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Proteases From *Talaromyces flavus* and *Trichoderma harzianum* : Purification , Characterization and Antifungal Activity Against Brown Spot Disease on Faba Bean

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ABSTRACT

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Protease were produced by *Trichoderma harzianum* and *Talaromyces flavus* when its were grown in the presence of casein. Proteases from the culture filtrate of *T. harzianum* and *T. flavus* were purified and tested for their antifungal activity against brown spot disease caused by *Botrytis fabae* on faba bean. The enzymes were purified in a 2-step procedure involving ammonium sulfate precipitation and Sephadex G-200 gel permeation chromatography. Both enzymes have an optimum temperature of 30℃ and optimal pH value of 6.5. T. flavus exhibited high levels of extracellular protease activity compared with *T. harzianum*. Germination and growth rate, extracellular polygalacturonase (PGase) and carboxymethyl cellulase (CMCase) activities of *Botrytis fabae* were inhibited by the purified protease at a concentration of 40-120 U/ml. Growth and extracellular production of *B. fabae* were completely inhibited by the protease enzyme of *T. flavus* at a concentration of 80 U/ml, while protease from *T. harzianum* was effective at 120 U/ml. Proteases were effective in reducing brown spot disease severity and pathogen sporulation on faba bean leaves inoculated with *B. fabae*. Proteases at a low concentration of 80 U/ml inhibited *B. fabae* on leaf surface, as determined by Scanning Electron Microscope (SEM) examination. When enzymes were applied as a foliar spray on faba bean plants grown under natural infested field conditions, the disease incidence was greatly reduced. Protease from *T. flavus* at 80 U/ml was more significant in reducing disease incidence on leaves and pod and increased pod yield /plant. These results indicated that protease can be a safe biodegradable biocontrol agent for control of brown spot disease on fabae bean .

Key words: Brown spot, *Botrytis fabae*, faba bean, protease, *Talaromyces flavus*, *Trichoderma harzianum*

INTRODUCTION

The importance of the faba bean (*Vicia faba* L.) plant is due to its high nutritive value in both energy and protein contents. Therefore, increasing the crop production is one of the most important targets of agricultural policy in several countries [11, 26].

Brown spot disease caused by *Botrytis fabae* is one of the most significant diseases affecting faba bean in Egypt [1, 2] and other countries [13, 26]. The disease appears as reddish or chocolate brown spots on leaves. The widespread use of chemical crop protection is becoming increasingly unsatisfactory due to public health and environmental concerns, and selection of more virulent isolates. Most sustainable and environmentally acceptable control may be achieved using biocontrol agents.

The antifungal activity of some compounds is due to their ability to affect function or the structure of the fungal cell. Such compounds include enzymes, antibiotics and proteins. Cell wall- degradating enzymes such as chitinases, β-1,3-glucanases, proteases and cellulases are involved in the antagonistic activity of biocontrol agents against phytopathogenic fungi [3, 5].

Fungal proteases may play a significant role in cell-wall lysis [17]. Proteolytic enzymes or proteases catalyze the cleavage of peptide bonds in proteins. They are enzymes of class 3, the hydrolases, and subclass 3.4, peptide hydrolases or peptidases [27]. Most commercial serine proteases, mainly neutral and alkaline are produced by microorganisms belonging to genus *Bacillus* , *Streptomyces*, and *Trichoderma* [20, 21, 27]. A role of the enzymes produced by the mycoparasitic fungi *Trichoderma* in biological control of fungal pathogens like *Botrytis* has been demonstrated [3, 9], and the overproduction of an inducible extracellular protease by copy number effect resulted in enhanced biocontrol efficacy [10, 24]. Further studies have shown that overproduction of the inducible *Stenotrophomonas maltophilia* extracellular protease resulted in enhanced biocontrol activity against *Pythium ultimum* [6]. The fungus species of *Teleromyces flavus* has been described as a biological control agent against several fungal pathogens including *Sclerotium rolfsii*, and *Verticillium dahliae* [16] and the agents of anthracnose and powdery mildew [19], and the action of fungal hydrolytic enzymes has been considered as the main mechanism involved in the antagonistic process [7, 16].

In the present study, we have evaluated the antifungal potential of proteases from *T. flavus* and *T. harzianum* against *Botrytis fabae* the causal agent of brown spot disease in faba bean. We have purified and characterized the proteases and determine their effect *in vitro* against spore germination and mycelium growth rate, extracellular polygalacturonase (PGase) and carboxymethyl cellulase (CMCase) activities of *Botrytis fabae*.

MATERIALS AND METHODS

Plant pathogen

Botrytis fabae was isolated from diseased plants of faba bean cv. Giza 429 grown at a field at Giza governorate . Faba bean leaves infected with brown spot were collected , surface sterilized with 0.5% sodium hypochlorite solution for 1-2 min and plated on Faba Bean Dextrose Agar (FDA) medium (200 g faba bean seeds or 400 g of faba bean leaves -autoclave and filter to obtain faba infusion- 20 g of dextrose and 18 g agar/ L water) at 20℃. Isolate was identified in Plant Pathology Department, National Research Center, Egypt. The spores were harvested after 4 days by washing with 5 ml sterile water, containing 0.01% Tween 80, filtered through a 30 μm nylon filter and centrifuged for 5 min at 2600 rpm. The resulting conidial pellet was adjusted to a concentration of 10^4 conidia / ml by heamocytometer. *T. flavus* and *T. harzianum* isolated from the phyllosphere of healthy faba bean plants grown in a field at Giza governorate were also identified in Plant Pathology Department.

