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Deciphering the Mechanism of Mycoparasitism of *Sclerotinia sclerotiorum* by *Trichoderma* spp.

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ABSTRACT

White rot of mustard caused by Sclerotinia sclerotiorum is an important disease and rising to an outbreak level in recent past. Trichoderma is a biocontrol agent which controls the pathogens by various mechanisms viz. mycoparasitism, antibiosis, competition, systemic induced resistance etc., of which hyper-parasitism is the most important mechanism employed by Trichoderma against several plant pathogens. Studies were conducted to decipher the mechanism of mycoparasitism of S. sclerotiorum by Trichoderma spp. A total of eight different species of Trichoderma viz. T. harzianum (Th3), T. harzianum (Th5), T. viride, T. virens, T. asperellum, T. longibrachiatum, T. koningi, T. atroviride were tested against four S. sclerotiorum isolates. Dual culture results revealed that T. viride and two isolates of Trichoderma harzianum Th3 and Th5 were found to be more effective against S. sclerotiorum by inhibiting 71.94, 80.23 and 71.99 per cent mycelial growth respectively. Scanning electron microscopy results shows that T. harzianum (Th3) and T. viride mycoparasitize the S. sclerotiorum by attaching and coiling around by formation of either hyphal coils or hooks and finally degrading the hyphae and sclerotia of pathogen.

Key words: *Mycoparasitism, Sclerotinia sclerotiorum, Trichoderma spp., Scanning electron microscopy*

INTRODUCTION

Of the several biotic stresses which are responsible for low productivity of mustard crop, white rot caused by *S. sclerotiorum* is an important one. *Sclerotinia sclerotiorum* (Lib.) de Bary is a soil-borne pathogen capable of infecting more than 400 host plants worldwide. Biological control through *Trichoderma* spp. is a environmentally safe and economically sound practice. It includes different economically important species *viz.*, *T. harzianum*, *T. asperellum*, *T. viride*, *T. atroviride*, and *T. virens* and *T. reesei*[8]. *Trichoderma* antagonize the many phytopathogenic fungi through mechanism of mycoparasitism, antibiosis, competition and induced systemic resistence. Mycoparasitism is the most important mechanism used by *Trichoderma* against plant pathogens.

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It involves tropic growth of biocontrol agent towards the target organism[1]. First it coils around and finally causes dissolution of target pathogen cell wall or membrane by the activity of enzymes.

MATERIALS AND METHODS

Pathogen cultures

Survey of different mustard growing areas were carried out at different crop growth stages during rabi season (2013-14) and samples were collected at pod formation stage. The fungal pathogens were isolated, identified and purified on PDA plates and further grouped into four different isolates *viz*. SS26, SS41, SS43, SSAco on the basis of morphology, cultural characteristics and sclerotial bodies. Cultures were routinely grown in potato dextrose agar plates and incubated at 28±2°C for 5-7 days.

Trichoderma cultures

Trichoderma spp. viz. *Trichoderma harzianum* (Th3) – ITCC-5593; *T. viride* – ITCC-8315; *T. atroviride* – ITCC-7445; *T. asperellum* – ITCC-8940 and *T. longibrachiatum* – ITCC-7444 were procured from ITCC. *T. konongi* and *T. virens* were procured from Biological Control Laboratory, Division of Plant Pathology, IARI, New Delhi and *Trichoderma harzianum* (Th5) was collected from IARI field. Dual culture studies were carried out to select the efficient *Trichoderma* spp. against *S. sclerotiorum* isolates. Cultures were maintained on potato dextrose agar plates at 28±2°C for 5-7 days.

