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ANTAGONISTIC EFFECT OF FUNGAL ENDOPHYTES AGAINST SEED MYCOFLORA ISOLATED FROM *PONGAMIA PINNATA*

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ABSTRACT
Endophytic *Trichoderma* species were used as biocontrol agents against various seed mycoflora of *Pongamia pinnata* (L.) Pierre. (Karanja). Samples of leafs, roots, and stems of *P. pinnata* were collected from *P. pinnata* trees and five endophytes *viz.* three isolates of *Trichoderma harzianum* and two isolates of *Trichoderma aurioviride* were isolated and evaluated as potential biocontrol agent against *P. pinnata* seed mycoflora *viz., A. oryzae, A. ustus, A. sydowii, A. fumigatus, A. flavus, A. niger, Rhizopus* sp., *Fusarium* sp., *Penicillium* sp., *C. gloeosporioides* and *Absidia* sp. in *in-vitro* conditions by poison food technique. Dual culture results revealed that maximum colony growth inhibition of different seed fungi was achieved by *T. harzianum* E1 isolate and *T. aureoviride* E5 among different fungal endophytic antagonists screened during the course of study.

KEYWORDS: Antifungal activity, Endophytes, *Pongamia pinnata,* Biocontrol.

INTODUCTION

Biodiesel has gained immense importance in India and two plant species *viz. Pongamia pinnata* (L.) Pierre and *Jatropha curcas* L. are being exploited for this purpose. Seeds of *Pongamia pinnata* are used to extract oil and is often mixed with *Jatropha* oil and used as a substitute to diesel. The increasing price of petroleum products and concerns about oil production are likely to have serious implications for the automobile industry in the future. The answer to the above requirement is to search for an alternative to the fast-depleting reserves of fossil fuel from renewable natural resources (Martini and Shell 1998; Srivastava and Prasad, 2000). The thick oil from the seeds of *P. pinnata* is used for illumination, as a kerosene substitute, water paint binder, pesticide. The seeds of *P. pinnata* are subjected to attack by different fungal species such as *Aspergillus* sp., *Penicillium* sp. and *Fusarium* sp. and cause seed deterioration leading to poor germination, survival and oil yield. However, in storage several fungal species attack *P. pinnata* seeds and cause seed deterioration (Jamaluddin *et al*., 1985; Pandey and Prasad, 1993; Kumar *et al*., 2007). Microbial endophyte is a non pathogenic microbial colony which resides inside the healthy plant tissues. Endophytic microbes have been recognizing as a chemical producer with a broad range biological activity. Endophytic fungi have been recognized as useful sources of bioactive secondary metabolites (Schulz *et al*., 2002; Strobel, 2003). Many endophytic fungi have the ability to produce antimicrobial substances. Among the Ascomycota, *Trichoderma* species have been extensively studied. They colonise root surfaces, penetrate the first cell layers and are used as opportunistic plant symbionts for disease control and yield enhancement (Harman *et al.,* 2004). *Trichoderma* has been shown to be capable of eliciting a systemic acquired resistance (SAR) response in plants, which can raise the levels of resistance to disease by stimulating phytoalexins, natural plant defence chemicals (Calderon *et al.,* 1994). *Trichoderma* isolates have shown antagonistic activity against pathogens by mycoparasitism, production of antibiotics and competition in soil (Chet *et al*., 1998; Hoitink and Boehm, 1999). In the present study different endophytic *Trichoderma* spp. isolate were screened for colony inhibition by dual culture tests.

MATERIALS & METHODS

Plant Sample Collection

Mature and healthy plant leaves, stems and roots of *P. pinnata* were collected by sampling different parts of the trees of New Forest area, Dehradun. All samples were stored in paper bags and used to isolate endophytic fungal species within 12 hours of collection.

Isolation of Endophytes

Plant materials were thoroughly washed with running tap water, cut under sterile conditions into small pieces of (2-3cm) and surface sterilized with 1% sodium hypochlorite followed by 90% ethanol. Traces of both the treatment *viz.* sodium hypochlorite and alcohol were removed with a rinse in sterile distilled water. The plant segments were then plated on different microbiological media such as water agar, potato dextrose agar and rose bengal agar (Difco). The plates were incubated at 25°C for one week. Hyphal tips of fungi, emerging out of the plant tissues, were picked and grown on potato dextrose agar in pure cultures. Resulting fungal colonies were purified and identified using their microscopic characters such as conidiphore and conidial

structure measurements etc. Slants were prepared and stored at 4^0C until use. After the proper incubation of the plates, one set of seven days old cultures on PDA slants were preserved in mineral oil.

Taxonomic Identification of Endophytic Fungi

For studying the cultural and morphological characters, the isolated fungi were grown on PDA. Colony morphology including its color and growth characterstics was recorded. Slides were prepared for Microscopic observations of each endophyte by staining in lactophenol-cotton-blue (Vainio *et al*., 1998) and examined under light microscope. Mycelial, characterstics conidiophores and conidia produced in the culture condition.

