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Biological control of *Botrytis cinerea* on tomato stem wounds with *Trichoderma harzianum*

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Abstract

The effectiveness of *Trichoderma harzianum* in suppression of tomato stem rot caused by *Botrytis cinerea* was examined on tomato stem pieces and on whole plants. Ten days after simultanous inoculation with *B. cinerea* and *T. harzianum*, the incidence of infected stem pieces was reduced by 62–84%, the severity of infection by 68–71% and the intensity of sporulation by 87%. Seventeen days after inoculation of wounds on whole plants, the incidence of stem rot was reduced by 50 and 33% at 15 and 26 °C, respectively, and the incidence of rot at leaf scar sites on the main stem was reduced by 60 and 50%, respectively. Simultanous inoculation and pre-inoculation with *T. harzianum* gave good control of *B. cinerea* (50 and 90% disease reduction, 10 days after inoculation). The rate of rotting was not reduced by the biocontrol agent once infection was established. However, sporulation by *B. cinerea* was specifically reduced on these rotting stem pieces. Temperature had a greater effect than vapour pressure deficit (VPD) on the efficacy of biocontrol. Suppression of *B. cinerea* incidence by *T. harzianum* on stem pieces was significant at 10 °C and higher temperatures up to 26 °C. Control of infection was significantly lower at a VPD of 1.3 kPa (60% reduction), than at VPD <1.06 kPa (90–100% control). Reductions in the severity of stem rotting and the sporulation intensity of grey mould were generally not affected by VPD in the range 0.59–1.06 kPa. Survival of *T. harzianum* on stems was affected by both temperature and VPD and was greatest at 10 °C at a low VPD and at 26 °C at a high VPD.

Introduction

Stem infection by *Botrytis cinerea* is a severe problem in greenhouse vegetable crops, including tomato. Stems can be infected either by invasion of the fungus through the petiole (Elad and Shtienberg, unpublished) or by direct infection of wounds after pruning and harvesting (Dik et al., 1995; Nicot et al., 1994; Verhoeff, 1968). Infections on stems may ultimately girdle the stem, kill the entire plant and cause substantial yield losses (O'Neill, 1994; Verhoeff, 1968; Yunis et al., 1991). The practice of climate control in greenhouses is currently used for tomato and other vegetable crops in countries with cold weather conditions. Excessive heating and ventilation to prevent canopy wetness reduces the intensity of grey mould (Morgan, 1984; Winspear et al., 1970) but, although this practice is generally effective against infection of leaves, flowers and fruits, it is less effective in preventing stem infections. In addition, heating regimes to prevent canopy wetness consume energy very heavily; more detailed knowledge on the influence of climate on *B. cinerea* would help to optimize this control measure. Since cultural methods fail to provide adequate control of stem infection, growers continue to use fungicides to manage the pathogen. Spraying of fungicides onto the foliage and stems and application directly to stem wounds are both practised. However, it has been shown that the pathogen may develop resistance within a relatively short period against specific fungicides such as benzimidazoles, dicarboximides, diethofencarb and two sterol biosynthesis inhibitors (Elad, 1992; Elad and Shtienberg, 1995; Elad et al., 1992; Yunis and Elad, 1989).

The economic importance of grey mould and the risk of *B. cinerea* developing resistance are both important reasons to investigate biological control as an alternative control measure. Infection by necrotrophic pathogens, including *B. cinerea*, can be reduced by pre-inoculation of the phylloplane with epiphytic fungi, bacteria or yeasts (Blakeman and Fokkema, 1982; Elad et al., 1995). Adequate control of *B. cinerea* by *Trichoderma harzianum* has been achieved on grapes (Dubos, 1984), apple (Tronsmo and Ystaas, 1980) and strawberries (Tronsmo and Dennis, 1977). *Cladosporium herbarum, Aureobasidium pullulans* and *Exophiala jeanselmia* have also been found effective in controlling *B. cinerea* (Bhatt and Vaughan, 1962; Redmond et al., 1987). Recent information indicates that stem infection by *B. cinerea* may also be controlled by antagonistic microorganisms (Dik et al., 1995; Elad et al., 1994, 1995; Nicot et al., 1995).

