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Mycoparasitism of potato black scurf pathogen (*Rhizoctonia solani* Kuhn) by biological control agents to sustain production

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ABSTRACT

Eight isolates of *Trichoderma harzianum* and six isolates of *Pseudomonas fluorescens* were tested *in vitro* for their antagonistic potential against *Rhizoctonia solani*. In dual culture both bioagents were found antagonistic to the growth of *R. solani*. The hyphal interaction studied using light microscopy revealed destructive mycoparasitism of *R. solani* by *T. harzianum* mycelium. The method of mycoparasitism was sparse to intense coiling of *R. solani* followed by disintegration, disorganization and death of *R. solani* mycelium. Similarly, addition of bacterial antagonist *P. fluorescens* inhibited the growth of *R. solani* by disintegrating mycelium even without contact of two colonies. When sclerotia of *R. solani*, parasitized by the antagonist *T. harzianum* and *P. fluorescens* were studied under microscope, the outer cells of sclerotia showed disintegrated breakdown of cell wall and extrusion of cytoplasm. Since, conidiophores and conidia of *T. harzianum* were also observed in sections of parasitized sclerotia, an active parasitization of sclerotia by antagonist is indicated. Sclerotia of *R. solani* was completely destroyed by bioagents within 25 days of interaction under laboratory conditions.

Key words: *Solanum tuberosum*, *Trichoderma harzianum*, *Pseudomonas fluorescens*, antagonist.

INTRODUCTION

Potato crop is susceptible to many pathogens, some of which are wide spread and others are locally situated. *Rhizoctonia solani* Kuhn is one of those destructive plant pathogens attacking a broad variety of hosts under diverse environmental conditions in every part of the world. The fungus causes damping off of seedlings, root rot and stem cankers of growing plant and black scurf of potato tubers. In potato, it reduces stand, yield quality and marketability of the produce.

Biological control is an attractive and promising approach to control plant diseases, specially the soil-borne ones. It is now widely recognized that biological control of plant pathogens is a distinct possibility in the future and can be successfully exploited in modern agriculture. This approach can be especially attractive for the management of *R. solani*, because it is unspecialized pathogen. The period of susceptibility of plants to the pathogen is relatively long thus protection is needed for long time during vegetative period. Therefore, the antagonist (bioagents) applied with the seeds will grow on tuber and subsequently in the rhizosphere and protect the infection courts by suppressing the population of the target pathogen. Among the fungal antagonist species, *Trichoderma* and *Gliocladium* and bacterial antagonist *Pseudomonas* have received maximum attention in the past (Gupta *et al*., 8; Wicks *et al*., 18). In view of the importance of biological agents in the disease management, present investigation was conducted to find out the mycoparasitism reaction of different biological agents with *R. solani* under *in vitro* conditions.

MATERIALS AND METHODS

Potato tubers showing characteristics symptoms of black scurf disease were collected from potato fields, washed thoroughly under running tap water and air-dried. The tuber skin having black sclerotial bodies was peeled with the help of sterilized blades and surface sterilized (mercuric chloride 0.1%) for about a minute followed by 2-3 washings with sterile water. These sclerotia were then placed onto PDA medium under aseptic conditions and incubated at 28 ± 2°C. From the growing young colonies the fungus was sub-cultured on PDA slants. Slants were kept for incubation at 28 ± 2°C for three days and then conserved at low temperature for future use. To establish pathogenisity, mass culture of *R. solani* was prepared on sorghum grains as described by Prasad and Rangeswaran (14). Mass culture of *R. solani* was mixed in sterilized soil by applying 100 g colonized grains per kg soil and was incubated until white mycelial growth appears on the surface of soil. Soil amended with *R. solani* was filled in pots and selected healthy tubers having 2-3 healthy eyes were

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planted. Sterilized soil without *R. solani* served as control. After 10 days when potato sprouts emerged from soil, they were checked for the presence of lesion and symptoms produced. Tissue containing symptom was incubated on PDA plates and checked for the growth of *R. solani*. *Trichoderma harzianum* and *P. fluorescens* were isolated from potato field soil. Dilution plate technique (Johnson, 10) was used for isolating *T. harzianum* using *Trichoderma* selective medium (Elad *et al*., 7). Bacterial antagonist *Pseudomonas fluorescens* was isolated in the same way as *Trichoderma* except that the dilutions used were 10^{-6} and 10^{-7} and *Pseudomonas* was grown on King's B medium.

