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Diversity and bioactive potential of fungal endophytes associated with *Ocimum tenuiflorum* L. grown under different shade net conditions

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ABSTRACT

Fungal endophytes were screened from tulsi (*Ocimum tenuiflorum*) grown under different shade net conditions. One hundred different isolates of endophytes (belonging to 31 species) were obtained from tulsi from all the three treatments viz., 75% shade, 35% shade and open conditions (no shade). Most number of isolates and species were recorded from tulsi grown under 75% shade net condition. *Phyllosticta capitalensis* was found to be a dominant endophyte. A comparison of endophyte assemblage across different treatments showed that maximum coefficient of similarity between any two treatments was 0.51. *Alternaria* sp. (isolates OTE2 and OTE4) showed activity against both bacterial pathogens with both the isolates inhibiting both Gram positive and Gram negative bacteria. Morphological and phylogenetic analysis using Internal Transcribed Spacer Sequence revealed that isolates OTE2 and OTE4 were *A. burnsii*.

Keywords: Medicinal plants, Endophytes, *Ocimum*, Shade net

INTRODUCTION

One of the important components of the plant microbiome are endophytes that asymptomatically colonize the living internal host tissues, most often intercellularly. Different types of associations exist between fungal endophytes and their host plants that may be defined as mutualistic or symbiotic, antagonistic or slightly pathogenic (Schulz and Boyle, 2005; Arnold 2007). These fungal endophytes mainly belong to *Ascomycetes* or mitosporic fungi (Petrini, 1986). *Pestalotiopsis, Phomopsis* and *Phyllosticta* belonging to *Coelomycetes* are frequently isolated as endophytes and are called as "almost exclusive" endophytes (Bills and Polishook, 1992). Suryanarayanan (2017) considers *Colletotrichum, Pestalotiopsis, Phomopsis, Phyllosticta* and *Xylaria* as multi-host endophytic fungi as these fungi occur in a wide range of plant hosts occurring in different geographic locations while mostly dominating the endophyte assemblages. *Ascomycetes* such as *Chaetomium, Sordaria*, and *Sporormiella* have been reported as endophytes from several hosts although species belonging to these genera are known to be coprophilous (Umali *et al.,* 1999). Phylloplane fungi are also known to occur as endophytes and these include *Alternaria, Aureobasidium* and *Cladosporium*. Sterile forms have been isolated as endophytes from many hosts, and occurrence of these forms is a universal problem faced by mycologists. Culture characteristics such as growth rate, colony morphology and pigmentation are used to differentiate these sterile forms (Bills and Polishook, 1994). Mycologists routinely use molecular techniques to assign some of the sterile endophytes to respective taxa (Sun *et al.,* 2012).

Endophytes are known to produce several bioactive compounds of significant importance with major applications in the field of agriculture, industry and modern medicine such as novel antibiotics, antimycotics, immuno-suppressant and anticancer compounds (Mitchell *et al.,* 2008). Fungal endophytes are being considered to have high ability to produce various novel and known enzymes which could be used in various biotechnological applications including bioremediation (Suryanarayanan *et al.,* 2009; Pimentel *et al.,* 2011).

Schulz and Boyle (2005) showed that endophytes associated with medicinal plants have the potential to produce bioactive chemical constituents that are widely considered to be host-specific and this observation has been corroborated by the fact that endophytes are known to co-evolve with plant hosts and undergo species-specific interactions that allow them to elaborate host-derived metabolites as in the case of taxol production by endophytes of *Taxus* (Garcia *et al.,* 2012; Kumar *et al.,* 2019). Yin *et al.* (2009) showed that an endophytic fungal strain QJ18 produced the bioactive compound gentiopicrin as its host plant *Gentiana macrophylla*. Similarly, extracts from endophytic fungus (Vm-J2) were shown to produce the bioactive ingredient vincamine, which is used in the pharmaceutical industry as a cerebral stimulant and vasodilator, as its host plant *Vinca minor* (Yin and Sun, 2011). Cui *et al.* (2012) isolated ginkgolide B from *Fusarium oxysporum* obtained as an endophyte from *Ginkgo biloba*. Selim *et al.* (2011) showed that 55 of 99 endophytes of medicinal plants showed a broad spectrum inhibitory activity against different pathogenic bacteria and yeasts. Fungal endophytes from medicinal plants have been recognized as a repository of novel metabolites of pharmaceutical importance (Strobel *et al.,* 2004; Wryakrutta *et al.,* 2004; Kumar *et al.,* 2005). The natural products produced by fungal endophytes possess unique structures and excellent bioactivities offering an enormous potential for exploitation in the field of medicine, agriculture and various industries (Tan *et al.,* 2001; Zhang *et al.,* 2006; Kumaresan *et al.,* 2021). Endophytic fungi associated with medicinally

