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Synergistic effect of *Trichoderma* and *Rhizobium* on Both Biocontrol of Chocolate Spot Disease and Induction of Nodulation, Physiological Activities and Productivity of *Vicia faba*

¹W.I.A. Saber, ²K.M. Abd El-Hai and ³K.M. Ghoneem

¹Department of Microbiology, Soils, Water and Environment Research Institute, Agricultural Research Center, Giza, Egypt

²Department of Leguminous and Forage Disease Research,

³Department of Seed Pathology Research, Plant Pathology Research Institute, Agricultural Research Center, Giza, Egypt

Abstract: Experiments were carried out to correlate the biochemical features of *Trichoderma* species and *Rhizobium leguminosarum* to both biocontrol of *Botrytis fabae* and improving the productivity of faba bean. Of several *Trichoderma* species, isolated from phyllosphere of faba bean, six isolates, which grew considerably faster than *B. fabae* and have moderate to very good antagonism against this pathogen, were selected. *Trichoderma*'s growth inhibiting properties of *B. fabae* were due to the combined action of non-volatile and volatile metabolites (with antibiotic nature) and the secretion of cell-wall degrading enzymes. *Trichoderma viride* (tag3 and tag4) and *T. harzianum* tag7 have shown to be efficient mycoparasites on *B. fabae* (in which the mycelium appeared to be fragmented hyphae, vacuolated and disrupted as a result of *Trichoderma* parasitism). These three *Trichoderma* isolates were further applied in field of faba bean combined with *R. leguminosarum* which, the chromatographical analysis of its supernatant showed activity in growth promoter substances. The dual inoculation of seeds with a mixture of *R. leguminosarum* and *T. viride* tag4 then foliar spraying of the developed plants with the spore suspension of the same *T. viride* tag4 at the 35th and 55th day from sowing reduced chocolate spot disease and enhanced nodulation, nitrogenase activity and nitrogen fixing bacterial population in the rhizosphere. In addition to the improvements in the physiological activities (photosynthetic pigments, total phenol and polyphenol oxidase), plant growth and yield. On average, this treatment recorded about 57% reduction in chocolate spot disease and 23% increase in faba bean yield, compared to control plants. Therefore, a commercial production of an inoculum based on a mixture of *Rhizobium* and *Trichoderma* is very encouraged.

Key words: *Trichoderma*, *Rhizobium*, *B. fabae*, nodulation, faba bean, biological control, cell wall degrading enzymes

INTRODUCTION

Trichoderma species are free-living imperfect fungi, which are highly interactive in root, soil and foliar environments. Some of *Trichoderma* members possess mycoparasitic abilities

Corresponding Author: W.I.A. Saber, Department of Microbiology, Soils, Water and Environment Research Institute, Agricultural Research Center, Giza, Egypt

against pathogenic fungi, which allow for the development of biocontrol strategies based on *Trichoderma* in order to restore the beneficial balance of natural ecosystems, which is often lost in the crop situation (Harman *et al.*, 2004; Kredics *et al.*, 2004). *Trichoderma* work against fungal phytopathogens either indirectly by competing for nutrients and space, modifying environmental conditions, promoting plant growth and plant defense mechanisms and antibiosis; or directly through mechanisms such as mycoparasitism (Shakeri and Foster, 2007; Reino *et al.*, 2008).

Recent evidences indicating induced localized and systemic resistance by *Trichoderma* species in a range of plants to a variety of phytopathogens, in addition to their substantial influence on plant growth and development (Harman *et al.*, 2004; Adams *et al.*, 2007; Reino *et al.*, 2008). Moreover, *Trichoderma* are active in the production of fungal cell wall degrading enzymes including pectinases, cellulases and chitinases that are involved in biocontrol process (Barbosa *et al.*, 2001). The dual inoculation of legume seeds with chitinolytic and cellulolytic microbes plus *Rhizobium* not only protects plants against several diseases but also, necessary to improve growth and nodulation (Sindhu and Dadarwal, 2001). Anyhow, now work available about coinoculation of *Rhizobium* and *Trichoderma*.

There is an urgent need to improve *Vicia faba* yield, since this plant remains an important part of the diet of both humans and domestic animals in many parts of the world, because of its high nutritive value in both energy and protein contents. Furthermore, faba bean supplies an important benefit to the crop by fixing atmospheric nitrogen in symbiosis with *R. leguminosarum* thus, reducing costs and minimizing impact on the environment, that is why increasing the plant production is one of the major targets of the agricultural policy in several countries (El-Fallal and Migahed, 2003; Mahmoud *et al.*, 2004).

Chocolate spot, caused by *Botrytis fabae*, is a worldwide disease capable of devastating the unprotected faba bean, result in harmful effects on growth, physiological activities and yield. 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 faba bean as well as on the accompanying microflora. Therefore, controlling *B. fabae* by biocontrol agents seemed to be better and preferred than the chemical control (El-Fallal and Migahed, 2003; Mahmoud *et al.*, 2004).

Herein, a novel strategy was conducted for controlling chocolate spot disease as well as, enhancing growth, nodulation, physiological activities and yield of faba bean by *Trichoderma* species and *R. leguminosarum*.

MATERIALS AND METHODS

***Rhizobium leguminosarum* and Faba Bean Seeds**

Rhizobium leguminosarum bv. *vicia* was kindly obtained from the Biofertilizer Production Unit, Agric. Res. Center, Giza, Egypt. Seeds of faba bean; Giza 2 (susceptible for chocolate spot infection) were purchased from Legumes Department, Field Crops Research Institute, Agricultural Research Center, Giza, Egypt.

Isolation of *B. fabae* and *Trichoderma* Species

Twenty-one isolates of *B. fabae* were isolated from naturally infected faba bean leaves (showed typical symptoms of chocolate spot disease) collected from different fields of Dakhliya governorate. Leaves were surface sterilized (with 0.5% sodium hypochlorite solution for 1 to 2 min) and plated on faba bean dextrose agar medium (400 g of faba bean leaves (autoclaved and filtered to obtain faba bean infusion), 20 g of dextrose and 18 g agar)

at 20°C (Haggag *et al.*, 2006). Isolates were purified and identified according to Morgan (1971). The most aggressive isolate was selected on the base of pathogenicity test.

Eleven *Trichoderma* isolates (tag1 to tag11) were isolated from the phyllosphere of healthy faba bean plants grown in different fields at Dakhlia governorate, Egypt. *Trichoderma* species were isolated on the selective medium of Elad *et al.* (1991). The developed colonies were transferred onto PDA slants and identified after growing them on malt extract agar for two days at 25°C according to Rifai (1969) and Bissett (1991).

