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Research Article

Influence of Some Plant Extracts and Microbioagents on Some Physiological Traits of Faba Bean Infected with *Botrytis fabae*

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Abstract: Laboratory and greenhouse experiments were conducted to assess the efficacy of *Eucalyptus citriodora* Hook., *Ipomoea carnea* Jacq., *Cuminum cyminum* L., *Allium sativum* L. and *Hyoscyamus muticus* L. leaf extracts, and of *Streptomyces exfoliatus* (Waksman & Curtis) Waksman & Henrici (S) and *Trichoderma harzianum* Rifai (T) in controlling *Botrytis fabae*, which causes chocolate spot disease in the faba bean.

Laboratory studies supported the use of leaf extracts of *E. citriodora* (Ex. 1) and *I. carnea* (Ex. 2) in preference to other extracts for controlling the mycelial growth of *B. fabae*. In addition, the mixture S + T was the best of inhibiting spore germination followed by Ex. 1 + Ex. 2 after 8 h of testing, whereas. Ex. 1 + Ex. 2 followed by S + T produced the lowest percentage of germination after 16 h. Moreover, Ex. 2 was more efficient than Ex. 1. However, after 4 days, the inhibiting order of the mycelial growth of *B. fabae* was S+T > Ex. 1 + Ex. 2 > T > Ex. 2 > Ex. 1 = S.

Greenhouse experiments showed the highest activities of peroxidase, catalase and pectinase in plants infected with *B. fabae*. These activities were markedly reduced in healthy plants and changed widely under different biocontrol treatments. Applying biocontrol agents to infected plants increased mineral levels (N, P, K and Mg), and both Chl biosynthesis and photosynthetic activity, which in turn led to the accumulation of metabolites (carbohydrates and proteins). This accumulation helped the plant to resist the detrimental effects of *B. fabae* on growth, productivity and yield. In this context, the efficiency of the test biocontrol agents was in the order: T + S > Ex. 1 + Ex. 2 > T > Ex. 2 > S > Ex. 1.

Key Words: Catalase, growth, pectinase, peroxidase, photosynthesis, plant extracts, productivity, *Streptomyces, Trichoderma, Vicia faba*, yield

Introduction

The importance of the *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.

Chocolate spot, caused by *Botrytis fabae* Ikata, is the most serious disease of beans and is capable of devastating an unprotected crop. The disease appears as reddish or chocolate brown spots on leaves. These spots may grow larger and merge as a black mass. Defoliation and lodging occur after warm moist conditions, which favor disease development. The spots result in harmful effects on growth, most physiological activities and the yield of the plant (Khaled et al., 1995). The mode and development of the fungal infection were reported by Mansfield and Deverall (1974). The problem of adequately protecting plants against the fungus by using fungicides has been complicated by the development of fungicidal resistance and/or adverse effects on growth and productivity of the host plant as well as on the accompanying microflora (Khaled et al., 1995). Therefore, controlling *B. fabae* by biocontrol agents seemed to be better than and preferable to the chemical control.

The presence of antifungal compounds, in higher plants, has long been recognised as an important factor in

disease resistance (Mahadevan, 1982). Such compounds, being biodegradable and selective in their toxicity, are considered valuable for controlling some plant diseases (Singh and Dwivedi, 1987). In addition, plant extracts might have inhibitors to enzymes from the invading pathogen, and the effects of different phenolic compounds on the germination and growth of many fungal pathogens were studied by Ismail et al. (1987). *Actinomycetes*, and particularly *Streptomyces,* play a major role in antagonistic interaction for different plant pathogens because of their greater capacity for antibiotic production (Rothrock and Gottleib, 1984). In addition, *Trichoderma* Pers. was considered as a biocontrol agent for phytopathogenic fungi, but the mechanism of this effect is not clearly understood. Proposed mechanisms of this biocontrol are antibiosis (Ghisalberti et al., 1990), mycoparasitism (Singh and Faull, 1990), and competition and/or fungicidal action because of the capacity of *Trichoderma* to produce antibiotics or hydrolytic enzymes (Lorito et al., 1994).

Despite the many studies performed on biological control, relatively little is known about the role of the plant extracts, *Streptomyces exfoliatus* (Waksman & Curtis) Waksman & Henrici and *Trichoderma harzianum* Rifai in controlling *B. fabae* which causes chocolate spot disease in beans. In this study; we hypothesised that biocontrol agents might reduce or nullify the negative effects of *B. fabae* on the growth, photosynthesis and yield of faba bean plants. Therefore, this study aimed at (1) studying the role of selected plant extracts (added singly or in combination*)*, and of *S. exfoliatus* and *T. harzianum* (added singly or in combination), in reducing the detrimental effects of *B. fabae* on faba bean plants, (2) finding an explanation for the above role based on test attributes, (3) evaluating the enhancement of plant yields, and (4) finding a recommendation for controlling the fungal disease.

