/peat/amendments (1:1:0.5, wt/wt/wt)^y

Amendment	Days after inoculation			
	5	10	20	30
None	0.02 a	0.08 a	0.11 a	0.06 a
Cereal grains				
Oat meal	5.82 bc	2.87 c	3.52 d	2.79 de
Barley meal	2.05 bc	1.42 c	2.15 cd	1.65 cde
Rice meal	0.95 bc	1.11 c	0.98 bcd	1.31 cd
Leguminous seeds				
Lentil meal	4.79 c	3.44 c	1.15 bcd	3.48 e
Chickpea meal	7.89 c	0.95 bc	2.87 cd	3.12 e
Vetch meal	7.67 c	2.15 c	1.67 bcd	2.6 de
Fruit peels				
Apple peel meal	0.42 b	0.23 b	0.44 b	0.31 b
Orange peel meal	0.47 b	0.29 b	0.65 bc	1.03 c
MS _{within} ^z	0.76	0.36	0.41	0.19

^y Data are the mean of three samples. Data of conidial production were subjected to log₁₀ transformation before analysis. Means followed by the same letter in each column are not significantly different ($P = 0.05$) by Student-Newman-Keul's range test.

^z MS_{within} = error mean square.

neck held seedlings. Four seedlings were placed in each flask. Inoculation with *F. oxysporum* f. sp. *lycopersici* was made by adding to the flasks a microconidial suspension of the pathogen in sterile distilled water just before transplanting, giving a final concentration on the flasks of 1×10^4 microconidia ml⁻¹. Seedlings in seedbeds were watered 7 days before transplanting with a conidial suspension of *P. oxalicum* (10^6 conidia g⁻¹ substrate), obtained from fermentation bags after 5 days of incubation such as described above (vermiculite/peat/lentil meal, 1:1:0.5, wt/wt/wt, with 40% moisture). Control treatments were (i) flasks not inoculated with *F. oxysporum* f. sp. *lycopersici* and plants treated with *P. oxalicum*, (ii) flasks inoculated with *F. oxysporum* f. sp. *lycopersici* and plants not treated with *P. oxalicum*, and (iii) 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 ≈ 3 weeks under the conditions described above.

When the initial nutrient solution was consumed in any flask, fresh nutrient solution was added to all flasks to give a final volume of 100 ml and the consumption of the nutrient 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. (15) 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; 5 (100%) = dead plant. The area under the disease progress curve (AUDPC) was also calculated as described in Campbell and Madden (7). The experiment was carried out twice.

The second experiment was carried out to see the biocontrol efficacy of conidia obtained in fermentation bags containing vermiculite/peat/lentil meal (1:1:0.5, wt/wt/wt, with 40% moisture)

TABLE 4. Conidial production (conidia $\times 10^8$ g⁻¹ of substrate) of *Penicillium oxalicum* in solid fermentation in plastic bags containing vermiculite/peat/amendments (1:1:0.5, wt/wt/wt)^y

Amendment	Days after inoculation			
	5	10	20	30
None	0.02 a	0.03 a	0.06 a	0.17 a
Barley meal	5.36 cd	4.42 b	4.46 b	4.57 b
Lentil meal	6.98 d	7.76 b	4.74 b	7.00 b
Barley + lentil meal	4.24 cd	2.45 b	1.62 b	2.16 b
Barley + orange peel meal	0.82 b	3.80 b	2.87 b	3.40 b
Lentil + orange peel meal	1.62 bc	2.82 b	1.89 b	2.58 b
Barley + lentil + orange peel meal	4.22 cd	5.70 b	6.47 b	4.01 b
MS _{within} ^z	0.27	0.38	0.61	0.44

^y Data are the mean of three samples. Data of the conidial production were subjected to (log₁₀ + 1) transformation before analysis. Means followed by the same letter in each column are not significantly different ($P = 0.05$) by Student-Newman-Keul's range test.

^z MS_{within} = error mean square.