Dual culture of *R. solani* and *T. harzianum* (Morton and Stroube, 13) was used for testing antagonistic potential of bio-agent against pathogen *R. solani*. The plates were inoculated with 6 mm mycelia disc of *R. solani,* the pathogen and the antagonist fungi taken from the growing edge of three days old cultures and placed towards the edge and also opposite to each other at approximately 4.0 cm distance and incubated at 28 ± 2°C. Observations on the growth of pathogen were taken by measuring the colony diameter every 24 h from the underside of the petri dishes. The antagonistic potential of *P. fluorescens* against *R. solani* was examined by placing six mm mycelial discs of *R. solani* in the centre of the petri dish and the bacterium was streaked on both the opposite sides approximately 4 cm apart. Observations on growth of pathogen were taken at 24 h interval by measuring the colony diameter of *R. solani* from the underside of the petri dishes.

To study the interaction at different stages of dual culture, petri dishes inoculated with *R. solani* and *T. harzianum* were incubated for 5 days. The mycelial mat from the overlapping region were placed on microscopic slides, properly stained with aniline blue and mounted in lacto-phenol. Observations were recorded under microscope and micrographs were taken. To observe the mechanism of interaction of *R. solani* and *P. fluorescens*, both organisms were grown in the same plate on King's B medium. The *R. solani* mycelium from interacting /overlapping zones in dual culture petri dish was placed on microscopic slides and properly stained with aniline blue and mounted in lacto-phenol for observations.

The sclerotial interaction with *Trichoderma* was examined by placing aseptically six mm disc of *Trichoderma* in the centre of petri dishes containing PDA medium and incubated at 28 ± 2°C for 24 h. The dried sclerotia were kept aseptically in *Trichoderma* grown dishes and were incubated at 28 ± 2°C. The sclerotia were taken out at a 5 day interval and sections were cut with the help of microtome. Cut sections were placed on microscopic slides, stained with aniline blue, mounted on DPX permanent mount and examined under microscope. Sclerotia from *Trichoderma* petri dishes were checked for their viability at 5 days interval on Ko and Hora medium (Ko and Hora, 11).

Nutrient broth was prepared in 100 ml conical flask and inoculated with the culture of *P. fluorescens*. After 24 h of incubation, washed and dried sclerotia were dipped in the broth and incubated at 28 ± 2°C at 5 day intervals. Then scelerotia were taken out and checked for viability on Ko and Hora medium. Sclerotia taken at 5 days interval were dehydrated and cut into sections with the help of microtome and examined. The experiments were laid out in CRD. Analysis of variance (ANOVA) was calculated to separate the means.

RESULTS AND DISCUSSION

The tow fungal colonies grew towards each other and the contact was established usually 48 h after inoculation. The radial growth of eight different isolates of *T. harzianum* at different intervals is given in Table 1. The most promising isolate (No. 1) from *T. harzianum* was selected and maintained for its use in further experiments. The antagonistic potential of the fungal antagonist (*T. harzianum*) was tested in dual culture of PDA. Within 48 h, the colonies of *R. solani* and *T. harzianum* came into contact, thereafter *T. harzianum* started to over grow the *R. solani* colony. Finally, entire plate was covered with dark green mycelium of *T. harzianum*. Attempts to re-isolate *R. solani* from the zone of interaction and also from the zone where that antagonist has totally over grown the test fungus failed, revealing that the antagonist is capable of controlling the pathogen. Microscopic examination of the slides prepared from the zone of interaction between *T. harzianum* and *R. solani* revealed that when hyphae of *Trichoderma* came into contact with *R. solani*, it produced loop like wavy structures (Fig. 1 A-B). The *T. harzianum* hyphae grew along the hyphae of *R. solani* and at intervals it produced several short branches which coiled around the *R. solani* hyphae making an intricate intertwining of both the hyphae around each other. Observations of fungal development in dual culture revealed that growth inhibition of the host mycelium occurred soon after the contact with *Trichoderma* hyphae suggest that mycoparasitism/ hyperparasitism is one of the processes operating in *T. harzianum* and *R. solani* interaction. There was no indication of impaired growth of *R. solani* before coming in contact with the antagonist (*T. harzianum*) as has been reported for other fungus-fungus interaction (Cherif and Benhamou, 3). Instead, it seems likely that cell surface interactions between

Table 1. Comparative growth of eight isolates of antagonist fungi *T. harzianum* in dual culture with *R. solani* light and dark brown isolates.

