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Antagonistic activity of rhizosphere fungi against *Fusarium solani* causing wilt in *Dalbergia sissoo*

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Thirty six fungi were isolated from rhizosphere soil of natural and healthy plantations of the *Dalbergia sissoo* and their antagonistic properties were studied *in vitro* against a wilt pathogen, *Fusarium solani*. Different grades of colony interaction were observed in dual culture between *F. solani* and antagonistic fungi inhibiting fungal growth from 25.56% to 88.04%. Various types of mycoparasitic activities against the pathogen were also observed viz., hyphal coiling by antagonistic fungi and formation of mycelial trap, endo-parasitism, ghost mycelium formation, hyphal collapsing and breakage, and hyphal shrinkage which was also confirmed by scanning electron microscopy. Cell-free culture filtrate of antagonists at 100% concentration effectively inhibited mycelial dry weight of pathogen by 79.17% to 96.57%. Conidia of pathogen germinated more in light followed by dark conditions. Deleterious effect of culture filtrates on conidia germination resulted in weak germ tube and secondary branches, ghost mycelium production and swelling in hyphal cells and condensation of cytoplasm.

Keywords: *Dalbergia sissoo, Fusarium solani,* mycoparasitic activities, rhizosphere, wilt

INTRODUCTION

Soil is a natural habitat of all types of microorganisms including both beneficial and pathogenic organisms which maintain an equilibrium state by their actions and interactions. Moreover, rhizosphere is a zone of intense microbial activity where both types of organisms are present in a balanced manner under the influence of plant roots. In normal conditions, beneficial microfloras dominate over pathogenic species, but soil-borne plant diseases initiate when this equilibrium gets disturb. These diseases need to be controlled to maintain the quality and abundance of food, feed and fiber produced by growers around the world. Different approaches are made to manage plant diseases including chemicals, which enhance the production of crop yields and also provide the cheaper and fast results [1]. But chemical pesticides may lead to development of resistance in pathogenic strains, deleterious effect of chemicals on non-target organisms, besides their accumulation in soil and plants and occurrence of serious ecological and health problems [2].

Moreover, biological control is a mechanism of disease control that operates naturally or accomplished by exploiting antagonistic microorganisms isolating from nature, testing *in vitro* and reintroducing in natural environment against pathogens to be controlled. Though biological control of plant diseases is slow, gives few quick profits, but can be long lasting, inexpensive and eco-friendly. The system eliminates neither pathogen nor disease but bring them again into natural balance [3]. The purposeful utilization of introduced or resident living organisms to suppress the activities and populations of one or more plant pathogens is referred as biological control. The objective of this study was the utilization of rhizosphere mycoflora of *Dalbergia sissoo* for the management of its root pathogen *Fusarium solani*, which results in a high mortality by causing wilt disease.

MATERIALS AND METHODS

Isolation of rhizosphere fungi and fungal pathogen

Microfungi were isolated from the rhizosphere region of naturally growing healthy plantations of *Dalbergia sissoo* in Forest Research Institute, Dehradun (N30º 20′36.8″; E077º 59′47.3″; 668 amsl) by serial dilution method [4]. Pathogen was isolated by blotter technique from diseased root samples collected from wilted *D. sissoo* seedlings [5]. Fungal strains were purified by repeated sub-culturing in potato dextrose agar (PDA) plates and pure cultures were maintained in PDA slants at 4ºC.

Identification

Fungal cultures were examined through the stereoscopic binocular and compound research microscope on the basis of shape and size of conidiophores, conidial/spore arrangement and sporulation and identified with the help of identification keys, standard monographs [6-11] and available expertise.

