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Combination of *Trichoderma* species and *Bradyrhizobium japonicum* in control of *Phytophthora sojae* and soybean growth

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Abstract: Soybean, *Glycine max* (L.) Merr is one of the most important oilseed plants in the world and Phythophthora root and crown rot is a significant limiting factor for its planting. In the present study the antagonistic effect of 12 *Trichoderma* spp. *in vitro* and these *Trichoderma* spp. in combination with *Bradyrhizobium japonicum in vivo* on *Phytophthora sojae* and soybean growth were tested. In laboratory tests the effects of *Trichoderma* isolates were studied in dual culture, volatile compounds and culture filtrate metabolites. The most hyphal growth inhibitions were obtained using *T. virens, T. orientals* and *T. brevicompactum* in dual culture tests and *T. atroviride* in volatile compounds test. The effects of *Trichoderma* culture filtrates on *P. sojae* hyphal growth were studied at six concentrations in CMA medium and the results showed that culture filtrates of all species inhibited the hyphal growth and that different concentrations had different inhibitory effects. The most inhibition was obtained by *T. virens* and *T. brevicampactom* culture filtrates. The greenhouse tests were carried out as two experiments. In the first experiment the effects of coated seeds with *Trichoderma* isolates and *B. japonicum*, alone and in combinations, on control of *P. sojae* and in the second experiment the effect of these two biocontrol agents on soybean growth, alone and in combinations, were assayed. In the first experiment, germination percentage, damping-off, seedling vigour index (SVI) and disease severity were measured and results showed that *T. brevicompactum* as alone and in combinations, was the most effective species. In the second experiment, coated seeds with *Trichoderma* isolates and *B. japonicum, as* alone and in combinations, significantly promoted the growth of treated seeds and the most effective species were *T. orientals, T. brevicompactum and T. spirale.* Hence, results indicate that *T. brevicompactum*, as the second most common species after *T. harzianum* in Iran, was the most successful species applied individually and in combinations with *B. japonicum* to act as biocontrol agent for *P. sojae* and was also able to promote plant growth.

Keywords: biocontrol, *Trichoderma brevicompactum, Phytophthora sojae, Bradyrhizobium japonicum*

Introduction

Phytophthora sojae Kaufmann & Gerdemann causes root and stem rot of soybean (*Glycine max* L. Merr.), a serious and widespread disease (Grau *et al*., 2004) and is one of the most destructive diseases of soybean in Iran (Mohammadi *et al.* 2007; Mirabolfathy *et al*., 2000). Infected seeds may fail to emerge and infected seedlings are killed shortly after emergence. Plants infected at the primary leaf stage display typical "damping-off" and root and stem rot symptoms (Erwin and Ribeiro 1996).

Species of the genus *Trichoderma* are well documented as fungal biocontrol agents

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(Papavizas, 1985; Howell, 2002). These species are useful, avirulent plant symbionts that act as bicontrol agents against phytopathogenic fungi via mechanisms of competition, rhizosphere competence, mycoparasitism, antibiotic and enzyme production, induced resistance, and promoting plant growth (Harman *et al*., 2004; Howell, 2003). The majority of *Trichoderma* species are antagonists for phytopathogenic fungi and have been broadly used as the most important biocontrol agents (Tjamos *et al*., 1992)

Bradyrhizobium japonicum is an agronomically important gram-negative bacterium that has the ability to form root nodules on soybeans and to fix atmospheric nitrogen (Isawa *et al*.1999).

Biological control using antagonistic microbes alone, or as supplements to minimize the use of chemical pesticides in a system of integrated plant disease management, has become more important in recent years .Beneficial microbes, including antagonistic bacteria and fungi, applied as seed treatments provide unique opportunities and benefits for crop protection especially against soil-borne fungal pathogens (Harman 1991; Mao *et al*. 1997). However, the use of antagonistic fungi, especially *Trichoderma* and *Gliocladium* spp., has been more extensive than their bacterial counterparts (Ganesan *et al*., 2003). Bacteria isolated from the rhizosphere and belonging to a wide variety of genera have the potential to suppress diseases caused by a diversity of soil-borne plant pathogens. Some symbiotic N2 fixing *Rhizobium* strains not only fix atmospheric N2 in the nodules but also show an antagonistic effect against soil-borne pathogens (Ganesan *et al*. 2007).

Saber *et al*. (2009) found dual inoculation of faba bean seeds with a mixture of *R. leguminosarum* and *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. Based on their data, the use of *T. viride* 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.

Considerable research has been done to investigate combination of *Rhizobium* and *Trichoderma* for protection against many soil borne plant pathogens. In this research, biocontrol effects of native *Trichoderma* isolates and *B. japonicum* on root and stem rot of soybean were evaluated.