TABLE 5. Viability (%) of *Penicillium oxalicum* conidia produced in solid fermentation in plastic bag containing vermiculite/peat/barley meal or lentil meal (1:1:0.5, wt/wt/wt)^y

Amendment	Days after inoculation			
	5	10	20	30
Lentil meal	90.11 a	90.89 a	83.11 a	82.4 a
Barley meal	84.56 a	81.32 b	51.5 b	53.5 b
MS _{within} ^z	71.41	53.99	29.34	171.76

^y Data are the means of three samples. Means followed by the same letter in each column are not significantly different ($P = 0.05$) by Student-Newman-Keul's range test.

^z MS_{within} = error mean square.

after being stored at -80, -20, 4, or 25°C or at room temperature for 180 days. The experiment was similar to the first experiment, but treatments of tomato plants in this case were made with conidia from bags stored at different temperatures. The experiment was carried out twice.

Data analysis. Data were analyzed by analysis of variance. Prior to analysis, data were subjected to different transformations ($\log_{10}$) or ($\log_{10} + 1$) in the case of conidia production, ($\log_{10}$) in the case of AUDPC, and (arcsin) in the case of percentage germination and solution consumption to improve homogeneity of variances. When an *F* test was significant at *P* = 0.05, means were compared by Student-Newman-Keul's multiple range test (49). All the experiments were repeated twice. The repeat confirmed the results obtained in the first one; thus, only results from the first experiment will be reported and discussed here.

RESULTS

P. oxalicum produced significantly (*P* ≤ 0.02) more conidia in solid fermentation than in liquid fermentation during the experiment (Table 2). Conidial production was 250-fold higher in solid fermentation than in liquid fermentation at 30 days after inoculation.

Addition of amendments to PV significantly increased (*P* ≤ 0.01) conidial production of *P. oxalicum* in solid fermentation at every time assayed (Table 3). The best production was obtained on PV plus meal of cereal grains or leguminous seeds (100-fold higher), followed by PV plus apple or orange peel meals (10-fold higher). Conidial production obtained at 5 days after inoculation was similar or even higher than that recorded at the end of assay in PV plus meal of cereal grains or leguminous seeds.

Barley meal (as cereal grain) or lentil meal (as leguminous seed) were selected as the best representative amendments. Combinations of them or of orange peel meal did not enhance the conidial production in solid fermentation (*P* ≤ 0.04) (Table 4).

The conidial viability of *P. oxalicum* produced in PV plus lentil meal was higher than that of conidia produced in PV plus barley meal at every time period assayed (Table 5) (35% higher at 30 days after inoculation).

Changes in proportions of peat and vermiculite in the substrate did not enhance production and viability of conidia of *P. oxalicum* in solid fermentation (Table 6). However, when lentil meal was added to PV in a weight proportion higher than 0.5 (wt), a reduction of viability of conidia was observed at every time period assayed, and production of conidia was reduced after 30 days.

Initial moistures in the substrate of 30 and 40% were optimal for production and viability of conidia. At lower moistures, significant reductions in production and percent viable conidia were observed, particularly at 10% (Table 7).

At each date of sampling, there were not significant differences in the amount of conidia present in substrates stored at any temperature (Fig. 1), but there was a general decline in the number of conidia in bags with time of storage after 60 days. Conidial viability at -80°C already decreased after 2 days of storage. Conidial viability tended to be least affected by storage at -20°C, though treatment means fluctuated widely. The moisture on the storage substrate was maintained at ≈40% in bags stored at -80 and -20°C; whereas, at 25°C or at room temperature, moisture declined at 30 days, and at 4°C it decreased after 60 days.

Seedlings infected with *F. oxysporum* f. sp. *lycopersici* showed similar symptoms of Fusarium wilt described in De Cal et al. (17). Infected tomato plants watered with a conidial suspension of *P. oxalicum* produced in solid fermentation (fermentation bags containing vermiculite/peat/lentil meal, 1:1:0.5, wt/wt/wt, with 40% initial moisture) 7 days before transplanting resulted in a 49% reduction in AUDPC in the first experiment (Table 8). In addition, *P. oxalicum* treatment increased the solution consumption in infected plants. Uninfected plants treated with *P. oxalicum* did not show disease symptoms.

