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Pseudofusicoccum adansoniae isolated as an endophyte from *Jatropha podagrica:* new record for India

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Abstract — *Pseudofusicoccum adansoniae* (*Botryosphaeriaceae*), representing a new generic record for India, is described and illustrated. The species, isolated as an endophyte from healthy leaf midrib and fruit of *Jatropha podagrica,* is characterized by large conidiomata and hyaline ellipsoid conidia with round apices. Its teleomorph is unknown. Comparison of the internal transcribed spacer 1 and 2 loci and 5.8S rDNA sequences showed 100% sequence similarity with that of CBS 122055, the ex-type strain of *P. adansoniae*.

Key words — anamorph, *Botryosphaeriaceae*, ITS, phylogeny, taxonomy

Introduction

Pseudofusicoccum Mohali et al. (*Botryosphaeriaceae*) is a relatively new genus typified by *Pseudofusicoccum stromaticum* (Mohali et al.) Mohali et al. (Crous et al. 2006). Recent morphological studies and ITS1-5.8S-ITS2 sequence analyses indicate that *Pseudofusicoccum* species occur in Venezuela and Brazil (*P. stromaticum*), Western Australia (*P. adansoniae* Pavlic et al., *P. ardesiacum* Pavlic et al., *P. kimberleyense* Pavlic et al.), and South Africa (*P. olivaceum* J.W.M. Mehl & B. Slippers, *P. violaceum* J.W.M. Mehl & B. Slippers) (Mohali et al. 2006, 2007; Pavlic et al. 2008; Mehl et al. 2011; Marques et al. 2012) (Table 1). Although some species have been isolated from dead wood, most have been isolated from healthy plant tissues where these fungi occur seemingly as endophytes. Recently, during an investigation of endophytic fungal diversity of *Jatropha podagrica* Hook. (*Euphorbiaceae*), two isolates were recovered with the morphological characteristics of *Pseudofusicoccum adansoniae.* The plant is considered toxic (Ojewole & Odebiyi 1980) and used for tanning leather and the production of a red dye (Bassam 2010). A literature survey indicated

that *Pseudofusicoccum* has not previously reported from India (Bilgrami et al. 1991, Jamaluddin et al. 2004), and *P. adansoniae* has been reported only from Australia. The present communication describes and illustrates these isolates in light of the molecular sequence data from ITS rDNA loci.

Materials & methods

The fungus was isolated from healthy leaf midrib and fruit of the perennial plant *Jatropha podagrica* (Buddha belly or Gout plant) from Pune (Pimpri), Maharashtra, India (18°37′07.04″N 73°48′13.43″E). Plant parts were washed in running tap water, sterile distilled water (3 washes) and cut into fragments (1–2 cm). Pieces of the plant were dipped and washed with 75% ethanol (5 min, twice) and 100% ethanol (30 sec), placed on Potato Dextrose agar (PDA) (Hi-Media, India) and Czapek Dox agar (CDA) (Hi-Media, India) media, and incubated at room temperatures (RT; c. 28–30 °C). After 4–5 days, mycelial growth was observed on the CDA Petri dish. The cultures were purified on 2% Malt Extract agar (MEA) plates and strains designated as MMI00062 (from leaf midrib) and MMI00064 (from fruit). Unstained microscopic observations were made using lactophenol (Hi-Media, India) mounts and observed under a Nikon YS100 microscope (Nikon, Japan). Measurements of morpho-taxonomic characters were recorded and compared with type descriptions of known species (Mohali et al. 2006, Pavlic et al. 2008, Mehl et al. 2011). Rayner's colour chart (1970) was used as a reference for culture colours.

Scanning Electron Microscopy (SEM) of air-dried pycnidia was done by coating with platinum in Jeol sputter coater (JFC 1600) and examining on a Jeol-JSM 6360A SEM at 10 kV. The culture MMI00064 has been deposited in the Microbial Culture Collection (WDCM-930), Pune, India as MCC 1020.