Material and Methods

Pathogen

Soybean plants showing root and stem rot symptoms were collected from fields of Lorestan province (Iran). The isolation of the pathogen from diseased plants was performed on Corn Meal Agar (CMA) and CMA-PARPH media. Identification was achieved according to features of sexual and nonsexual reproduction organs and colony characteristics of isolates (Erwin and Ribeiro, 1996). The pathogenicity of the obtained isolates of *P. sojae* was studied in a pot culture experiment as described by Xiao *et al.* (2002). Then a typical isolate of the *P. sojae* was selected for further studies.

Trichoderma and *B. japonicum* isolates

The *Trichoderma* isolates used in this study were *Trichoderma ceramicum, T. virens, T. pseudokoningii, T. koningii, T. koningiosis, T. atroviridae, T. viridescens, T. asperellum, T. harzianum, T. orientalis*, *T.brevicompactum and T. spirale* that have been isolated from fields of Iran and provided by Zafari (Plant Protection Department of Bu-Ali Sina University). *B. japonicum* used as biocontrol bacterium in this study was produced by Fanavari Zisti Tabiatgara Company. This product was used at the rate of 1 lit/kg seed for seed coating treatment.

In vitro evaluation of _Trichoderma_ spp. against _P. sojae_

Dual culture technique

Five millimeter discs of *P. sojae* mycelium, that were aseptically punched with sterile cork borer (No. 5) from the periphery of actively-growing cultures, were placed at the periphery on surface of Petri dishes containing CMA medium and incubated at 25 ± 1 °C. After two days of mycelial growth, each plate was inoculated at equal distance on the opposite side of Petri dish with mycelial plug 5-mm diameter of the *Trichoderma* isolates. *Trichoderma* isolates for inoculation were obtained from the margin of actively growing 7-day old cultures on PDA. A completely randomized experimental design with four Petri dishes for each isolate was used. In control plates (without *Trichoderma*), a sterile agar disc was placed at the opposite side of the *P. sojae* plates. Inoculated plates were incubated at 25 ± 1°C until the end of the incubation period (7 days after inoculation). Two, four and six days after the incubation, radial growth of pathogen was measured and percent inhibition of average radial growth was calculated relative to growth of the controls as follows (Datta *et al*., 2004):

$$I(\%) = \frac{C - T}{C} \times 100$$

Where:

I = Percentage inhibition of pathogen by antagonists

C = Radial growth in control

T = Radial growth in the treatment

Effect of volatile inhibitors

The effect of volatile metabolites produced by *Trichoderma* isolates following the method described by Dennis and Webster (1971b) with slight modifications was carried out. Five millimeter plugs of *P. sojae* mycelia were placed in the center of the Petri dish containing CMA, after 2 days of growth, the bottom of Petri dish (*P. sojae*) was removed and placed on another plate containing CMA and Five millimeters mycelial discs of *Trichoderma* spp. and the rims of the 2 plates were sealed together by adhesive tape. In the controls, bottom *P. sojae* petri plates were removed and turned over another CMA petri dish without *Trichoderma* spp. All of the plates were incubated at 25 ± 1 ºC for 7 days and the growth of p. sojae colonies was recorded daily (every 24 hours).The percentages of growth inhibition were calculated using the above mentioned formula and were analyzed according to completely randomized design by SAS software.

Effect of non-volatile inhibitors

Mycelial discs of each *Trichoderma* isolate grown on PDA was separately inoculated into 100 ml flasks containing potato dextrose broth and incubated at 28 ± 2° C and 120 rpm in rotary shaker incubator for 10 days. Mycelial mats then were separated from the broth by filtering through ashless Whatman no. 1 filter paper. The filtrates were vacuum-filtered through sterile 0.22 mm millipore filters. Filter-sterilized culture filtrates from each *Trichoderma* sp. were added to autoclaved CMA to give concentrations of 5, 10, 20, 30, 35 and 40 % (V/V). After cooling to 45 ºC, 15 ml of each concentration were poured into 9-cm diameter culture plates, allowed to solidify, and inoculated with a 5-mm mycelial plug of a 5-day-old CMA culture of *P. sojae* (Dennis and Webester, 1971a). Plates containing CMA without culture filtrates served as controls. All plates were incubated for 7 days at 25 ºC. The percentage inhibition of radial growth was calculated and evaluated using completely randomized design by SAS software. CMA amended with sterile PDB was used to determine what effect, if any, PDB had on mycelia growth.

Evaluation of *Trichoderma* spp and *B. japonicum* in greenhouse

Preparation of *P. sojae* inocula

P. sojae grown on CMA at 25 °C for 7 days was used as inoculum for infesting the soil by blending the contents of 14 plates in one liter of deionized water.

Preparation of Conidial Suspensions of *Trichoderma* spp.

A conidial suspension of each *Trichoderma* isolate was prepared from a 10-day old culture of the isolate on PDA. The plate (9cm diameter) was flooded with 10ml of sterilized distilled water and shaken for a few minutes. The resulting suspension was filtered through muslin cloth. After filtering the suspensions through double layer of cheese cloth, the conidial concentration of the filtrate was adjusted to 4×10^5 conidia/ ml.

