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Biological control of chocolate spot disease in Vicia faba L. using the synergism among certain bio-agents

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Abstract: All of twenty five *Botrytis fabae* isolates isolated from naturally infected faba bean plants collected from five different districts in Dakahlia governorate were found to be pathogenic and caused typical symptoms of chocolate spot disease while, the isolate B4 was the most aggressive. Twenty five isolates of *Trichoderma* related to four species (*T. viride*, *T. harzianum*, *T. hamatum*, *T. album*) were screened for the antagonistic activity against the most aggressive *B. fabae* B4. All the twenty five isolates of *Trichoderma* showed some sort of antagonistic activity against the tested *B. fabae* B4 while The isolate *T. viride* T2 was the most potent antagonist to the pathogen *B. fabae* B4 followed by *T. harzianum* E3. Among the three bio-agents; *T. viride* T2, *T. harzianum* E3 and yeast extract, yeast extract was the most potent antagonist against *B. fabae*giving the highest %inhibition of growth parameters and sporulation followed by *T. harzianum* E3 then *T. viride* T2. Both non-volatile and volatile metabolites of *T. viride* T2 and *T. harzianum* E3 significantly inhibited the linear growth of *B. fabae*. The non–volatile metabolites of both *Trichoderma* species were more potent in inhibition of mycelial growth of *B. fabae* than the volatile metabolites. Ethyl acetate extracts of both *T. viride T2* and *T. harzianum E3* had antifungal activity and inhibited the growth and sporulation of *B. fabae* while, *T. harzianum* E3 was the most effective. Both of *T. viride* T2 *and T. harzianum* E3 produced cellulolytic and chitinolytic activity more than *B. fabae*. On the other hand, *B. fabae* produced much more proteolytic activity than them. There was no significant difference in amylolytic activity of all the three tested fungi. The green house experiment indicated that the treatment of Faba bean seeds and foliar spraying with the combination of *Rhizobium*, *T. viride* T2 and yeast was the best bio-agents combination in reduction of chocolate spot disease severity while, the individual treatment with *T. viride* T2 or *T. harzianum* was the most effective against disease incidence. The combination of *Rhizobium*, *T. viride* T2 and yeast and the combination of *Rhizobium*, *T. harzianum* E3 and yeast gave the highest increase in productivity traits.

keywords: Rhizobium, Trichoderma, yeast, B. fabae, faba bean

1.Introduction

The cultivation importance of faba bean around the world based on its high nutritional value of protein (about 28% in dried seed), carbohydrates (56%), vitamins and some other compounds, so it is an available rich source for human food and animal feed alike [1,2]. Its nutritional value is high, and believed to be superior to peas and other grain legumes [3]. Moreover, [4] added that not only faba bean is an excellent source of protein in human diets, but it is also a good source of iron, zinc, calcium, vitamin B group, health benefiting antioxidants, plant-sterols and dietary fibers. Also, faba bean leads to improve both of the soil texture and fertility, as well as the seeds are considered as a valuable source for proteins and energy [5].

In Egypt, faba bean is the most important legume food that is being eaten by rich and poor alike. Recently, Egypt is considered one of the first dry faba bean importing countries; its production decreased from 246801 tonnes in

2008 to119104 in 2016 (which is not enough to local consumption) and the cultivated area of faba bean decreased gradually from 71.445 ha in 2008 to 34.314 ha in 2016 [6].

Faba bean is subjected to many abiotic and biotic stresses along its all life stages. Diseases are the major responsible cause of faba bean yield and quality [7], and the most important factors that determine the yield level and stability of faba bean production [8].

Among all faba bean diseases, chocolate spot is the most important one worldwide which can be seed-borne or residue-borne [9]. As reported by [10], the fungus attacks all the above ground parts of faba bean plant and cause losses in both seed yield and seed quality. The caused losses by chocolate spot are mainly due to a decreased number of pods per plant [11], foliage damage, photosynthetic activity limitation and reduction of the crop production globally [12]. Estimation of losses in yield due to chocolate spot disease in the Nile Delta, may range 60-80% among susceptible cultivars, and up to 34% among the resistant ones [13,14]. So, chocolate spot disease is a limiting factor for the production of faba bean in north and middle Egypt [15].

Recently, biological control is considered as an important approach of agricultural biotechnology to control many fungal plant pathogens [16]. Concerning plant diseases, most bio-control methods have used a single bio-control agent as an antagonist to a single pathogen [17], which may not result in a good performance of the bio-control preparation because the single bio-control agent may not be active in all soil environments. Consequently, application of a mixture of bio-control agents is more closely imitate the natural situation, broaden the spectrum of the bio-control activity and enhance the efficacy and the reliability of the control [18]. A number of fungi and bacteria are well known to be very effective against soil borne diseases, as many researches showed that the foliar diseases can be managed effectively through microorganisms especially chocolate spot [19,2].