Growth Rate and Dual Culture Tests

For growth rate, plates of PDA were inoculated in the center with a 5 mm disc of individual *Trichoderma* isolate or *B. fabae*. After 3 days of incubation at 26°C, the colony diameter of each fungus was measured and the growth rate per day was calculated. For dual culture test, plates of PDA were inoculated with a 5 mm disc of *B. fabae*, 10 mm from the edge of Petri dish. A 5 mm disc of the tested *Trichoderma* isolate was placed 60 mm from the *B. fabae* disc, 24 h after the original inoculation. Paired cultures were incubated at 26°C in the light for 5 days. Plates inoculated with *B. fabae* only served as control. The growth of individual and dual cultures of the fungi were recorded and interaction between dual mycelia scored for degree of antagonism using a scale of 1 to 5 (Bell *et al.*, 1982), where 1 = *Trichoderma* overgrowing *B. fabae* and 5 = *B. fabae* overgrowing *Trichoderma*. *B. fabae* developed from plates of dual cultures were then, microscopically investigated and the changes in the mycelium of *B. fabae* were recorded.

Survival of *B. fabae* Mycelium when Paired with Conidia of *Trichoderma*

Mycelial discs (5 mm) of *B. fabae* were soaked in a conidial suspension of each of *Trichoderma* isolates (8 day old culture) and then placed in Petri dishes on moistened filter paper for 12 h. Discs were then removed to PDA supplemented with benomyl (1%) to suppress the growth of *Trichoderma*. Survival was expressed as the percentage of the mycelial discs from which *B. fabae* grew (Sivan and Chet, 1989).

Antagonistic Activity of Non-Volatile and Volatile Metabolites of *Trichoderma*

The effect of non-volatile and volatile metabolites of *Trichoderma* species against *B. fabae* was tested following the method of Barbosa *et al.* (2001). For the non-volatile metabolites, mycelial agar plugs removed from the edge of a young culture of *Trichoderma* species were transferred to the center of Petri dishes (9 cm diameter) containing PDA and a sterilized cellophane disc (9 cm diameter) adjusted on the medium surface, where the antagonist grown for 60 h. Then the cellophane containing the *Trichoderma* growth was removed and on the same medium, a disc of *B. fabae* was placed. The control treatments had *B. fabae* growing similarly on PDA medium where previously there was a cellophane disc without antagonist. For volatile metabolites, two bottoms of Petri dishes containing PDA were individually inoculated with a disc of pathogen and antagonist; the two bottoms were adjusted and attached by tape. The control sets did not contain the antagonist. The cultures were incubated at 26°C and 60% relative humidity under alternating luminosity (12 h light/12 h darkness). Percent of growth inhibition in both assays were estimated after 3 days.

Extraction of Metabolites with Antibiotic Activity from *Trichoderma*

Trichoderma isolates were grown in Erlenmeyer flasks (1 L) containing 250 mL PD broth. The cultures were incubated at 28°C on shaker at 140 rpm in the dark for 7 days. Culture filtrates were extracted twice by shaking in a separatory funnel with two third of its volume of ethyl acetate (EtOAc). The EtOAc phases were then pooled and concentrated to give a

final volume equivalent to a 250-fold concentration of the original volume of the filtrate (Melo and Faull, 2000). Antifungal activity was tested *in vitro* against *B. fabae*. The percentage of reduction in growth and sporulation were calculated.

Extracellular Enzymes of *Trichoderma* and *B. fabae*

The activities of some extracellular hydrolytic enzymes of *Trichoderma* isolates and *B. fabae* were detected by placing mycelial disks of the tested fungi on solid media containing the enzyme substrate and measuring the zone of degraded substrate formed around the colony. Cellulase and protease activities were studied using microcrystalline cellulose and milk agar as substrates, respectively (Barbosa *et al.*, 2001), chitinase using chitin (Ulhoa and Peberdy, 1991) and amylase activity, using soluble starch (Hankin and Anagnostakis, 1975).

Detection of Growth Substances Secreted by *R. leguminosarum*

Yeast Mannitol medium (Somasegaran and Hoben, 1985) was used for *R. leguminosarum* cultivation and inoculum preparation. Auxin-like substances in the supernatant of *R. leguminosarum* were detected colorimetrically at 530 nm using Ehrlich reagent (Larsen *et al.*, 1962). The promoter and inhibitor substances were determined chromatographically according to the method of Hartmann *et al.* (1967).

Field Experiment

Experiments were carried out at Tag El-Ezz, Agric. Res. Station, Dakhlia, Egypt, during 2007/2008 and 2008/2009 seasons. Soil is clayey in texture containing 54.1 clay, 36.5 silt and 9.4% sand. The E_c in soil paste = 6.04 dS m⁻¹, pH in water suspension (1:2.5) = 8.0, ESP = 8.16%, organic matter = 1.67% and CaCO₃ = 2.24%. Available N, P and K were 45.64, 11.21 and 283 mg kg⁻¹, respectively. The soil analysis was carried out in Mansoura Lab. for soil fertility.

The aim was to study the effect of seed inoculation (*Rhizobium* plus *Trichoderma*) in combined with foliar spraying of faba bean with *T. viride* tag3, *T. viride* tag4 or *T. harzianum* tag7 on natural infection by chocolate spot disease, as well as, on nodulation, physiological aspects, growth and yield. Before seed inoculation, an antagonism test was carried out between *Rhizobium* and *Trichoderma* to insure the compatibility between both microbes. At sowing, *R. leguminosarum* (10⁸ cfu mL⁻¹) was used individually or mixed in equal portion with each of *Trichoderma* sp. (10⁷ conidia⁻¹) to inoculate faba bean seeds in the presence of Arabic gum (16%). Inoculated seeds were air dried and sown immediately. In addition to seed inoculation, developed plants from each assigned treatment were sprayed with the spore suspension of the same *Trichoderma* sp. that was previously used in seed inoculation. Spraying was performed two times with 20 day interval beginning from 35 day after sowing. All other agricultural practices were carried out as usual. Complete randomized plot design was used. Accordingly, the following five treatments were allocated:

- Dual inoculation of seeds with a mixture of *R. leguminosarum* and *T. viride* tag3 then foliar spraying of the developed plants with the spore suspension of the same *T. viride* tag3 after 35 and 55 day from sowing (R+Tv.tag3)
- Dual inoculation of seeds with a mixture of *R. leguminosarum* and *T. viride* tag4 then foliar spraying of the developed plants with the spore suspension of the same *T. viride* tag4 after 35 and 55 day from sowing (R+Tv.tag4)
- Dual inoculation of seeds with a mixture of *R. leguminosarum* and *T. harzianum* tag7 then foliar spraying of the developed plants with the spore suspension of the same *T. harzianum* tag7 after 35 and 55 day from sowing (R+Th.tag7)

- Inoculation of seeds with *R. leguminosarum* only then foliar spraying of the developed plants with the fungicide; kocide 101 (2.5 g L⁻¹) after 35 and 55 day from sowing (R+ kocide)
- Control; inoculation of seeds with *R. leguminosarum* only then foliar spraying of the developed plants with tap water after 35 and 55 day from sowing (R)

Disease Assessment

At 50 and 70 day from sowing (15 days after each spray), plants were rated for both Disease Incidence (DI); as the percentage of infected leaves and Disease Severity (DS) according to the scale (class rate from 1 to 9) of Bernier *et al.* (1993) following the next formula:

$$DS \% = \frac{\sum (NPC \times CR)}{NIP \times MSC} \times 100$$

where, NPC is the number of plants in each class rate, CR is the class rate, NIP is the number of infected plants and MSC is the maximum severity class rate.

