

BIOCONTROL OF SOYBEAN CHARCOAL ROOT ROT DISEASE BY USING *TRICHODERMA* SPP.

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DISEASE BY USING *TRICHODERMA* SPP.H. BARARI<sup>1\*</sup>, A. FOROUTAN<sup>1</sup>\* E-mail: [hosseinbarari1385@yahoo.com](mailto:hosseinbarari1385@yahoo.com)

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**ABSTRACT.** *Macrophomina phaseolina* (Tassi) Goid, causing charcoal rot disease of soybean, is one of the major factors threatening soybean production, especially in dry years. This pathogen remains the prevailing causal agent of charcoal rot disease that significantly suppresses the yield of a variety of oilseed crops. Its wide host range and ability to survive under arid conditions, coupled with the ineffective use of fungicides against it, have spurred scientific endeavours for alternative avenues to control this phytopathogen. Hence, the present study aimed to provide empirical evidence of the efficacy of fungal isolates of *Trichoderma* spp. as biological control agents against charcoal rot in soybean (*Glycine max* L.). In this study *Trichoderma harzianum* strains 6, 14, 17, 21, 44, *T. asperellum* 26 and *T. virens* 32 were evaluated as potential biological agents for control of this disease. Mycelial growth of *M. phaseolina* strain h-7 was reduced by cell-free and volatile metabolites of *Trichoderma* strains by 16.4 to 64.8%. *T. harzianum* strain Tj17 significantly ( $p \leq 0.05$ ) reduced the incidence (to 7.3%)

and severity (to 3%) of disease 42 days after inoculation and increased the 1000 grain weight (to 178 g) in greenhouse conditions. For confirmation of the greenhouse tests, the selected antagonists were re-examined in field trials, where this isolate reduced the disease incidence (to 10%) and severity (to 3%). The overall results of this study show high capability of used antagonists in reduction of disease severity and incidence, and resulting in increased weight of the product. Hence, the findings reported in the present study supported the applicability of Tj17 isolate as possible alternative to fungicides for the control of charcoal rot in soybean.

**Keywords:** *Macrophomina phaseolina*; *Trichoderma harzianum*; *Trichoderma asperellum*; *Trichoderma virens*.

## INTRODUCTION

Charcoal rot of soybean, caused by *Macrophomina phaseolina* (Tassi.) Goid is a seed, as well as soil-borne disease, occurring in most of the

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soybean-growing regions of the world, including Iran. Dry conditions, relatively low moisture and nutrients (NPK) and high temperature ranging from 25-35°C are favourable for the disease at pod-setting and filling stages. There are no resistant lines; hence the disease has to be managed by other methods (Ansari, 2010). The fungus can infect the root and lower stem of over 500 plant species and is widely distributed in the United States (Wyllie, 1988). Micro-sclerotia of the fungus are primary sources for the infection of the roots and survive in soil and plant residue for two to 15 years (Wyllie, 1988).

Charcoal rot symptoms usually appear after mid-season. In severe infections due to the production of fungal toxins, such as phaseolinone and vascular blockage by the fungus, organs are destroyed (Bhattacharya *et al.*, 1994). Because of soil-borne and high power saprophytic of fungal pathogens, effective strategies for disease control are not available. The control methods used are generally for reducing the number of micro-sclerotia in soil and minimizing their contact with the root of the host. The use of chemicals is not recommended due to environmental pollution, but appropriate chemical agents that can protect the plant from the pathogen have been identified (Valinte *et al.*, 2007). Control of the disease is difficult. Soil fumigation is effective but costly. Limitations in the development of resistant cultivars also

exist. Traditional methods, such as long-term crop rotation, clean tillage, and organic amendments of soil were useful for soil-borne disease control, but these practices are being replaced with short rotations, monocultures, and intensive cropping to increase productivity (Cook *et al.*, 1973). So, finding an additional method for disease control is an essential work.

