

Morphological and molecular characterization of endophytic fungi associated with Cocoa (*Theobroma cacao* L.) in India

ABSTRACT

Aim: To identify cocoa associated endophytic fungi through morphological and molecular techniques.

Place and duration of study: Department of Plant Pathology, TNAU, Coimbatore, Tamil Nadu during April 2018 to November 2019.

Methodology: Endophytic fungal isolates were isolated from different parts of cocoa using Petrini method. Isolated endophytic fungal strains were grown in Malt extract broth and total genomic DNA was isolated and amplified using universal primers ITS1F and ITS4R. Amplified rDNA was visualized and documented.

Results: Morphological and molecular characterization of six endophytic fungi revealed that they are from four different taxa viz., *Lasiodiplodia pseudotheobromae* PAK-7, *Arthrinium rasikravindrae* P11, *Arthrinium rasikravindrae* P12, *Diaporthe* sp. Vef-3, *Lasiodiplodia theobromae* TN-R-3, *Colletotrichum* sp. TN-9-2 belonging to four different families viz., Botryosphaeriaceae, Apiosporaceae, Diaporthaceae and Glomerellaceae. under Phylum Ascomycota.

Conclusion: Present study indicates distribution and diversity of fungal endophytes in different plant parts of cocoa tree in south India.

Key words: cocoa, endophytic fungi, ITS.

1. Introduction:

Endophytes are diverse group of microbes living inside plants without any external symptoms. They influence plant health by the production of phytohormone and antimicrobial compounds. They also compete with plant pathogens for ecological niches and nutrients. Major group of endophytic fungi belongs to Ascomycetes and anamorphic fungi [1]. Basidiomycetes and zygomycetes are rarely encountered. These fungi have been identified in nearly 3,00,000 plant species and they dwell in all plant parts (leaf, flower, petiole, root, fruit) [23]. There existed a co-evolution between endophyte and its host which leads to low virulence [17]. The interaction between endophyte and host ranges from symbiotic mutualistic, to pathogenic [21, 20] depending on environmental conditions and plant health [15]. Cocoa (*Theobroma cacao* L.) is an introduced tropical rainforest crop grown as an intercrop or mixed crop within coconut, arecanut or oil palm plantations in India. In India Cocoa is being cultivated in the States of Kerala, Karnataka, Andhra Pradesh and Tamil Nadu in an area of 78,000 ha with total production of 16,050 MT. The average productivity of cocoa in India is 475 Kg/ha (www.dccd.gov.in). The present investigation was carried out with a view to isolate and identify fungal endophytes of cocoa both from morphologically and molecularly using PCR-based molecular techniques which helps to know endophytic fungal diversity in healthy cocoa tree.

2. Materials and method

2.1 Sample collection

During September 2017 asymptomatic plant from a disease prone area was located and marked. Different plant parts were excised with sterile knife and samples were carried to the lab in a zip-lock cover and kept at 4°C until further use (Table 1).

Table 1. Place of collection and isolation source of endophytic fungi.

Sl.no	Isolate	Intercropping system	Place of collection	Plant part
1.	Pak-7	Coconut and cocoa	Palakad, Kerala	Petiole
2.	P11	Coconut and cocoa	Palakad, Kerala	Petiole
3.	P12	Coconut and cocoa	Palakad, Kerala	Petiole
4.	VEF-3	Arecanut and cocoa	CPCRI,RS, Vittal, Karnataka	Petiole
5.	TN-R-3	Coconut and cocoa	TNAU, Coimbatore,TN	Root
6.	TN-9-2	Coconut and cocoa	TNAU, Coimbatore, TN	Root

2.2 Isolation of endophytic fungi

Isolation of endophytic fungi from cocoa was done by following Petrini method [11]. Sterilization involved washing of collected plant sample in running tap water followed by drying. Washed plant parts viz, stem, leaf, petiole and roots were excised into small bits with sterile scalpel and surface sterilized with 4% NaOCl for 30sec-1min followed by two times rinse in sterile distilled water. A second wash was given with 70% ethanol for 1-2 min. the surface sterilization efficiency was checked by plating last wash water on malt extract agar which served as a control. Plant parts were properly dried before placing on MEA and incubated at 27±2°C for 7-14 days depending on the growth of the endophyte.

