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Antifungal and enzyme activity of endophytic fungi isolated from *Ocimum sanctum* and *Aloe vera*

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The present study was carried out to isolate endophytic fungi from *Ocimum sanctum* and *Aloe vera* and to assess their antifungal activity against *Rhizoctonia solani*, *Fusarium oxysporum*, *Colletotrichum falcatum* and *Helminthosporium maydis.* The isolated fungi were further evaluated for production of extracellular enzymes such as amylase, cellulase, chitinase, pectinase, laccase, lipase and urease. During the study 18 endophytic fungi were isolated from leaves, stem and roots of *O. sanctum* and *Aloe vera* which are different from each other morphologically. AVR1 and AVR3 show antagonistic activity towards the *Fusarium oxysporum*. T2S1 and AVL1 were found positive for amylase, TL1, AVR1, and AV2L1 were found positive in cellulase production while only AVR3 was found positive for chitinase production. AVR3, TL2, TL3, TS1, AV2R1, T2S1 and T2L1 were positive in pectinase production, none of the isolates were found positive in laccase production, only AVL1 was found positive in lipase production and TL2, T2R1, AVL1, AVL4, T2L1 and TL3 were positive in urease production. The results of the study suggest that endophytic fungi associated with *O. sanctum* and *Aloe vera* are potential agents for antimicrobial activity and a vast source of enzyme.

Keywords: Endophytic fungi, antifungal activity, extracellular enzymes.

INTRODUCTION

Endophytes are ecological group of fungi that colonize living, internal tissue of plants without any discernible features of their presence (Gehlot and Soytong, 2008; Hyde et al., 2008). They are ubiquitous, share symbiotic relationships with their hosts (Tejesvi et al., 2005) and are found in all plant species (Naik et al., 2008; Stone et al., 2000). Infected plants benefited by exhibiting increased resistance to herbivore grazing through the production of various phytochemicals (Naik et al., 2008; Owen and Hundley, 2004). Endophytes are known to produce metabolites such as alkaloids, terpenoids, steroids, quinones, isocoumarin derivatives, flavanoids, phenols, phenolic acids, and peptides (Zhang et al., 2006). *Ocimum sanctum* and *Aloe vera* can serve as good model plants for studying the effects of fungal endophytes colonization on secondary metabolism. They have significant economic importance, a well-documented chemical profile, and some of its therapeutic chemicals are known to be affected by endophytic fungi colonization (Bauer and Wagner, 1991; Lata et al., 2003;

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Araim et al., 2009). Endophytes are symbiotic microorganisms of living plants and potential sources of biologically active natural products which are useful in medical, agricultural and industrial application. The main industries that used microbial enzymes are the food, textile, leather, pharmaceutical, cosmetics, fine chemicals, energy, biomaterials, paper, cellulose and detergent industries. The present study was carried out to evaluate the antagonistic activity of endophytes and to find the new sources of valuable extracellular enzymes from endophytic fungi to understand their functional role in the host.

MATERIALS AND METHODS

Collection of plant samples

Healthy plants of *O. sanctum* and *Aloe vera* were collected from two different places of Govind Ballabh Pant University Of Agriculture And Technology Pantnagar India that is medicinal plant research and development centre (MRDC) and garden section in sterile zip lock polythene bags.

Surface-sterilization and isolation of endophytic fungi

Isolation of endophytic fungi from *O. sanctum* and aloe vera was carried out using the protocol of Sunitha et al. (2013). Different parts of the medicinal plants such as leaves, stem and roots were cut into small pieces of 1 cm and washed under running tap water for 10 min, and sterilized in series with 70% ethanol for 1 min, 1.0 % sodium hypochlorite (NaOCl) for 1 min and further cleaned by passing through two sets of sterile distilled water. The sterile samples were placed on plate containing potato dextrose agar (PDA) media with 50 mg/l concentration of ampicillin to suppress the bacterial contamination. The parafilm wrapped Petri dishes were incubated at 25± 2 °C till the fungal mycelia starts growing from the samples.

