JJBS

Volume 13, Number 4, December 2020
ISSN 1995-6673
Pages 543 -550

Jordan Journal of Biological Sciences

Fungal Endophytes from *Tabernaemontana heyneana* Wall. (Apocynaceae), their Molecular Characterization, L-asparaginase and Antioxidant Activities

Naguvanahally S. Bhavana[1], Harischandra S. Prakash[2] and Monnanda S. Nalini[1,*]

[1]*Department of Studies in Botany,* [2]*UGC- BSR Fellow, Department of Studies in Biotechnology, University of Mysore, Manasagangotri, Mysore – 570 006, Karnataka, India*

Received: December 24, 2019; Revised: January 19, 2020; Accepted: February 1, 2020

Abstract

Endophytic fungi are an interesting group of microorganisms associated with the healthy tissues of medicinal plants used in folk medicines for health benefits. Fifteen fungal endophytes were isolated from the plant parts of the ethnomedicinal plant of Western Ghats, *Tabernaemontana heyneana* Wall., and evaluated for their L-asparaginase and antioxidative potentials. They were identified by rDNA sequencing of the ITS region and characterized as strains of *Fusarium*, *Colletotrichum*, *Curvularia*, *Nigrospora*, *Plectospharella*, *Neocosmospora*, *Trichoderma* and *Endomelanconiopsis*. *Trichoderma hamatum* and *Fusarium* sp., colonized the bark, leaf and stem tissue fragments. Secondary metabolites from the endophytic strains were extracted in ethyl acetate and evaluated for total phenolic content and antioxidant activity. The total phenolic content of the extracts from all the fungal strains ranged from 17.29±12.92 to 90.41±4.56 mg Gallic acid equivalent/g (mg GAE/g) dry extract and IC_{50} values in DPPH radical scavenging activity ranged from 51.64±8.91 µg/ml to 764.05±27.67 µg/ml. Eleven strains were positive for L-asparaginase enzyme activity in the range of 1.44 to 3.42 IU/ml. *Fusarium tricinctum* (MK752630) strain potentially produced high enzyme activity of 3.423 IU/ml. The highest total phenolic content (90.14±4.56 mg GAE/g dry extract) and scavenging activity (IC_{50} value of 51.64±8.91 µg/ml) were detected in *F. tricinctum*.

Keywords: Endophytic fungi; L-asparaginase; *Fusarium tricinctum*; total phenolic content, DPPH radical scavenging, Western Ghats

1. Introduction

Plants are known to harbor endophytic fungi that reside in internal tissues without showing any disease symptoms (Frohlich and Hyde, 1999). The infected host tissues are transiently symptomless, and the microbial colonization can be demonstrated through histological means, by isolation from strongly surface disinfected tissue, or, most recently, through the direct amplification of fungal nuclear DNA from colonized plant tissue (Stone *et al*., 2000). Fungal endophytes represent an important component of fungal biodiversity and are known to have mutualistic association with host plants (Selim *et al*., 2012). Endophytes are known to influence population dynamics, plant community diversity and ecosystem functions (Saikkonen *et al*., 1998). This group of microbes not only synthesizes metabolites to compete first with epiphytes and then with pathogens in order to colonize the host, but also regulates metabolism of the host in a balanced association (Schulz *et al*., 2002). Once inside host tissue, they assume a quiescent state either for the whole time or for an extended period of time until environmental conditions are favorable for the fungus or the ontogenetic state of the host changes to the advantage of the fungus (Sieber, 2007).

Endophytic fungi are known to have profound effects on plant ecology, fitness, evolution (Brundett, 2006) and play vital roles in host plants being chemical synthesizers inside plants (Owen and Hundley 2004), producing phytohormones (Schulz and Boyle, 2005), withstanding environmental stress (Malinowski and Belesky, 2000) and providing protection against pathogens (Akello *et al*., 2007). In recent years, endophytes from medicinal plants have demonstrated the ability to produce bioactive compounds which are potential sources of novel natural products (Nalini *et al*., 2019).

Asparaginase is widely distributed in nature from bacteria to mammals and plays a central role in the amino acid metabolism and utilization. L-asparaginase (EC 3.5.1.1.) belongs to the group of homologous amidohydrolases family, which catalyses the hydrolysis of the amino acid L-asparagine to L-aspartate and ammonia. L-asparaginase is the first enzyme with anti-leukemic activity studied thoroughly in human beings (Savitri *et al*., 2003). Asparaginase is used in food industry to prevent the formation of acrylamide when foods are processed at high temperatures (Cachumba *et al*., 2016).

Naturally occurring phenolic compounds are plant secondary metabolites which are good electron donors since their hydroxyl groups can directly contribute to antioxidant action. Currently, there is a great interest in finding natural antioxidants from endophytes associated with plant species. Plant-endophyte interaction and endophyte-endophyte interaction studies provide understandings of metabolite production by fungi. Endophytes are the repository of natural products

* Corresponding author e-mail: nmsomaiah@gmail.com.

displaying broad spectrum of biological activities like anticancer, antibacterial, antiviral, anti-diabetic, antioxidant and anti-inflammatory (Rajamanikyam *et al*., 2017).

Tabernaemontana heyneana Wall., is endemic to southern Western Ghats and is listed as lower risk/ near threatened species in the "IUCN Red List of Threatened Species" (World Conservation Monitoring Centre, 1998). *T. heyneana* possess antimicrobial and anthelmintic properties against skin venereal, respiratory and nervous disorders (Duraipandiyan *et al*., 2006) and get used in the traditional Indian system of medicine as a cure for cancer (Baskar *et al*., 2012). The medicinal plants inhabiting Western Ghats are major sources of traditional pharmaceuticals and therapeutics of significant potentialities. Therefore, we have investigated the endophytic fungi residing in *T. heyneana*, their molecular identification and evaluation of their L-asparaginase and antioxidative capacities by *in vitro* methods.

2. Materials and methods

2.1. Collection of host plant

Healthy plant samples of *T. heyneana* Wall., were collected in the forests of Kodagu district, in the Talacauvery area of Western Ghats (12º17´to 12º27´ N and 75º26´ to 75º33´E), Karnataka, India, during the month of June 2014. The habit of plant and collection locality is shown in Figure 1. A total of five leaves, stem and bark samples were collected separately from five plants. Bark samples from the trunk were cut 1.5 m above the ground level with help of sterile machete. The samples were placed in pre-sterilized zip-lock polythene bags, stored at 4ºC and transported to the laboratory. Fresh plant materials were used for the isolation of fungal endophytes to reduce the chance of contamination. Thus collected plant materials were subjected to surface sterilization within few hours after sampling.