Thus, the present study aimed to evaluate the efficacy of the integration of the bio-control agents *Rhizobium leguminosarum*, *Trichoderma* spp and yeast in management of chocolate spot disease of faba bean caused by *Borrytis fabae* as well as improving its growth, and productivity.

2.Materials and Methods

Isolation, purification and identification of the pathogen:

Samples of naturally infected *Vicia faba* leaflets that showed typical symptoms of chocolate spot disease were collected from five sites inside each of five different districts in Dakahlia governorate. The sampling destricts and sites were (Temi El-amdid; T1, T2, T3, T4 and T5, El-senblaween; E1, E2, E3, E4 and E5, Dekernis; D1, D2, D3, D4 and D4, Bilkas; B1, B2, B3, B4 and B5 & Aga; A1, A2, A3, A4 and A5). Samples were collected from 5 sites inside each district. Samples from each site were cut into small pieces, surface sterilized with 0.5% sodium hypochlorite solution for 1 to 2 minutes followed by 3 successive rinses in sterilized tap water and placed between two layers of sterilized tissue papers until dryness. Then plated on 9cm diameter petri dishes contain faba bean dextrose agar (FBDA) medium (400gm of autoclaved and filtrated faba bean leaves, 20gm dextrose and 18gm agar). Three replicates were used for each isolate. The plates were incubated at 20±2ºC for 7 days under 12 hours alternating cycles of light and darkness [20]. Purification of fungi was carried out by using the single spore or hyphal tip techniques and maintained on potato dextrose agar medium (PDA) for further studies [21]. The pure fungal isolates were identified according to [22].

Isolation of *Trichoderma*:

Trichoderma spp. were isolated from phyllosphere of healthy faba bean plants grown in different fields of the same sites of pathogen isolation on selective medium according to [23]. The developed colonies of *Trichoderma* were transferred onto PDA slants and identified on the basis of cultural and microscopic morphological characters according to [24, 25]. The isolates names were derived from the district of the sampling and the isolate number; isolate E1 means the *Trichoderma* number 1 that isolated from El-senblaween district.

Rhizobium leguminosarum and faba bean seeds:

R. leguminosorum had been kindly obtained from The Bio-fertilizer Production Unit, Agric., Res. Centre, Giza, Egypt. Seeds of faba bean; Giza 402 cultivar (susceptible to chocolate spot disease infection) were purchased from Legumes Department, Field Crops Research Institute, Agric., Res. Centre, Giza, Egypt.

Commercial yeast as a source of *Saccharomyces cerevisiae*:

The commercial bread yeast was used as a source for *Saccharomyces cerevisiae* to obtain yeast extract. According to [26], a flask containing 150 ml PDB (Potato Dextrose Broth) medium was autoclaved, allowed to cool, then *Saccharomyces cerevisiae* (active dry bread yeast) was added to the flask under aseptic conditions, then incubated for two days at 23±2^{o}C. The two days old liquid culture of yeast growth was frozen for 24 h, then left to melt before use.

Pathogenicity test:

FBLA medium (50gm Faba bean, 30gm sucrose, 20gm sodium chloride and 20gm agar in one liter of distilled water) was prepared, autoclaved for 15 minutes and poured into sterilized Petri dishes [27]. Plates were inoculated with discs of 5mm diameter of the *B. fabae* isolates and incubated at 20ºC for 12days alternating photoperiod 12h light/12h dark (three replicates for each isolate). 10ml aliquots of sterilized water were added to the cultures after incubation and the fungal colonies were fragmented and suspended in water using a fine brush. For each pathogen isolate, a concentration of ($2.5x10^5$conidia/ml) was prepared in distilled water containing 0.05% Tween 20.

Pots of 20-cm diameter were filled with disinfested soil at rate of 2.5kg/pot, clay: sand (2:1, V/V). Five faba bean seeds (cultivar Giza 402) of healthy appearance were sown in each pot (5 replicates for each isolate). After thirty-five days of sowing, the growing plants were sprayed with the spore suspension ($2.5x10^5$spore/ml) of each fungal isolate and covered with polyethylene bags for 24 hours to maintain a high relative humidity [28]. The control plants were sprayed with distilled water. Both inoculated and un-inoculated plants were maintained for 48 hours at 20ºC under greenhouse conditions. The disease severity (infection type) and the severity of leaf damage were assessed after 48 hours using scale (class rate from 1 to 9) of [29] depending on the extent of the lesions. Disease severity % was calculated for each replicate using this formula:

$$DS\% = \frac{\sum (NPC \, x \, CR)}{NIP \, x \, MSC} x100$$

where, (DS) is the disease severity, NPC is the number of plants in each class rate, CR is the class rate, NIP the number of infected plants and MSC is the maximum severity class rate (in this case = 9).