In biological control of the pathogens, some fungi, including *Trichoderma* and *Gliocladium* species have shown promise. The antagonistic activity of *Trichoderma* species against plant pathogens has been studied extensively (Hjeljord *et al.*, 2001; Krause *et al.*, 2001). A number of commercial formulations, based on *T. harzianum* and *T. virens* are available for the control of soil-borne and foliar diseases in a range of horticultural crops (Harman *et al.*, 1990; Lumsden *et al.*, 1992; Samuels *et al.*, 1996), but there is little information on the efficacy of *Trichoderma* isolates on charcoal rot of soybean in Iranian conditions.

The objective of this investigation was to evaluate the potential of some isolates of *Trichoderma harzianum* and *T. virens* for the biological control of soybean charcoal rot in Mazandaran, Iran.

## MATERIALS AND METHODS

### Isolation of pathogen and antagonist strains

The pathogen and antagonist strains used in this study were isolated from the diseased soybean plants and dry soil samples separately, which collected from

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different fields of Mazandaran, including Dashtenaz (36:62N and 52:18E), Kohkheil, Larim (Jouibar, 33:62N and 49:52E), Serajmohaleh (36:45N and 53:35E), Rostamkola (36:51N and 54:18E), Baiekola (36:18N and 54:03E), Estakhrposht (36:30N and 54:44E), Nozarabad (Neka, 36:25N and 53:18E), Gharakheil (36:43N and 52:82E), Arateh, (Ghaemshahar, 36:30N and 52:53E), during 2013-2014 cropping season.

*Macrophomina phaseolina* was isolated from infected soybean roots and crowns on PDA. For isolation of the *Trichoderma* strains, soil samples of the rhizosphere area were dried by keeping them at room the temperature for 8 days. Then dried samples were serial diluted in sterile distilled water (Wijesundera *et al.*, 1991). After dilution, 100  $\mu$ L aliquots of  $10^{-4}$  to  $10^{-6}$  dilutions were separately plated out on selective media of McFadden and Sutton (McFadden and Sutton, 1975). In the primary test, 12 *M. phaseolina* and 63 *Trichoderma* were isolated from the infected collected soybean plants and soil samples. Pathogenicity of the *M. phaseolina* isolates were tested on soybean cultivar "Sahar", then the most virulent strain, h-7, was selected for further studies.

All the antagonists and pathogen isolates were maintained on potato dextrose agar (PDA) and incubated at 25°C.

### Effect of *Trichoderma* spp. on mycelial growth of *M. phaseolina* h-7

Dual culture and cellophane overlays were used to determine the effect of *Trichoderma* isolates on the pathogen (Dennis and Webster, 1971). All antagonist pathogen combinations were examined on 15 mL of PDA in 90 mm Petri dishes.

For dual culture, a 5 mm diameter mycelial plug was taken from the actively growing 3-day-old colonies of *Trichoderma* or pathogen isolates and placed 5 cm apart on the agar.

For determining the effects of cell-free extracts, the cellophane overlay technique was used. Cellophane membranes (Australia Cellophane, Victoria), 9 cm diameter, were boiled in distilled water, interleaved with filter papers and autoclaved before being placed on the agar medium. For the control, a plug of sterile PDA medium was used instead of the antagonist. After 48 h, the cellophane membrane and adhering fungus or agar plug were removed (Etebarian *et al.*, 2000).

The culture plates were incubated at 25°C in the dark. After 7 days, inhibition zones were measured, compared with the controls, and percentage inhibition of growth was calculated. The percent growth inhibition was calculated using the formula as:

$$\% \text{ GI} = \frac{a - b}{a} \times 100 \%,$$

where % GI is the percent growth inhibition, a is the *M. phaseolina* uninhibited colony area or control, and b is the inhibited colony area. Completely randomized design with three replications was used in this study.

The effect of volatile metabolites produced by the effective *Trichoderma* isolates on mycelial growth was determined by the method of Dennis and Webster (Dennis and Webster, 1971). The two-day old culture of *Trichoderma* isolate and *M. phaseolina* culture plates were assembled. After incubation period of 72 h open the assembled plates and measure the *M. phaseolina* growth in test plates, compared with control. The inhibition of radial growth of pathogen at this time, according to the above formula

was calculated and recorded. The test was conducted in a completely randomized design with three replications.