2.3 Morphological characterization

Initial identification of fungal endophytes were done based on morphological observations. A loop full of mycelium was taken on a cavity slide and observed under stereobinocular phase contrast microscope (LEICA DM2000 LED) for the presence of structures like mycelium (color & septation) sexual and asexual structures/spores. Images were captured and analyzed using image analyzer.

2.4 ITS-PCR and sequencing

Molecular characterization was done following ITS-PCR and sequencing of amplified DNA fragment. Cultures were grown in MEB ((Malt extract broth) for 7 days at 27±2°C inside a BOD incubator. Approximately 200mg of freeze dried (-20°C) mycelium was macerated with 1-2ml of CTAB buffer (10%CTAB- 10ml, 1M Trisbase-5ml, 5M EDTA-2ml (pH-8), mercaptoethanol-1ml and dw-18ml) warmed at 60°C. Transferred 700µl of macerate into a eppendorf tube (2ml) and warmed at 65°C for 10min. Added 750 µl of Phenol: Chloroform: Isoamyl alcohol (25:24:1) and inverted it to form an emulsion. Centrifuged the content @10000rpm for 10 min. supernatant was transferred into sterile eppendorf tube and 0.5vol of 5M NaCl (150µl) and 2vol (600µl) of ice cold Isopropanol was added and incubated @ -20°C for 12h/overnight. Later tubes were inverted 2-3 times and centrifuged @13000rpm at 4°C for 10min. supernatant was discarded and the pellet was washed with 70% ethanol. After proper drying pellet was suspended in 50µL of TE buffer (10 mM Tris HCl pH 8.0, 1 mM EDTA). The quantity of DNA was checked by electrophoresis in 0.8 % agarose (HiMedia pvt Ltd. India) gel supplemented with 2µl of ethidium bromide for 45min at 90V in 1X TAE buffer (Tris base - 4.84g, acetic acid-1.09ml, EDTA-0.292g, dw-100ml, pH-8.1—8.2) and visualized under UV transilluminator.

Before PCR the concentration of genomic DNA was adjusted to 200ng. Later ITS-PCR was carried out using internal transcribed spacer primers ITS1F (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4R

(5'-TCCTCCGCTTATTGATATGC-3') with a final reaction mixture concentration of 55µl (smart primer - 20 µl, ITS1F- 4 µl, ITS4R- 4 µl, d2H20- 8 µl and DNA-8 µl). Amplification was carried out in Eppendorf thermal cycler with initial denaturation at 95°C for 3 min, denaturation at 95°C for 1min, annealing at 55°C for 1 min, extension at 72°C for 1 min followed by final extension at 72°C for 5 min with 35 cycles. The PCR amplified products were visualized in 1.2% agarose gel and documented. Amplified product was sent to AgriGenome Lab Pvt Ltd for purification and sequencing.

3. Results and discussion

The current study was carried out to find the associated endophytic fungi in apparently healthy cocoa tree in a disease prone area. Isolated endophytic fungi were morphologically and molecularly characterized following microscopic observations and rDNA sequence blast in NCBI database USA.

3.1 Morphological characterization

Morphological characterization is one of the oldest and reliable method for identification of any microorganisms. In the present investigation Cocoa endophytic fungi were characterized morphologically by studying mycelial and spore characters. Isolate PAK-7 (*Lasiodiplodia pseudotheobromae*) and TN-R-3 (*Lasiodiplodia theobromae*) produced coloured and septate mycelium without any sexual or asexual structures. Similarly morphological characterization of endophytic and saprobic isolates of *Lasiodiplodia pseudotheobromae* NI173 was studied where it produced brown coloured conidia with 19–25×12–15µm size [3].

The genus *Arthrinium* is widespread and occurs as saprobe on different range of substrate [1]. It is a plant pathogen reported to cause diseases like kernel blight of barley [9], damping-off of wheat [10]. It's endophytic nature has been reported in lichens, marine algae plant tissues [12,19,7]. Hanada et al. [6] reported endophytic nature of *Arthrinium sp* in cocoa tree. In present study *Arthrinium rasikravindrae* isolates P11 and *Arthrinium rasikravindrae* P12 produced olive green coloured lenticular single celled conidia with septate olive green coloured mycelium. The present finding is in agreement with Singh et al. [18] wher he isolated *Arthrinium rasikravindrii* sp. nov. from soil and it produced dark brown lenticular conidia with hyaline equatorial germ slits together with balloon-shaped, anomalous conidia.