Antagonistic activity of *Trichoderma* spp. against *B. fabae*:

Evaluation of the antagonistic effect of *Trichoderma* spp. against *B. fabae* was carried out using the dual culture technique [30]. The most virulent *B. fabae* isolate (B4) was cultured on PDA medium for 7 days at 20±2°C through inoculation of pathogen 5mm disc on the center of PDA Petri-dish. While the antagonist *Trichoderma* 5mm disc was cultured separately on PDA medium at 25°C for 7 days. The colony diameter of both *B. fabae* and *Trichodema* was measured and the growth rate per day was calculated. Also for dual culture technique, sterilized Petri dishes (90 mm in diameter) containing PDA medium were inoculated with 5mm disc of *B. fabae* at 10mm from the edge of Petri dish. Another 5mm disc of the tested *Trichoderma* isolate was placed on the opposite side of the *B. fabae* disc at a distance of 60mm from the pathogen, 24 hours after the original inoculation. Consider plates that inoculated only with *B. fabae* as control. Inoculated petri dishes were incubated at 20±2°C for 5 days. The dual and individual growth rates were recorded and the interaction between dual mycelia was scored for degree of antagonism using a scale of 1 to 5 [31], where 1 = *Trichoderma* over growing *B. fabae* and 5 = *B.fabae* overgrowing *Trichoderma*. The developed *B. fabae* from dual cultured plated were microscopically investigated and the changes in the *B. fabae* mycelia were recorded.

Antagonistic activity of non-volatile and volatile metabolites of *Trichoderma* spp.

Effect of non-volatile metabolites of *Trichoderma* on the linear growth of *B. fabae* (direct confrontation):

Direct confrontation was carried out using the method of (Barbosa *et al*., 2001) [32] in 90mm diameter petri dishes that contains PDA medium. Five mm in diameter agar discs for both pathogen (*Botrytis fabae* B4) and antagonists were used. Both pathogen and antagonist discs were put following diametrical axis to 5cm apart and distant from the center of the dish. Three petri dishes were used as replicates. The duel inoculated Petri dishes were incubated at 20±2°C for 6 days. The expansion of the pathogen on the edge of the dish is the indicator [33]. The mycelial growth of the pathogen was evaluated every 24 hour by measuring the diameter of the Petri dish and the radius of the pathogen on the side of the antagonist. The width of the inhibition zone between the two colonies was measured after 6 days of incubation.

Effect of volatile metabolites of *Trichoderma* spp. on the linear growth of *B. fabae* (indirect confrontation test):

The indirect confrontation was carried out using the technique of [33] and [34]. A 5mm diameter mycelial disc of both pathogen and antagonist were put in the center of a Petri dish containing PDA medium. After lids removal of Petri-dishes, the bottom of each antagonist containing dish was placed below another one that contains the pathogen and was enclosed by three layers of parafilm in order to prevent leakage of volatile metabolites. PDA containing Petri dishes without antagonist served as control. All treatment and control dishes were incubated at 20±2°C for 6 days. The diameter of mycelial pathogen growth was measured every day during the incubation period. An inhibition of mycelial growth was observed and calculated using the following formula:

I % = (1- cn/ co) x 100

Where: **I**: the inhibition of pathogen mycelial growth, **cn**: the average diameter of colonies in presence of the antagonist, **co**: the average diameter of control.

Effect of yeast extract on linear growth of *B. fabae*:

The commercial yeast *Saccharomyces cerevisiae* extract was assayed in vitro for inhibition of *B. fabae*. The assay of initial inhibition was carried out on PDA plates (1 ml of melted yeast extract and PDA medium were shacked gently to mix well then, left to solidify). Pathogen was grown on PDA petri dishes at 20±2°C for 2-3 days. A disc of 5mm diameter was taken from the margins of the growing pathogen colonies and placed on the center of the plates, then incubated for 6 days at 20±2°C. The distance was measured from the fungal colony center to its edge. This was compared to the growth of control treatment to determine the degree of inhibition of pathogen growth. Three replicates were used.

Evaluation of sporulation of *B. fabae*:

Sporulation in all treatments was estimated for the cultures of *Botrytisfabae* plates aged 12 days. For releasing all pathogen spores, 10 ml of sterile distilled water were added to each plate containing the fungal growth, then the spore suspension was poured into a beaker and made up to 50 ml using sterile distilled water [35]. Spores number was counted by using the haemocytometer slide under an optical microscope.

Extraction of Metabolites with antifungal activity from *Trichodema* isolates:

Trichoderma isolates were grown in Erlenmeyer flasks (1L) containing 250ml PD broth then incubated at 28°C on shaker at 140 rpm in dark condition for 7 days. To obtain the culture filtrates, extraction was performed twice by shaking in a separating funnel with two thirds of its volume of ethyl acetate (EtOAc). The phases of EtOAc were then pooled and concentrated to give a final volume equivalent to a 250-fold concentration of the original volume of the filtrates [36]. The antifungal activity of *Trichoderma* culture filtrates was tested in *vitro* against *B. fabae* and the percentages of reduction in growth and sporulation were calculated.

