

RESEARCH PAPER

## Effectiveness of biological control of *Phytophthora capsici* in pepper by *Trichoderma asperellum* strain T34

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**Summary.** Pepper (*Capsicum annuum* L.), one of the most widely grown vegetables worldwide, is susceptible to root rot caused by *Phytophthora capsici*. Many biocides have recently been banned in Europe because of human health and environmental concerns. Integrated pest management is a European priority, where biological control together with other agronomic practices should replace pesticide management of plant diseases in the future. Application of different concentrations of the fungus *Trichoderma asperellum* strain T34 (the in T34 Biocontrol<sup>®</sup>) on incidence of disease caused by *P. capsici* in pepper was studied. Different methods of application of the microbial control agent and inoculation of the pathogen were examined. T34 and etridiazole (Terrazole<sup>®</sup>) were compared for their ability to suppress *P. capsici*. T34 reduced disease in most of the assayed situations (up to 71% disease reduction), while etridiazole was effective only when applied at the same time as the pathogen. The results obtained are discussed on the basis of the different modes of action of T34 and etridiazole. T34 is a useful biological alternative to chemicals for the control of *P. capsici* in pepper.

**Key words:** *Capsicum annuum*, etridiazole, zoospores, root rot.

### Introduction

The oomycete *Phytophthora capsici* occurs worldwide and causes root and crown rot as well as aerial blight on leaves, fruit, and stems of bell pepper, tomato and cucurbits (Larkin *et al.*, 1995). This pathogen produces different types of propagules that are involved in infection and dispersal. Zoospores are short-lived propagules that survive for short periods, generally from days to weeks. In contrast, sporangia and hyphae (vegetative stages of the pathogen) survive in soil for between 4 to 8 weeks. Oospores are the primary overwintering propagules and persist for longer (Larkin *et al.*, 1995). Pepper (*Capsicum annuum* L.), one of the most widely grown vegetables, is

susceptible to root rot caused by *P. capsici*, and this disease can cause substantial crop losses (Sang *et al.*, 2008). Since pepper varieties with only intermediate resistance are known, the disease was controlled by soil fumigation with methyl bromide up to 2005, when this substance was banned (Ezziyyani *et al.*, 2007). Several of the chemicals used to manage this pathogen usually fail as a result of the development of fungicide resistance (Silvar *et al.*, 2006) or variable efficacy against the diverse propagules of the pathogen (Stasz and Martin, 1988; Kuhajek *et al.*, 2003). Alternatives that ensure more sustainable disease management are required in response to increased environmental awareness and legal restrictions on chemical fungicides in Europe. One alternative is biological control, in which microorganisms are selected on the basis of their ability to control diseases. For instance, *Trichoderma harzianum* strain 2413 was shown to reduce pepper root rot caused by *P. capsici*,

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but this microbial control agent is not commercially available (Sid Ahmed *et al.*, 1999). The microbial control agent *Trichoderma asperellum* strain T34 (commercialised as T34 Biocontrol®, Biocontrol Technologies S.L., Barcelona, Spain), isolated from *Fusarium* suppressive compost, is used as a biocontrol for *Fusarium oxysporum* f. sp. *dianthi* on carnations, based on results of laboratory and field trials (Sant *et al.*, 2010). It also significantly reduced disease caused by the soilborne fungus *F. oxysporum* f. sp. *lycopersici* in tomato plants when applied to roots (Cotxarrera *et al.*, 2002), and *Rhizoctonia solani* in cucumber (Trillas *et al.*, 2006). When applied to roots, *T. asperellum* strain T34 also induced plant resistance and reduced the proliferation of the foliar pathogen *Pseudomonas syringae* pv. *lachrymans* in cucumber (Segarra *et al.*, 2007), *Pseudomonas syringae* pv. *tomato*, the biotrophic oomycete *Hyaloperonospora parasitica* and the necrotrophic fungus *Plectosphaerella cucumerina* in *Arabidopsis thaliana* (Segarra *et al.*, 2009).

