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Studies on Biological Control of Sugarbeet Pathogen *Rhizoctonia solani* Kühn

Tarek A.A. Moussa
Department of Botany, Faculty of Science, University of Cairo, Giza 12613, Egypt

Abstract: A field experiment was carried out to investigate the biological control of *R. solani* by different organisms including bacteria and fungi. Three methods of application were used during the study. Control results differed with the method of application used. The present study indicated that all the antagonists tested inhibited infection by *R. solani* and that the efficacy of prevention depended on the application method used. Coating sugarbeet seeds with antagonists was produced results for applying the antagonist to control *R. solani*. A soil preinoculation that contained the antagonist was better than treating them with extracts of antagonists although antimicrobial compounds in the extracts have been found to control the disease to the same extent. The most effective biocontrol agent was *Trichoderma harzianum*. Hyphal interactions between *T. harzianum* and *R. solani* were observed by scanning electron microscopy. *T. harzianum* attached to the host by hyphal coils.

Key words: Biological control, sugarbeet, *Rhizoctonia solani*, antagonists, electron microscopy, mycoparasitism

Introduction

Pathogenic fungi of the genera *Aphanomyces*, *Polymyxa*, *Pythium*, *Phytophthora*, *Rhizoctonia*, *Sclerotium*, *Fusarium* and *Phoma* usually attack sugarbeet seedlings (Whitney and Duffus, 1986).

In Egypt, sugarbeet is attacked by several root-rot pathogens; the most serious of which are those caused by *Rhizoctonia solani* Kuhn and *Sclerotium rolfsii* Sacc. These two pathogenic fungi are subjected to two major environmental stresses: salinity and application of the herbicide pyradur for the control of weeds in sugarbeet. The impact of salinity stress on the pathogenicity and growth activities of these fungi has been studied (El-Abyad *et al.*, 1988a,b). The impact of salinity and herbicide pyradur on pathogenicity and production of cell wall degrading enzymes by these fungi have been also studied (Moussa, 1994; El-Abyad *et al.*, 1996, 1997).

Antagonism between soil microorganisms is a common phenomenon. Few articles were concerned with the existence and importance of the mechanisms of antagonism (Schroth and Hancock, 1981; Turhan, 1981; Hayashida *et al.*, 1989; El-Abyad *et al.*, 1993a, b; El-Shanshoury, 1994; Moussa, 1999; Moussa and Rizk, 2002).

Chemical and cultural methods to reduce the soil borne and tuber borne sources of inoculum were used, although research has been directed towards the use of antagonists for the biocontrol of *R. solani* on potatoes (Aluko and Hering, 1970; Chu and Wu, 1980; Clifford and Elenwa, 1983; van den Boogert and Jager, 1983) on sugarbeet (Moussa, 1999). Jager *et al.* (1979) and Jager and Velvis (1983a, b) reported the suppression of *R. solani* in potato fields and attributed it to the presence of antagonistic fungi in the soil. *Gliocladium roseum*, *G. virens*, *G. nigrovirens*, *Trichoderma hamatum* and *Verticillium biguttatum* were common hyperparasites of sclerotia and reduced their viability (Jager *et al.*, 1979; Jager and Velvis, 1983a, b; Velvis and Jager, 1983). Pleban *et al.* (1995) found that isolates of different endophytic bacteria were recovered from surface-disinfected seeds obtained from commercial companies, plants in the field and tissue culture. The bacteria were isolated such as *Bacillus cereus* from Sinapis inhibited growth of *R. solani*, *Pythium ultimum* and *S. rolfsii* and also exhibited chitinase activity. *B. subtilis* from onion tissue culture inhibited *R. solani* and *P. ultimum* growth. *B. cereus* from cauliflower inhibited growth of *R. solani*.

The present investigation, explored the biological control of *R. solani* by different microorganisms including bacteria and fungi, which of the antagonist is a powerful biocontrol agent to *R. solani*. and observed the mechanism of mycoparasitism between the powerful biological control agent and *R. solani*.

