

Evaluation of *Trichoderma* Isolates for Biological Control of Charcoal Stem Rot in Melon Caused by *Macrophomina phaseolina*

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ABSTRACT

Trichoderma harzianum (T39), *T. virens* (DAR74290), *T. viride* (MO), *T. harzianum* (M) and Trichodermin B a commercial formulation of *T. harzianum* (Bi) were evaluated as potential biological agents for the control of charcoal stem rot in melon caused by *Macrophomina phaseolina*. Cell-free metabolites of *T. harzianum* (M), *T. harzianum* (T39) and *T. virens* (DAR 74290) inhibited growth of *M. phaseolina* completely *in vitro* and appeared to be fungicidal. *T. viride* (MO) inhibited fungal growth from 34.9% to 71%. *T. harzianum* (T39), *T. harzianum* (M), *T. virens* (DAR 74290), *T. viride* (MO) and *T. harzianum* (Bi) were tested for their ability to protect melon plants from disease caused by *M. phaseolina* in a glasshouse experiment. The percentage of stand plants with the antagonist alone or in combination with the pathogen was significantly ($p < 0.05$) greater than in plants inoculated with the pathogen alone. The percentages for the stand plants in treatments were as follows: *T. harzianum* (T39), *T. harzianum* (M), *T. virens* (DAR 74290), *M. phaseolina*, *M. phaseolina* + *T. virens* (DAR 74290), *M. phaseolina* + *T. harzianum* (T39) and *M. phaseolina* + *T. harzianum* (M), at 95, 100, 97.5, 15, 64.25, 75.25 and 47.55 percent respectively. The percentage for the stand plant in the commercial Trichodermin B + *M. phaseolina* treatment (96.7%) was greater than for *M. phaseolina* alone (46.7%) and demonstrated the best result in the control of charcoal stem rot in melon.

Keywords: Biocontrol, Charcoal stem rot, Melon diseases, Soilborne pathogen, *Trichoderma*.

INTRODUCTION

Charcoal stem rot caused by the soilborne fungus *Macrophomina phaseolina* (Tassi) Goidanich is a serious disease of many cucurbit crops associated with drought stress. Charcoal rot affects all cucurbits. It is also major disease of melon in Iran and has been reported in different areas of Iran (Ershad, 1995). The crown leaves of infected plants turn yellow and wither and the veins may wilt and die, depending on the extent of infection. A green water-soaked lesion forms on the stem near ground level and may produce amber gumming. The lesion may extend 5-15 cm up the vein and, as it dries, the color changes to tan.

Eventually, small black microsclerotia (and sometime pycnidia) form within the lesion giving it a dusty, charcoal appearance. There are a few effective measures for controlling the disease including maintaining optimal soil moisture to avoid plant stress and rotation of cucurbits with a small grain crop. Some newly released hybrids show a high level of vein decline. Fumigation has shown some success in the controlling charcoal rot caused by *Macrophomina phaseolina* (Nyvall, 1989, Bruton *et al.* 1987). Cucurbit should be well supplied with N, P and K and, in particular, with the minor elements to maintain a well-balanced soil fertility to encourage vigorous growth (Sinclair and Backman, 1989; Sherf and MacNab, 1986).

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Biological agents could be an important component in the control of *M. phaseolina* if effective and reliable formulations are readily available, and could be integrated with chemical fungicides. The antagonistic activity of *Trichoderma* and *Gliocladium* species against plant pathogens has been studied extensively (Burgess and Hepworth 1996; HjeliJord *et al.*, 2001; Krause *et al.*, 2001; Sutton *et al.*, 1997; Lumsden *et al.*, 1996; Buns and Benson, 2000; Okhovat *et al.*, 1996 Etebarian *et al.*, 2000). A number of commercial formulations, based on *T. harzianum* and *T. virens* (formerly *Gliocladium virens*), are available for the control of soilborne and foliar disease in a range of horticultural crops (Harman *et al.*, 1996; Lumsden *et al.*, 1992; Samuels, 1996). *T. harzianum* isolate (T39) is the active ingredient of Trichodex, which is reported to control botrytis grey mold on a range of crops (Elad 1994; O'Neill *et al.*, 1996 a, b.). *T. harzianum* has been evaluated for the control of charcoal stem and root rot of melon in Egypt (Khalifa and Linddel, 1995) but there is little information on the efficacy of *Trichoderma virens* and other isolates of *T. harzianum* against *M. phaseolina*.

