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Bioprospecting Potential of Foliar Endophytic Fungi Associated with Commonly used Indian Medicinal Plants

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In the current study we have explored bioprospecting potentials (antimicrobial activity, antibiotic susceptibility pattern, enzyme activity and dye degradation ability) of endophytic fungi isolated from different medicinal plants. Total twenty fungal endophytes were isolated from the leaves of selected plants by using sterilization treatment followed by serial dilution agar plate technique. All isolates were tested for the antimicrobial activity against seven pathogenic strains including two Gram positive bacteria (*Staphylococcus epidermidis* and *Bacillus amyloliquifaciens*) and two Gram-negative bacteria (*Escherichia coli* and *Salmonella enterica ser. typhi*) and three fungi (*Aspergillus fumigatus*, *Aspergillus* sp. and *Candida albicans*) using agar well diffusion method. Out of twenty fungal isolates, fourteen endophytic fungal isolates exhibited both antifungal and antibacterial activity. It was observed that 55% of isolates exhibited urease activity, 40% amylase activity, while 45% esterase activity. Malachite green degradation was observed in 20% endophytic fungal isolates. Whereas one isolate was found sensitive to Ketoconazole and Itraconazole in antibiotic susceptibility studies. All other isolates were found resistant to all antibiotics. Further investigations are suggested in order to classify the microorganisms and exploit the potential of the compounds produced to inhibit pathogenic microorganisms and to explore the bioprospecting potential like nitrogen fixation and plant growth promoting activities etc.

Key words: *Candida*, pathogens, antibacterial and antifungal activity, malachite green.

Endophytes are those microflora which live in the interior parts of plants like leaves, stems and roots causing no apparent harm to host[1]. Different groups of fungi, bacteria, actinomycetes and mycoplasma are reported as endophytes of plant[2]. Endophytes have been known for more than hundreds of years. The word endophyte is derived from Greek, 'endo' from 'endon' meaning within, and 'phyte' from 'phyton' meaning plant. The world of endophytic fungi is highly diversified due to which they are being explored by researchers from historical time[3-4]. It is noteworthy that nearly 300,000 plant species exists on earths which are host to one or more endophytes[5]. However, only a handful of plants, mainly grass species, have been completely studied in relation to their endophytic biology[6].

Endophytes provide a broad variety of bioactive compounds with unique structure, including secondary metabolites alkaloids, benzopyranones, chinones, flavonoids, phenolic acids, quinines, steroids, terpenoids, tetralones, xanthones and others[7]. Such bioactive secondary metabolites are being used as agrochemicals, antibiotics, immunosuppressants, antiparasitics, antioxidants and anticancer agents[8]. Many researchers have demonstrated that the endophytes isolated from medicinal plants are excellent producers of strong fungicides, bactericidal and cytotoxic metabolites[9-10]. Keeping

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in view the above facts, the current study was designed to explore bioprospecting potential of endophytic fungi isolated from indigenous plants of Ambala, Haryana (India).

MATERIALS AND METHODS

Sample collection

For isolation of endophytic fungi, healthy and fresh leaves of 20 plants namely Tomato, Aloe vera, Chili, Radish, Cauliflower, Cabbage, Arjun, Pomegranate, Grass, Carrot, Coriander, Guava, Stevia, Mint, Garlic, Peas, Giloy, Turmeric, Neem and Rose were collected from different areas of Ambala, Haryana. Each sample was tagged and placed in separate polythene bags and transported aseptically to the laboratory and processed within 24 hours of collection. Fresh plant materials were used for isolation to reduce the chance of contamination[11].

Sample Pre-treatment

For the pre-treatment of leaf samples and isolation of endophytic fungi all leaf samples were excised and subjected to a surface sterilization procedure described by[6,12]. The procedure for sample pre-treatment is shown below in figure 1.

