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Evaluation of *Streptomyces* strains for Biological Control of Charcoal Stem Rot of Melon Caused by *Macrophomina phaseolina*

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Abstract: Biological control of causal pathogen (*Macrophomina phaseolina)* was investigated by four strains of *Streptomyces*. Dual culture and cellophane overlay technique were used *in vitro* assay. All antagonist –host combination were carried out in 4 replicates. Colony area was recorded daily, compared with controls and percentage of growth inhibition was calculated. Glasshouse studies were performed to test the ability of *Streptomyces* strains incorporated to the soil as a rate of 10g kg^{-1} potting mix. In other experiment seeds of melon were soaked in *Streptomyces* suspension and planted in infected soil with pathogen. *Streptomyces* strains significantly inhibited mycelial growth of *Macrophomina phaseolina* in dual culture. *Streptomyces* strains, STL, A20, and A15 inhibited the growth of pathogen by 88.16 to 89.3% and mycelial growth of *M. phseolina* was reduced 69.99% by *Streptomyces* strain A22. Cell free metabolites produced by 4 strains of *Streptomyces* reduced colony area by 80.14-99.66%. The results of glasshouse experiment indicated that when soil of pots inoculated with pathogen + *Streptomyces* or *Streptomyces* alone, percentage of healthy plants were significantly greater than in those of pathogen control ($p<0.05$). Similar results were obtained in seed treatment test.

Key words: Melon, charcoal rot, *Macrophomina phaseolina*, *Streptomyces*, biological control

INTRODUCTION

Charcoal stem rot, caused by the soilborne fungus *Macrophomina phaseolina,* is a serious disease of many crops associated with drought stress[1]. This disease also is an important disease of melon in Iran and has been reported from different areas of Iran[2]. Charcoal rot affects all cucurbits. Brown leaves of infected plants turn yellow and whiter. The vines may wilt and die, depending on the extent of infection. A green water soaked lesion forms on the stem near the ground level and may produce amber gumming. The lesion may extend 5-15 cm up the vine 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 control measure for this disease which include maintaining optimal soil moisture to avoid plant stress, rotation of cucurbits with a small grain crop. Some newly released hybrids show a high level of vine decline. Fumigation has shown some success in the controlling charcoal rot caused by *Macrophomina phaseolina*[3,4]. Cucurbit should be well supplied with N, P and K and especially with the minor elements and maintain a well-balanced soil fertility to encourage vigorous growth[1,5]. Biological agents could be an important component in the control of *M. phaseolina* if effective and reliable formulations were readily available and could be integrated with chemical fungicides. Khalifa and Lindel[6] reported that *Trichoderma harzianum* reduces disease severity of *Macrophomina* root rot of Melon in Egypt. *Streptomyces* species also warrant investigation as potential biological control agents for charcoal stem rot, as they have been reported to suppress a number of disease including pink rot of potato caused by *Phytophthora erythroseptica*[7] , root rot of *Bakansia grandis* caused by *Phytophthora cinammomi*[8], damping off of alfalfa seedlings by *Phoma medicaginis*[9]. Toussaint *et al.*[10] showed that 11 strains of *Streptomyces* species protected raspberry plants from disease caused by *P. fragariae* var rubi. Actinomycetes isolated from soils collected from *capsicum* crops in Korea have been shown to produce antibiotics active against *P. capsici*[11]. Faroughi *et al*[12]. tested 13 strains of *Streptomyces* for biocontrol of cantaloup root rot caused by *Phytophthora drechsleri*, they concluded seed treatment or adding of the mixture of 13 *Streptomyces* strains to infected soil with pathogen increased seedling survival by 43-50%. However, there is little or no information on the efficacy of *Steptomyces* against charcoal stem rot of melon. The aim of this investigation was to determine the potential of some strains of *Streptomyces* for biological control of *Macrophomina phaseolina* through *in vitro* and glasshouse experiments.