Materials and Methods

Laboratory and greenhouse experiments were carried out in Tanta, Middle Delta, Egypt 30° 47´ N (Lat.), 31° 00´ E (Long.)] during 2001 and 2002. Grains of *Vicia faba* cultivar Giza 429, obtained from the Agricultural Research Centre (Giza, Egypt), served to produce sensitive host plants for *B. fabae*. Test biocontrol agents included plant leaf extracts, *Streptomyces exfoliatus (S)* and *Trichoderma harzianum (T)*.

Preparation of *Botrytis fabae* spore suspension

B. fabae was isolated on PDA agar medium from infected faba bean leaves, and identified. It was compared with a reference strain given by the Agricultural Research Centre (Giza, Cairo, Egypt). A pathogenicity inoculum was prepared by growing the isolate in Petri dishes on potato dextrose agar for 5 days. The fungus was then homogenised and the spores counted (4×10^4 CFU/ml).

Preparation of plant extracts

Crude extracts of leaves of 5 plant species collected from different locations in Egypt were prepared. These plants were: 1) *Eucalyptus citriodora* Hook., 2) *Ipomoea carnea* Jacq., 3) *Cuminum cyminum* L., 4) *Allium sativum* L. and 5) *Hyoscyamus muticus* L. All extracts were prepared by grinding leaves (100 g) in 200 ml of distilled water. After squeezing the pulp through muslin, the filtrate was centrifuged at 3000 rpm for 15 min, lyophilised and further re-extracted with methanol. The organic layer was collected and evaporated at 40 °C to dryness. The obtained dry matter was dissolved in about 10 ml of distilled water, and then cleared by centrifugation for 15 min at 3000 rpm. Crude extracts were kept without further dilution and were used to evaluate their anti-*Botrytis* activities.

Preparation of microbioagent suspensions

Streptomyces exfoliatus was isolated from soil samples collected from Egyptian soil on Olson agar medium containing 25 µg/ml of each of ampicillin, streptomycin and nystatin and identified following the method of Agwa et al. (2000). Two milliliters of *Streptomyces exfoliatus* (5×10^6 spores/ ml) were grown in 500 ml of starch nitrate agar (Shirling and Gottlieb, 1966) for 7 days at 30 °C and shaken at 220 rpm.

Trichoderma harzianum NRRC-143 was obtained from the Microbial Properties Research Unit, USDA, USA. Two milliliters of *Trichoderma harzianum* (2×10^5 spores/ml) were grown in 500 ml of liquid Czapeks dox medium and shaken at 220 rpm for 7 days at 25 °C.

Spores and mass cakes of each of the 2 micro-organisms were collected by centrifugation at 5000 rpm for 15 min, washed several times with distilled water and extracted with methanol (24 h, 2 successive times). Thereafter, the methanol was evaporated and microbioagent residues were suspended in sterile distilled

water and used in laboratory experiments. Microbioagent extracts were mixed with water-agar medium to determine their effects on *B. fabae* spore germination. Spores of both micro-organisms were adjusted in distilled water to about 4 x 10^6 and 2 x 10^6 CFU/ml for *Streptomycetes exfoliatus* and *Trichoderma harzianum*, respectively, and then used for plant treatments (foliar application).

Laboratory experiments

Two laboratory (in vitro) experiments were performed to assess the sensitivity of *B. fabae* to test bioagents.

In the first experiment, Petri dishes (10 cm diameter), containing potato dextrose medium, were inoculated with spore suspension (1 ml per dish) of *Botrytis fabae* (4 x 10^4 CFU/ml). Paper discs (5 mm diameter) saturated with *Eucalyptus citriodora* (Ex. 1), *Ipomoea carnea* (Ex. 2), *Cuminum cyminum* (Ex. 3), *Allium sativum* (Ex. 4), *Hyoscyamus muticus* (Ex. 5) or sterile distilled water (control) were placed in the centre of the Petri-dishes. Thereafter, the mean diameter of the inhibition zone was measured after 4 days at 30 °C. This experiment confirmed that leaf extracts of *Eucalyptus citriodora* (Ex. 1) and *Ipomoea carnea* (Ex. 2) were the most efficient at controlling the mycelial growth of *B. fabae*. Therefore, both extracts were selected for the subsequent experiments.

