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Seasonal distribution and biopotential of endophytic fungi recovered from photosynthetic root of *Tinospora cordifolia*

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ABSTRACT

A total of 263 endophytic isolates were recovered from 1800 segments plotted in three different seasons (winter, summer, and monsoon) at three different locations (BHU, Ramnagar, and Maruadih) representing the 20 different fungal taxa. The colonization frequency was maximal during monsoon (20.5%) followed by winter (13.83%) and minimal during summer (9.5%). However, among sites, it was maximum at location 3 (Ramnagar) (18.66%) followed by location 1(BHU) (17.16%) and location 2 (Maruadih) (8.0%). A maximum CF of 1.5% was observed for *Cladosporium cladosporioides* followed by *Alternaria alternata* and *Colletotrichum gloeosporioides* 1.27%, *Nigrospora oryzae* 1.22%, and *Phomopsis tersa* 1.1% while *Aspergillus tubingensis* followed by *Fusarium brachygibbosum* were recovered as a rare taxon with 0.16% and 0.22% colonization frequency, respectively. The MANOVA and Jaccard's distance (Jc) clearly indicate that the effect of season was more pronounced than the location in respect to species diversity. Out of total endophytic fungi isolated, 50% of them were active against at least either one or more human pathogenic bacteria tested. Among all active isolates, *Pseudofusicoccum adansoniae* exhibited an impressive antibacterial activity against all pathogenic bacteria. Eleven endophytic fungi (55.00 %) were found to be active against one or more fungal pathogens. Many endophytic fungi exhibited the production of amylase, cellulase, lipase, pectinase, protease, and xylanase. Out of 20 endophytic fungi, 20% were found to show antioxidant activity, 45% of endophytic isolates exhibited siderophore production while none of the fungi was found to solubilize phosphate in solid agar medium

Keywords : Antimicrobial activity, endophytes diversity, colonization frequency, enzyme activity, siderophores

INTRODUCTION

Microorganisms of special habitat possess the special characters to maintain their very existence in that particular environment and one of such microbiota are endophytes. They are unseen, poorly exploited, and less studied biota of the microbial world residing inside healthy plant tissues. Therefore, there are great possibilities of their use in humans, plants wellness and sustainable existence. In 1866 de Bary introduced the term "endophyte" for any microbes residing inside the plant, but they were first isolated by Freeman in 1902 from the seeds of darnel *Lolium temulentum*. Actually, they are the microbes that reside inside the healthy living tissue of plants without causing any negative effect (Bacon and White, 2000). Endophytes include great group diversity *viz.,* bacteria, actinomycetes, cyanobacteria, and fungi (most studied) and known to have interactions in a variety of ways such as endophytes, seed dispersers, herbivores, and pathogens (Clay, 1988; Faeth, 2002; Gond *et al.,* 2010). They establish a variety of intricate biological intra- and inter-relationships among them and with their hosts, respectively. Endophytes are able to produce a multitude of secondary metabolites with diverse biological activities. However, merely 0.75-1.50% of known plant species have been explored for their endophytes yet. So, the opportunity to find new potential bioactive metabolites from cryptic endophytic microorganisms of nearly 374,000-400,000 plant species congruently occupying millions of biological niches is considered valuable (Singh *et al.,* 2021). This opportunity has increased further with the innovative discovery of bio-synthesis of *Taxus*-derived anticancer compound 'taxol' from its endophytic fungus *Taxomyces andreanae* (Stierle *et al.,* 1993). Later, a series of works revealed that a reasonable number of plant-derived compounds are synthesized by endophytes rather than hosts. About more than a hundred anticancer compounds including 57 novel or analogs of known compounds (Kharwar *et al.,* 2011) and a number of other bioactive compounds like antifungal, antibacterial, antimalarial, insecticidal, antioxidant and antiviral, and immunomodulators (Mishra *et al.,* 2012) have been isolated from endophytic mycoflora.

Comparatively very few Indian medicinal plants have been studied for endophytic diversity. In the present study, the endophytic mycoflora residing in the photosynthetic aerial roots (a unique character of the plant) of an Indian medicinal plant *Tinospora cordifolia* has been studied for the diversity as well as their biopotential. *T. cordifolia* is commonly known as Guduchi, Gurch, Giloe, or Amrita. This plant, native to India and distributed throughout the tropical Indian subcontinent, has a long history of use in Indian Ayurveda. It is a liver tonic, antidiabetic agent, anti-dyspepsia, anti-inflammatory, antiperiodic, anti-fever, anti-arthritic, and anti-allergic. Besides these medicinal values, the plant also possesses immunomodulatory constituents (Singh *et al.,* 2003).

MATERIAL AND METHODS

Sample collection from selected sites

Mature, healthy, symptomless photosynthetic roots of *T. cordifolia* collected from three individual plants from each of three locations of Varanasi (Location 1-Banaras Hindu University; location 2- Maruadih; location 3-Ramnagar) with almost similar climatic conditions but with different pollution level: the average rainfall was 5 mm, 50 mm, and 1010 mm; mean temperature was 20°C, 30°C, and 28°C; relative air

humidity was 65%, 45%, and 85% in winter, in summer, and during monsoon at location 1, 2 and 3, respectively. Sampling was done in three seasons *viz.* winter, monsoon, and summer between January to December. Samples were collected from the same plants in each season. *T. cordifolia* is a climber vine and all the plants examined in this study were growing upon mango trees (*Mangifera indica*). All samples were taken at a height of at least 1.5 m above ground level and put separately in sterile poly bags, brought to the laboratory in an icebox, stored at 4°C, and processed within 48 h.

