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RESEARCH ARTICLE

ENDOPHYTIC FUNGI: A SOURCE OF NOVEL ENZYMIC ANTIOXIDANTS AND BIOLOGICALLY ACTIVE SECONDARY METABOLITES

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ABSTRACT

Bacterial and fungal endophytes are an attractive group of microorganisms harbouring a number of bioactive natural products. *Ocimum sanctum* is known to have endophytes. Therefore the present project was carried out to evaluate the presence of endophytic fungi from *Ocimum sanctum* and the presence of enzymic antioxidants and phytochemicals. Black tulsi have shown mainly the presence of three enzymes amylase, protease and lipase as compared with that of the green tulsi which produces only lipase. Phytochemical analysis have shown that alkaloids and flavonoids were present both in black and green tulsi endophytes while cardiac glycosides and terpenoids were totally absent in both the types of the tulsi leaves.

INTRODUCTION

An endophytic fungi is a fungal microorganism, which spends the whole or part of its life cycle colonizing inter and /or intra-cellularly inside the healthy tissues of the host plants, typically causing no apparent symptoms of diseases (Petrini, 1991). Endophytes are microbes which colonize living, internal tissues of plants without causing any harm to their host (Bacon and White, 2000). These endophytes protect their hosts from infectious agents and adverse conditions by secreting bioactive secondary metabolites (Carroll and Carroll, 1978; Azevedo *et al*., 2000; Strobel, 2003). Fungal endophytes show a variety of associations with their host plants including from symbiotic or mutualistic or antagonistic or slightly pathogenic (Padhi *et al*., 2013). Their associations with host plant influence ecology and evolution of fungal endophytes and their host plant (Saikkonen *et al*., 1998). Fungal endophyte occurrence is not a host specific rather than single endophytes may be inhabitant of different host plant (Cohen, 2006). Their distribution in the plant tissues affected by their ability to utilize nutritional substances synthesize in different part of the same host (Carroll and Petrini, 1983).

Fungal endophytes are an attractive group of microorganism harbouring a number of bioactive natural products includes flavonoids, alkaloids, terpenoids, peptides, steroids and phenols etc. which could be utilized for exploitation in medical, agricultural and pharmaceuticals. However, most of the fungal endophytes yet to remain discovered. According to an estimate about 4,000 secondary metabolites having an active role in different aspects had been reported from fungi so called "creative fungi" which include species of *Penicillium*, *Fusarium*, *Aspergillus* and *Acremonium* (Dreyfuss and Chapela, 1994; Padhi *et al*., 2013).

Now a day's herbal drugs are prescribed widely even when their biologically active compounds are unknown because of their effectiveness, minimal side effects in clinical experience and relatively low cost (Valiathan, 1998). Medicinal plants provide a special environment for endophytes. Tulsi is known as "The Mother Medicine of Nature", and "The Queen of Herbs". *Ocimum sanctum* (Krishna Tulsi) belongs to the family Lamiaceae which was well known for its medical use. The stems are slender; leaves are oppositely arranged, lanceolate and are usually greenish or reddish, underneath measuring 5cm long and often covered with yellowish bristly hairs especially in the younger parts. Tulsi leaves contain bright, yellow coloured and pleasant odour of volatile oil (0.1 to 0.9%) (Panda and Kar, 1998). The plant has been widely acknowledged for the treatment of coryza, hay asthma, bowel complaints, worm

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infestations, kidney stones in traditional medicine (Bauer *et al.*, 1966; Sood *et al.*, 2005).

Ocimum sanctum plants are considered as sacred plants in India and known for its antimicrobial, immunomodulatory, antistress, anti-inflammatory, antipyretic, antiasthmatic, hypoglycemic, hypotensive and analgesic activities (Dhar *et al.*, 1968; Bhargava and Singh, 1981; Chattopadhyay, 1993). In view of this, *O. sanctum* was selected for isolation of endophytes and their functional characterization. It is also reported that *Ocimum sanctum L* exhibited antibacterial activity against *Klebisella*, *Escherichia coli*, *Proteus*, *Staphylococcus aureus* and *Vibrio cholerae* (Khalid *et al.*, 2008).

The need for new antimicrobial agents, in general, comes from the increasing rate of resistance to existing antibiotics. Thus there is a need to isolate and synthesize the antibiotics, therapeutic agents and agrochemicals from endophytes, which are highly effective, low toxic and have minor environmental impact. Therefore the present project was carried out to evaluate the presence of endophytic fungi from *Ocimum sanctum* and the presence of enzymic antioxidants and phytochemicals.

MATERIALS AND METHODS

Sample Collection

Healthy and mature plants of *Ocimum sanctum* were carefully chosen for sampling. Leaves of black and green tulsi plant were collected in sterile polythene bags and immediately transferred to the microbiology laboratory for further study.