important plant hosts are poorly investigated group of microorganisms that represent untapped pool of bioactive and novel chemical compounds that have tremendous applications in various domains (Rajagopal *et al.,* 2012). Since, fungal endophytes from medicinal plants have been recognized as a repository of novel metabolites of pharmaceutical importance, an attempt was made to identify endophytes associated with *Ocimum tenuiflorum* L. (holy basil or tulsi) and to study their antimicrobial activity *in vitro*. Although endophytes have been isolated from different host plants including several medicinal plants, the present study was carried out to know the effect of different shade (by growing plants under different agro shade nets) conditions on the occurrence of fungal endophytes in tulsi plants.

MATERIALS AND METHODS

Collection of samples

Leaves of tulsi (*Ocimum tenuiflorum* L.) were sampled from 4-month old plants that were grown in mixture of soil, coir pith, dung manure, leaf compost and vermicompost in the ratio of 6:1:1:1:1 under the conditions as mentioned below (Ambujavalli *et al.,* 2019). In treatment 1, the plants were grown in open condition; plants were grown in 35% shade net and 75% shade net in treatments 2 and 3, respectively. Ten leaves were sampled from each plant for each treatment and leaves were collected from 5 plants. Leaves were transported in separate, closed, sterile polythene bags and processed within 2 hours of collection.

Surface sterilization of plant tissue

Leaves collected from the plants were washed thoroughly in running tap water before processing. Lamina segments ($0.5cm^2$) were cut from the middle portion (including the midrib) of healthy leaves and were surface sterilized following a modified method of Suryanarayanan *et al.* (1998). The segments were dipped in 50% ethanol for 30 seconds, followed by 4% NaOCl for 60 seconds and 50% ethanol for 10 seconds. Segments of lamina after surface sterilization were plated on potato dextrose agar (PDA) medium, amended with antibiotic (chloramphenicol 150mg/l), contained in 9 cm diameter Petri dishes. Petri dishes were then incubated for 4 weeks to isolate the endophytes.

Statistical analyses: Coefficient of similarity

Similarity coefficient of endophyte assemblages of any two host species was calculated by following the method of Carroll and Carroll (1978). The coefficient is computed as similarity coefficient = 2w/(a+b), where,

a = sum of density of colonization for all endophytes in a particular treatment.

b = the similar sum for another treatment, and

w = the sum of lower densities of colonization in common between treatments.

Antimicrobial activity

Bacillus subtilis and *Escherichia coli* were used for studying the antimicrobial activity of isolated endophytes. The bacterial cultures were maintained in nutrient agar/broth.

Agar plug method

Fungal isolates were grown on PDA plates and incubated for 10 days at 28-30°C. From a well grown fungal colony, 8 mm agar discs were cut using sterile cork borer. The agar discs were used for bioassay by aseptically transferring on PDA plates containing a lawn of bacterial pathogens. The plates were incubated overnight at 4°C and subsequently kept at room temperature. Control plates contained PDA discs without any fungal growth. Inhibitory activity of the metabolite was evaluated by measuring diameter of the inhibition zone (in mm). The experiments were performed in triplicates.

Partial purification of the active metabolites

Four 250 ml conical flasks containing 100 ml potato dextrose broth (PDB) were prepared. Each flask was inoculated with fungal discs of endophytic *Alternaria burnsii* isolates (OTE2 and OTE4) obtained in the present study. Two flasks for each culture were incubated under static conditions. After fifteen days, the contents of the flasks were filtered through a cotton pad to separate the culture broth from the mycelial mass. The filtrate was extracted in equal volume of different solvents including acetone, ethyl acetate, methanol, benzene, petroleum ether and hexane. The solvent fractions were separated and condensed by evaporation. The dry residue was reconstituted in 2 ml of the same solvent. These fractions were tested for activity using disc diffusion assay (Selim *et al.,* 2011).