Seed treatment: Undamaged and clean seeds of soybean cv. Williams were surface sterilized (alcohol 70 %, 3 min; sodium hypochlorite 3%, 3 min), rinsed 6 times with sterile water, and shade dried. Then, eight ml of a *Trichoderma* conidial suspension containing 4×10^5 conidia/ml was used for coating 100 g of sterilized soybean seeds that were immersed for 30 min in 1 % carboxymethylcellulose (CMC, Merck) suspension of *Trichoderma* spp. The seed coating procedure with *B. japonicum* was conducted according to the method described by manufacturer. For coating of seeds by both antagonists, the two suspensions were mixed together. Seeds soaked in sterile water and treated with CMC without antagonist were used as control.

Pot experiments

Experiment 1

Effects of *Trichoderma* isolates and *B. japonicum* (singly and in combinations) as biocontrol agents for *P. sojae.*

Coated seeds with *Trichoderma* isolates and *B. japonicum,*as alone and in combinations,were planted in infested soil. Coated seeds planted in non infested soil were used as control. Pathogen inocula were added to soil at a rate of 250 ml per kg soil, pots were maintained for 3 weeks and the following parameters were estimated:

Germination percentage: Three weeks after sowing, the germinated seeds were counted and the germination percent was calculated.

Damping-off: number of seedlings that died after germination

Seedling Vigour Index (SVI): The germination percentage was calculated as mentioned above. The morphological parameters like shoot length and root length were measured and the vigour index (*VI*) of the seedlings was estimated as suggested by Abdul-Baki and Anderson (1973): Vigour Index = Germination percentage X Seedling length.

Disease severity: Seedlings were rated using a 5-class scale system: 0, healthy or no apparent root discoloration; 1) less than 25 % discoloration of the roots; 2) 25 – 50 % discoloration of the roots; 3) 50 – 75 % discoloration of the roots; and 4) more than 75 % discoloration of the roots or dead plants.

Experiment 2

Effects of *Trichoderma* isolates and *B. japonicum* (singly and in combinations) on plant growth. In this experiment coated seeds with *Trichoderma* isolates and *B. japonicum,* as alone and in combinations, were planted in sterile soil and grown for 30 days. The measured parameters in this experiment were seedling height (cm), root length (cm), shoot fresh weight (gr), root dry and fresh weights (gr).

In both experiments silty loam soil was collected from the field and pasteurized with water vapor for 4 h at 95 ± 5 °C .The infested soil and non infested soil were poured into 17-cm-diameter plastic pots and five of the treated or untreated seeds were planted in each pot. Pots were maintained at the photo periods of 12 h light at 25 °C and 12 h dark at 20 °C. Subsequently all pots were watered

when the soil surface dried. Three pots were maintained for each treatment.

Data analysis

Each experiment was done two times and the data from the trials were combined for statistical analysis using (SAS).

Results

In vitro evaluation of *Trichoderma* spp. against *P. sojae*:

In studying *Trichoderma* isolates and the *P. sojae* in dual culture, all of the *Trichoderma* isolates had marked significant inhibitory effect on the growth of the pathogen compared with their controls. Maximum *P. sojae* growth inhibition occurred in interacting with *T. virens, T. orientalis* and *T. brevicampactom* (57 % approximately).

However, in dual cultures, *P. sojae* grew slower than *Trichoderma* spp. and the area occupied by the colony was less than 50 %. *Trichoderma* isolates covered larger area on the plate and overgrew *P. sojae* colony within a period of four days. Green colour mycelial and conidial masses were observed on *P. sojae* colony. There was no zone of inhibition in between the colonies of *Trichoderma* species and *P. sojae*. The inhibition varied in dual tests from 47.45 to 58.87 % (Table 1).

In volatile metabolites test, all of the isolates significantly reduced the pathogen colony growth while the maximum growth reduction was observed in *T. atroviride* (49.08 %) and minimum in *T. viridescens* (6.17) (Table 1).

Culture filtrates of the *Trichoderma* isolates varied in the amount of inhibition of mycelial growth of *P. sojae* and different concentrations had different inhibition effects. Maximum growth reductions were observed with increasing concentrations. Culture filtrates of *T. virens* and *T. brevicampactom* showed the highest inhibition in all concentrations and at 20, 30, 35 and 40% concentrations caused up to 100 % growth inhibition. *T. asperellum* had the least inhibition (Fig. 1).

Table 1 Mean comparison of the effect of *Trichoderma* species on mycelial growth reduction of *P. sojae in vitro*.

