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In vitro evaluation of biological control agents against *Rhizoctonia solani*

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The efficacy of four fungal and one bacterial bioagents viz, *Trichoderma viride, Trichoderma harzianum, Aspergillus niger, Penicillium s*pp. and *Bacillus subtilis* were evaluated *in vitro* condition against the tobacco sore shin pathogen, *Rhizoctonia solani*. In the dual culture assay, the percentage inhibition of growth by *T. viride, T. harzianum,* , *A. niger, B. subtilis* and *Penicillium s*pp. on *R. solani* were 70%, 67%, 57%, 50% and 44% respectively. All the antagonists suppressed the formation of sclerotia. The volatile metabolite studies revealed that *T. viride* and *T. harzianum* showed 50% and 40% inhibition in mycelial growth respectively. Microscopic observations of the dual cultures revealed the inhibitory effect was caused by the hyphal interaction between the biocontrol agent and the pathogen causing lysis of pathogen hyphae. This resulted in the reduction of the mycelial growth of the *R. solani*. The results implied that the extent of inhibition by *T. viride* and *T. harzianum* provides the use of excellent potential antagonists capable of controlling the pathogenicity of *R. solani* on tobacco seedlings.

Key words: *T. viride, T. harzianum, Rhizoctonia solani,* bacterial bioagents

Introduction

Rhizoctonia solani Kuhn is the causal agent of sore shin disease, the most important seedling disease of tobacco worldwide (Lucas, 1975). This disease was recorded for the first time in Karnataka Light soil (KLS) nurseries during the nursery survey conducted in 2005 (Anonymous, 2006). In KLS losses were upto 10%. As a result the farmers suffer from transplant shortages for taking up timely planting in the zone. The pathogen is known to be soil borne and sclerotia are often found in the soil. Yet, limited information is available on its sustainable management and is generally treated by chemical applications. Overuse of the chemical may result in environmental, human health and pest resistance problem. The increasing awareness of fungicide-related hazards has

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emphasized the need for adopting biological methods as an alternative disease control method, which is also ecofriendly (Khare *et al*., 2010). Biological control appears to be the best solution for long term sustainability and effective management of soil borne disease which can considerably minimize the disease (Howell, 2003). Successful management of *R. solani* on various crops by bioagents has been previously reported (Meena *et al*., 2003; Atef, 2008; Hajieghrari *et al*., 2008). In view of the increasing importance of sore shin disease and the growing environmental concerns, the present *in vitro* study was carried out *t*o control the *R. solani* on tobacco by *T. viride, T. harzianum,* , *A. niger, B. subtilis* and *Penicillium s*pp.

Material and methods

Isolation of the pathogen: Isolation was done using diseased root pieces collected from different nurseries. Root pieces were washed under tap water for about 5 minutes to remove soil particles. The root pieces were dipped in 70% ethyl alcohol for 2 minutes and then transferred to sterile distilled water for 2-3 minutes twice. The treated root pieces were blot dried and then transferred to petri plates containing sterilized potato dextrose agar (PDA) medium with five pieces per plate. All plates were kept at 25 ± 2°C for 7 days and from these plates pure cultures of *R. solani* isolates were maintained. For confirmation the isolates were sent to IARI, New Delhi, India. The fungus was then sub cultured whenever needed during the present study.

Isolation and Maintenance of Biocontrol agents : The fungal antagonists *T. viride* and *T. harzianum* used in present investigation were obtained from Division of Plant pathology, GKVK, Bengaluru and *A. niger, B. subtilis and Penicillium s*pp. were isolated from the native rhizosphere soils collected around the healthy tobacco seedlings following the method of Lodha and Webster (1990). Tobacco seedlings with roots and adhering soil were carefully uprooted, placed in polythene bag, labeled and brought to the laboratory. Roots were separated from the seedlings and air dried in shade. Rhizosphere soil was collected from them by gentle scraping the roots with a fine scalpel. The isolation was done by serial dilution method (Aneja, 2005). By this method cultures of *A. niger, B. subtilis and Penicillium s*pp. were recovered. The cultures of *T. harzianum, T. viride, A. niger and Penicillium s*pp. were maintained on PDA and *Bacillus subtilis* on Nutrient Agar.

