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The role of *Trichoderma harzianum* protease in the biocontrol of *Botrytis cinerea*

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Abstract

The role of protease of *Trichoderma harzianum* in the biocontrol of *Botrytis cinerea* was examined. Two isolates of *T. harzianum* were compared for their ability to produce protease in liquid culture medium and on the surface of bean leaves. The biocontrol agent *T. harziaum* T39 produced 58 mU/ml of protease and *T. harzianum* NCIM1185 produced 54 mU/ml on the 5th day of growth in liquid culture medium. On bean leaves, combinations of *B. cinerea* and *T. harzianum* isolates were examined for the synthesis of protease. The protease activities were 0.9 and 0.6 mU/ml for *T. harzianum* T39 and NCIM1185, respectively, and 0.5 mU/ml for *B. cinerea* alone after 48 h of incubation. In the presence of *T. harzianum* T39 culture liquid containing protease, a 55% reduction in *B. cinerea* germination and a 80% reduction in the germ tube length were observed after 17 h of incubation *in vitro*. When *T. harzianum* isolates were added to *B. cinerea* on bean leaves, increased synthesis of protease was observed (1.0 and 1.2 mU/ml for T39 and NCIM1185, respectively). In the presence of *T. harzianum* NCIM1185 protease, although the rate of germination was reduced, *B. cinerea* attained 98% germination after 17 h of incubation. The hydrolytic enzymes produced by *B. cinerea*, endo-polygalacturonase (PG) and exoPG were partially deactivated by protease from the *T. harzianum* isolates. Carboxymethyl cellulase was deactivated only by protease of NCIM1185. On the surface of bean leaves, the protease (obtained from liquid culture medium of *T. harzianum* isolates) resulted in 56–100% reduction of disease severity. The culture liquid containing protease synthesized on the surface of bean leaves treated with *B. cinerea* and with *T. harzianum* was collected and added to fresh leaves infected by *B. cinerea*. There was 56–100% and 30–75% reduction of disease severity with liquid droplet collected from the leaves treated with *T. harzianum* T39 and NCIM1185, respectively. Increased control of disease was obtained by combining the conidia of *T. harzianum* isolates with protease obtained from culture media. Protease inhibitors, trans-epoxysuccinyl-L-leucylamido-(4-guanidino)butane (E64), antipain hydrochloride, and a mixture of inhibitors, but not pepstatin A, fully or partially nullified the biocontrol effect of T39. T39 was found to be a poor producer of chitinase and β-1,3-glucanase *in vitro*. These enzymes were not detected on leaves treated with T39. Involvement of protease in biocontrol of *B. cinerea* is suggested.

Introduction

Botrytis cinerea Pers.: Fr., the causal agent for grey mould, inflicts serious food and ornamental crop losses, particularly after harvest (Elad, 1997). *Trichoderma harzianum* has been extensively used as a biocontrol agent because it affects a large variety of phytopathogenic fungi that are responsible for major crop diseases (Elad and Chet, 1995). Several alternative mechanisms may be responsible for biocontrol, including the production of volatile and non-volatile antibiotics, production of fungal cell-wall-degrading enzymes, competition for key nutrients or elements, including those responsible for activating germination of quiescent pathogen propagules, and induced resistance (Elad, 1996).

T. harzianum T39 competes with germinating *B. cinerea* conidia in the early stages of the interaction

(Elad, 1996). It does not act by means of volatile or non-volatile antibiotics. The role of chitinases and β-1,3-glucanase in the control achieved by *T. harzianum* T39 was assessed by comparing the enzymatic activities of five isolates of the same species and correlating them with control achievements; there was no correlation between the ability to degrade *B. cinerea* cell-wall polymers and biocontrol activity. In any case, no mycoparasitism has been observed in the interaction between T39 and *B. cinerea* (Elad, 1996). Therefore, no role for chitinases and glucanases in the control of *B. cinerea* by *T. harzianum* T39 has been demonstrated.

Interference with pathogenicity processes has been suggested as a mode of interaction between *T. harzianum* T39 and *B. cinerea*. The effect of *T. harzianum* T39 on *B. cinerea* germination and on lesion production by the pathogen has been tested on bean leaves (Zimand et al., 1996). Initially, 20 h after application, the biomass of germ tubes of the pathogen was reduced by the biocontrol agent, possibly due to nutrient competition, but no difference in the amount of pathogen mycelium could be observed 48 h after inoculation. Nevertheless, the activity of the pectolytic enzymes, polygalacturonase (PG), pectin methyl esterase (PME) and pectate lyase (PL), produced by *B. cinerea* on bean leaves, was reduced in the presence of *T. harzianum* T39 and disease was significantly reduced by the antagonist (Zimand et al., 1996). These reductions in enzyme activities were detected even 4 days after inoculation (Zimand et al., 1996). The activities of chitinase, β-1,3-glucanase and cutinase, but not that of cellulase, were also reduced in the presence of the antagonist (Kapat et al., 1998b). This indicates a mechanism of biocontrol by which the antagonist interferes with the pathogenicity process. It could be a direct action of T39 on *B. cinerea* hydrolytic enzymes, or it could be an indirect effect, by affecting plant factors that induce enzymatic activity in *B. cinerea*.

Protease production is common for microorganisms including fungi (North, 1982), among which *Trichoderma* spp. are well known producers (Haab et al., 1990). Proteolytic activity of *T. viride* was claimed to be involved in biocontrol of *Sclerotium rolfsii* in autoclaved soil (Rodriguez-Kabana, 1978). Endoproteinase of *Bacillus megaterium* can inactivate extracellular enzymes of *Rhizoctonia solani* (Bertagnolli et al., 1996). One member of the group of hydrolytic enzymes that is believed to play an important role in mycoparasitism by *T. harzianum* was purified and identified as a serine protease (Geremia et al., 1993). Autoclaved mycelium, fungal cell wall preparations or chitin induces this enzyme and its production is repressed by glucose: it is not induced in the presence of glucose. Protease activity of *T. harzianum* increases when the liquid culture medium contains organic nitrogen sources (Kapat et al., 1996). It has been suggested that this protease is involved in the degradation of pathogen cell walls, membranes and even proteins released by the lysis of the pathogen, thus making nutrients available for the mycoparasite (Goldman et al., 1994). However, the role of proteases in the control of *B. cinerea* by *T. harzianum* T39 has not been demonstrated.

We report here the effect of protease of the biocontrol agent *T. harzianum* T39 and of *T. harzianum* NCIM1185 (Arnab et al., 1996) on the control by *B. cinerea*.

