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Biological Control of Sclerotinia Stem Rot (*S. minor*) of Sunflower Using *Trichoderma* Species

¹J. Abdollahzadeh, ¹E. Mohammadi Goltapeh and ²H. Rouhani

¹Department of Plant Pathology, Faculty of Agriculture,
Tarbiat Modarres University, Tehran, Iran

²Department of Plant Pathology, College of Agriculture,
Bu Ali University, Hamadan, Iran

Abstract: Based on *in vitro* results, three isolates: *T. harzianum* (J1), *T. harzianum* (J6) and *T. virens* were selected for greenhouse studies. Biocontrol efficacy of these isolates as well as the combination of *T. harzianum* (J1) and *T. virens* on crown and root rot of sunflower were studied in series of experiments using three methods; incorporation of conidial suspension into the soil (10^7 conidia g⁻¹ soil), incorporation of conidial suspension mixed with compost (10^7 conidia g⁻¹ compost) into the soil and impregnating of sunflower seeds with conidial suspension (10^7 conidia mL⁻¹). Experimental data were analyzed with Randomized Complete Block Design (RCBD) and means compared by Duncan's multiple range test. Greenhouse experiments (*in vivo*) showed that, the combination of *T. harzianum* (J1) and *T. virens* using incorporation of conidial suspension mixed with compost into the soil had the most efficiency on biocontrol of *S. minor* and reduced disease incidence up to 50%.

Key words: Biological control, *Sclerotinia minor*, *Trichoderma virens*, *T. harzianum*, sunflower

INTRODUCTION

Sclerotinia minor and *S. sclerotiorum* are important soil borne pathogens of sunflower (*Helianthus annuus*). Both species can infect the root system and cause a basal stem rot and wilt (Clarke *et al.*, 1990). For the first time, this disease was reported from Western Azarbaijan, Iran by Ale Agha (1971). Control measures are currently limited to the use of long rotations and the early sowing of tolerant hybrids (Clarke *et al.*, 1993), as commercial cultivars with adequate resistance are not yet available. Also, chemical control using dicarboximide fungicides is cost-effective only for crops where disease incidence is likely to reach 20% (Clarke *et al.*, 1990). Furthermore, recently some strains of *Sclerotinia* with resistance to this fungicides have been detected (Burgess and Hepworth, 1996). Biological control so can be considered as a valuable method to study. Study on different mycoparasites known to infect the sclerotia of *S. sclerotiorum* showed that *Trichoderma virens* was one of the most effective antagonist in reducing sclerotia viability *in vitro* (Whipps and Budge, 1990). Preliminary tests using an isolate of *T. virens* from a sunflower field in Northern Victoria indicated its potential for biocontrol of *S. minor*. *Trichoderma* species such as *T. harzianum* and

T. virens have also been shown attack the sclerotia and mycelium of *Sclerotinia* spp. (Whipps and Budge, 1990; Knudsen *et al.*, 1991). In order to identification of some effective isolates and use of them against disease caused by *S. minor* on sunflower, this study was conducted. Different methods of *Trichoderma* application were also investigated.

MATERIALS AND METHODS

Production of sclerotinia inoculum: *S. minor* isolate collected from infected sunflower of Khoy. Plates containing PDA medium were inoculated with mycelium from a 3-day-old culture of *S. minor*. The culture were incubated at 25°C in darkness for 2 weeks and sclerotia were recovered and dried on blotting paper for 30 min and stored in plates at room temperature.

Production of trichoderma inoculum: In experimental tests, three isolates of *Trichoderma* namely, *T. harzianum*(J1) isolated from soil of infected field of sunflower and *T. harzianum* (J6) and *T. virens* from substrate (compost) of edible fungi (*Agaricus bisporus*), were collected and used as the biological control agent in this work. Conidial suspension were prepared by adding

sterile distilled water (SDW) to a 4-week-old culture of *Trichoderma* isolates on PDA and gently scraping the surface with a sterile rod. The conidial concentration was adjusted at 10^8 conidial mL^{-1} for soil treatment and 10^7 conidial mL^{-1} for seed treatment.