In the second experiment, microbioagent extracts were mixed with water-agar medium to determine their effects on *B. fabae* spore germination. Thereafter, the germination (%) of *B. fabae* was calculated, after 8 and 16 h, using a light microscope. To determine the effect of test bioagents on mycelial the growth of *B. fabae*, Petri dishes containing potato dextrose medium were inoculated with spore suspension (1 ml per dish) of *B. fabae* (4 x 10^4 CFU/ ml) and then treated with bioagents. Paper discs (5 mm) saturated with sterile distilled water (control), Ex. 1, Ex. 2 or Ex. 1 + Ex .2 as well as mycelial discs (5 mm) of *Streptomycetes exfoliatus (S)*, *Trichoderma harzianum (T)* or *S + T* were placed in the centre of the dishes. The inhibition zone (cm) of the mycelial growth was measured after 4 days. This experiment mirrored the relative effects of test bioagents on the growth and germination of *B. fabae.*

Greenhouse experiments

Following to the laboratory study, a greenhouse experiment was conducted to evaluate the effect of test bioagents on the growth, yield and some physiological activities of *Vicia faba* infected with *B. fabae*.

Growth conditions

Clay–loam soil (collected from fields, field capacity = 41.57 %, EC of 1:5, soil extract at 25 °C = 2.05 mmohs/cm, pH 1: 2.5 soil suspension = 7.8, and available NPK = 33, 12.1 and 435 mg/kg, respectively) was used and dispensed in plastic pots (28 cm diameter, 20 cm depth, 4 kg soil/pot).

Pots were divided into 2 groups. The first consisted of healthy faba bean plants and the second included infected plants. Infected plants were subdivided into 7 subgroups: 1) non-biocontrol treated (untreated), 2) treated with *Eucalyptus citriodora* leaf extract (Ex. 1), 3) treated with *Ipomoea carnea* leaf extract (Ex. 2), 4) treated with both extracts (Ex. 1 + Ex. 2), 5) treated with *Streptomyces exfoliatus* (*S*), 6) treated with *Trichoderma harzianum* (*T*), and 7) treated with both *S* and *T* (*S + T*).

Grains of *Vicia faba* were disinfected in 2% (v/v) Na-hypochlorite for 10 min followed by washing with sterile distilled water. Ten seeds were sown per pot, and then thinned to 3 seeds at 15 days after sowing. The sowing date was November 4 2001 and the experiment was conducted for about 4 months. Pots were irrigated with tap water whenever necessary but in equal amounts.

NPK fertilisers were applied at rates of 0.6 g of urea/pot, 0.75 g of Ca-super-phosphate/pot, and 0.25 g of K-sulphate/pot. Phosphorus was added during soil preparation (i.e. before sowing). Each of N and K were applied, in 2 equal doses, at thinning and 2 weeks after thinning.

Faba bean plants were infected by spraying 20 ml of *B. fabae* spore suspension, containing 4 x 10^4 spores/ml with (1%) Tween 80, onto the shoots of 20-day-old bean plants.

At 1 and 2 weeks after infection, infected plants of each pot were sprayed with 20 ml of each bioagent. In the case of mixtures, 10 ml was taken from each component of the mixture. Thereafter, plants in each pot were left to be air-dried, sprayed with 15 ml of distilled water and covered with plastic bags for 2 h to maintain the high humidity atmosphere around the leaves.

Physiological measurements

At 75 days after sowing, plants were harvested and prepared for the following measurements:

Enzyme assay

Peroxidase (EC 1. 11. 1.7), catalase (EC 1. 11. 1. 6) and pectinase (EC 3. 2. 1. 15) enzymes were assayed at 26 ºC and expressed as units/mg of protein, where 1 unit is defined as the amount of enzyme converting 1 mmole of substrate to product during 1 min. Protein concentration was determined by the method of Lowery et al. (1951). Green leaves (0.5 g) were homogenized in 8 ml of 50 mM cold phosphate buffer (pH 7). Then the homogenate was centrifuged at 4000 rpm for 20 min. The supernatant was used as a crude extract for enzyme assay.

In the case of peroxidase, the assay mixture contained 0.1 M sodium phosphate buffer (pH 5.8), 7.2 mM tetraguaiacol, 11.8 mM H_2O_2 and 0.1 ml of crude extract in a final assay volume of 3 ml (Kato and Shimizu, 1987). The reaction was initiated by adding H_2O_2 and the change of absorbance was recorded at 470 nm. Peroxidase activity was calculated using the extinction coefficient (26.6 mM/cm at 470 nm) for tetraguaiacol.