Sample surface-sterilization, isolation and identification

Samples were washed thoroughly in running tap water, rinsed with double distilled water, and surface-sterilized according to standard protocol (Petrini *et al.,* 1992). Samples were dipped in 70% ethanol for 1 minute, immersed in aq. solution of NaOCl (4% available chlorine) for 3 min followed by immersion in 70% ethanol for 10 seconds. The samples were then rinsed in double distilled sterile water and dried under aseptic conditions. Two hundred segments of samples from each location in each season were cut into small pieces of approximately 0.5 cm long segments and placed on to Petri plates containing potato dextrose agar (PDA) medium supplemented with streptomycin (200mg/l) and incubated for 21 days at 26 ± 2°C in BOD (Caltan BOD Incubator-152, Narang Scientific Works, New Delhi). The tissues were checked every alternate day, and actively growing fungal mycelia were transferred onto new PDA plates for purification and identification. The leaf imprint method was applied to check the effectiveness of surface sterilization (Schulz *et al.,* 1998). The fungi were identified to the genus and/or species level based on colony morphology and micromorphology (conidia, conidiophores, and fruit body morphology) using standard fungal taxonomic manuals (Ainsworth *et al.,* 1973; Ellis, 1976; Von Arx, 1981; Barnett and Hunter, 1998). Those which did not sporulate on PDA were identified through molecular techniques. All isolates received a specific code number (TCPR) and were deposited at the Department of Botany, Banaras Hindu University, Varanasi, India in lyophilized form in separate cryovials at -20°C (Blue Star).

Statistical analysis

The frequency of colonization (CF %) was calculated as the number of segments colonized by a particular endophyte divided by the total number of segments examined × 100 (Hata and Futai, 1995). Shannon Wiener index, Whittaker's evenness, species richness, and Jaccard's index were calculated through PAST software. Box plot was made through R software whereas MANOVA and standard errors were calculated through SPSS 16.

Fermentation and extraction of metabolite

The endophytes were transferred to fresh PDA plates and allowed to grow at 26 ± 2°C for 7-14 days. Some colonized plugs of PDA (5 mm in diameter) were transferred into a 2000 ml Erlenmeyer flask containing 1000ml potato dextrose broth (PDB). Flasks were put on a shaker in an incubator (orbital shaking incubator Remi-RIS-24BL) at 120 rpm for 14-21 days at 26 ± 2°C. Metabolites were extracted thrice with ethyl acetate (with equal volume) at room temperature and concentrated in a rotary vacuum (IKA, RV10) evaporator (40°C) to get the residue dry (crude) for antimicrobial assays.

Antimicrobial bioassay

Antibacterial activity: The paper disc diffusion bioassay was done adopting the methodology by Hadacek and Greger (2000). The crude compound isolated from different endophytic fungi was dissolved in methanol (MeOH) to make the final concentration of 200mg/ml. The 10µl aliquots were applied on each 5 mm (2mg/disc) diameter paper disc, after evaporating the organic solvent the discs were placed to the center of 9 cm diameter Muller Hinton agar plates previously inoculated with 0.5 ml spore suspension (104 CFU/ml) of different human bacterial pathogens. After 2-3 days, the widths of the inhibition zones were measured (in mm) with a ruler scale.

Antifungal activity: The dual culture technique was used to assay the antifungal activity of endophytic fungal isolates. Antagonistic activity of the isolated endophytic fungi was observed against five pathogenic (3 phytopathogens and 2 human pathogens) fungi. Inhibition of pathogens by the antagonistic endophyte was carried out on PDA plates. Mycelial plugs of actively growing endophytic fungus were placed at the periphery of the culture plate and incubated for 2 days at 26 ± 2°C. After two days, the plate was inoculated with a mycelial plug of the pathogen placed 4 cm away from the growing endophyte. The dual culture plate was incubated for additional 7 days at 26±2°C. The percentage growth inhibition of the pathogen was calculated with the help of the formula given by Whipps (1997).

$$\% \text{ inhibition of radial growth} = \frac{R_1 - R_2}{R_1} \times 100$$

Where R_1 is the uninhibited radial growth of the pathogen and R_2 is the inhibited radial growth of the pathogen.

Enzymes and antioxidant activity

Enzymes assay for, amylase, lipase, pectinase, protease, and xylanase were performed as per Hankin and Anagnostakis (1975) while cellulase by Lingappa and Lockwood (1962). Phosphate solubilization by Pikovskaya (1948) and siderophore as per Schwyn and Neilands (1987) were performed. Each test for the enzymatic assay was performed in triplicates. *In vitro* antioxidant activity of EtOAc isolated crude extract of *P. adansoniae* was performed using the standard methodology (Shen *et al.,* 2010) with certain modifications.

