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Biocontrol of tomato wilt by *Penicillium oxalicum* formulations in different crop conditions

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Abstract

Eight formulations of *Penicillium oxalicum* (FOR1 to FOR8) were obtained by the addition of various ingredients, in two separate steps of the production and drying of *P. oxalicum* conidia. These formulations were then evaluated against tomato wilt in three glasshouse (G1 to G3) and two field (F1 and F2) experiments. All formulations were applied to seedlings in seedbeds 7 days before transplanting at a rate of 10^7 spores g^{-1} seedbed substrate. The conidial viability of each formulation was estimated by measuring germination just after fluid bed-drying, before seedbed application and after 1 and 2 years of storage at 4 °C under vacuum. The densities of *P. oxalicum* were estimated in the seedbed substrate and in the rhizosphere of three plants per treatment just before transplanting. Initial conidial viability of formulations just after fluid bed-drying was approx. 80%, except for FOR1, FOR4, and FOR7 which were 60%. The initial viability was maintained up to 40–50% for 2 years of storage at 4 °C under vacuum, except for FOR1. All formulations had $\geqslant 50\%$ viability at application time. Populations of *P. oxalicum* in the seedbed substrate just before transplanting were $>10^6$ cfu g^{-1} soil in G3 and F2; populations in rhizosphere were also $>10^6$ cfu g^{-1} fresh root, except for FOR3, FOR5, and FOR6 in G2. A range of 22–64% of disease reduction was observed with all formulations, although these reductions were not significant ($p = 0.05$) for FOR1, FOR4, and FOR5 in any experiment. Contrast analysis showed significant differences between biological treatments and untreated control ($p = 0.05$) in all experiments, but no significant differences between biological and chemical treatments. Initial conidial viability of *P. oxalicum* in formulations and populations of *P. oxalicum* in the seedbed substrate explained 78.26% of the variability in *P. oxalicum* populations in tomato rhizosphere before transplanting. Disease incidence in untreated plants was negatively correlated ($r = -0.54$) with the percentage of disease control. The relationship between the viability of formulations, the populations of *P. oxalicum* in seedbed and rhizosphere, and the control of tomato wilt is discussed.

Keywords: Biocontrol; Biological agent; *Fusarium oxysporum* f. sp. *lycopersici*; *Verticillium dahliae*; Biofungicide; Viability

1. Introduction

Penicillium oxalicum Thom. reduced vascular wilts caused by *Verticillium dahliae* Kleb and *Fusarium oxysporum* f. sp. *lycopersici* (Sacc.) Snyder & H.N. Hansen under glasshouse and field conditions (Larena et al., 2003a). Biocontrol of tomato wilt is based on application of *P. oxalicum* conidia (Pascual et al., 2000), which must contact tomato roots (De Cal et al., 1997a, 2000). A range of 10^6–10^7 conidia g^{-1} in seedbed substrate and rhizosphere before transplanting is required for effective control of tomato wilts (Larena et al., 2003a). Induction of resistance in tomato plants was demonstrated as the main mode of action of *P. oxalicum* against *F. oxysporum* f. sp. *lycopersici* (De Cal et al., 1997a).

A method for mass production of conidia has been developed and these conidial applications have reduced tomato wilt (Larena et al., 2002). Unfortunately, the viability of these conidia declined after 30 days at room temperature (Larena et al., 2002). Adequate shelf-life of a biological agent formulation

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requires stability for at least 1 year (Rodham et al., 1999). Conidia of *P. oxalicum* can be prepared by fluid bed-drying, with 100% viability at the end of the process and 40–50% viability after 180 days of storage at room temperature (Larena et al., 2003b). *P. oxalicum* conidia prepared by fluid bed-drying reduced the incidence of tomato wilt under glasshouse and field conditions (Larena et al., 2003b). However, these conidia are highly hydrophobic and thus difficult to dissolve in water.

Non-dusty, highly miscible, wettable powder *P. oxalicum* conidia were developed to obtain formulations that could be easily applied and efficiently distributed to reduce tomato wilt (Sabuquillo et al., 2005). These formulations reduced tomato wilt caused by *Fusarium* spp. under greenhouse and, in a preliminary trial, wilt caused by *Verticillium* spp. in a field assay (Sabuquillo et al., 2005). Improvements in formulation may further enhance and stabilize the performance of *P. oxalicum*. Commercial application depends upon the development of treatments that perform consistently under glasshouse and field conditions. Our aim was to develop suitable formulations of *P. oxalicum* that would improve conidial stability and increase contact with tomato roots, and also to evaluate these formulations in conditions of commercial usage.