Nodulation Status and Nitrogenase Activity

Samples of faba bean roots were collected after 50 and 70 days from sowing (20 days after each spray) for the determination of number and dry weight (at 70°C until constant weight) of nodules. Nitrogenase activity was determined (Lethbridge *et al.*, 1982) in the second season only.

Physiological Activities of Faba Bean Plants

After 70 days from sowing in the second season, the following physiological activities were tested; (1) Photosynthetic pigments (chlorophyll (Chl) a, b and total Chl and carotenoids) were determined in the blade of the third leaf of plant tip (terminal leaflet) (Mackinney, 1941), (2) Total phenolic compounds were determined in fresh shoot using the folin-ciocalteau reagent (Malik and Singh, 1980) and (3) Polyphenol oxidase was assayed colorimetrically using catichol as substrate (Matta and Dimond, 1963), the activity was expressed as the change in the absorbance of the reaction mixture every 50 sec for 5 min period at 495 nm.

Morphological Characters and Yield and its Components

After 70 days from sowing, growth parameters per plant (height and number of branches and leaves) were measured. At harvest, pods number plant⁻¹ and seeds number pod⁻¹, seed index (weight of 100-seed) and seed yield were recorded.

Microbial Counts

The media of Bridson (1978), Watanabe and Barraquio (1979) and Martin (1950) were used for counting the total bacteria, nitrogen fixers and total fungi, respectively, in the rhizospheric soil of faba bean plants in the second season at the 50th and at 70th day from sowing.

Statistical Analysis

All data were subjected to analysis of variance using the statistical analysis software; CoStat v6.4. Compare among means was carried out using either Tuckey test (for laboratory) or Duncan's new multiple range test (for field).

RESULTS AND DISCUSSION

Growth Rate of *Trichoderma* Isolates and the Pathogen

From the biological control view point, one of the most important principals for choosing the antagonistic organism is its capability to grow fast and occupy most of the place in the site of probable exist of the pathogen. For this reason, 11 *Trichoderma* species (isolated from faba bean phyllosphere) and aggressive isolate (isolated from infected faba bean plants) of chocolate spot causal pathogen (*B. fabae*) were investigated for the growth rate (Fig. 1). Significant difference in growth was observed between individual *Trichoderma* isolates. However, all *Trichoderma* isolates grew considerably faster than *B. fabae*. The rapid growth gives *Trichoderma* an important advantage in the competition for space and nutrients with plant pathogenic fungi, even before it deploys its arsenal of mycotoxins (Barbosa *et al.*, 2001). Accordingly, the fastest six *Trichoderma* isolates in growth rate were identified to species level and used for further studies.

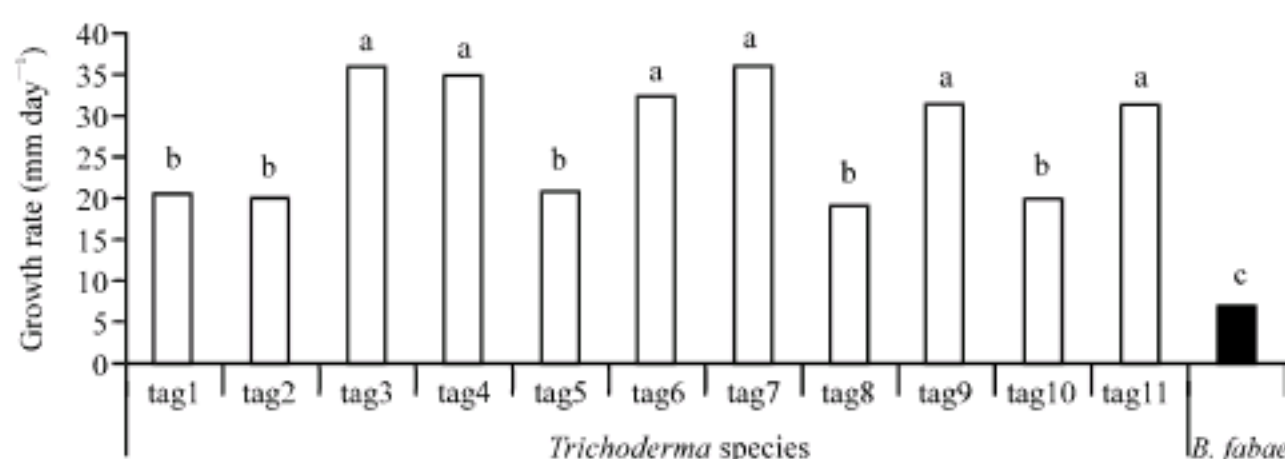


Fig. 1: Growth rate of various *Trichoderma* species and *B. fabae* on PDA. Different letters indicate significant difference at $p \leq 0.01$ (Tuckey test)

Direct Antagonism of *Trichoderma* Against *B. fabae*

In dual culture test, the six *Trichoderma* isolates inhibited the growth of *B. fabae* (Table 1). *T. viride* (tag3 and tag4) and *T. harzianum* tag7 showed high degree of antagonism and reduced the growth of *B. fabae* by over 60% after 5 days of the dual growth. The best antagonism reaction (1) was recorded by *T. viride* tag3 and *T. harzianum* tag7, which means the occurrence of strong mycoparasitism. The light microscope investigation showed *B. fabae* mycelium to be fragmented hyphae, vacuolated and disrupted. Moreover, when

Table 1: Growth of *B. fabae* as affected by different *Trichoderma* species in dual culture test

Antagonistic fungus	Growth of <i>B. fabae</i> (%) [*]		Antagonism reaction ^{**}
	After 3 days	After 5 days	
<i>T. viride</i> tag3	38.19c	29.50f	1
<i>T. viride</i> tag4	46.37b	39.64d	2
<i>T. polysporum</i> tag6	62.27a	58.89b	3
<i>T. harzianum</i> tag7	45.29bc	35.28e	1
<i>T. hamatum</i> tag9	61.25a	62.22a	3
<i>T. sp.</i> tag11	60.86a	55.73c	2

^{*}(Radius growth of *B. Fabae* in the direction of *Trichoderma*/Radius of growth in the absence of *Trichoderma*) $\times 100$.

