Effect of *Trichoderma harzianum* on *Botrytis cinerea* Pathogenicity

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ABSTRACT

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Germination and germ-tube elongation of conidia of the pathogen *Botrytis cinerea* on bean leaves were reduced in the presence of the biocontrol agent *Trichoderma harzianum* T39. A reduction of 20 to 50% in germ-tube biomass was observed 20 h after inoculation. This reduction in germination did not result in complete prevention of disease development on the leaves. One day after inoculation, disease severity on leaves infected by the pathogen with and without the biocontrol agent was similar (~10% necrotic area). Subsequently, the disease developed rapidly in the control leaves and caused almost complete necrosis, whereas in the presence of *T. harzianum* T39 the necrotic area reached only ~50% of the leaf surface. The production of pectin-degrading enzymes by *B. cinerea* was measured up to 4 days after inoculation. Up to 1.3 enzyme units of polygalacturonase (PG), 9 microequivalents of NaOH, which express the activity of pectin methyl esterase (PME), and up to 1.5 units of pectate lyase (PL) were detected on bean leaves. Under the same conditions, the biocontrol agent, *T. harzianum* T39, did not produce any of these enzymes. On leaves infected with *B. cinerea* in the presence of the biocontrol agent, the activity of the pathogen's PG was reduced by 40 to 83%. This was reflected on an activity gel by the faintness of these PG isoenzymes and the delay in their appearance. An up to 100% reduction in PME activity and a ~30% reduction in PL activity also were recorded. We suggest that *T. harzianum* T39 acts by reducing the enzyme activities of the pathogen. An indirect effect of enhancing the defense mechanism of the host plant is discussed.

Additional keywords: cell wall-degrading enzymes, gray mold.

The fungus *Botrytis cinerea* Pers.:Fr. infects stems, leaves, flowers, and fruits of various crops (18). *Trichoderma harzianum* T39 is an efficient biocontrol agent that is used against this pathogen under commercial conditions (19,20,37,44). Several mechanisms have been suggested as being responsible for the control of plant diseases by *Trichoderma* spp. (6). Competition for space and nutrients is thought to control *B. cinerea*, because the pathogen requires exogenous nutrients for germination and germ-tube elongation over a period of several hours on the phyllosphere before penetrating the host plant (4,13). Mycoparasitism against *B. cinerea* has been suggested by Bèlanger et al. (2) and Tronsmo and Raa (39). Lorito et al. (31) reported antifungal activity against *B. cinerea* by chitinolytic enzymes of *T. harzianum*. Production of inhibitory compounds (7) or antibiotics (14,27), suppression of sporulation (25), and induced resistance (17) all may be involved in the control of this disease.

Extracellular pectolytic enzymes of fungal pathogens play an important role in pathogenesis (8,16). A variety of pectic enzymes are produced by *B. cinerea*, among them hydrolases such as polygalacturonase (PG) (42,23,28,33) and pectin methyl esterase (PME) (21) and lyases such as pectate lyase (PL) (35). Pectin-degrading enzymes disrupt the structure of plant cell walls (1). Fragments released from the cell wall by these enzymes can elicit plant defense responses (5,22). The effect of *T. harzianum* T39 on the activity of the pectin-degrading enzymes of *B. cinerea* and on disease development on bean leaves was studied to elucidate a new potential mechanism of biological control.

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MATERIALS AND METHODS

Fungal isolates. The fungal pathogen, *B. cinerea* (isolate 16), was isolated from infected cucumber fruits in Israel (15) and maintained on potato dextrose agar (PDA) at 18 to 20°C. The biocontrol agent, *T. harzianum* T39, which was isolated from a cucumber canopy in a greenhouse, and the isolates *T. viride* T99 and *T. harzianum* T28, which were obtained from a collection (46), were maintained on PDA at 20 to 23°C.

Plant material. Bean plants (*Phaseolus vulgaris* L.) were planted in 2-liter polyethylene pots and grown in the greenhouse at 20 to 25°C. Leaves were collected from 3- to 4-week-old plants and incubated horizontally in polyethylene boxes on a polyethylene grid overlaid with water-soaked filter paper. The boxes were kept in transparent polyethylene bags to allow for condensation at vapor pressure deficit 0.104 to 0.118 kPa and 95% relative humidity. The boxes were held in a walk-in growth chamber at 18 to 20°C throughout the experiment (17).