2.2. Surface sterilization and isolation of fungal endophytes

The leaf, stem and bark samples were rinsed gently in running tap water to remove dust and debris. Before surface sterilization, the cleaned stems were cut into pieces of 5 cm length and bark samples were halved. Samples were first immersed in 70% (v/v) ethanol for one minute, 3.5% (v/v) Sodium hypochlorite for two minutes. The samples were rinsed three times with sterile distilled water and dried on sterile blotters under laminar airflow to ensure complete drying (Schulz *et al*., 1993). Bits of 1.0X0.1 cm size were excised with the aid of sterilized blade. 200 segments of leaf, stem and bark were evenly placed on water agar (WA) medium (15 g/L). The Petri dishes were sealed using Parafilm ™ and incubated at 27±2º C in a light chamber with 12 hours of light followed by 12 hours of dark cycles for 4-6 weeks. The Petri dishes were monitored periodically to check the growth of endophytic fungal colonies from the segments. The hyphal tips which grew out from the segments were transferred separately onto fresh Potato Dextrose Agar (PDA) slants with a sterile fine tipped needle under stereo binocular microscope and incubated at 27±2 °C for 10-15 days and pure cultures were maintained at 4 °C for further use.

2.3. Identification of endophytic fungi

Morphological identification was done by inoculating the endophytic fungi on PDA plates followed by seven days of incubation and observation of colony and spore morphology. The slides of each fungal endophytes were prepared by tease mount method using lactophenol cotton blue staining [NICE, Kerala, India] and observed under the light microscope (Quasmo, Haryana, India) with 400X magnification. The identification was based on the observation of mycelia, fruiting bodies, conidial characters according to the standard identification manuals (Domsch *et al*., 1980; Singh *et al*., 1991; Barnett and Hunter, 1998; Mathur and Kongsdal, 2003).

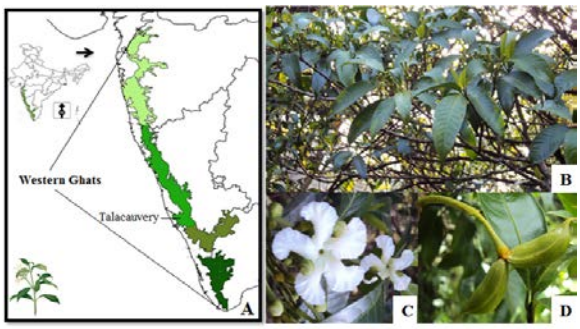

Figure 1.A. Map showing the location of plant sample collection from Talacauvery site of Western Ghats; B. *Tabernaemontana heyneana* Wall. habit; C-Flowers; D- A pair of follicles.

2.4. Molecular characterization of fungal endophytes

Hyphal tips from 15 morphologically different endophytic fungi, each representing individual morphospecies, were inoculated into conical flask containing Potato Dextrose Broth (PDB, Hi Media, Mumbai, India) and incubated at 27±2 °C for 7-10 days. The resulting mycelia were separated and placed in polythene zip lock covers. The mycelia were then freeze dried before being ground in a pestle and mortar containing liquid nitrogen. The powdered mycelia were stored at -20 °C until required. The genomic DNA was extracted using cetyltrimethylammonium bromide (CTAB) method with minor modifications (Ausubel *et al*., 1994). The DNA concentration was measured using Nanodrop Spectrophotometer (Thermo Fischer 2000c, Bangalore, India) at 260 and 280 nm.

PCR amplification in ITS rDNA region of isolates was conducted using PCR 18 kit (Chromous Biotech Pvt. Ltd. Bangalore, India). The target ITS 1, ITS 2 regions and 5.8s gene were amplified using ITS 1 (5´-TCCGTAGGTGAACCTGCG - 3´) and ITS 4 (5´-TCCTCCGCTTATTGATATGC - 3´) primers. Amplification reactions were performed in a thermal cycler (Master cycler gradient, Eppendorf, Germany) with the following cycling conditions 94ºC for 2 min (Initial denaturation), 35 cycles of 94ºC for 1 min (Denaturation), 47ºC for 15 sec (Primer annealing), 72ºC for 30 sec (Primer extension), followed by 10 min of final extension at 72ºC. Following amplification, the PCR products were analyzed by horizontal agarose gel electrophoresis through 1% agarose gel supplemented with ethidium bromide along with 100bp DNA marker. DNA bands on the gel were visualized under UV light trans-illuminator (Geldoc XRT, BioRad USA) and documented.

The PCR products were sent to Chromous Biotech Pvt. Ltd., Bangalore, India for purification and sequencing. Sequencing similarity searches were achieved for the obtained fungal sequences and compared with ITS sequence data from strains available in the database GenBank (National Centre for Biotechnology Information website; http://www.ncbi.nlm.nih.gov/) by using the BLAST sequence match routines.

2.5. Data Analysis

The relative colonization frequency (%CF) was calculated as the number of segments colonized by a fungus divide by total number of segments plated x 100.

2.6. L-asparaginase activity of fungal endophytes

Fifteen fungal endophytes isolated from leaf, stem and bark of *T. heyneana* were subjected to rapid screening of primary plate assay method using modified Czapek Dox (MCD) agar medium using phenol red as an indicator (Gulati *et al*., 1997). The composition of the prepared medium was as follows: glucose- 2.0 g/l, L-asparagine- 10 g/l, potassium dihydrogen phosphate (KH_2PO_4)- 1.52 g/l, potassium chloride (KCl)- 0.52 g/l, magnesium sulphate ($MgSO_4 \cdot 7H_2O$)-0.52 g/l, copper nitrate ($CuNO_3 \cdot 3H_2O$)-0.001 g/l, zinc sulphate ($ZnSO_4 \cdot 7H_2O$)- 0.001 g/l, ferrous sulphate ($FeSO_4 \cdot 7H_2O$)- 0.001 g/l and agar 20g/l. 2.5% (w/v) stock solution of the phenol red dye was prepared and MCD medium was supplemented with 0.009% phenol red dye indicator to check the ability of test fungi that grow in the medium. Final pH of the medium was adjusted to 6.2. The control plates were prepared with KNO_3-0.001 g/l as the nitrogen source and lacking in phenol red dye indicator. The prepared media was autoclaved and poured into pre–sterilized plates and marked into four quadrants. Mycelial plugs from four different endophytic fungi were inoculated and incubated at 27±2 °C for five days. The colonies showing pink zones were inoculated individually on MCD agar plates to approve the activity of enzyme.