Materials and methods

Fungi and plants

Two isolates of *Trichoderma harzianum* Rifai were used: the biocontrol agent *T. harzianum* T39 (Elad, 1994; Zimand et al., 1996) and *T. harzianum* NCIM1185 (Biochemical Engineering Laboratory, Department of Chemical Engineering, Indian Institute of Technology, Madras, India). The pathogen, *Botrytis cinerea* Pers.: Fr., originally isolated from a cucumber fruit (Elad, 1988), was maintained on potato dextrose agar (PDA) at $22 \pm 2\,^{\circ}$C. Conidial suspensions were prepared by washing 4-day old cultures of *T. harzianum* or 14-day old cultures of *B. cinerea* in sterilized water. The *T. harzianum* isolates are referred to as T39 and NCIM1185 hereafter. Dead cells were obtained by heating the conidial suspensions for 10 min at $100\,^{\circ}$C, centrifuging the suspensions and resuspending the cells in sterilized water.

Plants of bean (*Phaseolus vulgaris* L.) were planted in 1-l plastic pots containing peat, vermiculite and volcanic gravel (2 : 1 : 2) and grown in a greenhouse at $22 \pm 3\,^{\circ}$C. Leaves were collected from 3 to 4-week-old plants. Detached leaves were incubated in $30 \times 45 \times 5$ cm plastic trays on a plastic grid that was laid over water-soaked filter paper. The trays were kept inside a transparent polyethylene bag to prevent desiccation and incubated at $22 \pm 2\,^{\circ}$C for 7 days.

Treatment of plant material

Plant material was inoculated with a 10^5 ml^{-1} conidial suspension of *B. cinerea* containing 0.02 M glucose and 0.02 M KH_2PO_4 to promote infection (Leone and Tonneijick, 1990). Detached leaves were inoculated with 30-µl drops containing suspension of *B. cinerea*. The droplets were harvested every 24 h for 3 days. The liquid collected from the surface of the leaves was centrifuged at 1000*g* for 10 min before being stored at −20 °C. The sample was assayed for protease. Experiments were conducted on the surface of bean leaves with conidia of *B. cinerea* alone and with various combinations of *B. cinerea* conidia, conidia of *T. harzianum* (10^6 ml^{-1}) and protease from *T. harzianum*. Since nutrients promote *B. cinerea* germination and infection (Elad, 1997), in experiments involving the addition of culture filtrate a second *B. cinerea* control treatment was included, in which the pathogen was also supplemented with potato dextrose broth (PDB) as rich nutrient source.

The extent of germination of *B. cinerea* conidia in droplets on detached leaves was monitored up to 17 h of incubation. Leaf pieces bearing a drop of the interacting microorganisms were placed on glass slides, dyed with aniline blue, incubated at room temperature for 5 min and observed under a light microscope. The germ-tube lengths were measured with an ocular scale (Elad et al., 1994a,b).

Symptoms of the drop-inoculated leaves were evaluated beneath the inoculation droplets according to an eight-level rot intensity index (0–7) where 0 = symptomless leaf tissue, 1 = 1–12% rot, 2 = 13–25% rot, 3 = 26–50% rot, 4 = 51–100% rot, 5 = rot extending about 1 mm around the droplet, 6 = rot extending 1–3 mm and 7 = rot extending beyond 3 mm (Elad et al., 1994a,b).

Production of protease in vitro

Conidia from 4-day-old potato dextrose agar (PDA) cultures of the *T. harzianum* isolates (10^6 ml^{-1}) were placed aseptically into 250-ml Erlenmeyer flasks containing 50-ml liquid of production medium (PDM). The growth medium contained (g l^{-1}): $(NH_4)_2SO_4$, 1.4; KH_2PO_4, 2.0; NaH_2PO_4, 6.9; $MgSO_4 \cdot 7H_2O$, 0.3; dextrose, 1.0; peptone, 10; urea, 0.3 (Anjanikumari and Panda, 1985). The initial pH was adjusted to 5.0 using 1 N HCl. The organisms were grown for 36 h at 22 °C on a reciprocal shaker, 180 cycles/min. After the specified incubation period, mycelial pellets were transferred to new PDM. The fermentation broth was sampled once every 24 h and was centrifuged at 1,000*g* at 4 °C for 10 min. The culture filtrate was dialyzed and stored at −20 °C until it was assayed for protease activity.

Assay of protease

A reaction mixture containing 0.8 ml of Hammerstein casein (6.0 g l^{-1}, dissolved in 0.05 M phosphate buffer, pH 6.0) and 0.2 ml of culture filtrate was incubated without shaking at 37 °C for 15 min. The sample was then centrifuged at 1,000*g* for 10 min. One ml of the supernatant was used for the assay of tyrosine, according to Lowry et al. (1951). One unit of enzyme activity was defined as the amount of enzyme required for the formation of 1 µmol of the product per minute of the reaction, under the standard assay conditions. The activities of the dialyzed protease solutions used throughout the study are mentioned in the relevant Results sections.

Deactivation study

The *in vitro* study of enzyme deactivation was carried out with three hydrolytic enzymes synthesized by *B. cinerea* (exo- and endo-polygalacturonase (PG) and carboxymethyl cellulase (CMCase)). Liquid culture containing the hydrolytic enzymes were obtained from the liquid culture of *B. cinerea* grown on specific carbon source (Kapat et al., 1998a). Equal volumes of the culture filtrate containing a specific hydrolytic enzyme and *T. harzianum* growth liquid containing protease were incubated for 16 h at 22 °C. A mixture of an equal volume of sterile distilled water and culture filtrate containing protease was used as a control. The enzyme activities were measured at $t = 0$ min. Samples were taken at 30 min intervals and assayed for the residual activities of the enzymes.

Assays of polygalacturonases and carboxymethylcellulase

ExoPG activity was determined by measuring the rate of increase of galacturonic acid concentrations in enzyme–substrate reaction mixture with dinitrosalicylic (DNS) acid reagent (Miller, 1959). The reaction mixture contained 0.4 ml of 0.25% polygalacturonic acid dissolved in sodium acetate buffer (0.05 M, pH 5.2) and 0.1 ml of culture filtrate. The net increase of

reducing sugar in the reaction mixture was determined by comparing the measured optical densities with a standard curve prepared with pure galacturonic acid. One unit of enzyme activity was defined as the amount of enzyme that catalyzed the release of 1 μmol of galacturonic acid per minute per ml of the culture filtrate under the assay conditions.