Treatment of plant material. Detached leaves were inoculated with 50-µl drops containing suspensions of *B. cinerea* alone (5×10^5 conidia per ml) or with *T. harzianum* T39 (10^7 conidia per ml). To enhance disease development, the suspensions were supplemented with KH_2PO_4 as specified for each experiment. According to Van den Heuvel and Waterreus (41), phosphate stimulation of *B. cinerea* infection in French bean leaves is due to stimulated penetration via enhancement of the activities of certain pectic enzymes. Noninoculated leaves were treated with water. Each treatment contained five leaves as replicates; each replicate was inoculated with 25 drops. At each sampling (one a day for 5 days after inoculation), the severity of the symptoms under the drops was evaluated. Drops of each replicate were collected and centrifuged

to allow sedimentation of conidia. After centrifugation, pectin-degrading enzyme activity was monitored in the supernatant: activity gels were used to detect PG isoenzymes, and Western blot analysis was used to detect endo-PG.

Estimation of the pathogen biomass. Five drops of each treatment were observed under a light microscope (17) to estimate the *B. cinerea* biomass. Aniline blue was added to the drops 15 min before observation. Conidia (100 from each treatment, with or without *T. harzianum*) were evaluated for germination, and the length of the germ tubes was measured. The relative biomass was calculated by multiplying the percentage of germinating conidia by the average germ-tube length (17). Because variation in the latter parameter was small among replicates within treatments, this calculation was deemed a good estimation of the germ-tube biomass. After the first day of incubation the amount of hyphae on the leaf surface was estimated by light microscopy.

Evaluation of disease severity. Symptom severity on the drop-inoculated leaves was evaluated based on a 0 to 7 index, where 0 = symptomless leaf tissue; 1 = 1 to 12%, 2 = 13 to 25%, 3 = 26 to 50%, and 4 = 51 to 100% necrotic area under the drop; and 5 = expansion zone around the drop <2 mm, 6 = expansion zone 2 to 5 mm, and 7 = expansion zone >5 mm (12).

Statistical analysis. Experiments were arranged in a completely randomized design and repeated at least three times. Treatments were replicated at least four times. Data were arcsine transformed, subjected to analysis of variance, and tested for significance by the Student-Newman-Keuls multiple range test. Only the findings of one representative experiment are presented below.

Enzymatic activity assays and protein determinations. PG (EC 3.2.1.15) activity was tested by a modified Nelson-Somogyi assay (23). The reaction mixture contained 2% (wt/vol) polygalacturonic acid (Sigma Chemical Company, St. Louis) in 50 mM acetate buffer (pH 5.2) as the substrate. One unit was defined as the amount of enzyme that released 1 µM galacturonic acid in 1 h. PME (EC 3.1.1.11) activity was determined by a modification of the continuous titration method. A solution of 1.2% (wt/vol) pectin (Sigma), adjusted to pH 5.5 with 1 N NaOH, served as the substrate. After the addition of 1 ml of the crude enzyme to 3 ml of the substrate, the exact pH was measured (Spectronic 401, Spectronic Instruments, Rochester, NY). After 1 h of reaction at 30°C, the enzyme-substrate mixture was titrated with 0.02 N NaOH to the pH recorded at zero time. Data are expressed as microequivalents of NaOH absorbed per hour per milliliter of crude enzyme (21). PL (EC 4.2.2.2) activity was determined spectrophotometrically at 235 nm. The reaction was a modification of the assay suggested by Nasumo and Starr (36). The reaction mixture contained 0.3% (wt/vol) polygalacturonic acid in 50 mM Tris-HCl buffer (pH 8.5) in the presence of 1 mM $CaCl_2$ as the substrate. One unit of activity was defined as the amount of enzyme that caused an increase of 2.6 in absorbance at 235 nm, equivalent to the release of 1 µmol aldehyde groups (36). Protein concentration was measured spectrophotometrically at 595 nm with Bradford reagent (Bio-Rad Laboratories, Munich).