Dual culture assay

Antagonistic potential of *Trichoderma* spp. was studied by dual culture assay against *S. sclerotiorum* by keeping the pathogen inoculated PDA plates as control[2]. In this previously prepared pure culture of pathogen isolates as well as different *Trichoderma* spp. were used. Each *Trichoderma* spp. was inoculated against each S. *sclerotiorum* isolate in triplicate on PDA plates and incubated at 28±2°C. Antagonism activity was monitored by performing both daily measurements of fungal colony growth and direct observation of the plates, during seven days. The radial growth of the pathogen was measured after full growth and the percent inhibition was calculated as follows: PI = (C - T) × 100 / C, where PI is the percent inhibition of mycelia growth; C is the radial growth of pathogen in control plates (cm) and T is the radial growth of pathogen in dual culture (cm).

Scanning Electron Microscopy (SEM) analysis of dual cultured plates

For SEM analysis of dual culture plates of *Trichoderma* with *Sclerotinia sclerotiorum* the procedure given by Pisi *et al.*[7] was followed. Small pieces of agar were taken from dual cultures at the interaction zones, when the fungi were at their early stage of interaction. Excess of agar was removed with a razor blade prior to further preparation. Fixation of specimens was done in 3% glutaraldehyde in 0.1 M phosphate buffer (P^H 7.0). After 12 hours of refrigeration, the specimens were dehydrated in a graded acetone series at 30%, 50%, 60%, 70%, 80%, 90%, and 95% concentration. The critically dried samples were further processed for SEM analysis.

Statistical analysis

Data were analyzed by one-way analysis of variance (ANOVA) using OPSTAT software (www.hau.ernet.in/about/opstat.php).

Differences between treatment mean values were determined following least significant difference (LSD) test at $P < 0.05$.

RESULTS

Selection of efficient *Trichoderma* spp. against *Sclerotinia sclerotiorum*

The radial growth measured in dual culture plates were used to select efficient *Trichoderma* spp. against *Sclerotinia sclerotiorum*. The observed radial diameter was used in calculating the per cent growth inhibition of pathogen. Per cent inhibition data shows that all the species of *Trichoderma* inhibited the growth of all the four isolates of *Sclerotinia sclerotiorum. Trichoderma viride, Trichoderma harzianum* (Th3), and *Trichoderma harzianum* (Th5) inhibited the growth up to 71.94, 80.23 and 71.99 per cent respectively after 7 days, were highest among all the eight species. *T. longibrachiatum* was

least effective with 46.28 per cent growth inhibition (Table 1, Figure 1).

The data shows that *Trichoderma viride*, *Trichoderma harzianum* (Th3), and *Trichoderma harzianum* (Th5) were superior over other species and inhibited the *Sclerotinia sclerotiorum* growth significantly. Therefore *Trichoderma viride, Trichoderma harzianum* (Th3), and *Trichoderma harzianum* (Th5) were selected for further studies.

Scanning Electron Microscopy (SEM) studies

The dual culture plates showing effective inhibition were viewed under Scanning Electron Microscopy(SEM). SEM images shows that both *Trichoderma harzianum* (Th3) and *T. viride* mycoprasitize the hyphae of *S. sclerotiorum* by attaching, coiling around, invading and finally degrade it. Firstly the hypha of *Trichoderma harzianum* (Th3) and *T. viride* comes in contact with hyphae of *S. sclerotiorum*. Then it coils around the hyphae and invades it. Finally the hyphae of*S. sclerotiorum* is degraded by hyphae of biocontrol agent. Study of mycoparasitism of sclerotia by *Trichoderma* spp. was also done with scanning electron microscopy. Results show that hyphae of *Trichoderma* spp. penetrate the sclerotial bodies and mycoparasitize it (Fig 1).