Extraction of total genomic DNA and PCR amplification for ITS rDNA

Genomic DNA was extracted and amplified from unidentified and mycelia-sterilia following the slightly modified standard protocol (Mishra *et al.,* 2012). The universal primers ITS1 5′ TCCGTAGGTGAACCTGCGG 3′ and ITS4 5′ CCTCCGCTTATTGATATGC 3′ (Metabion International, Martinsried, Germany) were used to amplify the 5.8S rDNA and two ITS regions flanked between the 18S

and 28S rRNA genes. Total PCR mixture of 25µl, each contains 1µl (100 ng/µl) of DNA template, 1µl of each primer, 0.33µl (3 unit) Taq polymerase, 1.5µl $MgCl_2$, 0.25µl dNTPs, buffer (10X) 2.5µl and 17.42µl MQ water for each reaction mixture. The PCR reactions were performed in icycler (BioRad) with following conditions: pre-denaturation at 94°C for 4 min, 35 cycles at 94°C (denaturation) for 1 min, 55°C (annealing) for 1 min, 72°C (extension) for 1 min and then a final extension for 5 min at 72°C. Amplified PCR products were resolved by electrophoresis in 1.5% (w/v) agarose gels stained with ethidium bromide (0.5 µg/ml) for visual examination. PCR product was sent to First BASE Laboratories (Malaysia) for sequencing. Obtained ITS rDNA sequence of each fungus was compared by Blast search among mention sequences at the NCBI GenBank for the identification of sequences with the highest similarity.

RESULTS

A total of 263 endophytic isolates belonging to 20 fungal taxa was obtained from 1800 healthy symptomless segments of the photosynthetic root of *Tinospora cordifolia* (**Table 1**). Out of 20 fungal species, the colonization frequency was maximal during monsoon (20.5%) followed by winter (13.83%) and minimal during summer (9.5%). However, among sites, the maximum CF was at location 3 (Ramnagar) (18.66%) followed by location 1 (BHU) (17.16%) and least to location 2 (Maruadih) (8.0%) (**Fig. 1**). A maximum CF of 1.5% was observed for *Cladosporium cladosporioides* followed by *Alternaria alternata* and *Colletotrichum gloeosporioides* 1.27%, *Nigrospora oryzae* 1.22% and *Phomopsis tersa* 1.1% while *Aspergillus tubingensis* followed by *Fusarium brachygibbosum* were recovered as a rare taxon with 0.16% and 0.22% colonization frequency, respectively (**Table 1**). Species richness, Shannon and Simpson diversity indices were found maximum for monsoon at location 3, but at location 1 in winter it was found more even than the others (**Table 2**).

Table 1: Recovery of endophytic fungi from photosynthetic root of *Tinospora cordifolia* at three different season and location

Endophytic fungi	Winter			Summer			Monsoon			Total	CF%
	Loc1	Loc2	Loc3	Loc1	Loc2	Loc3	Loc1	Loc2	Loc3		
Alternaria alternaria	4	2	3	2	0	3	3	2	4	23	1.27
Aschersonia sp.	3	2	2	0	0	0	0	0	0	7	0.38
Aspergillus flavus	0	1	2	2	2	2	4	1	2	16	0.88
Aspergillus terreus	0	0	1	1	3	2	0	0	3	10	0.55
Aspergillus tubingensis	0	0	0	0	0	0	1	0	2	3	0.16
Botryosphaeria rhodina	4	0	2	2	0	0	0	0	5	13	0.72
Chaetomium globosum	0	0	0	2	1	2	5	4	3	17	0.94
Cladosporium cladosporioides	4	3	4	3	2	2	4	2	3	27	1.5
Colletotrichum gloeosporioides	4	4	4	0	0	0	3	4	4	23	1.27
Drechslera graminea	2	0	2	0	0	0	5	0	3	12	0.66
Fusarium brachygibbosum	0	0	0	0	0	0	3	0	2	5	0.27
Nigrospora oryzae	2	0	6	2	3	3	2	1	3	22	1.22
Monilia sp.	0	0	0	0	0	0	2	2	2	6	0.33
Pestalotia sp.	3	2	2	1	0	2	0	0	0	10	0.55
Phoma putaminum	0	0	0	3	1	4	0	0	0	8	0.44
Phomopsis tersa	3	2	2	3	0	0	6	0	4	20	1.11
Phomopsis sp.	0	0	0	0	0	0	4	2	4	10	0.55
Pleosporales sp.	0	0	0	0	0	0	2	0	5	7	0.38
Pseudofusicoccum adansoniae	3	0	5	0	0	4	4	0	2	18	1.00
Veronaea sp.	0	0	0	0	0	0	2	2	2	6	0.33
Total	32	16	35	21	12	24	50	20	53	263	14.61

