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RESEARCH PAPER

Enzymatic Activity of Fungi Endophytic on Five Medicinal Plant Species of the Pristine Sacred Forests of Meghalaya, India

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Abstract Fungal species that establish an endophytic role inside the tissues of medicinal plants are known to produce a wide range of biologically active metabolites and enzymes. In the present study, the most dominant and representative endophytic fungal species of five ethno-medicinal plants prevalent in the pristine sacred forests of Meghalaya, were screened for their ability to produce amylase, cellulase, protease, lipase, and xylanase. Each of endophytic fungal isolates showed a wide range of enzyme activity. Mycelial biomass generation and root colonization, in addition to the enzyme activity of the endophytic fungal isolates, provided insights into their probable origin and ecological roles within the plant host.

Keywords: endophytic fungi, amylase, cellulase, protease, lipase, xylanase, sacred forests

1. Introduction

Enzymes of microbial origin have high biotechnological importance in the processing of foods, manufacturing of detergents, textiles, pharmaceutical products, medical therapy, and in molecular biology [1-3]. Among a large number of non-pathogenic microorganisms capable of producing useful enzymes, filamentous fungi are particularly interesting due to their easy cultivation and high production of extracellular enzymes of large industrial potency. Endophytic fungi occupy a relatively unexplored area in microorganism isolation, and thus represent a new source for obtaining enzymes with different potentialities [4]. Fungi endophytic on medicinal plants or plants that grow in unique and extreme habitats are likely to possess novel enzyme systems that may help in the understanding of their host tissue colonization ability, in view of the competition provided by saprophytes and plant pathogenic fungi. Further, the endophytes colonizing the tissues of plants of economic importance have drawn larger interest after the discovery of *Taxomyces* sp on the cortical tissue of *Taxus baccata*, which is known to produce the anti-cancer drug Taxol [5]. Despite all the anthropogenic disturbances occurring in the natural environment in Meghalaya, especially because of over-exploitation of the ethno-medicinal plants, an estimated forest cover of about 1,000 sq km, accounting for about a five percent area of the state, has been left undisturbed since ancient times due to religious beliefs. These forests, known as 'sacred forests' or 'sacred groves', constitute a substantial portion of private and community forests in the region and also serve as reservoirs of endemic and rare medicinal plants. The present study was undertaken to explore the prevalence of endophytic fungi on the traditionally used medicinal plants of the sacred forests and to assess the potentialities of these fungi in the production of industrially relevant enzymes.

2. Materials and Methods

2.1. Isolation of endophytic fungi from medicinal plants

Five selected ethno-medicinal plants, namely *Potentilla fulgens*, *Osbeckia stellata*, *Osbeckia chinensis*, *Camellia caduca*, and *Schima khasiana* were collected from the Lum Shyllong, Sohra, Mawphlang and Umsaw Nongkhrai 'sacred groves' spread over the state of Meghalaya, India. About 100 surface sterilized root and stem pieces of each

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of the five plants were air dried and flamed before removing the outer layers. Two centimeter long pieces of these roots and stems were placed on petri plates containing water agar as described by Strobel *et al*. (1996) [6] and incubated at 24°C for 7 days. After incubation for 7 days, the hyphal tips of the developing fungi were aseptically removed and placed on potato dextrose agar (PDA) according to the procedure described by Strobel *et al*. (2005) [7]. Growth characteristics in liquid potato dextrose medium, of the most dominant fungal endophyte species isolated from each plant, was measured spectrophotometrically using a protocol modified from Meletiadis *et al.*, (2001) [8] at 450 nm in a UV visible spectrophotometer.

2.2. Plate based assay for extracellular enzymes

The five endophytic fungal isolates isolated from the respective medicinal plants were tested for cellulose and xylanase production using 1% carboxy-methyl cellulose, 1% xylan as carbon source respectively [9,10]. An agar diffusion method incorporating methyl red dye was used as a qualitative assay modified from Downie *et al.* 1994 [9]. Amylase activity was tested using starch-agar plates and lipase activity by Tween-20 (10%) incorporated agar plates. Protease activity was assayed using casein hydrolysis medium, which contained 1% skimmed milk. After incubation at 25ºC for 5 days, the diameter of the clear zone was measured.