For sequencing of the ITS loci, DNA was extracted from pure cultures grown in Petri dishes using the QIAamp® DNA Mini Kit (Qiagen, Inc., Valencia CA). DNA concentration was estimated using a Nanodrop ND-1000 machine (Thermo scientific, USA). The genomic DNA was amplified using ITS1 and ITS4 primers (White et al. 1990) in a PE 9700 thermocycler (PE, Applied Biosystems, Singapore) with the following conditions: initial denaturation at 94 °C for 2 min, 35 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min, extension at 72 °C for 1 min, and a final extension step at 72 °C for 10 min. The positive amplicons were purified using a PCR Cleanup Kit (Qiagen, Inc., Valencia CA) and purified PCR products were sequenced (both strands) on an ABI 3730 xl DNA analyzer using the Big Dye terminator kit (Applied Biosystems, Inc., Foster City, CA). An NCBI BLASTn search was conducted for sequence similarity (Zhang et al. 2000).

For phylogenetic relationships with *Pseudofusicoccum adansoniae* MCC 1020, 11 related sequences were retrieved from GenBank database. *Neoscytalidium novaehollandiae* CBS 122071 (EF585540.1) was used as the outgroup and sequence alignment carried out using MUSCLE (Edgar 2004). Maximum parsimony (MP) analysis and phylogenetic tree construction was conducted using MEGA 5.0 (Tamura et al. 2011).

Table 1: Morphological comparison of *Pseudofusicoccum adansoniae* MCC 1020, *P. adansoniae* CBS 122055, and other species.

	P. adansoniae MCC 1020	*P. adansoniae* Pavlic et al. 2008	*P. kimberleyense* Pavlic et al. 2008	*P. ardesiacum* Pavlic et al. 2008	*P. violaceum* Mehl et al. 2011	*P. olivaceum* Mehl et al. 2011	*P. stromaticum* Mohali et al. 2006
Colony diam.	90 mm	90 mm	90 mm	90 mm	—	—	70–75 mm
Colony colour	Gray olivaceous to olivaceous black	Gray olivaceous to olivaceous black	Olivaceous black to black	Violaceous gray to slate blue	Olivaceous to greenish black	White to olivaceous	—
Optimum temp.	28–30°C	30°C	30°C	30°C	30°C	25°C	—
Pycnidia	200–500 µm (in culture)	500 µm (on host)	510 µm (on host)	500 µm (on host)	500–620 µm (on host)	530–630 µm (on host)	—
Conidia (mean, in µm)	Ellipsoid, occas. bent or irreg. shaped (16.5 × 7.3)	Apices rounded, smooth with fine granular content (22.5 × 5.2)	Ellipsoid, straight / slightly curved (30.7 × 7.4)	Ellipsoid to rod-shaped, straight / sl. bent (25 × 7.5)	Bacilliform, granular, guttulate (33.0 × 9.5)	Guttulate (22.8 × 7.0)	Bacilliform (21.7 × 5.4)
Conidiogenous cells (mean, in µm)	Cylindrical (10.2 × 2.0)	Cylindrical (12.7 × 2.4)	(Sub)cylindrical (9.8 × 3.3)	Cylindrical (8.6 × 3.5)	Cylindrical (8.6 × 4.3)	Cylindrical-guttulate-circular (6.6 × 3.7)	Cylindrical (13 × 2.5)
% Similarity with MCC 1020	—	100% (0 nt)	98.8% (6 nt)	98.8% (6 nt)	98.6% (7 nt)	98% (10 nt)	98.4% (8 nt)
Hosts	*Jatropha podagrica*	*Adansonia gregorii, Acacia synchronicia, Eucalyptus* sp., *Ficus opposita*	*A. gregorii, Ac. synchronicia, Eucalyptus* sp., *F. opposita*	*A. gregorii, Eucalyptus* sp.	*Pterocarpus angolensis*	*Pt. angolensis*	*Eucalyptus* sp.
Distribution	India	Australia	Australia	Australia	South Africa	South Africa	Venezuela