Gel electrophoresis. Polyacrylamide gel electrophoresis was performed by PhastSystem (Pharmacia LKB Biotechnology, Uppsala, Sweden) with 7.5% native gels according to the manufacturer's directions. The drops collected from inoculated leaves were concentrated by Cintercon-10 (Amicon Co., Beverly, MA). Proteins were quantified, and samples were brought to the same protein concentration, i.e., 0.1 to 0.5 µg. Sucrose (10%, wt/vol) was added to the analyzed samples. Identification of PG on polyacrylamide gels was carried out as reported by Lisker and Retig (30). For

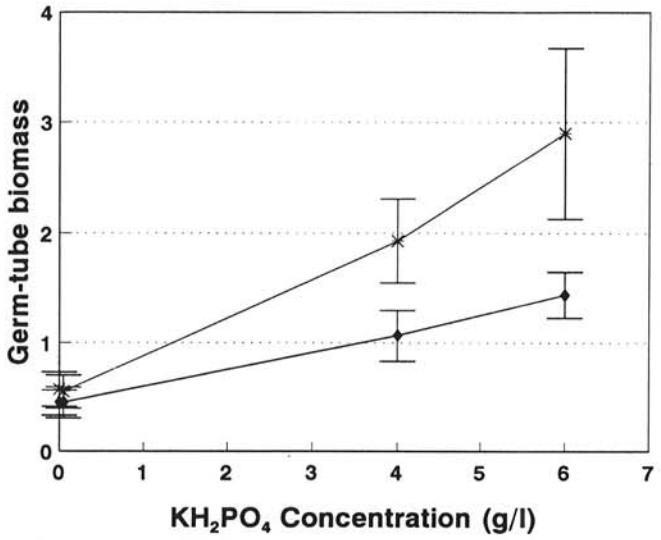

Fig. 1. The effect of KH_2PO_4 concentration on the biomass of *Botrytis cinerea*, with (◆) or without (∗) *Trichoderma harzianum* T39, on bean leaves 20 h after inoculation. Germ-tube biomass = percent germination × germ-tube length. Vertical bars indicate standard error.