2.3. Estimation of the extracellular enzymes in liquid culture medium

Estimation of extracellular lipase and protease enzymes of the endophytic fungal isolates using Skim milk and Tween 20 (Polysorbate 20) broth mediums was carried out as per the protocol described by Ivanova *et al.*, 2009 [11] with minor modifications. The flasks containing the fungal cultures were incubated at 23°C and kept at 120 rpm in a New Brunswick shaker incubator. Starch, carboxy-methyl-cellulose, and xylan broth mediums were used to culture the fungal isolates for estimation of extracellular amylase, cellulase, and xylanase enzymes and the overall amylase, cellulase, and xylanase enzyme production in the broth culture was determined by the 3-5, dinitrosalicylic acid (DNS) method of Miller (1959) [12]. Readings for enzyme production by the endophytic fungal isolates were taken at the time of inoculation *i.e*., 0, 24, 48, 72, 96, and 120 h. Absorbance readings were taken at 540 nm in a UV visible spectrophotometer (Cecil) for lipase and protease enzymes and 600 nm in the case of the DNS method for amylase, cellulase, and xylanase enzymes. One unit of amylase, cellulase, and xylanase was defined as the amount of enzyme required to liberate 1 μg of glucose from the soluble substrate per hour per mL of the culture medium under the assay conditions. One unit of lipase and protease was defined as the amount of enzyme required to decrease the O.D. (optical density) value by 0.001 units per hour per mL of the liquid culture media containing the enzyme substrate under the assay conditions. Mycelial biomass generation by the endophytic fungal isolates was also determined by taking the fresh weight of the fungal mycelia after completion of 120 h of inoculation into the respective media used for each of the enzyme assays.

2.4. Root colonization studies

The endophytic fungal isolates were tested for their ability to colonize host plant tissues (root tissues) upon inoculation of the specific endophyte into the host plant grown in pot cultures. This was carried out to observe their root colonization ability upon selective introduction into host plants made sterile by the use of commercially available bleach as described by Medina *et al.*, 2000 [13]. Control plants not inoculated by the specific endophyte were similarly sterilized by the same protocol and grown along with the treated plants. The host plants selected for the study were under the seedling stage. The soil in which the bleach-sterilized plants were planted was freed from microbial infestation by repeated autoclaving at 121°C at 15 psi pressure at intervals of 5 days. This was carried out to ensure that the spores of bacteria and fungi that may have escaped an initial cycle of steam sterilization will germinate and subsequently become eliminated in the subsequent cycles of autoclaving. Plant roots/seedling stem tissues collected from the wild were also tested for the presence of fungal endophytes. These served as the positive controls for the experiment. The colonization of the endophytic fungal species was visualized with the help of a light microscope upon specifically staining the fungi in the root tissues as per the protocol described by Rai and Varma, 2005 [14]. For assessment of root colonization, the slide method proposed by Giovannetti and Mosse (1998) [15] was followed. The root-pieces (1 cm long) were selected at random from the stained samples and mounted on microscopic slides in groups of 10. The presence of infection was recorded in each of the 10-pieces, and the percentage of infection was calculated as:

$$\% \text{ Colonization} = (\text{Number of root segments colonized} / \text{Total number of root segments observed}) \times 100$$

2.5. Fungal identification

The endophytic fungal isolates were stained with aniline blue, as per the standard protocol described by Cappucino and Sherman (1992) [16], and were morphologically identified with the help of a Motic phase contrast trinocular research microscope model BA-450PH (Feintechnik,

Germany). Molecular characterization of the endophytic fungal isolates, *i.e*., RS07PF, RS07OS, RS07OC, RS07CC, and RS07SK from each of the 5 selected medicinal plants was carried out by PCR amplification of the β-tubulin gene using the universal primers of btub3 and btub4r, as per the protocol described by Huang *et al*. (2009) [17]. The amplicons obtained were sequenced in an Applied Biosystems 3700 Genetic Analyser with BigDye Terminator ver. 3.1. Alignments and phylogenetic analyses were performed using MEGA4 software (Tamura *et al*., 2007) [18].