Enzymes production

For enzyme production, *T. flavus* and *T. harzianum* were grown in TLE medium [5] at 28℃. Erlenmeyer flasks containing 500 ml of TLE liquid culture medium containing 0.5% casein were inoculated with 2×10^5 spores of each biocontrol organism and incubated for four days on a rotary shaker (120 rpm) at 28℃. The culture supernatant was then collected by centrifugation at 4000 rpm under cooling. The supernatant was used as a crude enzyme preparation for the purification of the enzyme.

Enzymes assay and protein determination

It was carried out according to the modified method of Soad [23]; the reaction medium contains 500 μl of 1% soluble casein , 100 mM phosphate buffer, pH 6.0 and enzyme source (from culture supernatants), in a total volume of 2.5 ml. The reaction mixture was incubated at 40 ℃ for 30 min after which the reaction mixture was stopped by addition of 2 ml of 15% trichloroacetic acid . The reaction mixture was then centrifuged at 5,000 rpm for 10 min and the precipitated proteins were discarded. From the resulting supernatants 0.5 ml was withdrawn and pipetted in a test tube containing 1.5 ml of 0.33 M sodium hydroxide. Control sample was the mixture with reaction

stopped at zone time. The absorbance of the supernatant was determined at 280 nm [24] during growth periods. One unit of protease activity was defined as the quantity of enzyme required to release one μmol of tyrosine per minute under the experimental conditions. Protein concentration was determined by the method of Lowry *et al.* [12].

Partial purification of protease

The crude enzyme preparation was lyophilized, dialyzed against distilled water at 4℃ for 24 hr and adjusted to fractional saturation with ammonium sulfate. The resulting precipitates (20, 40, 60, 80 and 100% saturations) were separated, dissolved in small amounts of 50 mM phosphate buffer at pH 6.0 and dialyzed against the same buffer at 4℃ for 24 hr. The protein pellet obtained after saturation with ammonium sulphate between 50% and 70% was dissolved in 0.1M Tris-HCl buffer and loaded onto a column of Sephadex G-200 (1.5×24 cm) equilibrated with Tris-HCl buffer, pH 7.8. The column was eluted at a flow rate of 60 mL/h with a 1:1 volume gradient from 0.1M to 1M NaCl in the same buffer. From the elution profile, it was observed that the protease was eluted as a well-resolved single peak of caseinase activity coinciding with a single protein peak at a NaCl concentration of 0.6 M. Fractions (19-23) with high protease activities were pooled, dialyzed, and concentrated by lyophilization (Lyophilizer, Vertisis, South Africa) and used for further studies.

Effect of pH and temperature on the activity of protease

To determined the optimal pH of protease activity, different pH values were assayed in the pH range of 4-8 using 50 mM citrate phosphate buffer (pH 4.0-7.0) and phosphate buffer (pH 7.0- 8.0) under the experimental conditions. Optimal temperature of proteases were determined in the range of 10℃ to 60° for 30 min. under the experimental conditions.

Effect of protease on *Botrytis* growth

The effect of the protease enzyme on inhibition of conidial germination and growth of *B. fabae* was examined on FDA medium. Medium was amended with 4, 8, 16, 40, 80 and 120 U / ml concentrations of protease or distilled water (control) and 50 μl of conidial suspension (10^4) of *B. fabae* in Petri dishes (90 mm diameter). The percent of reduction of conidia germination was examined after 24 h of incubation under alternating 12 h light: 12 h dark at 20 ±1℃ with light microscope. The percent of growth reduction was estimated after four days.

Effect of protease on the hydrolytic enzymes production by *Botrytis fabae*

Conidia suspension of *B. fabae* was placed aseptically into 250 ml Erlenmeyer flasks containing 50 ml liquid of FD and amended with 4, 8, 16, 40, 80 and 120 U / ml concentrations of enzymes or distilled water (control). After four days of incubation at 20 ±1℃, the culture filtrate was collected, dialyzed and stored at 20℃ until use.

I. Polygalacturonase (PG)

Polygalacturonase activity was determined by measuring the rate of increase of galacturonic acid concentrations using dinitro salicylic (DNS) acid reagent [19]. 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. 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.

II. Carboxymethyl Cellulase (CMCase)

Carboxymethyl Cellulase activity was measured as the increase of reducing sugars released determined by Miller method [19] in a reaction mixture containing 0.3 ml of substrate (1.0% carboxymethyl cellulase 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 of glucose per minute per ml of the culture filtrate under the assay conditions.

