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Biological Management of Black Scurf of Potato Caused by *Rhizoctonia solani* Kühn.

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ABSTRACT

Black scurf caused by *Rhizoctonia solani* Kühn is one of the destructive diseases of potato. In present study, effect of bioagents and cow urine was evaluated for management of black scurf of potato under lab conditions. Two isolates of *Trichoderma harzianum,* 10 isolates of *Pseudomonas fluorescens* and three concentrations of cow urine were examined against the mycelial growth of *R. solani.* Among the two isolates of *T. harzianum*, Th-39 inhibited 71.85% mycelial growth of *R. solani*. PF-2 isolate of *P. fluorescens* was found most inhibitory (94.42%) followed by PF-4 isolate (76.74%) and PF-11 isolate (70.00%).Out of the three concentrations of cow urine, 100% concentration was found most promising with 87.53% inhibition. The sclerotial parasitization of *R. solani* by *T. harzianum* was also studied through microtomy. Microtomed sections of *T. harzianum* parasitized sclerotia of *R. solani* showed the disruption and disintegration of cells of sclerotia. Nuclear staining of *R. solani* confirmed its multinucleate nature. Thus, this investigation helps to develop an economical and environment friendly method for the farmers to overcome the harmful effect of *Rhizoctonia solani*.

Keywords: Biocontrol agents, black scurf, cow urine, *Rhizoctonia solani*

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INTRODUCTION

In India the total production of potato for the year 2014-15 is estimated to be 44.89 million tons [1]. Throughout the world, the production of potato is deprived by pests, pathogens and weeds. Losses due to pathogens can occur during pre-harvest and post-harvest time including transport, storage and marketing of potatoes [2]. The fungus *Rhizoctonia solani* Kühn (*Thanetophorus cucumeris* (Frank) Donk) is one of the destructive plant pathogens, also causing severe diseases in a wide variety of plants under diverse environmental conditions worldwide [3]. Black scurf disease is known to reduce stand, quality, yield and price of tubers produced. The typical disease symptoms are death of pre-emerging sprouts, canker formation on underground stem parts and stolons, diminished root systems, and formation of sclerotia on progeny tubers (typical black scurf symptoms). The fungus spreads to new growing areas by sclerotia present on seed tubers [4]. As the pathogen is both seed (tuber) and soil borne in nature, the management of this disease by a single control measure is a difficult task. Because of its accessibility for control agents, tuberborne *R.solani* is relatively easy to control as compared with soil borne inoculums [5]. Therefore, integrated disease management (IDM) including biological, chemical and cultural methods is needed for effective management. Biological control gives promise as a strategy for disease management and it is environment friendly too. Antagonistic fungi (especially *Trichoderma* spp.) and bacteria (fluorescent pseudomonads) have been found widely useful in agriculture against a number of phytopathogens [6, 7]. The use of animal waste (cow urine and dung) is one of the exciting and attractive approaches for management of *R. solani* because it can target tuberborne as well as soilborne inoculums [8, 9]. Keeping these aspects in mind, in present investigation, biocontrol agents and cow urine were evaluated for better management of black scurf of potato.

MATERIALS AND METHODS

Selection of biocontrol agents and their antagonistic activity

Two isolates of the antagonistic fungus *Trichoderma harzianum* (Th-39 and Th-ranichauri), and ten isolates of bacterium *Pseudomonas fluorescens* viz. PF-2, PF-3, PF-4, PF-6, PF-11, PF-12, PF-18, PF-28, PF-31 and PF-173 were used as biocontrol agents against *R. solani*. Both *Trichoderma harzianum* and *Pseudomonas fluorescens* isolates were obtained from Biocontrol Laboratory in Department of Plant Pathology, G.B. Pant University of Agriculture and Technology, Pantnagar. *T. harzianum* isolate Th-39 originally isolated from Tarai region of Uttarakhand from rice crop while Th-ranichauri isolate originally isolated from Ranichauri area of Uttarakhand. The antagonistic ability of biocontrol agents against *R. solani* was tested using the dual culture technique [10].