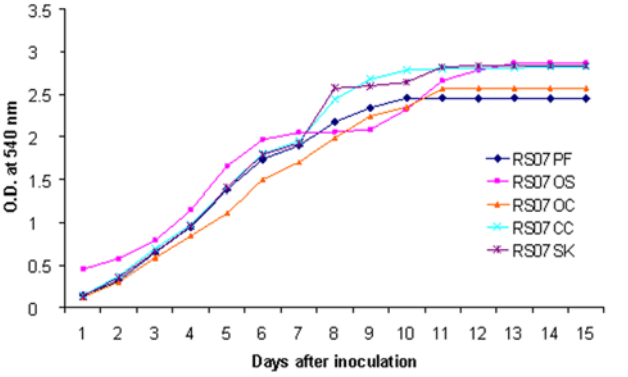

Fig. 1. Growth pattern of the endophytic fungal isolates in liquid potato dextrose media. RS07PF: endophytic fungal isolate of *Potentilla fulgens*; RS07OS: endophytic fungal isolate of *Osbeckia stellata*; RS07OC: endophytic fungal isolate of *Osbeckia chinensis*; RS07CC: endophytic fungal isolate of *Camellia caduca* and RS07SK: endophytic fungal isolate of *Schima Khasiana.* Values are the mean of three replicates.

3. Results and Discussion

There is a considerable variation in the production of extracellular enzymes by the endophytic fungal isolates (Table 1). Endophyte isolated from *Potentilla fulgens i.e.* RS07PF shows high production of lipase and xylanase enzymes with enzyme production ratios of 3 and 2, respectively, whereas the endophytic fungal isolate from *Osbeckia stellata* shows intermediate enzyme production for cellulase, protease, lipase and xylanase with values of 1.72, 1.45, 1.40, and 1.37, respectively. RS07OC showed the highest xylanase production with a value of 2.08 (Table 1). The enzyme production ratios for the two endophytic fungal isolates from *Camellia caduca* and *Schima khasiana* were quite similar (Table 1). These two endophytes produced a strong reaction in terms of the enzyme production ratio in the case of protease, while cellulase, lipase and xylanase production ratios were of medium reaction strength (Table 1). Production of amylase was not detected in the case of all the isolates, except for isolate RS07OC which showed an intermediate reaction with a value of 1.5. Production of cellulase was not detected in the case of endophytic fungal isolates RS07PF and RS07OC (Table 1). The extracellular enzyme production profiles of the endophytic fungi also often suggest their ecological roles as endophytes/latent pathogens or saprobes in their natural environment. The highest cellulase production was shown by the endophytic *Mortierella hyalina* (RS07OS) isolated from *Osbeckia stellata* Buch. -Ham. ex D. Don and the highest xylanase activity was shown by the endophytic isolate RS07CC isolated from *Camellia caduca.* All of the five endophytes showed proteolytic activity, suggesting their possible role as entomo-pathogens. The highest protease activity was shown by the two *Penicillium* sp. isolated from *Camellia caduca* and *Schima khasiana*, which interestingly also belong to the same plant family *i.e*. Theaceae. It is also interesting to note that nearly all of the endophytic isolates failed to produce any visual signs for amylolytic enzymes in agar plate based assays, except for *Paecilomyces variabilis* isolated from *Osbeckia sinensis* which was of medium reaction strength. *Talaromyces flavus* showed a very strong reaction for the production of lipase followed by *Paecilomyces variabilis*, *Penicillium* sp. and *Mortierella hyalina* in respective order. Lumyong *et al.* 2002 [19], while studying the enzymatic activity of the 26 strains of endophytic fungi of six native seedling plant

Table 1. Extracellular enzyme production by the endophytic fungal isolates in agar plate based assay

Endophytic fungal isolate	Morphological identity	Host plant	Enzyme production ratio				
			Amylase	Cellulase	Lipase	Protease	Xylanase
RS07PF	*Talaromyces flavus*	Pf	0	0	3	1.5	2
RS07OS	*Mortierella hyalina*	Os	0	1.72	1.40	1.45	1.37
RS07OC	*Paecilomyces variabilis*	Oc	1.5	0	2.2	1.6	2.08
RS07CC	*Penicillium* sp.	Cc	0	1.62	1.47	2.3	1.6
RS07SK	*Penicillium* sp.	Sk	0	1.55	1.50	2.3	1.45

All values are mean of three replicates. Enzyme production ratio = the ratio of clear zone diameter to that of colony diameter. The extracellular enzymatic reactions were classified into the following four types: (i) strong reaction, the extracellular enzyme ratio was higher than or equal to 2; (ii) medium reaction, the extracellular enzyme ratio was less than 2 but more than 1; (iii) weak reaction, the extracellular enzyme ratio was equal to or less than 1; and (iv) no reaction, there is no reaction at all or the enzyme ratio is 0.
Pf, *Potentilla fulgens*; Os, *Osbeckia stellata*; Oc, *Osbeckia chinensis*.; Cc, *Camellia caduca*.; and Sk, *Schima khasiana*.

