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

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Laboratory and greenhouse experiments were conducted to assess the efficacy of *Eucalyptus citriodora, Ipomoea carnea, Cuminum cyminum, Allium sativum and Hyoscyamus muticus leaf extracts, and Streptomyces exfloliatus* (*S*) and *Trichoderma harzianum* (*T*) for controlling *Botrytis fabae* causing chocolate spot disease of faba bean. Laboratory study supported the use of *E. citriodora* (Ex 1) and *I. carnea* (Ex 2) extracts than the others for controlling the growth of *B. fabae*. *S*+*T* was the best for inhibiting spore germination followed by Ex 1+Ex 2 after 8 h of testing, whereas Ex 1+Ex 2 produced the lowest percent of germination after 16 h. After 4 days, the inhibiting order of the 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 the infected plants. These activities were markedly reduced in healthy plants and widely changed by the biocontrol treatments. Applying biocontrol agents to the infected plants increased minerals (N, P, K and Mg), and both Chl biosynthesis and the photosynthetic activity, which in turn led to accumulation of metabolites. This served the plant to resist the detrimental effects of *B. fabae* on the plant growth and yield. In this concern, the efficiency of test biocontrol agents seemed to be in the order: *T*+*S* > Ex 1+Ex 2 > *T* > *S* > Ex 2 > Ex.

Keywords: photosynthesis, plant extracts, *Streptomyces*, *Trichoderma* , *Vicia faba*

The importance of *Vicia faba* plant is due to its high nutritive value in both energy and protein contents. Therefore, increasing the plant crop production is one of the most major targets of the agricultural policy in several countries.

Chocolate spot, caused by *B. fabae*, is the most serious disease of beans and 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 the disease development. The spots result in harmful effects on growth, the most physiological activities and 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 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 and preferred than the chemical control.

The presence of antifungal compounds, in higher plants, has long been recognized as an important factor to disease resistance. Such compounds, being biodegradable and selective in their toxicity, are considered valuable for controlling some plant diseases (Singh and Dwivedi, 1987). Also, plant extracts might have inhibitors to enzymes from the invading pathogen, where effects of different phenolic compounds on germination and growth of many fungal pathogens have been studied by Ismail et al. (1987). Actinomycetes, particularly *Streptomycetes* 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* 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 thought to be antibiosis (Ghisalberti et al., 1990), mycoparasitism (Singh and Faull, 1990), competition and/or fungicidal action because of the capacity of *Trichoderma* for production of antibiotics or hydrolytic enzymes (Lorito et al., 1994).

Despite the great many studies on biological control, relatively little is known about the role of plant

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extractes, *S. exfloliatus* and *T. harzianum* to control *B. fabae* causing chocolate spot disease of beans. In this work, we hypothesized that biocontrol agents might reduce or nullify the negative effects of *B. fabae* on 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), *S. exfloliatus* and *T. harzianum* (added singly or in combination), to reduce 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 yield of plants, and (4) finding a recommendation for controlling the fungal disease.

MATERIALS AND METHODS

Plant Materials

Laboratory and greenhouse experiments were carried out in Tanta, Middle Delta, Egypt [geographical coordinates 30°C 47′ N (Lat.), 31°C 00′ E (Long.)] during 2001 and 2002. Grains of *V. faba* cultivar Giza 429, obtained from the Agricultural Research Center (Giza, Egypt), served for producing sensitive host plants for *B. fabae* causing chocolate spot disease of beans. Test biocontrol agents included plant leaf extracts, *S. exfloliatus* (*S*) and *T. harzianum* (*T*).

Preparation of *B. fabae* Spore Suspension

B. fabae was isolated on PDA agar medium ,from the infected faba bean leaves, and identified. It was compared with a reference strain gifted from the Agricultural Research Center. Pathogenicity inoculum was prepared by growing the isolate in Petri-dishes on potato dextrose agar for 5 days, then the fungus was homogenized and the spores counted to be 4×10^4 CFU ml^{-1}.

Preparation of Plant Extracts

Crude extracts of leaves of five plant species collected from different locations in Egypt were prepared. These plants included: 1) *E. citriodora*, 2) *I. carnea*, 3) *C. cyminum*, 4) *A. sativum*, and 5) *H. muticus*. All extracts were prepared by grinding leaves (100 g) in 200 ml distilled water. After squeezing the pulp through muslin, the filtrate was centrifuged at 3000 rpm for 15 min (Model 3510 centrifue, Hettich, Germany), lyophilized 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, then cleared by centrifugation for 15 min at 3000 rpm. Crude extract was kept without further dilution and used to evaluate their anti-*Botrytis* activities.