Recently, an isolate of *T. harzianum* (T39) effectively controlled *Botrytis* diseases in greenhouse crops and grapes in Israel and elsewhere (Elad et al., 1993; Elad et al., 1994a; Elad and Shtienberg, 1995). We report here on the effects of this biocontrol agent on infection of tomato stem wounds by *B. cinerea*. The effects of microclimate and time of application on the activity and survival of the biocontrol agent on stem wounds were also studied.

Materials and methods

Plant materials

Tomato (*Lycopersicum esculentum* Mill., cv. 144) plants were grown in a peat/perlite/sand mixture (1:1:1) in 17-cm pots in a greenhouse at 15–26 °C, and under commercial conditions in tufa slabs in a polyethylene greenhouse. Plants in pots were fed weekly with a proprietary tomato feed; those in the commercial greenhouse with every watering. No fungicides were applied to either crop. Stems and side shoots were collected for inoculation experiments as required; stems were from plants about 1 m high, and side shoots were usually 40–50 cm in length. Six week-old plants were used to study interactions of *B. cinerea* and *T. harzianum* on wounds on intact plants. Paste treatments were applied to *Botrytis* lesions in a 24-week-old crop in a commercial greenhouse.

Biocontrol agent

A preparation of *T. harzianum* T39, originally isolated from a cucumber fruit (Elad et al., 1993), was used in all experiments. The preparation (Trichodex 25P) was grown and formulated by Makhteshim Chemical Works Ltd. (Be'er Sheva, Israel).

Stem piece bioassay

The top 10-cm lengths of stem and side shoots were discarded and the remainder of each was cut into ten pieces, each 3 cm in length. Twenty pieces were inserted vertically with the youngest end uppermost, into a 10-cm diameter pot containing autoclaved perlite, previously wetted with distilled water to a known weight. Each of four replicate pots contained equal numbers of pieces of tissue of different age. Pots of stem pieces were placed in plastic boxes (380×330×160 mm) and these were enclosed in a polyethylene bag and placed in an illuminated (10 J/cm^2/h at table level) walk-in growth room maintained at about 20 °C.

Inoculation

B. cinerea had been previously isolated from a naturally infected cucumber flower (Elad, 1988). The fungus was maintained and grown on potato dextrose agar (PDA, Difco). Suspensions of conidia (5×10^5/ml) of *B. cinerea* were prepared from 10–14-day cultures by agitating a piece of sporulating culture in sterile distilled water (SDW) and filtering through folded muslin. Spore concentration was determined with a hemocytometer and adjusted as necessary. Wounds on stem pieces were individually inoculated with 20 μl of spore suspension through an Eppendorf pipette; controls were inoculated with water. The biocontrol agent was prepared as a suspension of the commercial formulation in water (0.1 g/100 ml), equivalent to 10^6 cfu/ml. A total volume of 20-μl was applied to each stem piece. Unless stated otherwise, droplets of *B. cinerea* and *T. harzianum* were applied at the same time. *T. harzianum* was applied up to 3 days before *B. cinerea* or up to 3 days after *B. cinerea* in one experiment.

Whole-plant experiments

Plants were inoculated at wound sites which had been created by cutting off leaf petioles adjacent to the stem, or cutting off stem tops immediately above a leaf. There was a total of six inoculation sites on each plant: three leaves, the top of the stem and two side shoots were removed from each plant. Wounds were inoculated with a 15-μl droplet of spore suspension in water and plants were then covered with a clear polyethylene bag for 5 days.

A paste was prepared from a commercial preparation of *T. harzianum* (500 g) in water (1 litre) and applied to tomato stem lesions (10–30 mm in length) with a 10-mm paint brush. Stems were painted covering the Botrytis lesion and about 5 mm beyond the lesion edge. Lesion length was measured immediately before treatment and after 3 weeks. A total of 24 lesions on 12 plants were painted with *T. harzianum* paste. An equal number of lesions was left untreated.