In vitro studies

Antagonism by trichoderma: Antagonism of *S. minor* was studied in co-culture on PDA by one, two and six isolates of *T. virens*, *T. viride* and *T. harzianum*, respectively. Disks of antagonist isolates and *S. minor* were cut of the margin of 5-day-old cultures on PDA and placed 6 cm apart on fresh PDA plates. The cultures were incubated at 22°C for two weeks. The mycelia in the interface of antagonist and pathogen colonies were examined by microscope (Magnification, 40×10) after incubation for 2 days. In order to study the colonization ability of antagonist isolates, 3 and 14-day-old cultures of *S. minor* were inoculated with disks of antagonist and incubated for two weeks. Both experiments were carried out in four replicate.

Greenhouse studies: Studies were done with sunflower (*Helianthus annuus*) cv. Record in agriculture research center of Urmia (Western Azarbaijan, Iran) in winter of 2001. Four seeds were sown in pots containing clay-loam soils. The pots were maintained in greenhouse condition at $22\pm 5^\circ\text{C}$ and watered every 3 days. All treatments were replicated 3 times and data were analyzed using Randomized Complete Block Design (RCBD) and means separated using Duncan's multiple range test. Data were transformed before the analysis according to:

$$\sqrt{x + \frac{1}{2}}$$

Soil incorporation of conidial suspension: In this experiment, a conidial suspension (100 mL) of *Trichoderma* isolates were added to 1 kg soil on each pot. In order to infect the pots, 100 sclerotia of *S. minor* were added to 1 kg soil on each pot. Treatments were as the follows:

a) *T. harzianum* (J1) b) *T. harzianum* (J6) c) *T. virens* d) *T. harzianum* (J1) + *T. virens*

In the later treatment 50 mL of conidial suspension of each isolate was added to 1 kg soil on each pot. Controls were treated with tap water. After 7 days sunflower seeds were sown. The pots were then observed daily for the development of basal stem lesion.

Soil incorporation of conidial suspension with support: In this experiment, at first 100 g ground pasteurized button

mushroom compost were mixed with 100 mL conidial suspension of *Trichoderma* isolates and incubated in lab condition for 1 week. Then the compost was added to 1 kg soil on each pot containing 100 sclerotium of *S. minor*. Treatments were as follows:

a) *T. harzianum* (J1) b) *T. harzianum* (J6) c) *T. virens* d) *T. harzianum* (J1) + *T. virens*

In the later treatment, 50 mL^{-1} of conidial suspension of each isolate was added to 100 g ground pasteurized button mushroom compost. Controls were treated with uninoculated compost (100 g) and tap water. After 7 days sunflower seeds were sown. The pots were then observed daily for the development of basal stem lesion.

Seed treatment: In this experiment, after preparation of conidial suspension (10^7 conidium mL^{-1}), the Arabic gum (3 g L) was added in order to stick conidia on seed coat. Then the seeds were impregnated for at least 2 h in the conidial suspension and sown in the soil infected with 100 sclerotium of *S. minor*. Treatments were as the follows:

a) *T. harzianum* (J1) b) *T. harzianum* (J6) c) *T. virens* d) *T. harzianum* (J1) + *T. virens*

The later treatment was containing of 50 mL of conidial suspension of each isolate.

RESULTS

In vitro studies

Antagonism by trichoderma: Examination of mycelia in the interface of all *Trichoderma* isolates revealed the absence of cytoplasmic contents due to destruction and endolysis of hyphal tips of *S. minor* (Fig. 1). Invasion of hyphae of *S. minor* was not detected. Study on colonization ability of antagonist isolates on 3-day-old cultures of *S. minor* showed that *T. virens* and *T. harzianum* (J1) overgrows the mycelium of *S. minor* on PDA and colonized sclerotial initials and preventing melanization of sclerotia (Fig. 2). Study on colonization ability of *Trichoderma* isolates on 14-day-old cultures of *S. minor* showed that *T. harzianum* (J1) and *T. harzianum* (J6) overgrows the clony of *S. minor* and also colonized, sporulated on mature sclerotia and lysed them on PDA (Fig. 3).