^{**}Numbers refer to antagonism reactions of *Trichoderma* with *B. fabae* based on the antagonism scale of Bell *et al.* (1982) after 5 days of dual growth. Different letters within a column indicate significant difference at $p \leq 0.01$ (Tuckey test)

plates were observed for more than 5 days, three species of *Trichoderma* (tag3, tag4 and tag7) were found to produce inhibition halos and sporulated over the colonies of *B. fabae*. These observations were confirmed by studying the survival of *B. fabae* mycelium when paired with the conidial suspension of *Trichoderma* (Fig. 2) in which, remarkable inhibition of mycelial growth of *B. fabae* was observed. *T. viride* (tag 3 and tag4) and *T. harzianum* tag7 totally inhibited the mycelial growth of *B. fabae*. Melo and Faull (2000) recorded similar observations.

Once the fungal pathogen come into contact, *Trichoderma* sp. attach to and can coil around it, in some cases, form appressoria on the host surface, wherein *Trichoderma* produce several cell wall degrading enzymes and probably also antibiotics, the combined activities of these compounds result in parasitism and dissolution of the cell walls forming holes which acts as direct entry of *Trichoderma* hyphae into the target fungus (Melo and Faull, 2000; Harman *et al.*, 2004). This interpretation could be applied only for *T. viride* (tag3 and tag4), *T. harzianum* tag7 and *Trichoderma* sp. tag11. The other two isolates persisted strong antibiosis zone against *B. fabae* without mycoparasitism.

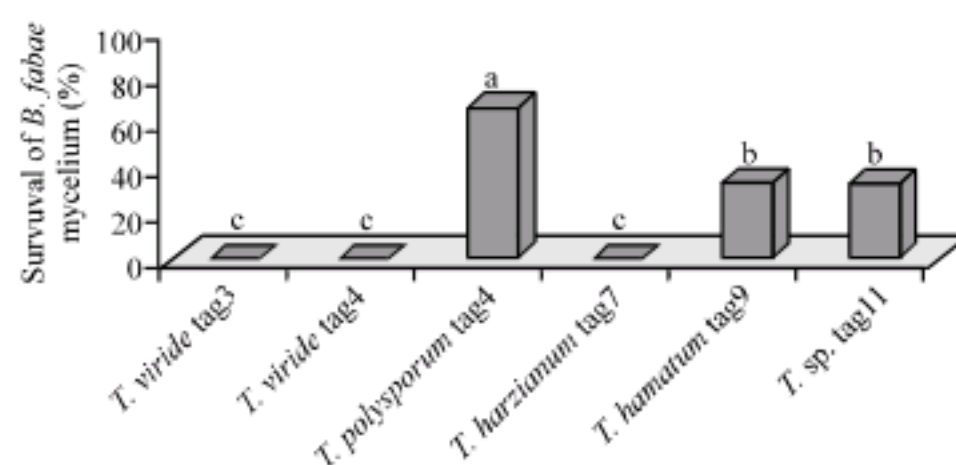


Fig. 2: Survival of *B. fabae* mycelium when paired with conidia of different *Trichoderma* species. Different letters indicate significant difference at $p \leq 0.01$ (Tuckey test)

Antagonistic Activity of *Trichoderma* Metabolites

Data of Fig. 3 show the effect of non-volatile and volatile metabolites of *Trichoderma* on *B. fabae* growth. The non-volatile substances were responsible for most of the growth

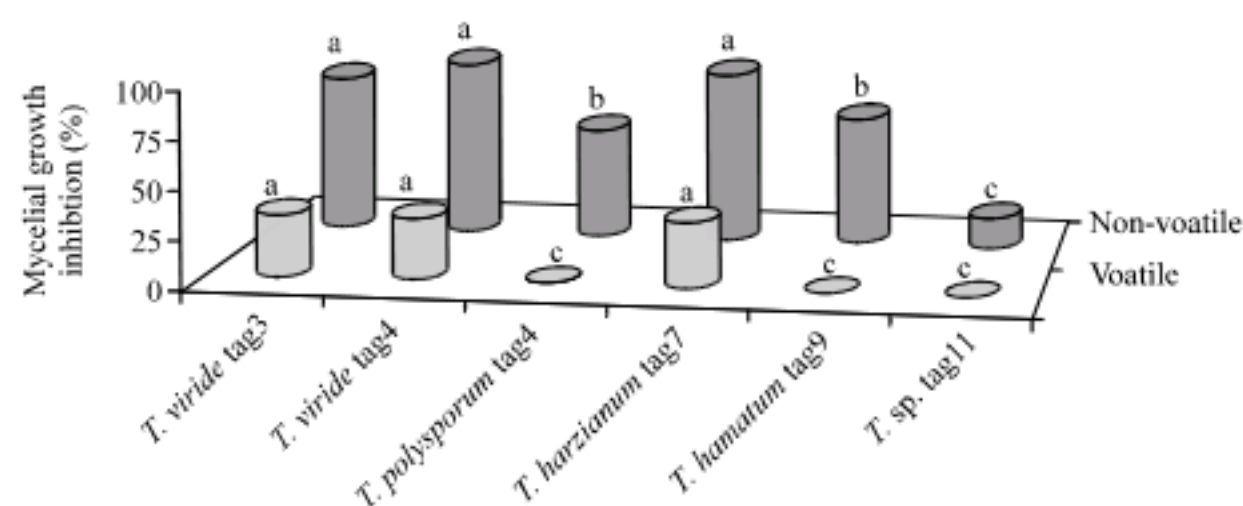


Fig. 3: Inhibition of *B. fabae* growth by non-volatile and volatile metabolites of different *Trichoderma* species. For each metabolite. Different letters indicate significant difference at $p \leq 0.01$ (Tuckey test)

inhibition, when *B. fabae* was cultivated in the same Petri dish, where *Trichoderma* was previously grown, antagonism was readily observed. *T. viride* (tag3 and tag4) and *T. harzianum* were more efficient than the other isolates; they reduced the growth of *B. fabae* by over 70%. For the volatile metabolites, *T. viride* (tag3 and tag4) and *T. harzianum* tag7 were the only ones to produce some growth inhibition by volatile substances. These *Trichoderma* antibiotics were extracted with EtOAc and their impact on the growth of *B. fabae*, were evaluated. Data of Table 2 show that EtOAc phase extracts of *T. viride* (tag3 and tag4) and *T. harzianum* tag7 possessed strong antifungal activity and inhibited *B. fabae* growth and sporulation by over 73 and 90%, respectively. *Trichoderma hamatum* did not produce any antibiotic activity.

