

Opportunistic endophytism of *Trichoderma* species in rice Pusa Basmati-1 (PB1)

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Microbes that colonize internal tissues of plants are called endophytes, and are known for their functional role against biotic/abiotic stress and growth promotion activity in plants. The ascomyceteous fungus *Trichoderma* spp. (Teleomorph: *Hypocrea*) are well known antagonists cum biocontrol agents. In this study, a total of five *Trichoderma* isolates from two different species viz., *T. asperellum* (TaR1, TaR2 and TaR3) and *T. asperelloides* (TaR4 and TaR5) collected from different agro-climatic zones in Rajasthan, India were evaluated for their endophytism in rice variety Pusa Basmati-1 (PB-1) through soil and seed treatment. An attempt was made to re-isolate the fungus from rice roots (seed and soil treated) and further subjected to microscopic and molecular analysis. Re-isolation results revealed that culture growth of *Trichoderma* spp. isolated was similar to that of the inoculated ones. The microscopic analysis (light and scanning electron microscopy) results also confirmed that the re-isolated endophytic fungus were identical to the inoculated ones. These results were further confirmed by polymerase chain reaction (PCR) amplification of the rDNA region (18SrRNA, ITS1, 5.8SrRNA, ITS2 and 28SrRNA) and translation elongation factor 1 (*Tef1*) with the re-isolated *Trichoderma asperellum* and *T. asperelloides* isolates. In this study, it has been confirmed that *Trichoderma asperellum* and *T. asperelloides* turns endophytic in rice after introduction through seed and soil treatment.

Keywords: Ascomyceteous fungus, Molecular phylogeny, Mycoparasitism, Pusa Basmati-1 (PB-1), Rajasthan, SEM, Translation elongation factor 1 (*Tef1*)

Endophytes that colonize the plants internal tissues are ubiquitous in nature and known to occur in all plant species¹. Mostly they belong to a fungi or bacterium. The major interest with endophytes is that they produce active secondary metabolites which have antimicrobial properties. Secondary metabolites produced by endophytes (in stems, leaves and roots) are mainly used for their survival purpose against abiotic and biotic stress. It also helps in disease resistance, water preservation and improved quantity of biomass. Endophytes occupy ecological niches in the living internal tissues of their hosts without any adverse effects².

Fungal endophytes are classified into two major groups (clavicipitaceous and non-clavicipitaceous) with respect to the phylogeny and life history traits. Clavicipitaceous endophytes have been well studied and are known to infect economically important grass viz., *Festuca* sp. and *Lolium* sp.³. Taxonomically distinct (non-clavicipitaceous) and diverse group of

fungi are known to be associated with monocot and dicots. They have a broader host range and isolated from various geographic and climatic zones. It is thought that endophytes enter the plant tissues through the root zone, flower, leaf and cotyledons.

The ascomyceteous fungal genus *Trichoderma* (Teleomorph: *Hypocrea*) is a well known antagonist and widely used biological control agent (BCA) against several economically important plant pathogens⁵. It is also playing vital role in the industrial application for production of hydrolytic enzymes viz., Cellulase⁶, Chitinase^{7,8} and Glucanase⁹. Some of the *Trichoderma* species viz., *T. harzianum* (T-22 Root shield and T-39 Trichodex), *T. viride* (Bip T), *T. atroviride* (Binab TF WB) and *T. virens* (G-41 Technical, BW240G Biological fungicides) are already in market as successful biocontrol agents¹⁰. *Trichoderma* as endophytes have been reported in *Hevea* sp. with *T. amazonicum*¹¹, Cocoa with *T. martia*¹², Cocoa with *T. hamatum*¹³, Cocoa with *T. asperellum*¹¹, Coffee with *T. flagellatum*¹⁴, Lentil with *T. gamsii*¹⁵ and *Dendrobium nobile* with *T. chlorosporum*¹⁶. Though fungal endophytes have been investigated much in case of grasses¹⁷, similar

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works on the endophytic behavior of *Trichoderma* species on rice (*Oryza sativa* L.) are scarce. Hence, endophytism of *Trichoderma* in rice needs to be investigated in depth for better understanding its mechanism against biotic and abiotic stresses. Most of the studies on fungal endophytes in rice have used morphological features to characterize it, but literatures have shown that conformity should be at molecular level to distinguish the closely related species or species complexes¹⁸.

