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Athelia rolfsii associated with mulberry root rot disease in Tamil Nadu, India

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Abstract

During a survey (2019-2021), root rot symptoms were observed in the established mulberry gardens located in Harur and Kinathukadavu (GPS coordinates: 12.19750° N 78.28333° E and 10.81780° N 77.02340° E) of Tami Nadu, India. Dried foliage and rotten/ decayed root portions along with strands of mycelia were observed in the affected plants. Isolations from diseased roots yielded *Sclerotium* sp. and morphological characteristics were recorded on Potato dextrose agar. Molecular characterization was done by sequencing the internal transcribed spacer (ITS) region and confirmed as *Athelia rolfsii.* Further pathogenicity was proved by detached root cortex technique and potculture experiments on the mulberry saplings. Artificially inoculated mulberry saplings showed wilting, yellowing with discoloured rotten root portions similar to a real-time field infection and re-isolation of *A. rolfsii* confirmed Koch's postulates. Cryo-microtomy revealed the histopathology of rotten mulberry roots and compared them with healthy roots. The current research was done to document the occurrence of *A. rolfsii* causing root rot in mulberry to develop effective management to encompass the disease.

Keywords – Cryo-microtomy – decayed root – pathogenicity – *S. rolfsii* and survey

Introduction

Mulberry (*Morus indica* L.), is a perennial crop that can be grown in a variety of agro-climatic conditions. Intensive mulberry cultivation is indispensable, as the bivoltine double hybrids of the silkworm (*Bombyx mori* L.) are bred commercially for cocoon production in tropical southern India. Continuous and huge production of biomass in the challenging system might have become threat to the mulberry crop.

Many foliar and root diseases are recorded in mulberry (Sharma et al. 2003, Maji 2011). Foliar diseases mainly results in leaf yield loss whereas root diseases lead to complete mortality of young and aged plantations as well. Unlike foliar diseases of mulberry, root diseases occur throughout the year with varied disease severity. Pinto et al. (2018) mentioned the difficulties in managing root rot diseases in comparison with foliar diseases and they are the major obstacles around the world to the sericulturists.

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There are about nine types of root rot diseases reported in mulberry *viz.,* dry rot (*Fusarium* sp.), charcoal rot (*Macrophomina phaseolina*), black rot (*Lasiodiplodia theobromae*), white rot (*Rosellinia necatrix*), violet rot (*Helicobasidium mompa*), *Armilaria* rot, *Rhizopus* rot, *Rhizctonia* rot and bacterial rot (*Psuedomonas solanacearum).* The root rot associated pathogens could cause leaf yield loss of maximum 70% and complete mortality of plants. The complex biodiversity, interplay and the soil-borne nature of root rot pathogens hinder disease management in almost all mulberry growing countries. Moreover, nematodes also have role in occurrence of root rot disease complex (Sharma et al. 2003, Ganeshamoorthi et al. 2008, Sutthisa et al. 2010, Sowmya 2018, Gnanesh et al. 2020).

In India, mulberry is cultivated in more than 6.175 lakh acres by mostly small and marginal farmers (CSB 2021). They largely depend on the high yielding mulberry cultivar Victory-1 (V1), which is highly prone to root rot diseases and the management of root diseases is a challenging task. In order to develop strategies to combat root rot disease, it is essential to have complete facts about the casual agents. Therefore, the present study focused on documenting the occurrence *A. rolfsii* associated with mulberry root rot disease.

Materials & Methods

Survey, sample collection and isolation of pathogens

During the survey 2019-2021, infected mulberry root samples were collected from the plants that showed typical root rot symptoms along the rhizosphere soil in the established mulberry gardens located in Harur and Kinathukadavu of Tamil Nadu, India. Information regarding variety and age of mulberry plants were recorded. Disease incidence was also recorded and Per Cent Disease incidence (PDI) calculated using the formula:

$$\text{Percent Disease Incidence (PDI)} = \frac{\text{Number of infected plants}}{\text{Total number of plants}} \times 100$$

Symptomatic root samples were subjected to isolation of the pathogens. Root bits of approximately 1cm were taken at the junction of healthy and rotten zones, surface sterilized and inoculated on Potato Dextrose Agar (PDA) supplemented with streptomycin sulphate (0.03%) (Rangaswami & Mahadevan 1999). The plates were incubated at 25±2°C and pure cultures were obtained by the hyphal tip method.

Morphological and molecular characterization

Macromorphological characteristics of *A. rolfsii* such as colony color and growth rate, sclerotial shape, colour, size and production were recorded on PDA after seven days of inoculation. Further micromorphological characteristics were recorded with the aid of phase contrast microscope (Leica DM2000, Germany). For molecular confirmation, DNA of the isolates was extracted using the CTAB method (cetyltrimethylammonium bromide method) (He 2000). Species identity was confirmed by amplifying the ITS 1 and ITS 4 regions of rDNA (White et al. 1990). The amplification conditions were (i) 10 min at 95°C of initial denaturation, (ii) 35 cycles of denaturation at 94°C for 1min, primer annealing at 55°C for 1min, primer extension at 72°C for 1min and (iii) 10min at 72°C of final extension. The final PCR product was sequenced and submitted to the NCBI database.