Extraction of extracellular enzymes of both *Trichoderma* spp. and *B. fabae*:

To detect the activities of some extracellular hydrolytic enzymes of *Trichoderma* isolates and *B. fabae*, 5mm mycelial discs of the tested fungi (the pathogen and *Trichoderma*) were placed on solid media containing the enzyme substrate. The lytic zone of degraded substrate formed around the colony was measured. Activities of cellulase and protease were studied by using micro crystalline cellulose and milk agar as substrates, respectively [32],

chitinase activity by using chitin [37] and amylase activity by using soluble starch [38].

Field experiment

Two field experiments were carried out at Tag el-Ezz, Agric, Research Station, Dakhlia, Egypt, during 2014/2015 and 2015/2016 seasons. The field experiment was designed to evaluate the capabilities of the bio-control agents *Rhizobium leguminosarum (R), Trichoderma viride*T2, *Trichoderma harizianum* E3, yeast extract (Y) in comparison with control (non-treated plants) and chemical treatments (Kocide 101 as a fungicide) to control chocolate spot disease of faba bean caused by *B. fabae*.

1. Control, tap water (C)
2. *Rhizobium leguminosarum* (R)
3. *Trichoderma viride*T2 (*T. viride*T2)
4. *Trichoderma harizianum*E3 (*T. harizianum*E3)
5. Yeast extract (Y)
6. R + *T. viride T2*
7. R + *T. harizianum*E3
8. R + Y
9. Y + *T. viride T2*
10. Y + *T. harizianum* E3
11. R + *T. viride*T2 + Y
12. R + *T. harizianum* E3 + Y
13. Kocide 101; 2.5 g l^{-1} (F)

Planting and growth conditions:

Before seed inoculation an antagonism test was carried out between rhizobium, *Trichoderma* sp. and yeast extract to insure the compatibility between the three microbes. For both soaking and spraying treatments equal volumes of each antagonist suspension were used where *R. leguminosarum* used at 10^{8}cfu ml^{-1} concentration, each *Trichoderma* sp was used at concentration of 10^{7} conidia ml^{-1} and *S. cerevisiae* extract was used at concentration of 10^{11} cell ml^{-1}. Inoculation of faba bean seeds with each individual, dual or trio treatments occurs in presence of 16% Arabic gum. The inoculated faba bean seed were air dried and sown immediately. The developed plants from each treatment were sprayed with the same spore suspension mixture that was previously used in seed inoculation. Spraying was performed twice with 20 days interval (at 35th day and 55th day of sowing). The other agricultural practices were carried out as usual. Complete randomize plot design was used.

Disease incidence and disease severity:

After 15 days of the second spray, the developed plants were rated for both disease incidence (DI) as the percentage of infected leaves and disease severity (DS) using 1-9 scale where: 1 = no disease symptoms or very small specks, 3 = few small discrete lesions, 5 = some coalesced lesions with some defoliation, 7 = some coalesced sporulating lesions, 50% defoliation and some dead plants, and 9 = Extensive lesions on leaves, stems and pods, severe defoliation, heavy sporulation, stem girdling, blackening and death of more than 80% of plants [29]. The disease data recorded based on scoring scale mentioned above was converted to percentage severity index according to the next formula by [39]:

$$DS\% = \sum \frac{(NPC \, x \, CR)}{Nip \, x \, MSC} \, x100$$

Where: **NPC**: Number of plants in each class rate, **CR**: class rate, **NIP**: No of infected plants, **MSC**: the maximum severity class rate.

Analysis of yield parameters:

The yield components were recorded at the harvest as follow:

* Number of pods/plant

*Seed yield g/plant

* Seed index (weight of 100-seed)

Statistical analysis:

Data were subjected to analysis of variance using CoStat software. The means were compared by using Tuckey test (for laboratory experiments) and Duncan`s new multiple range test (for field experiments) [40].

3. Results and Discussion

Isolation and pathogenicity test of *B. fabae*

Data in Table (1) showed that all tested *B. fabae* isolates (twenty five isolates) were pathogenic and caused typical symptoms of chocolate spot disease while, the isolate B4 was the most aggressive and caused The highest values of disease severity (83.63 %). So, it was used for the further experiments.

Direct antagonism of the isolated *Trichoderma* spp. against the most aggressive *B. fabae* isolate B4.

Twenty five isolates of *Trichoderma* related to four species (*T. viride, T. harzianum, T. hamatum, T. album)* were screened for the antagonistic activity against the most aggressive *B. fabae* B4. The results presented in Table (2) showed that all the twenty five isolates of *Trichoderma* showed some sort of antagonistic activity against the tested *B. fabae* B4 while the isolate *T. viride* T2 was the most potent antagonist to the pathogen *B. fabae* B4 causing 84.01% inhibition of its mycelial growth followed by *T. harzianum* E3 which caused 78.23% inhibition in pathogen mycelial growth.