Preparation of Microbioagent Suspensions

S. exfloliatus was isolated from soil sample collected from Egyptian soil (Kafr El-Sheikh, North Delta) on Olson agar medium containing 25 μg ml^{-1} of each of ampicillin, streptomycin and nystatin. It was identified by Agwa et al. (2000). Two ml of *S. exfloliatus* (5×10^6 spores ml^{-1}) was grown in 500 ml of starch nitrate agar (Shirling and Gottlieb, 1966) for 7 days at 30°C and shaken to 220 rpm. *T. harzianum* NRRC-143 was obtained from the Microbial Properties Research Unit (USDA, USA). Two ml of *T. harzianum* (2×10^5 spores ml^{-1}) was grown in 500 ml liquid Czapeks dox medium and shaken at 220 rpm for 7 days at 25°C. Spores and mass cakes of each of the two microorganisms were collected by centrifugation at 5000 rpm for 15 min, washed several times with distilled water and extracted with methanol for 24 h each for two successive times. Thereafter, methanol was evaporated and microbioagent residues were suspended in sterile distilled water and used in the laboratory experiment. Microbioagent extracts were mixed with water-agar medium to determine their effects on *B. fabae* spore germination. On the other hand, spores of both microorganisms were adjusted in distilled water to about 4×10^6 and 2×10^6 CFU ml^{-1} for *S. exfloliatus* and *T. harzianum*, respectively, and then used for plant treatments (foliar application).

Laboratory Experiments

Two laboratory 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 *B. fabae* (4×10^4 CFU ml^{-1}). Paper discs of 5 mm in diameter saturated with either *E. citriodora* (Ex 1), *I. carnea* (Ex 2), *C. cyminum* (Ex 3), *A. sativum* (Ex 4), *H. muticus* (Ex 5) or sterile distilled water (control) were placed in the center of Petri-dishes. Thereafter, the mean diameter of the inhibition zone was measured after 4 days at 30°C. This experiment certified that leaf extracts of *E. citriodora* (Ex 1) and *I. carnea* (Ex 2) were the most efficient ones for 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, by using the light microscope (WZ 25 IC 280, Type 020-435.037-512794/040879, GMBH, Germany). To determine the effect of test bioagents on mycelial growth of *B. fabae*, Petri-dishes containing potato dextrose medium, were inoculated with spore suspension (1 ml per dish) of *B. fabae* (4×10^4 CFU ml^{-1}), then treated with the 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 *S. exfloliatus* (*S*), *T. harzianum* (*T*) or *S*+*T* were placed on the center of Petri-dishes. Thereafter, the inhibition zone (cm) of the mycelial growth was measured after 4 days. This experiment mirrored the relative effects of test bioagents on growth and germination of *B. fabae*.

Greenhouse Experiment

As a consequence to the laboratory study, a greenhouse experiment was conducted to evaluate the effect of test bioagents on growth, yield and some physiological activities of *V. 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^{-1}, pH of 1: 2.5 soil suspension = 7.8, and available NPK = 33, 12.1 and 435 mg kg^{-1}, respectively) was used and dispensed in plastic pots (28-cm diameter, 20-cm depth, 4 kg soil pot^{-1}). Pots were divided into two groups: the first represented 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 *E. citriodora* leaf extract (Ex 1), 3) treated with *I. carnea* leaf extract (Ex 2), 4) treated with both extracts (Ex 1+Ex 2), 5) treated with *S. exfloliatus* (*S*), 6) treated with *T. harzianum* (*T*), and 7) treated with both *S* and *T* (*S*+*T*). Grains of *V. 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 three at 15 days after sowing. The sowing date was Nov. 4, 2001 and the experiment was conducted for about 4 months. Pots were irrigated with tap water whenever they needed, but with equal amounts. NPK fertilizers were applied at rates of 0.6 g urea, 0.75 g Ca-super-phosphate, and 0.25 g K-sulphate per. Phosphorous was added during soil preparation (i.e., before sowing). Each of N and K was applied, in two equal doses, at thinning and two weeks after thinning. Faba bean plants were infected by spraying 20 ml of *B. fabae* spore suspension, containing 4×10^4 spores ml^{-1} with 1% Tween 80, onto shoot of 20 day-old bean plants. At one and two weeks after infection, infected plants of each pot were sprayed with 20 ml of each bioagent. In 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 distilled water and covered with plastic bags for two hours to maintain high humidity atmosphere around the leaves.