Table 2: Antibiotic activity of ethyl acetate extracts (EtOAc phase) of different *Trichoderma* species against growth and sporulation of *B. fabae*

Fungus	Growth inhibition (%)	Sporulation inhibition (%)
<i>T. viride</i> tag3	80.1a	96.7a
<i>T. viride</i> tag4	73.7b	90.3a
<i>T. polysporum</i> tag6	33.3c	50.1b
<i>T. harzianum</i> tag7	79.5a	95.5a
<i>T. hamatum</i> tag9	00.0e	00.0c
<i>T. sp.</i> tag11	12.8d	47.8b

Different letters within a column indicate significant difference at $p \leq 0.01$ (Tuckey test)

Species of *Trichoderma* are known to have about 20 to 30 genes, proteins, enzymes and other metabolites that are directly involved in the interaction between *Trichoderma* and target fungi (Harman *et al.*, 2004). The antifungal activity of *Trichoderma* metabolites is achieved by biosynthesizing a wide array of non-volatile metabolites e.g., anthraquinones, koniginins, trichodermamides, steroidal antibiotic, viridifungins, harzialactones, trichothecenes, peptaibols, statins, acoranes and miscellanea, in addition to, volatile metabolites, with strong coconut smell, e.g., simple aromatic compounds, some polyketides (e.g., pyrones (6-pentyl-2-pyrone) and the butenolides), volatile terpenes and the isocyanate metabolites (Barbosa *et al.*, 2001; Harman *et al.*, 2004; Shakeri and Foster, 2007; Reino *et al.*, 2008).

Extracellular Enzymatic Activity

The secretion of the extracellular cell wall degrading enzymes were tested for both *Trichoderma* species and *B. fabae* (Fig. 4). Species of *Trichoderma* produced much more cellulolytic, amylolytic and chitinolytic activities than *B. fabae*. Only the species of *Trichoderma* secreted amylase and chitinase. With exception to the low proteolytic activity of *T. viride* (tag3 and tag4) and *T. harzianum* tag7, protease was, exclusively, secreted by *B. fabae* only.

Several reports have demonstrated positive relationship between the production of cellulase, chitinase and protease and the ability to control plant diseases, these enzymes are induced in *Trichoderma* during the parasitic interaction and can inhibit the growth of several fungal plant pathogens by degrading cell walls (Melo and Faull, 2000; Harman *et al.*, 2004; Shakeri and Foster, 2007). The ready expression of amylolytic enzymes in *Trichoderma* than *B. fabae* is partially responsible for the rapid growth of *Trichoderma* on PDA as, previously, shown in Fig. 1. Additionally, there is no immediate explanation for the negative proteolytic activity of some *Trichoderma* species, except the possible attachment of protease to the mycelium of *Trichoderma* (Barbosa *et al.*, 2001). Microbes with these enzymatic activities positively induce the nodulation when used in combined with rhizobia to inoculate legumes seeds (Sindhu and Dadarwal, 2001).

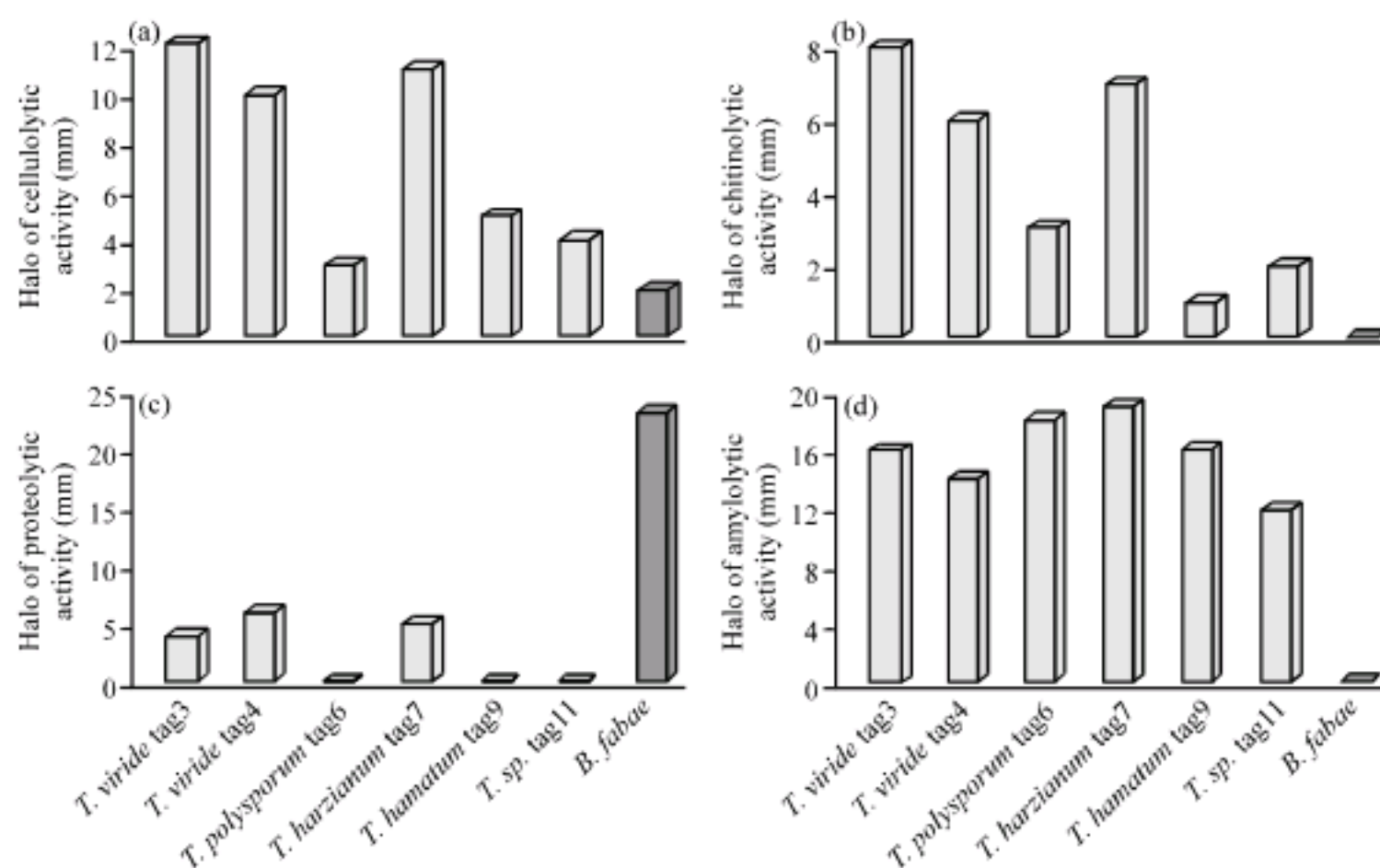


Fig. 4: Extracellular enzymatic activities for both *Trichoderma* species and *B. fabae*

Based on the previous results, *T. viride* (tag3 and tag4) and *T. harzianum* tag7, as the most efficient mycoparasites and antibiotic producers, were selected to be applied as seed inoculants combined with *R. leguminosarum* and, at the same time, as foliar spraying on grown faba bean plants.