The efficacy of *Trichoderma asperellum* strain T34 (T34) for control of disease caused by *P. capsici* in pepper compared with chemical control was tested in the present study. T34 was applied at different concentrations and at different time points between application of the microbial control agent and the inoculation of the pathogen. T34 was compared with the fungicide etridiazole, a pesticide commonly used in commercial practice against *P. capsici*.

## Materials and methods

### Plant material

Seeds of green pepper (*Capsicum annuum* cv. Dulce Italiano) were sown in 20 mL multipot trays filled with neutralised peat. For a growth chamber experiment, 2-week-old seedlings were transferred to 400 mL pots filled with neutralised peat, with five pots per treatment. The experiment was conducted in a controlled environment chamber (EGC walk-in chamber). The photosynthetically active photon flux density in the growth chamber was 200  $\mu\text{mol m}^{-2} \text{s}^{-1}$  during the 16 h photoperiod and the temperature was maintained at 25°C.

For greenhouse experiments, 4- or 3-week-old plants (2009 and 2010, respectively) were transferred to 1 L pots. One plant was placed in each pot, and pots were filled with neutralised peat. Six pots per treatment were randomly distributed along a north/

south orientated table in a greenhouse located at the University of Barcelona. Each growth chamber and greenhouse experiment was performed twice in identical growing conditions.

### *Phytophthora capsici* inoculum preparation

Agar fragments colonised with *P. capsici* were stored in water stocks at 10°C. The pathogen recovered from these stocks was grown on medium in Petri plates, containing (per L) 200 mL of clarified tomato juice, 800 mL of distilled water, 3 g of  $\text{CaCO}_3$  and 20 g of agar. Plates were stored at 25°C in the dark for 14 d. They were then flooded with 20 mL of sterile 0.1%  $\text{KNO}_3$  solution and stored under fluorescent light (a minimum of 16 h light per d) for 7 d. The  $\text{KNO}_3$  solution was then replaced by sterile deionised water (SDW) and the plates were cold treated by leaving them for 2 h at 4°C, followed by 2 h at 25°C. This treatment induced zoospore release from the sporangia. The plates were each scraped with a glass rod. Water from the plates containing the zoospores was pooled and zoospores were counted with a haemocytometer. Approximately 600 zoospores per mL of peat were applied. The protocol for the production of zoospores was adapted from Chae *et al.* (2006).

### Treatments

In all cases, the first treatment was applied when plants were transplanted. The pathogen was applied 1 week later (growth chamber experiments), 1 month after transplanting (2009 greenhouse experiments) and at the time of transplanting (2010 greenhouse experiments). The second treatment was applied when the first symptoms of infection appeared.

Treatments consisted of *Trichoderma asperellum* strain T34 at three application rates:  $10^3$ ,  $10^4$  and  $10^5$  conidia  $\text{mL}^{-1}$  of peat. In the 2010 experiments, as a consequence of the experience with the previous experiments, a narrower range of T34 application rates was assayed ( $10^3$ ,  $3 \times 10^3$  and  $10^4$  conidia  $\text{mL}^{-1}$  of peat). T34 (Trillas and Cotxarrera, 2003) stored in silica gel at 4°C was grown in a liquid medium containing 10 g  $\text{L}^{-1}$  malt for 5 d at 25°C. The medium was agitated in a horizontal shaker at 150 rpm. The culture was filtered through a 50  $\mu\text{m}$  nylon mesh to remove the mycelium and centrifuged at  $10,000 \times g$  (4°C). The pellet was washed twice in SDW to obtain

medium-free conidia. Etridiazole (commercialised as Terrazole<sup>®</sup>, 48% a.i. Crompton-Uniroyal Chemical, Slough, United Kingdom) was used for comparison. The chemical treatment was applied at the recommended rate of 2 L ha<sup>-1</sup>.