Direct confrontation of *Trichoderma* spp. and yeast **against *B. fabae***

The effects of *T. viride* T2, *T. harzianum* E3 and yeast extract on linear growth, mycelial dry weight and sporulation of *B. fabae* were presented in Table (3) and Figure (1). Data showed that the fungicide Kocide101 completely inhibited both growth and sporulation of *B. fabae.* Among the three bio-agents, yeast extract was the most potent antagonism against *B. fabae* giving the highest % inhibition of growth parameters and sporulation followed by *T. harzianum* E3 then *T. viride* T2.

Table (1): Pathogenicity test of each *Botrytis fabae* isolate on faba bean plant under green house conditions.

Site of sampling	Isolate	Disease severity %
Control (Dist. water)		28.80 n
Temae-El-Amdid	*B. fabae*T1	62.27 fg
	*B. fabae*T2	75.30 b
	*B. fabae*T3	70.03 d
	*B. fabae*T4	60.03 g
	*B. fabae*T5	53.97 h
El-Senblawin	*B. fabae*E1	48.83 i
	*B. fabae*E2	45.10 j
	*B. fabae*E3	50.47 i
	*B. fabae*E4	55.10 h
	*B. fabae*E5	45.53 j
Dekernis	*B. fabae*D1	67.17 e
	*B. fabae*D2	68.77 de
	*B. fabae*D3	75.70 b
	*B. fabae*D4	67.63 e
	*B. fabae*D5	63.70 f
Bilkas	*B. fabae*B1	70.37 cd
	*B. fabae*B2	70.13 cd
	*B. fabae*B3	70.80 cd
	*B. fabae*B4	83.63 a
	*B. fabae*B5	72.37 c
Aga	*B. fabae*A1	35.43 m
	*B. fabae*A2	41.00 k
	*B. fabae*A3	36.93 lm
	*B. fabae*A4	38.33 l
	*B. fabae*A5	42.73 k

*Means followed by different letters in above columns are significantly different (high), while similar letters indicates small significance according to Duncan's multiple range test at *p=0.05*

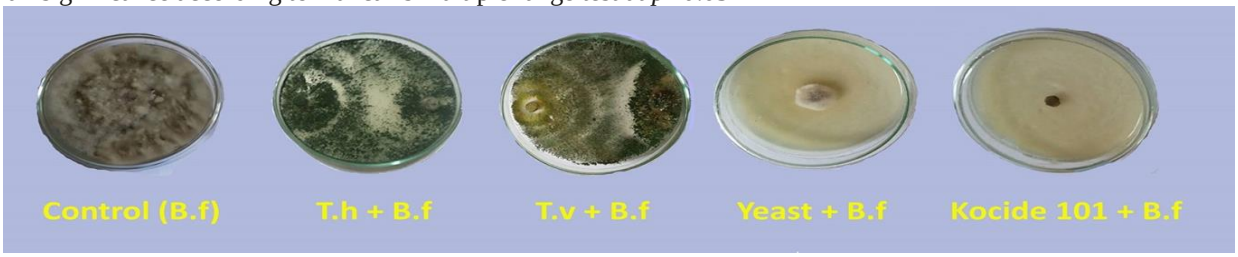

Figure (1): Effect of *Trichoderma* spp. and yeast extract on linear growth of *B.fabae* Antagonistic activity of non-volatile and volatile metabolites of *Trichoderma*

Table (2): Effect of *Trichoderma* isolates on mycelial growth of *Botrytis fabae* B4 isolate

***Trichoderma* spp.**	**Isolate**	**Mycelial growth (cm)**		**Inhibition%**
Control		8.13	A	0.00
Trichoderma viride	T1	2.50	h-j	69.25
	T2	1.30	M	84.01
	E3	2.53	Hi	68.88
	E4	2.63	Gh	67.65
	B5	2.93	F	63.96
	B6	3.13	d-f	61.50
	B7	3.07	Ef	62.24
	B8	2.90	Fg	64.33
	A9	3.10	d-f	61.87
Trichoderma harzianum	T1	2.10	K	74.17
	T2	2.23	Jk	72.57
	E3	1.77	L	78.23
	D4	3.37	Cd	58.55
	D5	3.23	De	60.27
	D6	2.00	Kl	75.40
	D7	2.23	Jk	72.57
	D8	2.07	K	74.54
	A9	2.27	i-k	72.08
	A10	4.33	B	46.74
Trichoderma hamatum	E1	4.37	B	46.25
	E2	4.10	B	49.57
	B3	4.13	B	49.20
	A4	4.30	B	47.11
T. album	T1	3.53	C	56.58
	A2	3.53	C	56.58

*Means followed by different letters in above columns are significantly different (high), while similar letters indicates small significance according to Duncan's multiple range test at *p=0.05*