The Isolate TN-9-2 (*Colletotrichum sp.*) produced hyaline dumble shaped single celled conidia this is in agreement with Rojas et al. [13]. Endophytic nature of *Diaporthe sp.*, *Colletotrichum sp* and *Lasiodiplodia sp* in cocoa was reported by Rubini et al. [14]. Ding et al.[4] studied the morphological characters of endophytic *Diaporthe sp.* Isolated from *Camptotheca acuminata* from China. It produced gray colored colony with branched and septate mycelium. This finding matching with that of present study where isolate VEF-3 (*Diaporthe sp.*) produced light brown to dark colored septate mycelium with few alpha conidia in MEA medium.

3.2 Molecular characterization and phylogeny

3.2.1 ITS-PCR and sequencing

Isolated and amplified rDNA from endophytic fungi were subjected to PCR analysis. The DNA band size ranged from 336-457bp (Fig 2). ITS rDNA sequence analysis of PAK-7 and TN-R-3 showed cent percent similarity with *Lasiodiplodia pseudotheobromae* and *Lasiodiplodia theobromae* respectively (Table 2). Similarly de Silva et al.[3] did molecular characterization of *Lasiodiplodia pseudotheobromae* isolated from Magnolia forest plants using combined ITS, tef1 and tub2 genes.

Endophytic *C. gloeosporioides* strains from asymptomatic leaves, anthracnose lesions on leaves and fruits of *Theobroma cacao* (cacao) were subjected to multilocus phylogeny to distinguish host-associated pathogens from asymptomatic cocoa plant parts [13]. Endophytic Isolate TN-9-2 from the present investigation produced dumble shaped hyaline conidia which is the characteristic feature of genera *Colletotrichum*. The sequencing result showed that it matched with that of *Colletotrichum sp.* voucher UOM 1290T with 100% identity (Table 2). Lima et al.[8] identified *C. gloeosporioides*, *C. boninense*, and *C. simmondsii* in Brazilian pepper tree using combined morphological and PCR

taxon-specific primer with CaInt/ITS4, CgInt/ITS4, and Col1/ITS4, which amplify specific bands in *C. acutatum*, *C. gloeosporioides lato sensu*, and *Colletotrichum boninensis*, respectively.

Hanada et al.[6] reported endophytic nature of *Arthrinium sp* in cocoa tree. In present study endophytic isolates P11 and P12 were isolated from cocoa and they were molecularly characterized using ITS1F and ITS4R primers. The sequence result showed 100% identity with *Arthrinium rasikavindii* (Table 2). Ghasemi et al. [5] identified endophytic *Arthrinium arundinis* from oak trees in Arasbaran forest based on morphological characters and ITS-rDNA and Tub region sequence data.

There are many reports suggesting endophytic nature of *Diaporthe sp.* In cocoa as well as in forest tree. In present study endophytic fungal isolate Vef-3 sequence data matched with taxa *Diaporthe sp.* with 100% similarity.