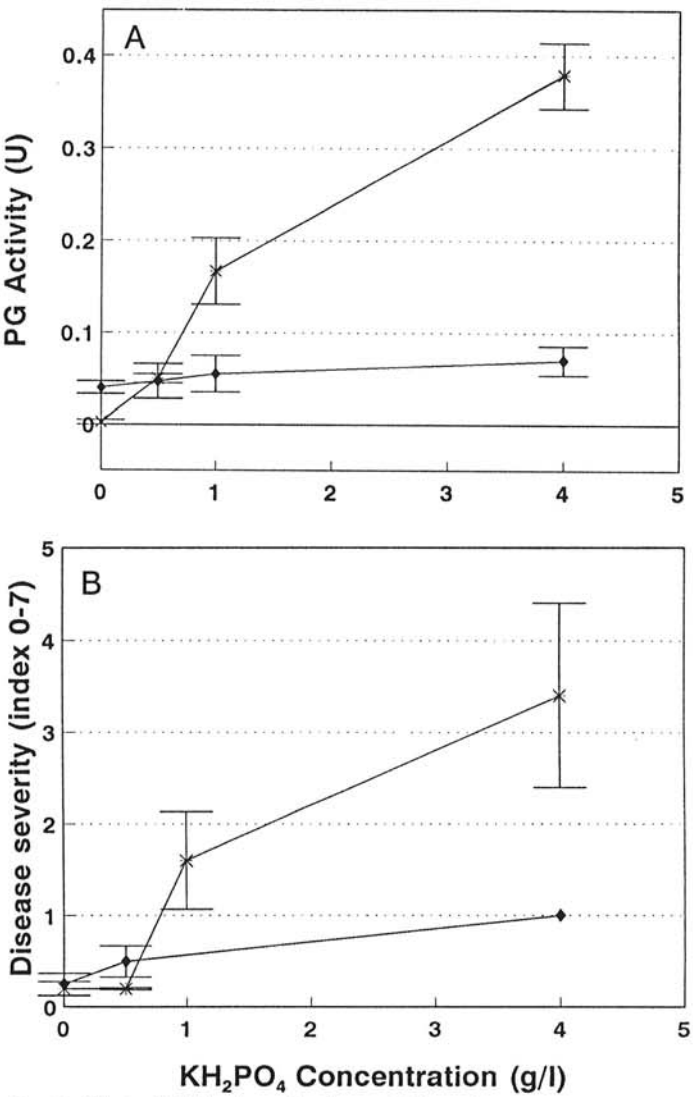

Fig. 2. Effect of *Trichoderma harzianum* T39 on *Botrytis cinerea* supplemented with various KH_2PO_4 concentrations 2 days after inoculation on bean leaves. **A,** Effect on polygalacturonase (PG) activity. One unit = the amount of enzyme that released 1 µM galacturonic acid in 1 h. *B. cinerea* alone (∗); *B. cinerea* and *T. harzianum* T39 (◆). Vertical bars indicate standard error. **B,** Effect of *T. harzianum* and KH_2PO_4 concentrations on disease severity. Disease severity was indexed on a scale of 0 to 7, where 0 = healthy leaf and 7 = expansion zone of necrotic lesions >5 mm. *B. cinerea* alone (∗); *B. cinerea* and *T. harzianum* T39 (◆). Vertical bars indicate standard error.

staining, gels were incubated for 30 min with 1.2% (wt/vol) polygalacturonic acid and immersed for an additional 15 min in 0.05% (wt/vol) ruthenium red (Sigma) solution. Destaining in water enhanced the appearance of the bands.

Western blot analysis. Samples containing 10 µg of protein were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (26) by the Mini Protean II dual slab cell system (Bio-Rad, Hercules, CA). Electrophoresis was carried out at 150 to 200 V for 1 h. Proteins were electroblotted onto a nitrocellulose membrane with a Bio-Rad minitransfer cell for 45 min. The membrane was blocked with 3% (wt/vol) skim milk in Tris-HCl–buffered saline (TBS), pH 7.6, for 2 h at room temperature. The membrane was probed in TBS for 2 h with specific sera (provided by B. Williamson, Scottish Crop Research Institute, Invergowrie, Scotland). The membrane was washed three times, 10 min each, in TBS containing 0.5% (vol/vol) Tween 20 (TBST). The membrane was incubated for 2 h with alkaline phosphatase-labeled goat anti-rabbit antibody (Sigma) in TBS. After three 10-min washes in TBST, color was developed in Tris-NaCl buffer, pH 9.8, containing 5 mM $MgC_{12}{\cdot}6H_2O$, utilizing 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium alkaline-phosphatase substrates (Boehringer GmbH, Mannheim, Germany) (24). The size of the enzyme was estimated by comparison to prestained SDS-PAGE, standard low range molecular weight markers (Bio-Rad, Hercules, CA).

RESULTS

Effect of *T. harzianum* on *B. cinerea* germination and biomass on bean leaves. In the presence of *T. harzianum* T39, *B. cinerea* biomass was reduced by 20 to 50% at all KH_2PO_4 concentrations tested (Fig. 1). This reduction was due mainly to an effect on germ-tube elongation; the germination rate was only partially affected. Although *T. harzianum* T39 reduced the biomass of the pathogen after 20 h, many conidial germ tubes invaded the host tissue, and disease symptoms appeared. Symptom severity on leaves treated with *B. cinerea* with or without *T. harzianum* was similar after 24 h (up to 12% necrotic area). However expansion of the lesions was slowed (by as much as 50%) in the presence of T39. Therefore, we speculated that *T. harzianum* T39 may affect the activity of the pectin-degrading enzymes of *B. cinerea*.

Effect of KH_2PO_4 on PG activity and disease severity of *B. cinerea* on bean leaves. *B. cinerea* exhibited high PG activity in vitro (G. Zimand, A. Kapat, Y. Elad, and I. Chet, *unpublished data*) and on bean leaves (Fig. 2A). The highest level of *B. cinerea* PG activity was observed in the presence of 4 g of KH_2PO_4 per liter after 2 days of incubation (Fig. 2A). *T. harzianum* also exhibited high PG activity in vitro (G. Zimand, A. Kapat, Y. Elad, and I. Chet, *unpublished data*) but not on bean leaves. In the presence of *T. harzianum*, the activity of *B. cinerea* PG was reduced. The amount of this reduction was affected by the KH_2PO_4 concentration, with PG activity more pronounced at the highest concentration. The reductions in enzyme activity at 0.5, 1, and 4 g of KH_2PO_4 per liter were 6, 66, and 81%, respectively. Disease severity on control leaves inoculated only with *B. cinerea* reached a symptom-severity index value of 3.4, whereas leaves treated with *T. harzianum* T39 only reached a value of 1 after 2 days of incubation (Fig. 2B). The greatest reductions in disease severity as well as in PG activity (81%) were achieved at the highest concentration of KH_2PO_4 (4 g/liter) tested.