Catalase was assayed according to the method of Kato and Shimizu (1987) by measuring the initial rate of H_2O_2 disappearance. A sample of 0.1 ml of crude extract was added to 3 ml of the reaction mixture containing 0.1 M sodium phosphate buffer (pH 7), and 2 mM H_2O_2. The breakdown of H_2O_2 was followed by measuring the absorbance change at 240 nm and the enzyme activity was calculated using the extinction coefficient (40 mM /cm at 240 nm) for H_2O_2.

Pectinase activity was assayed as described by Somogyi (1952). The reaction mixture contained 0.8 ml of 0.5% sodium polypectate in 0.2 M sodium acetate buffer (pH 4.8), and 0.2 ml of crude extract. After 1 h incubation at 30 °C, pectinase activity was determined by measurement of the release of reducing groups.

Mineral concentration

The mixed-acid digestion method was used in preparing the sample solution used for determination of mineral ions. Total nitrogen concentration was determined using the micro-Kjeldahl method (Jacobs, 1958). Phosphorus concentration was spectrophotometrically determined by the molybdenum-blue method (Page, 1982). K and Mg were determined according to the method described Allen et al. (1974). A flamephotometer (Corning Scientific Instruments, Model 400) was used for K determination, while an atomic-absorption spectrophotometer (Perkin-Elmer, 2380) was used for determination of Mg.

Chlorophyll (Chl) concentraion

Chl was extracted, from 0.5 g fresh weight of green leaves, in 10 ml of pure N, N-dimethyl formamide (Ebrahim et al., 1998). The extract was kept in darkness for 2 days at 4 °C, and then centrifuged for 15 min at 4000 rpm. Thereafter, Chl a + b concentration in the supernatant was spectrophotometrically determined according to the equations of Moran and Porath (1980).

Photosynthetic (Hill-reaction) activity

Photosystem II (PSII) activity of chloroplasts isolated from faba bean leaves, expressed as the electron-transport rate, was determined by using 2, 6-dichlorophenol indophenol (DCPIP) as an electron acceptor (Biswal and Mohanty, 1976). Chloroplasts were isolated, under cold conditions, as described by Osman and El-Shintinawy (1988) with minor modifications. All materials used were previously cooled in a refrigerator for 15 min. Green leaves were kept in darkness for 24 h, then a sample of 10 g was macerated and homogenized in a mixer for 8 s. (2 intervals, 4 s each) in 60 ml of an ice-cold isolation buffer (pH 7.8) containing 50 mM tricin, 50 mM NaCl, 3 mM $MgCl_2$. 6 H_2O, and 0.5 mM EDTA. The homogenate was filtrated through 8 layers of cheesecloth and centrifuged for 2 min at 4000 rpm. The resulting chloroplast pellet was suspended in 20 ml of a suspension buffer (pH 7.5) containing 40 mM tricin, 10 mM NaCl, 400 mM sorbitol, and 0.1% (w/v) bovine serum albumin. The suspension was again centrifuged as described above. The new pellet was resuspended in 10 ml of a reaction buffer (pH 7.8) containing 4 mM $MgCl_2$.6 H_2O, 400 mM sorbitol, 60 mM KH_2PO_4, and 0.1 ml of the reaction mixture, in 3 ml of 80% acetone. The extract was centrifuged for 5 min at 4000 rpm. The concentration of Chl a + b in the supernatant was determined according to the equation of Arnon (1949). For measuring the PSII activity, an assay sample was prepared by mixing 1.6 ml of 10 mM DCPIP (dissolved in 96% ethanol) with 50 µg of Chl, and then the volume was made up to 3 ml with the reaction buffer. The sample was illuminated (at right angles) with red actinic light (300 Wm^2, 10 min) provided by a slide projector. The DCPIP photoreduction was spectrophotometrically assayed by recording the absorbance at 260 nm. The

difference between the absorbance of dark (Ad) and illuminated (Ai) samples of each treatment was used as a measure of the electron-transport rate (PSII activity), which was expressed as µmol DCPIP reduced (mg/ Chl/ h).

PSII activity = [(Ad-Ai) (F.dil) (1000 x 6)] / [Chl conc. x time]

where F was calculated using a calibration curve of DCPIP against the absorbance.

Metabolite concentration

Metabolites in leaves were extracted in borate buffer (pH 8). Carbohydrate fractions were estimated according to Naguib (1963, 1964), while the total-soluble proteins were estimated according to the method adopted by Lowry et al. (1951).