Pots experiments

Faba bean cultivar Giza 429 obtained from the Agricultural Research Centre (Giza, Egypt) were sown in plastic pots (25 cm^2 in diam.) containing peat, vermiculite and sand (1:1:1) and grown in a glasshouse at 22 ±2 ℃. Pots (5 replicates) were sown with five seeds and irrigated daily. Faba bean plants were inculcated by spraying 20 ml of *B. fabae* spore suspension, containing 4×10^4 spores/ml with (1%) Tween 80, .02 M glucose and 0.02 M KH_2PO_4 onto the shoots of 20-day-old bean plants. After 72 hrs, leaves were sprayed with 20 ml of protease at 4, 8, 16, 40, 80 and 120 U/ ml concentrations. Control plots were treated with 20 ml of water. Treatments were arranged in a completely randomized block. Plants were incubated for 18-24 h at greenhouse temperatures in a dark room. Plants were transferred to natural light greenhouse chambers at 22 ±2 ℃.

I. Disease severity measurement

Brown spot disease severity was assessed during 50 days after treatment using the 0-7 disease index proposed by Elad *et al*.,1994 ,where 0= symptomless leaf tissue, 1= 1-12% of the area under the droplet is necrotic, 2= 13-25% ; 3= 26- 50% ; 4= 51-100% ; 5 = necrotic area diameter exceeds the droplet diameter by up to 1 mm ; 6= necrotic area diameter exceeds droplet diameter by 1-3 mm; and 7= necrotic area diameter exceeds droplet diameter by more than 3 mm. Population counts of *Botrytis* was measured on infected lesions using a haemocytometer and expressed as no. of spores per cm^2.

II. Scanning Electron Microscope (SEM)

Samples (1cm) 10 days old after treated with protease at 80 U/ ml concentration were immersed in 5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.2 , washed with the same phosphate buffer , dehydrated by passages through grade aqueous ethyl alcohol series (10, 30, 50, 75 and 95%), and then placed in 100% ethanol at room temperature for few minutes. Critical point dried specimens were coated with gold palladium. Micrographs were taken on Polaroid type positive film with Uv- haze and 0.2 orange filters. Two pre-treatment samples were examined

Field experiments

During 2003 / 2004 and 2004 / 2005 seasons two experiments were carried out on faba bean plants cultivar Giza 429 under natural field infection by *B. fabae* at El-Gezerit El Dahab, Giza governorate. Seeds were planted in a sandy loam soil with 50 cm distance between plants and 50 cm between rows. Each plot was about 5 m^2 area. Plots with five replicates were arranged in a randomized block design. Fifty seeds were used for each replicate. Protease was sprayed at 4, 8, 16, 40, 80 and 120 U/ ml concentrations when plants were grown up to the 2 leaf stage. The protease were mixed with distilled water containing 0.01 % surfactant and sprayed to run-off on all leaves. The plants were sprayed three times at 15 days intervals. Control plots were treated with water. Disease severity of each treatment was measured and classified on a scale of 0-7 as previously mentioned [8]. Disease assessment on leaves and fruits was done at 15-day intervals. *Botrytis fabae* sporulation was measured via counting the spores by using a haemocytometer and expressed as number of spores per cm^2.

The percentages of disease severity were arcsin transformed before analysis of variance to improve homogeneity of variance. Duncan's multiple range test was applied to determine whether differences between treatments were significant ($P< 0.05$).

RESULTS

Time course of the protease production by *T. flavus* and *T. harzianum*

The results presented in Fig.1 show that the amount of protease enzymes was highest on produced at 6 to 8 day incubation for both organisms. The maximum enzyme activity was on the 6th day and started to decline at the 10th day of incubation (Fig.1).

Partial purification of the crude enzyme

Trials to purify the crude enzyme solution (culture supernatant) with acetone or ethanol proved to be

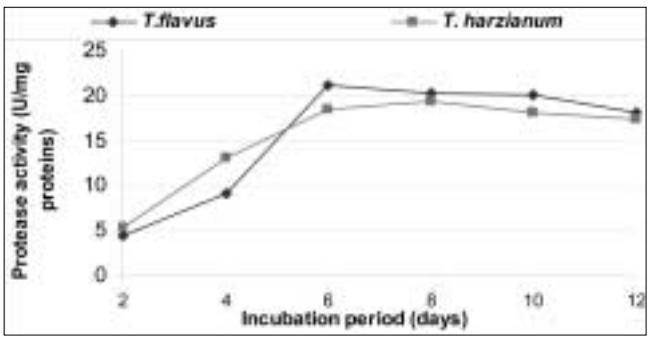

Fig. 1. Protease production by *Talaromyces flavus* and *Trichoderma harzianum* at different growth periods.

Table 1. Purification of protease from the cultural supernatant of *Taralomyces flavus* and *Trichoderma harzianum*.