EndoPG breaks down polygalacturonic acid randomly to form polymer fragments with a reducing group at the end; this was measured by the Nelson Somogy method (Collmer et al., 1988). The reaction mixture contained 1 ml of substrate (2% polygalacturonic acid : 0.4 M NaCl : 0.2 M sodium acetate buffer pH 5.2 : distilled water 1 : 1 : 1 : 1) and 50 μl culture filtrate. One unit of enzyme activity was defined as the amount of enzyme that released 1 μmol of reducing groups per min per ml of culture filtrate, under the assay conditions.

CMCase was measured in terms of the reducing sugar released as a result of enzyme–substrate reaction by Miller's (1959) method. The reaction mixture contained 0.3 ml of substrate (1% CMC dissolved in 50 mM acetate buffer, pH 5.2) and 0.2 ml of culture filtrate. One unit of enzyme activity was defined as the amount of enzyme that catalyzed the release of 1 μmol glucose per min per ml of culture filtrate, under the assay conditions.

Production of chitinase and β-1,3-glucanase

The fungi were grown in liquid culture as mentioned above for protease production except that colloidal chitin and laminarin served as sole carbon sources for the induction of the respective enzymes and urea was omitted from the medium. In a separate set of experiments the production of these enzymes in the above mentioned PDM was tested.

Colloidal (acid swollen) chitin was prepared as follows: one gram of chitin (crab shell) was added to 10 ml of 85% orthophosphoric acid. The mixture was stirred to make a gelatinous paste and stored at 0 °C for 24 h. The gelatinous mixture was reprecipitated into an excess of cold (15 °C) distilled water. It was made to a paste using a pestle and mortar and then resuspended in Na-acetate buffer (50 mM, pH 4.75). The suspension was stored in a sterile container at 10 °C.

Chitinase in liquids originating from *in vitro* culturing or from the surface of bean leaves was assayed as follows: a tube containing reaction mixture [0.55 ml of acid swollen chitin (5 gl^{-1}, suspended in 50 mM Na-acetate buffer, pH 4.75), 0.30 ml of acetate buffer (50 mM, pH 4.75) and 0.15 ml of culture filtrate] was incubated at 47 °C for 1 h in unstirred condition. The reaction was terminated by adding 1 ml of potassium sodium-tartrate reagent to the reaction mixture (Monreal and Reese, 1969). A control was taken where the enzyme was deactivated by adding 1 ml of potassium sodium-tartrate reagent before the commencement of incubation. The alkaline tartrate reagent was prepared by mixing 270 g potassium sodium-tartrate dissolved in 400 ml distilled water and a solution of 6.25 g of phenol in 10% sodium hydroxide solution (17.5 g NaOH in 175 ml distilled water). The reagent was stored in a dark bottle. One milliliter of dinitrosalicylic acid (DNS) reagent was added to the tubes and the tubes were kept in a boiling water bath for 5 min. DNS reagent was prepared by dissolving 1.5 g of 3,5-dinitrosalicylic acid in 100 ml of distilled water. The reaction mixture was cooled to 30 °C. Seven milliliters of distilled water were added to each tube to make up the final volume to 10 ml. The mixture was centrifuged at 5000 rpm for 5 min and the intensity of colour (formed due to the reaction of N-acetyl-D-glucosamine released with dinitrosalicylic acid) was measured at 540 nm by a spectrophotometer (Miller, 1959).

β-1,3-Glucanase in liquids originating from *in vitro* culturing or from the surface of bean leaves was measured in a 0.7 ml reaction mixture contained 0.2 ml of enzyme sample and 0.5 ml of laminarin (50 μg) in citrate buffer (50 nM, pH 4.8). The reaction mixture was incubated at 50 °C for 10 min (Elad et al., 1982). The reducing sugars liberated were estimated by the dinitrosalicylic acid method (Miller, 1959).

Protease inhibitors

The protease inhibitors (Wetherall et al., 1995) antipain hydrochloride (Sigma), trans-epoxysuccinyl-L-leucylamido-(4-guanidino)butane = E64 (Sigma) and pepstatin A (Sigma) and the protease inhibitor cocktail Complete™ (Boehringer Mannheim) were added to the drops of suspensions of *B. cinerea* or *B. cinerea* + *T. harzianum* conidia on bean leaves.

Statistical analysis

Experiments were arranged in a completely randomized design and repeated at least twice. Treatments were replicated at least six times. Data were arcsin transformed, subjected to analysis of variance, and

compared statistically according to Fisher's protected least significant difference test at $P \leq 0.05$.

Results

Effect of live or dead T. harzianum *conidia on* B. cinerea

Leaves of bean were treated with droplets containing suspensions of live or dead *T. harzianum* isolates and *B. cinerea* (Figure 1). The presence of killed or live T39, however, reduced disease severity by 59 and 77%, respectively after 3 days. The disease reduction by NCIM1185 was low (41% reduction) compared with the effect obtained with T39. The killed NCIM1185 did not reduce the disease (Figure 1).

Production of chitinase, β-1,3-glucanase and carboxy methyl cellulase by Trichoderma harzianum *in liquid culture medium and on bean leaves*

Isolate NCIM1185 was found to be a producer of chitinase and β-1,3-glucanase *in vitro*. Levels of chitinase ($\pm$S.E.) were 0, 0.45 ± 0.03, 0.61 ± 0.05, 3.32 ± 0.21, 15.98 ± 0.12 and 8.14 ± 0.06 mU/ml and levels of β-1,3-glucanase were 0, 0, 0, 1.48 ± 0.09, 0.37 ± 0.04 and 0.74 ± 0.06 mU/ml after 1, 2, 3, 4, 5 and 6 days of incubation. Whereas T39 was found to be a poor producer of chitinase and β-1,3-glucanase. Levels of chitinase were 0, 0, 0, 0.31 ± 0.02, 0.6 ± 0.07 and 0.68 ± 0.05 mU/ml and levels of β-1,3-glucanase were 0, 0, 0, 0, 1.11 ± 0.03 and 1.11 ± 0.02 mU/ml after 1, 2, 3, 4, 5 and 6 days of incubation. Forty to 65% lower activities of the enzymes were detected in PDM cultures.