The present study focuses on the endophytism of *Trichoderma* spp. in Pusa Basmati-1 (PB-1) variety of rice after their introduction through seed and soil.

Material and Methods

Isolation of endophytic fungi from rice fields

Twenty-eight days old healthy Pusa basmati-1 (PB-1) rice plants were collected from different plots in Division of Genetics, ICAR-IARI, New Delhi. In the lab, the samples were washed thoroughly with tap water and different plant parts (leaves, stems and roots) were cut into small pieces (1 cm length). All the parts were surface sterilized (75% alcohol for five minutes followed by two minutes in 2.5% sodium hypochlorite) and washing five times in sterilized distilled water (SDW).

Sterility checks of the root samples were done by pouring 0.1mL of fifth rinsed water and plated out on Potato Dextrose Agar (PDA) and *Trichoderma* specific media (TSM). Both the plates were incubated at 28°C for 5-7 days. The roots dried on sterile blotter paper and plated on PDA with the special precautions to avoid *Trichoderma* as a contaminant. Plates were incubated at 28°C till the hyphal tips appear from plant tissues. Pure culture was obtained by sub culturing mycelium emerging from plant tissues. The fungus was identified by their microscopic and

macroscopic characters with the help of light microscopy¹⁹.

Trichoderma cultures: Morphological and molecular characterization

Three isolates of *Trichoderma asperellum* and two isolates of *Trichoderma asperelloides* available in Biological control laboratory, Division of Plant Pathology, ICAR-IARI, New Delhi was used for the endophytic study (Table 1).

Morphological identification of the isolates (inoculated and re-isolated) was carried out based on conidia shape, colony appearance, phialides characteristics and branching patterns of conidiophores. To confirm the molecular identity, all the isolates (inoculated and re-isolated) were subjected to PCR amplification of ribosomal DNA (rDNA) region (28SrDNA, 18SrDNA, 5.8SrDNA, ITS1 and ITS2) and translation elongation factor1 (*Tef1*) region. DNA isolation was done by following cetyl trimethyl ammonium bromide (CTAB) method with slight modifications. Polymerase chain reaction (PCR) was performed following the programme for ITS (Initial denaturation at 95°C for 5min; followed by 30 cycles of denaturation at 95°C for 30 s, annealing at 60°C for 30 s and extension at 72°C for 1 min; followed by final extension at 72°C for 10 min) and *Tef1* (Initial denaturation at 95°C for 5 min; followed by 30 cycles of denaturation at 95°C for 30 s, annealing at 60°C for 30 s and extension at 72°C for 2 min; followed by final extension at 72°C for 10 min) using Gene Pro thermal cycler, Bioer 2 Technology Co., Ltd., China. Each PCR reaction was carried out in a final volume of 50 µL containing the following: 2 µL of genomic DNA (50 ng/µL), 1 µL of forward and reverse primer each (10 pM), 1.0 µL of 10 mM dNTP's, 5.0 µL of 10X PCR buffer with

Table 1 — Details of *Trichoderma* cultures used in this study

| Isolate | Isolate Code | Source & Place of collection | ITCC No. | NCBI No. | |
|----------------------------------|--------------|---|----------|----------|-------------|
| | | | | ITS | <i>Tef1</i> |
| <i>Trichoderma asperellum</i> | TaR1 | Soil & Vegetable field, Jaipur, Rajasthan | 7845 | KT001076 | KT722733 |
| <i>Trichoderma asperellum</i> | TaR2 | Soil & Vegetable field, Jaipur, Rajasthan | 7846 | KT001077 | KT722734 |
| <i>Trichoderma asperellum</i> | TaR3 | Soil & Vegetable field, Jaipur, Rajasthan | 7847 | KT001078 | KT722735 |
| <i>Trichoderma asperelloides</i> | TaR4 | Soil & Vegetable field, Jaipur, Rajasthan | 7848 | KT722738 | KT722736 |
| <i>Trichoderma asperelloides</i> | TaR5 | Soil & Vegetable field, Jaipur, Rajasthan | 7849 | KT722739 | KT722737 |

MgCl₂, 0.5 µL of DyNAzyme II DNA Polymerase (2U/µL) and 39.5 µL of molecular biology grade water (Thermo Fisher Scientific, USA).