2.7. Quantitative assay for enzyme production

For the production of L-asparaginase under liquid state, the mycelial discs from positive agar plates were inoculated into modified Czapek Dox's broth and incubated at 30ºC in orbital shaker (GeNei™, Bangalore) for 5 days at 120 rpm. The crude enzyme sample was extracted by centrifugation and supernatant that contained the enzyme used for further analysis by Nesslerization. The reaction mixture consisting of 0.5 ml of 0.04 M L-asparagine, 0.5 ml of 0.5 M TrisHCl buffer (pH 8.2), 0.5 ml of enzyme was obtained from the culture filtrate and 0.5 ml of distilled water. The samples were incubated for 30 min at 27ºC. To stop the enzymatic reaction, 0.5 ml of 1.5 M trichloroacetic acid (TCA) was added. This was followed by pipetting 0.1 ml of the mixture into fresh tubes containing 3.7 ml of distilled water and 0.2 ml of Nessler's reagent and incubated at 27 ºC for 20 minutes, after which the absorbance of the samples was measured at 450 nm using UV-Visible spectrophotometer (TPL Technology Pvt. Ltd.,Bangalore). Blank was prepared by adding TCA followed by enzyme sample. One international unit (IU) of L-asparaginase was expressed as the amount of enzyme that catalyzes the formation of 1µmol of ammonia per minute at 27 ºC (Imada *et al*., 1973).

2.8. Submerged fermentation and extraction of secondary metabolites

The sequential steps involved in fermentation and extraction of secondary metabolites is represented in Figure 2. At first the endophytic fungi were cultivated by inoculating the actively growing mycelia from 7-days-old pure culture into Erlenmeyer flask containing 500 ml of Potato Dextrose Broth. The flasks were then incubated for 21 days at 27±2 ºC. After incubation, the culture filtrates were filtered through muslin cloth to separate mycelia. To the culture filtrate, equal volume of ethyl acetate was added and mixed well for 10 minutes and kept for 5 minutes till two clear immiscible layers formed. The upper layer of the solvent containing extracted compounds was then separated, evaporated and dried in Rotary flash evaporator (Superfit Model PBU-6D, India). The dry solid residue was dissolved in ethanol and stored as crude extract in glass vials.

2.9. Determination of total phenolic content

The total phenolic content of ethyl acetate extracts of endophytic fungi was estimated using Folin-Ciocalteu (FC) reagent based assay using Gallic acid standard (Liu *et al*., 2007). One ml of FC reagent and two ml of (20%, w/v) Na_2CO_3 was mixed with methanolic crude extracts. The mixture was incubated at room temperature in the dark for 45 minutes, and the absorbance of the developed color was recorded at 765 nm using UV-vis spectrophotometer (DU 730 "Life sciences", Beckman Coulter). Gallic acid was used as a reference standard for plotting the calibration curve. The TPC of the extracts was expressed as mg of Gallic acid equivalent (GAE)/ g of the dry extract.

2.10. Determination of antioxidant capacity by DPPH (2, 2-diphenyl-1-picrylhydrazyl) assay

The antioxidant activity of endophytic fungal extracts was determined by free radical scavenging activity of DPPH by the method of Pannangpetch *et al*. (2007) with slight modifications. A total of two ml of DPPH solution (DPPH, Sigma-Aldrich, Bangalore, India) (0.001 mM) was added into the endophytic fungal extracts and incubated for 20 minutes at room temperature in the dark. After incubation, the absorbance was measured at 517 nm. Ascorbic acid was used as reference antioxidant compound. The scavenging activity was expressed as inhibitory concentration 50% (IC_{50}) (µg/ml). The scavenging activity was calculated using the following equation:

$$\% \text{ Scavenging} = \frac{Ac - As}{Ac} X100$$

Ac= Absorbance of control; As= Absorbance of test sample

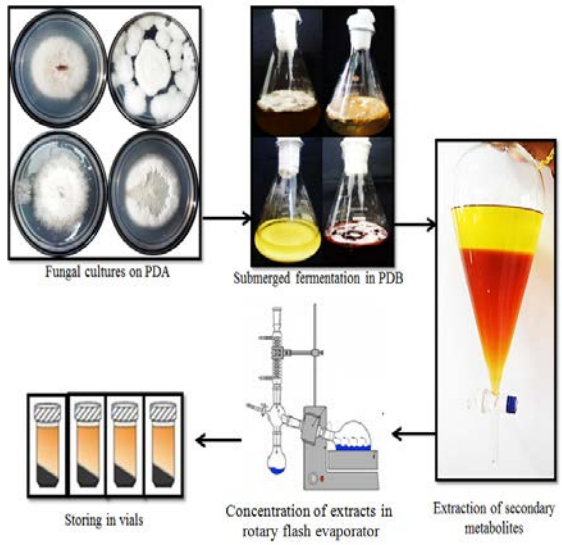

Figure 2. Fermentation and extraction of secondary metabolites from the fungal endophytes of *T. heyneana*

3. Results

3.1. Isolation and the identification of fungal endophytes

A total of 600 tissue segments yielded 477 isolates were obtained, which were distributed in 15 endophytic species. The results revealed that leaf fragments were colonized by more number of fungal endophytes (186) followed by stem (171) and bark segments (120). The analysis of distribution frequencies of endophytes indicated that the fungal communities in the host contained a few frequent genera and some infrequent species.