Disease development on bean leaves as affected by the biocontrol agent and 4 g of KH_2PO_4 per liter. Disease severity was low (index value of 1 to 2) in treatments with or without *T. harzianum* T39 after 1 day of incubation. Disease severity increased to an index value of 4.5 and 7 in the control treatment and to 1.6 and 3.2 in the presence of *T. harzianum* T39 after 2 and 4 days, respectively. The differences between the treatments with and without *T. harzianum* were not as pronounced after 2 to 4 days relative to the first day of incubation (Fig. 3).

Electrophoresis of proteins collected from the surface of infected bean leaves. PG activity in crude protein samples collected from bean leaves infected by *B. cinerea* in the presence or absence of *T. harzianum* T39 was analyzed on native polyacrylamide gels (Fig. 4). After 1 day of incubation in the absence of *T. harzianum* T39, *B. cinerea* had produced three distinct PG isoenzymes. In contrast, isoenzyme activity was weak in the samples taken from leaves infected with *B. cinerea* in the presence of *T. harzianum*. After 2 days on bean leaves, *B. cinerea* had produced six isoenzymes, whereas in the presence of *T. harzianum* only three of these were evident, the same number that was observed on the first day in the *B. cinerea* treatment. After 5 days on bean leaves, the six isoenzymes observed on the second day were most pronounced. In the presence of T39, six weak isoenzymes were observed (Fig. 4).

Western blot analysis with specific endo-PG antibodies of the crude proteins collected from the leaves. Western blot analysis of drops collected from the treated leaves revealed one band with

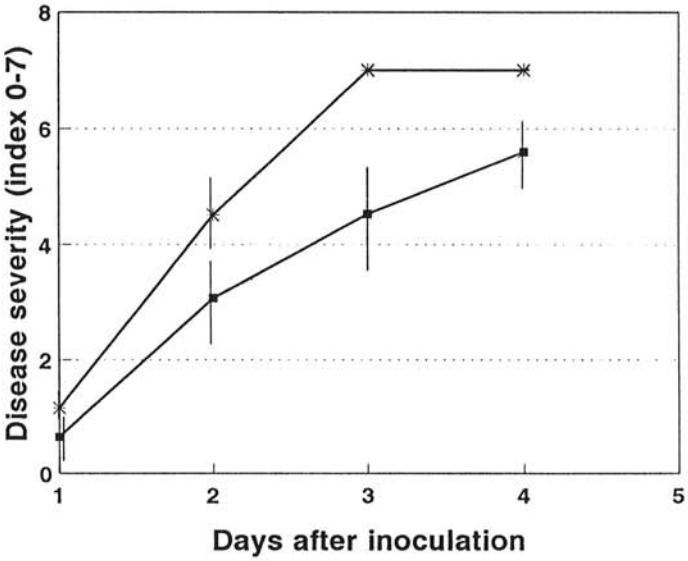

Fig. 3. Effect of *Trichoderma harzianum* T39 on severity of disease caused by *Botrytis cinerea* 1 to 4 days after inoculation: *B. cinerea* alone (*) or with *T. harzianum* T39 (■). Disease severity was indexed on a scale of 0 to 7, where 0 = healthy leaf and 7 = expansion zone of necrotic lesions >5 mm. Vertical bars indicate standard error.

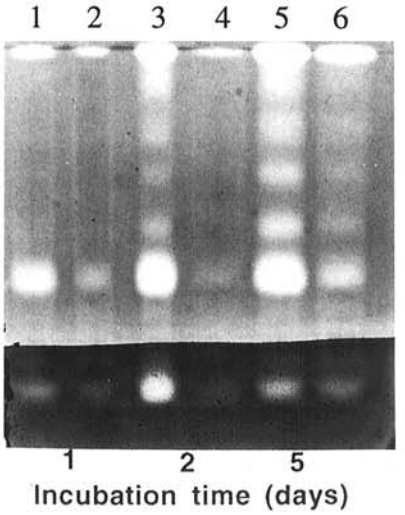

Fig. 4. Electrophoretic patterns on native gel of polygalacturonase produced by *Botrytis cinerea* on bean leaves with and without *Trichoderma harzianum* T39 1, 2, and 5 days after inoculation. Lanes 1, 3, and 5, *B. cinerea* alone; lanes 2, 4, and 6, *B. cinerea* and *T. harzianum* T39.