Purification	Total activity	Total protein (mg)	Specific activity (U/mg)	Yield (%)	Purification fold
Terolomyces flavus					
Culture supernatant	3413.3	160.2	21.3	100	-
Ammonium sulfate precipitation	2324.5	28.9	80.4	68.1	3.8
Trichoderma harzianum					
Culture supernatant	2984.2	129.7	23.0	100	-
Ammonium sulfate precipitation	1647.3	28.6	57.6	55.2	2.5

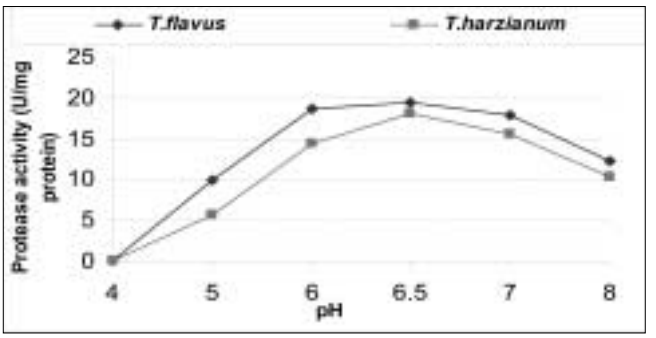

Fig. 2. Activity of the partial purified protease at different pH.

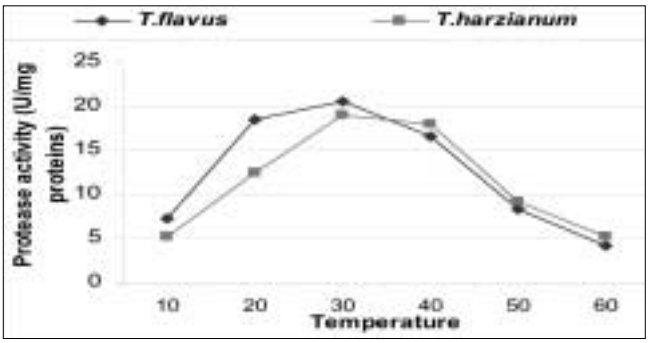

Fig. 3. Activity of the partial purified protease at different temperature.

unsatisfactory. Saturation with ammonium sulphate of 60% afforded a precipitate with a purification fold increase of 3.8 and 2.5 for *T. flavus* and *T. harzianum* , and 68.1 and 55.2% recovery of enzymatic activity, respectively (Table 1).

Effect of pH on the partial purified enzyme activity

The activities of the partial purified enzymes at different pH values are shown in Fig. 2. The highest protease activity of *T. flavus* and *T. harzianum* was at pH 6.5 using citrate phosphate buffer. The enzyme activity decreased gradually at increasing pH values (Fig.2).

Effect of Temperature on Enzyme Activity

The activity of the crude and purified enzyme was determined at different temperatures ranging from 10℃to 60℃. The highest activity was in the temperature range from 20 to 40℃ with *T. flavus* protease and from 30 to 40 ℃ with *T. harzianum* protease. The activity declined at temperatures over 40℃ (Fig.3).

Effect of protease on *Botrytis* growth

The germination and growth of *Botrytis* conidia were observed and quantified in the presence of protease from *T. flavus* and *T. harzianum* (Fig.4). Germination of *Botrytis* conidia was inhibited in the presence of *T. flavus* and *T. harzianum* protease. Inhibition increased with increasing concentration of enzyme. In the presence of protease of *T. flavus*, 100% inhibition was recorded at 40 U/ml. *T. harzianum* protease; 56 and 100% inhibition was observed with 40 and 120 U /ml, respectively. Growth of *B. fabae* was also inhibited by protease from *T. flavus* and *T. harizanum* (Fig.4). Protease from *T. harzianum* gave levels of inhibition comparable to, or slightly lower than those obtained by protease from *T. flavus*. Complete inhibition occurred with protease of *T. flavus* at 40 U /ml.

Effect of protease on the hydrolytic enzymes produced by *Botrytis fabae*

The ability of *B. fabae* to produce extracellular PGase and CMCase was studied the growing the pathogen on a FD medium amended with different concentrations of protease produced by *T. flavus* and *T. harzianum* (Fig.5). *B. fabae* produced less PGase activities when grown on a

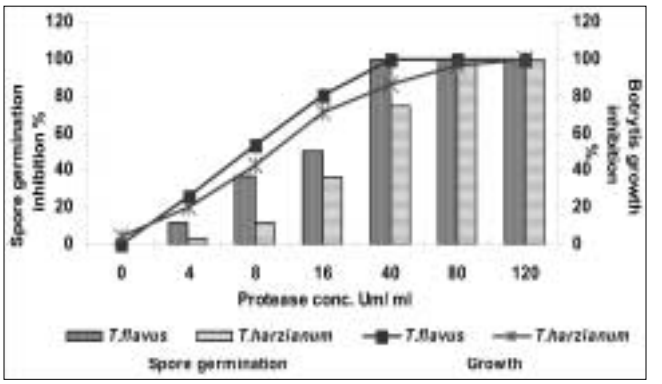

Fig. 4. Inhibitory effect of protease from *Talaromyces flavus* and *Trichoderma harzianum* on germination and growth of *Botrytis* fabae.