Physiological Measurements

At 75 days after sowing, plants were harvested and prepared for the measurements of enzyme activities, mineral concentration, chlorophyll concentration, photosynthetic activity, and metabolite concentration.

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°C and expressed as units in mg protein^{-1}, where 1 unit is defined as the amount of enzyme converting one (mole 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 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 crude extract in the 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 $mM^{-1}cm^{-1}$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 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 fol-

lowed by measuring the absorbance at 240 nm and the enzyme activity was calculated using the extinction coefficient (40 $mM^{-1}cm^{-1}$ at 240 nm) for H_2O_2. Pectinase activity was assayed as described by Somogyi (1952). The reaction mixture contained 0.8 ml 0.5% sodium polypectate in 0.2 M sodium acetate buffer (pH 4.8), and 0.2 ml crude extract. After 1 h incubation at 30°C, pectinase activity was determined by measurement of the release of reducing groups.

Mineral Concentration

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 molybdenum-blue method (Page, 1982). Potassium (K) and Mg were determined according to Allen et al. (1974). Flamephotometer (Model 400, Corning Scientific Instruments, USA) was used for K determination, while Atomic-Absorption Sepctrophotometer (Model 2380, Perkin-Elmer, USA) 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 dark for 2 days at 4°C, and then centrifuged for 15 min at 4000 rpm (Model A8-24, Rotor-Kontron, Germany). 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 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 dark for 24 h, then a sample of 10 g was macerated and homogenized in a mixer for 8 sec (2 intervals, 4 sec in each) in 60 ml of an ice-cold isolation buffer (pH 7.8) containing 50 mM Tricin, 50 mM NaCl, 3 mM $MgCl_2 \cdot 6\ H_2O$, and 0.5 mM EDTA. The homogenate was filtrated through 8 layers of cheesecloth and centrifuged for 2 min at 4000 rpm (Rotor-Kontron, Model A8-24). 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) of 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 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, assay sample was prepared by mixing 1.6 ml of 10 mM DCPIP (dissolved in 96% ethanol) with 50 µg Chl, and then the volume was completed to 3 ml by the reaction buffer. The sample was illuminated (at right angle) with red actinic light (300 $W \cdot m^{-2}$, 10 min) provided from 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 $(mg\ Chl)^{-1}\ h^{-1}$.

$$\text{PSII activity} = [(Ad\text{-}Ai)\ (Fxdil)\ (1000 \times 6)] / [\text{Chl conc.} \times \text{time}]$$

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-month old, plant samples were separated into shoots and leaves, then shoot height and leaf number were recorded. Shoots and leaves were oven-dried at 70°C to constant weights, then dry weights of both were recorded. At 4-month old, pods were separated, oven-dried, then seed yield was determined.

Statistical Analysis

All experiments were conducted by using a completely randomized design in factorial arrangement with 4 replicates at least. All data were averaged and statistically analyzed using one- and two-way analysis of variance. In 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 between means by multiple range test of Duncan (Duncan, 1955).

RESULTS

In Vitro Growth of *B. fabae* as Affected by Plant Extracts

Leaf extracts from *E. citriodora* (Ex 1), *I. carnea* (Ex 2), *C. cyminum* (Ex 3), *A. sativum* (Ex 4) and *H. muticus* (Ex 5) were tested for their inhibitory effect on *B. fabae* (Table 2). 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 zone, respectively. However, Ex 4 and Ex 5 came in the last rank where they produced 1.6 and 1.5 cm of inhibition zone. 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. exfloliatus*, *T. harzianum*, and both (*S*+*T*)] was tested against *B. fabae* spore germination as a step to control the pathogen infection before disease development. The mixture of *S*+*T* gave the highest inhibition of spore germination followed by the mixture of Ex 1 + Ex 2 then Ex 2 after 8 h of testing (Table 1). Whereas, Ex 1 + Ex 2 followed by *S*+*T* produced the lowest percent of germination after 16 h. Moreover, Ex 2 seemed to be more efficient than Ex 1. However, after 4 days of testing on solid medium, the order of inhibiting the mycelial growth of *B. fabae* was *S*+*T* > Ex 1 + Ex 2 > *T* > Ex 2 > Ex 1 = *S*.