TABLE 1. Effect of *Trichoderma* isolates on polygalacturonase (PG) activity and disease severity of *Botrytis cinerea*

	Incubation time (days)					
	1		2		3	
Trichoderma isolate	Disease severity[x]	PG (U)[y]	Disease severity	PG (U)	Disease severity	PG (U)
Control	1.47 a[z]	0.18	3.18 a	1.20	6.2 a	1.00
T39	0.46 b	0.03	1.58 b	0.70	2.5 b	1.10
T28	1.85 a	0.90	3.65 a	1.34	6.4 a	1.30
T99	1.62 a	0.15	2.70 a	1.17	5.5 a	1.28

[x] Disease severity index on a scale of 0 to 7, where 0 = healthy leaf and 7 = expansion zone of necrotic lesions >5 mm.
[y] One unit = the amount of enzyme that released 1 μM galacturonic acid in 1 h.
[z] Numbers within columns followed by the same letter do not differ significantly at $P \leq 0.05$. The Student-Newman-Keuls multiple range test was used for analysis. No variability in enzymatic activity was detected in any of the treatments under these conditions.

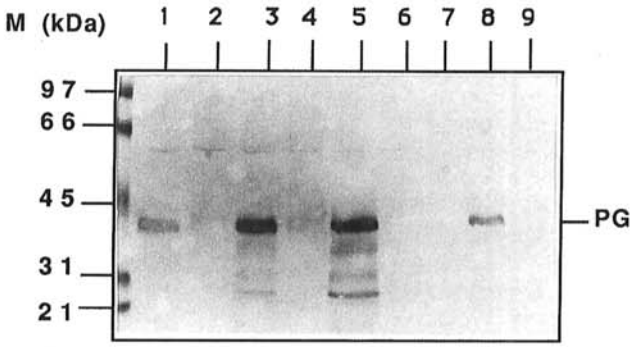

Fig. 5. Western blot analysis (10% gel) of *Botrytis cinerea* polygalacturonase (PG) with and without *Trichoderma harzianum* T39 on bean leaves. The enzyme samples were: lanes 1, 3, and 5, *B. cinerea* alone; lanes 2, 4, and 6, *B. cinerea* with *T. harzianum* T39, supplemented with 4 g of KH_2PO_4 per liter; lane 8, *B. cinerea* alone; and lane 9, *B. cinerea* with *T. harzianum* T39, supplemented with 0.5 g of KH_2PO_4 per liter. Pairs of samples for lanes 1 and 2, 3 and 4, and 5 and 6 were taken 1, 2, and 5 days after inoculation, respectively. Samples for lanes 8 and 9 were taken 4 days after inoculation.

a molecular mass of ~36 kDa in control leaves infected with *B. cinerea* alone, supplemented with 4 g of KH_2PO_4 per liter (Fig. 5). This band was similar to the endo-PG band described by Johnston and Williamson (23). It appeared weakly after 1 day of incubation in the control treatment (lane 1) and did not appear when *T. harzianum* was present on the leaves (lane 2). After 2 days of incubation, this band was stronger in the control treatment (lane 3) and was faintly apparent in the treatment with *T. harzianum* (lane 4). A similar phenomenon was observed after 5 days of incubation (lanes 5 and 6). The results were similar when the leaves were inoculated with 0.5 g of KH_2PO_4 per liter (lanes 8 and 9) (Fig. 5).

Effect of other *Trichoderma* spp. isolates on *B. cinerea* disease development and PG activity. The effects of *Trichoderma* isolates T99 and T28 were compared to that of the biocontrol agent, T39 (Table 1). Only T39 significantly reduced disease severity and PG activity of the pathogen after 1 and 2 days of incubation. Although PG activity was not reduced by T39 on the third day of incubation, disease severity was affected by the previous reductions in enzyme activity (Table 1).

Effect of *T. harzianum* on *B. cinerea* PME and PL activities on bean leaves. *T. harzianum* T39 did not produce any PME or PL on bean leaves. The activities of PME and PL produced by *B. cinerea* on bean leaves supplemented with 4 g of KH_2PO_4 per liter and *T. harzianum* were reduced by approximately 30 and 100%, respectively, relative to the corresponding activities in the absence of the biocontrol agent (Fig. 6).