Isolate / Days	Colony diameter (cm)									
	Light brown					Dark brown				
	2	4	6	8	Mean	2	4	6	8	Mean
1	3.42	4.67	6.55	8.80	5.86	3.22	4.51	6.49	8.56	5.70
2	3.12	4.03	6.16	7.24	5.14	3.01	4.21	6.18	7.24	5.16
3	3.01	3.96	6.01	7.05	5.01	2.90	4.01	6.11	7.00	5.01
4	2.80	3.68	5.93	6.88	4.82	2.93	3.91	6.01	7.12	4.99
5	2.96	3.78	5.96	7.01	4.93	3.01	3.93	5.73	7.01	4.92
6	3.13	4.01	6.23	8.08	5.36	3.01	3.88	5.89	7.95	5.18
7	2.66	3.58	5.73	6.73	4.67	2.73	3.64	5.82	7.00	4.80
8	2.91	3.73	5.82	7.45	4.98	3.02	3.80	6.00	7.51	5.08
Mean	3.00	3.93	6.05	7.41		2.98	3.99	6.03	7.42	
LSD ($P \leq 0.05$)	Isolate: 0.22 Day : 0.15 Isolate × day : 0.62					Isolate: 0.18 Day : 0.14 Isolate × day : 0.54				

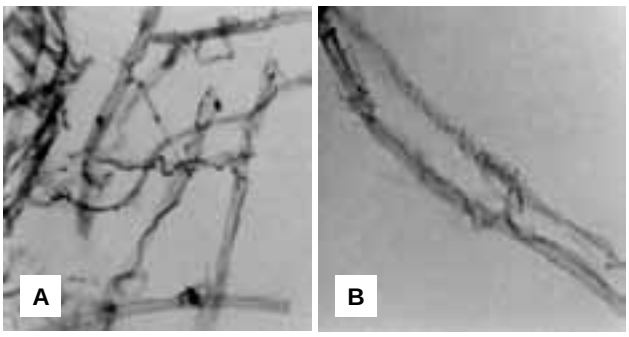

Fig. 1. *In vitro* interaction of *Rhizoctonia solani* and *Trichoderma harzianum*: parasitization of *R. solani* by *T. harzianum* (A), invasion and wall lysis of *R. solani* mycelia growth by intense coiling of *T. harzianum* (B).

both fungi are crucial for subsequent steps leading to growth inhibition of the pathogen. Examination of sections of non-parasitized healthy sclerotia showed that sclerotia structures were composed of single type brown coloured, unbroken mature cells. Whereas, *T. harzianum* colonized sclerotia revealed that cell walls of most of the cells were broken and cytoplasm was lacking. The outer layers were usually hyaline, empty, collapsed and sometimes broken, whereas, the inner layers were brown in colour with cell wall sometimes broken. Conidia and conideophore of *T. harzianum* were seen emerging out from the outer cells of sclerotia. This again confirms the parasitism of sclerotia bodies of *R. solani* by antagonist fungus *T. harzianum*. Antagonism of *Trichoderma* spp. against *R. solani* is known and has been reported by several workers (Dutta and Das, 4; Elad *et al.*, 7; Manzali *et al.*, 12; Prasad and Rangeshwaran, 14).

Six strains of *P. fluorescens* isolated from potato rhizosphere were tested against *R. solani* for their antagonist potential using dual culture technique. All the *Pseudomonas* isolates inhibited the growth of *R. solani* but the isolate No. 1 was found most effective (Table 2). Slides of the mycelium of *R. solani* from the dual culture petri dishes were observed under microscope. It was observed that mycelium of *R. solani* was broken and disintegrated from which

Table 2. Comparative growth of *R. solani* light brown isolate and dark brown isolate in dual culture plates along with six isolates of antagonist bacteria *P. fluorescens* (after 60 h).