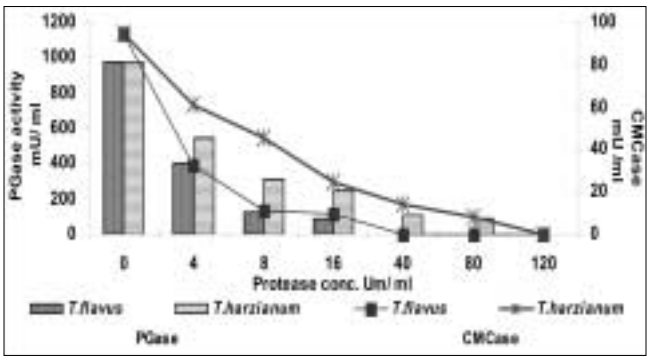

Fig. 5. Inhibitory effect of protease of *Talaromyces flavus* and *Trichoderma harzianum* on extracellular PGase and CMCasae activity of *Botrytis fabae*.

Table 2. Disease severity of brown spot and sporulation of *Botrytis fabae* on faba bean leaves treated with protease of *Talaromyces flavus* and *Trichoderma harzianum* under artificial infested conditions

Protease conc. U/ ml	Disease severity (%)[2]					Sporulation (no / cm^2)				
	10[1]	20	30	40	50	10[1]	20	30	40	50
Control	3.50	6.48	16.38	21.6	32.60	6.63	26.78	59.80	156.30	351.50
Taralomyces flavus										
40	0.00	0.58	2.08	4.3	6.40	0.00	1.60	10.27	21.30	51.60
80	0.00	0.00	1.18	1.8	2.60	0.00	0.00	7.50	15.20	27.20
120	0.00	0.00	0.00	0.9	1.20	0.00	0.00	0.00	1.68	6.28
Trichoderma harzianum										
40	0.78	2.18	4.58	8.5	12.50	0.00	5.18	13.48	29.77	64.80
80	0.00	1.18	3.47	5.8	7.18	0.00	0.00	9.57	18.28	35.57
120	0.00	0.00	0.27	1.2	2.40	0.00	0.00	0.00	2.37	9.68
LSD (P< 0.05)	0.58	1.03	1.69	3.02	4.65	0.86	3.06	3.87	5.32	11.58

[1] Days after sawing
[2] Brown spot disease severity was assessed during 30 days as disease index (0-7) (Elad *et al*.,1994).

medium amended with protease in comparing with unamended one. The highest reduction of enzyme activity produced by *B. fabae* was achieved in the presence of protease of *T. flavus*, especially at 40 U/ml. In the presence of protease from *T. harzianum*, a maximum reduction of PGase was obtained at 120 U/ ml.

The same results were also observed in CMCase. The enzyme activity was gradually decreased with increasing concentration of protease of *T. flavus* and completely inhibited at 40 U/ ml. When *B. fabae* grown in a medium amended with protease of *T. harzianum*, complete inhibition occurred at the concentration of 120 U /ml.

Pots experiments

Evaluation of different concentrations of protease produced by *T. flavus* and *T. harzianum* on suppression of *B. fabae* disease incidence was investigated under artificial inoculation conditions (Table 2). Data clearly indicated that in untreated plants, brown spot infection gradually increased on leaves during growth periods. Greatest differences were obtained among treatments of protease and untreated control. Brown spot disease was reduced by all protease concentrations, especially at 120 U/ ml of protease from *T. flavus* followed by *T. harzianum.*

Data presented in Table 2 indicated that under artificially infested conditions, protease produced by *T. flavus* and *T. harzianum* at 120 U/ ml reduced the mean number of spores/ cm^2 of leaf compared with untreated plants during growth periods. Protease at 80 U/ ml, was also very effective in reducing the spore number.

Scanning Electron Microscope (SEM)

