Short Communication

MORPHOLOGICAL AND MOLECULAR CHARACTERIZATION OF *SCLEROTIUM ROLFSII* ASSOCIATED WITH LEAF BLIGHT DISEASE OF *PSYCHOTRIA NERVOSA* (WILD COFFEE)

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SUMMARY

Psychotria nervosa (Rubiaceae), commonly known as wild coffee, was found affected by leaf blight disease in the forest regions of Mysore, Madikeri and Hassan districts of Karnataka state (India). Characteristic leaf blight symptoms were observed at the centre of leaf lamina of basal leaves and tiny sclerotial bodies entangled in mycelia were prominent on the adaxial leaf surface. The associated fungal pathogen was isolated and identified as *Sclerotium rolfsii* on the basis of morphological characters and sequence analysis of ITS region of rDNA. The sequences shared 100% similarity with reliable sequence of *S. rolfsii* retrieved from GenBank. The detached leaf assay produced leaf blight symptoms seven days after inoculation. *S. rolfsii* known to infects a wide range of host plants including forest tree species. This is the first report of *S. rolfsii* causing leaf blight disease of wild coffee in India.

Psychotria nervosa Sw. (Rubiaceae) is an evergreen, shade tolerant shrubby plant native to Florida and Central and South America (Gilmen, 2011; Liogier, 1997; Stevens *et al*., 2001). It is commonly called wild coffee as the fruit resembles the true coffee bean, also found distributed in the forest regions of southern Karnataka (India). Wild coffee widely cultivated in gardens as an ornamental for its lush and evergreen foliage. It is used in ethno-medicinal practices by tribes of Southern India for the treatment of dysentery, colds, stomach ache, asthma, sores, boils, swollen feet, and fungal infection (Biswas *et al*., 2008; Porto *et al*., 2009). *P. nervosa* plant found infected with a leaf blight disease in the year 2013-14. Therefore, the present study was conducted to investigate and identify the pathogen associated with blight symptoms and to prove Koch postulates to confirm the fungal association with leaf blight disease. During a survey of forest tree, we observed a characteristic leaf blight disease on wild coffee plants. Therefore, in the present investigation the disease incidence, isolation, identification, morpho-cultural and molecular characterization of fungal pathogen associated with leaf blight disease was performed along with pathogenicity assay.

During a field survey conducted from March-June 2013 to 2014 in major forest regions of Mysore, Madikeri and Hassan districts of Karnataka state (India), a characteristic leaf blight disease was observed on this plant and the disease was prominent on basal leaves. Under severe infection the basal stem was also affected. A systematic investigation was done to isolate and identify the fungal pathogen associated with this disease. The disease incidence was determined as number of plants with leaf blight symptoms and total number of plants examined in an approximately 3 square kilometers transect area of forest regions. Leaf blight affected leaves were collected from all three districts and leaf pieces (1.5 cm), surface sterilized with 1% (v/v) sodium hypochlorite (NaOCl) solution for two min, air dried, placed on potato dextrose agar (PDA) medium amended with chloramphenicol (120 mg/l) and incubated for seven days at room temperature. Fungal colonies were sub-cultured on PDA and colony growth, colour and development of reproductive structures were observed.

Pathogenicity test was conducted using detached leaf assay (Sharma *et al*., 2012). Three representative isolates, one from each district, were used and nine healthy leaves were washed with sterile water and placed in a moist chamber. Inoculation (three leaves per isolate) was made on each leaf by placing a single mature sclerotium from 15 day old culture at the centre of the leaf lamina and incubated at 24 ± 2°C for seven days. The experiment was conducted in triplicates of three leaves each and appearance of leaf blight symptom was recorded after five days of incubation. Healthy leaves treated only with sterile water served as controls.

Genomic DNA was extracted from 15 days old fungal culture by CTAB extraction method (Zhang *et al*., 1998). PCR was performed using Advanced Thermus25 Thermocycler (Peqlab, Germany). The universal primers for PCR were obtained from Bangalore Genie, India. The primer pairs ITS1-5'-CGGATCTCTTGGTTCTGGCA-3' and

