Research Article

Phytochemical Screening and Extracellular Enzymatic Enumeration of Foliar Endophytic Fungal Isolates of *Centella asiatica* (L.) Urban

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ABSTRACT

The escalating demand of bioactive compounds in the global market poses a serious threat to medicinal plants and their natural habitat. Bioprospecting endophytic fungi materialize promising frontage to come over these tribulations since these are agile in producing imperative and novel bioactive compounds. During present investigation eight foliar endophytic fungi were isolated from *C.asiatica* (L.) Urban (Mandookparni/Indian Pennywort) belonging to apiaceae family. The fungal isolates were screened for extracellular enzymes viz., amylase, cellulase, laccase, lipase, and protease on solid media. 62 % of endophytic fungi showed positive results for lipase; 87% for amylase; 37% for both laccase and protease and none for cellulase. The qualitative screening for phytochemicals from the ethyl acetate extract of the endophytic fungi revealed presence of phenols, flavonoids, tannins, alkaloids, terpenoids, saponins, steroids, carbohydrates, fats and protein. The study clearly suggests that different endophytic fungi hold good potential of producing diverse range of phytochemicals and extracellular enzymes which mediate plant defense.

Keywords: Endophytic fungi, *Centella asiatica,* Extracellular enzyme, Phytochemicals.

INTRODUCTION

The term "endophytic fungi" refers to an organism that lives within plant tissue by forming symbiotic relationship with the host.[1] Endophytic fungi are among the most resourceful groups of secondary metabolite producers that play important biological roles for human life. They are potential sources of novel secondary metabolites for exploitation in pharmaceutical industry, agriculture, and in environmental applications. Fungal endophytes indirectly promote plant growth by producing various secondary metabolites and enzymes, which are responsible for adaptation of plants to abiotic stresses and biotic stresses.[2,3] Large number of enzymes are derived from fungi including pectinases, xylanases, cellulases, lipases, proteinases and laccases etc. These are widely used in food, beverages, confectionaries, textiles and leather industries. Endophytic fungi seem to be an emerging source of medicinally and industrially important metabolites. Therefore, the present study was undertaken to determine the ability of endophytic fungi derived from leaf of *C. asiatica* (L.) Urban for production of various phytochemicals and extracellular enzymes.

MATERIALS AND METHODS

Isolation of endophytic fungi

The green plants of *C. asiatica* were collected from Medicinal Plant Research and Development Centre, Pantnagar, India in the month of April. Leaf explants of *C. asiatica* were washed under running tap water followed by immersion in 2% Tween 20 for 10-15 min. with recurrent agitation. This is eventually followed by washing with sterile water 4-5 times. It was further surface sterilized using 70% ethanol for 1 min. followed by 2% sodium hypochloride for 30 sec, then again in 70% ethanol for 30 seconds and then washed with sterile water 3-4 times. Pieces of the leaf were then placed on potato dextrose agar (PDA) medium amended with streptomycin (50mg/L) to eliminate bacterial growth it was then incubated at 28±1°C until fungal growth appeared on the plate. Hyphal tips of the fungus were then picked up from each plate, inoculated onto another PDA medium plate, and incubated at 28±1°C.

Fungal identification

The endophytic fungal isolates were stained with lactophenol cotton blue, and were morphologically identified with the help of a microscope.

Phytochemical screening

Phytochemical screening of the crude extract of foliar endophytic fungi isolated from *C. asiatica* (L.) Urban was performed to assess its major primary and secondary metabolites. The standard methods were adopted to screen the phytochemicals proposed by Harborne and Sofowora.[4,5]

Extracellular enzyme assay

The endophytic fungi isolated from leaf of *C. asiatica* were qualitatively analysed for five different extracellular enzyme assays *viz.* cellulase, amylase, protease, lipase and laccase following Hankin and Ananostakis, (1975) with slight modifications.[6] The functional role of extracellular enzymes produced by the fungal endophytes was evaluated by inoculation of discs of fungal hyphae on the solid media with dissolved substrates for 3-5 days and adding reagents to the plates to detect the remaining test substrate. Amylase activity was determined using soluble starch in glucose yeast extract peptone agar (GYP) and lipase activity by supplementing tween-20 medium in

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peptone agar medium. Protease and laccase activity was assayed using gelatin and 1-napthol respectively in GYP agar media. Cellulase activity was determined using Na-carboxymethyl in cellulose yeast extract peptone agar medium. After incubation for 3-5 days at 28±1°C, enzyme production by endophytic fungi was measured by measuring the width of clearing zone or colour reaction zone in mm.[7] The total of three replicates of each treatment were assayed taking non-inoculated plates with substrates as negative controls.

Statistical Analysis: All the experiments were performed in triplicates and the means were analyzed statistically with two way Analysis of Variance (ANOVA).