### Inhibition of zoospore germination

Equal volumes of the pathogen and biological control agent treatments (at the same concentrations used in the 2010 experiments) were mixed. This mixture was kept at room temperature for 24 h and samples were observed under a light microscope to count the percentage of non-germinated zoospores. A minimum of 50 zoospores was counted per treatment.

### Evaluation of disease

In the growth chamber and the 2010 greenhouse studies, disease was evaluated on the following scale 21 d after pathogen infestation: 0, healthy plant; 1, less than half the leaves wilted; 2, more than half the leaves wilted; and 3, dead plant.

In the greenhouse experiments conducted in 2009, disease severity was scored 45 d after pathogen inoculation using a different symptom scale. In those experiments the plants were older when inoculated with the pathogen and consequently the symptoms and the development of the disease were different. The symptom scale used was as follows: 0, healthy plant; 1, small canker (less than 2.5 cm); 2, medium canker (2.5-5 cm); 3, large canker (more than 5 cm); and 4, dead plant.

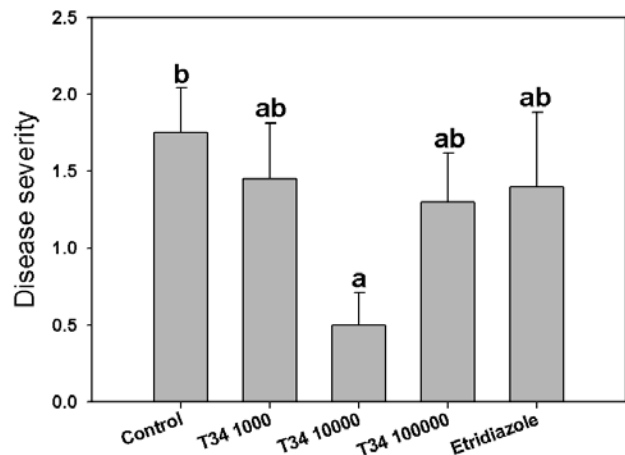
In addition, at the end of the 2009 and 2010 experiments, the dry weight of each plant was determined.

### Statistical analyses

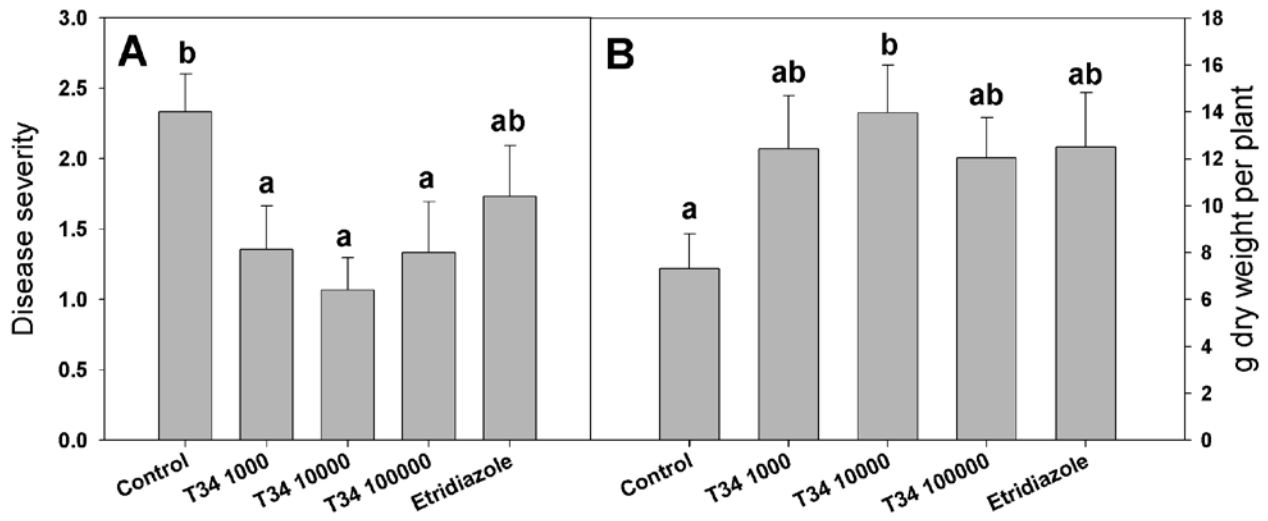
All experiments were performed twice, and data from the two repetitions were pooled as there was no statistically significant experiment × treatment interaction. All data were analysed with SPSS (version 16.0 from SPSS, Inc.). The variables disease severity, dry weight and percentage inhibition of zoospore germination were examined by analysis of variance, with the Duncan's multiple range test applied when the ANOVA was significant ( $P < 0.05$ ).