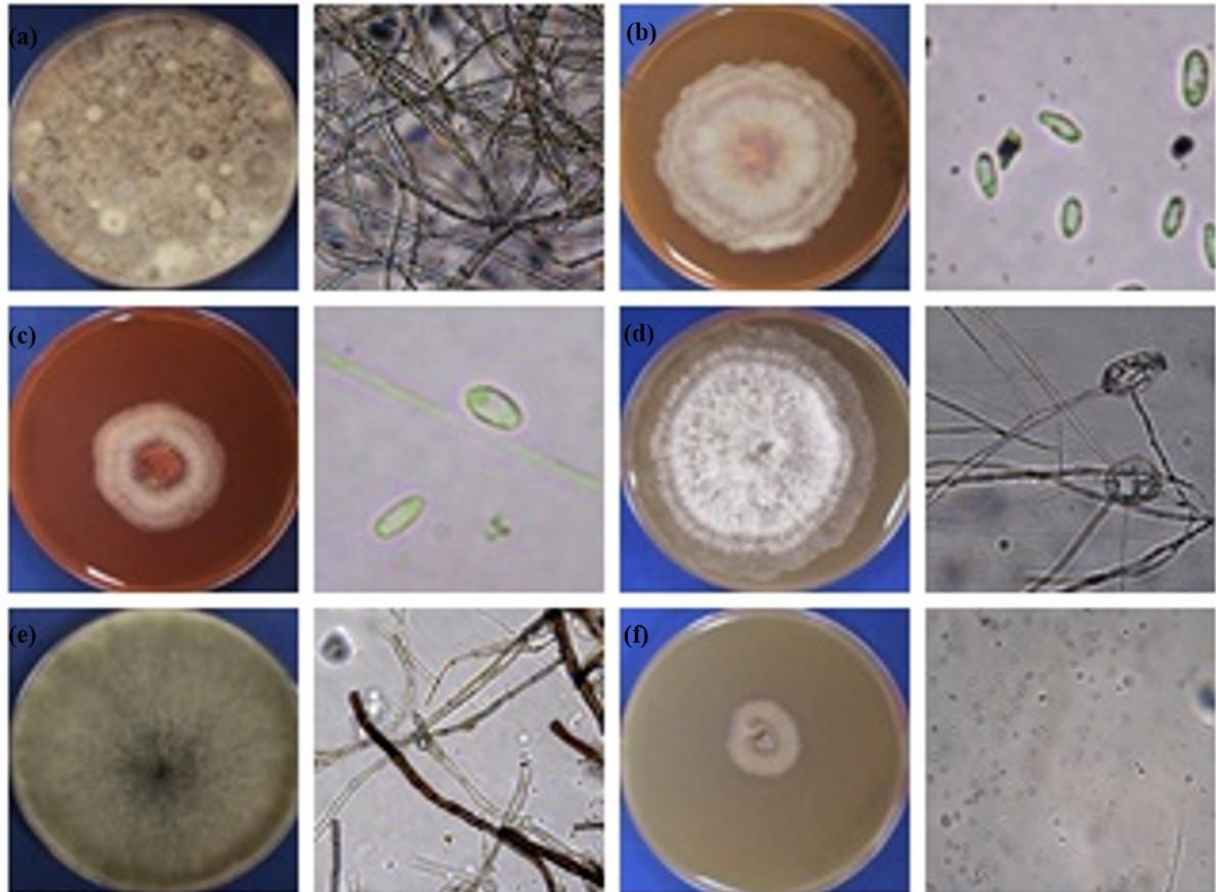


Fig 1. Morphological characters of endophytic fungi a-PAK-7 (*Lasiodiplodia pseudotheobromae*), b- c *Arthrinium rasikravindrae* P11 and P12 isolates conidia, d- Vef-3 (*Diaporthe sp.*) melanized mycelium, e- TN-R-3 (*Lasiodiplodia theobromae*) melanized mycelium, f- TN-9-2 (*Colletotrichum sp.*) conidia.

Table 1. Morphological characterization of endophytic fungi of cocoa.

Sl. no	Isolate	Organism	Culture colour	Margin	Mycelium	Spore
1	PAK-7	<i>Lasiodiplodia pseudotheobromae</i>	Lichen Green	Entire	Light brown color	Sterile
2	P11	<i>Arthrinium</i>	Pinkish	Irregular/Wa	Olive green	Olive green

		<i>rasikravindrae</i>	white	vy	color	color conidia
3	P12	<i>Arthrinium rasikravindrae</i>	Pinkish white	Fringed	Olive green color	Olive green color conidia
4	Vef-3	<i>Diaporthe</i> sp.	Chalk white	Fringed	Light brown to dark brown	Colorless Alpha conidia
5	TN-R-3	<i>Lasiodiplodia theobromae</i>	Ash gray	Irregular	Chocolate brown and septate	Sterile
6	TN-9-2	<i>Colletotrichum</i> sp	Ash gray	Irregular	Hyaline	Dumble shaped single celled hyaline conidia

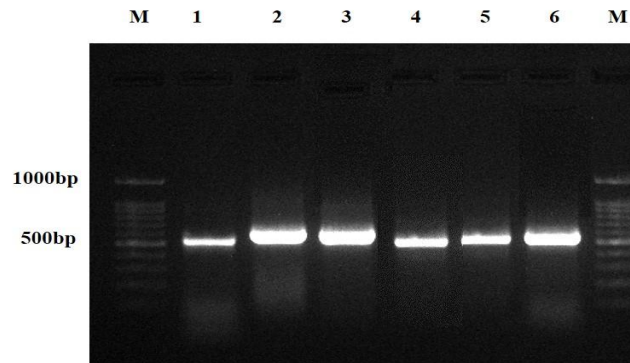


Fig 2. Molecular characterization of endophytic fungi of cocoa using ITS1F and ITS4R primers: M- 1.5kb Marker, 1- PAK-7 (*Lasiodiplodia pseudotheobromae*), 2- P11 (*Arthrinium rasikravindrae*), 3- P12 (*Arthrinium rasikravindrae*), 4- Vef-3 (*Diaporthe* sp.), 5 -TN-R-3 (*Lasiodiplodia theobromae*), 6 - TN-9-2 (*Colletotrichum* sp.).