Antagonistic activities of fungal isolates against *F. solani in vitro*

Colony growth inhibition

Antagonistic property of all fungal isolates was tested against *F. solani* following dual culture method [12]. A 5 days-old mycelial disc of pathogen (5 mm dia.) was cut from actively growing colony margin and placed in one side of sterilized PDA plate (7 cm dia.). After 48 h of incubation at 27±2ºC, mycelial disc of another fungus used as antagonist was transferred to the opposite side of the Petri plates. The inoculated plates were incubated at 27±2ºC for 7-10 days and observed periodically. The zone of inhibition was recorded and growth inhibition (%) was calculated by using the formula: 100 × (C – T) / C, where, C = radial growth of pathogen in control, T = radial growth of pathogen in dual culture.

Microscopic examination of fungal hyphae

A compound microscope was used to observe distortion, abnormality and defect caused due to antagonistic activity of fungal isolates in the hyphae of *F. solani*. Fungal hyphae were picked up with the help of a sterile needle or forceps from the edge of zone of inhibition/interaction and placed onto a clean glass slide upon which a drop of lacto-phenol was put. Slide was observed under a stereoscopic binocular and compound research microscope.

Scanning electron microscopy of fungal hyphae from zone of interaction

For preparation of scanning electron microscope (SEM) samples, hyphae were collected with the help of a sterile needle from the zone of interaction between two fungi. Mycelia were fixed overnight using 4% glutaraldehyde in 0.05 M phosphate buffer (pH 7.3) at 48ºC and washed thrice in phosphate buffer (10 min each). Then, samples were dehydrated by serially passing through 70, 80, 90 and 100% ethanol (5 min at each stage) and finally in 100% ethanol at room temperature. Thereafter, ethanol was replaced with liquid CO_2 and the samples were air dried following [13]. The samples were mounted on stubs followed by gold coating using BAL-TEC-SCD-005 Sputter Coater, BAL-TEC AG, Balzers, Liechtenstein; Germany; the coated specimens were analyzed by xT Microscope Server software and photomicrographs showing deformities/abnormalities in fungal hyphae were taken.

Growth inhibition of *F. solani* by fungal Cell-free culture filtrates

Preparation of Cell-free culture filtrate

Potato dextrose broth (PDB) was inoculated with antagonistic fungal isolates after screening by dual culture technique. Flasks were incubated in rotating shaker at 150 rpm at 27±2^{o}C up to 15 days. Then broth cultures of fungal isolates were filtered first with Whatman No. 1 filter paper and then with a bacterial syringe filter (0.2µm). The filtrates were used directly for measuring inhibition (%) in mycelial dry weight and conidial germination.

Mycelial biomass inhibition

PDB was prepared and sterilized at 121±1ºC for 20 min. For estimation of percent mycelial biomass inhibition Cell-free culture filtrate of all strains was separately added in pre-sterilized PDB in three replicates to get 25, 50, 75 and 100% concentration (v/v) and to make final volume 50 ml. The flasks were inoculated with 5 mycelial discs (5 mm dia.) cut from actively growing margin of *F. solani* colony. Each culture was filtered through pre-weighed Whatman filter paper No. 1 after incubation at 27±2ºC for 15 days. The mycelial mat of each treatment was dried at 80ºC for 24 h. Mycelial growth inhibition (%) was determined by using the formula: 100× (C-T)/C, where, C = mycelial dry weight in control, T= mycelial dry weight in treatment.

Conidia germination

A humid chamber for conidial germination was prepared by using a germination paper according to the size of the Petri plate (14 cm dia.), moistened with distilled water and then sterilized properly. Culture filtrate (20µl) of each antagonist was taken in the wells of cavity slides separately and conidia from 7-days old culture of pathogen were introduced and mixed. Sterilized distilled water was used in place of culture filtrate in control sets. Humid chamber containing cavity slides was incubated at room temperature in light as well as in dark condition. Conidial germination was observed after 6 h to 48 h.

Conidial germination and characteristics of the germ tube were determined and compared with that of control [14]. Conidia germination (%) was calculated by counting a total of at least 100 conidia (both germinating

and non-germinated) in five microscopic fields of a microscope by the formula: 10 × total number of germinated conidia/ total number of conidia.