Plant Growth Substances of *R. leguminosarum*

Before using *R. leguminosarum* in the dual inoculation with the selected *Trichoderma* species, the growth substances in the supernatant of *R. leguminosarum* was tested. It is clear from the chromatographical analysis (Fig. 5) that there were growth-promoting substances existing at all values of retention factor (Rf). The net unites of promoter substances being 29.5, while the auxin-like substances being 2.92 mg mL⁻¹. This means that *R. leguminosarum* has biological activity on faba bean growth. Ahmad *et al.* (2008) found that 80% of the nitrogen-fixing bacteria produce indol acetic acid. These growth substances

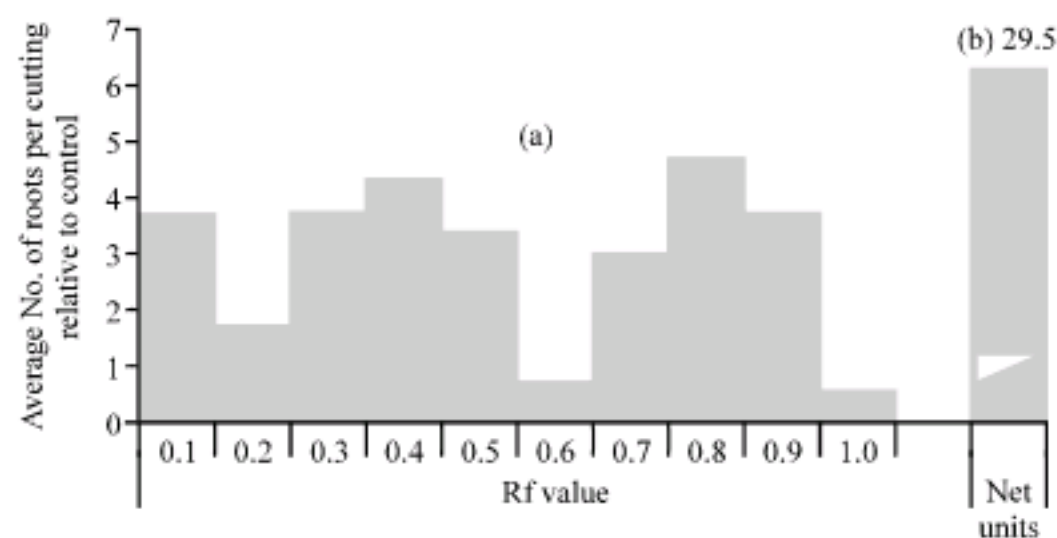


Fig. 5: The biological activity of growth substances secreted by *R. leguminosarum* at (a) different Rf values and (b) net units of growth promoters

lead to increase in total phenol, calcium content and polyphenol oxidase activity, which protect plants against pathogens and improve the growth (Chowdhury, 2003).

The antagonism between *R. leguminosarum* and the three *Trichoderma* isolates that were previously selected from the comparative *in vitro* experiments was tested. No antagonistic action was observed between *R. leguminosarum* and any of *T. viride* (tag3 and tag4) or *T. harzianum* tag7. So, *R. leguminosarum* was used to inoculate faba bean seeds in combined with the selected *Trichoderma*.

Field Evaluation of the Dual Application of *R. leguminosarum* and *Trichoderma* Incidence (DI) and Severity (DS) of Chocolate Spot Disease on Faba Bean Plants

Data (Table 3) of the two growing seasons show that, the dual inoculation of seed (*R. leguminosarum* and *Trichoderma*) and foliar spraying of grown faba bean with each of *Trichoderma* isolate (as mentioned in materials and methods) significantly reduced chocolate spot DI and DS caused by *B. fabae* as compared to control (R). Treatment of faba bean with R+Tv.tag4 reduced DI and DS by 45.3 and 59.6 and 32.8 and 51.0% (average of both seasons) after 50 and 70 days from sowing, respectively. The range of infection level, in the presence of *Trichoderma*, is economically accepted and could be comparable with that of the treatment with fungicide. In spite of the later recorded the highest reduction in disease parameters. This may be referred to the ability of *Trichoderma* to cause an inhibition of *B. fabae* growth (Table 1, 2 and Fig. 2, 3) and secretion of extracellular enzymes (Fig. 4) that can degrade the cell wall of the pathogen (Melo and Faull, 2000).

Table 3: Efficacy of dual inoculation of faba bean with *R. leguminosarum* and different *Trichoderma* species on chocolate spot disease development in field

Treatments	After 50 day				After 70 day			
	DI		DS		DI		DS	
	1 st season	2 nd season	1 st season	2 nd season	1 st season	2 nd season	1 st season	2 nd season
R+Tv.tag3	33.2b	26.0ab	9.9b	8.0b	40.6b	35.5b	21.7b	19.5b
R+Tv.tag4	28.9b	21.7b	9.0b	7.3b	35.0b	28.3c	16.3bc	13.5bc
R+Th.tag7	31.4b	22.8ab	9.8b	9.3b	45.0b	31.2bc	20.7bc	18.3b
R+kocide	15.9c	15.2c	4.8c	5.6b	13.5c	12.8d	7.3c	7.0c
R (control)	47.8a	29.0a	15.2a	14.6a	71.8a	58.2a	38.3a	35.9a

Different letters within a column indicate significant difference at $p \leq 0.05$ (Duncan test)

Nodulation Status of Faba Bean

As shown in Table 4 and Fig. 6, the dual inoculation of faba bean seeds with a mixture of *R. leguminosarum* and each of *Trichoderma* species followed by foliar spraying with *Trichoderma* led to significant enhancements in number and dry weight of nodules and nitrogenase activity during the growth period of faba bean. R+Tv.tag4 recorded the highest values of the tested parameters after 50 and 70 days from sowing than that of both fungicide (R+ kocide) and control (R) treatments. Generally, the species of *Trichoderma* had potential effect on nodulation status.

Chitinase and cellulase may contribute to promotion of nodule formation by the rhizobia after coinoculation with *Trichoderma* by improving the attack of rhizobia to the specific legume plant and facilitate the penetration of rhizobia into the root hair tissues, causing root hairs more softening and easily curling. Additionally, inoculation by *Trichoderma* lead to enlargement in root size and rooting depth, all these effects lead to increase the number and biomass of nodules, hence, nitrogenase activity (Sindhu and Dadarwal, 2001; Adams *et al.*, 2007).