The amplified products were separated electrophoretically (Best Lab International Inc., China) on 1.5% agarose gel stained with 0.5 µg/µL ethidium bromide for 1 h in 1X TAE at constant 80V²⁰. The PCR products were visualized under UV light and gel documented (Bio-Rad Laboratories, CA, USA). Amplified fragments were gel purified (MACHEREY-NAGEL GmbH & Co. KG), sequenced (Xcelris Labs limited, Ahmedabad, India) and submitted to NCBI.

Trichoderma inoculum preparation and treatment

To study the endophytism, all the *Trichoderma* isolates (TaR1, TaR2, TaR3, TaR4 and TaR5) were treated with soil and seed. A disc (5 mm) of *T. asperellum* and *T. asperelloides* isolates were inoculated in 100 mL Potato Dextrose Broth (PDB) and kept at 28±2°C for 5-7 days. The mycelial mat was harvested by using sterile muslin cloth and the suspension was used further for soil and seed treatment. Soil was sterilized by autoclaving (121.6°C at 15psi for 20 min) for two consecutive days and then treated with *T. asperellum* and *T. asperelloides* spore suspension (colony forming unit-2×10⁶ spores/mL) before five days of seed sowing. Soil was properly mixed with the spore suspension by sprinkling water and inverted at once in a day. PB-1 seeds were sown after 5 days of soil treatment with *Trichoderma* isolates²¹.

For seed treatment, 3mL of conidial suspension (CFU-2×10⁶ spores/mL) was used per 10 g of seed and mixed thoroughly in a small plastic container with constant shaking for uniform coating. The seeds were kept overnight and the next day it was sown. Seeds for both treatments were sown at the rate of 20 seeds/pots. The pots were prepared by using sterilized soil (pH: 6.5-7.5 and moisture 100%) along with FYM (4:1) for avoiding microbial contaminations. Control pots were also made with untreated seeds and pots.

Light microscopy analysis

Light microscopy was performed to identify the fungus inside the rice roots. Twenty-eight days old root samples were taken from both soil and seed treated samples and cut into small pieces (0.5-1.0 cm). Samples were fixed in 50% ethanol for 12 h followed by heating in 5% KOH at 90°C in 5 min. Samples were then washed with tap water and acidified with

0.1N HCl for 5-10 min followed by staining with 0.001% acid fuchsin in solution of acid-glycerine-water (87.5 mL lactic acid, 6.3 glycerine and 6.3 mL water) for 1 h at 55°C. Excess dye amount was removed in 100% glycerine. The root samples were cut in horizontal thin sizes and stained lactophenol cotton blue. The sample were fixed in centre of the slide along with cover slip without any air bubbles and observed under light microscope. *T. asperellum* and *T. asperelloides* were identified on the basis of their morphological conidia, colony texture and shape².

Re-isolation of endophytic fungi from treated roots

Endophytic fungi were re-isolated from roots to confirm endophytism in rice. Freshly uprooted roots were washed under tap water to remove the soil debris, dried and washed with SDW. The root was allowed to dry on blotter paper followed by cutting them into small pieces (~1 cm) and then immersed in 70% ethanol. Surface sterilization process continued by dipping the root pieces in 2.5 per cent sodium hypochlorite for 2 min. The roots were then rinsed 5 times in SDW²². Sterility checks for the root samples were done by plating 0.1 mL from the fifth rinse on PDA and TSM and kept at 28±2°C for 5-6 days.

Surface sterilized roots were dried on sterilized blotter papers, crushed under aseptic conditions and finally plated on PDA. The plates were incubated for 5 days at 28±2°C. After the appearance of fungal growth, subculturing was done for all the isolates.