Isolation of Endophytic Bacteria and Fungi from Ocimum sanctum

Isolation of endophytic fungi from *Ocimum sanctum* was carried out using the protocol described by Strobel *et al.*, (1996). The leaves were washed under running tap water. Surface sterilization was carried out with 1.0% sodium hypochlorite (NaOCl) (v/v) for 2 minutes followed by treatment with 90% ethanol for 30 seconds. After this the plant materials were further cleaned by passing through two sets of sterile distilled water to remove the trace amount of disinfectant used for surface sterilization process. For the isolation of bacterial and fungal endophytes, leaf sections were placed on nutrient agar plates and potato dextrose agar plates respectively. The media plates were incubated at 37^0C and 28^0C for bacterial and fungal growth respectively. After the isolates obtained from Nutrient Agar and PDA, the isolates were transferred on respective slants and stored at 4^0C for further analysis.

Enzyme Analysis for Fungal Endophytes

Extracellular enzymes assay was conducted to investigate the production of enzymes by the endophytic fungi. It was assessed by digestion of suspended or dissolved substrate in agar plates after inoculation with 3 mm mycelia plug and incubation for 3-5 days at 28^0C. The diameter of the clear zone was used as a measurement of the amount of enzyme production (Devi *et al.*, 2012; Pavithra *et al.*, 2012).

Amylase

The plate of glucose yeast extracts peptone agar medium (GYP) containing 1% starch solution was prepared (glucose 1gm, yeast extract 0.1 gm, peptone 0.5 gm, agar 10 gm, distilled water 1litre, pH 6). The isolated fungal endophyte was inoculated on the plate and kept for incubation. After 5 days of incubation, the plate with fungal colony was flooded with 1% iodine in 2% potassium iodide. The clear zone surrounding the colony was considered positive for amylase enzyme.

Lipase

The fungus was allowed to grow on peptone agar medium supplemented with tween 20 (peptone 10 gm, NaCl 5 gm, agar 14 gm, distilled water 1 litr, pH 6). The plate was incubated for 5 days at 28^0C. After incubation a clear zone around the colony indicated the presence of lipase enzyme.

Protease

The fungus was inoculated on GYP agar medium amended with 0.4% phenol red and pH 6. The plate was incubated for 3 days, and then was flooded with saturated ammonium sulphate. After incubation undigested phenols were precipitated with ammonium sulphate and digested area around colony would appear as a clear zone.

Phytochemical Analysis of Fungal Endophytes

Fermentation and Extraction

In 250ml Erlenmeyer flask containing 100ml of potato dextrose broth, the fungal endophyte was inoculated. The flask was kept at room temperature for 21 days under stationary conditions with intermittent shaking. After incubation the broth culture was filtered and the mycelia and filtrate were separate out. To the filtrate equal volume of ethyl acetate was added, mixed well for 10 minutes and kept for 5 minutes till the two clear immiscible layers formed. The upper layer of ethyl acetate containing the extracted compounds was separated using separating funnel. The mycelium was grinded properly in a Mortar and Pestle using ethyl acetate as a solvent and then it was filtered using cheese cloth. Both mycelia and culture filtrate extract were pooled together and evaporated to dryness in hot air oven. The extract residue were dissolved in DMSO and stored at 4^0C for further analysis. This extract was used for the presence of secondary metabolites such as alkaloids, cardiac glycosides, flavonoids and terpenoids (Haque *et al.*, 2005; Radji *et al.*, 2011).

Phytochemical Tests

The presence of phytochemicals was carried out according to Handunnettiet *et al.*, (2009); Maniyar and Bhixavatimath (2011).

Alkaloids

Fungal crude extract was dissolved in 2 N HCl. The mixture was treated with a few drop of Mayer's reagent (3ml potassium iodide mixed with 2ml mercuric chloride solution). The creamish precipitate indicates the presence of alkaloids.

Cardiac glycosides

The crude fungal extract was treated with 1ml of $FeCl_3$ reagent. To this solution few drops of conc. H_2SO_4 was added. Greenish blue colour within few minutes indicates presence of cardiac glycosides.

Flavonoids

In a test tube containing 5ml of fungal crude extract, 5-10 drops of diluted HCl and small piece of zinc dust was added and solution was boiled for few minutes. Reddish pink or dirty brown colour was produced indicating presence of flavonoids.

Terpenoids

A 5ml of fungal fungal crude extract was taken in a test tube, to which 2ml chloroform and 3ml conc. H_2SO_4 was added. This solution was allowed to form layer. A reddish brown precipitate at interface indicated the presence of terpenoids.