MATERIALS AND METHODS

Pathogen and antagonist strains: The isolate of *Macrophomina phaseolina* was isolated from diseased plants of melon from the Garmsar area of Iran and maintained on Potato Dextrose Agar (PDA) in the dark at 4°C.

Streptomyces strains A22, A20, A15 and STL were obtained from in ground glasshouse crops of Capsicum near Virginia, South Australia[13]. The strains inhibited the growth of *Sclerotium rolfsii* Sacc and *Phtyophthora erythroseptica in vitro* and potting mix[7,13]. *Streptomyces* strains were maintained on 1/5 M32 agar (1/5 strength of M32 agar medium)[14] in the dark at 4°C .

***In vitro* inhibition assay:** Dual culture[15] and cellophane overlay[16] techniques were used to examine the effects of *Streptomyces* strains A15, A20 , A22 and STL on mycelial growth of *Macrophomina phaseolina.* Culture were grown on 15 ml of casein glycerol medium(CGM) agar medium[7,17] in 9 cm petri plates. For dual culture, half of the agar surface was smeared with a suspension of one *Streptomyces* strain in sterile distilled water (SDW) using a sterile cotton bud. After incubation at 7 days, a plug (5 mm diameter), cut from the leading edge of 4 day-old culture of *M. phaseolina* on CGM agar medium was placed on the other half of the plate. For controls, CGM agar was inoculated with the pathogen alone. Plates were incubated at 25°C in the dark for 7 days. The surface area of the colonies *M. phaseolina* was recorded daily, compared with the controls and the percentage of growth inhibition was calculated.

For the cellophane overlay technique, cellophane membrane (Australia Cellophane, Victoria), 9 cm in diameter were boiled in distilled water interleaved with filter paper and autoclaved. A cellophane membrane was placed on the agar in each petri plate and dried in a lamina flow cabinet for 15 min. A 7 day -old culture of one *Streptomyces* strain, suspended in SDW, was smeared the entire surface of the cellophane. For controls SDW was applied. The plates were incubated in the dark at 25°C for 7 days, after which the cellophane membrane with adhering *Streptomyces* culture was removed. A plug(5 mm diameter) of *M. phaseolina* was placed on the culture of the plate previously occupied by the antagonist. The plates were then incubated in the dark at 25°C for 7 days. The surface area of *M. phaseolina* colonies was recorded daily, compared with the controls and the percentage of growth inhibition was calculated. The data for percentage inhibition of growth at 1, 2, 3 and 7 days in dual culture and the data for 4 and 7 days after inoculation of the pathogen in cellophane technique were pooled and analyzed statistically.

Glasshouse assay for biological control of *M. phaseolina* on melon

Soil treatment: The ability of *Streptomyces* isolates to reduce incidence of charcoal and stem rot of melon in glasshouse was investigated. *M. phaseolona* isolate was grown on potato dextrose agar (PDA) and when they were growing rapidly (in about 10 days) pieces of culture 5x2 cm in size were transferred to 125 mL Erlenmeyer flasks containing autoclaved sand -corn meal medium (110 g sand, 6 g corn meal, 20 mL sterilized water). The flasks incubated at 25°C for 30 days. Containing of each flask were mixed with 2500 g of autoclaved potting mix and placed in 20 cm pots.

Inoculum of *Streptomyces* isolates was prepared as follows: wheat barn were soaked for 1 h and then transferred to 125 Erlenmeyer flask and autoclaved for 1 h at 121°C on two successive days. Isolates A13, A15, A20 and STL were grown separately on CGM at 25°C for 7 days. And ¼ of the contents of one petri plate were added to each flask, mixed with wheat barn and incubated at 25°C for 21 days. Wheat barn infected with the *Streptomyces,* were combined and blended in SDW to make slurry. The rate of inoculum which were applied to potting mix, was 10 g infected barn kg^{-1}. Treatments comprised: *Streptomyces* alone, mixed *Streptomyces* isolates + *M. phaseolina* and control without antagonist and pathogen. Seeds of Garmsar native melon cultivar were surface disinfected by soaking in 0.5% sodium hypochlorite for 3 min then rinsed three times in SDW. Fifteen seeds were sown in each pot. There were four replicate pots per treatment arranged in a completely randomized design. Plants were maintained in the glasshouse without supplementary lighting from April to June(spring) in Pakdasht Tehran. Pots were watered at 2 or 3 days intervals until emerge and daily thereafter.