Identification of fungal isolates

The sample of fungal isolates were mounted on the sterile slides then it was stained with lactophenol cotton blue and examined in 40X light microscopy. The fungal cultures were identified on the basis of microscopic characters, for spore shape and phenotypic characteristics, for spore type, growth colour, growth rate using standard manual (Barnett and Hunter, 1998).

Antibiosis activity

Antifungal activity was screened using dual culture method described by Fatma et al. (2010) in which both endophytic fungi and test fungi were inoculated in single Potato Dextrose Agar media. Antifungal activity was checked against four plant pathogens, *Rhizoctonia solani* which mainly causes sheath blight of rice, *Fusarium oxysporum* which mainly causes panama disease of banana, *Colletotrichum falcatum* which mainly causes red rot of sugarcane and *Helminthosporium maydis* mainly causing leaf blight in maize. Test fungi were inoculated at one side of potato dextrose agar plate and endophytic fungi was inoculated at other side of the plate and incubated for five days at 27°C. Antifungal activity was indicative as mycelia growth of test fungus in the direction of active endophyte.

Extracellular enzyme production

Amylolytic activity

Amylase activity was assessed according to method described by Sunitha *et al., (*2013**),** by growing the fungi on Glucose Yeast Extract Peptone Agar (GYP) medium (glucose-1g, yeast extract -0.1g, peptone- 0.5g, agar -16g, distilled water-1L) with 0.2% soluble starch pH 6.0. After incubation, the plates were flooded with 1% iodine in 2% potassium iodide.

Cellulase activity

Glucose Yeast Extract Peptone Agar medium containing 0.5% Carboxy-methyl cellulose was used. After 3 days of fungal colony growth, the plates were flooded with 0.2% aqueous Congo red solution and destained with 1 M NaCl for 15 min. Appearance of yellow areas around the fungal colony in an otherwise red medium indicated cellulase activity.

Chitinolytic activity

To check the chitinolytic activity, fungi were grown in colloidal chitin prepared by the method of Rodriguez-Kabana et al. (1984), by partial hydrolysis with 10 N HCl for 1.5 h at room temperature. The colloidal chitin was then washed several times with large volumes of tap water and then washed with distilled water for 5 to 7 times to adjust the pH. Chitin agar media was prepared (yeast extract, 1.5 g; chiti, 2.0 g; agar, 20 g; distilled water, 1 L). Plates were inoculated with test cultures and then incubated at 26°C upto 72 h. Appearance of clear zone surrounding the culture showed chitinase activity.

Pectinolytic activity

Pectinolytic activity was determined by growing the fungi in Pectin Agar medium (Pectin -5g, yeast extract-1g, agar- 15g, pH 5.0 in 1L distilled water). After the incubation period, the plates were flooded with 1% aqueous solution of hexadecyl trimethylammonoium and a clear zone was formed around the fungal colony indicated pectinolytic activity.

Laccase activity

To assess the laccase activity Glucose Yeast Extract Peptone Agar medium with 0.05 g 1-napthol/ L, pH 6.0 was used. As the fungus grows the colourless medium turns blue due to oxidation of 1-napthol by laccase.

Lipolytic activity

For lipase activity, the fungi were grown on peptone agar medium (peptone, 10 g; NaCl, 5 g; $CaCl_2$ $2H_2O$, 0.1 g; agar, 16 g, distilled water, 1 L; pH 6.0) supplemented with Tween 20 separately sterilized and added 1% to the medium. At the end of the incubation period, a visible precipitate around the colony due to the formation of calcium salts of the lauric acid liberated by the enzyme indicated positive lipase activity.

Urease production

The ability of the isolates to attack nitrogen and carbon bonds in amide compounds was determined using Christensen's urea broth

Table 1. Fungal isolates from different parts of plants.

Region	Site of selection			
	MRDC		**Garden section**	
Pantnagar	TULSI	ALOE	TULSI	ALOE
	LEAF TLI, TL2,TL3	LEAF AVLI,AVL2, AVL3, AVL4	LEAF T2L1	LEAF AV2L1
	STEM TSI, TS2	STEM	STEM T2S1	STEM
	ROOT TRI	ROOT AVR1, AVR2, AVR3	ROOT T2RO1	ROOT AV2RO1

Table 2. Morphological characteristics' of fungal isolates.