Growth criteria and seed yield

At 3 months old, plant samples were separated into shoots and leaves, and shoot heights and leaf numbers were recorded. Shoots and leaves were oven-dried at 70 ^{o}C to constant weights, and dry weights of both were recorded. At 4 months old, pods were separated, oven-dried, and the seed yield was determined.

Statistical analysis

All experiments were conducted using a completely randomized design in a factorial arrangement with at least 4 replicates. All data were averaged and statistically analysed using 1 and 2 way analysis of variance. In the case of percentages, the original data were arcsine-transformed prior to analysis. The least significant difference (LSD) at the 5% level was used to compare means using multiple range test Duncar's (Duncan, 1955).

Results

In vitro growth of *B. fabae* as affected by plant extracts

Leaf extracts from *Eucalyptus citriodora* (Ex. 1), *Ipomoea carnea* (Ex. 2), *Cuminum cyminum* (Ex. 3), *Allium sativum* (Ex. 4) and *Hyoscyamus muticus* (Ex. 5) were tested for their inhibitory effect on *B. fabae* (Table 1). Ex. 2 produced a 4 cm inhibition zone for the fungal mycelial growth, followed by Ex. 1 and Ex. 3 which gave 3.2 and 3 cm inhibition zones, respectively. Ex. 4 and Ex. 5 were last, producing 1.6 and 1.5 cm inhibition zones. Therefore, Ex. 2 and Ex. 1 were evaluated further for in vivo assays for controlling *B. fabae*.

In vitro bioassay of germination and growth of *B. fabae* as affected by bioagents

Efficiency of plant extracts (Ex. 1, Ex. 2 and both) and microbioagents [S. *exfoliatus, T. harzianum*, and both (*S + T*)] was tested against *B. fabae* spore germination as a step to controlling the pathogen infection before disease development. *S + T* gave the highest inhibition of spore germination followed by Ex. 1 + Ex. 2 and then Ex. 2 after 8 h of testing (Table 2). Ex. 1 + Ex. 2 produced the lowest percent of germination after 16 h followed by *S + T*. Moreover, Ex. 2 was more efficient than Ex. 1. However, after 4 days of testing on solid medium, the order of inhibition of the mycelial growth of *B. fabae* was *S + T* > Ex. 1 + Ex. 2 >*T* > Ex. 2 > Ex. 1 = *S*.

In vivo plant defence against spot development

The role of peroxidase and catalase enzymes in defence against *Botrytis* pathogenicity was investigated. In addation, pectinase activity was assayed for healthy and infected plants (Table 3). Peroxidase and catalase

Table 1. Inhibition zone (cm) of *Botrytis fabae* as affected by plant leaf extracts from *Eucalyptus citriodora* (Ex. 1), *Ipomoea carnea* (Ex. 2), *Cuminum cyminum* (Ex. 3), *Allium sativum* (Ex. 4) and *Hyocyamus muticus* (Ex. 5).

Plant extract	Control	Ex. 1	Ex. 2	Ex. 3	Ex. 4	Ex. 5
Inhibition zone	0.0 d	3.2 b	4.0 a	3.0 b	1.6 c	1.5 c

* Means followed by the same letter are not significantly different at the 0.05 level according to LSD.

Table 2. Germination of *Botrytis fabae* spores (%) and the inhibition zone (cm) as affected by different biocontrol agents involving: *Eucalyptus citriodora* leaf extract (Ex1), *Ipomoea carnea* leaf extract (Ex. 2), Ex. 1 + Ex., *Streptomyces exfoliatus* (S), *Trichoderma harzianum* (T), and S + T.

Test character	Time	Biocontrol agent						
		Control	Ex. 1	Ex. 2	Ex. 1 + Ex. 2	S	T	S + T
Germination	8 h	50.5 a	33.8 c	24.5 de	20.0 ef	28.3 d	41.3 b	16.5 f
	16 h	88.3 a	67.5 d	63.3 de	44.8 f	72.8 c	83.0 b	60.0 e
Inhibition zone	4 days	0.00 d	3.60 c	3.73 bc	4.43 a	3.60 c	4.03 b	4.78 a

* Means in the same row followed by the same letter are not significantly different at the 0.05 level according to LSD.

Table 3. Activities of peroxidase, catalase and pectinase [unit /mg of protein] in leaves of 75-day old faba bean plants infected with *Botrytis fabae* with respect to some biocontrol agents involoving: *Eucalyptus citriodora* leaf extract (Ex. 1), *Ipomoea carnea* leaf extract (Ex. 2), Ex. 1 + Ex. 2, *Streptomyces exfoliatus* (S), *Trichoderma harzianum* (T) and S + T.