Isolation of endophytic fungi

For the isolation of endophytic fungi, pre-treated dried leaf sample were crushed with sterile distilled water using sterile mortar and pestle. About 1mL of crushed sample was serially diluted up to 10^{-5} dilutions. Potato dextrose agar medium supplemented with streptomycin to suppress bacterial growth was prepared and used for the isolation of endophytic fungi, about 0.1 ml of aliquot from 10^{-2} to 10^{-5} dilutions were taken and spreaded on potato dextrose agar medium using sterile cotton swab. All the plates were incubated in incubator for 3-4 days at 25^0C. In case of undried samples, leaves were cut into small pieces by using sterile knife and placed on PDA plates supplemented with streptomycin and incubated in incubator for 3-4 days at 25^0 C for fungal growth. The plates were observed for appearance of colonies and number of colonies produced on each plate. Highly sterile conditions were maintained for isolation of endophytic fungi[12].

Purification and maintenance of endophytic fungi

Fungi were purified by streak plate method on PDA and incubated at 25^0C for 3-4 days and transferred to PDA slants and then maintained in refrigerator at 4^0C till further analysis[13].

Screening

1. Screening for Antibacterial and antifungal activity
2. Screening for Enzymatic activity
3. Screening for Dye degradation
4. Screening for Antibiotic susceptibility pattern

Screening for antibacterial and antifungal activity

Test microorganisms

A total of seven human pathogenic strains such as three test fungi *Aspergillus fumigatus*, *Aspergillus* sp.(molds), *Candida albicans* (yeasts) and four test bacteria including Gram negative *Escherichia coli, Salmonella enterica ser. typhi,* and *Gram positive Staphylococcus epidermidis, Bacillus amyloliquifaciens* were procured from MTCC, IMTECH (Chandigarh). All the test fungal and bacterial strains were as given in table 1 are maintained in their respective media (Czapek dox broth for *Aspergillus* sp., *Aspergillus fumigatus*; Potato dextrose broth for *Candida albicans*;

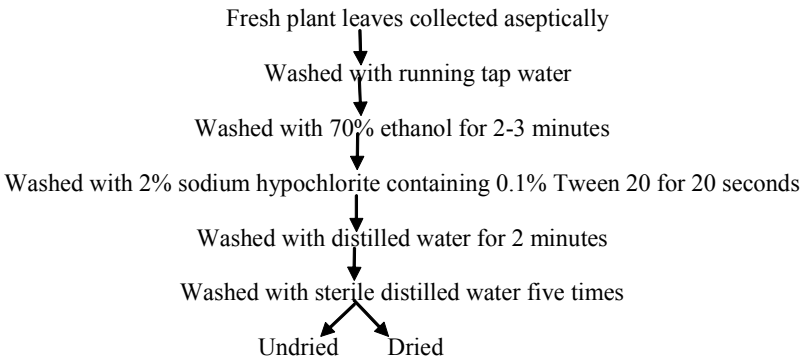

Fig. 1. The procedure for sample pre-treatment is shown below.

Nutrient broth for *Bacillus amyloliquifaciens*, *Staphylococcus epidermidis, Escherichia coli*; Trypticase soy broth for *Salmonella enterica ser. typhi*) for further study.

Standardisation of tested microorganisms

The test microorganisms were standardised by using 0.5 McFarland standards. McFarland Standards was used as reference to adjust the density of microbial suspensions so that their number would be within a given range. 0.5 McFarland gives approximate cell density of 1.5 x 10^8 CFU/ml, having absorbance of 0.132 at wavelength of 600 nm. For preparation of the 0.5 McFarland standard, 0.05mL of barium chloride ($BaCl_2$) (1.17% w/v $BaCl_2.2H_2O$) was added to 9.95 ml of 0.18M H_2SO_4 (1.0% w/v) with constant stirring. The McFarland standard tube was tightly sealed to prevent loss by evaporation and stored for up to 6 months. To aid comparison the test and standard were compared against a white background with a contrasting black line or by measuring the absorbance with that of the standard[14].

Production and evaluation for antimicrobial metabolite from fungi

Potato dextrose broth was used for antimicrobial metabolite production from endophytic fungi, 500mL Erlenmeyer's flasks each containing 200 mL PDB autoclaved at 121^{0}C for 15 minutes and inoculated with five days old colony of a fungal isolate grown on PDA. The inoculated flasks were incubated at 25^{0}C for 5-7 days under stationary condition. Then centrifuged at 10000 rpm for 10 min. Antimicrobial activity of culture supernatant (100µL/well) and broth (100µL/well) was tested by agar well diffusion method against test organisms.