***Trichoderma* spp.**	**Dual culture test (%)**	**Volatile compounds test (%)**
T.brevicompactum	57.88a*	29.58c
T. orientalis	57.82a	16.33d
T. virens	56.81a	14.87de
T. pseudokoningii	56.59a	14.63de
T. atroviride	56.22a	49.08a
T. spirale	56.01a	9.85ef
T. koningiopsis	55.11ab	40.62 b
T. ceramicum	54.89ab	38.95b
T. koningii	54.53ab	17.45d
T. viridescens	53.28ab	6.17f
T. asperellum	50.99ab	29.50c
T. harzianum	47.46b	18.66d

***** values are average of 3 replicates. Means with the same letter in each column are not significantly different in Duncan at ($P \leq .05$).

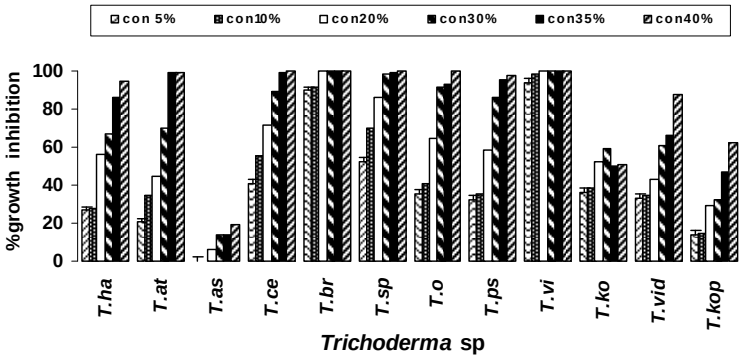

Figure 1 Mean comparison of inhibitory effects of liquid culture filtrates of *Trichoderma* species on *P. sojae* mycelial growth at 5, 10, 20, 30, 35 and 40 % concentrations, in CMA medium, *in vitro* [abbreviations for Trichoderma species in this figure are as follows: *T. virens (T. vi), T. orientalis (T. o), T. ceramicum (T. ce), T. atroviride (T. at), T. koningii (T. ko), T. brevicompactum (T. br), T. spirale (T. sp), T. viridescens (T. vid), T. pseudokoningii (T. ps), T. harzianum (T. ha), T. asperellum (T. as), T. koningiopsis (T. kop)*].

Evaluation of *Trichoderma* spp. and *B. japonicum* in greenhouse

Experiment 1

Effects of *Trichoderma* isolates and *B. japonicum*, singly and in combinations.

The results showed that there were no significant differences in any of the parameters between each *Trichoderma* treatment alone and *Trichoderma* + *Bradyrhizobium* ones. The germination percentage of treated seeds in non infested soil was not significantly different from the untreated control, but the treated seeds germinated faster than the untreated seeds. Germination percentage of all treated seeds in infested soil, except that of *Bradyrhizobium* alone increased significantly in comparison with untreated control in infested soil. The maximum germination percentage was recorded for *T. brevicompactum* individually and in combination with *Bradyihizobium* as 80. 4 % and 81.7 % respectively (fig 2).

The percentage of damping-off dropped to zero in inoculated plants treated with *T. ceramicum, T. atroviride, T. koningii, T. virens, T. brevicompactum* and *T. orientalis* species solely and in combination with *Bradyrhizobium* while disease incidence was 100% in inoculated plants treated with *Bradyrhizobium* solely (fig 3).

In the non infested soil, all *Trichoderma* spp. except *T. koningiopsis* significantly increased the SVI compared with untreated control and *Bradyrhizobium* treatment which were equal. The highest SVIs were recorded in *T. brevicompactum* and *T. orientalis* treatments, 12653.33 and 12983.33 respectively.

In the infested soil, combined application of *Trichoderma* spp. and *Bradyrhizobium* significantly increased the SVI compared with using each one alone. *T. brevicompactum* was the most successful species which increased SVI up to 6674.31 (Table 2).

Although based on seeding Vigour Index (SVI) parameters of plants treated with *Trichoderma* and *Bradyrhizobium each one* alone and in combination were not as vigorous as healthy control plants, all *Trichoderma* and *Bradyrhizobium* treatments except *Bradyrhizobium* exclusive treatment reduced disease severity compared with infected control plants. *T. brevicompactum* solely and in combination with *Bradyrhizobium* was the most effective species to reduce disease severity by 30.0 and 30.6, respectively, (fig 4).

Experiment 2

In this experiment Effects of *Trichoderma* isolates and *B. japonicum* (singly and in combinations) on plant growth were evaluated. Coated Seeds with *Trichoderma* isolates and *Bradyrhizobium japonicum,* as alone and in combinations, significantly promoted the growth of treated seeds based on measured growth

factors. Fresh and dried root weights, root and shoot heights and fresh shoot weight increased nearly 2 - 2.5 folds over that of control. The most effective species were *T. orientalis, T. brevicompactum and T. spirale* (table 3).

Increased growth parameters in combined treatments may be due to synergistic relationship between *Trichoderma* and *Bradyrhizobium.* Coating with the adhesive material alone neither affected disease nor plant growth.