RESULTS AND DISCUSSION

A total of 8 endophytic fungi were isolated from leaves of *C. asiatica*. The isolated strains were identified as *Colletotrichum gloeosporioides, Colletotrichum* sp. *Fusarium* sp., *Curvularia* sp., *Nigrospora* sp., *Alternaria* sp., *Aspergillus* sp., *Fusarium equiseti*. All the identified endophytic fungal isolates belonged to Ascomycota. Other workers also reported endophytic isolates belonged to Phylum Ascomycota.[8,9]

Phytochemical Screening

The Phytochemical analysis has been carried out in several plant species but very few reports are available on endophytes.[10] Therefore, in the present study, ethyl acetate extract of foliar endophytic fungi of *C. asiatica* was screened to validate the status of phytochemicals present in the endophytic fungal extracts. The phytochemical analysis showed the presence of different phytochemicals listed in table 1.

Interestingly, endophytic fungi viz. *C. gloeosporioides* and *Curvularia* sp. showed presence of all the phytochemicals screened. *Fusarium* sp., *Colletotrichum* sp., *Nigrospora* sp. and *Alternaria* sp. also contained most of the tested phytochemicals. *Aspergillus* sp. harboured phenolics, flavonoids, proteins and carbohydrates.

Alkaloids, terpenoids and proteins were absent in *F. equiseti*. The present finding is in consistence with earlier studies where different endophytes showed the presence of different phytochemicals *viz*., saponins, phenolics, alkaloids, flavonoids, tannins, fats, steroids, proteins, carbohydrates and terpenoid compounds.[11-17]

The use of endophytic fungi as a storehose of phytochemicals could be a valuable source for the development of antimicrobial, insecticidal, antioxidant and anticancer agents.[18]

A catalog of the various phytochemicals produced by each endophytic fungi helps in the assortment of endophytes for extraction of the useful metabolites.

Table 1: Phytochemical analysis of ethyl acetate extract of foliar endophytic fungi of *C. asiatica.*

Isolates	Phytochemicals									
	Phenolics	Flavonoids	Alkaloids	Terpenoids	Saponins	Proteins	Tannins	Steroids	Carbo-hydrates	Fats
Colletotrichum gloeosporioides	+	+	+	+	+	+	+	+	+	+
Colletotrichum sp.	+	+	+	-	+	+	-	-	+	-
Fusarium sp.	+	+	+	+	-	+	+	+	+	-
Curvularia sp.	+	+	+	+	+	+	+	+	+	+
Nigrospora sp.	+	+	+	-	-	+	-	-	+	+
Alternaria sp.	+	+	+	+	-	+	+	-	+	+
Aspergillus sp.	+	+	-	-	-	+	-	-	+	-
Fusarium equiseti	+	+	-	-	+	-	+	+	+	+

+= Presence, – =Absent; Data is three replicates of each sample.

Table 2: List of foliar endophytic fungal isolates producing enzyme

S. No	Endophytic fungal isolates	Extracellular Enzyme				
		Lipase	amylase	Protease	Laccase	Cellulase
1	*Colletotrichum gloeosporioides*	+	+	+	+	-
2	*Colletotrichum sp.*	-	+	-	-	-
3	*Fusarium* sp.	+	+	+	-	-
4	*Curvularia* sp.	+	-	-	-	-
5	*Nigrospora* sp.	+	+	+	+	-
6	*Alternaria* sp.	-	+	-	-	-
7	*Aspergillus* sp.	+	+	-	-	-
8	*Fusarium equiseti*	-	+	-	+	-

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Extracellular Enzyme Production

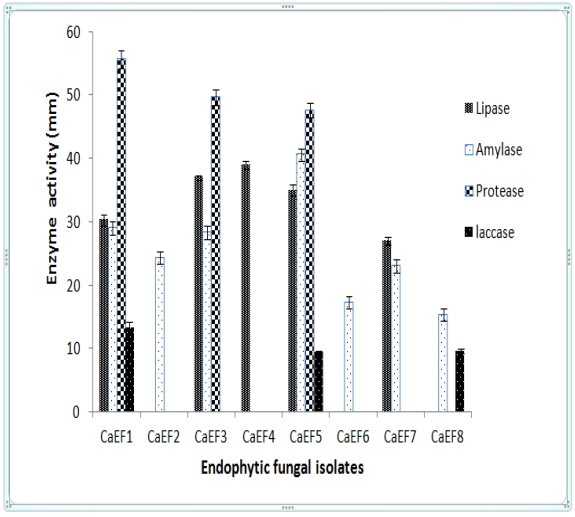

Figure 1: Production of fungal enzymes by foliar endophytic fungi from *C. asiatica.* CaEF1: *C. gloeosporioides*; CaEF2: *Colletotrichum* sp. CaEF3: *Fusarium* sp.; CaEF4: *Curvularia* sp.; CaEF5: *Nigrospora* sp.; CaEF6: *Alternaria* sp; CaEF7: *Aspergillus* sp.; CaEF8: *Fusarium equiseti*. Critical Difference at 5% and Standard error mean of Endophytic Fungi (EF), Extracellular Enzyme (EE) and interaction (EF×EE) is 1.052, 0.744 and 2.105; 0.372, 0.263 and 0.754 respectively.