In Vivo Plant Defense against Spot Development

The role of peroxidase and catalase enzymes in defense against *Botrytis* pathogenicity was investigated. Also, pectinase activity was assayed for healthy and infected plants (Table 3). Peroxidase and catalase activities were the lowest in healthy plants, whereas they reached the highest levels in infected untreated faba bean leaves. Moreover, activities of both enzymes, in the leaves of infected plants, decreased under different biocontrol treatments. However, the activity of pectinase enzyme recorded the highest level in infected untreated faba bean (7.35 units mg^{-1} protein) where the pathogen invaded the faba bean tissues. This activity has widely decreased in infected plants under different biocontrol treatments.

In Vivo Plant Minerals

The pathogen has significantly decreased mineral concentrations (N, P, K and Mg) of faba bean (Table 4). Pathogen infection decreased the nitrogen contents of faba bean by 30%, whereas about 20%

Table 1. Inhibition zone (cm) of *B. fabae* as affected by plant leaf extracts from *E. citriodora* (Ex 1), *I. carnea* (Ex 2), *C. cynum* (Ex 3), *A. sativum* (Ex 4) and *H. 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 alphabetical letter (e.g., a, b, ...or d) are not significantly different at the 0.05 level accordingto LSD.

Table 2. Germination (%) of *B. fabae* spores and the inhibition zone (cm) as affected by different biocontrol agents involving: *E. citriodora* leaf extract (Ex 1), *I. carnea* leaf extract (Ex 2), Ex 1 + Ex 2, *S. exfloliatus* (*S*), *T. 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	21.3 ef	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 alphabetical letter (e.g., a, b, ...or f) are not significantly different at the 0.05 level according to LSD.

Table 3. Activities of peroxidase, catalase and pectinase [unit (mg protein)$^{-1}$] in leaves of 75-day old faba bean infected with *B. fabae* with respect to some biocontrol agents involoving: *E. citriodora* leaf extract (Ex 1), *I. carnea* leaf extract (Ex 2), Ex 1 + Ex 2, *S. exfloliatus* (*S*), *T. 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 a	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 2	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 alphabetical letter (e.g., a, b, ...or d) are not significantly different at the0.05 level according to LSD.

reduction was observed in the content of P, K and Mg. Over the time course of plant growth, the mixture of *S* and *T* was proved to be the best mean to control the pathogen infection where it gave about 90% of N, P, K and Mg as that of healthy faba bean. On the other hand, the mixture of Ex 1+Ex 2 and *T. harzianum* came in the second and third rank, respectively, with respect to pathogen treatment efficiency.

Photosynthetic Performance and Metabolite Accumulation in Plant Leaves

B. fabae has significantly affected faba bean Chl content. Also, significant increase 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 most pronounced in case of *S*+*T* than the other bio-

Table 4. Nitrogen (N), phosphorus (P), potassium (K) and magnesium (Mg) concentrations [mg g^{-1} (d.m.)] in leaves of 75-day old faba bean infected with *B. fabae* with respect to some biocontrol agents involoving: *E. citriodora* leaf extract (Ex 1), *I. carnea* leaf extract (Ex 2), Ex 1 + Ex 2, *S. exfloliatus* (*S*), *T. 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 Ex1	15.1 f	13.3 de	11.6 cd	5.1 cd
Infected and treated with Ex2	15.7 e	13.3 de	11.7 cd	5.1 cd
Infected and treated with Ex1+Ex2	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 *T*	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 alphabetical letter (e.g., a, b, ...or f) are not significantly different at the0.05 level according to LSD.

Table 5. Chlorophyll (Chl) concentration [mg (g d.m.)$^{-1}$], photosystem II (PSII) activity {μmol DCPIP reduced (mg Chl)$^{-1}$ h^{-1}}, and concentrations of total-soluble sugars (TSS), polysaccharides (PS) and total soluble proteins (TSP) [mg g^{-1} (d.m.)] in leaves of 75-day old faba bean infected with *B. fabae* with respect to some biocontrol agents involoving: *E. citriodora* leaf extract (Ex 1), *I. carnea* leaf extract (Ex 2), Ex 1 + Ex 2, *S. exfloliatus* (*S*), *T. 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 Ex1	7.6 e	80.9 cd	149 ef	242 e	97.5 cd
Infected and treated with Ex2	7.8 de	81.5 cd	153 de	247 e	102 cd
Infected and treated with Ex1+Ex2	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 alphabetical letter (e.g., a, b, ...or f) are not significantly different at the0.05 level according to LSD.

Table 6. Some growth criteria (3-months old) and seed yield (4-month old) of faba bean infected with *B. fabae* with respect to some biocontrol agents involoving: *E. citriodora* leaf extract (Ex 1), *I. carnea* leaf extract (Ex 2), Ex 1 + Ex 2, *S. exfloliatus* (*S*), *T. harzianum* (*T*) and *S*+*T*.