Isolation of seed mycoflora

The seed fungi were isolated by the agar plate method (Agarwal and Sinclair, 1997). Seeds were surface sterilized in 0.1% mercuric chloride solution for 30 sec followed by 3 to 4 successive washing in sterilized water and absorption of extra moisture by keeping the seeds on sterilized blotting paper in aseptic condition. In the Potato dextrose agar (PDA) culture plates 3 seeds per plate were placed and incubated at 25 ± 2^0c for 7days. After incubation Taxonomic identities of the isolated fungi were determined using standard keys by (Booth, 1971; Ellis, 1971; Barnett and Hunter, 1972).

Antagonistic Test

Three different isolates of *Trichoderma harzianum* and two isolates of *Trichoderma aureoviride* were isolated from healthy leaves, roots and stems of *Pongamia pinnata* from New Forest area, Dehradun. Screening endophytic isolates for their antagonistic property was carried out by inoculating each fungal endophyte in dual culture with isolated fungal species from *P. pinnata* seeds in 7 cm Petri dishes on PDA (Nuangmek *et al*., 2008). Each agar plate was inoculated with a 5 mm disk from the margin of actively growing 5 days old endophytic fungal colony and positioned opposite to the pathogen disk. The distance between discs was approximately 5 cm. Experiments were conducted in triplicate and data were subjected to one way ANOVA.

Plates inoculated with pathogen alone were kept as control. Culture plates were then incubated at room temperature (25-30°C) and after 7 days, the colony diameters of the pathogens in the control and in each paired cultures dual cultures were recorded. The pathogen colony inhibition percentage was calculated by following equation (Vincent, 1947):

$$Percentage\ of\ Inhibition = \frac{A - B \times 100}{A}$$

A= Radius of pathogen in control plate

B= Radius of pathogen in dual culture plate

RESULTS

Five endophytic *Trichoderma* sp. isolates were isolated from leaf, stem and root samples (Table1). From the leaf samples two *Trichoderma* sp. *viz. T. harzianum* and *T. aureoviride* were isolated where as *T. harzianum* was isolated from stem and root sample. Three different isolates of *Trichoderma harzianum* and two isolates *of Trichoderma aureoviride* were *in-vitro* evaluated for their antagonistic effect against seed mycoflora *viz. Penicillium* sp., *Aspergillus oryzae, Aspergillus ustus, Aspergillus sydowii, Aspergillus fumigatus, Aspergillus flavus, Aspergillus niger, Rhizopus* sp., *Fusarium* sp., *Colletotrichum gloeosporioides* and *Absidia* sp. by dual culture technique (Fig. 1 and Fig. 2).

TABLE1: Endophytic fungal isolates obtained from different *Pongamia pinnata* plant parts

S. NO.	P. PINNATA SAMPLE	ENDOPHYTIC FUNGAL SPECIES ISOLATED	ISOLATE CODE
1	Leaf	*Trichoderma harzianum*	E1
2	Stem	*Trichoderma harzianum*	E2
3	Root	*Trichoderma harzianum*	E3
4	Leaf	*Trichoderma aureoviride*	E4
5	Leaf	*Trichoderma aureoviride*	E5

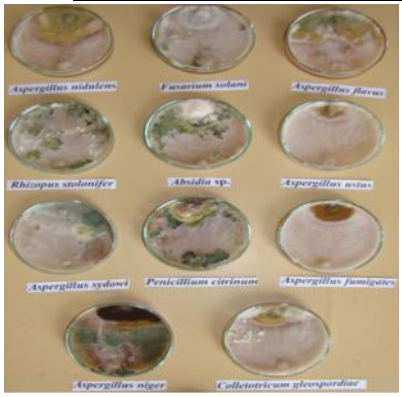

FIGURE 1. Evaluation of antagonistic potential of *T. harzianum* (E1) against *Pongamia pinnata* seed mycoflora**.**

FIGURE 2. Evaluation of antagonistic potential of *T. aureoviride* (E5) against *Pongamia pinnata* seed mycoflora.

Based on inhibition zone, inhibition percentage of antagonists was recorded. The results thus obtained are presented in the (Table 2). Maximum colony growth inhibition was recorded by the endophytic antagonist *Trichoderma harzianum* (E1) (69.13%), irrespective of pathogenic species followed by *T. aureoviride* (E5) (67.80%), *T. aureoviride* (E4) (67.27%) and *T. harzianum* (E2) (66.53%) and least inhibition was recorded with *T. harzianum* (E3) (65.49%). Maximum pathogen inhibition irrespective of antagonists, was observed against *Aspergillus ustus* (72.66%) followed by *Absidia* sp. (71.42%), *A. niger* (71.33%), *Fusarium sp*. (71.20%), *C. gloeosporioides* (69.28%), *A. fumigatus* (68.19%), *A. sydowii* (65.42%), *A. flavus* (64.28%), *A. oryzae* (63.81%), and *Penicillium* sp. (62.28%). Least inhibition was observed against *Rhizopus* sp. (59.81%). Even the least efficacy of isolate E3 showed more than 60% inhibition. All the five isolates of *Trichoderma harzianum* and *Trichoderma aureoviride* showed biological control potential agent against all the seed mycoflora. On interaction with *T. harzianum*, isolates labelled (E1, E2 and E3) growth were inhibited at the line of contact. However, *A. niger* showed aversion on interaction with *T. aureoviride*. The antagonist produces enzymes which causes lysis of cell wall components of the pathogenic fungi. Microscopic examination at the point of contact of two fungi revealed that the overgrowing mycelium of the antagonist penetrated the mycelium of the pathogen and the tip of the hyphea of the pathogen swelled and curled making it ineffective. The data revealed that all the pathogens may be inhibited by *T. harzianum* E1 isolate and *T. aureoviride* E5 and must be tested for their efficacy in storage as seed mycoflora treatment before using them on large scale.