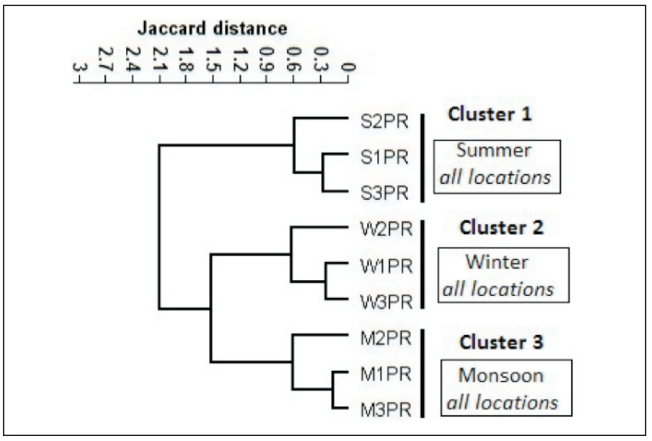

Fig. 1: Jaccard's similarity between seasons and locations

Dissimilarity of endophytic communities in regards to species composition as expressed by Jaccard's distance (Jc) was the highest among seasons (**Fig. 2**). Some species frequently occurred preferentially in one or two seasons. For example, *Aschersonia* sp. only occurred in winter, *A. tubingensis, F. brachygibbosum, Monilia* sp., *Phomopsis* sp., *Pleosporales* sp., and *Veronaea* sp., were registered only in monsoon, whereas *Phoma putaminum* reported in summer only (**Table 1**). The study of MANOVA reveals that both the factors (season, and location) have a significant influence on all the diversity indices *viz.* species richness ($p \leq 0.001$), Shannon ($p \leq 0.001$), and evenness ($p \leq 0.05$) while the interaction between season and location was found significant only to species richness ($p \leq 0.05$), (**Table 3**). Six endophytic taxa out of 20 were morphologically unidentified and, therefore, shifted to molecular identification. Two fungal mycelia coded TCPR 101 (NCBI GenBank accession no. JX628753) and TCPR 116 (accession no. Jx951181) showed 100% sequence similarity with *Alternaria alternata* and *Pseudofusicoccum adansoniae* while the other four sterile mycelia coded as TCPR 106 (accession no. JX951175), TCPR 109 (accession no. JX951176), TCPR 114 (accession no. JX951179) and TCPR 115 (accession no. JX951180) showed 99% sequence similarity with *Colletotrichum gloeosporioides*, *Fusarium brachygibbosum, Phomopsis tersa*, and *Pleosporales* sp., respectively (**Fig. 3**). Out of total endophytic fungi isolated 50% of them were active against at least 1 human pathogenic bacterium tested. Among all active isolates, *P. adansoniae* exhibited impressive antibacterial activity against all human pathogenic bacteria tested followed by *B. rhodina* which exhibited activity against 4 pathogenic bacteria. Other potential endophytic isolates like *C. globosum, F. brachygibbosum* and *Pestalotia* sp. showed significant antibacterial property against 3 bacteria. The pathogenic isolate *Shigella flexnii* IMS/GN1was found most susceptible as inhibited by 8 out of 10 active endophytic fungi followed by *Proteus vulgaris* IMS/GN7 and *E. coli* ATCC 25922 while *Morganella morganii* IMS/GN6 was found most resistant pathogenic bacterium and showed susceptibility for only *P. adansoniae* (**Table 4**). The antifungal activity of all 20 endophytic fungal isolates was screened out against five pathogenic fungi in a dual culture assay (**Table 5**). Eleven endophytic fungi (55.00%) were found to be active against either one or more fungal pathogen(s). *P. adansoniae* was the most active member which inhibited the growth of three out of five pathogens tested except the *Trichophyton rubrum* and *Alternaia alternata*. The maximum inhibition was 42.5% displayed by *P. adansoniae* against *C. lunata*. *Phomopsis* sp. inhibited 35.7% radial growth of *F. oxysporum* whereas *Candida* was inhibited the maximum by *B. rhodina*. The pathogenic *C. lunata* was found most susceptible as inhibited by eight endophytic fungi while *Trichophyton rubrum* was noticed resistant against all endophytic fungi

Table 2: Diversity indices of endophytic fungi of photosynthetic root of *T. cordifolia.*

Indices	WL1	WL2	WL3	SL1	SL2	SL3	ML1	ML2	ML3
Species richness	10	7	12	10	6	9	15	9	17
Shannon_H	2.274	1.873	2.37	2.244	1.705	2.152	2.623	2.095	2.78
Simpson_1-D	0.895	0.8359	0.896	0.889	0.806	0.879	0.922	0.865	0.9349
Evenness	0.972	0.9301	0.891	0.943	0.917	0.956	0.918	0.903	0.9484

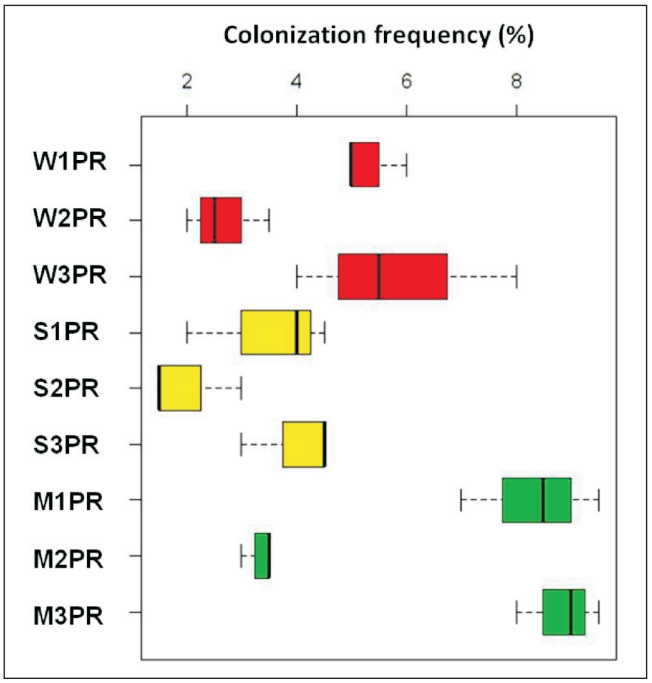

Fig. 2: Percent recovery of endophytic fungi from photosynthetic root of *T. cordifolia*