Table (3): Effect of *Trichoderma* and yeast extract on growth and sporulation of *B.fabae*

Treatments	**Linear growth**		**% Inhibition**	**Mycelium D.W. (mg)**		**% Inhibition**	**Sporulation**		**%Inhibition**
Control	9.00	a	0.00	229.67	a	0.00	36.00	A	0.00
***T.viride* T2**	2.63	b	70.78	71.67	b	68.79	21.33	B	40.75
T.*harzianum* E3	2.27	c	74.78	69.33	b	69.81	16.00	C	55.56
Yeast extract	1.10	d	87.78	60.33	c	73.73	11.67	D	67.58
Kocide 101	0.00	e	100.00	0.00	d	100.00	0.00	E	100.00

*Means followed by different letters in above columns are significantly different (high), while similar letters indicates small significance according to Duncan's multiple range test at *p=0.05.*

Data represented in Table 4 showed that both non-volatile and volatile metabolites of *T.viride* T2 and *T. harzianum* E3 significantly inhibited the linear growth of *B. fabae.* The non–volatile metabolites of both *Trichoderma* species were more potent in inhibition of mycelial growth of B. fabae than the volatile metabolites

Table (4): Effect of non-volatile and volatile metabolites of *Trichoderma* on linear growth of *B.fabae*.

Antagonistic fungi	Non-volatile metabolites			Volatile metabolites		
	Linear growth		**% Inhibition**	**Linear growth**		**% Inhibition**
Control	9.00	a	0.00	9.00	a	0.00
***T.viride* T2**	2.10	b	76.67	4.23	b	53.00
***T. harzianum* E3**	1.40	c	84.44	3.40	c	62.22

* Means followed by differ*Means followed by different letters in above columns are significantly different (high), while similar letters indicates small significance according to Duncan's multiple range test at *p=0.0*

Antifungal activity of *Trichoderma*

Data represented in Table (5) showed that ethyl acetate extracts of both *T. viride* T2 and *T. harzianum* E3 had antifungal activity and inhibited the growth and sporulation of *B. fabae*. *T. harzianum* E3 was the most effective and inhibited the linear growth and sporulation of *B. fabae* by 87% and 72 %, respectively

Table (5): Antifungal activity of ethyl acetate extract of *Trichoderma* spp. against linear growth and sporulation of *B.fabae*

Ethyl acetate extract of	Linear growth		% Inhibition	Sporulation		% Inhibition
Control	9.00	a	0.00	53.00	a	0.00
***T.viride* T2**	1.67	b	81.44	18.33	b	65.42
***T. harzianum* E3**	1.13	c	87.44	14.33	c	72.96

* Means followed by different letters in above columns are significantly different (high), while similar letters indicates small significance according to Duncan's multiple range test at *p=0.05*

Extracellular enzymatic activity

Data presented in Table (6) showed that both of *Trichoderma* species produced cellulolytic and chitinolytic activity more than *B. fabae*. On the other hand, *B. fabae* produced much more proteolytic activity than *Trichoderma* spp.There was no significant difference in amylolytic activity of all the three tested fungi

Table (6): Extracellular enzymatic activities for *Trichoderma* and *B. fabae*

Treatments	Proteolytic activity (mm)		Cellulolytic activity (mm)		Chitiolytic activity (mm)			Amylolytic activity (mm)
B.fabae	20.67	a	1.83	C	0.00	C	10.67	A
***T.viride* T2**	8.67	b	9.00	B	8.00	A	10.17	A
***T. harzianum* E3**	6.33	c	11.33	A	6.33	b	10.67	a

* Means followed by different letters in above columns are significantly different (high), while similar letters indicates small significance according to Duncan's multiple range test at *p=0.05*

Table (7): Effect of bio-agents on chocolate spot disease

Treatments	Disease Severity				Disease Incidence			
	2014/2015		2015/2016		2014/2015		2015/2016	
Control (*B. fabae*)	26.99	a	33.13	a	18.74	a	22.05	A
Rhizobium	22.65	b	25.29	b	15.43	b	18.16	B
T. viride T2	19.08	d	21.73	d	7.38	h	8.18	I
T. harzianum E3	20.32	c	23.80	c	7.86	g	8.87	H
Yeast	18.13	d	20.66	d	12.55	d	13.70	E
R + *T. viride* T2	13.56	g	16.08	g	8.72	f	9.51	G
R + *T. harzianum* E3	15.22	f	17.57	f	8.89	f	9.79	G
R + yeast	13.03	g	15.07	g	15.16	b	17.40	C
Yeast + *T. viride* T2	9.17	i	10.72	i	10.31	e	12.25	F
Yeast + *T. harzianum* E3	16.39	e	19.24	e	10.65	e	12.77	F
R+*T. viride* T2+ yeast	7.39	j	8.89	j	13.76	c	15.61	D
R+ *T. harzianum* E3+ yeast	11.00	h	13.55	h	13.93	c	15.91	D
Koside 101	6.05	k	6.95	k	4.70	i	5.15	J

* Means followed by different letters in above columns are significantly different (high), while similar letters indicates small significance according to Duncan's multiple range test at *p=0.05*.