Materials and Methods

Pathogenic fungus: Rhizoctonia solani (AG 2-2) Kuhn was isolated from diseased sugarbeet roots (El-Abyad *et al.*, 1988a) described by El-Abyad and Abu-Taleb (1990) and maintained on the medium composed of (gl^{-1}): dextrose, 30; KH_2PO_4, 1; $MgSO_4.7H_2O$, 0.5; KCl, 0.5; KNO_3, 2 and 1 ml of each of stock solutions (1 gl^{-1}) of $FeSO_4.7H_2O$, $MnSO_4.7H_2O$, $ZnSO_4.7H_2O$ and thiamine; agar, 20 g (Johnson and Curl, 1972).

Host plant: Seeds of sugarbeet (*Beta vulgaris* L.) cultivar was obtained from the North Delta Sugar Company, Egypt. These were surface-sterilized with 0.1% $HgCl_2$ for 30 sec, then thoroughly washed with sterile distilled water.

Experimental soil: The soil used in the greenhouse experiments, obtained from a sugarbeet field at Kafr El-Shikh governorate, Egypt was a sandy clay loam of the following mechanical analysis: 17% silt, 30% clay, 31% fine sand and 19% coarse sand. The other properties were 0.8% organic carbon, 4.2% total carbonates and 0.3% total water-soluble salts (pH 8).

Test organisms: The test organisms used in this study were obtained from the culture collection of Microbial Resources Center (MIRCEN), Faculty of Agriculture, Ain Shams University, Cairo, Egypt.

Bacillus cereus 1080 and *B. subtilis* 1020 were maintained on nutrient agar medium; *Candida utilis* and *Saccharomyces cerevisiae* 1883 were maintained on universal medium described by Gibriel *et al.* (1987).

Pathogenicity experiment: The fungus grew rapidly and produced abundance mycelia on yeast extract agar. It grew to maturity in a (petri dish 9 diameter) four days after inoculation. The pathogenicity of *R. solani* was tested on sugarbeet planted in plastic pots (20x8 cm) containing 5 cm of soil and infected with a soil-potato inoculum placed in the middle of the pot. *R. solani* was inoculated into the soil @ 0.5, 1.0, 1.5 and 2.0 g pot^{-1} (Rothrock and Gottlieb, 1984).

Antibiotic activities of antagonists of *R. solani*: Antagonists were grown in liquid media to obtain their antibiotic metabolites. The substances produced by the nine antagonists were tested on diffusion plates against different test microorganisms, *B. cereus*, *B. subtilis*, *Candida utilis* and *Saccharomyces cerevisia*.

Control of *R. solani* by coating sugarbeet seeds with antagonists: The antagonists were grown at 28°C for 7 days. The hyphae and spores of three plates were suspended in 15 ml sterile distilled water in which seeds were coated as described by Singh and Mehrotra (1980). Both pathogen inoculation and treated seeds were done simultaneously. Treated seeds were air-dried for 30 min before sowing.

Control of *R. solani* by soil preinoculation with antagonists: The antagonists were grown in liquid medium on a rotatory shaker (200 rpm) at 28°C, the hyphae were harvested by centrifugation at 3000g for 20 min and the pellets resuspended in 10 ml of sterile distilled water. The antagonists were inoculated into the soil 7 days prior to the inoculation of pathogen and seed sowing at a rate of 1%.

Control of *R. solani* by seed soaking with antagonist's filtrates: The treatment with seeds soaked in the filtrate of antagonists was done with filtrates or distilled water as control for 30 min prior to the sowing and infestation of soil. Control treatments included seeds soaked in sterile distilled water.
The pots were kept in the greenhouse for 60 days and the percentage of disease incidence was calculated.

SEM procedures: Cellophane membranes from the interaction area were removed and the organisms were fixed in 3% glutaraldehyde (Sigma Chemicals Co., St. Louis, MO63178) in 0.1M phosphate buffer (pH 7). After 12 h of refrigeration, the specimens were dehydrated in a graded acetone series. Critical point dried specimens were coated with gold palladium and viewed in a scanning electron microscope (JEM-100S).

Results

Pathogenicity experiment: In order to select the proper inoculum size that may yield about 60% infection in the developed seedlings, the seeds of sugarbeet cultivar were sown in pots containing autoclaved soil and inoculated with inocula of *R. solani* as previously described.
The results obtained (Table 1) revealed that in the absence of pathogen, the disease incidence was nil. The results also indicated that disease incidence increased (63.37%) in proportion to the weight of inoculum of *R. solani*. 1.5 g pot^{-1} inoculum size for the pathogen *R. solani* was chosen for the biological control experiments.