TABLE 2: Colony growth inhibition of *P. pinnata* seed mycoflora by antagonistic *Trichoderma* sp. isolates

Endophytic Antagonists	Pathogens											Mean
	A. ustus	*Absidia* sp.	*Rhizopus* sp.	*A. oryzae*	*A. flavus*	*Fusarium* sp.	*A. niger*	*Colletotricum gloeosporioide*	*A. sydowi*	*A. fumigatus*	*Penicillium* sp.	
T. harzianum (E1)	70.47	74.76	60.47	69.52	65.23	70.47	76.66	69.01	63.33	70.95	69.52	69.13[1]
T. harzianum (E2)	70.95	70.95	58.09	50.00	65.23	70.95	71.90	68.05	69.01	70.47	66.19	66.53[5]
T. harzianum (E3)	74.28	64.76	58.57	60.00	68.09	70.47	78.09	64.76	61.42	68.09	51.90	65.49[4]
T. aureoviride (E4)	71.90	71.90	58.09	69.05	63.33	70.47	65.71	70.95	68.57	64.76	65.23	67.27[3]
T. aureoviride (E5)	75.71	74.76	63.80	70.47	59.52	73.61	64.28	73.61	64.76	66.66	58.57	67.80[2]
Mean	72.66	71.42	59.81	63.81	64.28	71.20	71.33	69.28	65.42	68.19	62.28	
$CD_{(0.05)}$	Endophytic antagonist =.570				Pathogen =.845			Pathogen x Endophytic antagonist =1.890				

DISCUSSION

The results of the present findings showed that endophytic *Trichoderma* sp. isolated from leaf, stem and root restricted the growth of seed mycoflora of *Pongamia pinnata*. The activities of endophytes in suppressing fungal pathogens were confirmed by (Poling *et al*., 2008; Salehpour *et al*., 2005; Muhammad and Amusa, 2003). Different isolates of *Trichoderma* spp. *viz. T. harzianum* and *T. aureoviride* overgrew the pathogens in dual culture tests. *Trichoderma* species have been successfully used as biocontrol agents due to their high reproductive capacity, efficient utilization of nutrients, and strong aggressiveness against other phytopathogens, efficiency in promoting plant growth and defense mechanism and ability to modify the rhizosphere (Kleifeld and Chet, 1992). *Trichoderma* isolates have shown antagonistic activity against pathogens by mycoparasitism, production of antibiotics and competition in soil (Chet *et al*., 1998; Hoitink and Boehm, 1999). However, additional attributes were observed as for instance increase of plant growth and induction of disease resistance. Maize plants colonized by *Trichoderma harzianum* (T22) increased yield and growth up under low levels of nitrogen fertilizer (Harman, 2000). Not only strains from different species as *T. asperellum, T. hamatum, T. harzianum* and *T. virens* are able to colonize roots, but also a single Trichoderma strain can also interact with different plant hosts and induces resistance against different diseases such as Phytophthora blight on cucumber, Botrytis blight on begonia and Botryosphaeria dieback on ericaceous plants (Harman *et al*., 2004; Khan *et al*., 2004; Horst *et al*., 2005; Hoitink *et al*., 2006; Harman and Shoresh, 2007). However, when a biocontrol agent is applied in field competition by other microorganisms in the environment pose a serious threat to the very survival of the antagonist. Thus in field condition the most effective antagonist must be used. However, since our basic aim to conduct this experiment was to identify an antagonist non-pathogenic to plants and to evaluate their efficiency as seed dressing agent before storage. For *T. harzianum* E1 isolate and for *T. aureoviride* E5 may be further tested for seed dressing in field experiments involving seed storage conditions in depots and nurseries. The results thus obtained may lead to the formulation and commercial application thus improving the *P. pinnata* seed viability, health and oil yield/ quality.

CONCLUSION

Thus from the above study it is concluded different isolates of *Trichoderma* species have been found to be effective in controlling the seed mycoflora. To this end, further work is needed to employ all the available means of maximally putting endophytes to beneficial use especially for seed dressing in storage. *Trichoderma* sp. *viz. T. harzianum* and *T. aureoviride* showed the potential as biocontrol agents

requiring for further testing and application for use on the field.

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