African Journal of Microbiology Research Vol. 4(9), pp. 766-770, 4 May 2010
Available online http://www.academicjournals.org/ajmr
ISSN 1996-0808 © 2010 Academic Journals

Full Length Research Paper

Penicillium verruculosum RS7PF: A root fungal endophyte associated with an ethno-medicinal plant of the indigenous tribes of Eastern India

R. K. Bhagobaty, S. R. Joshi* and R. Kumar

Microbiology Laboratory, Department of Biotechnology and Bioinformatics, North Eastern Hill University, Umshing, Shillong-793 022, Meghalaya, India.

Accepted 11 March, 2010

***Potentilla fulgens* L., an ethno-medicinal plant of the ethnic tribes of Meghalaya in the Eastern Himalayan range of India was screened for root fungal endophytes. This plant is distributed in the wild habitats and in the traditionally preserved 'Sacred Groves' of Meghalaya in Eastern India, which is of late getting over-exploited for its medicinal value. Isolation of endophytes in water agar plates yielded a unique fungus that had similar morphological characteristics with the members of the genus *Talaromyces*. Scanning and transmission electron microscopic studies of the fungus revealed its surface morphological features, close association and extensive colonization of plant root tissues. Molecular characterization using fungal specific 18S rRNA primers showed that the fungus shared a 98% homology with *Penicillium verruculosum* (Genbank accession number AF510496). The isolate investigated is likely to be a novel endophytic strain of *P. verruculosum* which has evolved a very close symbiotic association with the host plant roots. The fungal endophyte characterized is being explored as an early colonizer in the establishment of seedlings and successful micropropagation of the plant.**

Key words: *Potentilla fulgens* L., ethnic tribes, Sacred Grove, fungal endophyte, molecular characterization, *Penicillium verruculosum.*

INTRODUCTION

Potentilla fulgens L. of the Rosaceae family, commonly found at higher altitudes (1500 - 2000 m MSL) in Khasi hills of Meghalaya in the fringes of Eastern Himalayan range, India, has been used as a folk remedy for a variety of ailments by the ethnic tribal population. Traditionally, pieces of tap roots of *P. fulgens* (laniang kynthei in Khasi) are chewed along with raw areca nut (*Areca catechu* L.*)* and betel leaves (*Piper betel* L.*)*. This plant is found to be prevalent in the Sacred Groves as well as in wild habitats. These habitats are known to receive highest rainfall in the world being in the vicinity of Cherrapunjee. There is extensive exploitation of this plant especially from the wild habitats as 'Sacred Groves' are spared from human intervention for traditional beliefs. This plant in its wild habitat has certain adaptive features and one among these is the association with endophytic fungal species which could help in its survival in and around the areas receiving the highest rainfall in the world. It is known that there are several symbiotic relationships in nature. The present study was aimed at exploring the endophytic association of the plant especially in the root region which may be contributing to the vigorous growth of the plant in such extreme rainfall habitats. The compositions of endophytes are known to create the distinctiveness of each plant (Agusta et al., 2006). Reports on fungal endophytes associated with *P. fulgens* have been very scanty. There is high probability of encountering fungi in soil- and plant-associated samples that have not yet been taxonomically described or that present other

*Corresponding author. E-mail: srjoshi2006@yahoo.co.in. Tel: +91 364 2722405 (O) Fax: +91-364- 2550076.

identification problems, often preventing identification below the genus level. The likelihood of unfamiliar and un-described species is increased when little studied substrates, hosts or habitats are investigated, which may impose limitations on diversity studies in habitats of great interest. It was therefore, undertaken to isolate and characterize fungal endophyte(s) associated with the roots of the plant considering the ethno-medicinal uses of the roots of this plant by Traditional Medical Practitioners (TMPs). Further, this initial study was also aimed at providing an insight into the possibility of finding growth promoting endophytic symbionts of the plant so that hardening and growth enhancement of this endangered plant may be successfully undertaken through micro-propagation and tissue culture techniques.