Table 2 Effect of *Trichoderma* and *Bradyrhizobium* seed treatment on seedling vigour index.

Treatment	***Trichoderma* + *Phytophthora***	***Trichoderma***	***Trichoderma + Bradyrhizobium* + *Phytophthora***
Untreated control	7041.25a	7041.25h	7041.25a
CMC *	1347.45f	1347.45i	1347.45gh
T. spirale	3444.99e	11545.42b	3833.65def
T. orientalis	5742.49bc	12983.33a	6166.73abc
T. brevicompactum	6670.52ab	12653.33a	6674.31ab
T. viridescens	2125.61f	9859.72def	3423.49ef
T. virens	2300.96f	10250.00cde	2624.033fg
T. pseudokoningii	5120.29cd	10660.00c	5149.30bcd
T. koningiopsis	3793.98e	7225.00h	3770.28def
T. koningii	3775.88e	8623.25g	3778.27def
T. harzianum	4496.24de	10568.33cd	4443.00de
T. atroviride	4186.11de	8874.00g	4186.11de
T. asperellum	4478.18de	9670.17ef	4796.89cde
T. ceramicum	4523.19de	9201.67fg	4523.19de
Phytophthora alone	0g	0j	0h
Bradyrhizobium + *Phytophthora*	150.42g	150.42j	150.42h
Bradyrhizobium alone	6681.25ab	6681.25h	6681.25a

***** Cmc: coated seeds with carboxy-methylcellulose suspension.
Means with the same letters in each column are not significantly different (P < 0.05) by Duncan's multiple range test.

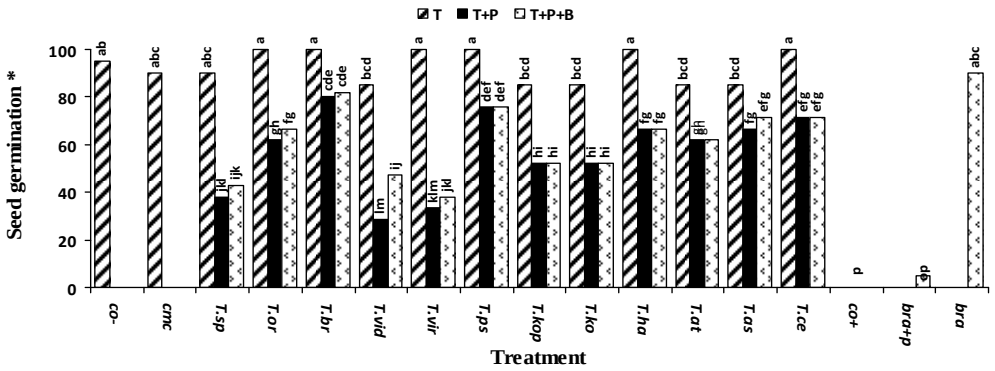

Figure 2 Percentage of seed germination in soybean seeds treated with *B. japonicum* and *Trichoderma* species applied singly and in combination for controlling seed rot caused by *P. sojae.*
Diagram values are average of 3 replicates. Bars with the same letters are not significantly different (P ≤.05) according to Duncan's multiple range test. co + : control with pathogen only. co -: control with neither antagonist nor pathogen. cmc: coated seeds with carboxy-methylcellulose suspension Bra: coated seeds with *Bradyrhizobium* and planted in non infested soil. Bra+p: coated seeds with *Bradyrhizobium* and planted in infested soil. T: coated seeds with *Trichoderrma* and planted in noninfested soil. T+P: coated seeds with *Trichoderrma* and planted in infested soil. T + P + B: coated seeds with *Trichoderma* + *Bradyrhizobium* and planted at infested soil. Abbreviations of *Trichoderma* species in this experiment are shown as: *T. virens (T. vi)*, *T. orientalis (T. or)*, *T . ceramicum (T. ce)*, *T. atroviride (T. at)*, *T. koningii (T. ko)*, *T. brevicompactum (T. br)*, *T. spirale (T. sp)*, *T. viridescens (T. vid)*, *T. pseudokoningii (T. ps)*, *T. harzianum (T. ha)*, *T. asperellum (T. as)*, *T. koningiopsis (T. kop).*