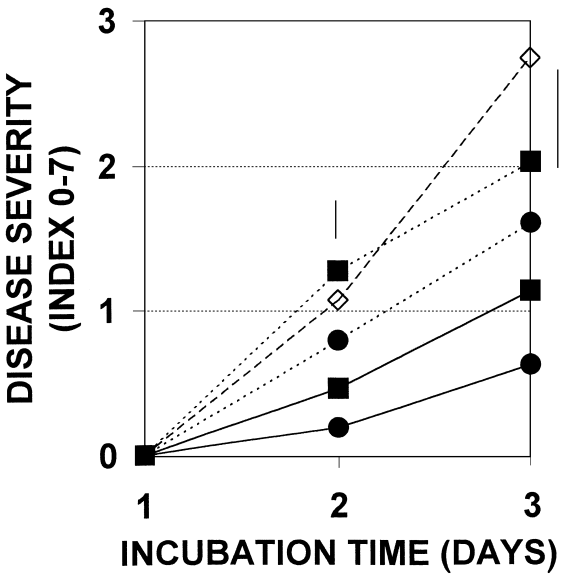

Figure 1. Effect of live (●) or dead (■) *Trichoderma harzianum* (—— = T39, - - - = NCIM1185) conidia on disease inflicted by *Botrytis cinerea* on bean leaves. - - -◇ = control. Disease was evaluated according to a 0–7 scale where 0 = symptomless. Bar = LSD for each sampling date.

The production of chitinase, β-1,3-glucanase and CMCase on bean leaves by live *B. cinerea* was significant (Table 1). The enzymes were not detected when suspensions of the *T. harzianum* isolates were placed on the leaves. Chitinase and β-1,3-glucanase of *B. cinerea* were found in lower levels when the pathogen was co-inoculated with live conidia of each of the *T. harzianum* isolates on the leaves whereas CMCase of *B. cinerea* was not suppressed.

Production of protease by Trichoderma harzianum *in liquid culture medium and on bean leaves*

Isolates T39 and NCIM1185 were good producers of protease *in vitro*, and the enzyme was already found in 1-day-old cultures. The level of enzymes activity gradually rose as fermentation progressed in both cases (data not shown). The maximum levels of protease were 58 mU/ml for T39 and 54 mU/ml for NCIM1185 on day 5.

Different combinations of *B. cinerea* and the *T. harzianum* isolates were tested for the synthesis of extracellular protease on the surface of the bean leaves (Figure 2). When live *B. cinerea*, T39 and NCIM1185 were grown alone, the protease levels (after 48 h) were 0.7 mU/ml, 0.9 mU/ml and 0.6 mU/ml, respectively. Application of killed *B. cinerea*, T39 and NCIM1185 resulted in similar levels of protease on the bean leaves. All the above mentioned levels were significantly not different from levels detected on untreated leaves. The isolates of *T. harzianum*. T39 and NCIM1185 synthesized protease on the leaves when applied alone (0.95 mU/ml and 2.25 mU/ml after 48 h, respectively, the later significantly different from the untreated control). The presence of live *B. cinerea* conidia in combination with conidia of *T. harzianum* T39 resulted in a significant increased level of extracellular protease on the surface of the bean leaves (1.35 mU/ml after 72 h) (Figure 2).

Table 1. Production of chitinase, β-1,3-glucanase and carboxymethylcellulase (mU ± SE) by *Trichoderma harzianum* isolates and by *Botrytis cinerea* on the surface of bean leaves

B. cinerea state	*T. harzianum* isolate and state	Incubation time (days)	Enzyme		
			Chitinase	β-1,3-glucanase	Carboxymethylcellulase
Water control	—	1–3	0	0	0
+, Heat killed	—	1–3	0	0	0
—	T39, heat killed	1–3	0	0	0
—	NCIM1185, heat killed	1–3	0	0	0
+, Live	—	1	24.8 ± 4.7	58.4 ± 9.3	57.4 ± 5.1
		2	20.7 ± 2.9	55.6 ± 8.4	59.7 ± 4.6
		3	14.1 ± 1.7	28.7 ± 5.6	42.4 ± 4.1
—	T39, live	1–3	0	0	0
—	NCIM1185, live	1–3	0	0	0
+, Killed	T39, live	1–3	0	0	0
+, Killed	NCIM1185, live	1–3	0	0	0
+, Live	T39, killed	1	20.4 ± 4.1	46.3 ± 6.5	49.3 ± 3.9
		2	22.6 ± 3.1	44.4 ± 6.8	55.6 ± 4.7
		3	13.6 ± 1.3	22.2 ± 4.3	44.1 ± 4.1
+, Live	NCIM1185, killed	1	14.7 ± 3.2	37.0 ± 6.5	46.2 ± 3.9
		2	9.05 ± 2.1	28.8 ± 5.1	43.4 ± 3.8
		3	6.4 ± 1.0	22.3 ± 4.7	40.4 ± 3.3
+, Live	T39, Live	1	7.9 ± 1.3	6.1 ± 0.7	49.1 ± 5.3
		2	3.9 ± 0.5	2.7 ± 0.2	59.3 ± 5.1
		3	0.5 ± 0.01	6.5 ± 0.8	52.0 ± 4.3
+, Live	NCIM1185, Live	1	4.3 ± 0.7	4.3 ± 0.5	38.0 ± 3.7
		2	3.8 ± 0.4	8.0 ± 0.9	48.0 ± 5.9
		3	3.5 ± 0.3	5.6 ± 0.6	34.6 ± 4.5

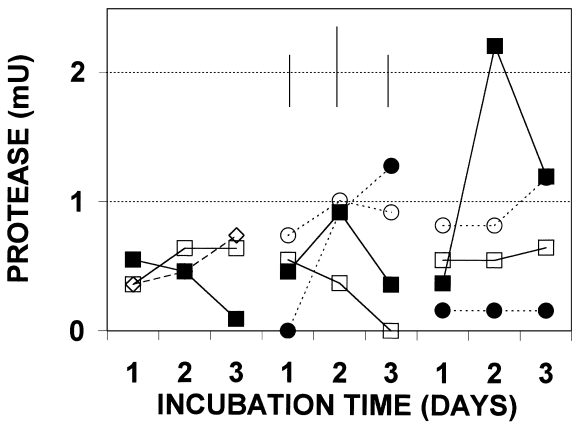

Figure 2. Presence of extracellular protease on the surface of bean leaves treated with *Trichoderma harzianum* isolates and with *Botrytis cinerea*. Bar = LSD for each sampling date. Left: - - -◇ = water control with no added fungus, ■ = *B. cinerea* alone and □ = killed *B. cinerea* alone. Center: *T. harzianum* T39 alone killed (□) or live (■), each alone — or with *B. cinerea* - - - (Killed T39 = ◯ and live T39 = ●). Right: *T. harzianum* NCIM1185 alone killed (□) or live (■), each alone — or with *B. cinerea* - - - (Killed NCIM1185 = ◯ and live NCIM1185 = ●).