Isolate	*R. solani* colony diameter (cm)	
	Light brown	Dark brown
Control	3.66	4.10
1	1.53	1.23
2	2.56	2.70
3	2.23	2.13
4	2.30	2.16
5	2.76	2.20
6	2.46	1.93
LSD ($P \leq 0.05$)	0.21	0.35

cytoplasm tended to exude out (Fig. 2 A-B). Results indicated that *P. fluorescens* was antagonistic to *R. solani* and inhibited the growth without touching its mycelium. The antagonistic effect of *P. fluorescens* was might be due to production of diffusible toxins/ antibiotics/enzymes by the antagonists, destructing the pathogen mycelium. Observations recorded on sclerotial interaction with *P. fluorescens* clearly indicated that up to 15 days all sclerotia germinated, when kept on Ko and Hora medium aseptically. However, on 20th day 60% sclerotia did not germinate and on 25th day all sclerotia lost their viability. Sections of sclerotia incubated for 25 days were cut and studied under microscope (Table 3). In sections of healthy sclerotia, there was only a single type cells and all the cells were healthy, mature and brown in colour. Cells of antagonist invaded sclerotia were divided into two parts, outer layer disorganized and hyaline whereas inner ones were with thick cell wall and brown in colour. The cells of outer layers were broken and cytoplasm exuded out. The cells of inner layers were more intact as seen by their organization and colour. The disintegration of cells indicated the production of some metabolite by *Pseudomonas* cells which disintegrated the cell walls of sclerotia of *R. solani*. The presented results are consistent with the observations of several workers (Ahmadzadeh, 1; Berta *et al*., 2; Rajendran and Samiyappan, 15; Siddiqui and Shaukat, 16; Wang *et al*., 17). An antibiotic strongly inhibitory to *R. solani* was isolated from *P. fluorescens* cultures and identified as pyrrolnitrin. The antibiotic also inhibited the growth of other fungi associated with the cotton seedling disease complex (Howell and Stipanovic, 9). Another antibiotic, pyoluteorin was isolated from the cultures of strain pf5 and is inhibitory to *Pythium* but not to *R. solani*. Elad and Baker (5), and Elad and Chet (6) suggested the competition for nutrients and iron between bacteria and pathogen as one of the controlling mechanism. These results provide an option for treating potato seed tubers, bearing sclerotia on surface with bioagent *P. fluorescences* before keeping in cold storage for inactivating the primary inocula of pathogen *R. solani*. Bacterial antagonist *P. fluorescens* was able to parasitize or destroy the *R. solani* sclerotia after incubation of >20 days under laboratory conditions. This principal may be exploited for reduction in initial/primary inocula which come along with infected tubers, kept in cold storage. Therefore, seed potatoes should be treated with *T. harzianum* or *P. fluorescens* before keeping in cold storage for inactivating the primary inocula of the pathogen *R. solani*. These results were consistent with the results obtained by Gupta *et al.* (8) in tomato. Bacterial antagonist *P. fluorescens* was able to parasitize or destroy the *R. solani* sclerotia after incubation of >20 days under laboratory conditions. Our results suggest that *T. harzianum* and *P. fluorescens* are potential antagonists against *R. solani* but needs further confirmation under field conditions.

Table 3. Effect of most effective isolate of *P. fluorescens* on *in vitro* viability of sclerotia of *R. solani* (n = 10).

Germination (%) of *R. solani* sclerotia at different intervals				
5th day	10th day	15th day	20th day	25th day
100	100	100	40	0

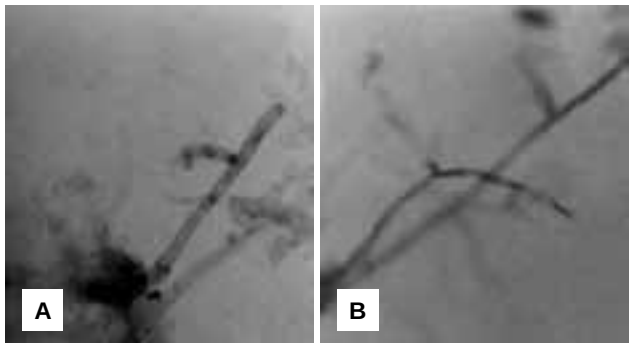

Fig. 2. *In vitro* interaction of *Rhizoctonia solani* and *Pseudomonas fluorescens*: disintegration of mycelium wall of *R. solani* by *P. fluorescens* (A), disintegration of mycelium cell wall and extruding hyphal cytoplasm of *R. solani* (B).

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