For fungal biocontrol assay, from full grown plates of antagonist (*T. harzianum*) and pathogen (*R. solani*) 6 mm discs were punched and placed opposite to each other in 90 mm petriplates poured with semi-synthetic media (PDA). Similarly for bacterial biocontrol assay, 20 ml of sterilized equimixture of sterilized PDA and sterilized King's B medium was poured into petriplates under aseptic conditions. Filter paper discs cut with the help of a paper punch were autoclaved twice and were soaked in 48 hrs old *Pseudomonas fluorescens* liquid culture in King's B broth. In each petriplate, one filter paper disc was kept opposite to *R. solani* disc. For both bacterial and fungal antagonists three replicates were taken and inoculated plates were incubated at 28±2ºC temperature. After full growth in the check plate in three days, the diameter of fungal colony was measured. On the basis of colony diameter in dual culture and control (having only *R. solani*), the inhibition of mycelial growth of *R. solani* by *T. harzianum* and *P. fluorescens* was calculated.

In vitro study for the antifungal activity of cow urine against *R. solani*

To evaluate the activity of cow urine on the radial growth of *R, solani,* Poisoned food technique [11] on potato dextrose agar (PDA) medium was used. Under aseptic conditions in the laminar air flow chamber, the freshly collected cow urine was filtered with the help of bacterial filter paper (Whatman filter paper No. 43). The filtrate obtained was mixed with sterilized water in the ratio of 1:0, 1:1 and 1:3 respectively to make the final concentrations of 100%, 50% and 25% respectively. These three concentrations were added at the rate of 1.5 ml, 3 ml, 4.5 ml, and 6 ml per 60 ml PDA. The required amount of cow urine was added in PDA and mixed properly before pouring. Ammended media with cow urine, were poured into PDA petriplates. From 4 days old culture of *R. solani*, 6 mm mycelial disc was cut with the help of a sterile cork borer and placed in the centre of each petriplate. The plates were incubated at 28±2ºC for 2 days. Diameter of fungal colony was

measured when the check growth was full. Percent inhibition was calculated with the help of mean colony diameter [12].

Parasitization of *R. solani* sclerotia

To study parasitization of pathogen (*R. solani)* by biocontrol agent (*T. harzianum*), microtomy of sclerotia of *R. solani* was performed. From one week old *R. solani* cultures grown on PDA, sclerotia were harvested and surface sterilized for 1 min. with 0.1 percent sodium hypochlorite (NaOCl) solution [13]. For the microtomy of *T. harzianum* parasitized sclerotia, 20 mm of PDA was poured in petriplates. Under aseptic conditions, 6 mm disc of *T. harzianum* was inoculated in the centre of the plate and incubated at 28±2ºC for 24 hrs. Potato tubers having *R. solani* sclerotia on their surface were washed in running tap water. Sclerotia were removed with the help of sterilized blade and washed in 0.1 percent NaOCl solution and sterilized water respectively. Sclerotia were dried on sterilized blotting paper and kept in *T. harzianum* grown plate aseptically. Inoculated plates were incubated at 28±2ºC. After 15 days, sclerotia were taken out and surface sterilized with 0.1 percent NaOCl solution for 1 minute. These surface sterilized sclerotia (both healthy and *T. harzianum* parasitized) were then rinsed with sterilized distilled water for 3 times, dried on sterilized blotting paper and fixed in FAA (formalin acetic acid) for one week. After this, the sclerotia were thoroughly washed in 50% ethanol twice and dehydrated by dipping them in 50% ethanol, 70% ethanol and 95% ethanol, each for 30 minute duration followed by three times 100% ethanol wash for 30 minutes duration and then dipped in xylene for 30 minutes. Infilteration was done by dissolving paraffin wax in xylene containing the sclerotia and gradually increasing the concentration of paraffin. Finally, the mixture was kept in an oven at 60ºC to evaporate xylene. Then sclerotia were transferred to fresh melted wax and this step was repeated for 3 times. Along with the melted wax the processed sclerotia were placed to molds. When the blocks were solidified, they were removed from molds kept in refrigerator for 45 minutes. The hardened wax blocks were fixed on the stage of a rotary microtome and 10 microns thick sections were cut. The sections were floated in a warm water bath and fixed to microscopic slides by using 2 percent gelatin and a gentle heat. These sections were rehydrated (reverse process of dehydration), stained with aniline blue and observed under microscope [14].