Table 2. Molecular characterization of endophytic fungi isolated from cocoa

Sl. no	Isolate	Accession number	Closest match	Query coverage (%)	Percent identity
1.	Pak-7	MN418007	<i>Lasiodiplodia pseudotheobromae</i>	100	100
2.	P11	MN414159	<i>Arthrinium rasikravindrae</i>	100	100
3.	P12	MN413149	<i>Arthrinium rasikravindrae</i>	100	100
4.	VEF-3	MN420881	<i>Diaporthe</i> sp.	100	100
5.	TN-R-3	MN400974	<i>Lasiodiplodia theobromae</i>	100	100
6.	TN-9-2	MN412519	<i>Colletotrichum</i> sp	100	100

3.2.2 Phylogenetic analysis

Phylogenetic tree is used to represent evolutionary relationships between organisms which are having ancestors in common. In the present study evolutionary tree was constructed using MEGA7 software with four clades representing four different genera *Lasiodiplodia*, *Diaporthe*, *Arthrinium* and *Colletotrichum* belonging to the same Phylum Ascomycota representing four different families (Fig 3). The genus *Lasiodiplodia* was from Botryosphaeriaceae, *Arthrinium* from Apiosporaceae, *Colletotrichum* from Glomerellaceae and *Diaporthe* from Diaporthaceae. The first clade consisted of

two sequences of *Lasioidiplodia* which clustered with twelve sequences from NCBI data base (MN046825, MN173966, MK808536, MK883476, MN398978, MN256461, MK808133, MH731278, KY969640, KF913502, KF164311, JX868715) with 95% bootstrap value. The second clade comprised by the genus *Arthrinium*, which consisted two sequences of endophytic fungi of cocoa which clustered with five NCBI database sequences (MK304236, KT722600, MK014896, MH498538 and MK850243) with 88 % bootstrap value. The third clade comprised *Colletotrichum* genus which consisted one sequence of endophytic fungi which clustered with four sequences from NCBI database (MK914629, MH883641, MH388336 and MK300802) with 90% bootstrap value. The fourth clade comprised by the genus *Diaporthe* with one endophytic fungal sequence which clustered with four NCBI database sequences (KF435362, KF688124, MK335817 and KU712435) with boot strap value 65%.

