

Bio-prospecting fungal endophytes of high altitude medicinal plants for commercially imperative enzymes

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ABSTRACT

Endophytic fungi have been a focal point of research as repository of extreme chemical diversity. Isolation of fungal endophytes from medicinal plants has led to detection of plethora of novel agents encompassing bioactive potential. In the present work, *Withania somnifera*, *Ocimum basillicum* and *Syzygium aromaticum* from high altitude region were selected for bio-prospecting of fungal endophytes. 14 fungal endophytes were recovered from different parts of *Withania somnifera*, *Ocimum basillicum* and *Syzygium aromaticum*. Maximum fungal colonization was recovered from *Withania somnifera* and *Syzygium aromaticum* (42.8 %). In the preliminary screening for production of commercially important enzymes including protease, amylase, cellulose and asparaginase activity, all fungal endophytes of *Ocimum basillicum* exhibited potent activity. However, In case of proteolytic activity, #9SASTD exhibited maximum proteolytic potential. Maximum amylase and cellulase production was observed in #2SASTD and #14WSLF respectively. Interestingly, isolates from *Withania somnifera* and *Syzygium aromaticum* exhibited potent asparaginase activity with maximum potential in #22WSLD. Thus, the data clearly indicates the potential of high altitude medicinal plants as source of endophytic repository that can be taken as measure to prevent exploitation of endangered medicinal plants for commercial use. Further studies are warranted for characterization of the fungal isolates.

KEY WORDS: ASPARAGINASE, ENDOPHYTE, *OCIMUM BASILICUM*, *SYZYGIUM AROMATICUM*, *WITHANIA SOMINIFERA*

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INTRODUCTION

Endophytic fungi comprise a group of heterogeneous fungi which live asymptotically inside the plant tissues without showing any sign of their existence (Strobel and Daisy, 2003; Muller *et al.*, 2016). Fungal endophytes are considered to be “Gold Mine” of novel bioactive compounds with immense therapeutic potential in the pharmaceutical sector. Various anticancer, anti-inflammatory, antioxidant, antimalarial drugs have been reported from fungal endophytes that are currently being exploited as control measure for various medical conditions such as hypercholesterolemia, Leukemia, renal failure, cardiovascular disorders (Gouda *et al.* 2016; Raviraja *et al.* 2006). Fungal endophytes are also proved to mimic the bioactive properties of the host plant due to the horizontal gene transfer (Strobel, 2002; Strobel *et al.* 2004; Jia *et al.* 2016; Venieraki *et al.* 2017; Huang *et al.* 2018). Potential of medicinal plants from high altitude region in pharmaceutical industry is undebatable. Over exploitation of medicinal plants has been a threat of increase in number of endangered plants. Hence, fungal endophytes inhabiting the plants with medicinal values are under exploitation by various groups of researchers in the process of finding a novel chemical scaffold with therapeutic potential (Kapoor and Saxena, 2016; Kapoor and Saxena, 2018).

The present study was oriented towards the exploration of fungal endophytes inhabiting *Withania somnifera*, *Syzygium aromaticum* and *Ocimum basilicum* of Uttarakhand region, India. The main rationale behind selection of these plants from Uttarakhand region lays within the fact that endophytic mycoflora of Uttarakhand region is very sparsely explored for bioactive entities.

MATERIALS AND METHODS

PLANT SAMPLE COLLECTION AND ISOLATION OF ENDOPHYTIC FUNGI

Healthy parts (stem and leaves) of medicinal plants viz. *Withania somnifera*, *Syzygium aromaticum* and *Ocimum basilicum* were collected from Kanhaiya Vihar region, Dehradun during winter season. The samples were transported to laboratory in sealed pouches and stored at 4°C. For the isolation of endophytes, surface sterilization of stems and leaves were carried out by dipping in 0.1% sodium hypochlorite solution for 2-3 min followed by 70% Ethanol for 1 min and then subsequent washing in 30% Ethanol for 30-45 sec. The surface sterilized samples was cross sectioned into small pieces of 1-2 mm size aseptically and were inoculated on to pre sterilized Potato Dextrose Agar (PDA) plates.

The plates were then incubated at 26±2°C, 16h/8h light/dark condition for 8-10 days. The plates were regularly monitored for any fungal growth. The fungal hyphae emerging out of the segment was transferred to fresh PDA plate aseptically with the help of inoculation loop to obtain pure culture (Mitchell *et al.* 2008).