Table 4: Nodulation status of faba bean as a response to dual inoculation with *R. leguminosarum* and different *Trichoderma* species after 70 days from sowing

Treatments	Nodulation after 50 day				Nodulation after 70 day			
	No. per plant		Dry wt. (mg plant ⁻¹)		No. per plant		Dry wt. (mg plant ⁻¹)	
	1 st season	2 nd season	1 st season	2 nd season	1 st season	2 nd season	1 st season	2 nd season
R+Tv.tag3	154.0b	107.0bc	0.177a	0.160b	228.3c	242.7b	0.236c	0.240b
R+Tv.tag4	169.7a	138.0a	0.171a	0.173a	349.3a	336.0a	0.280a	0.265a
R+Th.tag7	143.7b	106.3c	0.155b	0.160b	277.0b	254.0b	0.260b	0.246b
R+kocide	109.7c	112.3b	0.115c	0.111c	205.7d	208.0c	0.173d	0.152d
R (control)	112.3c	112.0b	0.114c	0.113c	189.3e	199.3c	0.178d	0.170c

Different letters within a column indicate significant difference at p≤0.05 (Duncan test)

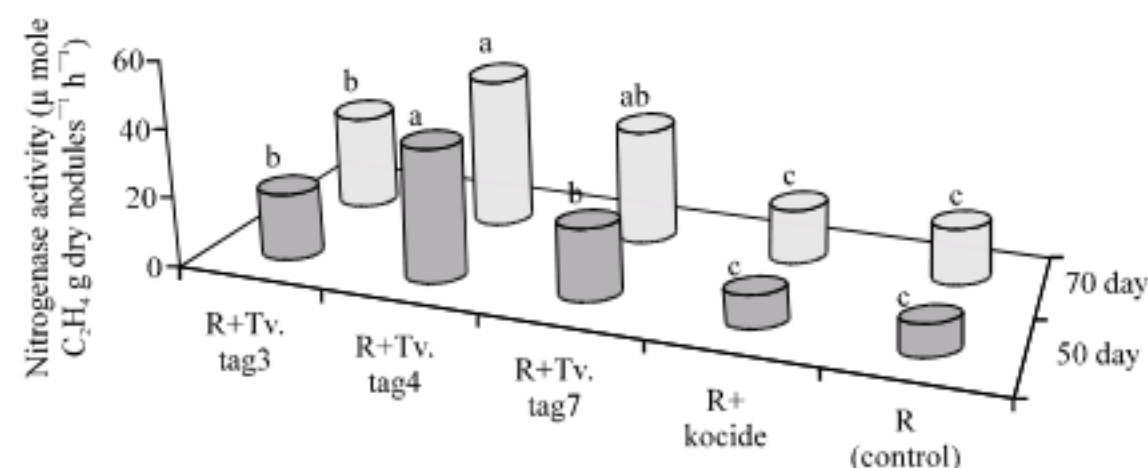


Fig. 6: Nitrogenase activity of faba bean nodules after 50 and 70 days from sowing. For each period of time, different letters indicate significant difference at p≤0.05 (Duncan test)

Physiological Activities of Faba Bean Plants

Photosynthetic pigments is a good parameter reflecting the health condition of plant. As shown in Table 5, Chl a and carotenoids did not significantly affected by any of the treatments. On the other hand, Chl b and total Chl significantly increased in plants developed from faba bean treated with R+Tv.tag4. This increment is by enhancing the efficacy of photosynthetic apparatus with a better potential for disease resistance and decrease in photophosphorylation rate usually occurring after infection (Chandra and Bhatt, 1998).

Table 5: Photosynthetic pigments (mg g⁻¹ fresh weight) of faba bean plants as a response of dual inoculation with *R. leguminosarum* and different *Trichoderma* species after 70 days from sowing

Treatments	Chl. a	Chl. b	Total Chl.	Carotenoids
R+Tv.tag3	0.967a	0.483b	1.425bc	0.156a
R+Tv.tag4	0.942a	0.712a	1.679a	0.236a
R+Th.tag7	0.949a	0.439b	1.388bc	0.253a
R+kocide	0.988a	0.572ab	1.560ab	0.263a
R (control)	0.797a	0.464b	1.261c	0.168a

Different letters within a column indicate significant difference at p≤0.05 (Duncan test)

Total phenols and polyphenol oxidase activity in faba bean plants were measured (Fig. 7) since they play important role in plant protection against pathogens, both of them were induced greatly because of the dual application of *R. leguminosarum* and *Trichoderma*, compared to the fungicide (R+kocide) and control (R) treatments.

The first step of the defense mechanism in plants involves a rapid accumulation of phenols at the infection site, which restricts or slows the growth of the pathogen because of its action as antioxidant, antimicrobial and photoreceptor (Lamba *et al.*, 2008). The

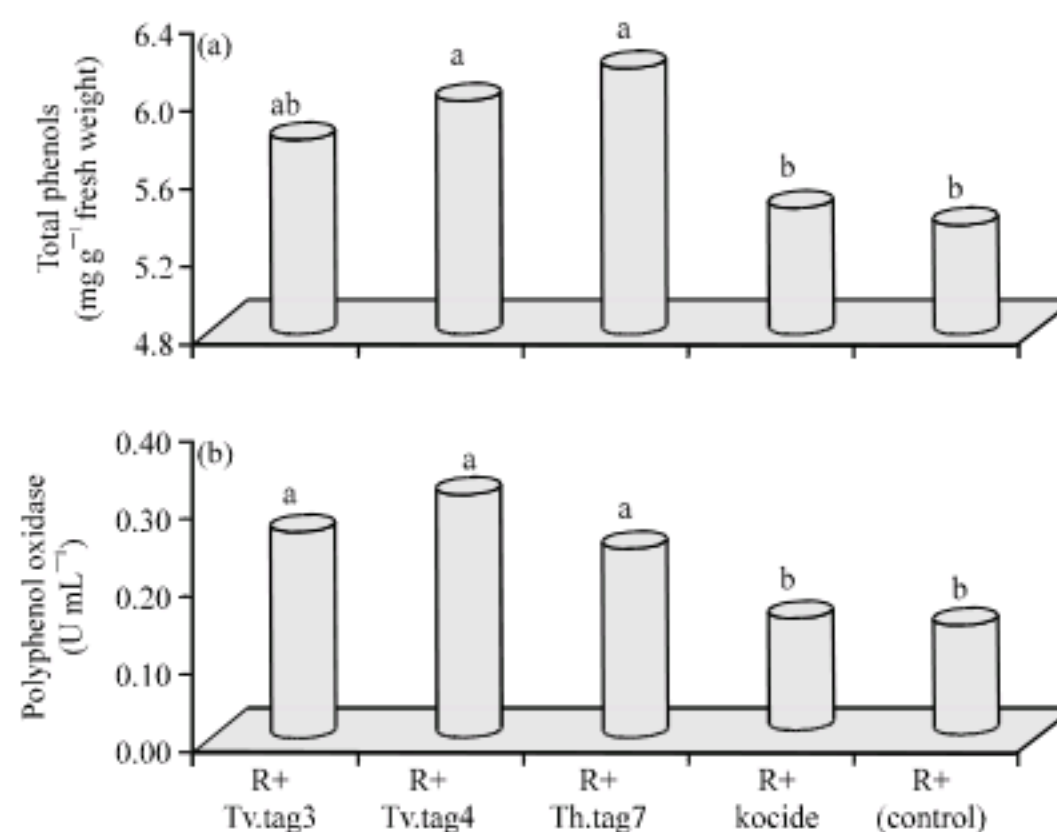


Fig. 7: (a) Total phenols and (b) polyphenol oxidase activity in faba bean plants as affected by dual inoculation with *R. leguminosarum* and different *Trichoderma* species after 70 days from sowing. In each histogram, different letters indicate significant difference at $p \leq 0.05$ (Duncan test)