RESULTS

Healthy and mature plants of *Ocimum sanctum* were carefully chosen for sampling. Leaves of black and green tulsi of the plant were evaluated for the endophytic bacteria and fungi.

Bacterial Endophytes

Total 16 bacterial endophytes were isolated from Tulsi leaves. Out of which 10 isolates were found to be Gram positive bacteria whereas 6 isolates were Gram negative bacteria after performing Gram staining (Table 1).

Table 1 Bacterial Endophytes Isolated from Tulsi

Type	Gram +ve	Gram –ve
Black Tulsi (BT)	5	3
Green Tulsi (GT)	5	3

Fungal Endophytes

Total 7 fungal endophytes were isolated from Tulsi leaves. Out of which 3 isolates were found to be White colour fungi whereas 4 isolates were Green colour fungi after incubation of 7 days at 28^0C (Table 2).

Table 2 Fungal Endophytes Isolated from Tulsi

Type	White	Green
Black Tulsi (BT)	1	2
Green Tulsi (GT)	2	2

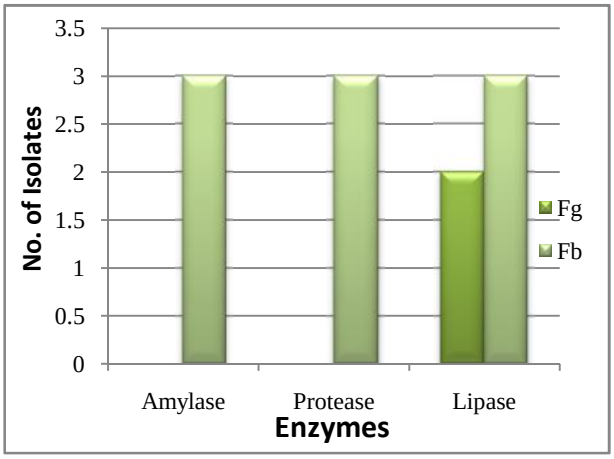
Enzyme Activity of Fungal Endophytes

All 7 fungal endophytes were tested for enzyme assay. The maximum activity of fungal endophytes was observed for the presence of amylase, protease and lipase of Black Tulsi while for Green Tulsi only lipase was found to be produced (Table 3) (Graph 1) (Figure 1-3).

Table 3 Enzyme activity of Fungal Endophytes

Endophytes	Amylase	Protease	Lipase
Fb 1	+	+	+
Fb 2	+	+	+
Fb 3	+	+	+
Fg 1	-	-	-
Fg 2	-	-	+
Fg 3	-	-	+
Fg 4	-	-	-

Where, Fb: Fungal Black Tulsi; Fg: Fungal Green Tulsi.



Graph 1 Enzyme activity of Fungal Endophytes

Figure 1 Amylase Activity by Fungal Endophytes

Figure 2 Protease Activity by Fungal Endophytes

Figure 3 Lipase Activity by Fungal Endophytes

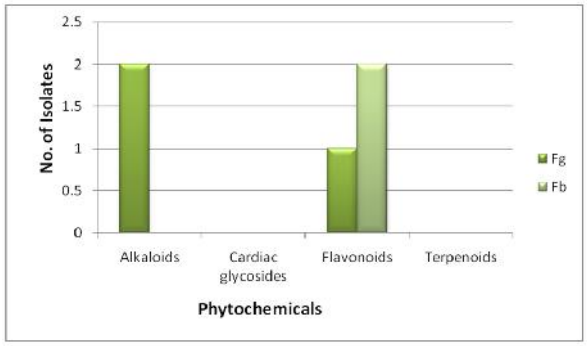
Phytochemical Analysis of Fungal Endophytes

In this study of phytochemical analysis, the fungal endophytes were found to be good source of secondary metabolites. The fungal isolates of some Black and Green Tulsi were found to be positive for alkaloids and flavonoids while none of the isolates showed presence of cardiac glycosides and terpenoids (Table 4) (Graph 2).

Table 4 Phytochemical Analysis of Endophytic Fungi

Endophytes	Alkaloids	Cardiac Glycosides	Flavonoids	Terpenoids
Fb 1	-	-	+	-
Fb 2	-	-	+	-
Fb 3	-	-	-	-
Fg 1	+	-	-	-
Fg 2	-	-	+	-
Fg 3	+	-	-	-
Fg 4	-	-	-	-

Where, '+' indicates presence while '-' indicates absence



Graph 2 Phytochemical Analysis of Endophytic Fungi

DISCUSSION

In the present study *Ocimum sanctum* plant were taken for the isolation of endophytic bacteria and fungi. Black and green colour tulsi leaves were analysed for the isolation of endophytes. It was found that a total of 16 bacterial endophytes were isolated from tulsi leaves which included 10 Gram positive bacteria and 6 Gram negative bacteria. In the present study total 7 fungal endophytes were isolated from tulsi leaves. On the basis of cultural characteristics on Potato Dextrose agar, 3 fungi were found to be white coloured whereas, 4 fungi were appeared as green coloured fungi. These results were correlated with the previous findings of Pavithra *et al*., (2012). In this study there was occurrence of different types of endophytic fungi in media plates.