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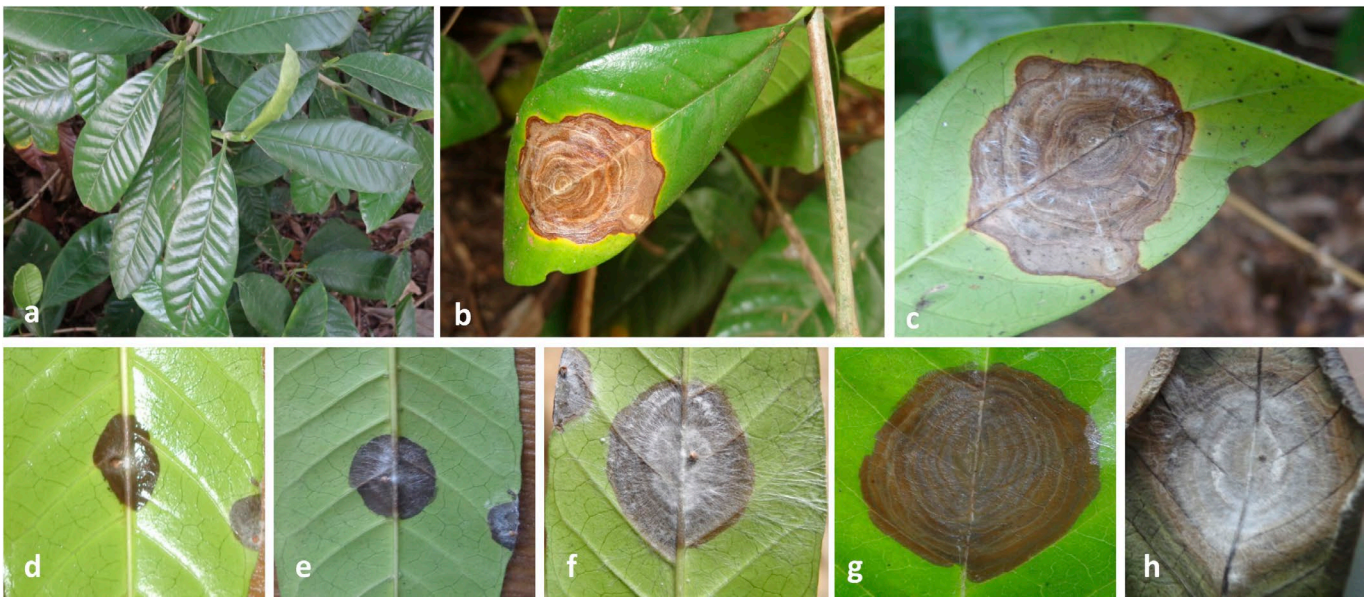

Fig. 1. a. Healthy branch of *P. nervosa*; b. Leaf blight on abaxial leaf surface; c. on adaxial leaf surface; d-g. Development of leaf blight on artificially inoculated leaves, after 5-7 days after inoculation; h. blight symptom after 15 days of incubation similar to naturally expressed symptom.

ITS4-5'-GACGCTCGAACAGGCATGCC-3' were used for r-DNA amplification (White *et al*., 1990). The PCR amplification was carried out in 25 µl reaction mixture containing 1 µl of DNA sample, 2.5 µl of 10× PCR buffer, 2.5 mM $MgCl_2$, 2.0 µl of 2 mM dNTPs, 20 pmol of each forward and reverse primer (1.0 µl) and 0.2 µl of Taq DNA Polymerase and made up to 25 µl with 14.8 µl of nuclease free water. The PCR conditions include initial denaturation at 94ºC for 3 min, 30 cycles of denaturation at 94ºC for 30 s, primer annealing at 55ºC for 30 s, followed by primer extension for 30 s at 74ºC and final extension at 74ºC for 10 min. The amplified PCR products were sequenced using an ABI3730xI DNA analyzer (Applied Biosystems, Foster City, CA, USA). ITS-rDNA sequences were aligned using the CLUSTALW2 program (Larkin *et al*., 2007). Phylogenetic tree was constructed based on the Neighbour-joining [NJ] method as implemented in MEGA version6 (Tamura *et al*., 2011) using Kimura-two-parameter model with 1000 bootstrap replications.

Leaf blight disease was observed on basal leaves only on young wild coffee plants of about 1-2 ft height and the disease incidence was 28.81% (Madikeri), 39.39% (Hassan) and 47.36% (Mysore) respectively (Table 1). First symptoms appeared as small, necrotic spots and the spots enlarged and coalesce to form a characteristic leaf blight symptoms measuring up to 2-4 cm (Fig. 1a-c). The fungal pathogen was isolated consistently from all symptomatic leaf samples. The dense, aerial, whitish, cottony mycelium with globoid sclerotia was observed after 12-15 days of incubation. Sclerotia (0.5-2.5 mm) were whitish in the beginning and turned to brownish as they matured. Based on morphology and cultural features, the fungal pathogen was identified as *Sclerotium rolfsii* Sacc. teleomorph *Athelia rolfsii* (Curzi) Tu and Kimbrough (Saccardo, 1931; Mordue, 1974; Punja *et al*., 1982). *S. rolfsii* was differentiated from *S. delphinii* by number and size of sclerotia. *S. delphinii* produces 20-30 sclerotia/plate which measures 3-5 mm in diameter (Stevans, 1931; Punja and Damiani, 1996).