The isolates were identified using morphological and microscopic characteristics with the support of molecular analysis. 15 endophytic species belonged to Sordariomycetes (87%) and Dothideomycetes (13%) were obtained. Molecular characterization of isolated endophytic fungi with GenBank accession numbers, the closest match of ITS sequence in the NCBI database and their sequence similarity is depicted in Table 1. Among the isolated endophytic fungi, seven different species of *Fusarium* and two species of *Colletotrichum* were recovered. Five species of *Fusarium* viz., *Fusarium begoniae*, *Fusarium commune*, *Fusarium culmorum*, *Fusarium tricinctum*, *Fusarium babinda* were associated with the stem segments. In addition, *Nigrospora sphaerica* and *Curvularia coicis* were also recovered from the stem segments. *Neocosmospora haematococca*, *Plectosphaerella cucumerina*, *Colletotrichum fructicola* exhibited tissue specificity by colonizing the leaf segments, while *Fusarium petroliphilum*, *Endomelanconiopsis endophytica* were isolated from bark segments. *Colletotrichum gloeosporioides* colonized both leaf and the stem fragments, whereas *Fusarium* sp. and *Trichoderma hamatum* showed colonization in all plant segments. The percent colonization frequency of endophytic fungi in bark, leaf and stem parts along with their isolation code are represented in Table 2. The highest percent colonization was exhibited by *Fusarium* sp. (15.3) followed by *T. hamatum* (14.7) whereas *F. commune* showed the least percent colonization of 2.0. All endophytes from *T. heyneana* were assembled into eight different genera and assigned to Nectriaceae, Glomerallaceae, Plectosphaerellaceae, Hypocreaceae, Pleosporaceae, Botryosphaeriaceae and Trichosphaeriaceae.

3.2. Qualitative analysis of endophytic fungi for L-asparaginase activity

Of the 15 fungal endophytes screened for enzyme activity by preliminary plate assay method, 11 could grow on MCD agar producing pink zone with phenol red, a dye indicator that changes from yellow to pink (acidic to alkaline condition). The formation of pink zone around each fungal colony specified variations in pH due to the accumulation of ammonia in the medium.

3.3. Quantitative analysis by Nesslerization

Positive isolates from preliminary screening were further evaluated by Nesslerization using spectrophotometric methods. Eleven positive isolates exhibited asparaginase activity in the range of 1.448±0.07 to 3.423±0.05 IU/ml (Figure 3). *F. tricinctum* from stem showed highest asparaginase activity among all the tested endophytic fungi with 3.423±0.05 IU/ml of enzyme. *C. fructicola* from stem and *F. commune* from leaf also demonstrated high asparaginase activity of 3.166±0.07 and 3.036±0.11 IU/ml respectively. *Fusarium* sp., *F. begoniae* and *E. endophytica* showed moderate L-aspraginase activity in the range of 2.827±0.09 – 2.388±0.06 IU/ml of enzyme.

3.4. Determination of total phenolic content

Total phenolic content of all the fungal extracts ranged from 18.14±8.62 to 90.41±4.56 mg GAE/g dry extract (Table 3). The extracts of *F. tricinctum* displayed highest TPC (90.41±4.56 mg GAE/g dry extract) followed by *C. coicis* (88.99±38.75 mg GAE/g dry extract) and *N. sphaerica* (87.52±34.44 mg GAE/g dry extract).

3.5. Antioxidant activity

The antioxidant activity of the endophytic extracts was determined following DPPH radical scavenging activity. The free radical scavenging activity is represented as 50% scavenging activity (IC_{50}) and is depicted in Table 3. The fungal extracts indicated IC_{50} values ranging from 51.64±8.91 µg/ml to 764.05±27.67 µg/ml. *F. tricinctum* extract depicted high scavenging activity with 51.64±8.91 µg/ml, followed by *F. commune* (124.16±9.12) and *N. haematococca* (129.20±15.12 µg/ml).

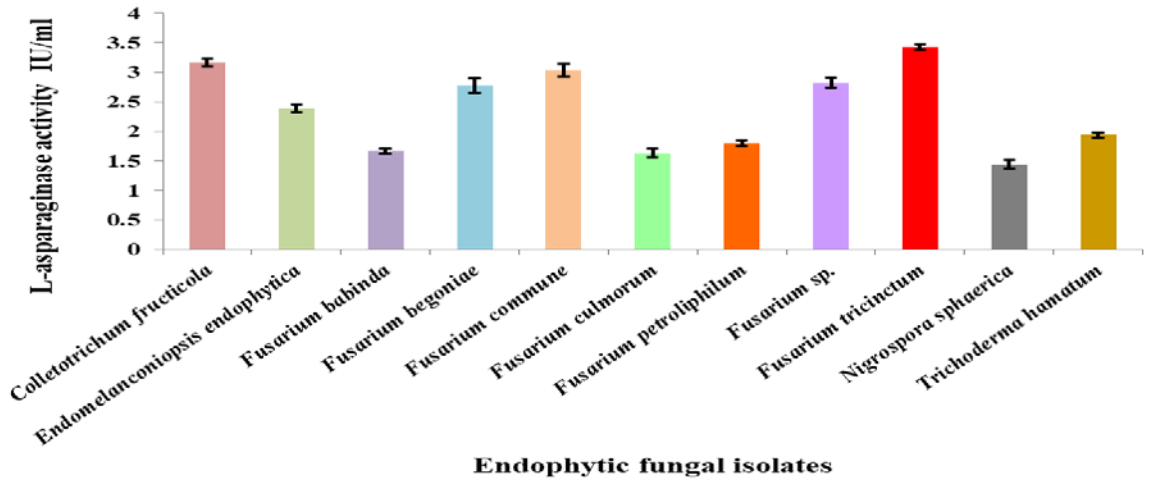

Figure 3. L-asparaginase producers of endophytic fungal species isolated from *T. heyneana.*

Data are reported as mean±SD of three independent analyses (n=3).

Table 1. Molecular characterization of fungal endophytes isolated from *T. heyneana* with GenBank accession numbers, ITS sequence match in the NCBI database and their percent sequence similarity

Fungal endophytes	Isolate code	GenBank accession number	Closest match ITS	Sequence length (bp)	Sequencing similarity
Colletotrichum gloeosporioides	TH-WG-03	MK767027	KU682216	441	441/441 (100%)
Colletotrichum fructicola	TH-WG-11	MK976025	MK041495	516	507/517 (98%)
Curvularia coicis	TH-WG-12	MK976026	MG589634	555	532/553 (96%)
Endomelanconiopsis endophytica	TH-WG-13	MK991793	MK075024	527	527/527 (100%)
Fusarium tricinctum	TH-WG-04	MK752630	MK102656	504	495/504 (98%)
Fusarium sp.	TH-WG-06	MK776870	MH935958	293	290/290 (100%)
Neocosmospora haematococca	TH-WG-07	MK911734	KX099641	512	459/464 (99%)
Nigrospora sphaerica	TH-WG-15	MN066344	MF380852	493	484/500 (97%)
Plectosphaerella cucumerina	TH-WG-08	MK940863	MH063586	499	485/487 (99%)
Trichoderma hamatum	TH-WG-09	MK940897	MK304047	551	553/553 (100%)
Fusarium babinda	TH-WG-10	MK940898	MH862578	404	381/406 (94%)
Fusarium begoniae	TH-WG-01	MK720625	KM577645	483	475/483 (98%)
Fusarium commune	TH-WG-02	MK723994	KX878889	473	473/473 (100%)
Fusarium culmorum	TH-WG-05	MK767026	KU375665	468	468/468 (100%)
Fusarium petroliphilum	TH-WG-14	MN066343	LS999414	506	501/509 (98%)