Plant treatment	Growth criteria				Seed yield (g plant^{-1})
	Shoot height (cm plant^{-1})	Leaf number per plant	Shoot DW (g plant^{-1})	Leaf DW (g plant^{-1})	
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 alphabetical letter (e.g., a, b, ...or g) are not significantly different at the0.05 level according to LSD.

agents which came in this sequence Ex 1 + Ex 2 > *T* > Ex 2 > Ex 1 > *S*, but in all cases the healthy (control) plants achieved the highest values of all test characters (Table 5).

Plant Growth, Productivity and Yield

The mixture of *S*+*T* as well as this of 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, where the infected plants gave 6.2 g per plant as seed yield, however the healthy plants produced 9.6 g per plant. Treating faba bean plants with plant extracts and microbioagents improved most tested growth criteria as well as the plant productivity and seed yield. The magnitude of the response was more pronounced in case of *S*+*T* than Ex 1 + Ex 2 than *T* than Ex 2 than *S* than Ex 1. In this respect, the treatment *S*+*T* kept 90% of seed yield achieved by healthy plants. On the contrary, it could be shown that the leaf number was not significantly influenced by most treatments.

DISCUSSION

Faba bean cultural practice modifications and fungicides provide partial crop protection only, i.e., without attention to 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 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).

Leaf extract of *I. carnea* was the most efficient one, followed by this of *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, flavonoides and terpenoides) which could adversely influence the pathogen growth and development (Cown, 1999). Some plants antagonise the growth and/or development of others by releasing some chemical compounds (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 percent of pathogen spore germination was formed under the effect 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).

Investigations of pathogen-host interaction problems often encountered where a number of factors play a part; 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 detrimental effects of pectinase on the plant cell walls. Severity of leaf invasion with *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 wall breakdown and

the rate of cell injury supports the view that the 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 the infected-untreated faba bean plant reflects the plant response to disease, where this increase could be higher around the penetration sites of pathogen. In this regard, it was reported that catalase activity reduce the level of hydrogen peroxide which may accumulate up to toxic levels in diseased tissues and turns it into water and free oxygen which has microbiocidal activity (Misaghi, 1982). The obtained results indicated significant differences in the activity of oxidative enzymes that 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 for 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 or inhibition of the pathogen pectinase by biocontrol treatments.

The variation in mineral (N, P, K and Mg) concentrations of 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 faba bean. The adverse effect of *B. fabae* on mineral accumulation by plant might be due to 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 decreased 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 referred to the influence of pathogen, plant extracts and/ or microbioagents on chloroplast enzyme activities. Furthermore, the change in Chl concentration under plant treatments was mirrored by the variation in N and Mg concentrations (see Table 4). Nitrogen and Mg are major components of chlorophyll molecules (Abu-Grab and Ebrahim, 2000). Regarding PSII activity, results obtained might be interpreted as due to the effect of the plant treatment on (1) manganese (Mn) concentrations, (2) structure and composition of the light-harvesting complex of PSII, (3) efficiency of energy transfer from the light-harvesting complex to the reaction center of PSII (P680), and/or (4) the ability of P680 to accept light energy. In this respect, it was stated that Hill-reaction takes place in a system called water-splitting system (Krause and Santarius, 1975). This system contained 4 Mn atoms, which locate on D_1 and D_2 proteins of P680 and play a central role in cleavage of water molecules leading to production of molecular oxygen (Ebrahim et al., 1998). Changes in carbohydrate concentration with plant treatments could be attributed to their effects on: (1) Chl content of leaves (Aly et al., 2003), and/or (2) 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 contrary effects of *B. fabae* and the biocontrol agents on growth, productivity and yield of faba bean might referred to: (1) the pathogenicity of *B. fabae* (Williams, 1978), (2) the allelopathy effect of leaf extracts, and/or (3) the anti-*Botrytis* effect of both *Trichoderma* and *Streptomyces*. The pronounced recovery of growth, productivity and yield of infected plants, by adding *T+S* or Ex 1 + Ex 2 than adding individual treatments, could be ascribed to additive effects of both bioagents in minimizing chocolate spots caused by *B. fabae*. Therefore, we recommend the use of *S. exfloliatus+T. harzianum* , *E. citriodora* + *I. carnea* leaf extracts, *T. harzianum*, *S. exfloliatus*, *I. carnea* leaf extract, and *E. citriodora* leaf extract in the same order to control the growth and development of *B. fabae* causing chocolate spots of faba bean plants.

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