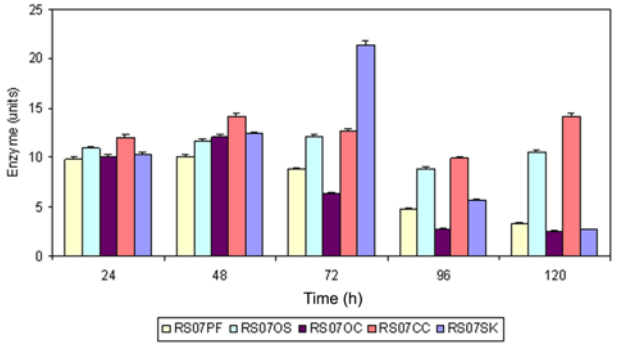

Fig. 2. Extracellular amylase production by the endophytic fungal isolates in liquid media. RS07PF: endophytic fungal isolate of *Potentilla fulgens*; RS07OS: endophytic fungal isolate of *Osbeckia stellata*; RS07OC: endophytic fungal isolate of *Osbeckia chinensis*; RS07CC: endophytic fungal isolate of *Camellia caduca* and RS07SK: endophytic fungal isolate of *Schima khasiana.* Values are the mean of three replicates.

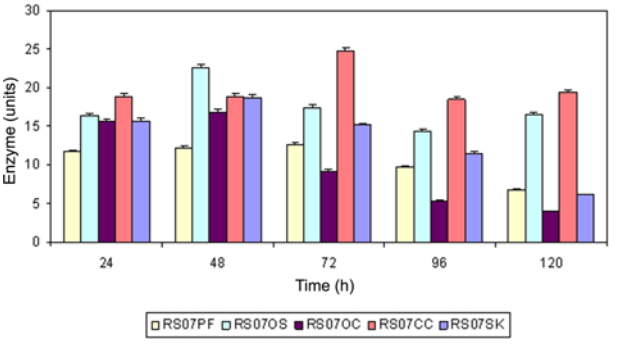

Fig. 4. Extracellular xylanase production by the endophytic fungal isolates in liquid media. RS07PF: endophytic fungal isolate of *Potentilla fulgens*; RS07OS: endophytic fungal isolate of *Osbeckia stellata*; RS07OC: endophytic fungal isolate of *Osbeckia chinensis*; RS07CC: endophytic fungal isolate of *Camellia caduca* and RS07SK: endophytic fungal isolate of *Schima khasiana.* Values are the mean of three replicates.

species of the Doi Suthep-Pui national park in Thailand, reported the ability to produce cellulase, mannanase, proteinase, and xylanase was distributed among the test strains. They further stated that the ability to produce enzymes may be related to the lifestyle abilities of the endophytic fungi being tested. The knowledge of enzyme production by endophytic fungi may provide insights into their possible biotechnological applications and also provide an idea about their life cycles within the plant tissues.

The production of extracellular amylase, cellulase, xylanase, lipase and protease in liquid culture medium is shown in Figs. 2, 3, 4, 5, and 6, respectively. All the endophytic fungal isolates showed the production of amylase in submerged liquid culture conditions, with RS07SK showing the highest production after 72 h of inoculation into the culture medium (Fig. 2). The highest cellulase