Table 2. Colonization frequency of endophytic fungi associated with *T. heyneana*

.Endophytic strains	Isolate code	Plant parts						
		Bark*		Leaf*		Stem*		Total % CF
		I	% CF	I	% CF	I	% CF	
Colletotrichum gloeosporioides	TH-WG-03	-	-	29	14.5	3	1.5	5.3
Colletotrichum fructicola	TH-WG-11	-	-	17	8.5	-	-	2.9
Curvularia coicis	TH-WG-12	-	-	-	-	17	8.5	2.9
Endomelanconiopsis endophytica	TH-WG-13	35	17.5	-	-	-	-	5.9
Fusarium tricinctum	TH-WG-04	-	-	-	-	36	18	6.0
Fusarium sp.	TH-WG-06	8	4.0	75	37.5	9	4.5	15.3
Neocosmospora haematococca	TH-WG-07	-	-	18	9.0	-	-	3.0
Nigrospora sphaerica	TH-WG-15	-	-	-	-	19	9.5	3.1
Plectosphaerella cucumerina	TH-WG-08	-	-	15	7.5	-	-	2.5
Trichoderma hamatum	TH-WG-09	49	24.5	32	16	7	3.5	14.7
Fusarium babinda	TH-WG-10	-	-	-	-	27	13.5	4.5
Fusarium begoniae	TH-WG-01	-	-	-	-	13	6.5	2.1
Fusarium commune	TH-WG-02	-	-	-	-	12	6.0	2.0
Fusarium culmorum	TH-WG-05	-	-	-	-	24	12	4.0
Fusarium petroliphilum	TH-WG-14	28	14.0	-	-	-	-	4.7

* 200 segments were plated from bark, leaf and stem respectively for frequency analysis. I: Number of isolates; CF-Colonization frequency; '-': Not detected

Table 3. Estimation of total phenolic content and radical scavenging potentials of *T. heyneana* endophytes

Fungal strains	Total phenolic content (mg GAE/ g dry extract)	DPPH radical scavenging capacity (IC_{50} µg/ml)
C. gloeosporioides	37.82±25.82	145.48±14.73
C. fructicola	33.51±16.02	413.36±26.40
C. coicis	88.99±38.75	374.15±30.50
E. endophytica	64.77±11.39	736.04±31.65
F. babinda	25.30±3.01	175.25±21.35
F. begoniae	37.87±3.44	138.68±5.71
F. commune	76.84±16.20	124.16±9.12
F. culmorum	35.96±21.52	378.04±17.48
F. petroliphilum	78.35±3.80	764.05±27.67
F. tricinctum	90.41±4.56	51.64±8.91
Fusarium sp.	18.14±8.62	336.03±22.71
N. haematococca	17.29±12.92	129.20±15.12
N. sphaerica	87.52±34.44	155.26±33.71
P. cucumerina	31.25±3.88	670.37±26.51
T. hamatum	68.65±12.62	481.24±17.99

Abbreviations: DPPH – 1,1-diphenyl-2-picrylhydrazyl; IC_{50} – Inhibitory concentration 50%. Data are reported as mean ± SD of three independent analyses.

4. Discussion

The Western Ghats of India has an exceptionally high level of biological diversity and is recognized as one of the world's 'hot spots' of biological diversity. *T. heyneana* is one of the medicinally important species of apocynaceae known to possess active pharmacological principles. This study has aimed to isolate endophytic fungi from *T. heyneana* and evaluate their L-asparaginase activity, total phenolic content and antioxidant activity.

Fifteen endophytic fungal strains such as species of *Fusarium*, *Colletotrichum*, *Neocosmospora*, *Plectosphaerella*, *Trichoderma*, *Curvularia*, *Endomelanconiopsis* and *Nigrospora* were identified using ITS sequencing method. The occurrence of seven different *Fusarium* species is remarkable as these have adapted to a range of geographical sites, climatic conditions, ecological habitats and host plants. Researchers suggest that there are similar positive interactions of endophytic *Fusarium* species with plants (Blok and Bollen, 1995). *Fusarium* sp. and *Colletotrichum* sp. are also reported as endophytes from earlier studies in *T. heyneana* (Manasa and Nalini, 2014). In our quest to identify fungal endophytes from medicinal species of *Rauvolfia*, both *Fusarium* and *Colletotrichum* spp. were documented as endophytes (Bhavana *et al*., 2019). Preceeding findings also reveal the evaluation of *T. divaricata* for the isolation of endophytes like *Colletotrichum*, *Gliocladium*, Mycelia sterilia, *Phoma*, *Phomopsis*, *Xylaria* sp. (Huang *et al*., 2008) as well as for arbuscular mycorrhizal and dark septate endophyte fungal associations (Debnath *et al*., 2015).

It was interesting to document that some of the fungal genera such as *Plectrosphaerella*, *Trichoderma*, *Curvularia*, *Endomelanconiopsis* and *Nigrospora* were not previously reported from *T. heyneana*. The variation in the colonization potential of the endophytic fungi shows that, each endophyte exhibited different degrees of affinity towards different tissues of the plant. *Fusarium* sp., and *Trichoderma* showed colonization in leaf, stem and bark tissues while the remaining endophytes were specific to tissue types.

Of the 15 fungal endophytes, the highest percent colonization was recorded in *Fusarium* sp. (15.3) followed by *T. hamatum* (14.7) whereas *F. commune* showed low percent colonization (2.0). *Fusarium* spp. as endophytes are documented from *T. heyneana* (Manasa and Nalini, 2014), *Catharanthus roseus* (Ayob and Simarani, 2016), Himalayan Yew plants (Garyali *et al*., 2013) and from Malaysian anticancer plants (Chow and Ting, 2015). Similarly, *Trichoderma* spp. as endophytes have been recorded from three medicinal plants ie., *Solanum surattense* (Ikram *et al*., 2019), *Salvia militiorrhiza* (Zhou *et al*., 2019) and *Elettaria* (Munir *et al*., 2019).