## Results

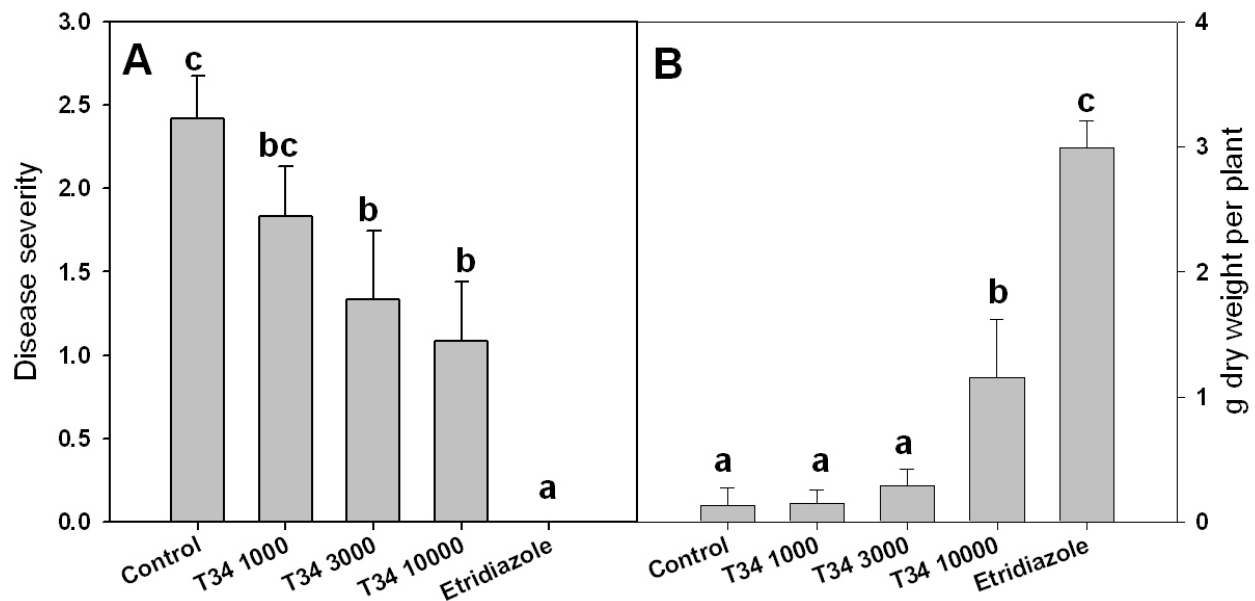
In the growth chamber experiment, disease severity was reduced by T34 when applied at 10<sup>4</sup> conidia mL<sup>-1</sup> of peat (Figure 1). At this concentration, this product achieved a 71% decrease of disease compared with the control. The other treatments (T34 at 10<sup>3</sup> and 10<sup>5</sup> conidia mL<sup>-1</sup>, and etridiazole) failed to control the disease. In the 2009 greenhouse experiment, the application of 10<sup>3</sup>, 10<sup>4</sup> or 10<sup>5</sup> conidia of T34 per mL of peat reduced disease severity compared to controls (Figure 2A), with a 42, 54 and 43% reduction in disease for the three concentrations, respectively. Treatment with etridiazole did not significantly affect disease severity. Although plants from all the treatments had greater dry weights than controls, only those treated with 10<sup>4</sup> conidia mL<sup>-1</sup> of T34 were significantly heavier (191%) than controls (Figure 2B). In the 2010 greenhouse experiment, the application of T34 at 3 × 10<sup>3</sup> and 10<sup>4</sup> conidia mL<sup>-1</sup> significantly reduced disease sever-