SEM analysis

The surface sterilized root samples were fixed overnight at room temperature in 4% glutaraldehyde in 0.05M phosphate buffer (pH 7.3). They were washed in phosphate buffer thrice for 15 min and dehydrated through 30, 50, 70, 80, 90 and 100% ethanol for 15 min. The samples undergone critical point drying for 5 min and immediately fixed on the stubs and coated. Samples were immediately observed in Zeiss EVO10, Germany. Same protocol was followed to observe the morphological characteristics of the re-isolated fungus as well as to investigate mycoparasitism in dual culture assay against *Rhizoctonia solani*²³.

Molecular phylogenetic analysis

ITS and *tef1* gene sequences of *T. asperellum* and *T. asperelloides* were obtained from GenBank and aligned with the generated sequences using ClustalW

multiple sequence alignment for constructing the phylogenetic tree²⁴ using the MEGA5 software program²⁵, and a maximum likelihood (ML) tree was constructed using the Kimura 2-parameter distance model²⁶. Finally, the CONSENSE program was used to construct the tree.

Data analysis

OPSTAT software programme was used for statistical analysis of the data obtained. All the experiments were repeated once again to confirm the results. Data deviating from the mean were considered as standard deviation.

Results

Isolation of endophytic fungi from rice grown under natural conditions

The different parts of rice tissues like leaves, stems and roots were used. Four different fungal genera were isolated viz., *Penicillium* sp., *Alternaria* sp., *Fusarium* sp. and *Aspergillus* sp. from rice tissues (Fig. 1).

Trichoderma spp. was not observed in the leaves and stems of the untreated crop.

Morphological characterization: Macroscopic features

Trichoderma cultures (*T. asperellum*-TaR1, TaR2 and TaR3; *T. asperelloides*-TaR4 and TaR5) obtained from the biological control laboratory, Division of Plant Pathology, ICAR-IARI, New Delhi was sub-cultured. After 7 days, culture plates were observed to be dark green in colour and all the isolates exhibited fast growth and covered the entire plate surface within 3 days of incubation. Some of the isolates formed concentric rings (TaR2, TaR3 and TaR4) and all the isolates were observed to be changed in colour from whitish to greenish (either dark or light) during the growth period (Fig. 2).

Microscopic features

Light microscopy results revealed that conidia of all the 5 isolates were green colour with sub-globose to globose in shape. Conidiophores were observed to be

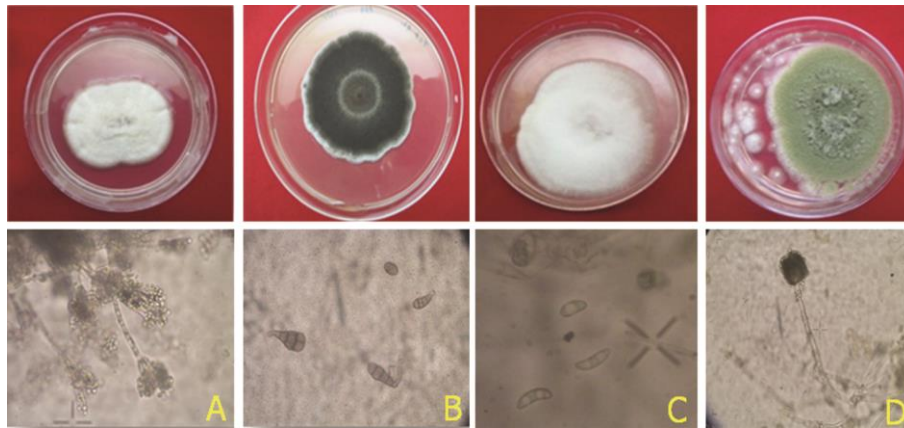


Fig. 1 — Seven days old cultures on PDA (top) and microscopic images (bottom) of endophytic fungi isolated from Pusa Basmati-1 (PB-1) rice plants grown under natural condition: (A) *Penicillium* sp.; (B) *Alternaria* sp.; (C) *Fusarium* sp.; and (D) *Aspergillus* sp.