Nuclear staining

To confirm multi- or binucleate nature of *R. solani,* nuclear staining was performed using DNA-intercalating fluorochrome ethidium bromide (2, 7-diamino-10-ethyl-9-phenyl phenanthridium bromide) [15]. A bit of 3-day old unfixed mycelium of *R. solani* was immersed for 15 min in 0.2 ml of 0.1% solution of ethidium bromide in ethanol: water (3:1, v/v) on a microscopic slide. Afterward, excess stain was removed by tilting the slide followed by gradual replacement with distilled water carefully and finally absorbing the excess stain with blotting paper from one end. To reduce background fluorescence, stained mounts were examined microscopically using reflected light fluorescence (AHL-RFL) of the Vanox-S AHBS (Olympus BX 40) microscope. Observations were recorded under G-excitation (456 to 500 nm) with the Dm 480 dichronic mirror, B 545 excitation filter and O 599 barrier filter.

RESULTS AND DISCUSSION

Antagonistic bioassay

Bioassay for evaluation of different biocontrol agents against *R. solani* showed their significant impact on control of *R. solani* pathogen. In fungal biocontrol bioassay both isolates were found to have more than 50% inhibition of pathogen. As compared to Th-ranichauri (50.74%), Th-39 isolate showed significant inhibition upto 71.85% (Table 1).

Table 1: Effect of different isolates of *Trichoderma harzianum* on the mycelial growth of *R. solani* in dual culture after 72 hrs of incubation (values in the bracket represent angular transformation)

Isolates of *T. harzianum*	Percent inhibition of mycelial growth of *R. solani**
Th-ranichauri	50.74 (45.42[a])
Th-39	71.85 (57.95[b])
CD at 1% = 6.25	CV = 2.56

* Mean of three replicates

Bacterial bioassay results showed that all the isolates were having biocontrol efficacy against the pathogen. Statistically, *Pseudomonas fluorescens* isolate PF-2 was found most inhibitory (94.42%) followed by PF-4 isolate (76.74%) and PF-11 isolate (70.00%) (Table 2).

Table 2: Effect of different isolates of *Pseudomonas fluorescens* on the mycelial growth of *R. solani* in dual culture after 96 hrs of incubation (values in the bracket represent angular transformation)

Isolates of *P. fluorescens*	Percent inhibition of mycelial growth of *R. solani**
PF-2	94.42 (37.04[a])
PF-3	46.71 (38.34[ab])
PF-4	76.74 (40.94[ab])
PF-6	38.50 (42.02[ab])
PF-11	70.00 (43.08[b])
PF-12	50.72 (45.42[b])
PF-18	53.00 (46.70[b])
PF-28	44.82 (56.82[c])
PF-31	43.01 (61.15[c])
PF-173	36.33 (76.41[d])
CD at 1% = 5.22	CV = 7.86

* Mean of three replicates

In present investigation, both the isolates of *T. harzianum* and five out of 10 isolates of *P. fluorescens* were found to inhibit mycelial growth of *R. solani in vitro.* Similar observations on the effectiveness of *Trichoderma* and *Pseudomonas* species have been reported by earlier workers [16-21].