**Figure 1.** Disease severity produced by *Phytophthora capsici* in pepper plants on the last day of the growth chamber experiment (21 d after inoculation with the pathogen). Disease severity was scored using the following symptom scale: 0, healthy plant; 1, less than half the leaves wilted; 2, more than half the leaves wilted; and 3, dead plant. Control, water; T34 1,000, 10,000 and 100,000, *Trichoderma asperellum* strain T34 at 10<sup>3</sup>, 10<sup>4</sup> and 10<sup>5</sup> conidia mL<sup>-1</sup>; Etridiazole, Terrazole<sup>®</sup> at 2 L ha<sup>-1</sup>. All the plants were inoculated with *P. capsici*. Treatments were applied 7 d before the pathogen and a second treatment of biocontrol agent was applied upon detection of symptoms of disease. Different letters indicate significant differences within treatments, Duncan's multiple range test ( $P < 0.05$ ).

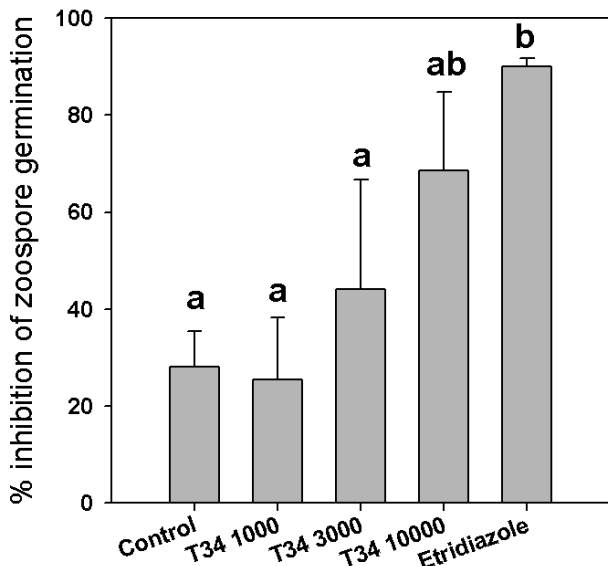


**Figure 2.** Disease severity produced by *Phytophthora capsici* in pepper plants (A) and dry weight of the pepper plants (B) on the last day of the 2009 greenhouse experiment (45 d after inoculation with the pathogen). Disease severity was scored using the following symptom scale: 0, healthy plant; 1, small canker (less than 2.5 cm); 2, medium canker (2.5–5 cm); 3, large canker (more than 5 cm); and 4, dead plants. Control, water; T34 1,000, 10,000 and 100,000, *Trichoderma asperellum* strain T34 at  $10^3$ ,  $10^4$  and  $10^5$  conidia  $\text{mL}^{-1}$ ; Etridiazole, Terrazole® at  $2 \text{ L ha}^{-1}$ . Treatments were applied 30 d before inoculation with the pathogen and a second treatment was applied upon detection of symptoms of disease. Different letters indicate significant differences within treatments, Duncan’s multiple range test ( $P < 0.05$ ).