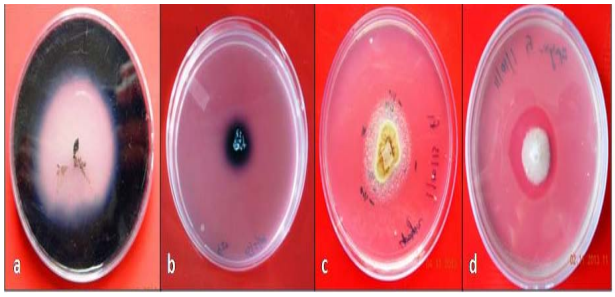

Figure 2: Production of extracellular enzymes by endophytic fungi from *C. asiatica* (a) Amylase, (b) Laccase (c) Lipase and (d) Protease

The significant variation was found in the production of extracellular enzymes by different endophytic fungal isolates (Table 2; Fig.1, Fig. 2). Most of the selected endophytic fungi except *Curvularia* sp. showed significant amylase activity. The maximum amylase production was from *Nigrospora* sp. (40.66 mm) followed by *C. gloeosporioides* (29.0 mm). Choi (2005) reported that all endophytic strains were able to degrade starch.[19] While Sunitha (2013) reported that only 62% of the fungal endophytes were able to produce amylase.[20] The amylase activity exhibited by these endophytic fungi may help the host plant to degrade starch during plant senescence before the appearance of other new colonies. Unlike amylase, protease activity was efficiently shown by only three isolates i.e. *C. gloeosporioides, Fusarium* sp., and *Nigrospora* sp. Maximum protease activity (55.66 mm) was exhibited by *C. gloeosporioides* followed by *Fusarium* sp. (49.67 mm). The result coincides with Venkatesagowda (2012) where high proteolyic activity of endophytic fungi *C. gloeosporioides* was observed.[21]

In the present study most of the endophytic fungi viz., *C. gloeosporioides, Fusarium* sp., *Curvularia* sp., *Nigrospora* sp., and *Aspergillus* sp., were involved in the production of lipase. This is in conformity with the study of Amirita (2012) who reported lipolytic activity of *Curvularia brachysopra*, *C. gloeosporioides* and other endophytic fungi isolated from the medicinal plants.[22] In the current study *Curvularia* sp. was found to be the most efficient producer of lipase, having 39.0 mm enzyme activity. However Colen (2006) found *C. gloeosporioides* as the best producer of lipase.[23]

Laccase are the other important fungal enzymes that are implicated in decomposition of lignins and a variety of lignin model compounds (Archibald and Roy, 1992).[24] Laccase producers are responsible for the removal of toxic phenols from the medium in which these fungi grow under normal conditions (Pragathi).[25] This property may confer the endophytic fungi with antioxidant properties. Only three endophytes isolated from *C. asiatica viz., C. gloeosporioides, Nigrospora* sp. and *Colletotrichum* sp. showed positive result for laccase. *C. gloeosporioides* was found to be significant producer of laccase (13.33 mm) followed by *Colletotrichum sp.* and *Nigrospora* sp. The results are in conformity with Bucher (2004) where not many isolates produced laccase.[26] None of the fungal isolates were efficient in cellulase production. The lack of cellulase activity can be due to endophytic localization of the fungi. An active nature of cellulase might be harmful to the host plant. Other researchers also reported lack of certain extracellular enzymes by endophytic fungi.[27]

Interestingly, many of the endophytic fungi showed positive growth on specific media but were inefficient in producing extracellular enzyme. The most obvious reason to this could be the ability of the fungi to use supplementary materials in the medium rather than the test substrate, or the fungi may be growing exclusively from the carbon source present in the inoculum disc.[7] The phytochemicals and extracellular enzyme production by the endophytic fungi may provide host plant with resistance against pathogens.[28]

CONCLUSION

Endophytic fungi inhabit moderately unexplored area in microbial realm representing a novel source of secondary metabolites. To the best of our knowledge this is the first report of phytochemical screening and extracellular enzyme production by foliar endophytic fungi of *C. asiatica.* The use of endophytic fungi for production of bioactive compounds would reduce the need to harvest the slow growing and endangered plants. Moreover, fungal enzymes are often more stable than the enzymes obtained from plants and animals. Therefore, screening endophytic fungal resources for novel metabolites and enzymes and their impending applications will definitely help to achieve eco-friendly industrial applications.

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