Among the three concentrations of cow urine, inhibition of mycelial growth was found to be maximum in 100% concentration (87.53%) and minimum in 25% concentration (0.33%). 100% concentration of cow urine showed significant increase in inhibition of mycelial growth when added at the rate of 6ml and 4.5 ml (both 100.00%) compared to 3 ml (78.52%) and 1.5 ml (71.85%) (Table 3). Cow urine acts as an antifungal agent. Similar results have been reported by previous researchers. Cow urine distillate has been found effective against *Rhizoctonia solani in vitro* [8] and it also caused highest reduction in disease severity (69.6%), maximum increase grain yield (33.1%) as well as maximum 1000-grain weight (49.7%.) in case of soybean web blight disease [9].

Table 3: Effect of different doses of cow urine on the mycelial growth of *R. solani in vitro*

Treatments	Percent inhibition at different doses after 48 hrs of incubation*				
	1.5 ml/60ml PDA	3.0 ml/60ml PDA	4.5 ml/60ml PDA	6.0 ml/60ml PDA	Mean
25% Cow urine	0.93	0.37	0.00	0.00	0.33[a]
50% Cow urine	0.00	0.00	0.00	87.96	21.99[b]
100% Cow urine	71.85	78.52	100.00	100.00	87.53[c]
Check	0.00	0.00	0.00	0.00	0.00[a]
CD at 5% Cow urine concentrations CD1 = 0.44 Doses CD2 = 0.44 Interaction (Concentrations × doses) CD3 = 0.88 CV = 1.93					

* Mean of three replicates

Parasitization

The examination of microtomed sections of healthy (non-parasitized) sclerotia of *R. solani* revealed that the sclerotial bodies were made up of single type of brown coloured, mature and compact monilloid cells (Figure 1A). While the microtomed sections of *T. harzianum* parasitized sclerotia of *R. solani* showed that the cell walls of the cells were broken and cytoplasm was lacking (Figure 1B and 1C). The cells were hyaline or

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brown coloured, collapsed andhaving broken cell walls. From the walls of sclerotia, the conidia and conidiophores of *T. harzianum* were seen protruding out that confirmed the parasitization of *R. solani* sclerotial bodies by the antagonistic fungus *T. harzianum*.

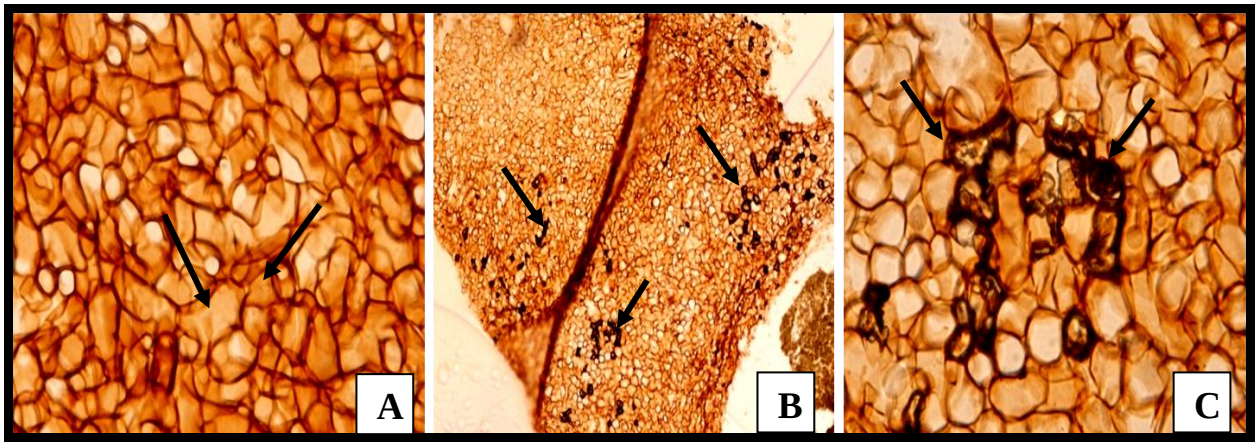

Figure 1: (A) Section revealing the structures of healthy sclerotia of *R. solani* (B) and (C)- Section of *T. harzianum* parasitized sclerotia: the sclerotial tissues shows invasion, break down and disintegration by the antagonistic fungus