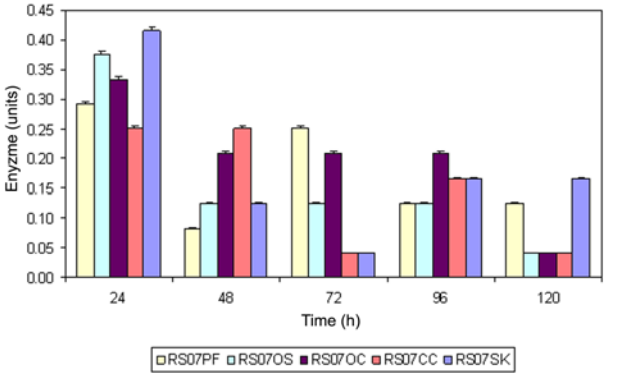

Fig. 5. Extracellular lipase production by the endophytic fungal isolates in liquid media. RS07PF: endophytic fungal isolate of *Potentilla fulgens*; RS07OS: Endophytic fungal isolate of *Osbeckia stellata*; RS07OC: Endophytic fungal isolate of *Osbeckia chinensis*; RS07CC: endophytic fungal isolate of *Camellia caduca* and RS07SK: endophytic fungal isolate of *Schima khasiana.* Values are the mean of three replicates.

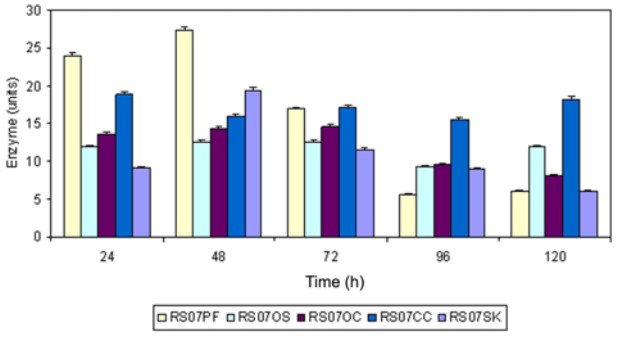

Fig. 3. Extracellular cellulase production by the endophytic fungal isolates in liquid media. RS07PF: endophytic fungal isolate of *Potentilla fulgens*; RS07OS: endophytic fungal isolate of *Osbeckia stellata*; RS07OC: endophytic fungal isolate of *Osbeckia chinensis*; RS07CC: endophytic fungal isolate of *Camellia caduca* and RS07SK: endophytic fungal isolate of *Schima khasiana.* Values are the mean of three replicates.

activity (27.3 U/h/mL) was shown by endophytic fungal isolate of *Potentilla fulgens* after 48 h of incubation (Fig. 3). The highest xylanase activity (24.7 U/h//mL) was shown by the endophytic fungal isolate RS07CC after 72 h of incubation (Fig. 4). The production of lipase enzyme by the fungal endophytes gradually declined as the time after inoculation increased from 24 to 120 h. The highest lipase activity was observed in the case of RS07SK at 24 h after inoculation (Fig. 5). The production of protein degrading enzyme(s) peaked at 48 h after inoculation followed by a drastic reduction at 120 h. The highest protease activity was shown by RS07PF (34.9 U/h//mL) (Fig. 6). Maria *et al.* 2005 [20], while studying the enzymatic activity of the mangrove endophytic fungi of the southwest coast of India, reported that cellulase and lipase activity was present in all fungi, while amylase and protease was present in a few.

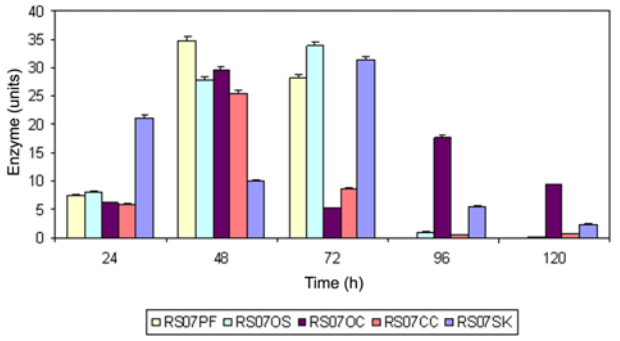

Fig. 6. Extracellular protease production by the endophytic fungal isolates in liquid media. RS07PF: endophytic fungal isolate of *Potentilla fulgens*; RS07OS: endophytic fungal isolate of *Osbeckia stellata*; RS07OC: endophytic fungal isolate of *Osbeckia chinensis*; RS07CC: endophytic fungal isolate of *Camellia caduca* and RS07SK: endophytic fungal isolate of *Schima khasiana.* Values are the mean of three replicates.