The effect of protease on the suppression of *B. fabae*

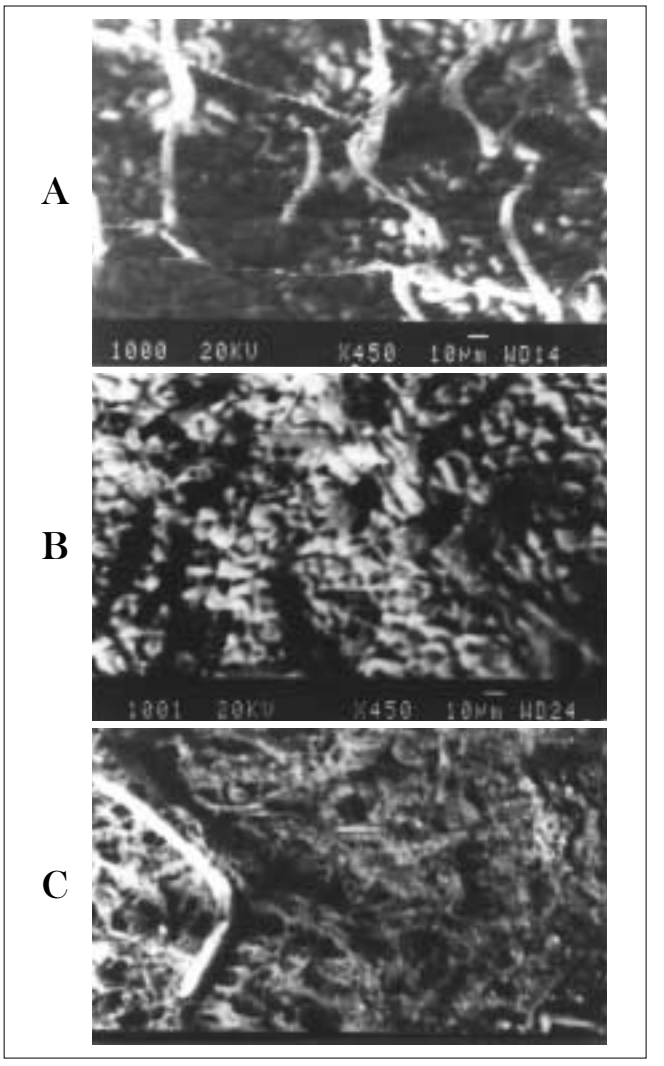

Fig. 6. Scanning electron microscope micrographs showing mycelium lysis of *B. fabae* in the present of protease at 80 U / ml from *Talaromyces flavus* and *Trichoderma harzianum*. A) Control B) *Talaromyces flavus* C) *Trichoderma harzianum.*

Table 3. Disease severity of brown spot on fabae bean treated with protease (0, 40, 80 and 120 U / ml) from *Talaromyces flavus* and *Trichoderma harzianum* under naturally infested conditions.

Protease conc. U / ml	2003/ 2004 season				2004/2005 season			
	Leaves			Pods			Leaves	Pods
	30[1]	70	110	110	30	70	110	110
Control	4.21[2]	14.50	19.60	10.50	3.21	13.30	18.40	9.54
Taralomyces flavus								
40	0.81	2.35	4.63	1.25	0.65	2.01	3.65	1.33
80	0.00	1.35	2.91	0.58	0.00	1.12	2.65	0.65
120	0.00	0.24	1.24	0.00	0.00	0.33	0.98	0.00
Trichoderma harzianum								
40	1.20	3.65	6.23	2.36	1.45	3.54	4.65	1.87
80	0.50	2.35	3.33	1.35	0.00	2.65	3.33	1.25
120	0.00	0.87	2.54	0.50	0.00	0.98	1.32	0.57
LSD (P< 0.05)	0.54	0.74	0.98	1.45	0.32	0.64	0.87	1.01

[1] Days after sawing

[2] Brown spot disease severity was assessed during 30 days as disease index (0-7) (Elad *et al*.,1994) .

Table 4. Sporulation of *Botrytis fabae* on fabae bean treated with protease (0, 40, 80 and 120 U / ml) from *Talaromyces flavus* and *Trichoderma harzianum* under naturally infested conditions.

Protease conc. U / ml	2003/ 2004 season				2004/2005 season			
	Leaves			Pods	Leaves			Pods
	30[1]	70	110	110	30	70	110	110
Control	11.20	25.30	125.3	56.8	9.65	22.6	113.6	52.3
Taralomyces flavus								
40	2.30	6.65	19.2	12.3	2.65	6.45	14.5	11.3
80	0.00	2.33	10.3	6.5	0.00	1.65	8.65	4.3
120	0.00	0.00	5.2	3.3	0.00	0.00	3.33	2.3
Trichoderma harzianum								
40	3.30	8.30	23.6	15.6	2.12	7.32	14.3	13.6
80	0.00	5.60	16.3	6.2	0.33	2.31	16.3	9.2
120	0.00	0.00	10.3	8.6	0.00	0.00	10.3	7.32
LSD (P< 0.05)	0.64	0.95	5.48	3.28	0.55	0.91	5.32	3.01

[1] Days after sawing

growth was tested by application of pathogen and the enzymes at 80 U /ml (Fig. 6). Growth of *B. fabae* on plant surface was inhibited by protease produced by either *T. flavus* or *T. harzianum*. Protease of *T. flavus*, inhibited growth of *B. fabae* more than protease of *T. harzianum*. Some morphological changes in mycelium of *B. fabae* were induced by protease of *T. harzianum*, i.e. hyphal distortion and lysis.

Field experiments

Under naturally infested conditions, the effect of various concentrations of protease produced by either *T. flavus* or *T. harzianum* on inhibition of *B. fabae* was also investigated (Table 3). The disease, expressed as infected area (%) of leaves and pods, was very severe in both seasons. Significant differences were obtained among treatments of protease and untreated control concerning control of brown spot disease on fabae bean plants. Analysis of data indicated that all tested concentrations of protease from both *T. flavus* and *T. harzianum* significantly reduced disease severity in 2003 /2004 and 2004 / 2005 seasons. Greatest control was achieved using 120 U/ ml of protease from *T. flavus* which greatly reduced brown spot disease of leaves of *fabae bean* and completely inhibited pod disease after 110 days of planting in both seasons. Data also showed that *T. harzianum* protease treatment at 120 U/ ml gave the better effect. Moreover, data revealed that spraying plants with protease at 80 U/ ml either from *T. flavus* or *T. harzianum*, had a positive control effect.