Preparation of fungal extract

The isolated fungi were grown in a PDB medium separately. After 21 days of incubation, the fungal extracts were filtered by using Whatman No.1 filter paper, and then separated from the fungal biomass. The filtrate was transferred to a separating funnel and mixed with equal amount of ethyl acetate to separate the active fractions (Nanda *et al.,* 2018; Dalinova *et al.,* 2020).

GC-MS analysis

The GC-MS analysis was carried out for screening of phytocompounds present in the ethyl acetate extracts of endophytic fungus *Alternaria burnsii*. GC-MS analysis was performed at Indian Institute of Food Processing Technology, Thanjavur, Tamil Nadu, India in GC-MS Equipment Scion 436-GC Bruker system following the method of Pakkirisamy *et al.* (2017).

DNA isolation and PCR analysis

About 100 mg of the tissue/mycelium was homogenized using liquid nitrogen and the powdered tissue was transferred to a micro centrifuge tube and processed to obtain the genomic DNA (Sawmya *et al.,* 2013). The PCR amplification of the ITS region was performed using the ITS1 and ITS4 primers (White *et al.,* 1990). The PCR amplification was carried out in a PCR thermal cycler (GeneAmp PCR System 9700, Applied Biosystems) with the following conditions: 98°C for 30s, thermal cycling for 40 cycles (98°C, 58°C, and

72°C for 5, 10 and 15 s respectively), and finally at 72°C for 60s. The amplified products were sequenced and the obtained sequences have been deposited in GenBank database with the accession numbers MN559406 (OTE2) and MN559407 (OTE4).

Phylogenetic analysis

The ITS sequences of the two fungal endophytes were taken up for phylogenetic analysis. A preliminary blast analysis was performed and based on the sequence similarity, a total of 30 nucleotide sequences were selected for further analysis. A multiple sequence alignment was performed using CLUSTALW and manually edited using Mega6 software. By using the Maximum Likelihood method based on the Kimura 2-parameter model and 1000 bootstrap replicates, a final tree was constructed with branch lengths and were measured as the number of substitutions per site. The final tree was generated with branches showing percent of trees in which the taxa were found to cluster together. All positions with less than 95% site coverage were eliminated and the final dataset included 458 positions.

RESULTS

One hundred and fourteen isolates of endophytes (belonging to 31 species) were obtained from tulsi from all the three treatments. Most number of isolates and species were recorded from tulsi grown under 75% shade net condition (**Table 1**). In open conditions, while sterile forms dominated the endophyte assemblage, an isolate of *Phyllosticta capitalensis* along with other hyphomycete forms were recorded. Under 35% shade net conditions, *P. capitalensis* dominated the endophyte assemblage, while *Colletotrichum* sp. was frequently observed. *P. capitalensis* and *Trichothecium* sp. dominated the endophyte assemblage of plants grown under 75% shade net. In total, forty one isolates of sterile forms were recorded. A comparison of endophyte assemblage across different treatments showed that maximum coefficient of similarity (0.51) was observed between treatment 1 and 3 (**Table 2**).

Table 1: Fungal endophytes from tulsi grown open and shade-net conditions

Endophyte	Number of isolates		
	Open	35% Shade-net	75% Shade-net
Alternaria burnsii	-	2	2
Aspergillus fumigatus	3	2	-
Aspergillus niger	1	-	1
Cladosporium cladosporioides	1	1	1
Colletotrichum sp.	-	1	-
Curvularia lunata	1		-
Nigrospora sp.	1	1	1
Phyllosticta capitalensis	1	8	4
Sporormiella minima	-	1	-
Trichothecium sp.	-	1	4
Sterile forms	27(8)	8(2)	41(13)
No. of Isolates	35	25	54
No. of species	14	10	19

Table 2: Comparison of endophyte assemblage of *O. tenuiflorum* grown in different conditions.