Effect of Rhizobium, *Trichoderma* spp. and yeast extract and their combination on chocolate spot disease of faba bean in green house

Results in Table (7) showed that all tested bio-agents and their combinations as well as Kocide101 fungicide significantly reduced disease severity and disease incidence of chocolate spot during two growing seasons. The maximum reduction in disease was by the fungicide Kocide101. The treatment of seed and foliar spraying with the combination of *R+T.viride* T2+ yeast was the best bio-agents in reduction of disease severity while, the individual treatment with *T.viride* T2 or *T. harzianum* was the most effective against disease incidence.

Effect of Rhizobium, *Trichoderma* spp. and yeast extract and their combinations on the productivity of faba bean plant infected with chocolate spot in green house

It is clear from Table (7) that, the three bio-agents and their combinations significantly increased faba bean yield components [No. of

pods plant^{-1}, seed yield (g plant^{-1}) and weight of 100 seed (g)] in the two growing seasons. In this respect, the trio combination of *R+T. viride* T2+ yeast and *R+ T. harzianum* E3+ yeast gave the highest increase in productivity traits. On the other hand, the fungicide Kocide101 had not significant effect on yield parameters

Table (8): Effect of *Rhizobium*, *Trichoderma* spp. and yeast extract and their combinations on the productivity of faba bean plant infected with chocolate spot in green house

Treatments	No. of pods/plant				Seed yield g/plant				Weight of 100 seeds (g)			
	2014/2015		2015/2016		2014/2015		2015/2016		2014/2015		2015/2016	
Control (*B. fabae*)	31.67	h	23.67	j	55.62	J	41.47	i	62.79	h	62.96	h
Rhizobium	45.67	d	36.33	ef	76.50	Fg	65.99	ef	66.26	d	66.25	e
T.viride T2	39.67	ef	31.00	gh	73.60	Gh	63.74	fg	64.41	f	64.30	g
T. harzianum E3	36.67	fg	29.67	h	71.38	H	61.78	g	63.67	g	63.81	g
Yeast	34.00	gh	28.00	hi	65.32	I	57.95	h	65.40	e	65.32	f
R + T.viride T2	57.33	b	44.67	c	87.40	Bc	77.05	bc	68.05	bc	67.65	cd
R+ T. harzianum E3	52.33	c	40.33	d	84.72	cd	74.71	c	67.63	c	67.06	d
R + yeast	43.00	de	34.33	fg	75.48	Fg	65.41	f	68.36	b	68.21	bc
Yeast + *T.viride* T2	49.67	c	39.33	de	80.82	De	70.92	d	65.90	de	65.68	ef
Yeast + *T. harzianum* E3	46.00	d	38.00	de	78.93	Ef	68.44	de	65.48	e	65.36	f
R+T.viride T2+ yeast	62.33	a	52.00	a	91.70	A	81.28	a	69.65	a	69.65	a
R+ T. harzianum E3+ yeast	60.67	ab	48.33	b	90.67	ab	78.35	b	69.28	a	68.45	b
Koside 101	34.33	gh	26.00	ij	57.45	j	42.44	i	62.29	h	62.79	h

* Means followed by different letters in above columns are significantly different (high), while similar letters indicates small significance according to Duncan's multiple range test at *p=0.05*

Discussion

Using antagonistic microorganisms as a biological control leads to decrease the fungicides use and minimize their residues in agriculture products. At the same time, it is easy in application, not expensive, safe, un-hazardous for human and avoids environmental pollution [41]. The results of the present work revealed that all of twenty five *Botrytis fabae* isolates isolated from naturally infected faba bean plants collected from five different districts in Dakahlia governorate were found to be pathogenic and caused typical symptoms of chocolate spot disease while, the isolate B4 was the most aggressive. These results were in accordance with that of [42] who isolated the causative organism of chocolate spot disease from diseased fababean leaves collected from different counties of Dakahlia governorate namely; Aga, Mansoura, Sinbllawin and Sherbeen. Also, These results were in accordance with those of [43,44].

The present result indicated that all the tested twenty five *Trichoderma* isolates showed some sort of antagonistic activity against *B. fabae* B4 while the isolate *T. viride* T2 was the most potent antagonist to the pathogen *B. fabae* B4 followed by *T. harzianum* E3. This result was in accordance with that previously confirmed by several researchers that *Trichoderma* spp have antagonistic effect against several plant pathogens [45,10]. *Trichoderma* spp inhibits the fungal growth due to competition, parasitism and antibiosis [46]. In this investigation both *Trichoderma* sp. grew considerably faster than *B. fabae*. So, it has an important advantage in competition for space and nutrients with the pathogen, even before it deploys its arsenal of mycotoxins [32, 10].