MATERIALS AND METHODS

Collection of plant material

*P. fulgens L. (*Figure 1) was collected during the month of July 2007 to July 2009 from wild habitats and Shillong Peak Sacred Grove. Shillong, Meghalaya, India, (Altitude 1700 m above MSL. 25°34′ North latitude and 91°54′ East longitude) spread over an area of approximately 10 km^2.

Isolation of endophytic fungi

About 100 surface sterilized root pieces of *P. fulgens* collected from various sites were air dried and flamed before removing the outer layers. Two centimeter long pieces of these roots were placed on Petri plates containing water agar as described by Strobel et al. (1996) and incubated at 24°C for 7 days. After incubation for 7 days, hyphal tips of developing fungi were aseptically removed and placed on potato dextrose agar (PDA) according to the procedure described by Strobel et al. (2005).

Fungal growth and storage

The endophytic fungus was subsequently grown on PDA plates and also in PD broth. Pure culturing of the fungus was done on 14 days interval from the working culture plates. Agar plugs containing the fungus were placed in sterile distilled water and stored at 4°C. Fungal viability was determined according to the protocol described by Strobel et al. (2005).

Morphological characterization of the endophytic fungus

The fungus was stained using aniline blue according to the standard protocol described by Cappucino and Sherman (1992) and observed under a Motic phase contrast trinocular research microscope model BA-450PH (Feintechnik, Germany).

Scanning electron microscopy of the endophytic fungus

The samples were mounted on the specimen Aluminium stub (10 mm dia. x 30 mm high) using double coated carbon tap taking care to avoid any trapped air bubble. A thin conductive coating of gold was applied to the sample using sputter coater unit (Polaron, SC7600) at a relatively low vacuum of 10^{-2} torr on the sputtering chamber. The coated samples were examined with a LEO-1430VP scanning electron microscope (Carl Zeiss). The tilt control was used to set the working distance (WD) of 12 mm.The morphology was analyzed under Back Scattering Detector.

Transmission electron microscopy (TEM) of the endophytic fungus

TEM studies were carried out using the root pieces of *P. fulgens* which were inoculated into the water agar plates. Fungal hyphae of the endophytic fungi, under investigation were seen emerging out of the root pieces after 7 days of incubation at 24°C. Specimen sized 1 - 2 mm were fixed in 1% modified Karnovsky's fixative for 2 h. This was followed on the second day by a wash with 0.1 M sodium cacodylate buffer after which a secondary fixation with 1% OsO_4 for 2 h was given to the specimen. Dehydration of the specimen was performed with varying concentrations of acetone followed by clearing with two changes of propylene oxide for 30 min on the third day of the specimen preparation. Infiltration of the specimen was carried out with 1:3 rations of embedding medium (araldite Cy212) and propylene oxide at room temperature for 12 h. Infiltration of the specimen was continued on day four with 1:1 and 3:1 ratio of araldite Cy212 and propylene oxide for one hour each. The treatment with 3:1 ratio of the embedding medium with propylene oxide was carried out in vacuum.

Finally, pure embedding medium was used to infiltrate the specimen. On the fifth day, polymerization of the block was achieved by raising the temperature to 60°C for 24 h. Cured (polymerized) blocks were then roughly hand-trimmed to remove the excess embedding medium from the tip containing the sample. Fine trimming was done in the ultra-microtome to prepare the block for sectioning. The face of the block was cut into the shape of a trapezoid. Sections were cut at a thickness range of 60 to 90 nm and made to float in double-distilled water before picking them up on to a copper grid and allowed to dry. The dried grids containing the section were then floated in staining solutions of uranyl acetate and lead citrate. Stained grids were then allowed to dry before they were viewed under the TEM JEOL 100 CX II with a working voltage of 80 Kv.