SECONDARY METABOLITE PRODUCTION

All the fungal endophytes were subjected to secondary metabolite production by following the method of Raviraja *et al.* 2006. Briefly describing, 5mm mycelial plug of 4-5 day old active culture was inoculated into 100 ml pre-sterilized Potato Dextrose Broth (PDB) followed by incubation at 26±2°C, 120 rpm for 7-10 days. After the culmination of incubation period, the culture filtrate was separated from mycelial mass by filtration through Whatman filter paper No. 4 followed by centrifugation at 10,000 rpm for 10 min. The supernatant so obtained was stored at 4°C till further use.

SCREENING OF ENZYMATIC ACTIVITIES

For the protease activity, 1 % skim milk agar plates were prepared and 5 mm wells were punched out by sterile cork borer. To each well, 30 µl of each culture filtrate was added followed by incubation at 37°C degrees for 24 h. Non-inoculated PDB served as control. After incubation, a clear zone around the wells indicates the proteolytic activity. The zone diameter was measured and expressed as Mean ± SD.

(a) Cellulolytic activity:

Cellulase activity was assessed by preparing Modified Czepak Dox (MCD) agar plates supplemented with 1 % carboxymethyl cellulose (CMC). 30 µl of each culture filtrate was dispensed in 5 mm well prepared by sterile cork borer in MCD-CMC agar plates. The plates were incubated at 37° C for 18-24 h. Un-inoculated PDB served as control. After the incubation is over, the plates were flooded with aqueous congo red solution. After 15 min, appearance of yellow zone around the fungal colony indicated Cellulolytic activity (Lingappa *et al.* 1962). The zone diameter was measured and represented as Mean ± SD.

(b) Amylolytic activity:

Amylase activity was assessed by preparing the 1% starch agar plate followed by preparation of 5 mm well with the help of pre-sterilized cork borer. Briefly, 30 µl culture filtrate of each fungus was loaded into the wells followed by incubation at 37° C for 18-24 h. Un-inoculated PDB served as control. After the incubation, the plates were flooded with 1 % iodine solution. Appearance of clear zone around the well indicated amyolytic

activity (Hankin and Anagnostakis, 1975). The zone diameter was measured and represented as Mean \pm SD.

(c) Asparaginase Activity assay:

Production of Asparaginase by the fungal endophytes was assessed by modified Ditch plate assay (Mahajan *et al.* 2013). Briefly, L- asparaginase-agar (2 %) plates containing phenol red (0.009 %) were prepared and each plate was divided into four quadrants followed by preparation of 5mm wells in each quadrant using sterilized cork borer. Further, 30 μ l of culture filtrates of each fungal endophyte were loaded onto the premade wells in L-asparaginase agar plates. The plates were incubated at 37°C for 24h. After the culmination of incubation period, the plates were observed for the pink halo formation around the wells. The zone diameter was recorded and expressed as Mean \pm SD.

RESULTS

A total of 14 fungal endophytes were recovered from different parts of *Withania somnifera*, *Ocimum basillicum*

and *Syzygium aromaticum* (Table 1, Figure 1). Maximum fungal colonization was recovered from *Withania somnifera* and *Syzygium aromaticum*(42.8%) followed by *Ocimum basillicum*(14.2%). The host tissue of each plant sample exhibited a variation in colonization of the endophytic mycoflora. The maximum colonization of fungal endophytes was observed in leaf (57.1%) followed by stem (28.5%) and internal tissue/vascular tissue in the stem (14.2%). No endophyte was recovered from the stem and stem internal tissue of *Withania somnifera* and leaf of *Ocimum basillicum* (Table 2).

SCREENING OF BIO-ACTIVITIES:

In the preliminary screening of protease, amylase and cellulase activity, both the isolates recovered from *Ocimum basillicum* were found to be positive for proteolytic, amylolytic and cellulolytic activity. Further, the isolates recovered from *Syzygium aromaticum* were potent producers of amylase and cellulase. Out of 14 isolates, 7 fungal endophytes were found to exhibit proteolytic activity with maximum potential in #9SASTD

Table 1. Showing culture code of fungal endophyte, plant name, plant part and place of plant collection