Plant treatment	Peroxidase	Catalase	Pectinase
Healthy (control)	3.72 c	3.17 d	3.43 b
Infected untreated	9.00 c	6.05 a	7.35 a
Infected and treated with Ex. 1	3.90 c	3.45 cd	6.70 a
Infected and treated with Ex. 2	4.10 c	4.10 c	6.55 a
Infected and treated with Ex. 1 + Ex.	5.30 bc	5.65 ab	4.60 b
Infected and treated with S	4.15 c	5.00 b	6.55 a
Infected and treated with T	4.25 c	5.05 b	5.98 a
Infected and treated with S + T	6.07 b	5.95 a	4.18 b

* Means in the same column followed by the same letter are not significantly different at the 0.05 level according to LSD.

activities were lowest in the healthy plants, and they reached the highest levels in infected untreated faba bean leaves. Moreover, activities of both enzymes, in leaves of infected plants, decreased under different biocontrol treatments. However, the activity of pectinase enzyme recorded the highest level in infected untreated faba beans (7.35 units /mg of protein) where the pathogen invaded the bean tissues. This activity has decreased widely in infected plants under different biocontrol treatments.

In vivo plant minerals

The pathogen significantly reduced mineral concentrations (N, P, K and Mg) in the faba bean (Table 4). Pathogen infection reduced the N contents of faba beans by 30%, whereas 20% or so reduction was observed in the content of P, K and Mg. During plant growth, the mixture of *S + T* was proved to be the best means to control the pathogen infection, giving about 90% of the N, P, K and Mg given by a healthy faba bean. Ex. 1+ Ex. 2 and *T. harzianum* came in second and third place respectively, with respect to pathogen treatment efficiency.

Photosynthetic performance and metabolite accumulation in plant leaves

B. fabae significantly affected the faba bean Chl content. In addition, significant increases in Chl, PSII activity, total soluble sugars, polysaccharides and total soluble protein concentrations were observed after the plant treatment with all biocontrol agents. This increase was more pronounced in the case of *S + T* than in the

Table 4. Nitrogen (N), phosphorus (P), potassium (K) and magnesium (Mg) concentrations [mg/g (d.m)] in leaves of 75-day-old faba bean plants infected with *Botrytis fabae* with respect to some biocontrol agents involoving *Eucalyptus citriodora* leaf extract (Ex. 1), *Ipomoea carnea* leaf extract (Ex. 2), Ex. 1+Ex. 2, *Streptomyces exfoliatus* (S), *Trichoderma harzianum* (T) and S + T.

Plant treatment	N	P	K	Mg
Healthy (control)	20.3 a	16.2 a	13.9 a	6.1 a
Infected untreated	14.7 f	12.9 e	11.2 d	4.8 d
Infected and treated with Ex. 1	15.1 f	13.3 de	11.6 cd	5.1 cd
Infected and treated with Ex. 2	15.7 e	13.3 de	11.7 cd	5.1 cd
Infected and treated with Ex. 1+Ex. 2	18.9 b	14.9 b	12.7 b	5.6 b
Infected and treated with S	16.3 d	13.6 cd	12.1 bcd	5.3 bc
Infected and treated with	18.4 c	14.1 c	12.4 bc	5.5 b
Infected and treated with S + T	19.3 b	15.1 b	12.7 b	5.6 b

* Means in the same column followed by the same letter are not significantly different at the 0.05 level according to LSD.

Table 5. Chlorophyll (Chl) concentration [mg (g d.m.)-1], photosystem II (PSII) activity {µmol DCPIP reduced (mg/Chl/h}, and total-soluble sugars (TSS), polysaccharides (PS) and total soluble proteins (TSP) concentrations [mg/ g (d.m)] in leaves of 75-day old faba bean plants infected with *Botrytis fabae* with respect to some biocontrol agents involving: *Eucalyptus citriodora* leaf extract (Ex. 1), *Ipomoea carnea* leaf extract (Ex. 2), Ex. 1+Ex. 2, *Streptomyces exfoliatus* (S), *Trichoderma harzianum* (T) and S + T.

Plant treatment	Chl a + b	PS II activity	TSS	PS	TSP
Healthy (control)	10.0 a	93.1 a	213 a	327 a	132 a
Infected untreated	7.6 e	77.7 d	144 f	240 e	94.0 d
Infected and treated with Ex. 1	7.6 e	80.9 cd	149 ef	242 e	97.5 cd
Infected and treated with Ex. 2	7.8 de	81.5 cd	153 de	247 e	102 cd
Infected and treated with Ex. 1+Ex. 2	9.3 b	85.9 bc	185 b	281 c	121 b
Infected and treated with S	8.2 d	82.0 cd	159 d	254 de	104 c
Infected and treated with T	8.7 c	83.8 c	171 c	267 cd	117 b
Infected and treated with S + T	9.6 a	89.0 ab	188 b	303 b	124 a

* Means in the same column followed by the same letter are not significantly different at the 0.05 level according to LSD.

other bioagents, which followed in the sequence Ex. 1 + Ex. 2 > *T* > Ex. 2 > Ex. 1 > *S*, although in all cases the healthy (control) plants achieved the highest values of all test characters (Table 5).