Molecular characterization of the endophytic fungus

DNA was isolated using Genei Fungal DNA Isolation Kit (Bangalore Genei KT125A) according to the manufacturer's instructions. PCR was done in a volume of 50 µl containing 1X PCR buffer (Tris HCl), 10 mM dNTP (2.5 mM each), 10 pmol of each primer, 3U Taq Polymerase and 50 ng Template. DNA amplification was carried out in Applied Biosystems 2720 thermal cycler with the following thermal cycling profile: an initial denaturation step at 94°C for 5 min was followed by 40 cycles of amplification (denaturation at 94°C for 30 s, annealing at 56°C for 45 s, and extension at 72°C for 60 s) ending with a final extension step at 72°C for 10 min. After PCR amplification, 2 µl was removed and subjected to agarose gel electrophoresis and the gel was stained with ethidium bromide, and the amplicons were visualized using a Kodak Gel Logic 100 gel documentation system. The PCR primers used for the sequencing were BGIT5 and BGNL4 obtained from Bangalore Genei, India. The PCR product was purified and cloned in TA vector using Instant Ligation Kit (Bangalore Genei KT91) and DH5α cells transformed as per manufacturer`s instruction. Positive clones were screened and sequenced using SP6 and T7 primers in Applied Biosystems 3100 Genetic analyzer. The sequence data was analyzed using

Figure 1. *P. fulgens* L. in flowering stage in Sacred Grove at Shillong peak, Meghalaya, India.

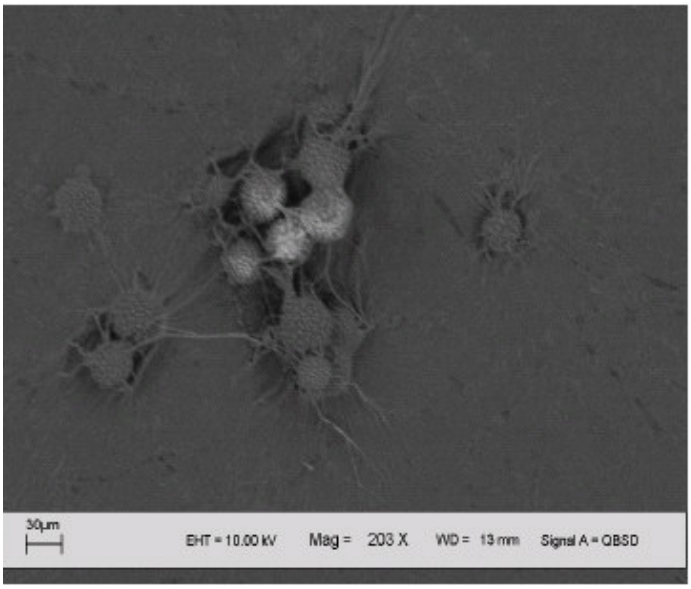

Figure 2. SEM micrograph of *P. verruculosum* RS7PF fruiting bodies.

BLAST with the available databases in NCBI.

RESULTS

Isolation and growth characteristics

Small root pieces of *P. fulgens*, after being subjected to procedures for endophyte isolation,, consistently yielded one microorganism that outwardly resembled a *Talaromyces* sp. The isolation procedure was repeated for all the root samples collected from various sites. White cottony