Assessment

Stem pieces were assessed at several intervals, usually 3, 4, 5, 6, 8 and 10 days after inoculation, for the extent of tissue rotting and the degree of sporulation of *B. cinerea*. The following scales were used. Stem infection: 0, stem end green; 1, stem end brown or black; 2, up to 5 mm of stem length discoloured; 3, 6–15 mm of stem length discoloured; 4, >15 mm of stem length discoloured. Sporulation: 0, no sporulation; 1, sporulation on stem end only; 2, sporulation on up to 0.25 of stem length; 3, sporulation on 0.25–0.75 of stem length; 4, sporulation on >0.75 of stem length. Infection and sporulation severity indices (0–4) were calculated for all stem pieces including non-infected ones. The proportion of infected stem pieces (infection incidence) was also determined. A stem was regarded as infected if it had an infection and/or a sporulation index >1. Percentage reduction (control efficacy) of the infection incidence, infection severity and sporulation severity were calculated for each assessment.

Wounds on whole plants inoculated with *B. cinerea* and/or *T. harzianum* were assessed 6 and 17 days after inoculation. Stems were regarded as infected when lesions greater than 5 mm in length were evident on cut stem ends or when stem discoloration and/or rotting extended more than 5 mm from the edge of a leaf scar. The length of lesions was measured.

Humidity regimes

Several different vapour pressure deficits (VPD) were produced by means of saturated salt solutions in distilled water, as previously described (Winston and Bates, 1960). Pots of stem pieces were stood in glass Petri dishes and the bases of incubation boxes were flooded to a depth of 5 mm with distilled water (100% RH), NaCl (85% RH), KCl (75% RH) or glucose (55% RH) solutions and maintained at 20 °C. A very low RH (<40%) was produced by placing uncovered boxes in a growth room with fan ventilation. The corresponding VPDs were 0, 0.35, 0.59, 1.06 and >1.30 kPa, respectively.

Wound age

Pots of stem pieces were prepared, covered with polyethylene bags (VPD <0.2 kPa) and maintained at 20 °C until the desired wound age had been reached.

Spore germination and germ tube growth

Spore suspensions (10^5/ml) of *B. cinerea* or *T. harzianum* in sterile water were spread over a PDA plate and plates were incubated at the required temperature (5, 10, 15, 22, 26, 30, 37 °C). Spore germination and germ tube growth were determined by microscopic examination of three replicate plates per treatment.

Monitoring of T. harzianum *and* B. cinerea *populations*

The top 10-mm of 10 inoculated stem pieces were removed and placed in 50 ml of SDW in 250-ml flasks. Stem pieces without obvious soft rotting and without *Botrytis* sporulation were selected. Two drops of Tween 80 were added and the flasks were shaken for 2 h on a linear shaker. Aliquots (1 ml) were removed and placed, directly or after dilution (1:10) in SDW, on potato dextrose agar containing 50 mg/ml Rose Bengal and on a *B. cinerea*-selective medium (Adapted from Seddon, B. – Elad and Shtienberg, 1995). The plates were incubated at 20 °C and the numbers of *T. harzianum* colonies and *B. cinerea* colonies were counted after 6 days. There were three replicate plates per treatment.

Experimental design and data analysis

Each of the experiments was repeated at least once. Since conclusions based on different trials of repeated experiments were similar, findings of one experiment are presented in the results. There were four to six replicates per treatment for the stem pieces experiments (4–6 pots with 20 stem pieces each) and eight replicates plants per treatment for the whole-plant experiments. Experiments were laid out in a completely randomized design. Data in percentages were arcsin transformed and treatments were compared statistically using Fisher's protected LSD Test at $P \leq 0.05$. Statistical differences are indicated in the figures by asterisks, since the scales used (percentage or index) are not linear.