Antimicrobial activities of antagonists of *R. solani*: Results indicated that all antagonists produced antimicrobial substances and that most of the antagonists inhibited growth of fungi (Table 2). The antimicrobial activities were related to the growth media used to culture the antagonists and the sensitivity of the test microorganisms. Because antibiotic inhibition of *B. megaterium* and *E. coli* tested by metabolites of the antagonists were visible to the eye, the microorganisms were considered suitable as test microorganisms for checking the production of antimicrobial substances by the antagonists.

Control of *R. solani* by coating sugarbeet seeds with antagonists: When the seeds of sugarbeet were coated with antagonists the seeds planted in sand infected with *R. solani*, seedling root-rot was inhibited (Table 3). The disease incidence was reduced 39.01, 39.7, 63.5, 65.1, 68.2, 74.9, 76.2, 79.3 and 84.8% by *Trichoderma pseudokoningii, T. viride, Gliocladium deliquescens, Bacillus cereus, T. koningii, B. subtilis, Penicillium vermiculatum, Paecilomyces marquandii* and *T. harzianum*, respectively related to the control. The effect of *P. marquandii* and *T. harzianum* were significantly greater than *B. subtilis*.

Control of *R. solani* by soil preinoculation with antagonists: The results indicated that the degrees of control root rot in sugarbeet depend on the type of antagonist used. Out of the 9 antagonists investigated, *Trichoderma harzianum* and *G. deliquescens* were the most effective in controlling the disease where the disease incidence was reduced 77.3 and 72.7%, respectively; followed by *T. koningii, T. viride* and *Bacillus cereus* where the disease incidence was reduced to 36.3 for the three antagonists (Table 3).

Control of *R. solani* by seed soaking with antagonist's filtrates: Sugarbeet seeds were treated with culture filtrates of the various antagonists before planting in sand beds infected with *R. solani*. Results of growth studies indicated that the extracts protected the sugarbeet from infection by *R. solani* for some instances (Table 3). The effects of *G. deliquescens* and *P. marquandii* were slightly greater than those of the other antagonists. The antimicrobial effects from some fungal antagonists were greater than those of the bacterial antagonists.

SEM observations: Mycelial samples from the interaction region of dual cultures of *R. solani* and *T. harzianum* were observed in a scanning electron microscope. The diameter of hyphae of *T. harzianum* was 1.5-3 μm and the diameter of the plant pathogen was 5-7 μm, so they could easily be distinguished from each other (Figs. 1-3). Hyphae of *T. harzianum* frequently grew parallel to the host and attached itself to host mycelium by forming hooks (Fig. 1). Following this interaction, the mycoparasite sometimes penetrated the host mycelium (Fig. 2) apparently by partially degrading its cell wall (Figs. 2, 3).

Discussion

Results of the present study showed that the disease incidence increased in correlation with the inoculum size of pathogen *R. solani*. The antimicrobial activities were related to the type of the antagonists and the sensitivity of the test microorganisms. Production of secondary metabolites e.g., antibiotics, Fe-chelating siderophores and cyanide is most often associated with fungal suppression by fluorescent pseudomonads in the rhizosphere of sugar beets (Shanahan *et al.*, 1992; Lovic *et al.*, 1993) and other crops (Howell and Stipanovic, 1979, 1980; Voisard *et al.*, 1989; Thomashow *et al.*, 1990). In addition, cellulytic and chitinolytic activity have been reported in bacteria and fungi (Fuchs *et al.*, 1986; Watanabe *et al.*, 1990; Romaguera *et al.*, 1992; Schirmböck *et al.*, 1994; Tweddell *et al.*, 1994; Chernin *et al.*, 1995; Trachuk *et al.*, 1996).
The results indicated that the control of the pathogen differed according to the treatment and the type of antagonistic microorganism. The best treatment for controlling the pathogen by all antagonistic microorganisms was the seed coating treatment. This may be due to the spores of the antagonist in contact with seeds of sugarbeet, which continuously supplied the seeds with the antimicrobial compound (s). Similar results were obtained by other workers in the control of maize root rot (Singh and Mehrotra, 1980); *Fusarium* root rot of faba bean (Yehia *et al.*, 1982); damping-off of sugarbeet (Martin and Hancock, 1987); tomato wilt (El-Abyad *et al.*, 1993a); sugarbeet disease (Moussa and Rizk, 2002). In all these studies and in present work, the seed coating treatment improved plant growth may be due to the growth regulators produced by the antagonist together with their continuous supply to the developing plants as a result of the intimate contact between the seeds and the antagonist.
Inoculation of soil with the antagonist prior to sowing was less effective in controlling the pathogen than the seed coating treatment. At the late stage of cultivation in the soil pre-inoculation treatment may indicate that *R. solani* was more tolerant to the environmental conditions. This may be also due to the decline of the antagonistic isolate population resulting in a decreased production of antimicrobial substances. Similar studies were applied for the control of root rot of pea seedlings (Rothrock and Gottlieb, 1984); damping-off of cauliflower (Kundu and Nandi, 1993); sugarbeet disease (Moussa and Rizk, 2002).