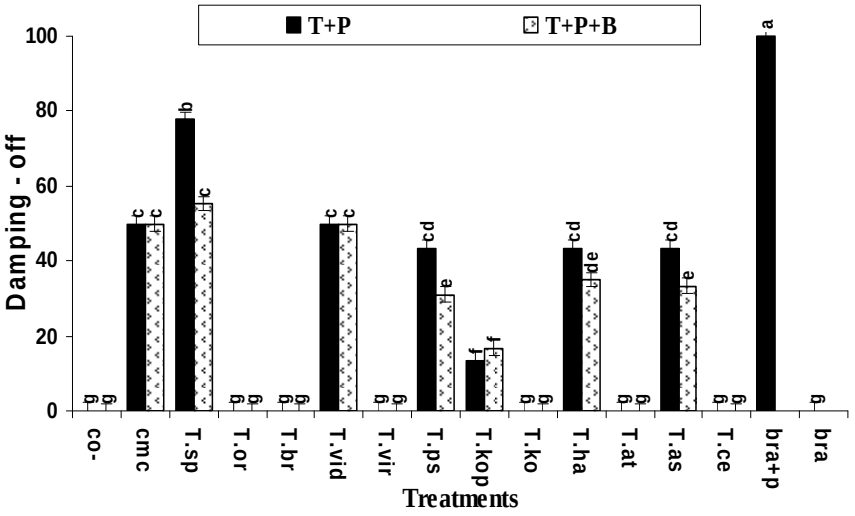

Figure 3 Percentage of damping-off caused by *P. sojae* on soybean seedlings treated with *B. japonicum* and *Trichoderma* species applied individually and in combination.

*Diagram values are average of 3 replicates. Means followed by the same letter are not significantly different (P ≤.05) according to Duncan's multiple range test. Treatments are as follows: co +: control without antagonist and with pathogen. co -: control with neither antagonist nor pathogen. cmc: coated seeds with carboxy methylcellulose suspension bra: coated seeds with Bradyrhizobium and planted in non infested soil. bra + p: coated seeds with *Bradyrhizobium* and planted in infested soil. T + P: coated seeds with *Trichoderrma* and planted in infested soil. T + P + B: coated seeds with *Trichoderma* + *Bradyrhizobium* and planted at infested soil. Abbreviations of Trichoderma species in this figure are shown as: *T. virens (T. vi)*, *T. orientalis (T. or)*, *T . ceramicum (T. ce)*, *T. atroviride (T. at)*, *T. koningii (T. ko)*, *T. brevicompactum (T. br)*, *T. spirale (T. sp)*, *T. viridescens (T. vid)*, *T. pseudokoningii (T. ps)*, *T. harzianum (T. ha)*, *T. asperellum (T. as)*, *T. koningiopsis (T. kop)*].

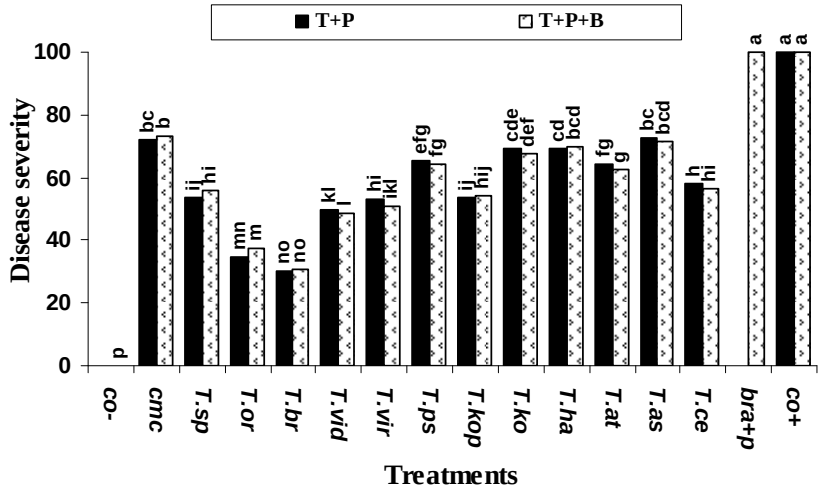

Figure 4 Percentage of *P. sojae* disease severity on soybean seeds treated with *B. japonicum* and *Trichoderma* species applied individually and in combination.

* Diagram values are average of 3 replicates. Means marked with the same letter(s) are not significantly different according to Duncan's Multiple Range test (P ≤.05). co + : control without antagonist and with pathogen. co -: control with neither antagonist nor pathogen. cmc: coated seeds with carboxy-methylcellulose suspension bra: coated seeds with *Bradyrhizobium* and planted in non infested soil. Bra + p: coated seeds with Bradyrhizobium and planted in infested soil. T + P: coated seeds with *Trichoderrma* and planted in infested soil. T + P + B: coated seeds with *Trichoderma* + *Bradyrhizobium* and planted in infested soil. Abbreviation of Trichoderma species in this experiment showed as: *T. virens (T. vi)*, *T. orientalis (T. or)*, *T . ceramicum (T. ce)*, *T. atroviride (T. at)*, *T. koningii (T. ko)*, *T. brevicompactum (T. br)*, *T. spirale (T. sp)*, *T. viridescens (T. vid)*, *T. pseudokoningii (T. ps)*, *T. harzianum (T. ha)*, *T. asperellum (T. as)*, *T. koningiopsis (T. kop)*].