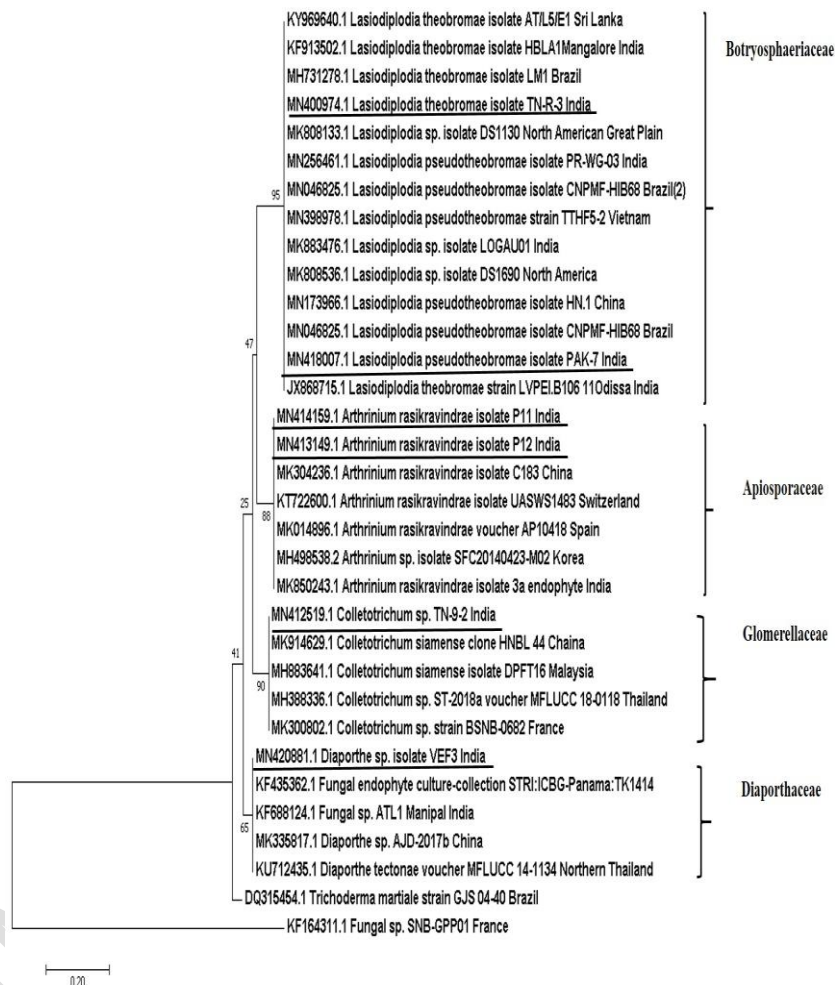


Fig 3. Phylogenetic relationship between six endophytic fungal isolates of *Theobroma cacao* based on ITS rDNA sequence by Neighbour joining (NJ) method following Bootstrap test(1000 replication).The evolutionary distances were computed using the Maximum Composite Likelihood method following Gamma distribution using MEGA7 Software.

In present investigation all six isolated endophytic fungal taxa belonged to the phylum Ascomycota. This is in agreement with the findings of EVANS1et al.[8] and Crozier et al.[3] where they isolated and characterized fungal community inhabiting in root, stem and branches of cocoa. The studies on fungal endophytic community in Cocoa is not well known so efforts may be taken to characterize these microbes with a view to characterize it both morphologically and molecularly.

4. Conclusion

The aim of this study is to identify endophytic fungal isolated of cocoa both from morphologically and molecularly. Total six endophytic fungal isolates were studied and they were from same phylum

Ascomycota. Morphological characterization of cocoa isolates revealed that they are from different taxa. Isolates PAK-7 and TN-R-3 are from Botryosphaeriaceae, P11 and P12 were from Apiosporaceae, Vef-3 was from Diaporthaceae and *Colletotrichum* was from Glomorellaceae. Molecular characterization of ribosomal DNA revealed that the isolates PAK-7 and TN-R-3 belonged to *Lasiodiplodia*, isolates P11 and P12 were belonging to taxa *Arthrinium*, isolate Vef-3 belonged to taxa *Diaporthe* and isolate TN-9-2 belonged to taxa *Colletotrichum*.

Studies on endophytes is a new field of research which gives us the way to understand the diversity of endophytic community in a plant. Present study indicated the presence of diverse taxonomic groups of fungi living inside cocoa tree. To understand it in a better way further studies may be carried out to confirm endophytic nature of asymptomatic fungal pathogens as well as antagonistic microbes in cocoa.

Reference

1. Agut M, Calvo MA. *In vitro* conidial germination in *Arthrinium aureum* and *Arthrinium phaeospermum*. Mycopathologia.2004;157: 363–367.
2. Arnold AE. Understanding the diversity of foliar fungal endophytes: progress, challenges, and frontiers. Fungal Biol. Rev. 2007; 21: 51–66.
3. Croziera J, Thomasa SE, Aimeb MC, Evansa HC, Holmesa KA. Molecular characterization of fungal endophytic morphospecies isolated from stems and pods of *Theobroma cacao*. Plant Pathol.2006.55;783–791.
4. de Silva NI, Phillips AJL, Liu J, Lumyong S, Hyde KD. Phylogeny and morphology of *Lasiodiplodia* species associated with Magnolia forest plants. Scientific Reports.2019;9:14355
5. Ding X, Liu K, Deng B, Chen W, Li W, Liu F. Isolation and characterization of endophytic fungi from *Camptotheca acuminata*. World J Microbiol Biotechnol .2013;29(10):1831-1838.
6. Ghasemi ES, Arzanlou M, Babai, AA, Narmani A. Morphological and Molecular Characterization of Endophytic Fungi from Oak Trees in Arasbaran Forests. J App Res Plant Prot. 2019;8(1):1-17.
7. Hanada RE, Pomella AWW, Costa HS, Bezerra JL, Loguercio LL, Pereira JO. Endophytic fungal diversity in *Theobroma cacao* (cacao) and *T. grandiflorum* (cupuaçu) trees and their potential for growth promotion and biocontrol of black-pod disease. Fungal Biol. 2010;114(11-12):901-910.
8. Evans HC, Holmes KA, Thomas SE. Endophytes and mycoparasites associated with an indigenous forest tree, *Theobroma gileri*, in Ecuador and a preliminary assessment of their potential as biocontrol agents of cocoa diseases. Mycol Prog.2003. 2(2): 149–160.
9. He Y, Zhang Z. Diversity of organism in the *Usnea longissima* lichen. Afr. J. Microbiol. Res. 2012; 6(22):4797-4804.
10. Lima JS, Figueiredo JG, Gomes RG, Stringari D, Goulin EH, Adamoski D, Glienke C.2012. Genetic diversity of *Colletotrichum* spp. an endophytic fungi in a medicinal plant, Brazilian pepper tree. ISRN Microbiol.2012;215716:1- 7.
11. Martínez-Cano C, Grey WE, Sands DC. First report of *Arthrinium arundinis* causing kernel blight on barley. Plant Dis.1992;76: 1077.
12. Mavragani DC, Abdellatif L, McConkey B *et al.* First report of damping-off of durum wheat caused by *Arthrinium sacchari* in the semi-arid Saskatchewan fields. Plant Dis .2007;91:469. doi:10.1094 /PDIS-91-4-0469A.