RESULTS

Identification of rhizosphere fungi and *F. solani*

A total of thirty six (DSRF1-DSRF36) fungal species were isolated from the rhizosphere region of *D. sissoo*, which belong to the genera *Aspergillus*, *Cladosporium*, *Cuninghamella*, *Fusarium*, *Mucor*, *Paecilomyces*, *Penicillium*, *Phymatotrichopsis*, *Phoma*, *Rhizopus*, *Trichoderma*. etc. on the basis of identification keys and standard monographs. The colonies of wilt pathogen, *Fusarium solani*, were sparse to dense and floccose with greyish white to pink mycelia with numerous oval shaped microconidia and fusoid macroconidia.

Antagonistic properties of fungal isolates against *F. solani*

Fungal isolates (DSRF1-DSRF36) tested by dual culture method against the pathogens *F. solani* showed varying degree of inhibition. Maximum number of isolates was found effective in inhibiting the growth by 40-50% (Figure **1**).

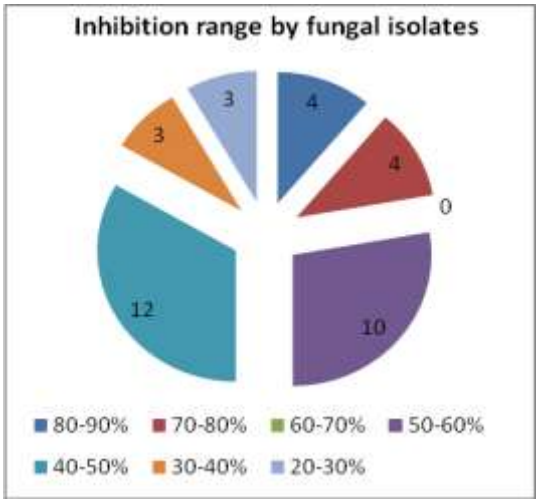

Figure 1. Inhibition range for *F. solani* by rhizosphere fungi.

The maximum growth was inhibited by *Trichoderma virens* DSRF33 (88.04±0.55) followed by *Trichoderma koningii* DSRF15 (84.44±4.19), *Aspergillus niger* DSRF19 (81.11±0.96), *Penicillium frequentans* DSRF11 (80.56±0.96), *Penicillium decumbens* DSRF7 (79.45±6.94), *Aspergillus humicola* DSRF12 (76.11±0.96), *Paecilomyces lilacinus* DSRF26 (75.00±1.67) and *Aspergillus alliaceus* DSRF21 (73.33±1.67) (Figure **2**).

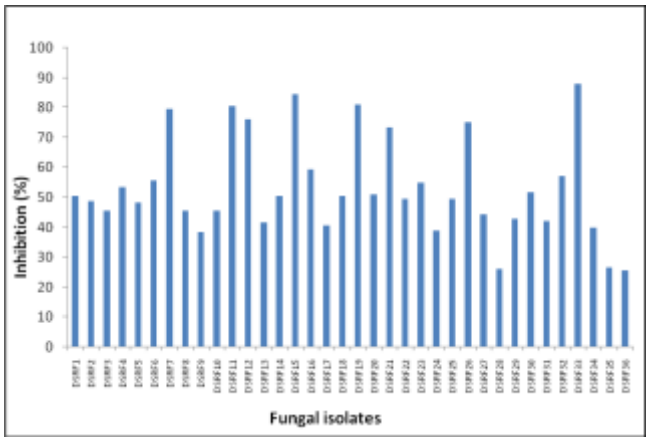

Figure 2. Effect of fungal isolates on growth inhibition of *F. solani* in dual culture.