Table 3: Summary of MANOVA of different diversity indices for endophytic fungi of *T. cordifolia* root

Indices	Season ($F_{2,18}$)	Location ($F_{2,18}$)	Season X Location ($F_{4,18}$)
Species richness	34.111***	25.063***	3.648**
Shannon	14.653***	11.694**	1.400^{ns}
Evenness	4.214**	5.695**	1.520^{ns}
*** Significant at P<0.01, ** Significant at P<0.05, NS-non significant			

tested (**Table 5**).

Six extracellular enzymes production was assayed by the

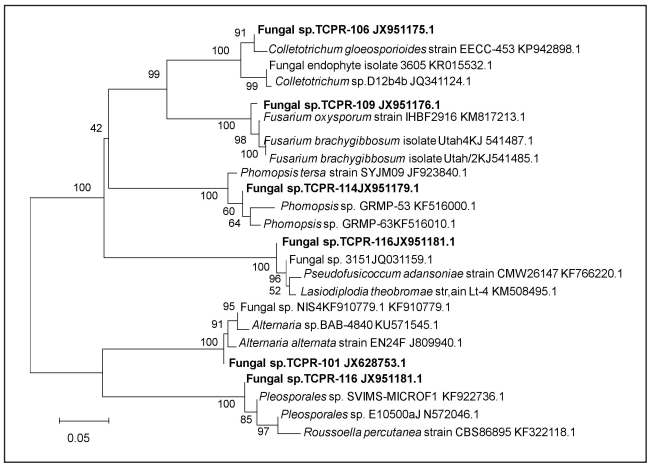

Fig. 3: Phylogenetic relationship of fungal endophytes identified through molecular method. The tree as constructed and evaluated by performing bootstrap (1000) analysis with the evolutionary distances using the neighbour joining method

Table 4: Antibacterial activity of crude extract of endophytic fungi recovered from photosynthetic root of *T. cordifolia*

Endophytic fungi	Diameter of inhibition zone (mean±SE in cm) of human pathogenic bacteria* (5mg/ml)							
	A	B	C	D	E	F	G	H
Alternaria alternata	-	-	-	0.90± .057	-	-		
Aschersonia sp.	-	-	-	-	-	-	-	-
Aspergillus flavus	-	-	-	-	-	-	-	-
Aspergillus terreus	-	-	-	-	-	-	-	-
Aspergillus tubingensis	-	-	-	-	-	-	-	-
Botryosphaeria rhodina	1.10± .057	-	-	-	2.26± .033	1.50± .100	-	1.23± .033
Chaetomium globosum	1.06± .066	-	1.13± .033		-	-	-	1.93± .0.33
Cladosporium cladosporioides	-	-	-	-	-	-	-	-
Colletotrichum gloeosporioides	1.03± .033	-	-	-	-	-	-	1.06± .088
Drechslera graminea	-	-	-	-	-	-	-	-
Fusarium brachygibbosum	0.95± .028	1.10± .057	-	-	-	-	-	1.03± .033
Nigrospora oryzae	-	0.86± .057	-	-	-	-	-	-
Monilia sp.	-	-	-	-	-	-	-	-
Pestalotia sp.	1.60± .057	1.83± .033	-	-	-	-	-	3.91± .044
Phoma putaminum	-	-	-	-	-	-	-	-
Phomopsis tersa	-	-	-	-	-	-	-	-
Phomopsis sp.	0.93± .033	0.91± .044	-	-	-	-	-	-
Pleosporales sp.	2.43± .033		-	-	-	-	-	-
Pseudofusicoccum adansoniae	1.96± .033	1.85± .028	2.30± .066	3.10± .057	1.98± .016	1.35± .028	2.50± .050	2.13± .033
Veronaea sp.	-	-	-	-	-	-	-	-
Controls								
Methanol	0	0	0	0	0	0	0	0
Ampicillin (10µg/disc)	0	0	0	0	0	0	0	0
Ciprofloxacin (5µg/disc)	18.00 ±0.00	39.00 ±0.00	40	40	34.00 ±0.00	32.00 ±0.00	30	32.00 ±0.00

*A- *Shigella flexnerii* IMS/GN1, B- *E. coli* ATCC 25922, C- *Salmonella enteritidis* IMS/GN3, D- *S. paratyphi* IMS/GN4, E- *Pseudomonas aeruginosa* ATCC 27853, F- *Citrobacter freundii* IMS/GN5, G- *Morganella morganii* IMS/GN6, H- *Proteus vulgaris* IMS/GN7 ; - = not detected.

isolated fungi of the photosynthetic root of *Tinospora cordifolia* and eight endophytic fungi exhibited the production of amylase (40%) on substrate amended growth medium. The cellulase, lipase, pectinase, protease, and xylanase production was found by 25%, 35%, 10%, 35%