suggested mechanisms for the pathogen defense role of polyphenol oxidases (PPO), include; (1) general toxicity of PPO-generated quinones to pathogens and plant cells, accelerating cell death (2) alkylation and reduced bioavailability of cellular proteins to the pathogen (3) cross-linking of quinones with protein or other phenolics, forming a physical barrier to pathogens in the cell wall and (4) quinone redox cycling leading to H_2O_2 and other reactive oxygen species, which are known to be important factors in plant pathogen interactions and defense signaling (Li and Steffens, 2002; Raj *et al.*, 2006). That is why the levels of both total phenols and PPO are naturally high in resistant varieties (Gogoi *et al.*, 2001; Raj *et al.*, 2006).

Faba Bean Growth, Productivity and Yield

As shown in Tables 6 and 7, seed inoculation with *R. leguminosarum* plus each of *Trichoderma* isolate followed by foliar spraying of the grown faba bean plants with each of same *Trichoderma* isolate increased significantly the growth, productivity and yield of faba bean, in this respect, R+Tv.tag4 followed by R+Tv.tag3 gave the highest values of the tested parameters compared with all other treatments. However, number of seeds pod^{-1} was not affected by any of the treatments. Application of R+Tv.tag4 recorded about 23% (average of the two seasons) increase in faba bean yield compared with that of seed inoculation with *R. leguminosarum* plus spraying developed faba bean plants with the fungicide (R+kocide).

These improvements may be back to the combined action of both *R. leguminosarum* that stimulated plant growth by nitrogen fixation (Fig. 6) and secretion of growth promoter substances (Fig. 5) and also, due to *Trichoderma* which have been, recently, reported as plant growth promoting fungi (Adams *et al.*, 2007). However, it is important to note that growth promotion by *Trichoderma* is not simply a result of reduced pathogenicity (although it is undoubtedly a contributing factor) but it is a combination of direct *Trichoderma* effects i.e., *Trichoderma* colonization, control of deleterious microflora, increasing the dissolution of nutrients, increasing the nitrogen usage efficiency, increasing the root size and rooting

Table 6: Efficacy of dual inoculation with *R. leguminosarum* and different *Trichoderma* species on vegetative growth of faba bean after 70 days from sowing

Treatments	Height plant ⁻¹ (cm)		No. of branches plant ⁻¹		No. of leaves plant ⁻¹	
	1 st season	2 nd season	1 st season	2 nd season	1 st season	2 nd season
R+Tv.tag3	86.6b	79.3b	6.0b	5.7ab	54.0b	52.0b
R+Tv.tag4	96.4a	84.7a	7.3a	6.4a	59.6a	60.4a
R+Th.tag7	80.4cd	73.4c	4.6c	5.0b	42.6c	46.3c
R+kocide	83.3bc	80.2ab	6.3ab	6.0a	52.7b	49.0bc
R (control)	78.4d	76.7c	4.6c	5.0b	43.0c	46.3c

Different letters within a column indicate significant difference at $p \leq 0.05$ (Duncan test)

Table 7: Efficacy of dual inoculation with *R. leguminosarum* and different *Trichoderma* species on faba bean yield and its attributes

Treatments	Yield attributes							
	No. of pods plant ⁻¹		No. of seeds pod ⁻¹		Seed index		Seed yield (Ardab/Fed)	
	1 st season	2 nd season	1 st season	2 nd season	1 st season	2 nd season	1 st season	2 nd season
R+Tv.tag3	32.7b	31.5b	3.0a	3.0a	59.7ab	61.4ab	9.8b	10.0b
R+Tv.tag4	35.4a	36.0a	3.3a	3.7a	62.6a	62.4a	10.9a	11.3a
R+Th.tag7	25.3c	24.0d	3.0a	3.0a	62.7a	60.0b	9.2b	9.6b
R+kocide	24.4c	27.0c	3.0a	3.3a	60.3ab	60.3ab	9.0b	8.9c
R (control)	21.7d	19.0e	3.0a	3.0a	57.0b	57.3c	7.6c	7.0d

Different letters within a column indicate significant difference at $p \leq 0.05$ (Duncan test)

depth, (which aid nutrient absorption and increase resistance to drought) and increasing the capable of tolerating high levels of metals in metal contaminated soils (Harman, 2000; Harman *et al.*, 2004; Adams *et al.*, 2007).

Counts of Microbial Groups

Microbial groups were enumerated in the rhizospheric soil of faba bean plants (Table 8). There were remarkable increases in the counts of total bacteria, total fungi and nitrogen fixers by the time and by the inoculation with *Rhizobium* combined with *Trichoderma*. The presence of *Trichoderma* improved the tested microbial populations, especially, the ratio of nitrogen fixers to total bacteria which reached its maximum in the presence of *Trichoderma* inoculation as in the case of R+Tv.tag4; this was in turn reflected on nitrogen fixation and the health and productivity of the plants. These increments in counts of microbial groups may be due to the action of *Trichoderma*, which colonize on plant root and can control the deleterious microflora as well as increasing root size and depth (Harman *et al.*, 2004; Adams *et al.*, 2007).

Table 8: Changes in some microbial populations in the rhizospheric soil of faba bean as a response to dual inoculation of *R. leguminosarum* and different *Trichoderma* species

Treatments	Bacterial count*							
	Total bacterial		N ₂ -fixers		N/T**		Total fungal count*	
	50 day	70 day	50 day	70 day	50 day	70 day	50 day	70 day
R+Tv.tag3	5.845	5.898	4.653	4.740	6.4	7.00	4.477	4.643
R+Tv.tag4	5.602	5.903	4.602	4.954	10.0	11.30	4.602	4.771
R+Th.tag7	5.845	5.968	4.778	4.908	8.6	8.70	4.602	4.672
R+kocide	5.778	5.785	4.000	4.041	1.7	1.80	3.845	3.851
R (control)	5.863	5.875	4.301	4.342	2.7	2.90	3.869	3.881

*The counts of microbial groups were expressed as logarithm cfu g⁻¹. **N/T = (N₂-fixers count/total bacterial count)×100. This ratio was calculated from the natural numbers of cfu not from the logarithmic numbers

Based on the presented data, the use of *T. viride* tag4 in combined with *R. leguminosarum* is an effective strategy for an integrated management of chocolate spot

disease as well as increasing growth and productivity of faba bean. Therefore, a commercial production of an inoculum (composed of both microbes) and its incorporation in the production system of *Vicia faba* is of great benefit.

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