**Figure 3.** Disease severity produced by *Phytophthora capsici* in pepper plants (A) and dry weight of the pepper plants (B) on the last day of the 2010 greenhouse experiment (21 d after inoculation with the pathogen). Disease severity was scored using the following symptom scale: 0, healthy plant; 1, less than half the leaves wilted; 2, more than half the leaves wilted; and 3, dead plant. Control, water; T34 1,000, 3,000 and 10,000, *Trichoderma asperellum* strain T34 at  $10^3$ ,  $3 \times 10^3$  and  $10^4$  conidia  $\text{mL}^{-1}$ ; Etridiazole, Terrazole® at  $2 \text{ L ha}^{-1}$ . Treatments were applied at the same time as the pathogen and a second treatment was applied on detection of symptoms of disease. Different letters indicate significant differences within treatments, Duncan’s multiple range test ( $P < 0.05$ ).

ity (45 and 55% decrease, respectively) compared with the control, as did etridiazole (Figure 3A). The chemical treatment achieved 100% suppression of disease. The application of T34 at  $10^3$  conidia  $\text{mL}^{-1}$  did not significantly reduce disease severity. Disease control was similar for T34 at  $3 \times 10^3$  and  $10^4$  conidia  $\text{mL}^{-1}$ . Plants treated with this product at  $10^4$  conidia  $\text{mL}^{-1}$  and with etridiazole had significantly greater dry weights than controls (8.5 and 22 times greater, respectively), plants treated with the chemical product had greater dry weights than the plants treated with T34 (Figure 3B). In addition, zoospores that were treated *in vitro* with etridiazole showed significant inhibition of germination after encystment compared with controls; almost 90% of the zoospores did not germinate after 24 h (Fig. 4). The germination of zoospores treated with T34 at  $10^3$ ,  $3 \times 10^3$  and  $10^4$  conidia  $\text{mL}^{-1}$  was not significantly different from controls after 24 h; however, the  $10^4$  conidia  $\text{mL}^{-1}$  treatment reduced germination by 69%, which was not statistically different to the results obtained with etridiazole (Figure 4).



**Figure 4.** Inhibition of zoospore germination (%) after 24 h of incubation of a mixture of equal volumes of *Phytophthora capsici* and treatments. Control, water; T34 1,000, 3,000 and 10,000, *Trichoderma asperellum* strain T34 at  $10^3$ ,  $3 \times 10^3$  and  $10^4$  conidia  $\text{mL}^{-1}$ ; Etridiazole, Terrazole® at  $2 \text{ L ha}^{-1}$ . Different letters indicate significant differences within treatments, Duncan's multiple range test ( $P < 0.05$ ).

## Discussion

Evidence supporting the use of T34 as an alternative to the chemical etridiazole to control *P. capsici* in pepper plants is reported here. In all studies, the optimal concentration was  $10^4$  conidia  $\text{mL}^{-1}$  of peat. The only experiment where all the T34 concentrations tested gave significant decreases in disease severity was the greenhouse experiment conducted in 2009. In that experiment, the microbial control agent colonized the peat for 1 month before the pathogen was applied and thus reached an optimal concentration (unpublished data suggest that this optimum is c.  $10^4$  conidia  $\text{mL}^{-1}$ ). It has been reported that *T. harzianum* strain 2413 reduced disease produced by *P. capsici* by 29, 30, 32 and 38% when applied at  $1.3 \times 10^4$ ,  $2 \times 10^5$ ,  $8 \times 10^6$  and  $3.5 \times 10^8$  conidia  $\text{mL}^{-1}$ , respectively (Ezziyyani *et al.*, 2007). T34 was able to reduce disease by 54–71% when applied at  $10^4$  conidia  $\text{mL}^{-1}$ , thus showing greater efficacy than strain 2413. Concentrations of T34 greater than  $10^5$  conidia  $\text{mL}^{-1}$  were not assayed because this amount of biocontrol agent is not considered to be commercially viable. Similar to T34, *T. hamatum* strain 382 applied at approximately the same concentration also controlled *P. capsici* damping-off of cucumber when this plant was treated 7 days before inoculation with the pathogen (Khan *et al.*, 2004). In addition, induction of systemic defence by *T. hamatum* strain 382 was demonstrated using a leaf blight system where the biological control agent and the pathogen were spatially separated. Similarly, the reduction in *P. capsici* in pepper by pre-treatment with *T. harzianum* strain 2413 has been related both to a decrease in the pathogen population over time and induction of plant resistance (Sid Ahmed *et al.*, 1999, 2000). Although we did not address the induction of resistance in this study, T34 was shown to induce systemic acquired resistance against various foliar diseases when applied to the roots of *Arabidopsis* and cucumber (Segarra *et al.*, 2007, 2009). While the inhibition of zoospore germination by T34 was not statistically significant, there was a clear correlation between the amount of T34 and the percentage of inhibition. On the basis of this observation, the possibility that this agent inhibits zoospore germination cannot be ruled out. Given that the inhibition of zoospore germination was not significant, but disease was reduced at the three concentrations assayed, other mechanisms, such as mycelial hyperparasitism or competition, may have occurred. *T. harzianum* strain 2413 reduced *Phytophthora* root rot of pepper