Screening of endophytic fungi for antimicrobial activity by agar well diffusion method

In the agar well diffusion method, plates containing the media according to the test organism were inoculated with test organism and spreaded with sterile swabs. Wells of 7mm were made with sterile cork borer into agar inoculated plates. 100µL of test metabolite from each of the fungal isolate was poured into a well of the inoculated plates. The plates thus prepared were left at room temperature for ten minutes allowing the diffusion of the extract into the agar[15]. After incubation for 24 hrs at 37^0 C, for bacteria and 3-4days at 27^0 C for fungi, the plates were observed. If antibacterial and antifungal activity was present on the plates, it was indicated by an inhibition zone surrounding the well containing the fungal metabolite. The zone of inhibition was measured and expressed in millimetres. Antibacterial and antifungal activity was recorded if the zone of inhibition was greater than 8 mm [16].

Screening for enzymatic activity

Determination of urease activity

Fungal isolates were inoculated onto the urea agar slants and incubated for 3-5 days at 25^{0}C. After incubation slants were observed for change in redish pink colour indicating positive urease activity[17].

Determination of amylolytic activity

The isolates were inoculated on the sterile plate containing nutrient agar (NA) supplemented with 0.2% starch as substrate (pH 6.0). After incubation the culture were treated with Gram's iodine, which allow the formation of clear halos around the colony[18].

Determination of esterase activity

The medium containing peptone 10.0g/l, NaCl 5.0g/l, $CaCl_2.2H_2O$ 0.1g/l, agar 18.0g/l, pH 7.4 was prepared for determining esterase activity. To the sterilized culture medium, previously sterilized Tween 80(as lipid) was added in a final concentration of 1% (v/v). Endophytic fungal isolates were inoculated to the sterile medium. The precipitation of ester compound around the colony indicates the presence of esterase enzyme[18].

Screening for dye degradation

Endophytic fungal isolates were spot inoculated onto screening medium supplemented with malachite green (0.01%) and incubated at 25^{0}C for 3-5 days. Clear zone around the fungal spot indicated dye degradation[11,19].

Antibiotic susceptibility pattern of endophytic fungi

Antibiotic susceptibility pattern of those endophytic fungal isolates which showed best antimicrobial activity and enzymatic activity was determined by adopting Kirby-Bauer disc diffusion method[20] by antibiotics obtained from Hi media Laboratories Pvt. Ltd. Mumbai, India. Broth cultures of endophytic fungus was prepared using Potato dextrose broth and adjusted to 0.5 Macfarland standards. All the cultures were inoculated into potato dextrose agar plates using sterile cotton

swab. Standard antibiotic discs, Nystatin, Miconazole, Clotrimazole, Ketoconazole, Fluconazole and Itraconazole were placed on PDA plates and incubated at 25^{0}C for 3-4 days. After incubation, antibiotic susceptibility pattern was determined by measuring the zone of inhibition[11].

Identification of endophytic fungi

For the identification of endophytic fungal isolates, fungal colonies were grown on PDA medium at 25^{0}C for 7 days and following characteristics: colony characteristics (i.e. colour, exudates produced, growth of the colony), sporulating structures (conidial head, types of conidiogenous cells, arrangement of conidia, sporangial head, types of spores, pycnidia, accervuli, sporodochia, ascocarps etc.) were recorded and identified by following various manuals and monographs

RESULTS AND DISCUSSION

Isolation and antimicrobial activity of endophytic fungal isolates

Endophytic fungi were isolated from different plants. The isolates were differentiated on the basis of their colony morphologies; two-three distinct colonies were observed and selected from each sample. A total of twenty endophytic fungal isolate were finally selected, purified and maintained and used for further analysis. The agar

Table 1. A list of bacterial and fungal strains used in present study

Test Bacterial Srains				Test Fungal strains	
Gram negative Bacteria	MTCC No.	Gram positive Bacteria	MTCC No.	Fungal strains	MTCC No.
Escherichia coli	723	*Staphylococcus epidermidis*	435	*Aspergillus fumigates*	4163
Salmonella enterica ser. Typhi	3216	*Bacillus amyloliquifaciens*	1488	*Aspergillus* sp.	1344
				Candida albicans	3017