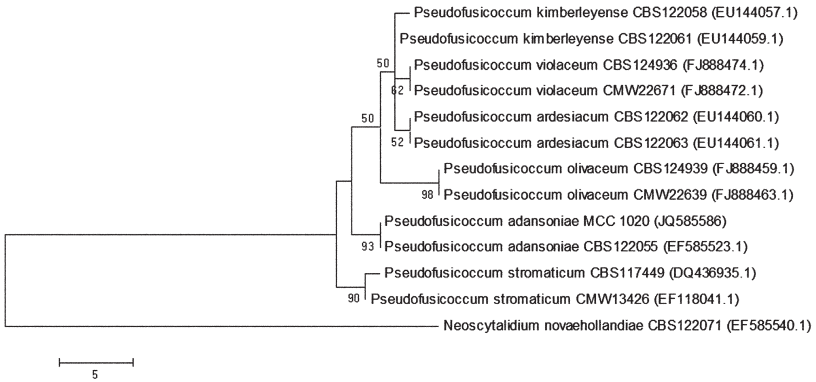


Plate 1. Maximum parsimony tree (by complete deletion of gaps) based on ITS sequences (ITS1-5.8S-ITS2) depicting the position of *P. adansoniae* MCC 1020 within *Pseudofusicoccum* and its relation with other closely related phylospecies. Bootstrap values (1000 replicates) shown above branches.

Results

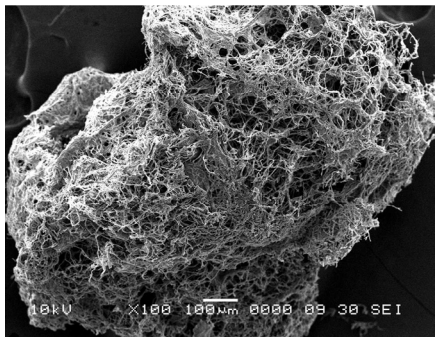
The cultures isolated were identified as *Pseudofusicoccum adansoniae* based on colony size and colour, conidial size, and conidiophore morphology as described by Pavlic et al. (2008). When the ITS sequence data of six *Pseudofusicoccum* species procured from GenBank were aligned with our isolate (JQ585586) sequence, 44 ambiguous base pairs bp were removed from the total 568 (bp) prior to analysis. Of the 524 bp used to reconstruct the phylogeny, 457 were conserved, 64 were variable, 16 were parsimony-informative, and 48 were singletons. Maximum parsimony analysis produced eleven equally parsimonious trees with a tree length of 68 (CI = 0.941176, RI = 0.857143, and RCI = 0.806723 for all sites). One such most parsimonious tree (Plate 1) exhibits three major clades: Clade 1 (*P. kimberleyense, P. violaceum, P. ardesiacum*), Clade 2 (*P. stromaticum*, *P. adansoniae* MCC 1020, *P. adansoniae* CBS 122055), and Clade 3 (consisting of *P. olivaceum*). The Maximum parsimony (MP) tree showed *P. adansoniae* MCC 1020 forming a separate clade along with *P. adansoniae* CBS 122055, but forming a sister branch to *P. stromaticum*. Moreover, the strain MCC 1020 and *P. adansoniae* CBS 122055 showed 100% similarity within 507nt of the ITS region when compared in MEGA 5.0 (Tamura et al. 2011).

Taxonomy

Pseudofusicoccum adansoniae Pavlic, T.I. Burgess & M.J. Wingf., Mycologia 100: 855. 2008. Plate 2

Teleomorph: Unknown.

Colonies initially form a moderately dense mycelial mat. Initially mycelium pale olivaceous grey (21′′′′′d) to olivaceous grey (21′′′′′b) from the middle of



1

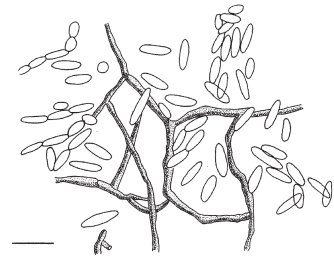


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Plate 2. *Pseudofusicoccum adansoniae*. Fig. 1. Conidiomata detail showing mycelial hairs covering the surface (SEM). Fig. 2. Conidia. Scale bar = 25µm.