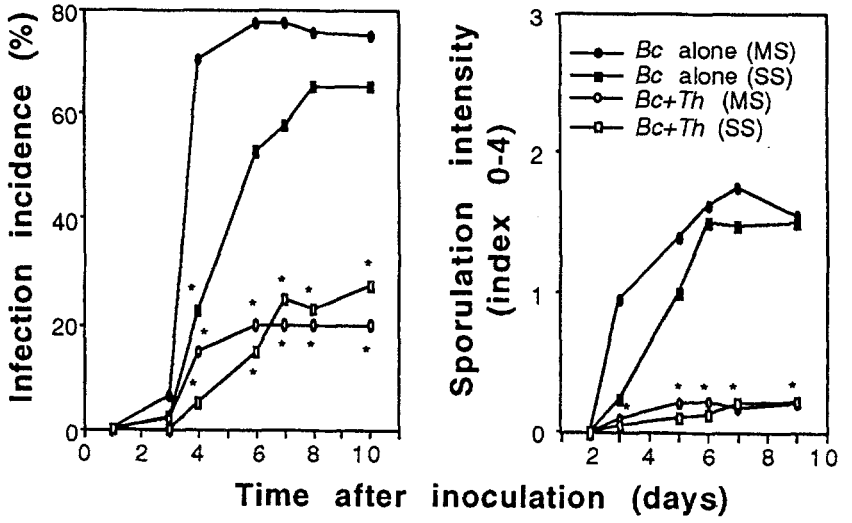

Figure 1. Effect of *Trichoderma harzianum* (Th) on incidence of infection and intensity of sporulation caused by *Botrytis cinerea* (*Bc*) on tomato stem pieces. Stem pieces originated from main stems (MS) of tomato plants or from side-shoots (SS) of the same plants. (* = significantly different from the untreated control at $P \leq 0.05$).

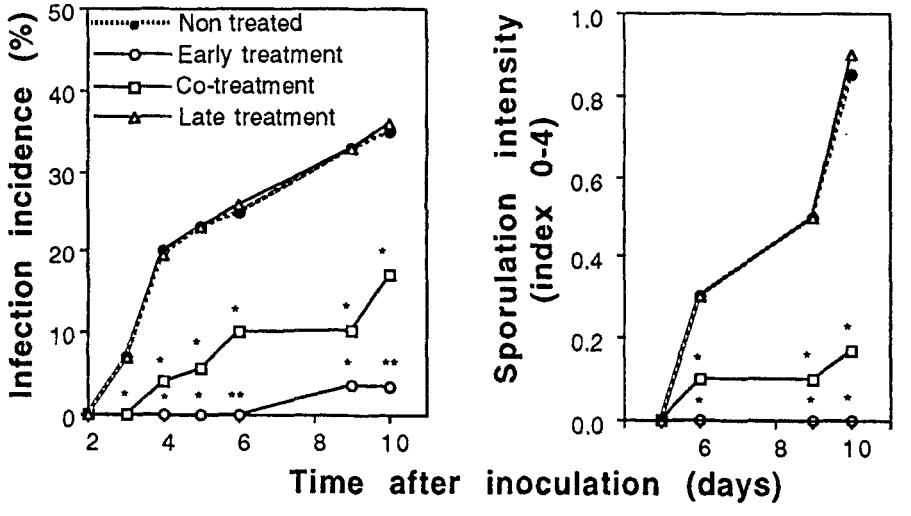

Figure 2. Effect of the timing of *Trichoderma harzianum* application on suppression of infection and sporulation caused by *Botrytis cinerea* in tomato stem pieces. *T. harzianum* was applied 8 h earlier than *B. cinerea*, at the same time, or 24 h after *B. cinerea*. (* = significantly different from the untreated control at $P \leq 0.05$, ** = significantly different from the co-inoculation treatment at $P \leq 0.05$).

Results

Effect of T. harzianum *on infection of stem wounds*

Stem pieces co-inoculated with *T. harzianum* and *B. cinerea* and incubated at 20 °C developed less Botrytis stem rot than those inoculated with *B. cinerea* alone. Control of *B. cinerea* by *T. harzianum* occurred to a similar extent on main-stem and side-shoot tissues (Figure 1). Ten days after inoculation, the incidence of infected stem pieces was reduced by 62–84% and the intensity of sporulation by 87% (Figure 1). The severity of infection was reduced by 68–71% (results not shown). Characteristically, tissue rotting and sporulation, where they occurred on stems treated with the biocontrol agent, remained confined to the stem end.

The timing of *T. harzianum* treatment in relation to *B. cinerea* inoculation significantly affected the efficacy of control. After 12 days incubation at 20 °C, infection rate and the intensity of sporulation were significantly reduced in treatments where *T. harzianum*

was applied before *B. cinerea* or together with it (Figure 2). Inoculation of stem pieces with *T. harzianum* at intervals of 1 day or more before inoculation with *B. cinerea* was inconclusive, as wound susceptibility had decreased and very little stem rot occurred even in the absence of *T. harzianum* (data not presented). Inoculation of stems with *T. harzianum* 1–3 days after inoculation with *B. cinerea* had no effect on the incidence of stem infection, the rate of tissue rotting, or the development or extent of sporulation by *B. cinerea*.