Table 2. Antimicrobial activity of endophytic fungal isolates

S. No.	Isolate	Tested microorganisms (ZOI in mm)						
		B.amyloliquifaciens	*S.epidermidis*	*S.typhi*	*E.coli*	*C.albicans*	*Aspergillus sp.*	*A.fumigatus*
1.	P_1	-	-	-	-	-	-	-
2.	P_2	-	-	20	-	27	25	-
3.	P_3	-	-	-	-	20	-	13
4.	P_4	-	-	20	-	-	24	15
5.	P_5	-	-	-	-	-	-	-
6.	P_6	-	-	-	-	-	21	-
7.	P_7	-	-	-	-	-	-	-
8.	P_8	-	-	-	11	-	-	-
9.	P_9	12	-	-	-	-	-	-
10.	P_{10}	-	-	-	-	-	-	-
11.	P_{11}	-	-	-	-	28	-	-
12.	P_{12}	-	-	-	-	25	-	-
13	P_{13}	-	10	-	-	-	-	-
14.	P_{14}	-	-	-	-	-	10	-
15.	P_{15}	-	-	-	-	-	-	11
16.	P_{16}	-	-	-	-	-	-	-
17.	P_{17}		-	-	-	12	-	-
18.	P_{18}	-	-	18	-	-	-	-
19.	P_{19}	-	-	-	-	-	-	10
20	P_{20}	-	-	-	-	-	-	-

Number indicates the sizes of zone of inhibitions (ZOI) in mm and '-' No activity

well diffusion method was used to assess the antimicrobial activity of twenty isolated fungal cultures against 7 pathogenic strains including 4 bacterial (Gram-positive *Staphylococcus epidermidis* and *Bacillus amyloliquifaciens* and Gram-negative *Escherichia coli* and *Salmonella*

Table 3. Enzymatic and biodegradation activity of endophytic fungal isolates

S. No.	Isolate	Urease	Amylase	Esterase	Malachite green
1.	P_1	-	-	-	+
2.	P_2	-	-	-	-
3.	P_3	+	-	-	-
4.	P_4	+	+	+	+
5.	P_5	+	+	+	+
6.	P_6	+	-	+	-
7.	P_7	+	-	-	+
8.	P_8	-	-	-	-
9.	P_9	-	-	-	-
10.	P_{10}	-	+	+	-
11.	P_{11}	-	-	-	-
12.	P_{12}	+	-	-	-
13.	P_{13}	-	-	-	-
14.	P_{14}	-	+	+	-
15.	P_{15}	+	+	-	-
16.	P_{16}	+	-	-	-
17.	P_{17}	-	-	+	-
18	P_{18}	+	+	+	-
19.	P_{19}	+	+	+	-
20.	P_{20}	+	+	+	-

+ Producer; -Nonproducer

Table 4. Morphological characteristics of selected fungal isolates

S. No.	Isolates selected	Colony characteristics	Morphological characteristics
1.	P_2	Greenish blue	Conidospore arising from foot cell, conidia on phialides on vesicals
2.	P_3	Black colonies	Spherical conidia
3.	P_4	Blue green colonies	conidia in long chain on repeatedly branched conidiophores
4.	P_{18}	Whitish colony	cottony appearance, raised colony, spherical conidia

Table 5. Antibiotic susceptibility pattern of fungal isolates

S. No.	Antibiotics	Symbol	concentration	Selected endophytic fungal isoltates (ZOI in mm)			
				P_2	P_3	P_4	P_{18}
1.	Ketoconazole	KT	50 µg	R	R	R	15
2.	Clotrimazole	CC	10 µg	R	R	R	R
3.	Miconazole	MIC	30 µg	R	R	R	R
4.	Itraconazole	IT	30 µg	R	R	R	18
5	Nystatin	NS	50 µg	R	R	R	R
6	Fluconazole	FLC	10 µg	R	R	R	R

'R'- Resistance; 'ZOI'- Zone of inhibition

enterica ser. typhi) and 3 fungal (*Aspergillus fumigatus*, *Aspergillus* sp. and *Candida albicans*) strains. Out of 20 isolates tested, some isolates were found to exhibit antimicrobial activity against indicator strains as shown in table 2.