Table 1. Disease incidence in each surveyed area and morphological characteristics of *Sclerotium rolfsii* isolates isolated from *Psychotria nervosa*

Isolate	Geographical origin	Geographical coordinates	Disease Incidence	No. of isolations	No. of Sclerotia	Colony type	Diameter of sclerotia***	GB Acc. No
SrPnMKS1	Mysore	N 12°16'53.43" E76°28'41.85"	47.36 (38)*	3	350 ± 18**	Aerial Fluffy (brown)	0.5-2 mm	KP412464.1
SrPnMKS2	Madikeri	N 12°12'01.77" E75°45'23.58"	21.81 (55)	3	288 ± 12	Aerial Fluffy (brown)	0.2-1.8 mm	KP412465.1
SrPnMKS3	Hassan	N 13°00'45.32" E75°53'41.01"	39.39 (33)	2	324 ± 16	Aerial Fluffy	0.4-2.4 mm	KP412466.1

* Values in parenthesis indicates total number of plants observed from the study site; ** Average number of sclerotia per plate produced; *** Twenty sclerotia per isolate were measured.

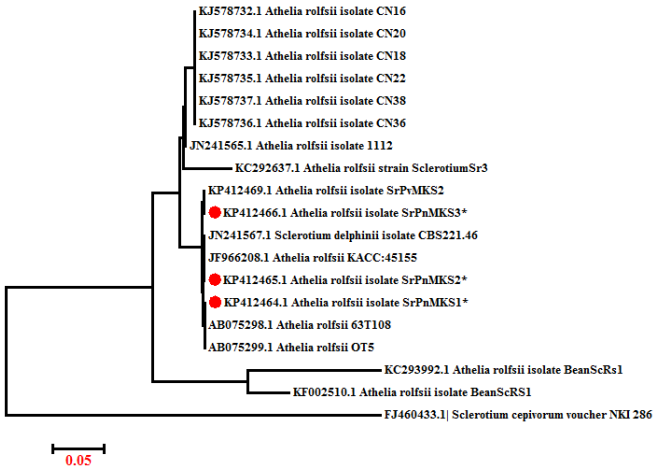

Fig. 2. Phylogenetic tree constructed by Neighbour-Joining method using ITS r-DNA regions of *S. rolfsii* isolates from Karnataka. Tamura-Nei Substitution model and nearest neighbour-interchange search options with 1000 bootstrap replicates were used. The tree is rooted with *S. cepivorum*.

Symptoms of leaf blight were reproduced on artificially inoculated leaves of wild coffee. Though the size of necrotic leaf spots on artificially inoculated leaves varied on upper and lower leaf surfaces, however spots were similar to the ones observed on leaves infected naturally (Fig. 1d-h) and the pathogen was re-isolated on PDA thus fulfilling Koch's postulates. No leaf blight symptoms were observed on control leaves inoculated with sterile water.

PCR amplification of ITS-rDNA yielded an expected amplicon size of 650 bp, as visualized in 1.5% agarose gel. nBlast analysis of sequence revealed 100% homology with reference strain *S. rolfsii* (AB075298.1) (Teleomorph: *Athelia rolfsii*). A total of three amplified DNA sequences, representing each isolate from all the three regions were deposited in the GenBank (KP412464.1, KP412465.1 and KP412466.1). Phylogenetic tree constructed using Neighbour-Joining method showed that the sequences grouped into a single clade along with reference sequence and the tree was rooted to *Sclerotium cepivorum* with 1000 boot strap replications (Fig. 2).

Sclerotium rolfsii causes serious disease on a wide variety of plants, including field crops, vegetable and fruit crops, ornamental crops and also on turf (Aycock, 1966; Punja, 1985). The disease caused by the sclerotial fungus is generally termed as southern blight and southern stem rot (Punja, 1985; Mullen, 2001). The fungus infects seedlings, herbaceous plants, woody plants, fleshy roots, bulbs and fruits. Most frequently, it infects lower stem near or at the soil surface, but it also infects other parts of a susceptible plant as long as environmental conditions are favourable (Mullen, 2001). The fungus *S. rolfsii* is known to cause diseases in nursery seedlings of various economically important forest tree species (Punja, 1985) such as collar rot of *Swietenia macrophylla*, leaf blight of *Pterocarpus santalinusin* web blight of *Azadirachta indica*, leaf blight of *Bombax ceiba*, *B. insigne* and *Eucalyptus grandis* (Maria Florence *et al*., 1985; Sankaran *et al*., 1985, 1986; Sharma and Mohanan, 1992). To the best of our knowledge, this is the first report of *S. rolfsii* causing leaf blight on wild coffee in India.

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