Table 5: *In vitro* antifungal activity of endophytic fungi isolated from photosynthetic root of *T. cordifolia*

Endophytic fungi	% growth inhibition of pathogenic fungi*				
	A	B	C	D	E
Alternaria alternata	-	27.5	-	-	-
Aschersonia sp.	29.5	-	32.4	-	-
Aspergillus flavus	-	-	-	-	9.0
Aspergillus terreus	-	-	-	-	-
Aspergillus tubingensis	-	-	-	-	-
Botryosphaeria rhodina	-	-	-	-	13.0
Chaetomium globosum	-	31.4	24.07	-	-
Cladosporium cladosporioides	-	-	-	-	-
Colletotrichum gloeosporioides	-	24.5	31.5	-	-
Drechslera graminea	-	-	-	-	-
Fusarium brachygibbosum	-	24.7	27.3	-	-
Nigrospora oryzae	-	31.5	-	-	-
Monilia sp.	-	-	-	-	-
Pestalotia sp.	-	40.4	-	-	11.0
Phoma putaminum	-	-	-	-	-
Phomopsis sp.	-	32.5	35.7	-	-
Phomopsis tersa	-		-	-	9.0
Pleosporales sp.	-	-	-	-	-
Pseudofusicoccum adansoniae	-	42.5	35.0	-	10.0
Veronaea sp.	-	-	-	-	-

*A-*Alternaria alternata*, B- *Curvularia lunata*, C- *Fusarium oxysporum*, D- *Trichophyton rubrum* and E- *Candida albicans* , # zone of inhibition (in mm) ; - = not detected.

and 30%, respectively from different endophytic fungi (**Table 6**). The *P. putaminum* gave the maximum zone for amylase and xylanase production, *P. tersa* for cellulase, *B. rhodina* for lipase, *Pleosporales* sp. for pectinase and *C. gloeosporioides* for protease, respectively (**Table 6**). Out of 20 endophytic fungi, only 20% were found able to show the antioxidant activity and among them *B. rhodina* was found the best. Forty-five per cent of endophytic isolates exhibited siderophore production and out of which *F. brachygibbosum* was found the best producer followed by *B. rhodina* and others on solid agar media, whereas none of the fungi could solubilize phosphate on solid agar medium (**Table 7**).

DISCUSSION

The present study concentrated on the diversity, distribution, and biopotential of endophytic fungi recovered from the photosynthetic root of Indian medicinal vine *T. cordifolia* in three different seasons at three different locations of the Varanasi region. The study reveals that diversity and frequency were greatly influenced by season than the location. The colonization frequency and diversity indices like species richness, Shannon and Simpson index were

Table 6: Extracellular enzyme production by endophytic fungi isolated from photosynthetic root of *T. cordifolia*

Endophytic fungi	Extracellular enzyme production by endophytic fungi zone diameter (mean±SE mm)					
	Amylase	Cellulase	Lipase	Pectinase	Protease	Xylanase
Alternaria alternaria	2.60±.057	-	-	-	-	-
Aschersonia sp.	4.03±.033	2.73±.033	4.06±.033	-	-	-
Aspergillus flavus	4.03±.033	-	-	-	-	-
Aspergillus terreus	1.70±057	-	-	-	-	-
Aspergillus tubingensis	-	-	-	-	-	-
Botryosphaeria rhodina	-	-	4.15±.050	-	-	-
Chaetomium globosum	-	1.30±.033	-	-	-	-
Cladosporium cladosporioides	4.53±.033	3.00±.057	2.96±.088	1.93±.066	1.56±.016	2.83±.088
Colletotrichum gloeosporioides	-	-		-	5.36±.088	4.15±.050
Drechslera graminea	-	-	-	-	-	-
Fusarium brachygibbosum	-	-	3.93±.088	-	-	-
Nigrospora oryzae	1.76±.033	-	-	-	-	1.10±.057
Monilia sp.	-	-	-	-	2.36±.088	-
Pestalotia sp.	-	-	3.26±.033	-	4.66±.088	-
Phoma putaminum	5.73±.066	-	-	-	5.00±.057	4.43±.088
Phomopsis sp.	-	-	2.16±.033	-	4.96±.033	4.33±.033
Phomopsis tersa	-	5.50±.057	-	-	-	-
Pleosporales sp.	2.76±.033	2.44±.057	3.06±.088	3.53±.66	-	2.73±.033
Pseudofusicoccum adansoniae	-	-	-	-	2.96±.088	-
- = not detected						

highest in monsoon than winter and summer (**Table 1, 2; Fig. 1**). Many workers reported that the degree of endophytic colonization increased during wet seasons and this is in accordance with our results. It might be due to the availability of proper temperature and humidity required for the growth and establishment of fungi inside the host (Yadav *et al.,* 2016; Mishra *et al.,* 2012; Singh *et al.,* 2017). Recovery of genera like *Cladosporium, Alternaria, Colletotrichum, Nigrospora*, and *Phomopsis* were the most frequently encountered endophytic fungi in this study, which corroborate with the earlier reports of their occurrence in the earlier well studied Indian medicinal plants. (Verma *et al.,* 2014; Mishra *et al.,* 2012: Verma *et al.,* 2007), and it may be due to the high spore production of these fungi and their cosmopolitan nature, which statistically increase their chance to get established as endophytes (Mishra *et al.,* 2012). The presence of some taxa like *A. tubingensis* and *F. brachygibbosum* as a rare/accidental species reveals the authenticity and adequacy of sampling size and surface sterilization. It was interesting that MANOVA and Jaccard's distance (Jc) clearly indicate that the effect of the season was more pronounced than the location in respect to species diversity (**Table 3; Fig. 2**), the result was in accordance with our earlier study (Mishra *et al.,* 2012; Singh *et al.,* 2017). In addition to this, Yadav *et al.* (2016) also reported that the effect of the season has a greater influence on the diversity of endophytic fungi of *Eugenia jambolana* in compression to site and tissue type.