The isolates P_2 and P_4 showed activity against three tested pathogenic strains whereas isolates P_3 exhibited activity against 2 tested strains out of 7. Isolate P_1, P_5, P_7, P_{10}, P_{16}, P_{20} did not exhibit antimicrobial activity against any of the test strains as shown in Figure 2.

The spectra of inhibition were different among the isolates selected. Out of the 20 isolates, isolate P_2 showed maximum zone of inhibition against *C. albicans* of 27mm, followed by *Aspergillus* sp. (25mm), and minimum against *S. typhi* (20mm); isolate P_4 exhibited maximum zone of inhibition against *Aspergillus* sp. (24mm), followed by *S. typhi* (20mm), and minimum against *A. fumigatus* (15mm) and P_3 isolate showed maximum zone of inhibition against *C. albicans* of 20mm, and minimum against *A. fumigatus* with zone of inhibition 13mm (Figure 3).

The isolates which showed activity against only 1 tested strain were isolate P_6, P_9, P_{11}, P_{12}, P_{13}, P_{14}, P_{15}, P_{17}, P_{18}, P_{19}. Isolate P_6 showed zone of inhibition against *Aspergillus* sp. with zone of inhibition of 21mm, but failed to show inhibition against all other; P_9 showed inhibition against *B. amyloliquifaciens* with zone of inhibition of 12mm; P_{11} and P_{12} showed inhibition against *C. albicans* with zone of inhibition of 28mm; P_{11} showed maximum inhibition against *E. coli* with zone of inhibition of 28mm, 25mm; and P_{13} showed zone of inhibition against *S. epidermidis* of 10mm, P_{14} showed inhibitory activity against *Aspergillus* sp.(10mm); P_{15} showed activity against *A. fumigatus* with zone of inhibition of 11mm; P_{17} showed activity against *C. albicans* with zone of inhibition of 12mm; P_{18} showed activity against *S. typhi* with zone of inhibition 18mm; P_{19} showed zone of inhibition against *A. fumigatus* with zone of inhibition 10mm as shown in (Fig 3). Similar work was performed by Radji *et al.* [24], they tested the antibacterial properties of isolated endophytic fungi isolated from surface sterilized leaves and small branches of *Garcinia mangostana* plant found in Indonesia against *Staphylococcus aureus* ATCC 25923, *Bacillus subtilis* ATCC 6633, *Escherichia coli* ATCC 25922, *Pseudomonas*

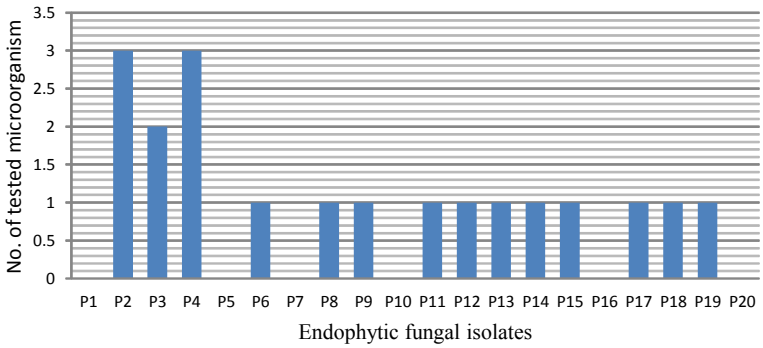

Fig. 2. Number of tested pathogenic strains inhibited by isolated endophytic fungi

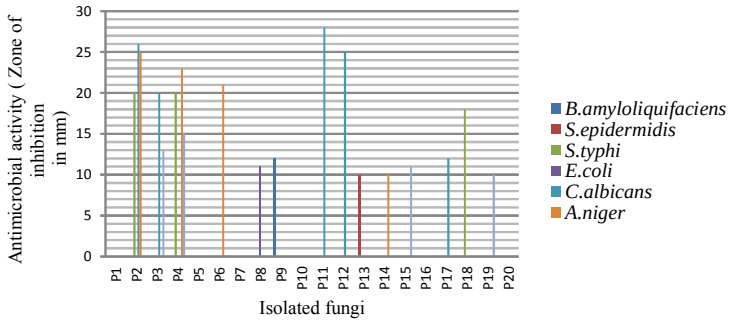

Fig. 3. Antimicrobial activity of fungal isolates cultures against pathogenic strains by agar well diffusion method