when applied 7 d before inoculation with the pathogen and its antagonistic effects were related to the *in vitro* capacity of *T. harzianum* to inhibit mycelial growth and to envelop and disintegrate *P. capsici* hyphae (Ezziyyani *et al.*, 2007). It is possible that the mechanism of control by T34 could be similar, but this was not investigated in the present study.

Chemical control of *P. capsici* with etridiazole was highly effective in the 2010 greenhouse experiment, in which this compound was applied at the same time as the pathogen, but failed when the pathogen was applied 7 and 30 da after the treatment. Etridiazole is extensively used for controlling damping-off, root rot and stem rot caused by *Phytophthora* and *Phytium* as described by the US Environmental Protection Agency (Liu *et al.*, 2009). This chemical is a contact fungicide with protective and curative action (Agriculture and Environment Research Unit, 2011). The mechanisms of action of the fungicide are related to the release of phospholipases in mitochondria (Casperson and Lyr, 1975; Lyr *et al.*, 1977) and inhibition of triglyceride and sterol ester synthesis (Lyr *et al.*, 1975). The germination of zoospores was inhibited by etridiazole in the experiments described here; however, there is some controversy in the literature regarding the efficacy of this product against oomycete structures. Stasz and Martin, (1988) demonstrated that thin-walled oospores and sporangia of *Pythium* were killed by etridiazole, while thick-walled oospores were not, and their viability was only affected by etridiazole at high concentration. The authors suggested that survival of the resistant structures may result in further disease outbreaks. In contrast, Kuhajek *et al.*, (2003) reported that etridiazole had limited efficacy against germinating spores but was a potent inhibitor of mycelium growth of *Phytophthora nicotianae*. Moreover, etridiazole has a variable DT<sub>50</sub> (period required for 50% decay of the compound) depending on the conditions. The DT<sub>50</sub> of this substance in a given soil (sandy loam) ranges from 0.59 (flooded substrate) to 36 d (45% soil water content) (European Food Safety Authority, 2010). The high water content in the substrate used in the present studies, which favours the occurrence of disease, therefore also enhanced the degradation of etridiazole. This could explain the failure of etridiazole to suppress disease when 7 or 30 d had passed between the treatment and the application of the pathogen.

Here we show that, in contrast to the chemical control by etridiazole, T34 reduced disease when applied preventively but also when applied simultane-

ously with the pathogen. This indicates that previous substrate colonisation of the microbial control agent is not required for successful control of the pathogen. In addition, this biological control agent consistently reduced wilting in small plants and canker development in larger ones. On the basis of these observations, we conclude that T34 is a suitable alternative to etridiazole for the management of *P. capsici* as it shows greater efficacy than the chemical treatment. There is an increasing body of evidence that various strains of microbial control agents are able to replace chemical pesticides in integrated pest management.

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