Table 7: Antioxidant, phosphate solubilization and siderophore production potential of endophytic fungi

Endophytic fungi	Antioxidant activity IC_{50} (µg/ml)	Phosphate solubilization	Siderophore production (in cm)
Alternaria alternaria	185	-	0.966±.066
Aschersonia sp.	177	-	-
Aspergillus flavus	-	-	-
Aspergillus terreus	-	-	1.06±.066
Aspergillus tubingensis	-	-	-
Botryosphaeria rhodina	135	-	4.90±.088
Chaetomium globosum	-	-	-
Cladosporium cladosporioides	-	-	1.33±.033
Colletotrichum gloeosporioides	-	-	-
Drechslera graminea	-	-	-
Fusarium brachygibbosum	-	-	5.06±.066
Nigrospora oryzae	-	-	3.26±.033
Monilia sp.	-		-
Pestalotia sp.	-	-	-
Phoma putaminum	-	-	-
Phomopsis sp.	-	-	-
Phomopsis tersa	-	-	4.10±.057
Pleosporales sp.	-	-	3.66±.057
Pseudofusicoccum adansoniae	155	-	4.33±.088
Veronaea musae	-	-	-
- = not detected			

In the present study, 70% of endophytic fungi were identified through their morphological characters, but the remaining 30% of endophytic fungi did not produce any identifiable spore and therefore subjected to molecular technique. ITS 1 (forward) and ITS 4 (reverse) primers were used for amplification of DNA followed by sequencing and identification after NCBI blast search (**Fig. 3**). A number of workers had successfully used rDNA in the phylogenetic analysis and identification of morphologically unidentifiable endophytic fungi (Wang *et al.,* 2005). There are many molecular techniques like DNA cloning, DGGE T-RFLP, pyrosequencing, and DGGE fingerprinting that have been used to clarify the taxonomic position of fungi (Verma *et al.,* 2014). The improper and enormous use of antibiotics developed resistance in pathogenic microbes against existing drugs. Now a days, it is a challenge for scientists to discover an alternative way for new and effective antibiotics. During the course of antibacterial activity, 50% of endophytic fungi showed antibacterial attributes against at least 1 human pathogenic bacterium tested while it was found 55% active against fungi (**Table 4, 5**). The report was in accordance with the previous reports of other Indian medicinal plants where Verma *et al.* (2014) found that 58.33% endophytic fungi were active against at least either one or more bacterial pathogens, whereas the crude extract of five endophytic fungi inhibited the growth of five or more than five (50%) of the fungal pathogens tested. Similarly, Kalyanasundaram *et al.* (2015) found 70% of endophytic fungi were found active against bacterial and fungal pathogens while Yadav *et al.* (2016) reported the