aeruginosa ATCC 27853, *Salmonella typhi* ATCC 14028 and *Micrococcus luteus* (ATCC 10240). The zone of inhibition of isolated endophytes was 6.6mm for *Escherichia coli and* 6.7mm for *Salmonella typhi* whereas in present study the zone of inhibition against *Escherichia coli* 11mm and against *Salmonella typhi* was 20mm. In present study the isolated endophytic fungal isolates exhibited more activity (in mm) against fungi (in mm) than bacteria. In vitro experiments on antifungal activities of the isolates against pathogenic fungi showed that endophytic fungi also posses the ability to inhibit the growth of several plant pathogenic fungi by the production of diverse microbial metabolites including antibiotics.

Other endophytic bacterial filtrates which showed low or lack of antimicrobial activity in the bioassays may have active compounds but probably in smaller amounts and/or the screened filtrates could yield more potent compounds once they had undergone some purification[21]. Also, extracts which showed no antimicrobial activity in these assays may be active against other microbes which were not tested[22].

Fig. 4. Antimicrobial activity of fungal isolate P_2 against (a) *C. albicans* (b) *Aspergillus* sp. (c) *S. typhi*

Fig. 5. Cultural and morphological characteristics of fungal isolate

Enzymatic and Biodegradation activity of fungal isolates

In the present study 11(55%) fungal isolates were urease producers, 8 (40%) were amylase producers, while 9 (45%) were esterase producers as shown in table 3. In case of 20 fungal isolates, only 4 (20%) isolates were positive for malachite green degradation. Clear zone around the colony indicates the degradation of dye as shown in table 3. Amylase positive isolates indicated starch degradation on starch agar plates. Plant tissue store starch as a food source and the endophytes can consume the starch before other new colonizers appear. Clear halo on Tween 80 plates indicated esterase activity of the endophytes. Esterase breaks down fats into fatty acid and glycerine, but it also brings the reversible reaction. In reversible reaction, esterase enzyme helps the plant in production of saponine which gives a significant medicinal property to the plant[18].

Identification of endophytic fungus

For fungus identification colony characteristics such as colour, exudates produced, growth of the colony, sporulating structures were recorded and identified (table 4 and figure 5). Tayung *et al.*[23] was identified endophytic fungi by observing their colony characteristics and molecular characterization. On the basis of morphological, Colonial and Lactophenol cotton blue P_2 were partially identified as *Penicillium* sp. and P_4 belongs to *Aspergillus* sp. Wang *et al.*[10] identified various genera of fungi from endophytes including *Phoma, Aspergillus, Pestalotiopsis* and *Alternaria.*

Antibiotic susceptibility pattern of fungal isolates

The results for antibiotic susceptibility pattern of fungal strains are shown in table 5 and figure 4. Ketoconazole was found to be active against isolate P18 with inhibition zone diameter of 15mm and was found to be effective against isolate P2, P3, and P4. Clotrimazole, Miconazole, Nystatin and Fluconazole did not show any activity against isolate P2, P3, and P4. Itraconazole was found to be active against P18 (18mm) and showed no activity against isolate P2, P3 and P4. Emergence of antibiotic resistance among the pathogenic microorganisms limits trement options. Antibiotic resistant genes, in addition to clinical pathogens, are also present in environmental isolates which are horizontally transferred to other microorganisms[12].

CONCLUSION

In conclusion, endophytic microorganisms are a very promising source for production of bioactive compounds. They are a poorly investigated group of microorganisms that represent an abundant and dependable source of bioactive and chemically novel compounds with potential for exploitation in a wide variety of medical, agricultural, and industrial arenas. The mechanisms through which endophytes exist and respond to their surroundings must be better understood in order to be more predictive about which higher plants to seek, study, and spend time isolating microfloral components. This may facilitate the product discovery processes. Further investigations are suggested in order to classify the microorganisms and exploit the potential of the substance produced to inhibit pathogenic microorganisms.

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