Antagonist species were identified on the basis of the morphological characterization of the colonies, measurement of hyphal diameter, conidiophores and conidia dimensions (Rifai, 1969; Bissett, 1991). *M. phaseolina* h-7 with the most virulence in pathogenicity test as the causal agent and *T. harzianum* G-6 (from Gharakheil), *T. harzianum* K-14 (from Kiakolla), *T. harzianum* J-17 (from Jouibar), *T. harzianum* B-21 (from Baiekola), *T. harzianum* D-44 (from Dashtenaz), *T. asperellum* B-26 (from Babol), *T. virens* B-32 (from Baiekola) with production of the most inhibition zone against the pathogen as the antagonists were selected in this study.

#### Biological control of *M. phaseolina* h-7 on soybean in greenhouse

The selected antagonists were tested for their ability to reduce the incidence and severity of root and crown rot in soybean.

For this propose, *M. phaseolina* h-7 and *Trichoderma* isolates were grown on PDA for one week. Inoculums of *M. phaseolina* h-7 was multiplied by transferring 5 cm diameter pieces of culture to 250 ml Erlenmeyer flasks containing 100 g of sand, 5g of corn meal and 20 ml of sterile distilled water, and inoculums of *Trichoderma* strains were multiplied by transferring 5 cm diameter pieces of culture to 250 ml Erlenmeyer flasks containing 100 ml of moist wheat bran. The inoculated substrates were incubated at room temperature for

3 weeks, until the surfaces were covered by mycelium.

Both multiplied inocula of the pathogen and antagonists were mixed with the autoclaved potted soil (field soil) at the rate of 5 g/kg of soil. *M. phaseolina* h-7 was used one day before sowing and *Trichoderma* strains were applied on the day of seeding.

Seeds of soybean cultivar "Sahar" were surface-disinfected by soaking in 1% sodium hypochlorite for 2 min, rinsed three times in sterile distilled water and sown in 20 cm diameter plastic pots containing the treated soils. Treatments were: *M. phaseolina* h-7 + *T. harzianum* T-g 6, *M. phaseolina* h-7 + *T. harzianum* T-k14, *M. phaseolina* h-7 + *T. harzianum* T-j17, *M. phaseolina* h-7 + *T. harzianum* T-b21, *M. phaseolina* h-7 + *T. harzianum* T-d44, *M. phaseolina* h-7 + *T. asperellum* T-b26, *M. phaseolina* h-7 + *T. virens* T-b32, inoculated control with *M. phaseolina* h-7 (diseased control) and uninoculated control with *M. phaseolina* h-7 (healthy control). Plants were maintained in the greenhouse of Gharakheil Crop Research Station of Mazandaran, Iran.

The disease incidence and severity were performed based on Agrawal and Sarbhoy (Agrawal and Sarbhoy, 1976). method. Accordingly, the soybean plants were scored in the R7 developmental stage from 1 (no stem discoloration) to 5 (observation of disease symptoms more than 50% on stem length). Numerical values of 0, 1.25, 2.5, 3.75 and 5, respectively, for score 1 to 5 were used and the disease severity was calculated using the following formula:

$$D.S. = \frac{[(1.25 \times y_2)(2.5 \times y_3)(3.75 \times y_4)(5 \times y_5)]}{\text{Total No. plants}} \times \frac{1}{0.025}$$

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The experiment was arranged as completely randomized design with three replications.

### Biological control of *M. phaseolina* h-7 on soybean in field

For confirmation of the green house tests, the selected antagonists were re-examined in field trials. All other treatments were the same as mentioned for the greenhouse, except the experimental design, which was arranged as randomized complete block with four replications and the disease incidence and severity according to what was described above were measured.

Analysis of variance and Duncan's Multiple Range Test was used to determine differences among treatments (Little and Hills, 1978).

## RESULTS

### Effect of *Trichoderma* spp. on mycelial growth of *M. phaseolina* in vitro

All tested *Trichoderma* isolates inhibited mycelial growth of *M. phaseolina* h-7 in dual culture. There were significant differences among the *Trichoderma* species. Growth of *M. phaseolina* h-7 was reduced by 31.5% by *T. harzianum* d44 up to 64.8% by *T. harzianum* j17 (Fig. 1).