The fungal strains were investigated for L-asparaginase production, total phenolic content and antioxidative potentials. *T. heyneana* revealed the presence of diverse endophytic fungal species with L-asparaginase activity. Of the 15 endophytic strains screened, 11 were positive for L-asparaginase. *F. tricinctum* isolated from stem fragments showed high asparaginase activity among all the tested fungi (3.423±0.05 IU of enzyme).

Fusarium endophytes differ in their ability to produce L-asparaginase. *F. verticilloides* from *T. heyneana*, showed considerably less enzyme activity of 1.136 IU/ml (Manasa and Nalini (2014). *Fusarium* spp. are known producers of L-asparaginase. *F. oxysporum* with 27.10 units/gram dry substrate (Pallem, 2019), *F. equiseti* with 8.51 IU (Hosamani and Kaliwal, 2011), *F. culmorum* with 7.21 units/gram dry substrate (Meghavaram and Janakiraman, 2017), *Fusarium* sp. with 11.4 U/min/ml (Gonçalves *et al*., 2016), *F. solani* and *F. oxysporum* with 0.04 IU/ml and 0.08 IU/ml respectively were also reported for their asparaginase activity (Gulati *et al*., 1997). Our results are on par with the original work of Theantana *et al.* (2009), wherein quantitative activity for 53 positive/82 strains of endophytes ranged from 0.014 to 1.5 Units/ml. It is interesting to note that *Trichosporon asahii* IBBLA1 isolated from soil and moss samples from Schirmacher hills, Antarctica regions exhibited significant enzyme activity of 20.57 IU/ml (Ashok *et al*., 2019).

In the present investigation, high TPC of 90.41± 4.56 mg GAE/g dry extract, was detected in *F. tricinctum*. *T. heyneana* is a well-known Indian medicinal plant with various phytochemicals. The total phenolic contents of fresh leaf extract of *T. heyneana* contained 11.4± 0.17 mg GAE/g dry extract (Sathishkumar and Baskar, 2012), whereas in the methanol extract, the TPC was 14.0± 0.45 mg GAE/g dry extract (Manasa and Chandrashekar, 2015). On the basis of these observations, it can be opined that the phenolic content of endophytic fungal extracts are high in comparison with the host plants.

Because of their capability to secrete bioactive metabolites, endophytic fungi can also reduce the oxidative stress (Khan *et al*., 2017). The anti-oxidative function of endophytic fungi is due to the secretion of phenolic compounds. Of the 15 endophytic fungal strains screened for the antioxidant activity, *F. tricinctum* was found have high antioxidative potentials with IC_{50} value of 51.64 ± 8.91 µg/ml., whereas the DPPH radical scavenging activity of *T. heyneana* carried out by Baskar *et al*. (2012), Sathishkumar and Baskar (2012) showed IC_{50} values of

340.17 ±26.04 µg/ml and 507 µg/ml respectively, lesser in comparison with the current study.

Endophytic *F. tricinctum* with unique bioactivity from several medicinal species are reported viz., *Taxus baccata* (Vasundhara *et al*., 2016) *Salicornia bigelovii* (Zhang *et al*., 2015), *Aristolochia paucinervis* (Wätjen *et al*., 2009) *Fritillaria unibracteata* var. *wabuensis* (Pan *et al*., 2017a), *F. cirrhosa* (Pan *et al*., 2017b), grasses (Przemieniecki *et al*., 2019) and *Paris polyphylla* var. *yunnanensis* (Zhang *et al*., 2011).

5. Conclusion

This study explores the endophytic fungi from *T. heyneana* and their L-asparaginase, total phenolic content and antioxidative potentials. The increasing importance of L-asparaginase in recent years due to its anti-carcinogenic applications encouraged us to screen the extracts of endophytes for L-aspraginase activity. *F. tricinctum* displayed significant L-asparaginase activity with remarkable total phenolic content and antioxidative capacities. Our study has shown that fungal endophytes from *T. heyneana* have potential bioactivities. The extracts are being evaluated for their anti-proliferative potentials in *in vitro* cancer cell lines.

Conflict of interest

The authors declare that there is no conflict of interest

Funding

This work was partially funded by the Department of Backward Classes Welfare, Government of Karnataka, India to the first author

Acknowledgements

The authors thank the Institution of Excellence (IOE) Biodiversity & Sustainable Development, Vijnan Bhawan, and the University of Mysore for providing the necessary facilities to carry out the present study.

References

Akello J, Dubois T, Gold CS, Coyne D, Nakavuma J and Paparu P. 2007. *Beauveria bassiana* (Balsamo) vuillemin as an endophyte in tissue culture banana (*Musa* spp.). *J Invertebr Pathol*., **96**: 34–42. http://doi.org/10.1016/j.jip.2007.02.004.

Ashok A, Doriya K, Rao JV, Qureshi A, Kumartiwari A and Kumar DS. 2019. Microbes producing L-asparaginase free of glutaminase and urease isolated from extreme locations of Antarctic soil and moss. *Sci rep*., **9**: 1423. https://doi.org/10.1038/s41598-018-38094-1

Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA and Struhl K. 1994. **Current Protocols in Molecular Biology**. Wiley, New York.

Ayob FW and Simarani K. 2016. Endophytic filamentous fungi from a *Catharanthus roseus*, identification and its hydrolytic enzymes. *Saudi Pharm J.*, **24**(3): 273-278.

Barnett HL and Hunter BB. 1998. **Illustrated Genera of Imperfect Fungi.** APS Press, Minnesota, Minn, USA, 4th edition.