Dual culture method: *In vitro* antagonistic activity of *T. viride, T. harzianum, A. niger* and *Penicillium s*pp. against *Rhizoctonia solani* was studied in dual culture technique by following the method by Kucuk and Kivanc (2003). Petridishes (90 mm) containing 20 ml of sterile PDA were inoculated with a 5mm diameter plug of 7- day- old pure culture of antagonistic

fungi and pathogens. One mycelial disc of each fungus was placed on opposite poles of PDA plates and incubated at 25°C in incubator and radial growth of pathogen was measured at 24 h intervals. In case of *B. subtilis*, parallel streaking was done on one side of the agar plate and incubated at 37°C for 24 h. After the incubation period a 5 mm diameter mycelia plug of actively growing *R. solani* was placed on the opposite pole and incubated at 25°C for 7 days.

Control petri dishes were inoculated with pathogens and a sterile agar plug. Three replications were maintained for each treatment. Percentage inhibition of pathogen was calculated by the following formula (Fokkema, 1973). R1-R2/R1x100, where R2 denotes the radial growth of the pathogen towards the opponent antagonist and R1 denotes the radial growth of the pathogen towards opposite side. The experiment was repeated thrice.

Slide culture method: For each pathogen - *Trichoderma* interaction, a clean slide was placed in 9 cm diameter plates and sterilized. Then a small amount of autoclaved melted PDA was spread over the slide to make a thin PDA film on the slide. The 5 mm diameter mycelia plug of one week old *R. solani* culture is cut from the margin and *T. harzianum and T. viride* were placed on opposite sides of the slides separately at 3 cm apart on PDA surface. Then 1 ml of double distilled water was added to the plate to prevent drying and then incubated at 25±1°C for 7 days. After incubation period the meeting area of pathogen-*Trichoderma* hyphae was observed under a light microscope.

Effect of volatile substance produced by *T. viride* and *T. harzianum on growth of the pathogen:* The method described by Dennis and Webster (1971) was adopted. Petriplates containing 20 ml of PDA were inoculated separately with 5 mm disc of antagonists and incubated for 5 hours. After this lid of each plate was replaced by a bottom containing PDA previously inoculated with the disc of the pathogen and sealed together with paraffin film. The control sets did not contain the antagonist. The cultures were incubated at 25°C.The studies were conducted in three replicates. Radial growth was measured at 24 hours intervals and percent inhibition was determined using the formula:

$$\text{Percent inhibition} = \frac{C2 - C1}{C2} \times 100$$

Where, C_2 means growth of *R. solani* in control and C1 means growth of *R. solani* in treatment.

Results and discussion

Differential biocontrol ability among the antagonists was noticed against *R. solani* (Fig.1). Results showed that among the 5 potential antagonists, *T.*

viride and *T. harzianum* proved to be the most potent bioagents against *R. solani*. Growth inhibition in the pathogen differed significantly. 7 days of incubation showed different degrees of mycelial growth inhibition of *R. solani* (Fig.2). Initially very less sclerotia were observed near the intermingled contact zone of *R. solani* and *Trichoderma*. However, after 4 days of incubation there was suppression of growth and sclerotia formation on antagonized portion of hyphae. *T. harzianum* and *T. viride* completely overgrew the pathogen with percentage inhibition of 67 % and 70 % respectively. *T. viride* was most potent in reducing growth of the pathogen than *T. harzianum*. Saikia *et al*., (1995) also reported *T. viride* as more effective than *T. harzianum* in reducing mycelial growth of *R. solani* causing Cauliflower stem rot. According to Papavizas and Lumsden (1982); Devaki *et al. (*1992)*,* the mechanisms involved in the control of pathogens by *Trichoderma* spp. are probably due to antibiosis, lysis, competition and mycoparasitism. The mode of mycoparasitism was observed to be entirely different between *T. viride* and *R. solani* (Pandey *et al*., 2005). The growth of *T. viride* hyphae was observed inside host mycelium. Due to this, shrinkage and coagulation of cytoplasm of the pathogen was observed (Fig. 3).