Cell free metabolites of *T. harzianum* d44, *T. harzianum* g6, *T. harzianum* k14, *T. harzianum* j17, *T. harzianum* b21, *T. asperellum* b26, *T. virens* b32 reduced the growth *M. phaseolina* h-7 at the rates of 26.2, 50.6, 39.3, 52.5, 46, 43.7 and 22.5%, respectively (Fig. 2).

Antifungal activity of volatile metabolites against *M. phaseolina* h-7 varied among the *Trichoderma* strains. Percentage reduction of *M. phaseolina* h-7 with the *T. harzianum* j17 was significantly greater than that of the other *Trichoderma* strains (Fig. 3).

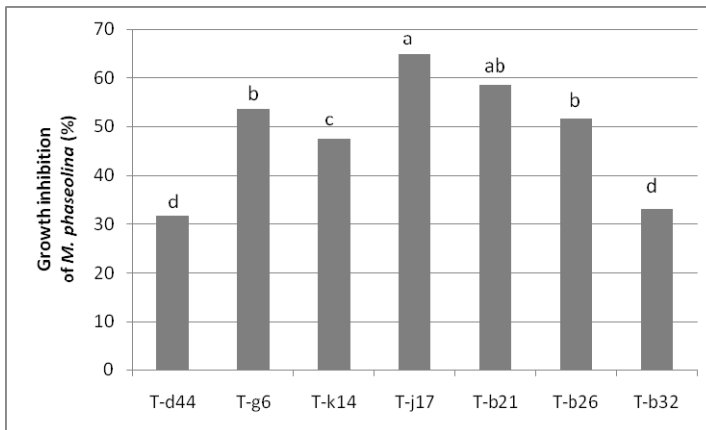
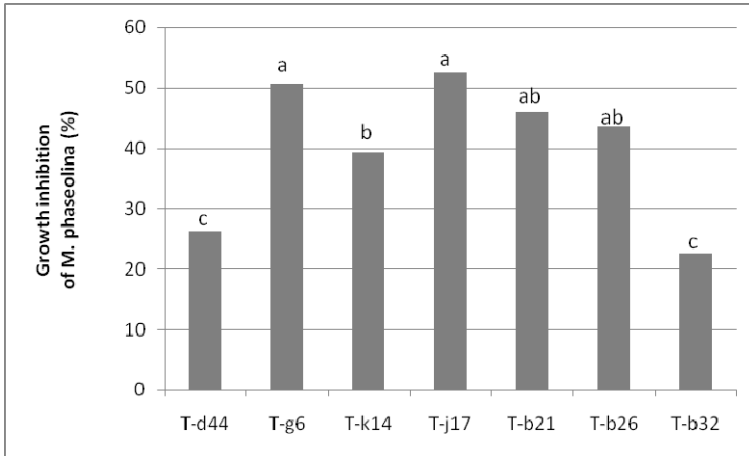
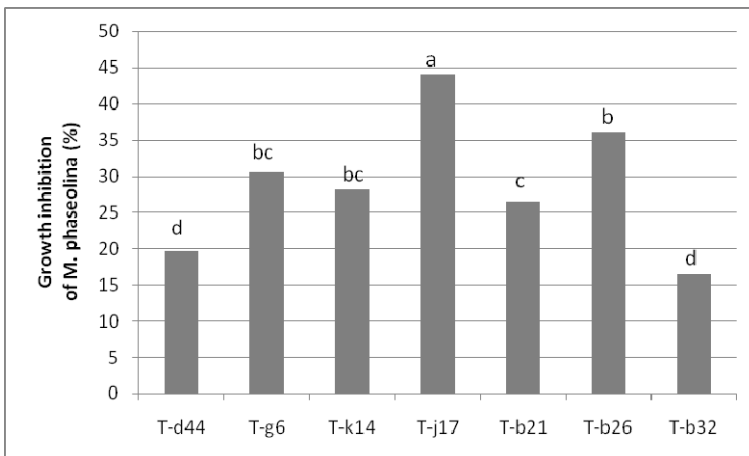


Figure 1 - Effect of *Trichoderma* strains on growth inhibition of *M. phaseolina* h-7 (dual culture). Treatments with the same letters do not differ significantly ( $p \leq 0.5$ ) according to the Duncan's multiple range test.