DISCUSSION

As mustard is an important crop and affected by many diseases, the biological control play an important role in integrated management of these diseases. *Trichoderma* is the most explored and studied biocontrol agent against many soil borne and foliar pathogens. White rot of mustard caused by *Sclerotinia sclerotiorum* is a threatening disease from previous 5-6 years in most of mustard growing regions of the country. A number of biocontrol agents have been characterized for the control of *S. sclerotiorum*[4]. Among them *Trichoderma* is most important and it mycoparasitize the target pathogen by attaching, coiling, and dissolution of target pathogens cell wall or membrane by the activity of enzymes. Many strains of *Trichoderma* produces extracellular cell wall degrading enzymes which were traditionally included in the concept of mycoparasitism[5]. The most common BCAs of the *Trichoderma* genus are strains of *T. virens*, *T. viride* and above all, *T. harzianum*, which is a species aggregates includes different strains used as BCAs of phytopathogenic and viral vector fungi[3]. In present investigations we also collected and tested different species of *Trichoderma* against *S. sclerotiorum*. Among the eight species tested, three were found most effective against *S. sclerotiorum.* Among these three *Trichoderma harzianum* (Th3) was most effective with 80 per cent growth inhibition of pathogen. Other two *T. viride* and *Trichoderma harzianum* (Th5) were also inhibited growth up to 72 per cent which was significant. So these three species were selected for further study in scanning electron microscopy.

Scanning electron microscopy results revealed that *T. harzianum* hyphae grew towards and coiled around the *S. sclerotiorum* hyphae. Dense coils of hyphae of *T. harzianum* and partial degradation of the *Sclerotinia* cell wall were observed in later stages of the parasitism[6]. The interaction between *Trichoderma* spp. and *Fusarium* spp. revealed that the mycoparasitic hyphae were usually attached longitudinally to the hyphae of the pathogens. Hyphal coilings, hooks, pincer-shaped structures, short contact branches were observed and hyphal depressions were also present[7]. Our results of scanning electron microscopy of dual culture assay also shows that at early stage of interaction at inhibition zones, hyphae of Th3 and *T. viride* attaches to the hyphae of *S. sclerotiorum* and coils around it either forming hyphal coils or hooks. Then these pathogenic hyphae were degraded by Trichoderma spp. hyphae. Study on mycoparasitism of sclerotial bodies of *S. sclerotiorum* by Th3 under scanning electron microscopy revealed that hyphae of *Trichoderma* parasitize the sclerotia by growing around, penetrating the sclerotia and finally degradation them.

Table 1: Bio-efficacy of *Trichoderma*spp against different isolates of *Sclerotinia sclerotiorum*

Inhibition of growth (%)					
***Trichoderma* Spp. / *S. Sclerotiorum* isolates**	**SS26**	**SS41**	**SS43**	**SSAco**	**Mean**
T.viride	77.45(61.65)	79.19(62.85)	68.99(56.16)	62.14(52.02)	71.94(58.17)
T. harzianum(Th3)	84.29(66.63)	83.11(65.73)	78.07(62.06)	75.47(60.29)	80.23(63.68)
T. harzianum(Th5)	73.92(59.31)	68.82(56.04)	76.53(61.04)	68.72(55.98)	71.99(58.09)
T.virens	64.59(53.46)	62.26(52.08)	58.62(49.95)	52.57(46.45)	59.51(50.49)
T. longibrachiatum	44.16(41.62)	47.95(43.80)	57.54(49.32)	35.49(36.54)	46.28(42.82)
T.asperellum	66.95(54.90)	61.55(51.66)	63.50(52.83)	35.16(36.34)	56.79(48.93)
T.koningi	61.41(51.57)	61.61(51.70)	55.84(48.34)	41.41(40.03)	55.07(47.91)
T.atroviride	64.22(53.24)	60.27(50.91)	55.85(48.34)	56.44(48.71)	59.19(50.30)
C.D.	3.25	3.38	3.47	3.77	3.47
SE(d)	1.52	1.58	1.62	1.76	1.62