The direct antagonism of against *B. fabae* might be due to producing several cell wall degrading enzymes and antibiotics which causes dissolution of the fungus cell walls forming holes which act as direct entry of *Trichoderma* hyphae into the target fungus [46]. Saber *et al*. [10] showed fragmented, vacuolated and disrupted hyphae of *B. fabae* mycelia due to strong mycoparasitism by *Trichoderma* which produced inhibition halos and sporulated over the colonies of the fungus. Benítez *et al*. [47] concluded that such mechanisms of *Trichoderma* which use a biocontrol on phytopathogenic fungi, as well as to the isolation of several genes encoding either enzymes and structural or regulatory proteins or components of signaling pathways that are involved in processes such as the specific recognition of hosts by *Trichoderma* strains. The various mechanisms of *Trichoderma* spp. including nutrient competition, antibiosis,

antagonism, inhibition of pathogen or plant enzymes; processes of biodegradation, carbon and nitrogen cycling; complex interactions with plants in the root zone of the rhizosphere, which involve various processes such as colonization, plant growth stimulation, bio-control of diverse plant pathogens, decomposition of organic matter, symbiosis and nutrient exchange [45,48].

The present result revealed that, among the three bio-agents; *T. viride* T2, T. *harzianum* E3 and yeast extract, yeast extract was the most potent antagonism against *B. fabae* giving the highest %inhibition of growth parameters and sporulation followed by *T. harzianum* E3 then *T. viride* T2. That inhibitory effect of yeast may be due to the production of soluble antifungal metabolites diffused into the medium where the action mechanisms of yeast against pathogenic fungi are either by the production of proteinaceous killer toxins [49,50]; or production of hydrolytic enzymes that degrade the cell wall of the pathogenic fungi [51]; or production of toxic volatile compounds [52] which linked with the reduction in growth of pathogenic fungi. *Saccharomyces cerevisiae* extract has inhibitory effect on different fungal genera during storage of peanut seed [53]. Also, it showed antifungal activity against the pathogens of soybean root and stalk rot diseases [54]. The antimicrobial action of yeast against the pathogenic fungi could be due to production of hydrolytic enzymes capable of degrading the cell wall of pathogen [51].

The obtained results indicated that both non-volatile and volatile metabolites of *T. viride* T2 and *T. harzianum* E3 significantly inhibited the linear growth of *B. fabae* where the non–volatile metabolites of both *Trichoderma* species were more potent in inhibition of mycelial growth of *B. fabae* than the volatile metabolites. These results were matching what mentioned by Prasad and Kumar [54] that the antagonistic effect of *Trichoderma* spp may be due to production the antimicrobial metabolites (non-volatile and volatile) which inhibited the mycelial growth and sporulation).

This work found that both of *T. viride* T2 *and T. harzianum* E3 produced cellulolytic and chitinolytic activity more than *B. fabae*. On the other hand, *B. fabae* produced much more proteolytic activity than them. There was no significant difference in amylolytic activity of all the three tested fungi. This was in agreement with the results obtained by Prasad and Kumar [55] who found that *Trichoderma* spp. secret extracellular enzymes that degrade the cell wall of the fungal pathogen.

In this study, the green house experiment indicated that the treatment of Faba bean seeds and foliar spraying with the combination of *Rhizobium, T. viride* T2 and yeast was the best bio-agents combination in reduction of chocolate spot disease severity while, the individual treatment with *T. viride* T2 or *T. harzianum* was the most effective against disease incidence. There is a synergistic effect between *Rhizobium* and *Trichoderma* due to the production of chitinase and cellulase enzymes by *Trichoderma*. These results highlighted the role of *R. leguminosarum* in decreasing the harmful effect of *B. fabae* may be due to its production of extracellular compounds with direct antimicrobial activities and auxin-like substances as well as lipopolysaccharides [10,56]. The auxin-like substances led to increase total phenols and calcium content which improve the growth and protect plants against pathogens [57]. These enzymes may contribute to promotion of nodule formation by rhizobia through improving the attack of rhizobia to specific legume plant and facilitate the penetration of rhizobia into the root hair tissue. In addition to, *Trichoderma* treatment leads to enlargement in root size and rooting depth which causes increase the number and biomass of nodules, hence, nitrogenase activity [58,59].

The increment in faba bean productivity under the application of *Rhizobium* and *Trichoderma* proved by this work may be back to the combined action of rhizobia that stimulated plant growth by nitrogen fixation and secretion of growth promote or substances and *Trichoderma* which have been, recently, reported as plant growth promoting fungi [59].

In conclusion, combinations among some biotic agents like the combination of *Rhizobium, T. viride* T2 and yeast and the combination of *Rhizobium, T. harzianum* E3 and yeast were highly recommended to produce commercial products to control chocolate spot

disease of faba bean and achieve the greatest productivity of the plant.

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