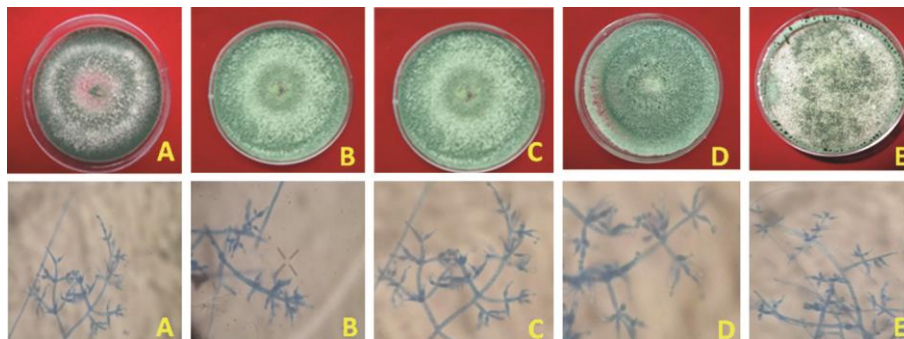


Fig. 2 — Seven days old culture plates (top) and microscopic images (bottom) of *Trichoderma asperellum*: (A) TaR1, (B) TaR2 and (C) TaR3; and *Trichoderma asperelloides*: (D) TaR4, and (E) TaR5 grown on PDA plates collected from different agro-climatic zone in Rajasthan, India.

loosely tufted and regularly branched with side branches arising at right angles. The conidiophores terminated with flask shaped phialides which were swollen at the middle. The phialides were either single or was seen to form a whorl of 2-4 divergent phialides (Fig. 2). Since the cultural and morphological characters are more or less similar in *T. asperellum* and *T. asperelloides* it almost becomes difficult to distinguish the isolates of these two species.

Molecular identification

Since morphological features were not sufficient to define the species correctly, molecular techniques are used more frequently for species identification. PCR amplification of all the 5 isolates with ITS and *Tef1* primers revealed that a single band was observed around 600 and 1000 bp, respectively (Fig. 3). Contig was prepared and submitted to the NCBI database (Table 1). Phylogeny tree also confirmed that three isolates (TaR1, TaR2 and TaR3) belonging to *T. asperellum* and two isolates (TaR4 and TaR5) belonging to *T. asperelloides* (Fig. 4).

Microscopic observation of treated roots

Internal colonization of roots with *Trichoderma* spp. was observed at 28 days of seed and soil treatment. Mycelium was visible but thorough observation of the endophytes was not clear since the

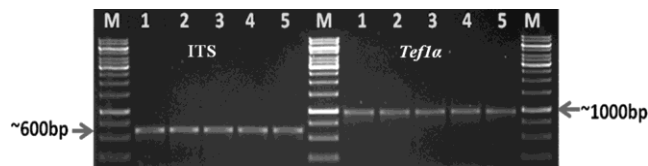


Fig. 3 — ITS and *Tef1a* gene amplification of *Trichoderma* isolates using primer pair ITS1/ITS4 and EF1F/TEF1R, respectively. [M: 1Kb DNA ladder; Lane 1: TaR1; Lane 2: TaR2; Lane 3: TaR3; Lane 4: TaR4 and Lane 5: TaR5]

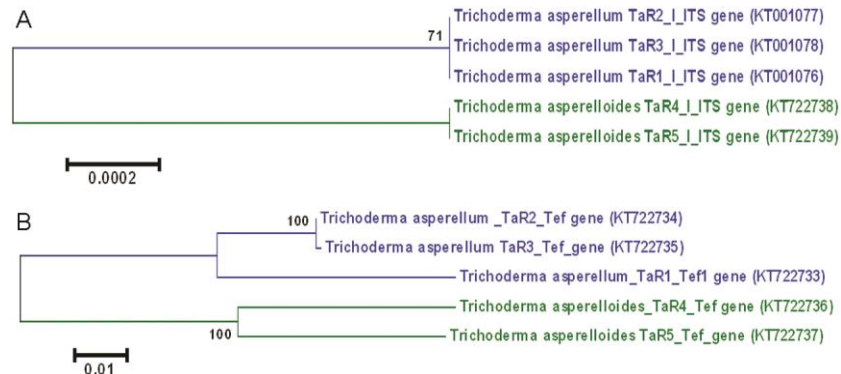


Fig. 4 — Phylogenetic relationships of two different *Trichoderma* spp. inferred by analysis of (A) ITS; and (B) *Tef1* gene sequences. [The Maximum Likelihood tree was constructed using two-parameter model implemented in the MEGA 6.06 program (I-Inoculated)]