T. harzianum hyphae coiled around the pathogen. Later on pegging started and knob like haustoria formed inside the hyphae of *R. solani.* The cytoplasmic contents may be taken through the haustoria and only cell wall was clearly visible without cytoplasm. Baker and Cook (1979) have reported that the enzymes may be produced that digest the mycelial walls and septal walls or antibiotics may be formed that inhibit growth or cause endolysis. Dennis and Webster (1971) have reported that *Trichoderma* spp. are known to produce a number of antibiotics such as Trichodermin, Trichodermol, Harzianum A and Harzianolide as well as some cell wall degrading enzymes such as Chitinases, glucanases that break down polysaccharide, chitins and β-glucans, thereby destroying cell wall integrity (Elad, 2000; Devaki *et al*., 1992). These may also play a major role in mycoparasitism because of changes in cell wall integrity.

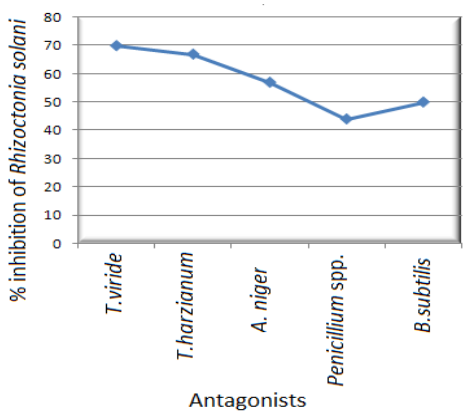

Fig. 1. Inhibition of growth of *Rhizoctonia solani* by different antagonists in 7-day-old culture

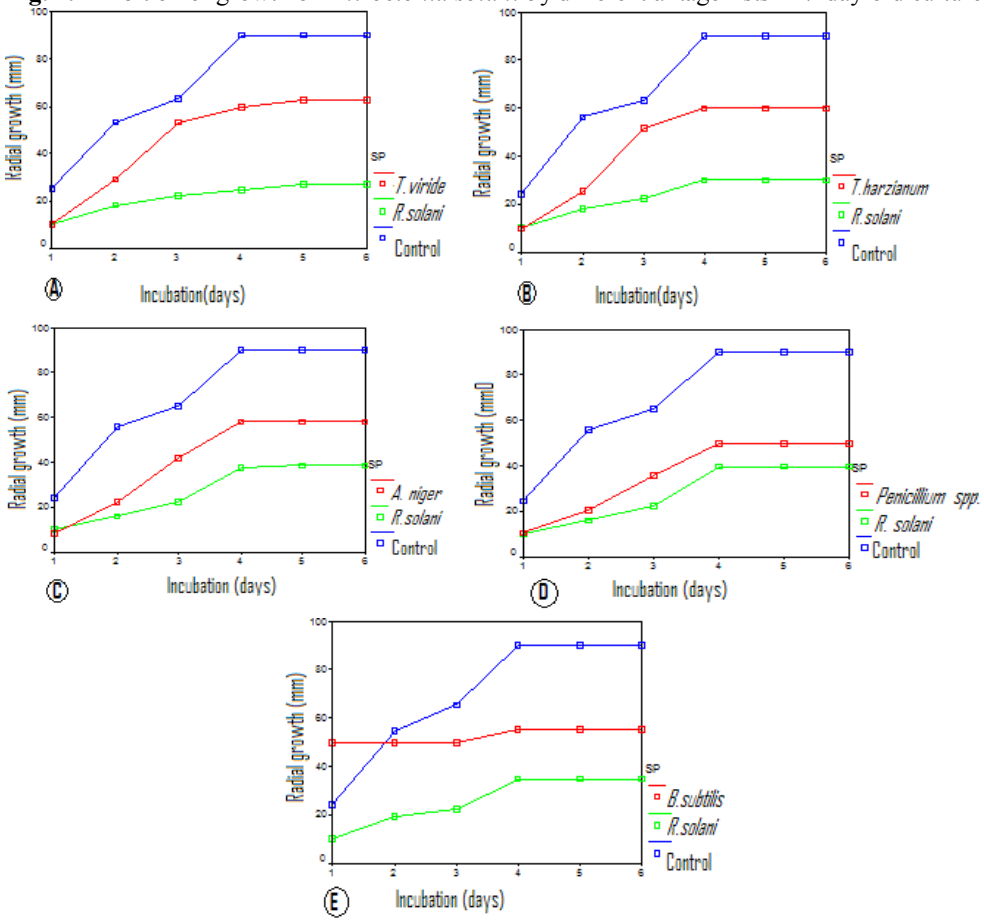

Fig. 2. Growth pattern of *Rhizoctonia solani* and the biological control agents in terms of radius of the colony. ■ - Control-Pathogen alone , ■- Biocontrol agent , ■ -*Rhizoctonia solani.* ■ - *T. viride* , B. ■ - *T. harzianum*, C. ■ -*A. niger,* D. ■ -*Penicillium* spp. and E. ■ -*B. subtilis*