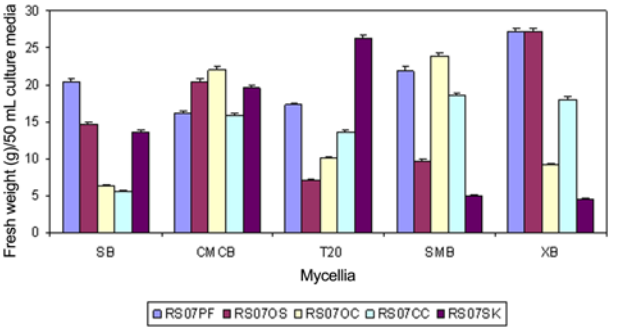

Fig. 7. Mycelial biomass production by the endophytic fungal isolates after 120 h of inoculation. RS07PF: Endophytic fungal isolate of *Potentilla fulgens*; RS07OS: endophytic fungal isolate of *Osbeckia stellata*; RS07OC: endophytic fungal isolate of *Osbeckia chinensis*; RS07CC: endophytic fungal isolate of *Camellia caduca* and RS07SK: endophytic fungal isolate of *Schima khasiana.* SB: Starch broth; CMCB: Carboxy-methyl cellulose broth; T20: Tween 20 broth; SMB: Skim milk broth; XB: Xylan broth. Values are the mean of three replicates.

The production of extra-cellular enzymes was greater in the liquid medium in comparison to the plate based assays (Figs. 2 ~ 6). Enzymes that were not detected in plate based assays for some particular isolates were shown to be produced in liquid culture conditions. This may be due to the increased availability of the substrate per surface area of the fungi under liquid culture conditions. Moreover, the enzyme production in the plate based assay is relative to the colony diameter with that of the clear/halo zone formation around the colonies. For fungi that have a high growth rate in terms of colony diameter, the clear zones or halos created in the agar plates with the specific substrate may be masked and thereby preventing detection of the particular enzyme. Solid media enzyme assays detect enzyme synthesis, release from the mycelium, and activity in the medium following production. Thus the lack of a positive result could mean that either the enzyme is not produced, or that it is produced and not released from the mycelium, or that it is produced and released, but the medium inhibits its detection. Thus, the absence of a reaction in solid media enzyme assays is not absolute confirmation of the inability of a species to produce a particular enzyme [21].

The endophytic fungal isolates showed typical fungal growth characteristics when grown in liquid potato dextrose media (Fig. 1) and also exhibited promising mycelial biomass generation in five different liquid media at different time intervals (Fig. 7). The mycelial biomass production after 120 h 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 (Fig. 7). The highest production of biomass (27.196 g/50 mL) was recorded in the case of the endophytic fungal isolate RS07OS in the xylanase broth medium. This was followed by RS07PF in the same culture media with a biomass of 27.167 g/50 mL. Endophytic fungal isolate RS07SK produced the maximum biomass in the lipase (Tween 20) broth with a value of 26.225 g/50 mL of culture media (Fig. 7). The highest root colonization percentage was shown by isolate RS07OS at 95 per cent after six months of introduction into the host root tissues (Table 2). In each particular case, in order to ascertain whether or not the same introduced fungal species had colonized the root tissues, we also tried to re-isolate the respective endophytic fungi from each of the host plants.

Table 2. Root colonization percentages of the endophytic fungal isolates in their respective host plant roots

Host plant	Endophyte*	Root colonization % after 3 months of inoculation	Root colonization % after 6 months of inoculation	Uninoculated control.	Roots of seedling stage host plants in their respective wild habitats (positive control)
Potentilla fulgens	*Talaromyces flavus*	57	84	Nil	67
Osbeckia stellata	*Mortierella hyalina*	68	95	Nil	56
Osbeckia chinensis	*Paecilomyces variabilis*	45	78	Nil	53
Camellia caduca	*Penicillium* sp.	65	89	Nil	54
Schima khasiana	*Penicillium* sp.	60	90	Nil	58