morphology could have been changed through slide preparation (Fig. 5). Conidia were not observed in both instances. The mycelium was seen as straight and sometimes the septation of the hyphae was observed. To further confirm the endophytism, samples were subjected to SEM analysis and the results revealed that fungal hyphae were seen in all the root samples for both the treatments (Fig. 6). It was to confirm that the fungus seen in SEM was *Trichoderma* spp. No other microbes were observed.

Re-isolation of endophytic fungi from the roots

To conclusively say that *Trichoderma* spp. used in this study is endophytic in rice roots, re-isolation of the fungi seen in microscopy was attempted. The results revealed that white fungal colonies were observed after 3 days of incubation in both treated and control samples. But after 7 days, growth of the treated plates was changed into dark green colour which was similar to the inoculated plates. In case of control plates there was no *Trichoderma* spp. growth and it was *Aspergillus* sp. was noticed (Fig. 6). SEM analysis results of the re-isolated fungus revealed that in all the treated (soil and seed) plates *Trichoderma* spp.

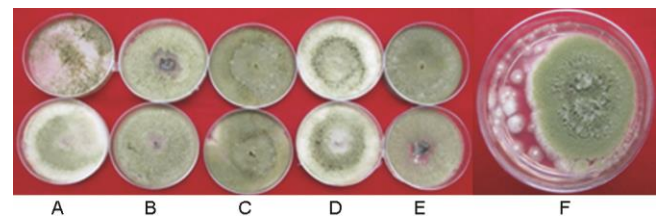


Fig. 5 — Seven days old endophytic fungi re-isolated from *Trichoderma* treated (Soil at the top and seed at the bottom) Pusa Basmati-1 rice roots on PDA plates: (A) TaR1; (B) TaR2; (C) TaR3; (D) TaR4; (E) TaR5; and (F) Control plate showing mycelial growth isolated from *Trichoderma* untreated Pusa Basmati-1 rice roots.