Microscopic examination of fungal hyphae

Light microscopic study and scanning electron micrographs showrd post-interaction events in the hyphae of *F. solani* caused by *T. virens* DSRF33 and *T. koningii* DSRF15 displaying several deformities besides colony growth inhibition, coiling around hyphae and formation of mycelial trap (A), endo-parasitism (B), ghost mycelium formation (C), hyphal collapsed and breakage (D), and hyphal shrinkage (E) (Figure **3**, A-E).

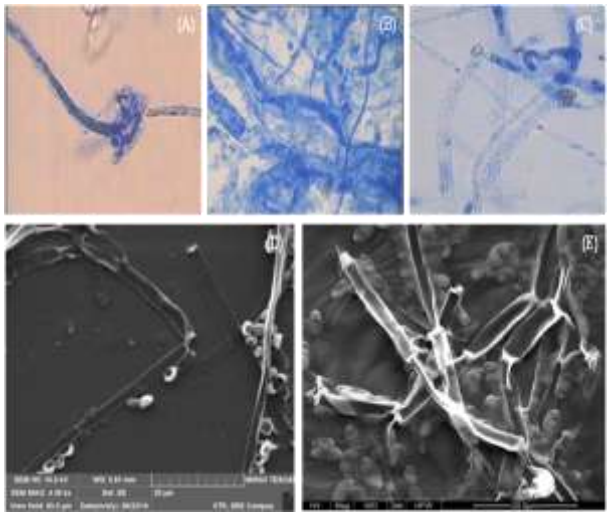

Figure 3. Light and scanning electron micrographs showing post-interaction events between *Trichoderma* and *F. solani*; ecto-parasitism (coiling around hyphae and formation of mycelial trap) (A), endo-parasitism (B), ghost mycelium formation (C); hyphal collapsed and breakage (D); hyphal shrinkage (E).

Effect of cell-free culture filtrate on inhibition of mycelial biomass

Pure culture filtrates (100% concentration) of all fungal strains significantly (P >0.5) inhibited the mycelial biomass yield, while mycelial biomass yield gradually declined with decrease in concentration of cell-free culture filtrates. The maximum inhibition was caused by *Trichoderma virens* DSRF33 (96.57%) followed by *Trichoderma koningii* DSRF15 (93.27%), *Aspergillus niger* DSRF19 (92.27%) and *Penicillium frequentans* DSRF11 (90.63%) at 100% concentration of culture filtrate (Figure **4)**.

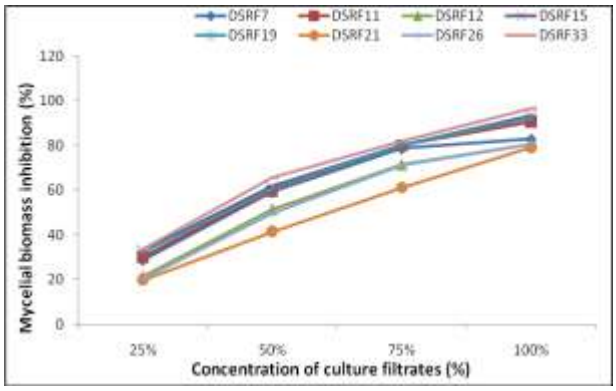

Figure 4. Effect of cell-free culture filtrates of fungal isolates on mycelial biomass inhibition of *F. solani.*

Effect of fungal cell-free culture filtrates on conidia germination of *F. solani*

Conidia germination of *F. solani* gradually increased with incubation time from 6 to 48 h in both the conditions. Minimum conidia germination of pathogen was recorded in dark as compared to light conditions (Table **1**). *Trichoderma virens* DSRF33 resulted in minimum spore germination and at par with *Trichoderma koningii* in both the conditions. Since conidial germination increased with incubation time, inhibitory effects of culture filtrates of *T. virens* DSRF33 and *T. koningii* DSRF15 was also precise. As compared to normal conidia germination in control (Figure **5**, A-B); *F. solani* conidia produced weak secondary germ tube (C), weak secondary hyphae (D), ghost mycelia (E), swelling in hyphal cells and condensation of cytoplasm (F), in the treatment of culture filtrates of *T. virens* DSRF33 and *T. koningii* DSRF15.

