SUMMARY

The state of Meghalaya in the north-eastern region of India falls under the realm of Indo-Burma mega biodiversity hotspot. In Meghalaya ‘Sacred forests’ that are conserved due to the traditional religious beliefs of the ethnic population, not only constitute a major reservoir of the floral and faunal biodiversity, but are also assumed to be treasure troves of novel endophytic fungi inhabiting the diverse plants growing in these forests.

The present study was undertaken to study the endophytic mycoflora associated with five ethno-medicinal plants prevalent in the traditionally preserved ‘Sacred forests’ of Meghalaya, India. These medicinal plants namely, *Potentilla fulgens*, *Osbeckia stellata*, *Osbeckia chinensis*, *Camellia caduca* and *Schima khasiana* were selected based upon their reported usage in the ethnic medicinal practices by traditional medicine practitioners (TMPs) in the state of Meghalaya. Isolation followed by morphological characterization of the endophytic fungi from the root and stem portions of the selected plants yielded a total of 702 fungal isolates. Morphologically, all the isolates were grouped into 16 taxonomic groups. The highest diversity of endophytic fungal taxa was observed for *Camellia caduca* followed by *Osbeckia chinensis*, *Potentilla fulgens*, *Schima khasiana* and *Osbeckia stellata*.

Majority of the endophytic fungal species isolated from the roots and stem portions of the five selected medicinal plants belonged to the Ascomycetes group of fungi. *Talaromyces flavus* was the most dominant fungal species with a total of 167 isolates in case of *Potentilla fulgens*. *Mortierella hyalina* was the most frequently isolated endophyte from *Osbeckia stellata* while *Paecilomyces variabilis* was the dominant fungi in case of
Osbeckia chinensis. *Penicillium* spp. were frequently isolated fungi from *Camellia caduca* and *Schima khasiana.*

The production of extracellular enzymes amylase, cellulase, xylanase, lipase and protease by the five dominant fungal isolates from each of the medicinal plant species was determined using agar-plate based assays and also in liquid media. The production of extra-cellular enzymes was found to be more in liquid medium when compared to the plate based assays. All the endophytic fungal isolates showed production of amylase in submerged liquid culture conditions, with RS07SK showing the highest production after 72 hours of inoculation into the culture medium. The highest cellulase activity (27.3 U hour\(^{-1}\)ml\(^{-1}\)) was shown by endophytic fungal isolate of *Potentilla fulgens* after 48 hours of incubation. The highest xylanase activity (24.7 U hour\(^{-1}\)ml\(^{-1}\)) was shown by endophytic fungal isolate RS07CC, isolated from *Camellia caduca,* after 72 hours of incubation. The production of lipase enzyme by the fungal endophytes gradually declined as the time after inoculation increased from 24 to 120 hours. The highest lipase activity was observed in the case of RS07SK, the isolate from *Schima khasiana,* at 24 hours after inoculation. The production of protein degrading enzyme(s) peaked at 48 hours after inoculation followed by a drastic reduction at 120 hours. The highest protease activity was shown by RS07PF, an isolate from *Potentilla fulgens,* with the value of 34.9 U hour\(^{-1}\)ml\(^{-1}\).