Baskar AA, Al Numair KS, Alsaif MA and Ignacimuthu S. 2012. *In vitro* antioxidant and antiproliferative potentials of medicinal plants used in traditional Indian medicine to treat cancer. *Redox Rep.,* **17**(4): 145-156. DOI: 10.1179/1351000212Y.0000000017

Bhavana NS, Prakash HS and Nalini MS. 2019. Antioxidative and L-asparaginase potentials of fungal endophytes from *Rauvolfia densiflora* (Apocynaceae), an ethnomedicinal species of the Western Ghats. *Czech Mycol* **71**(2): 187–203. DOI: https://doi.org/10.33585/cmy.71205

Blok WJ and Bollen GJ. 1995. Fungi on roots and stem bases of *Asparagus* in the Netherlands species and pathogenicity. *Eur J Plant Pathol.,* **101**(1): 15-24. DOI:https://doi.org/10.1007/BF01876090

Brundrett MC. 2006. Understanding the roles of multifunctional mycorrhizal and endophytic fungi. In: Schulz BJE, Boyle CJC, Sieber TN (eds.), **Microbial Root Endophytes**. Berlin, Germany: Springer-Verlag, PP. 281–293.

Cachumba JJ, Antunes FA, Peres GF, Brumano LP, Santos JC and Da Silva SS. 2016. Current applications and different approaches for microbial L-asparaginase production. *Braz J Microbiol.,* **47**(1): 77-85.

Chow YY and Ting ASY. 2015. Endophytic L-asparaginase producing fungi from plants associated with anticancer properties. *J Adv Res.,* **6**(6): 869-876. DOI:10.1016/j.jare.2014.07.005

Debnath A, Karmakar P, Debnath S, Roy Das A, Saha AK and Das P. 2015. Arbuscular mycorrhizal and dark septate endophyte fungal association in some plants of Tripura, North-East India.*Curr Res Environ Appl Mycol*., **5**(4): 398-407.

Domsch KH, Gams W and Anderson T. 1980. **Compendium of soil fungi**. Academic Press, New York, NY, USA.

Duraipandiyan V, Ayyanar M and Ignacimuthu S. 2006. Antimicrobial activity of some ethnomedicinal plants used by Paliyar tribe from Tamil Nadu, India. *BMC Complement Altern Med*., **6**: 35-41. DOI:10.1186/1472-6882-6-35

Fröhlich J and Hyde KD. 1999. Biodiversity of palm fungi in the tropics: are global fungal diversity estimates realistic?. *Biodivers Conserv*., **8**: 977–1004

Garyali S, Kumar A and Sudhakara Reddy M. 2013. Taxol production by an endophytic fungus *Fusarium redolens*, isolated from Himalayan Yew. *J Microbiol Biotechnol.,* **23**(10): 1372-1380.

Gonçalves AB, Maia ACF, Rueda JA and Vanzela APFC. 2016. Fungal production of the anti-leukemic enzyme L-asparaginase: from screening to medium development. *Acta Sci Biol Sci*., **38**(4): 387-394.

Gulati R, Saxena RK and Gupta R. 1997. A rapid plate assay for screening L-asparaginase producing microorganisms. *Lett Appl Microbiol.,* **24**: 23-26.

Hosamani R and Kaliwal BB. 2011. L-asparaginase an antitumor agent production by *Fusarium equiseti* using solid state fermentation. *Int J Drug Discov.*, **3**(2): 88-99.

Huang WY, Cai YZ, Hyde KD, Corke H and Sun M. 2008. Biodiversity of endophytic fungi associated with 29 traditional Chinese medicinal plants. *Fungal Divers.,* **33**: 61–75.

Ikram M, Ali N, Jan G, Hamayun M, Jan FG and Iqbal A. 2019. Novel antimicrobial and antioxidative activity by endophytic *Penicillium roqueforti* and *Trichoderma reesei* isolated from *Solanum surattense*. *Acta Physiol Plant.,* **41**(164): 1-11.

Imada A, Igarasi S, Nakahama K and Isono M. 1973. Asparaginase and glutaminase activities of micro-organisms. *J Gen Microbiol.,* **76**(1): 85-99.

Khan AL, Gilani SA, Waqas M, Al-Hosni K, Al-Khiziri S, Kim YH, Ali L, Kang SM, Asaf S, Shahzad R, Hussain J, Lee IJ and Al-Harrasi A. 2017. Endophytes from medicinal plants and their potential for producing indole acetic acid, improving seed germination and mitigating oxidative stress. *J Zhejiang Univ Sci B.,***18**(2): 125–137.

Liu X, Dong M, Chen X, Jiang M, Lv X and Yan G. 2007. Antioxidant activity and phenolics of an endophytic *Xylaria* sp. from *Ginkgo biloba*. *Food Chem.*,**105**(2): 548-554.

Malinowski DP and Belesky DP. 2000. Adaptations of endophyte-infected cool-season grasses to environmental stresses: mechanisms of drought and mineral stress tolerance. *Crop Sci*., **40**: 923–940. DOI: 10.2135/cropsci2000.404923x.

Manasa C and Nalini MS. 2014. L-Asparaginase activity of fungal endophytes from *Tabernaemontana heyneana* Wall. (Apocynaceae), endemic to the Western Ghats (India). *Int Sch Res Notices.*, 1-7. Article ID 925131, http://dx.doi.org/10.1155/2014/925131

Manasa DJ and Chandrashekar KR. 2015. Antioxidant and antimicrobial activities of *T. heyneana* Wall. And endemic plant of Western Ghats. *Int J Pharm Pharm Sci.*,**7**(7): 311-315.

Mathur SB and Kongsdal O. 2003. **Common laboratory seed health testing methods for detecting fungi.** International Seed Testing Association, Geneva, Switzerland.

Meghavaram AK and Janakiraman S. 2017. Solid state fermentation: An effective fermentation strategy for the production of L-asparaginase by *Fusarium culmorum* (ASP-87). *Biocat Agri Biotech.*, **11**:124-130. DOI:10.1016/j.bcab.2017.06.001

Munir E, Lutfia A and Yurnaliza 2019. Records of culturable endophytic fungi inhabiting rhizome of *Elettaria* in Hutan Sibayak, North Sumatera the 4th International Conference on Biological Sciences and Biotechnology. Pondicherry University, Kalapet, Pondicherry **305**: 1-6. DOI:10.1088/1755-1315/305/1/012004

Nalini MS, Prakash HS and Tejesvi MV. 2019. Bioactive potentials of novel molecules from the endophytes of medicinal plants. In: Egamberdieva D and Tiezzi A (Eds.), **Medically Important Plant Biomes: Source of Secondary metabolites**, **Microorganisms for Sustainability** 15, Singapore, Springer Nature Singapore Pte Ltd, PP. 293-351. https://doi.org/10.1007/978-981-13-9566-6_13

Owen NL and Hundley N. 2004. Endophytes-the chemical synthesizers inside plants. *Sci Pro*., **87**: 79–99. https://doi.org/10.3184/003685004783238553.