TABLE 6. Effects of different proportions of peat, vermiculite, and lentil meal in the substrate on production and viability of *Penicillium oxalicum* conidia in solid fermentation^w

Proportion ^x	Days of incubation ^y							
	5		10		20		30	
	Conidia	Via (%)	Conidia	Via (%)	Conidia	Via (%)	Conidia	Via (%)
1:1:0.5 (wt/wt/wt)	4.29 a	94.4 a	7.99 (8.85) a	96.4 a	2.99 a	87.1 a	6.37 (8.78) a	73.3 a
1:1:1 (wt/wt/wt)	3.20 a	60.7 b	4.21 (8.61) a	76.9 b	11.1 a	34.2 b	2.86 (8.44) b	25.8 b
1:0.5:0.5 (wt/wt/wt)	10.74 b	97.3 a	12.99 (9.03) a	94.9 a	8.7 a	88.9 a	15.95 (9.13) a	70.9 a
1:1:0.5 (vol/vol/vol)	6.99 ab	98.4 a	6.32 (8.79) a	92.4 a	11.16 a	87.8 a	12.06 (9.07) a	60.0 a
MS _{within} ^z	4.24 × 10 ¹⁶	19.1	(0.05)	21.0	2.68 × 10 ⁷	23.2	(0.03)	83.1

^w Data are the means of three samples. Data in parentheses are conidial production subjected to $\log_{10}$ transformation before analysis. Means followed by the same letter in each column are not significantly different (*P* = 0.05) by Student-Newman-Keul's range test.

^x Proportions of peat/vermiculite/lentil meal.

^y Conidia × 10⁸ g⁻¹; Via = viability.

^z MS_{within} = error mean square.

TABLE 7. Effect of initial moisture in the substrate on production and viability of *Penicillium oxalicum* conidia in solid fermentation^x

Moisture (%)	Days of incubation ^y							
	5		10		20		30	
	Conidia	Via (%)	Conidia	Via (%)	Conidia	Via (%)	Conidia	Via (%)
10	0.04 (6.37) a	47.7 a	0.037 (5.88) a	52.2 (32.08) a	0.048 (6.22) a	21.8 a	0.011 (5.86) a	9.4 a
20	0.27 (6.58) a	96.7 b	0.052 (6.32) a	88.9 (65.35) b	0.192 (6.72) a	83.1 b	0.128 (6.92) b	77.9 b
30	1.20 (7.87) ab	97.5 b	0.797 (7.85) b	87.9 (61.68) b	3.03 (8.40) b	86.4 b	1.56 (8.19) c	78.0 b
40	3.32 (8.5) b	98.4 b	11.68 (9.05) b	95.1 (72.25) b	10.34 (8.93) b	88.4 b	6.67 (8.73) c	78.2 b
MS _{within} ^z	0.48	5.4	(0.48)	(103.12)	(0.41)	96.2	(0.15)	232.2

^x Data are the means of three samples. Data in parentheses are conidial production or viability subjected to $\log_{10}$ and arcsin transformation, respectively, before analysis. Means followed by the same letter in each column are not significantly different (*P* = 0.05) by Student-Newman-Keul's range test.

^y Conidia × 10⁸ g⁻¹; Via = viability.

^z MS_{within} = error mean square.

Fig. 1. A, Amount, B, viability, and C, moisture of substrate of conidia of *Penicillium oxalicum* obtained after solid fermentation in peat, vermiculite, and lentil meal (1:1:0.5, wt/wt/wt) after 5 days of incubation at 20 to 25°C and storage for up to 180 days at -80 (●), -20 (+), 4 (▲), and 25°C (■), and at room temperature (×). Each point is the mean of three replicates. MS_{within} is error mean square.

In the second experiment, only treatment with conidia stored at -20°C increased solution consumption (Table 9). Treatment with conidia stored at room temperature also reduced AUDPC.