Effect of protease on the germination of B. cinerea *conidia*

The germination of *B. cinerea* conidia was observed and quantified in the presence of *T. harzianum* proteases (the activity of dialyzed protease was 54 and 58 mU/ml for T39 and NCIM1185, respectively). *B. cinerea* attained 100% germination (average germ-tube length, 100 μm) at 17 h of incubation (Figure 3). In the presence of T39 protease, a 55% reduction in germination was noticed at 17 h of incubation (average germ-tube length, 16 μm) and, in presence of NCIM1185 protease, although the germination was slowed, *B. cinerea* was able to overcome the inhibition in the later hours of incubation, attaining 98% germination (Figure 3).

Effect of protease on the hydrolytic enzymes produced by B. cinerea *in vitro*

Deactivation studies were carried out using a culture filtrate protease from *T. harzianum* isolates (Figure 4). Protease (50 mU/ml) was added to solutions of three

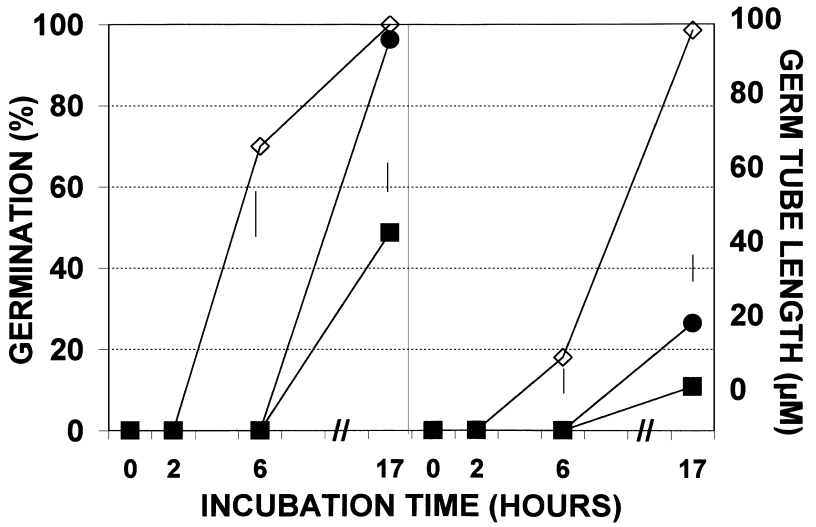

Figure 3. Effect of crude protease from cultures of *Trichoderma harzianum* T39 (■) and NCIM1185 (●) on the germination rate of conidia of *Botrytis cinerea* (left) and on the development of germ tubes (right) on the surface of bean leaves. Bar = LSD for each sampling date. ◊ = Control.

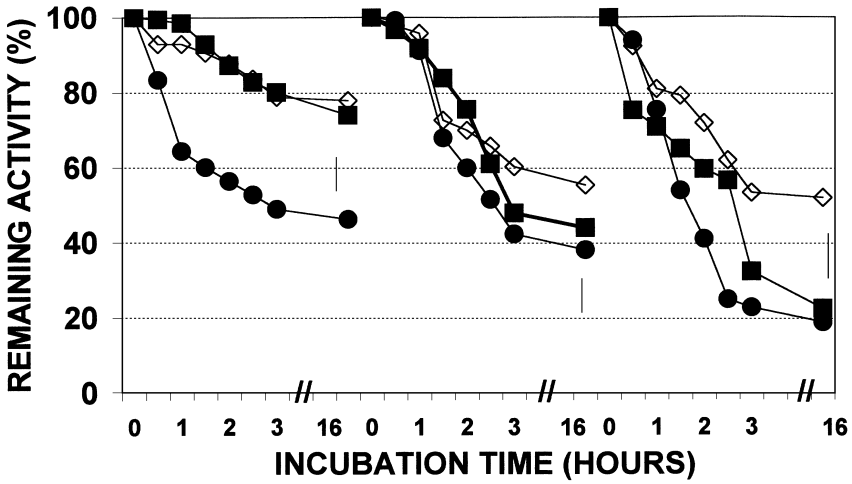

Figure 4. Effect of crude protease from cultures of *Trichoderma harzianum* T39 (■) and NCIM1185 (●) on the residual activity of the enzymes, carboxymethyl cellulase (left), endo-polygalacturonase (center) and exo-polygalacturonase (right) of *Botrytis cinerea*. ◊ = Control. Bar = LSD.

hydrolytic enzymes, exoPG, endoPG and CMCase, from *B. cinerea*. CMCase was affected only by protease from *T. harzianum* NCIM1185 (41% reduction in activity at the end of the incubation). Similar deactivation patterns were observed with endoPG and exoPG in the presence of proteases from T39 and NCIM1185. EndoPG was deactivated by 24–35% at the end of the incubation period and exoPG was deactivated by 58–62% at the end of the incubation period (Figure 4).

Effect of protease on the development of disease on bean leaves

In order to find out the effect of protease on disease severity, a series of experiments was conducted with deactivated or active protease contained in culture filtrates of *T. harzianum* (Figure 5). In the presence of deactivated proteases of either *T. harzianum* isolate, *B. cinerea* conidia inflicted severe necrosis on the bean

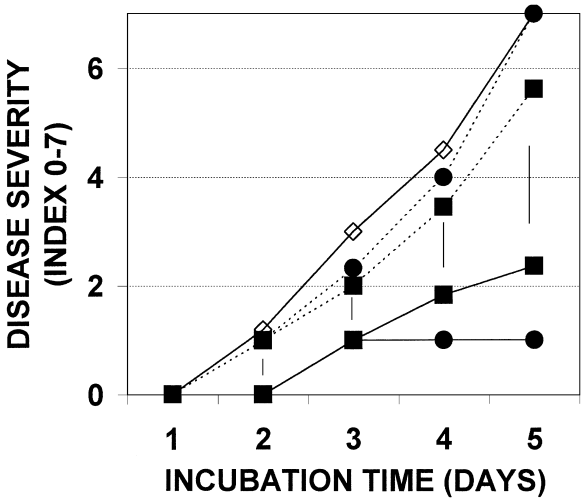

Figure 5. Effect of crude protease (untreated = — , heat deactivated = - - -) from cultures of *Trichoderma harzianum* T39 (■) and NCIM1185 (●) on the development of disease incited by *Botrytis cinerea* on bean leaves. Disease was evaluated according to a 0–7 scale where 0 = symptomless. ◊ = Control. Bar = LSD for each sampling date.