The objective of this investigation was to evaluate the potential of some isolates of *Trichoderma* for the biological control of *M. phaseolina*.

MATERIALS AND METHODS

Fungal Isolates

T. virens isolate (DAR 74290) (formerly *Gliocladium virens*), isolated from capsicum in south Australia was obtained from E. Scott in the Department of Applied Molecular Biology at the University of Adelaide. This isolate inhibited the growth of *Sclerotium rolfii* and *Phytophthora erythroseptica* under *in vitro* and glasshouse conditions (Na Lampang, 1994; Etebarian *et al.*, 2000). *T. harzianum* (T39) was isolated from Trichodex (Makhteshim Chemical Works, supplied by Abbot Australasia pry

Ltd. New South Wales, Australia) with the supplier's permission (Etebarian *et al.*, 2000). *T. harzianum* (M) (isolated from mushrooms in Hamedan), *T. harzianum* (Bi) (isolated from commercial Trichodermin B (Talphighe Daneh, Co. Iran)) and *Trichoderma viride*(MO) (formulated by Talphighe Daneh Co.) were obtained from Dr. Rohani of Bu-Ali Sina University, Hamedan. For the laboratory experiments, *T. viride*(MO) was isolated from formulated *T. viride*(MO). The *Trichoderma* species were maintained on potato dextrose agar (PDA). The *Macrophomina phaseolina* used in this study was isolated from diseased melon plants from the Garmsar area of Iran and maintained on PDA. The pathogenicity of the *Macrophomina phaseolina* isolate was proved on the Garmsar melon cultivar. All cultures were incubated at 25°C in darkness.

Effect of *Trichoderma* Species on Mycelial Growth of *M. phaseolina* *In vitro*

Dual culture (Dennis and Webster, 1971b) and cellophane overlays (Dennis and Webster, 1971a) were used to study the antagonist effects of the *Trichoderma* isolates on *M. phaseolina*. All antagonist-pathogen combinations were examined on 10-15ml of PDA (Sivasithamparam *et al.*, 1979) in 9-cm petri dishes, with four replicate plates per treatment. For dual cultures, a mycelial plug (5 mm in diameter), taken from actively growing, 3-day-old colonies of *M. phaseolina* or *Trichoderma* isolates placed 5 cm apart on the agar. For the control treatments, a plug sterile PDA medium was used instead of the antagonist. For cellophane overlays, cellophane membranes (Australia cellophane Victoria) 9 cm in diameter, were boiled in distilled water, then interleaved with filter paper and autoclaved before being placed on the agar medium. One 5-mm diameter plug of each of the *Trichoderma* isolates growing on PDA was placed on the centre of each cellophane membrane. For control treatments, a plug of sterile PDA medium

was used instead of the antagonist. The cellophane membrane and adhering fungus, or agar plug, were removed after two days (Etebarian *et al.* 2000). A plug of *Macrophomina phaseolina* was placed on the agar in the centre of the plate. The plates were incubated at 25°C in the dark for seven days when colony diameters were measured. The surface area of the colonies of *F.graminearum* was recorded daily, compared with the controls and the percentage inhibition of growth was calculated. The pathogen colony diameter was the average of two measurements with a ruler and the area was calculated. The percentage of growth inhibition was calculated using the formula $n = (a-b)/a \times 100$ where $n = \%$ growth inhibition, $a =$ the colony area of uninhibited *M. phaseolina* and $b =$ the colony area of treatment. Where *M. phaseolina* did not grow, the inoculum plug was transferred to fresh PDA to determine if the diffusible metabolites were fungicidal or fungistatic.

Effect of *Trichoderma* Species on Incidence of Charcoal Stem Rot in Melon

The ability of *Trichoderma* species to reduce incidence of stem and melon root rot in a glasshouse was investigated. The isolate of *M. phaseolina* was grown on potato-dextrose-agar (PDA) after seven days, and pieces of culture about 5 x 2 cm in size were transferred to 125ml Erlenmeyer flasks containing an autoclaved sand-corn meal medium (110g sand, 6g corn meal, 20ml sterile H₂O) and incubated at 25°C for 30 days. Thirty days later, 100 ml of *M. phaseolina* inoculum was mixed with approximately 2500 g autoclaved potting mix (two parts compost to one part of field soil) and placed in 20 cm pots. *Trichoderma* species were grown on PDA for nine days and 1/4 of contents of one petri plate were added to an Erlenmeyer flask containing 100 ml moist wheat bran and incubated at 25°C for 20 days. Wheat bran infested with