In the study of Gurupavithra and Jayachitra (2013) the endophytic fungi were isolated from *O. Sanctum* by using Potato Dextrose agar media. Thus, medicinal plants provide a special environment for endophytes. Many previous researchers reported endophytic bacteria and fungi with novel and bioactive natural products obtained from medicinal plants (Song *et al.*, 2005; Huang *et al.*, 2010).

The need for new antimicrobial agents in general comes from the increasing rates of resistance to existing antibiotics. This problem extends beyond the clinical application of antimicrobial drugs. Thus, endophytic fungi and bacteria produce various bioactive compounds. In vitro and in vivo studies would reveal the antibacterial activity of these organisms. Enzyme activity of fungal endophytes showed that black tulsi fungal endophytes have produced amylase and protease while green tulsi endophytes have not produced these enzymes. Apart from this, black tulsi fungal endophytes as well as some of the green tulsi endophytes have produced the enzyme lipase. According to Pavithra *et al*., (2012), endophytes are the potential source of enzyme producer. In their study, they have found amylase, lipase, pectinase and cellulase enzymes isolated from *O. Sanctum* fungal endophytes. Devi *et al.*, (2012) have revealed some enzymes such as amylase, cellulase, laccase, lipase and protease from the endophytic fungi of *Centellaa siatica* plant.

Screening for phytochemicals was carried out for the presence of alkaloids, cardiac glycosides, flavonoids and terpenoids. It was found that black fungal endophytes and some green fungal endophytes have produced flavonoids while alkaloids were present in only green fungal endophytes however neither black nor green fungal endophytes have produced cardiac glycosides and terpenoids.

The need for new bioactive compounds to overcome the growing problems of drug resistance in microorganisms and the appearance of new diseases is of increasing importance. The capability of fungi to produce bioactive metabolites has encouraged researchers to isolate and screen fungi from diverse habitat and environments to search for novel bioactive metabolites. Some endophytes produce phytochemical that were originally thought of as characteristic of the host plant. It appears that genetic interaction between the endophyte and the host has occurred over evolutionary time. In the past two decades, scientists mainly focused on the investigation of endophytic fungal diversity, relationships between endophytic fungi and their host plants, seeking for natural bioactive compounds originated from the endophytic fungi, and improving the productivity of some potential candidates by taking advantage of genetic engineering, microbial fermentation projects and other measures.

Endophytic fungi are a good and reliable source of novel natural compounds with a high level of biodiversity and may also produces several compounds of pharmaceutical

significance, which is currently attracting scientific investigations worldwide. In nature, plants seem to be in a close interaction with endophytic fungi. The production of bioactive compounds by endophytes, particularly those restricted to their host plants, are significant both from the biochemical and molecular point of view. Secondary metabolites produced by endophytes (including those produced by plants) nurtures expectations of utilizing them as alternative and sustainable sources of these compounds. However, the commercial implication of production of desirable compounds by endophytic fungi still remains a future goal.

A deeper understanding of host–endophyte relationships at the molecular and genetic levels, of biogenetic gene cluster regulation, and the effects of environmental changes and culture conditions on gene expression will be helpful for optimizing secondary metabolite production by endophytic fungi under laboratory conditions. Further research at advanced molecular level may offer better insights into endophyte biodiversity and the regulation of fungal secondary metabolism.

CONCLUSION

In the present study 16 bacterial endophytes including 10 Gram positive and 6 Gram negative bacteria were isolated. Total 7 fungal endophytes were isolated from the tulsi leaves. Black tulsi have shown mainly the presence of three enzymes amylase, protease and lipase as compared with that of the green tulsi which produces only lipase.

Phytochemical analysis have shown that alkaloids and flavonoids were present both in black and green tulsi endophytes while cardiac glycosides and terpenoids were totally absent in both the types of the tulsi leaves.

The present study thus, reinforced the assumption that endophytes of ethnomedicinal plants of Nagpur City could be a promising source of antimicrobial substances. The endophytes of medicinal plants provide a good source for compounds of biological activity and endophytes are an untapped reservoir of potentially novel effective drugs. It can be concluded that the antibacterial activity of endophytic fungi are varied from species to species. Endophytes in host plants can stimulate plant growth, increase disease resistance, improve plant's ability to withstand environmental stresses and recycle nutrient.

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