Results presented in Figure 1 refer to all inoculated stem pieces. Results of another experiment, employing a large number of stem pieces, were analysed differently: the infected stem pieces were considered separately (Table 1). The severity of disease and intensity of sporulation were observed 8 days after inoculation. Although the progress of rotting on infected stem pieces was not significantly slowed by the biocontrol agent, sporulation was specifically reduced on infected stem pieces previously treated with *T. harzianum* (Table 1).

Inoculation of *B. cinerea* on to fresh stem and leaf-scar wounds on 6-week-old tomato plants resulted in the development of a high incidence of stem lesions when plants were incubated at 15 and at 26 °C. In general, a higher incidence and larger lesions developed when inoculation was carried out on stem wounds (Figure 3). The incidence of stem rot was significantly reduced when wounds were co-inoculated with *T. harzianum*. Seventeen days after inoculation, the incidence of stem rot was reduced by 50 and 33% at 15 and 26 °C, respectively, and the incidence of rot at leaf-scar sites on the main stem was reduced by 60 and 50%, respectively (Figure 3a). Lesion length was similarly reduced by *T. harzianum* when all wounds were considered (results not shown). However, the lesion length of infected wounds (excluding non-infected wounds) was not reduced by the biocontrol treatment (Figure 3b). Sporulation was less on plants co-inoculated with *T. harzianum* (results not presented).

Lesions with sporulating *B. cinerea* on tomato stems in a commercial greenhouse were painted with a paste containing 67% (w/v) of a *T. harzianum* preparation in water. Three weeks after treatment, the surface area of lesions had increased by 2.12 and 2.58 cm^2 on untreated and *T. harzianum*-treated stems, respectively. These values do not differ significantly as determined by a t-test ($t = 0.16$; $P = 0.65$).

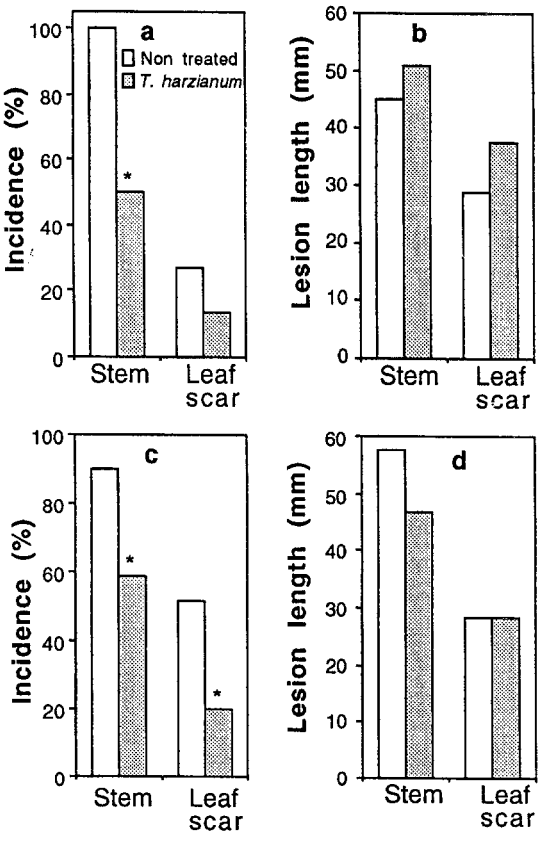

Figure 3. Effect of *Trichoderma harzianum* on rot caused by *Botrytis cinerea* on tomato stem wounds and leaf scars of 6-week-old plants. After treatment with the pathogen and the biocontrol agent plants were incubated at 15 (a b) or 26 °C (c d). Incidence of infection (a c) and lesion length (b d) were measured 17 days after treatment. Lesion length was calculated for infected wounds only. * significantly different from the untreated control at $P \leq 0.05$.

Effect of temperature and VPD on efficacy of T. harzianum

The degree of suppression of *B. cinerea* incidence by *T. harzianum* increased with increasing temperature up to 26 °C (Figure 4a). Reduction in the severity of Botrytis stem rot was greatest at 20 °C and least at 5 °C (results not shown). Reduction in the sporulation intensity increased with increasing temperature in the range 15 to 26 °C. No sporulation was observed after 20 days on any stem pieces incubated at 5–10 °C (Figure 4b).