DISCUSSION

Disease severity was similar on bean leaves inoculated with *B. cinerea* with or without *T. harzianum* T39, as determined 20 h after inoculation. At the same time, the biomass of the pathogen was reduced in the presence of *T. harzianum* T39, mainly due to an effect on germ-tube elongation and, to a lesser extent, on germination rate. In the presence of *T. harzianum* T39, germination of *B. cinerea* conidia and subsequent penetration of host tissue occurred, as was evident based on the initial formation of symptoms. However, although disease severity on the control leaves developed rapidly, it progressed much more slowly on the leaves treated with the biocontrol agent.

Because pectic enzymes are considered the principal enzymes involved in pathogenicity (41), we studied the effect of the biocontrol agent on the pectin-degrading enzymes of the pathogen on leaves. Borowicz et al. (3) previously suggested inhibition of fungal enzymes by the plant growth-promoting bacteria *Pseudomonas* spp. in vitro.

Very little information is available concerning the activity of the pectic enzymes of *T. harzianum* in vivo. In our study, no PG, PME, or PL activity was detected on leaves treated with *T. harzianum* T39 alone. These results are in contrast to findings under in vitro conditions (G. Zimand, A. Kapat, Y. Elad, and I. Chet, *unpublished data*) and demonstrate the fact that fungi can produce different sets of enzymes as a function of growth conditions, as suggested by Hancock et al. (21).

Activities of PG, PME, and PL in *B. cinerea*, as well as disease severity, were high on bean leaves (Figs. 2A and 6), similar to severity on other hosts, such as carrot, strawberry, raspberry, cabbage, and grapes (35). Two to four days after inoculation, while the amount of hyphae on leaves inoculated with *B. cinerea* alone and in the presence of *T. harzianum* T39 seemed to be the same, the activity of the pectin-degrading enzymes and disease severity were reduced in the presence of the biocontrol agent (Figs. 2A and 6). These reductions could be due simply to the reduction in *B. cinerea* biomass during the initial 20 h after inoculation, but they also could be a direct or indirect effect of *T. harzianum* T39 on the *B. cinerea* enzymes. Our previous results (45) indicated that *T. harzianum* T39 inoculated on bean leaves did not produce any detectable substances inhibitory to several microorganisms, including *B. cinerea*. For this reason, direct and indirect effects of *T. harzianum* T39 on the *B. cinerea* enzymes were considered here. *T. harzianum* T39 could manifest a direct effect on enzyme production, activation, or secretion of enzymes. Such an effect has been suggested by Milling and Richardson (34) and Daniels and Lucas (10) as the mode of action for the fungicide pyrimethanil against *Botrytis*. Alternatively the direct effect could be similar to that suggested by Pietr et al. (38), i.e., secretion of proteolytic enzymes that affect the pathogen's enzymes. Indirect effects of *T. harzianum* T39 could involve a leaf's defense mechanisms.

Many authors have reported that PG enzyme group activity is a major component of the infection process (1,9) and that they are the first enzymes secreted by germinating and nongerminating conidia of *B. cinerea* (29,43). For this reason, PG activity was selected for further study. *B. cinerea* produces a number of isoenzymes in vitro and in vivo belonging to the PG group (11,29,41). The production of several isoenzymes of PG has been reported for other pathogens, including *Sclerotinia sclerotiorum* (32).