Greenhouse studies

Soil incorporation of conidial suspension: In this experiment, the application of conidial suspension of *T. harzianum* (J6), *T. virens*, *T. harzianum* (J1) and *T. virens* + *T. harzianum* (J1) in 1 kg soil on each pot containing sclerotia of *S. minor* reduced disease incidence by 8.33, 16.66, 25 and 41.66%, respectively (Table 1).

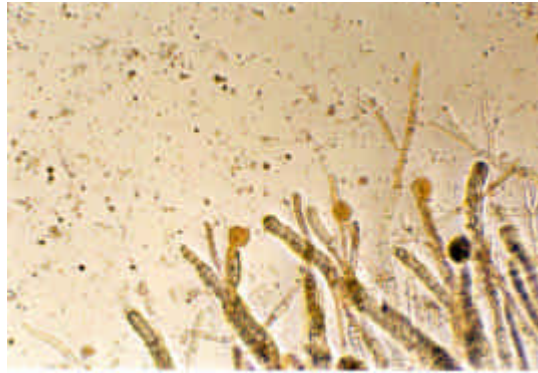


Fig. 1: Light microscopy of *S. minor* mycelium from the interface with all *Trichoderma* isolates in co-culture on PDA, showing empty hyphal tip of *S. minor* (Magnification 40×10)

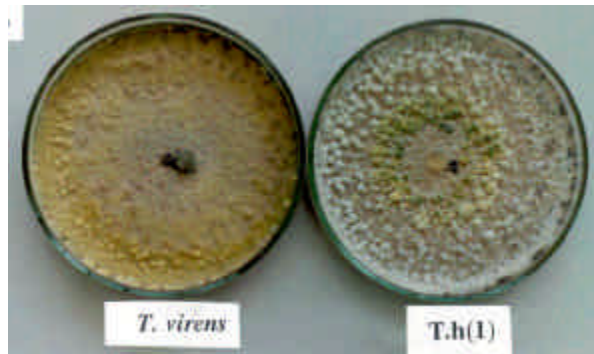


Fig. 2: Colonization of mycelia and sclerotial initials and preventing melanization of sclerotia of *S. minor* by *T. virens* and *T. harzianum*(J1) on 3-day-old cultures of *S. minor*

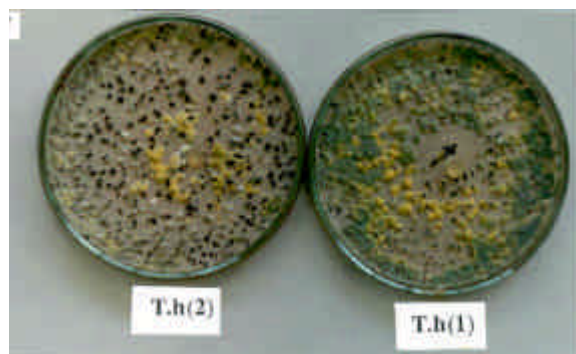


Fig. 3: Colonization and sporulation of mature sclerotia of *S. minor* by *T. harzianum* (J1) and *T. harzianum* (J6) on 14-day-old cultures of *S. minor*

Table 1: Effect of *Trichoderma* isolates on crown and root rot of sunflower caused by *S. minor* in greenhouse condition

Treatment	Disease incidence (%) ^x	
	A*	B*
Control	0.00a ^y	0.00a ^y
<i>S. minor</i>	91.66	91.66d
<i>T. virens</i> + <i>S. minor</i>	75.00bcd	66.66bcd
<i>T. harzianum</i> (J1) + <i>S. minor</i>	66.66bcd	58.66bcd
<i>T. harzianum</i> (J1) + <i>T. virens</i> + <i>S. minor</i>	50.00abc	41.66ab
<i>T. harzianum</i> (J6) + <i>S. minor</i>	83.33cd	75.00bcd

X: values averaged over three replicates with four seed per pot, Y: means followed by different letter are significantly different ($p>0.01$) according to Duncan's Multiple range test., A*: application method: soil incorporation of conidial suspension, B*: application method: soil incorporation of conidial suspension with support