DISCUSSION

P. oxalicum produced the highest number of viable conidia (>10⁸ conidia g⁻¹ dry weight of substrate, >80% viability) when it was grown in fermentation bags containing a sterilized mixture of peat/vermiculite/lentil meal (1:1:0.5, wt/wt/wt) with 30 to 40% initial moisture for 5 days. Although *P. oxalicum* produced conidia on liquid fermentation, volumetric yield of *P. oxalicum* conidia is 250-fold higher in solid fermentation than in liquid fermentation at 30 days after inoculation. Previous studies by Pascual et al. (43) showed that the maximum level of conidiation obtained in submerged cultures of *P. oxalicum* was 35 × 10⁶ conidia ml⁻¹. Other biological control agents sporulate poorly in liquid fermentation systems (32). Causes accounting for this are growth conditions more like natural habitat (8), aeration more easily obtained, and a better access to nutrients (1) in solid-state fermentation than in liquid cultures. Although inoculum produced by liquid fermentation generally is preferred by industry because of its high viability, long shelf life, and easy application (27,29), some solid fermentation systems have been developed that are economically competitive with the corresponding submerged culture production (35). Solid fermentation offers several advantages over conventional submerged culture: lower capital and operating costs, simpler

TABLE 8. Effect of *Penicillium oxalicum* on disease development caused by *Fusarium oxysporum* f. sp. *lycopersici* on tomato cv. Precodur plants grown in a growth chamber at 21 days after inoculation^w

Treatment	<i>F. oxysporum</i> f. sp. <i>lycopersici</i>	AUDPC ^x	Consumption ^y
<i>P. oxalicum</i>	1	365.9 b	1.78 b
<i>P. oxalicum</i>	0	0 a	3.74 d
Nontreated	1	717.2 c	1.23 a
Nontreated	0	0 a	3.07 c
MS _{within} ^z	...	9,678.84	0.20

^w *P. oxalicum* was applied 7 days before transplanting. Data are the mean of five replicates, with four plants per replicate. Data were analyzed using analysis of variance. Means were compared by Student-Newman-Keul's. Means within each recorded parameter with no common letter differed significantly at *P* = 0.05.

^x AUDPC = area under the disease progress curve.

^y Solution consumption, milliliters per plant per day.

^z MS_{within} = error mean square.

TABLE 9. Effect of conidia of *Penicillium oxalicum* obtained from substrate stored at -80, -20, 4, and 25°C and at room temperature (ambient) for 180 days on disease development caused by *Fusarium oxysporum* f. sp. *lycopersici* on tomato cv. Precodur plants grown in a growth chamber at 21 days after inoculation^y

<i>P. oxalicum</i> treatment storages	AUDPC	Solution consumption (ml/plant/day)
Ambient	130 (2.07) a	4.37 (54.775) ab
20 to 25°C	281.56 (2.42) b	3.76 (43.58) ab
4°C	344.49 (2.45) b	3.81 (48.96) ab
-20°C	110.31 (2.03) a	5.30 (71.08) a
-80°C	322.82 (2.49) b	3.11 (34.94) b
Untreated	284.18 (2.44) b	3.59 (41.16) b
MS _{within} ^z	(0.03)	(225.37)

^y *P. oxalicum* was applied 7 days before transplanting. Data are the mean of five replicates, with four plants per replicate. Data of area under the disease progress curve (AUDPC) and solution consumption in brackets were subjected to log₁₀ and arcsin transformation before analysis, respectively. Data was analyzed using analysis of variance. Means were compared by Student-Newman-Keul's. Means within each recorded parameter with no common letter differed significantly at *P* = 0.05.

^z MS_{within} = error mean square.

equipment and media, and easier downstream processing (39). These advantages increase if the final product can include the production substrate.

The optimal growing environment in solid fermentation includes many variables such as temperature, moisture content, substrate (inert support and nutrients), and length of time for fermentation (45). Vented, autoclavable, plastic bags are convenient containers to conduct solid-state fermentation (3). The solid substrate acts as a heterogeneous source of carbon, nitrogen, and minerals as well as growth factors, and also has a capacity to absorb water (37). Maximum levels of *P. oxalicum* conidia ($>10^8$ conidia g^{-1} dry weight of substrate) were obtained on peat/vermiculite/cereal grain meal or leguminous seed meal (100-fold higher than in PV without amendments), but viability of conidia was better when produced in leguminous seed meal (at a proportion of 0.5 wt) (Table 6). Protein content is higher in lentil seed than in barley grain, whereas the opposite is true in the case of carbohydrates (Table 1). This also can be due to the effect of substrates on solid fermentation conditions (10). Heat from fermentation and reduced O_2 supply can be restricted in solid fermentation process (31).