The figures are mean of three replicates.
*Morphological identity

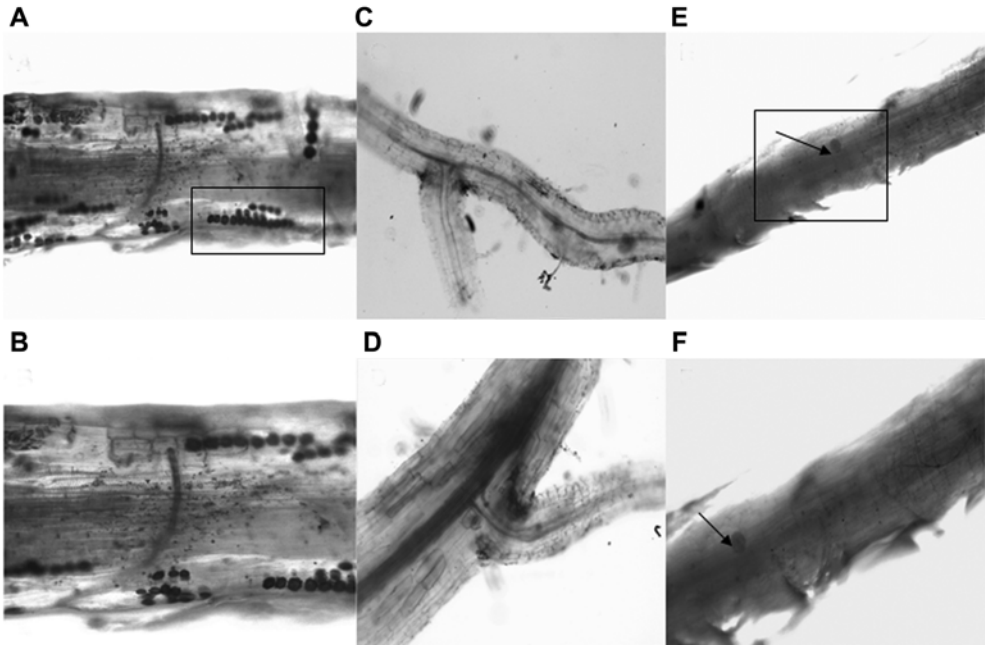

Fig. 8. Colonization of the root tissues of the host plant *Osbeckia stellata* by the endophytic fungal isolate RS07OS as visualized under light microscope. (A, B) *O. stellata* from its wild habitat, showing the presence of endophytic fungi (40 × magnification). (C, D) Roots of the sterilized control plant (*O. stellata*) (40 × magnification). (E, F) Roots of *O. stellata* after 3 months of inoculation with fungal endophyte RS07OS, showing internal colonization by the introduced fungal endophyte (40 × magnification).

All the isolates that were obtained on re-isolation from the host plant roots had the same cultural and morphological characteristics as those of the introduced isolates. Un-inoculated control and the roots of seedling stage host plants in their respective wild habitats served as the negative and positive controls for the root colonization study, respectively (Table 2, Fig. 8). Isolate RS07SK showed the highest biomass generation and lipase activity (Figs. 5 and 7). However, similar patterns could not be observed for other high mycelial biomass generating fungi (*i.e.* RS07OS & RS07PF) in terms of their enzyme activities. Similar observations were made by Johnson *et al.* 1993 [22], while studying the extracellular enzyme production and synthetic lignin mineralization by *Ceniponropsis subvermispora*. They reported that higher enzyme production levels observed with non-limiting nitrogen or carbon did not simply reflect differences in fungal biomass.

The root-colonization percentages of each of the re-introduced endophytic fungal isolates in their respective host plants increased considerably from 45 to 90 days of inoculation. The initial root colonization percentage observed after 45 days of inoculation for the isolates was close to about 60 percent, which increased to around 90 percent during the second sampling at 90 days after inoculation (Table 2). These results indicate that the endophytic fungal isolates are able to rapidly colonize most of their respective host roots within a very short time frame of around 2 months, when most of their natural competitors were eliminated under laboratory conditions. All the host plants were healthy after inoculation and there was a marked increase in the plant growth in the presence of the endophytic fungal association in comparison to the control plants. Moreover, in the uninoculated control plants that were studied for the presence of any fungal association over a period of time, microscopically visible endophytic association was not detected. However, in natural conditions such as the sacred forests of Meghalaya where most of the plants thrive, competition with other related strains of fungi predominant in the habitat of these plants cannot be ruled out. As such we attempted to assess the presence of any endophytic or mycorrhizal type of association with the roots of the host plants isolated from their respective habitats in the wild (Fig. 8). We selected at random host plants in the seedling stage in the wild habitats, of nearly the same height / age as that of our pot experiments and found that the root colonization was 67, 56, 53, 54, and 58 percent for *Potentilla fulgens*, *Osbeckia stellata*, *Osbeckia chinensis*, *Camellia caduca and Schima khasiana*, respectively. We were also successful at re-isolating strains that were morphologically similar to the endophytes under investigation from the roots of the host plants growing in their natural habitat. Rai and Varma, 2005 [14], while studying the growth promoting potential of *Piriformospora indica* on the medicinal plant *Adhatoda vasica*, reported that the root colonization of *A. vasica* by introduced *P. indica* increased with time from 53% after 2 months to