S. No.	Culture Code	Plant part	Plant Name	Place of collection
1.	#16OPSTD	Stem	<i>Ocimum basillicum</i>	Kanhaiyavihar, Dehradun
2.	#9OPSTITD	Stem internal tissue	<i>Ocimum basillicum</i>	Kanhaiyavihar, Dehradun
3.	#6WSLD	Leaf	<i>Withania somnifera</i>	Kanhaiyavihar, Dehradun
4.	#9 WSLD	Leaf	<i>Withania somnifera</i>	Kanhaiyavihar, Dehradun
5.	#10 WSLD	Leaf	<i>Withania somnifera</i>	Kanhaiyavihar, Dehradun
6.	#14 WSLD	Leaf	<i>Withania somnifera</i>	Kanhaiyavihar, Dehradun
7.	#22 WSLD	Leaf	<i>Withania somnifera</i>	Kanhaiyavihar, Dehradun
8.	#24 WSLD	Leaf	<i>Withania somnifera</i>	Kanhaiyavihar, Dehradun
9.	#7SALD	Leaf	<i>Syzygium aromaticum</i>	Kanhaiyavihar, Dehradun
10.	#16SALD	Leaf	<i>Syzygium aromaticum</i>	Kanhaiyavihar, Dehradun
11.	#2SASTD	Stem	<i>Syzygium aromaticum</i>	Kanhaiyavihar, Dehradun
12.	#9SASTD	Stem	<i>Syzygium aromaticum</i>	Kanhaiyavihar, Dehradun
13.	#15SASTD	Stem	<i>Syzygium aromaticum</i>	Kanhaiyavihar, Dehradun
14.	#16SASTITD	Stem Internal tissue	<i>Syzygium aromaticum</i>	Kanhaiyavihar, Dehradun

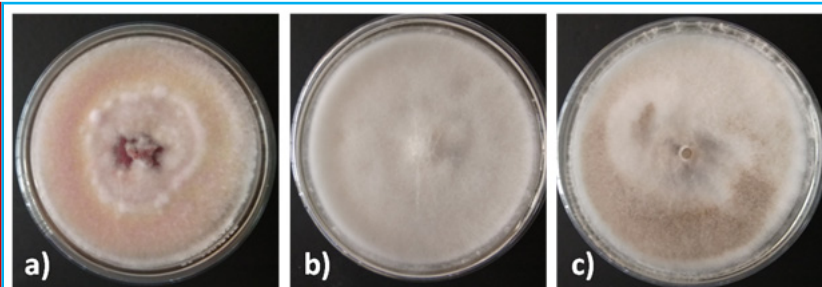


FIGURE 1. Fungal endophytes isolated from different parts of medicinal plants collected from Uttarakhand

Table 2. Summary of endophytic fungi isolated from different tissues of host plant

S. No	Host plant	Endophytic fungi			Total
		Leaf	Stem	Stem internal Tissue	
1.	<i>Withania somnifera</i>	6	0	0	06
2.	<i>Syzygium aromaticum</i>	2	3	1	06
3.	<i>Ocimum basilicum</i>	0	1	1	2
	Total	8	4	2	14

Table 3. showing bioactivity profiling of culture filtrates of fungal endophytes expressed as zone size in mm

S. No	Culture code	Mean zone size (mm)			
		Protease	Cellulase	Amylase	Asparaginase
1.	#16OPSTD	8 ± 0	11 ± 0	7 ± 0	-
2.	#9OPSTITD	11 ± 0	10 ± 0	9 ± 0	-
3.	#6WSLD	-	15 ± 0	12 ± 0	14.33 ± 0.57
4.	#9 WSLD	-	-	-	-
5.	#10WSLD	-	-	-	-
6.	#14WSLD	9 ± 0	16 ± 0	11 ± 0	18.67 ± 0.57
7.	#22WSLD	-	-	9.5 ± 0.70	21.67 ± 0.57
8.	#24 WSLD	-	-	-	-
9.	#7SALD	14.5 ± 0.70	8 ± 0	14 ± 0	-
10.	#16SALD	-	10 ± 0	8 ± 0	16 ± 0
11.	#2SASTD	15 ± 0	10 ± 0	16.5 ± 0.70	11.33 ± 0.57
12.	#9SASTD	15.5 ± 0.57	11 ± 0	13 ± 0	18.33 ± 0.57
13.	#15SASTD	-	10 ± 0	9.5 ± 0.70	-
14.	#16SASTTD	7 ± 0	9 ± 0	11 ± 0	-