The control of temperature and moisture has been reported to be the key factor in solid fermentation systems (47). *P. oxalicum* is a cosmopolitan fungus that is adapted to dry and warm conditions (44). The sporulation range temperature was between 15 and 35°C, with temperatures below 10°C inhibiting sporulation (44). In solid fermentation systems, the process of microbial growth can cause a rise in substrate temperature (23). *P. oxalicum* solid fermentation was started at 20 to 25°C. Although there may be an increase in the temperature later, as a result of the fermentation processes, the thermotolerance of the fungus would allow it to grow and survive without any problem through 5 days of incubation or for longer periods. Substrate moisture is an important factor for microorganism growth in solid fermentation (25). Water is necessary to cause swelling of the substrate and facilitates utilization of the medium by the organisms (45). However, water excesses cause substrates to stick together, limiting oxygen transfer and increasing the risk of bacterial contamination. In very low moisture levels, no growth of the organism will occur (40). Production of conidia of *P. oxalicum* was greater with 30 to 40% initial moisture than at lower values. Viability of conidia was low at 10% initial moisture. Temperature, aeration rate, and water content were important parameters on the sporulation of another species of *Penicillium* on solid fermentation systems, as with *P. roqueforti*, where the optimum conditions were 23 to 42°C, 1,263 liters h^{-1} , and 0.481 g^{-1} dry matter (30).

Optimal production of *P. oxalicum* conidia in substrates with lentil meal was obtained at 5 days after inoculation at 25°C. Solid-state fermentation held longer did not generally increase conidial numbers nor viability. Considerable cost savings in producing inoculum could be obtained by harvesting inoculum after only 5 days. Nevertheless, harvesting of conidia could be done over a wide period of time because the amount of conidia obtained at 25°C was maintained in the range of 10^9 conidia g^{-1} from 5 to 60 days, and survival of these conidia was higher than 70% until 150 days of incubation. This fact facilitates the scale-up of the process. Large scale-up production could easily be obtained by increasing the volume and the number of bags, and incubating them in a incubator at 20 to 25°C in the dark. This process requires low-cost equipment and, consequently, low total capital investments. Another factor to be considered is the cost of the substrate (8,9). Maximum levels of *P. oxalicum* conidia were obtained on peat/vermiculite/lentil meal, all of them with low cost. There are abundant agroproducts such as wheat bran (46), wheat bran-peat mixture (48), rice bran, and a range of grains (34,52) that have received a great deal of attention (5,33).

Conidia of *P. oxalicum* in solid fermentation had a long shelf life (>180 days) if stored at -20°C . Reduction on shelf life by

effects of high temperatures have been noted before for a range of inocula of *Trichoderma* spp. and *Gliocladium virens* (24), *Trichoderma harzianum*, *Gliocladium roseum* (50), and *Coniothyrium minitans* (33). Freeze drying, atomization, and bed-fluid dry methods may be able to preserve the inoculum for a long time with high viability (4). Reduction of viability of *P. oxalicum* conidia stored at -80°C could be due to the absence of a protectant such as glycerol in the stored mixture.

Fresh conidia produced in solid fermentation and conidia stored at -20°C for 180 days reduced Fusarium disease development in a manner similar to conidia produced in PDA plates (12,13,15,16). It is necessary to compare the efficacy of conidia from solid fermentation with that of inoculum produced on agar plates, because the efficacy of the biocontrol agent can be affected by the environmental conditions during its production. Pascual et al. (41) observed that conidia of *P. oxalicum* from agar plates were more effective than submerged ones against Fusarium wilt of tomato. In other fungus, such as *Talaromyces flavus* (anamorph *Penicillium dangeardii*), substrates that produced the most propagules were not the same ones that produced the most efficacious propagules (19).

P. oxalicum is a promising biological control agent for Fusarium wilt and other tomato diseases (12,13,15,16). The fungus also shows biocontrol against other crop diseases (21,26,51). Commercial application of this biocontrol agent depends on production of large quantities of conidia, which requires reliable mass-production systems. A solid-state fermentation process for conidia of *P. oxalicum* has now been described. Research is in progress to improve the shelf life of *P. oxalicum* conidia to stabilize the performance of a formulated product containing *P. oxalicum*.

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