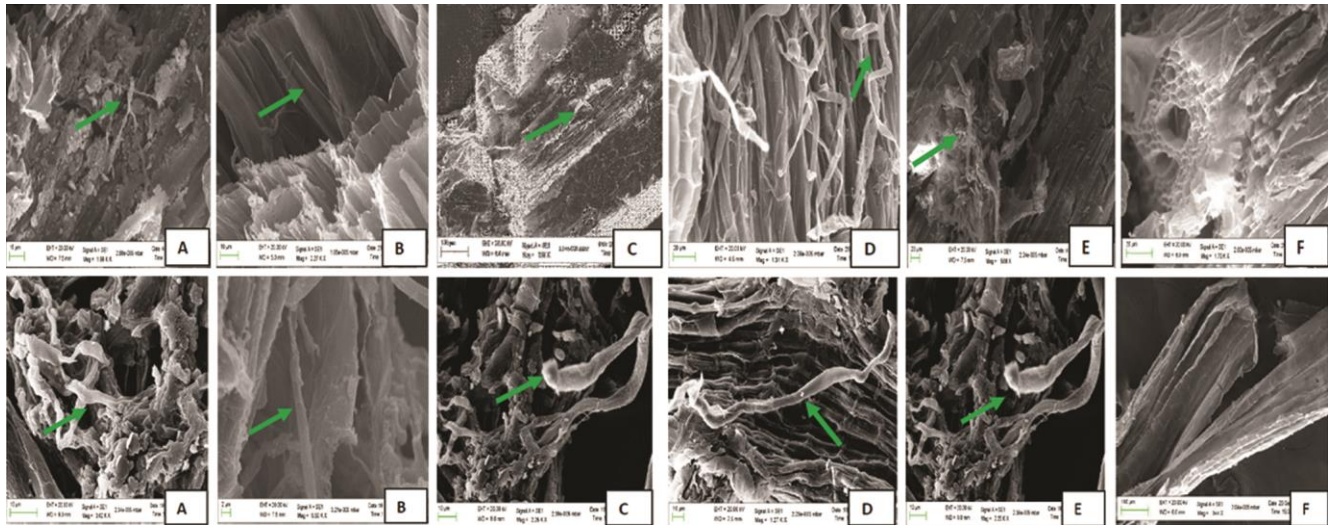


Fig. 6—SEM photos showing internal colonization of Pusa Basmati-1 (PB-1) rice roots treated (seed at the top and soil at the bottom) with *Trichoderma asperellum* isolates: (A) TaR1, (B) TaR2, (C) TaR3; and *Trichoderma asperelloides* isolates: (D) TaR4, (E) TaR5 and (F) control image showing no mycelial growth isolated from *Trichoderma* untreated Pusa Basmati-1 rice roots.

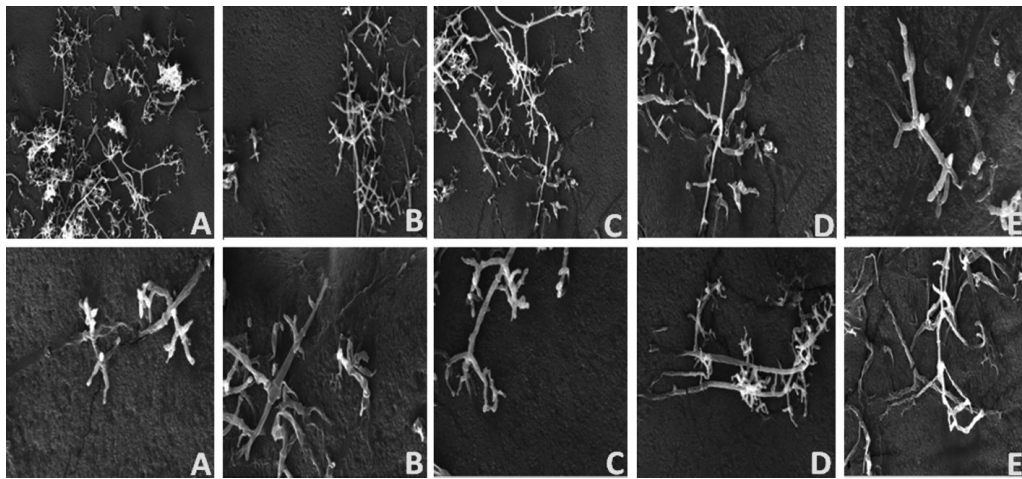


Fig. 7—SEM images showing conidiophores, phialides and conidial structures of three days old *Trichoderma asperellum* isolates (A) TaR1, (B) TaR2, (C) TaR3; and *Trichoderma asperelloides* isolates (D) TaR4, and (E) TaR5 re-isolated from seed (top) and soil (bottom) treated Pusa Basmati-1 rice roots.

was observed and it was confirmed by the observation of the conidiophores, phialides and conidial structures (Fig. 7).

Re-isolated fungus was further subjected to molecular analysis by amplification of ITS and *Tef1* gene and the results confirmed with the amplification of 600 and 1000 bp, respectively. Phylogenetic tree results for the ITS and *Tef1* gene also confirmed that both the inoculated and re-isolated *T. asperellum* (TaR1, TaR2 and TaR3) and *T. asperelloides* (TaR4 and TaR5) isolates were falling under the same cluster (Fig. 8).