Trichoderma was mixed and blended in sterile distilled water to make a slurry. The amount of inoculum applied to the potting mix was 5 g infested bran per Kg. The pathogen was applied one day before planting and the antagonist on the day of sowing the seeds. Ten seeds of Garmsar native melon cultivar were sown in each pot. Before sowing, seeds were surface disinfested by soaking in 0.5% sodium hypochlorite for 3 minutes and then rinsed three times in sterile distilled water. Treatments comprised: uninoculated control; *M. phaseoli* alone; *T. virens* (DAR 74290) alone, *M. phaseolina* plus *T. virens* (DAR 74290), *T. harzianum* (T39) alone, *T. harzianum* (T39) plus *M. phaseolina*, *T. harzianum* (M) alone and *T. harzianum* (M) plus *M. phaseolina*. There were four replicates per treatment, arranged in a completely randomized design. Plants were maintained in the glasshouse without supplementary lighting from April to June. Pots were irrigated at two or three days intervals. The total number of plants and number of stand plants were determined in each pot eight weeks after inoculation and the percentage of stand plants were calculated.

Effect of Trichodermin B and Formulation of *T. viride*(MO) on Incidence of Charcoal Stem Rot in Melon

The autoclaved potting mix in 20 cm diameter plastic pots was inoculated with *M. phaseolina* and prepared as above, or left uninoculated, and 10 seeds of Garmsar cultivar were sown in each pot. The antagonist was added at a rate of 2g/Kg (following the commercial formulation) to potting mix to give 3×10^4 CFU g⁻¹ at inoculation. The population of *Trichoderma* in Trichodermin B or the formulation of *T.viride* (MO) was 1×10^7 CFU g⁻¹. For the determination of CFU in potting mix, the weight of soil in each pot was determined and CFU was calculated. There were three



replicate pots per treatment. Treatments comprised: uninoculated control; *M. phaseolina* alone; *T. viride*(MO) alone, Trichodermin B (*T. harzianum* Bi) alone. *M. phaseolina* plus *T. viride*(MO) and *M. phaseolina* plus Trichodermin B. Plant were maintained in the glasshouse at temperatures ranging from 20 to 30° C during April and May without any supplementary lighting. Treatments were arranged in a completely randomised design and the percentage of stand plants was determined at ten- days intervals as above. The data obtained 4 weeks after inoculation was used for analysis.

Statistical Analysis

Data on the percentage reduction in area growth and the percentage of plants surviving were subjected to arcsin square root transformation. Analysis of variance was performed and the means were separated using Duncan's multiple Range Test at $p < 0.05$ (Little and Hills, 1978).

RESULTS

Effect of *Trichoderma* Isolates on Mycelial Growth of *M. phaseolina* In vitro

T. harzianum (T39), *T. harzianum* (M) and *T. virens* (DAR 74290) inhibited mycelial

growth of *M. phaseolina* in a dual culture. Mycelial growth of the pathogen was reduced by 48.6%, 57% and 56.6% by *T. harzianum* (T39), *T. harzianum* (M) and *T. virens* (DAR 74290), respectively. *T. viride*(MO) reduced the growth of the pathogen by 19.2%. In cellophane technique, no growth of *M. phaseolina* occurred overlaid with *T. harzianum* (M), *T. harzianum* (T39) and *T. virens*(DAR 74290) seven days after inoculation of the pathogen. Furthermore, the plugs of pathogen failed to grow when transferred to a fresh medium. *T. viride*(MO) inhibited growth of *M. phaseolina*, by 71% in terms of colony area, seven days after pathogen inoculation (Table 1).