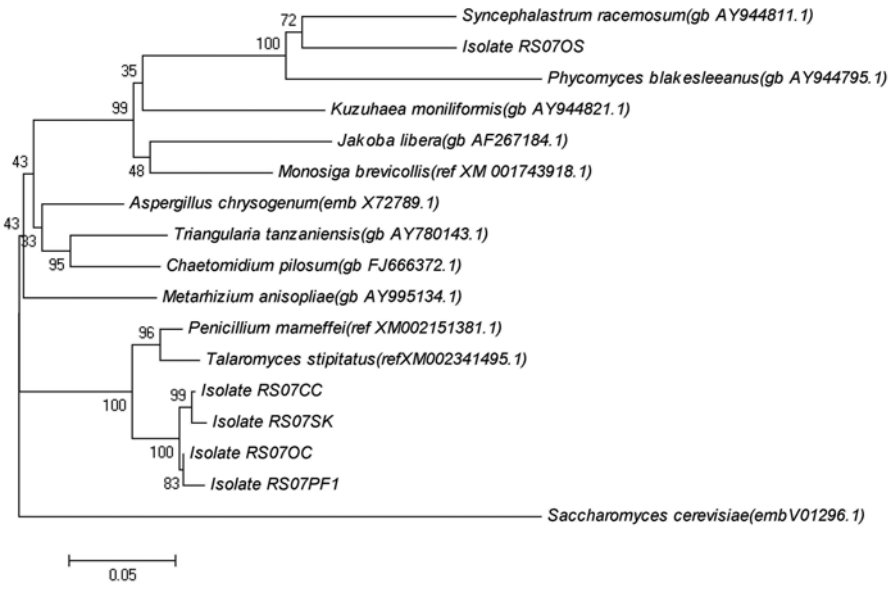

Fig. 9. Evolutionary position of the 5 endophytic fungal isolates with other related fungal species based on β-tubulin gene sequence similarity.

95% after 6 months. They also reported that there was a remarkable enhancement in the growth rate of the plant inoculated with *P. indica*. The growth was very fast up to 2 months and slowed down thereafter. At each observation time, growth was significantly higher for the plants inoculated with *P. indica* versus the control plants.

Molecular characterization of the partial β-tubulin gene revealed the evolutionary position of the five isolates with fungal sequences available in Genbank (NCBI) (Fig. 9). However, it should be noted that the endophytic fungal isolates in the present study demonstrated cryptic morphological characteristics and as such have been referred to in this paper only by their isolate numbers and morphological identity [23].

4. Conclusion

All the endophytic fungal isolates showed production of protease and lipases which suggests that they may be relevant as bio-control agents. To the best of our knowledge this is the first report of extra-cellular enzyme production by non-mycorrhizal filamentous endophytic fungi isolated from ethno-medicinally important plants of the sacred forests of the eastern himalayan state of Meghalaya in India. A process to sterilize plants using bleach treatment was also successfully adapted in this study to generate microbe-free-plant seedlings which may become a fast and inexpensive alternative to the micropropagation of the host plants for use in co-culture studies with endophytic fungi. From an evolutionary perspective, these endophytic fungal strains may have adapted to the respective metabolic machinery of the host tissues to produce biomolecules not only important for their own biology, but also for the host plant's requirements. Moreover, the genetic machinery required to produce cell wall degrading enzymes such as cellulase, may be already present in endophytic fungi prior to the establishment of the symbiotic relationship with the host plant. Further studies at the gene expression level using DNA micro-arrays may help us to elucidate the mechanisms by which the genes responsible for enzyme production are controlled, based upon the factors at play in the natural habitats of the endophytic fungi *i.e.* inside the plant tissues.

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