Pallem C. 2019. Solid-state fermentation of corn husk for the synthesis of asparaginase by *Fusarium oxysporum*. *Asian J Pharm Pharmacol.*, **5**(4): 678-681. DOI: https://doi.org/10.31024/ajpp.2019.5.4.5

Pan F, Su TJ, Cai SM and Wu W. 2017a. Fungal endophyte-derived *Fritillaria unibracteata* var. *wabuensis*: diversity, antioxidant capacities *in vitro* and relations to phenolic, flavonoid or saponin compounds. *Sci Rep.*, **7**:42008. DOI: 10.1038/srep42008

Pan F, Su TJ, Deng KL and Wu W. 2017b. Antioxidant activity and methanolic constituents of endophytic *Fusarium tricinctum* CBY11 isolated from *Fritillaria cirrhosa*. *Mycosystema*., **36**(6): 752-765. DOI: 10.16333/j.1001-6880.2017.3.002

Pannangpetch P, Laupatarakasem P, Kukonviriyapan V, Kukongriyapa C, Kongyinayoes B and Aromdee C. 2007. Antioxidant activity and protective effect against oxidative hemolysis of *Clinacanthus nutans* (Burm.f).Lindau. *Songklanakarin J Sci Technol.*, **29**(1): 1-9.

Przemieniecki SW, Damszel M, Kurowski TP, Mastalerz J. and Kotlarz K. 2019. Identification, ecological evaluation and phylogenetic analysis of non-symbiotic endophytic fungi colonizing timothy grass and perennial ryegrass grown in adjacent plots. *Grass Forage Sci.*, **74**: 42–52. DOI: 10.1111/gfs.12404

Rajamanikyam M, Vadlapudi V, Ramars amanchy and Upadhyayula SM. 2017. Endophytic fungi as novel resources of natural therapeutics. *Braz Arch Biol Technol.*, **60**: 1-26. http://dx.doi.org/10.1590/1678-4324-2017160542.

Saikkonen K, Faeth SH, Helander M and Sullivan TJ. 1998. Fungal Endohytes: A continuum of interactions with host plants. *Annu Rev Ecol Evol Syst.*, **29**(1): 319-343. https://doi.org/10.1146/annurev.ecolsys.29.1.319

Sathishkumar T and Baskar R. 2012. Evaluation of antioxidant properties of *Tabernaemontana heyneana* Wall.leaves. *Ind J Nat Prod Resour.*, **3**(2): 197-207.

Savitri, Asthana N and Azmi W. 2003. Microbial L-asparaginase a potent antitumor enzyme. *Ind J Biotech.*, **2**: 184-194.

Schulz B and Boyle C. 2005. The endophytic continuum. *Mycol Res.*, **109**: 661-686. https://doi.org/10.1017/S095375620500273X

Schulz BU, Wanke U, Drager S and Aust HJ. 1993. Endophytes from herbaceous plants and shrubs: effectiveness of surface sterilization methods. *Mycol Res.*, **97**: 1447-1450. https://doi.org/10.1016/S0953-7562(09)80215-3

Schulz B, Boyle C, Draeger S, Römmert AK and Krohn K. 2002. Endophytic fungi: a source of novel biologically active secondary metabolites. *Mycol Res.*, **106** (9): 996-1004 https://doi.org/10.1017/S0953756202006342

Selim KA, El-Beih AA, AbdEl-Rahman TM and El-Diwany AI. 2012. Biology of endophytic fungi. *Curr Res Environ Appl Mycol.*, 31-82. DOI: 10.5943/cream/2/1/3

Sieber TN. 2007. Endophytic fungi in forest trees: are they mutualists?. *Fungal Biol Rev*., **21**(2-3): 75-89. https://doi.org/10.1016/j.fbr.2007.05.004

Singh K, Frisvad JC, Thrane U and Mathur SB. 1991. **An illustrated manual on identification of some seed-borne *Aspergilli, Fusaria, Penicillia* and their mycotoxins**. Danish Government, Institute of Seed Pathology for Developing Countries, Hellerup, Denmark.

Stone JK, Bacon CW and White JF. 2000. An overview of endophytic microbes: endophytism defined. In: Bacon CW and White JF (Eds.), **Microbial Endophytes**. Marcel Dekker, New York, PP. 3-29.

Theantana T, Hyde KD and Lumyong S. 2009. Asparaginase production by endophytic fungi from Thai medicinal plants: cytotoxicity properties. *Int J Integr Biol*., **7**(1): 1-8.

Vasundhara M, Baranwal M and Kumar A. 2016. *Fusarium tricinctum*, an endophytic fungus exhibits cell growth inhibition and antioxidant activity. *Ind J Microbiol.*, **56**(4): 433–438. DOI: 10.1007/s12088-016-0600-x

Wätjen W, Debbab A, Hohlfeld A, Chovolou Y, Kampkötter A, Edrada RA, Ebel R, Hakiki A, Mosaddak M, Totzke F, Kubbutat MH and Proksch P. 2009. Enniatins A1, B and B1 from an endophytic strain of *Fusarium tricinctum* induce apoptotic cell death in H4IIE hepatoma cells accompanied by inhibition of ERK phosphorylation. *Mol Nutri Food Res.*, **53**(4): 431-40. DOI: 10.1002/mnfr.200700428

Zhang J, Liu D, Wang H, Liu T and Xin Z. 2015. Fusartricina sesquiterpenoid ether produced by an endophytic fungus *Fusarium tricinctum* salicorn 19. *Eur Food Res Tech.*, **240**: 805-814. DOI:10.1007/s00217-014-2386-6

Zhang Y, Zhao J, Wang J, Shan T, Mou Y, Zhou L and Wang J. 2011. Chemical composition and antimicrobial activity of the volatile oil from *Fusarium tricinctum*, the endophytic fungus in *Paris polyphylla* var. yunnanensis. *Nat Prod Commun*., **6**(11): 1759-1762.

Zhou J, Xu Z, Sun H and Zhang H. 2019. Smoke-isolated butenolide elicits tanshinone I production in endophytic fungus *Trichoderma atroviride* D16 from *Salvia miltiorrhiza*. *S Afr J Bot.*, **124**: 1-4. https://doi.org/10.1016/j.sajb.2019.04.005.