2. Materials and methods

2.1. Cultures

Penicillium oxalicum Currie & Thom (ATCC No. 201888) was stored on potato dextrose agar (PDA) (Difco, Detroit, MI, USA) slants at 4 °C and grown on PDA in Petri plates in the dark at 20–25 °C for 7 days for conidial inoculum production. Fresh conidia of *P. oxalicum* were produced on peat/vermiculite/lentil meal (1/1/0.5; wt/wt/wt) in plastic bags especially designed for solid-state fermentation (VALMIC^{R}, Sacherei de Pont-Audemer S.A., Pont-Audemer, France) (Larena et al., 2002). Fifty grams of mixture, enclosed in plastic bags of 600 cm^3, was inoculated with 20 ml of a conidial suspension of *P. oxalicum* in distilled water (10^5 conidia g^{-1} dry substrate). The bags were resealed and incubated in darkness at 20–25 °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 counted with a haemocytometer. Conidial suspensions were then passed through a 1 μm filter paper using a Büchner funnel. Conidial paste was dried by a fluid bed-dryer 350 s (Burkard Manufacturing Co. Ltd., Hertfordshire, UK) as previously described (Larena et al., 2003b) until conidia moisture content was lower than 10%. Conidial moisture content was measured using a humidity analyzer (BOECKEL, GmbH +Co, Hamburg, Germany).

An isolate of *F. oxysporum* f. sp. *lycopersici* race 2 (ATCC No. 201829) obtained from a tomato plant in southern Spain was stored at 4 °C in tubes containing sterile sand (Delcán et al., 2002). The fungus was grown on Czapek Dox Agar (CDA) (Difco; Detroit, MI, USA) in the dark at 25 °C for mycelial production. The aggressiveness of *F. oxysporum* f. sp. *lycopersici* was previously tested and recorded as type three according to Pineau's scale (75% of diseased plants after 30 days of inoculation) (Pineau, 1976). Microconidia of *F. oxysporum* f. sp. *lycopersici* were produced in Czapek-Dox broth (Difco; Detroit, MI, USA) in 5 days at 25 °C on a rotary shaker (Lab-Line Instruments, Inc., model 3527, Melrose Park, Illinois, USA) at 150 rpm (De Cal et al., 1995), and the culture was filtered through glass wool. Chlamydospores of *F. oxysporum* f. sp. *lycopersici* were produced in bags ($80 \times 40 \times 20$ cm^3) containing sterile peat. Bags were inoculated with a microconidial suspension in Czapek-Dox broth to a final density of 10^5 microconidia g^{-1} peat, and were left in a glasshouse without artificial light for 30 days at 20 °C (night) to 30 °C (day). At this time almost all the microconidia became chlamydospores (De Cal et al., 1997b). The population of *F. oxysporum* f. sp. *lycopersici* in the peat was estimated as number of colony forming units (CFU) per gram of dry peat, as previously described (De Cal et al., 1995, 1997b, 1999a; Larena et al., 2003a).

2.2. Plant material

Tomato cultivars San Pedro, Muchamiel, and Valencia1, all of them susceptible to races 1 and 2 of *F. oxysporum* f. sp. *lycopersici* and to *V. dahliae*, were used in glasshouse and field experiments.

Tomato seeds for glasshouse experiments G1 and G2 were sown in trays with 198 individual cells ($3 \times 3 \times 5$ cm^3 per cell) containing soil. Trays were maintained on benches in a glasshouse without artificial light at 20 °C (night) to 30 °C (day) for 5–6 weeks before transplanting.

Tomato seeds for glasshouse experiment G3 were sown in trays ($27 \times 42 \times 7$ cm^3) containing an autoclaved mixture of vermiculite and peat (1:1; v: v). Trays were maintained for 3 weeks in a growth chamber at 22 °C (8 h dark) and at 28 °C (16 h with fluorescent light, 100 μE/m^2 s) and 80–100% humidity, and then placed on benches in a glasshouse without artificial light for 7 days at 20 °C (night) to 30 °C (day) before seedlings were transplanted.

Tomato seeds for field experiments F1 and F2 were sown in trays with 40 individual cells ($4 \times 4 \times 6$ cm^3 per cell) containing an autoclaved mixture of vermiculite and peat (1:1; v:v). Trays were managed as for experi-