Table 1: Effect of *R. solani* inoculum size on the disease incidence of root rot in sugarbeet

Inoculum size (g pot^{-1})	Disease incidence (%)
Control	0.0
0.5	18.37
1.0	45.87
1.5	63.37
2.0	80.22

Table 2: Antimicrobial activities of antagonists of *R. solani* tested on several microorganisms

Antagonist	Test microorganism			
	A	B	C	D
Bacteria				
Bacillus cereus CB 22	-	+	+	+
B. subtilis F 29-3	+	+	+	+
Fungi				
Gliocladium deliquescens F-92	+	+	+	+
Paecilomyces marquandii CF302	+	+	+	+
Penicillium vermiculatum F-60	+	+	+	+
Trichoderma harzianum TVCN1	+	+	-	+
T. koningii T12	+	+	+	+
T. pseudokoningii T33	+	-	-	+
*T. viride*TD	+	+	+	+

A, *Bacillus megaterium* 1033; B, *Escherichia coli* 1357; C, *Candida utilis*; D, *Saccharomyces cerevisiae*
+, means positive; -, means no reaction tested with > 100 mg ml^{-1} crude antibiotic

Table 3: Control of sugarbeet root rot disease caused by *R. solani* with different antagonists

	Disease incidence (%)		
Antagonist	Seed coating	Seed soaking	Soil pre-inoculation
Control	42.53a	71.43	75.68a*
Bacteria			
Bacillus cereus	14.85defg	66.4bc	48.18a
B. subtilis	10.67efgh*	81.2a	52.91a
Fungi			
Gliocladium deliquescens	15.51def	51.9c	20.68b
Paecilomyces marquandii	8.8fgh**	52.3c	50.93a
Penicillium vermiculatum	10.12efgh	65.5bc*	50.93a
Trichoderma harzianum	6.48h	62.9bc	17.16b
T. koningii	13.53efg	69.5ab	48.18a
T. pseudokoningii	25.94b	63.6bc	52.9a
T. viride	25.63bc	60.5bc	48.18a

* Values within a row followed by the same letter are not significantly different at 5% level according to Duncan's multiple range test (DMRT). ** Values within the column followed by the same letter are not significantly different at 5% level (based on DMRT)