Biological Control of *M. phaseolina* on Melon in a Glasshouse Test

The percentage of healthy plants inoculated with the antagonist alone or in combination with the pathogen was significantly ($p < 0.05$) greater than in plants inoculated with the pathogen alone. The percentage of plants surviving 8 weeks after treatments with *M. phaseolina* plus *T. virens* (DAR. 74290), *M. phaseolina* plus *T. harzianum* (T39) and *M. phaseolina* plus *T. harzianum* (M) were 64.3%, 75.3% and 47.6%, respectively (Table 2). *M. phaseolina* was isolated from all inoculated plants showing symptoms but not from the one control pots treated with *T. harzianum* (T39) alone or *T. virens* (DAR

Table 1. Effect of *Trichoderma* isolates on mycelial growth of *M. phaseolina* 7 days after inoculation *in vitro*.

| Treatments | Cellophane technique, % growth inhibition | Dual culture, % growth inhibition |
|------------------------------|---|-----------------------------------|
| <i>T. harzianum</i> (T39) | 100 a | 48.6 a |
| <i>T. harzianum</i> (M) | 100 a | 57.1 a |
| <i>T. virens</i> (DAR74290) | 100 a | 56.6 a |
| <i>T. viride</i> (MO) | 71 b | 19.2 b |

Data are the means of 4 replicates, the percentage growth inhibition was calculated using the formula $n = (a-b)/a * 100$ where n = % growth inhibition, a = colony area of uninhibited control and b = colony area of treatment. Data were subjected to arcsin square root transformation before analysis. Means within columns followed by the same letters do not differ significantly at $p < 0.05$ according to Duncan's Multiple Range test.

74290) alone in which a few plants had wilted, possibly due to nutrient deficiency.

Effect of Trichodermin B and Formulation of *T. viride*(MO) on Control of Charcoal Stem Melon Rot in Glasshouse Condition

The percentage of healthy plants surviving inoculation with *M. phaseoli* alone was significantly less than with *M. phaseolina* plus *T. harzianum* Bi (Trichodermin B) and *T.viride* (MO)+pathogen ($p<0.05$). There were no significant differences between the uninoculated control , Trichodermin B plus *M. phaseolina* and Trichodermin B alone (Table 2).

DISCUSSION

Cell free metabolites produced by *T. harzianum* (T39), *T. virens*(DAR74290) and *T. harzianum* (M) completely inhibited growth of *M. phaseolina* and appeared to be fungicidal. The *T. virens*(DAR 74290)

isolate used in this study had previously been shown to produce gliotoxin (Na Lampang, 1994). Gliotoxin has been shown to be the major antibiotic inhibitory to *Pythium utlimum* and *Rhizoctonia solani* produced by *T. virens* in a soil-free culture (Lumsden *et al.* 1992). *T. virens* has also been shown to produce endochitinase, which acted synergistically with gliotoxin to inhibit the germination of conidia of *Botrytis cinerea* (Di Pietro *et al.*, 1993). Isolates of *T. harzianum* are known to produce a range of antifungal antibiotics (Ghisalberti and Sivasithamparam, 1991) and enzymes (Lorito *et al.*, 1994) whereas the strain (T39) is not identified as a producer of inhibitory compounds (Etebarian *et al.*, 2000). Results indicated that cell free metabolites produced by *T. viride*(MO) reduced the colony area of *M. phaseoli* by 19.2 and 34.9% in the dual culture and cellophane methods, respectively. It may be due to the slow growth of *T. viride*(MO), since the diameter of the *T. viride* (MO) colony before removing the cellophane membrane was

Table 2. Effect of *Trichoderma* isolates on stem and root rot in melon (*M. phaseolina*).

| Trichoderma were grown on wheat bran and applied to potting mix | |
|---|-----------------------------------|
| Treatments | % seedling surviving |
| Uninoculated control | 100 a |
| <i>T. harzianum</i> (T39) | 95 a |
| <i>T. harzianum</i> (M) | 100 a |
| <i>T. virens</i> (DAR 74290) | 97.5 a |
| <i>M. phaseolina</i> | 15 d |
| <i>M. phaseolina</i> + <i>T. virens</i> (DAR 74290) | 64.3 b |
| <i>M. phaseolina</i> + <i>T. harzianum</i> (T39) | 75.3 b |
| <i>M. phaseolina</i> + <i>T. harzianum</i> (M) | 47.6 c |
| Trichodermin B and formulated <i>T. viride</i> (MO) were applied to potting mix | |
| Treatments | % seedling surviving ^a |
| Uninoculated control | 83.3 a |
| <i>T. viride</i> (MO)(formulated) | 63.3 b |
| Trichodermin B | 83.3 a |
| <i>M. phaseolina</i> | 46.6 b |
| <i>T. viride</i> (MO) + <i>M. phaseolina</i> | 63.3 b |
| Trichodermin B + <i>M. phaseolina</i> | 96.6 a |