**Figure 2 - Effect of cell free metabolites of *Trichoderma* strains on growth inhibition of *M. phaseolina* h-7 (cellophane method). Treatments with the same letters do not differ significantly ( $p \leq 0.5$ ) according to the Duncan's multiple range test.**



**Figure 3 - Antifungal activity of the *Trichoderma* strains volatile metabolites on growth inhibition of *M. phaseolina* h-7. Treatments with the same letters do not differ significantly ( $p \leq 0.05$ ) according to Duncan's multiple range test.**

### Biological control of *M. phaseolina* h-7 on soybean in greenhouse

Disease incidence and severity in plants inoculated with the pathogen and *T. harzianum* j17 was significantly less than the pathogen control and other treatments (Fig. 4).

Thousand grain weight in soybean plants inoculated with the pathogen and *T. harzianum* j17 (178 g) was significantly more than the inoculated pathogen control and other treatments (Fig. 5).

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**Biological control of *M. phaseolina* h-7 on soybean in field**

Disease incidence and severity in plants inoculated with the pathogen

and *T. harzianum* j17 was significantly less than the pathogen control and other treatments (Fig. 6).

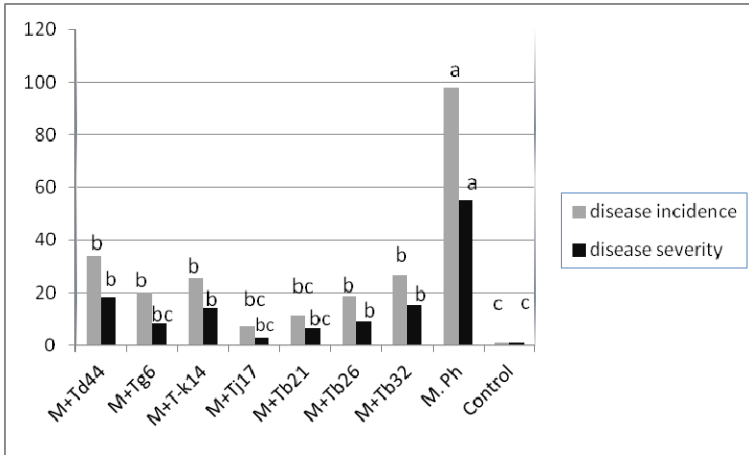


Figure 4 - Effect of *Trichoderma* strains on charcoal rot disease incidence and severity on soybean in a greenhouse experiment. Treatments with the same letters do not differ significantly ( $p \leq 0.05$ ) according to Duncan's multiple range test.

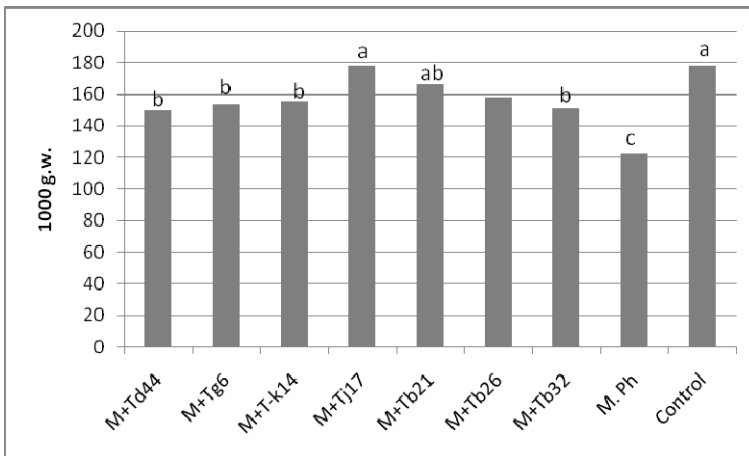
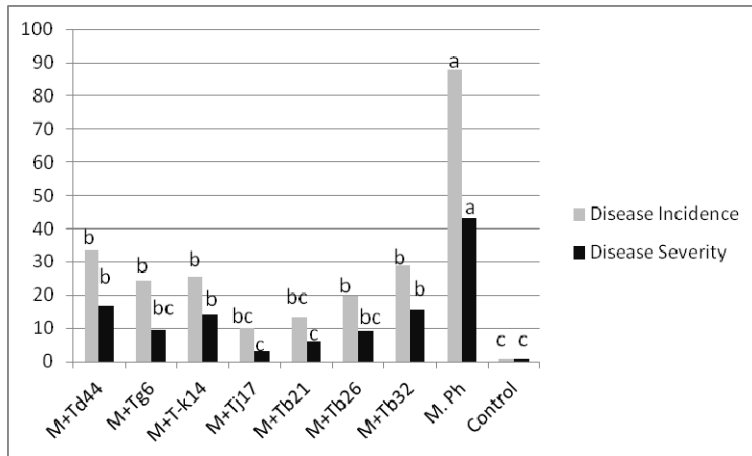