The parasitization of sclerotia (the survival structure of the pathogen) can be one of the biological control mechanisms. The results of microtomy indicated that interaction between the pathogen (*R. solani*) and antagonistic fungus (*T. harzianum*) also occurs at the level of sclerotial parasitization. Sclerotial parasitization and destruction may affect the inoculum potential by reducing both inoculum density as well as inoculum capacity. This approach can be employed for management of primary inoculum of the pathogen which comes from the sclerotia bearing infected potato tubers kept in cold stores. The treatment of these tubers with *T. harzianum* should be done before keeping for storage so that antagonistic fungus *T. harzianum* has enough time to destroy sclerotia. The parasitization of *R. solani* sclerotia by *T. harzianum* have also been reported by earlier workers [22, 23].

Nuclear staining

Results of nuclear staining of the pathogen *R. solani* with DNA-intercalating fluorochrome ethidium bromide stain showed that the nuclei of *R. solani* were fluoresce bright brick red under green excitation and also mycelial cells were found to be multinucleate (Figure 2A and 2B).

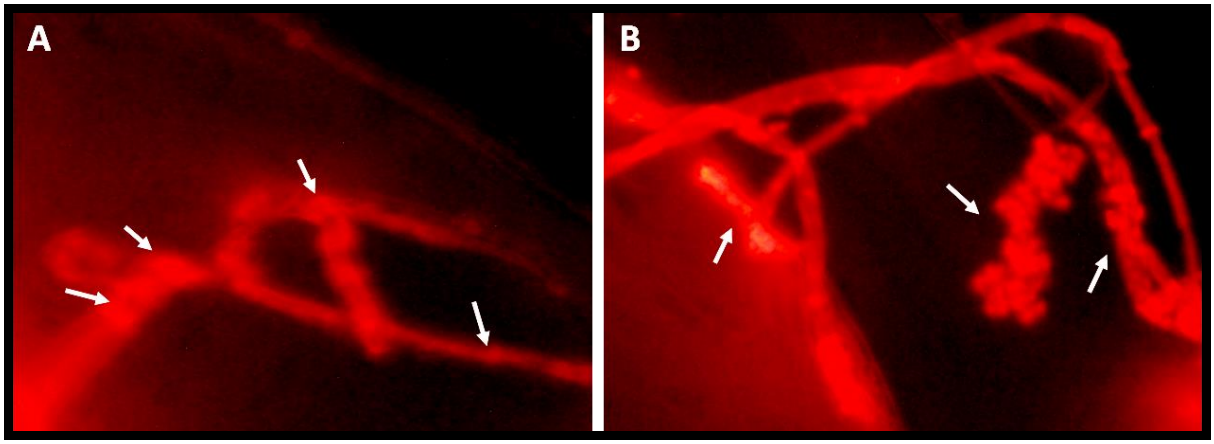

Figure 2: Nuclear staining of fungal mycelium (A) and (B) - Bright brick red colored nuclei of *R. solani* as observed under fluorescent microscope

The method used for nuclear staining of *R. solani* provided good results that confirmed the multinucleate nature of *Rhizoctonia solani* and has also been foundcost-effective. Similar observations have also been reported earlier [15].

CONCLUSIONS

In current work, we observed that management of black scurf of potato by a single control measure is difficult as the pathogen is both seed (tuber) and soil borne in nature. Therefore, integrated disease management (IDM) is needed for effective management of this disease that though does not decrease the yield but is known to deteriorate the quality of potato tubers making them unacceptable to be used for seed purpose and their market value is also reduced. Results of biocontrol agents like *Trichoderma* and *Pseudomonas sp.* in this study confirms their potential against pathogen control. However, in this study, application of cow urine is new and promising way to control black scruf of potato but still there is a need of understanding the mode of action of this antagonist and also its long term sustainability under natural/field condition. Therefore, in future more understanding about similar kind of biocontrol agents can help to boost their potential in the field of organic agriculture.

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