Plant growth, productivity and yield

S + *T* and Ex. 1 + Ex. 2 resulted in an increase in faba bean growth parameters (Table 6). Faba bean plants lost about 40% of their productivity due to *B. fabae* infection, infected plants giving 6.2 g/plant as seed yield, with healthy plants producing 9.6 g /plant. Treating faba bean plants with plant extracts and microbioagents improved most tested growth criteria as well as plant productivity and seed yield. The magnitude of the response was most pronounced in the case of *S +T,* followed by Ex. 1 + Ex. 2 ,*T* , Ex. 2, *S* and Ex. 1. in that order. In this respect, *S + T* kept 90% of the seed yield achieved by healthy plants. In contrast, it was also shown that leaf numbers were not significantly influenced by most treatments.

Table 6. Some growth criteria (3-months-old) and seed yield (4-month- old) of faba bean plants infected with *Botrytis fabae* with respect to some biocontrol agents involving: *Eucalyptus citriodora* leaf extract (Ex. 1), *Ipomoea carnea* leaf extract (Ex. 2), Ex. 1 + Ex. 2, *Streptomyces exfoliatus* (S), *Trichoderma harzianum* (T) and S + T.

Plant treatment	Growth criteria				Seed yield (g /plant)
	Shoot height (cm /plant)	Leaf number per plant	Shoot DW (g /plant)	Leaf DW (g /plant)	
Healthy (control)	50.8 a	14 a	5.02 a	1.96 b	9.6 a
Infected untreated	43.3 d	12 b	3.01 g	1.17 f	6.2 f
Infected and treated with Ex. 1	44.8 cd	11 b	3.17 bg	1.26 ef	6.4 f
Infected and treated with Ex. 2	45.2 bcd	11 b	3.22 f	1.29 de	6.8 e
Infected and treated with Ex. 1+Ex. 2	47.5 bc	12 b	4.11 c	1.64 c	8.3 c
Infected and treated with S	46.0 bcd	11 b	3.46 e	1.39 d	7.1 e
Infected and treated with T	46.1 bcd	12 b	3.83 d	1.54 c	7.9 d
Infected and treated with S + T	48.4 ab	12 b	4.49 b	1.77 a	8.7 b

* Means in the same column followed by the same letter are not significantly different at the 0.05 level according to LSD.

Discussion

Faba bean culture practice modifications and fungicides provide only partial crop protection (i.e. ignoring the subsidiary adverse effects of fungicides on the host plant as well as on the accompanying microflora). Therefore, effective means of protection should include bioagents as major components. Chocolate spot disease of the faba bean (developed by *B. fabae*) is individually quite destructive and damaging due to its interaction with rust yellow mosaic and/or bean leaf roll viral diseases (Omar et al., 1985).

I. carnea leaf extract was the most efficient treatment, followed by *E. citriodora*, with respect to in vitro inhibition of *B. fabae* mycelial growth. This may be attributed to the plant contents of secondary metabolites (e.g., phenolic, alkaloids, flavonoids and terpenoids) that could adversely influence pathogen growth and development (Cown, 1999). Some plants impact on the growth and/or development of others by releasing various chemical compounds called allelopathy (Jadhav et al., 1997).

The effect of plant extracts and microbioagents on *B. faba* spore germination was observed as a fungitasis, where the lowest percentage of pathogen spore germination was formed under the effects of *S + T* and Ex.1 + Ex. 2 after 8 h of incubation. However, extracts of *I. Carnea* plus *E. citriodora (*Ex. 1 + Ex. 2) followed by *S + T* produced the lowest percentage of *B. fabae* spore germination after 16 h. Several higher plants have been found to possess outstanding fungitoxicity against mycelial growth or spore germination of different phytopathogenic fungi (Sattar et al., 1995; Jadhave, et al. 1997; Kurucheve et al. 1997).