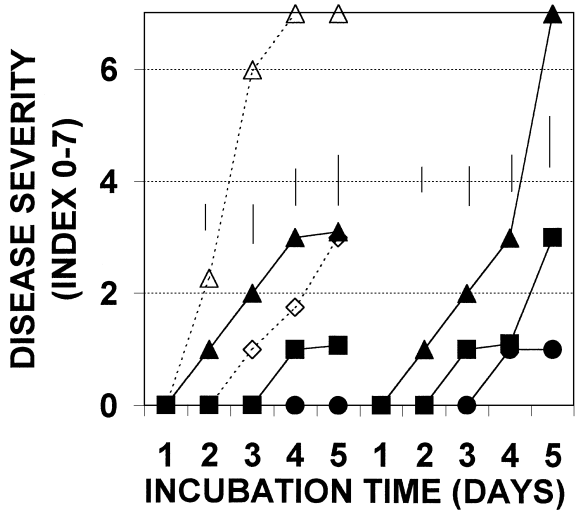

Figure 6. Effect of the combination of crude protease and conidia of *Trichoderma harzianum* T39 (left) and NCIM1185 (right) on the development of disease incited by *Botrytis cinerea* on bean leaves. - - - = controls of *B. cinerea* alone not supplemented with PDB (◊) or supplemented with PDB (△). — = *B. cinerea* with conidia of *T. harzianum* (■), supplemented with PDB (▲) or with protease (●). Disease was evaluated according to a 0–7 scale where 0 = symptomless. Bar = LSD for each sampling date.

leaves. The extent of disease control with untreated protease from *T. harzianum* T39 or NCIM was 56 or 86%, respectively after 5 days (Figure 5).

Combinations of dialyzed culture filtrates containing protease (activity of 52 and 59 mU/ml for T39 and NCIM1185 proteases, respectively) and live conidia of *T. harzianum* isolates were tested for their ability to suppress disease on bean leaves. *B. cinerea* was applied as conidia both in the presence and in the absence of PDB (Figure 6). In the presence of PDB, *B. cinerea* inflicted complete necrosis of the leaves by day 5, whereas in its absence the disease severity index was 3.5 on day 5. When protease of T39 and live conidia of the same organism were applied with *B. cinerea* conidia, complete control of disease was observed. Similarly, in the presence of protease of NCIM1185 and of the live conidia, the disease was controlled to the greatest extent (Figure 6).

Effect of liquid from bean leaves on B. cinerea

Experiments similar to those mentioned above were carried out with dialyzed droplets collected from the surface of bean leaves previously treated with *B. cinerea*, *B. cinerea* + T39 or *B. cinerea* + NCIM1185. The droplets were collected after 48–96 h of incubation and were applied to fresh leaves together with *B. cinerea* (Figure 7). When droplets from T39-treated leaves were applied, the disease severity was reduced as follows: 48-h droplets reduced severity by 39% and 72–96-h droplets reduced it by 53–56% on day 4. In the case of NCIM1185 + *B. cinerea* the 96-h droplets had a similar effect to the droplets originating from leaves treated with droplets of 96-h droplets of T39-treated leaves whereas 48–72 h droplets of NCIM1185-treated leaves were more effective in reducing the disease (Figure 7).

Delayed application of protease on bean leaves

In order to determine the time course of protease action in reducing the disease, the addition of culture filtrate from *T. harzianum* isolates containing protease was carried out at various times after inoculation with *B. cinerea* conidia on the surface of bean leaves. *B. cinerea* infection in either the absence or the presence of PDB (controls) reached disease severity indices of 2.5 and 7.0 on days 5 and 4, respectively. When the plant pathogen was inoculated together with heat-deactivated protease of either *T. harzianum* isolate from the beginning of incubation, no control of disease was observed (results not shown). The protease

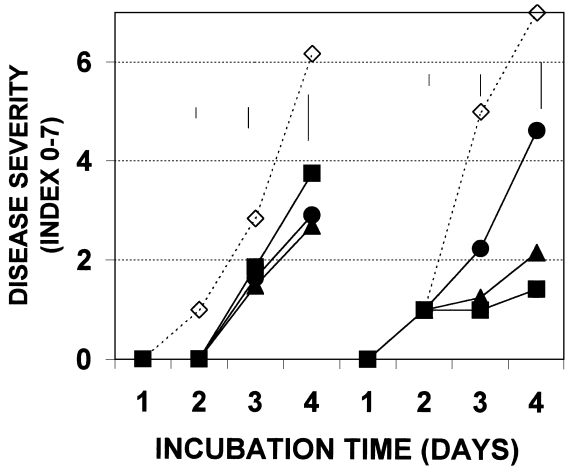

Figure 7. Effect of droplets collected from bean leaves treated with *Botrytis cinerea* and *Trichoderma harzianum* T39 (left) and NCIM1185 (right), after incubation of 48 h (■), 72 h (▲) and 96 h (●), on the development of disease incited by *Botrytis cinerea* on fresh bean leaves. - - - = *B. cinerea* inoculated with no additional liquid. Disease was evaluated according to a 0–7 scale where 0 = symptomless. Bar = LSD for each sampling date.

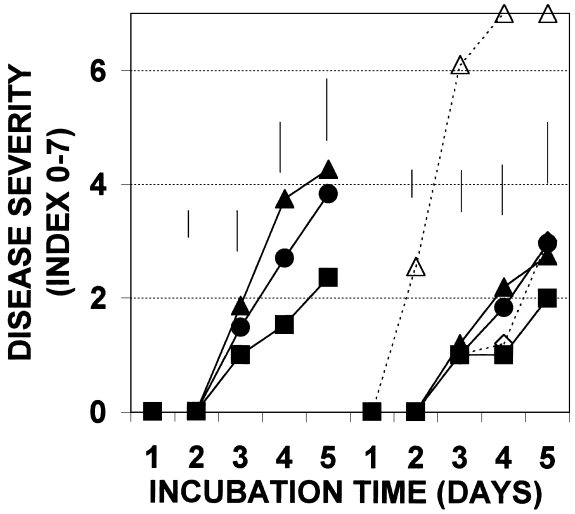

Figure 8. Effect of the time of application of crude protease from cultures of *Trichoderma harzianum* T39 (left) and NCIM1185 (right) on the development of disease incited by *Botrytis cinerea* on bean leaves. - - - = controls of *B. cinerea* alone, not supplemented with PDB (◊) or supplemented with PDB (△). — = *B. cinerea* with protease added at the same time (■), or protease added 2 (▲) or 3 (●) days after *B. cinerea* inoculation. Disease was evaluated according to a 0–7 scale where 0 = symptomless. Bar = LSD for each sampling date.

preparations of either isolate (42 and 48 mU/ml, respectively) had a substantial effect on the disease caused by *B. cinerea* (Figure 8). No change in the capacity to control the disease was noticed when NCIM1185 protease was added on day 2 or 3, as compared with earlier application of protease. Protease of T39 was more effective when applied on the day of inoculation with *B. cinerea* than 2–3 days later (Figure 8).