On PDA plates, a high level of spore germination (>90 %) was evident at 15, 20, 26 and 30 °C after 15 h. No germination had occurred after incubation for 2 days at 5 and 37 °C. Germ-tube growth and mycelial growth were greatest at 26 °C.

Table 1. Effect of *Trichoderma harzianum* on disease severity and on sporulation of *Botrytis cinerea* on infected tomato stem pieces[a]

Treatment	Number of stem pieces	Infection severity (index 0–4)		Sporulation intensity (index 0–4)	
		All pieces	Infected only	All pieces	Infected only
B. cinerea alone	168	2.92a[b]	3.52a	2.22a	2.85a
B. cinerea + *T. harzianum*	168	1.45b	3.07a	0.79b	1.54b
Control efficacy (%)		50	13	64	46

[a] Data were recorded 8 days after co-inoculation with *B. cinerea* and *T. harzianum*.
[b] Numbers representing severity or intensity at each column followed by a common letter do not differ significantly at $P \leq 0.05$.

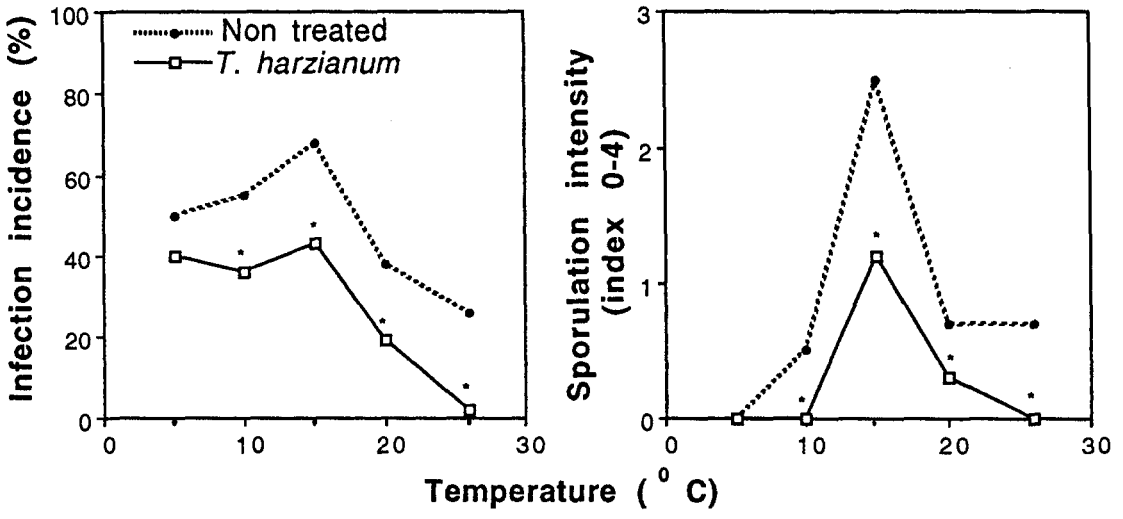

Figure 4. Effect of temperature on control efficacy of *Botrytis cinerea* infection and sporulation achieved by *Trichoderma harzianum* on tomato stem pieces. Stem pieces were co-inoculated with *T. harzianum* and *B. cinerea* and incubated for 12 days at VPD of 0.23 kPa. (* = significantly different from the untreated control at $P \leq 0.05$).

Incubation of inoculated stem pieces at different humidities slightly affected the degree of control exerted by *T. harzianum* (Figure 5). Control of infection was significantly lower at a VPD of 1.3 kPa: 60% reduction, as compared with 90–100% control at lower VPD values. Reduction of stem rot severity (results not shown) and sporulation intensity was generally not affected by VPD in the range of 0.59–1.06 kPa (Figure 5).