In ivestigations of pathogen-host interactions problems are often encountered where a number of factors are involved. One of these important factors is how the host defends itself. This might be by enzymes or metabolites. The high activities of peroxidase and catalase recorded in infected untreated plants could be considered as an antioxidant mechanism for protecting plants against the detrimental effects of pectinase on the plant cell walls. The severity of leaf invasion by *B. fabae* might be related to the fungal ability to form pectinase, which is clear in our results for infected-untreated faba bean plants. The close relationship between the rate of faba bean cell wall breakdown and the rate of cell injury supports the view that cell wall breakdown is responsible for cell death (Basham and Bateman, 1975). Activities of oxidative enzymes in any infected plant tissues are known to contribute to disease resistance mechanisms through the oxidation of phenols (Tarrad et al., 1993). The increase in peroxidase and catalase activities in infected-untreated faba bean plants reflects the plant response to disease, and this increase may be higher around the pathogen penetration sites. In this regard, it was reported that catalase activity reduces the level of hydrogen peroxide,

which may accumulate up to toxic levels in diseased tissues and turns it into water and free oxygen that possesses microbiocidal activity (Misaghi, 1982). The obtained results indicated significant differences in the activity of oxidative enzymes, which in turn could influence the oxidation of phenolic compounds such as quinones as well as the accumulation of free radicals. It is well known that high levels of quinone are highly toxic to plants and inactivate the pectic enzymes secreted by the pathogen. The fluctuation of pectinase activity under the different biocontrol agents might be due to the interference with or inhibition of the pathogen pectinase by biocontrol treatments.

The variation in mineral (N, P, K and Mg) concentrations in plant leaves under different treatments could be related to the influence of these treatments on the uptake and/or the metabolism of such minerals by the faba bean. The adverse effect of *B. fabae* on mineral accumulation by the plant might be due to the consumption of such minerals by the fungus to build its own metabolites. Alleviation of this adverse effect by spraying plants with either plant extracts or microbioagents could be ascribed to compounds produced by these agents and their antifungal effects on *B. fabae.*

B. fabae infection reduced photosynthetic criteria (Chl a + b, PSII activity), as well as metabolite concentrations (total soluble sugars, polysaccharides, and total soluble proteins), while the biocontrol agents increased all these criteria. The change in Chl concentration might be due to the effects of the influence of pathogens, plant extracts and/ or microbioagents on chloroplast enzyme activities. Furthermore, the change in Chl concentrations under the plant treatments was mirrored by the variation in N and Mg concentrations (Table 4). Nitrogen and magnesium are major components of chlorophyll molecules (Abu-Grab and Ebrahim, 2000). Regarding PSII activity the results obtained might be interpreted as being due to the effect of the plant treatment on (1) Mn concentrations, (2) the structure and composition of the light-harvesting complex of PSII, (3) the efficiency of energy transfer from the light-harvesting complex to the reaction centre of PSII (P680), and/or (4) the ability of P680 to accept light energy. In this respect, it was stated that the Hill reaction takes place in what is called the water-splitting system (Krause and Santarius, 1975). This system contains 4 Mn atoms, which are located on the D_1 and D_2 proteins of P680 and play a central role in the cleavage of water molecules leading to the production of molecular oxygen (Ebrahim et al., 1998). Changes in carbohydrate concentrations with the plant treatments could be attributed to their effects on (1) the Chl content of leaves (Aly et al. 2003), and/or (2)the activities of carboxylating (RuBP and PEP carboxylase) and/or dehydrogenase enzymes of CO_2-fixation (Katyal and Randhawa, 1983). However, the variation in protein content was ascribed to the effect on (1) the cytoplasmic ribosomes, (2) the synthesis of RNA by plant cells, which in turn play an important role in protein biosynthesis (Katyal and Randhawa, 1983), and/or (3) nitrate reductase activity in plant leaves (Kvyatkovskii, 1988).

The contrasting effects of *B. fabae* and the biocontrol agents on the growth, productivity and yield of faba bean may be due to (1) the pathogenicity of *B. fabae* (Williams, 1978), (2) the allelopathic effect of leaf extracts, and/or (3) the anti-*Botrytis* effect of both *Trichoderma* and *Streptomyces.* The pronounced recovery of the growth, productivity and yield of infected plants by adding *T + S* or Ex. 1+Ex. 2 rather than adding individual treatments could be ascribed to the additive effects of both bioagents in minimizing chocolate spots caused by *B. fabae*. Therefore, we recommend the use of *T. harzianum + S. exfoliatus*, *E. citriodora + I. carnea* leaf extracts, *T. harzianum, I. carnea leaf extract,* S. *exfoliatus* and *E. citriodora* leaf extract in that order to control the growth and development of *B. fabae* causing chocolate spots in faba bean plants.

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