DISCUSSION

Different rhizosphere fungi were identified on the basis of morphological characters, colony colour, arrangement of phialides and conidiophores, and shape and size of spores [6-11]. Presence of unicellular oval shaped microconidia in fresh isolates after 2-3 days from lateral conidiophores and multicellular macroconidia after 6-7 days with penultimate

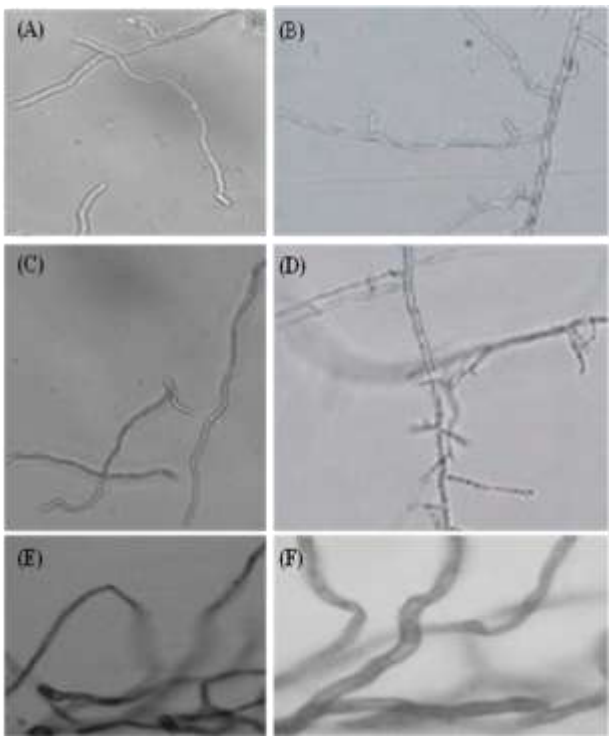

Figure 5. Effect of cell-free culture filtrates of fungal and bacterial isolates on conidia germination and mycelia growth of *F. solani*; (A-B) normal germination of conidia and hyphae; (c) conidia produced weak secondary germination tube; (D) hyphae produced weak secondary branches; (E) ghost mycelium production; (F) production of swollen hyphae and cytoplasm condensation in hyphae.

cell, a rounded foot cell with apical cell pointed and somewhat beaked confirmed the characteristics of *F. solani* [15].

Biological significance of rhizosphere fungi is very important and has been used by many workers. Fungal species isolated from rhizosphere have also been reported to have antagonistic activity against many phytopathogens, *e.g. Fusarium culmorum, Fusarium solani, Phytopthora parasitica, Pythium ultimum, Rhizoctonia solani, Sclerotinia sclerotiorum, Verticillium dalhae,* etc. [16-21]. The degree of inhibition of pathogen growth, in relation to growth in the absence of the potential control agent have been reported and used as a measure of effectiveness [14]. In present investigation rhizosphere fungi were used as antagonistic isolates against root pathogen and showed adequate results but *Trichoderma* species clearly dominated 88.04 and 84.44% inhibition. *Trichoderma* species are well known for their ability to produce a large number of lytic enzymes involved in general antibiosis or specific mycoparasitism [22-26]. Antibiosis and myco-parasitism are the well known mechanisms involved in biocontrol of pathogens by *Trichoderma*, competition for nutrition, space and dominance being equally important and mutually

Table 1. Effect of cell-free culture filtrates of fungal isolates on conidia germination of *F. solani* in light and dark conditions.