Survival of T. harzianum *and* B. cinerea *on stem wounds*

The populations of *T. harzianum* and *B. cinerea* separately, and of *B. cinerea* in the presence of *T. harzianum*, were recorded on stem pieces 14 days after inoculation. *T. harzianum* survived better at 10 °C and low VPD. Under high-VPD conditions, survival on stem pieces increased with increasing temperatures (Figure 6a). The population of *B. cinerea* was not affected by temperature in the range tested at high VPD, but decreased markedly at temperatures of 20 and 2 6 °C at low VPD (Figure 6b). In the presence of *T. harzianum*, the population of *B. cinerea* was generally less affected by temperature at high VPD than at low VPD. Under low-VPD conditions the *B. cinerea* population decreased with increasing temperature (Figure 6c).

Discussion

Stem infection by *B. cinerea* is a severe problem in greenhouse vegetable crops. It is observed on crops such as tomato, cucumber, pepper and eggplant (Elad

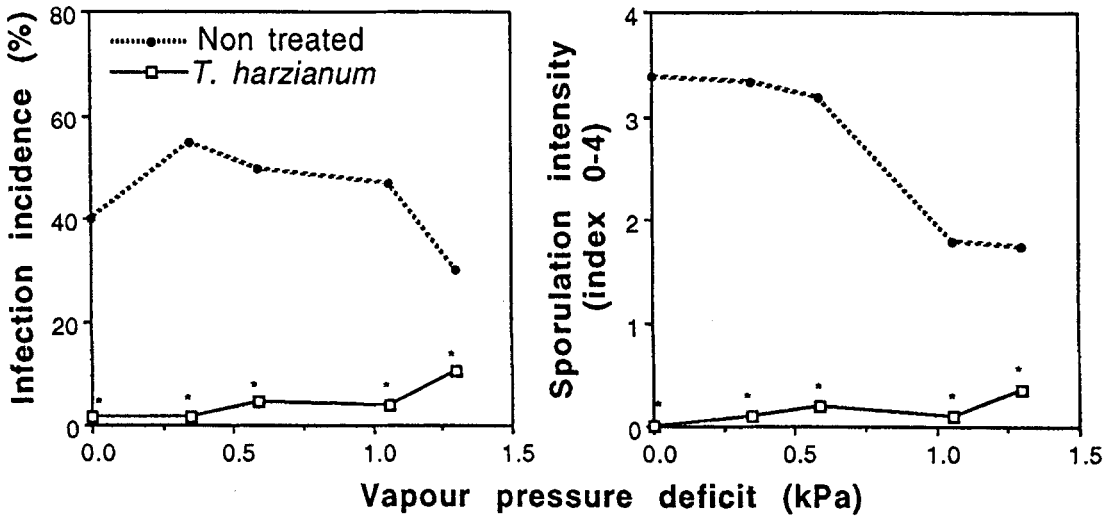

Figure 5. Effect of vapour pressure deficit (VPD) on control efficacy of *Botrytis cinerea* infection and sporulation achieved by *Trichoderma harzianum* on tomato stem pieces. Stem pieces were co-inoculated with *T. harzianum* and *Botrytis cinerea* and incubated for 12 days at 20 °C. (* = significantly different from the untreated control at $P \leq 0.05$).