Mycelial biomass production after 120 hours of inoculation in different culture media used for the assay of enzymes in terms of fresh weight was, in the range of 4.4 g/50 ml culture media to 27.1 g/ 50 ml culture media. The highest production of biomass (27.196 g/50 ml) was recorded in case of the endophytic fungal isolate RS07OS, an isolate from *Osbeckia stellata,* in xylan broth medium.
The anti-microbial activity of the extracts of the endophytic fungal isolates showed promising results against four bacterial test pathogens namely *Bacillus cereus*, *Salmonella typhi*, *Escherichia coli*, *Staphylococcus aureus* and a pathogenic yeast species, *Candida albicans*. The inhibition zone size for the fungal fermentation broth against the pathogens ranged from 10-32 mm. The anti-microbial activity increased when the fermentation broth of each of the endophytic fungal isolates were extracted and concentrated in ethyl acetate. The ethyl acetate extracts of the *Penicillium* sp. isolated from *Camellia caduca* showed the highest antimicrobial activity against *Bacillus cereus* with an inhibition zone of 21.3 mm. The highest antimicrobial activity for *Staphylococcus aureus* was exhibited by extract of *Paecilomyces variabilis* isolated from *Osbeckia chinensis* with an inhibition zone size of 29.7 mm. The highest antimicrobial activity for *Escherichia coli* was exhibited by the ethyl acetate extract of the *Penicillium* sp. (13.8 mm) isolated from *Camellia caduca*. The combination of the same *Penicillium* sp. and *Mortierella hyalina* isolated from *Osbeckia stellata* showed an inhibition zone size of 32.5 mm against *Salmonella typhi*, the other gram negative bacterial pathogen used in the study. Extracts of *Paecilomyces variabilis* showed the highest microbicidal activity against the pathogenic yeast strain under study with a inhibition zone size of 17 mm. The activity shown by the ethyl acetate extracts of the two *Penicillium* species in the present study therefore, indicates that these species may be very promising producers of antimicrobial compounds. However, the ability to produce antimicrobials among isolates of the same species may vary, as the *Penicillium* sp. isolated from *C. caduca* in the present study showed an inhibition zone of 32.5 mm against *Salmonella typhi* while the other *Penicillium* sp. isolated from *S. khasiana* produced an inhibition zone of 25 mm.
against the same pathogen. The test pathogens used in this study were resistant to standard antibiotics such as ampicillin and methicillin. The MIC’s of the crude ethyl acetate extracts ranged from 13 - 45 µg/ml. The extracts from Mortierella hyalina isolated from Osbeckia stellata showed the least MIC i.e. 27 µg/ml against B. cereus. It also showed low MIC value for the other gram-positive bacterial pathogen S. aureus. However, the best result of MIC was given by the endophytic fungi isolated from Osbeckia chinensis with a value of 22.3 µg/ml. The ethyl acetate extracts of the metabolites from Mortierella hyalina also showed the lowest MIC values for E. coli, S. typhi and the yeast pathogen Candida albicans with values of 34.67, 13.33 and 28.33µg/ml respectively.

The anti-oxidant potential of the fermentation broths of the endophytic fungal isolates were tested using the FRAP and DPPH assays. In both the assays, Mortierella hyalina isolated from the medicinal plant Osbeckia stellata showed a high anti-oxidant activity with a FRAP value of 1.316 and percent free radical scavenging activity of its fermentation broth at 79.7 %. FRAP values for the Penicillium sp. isolated from Schima khasiana was 1.417, which was the highest amongst the fungal isolates tested.

Molecular characterization of the endophytic fungal isolates using RAPD, PCR-RFLP approaches and sequencing of the ITS1, SSU rRNA and β- tubulin gene sequences revealed fungal isolate RS07OS isolated from Osbeckia stellata to be Syncephalastrum racemosum and isolate RS07OC isolated from Osbeckia chinensis to be a species of Talaromyces. Isolate RS07CC isolated from the medicinal plant Camellia caduca which showed similar colony characteristics as isolate RS07OS, was identified to be a species of
*Penicillium.* The endophytic fungal isolate RS07SK isolated from *Schima khasiana* was identified as *Penicillium purpurogenum.*

Studies on the metabolite production, by these five isolates was performed using agar plug paper chromatography, TLC and LC-MS followed by search in the mycotoxin and secondary metabolite database provided to us by Dr H.Z. Senyuva of Ankara Test and Analysis Laboratory, Turkey. Fusicoccin, an important antimicrobial agent was detected in the culture extracts of RS07PF, RS07OS and RS07SK. Abscissic acid was detected in all the isolates except isolate RS07PF. Shikimic acid was detected in the crude extract of the endophytic fungal isolate RS07SK. Caffeine was detected in the crude extracts of the isolates RS07PF, RS07OS and RS07OC. Indolacetic acid was detected in the isolates RS07PF and RS07OC. Gibberellic acid was also detected in the extracts of the fungal isolates RS07OS and RS07OC. Endophytic fungal isolate RS07CC isolated from *Camellia caduca* endemic to Meghalaya, India, was shown to be unique and distantly related to the other four isolates with regards to its secretome or metabolite profile when compared using the numerical taxonomy system (NTSys software, Exeter, UK).

The study revealed that plants, growing in the unique pristine habitats such as the ‘Sacred groves’ in Meghalaya which have established ethno-medicinal significance among the tribal populations, harbour endophytic fungal forms which show significance in their metabolite production profiles. Thus, these fungal isolates can become microbial factories for bioprospection of biomolecules of biotechnological relevance. It is therefore, suggested that fungal endophytes from diverse plants with ethno-medicinal relevance may be explored from such habitats to bioprospect them as microbial cell factories.

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