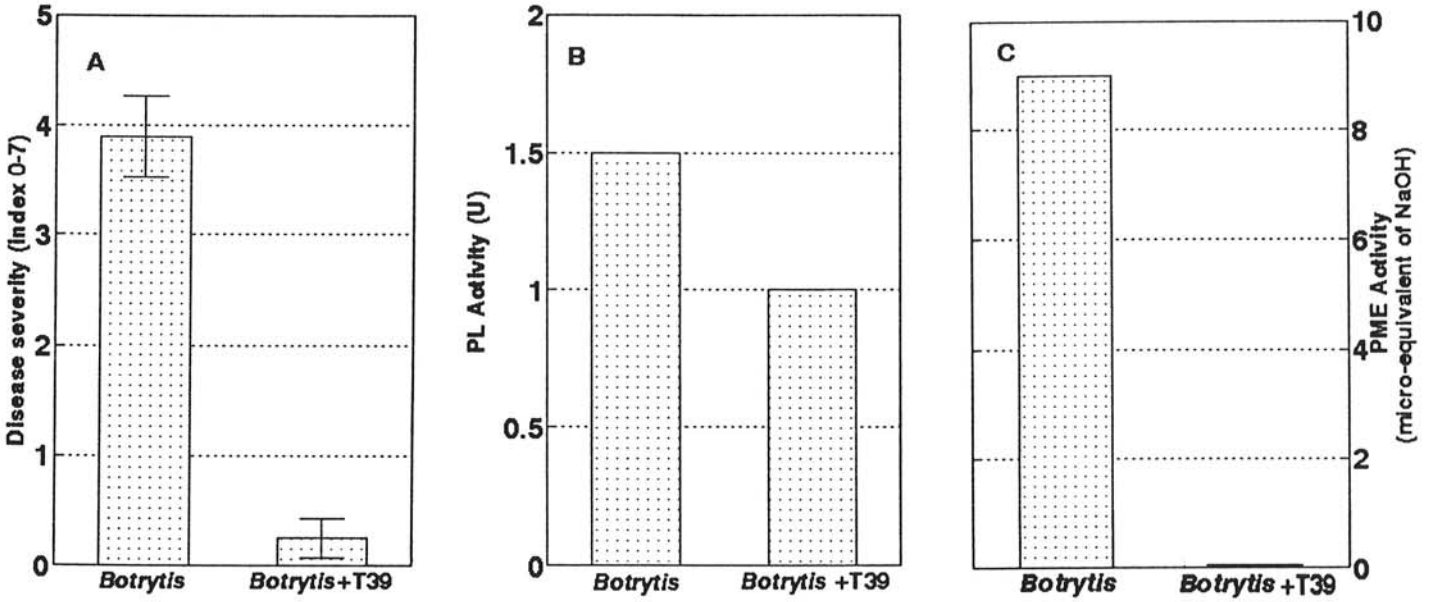

Fig. 6. Effect of *Trichoderma harzianum* T39 on *Botrytis cinerea*, supplemented with a concentration of 4 g of KH_2PO_4 per liter 3 days after inoculation. **A,** Disease severity was indexed on a scale of 0 to 7, where 0 = healthy leaf and 7 = expansion zone of necrotic lesions >5 mm. **B,** Pectate lyase (PL) activity. One unit = the amount of enzyme that caused an increase of 2.6 in absorbance at 235 nm, which is equivalent to the release of 1 μmol aldehyde groups. **C,** Pectin methyl esterase (PME) activity is expressed as microequivalents of NaOH absorbed per milliliter of filtrate produced on bean leaves per hour. Vertical bars indicate the standard error. No variability in enzymatic activity was detected in any of the treatments under these conditions.

The effect of *T. harzianum* T39 on the activity of the pathogen's enzymes was tested in the presence of various concentrations of KH_2PO_4. Van den Heuvel and Waterreus (41) suggested that KH_2PO_4 enhances disease development and PG activity. Therefore, we tested possible competition between the pathogen and biocontrol agent for this compound, which may cause a reduction in pectic enzyme activity. However, we found no evidence of such competition on bean leaves.

In the presence of *T. harzianum* T39 on bean leaves, the production of some PG isoenzymes of the pathogen are delayed (Figs. 4 and 5), and their activities are reduced (Fig. 4). We suggest that the effect on disease severity is a combination of two phenomena: the first is a direct effect of *T. harzianum* T39 on the enzyme's activities. However, because the direct effect on enzyme activity may not be sufficient to cause such disease reduction, a possible combination with an indirect effect on a leaf's defense mechanism is suggested. Urbanek et al. (40) found that *B. cinerea* PG elicits defense mechanisms in bean leaves. Cervone et al. (5) suggested that the accumulation of pectic enzyme products, i.e., oligogalacturonides with a degree of polymerization higher than nine, act as elicitors of defense mechanisms of plants. We suggest that in the presence of *T. harzianum* T39, the intensity of the pectolytic enzyme activity is reduced. Therefore, the bigger oligogalacturonides may accumulate and elicit the host plant's defense mechanisms, slowing the development of disease severity. Indeed, under conditions in which no control was achieved by the biocontrol agent, no reduction in PG activity was found. Moreover, other *Trichoderma* isolates did not reduce disease severity and did not affect PG activity (Table 1).

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