Table 3 Plant growth responses following seed treatments with *B. japonicum* and *Trichoderma* species applied individually and in combination.

treatment	fresh root weight (gr)		dried root weight (gr)		root height (cm)		shoot height (cm)		fresh shoot weight (gr)	
	T	T + B	T	T + B	T	T + B	T	T + B	T	T + B
Co-	0.87f	0.87f	0.31e	0.31 h	16.78abc	16.78f	57f	57g	6.5gh	6.5h
T. sp	1.96a	2.16a	0.56 a	0.69 a	21.83a	25.83a	106a	126a	10a	12.1a
T. or	1.70b	1.90b	0.49 b	0.62 bc	20.83ab	24.83ab	109a	129a	9.7b	10.9b
T. br	1.49b	1.79b	0.45 c	0.58 d	19.32bc	24.32abc	107a	127a	10.8a	12a
T. vid	1.59b	1.17de	0.51 b	0.43 fg	21.48ab	19.52e	95a	114b	9.59b	8.14fg
T. vi	0.97ef	1.79b	0.3 e	0.64 b	15.52e	25.48a	87c	106c	6.9fgh	10.7bc
T. ps	1.12de	1.32cd	0.36 d	0.5e	19.23bc	22.47cd	87c	107c	7.2fgh	8.4efg
T. kop	0.88f	1.14de	0.28 e	0.44f	15.1e	19.85e	70e	89e	8ed	8.95def
T. ko	0.94ef	1.08de	0.31 e	0.41 g	15.85e	19.1e	86c	105c	7.7def	9.3de
T. ha	1.19cd	1.39c	0.37 d	0.5 e	17.02de	21.02de	88c	108c	7.4efg	8.64ef
T. at	0.95ef	1.29cd	0.30 e	0.49 e	16.57de	22.27cd	87c	107c	9.1bc	10.32bc
T. as	1.09de	1.15de	0.36 d	0.43 fg	18.27cd	20.57de	96a	115b	9.2bc	10.4bc
T. ce	0.95ef	1.09de	0.31e	0.42 g	15.35e	20.97de	76d	96d	6.3h	10.7cd
B. japonicum		1.70c		0.60 cd		24.08abc		82e		9.7

Values are average of 3 replicates. Means with the same letter(s) in each column are not significantly different from each other according to Duncan's Multiple Range test (P ≤.05). Co -: neither antagonist nor pathogen. Bra: coated seeds with *Bradyrhizobium*. T: coated seeds with *Trichoderrma*. T + B: coated seeds with *Trichoderma* + *Bradyrhizobium*. abbreviation of Trichoderma species in this experiment showed as: *T. virens (T. vi)*, *T. orientalis (T. or)*, *T . ceramicum (T. ce)*, *T. atroviride (T. at)*, *T. koningii (T. ko)*, *T. brevicompactum (T. br)*, *T. spirale (T. sp)*, *T. viridescens (T. vid)*, *T. pseudokoningii (T. ps)*, *T. harzianum (T. ha)*, *T. asperellum (T. as)*, *T. koningiopsis (T. kop)*].

Discussion

Trichoderma species have long been recognized as agents for the control of plant disease and for their ability to improve plant growth and development. Their antagonistic nature may be due to antibiosis, nutrient competition and cell wall degrading enzymes. In the present study, we tested *Trichoderma* species for mycoparasitism in dual culture and their production of volatile and non-volatile compounds that inhibit the growth of *P. sojae in-vitro.*

In dual culture test, all isolates reduced the colony growth of the pathogen; due to the rapid growth of *Trichoderma* isolates that quickly colonized medium surface. These observations are similar to those of Kucuk and Kivance (2004) findings. Volatile and nonvolatile metabolites of *Trichoderma* isolates reduced the pathogen colony growth; however this reduction effect was more due to nonvolatile than volatile metabolites (Table 1). Previous studies have shown that most of *Trichoderma* strains produce volatile and nonvolatile metabolites that inhibit the growth of pathogenic fungi (Vey *et al.* 2001). These metabolites are composed of harzianic acid, alamthicins, tricholin, peptaboles, antibiotics, 6-phenthylalpha- pyrone, massoilactone, viridian, glioviridin, glisoprenins, heptelidic acid, and other suppressive compounds (Vey *et al.* 2001; Simon and Sivasithamparam, 1989; Kucuk and Kivance, 2004).

According to our results it is evident that volatile compounds from *Trichoderma atroviride* suppress the mycelial growth of *P. sojae* and are effective when compared to other spp. The earlier studies also revealed that antimicrobial metabolites *such as* aromatic pyrone antibiotics (Keszler *et al.* 2000) and peptides are produced by *T. atroviride* (Oh *et al.* 2000). The most successful species in culture filtrate production were *T.*

brevicampactom and *T. virens*. Nielsen *et al.* (2005) demonstrated that *T. brevicompactum* produced trichodermin and/or harzianum A on all media investigated, with liquid media yielding the largest amounts and Chet *et al.* (1997) showed that *T. virens* strains produced high amount of glioviridin antibiotics and protected cotton seedlings from seedling blight caused by *P. ultimum* .in greenhouse test.