Discussion

Trichoderma spp. has been well established as a potential fungal biocontrol agent widely used in various disease management programmes due to its multifaceted applications like mycoparasitism, plant growth promotion, induced systemic resistance²⁷ and pesticide degradations²⁸. In recent years, nano particle based bioformulations were developed from *Trichoderma* spp. and other biocontrol agents against important plant pathogens²⁹ and insects³⁰. The important feature of endophyte is that it has to be re-isolated from the introduced plant roots to

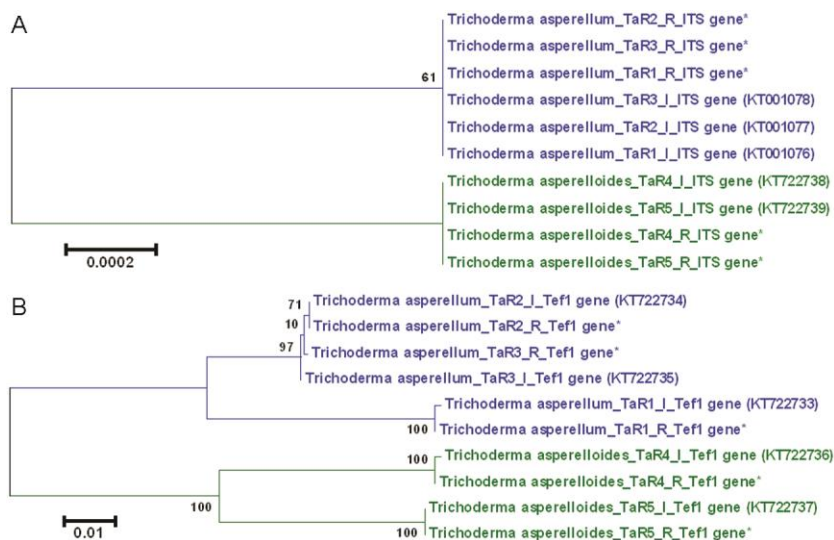


Fig. 8 — Phylogenetic relationships of two different *Trichoderma* spp. inferred by analysis of (A) ITS; and (B) *Tef1* gene sequences. [The Maximum Likelihood tree was constructed using two-parameter model implemented in the MEGA 6.06 program. ('I' and 'R' denotes inoculated and re-isolated, respectively)]

show that the fungus under investigation is endophytic.

In our study, we have used light microscopy, SEM analysis, re-isolation and PCR confirmation of the fungus using ITS and *Tef1* gene from *Trichoderma* introduced plant roots. Light microscopy and SEM assay have been used in many studies to investigate fungal endophytes. But in light microscopy and SEM analysis of rice roots, it was very difficult to show the internal colonization of the isolates. However, the best way of confirmation is by molecular methods (PCR confirmation and sequencing) of a known gene. Molecular methods are recommended for its accuracy and specificity. Hence, we relied on re-isolation of the fungus and molecularly confirm it using multi-gene phylogeny. Re-isolation of the fungus was made possible through surface sterilization, a method which ensures that epiphytic fungi or bacteria are eliminated to prevent biased results. This method has been used in most of the studies on endophytism. In this study, we have re-isolated the *Trichoderma* isolates (TaR1, TaR2, TaR3, TaR4 and TaR5) in pure form. According to ISTH (International Sub commission on *Trichoderma* and *Hypocera*) reference the ITS and *Tef1* genes were PCR amplified, sequenced and compared with the inoculated ones. Our results have shown similarity with several studies on endophytism in which they have also amplified ITS region to prove the endophytism^{9,10,22}. Since morphology was difficult to be distinguished between the closely related species

multi-gene phylogeny is mostly used for the identification of the fungal isolates up to species level³¹. *Tef1* region unanimously used to distinguish closely related *Trichoderma* species³².

Phylogenic tree of the ITS and *Tef1* has confirmed that both the inoculated and recovered isolates were one and the same *i.e.* *T. asperellum* (TaR1, TaR2 and TaR3) and *T. asperelloides* (TaR4 and TaR5). It clearly infers that all the 5 isolates are endophytic in rice roots. This observation is matching with a recent study, where *T. asperellum* isolates were re-inoculated in cacao seedlings through the roots and they were recovered from the roots and stems after one month³³. This is an example of opportunistic endophytism since the *Trichoderma* species is not endophytic but introduced through seed and soil enters the root system and survives in the roots. Studies on the survival of the *Trichoderma* in the form of endophytes after its application and their effect on the crop and pathogen are required.

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