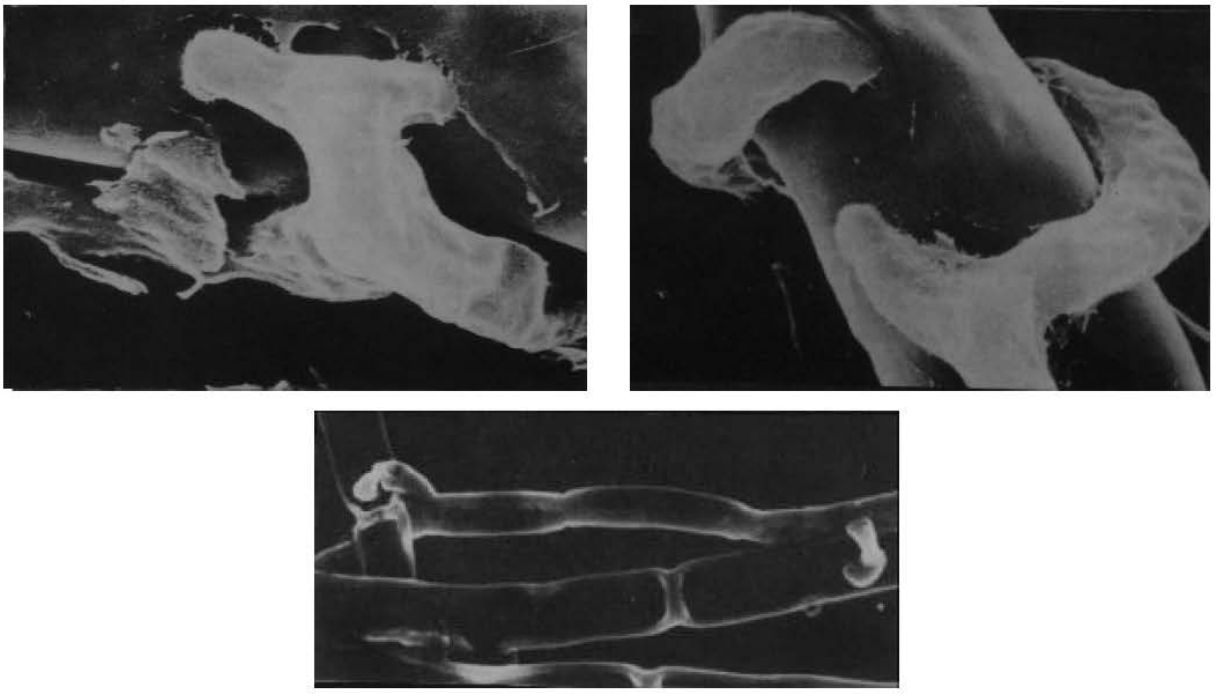

Fig. 1-3: Scanning electron micrographs of *Trichoderma harzianum* hyphae interacting with those of *Rhizoctonia solani*. 1, hooks of *T. hrzianum* attached to hyphae of *R. Solani* (X 2000). 2, appressorium-like structure, formed by *T. harzianum*, attached to a hype of *R. solani* with partial degradation of host cell wall (X 8500). 3, hypha of *T. Harzianum* coiling around and penetrating one of *R. solani*. Partial degradation of host cell wall can be observed (X 8500).

This investigation also showed that the seed soaking treatment was the least effective in controlling *R. solani* compared with the other treatments. This may be attributed to the decreased absorptive capacity of sugarbeet seeds for the antagonistic compounds due to their hard coat and hence low-level accumulation in the germinating seeds. Present findings are confirmed, to some extent, in controlling wilt of soybean and french bean (Khalid, 1987); root disease of cucumbers (Tahvonen, 1988); damping-off of sugarbeet (Rath and Wolf, 1992); wilt of tomato (El-Abyad *et al.*, 1993a), sugarbeet disease (Moussa and Rizk, 2002).

The results, indicated that the antimicrobial effects from fungal antagonists were greater than those of the bacterial antagonists. The control effects of mycoparasites appear to be associated with the inhibitory activities of their antimicrobial metabolites. When *T. harzianum* grew towards *R. solani* contact was made and mycoparasitism occurred. Chet *et al.* (1981) showed that hyphae of *T. hamatum* grew directly towards *R. solani*, which indicated that this was not a random phenomenon. Upon reaching host hyphae, the antagonistic fungus coiled around the host. Sometimes *Trichoderma* was also observed to penetrate host hyphae (Fig. 3). Scanning electron micrographs of the host-parasite interactions have shown contact cells of *Stephanoma phaeospora* parasitizing *Fusarium* sp. and penetration of other fungi by *Pythium acanthicum* (Hoch, 1978; Hoch and Fuller, 1977).

The cell walls of *R. solani* are composed of β-1,3-glucan (laminarin) and chitin (Bartnicki-Garcia, 1973; Chet *et al.*, 1967). *T. harzianum* releases active lytic enzymes, that can digest these components (Elad *et al.*, 1982; Hadar *et al.*, 1979). Microorganisms, that capable of lysing other organisms are widely spread in natural ecosystems (Mitchell and Alexander, 1963). Henis and Chet (1975) have suggested that the extracellular enzymes may play a role in microbiological control. Jones *et al.* (1974) have shown that *T. viride* solubilized hyphae of *Sclerotinia sclerotiorum* by β-1,3-glucanase activity.

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