antibacterial effect of 60% of endophytic fungi. It was very interesting that we found *P. adansoniae* ("TCPR-116" JX951181) active against all the pathogens tested. However, in addition, *B. rhodina, C. globosum* and *Pestalotia* sp. also exhibited significant antibacterial activity (**Table 4**). A bioactive compound such as (3S)-lasiodiplodin has been isolated from endophytic *B. rhodina* showed antibacterial activity against *S. aureus* (Rukachaisirikul *et al.,* 2009). Endophytic *C. globosum* of an aquatic plant *Nymphaea nouchaliis* was able to produce chaetoglobosin A which exhibited antibacterial activity against *B. subtilis, S. aureus,* and methicillin-resistant *S. aureus* (Dissanayake *et al.,* 2016). Pestalone isolated from *Pestalotia* exhibits moderate *in vitro* cytotoxicity against 60 human tumor cell lines. Pestalone also showed potent antibiotic activity against methicillin-resistant *S. aureus* and vancomycin-resistant *Enterococcus faecium* (Cueto *et al.,* 2001). Endophytic fungi survive inside the host tissue and secrete a variety of extracellular enzymes in order to utilize the available nutrients or food. Plant tissues store starch as a food source and this starch is one of the most easily digestive food sources within plant tissues. In the present study eight endophytic (40%) fungi produced amylase (**Table 6**). Our findings were similar to earlier reports in which authors reported the production of amylase by around 43% (Gulhane *et al.,* 2016) and 58 % (Prathyusha *et al.,* 2015) endophytic isolates, even there is also report that showed extracellular production of the same enzyme by 100% endophytic fungi (Job *et al.,* 2015). The amylase activity exhibited by these endophytic fungi may help the host plant to degrade starch during plant senescence before the appearance of other new colonies (Gupta and Chaturvedi, 2015). Cellulose is the world's most abundant natural biopolymer and a potentially important source for the production of industrially useful materials such as fuels and chemicals. Xylans are the main carbohydrate in the hemicellulosic fraction of vegetable tissues and form an interface between lignin and the other polysaccharides. In our study, a total of 25% of endophytic fungi produce cellulose whereas 30% produce extracellular xylanase (**Table 6**). Our experimental data is in accordance with others where they found the production of cellulase by 22% (Yadav *et al.,* 2015), 32% (Sunitha *et al.,* 2013) and xylanase 23% (Gond, 2011), however Suto *et al.* (2002) reported the production of later by 91.7 % endophytic fungi. The endophytes that are able to produce both cellulase and xylanase should have the capability to participate with other types of fungi surviving on dead wood and leaves and this type of endophytic fungi may also confer the quality of switching their work from endophytism to saprophytism as their host starts senescence (Carroll and Petrini, 1983). Lipases are enzymes that hydrolyze the ester bonds of water-insoluble substrates at the interface between the substrate and the water. They are produced by a variety of biota-like plants, animals, and microbes but most of them used in biotechnological applications were isolated from fungi (Torres *et al.,* 2003). Due to the high industrial demand for new lipase sources, there is an urgent need to find novel lipolytic microorganisms. Proteases are also an important industrial enzyme that catalyzes the protein molecules and have an important role in dairy, agriculture, and medicine. The present experiment reveals that both lipase and protease were produced by 35% of endophytic fungi (**Table 6**). The earlier reports also support our finding which suggests the production of protease (33%) (Prathyusha *et al.,* 2015), and lipase by 50% of endophytic fungi (Sunitha *et al.,* 2013), whereas Panuthai *et al.* (2012) reported 100% lipase production by endophytic fungi. Lipases and protease were also produced by mangrove endophytic fungi on the southwest coast of India. However, the high lipase activity suggests their ability to use fats as an energy source (Maria *et al.,* 2005). Pectin a structural heteropolysaccharide found in the primary cell walls of plants is catalyzed by pectinase. We found only 10% of endophytic fungi able to produce pectinase (**Table 6**). Some author reported the production of pectinase by more than 50% (Sunitha *et al.,* 2013) but Choi *et al.* (2005) found none of the endophytic fungi able to produce pectinase. However, it was suggested that an endophyte can degrade the pectic-substances, and this implies that the fungus is likely to be a latent pathogen (Choi *et al.,* 2005).

Antioxidants are the molecules that inhibit the oxidation of other molecules and therefore act as free radical scavengers. There are a number of synthetic antioxidants available, but their use involves toxic side effects thus there is a need to search for natural antioxidants and free radical scavengers (Dhankhar *et al.,* 2012) and therefore, naturally-derived antioxidants have received much attention in recent years (Schulz *et al.,* 2002). Out of total endophytic fungi isolated in this study, 4 (20%) endophytic taxa such as *A. alternata, Aschersonia* sp., *B. rhodina, P. adansoniae* exhibited antioxidant activity. Verma *et al.* (2014) found 55% endophytic fungi positive for antioxidant activity and among them they reported the best activity by *Aschersonia* sp. followed by *B. rhodina* isolated from *Madhuca indica*. Phongpaichit *et al.* (2007) also observed that 22.5% of the extracts of endophytic fungi isolated from *Garcinia* plants exhibited remarkable antioxidant activities.

Regarding the phosphate solubilization activity, none of the endophytic fungi found positive. It might be due to that these endophytes isolated from photosynthetic root (aerial root) instead of underground root. Iron is an essential nutrient for almost all biota (Johnson, 2008) and all microbes studied, with the exception of certain lactobacilli, require iron (Haas *et al.,* 1999), but in the aerobic habitat iron exists mainly as ferric (Fe^{3+}) ions, and therefore form insoluble hydroxides and oxyhydroxides (Lesuisse and Labbe, 1994) which in turn become unavailable for microorganisms. There are a number of microbes like bacteria and fungi that are able to produce siderophores which are of low molecular weight, iron-chelating ligands (Renshaw, 2002). In the present study, about

45% of endophytic fungi produced siderophore (**Table 7**). Our result is in accordance with other workers like Chadha *et al.* (2015) found 58% and Verma (2014) found about 41% of endophytic fungi to produced siderophore. Siderophore production is related to root-associated soil fungi, dark-septate endophytic fungi, and mycorrhiza. However, endophytic fungi survive in the aerial part of the host able to produced siderophore, and even pathogenic fungi that infect plant aerial tissues produce siderophores (Kajula, 2010). Literature suggests that siderophore produced by endophytic fungi may play a significant role in the symbiosis between host and fungi (Johnson, 2008).

CONCLUSIONS

Endophytic fungi could be utilized as an alternative resource for isolating the variety of biologically active molecules and may be used in different industries. Methods like NGS may help to explore the full structural diversity as culture-independent taxa do not appear through the culture-dependent methods. The other attributes of fungal endophytes could also be harnessed to promote plant growth against a variety of biotic and abiotic stresses. In order to enhance the known molecules and to isolate the cryptic compounds, techniques like OSMAC, co-culture, and epigenetic modulations may be adopted.

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