Isolate I.D.	Colour of growth surface on PDA plates	Spore shape /hyphae structure	Nature of growth
AVL1	White	Mycelium at 90^o	Fluffy
AVL2	White	Swellon hyphae	Fluffy
AVL3	White	Swellon hhyphae	Fluffy
AVL4	White	Septate cylindrical spore	Dry
AVR1	Black	Rounded	Powdery
AVR2	White	Rounded to eliptical	Velvety
AVR3	Black/orange	Rounded	Velvety
TL1	Black	Rounded	Powdery
TL2	White	Cylindrical	Moist
TL3	Black	Boomerang shaped	Velvety
TS1	Yellow/green	Rounded	Velvety
TS2	white	Swellon hyphae	Velvety
TR1	White/black	Mycelium at 90^0	Fluffy
AV2R1	Brown	Rounded	velvety
T2S1	White	Mycelium at 90^o	fluffy
T2R1	Pink	Canoe shaped	fluffy
T2L1	Creamish	Canoe shaped	velvety
AV2L1	Black	Rounded	powdery

(peptone, 1 g; Nacl, 5.0 g, KH_2PO_4, 2.0 g; phenol red, 2%; urea, 20% aqueous sol; pH-7.0) containing the pH indicator phenol red (Cappuccino and Sherman, 2002). Urea was separately sterilized by filter sterilisation and added aseptically to basal medium. A disc of isolated fungi were aseptically inoculated into sterile Christensen's urea broth using a cork borer and incubated for 48 h. Presence of red color shows positive for urease and yellow color as negative.

RESULTS AND DISCUSSION

A total of 18 endophytic fungi were isolated from leaves, stem and root of *O. sanctum* and *Aloe vera*, which are shown in Table 1. On the basis of different growth colour, spore type and nature of growth they are categorized using the standard manual (Table 2). Each isolate were sub cultured into a PDA agar and stored at 4°C for the further studies. Dennis and Webster (1971) describes that, fungi are known to produce a number of antibiotics, such as trichodermin, trichodermol, trichotoxin, harzianum and harzianolide. These compounds were responsible for most of the inhibition of fungal pathogen. The results of the antifungal activity showed that AVR1 and AVR3 were able to suppress mycelial growth of *F. oxysporum* (Figure 7*)* and no other fungal isolates was found active against plant pathogens *R. solani, F. oxysporum, C. falcatum* and *H. maydis*. Extracellular enzymes are synthesized inside the cell and then secreted outside the cell, where their function is to break down complex macromolecules into smaller units to be taken up by the organisms as a sole source of carbon, energy, and nutrients. Urease is a nickel-containing enzyme that catalyzes the hydrolysis of urea to yield ammonia and carbamat, the latter compound decomposes spontaneously to generate a second molecule of ammonia and carbon dioxide. The results of the urease activity (Figure 6) shows that AVL1, AVL4, TL2, TL3, T2R1 and T2L1 were found positive in urease production. Simmilar results has also been reported by Smith et al. (1993). Out of 18 endohpytic fungi only AVL3 and T2S were able to degrade starch by amylase production, which is shown by significant area of clear zone around fungal mycelia growth (Figure 1), while cellulase activity

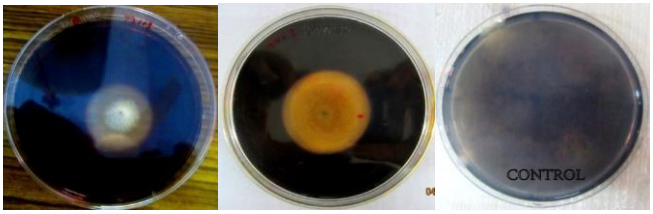

Figure 1. Amylolytic activity by endophytic fungi.