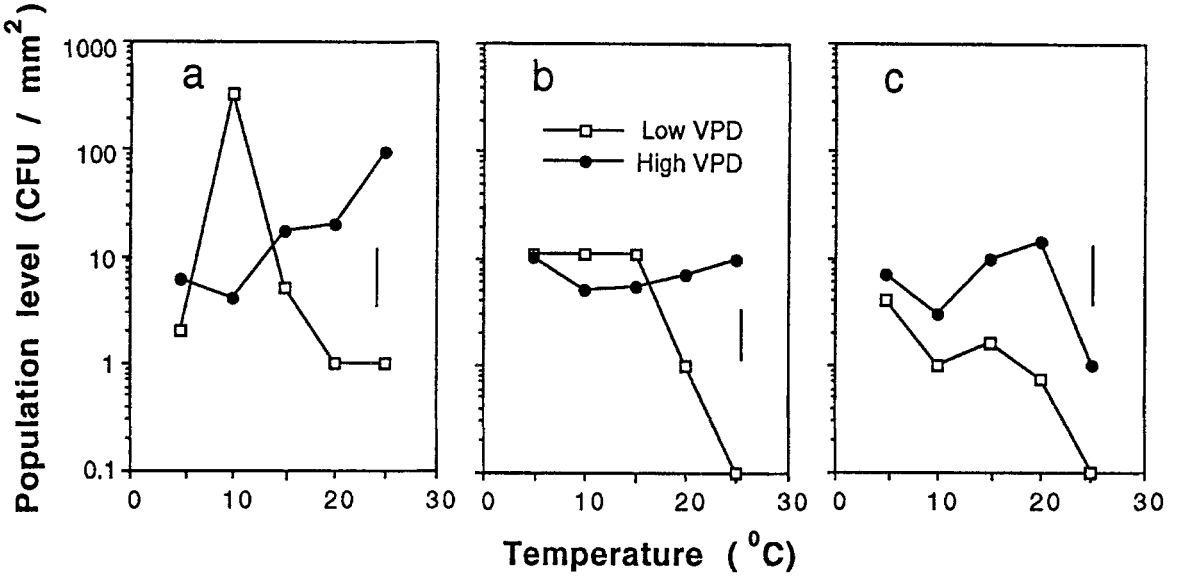

Figure 6. Levels of populations of *Trichoderma harzianum* (a) and of *Botrytis cinerea* applied alone (b) and of *B. cinerea* in the presence of *T. harzianum* (c) on wounds of tomato stem pieces showing no symptoms. The stem pieces were incubated for 14 days at low (<0.23 kPa) or high VPD (>0.95 kPa). Bars represent the largest LSD.

and Shtienberg, 1995). The damage inflicted on plants is severe in spite of intensive efforts by growers to control it with chemical fungicides or through expensive cultural practices. In the present investigation, *T. harzianum* was found capable of controlling infection on stem pieces or on wounds inflicted on whole plants (Figures 1–3). Early application of the biocontrol agent appeared to give better control. On the other hand, delayed application of the biocontrol agent did not control the disease (Figure 2) and, furthermore, the progress of rotting in infected stems was not reduced by the biocontrol agent applied together with the pathogen (Figure 3 and Table 1). It may be concluded that *T. harzianum* can prevent infection of wounds, but

once infection is established, rot development is not affected. These results resemble those of Mercier and Wilson (1995) who investigated biocontrol of apple grey mould by *Candida oleophila*. In the case of the yeast biocontrol agent, early application resulted in better control than delayed application.

Sporulation of *B. cinerea* was reduced in the presence of the biocontrol agent. This can be attributed to two possible effects: a direct influence on the sporulation process; and an indirect influence through delayed establishment of the pathogen in rotting stem tissues. Since *T. harzianum* did not slow the rate of rot development, once started, the direct influence on the sporulation process should not be excluded. Moreover, the total amount of conidia produced by *B. cinerea* on plant material was affected by both a reduction of infection rate and a reduction in sporulation capacity. The suppression of sporulation by biocontrol agents was suggested earlier by Köhl et al., (1992) and was found to be effective on strawberry leaves (Sutton and Peng, 1993). *T. harzianum* was among several microorganisms which significantly suppressed sporulation of *B. cinerea* on dead plant material (Elad et al., 1994b).

The efficacy of *T. harzianum* in controlling infection and sporulation was more affected by temperature (Figure 4) than by VPD (Figure 5). The biocontrol agent was more effective at high temperatures (15–26 °C) than at low temperatures (5–10 °C) in suppression of infection. Sporulation was not evident at the low temperatures; it was better suppressed with increasing temperatures up to 26 °C. As the optimum temperature for infection of tomato stem wounds by *B. cinerea* is around 15 °C, and the optimum temperature for growth of the *T. harzianum* strain used in this study was 26 °C, this may explain the greater degree of control we observed with increasing temperature from 15–26 °C. Possibly, strains of biocontrol agents with lower optima and minima temperatures of activity may provide better control at temperatures as low as those that allow rapid development of *B. cinerea*.

VPD as high as 1.3 kPa affected the efficacy of the biocontrol agent only slightly, which may be attributed to humidity supplied by tissue of the wounded host. This effect may also involve in supporting *T. harzianum* survival in the wound (Figure 6). Survival was also affected by microclimate conditions, as shown previously in work with leaves of various hosts (Elad and Kirshner, 1993). The decline in the population of the biocontrol agent in wounds not infected by *B. cinerea* within 14 days indicates that repeated applications are needed in order to protect the host from infection.

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