the colony. At maturity, grey olivaceous (21′′′′i) to olivaceous black (27′′′′m) and after 3–5 d becoming dark greenish-black (33′′′′′k) with age, covering the 90 mm diam Petri dish after 4 d in the dark on 2% MEA medium. Colonies slightly irregular, occasionally radially striated with zones or irregular circles. Aerial mycelium slightly fluffy, becoming dense and cottony with age, turning pale olivaceous grey (21′′′′′d). Radiations are evident on front and reverse of colony. Conidiomata 1–2 mm, some 5 mm in diam, readily formed from the middle of colony within 7–10 d, covering the entire surface of colony and deeply immersed in the medium (seen as a round black structures on the reverse side of Petri dishes 14–16 d after incubation), eustromatic, multilocular, immersed. Hyphae brown, branched. Conidiogenous cells holoblastic, smooth, cylindrical, hyaline, the first conidium produced holoblastically and subsequent conidia enteroblastically. Conidia ellipsoid, occasionally slightly bent or irregularly shaped, 9.7–25.8 × 6.5–9.7 µm (av. 16.5 × 7.3 µm), apices rounded, smooth with fine granular content, hyaline, thin-walled, unicellular, surrounded by a mucous layer.

Distribution and habitat: Western Australia (from infected *Adansonia gregorii* and *Mangifera indica*, and from asymptomatic *Acacia synchronicia*, *A. gregorii*, *Eucalyptus* sp., and *Ficus opposita*); Maharashtra, India (endophytic in *Jatropha podagrica*).

Material examined: **INDIA**, **Maharashtra State**, Pune, Pimpri, 18°37′07.04″N 73°48′13.43″E, endophytic in leaf midrib of *Jatropha podagrica*, 25.IV.2011, Rohit Sharma, culture MMI00062; endophytic in fruit of *J. podagrica*, 25.IV.2011, Rohit Sharma, culture MMI00064 (MCC 1020; GenBank JQ585586).

Discussion

Pseudofusicoccum adansoniae MCC 1020 is characterized by multilocular hairy conidiomata with several pycnidia whose inner margin is covered with hyaline mycelia. The fungus produces several conidiomata (10–15) in

culture covered by mycelial hairs (Fig. 1). It contains several hyaline, ellipsoid conidia (Fig. 2) formed on hyaline conidiophores covered in a mucous layer. However, the conidia of *P. adansoniae* MCC 1020 average 16.5 × 7.3 µm (Table 1) and are narrower and broader compared to the average 22.5 × 5.2 µm of *P. adansoniae* CBS 122055 (Pavlic et al. 2008). Table 1 clearly shows the similarity of a combination of characters (e.g., colony colour, growth characters, conidiogenous cell and conidial morphologies) for MCC 1020 and the other analyzed *P. adansoniae* strain. Phylogenetic analysis suggests that *P. adansoniae* is only distantly related to all other *Pseudofusicoccum* species. ITS1-5.8S-ITS2 sequence analyses place *P. adansoniae* MCC 1020 and *P. adansoniae* CBS 122055 together in a distinct clade forming a sister branch to *P. stromaticum*. The maximum parsimony analysis and resulting tree (Plate 1) clearly indicates that apart from the *P. adansoniae* and *P. stromaticum* branches, *Pseudofusicoccum* is divided into two major groups, one containing *P. violaceum, P. ardesiacum,* and *P. kimberleyense* and the other containing *P. olivaceum*. Neighbour Joining analysis also generates similar tree topologies.

Most species (including *Pseudofusicoccum*) of the *Botryosphaeriaceae* are endophytic, and some can be latent pathogens (Crous et al. 2006, Slippers & Wingfield 2007). *Pseudofusicoccum adansoniae* CBS 122055 was originally described from Derby, Western Australia, and isolated from infected branches of *Adansonia gregorii* F. Muell. (=*Adansonia gibbosa* Cunn. et al.) (*Malvaceae*) and healthy branches of *Acacia synchronicia* Maslin (*Leguminosae*), *Eucalyptus* sp., and *Ficus opposita* Miq. (*Moraceae*) (Pavlic et al. 2008). The fungus has also been isolated as an endophyte from *Adansonia gregorii* (Sakaladis et al. 2011a) and from diseased stems of *Mangifera indica* L. (*Anacardiaceae*) from Western Australia (Sakaladis et al. 2011b). Hence, detection of *P. adansoniae* MCC 1020 from healthy leaf midrib and fruit of *Jatropha podagrica* is not unusual, as it has been isolated from asymptomatic branches of other trees. As it represents a first species record outside Australia and the first *Pseudofusicoccum* record from India, it is reported as new to the Indian fungal flora. Reports of *Pseudofusicoccum* from other parts of world — especially as endophytes — should be expected.

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