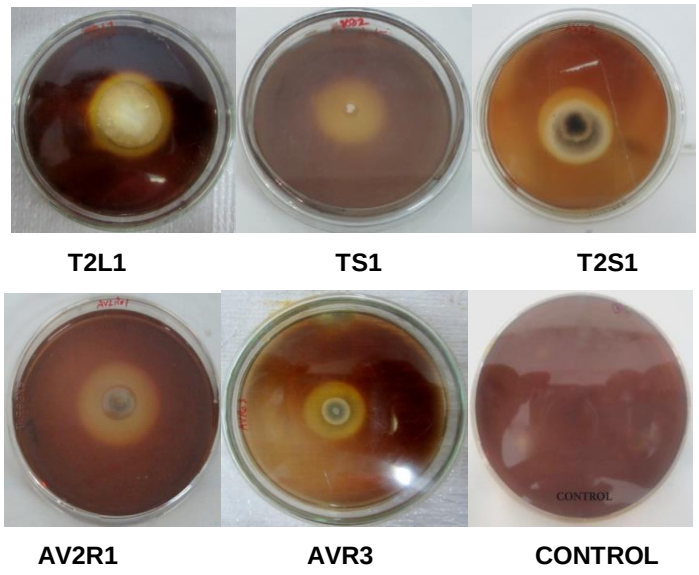

Figure 2. Pectinolytic activity by endophytic fungi.

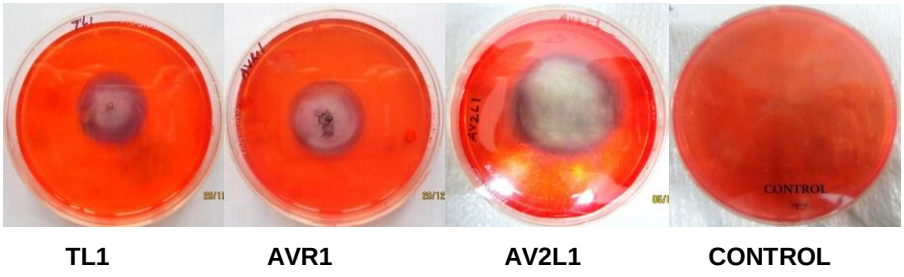

Figure 3. Cellulolytic activity by endophytic fungi.

(Figure 3) were found in four endophytes, in which AVR3 had maximum cellulase activity followed by AVR1, TL1 and TL3, which is shown by significant area of clear zone around fungal mycelia growth, Maria et al. (2006) describes the cellulose production in mangrove. Microbial pectinases are important in the decomposition of dead plant material, degradation of host tissue by phytopathogens generally begins with the production of pectinolytic enzymes. In pectinase production, maximum pectinase activity was observed in AV2R1 followed by T2L1, AVR3, TS1, T2L1, TL3, and TL2 (Figure 2). The ability of endophytic fungi to produce cellulase and pectinase may provide a resistance mechanism to the host against pathogenic invasion, to get nutrition from the host or to be a latent pathogen.(Choi et al., 2005). Lipase activity was found positive only in AVL1 (Figure 5). Amirita et al. (2012) reported lipolytic activity of *Curvularia brachyspora C. vermiformis, Drechslera hawaiiensis, C. falcatum and Phyllosticta sp.* isolated from medicinal plants. Previous report of Maria et al.

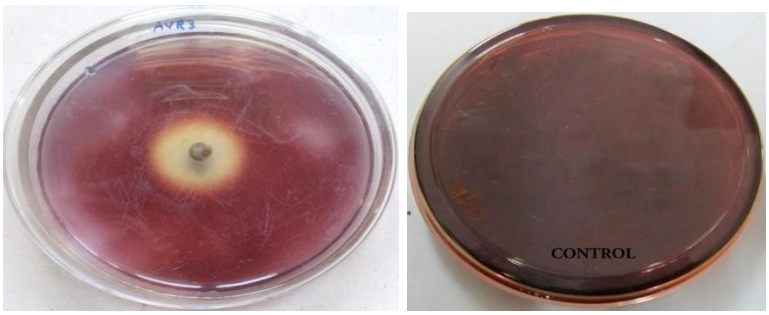

Figure 4. Chitinolytic activity by endophytic fungi.