Fungal isolates	Conidia germination (%)*				Mean
	Time (h)				
	6	12	24	48	
DSRF7	$2^{abc}(1)^{abc}$	$5^{bcdef}(2)^{bcde}$	$10^{ghijk}(5)^{fgh}$	$20^{no}(15)^{l}$	$9.2^{c}(5.8)^{c}$
DSRF11	$0^{a}(0)^{a}$	$2^{ab}(1)^{abcd}$	$6^{cdefg}(4)^{efg}$	$13^{klm}(6)^{hi}$	$5.3^{b}(2.8)^{b}$
DSRF12	$3^{abcd}(1)^{abcd}$	$8^{fghij}(4)^{efgh}$	$14^{klm}(7)^{ij}$	$23^{op}(19)^{m}$	$12^{d}(7.8)^{d}$
DSRF15	$0^{a}(0)^{a}$	$1^{ab}(0)^{a}$	$4^{abcde}(1)^{abc}$	$8^{efghi}(4)^{efg}$	$3.3^{a}(1.3)^{a}$
DSRF19	$0^{a}(0)^{a}$	$1^{ab}(1)^{abc}$	$6^{defgh}(3)^{cdef}$	$12^{jkl}(6)^{ghi}$	$4.8^{b}(2.5)^{b}$
DSRF21	$10^{hijk}(5)^{fgh}$	$15^{lm}(9)^{jk}$	$25^{pq}(14)^{l}$	$36^{r}(28)^{o}$	$21.5^{f}(14)^{f}$
DSRF26	$7^{defgh}(2)^{bcde}$	$11^{ijkl}(6)^{hi}$	$17^{mn}(10)^{k}$	$29^{q}(23)^{n}$	$16^{e}(10.3)^{e}$
DSRF33	$0^{a}(0)^{a}$	$0.7^{ab}(0)^{a}$	$3^{abcd}(1)^{abc}$	$6^{cdefg}(3)^{def}$	$2.4^{a}(1)^{a}$
Mean	$2.8^{a}(1.1)^{a}$	$5.5^{b}(2.9)^{b}$	$10.6^{c}(5.6)^{c}$	$17.1^{d}(13)^{d}$	

*****Values are mean of three replicates; values in parenthesis show germination in dark condition; superscript different alphabets show significant difference while similar superscripts shows no significant difference (P >0.5).

inclusive phenomena [27]. It is stated that *Trichoderma* is a hostile mycoparasite, which can control already established pathogens as well as newly entered pathogens [28]. During mycoparasitism cell wall degradation and penetration are frequently observed resulting in the direct destruction or lysis of propagules and structures [29]. Such type of destructions in the hyphae of *F. solani* were observed during post interaction study.

When pathogen was grown in culture filtrate of different fungal strains a continuous reduction in mycelial dry weight was observed with increasing concentrations of culture filtrate. Our these findings agree with previous report of similar observations against a pathogen of citrus fruit *Alternaria citri* when grown it in culture filtrate of different *Trichoderma* species [30]. In both the cases, 100% concentration of the isolate culture filtrate resulted in highest reduction. Similar findings have also been reported [31]. The study clearly reflects the inherent ability to induce antagonistic effects on fungal pathogen through a wide range of bioactive secondary metabolites produced by a variety of filamentous fungi [32, 33]. These metabolites include a number of substances, such as pyrones, gliovirin, gliotoxin, viridine, pyrones and viridiol.

Cell-free culture filtrate of fungal isolates inhibited conidial germination of *F. solani* which may be due to the presence of the toxic metabolites in culture filtrate secreted by the antagonistic fungi that inhibited spore germination and growth of germination tube. Such metabolites have also been reported in previous work [34, 35]. The complete inhibition of conidial germination of *Colletotrichum dematium* causative agent of antharacnose disease of soybean by the crude culture filtrate of *Trichoderma lignorum* also have been reported [36].

CONCLUSION

The present work shows that *D. sissoo* rhizosphere harbours many antagonistic microfungi with dominating species of *Trichoderma*, which can be used further to manage the wilt disease of *D. sissoo* caused by *F. solani*.

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