Effect of protease inhibitors on biocontrol of Botrytis cinerea

The effect of protease inhibitors on the suppression of *B. cinerea* by *T. harzianum* T39 was tested by application of the pathogen and the biocontrol agents along with various concentrations of the inhibitors and with heat treated compounds (Figure 9). Trans-epoxysuccinyl-L-leucylamido-(4-guanidino)butane did not significantly affect the infection potential of *B. cinerea* and the heat-treated higher concentrations of this inhibitor even increased it. Conversely, the inhibitor at 0.3–3.0 μg/ml, but not the heat-treated inhibitor, nullified the effect of the biocontrol agent (Figure 9A). Antipain hydrochloride inhibited the spread of *B. cinerea* symptoms, whereas the heat-treated inhibitor at all rates increased disease severity. This inhibitor nullified the effect of T39 on day 3 of incubation but not later and the heat-treated inhibitor had no effect on the capacity of the biocontrol agent to suppress disease (Figure 9B). Pepstatin A increased the severity of disease and did not nullify the effect of the antagonist (Figure 9C). The mixture of inhibitors, heat-treated or untreated, promoted the disease development, but only the non-treated mixture nullified the effect of T39 (Figure 9D).

Discussion

Live conidia of *T. harzianum* T39 achieved better control of *B. cinerea* on bean leaves than dead conidia. However, some suppression of disease was imposed by the dead conidia (Figure 1). This implies the possible involvement of induced resistance in the biocontrol of *B. cinerea* by T39, as previously suggested by De Meyer et al. (1998). In that work the resistance conferred to the root zone by treatment with T39 was observed in the foliage of bean, tomato and pepper plants, whereas in the present study, the possible resistance observed was locally induced.

This work is focused on the mechanism of biocontrol by *T. harzianum* by placing emphasis on the

effect of extracellular proteases synthesized on the germination and growth of *B. cinerea*. Since the capacity of *T. harzianum* isolates to produce extracellular protease had already been reported (Hagspiel et al., 1989; Haab et al., 1990; Manonmani and Joseph, 1993; Geremia et al., 1993), a detailed study on *T. harzianum* protease was carried out, in order to elucidate a protease-mediated biocontrol mechanism. Both T39 and NCIM1185 produced copious amount of protease in liquid culture and were able to synthesize protease on bean leaves. The production of protease by *T. harzianum* isolates was induced by the presence of live or dead conidia of the pathogen (Figure 2). The germination of *B. cinerea* conidia was affected by culture filtrates containing protease and other enzyme activities to a lesser extent. Assays with culture filtrates produced by T39 resulted in a substantial reduction in the rate of germination (Figure 3). Although the rate of

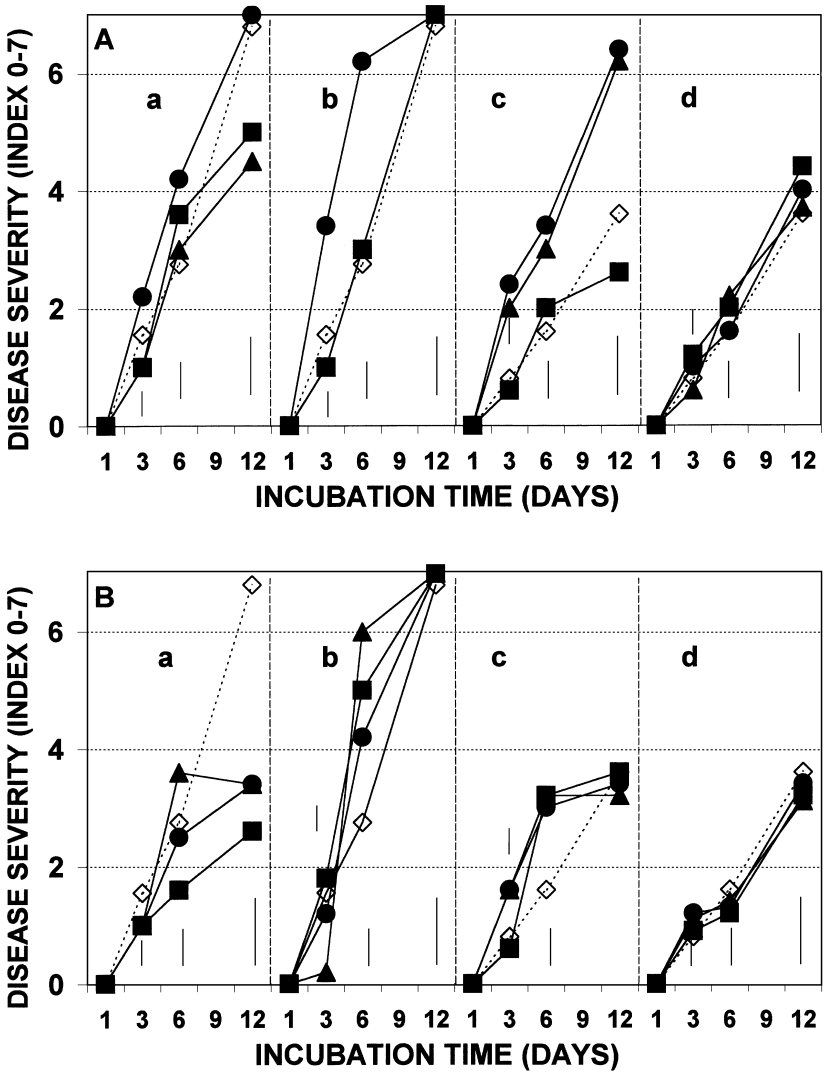

Figure 9. Effect of the protease inhibitors A – E64 = trans-epoxysuccinyl-L-leucylamido-(4-guanidino)butane, B – antipain hydrochloride, C – pepstatin A, and D – a mixture of inhibitors on disease incited by *Botrytis cinerea* alone (a and b) or with *Trichoderma harzianum* T39 (c and d). Untreated (a and c) or heat-treated (b and d) solutions of inhibitors were supplemented to the drops of the pathogen, alone or with the antagonist, at rates of 0 (- - -◇ 0.03 (■), 0.3 (▲) or 3.0 (●) μg/ml. Disease was evaluated according to a 0–7 scale where 0 = symptomless. Bar = LSD for each sampling date.