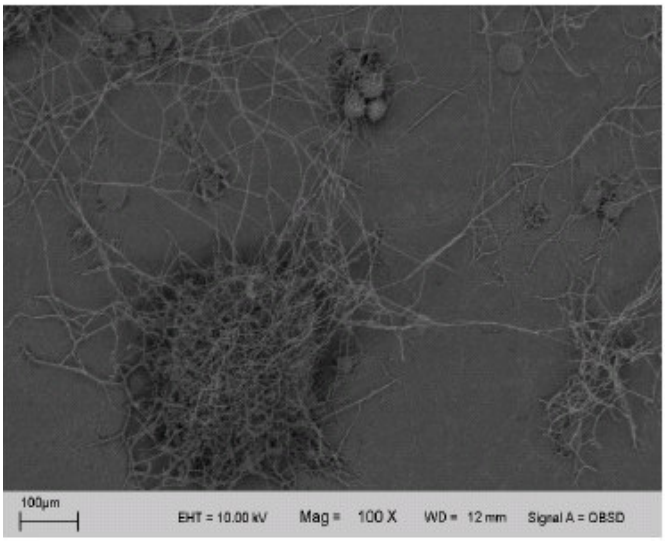

Figure 3. Branching pattern of *P. verruculosum* RS7PF as revealed by SEM

fungal mycelia were observed emerging out of the plant tissues after four days of incubation. The fungal colony fully covered the water agar plates after 7 days of incubation. This organism was initially labeled as isolate RS7PF. When transferred and grown on PDA, the organism produced a whitish cottony growth which gradually developed into a mycelium of yellowish coloration, and got more reddish brown within 3 weeks of incubation. The fungi exhibited resemblance to *Penicillium* and *Aspergillus* group in the branching behaviour of its hyphae and the fruiting bodies pattern.

Electron microscopic examination

Scanning electron microscopic examination of the organism's surface characters and hyphae revealed a mat like appearance of the hyphae and also fruiting bodies that had resemblances with those from the genus *Talaromyces* (Figures 2 and 3). Transmission electron microscopic examination of the healthy root pieces of *P. fulgens* from the wild habitat revealed the colonization of the internal root tissues by the fungal hyphae (Figure 4a and b).

Molecular characterization of the endophytic fungus

The characterized isolate RS7PF was studied for molecular relatedness to other organisms. 18S rRNA gene (1155 bp in length) of the isolate was amplified by PCR and sequenced. The sequence was compared for similarity with the available sequence information in the

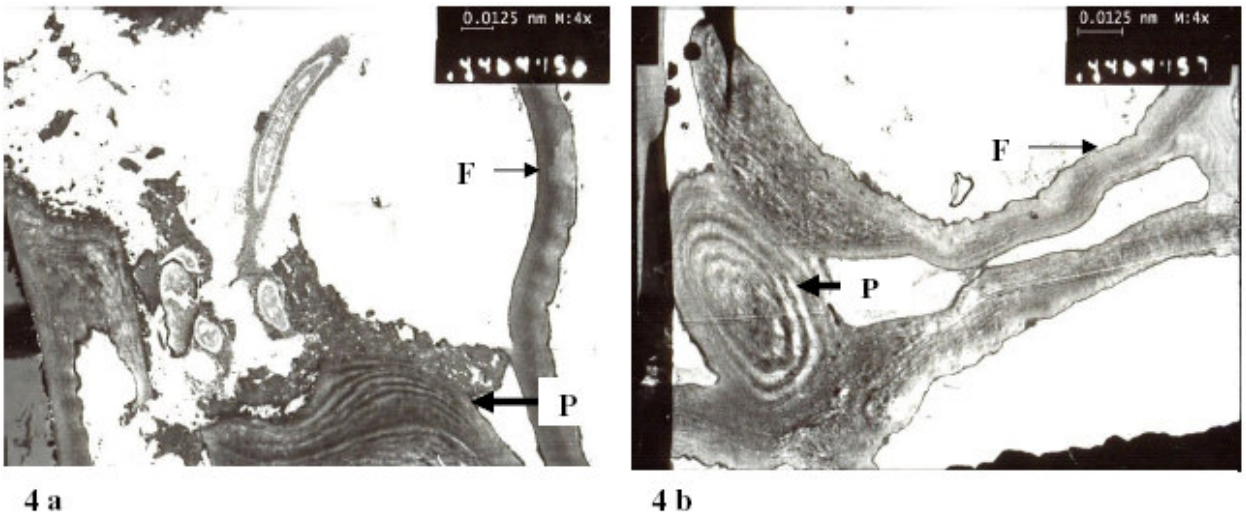

Figure 4. TEM micrographs showing *P. verruculosum* RS7PF colonizing the root tissues of *P. fulgens*. (F: fungal hyphae, P: plant tissue) (a and b).