* Value in parenthesis representing arc sin transformed value

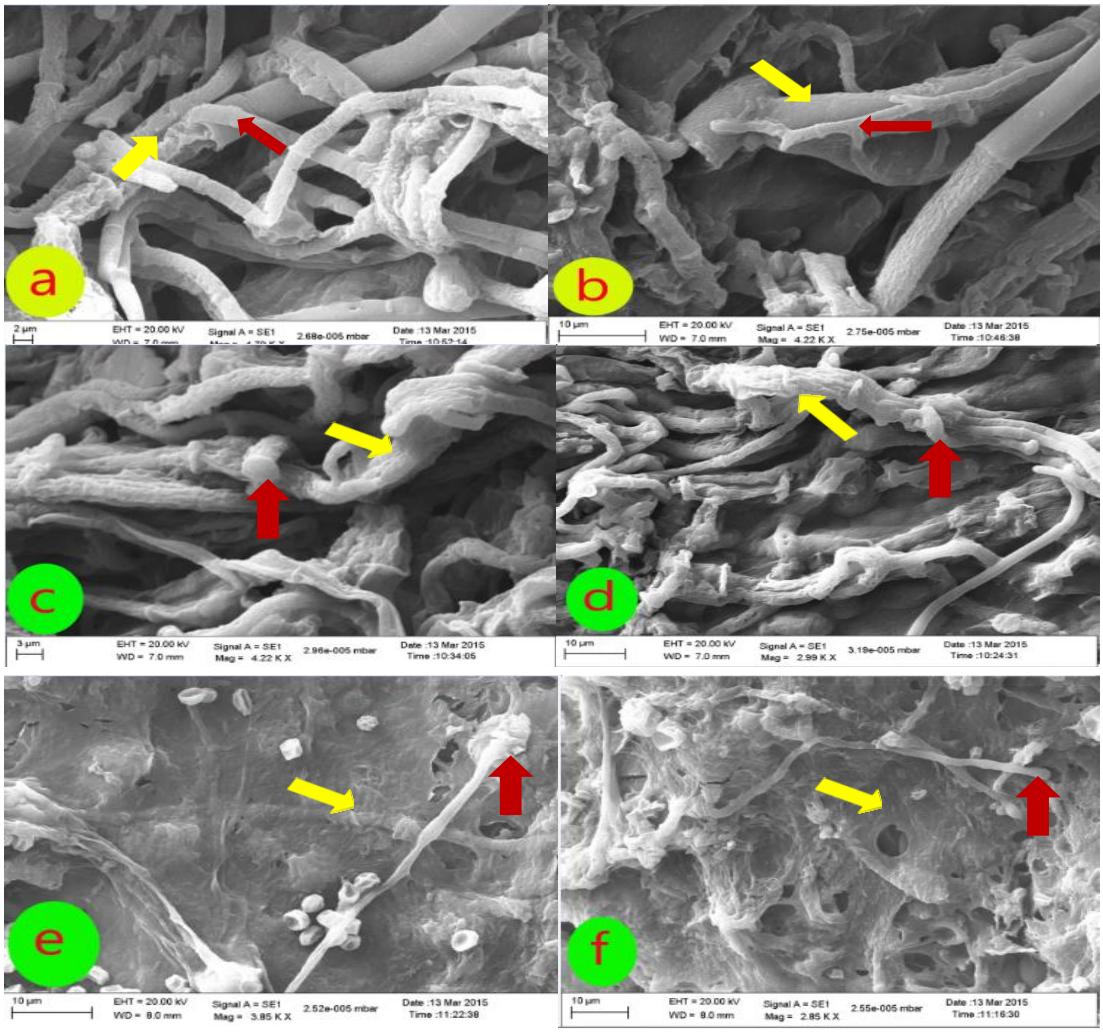

Fig 1: SEM studies of mycoparasitism

a – Coiling and degradation of *S. sclerotiorum* hyphae by hyphae of *T.harzianum* (Th3)
b – Adhesion of hyphae of *T. harzianum (*Th3) with *S. sclerotiorum* hyphae
c – Coiling of *S. sclerotiorum* hyphae by hyphae of *T. viride*.
d – Degradation of *S. sclerotiorum* hyphae by *T.viride* hyphae.
e – Hyphae of *T. harzianum (Th3)* mycoparasitizingsclerotial bodies of *S. sclerotiorum.*
f – Penetration of Th3 hyphae into sclerotia of *S. sclerotiorum*.

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