13. Petrini O. Taxonomy of endophytic fungi of aerial plant tissues. In: Microbiology of the phyllosphere, Edited by NJ Fokkema, J Van Den Heuvel. Cambridge University Press, Cambridge. 1986.
14. Ramos HP, Braun GH, Pupo MT, Said S. Antimicrobial Activity from Endophytic Fungi *Arthrinium* state of *Apiospora montagnei* Sacc. and *Papulaspora immersa*. Braz. Arch. Biol. Technol. 2010;53(3): 629-632.
15. Rojas EI, Rehner SA, Samuels GJ, et al.. *Colletotrichum gloeosporioides* s.l. associated with *Theobroma cacao* and other plants in Panamá: multi-locus phylogenies distinguish host-associated pathogens from asymptomatic endophytes. Mycologia. 2010;102(6):1318–1338.
16. Rubini MR, Silva-Ribeiro RT, Pomella AW, Maki CS, Araújo WL, Dos Santos DR, Azevedo JL. Diversity of endophytic fungal community of cacao (*Theobroma cacao* L.) and biological control of *Crinipellis perniciosus*, causal agent of Witches' Broom Disease. Int J Biol Sci. 2005;1(1): 24-33.
17. Schulz B, Boyle C. The endophytic continuum. Mycol Res. 2005;109:661–686
18. Sharma, R., Kulkarni, G., Sonawane, M. S., & Shouche, Y. S. A new endophytic species of *Arthrinium* (Apiosporaceae) from *Jatropha podagrica*. Mycoscience. 2014; 55(2):118-123.
19. Sieber TN. Endophytic fungi in forest trees: are they mutualists?. Fungal Biol Rev. 2007;21: 75–89.
20. Singh SM, Yadav LS, Singh PN, Hapat R, Sharma R, Singh SK. *Arthrinium rasikravindrii* sp. nov. from Svalbard, Norway. Mycotaxon. 2013;122(1):449-460.
21. Suryanarayanan TS, Venkatachalam A, Thirunavukkarasu N, Ravishankar JP, Doble M, Geetha, V. Internal mycobiota of marine macroalgae from the Tamil nadu coast: distribution, diversity and biotechnological potential. Bot Mar. 2010;53(5): 457-468.
22. Tellenbach C, Grünig CR, Sieber TN. Negative effects on survival and performance of Norway spruce seedlings colonized by dark septate root endophytes are primarily isolate dependent. Environ Microbiol. 2011;13:2508–2517.
23. Usuki F, Narisawa K. A mutualistic symbiosis between a dark septate endophytic fungus, *Heteroconium chaetospora*, and a nonmycorrhizal plant, Chinese cabbage. Mycologia. 2007;99: 175–184.
24. Vujanovic V, Mavragani D, Hamel C. Fungal communities associated with durum wheat production system: a characterization by growth stage, plant organ and preceding crop. Crop Prot. 2012; 37:26-34.
25. Yua J, Wub Y, Heb Z, Lib M, Zhub K, Gaoa B. Diversity and Antifungal Activity of Endophytic Fungi Associated with *Camellia oleifera*. Mycobio. 2018;46: 2: 85–91.

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