Our results indicated that combined application of *Bradyrhizobium* and *Trichoderma* species could control the pathogen significantly and promoted the growth of soybean seedlings. Harman *et al*. (1980) reported that *Rhizoctonia solani* and *Pythium* species were controlled by coating of *Trichoderma harzianum* on radish and pea seeds. *T. harzianum* seed coating gave the best results in reducing the disease severity compared with other treatments (Das *et al*., 2002). Mao *et al*. (1997) showed that seed treatment with *Gliocladium virens*, *Trichoderma viride* and *Burkholderia cepacia* increased seedling stand, plant height and fresh weight, also the root rot severity decreased in corn compared with those treated with a fungicide. Chakraborty *et al*. (2003) reported that combined application of *B. japonicum* and *Trichoderma harzianum* significantly reduced root rot disease in soybean, they also showed that activity of PAL, peroxidase enzymes and Phytoalexin (Glyceollin) activities increased in treated plants. In the present study also overall growth of the soybean plants increased in *Bradyrhizobium* and *Trichoderma* combined treatments.

These results may be due to the competition for space and nutrients, parasitism, production of enzymes, volatile and non-volatile metabolites or combinations of these mechanisms. The growth-promoting activity may be due to the solubilisation and sequestration of inorganic plant nutrients, production of unidentified growth promoting chemicals or by the N2 fixing activity of the *Bradyrhizobium*.

In conclusion, *T. brevicompactum* was found to be the most effective biocontrol agent against *P. sojae* in laboratory and glasshouse conditions among the studied species. Hence *T. brevicompactum* and *Bradyrhizobium* may be considered for use as biocontrol agents and biofertiliser for the management of *P. sojae*.

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ترکیب گونه‌های تریکودرما و باکتری *Bradyrhizobium japonicum* در کنترل *Phytophthora sojae* و رشد سویا

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چکیده: سویا *Glycine max* (L.) Merr. یکی از مهمترین دانه‌های روغنی جهان محسوب می‌شود. یکی از عوامل محدودکننده کشت سویا، بیماری پوسیدگی ریشه و طوقه است. در این بررسی اثر ۱۲ گونه *Trichoderma*، در شرایط آزمایشگاهی و اثر این گونه‌ها در ترکیب با *Bradyrhizobium japonicum* در گلخانه در کنترل *Phytophthora sojae* و رشد رویشی سویا ارزیابی شد. بررسی‌های آزمایشگاهی شامل: کشت متقابل، آزمون ترکیبات فرار، متابولیتهای خارج سلولی فیلتر شده بودند که گونه‌های *T. virens*،*T. orientalis* و *T. brevicompactum* در آزمون کشت متقابل، *T. atroviride* در آزمون ترکیبات فرار سبب بیشترین بازداری شدند. به‌منظور بررسی اثر ترکیبات فیلتر شده تریکودرما بر بازداری از رشد هیف *P. sojae* شش غلظت مختلف از این ترکیبات در محیط کشت CMA تهیه شد. نتایج نشان داد که ترکیبات فیلتر شده خارج سلولی تمامی گونه‌ها سبب بازداری رشد هیف شدند، غلظت‌های مختلف اثر بازدارندگی متفاوتی داشته و بیشترین بازدارندگی مربوط به *T. virens* و *T. brevicampactom* بود. بررسی‌های گلخانه‌ای به‌صورت دو آزمایش، شامل: بررسی اثر گونه‌های تریکودرما و *Bradyrhizobium japonicum* به‌تنهایی و در ترکیب با هم در کنترل بیماری پوسیدگی فیتوفتورایی سویا به روش تیمار بذر و اثر تریکودرما و *B. japonicum* بر شاخص‌های رشدی گیاه انجام گرفت. در آزمایش اول درصد جوانه‌زنی بذور، مرگ گیاهچه، شاخص قدرت گیاهچه و شدت بیماری اندازه‌گیری شد که گونه *T. brevicompactum* به‌تنهایی و در ترکیب با باکتری موثرترین گونه بود. در آزمایش دوم بذور آغشته به تریکودرما و باکتری به‌طور معنی‌داری باعث افزایش رشد گیاه سویا شدند که کارامدترین تیمارها ترکیب گونه‌های *T. spirale*، *T. brevicompactum* و *T. orientalis* با باکتری بودند. بنابراین نتایج نشان داد که گونه *T. brevicompactum* که بیشترین گونه جمع‌آوری شده بعد از گونه *T. harzianum* در ایران می‌باشد، به‌تنهایی و در ترکیب با باکتری موفق‌ترین گونه در کنترل *P.sojae* و افزایش رشد گیاه سویا می‌باشد.

واژگان کلیدی: کنترل بیولوژیک، *T. brevicompactum, Phytophthora sojae, Bradyrhizobium japonicum*