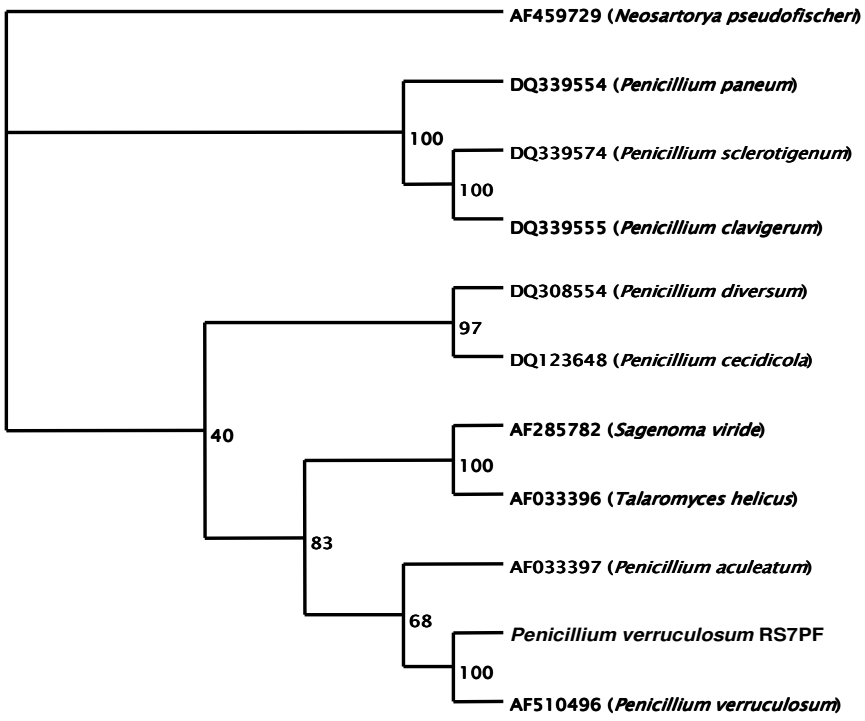

Figure 5. Phylogenetic tree of *P. verruculosum* RS7PF constructed with MEGA 4 software using the neighbor joining method.

NCBI GenBank database. There was 98% homology between its 18S rRNA and that of *Penicillium verruculosum* (Genbank accession number AF510496). The phylogenetic analysis was carried out using MEGA version 4 software (Tamura et al., 2007) with 1000 bootstrap replications (Figure 5).

DISCUSSION

The roots of *P. fulgens*, the ethno-medicinal plant used by the ethnic tribes of Eastern India, was found to be associated with the endophytic fungus *P. verruculosum* RS7PF throughout the sampling period of two years,

indicating that the endophyte had developed a continuous association with the plant in all seasons. Morphologically, the fungal isolate RS7PF represented the characteristics of *T. flavus* (Klöcker) (Bhagobaty and Joshi, 2008) and has been deposited in the microbial type culture collection (MTCC), Chandigarh, India. (MTCC accession no: 9073). It is important to note that taxonomic data for studies of microfungal diversity based on the traditional methods of dilution plating and sub-culturing for identification can be limited when a proportion of isolates fail to grow or sporulate in culture, preventing identification of isolates beyond the category of sterile mycelia (Watrud et al., 2006). Molecular phylogenetic study using the 18S rRNA gene analysis was to revalidate the morphological identity, and interestingly the results indicated that isolate RS7PF was more closely related with *P. verruculosum* (98% similarity). Marois et al. (1982) in a similar finding reported that *Penicillium dangeardii* Pitt was an anamorph of *T. flavus*, which is a potential biocontrol agent of several pathogenic fungi, including *Verticillium dahliae* (Kleb). Other organisms showing a high degree homology with the endophytic fungal isolate were *Penicillium aculeatum* (94% similarity) and *Talaromyces helicus* (93% similarity) (Figure 5).