Data revealed that sporulation of *B. fabae* gradually

increased on leaves during the growth periods of both seasons (Table 4). Spores number was consistently lower when plants were treated with protease of either *T. flavus* or *T. harzianum* at various concentrations. Plants treated with 120 and 80 U/ ml of protease showed the highest reduction of sporulation .

DISCUSSION

Reports on chitinases, glucanases, cellulase, ribosome-inactivating proteins, permatins and protease and the relative importance of any of these systems in the antagonistic process as antifungal agents are well documented [4, 6, 7, 9, 10, 27]. Proteases are one of the most important industrial enzymes accounting for nearly 60% of the total world-wide enzyme sales. However, few experiments have been conducted to evaluate this enzyme in controlling plant pathogens. Thus, study the antifungal effect of protease from *T. flavus* and *T. harzianum* against *B. fabae* in faba bean was investigated. Moreover, we have purified and characterized the proteases. Our results showed that *T. flavus* and *T. harzianum* produced high level of extracellular protease. Protease synthesis by the *T. flavus* occurred in the early stationary growth phases (72 h). Saturation with ammonium sulphate 60% afforded a precipitate with a purification fold of 3.8 and 2.5 for *T. flavus* and *T. harzianum* with 68.1 and 55.2% recovery of enzymatic activity, respectively. Protease was more active at pH range from 6 to 7 and at a temperature range from 2 to 30 ℃. *Bacillus spp*. and *Burkholderia* produced a protease with an optimal activity at 25 ℃ and pH of 6.0 [25, 14]. The purified enzymes was inhibitory to germination and growth as well as extracellular PGase and CMCasae activity of *B. fabae*. Protease from *T. flavus* showed a more pronounced inhibitory effect than *T. harzianum*. The highest reduction of enzyme activity of *B. fabae* was achieved in the presence of protease of *T. flavus*, especially at 40 U/ ml. It can be concluded that protease from both biocontrol agents can play a role in biological control of *B. fabae*. Under artificially infested conditions, growth, sporulation and disease incidence were greatly inhibited by protease treatment. SEM observation indicated that the 40 U/ ml protease degraded the mycelium of *B. fabae*. The results also showed that under natural conditions, disease incidence was greatly reduced by protease treatment. The highest reduction was recorded by protease from *T. flavus* at 80 U/ ml concentration .Whereas, complete inhibition by protease from *T. harzianum* needed the concentration of 120 U/ ml. Finally, our results strongly support that proteases plays an important role in the antagonism of *T. harzianum* and *T. flavus*. Different reports studies the direct use of antifungal protease enzyme to control plant pathogens by adding protease to the circulating water in the greenhouse or spraying plants in the field [9, 6]. A considerable amount of recent research has been devoted to study of the lytic systems produced by *T. harzianum*, including chitinases [5], -glucanases [22], and proteases [3], and the relative importance of any of these systems in the antagonistic process. In addition to secreating cell wall — degrading enzymes, *Talaromyces flavus* antagonizes *Sclerotium rolfsii*, *Verticillium dahliae* and others by parasitism and antibiosis [7, 15, 16].

In general, *T. flavus* produced high concentration of protease enzymes which appear to be more effective than protease from *T. harzianum*. This evidence indicates that differences between *T. flavus* and *T. harzianum* proteases may include sites of action influencing the biological efficacy. Clearly, the biochemical properties characterized *in vitro* and the antifungal activity of protease from *T. flavus* and *T. harzianum* indicated that this enzyme exhibits promising activity against the growth of *B. fabae*, rendering it a potentially useful agent for plant disease protection.

LITERATURE CITED

1. Abou- Zeid , A., Daboor, S., and Marwa, A. 2002. Application of molecular methods for characterization of *Botrytis fabae* and *Botrytis cinerea* of faba bean. Plant Prot. Sci. 38: 314-318.
2. Abou- Zeid , A., Sleim M., and Daboor, S. 2002. Characterization of an antibiotic produced by *Streptomces violaceus* T118 and its effect in controlling chocolate spot disease of Faba bean plants. Egyptian J. Microbiol. 37: 197-212.
3. Alvarez , S. M., Avila, G. L., Becerril, G. C., Fattel, F. L. F., Ortega, L. J., and Arroyo, R. 2000. A novel cysteine proteinase (CP65) of *Trichomonas vaginalis* involved in cytotoxicity. Microbial Pathogenesis 28: 193-202.
4. Dash, C., Vathipadiekal, V., George, S. P., and Rao, M. 2002. Slow-tight binding inhibition of xylanase by an aspartic protease inhibitor kinetic parameters and conformational changes that determine the affinity and selectivity of the bifunctional nature of the inhibitor. J. Biol. Chem. 277: 17978-17986 .
5. De Marco, J., Lima, L., Sousa, M., and Felix, C. 2000. A *Trichoderma harzianum* chitinase destroys the cell wall of the phytopathogen *Crinipellis perniciosa*, the causal agent of witches broom disease of cocoa. World J. Microbiol. and Biotechnol. 16: 383- 386.
6. Dunne, C., Loccoz, Y., Bruijn, F., O., and Gara, F. 2000. Overproduction of an inducible extracellular serine protease improves biological control of *Pythium*

ultimum by *Stenotrophomonas maltophilia* strain W81. Microbiology 146: 2069-2068.