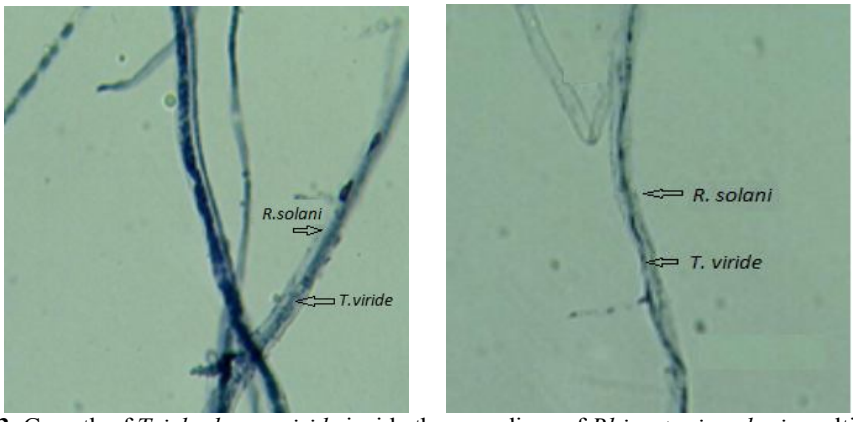

Fig. 3. Growth of *Trichoderma viride* inside the mycelium of *Rhizoctonia solani* resulting in shrinkage and coagulation of cytoplasm of *R. solani.*

Our study demonstrated the involvement of volatile metabolites in the inhibition of *R. solani*. After 7 days of incubation it was observed that volatile compounds from *T. viride* exhibited maximum growth inhibition of 33% on *R. solani* when compared to *T. harzianum* which exhibited 29% growth inhibition. The antagonistic properties of *Trichoderma* spp. producing volatile compounds inhibited the growth of various soil borne pathogens (Barbosa *et al*., 2001; Devaki, 1991; Kumar and Dubey, 2001). Several workers have reported that *Trichoderma* spp. produces large variety of volatile secondary metabolites such as Ethylene, Hydrogen cyanide, Aldehydes and Ketones which play an important role in controlling the plant pathogens. (Vey *et al*., 2001).

Inhibitory effect of *Aspergillus niger* on *R. solani* was also highly significant. Initially during the first two days the inhibitory effect of *A. niger* was slow but on 4 day it showed 58% inhibition the pathogen. This result is in congruent with work of Bosah *et al*. (2010) who have reported such effects of *A. niger* on *Sclerotium rolfsii*.

B. subtilis showed 50% of inhibition and the color of the media turned brown at the antagonized portion on the 5 day. This may be due to the presence of the antibiotic like Inturin A and Surfactin produced by *B. subtilis*. Akihiro *et al*. (1993) has reported that *B. subtilis* produced antifungal peptide antibiotic Inturin A and Surfactin. Inturin A has a strong antifungal activity when compared to Surfactin.

Penicillium spp. failed to produce significant inhibitory effect on *R. solani*. The pathogen overgrew the growth of *Penicillium* on 3rd day. However the mycelial growth of *Penicillium* picked up later and eventually suppressed the growth of *R. solani* with inhibition rate of 44.4%. The late antagonistic activity of *Penicillium* could be attributed to the late production of antibiotics.

It is clear from the investigation that *T. harzianum* and *T. viride* are more antagonistic in suppressing the mycelial growth and sclerotia formation of sore shin pathogen *R. solani.* Hence, further investigation with these two potential bioagents and their bioactive compounds effective against *R. solani* can be exploited for future plant disease management to control sore shin disease of tobacco.

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