It is likely that the specialized niche of the characterized isolate RS7PF inside the root tissues of *P. fulgens* growing in the wettest regions of the earth (Meghalaya, India) has put it under a directed evolutionary pressure for it to be different in the genetic level with type strains of *P. verruculosum,* since morphologically it showed resemblance with *T. flavus*. Therefore, it is proposed that our isolate RS7PF (Genbank accession number: EU579531) is a previously undescribed endophytic strain of *P. verruculosum* associated with *P. fulgens* L., growing in the wild habitats and the 'Sacred Groves' of the Khasi Hills of Meghalaya in the Eastern Himalayan ranges of India.

Further studies to ascertain the exact taxonomic position within the genus *Penicillium* are being undertaken together with a focus to generate biotechnologically relevant processes or products by using the novel and beneficial properties of our isolate RS7PF without just adding to taxonomic data. Our previous study on the seed germination potential of the crude fermentation broth of this endophytic fungal strain showed that it was capable of producing low but significant levels of endogenous auxins like Indole Acetic acid (I. A. A.) for enhancement of seed germination in non-host crops plants namely: Chick pea and Green gram (Bhagobaty and Joshi, 2009). The report on this fungal isolate which is first of its kind from this part of the world may therefore, prove to be an efficient endophytic associate in tissue culture approaches for extensive propagation of the already endangered ethno-medicinal plants like *P. fulgens*.

ACKNOWLEDGEMENTS

The authors are thankful to Dr. (Mrs.) B. Dkhar, Mr. J.P. Nongkynrih and Ms. A. Haloi of Sophisticated Analytical Instrumentation Facility, North-Eastern Hill University, Shillong for their help in TEM studies of the fungal sample and to K. K. Senapati of Central Instrumentation Facility, IIT-Guwahati for the SEM analysis of the fungus. We would also like to thank Prof. G. Strobel of Plant Sciences Division, Montana State University, Bozeman, USA for providing his constructive suggestions for improving our work. We acknowledge the grant received from UGC-UPE Biosciences Programme of our university for meeting laboratory requirements for the present study.

REFERENCES

Agusta A, Ohashi K, Shibuya H (2006). Composition of the endophytic filamentous fungi isolated from the tea plant *Camellia sinensis*. J. Nat. Med. 60: 268-272

Bhagobaty RK, Joshi SR (2008). DNA damage protective activity of the crude metabolites of endophytic fungi isolated from two ethno-pharmacologically important medicinal plants of the Khasi Hills of Meghalaya, India. Pharmacol. Online 3: 882-888.

Bhagobaty RK, Joshi SR (2009). Promotion of seed germination of Green gram and Chick pea by *Penicillium verruculosum* RS7PF, a root endophytic fungus of *Potentilla fulgens* L. J. Adv. Biotech. 8(12): 16-18.

Cappuccino JG, Sherman N (1996). Microbiology, A Laboratory Manual; 4th Ed. The Benjamin-Cumming Publishing Company, Inc, Menlopark, California p. 186.

Marois JJ, Dunn MT, Papavizas GC (1982). Biological control of *Verticillium* wilt of eggplant in the field. Plant Dis. 66: 1166-1168.

Strobel G, Manker DC, Mercier J (2005). Endophytic fungi and methods of use. [Online] Available from www.freepatentsonline.com/6911338.html [accessed 02 November 2007].

Strobel G, Yang X, Sears J, Kramer R, Sidhu RS, Hess WM (1996). Taxol from *Pestalotiopsis microspora*, an endophytic fungus of *Taxus wallichiana*. Microbiol.142: 435-440.

Tamura K, Dudley J, Nei M, Kumar S (2007). MEGA4: Molecular evolutionary genetic analysis software version 4.0.Mol Biol. Evol. 24(28): 1596-1599.

Watrud LS, Martin K, Donegan KK, Stone JK, Coleman CG (2006). Comparison of taxonomic, colony morphotype and PCR-RFLP methods to characterize microfungal diversity. Mycol. 98(3): 384-392.