Table 2: Effect of seed treatment with *Trichoderma* isolates on crown and root rot of sunflower caused by *S. minor* in greenhouse condition

Treatment	Disease incidence (%) ^x
Control	0.00a ^y
<i>S. minor</i>	91.66d
<i>T. virens</i> + <i>S. minor</i>	75.00bcd
<i>T. harzianum</i> (J1) + <i>S. minor</i>	83.33cd
<i>T. harzianum</i> (J1) + <i>T. virens</i> + <i>S. minor</i>	83.33cd
<i>T. harzianum</i> (J6) + <i>S. minor</i>	91.66d

X: values averaged over three replicates with four seed per pot, Y: means followed by different letter are significantly different ($p>0.01$) according to Duncan's Multiple range test

Soil incorporation of conidial suspension with support: In this experiment, the application of conidial suspension of *T. harzianum* (J6), *T. virens*, *T. harzianum* (J1) and *T. virens* + *T. harzianum* (J1) with support (pasteurized compost) in 1 kg soil on each pot containing sclerotia of *S. minor* reduced disease incidence by 16.66, 25, 33 and 50%, respectively (Table 1).

Seed treatment: In this experiment, the isolates of *T. harzianum* (J6), *T. virens*, *T. harzianum* (J1) and *T. virens* + *T. harzianum* (J1) reduced disease incidence caused by *S. minor* by 0, 16.66, 8.33 and 8.33%, respectively (Table 2).

DISCUSSION

The results of this study showed that all isolates exhibited antibiosis, evidenced by distortion and endolysis of hyphal tips of *S. minor* in co-culture. Antibiosis is well documented for *T. virens* (Lewis *et al.*, 1991; Robert and Lumsden, 1990) and parasitism of hyphae of *S. sclerotiorum* has been also reported (Tu, 1980). In present studies, hyphal invasion of *S. sclerotiorum* by all above mentioned *Trichoderma* isolates, was observed (In press). However, parasitism of *S. minor* hyphae by *T. virens* has not yet reported and was not also detected by any isolates in our investigation. In our studies, colonization of sclerotial initials and

preventing melanization of sclerotia on 3-day-old cultures as well as colonization, sporulation and lysis of melanized sclerotia on 14-day-old cultures of *S. minor* were observed and supported with results of other (Burgess and Hepworth, 1996). The results indicated that distortion and endolysis of *S. minor* hyphal tips by antibiosis and parasitism of sclerotia were two important antagonistic mechanisms of *Trichoderma* species on *S. minor*. Jones and Stewart (1999) reported that the soil incorporation of *T. harzianum* (C₅₂) was more effective than pellet information and transplant root spore dip. It reduced disease incidence of *S. minor* by 50% in lettuce.

Burgess and Hepworth (1996) also reported that application methods of *T. virens* (i.e., soil incorporation and seed treatment) reduced disease incidence of sclerotinia stem rot (*S. minor*) in sunflower by 44.2 and 14.8% in greenhouse condition, respectively. Present results, in consistent with previous authors showed that soil incorporation was more effective than seed treatment. Rouhani (2000) proved that out of 10 *Trichoderma* isolates only one could adjust to rhizosphere condition. The reason for little or no influence of *Trichoderma* isolates in the seed treatment could be due to slow stabilization of *Trichoderma* in rhizosphere as well as in crown of the host. In other words, rhizosphere and crown condition were not suitable to stabilize *Trichoderma* isolates. Furthermore, in this method, the number of conidia carried to the soil was greatly smaller in compare with other methods. In addition, these results showed that method of soil incorporation of conidial suspension with support (pasteurized compost) was more effective than application of them without support. May be it is due to that most of conidia were probably lysed without first germinating, or they germinated in response to some nutrients released from organic matter and subsequently lysed in the absence of food base adequate enough to sustain further growth and sporulation (Papavizas, 1985). In conclusion, the application of *Trichoderma* isolates by soil incorporation of conidial suspension with organic matter was more effective in crown and root rot disease incidence reduction caused by *Sclerotinia* on sunflower in greenhouse condition. But, it is necessary to evaluate the results of this study in the field.

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