Seed treatment test: The methods and materials for preparation of *M. phaseolina* inoculum were the same as described for soil treatment test. Inoculum of *Streptomyces* isolates were prepared as follows:

Isolates of *Streptomyces* A13, A15, A20 and STL were grown separately on 9 cm petri plate containing CGM medium at 25°C for 7 days. Bacteria were harvested from surface of plate and washed with 10 mL of SDW and suspended in 0.05% tween 20. Suspension of 4 isolates of *Streptomyces* were combined equally . The melon seeds were disinfected as mentioned above and soaked for 30 min in *Streptomyces* suspension and air died in laminar-air flow hood. For adhearing of bacteria to seed surface methyl cellulose was used[18]. The other methods and materials were the same as soil treatment test. In both experiments the percentage of healthy plants were determined 40 days after planting.

Statistical analysis: Data on percentage inhibition of growth and percentage of healthy plants were subjected to arsin square root transformation, before analysis, Analysis of variance was performed and means were separated using Duncan's Multiple Range Test at $p<0.05$ [19].

RESULTS AND DISCUSSION

Effect of *Streptomyces* isolates on mycelial growth of *M. phaseolina in vitro*: All isolates of *Streptomyces* inhibited mycelial growth of *M. phaseolina* in dual culture. Mycelial growth was reduced by 14.86-36.00 and 89.12-98.79 by *Streptomyces* isolates, 1 and 7 days after inoculation, respectively (Table 1)

Cell free metabolites produced by *Streptomyces* isolates reduced colony area of *M. phaseolina* by 80.14-99.50 and 83.56-99.66, 4 and 7 days after inoculation, respectively (Table 2).

Table 1: Percentage of growth inhibition of *M phaseolina* by different strains of *Streptomyces* in different times after inoculation (dual culture)

	Inoculation after (days)			
Streptomyces strains	1st	2nd	3rd	7th
A15	36.00a	77.97a	94.35a	98.79a
A20	28.95ab	73.33a	93.60a	98.63a
A22	14.86b	52.39b	81.76b	89.12b
STL	14.86b	66.02a	91.48a	98.57a

Data are means of four replicate plates. Numbers within columns followed by a common letter are not significantly different at $p<0.05$, according to Duncan,s Multiple Range Test. Data were subjected to arcsin square root transformation before analysis. Data are expressed as control without antagonists.

Table 2: Percentage of growth inhibition of *M phaseolina* by different strains of *Streptomyces* (cellophane overlay Technique)

	Inoculation after (days)	
Streptomyces strains	4th	7th
A22	99.50a	99.66a
A20	99.41b	99.56b
A15	98.67c	98.99c
STL	80.14d	83.56d

Data are means of four replicate plates. Numbers within columns followed by a common letter are not significantly different at $p<0.05$, according to Duncan,s Multiple Range Test. Data were subjected to arcsin square root transformation before analysis. Data are expressed as control without antagonists.

Table 3: Antagonistic effect of combined *Streptomyces* strains on the disease incidence caused by *M. phaseolina*

	% of healthy plants	% of healthy plants
Treatments	Seed treatment	Soil treatment
Streptomyces only	97.50a	100a
Streptomyces+*M. phaseolina*	100a	96.65a
M phaseolina only	50.50b	46.88b
Healthy control	100a	100a

Data are means of four replicate pots. Numbers within columns followed by a common letter are not significantly different at $p<0.05$, according to Duncan,s Multiple Range Test. Data were subjected to arcsin square root transformation before analysis. Data are expressed as control without antagonist and pathogen.