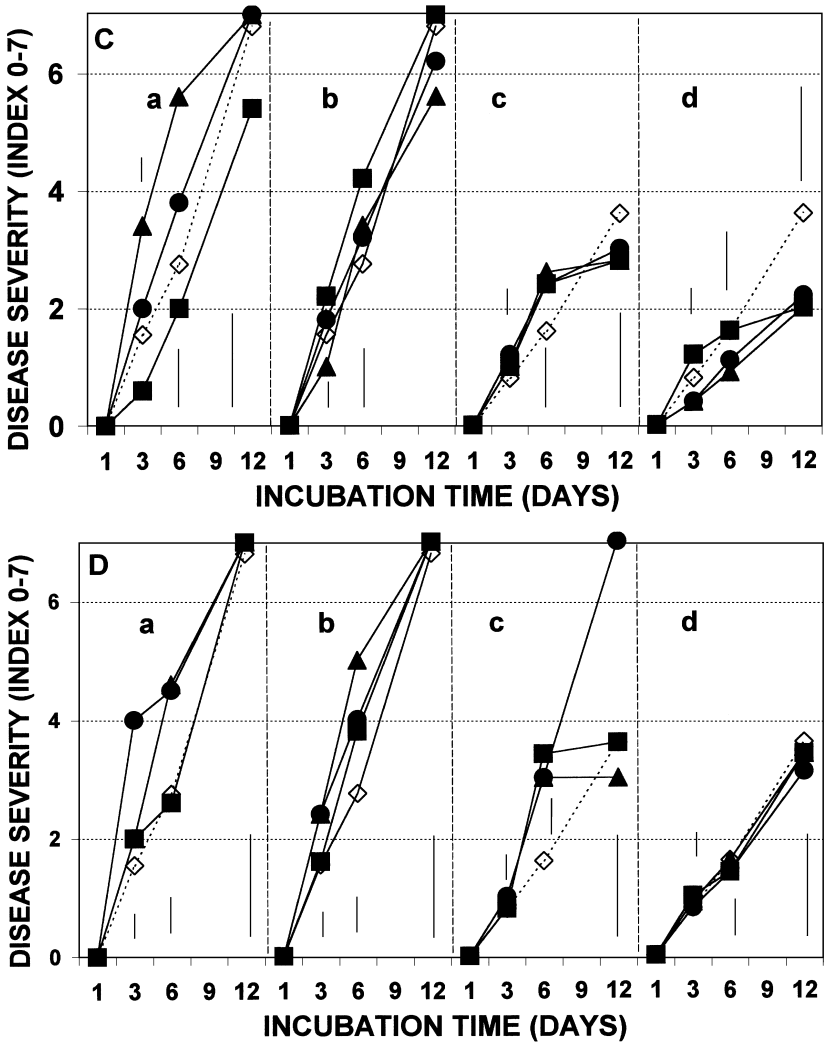

Figure 9. Continued.

germination was slow as compared to controls in the presence of NCIM1185 culture filtrate containing the effect was overcome by *B. cinerea* in the later phase of incubation. The difference in the inhibition achieved by the two isolates may stem from different protease profiles or from the presence of other compounds such as the hydrolytic enzymes chitinase, β-1,3-glucanase or cellulase in the liquid. However, for T39 it is certain that these enzymes are not involved since it is a very poor *in vitro* producer of them and these enzymes were not detected on bean leaves treated by the antagonist.

In vitro deactivation studies were performed to examine the inhibitory effect of protease on three hydrolytic enzymes (exoPG, endoPG and CMCase) produced by *B. cinerea*. The PGs were affected by protease produced by either isolate of *T. harzianum* (Figure 4) whereas CMCase was not affected in the presence of T39 protease. Indeed CMCase levels on *T. harzianum*-treated leaves were not affected by the presence of the antagonist (Kapat et al., 1998b). Similarly, the endoproteinase of *Bacillus megaterium* deactivated proteinase and pectin lyase but not cellulase and phospholipase A of *Rhizoctonia solani* (Bertagnolli et al., 1996).

To prove the capability of protease preparation to prevent the development of disease, both heat-deactivated and untreated *T. harzianum* proteases were

tested on the bean leaves. The culture filtrates containing proteases were able to prevent tissue necrosis (Figure 5), whereas the heat-treated proteases facilitated tissue necrosis to the greatest extent. It might be that the heat-deactivated proteases were used by the pathogen as an additional nutrient source. The combined effect of culture filtrate containing protease and live conidia of *T. harzianum* on *B. cinerea* was also examined. Complete control of the disease was observed in case of culture filtrate containing protease from T39 mixed with conidia (Figure 6) and a similar result was obtained when NCIM1185 protease was applied with live conidia. This effect may be due to limitation of *B. cinerea* enzymes and to other mechanisms exerted by live conidia of *T. harzianum*, such as competition for nutrients (Zimand et al., 1996).

Experiments were also carried out with the liquid containing protease collected at different times from droplets with either *B. cinerea* or *T. harzianum* conidia on the bean leaf surface. For both *T. harzianum* isolates, reduction of disease development was observed (Figure 7). The time course of protease action on the surface of bean leaves was monitored by applying protease at different times after inoculation. Early application of protease appeared to be most effective (Figure 8). The theory that protease has a role in the biocontrol of *B. cinerea* was confirmed by studies on the effects of protease inhibitors on the disease development. All three inhibitors nullified the effect of *T. harzianum* in untreated form (Figure 9), thereby increasing the severity of disease on bean leaves treated by T39. The most pronounced effect was obtained with E64. It was noted that heat-treated E64 did not inhibit T39. Heat treatment probably oxidized the active site to form an epoxy group.

The action of *Trichoderma* protease on the infection of *B. cinerea* on the surface of bean leaves and the development of disease symptoms could be bimodal. Firstly, protease may have an adverse effect on the germination of *Botrytis* conidia, and secondly it may be able to deactivate the pathogen hydrolytic enzymes that are responsible for plant tissue necrosis.

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