Figure 5 - Effect of *Trichoderma* strains on 1000 seed weight of soybean in a greenhouse experiment. Treatments with the same letters do not differ significantly ( $p \leq 0.05$ ) according to Duncan's multiple range test.



**Figure 6 - Effect of *Trichoderma* strains on disease incidence and severity in a field experiment. Treatments with the same letters do not differ significantly ( $p \leq 0.5$ ) according to Duncan's multiple range test.**

## DISCUSSION

In dual culture, all *Trichoderma* strains inhibited the growth of *M. phaseolina* h-16. Zones of inhibition were observed between the colonies of the pathogen and *Trichoderma* strains.

The inhibition zone could be due to the effect of diffusible inhibitory substances produced by the *Trichoderma* strains, which suppressed the growth of *M. phaseolina* h-7. The presence and size of the zone of inhibition have been used as evidence of the production of antibiotics by the *Trichoderma* strains (Jacson *et al.*, 1991; Crawford *et al.*, 1993).

Cell-free metabolites produced by the strains of *Trichoderma* could also reduce the colony area of *M. phaseolina* h-7. Even though the cellophane overlay technique has been used mainly for investigating non-

volatile metabolites of *Trichoderma* (Jacson *et al.*, 1991; Dennis and Webster, 1971). Although antibiotic substances from *Trichoderma* strains were not extracted and determined in this study, but some antibiotics, such as tubercidin, candicidin, phosphlactomycin, phenasin and 4-diacetylphloroglucinol, which have been produced by some antagonists, like *Pseudomonas fluorescens*, *Streptomyces* spp. and *Trichoderma* spp. have been reported by researchers (Lechevalier *et al.*, 1953; Fushimi *et al.*, 1989; Mazzola *et al.*, 1992; Hwang *et al.*, 1994).

Volatile metabolites produced by *Trichoderma* spp. inhibited mycelial growth of the pathogen in our study. Inhibitory effects of antagonists' metabolites have been reported in various studies (Ghisalberti and Sivasithamparam, 1991; Pal *et al.*, 2001; Monte and Liobell, 2003).



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Inoculation of soybean in the greenhouse conditions and field trials showed that treatments with *Trichoderma* strains could decrease root rot incidence and severity or increase the 1000 grain weight and total yield of tested soybean. The effectiveness of inoculation of soybean plants with antagonists in reduction of diseases and increase of yield was reported by investigators. Bochow and Fritzsche (1991) reported that Inoculation of plant with *Streptomyces* in greenhouse reduced the severity of *Phytophthora infestans*. This reduction was attributed to the induction of host resistance by the *Streptomyces* strain. Effectiveness inoculation of wheat plants with bacterial antagonists in reduction of root rot due to *Fusarium graminearum* and increase of yield were reported (El-Abyad *et al.*, 1993; Liu *et al.*, 1995; Jones and Samac, 1996; Okhovat *et al.*, 1996; Etebarian *et al.*, 2000; Luz, 2000; Nourozian *et al.*, 2006). Several commercially microbial agents like *P. fluorescens* and as Aspire are available for biological control (Jackson *et al.*, 1991). Some strains of *Streptomyces* spp. are also commercially available as microbial pesticides registered on some crops for biological control of soil-borne disease (Jones and Samac, 1996).

However, *Trichoderma* strains tested in this study could be used for control soybean charcoal rot.

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