Data are the means of 4 replicates, Means within columns followed by the same letters in each section of the table, do not differ significantly at $p<0.05$ according to Duncan's Multiple Range test. Trichodermin B and *T. viride* (MO)was formulated by Talphighe Daneh Co, but *T. viride* (MO)is not commercial (CFU = 10^7). ^a as compared with control



1.75 cm and those of *T. harzianum* (T39) and *T. virens* (DAR74290) were 5 cm and 4.5 cm, respectively. *Trichoderma* species were added to the potting mix as colonised wheat bran, a food base previously found to be suitable for *Trichoderma* species (Elad *et al.*, 1980) and *T. harzianum* Bi and *T. viride* (MO) were applied as a commercial formulation. The population of the antagonist was approximately 3×10^4 CFU per g of potting mix. This population is close to the 10^5 CFU g⁻¹ considered by Adams (1990) to be necessary to achieve control. However, Hadar *et al.* (1960) added 5×10^3 and 1×10^4 conidia of *T. harzianum* per gram of air-dried soil. Lewis and Papavizas (1987) found that a three- and eight-day-old inoculum of *T. harzianum* in wheat bran inhibited *R. solani* in soil more effectively than did a 15- or 40-day-old inoculum. The effect of the age of the inoculum and means of application on the ability of *T. harzianum* (T39), *T. virens* (DAR 74290), *T. viride* (MO), *T. harzianum* (M), *T. harzianum* Bi to control stem rot in melon should be investigated. In this investigation, the antagonists were applied at the time of planting to soil which had been infested one-day previously and it would be of value to examine the effects of infesting the soil both earlier than and at the time of planting. A biological control product which was effective when applied at the time of planting would be more likely to be accepted by growers than one which required additional cultivation. Information on the timing of infection of melon by *Macrophomina phaseolina* and also the effect *Trichoderma* species on the sclerotia of the pathogen in the field is required to optimise the application of the biological control agent.

In conclusion, the *Trichoderma* isolates and formulated products tested here reduced disease severity in melon plant seedlings in the glasshouse. Future research will involve studies of the mechanisms involved. These isolates warrant further investigation for

their ability to stem melon rot, especially in the field.

ACKNOWLEDGEMENTS

The financial support of the Vice Chancellor for research, University of Tehran, is gratefully acknowledged. The technical assistance of Maryam Nabizadeh is also appreciated.

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ارزیابی جدایه‌های *Trichoderma* در کنترل بیولوژیکی ساق سیاه خربزه در اثر
Macrophomina phaseolina

حسن رضا اعتباریان

چکیده

جدایه‌های *T. viride* (MO)، *T. virens* (DAR 74290)، *Trichoderma harzianum* (T39) و *harzianum* (M) و محصول تجارتي تریکودرمین (*T. harzianum* Bi) برای کنترل بیماری ساقه سیاه خربزه که عامل آن قارچ *Macrophomina phaseolina* می‌باشد، مورد ارزیابی قرار گرفت. مواد مترشحه *T. harzianum* (M)، *T. harzianum* (T39) و *T. virens* (DAR 74290) به طول کامل از رشد قارچ عامل بیماری در آزمایشگاه جلوگیری نمود و به نظر می‌رسد این مواد خاصیت قارچ کشی دارد. قارچ *T. viride* (MO) رشد قارچ را به میزان ۳۴/۹ تا ۷۱ درصد کاهش داد. نتایج آزمایش‌های گلخانه‌ای نشان داد که درصد گیاهان زنده در تیمارهای تریکودرما تنها یا همراه با قارچ عامل بیماری به طور قابل ملاحظه‌ای بیشتر از تیماری بود که فقط با قارچ عامل بیماری تلقیح شده بود ($P < 0.05$). درصد گیاهان زنده در تیمارهای *T. harzianum* (T39)، *T. harzianum* (M)، *T. virens* (DAR 74290)، *M. phaseolina* + *T. harzianum* (T39)، *M. phaseolina* + *T. virens* (DAR 74290)، *M. phaseolina* + *T. harzianum* (M) به ترتیب ۹۵، ۹۷/۵، ۱۵، ۶۴/۲۵، ۷۵/۲۵، ۴۷/۵۵ بود. بیشترین درصد گیاهان زنده با ۹۶/۷ درصد در تیمار *Trichodermin* B + *M. phaseolina* مشاهده شد که با تیمار شاهد غیر آلوده به بیماری تفاوت معنی‌داری نداشت.