7. Duo-Chuan, L. I., and Chen, S., and Jing, L. 2005. Purification and partial characterization of two chitinases from the mycoparasitic fungus *Tararomyces flavus*. Mycopathologia 159: 223-229.
8. Elad, Y., Kohl, J., and Fokkema N. 1994. Control of infection and sporulation of *Botrytis cinerea* on bean and tomato by saprophytic bacteria and fungi. Eur. J. Plant Pathol. 100: 315-336.
9. Elad ,Y., and Kapat, A. 1999. The role of *Trichoderma harzianum* protease in the biocontrol of *Botrytis cinerea*. Eur. J. Plant Pathol. 105: 177- 189.
10. Flores A., Chet, I., and Estrella, A. 1997. Improved biocontrol activity of *Trichoderma harzianum* by overexpression of the proteinase-encoding gene *prb1*. Curr. Genet. 31:30-37.
11. Liew, R. S., and Gaunt, R. E. 1981. Disease problems in the production of broad bean seed. Proceed- ings of the New Zealand Weed and Pest Control Conference 34: 55—58.
12. Lowery, O. H., Roserbrough,N. J., Fair, A. L., and Randall R. J. 1951. Protein measurement with the folin phenol reagent .J. Biol. Chem. 193: 255-257.
13. Grigaliunaite, B. 2001. Fungi of the genus *Botrytis* P. Micheli: Pers, Vilnius, Lithuania. Biologija 3: 14-17.
14. Jewell, S. N., and Falkinham, J.O. 2000. Purification and characterization of a novel protease from *Burkholderia casidae* strain 2.2N. 100th General Meeting of the American Society for Microbiology, Los Angeles, CA.
15. Madi, L., Katan, J. and Henis , Y. 1992. Inheritance of antagonistic properties and lytic enzyme activities in sexual crosses of *Talaromyces flavus*. Ann. Appl. Biol. 121: 565- 576.
16. Madi, L., Katan, J., and Henis, Y. 1997. Biological control of *Sclerotium rolfsii* and *Verticillium dahliae* by *Talaromyces flavus* is mediated by different mechanisms. Phytopathology 87: 1054-1060.
17. Mata, E., Magaldi, S., Hartung, D., Capriles, C., Dedis, L., Verde ,G., Perez ,C., and Capriles, C. 2001. *In vitro* antifungal activity of protease inhibitors. Mycopathologia 152: 135- 142.
18. Miller, G. L. 1959. Use dinitrosalicylic acid reagent for determination of reducing sugar. Analytical Chem. 31: 426-428.
19. Nozawa, H., Ishikawa, S., Nakayama, K., Ogawa, S., and Izu, S. 2004. Establishment of a procedure to control strawberry anthracnose and powdey mildew with Biotrust WP (*Talaromyces flavus*). Plant Protection Society 51: 37-42.
20. Pozo, M., Baek, J., Garcia, J., and Kenerley, C. 2004. Functional analysis of tvsp1, a serine protease-encoding gene in the biocontrol agent *Trichoderma virens*. Fungal Genetics and Biology 41: 336-348.
21. Rao, M. B., Tanksale, A. P., Ghatge, M. S., and Deshpande, V. V. 1998. Molecular and biotechnological aspects of microbial proteases. Microbiol. Mol. Biol. Rev. 62: 597-635.
22. Sanz, L., Montero, M., Redondo, J., Llobell, A. and Monte, E. 2005. Expression of an {alpha}-1,3-glucanase during mycoparasitic interaction of *Trichoderma asperellum*. FEBS J. 272: 493-499
23. Soad, M. M. 1995. Alkaline protease from *Streptomyces venezulae* DSM 4027. Egypt. J. Microbiol. 30: 355- 368.
24. Szekeres, A., Kredics, L., Antal, Z., Kevei, F., and Manczinger, L. 2004. Isolation and characterization of protease overproducing mutants of *Trichoderma harzianum*. FEMS Microbiol. Letters 233: 215-222.
25. Tsuchida, O., Yamagota, Y, Ishizuka, J., Arai J., Yamada J., Ta-keuchi M., and Ichishima, E. 1986. An alkaline proteinase of an alkalophilic *Bacillus* sp. Curr. Microbiol.14:7-12.
26. Ying, W. C.2000. Pathogen identification of leaf diseases and the study on the main diseases in spring broad bean in Gansu Province. Acta Phytophylacica Sinica 27: 121-125.
27. Veenekar, J. V., Tanksale, A., Ghatge, M., and Deshpande, V. 2001. Novel biofunctional alkaline protease inhibitor: protease inhibitory activity as the biochemical basis of antifungal activity. Biochem. Biophys. Res. Commun. 285: 1018- 1024.