Fig. 1: Effect of seed treatment of Streptomyces on disease incidence of charcoal stem rot (left; *Macrophomina phaseolina* + *Streptomyces*: right; *Macrophomina phaseolina* only).

Fig. 2: Effect of soil treatment of *Streptomyces* on disease incidence of charcoal stem rot (left; *Macrophomina phaseolina* + *Streptonyces*: right; *Macrophomina phaseolina* only).

Biological control of *Streptomyces* on melon in glasshouse conditions: Survival of seedling in pots treated with *M. phseolina* plus *Streptomyces* was similar to that in plants inoculated with *Streptomyces* only or control without antagonist and pathogen. Percentage of healthy plants in treatment *Streptomyces* + *M. phaseolina* were significantly greater than those *M. phaseolina* only in both seed and soil treatment tests (Table 3, Fig. 1 and 2). All strains of *Streptomyces,* inhibited growth of *M. phaseolina in vitro*, using dual culture and cellophane overlay techniques. The presence and size of the zone of inhibition suggested that this effect was due to the production of antibiotics by *Streptomyces* [7,9,20-22].

The mode of action of *Streptomyces* appeared to be antagonism by the production of Tubercidin, produced by *Streptomyces tubercidicus* and *S. violaceoniger* against *Phytophthora capsici*[23] , Geldamycin produced by *S. hygropicus* against *Rhizoctonia solani*[24].

The isolates tested here have also potential against other soil borne pathogens, Cell free metabolites of *Streptomyces* strains tested inhibited growth of *Phytophthora erythroseptica* the causal agent of pink rot of potato *in vitro*. The lesion size in Pontiac potato treated with *P. erythroseptica* and combined *Stretomyces* isolates (mean of 13.25 mm) were significantly less than of that pathogen alone (mean of 54.75 mm). The yield of tubers from Pontiac plants treated with combined *Streptomyces* and *P. erythroseptica* (mean 16.5 g fresh weight potato) were significantly greater than in control inoculated with the pathogen alone (mean 6.77 g/pot)[7].

The dry weight of shoots and roots of melon were not determined in this investigation, but in the absence of the pathogen, some actinomycetes isolates significantly increased shoot and dry weight. Such increases have been reported in cauliflower[25], wheat[26] and also increase of potato yield have been reported[7]. This phenomenon may be related to the ability of actinomycetes to produce growth regulator[27,28].

The use of actinomycetes to control fungal pathogens has advantages as they are not affected by fungicides and therefore, can be used as a component of integrated disease control including chemical control.

In this study percentages of healthy plants in treatments *Streptomyces* + *M. phseolina* were 100 and 96.65 in seed and soil treatment test, respectively. Seed treatment with *Streptomyces* is more acceptable than soil treatment because it is easier and low cost.

The effect of age of inoculum and means of application on the ability of *Streptomyces* to give consistent control charcoal stem root rot should be investigated. Similarly, the timing of the application of antagonists and the pathogen should be studied. In commercial crops, *M. phaseolina* are also present as sclerotia in infested soil prior to planting, however, the effect of *Streptomyces* on sclerotia has not been determined. In this study, the antagonists were applied , at the time of planting, to soil which had been infected one day previously and it would be of value to examine the effects of infecting the soil both earlier than, and 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. This means of application would also reduce the need for long-term survival of the antagonists in the soil, which may be limiting factor in the biological control of soil- borne fungal pathogens[29,30]. In conclusion, the *Streptomyces* strains tested here reduced disease severity in melon plant seedling in the glasshouse. Future research will involve studies of the mechanism involve. These isolates warrants further investigation for their ability to control of charcoal stem root rot especially in commercial situation. An integrated approach using a combination of *Streptomyces* and *Trichoderma* species such *T. harzianum* T39 and *T. virens* DAR 7420 (Etebarian unpublished) may allow reduction of disease incidence in different climate and different soils.

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