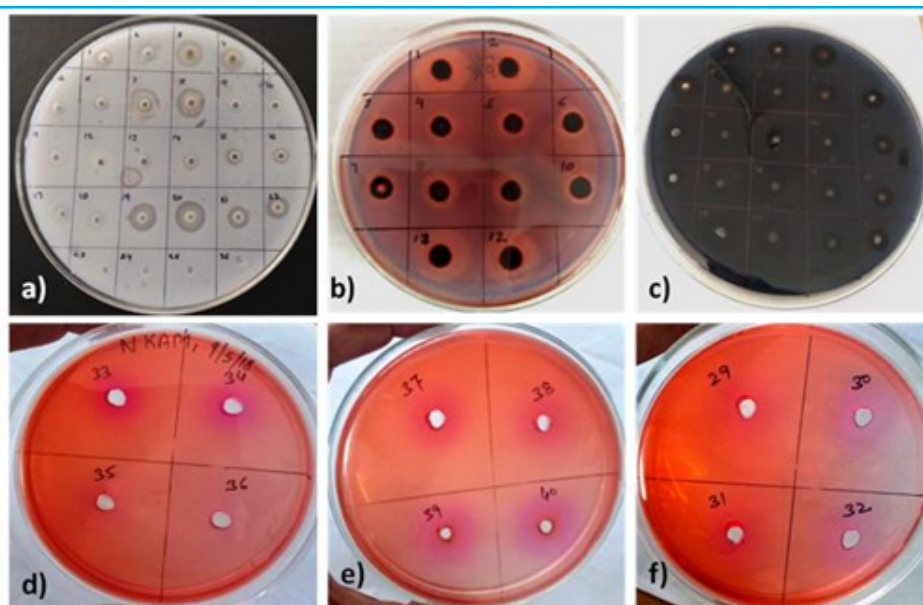


FIGURE 2. Enzyme production by endophytic fungal isolates: a) Proteolytic activity, b) cellulase activity, c) amylase activity, d)-f) L-asparaginase activity of culture filtrates of different fungal endophytes

closely followed by #2SASTD and #7SALD. Furthermore, #14WSLD and #16OBSTD was weak producer of proteolytic enzymes. Further, all the isolates of *Syzygium aromaticum* were found to positive in case of amylase and cellulase activity. However, maximum amylase production was observed in #2SASTD and maximum cellulase production was exhibited by #14WSLF followed by #6WSLF (Table 3, Figure 2). In case of asparaginase production assay, #22WSLD exhibited maximum asparaginase production with zone size of 21.3 mm followed by #14WSLD and #9SASTD with zone size of 18.6 mm and 17.6 mm respectively. Moderate level of enzyme production was observed in #16SALD and #6WSLD with zone size of 16 mm & 14.3 mm respectively (Table 3, Figure 2). Further, the isolates of *Ocimum basillicum* did not showed the activity.

DISCUSSION

Endophytes have been targeted as bioactive repository with immense potential for industrial and pharmaceutical interventions. Among the different types, fungal endophytes have found a niche of being a sublime resource for harnessing novel bioactive agents against spectrum of disorders (Strobel, 2003; Gunatilaka, 2006). Fungal endophytes produce diverse chemistry of molecules depending upon the host plant requirements against different kinds of stresses. Thus, choosing a plant for sampling to isolate a fungal endophyte is a crucial aspect. For this reason the medicinal plants documented in traditional indigenous preparations provide an ample group as repository of fungal endophytes. Among the geographically diverse medicinal plants, high altitude inhabiting medicinal plants have found their own niche in ethnopharmacology (Rajagopal *et al.* 2012). Owing to their immense bioactive potential, high altitude medicinal plants have been exploited to an extent of endangerment. Thus we hypothesize that, high altitude medicinal plants may be explored for endophytic fungal diversity and can further be screened for bioactivities (Chutuloo and Chalannavar, 2018)

The present study was undertaken to bio-prospect the fungal endophytes from medicinal of high altitude regions of Uttarakhand for pharmaceutical interventions. *Withania somnifera*, *Ocimum basillicum* and *Syzygium aromaticum* were selected for endophyte isolation based on their ethno-pharmacological potential. We observed higher colonization fungal endophytes in *Withania somnifera* and *Ocimum basillicum*. Isolates were further screened for industrially imperative enzymes. Interestingly, endophytic fungal isolates exhibited varying degree of enzyme production. These results corroborate our hypothesis that high altitude medicinal plants may

be explored for isolation of fungal endophytes with potent bioactivities. The present study shows promising signs for further purification and evenness of enzymes which may found application in pharmaceutical industry.

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