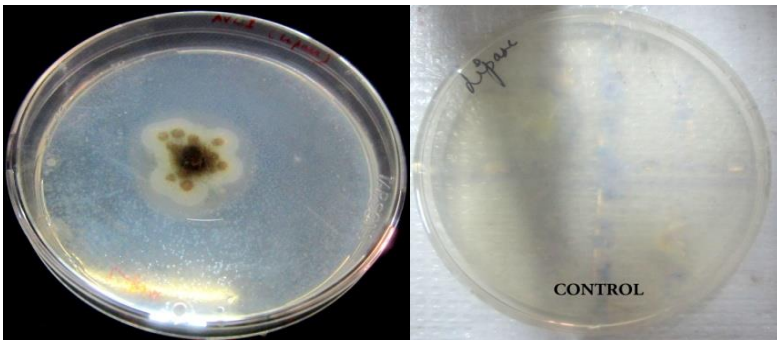

Figure 5. Lipase activity by endophytic fungi.

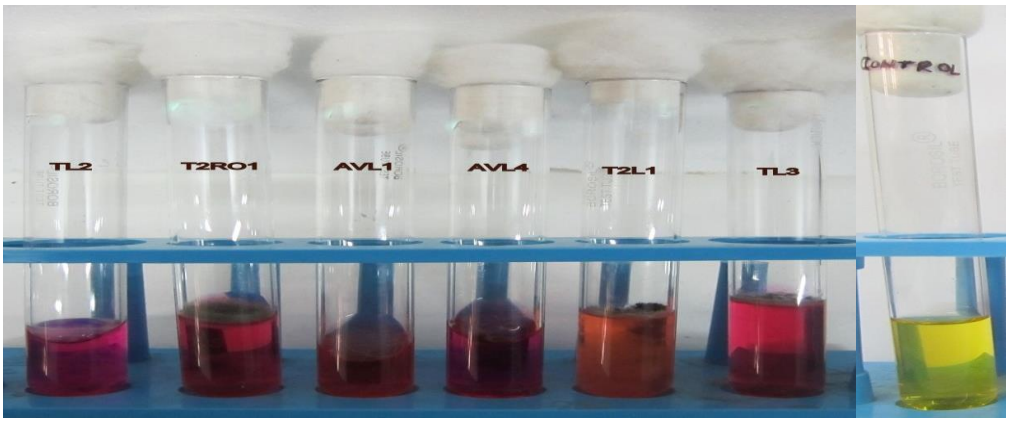

Figure 6. Urease production by endophytic fungi.

(2006), suggests that none of the isolates found active in laccase production. The endophytic nature of these fungi might be the reason for the lack of laccase activity. Out of the 18 fungal isolates only one isolate was positive for chitinase activity (Figure 4). Haran et al. (1996) stated that, chitinases play an important biological and physiological roles in fungi, containing autolytic, nutritional, morphogenetic, and parasitic roles. Chitinases in mycoparasitic fungi are most commonly suggested to be involved in mycoparasitism.

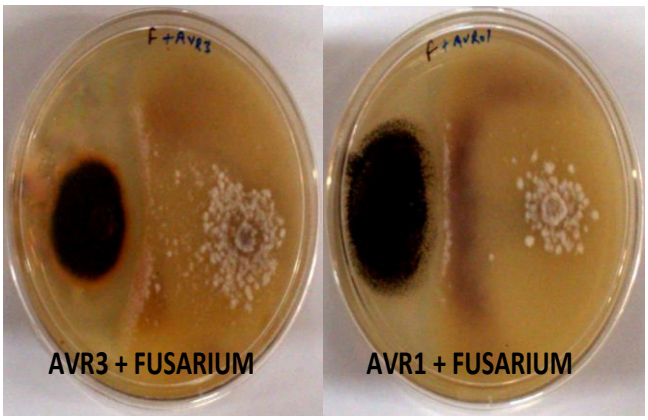

Figure 7. Antibiosis activity of endophytic fungi.

Conclusion

Endophytic fungal isolates was found to be associated with leaves, stem and root of the medicinal plant that is *O. sanctum* and *Aloe vera* and they differed significantly in their morphological, biochemical and functional characterstics. However, knowledge of the types, amounts and characteristics of enzymes produced by endophytic fungi would be useful for selecting organisms for industrial requirements. The potential endophytic fungi are being investigated quantitatively for extracellular enzyme production in liquid media.

Conflict of interests

The authors did not declare any conflict of interest.

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