Treatment	Open	35% shade	75% shade
Open	1		
35% shade	0.33	1	
75% shade	0.51	0.44	1

Antimicrobial activity of endophytes

Five fungal endophytes isolated from tulsi leaves grown under open and shade net conditions were tested against pathogenic strains of bacteria. *Alternaria burnsii* (isolates OTE2 and OTE4) showed activity against both bacterial pathogens with both the isolates inhibiting both Gram positive and Gram negative bacteria. Both the isolates OTE2 and OTE4 showed activity against all the pathogens when grown in PDA medium (**Table 3; Fig. 1**).

Table 3: Antibacterial activity of endophytic fungi isolated from leaves of tulsi

Endophyte	Inhibition zone (mm)	
	E.coli	***B. subtilis***
OTE2	27	25
OTE3	14	-
OTE4	28	22
OTE5	23	-
OTE14	-	30
OTE15	-	18

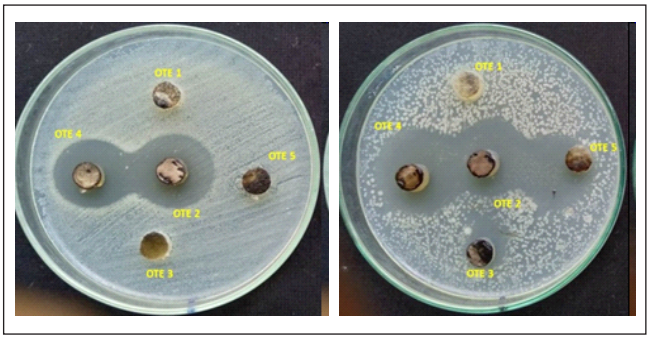

Fig. 1: Antibacterial activity of endophytic fungi isolated from leaves of tulsi against a) *Bacillus subtilis,* b) *Escherichia coli*

The endophytic fungal culture filtrate was extracted in equal volume of different solvents including polar solvents acetone,

ethyl acetate and methanol, and non-polar solvents benzene, hexane and petroleum ether. The solvent fractions were separated and condensed by evaporation. Polar solvent fractions of OTE2 and OTE4 showed better activity against *B. subtilis* and *E. coli* (**Table 4**).

Table 4: *In-vitro* antimicrobial activity of endophytic metabolites extracted using different solvents

Solvent	Activity of OTE2 against		Activity of OTE4 against	
	E.coli	*B. subtilis*	*E.coli*	*B. subtilis*
Acetone	+	+	+	+
Ethyl acetate	+	+	+	+
Methanol	+	+	+	+
Benzene	-	-	+	+
Hexane	+	+	+	+
Petroleum ether	-	-	-	-

+ = presence of a zone of inhibition; − = absence of a zone of inhibition

The chemical constituents in the ethyl acetate extracts of endophytic fungi *Alternatia burnsii* were identified using GC-MS. The active principles with their retention time (RT), molecular formula, molecular weight and concentration (Peak area %) were analysed. The chromatogram of *A. burnsii* extracts is shown in **fig. 2**.

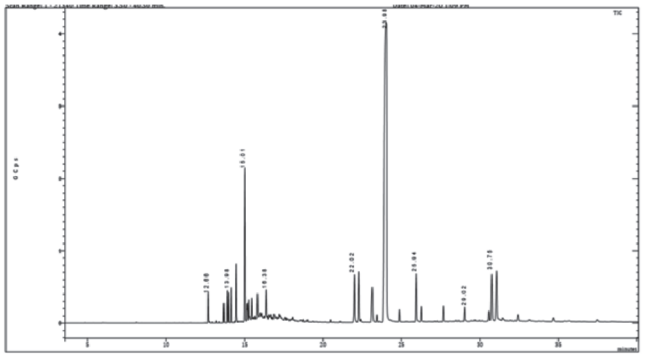

Fig. 2: GC-MS/MS chromatogram of *A. burnsii* (OTE4) extract

Phylogenetic analysis

Internal transcribed spacer sequence based evolutionary history was inferred for the two isolates OTE2 and OTE4 by using the Maximum Likelihood method based on the Kimura 2-parameter model. The tree with the highest log likelihood (-1636.7734) is shown (**Fig. 3**). Based on the maximum likelihood analysis of the ITS sequences, both the isolates OTE2 and OTE4 obtained in our study clustered together in a separate clade with high confidence score and were shown to group together with *Alternaria burnsii* (**Fig. 3**).

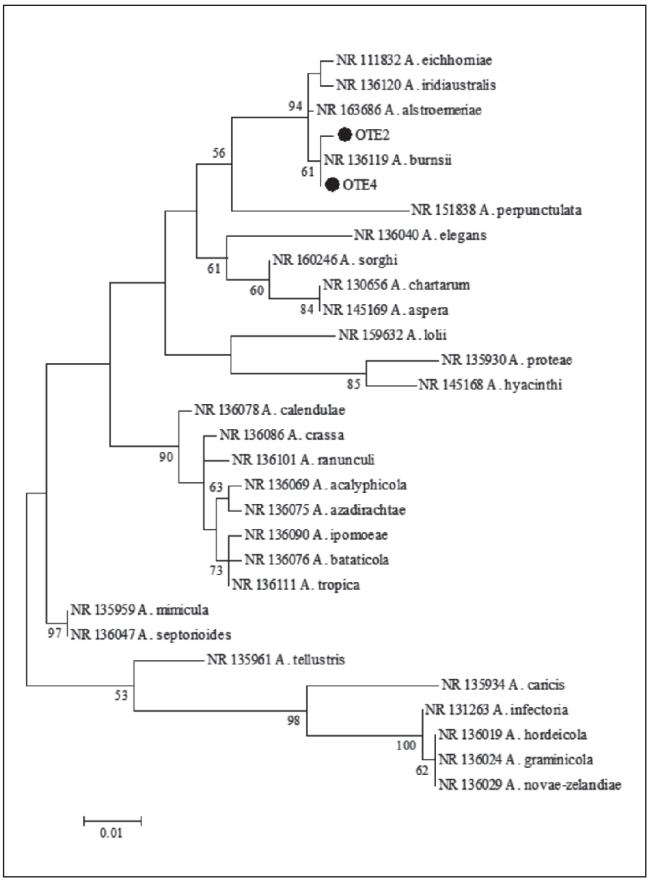

Fig. 3: Phylogenetic analysis of endophytic isolates OTE 2 and OTE 4 by Maximum Likelihood method

DISCUSSION

Mitosporic fungi belonging to *Hyphomycetes* and *Coelomycetes,* ascomycetous fungi and sterile forms were present as endophytes in tulsi. *Basidiomycetes* and *Oomycetes* were not encountered as these fungi rarely occur as endophytes (Petrini, 1986). The contribution by the different groups of fungi to the endophyte assemblages varied with the conditions the host plants were grown. Sterile forms dominated the endophyte assemblages while *Phyllosticta* occurred in plants grown in all three conditions i.e., tulsi grown in open, 35% and 75% agro shade net conditions and dominated the endophyte assemblage of tulsi leaves grown under 35% and 75% shade net (**Table 1**). *Phyllosticta* has been reported as a major endophyte associated with tropical hosts (Brown *et al.,* 1998; Murali *et al.,* 2007) and is a quint essential endophytic genus (Carroll, 1990). Further, *Colletotrichum* along with other genera including *Alternaria, Aspergillus, Cladopsorium, Nigrospora* have also been recorded from medicinal plants of Himachal Pradesh, India (Gautam, 2014). The fact that these fungi (*Colletotrichum* and *Phyllosticta*) occur in many tropical plants species (Suryanarayanan *et al.,* 2018) shows that these are well adapted to an endophytic mode of life.

Though hyphomycete genera such as *Aspergillus, Alternaria, Cladosporium* and *Curvularia* commonly occur as

phylloplane fungi, they are also known to occur as endophytes (Rashmi *et al.,* 2019); even in the present study, these fungi occurred as endophytes (**Table 1**). Earlier studies on fungal endophytes associated with *Ocimum sanctum* also revealed the presence of *Alternaria, Fusarium, Macrophomina* and *Colletotrichum* as frequent colonisers (Kaushik and Chowdhary, 2015). *Alternaria,* particularly the small-spored species, have been isolated frequently during the surveys of endophytes in the xylem and stem tissues of plants, such as *Pinus sylvestris* and *Fagus sylvatica*, as well as in the leaves of some important medicinal plants. Paul *et al.* (2015) isolated *Alternaria burnsii* as endophytes from seed pumpkin (*Cucurbita maxima*) and suggested that endophytic *Alternaria* can also cause disease as a latent pathogen. Reports suggest that phylloplane fungi are capable of penetrating the superficial layers of the leaf and grow out as colonies in plates by surviving the rigorous surface sterilization procedures used for isolating endophytes (Verhoeff, 1974; Cabral *et al.,* 1993). Intriguingly, some coprophilous genera such as *Podospora, Sordaria* and *Sporormiella* are frequently isolated as endophytes (Fisher *et al.,* 1986; Petrini, 1986; Pelaez *et al.,* 1998; Umali *et al.,* 1999; Kumaresan *et al.,* 2006; Rashmi *et al.,* 2019) and in the present study, *Sporormiella* sp., a coprophilous form, were found to occur as an endophyte.

Investigations carried out by Espinosa-Garcia and Langenheim (1990) showed that within a given site, individuals of host species show qualitative difference in their endophyte assemblages. In the present study, it was observed that the endophyte assemblages of tulsi grown under different conditions showed maximum similarity of 51% (**Table 2**) suggesting some preference to the conditions in which they were grown. Additionally, tulsi plants when grown under different shade net conditions showed different growth characters. The plants under open conditions that were exposed to higher light intensity, temperature and lower humidity produced leaves that were smaller in size; while plants kept in 75% shade treatment exhibited larger sized leaves. The difference in the microclimatic conditions may be responsible for the difference in endophyte assemblages found in different treatments. Giauque and Hawkes (2013) based on their studies on grass endophytes concluded that environmental factors related to past and current precipitation were the most important predictors of endophyte communities in the field, while a few studies have shown that the microclimate and microhabitat to influence endophyte richness and diversity (Currie *et al.,* 2014).

Apart from trying to understand the biology of endophytes, another motivation for mycologists to study these fungi is their ability to produce novel bioactive compounds (Gouda *et al.,* 2016). Two isolates of *Alternaria burnsii* obtained in the present study could inhibit the growth of gram negative and gram positive bacteria *in vitro*. In an earlier study, Taufiq and Darah (2018) isolated a total of 148 fungal endophytes from *O. sanctum* and found that 90.5% exhibited inhibitory activity towards at least one test microorganism. Hence, to identify the possible compounds involved in antimicrobial activity we performed a GC-MS analysis of the culture filtrates of the endophytic fungi. Gas chromatography (GC) coupled with a Mass Spectrometer (MS) is commonly used for phyto-chemical analysis due to their sensitivity, stability and high efficiency (Guo *et al.,* 2006). Twenty three compounds were recorded from *A. burnsii* of which diisooctyl phthalate showed a peak area (%) of 56.98. Phthalates are known to possess significant antimicrobial activity (Sulistiyania *et al.,* 2020) suggesting that antimicrobial activity in the present study could be due to higher proportion of disooctyl phthalate. Some of the other compounds identified include cannabinol, trifluoroacetate, benzene, (1-methyldodecyl)-, benzene, (1-ethylundecyl)-, bis (2-ethylhexyl) phthalate, phenol, 2,4-bis (1-phenylethyl)-, benzene, (1-methyldecyl)-, and benzene, (1-ethyldecyl). In an earlier study, a fungal endophyte isolate ST 9.1 isolated from *Nelumbo nucifera* which was shown to produce diisooctyl phthalate also showed significant antibacterial activity (Techaoei *et al.,* 2020).

This study adds on to our existing knowledge of fungal endophytes with regard to their occurrence in medicinal plants. The comparison of endophytes in tulsi in different shade net conditions showed that the endophyte colonization was influenced by microclimate conditions, and specific compounds elicited by fungal endophytes showed significant antibacterial activity.

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