Phylogenetic analysis was conducted in the MEGA-X software to confirm species identity. ITS-rDNA sequence of 14 isolates of *A. rolfsii* obtained from NCBI database was aligned using ClustalW and evolutionary history were analysed using the Neighbor-Joining (NJ) method. The genetic distances were computed using the Kimura-2-parameter method and the nodal support was assessed by bootstrap analysis with 1000 replicates (Mahadevakumar et al. 2017).

Pathogenicity tests

Experiments to demonstrate the pathogenicity were carried out both *in-vitro* and *in-vivo* using the isolate SR1. The detached root cortex method (Yoshida et al. 2001) with slight modifications was carried out as an *in-vitro* study. Surface sterilized, pencil-thickness, 8cm long healthy mulberry root bits were used. Under aseptic conditions, 2cm long root bark was removed using a sterile scalpel and 7 days old *A. rolfsii* mycelial disc of 6mm (Ø) was inoculated. Un-inoculated root samples were served as control. Three replications were maintained. The experiment was repeated twice and incubated at 25±2°C for 3 weeks.

Protocols reported by Pinto et al. (2018) were followed with some modifications for the *in-vivo* pathogenicity study. Autoclaved sorghum grains (*Sorghum bicolor*) were used for mass multiplication of *A. rolfsii* inocula. Four-month-old mulberry saplings (cultivar V1) were transplanted to the pots filled with sterile pot mixture (Sand: Soil: Farm Yard Manure- 1:1:1- 3 kg) and different concentrations of *A. rolfsii* inocula (1, 3 and 6% (w/w)). Three replications were maintained with two saplings per replication and the control plants were inoculated with autoclaved grains. The trial was conducted in glasshouse as a completely randomized design and repeated twice. Disease assessment was performed at regular intervals of 10 days to 90 days and the intensity of disease was calculated (Sharma & Gupta 2005, Mala et al. 2013, Pinto et al. 2018). Re-isolation from the inoculated saplings was carried out to establish Koch's postulates.

Histopathology

Healthy and *A. rolfsii* inoculated mulberry root samples were collected after 90 days to document the anatomy of the diseased root tissues. Root samples were washed to remove the adhered soil particles made into small pieces, blot dried and frozen. The frozen root samples were cut into thin sections of 30 micrometers width in a cryomicrotome (Leica CM1520, Germany) and observed (Wilson et al. 1977).

Results

Symptomology

During survey root rot symptoms were observed in established gardens comprising one to ten-year-old plantations as well. Affected plants showed typical wilting symptoms and they had partial to completely decayed root systems. Roots were observed as the primary site of infection and no symptoms seen on stumps/ shoots above collar region. Some infested roots were found with strands of mycelia (Fig. 1). The infection was seemed to be fast spreading due to rain after dry spell. Infection was vigorous inside xylem vessels as blockage and discolouration while outer cortex remained rigid. Details collected during the survey were presented in the Table 1.

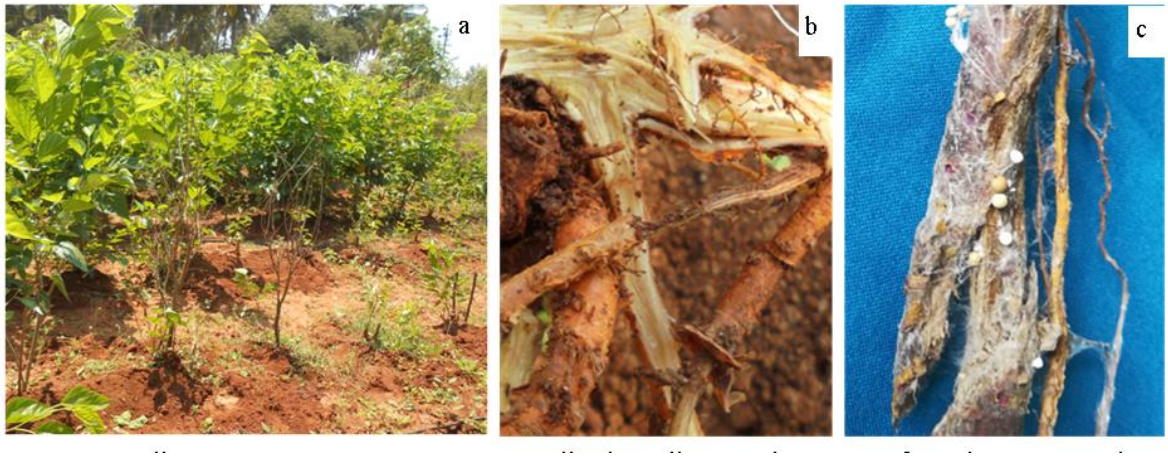

Fig. 1 – Mulberry root rot symtoms. a Wilted mulberry plant. b Infected root samples. c Mycelial strands and sclerotia of *A. rolfsii* on Mulberry roots.

Table 1 Survey details on the incidence of *A. rolfsii* pathogen causing root rot of mulberry.

GPS coordinates	Mulberry variety	Age (years)	Area (acre)	Soil type	Planting system	Irrigation type	Previous crop cultivated	PDI (%)
12.19750°N 78.28333°E	V1	10	1	Sandy clay loam	Paired row	Drip	Sugarcane	0.1
10.81780°N 77.02340°E	V1	2	1	Silt clay loam	4X4 tree type	Drip	Tomato & Banana	6.77

Morphological and molecular characterization

This putative pathogen grew vigorously on PDA with fan-like wavy, coarse and bright white mycelium (Fig. 2a). Large numbers of mycelial tufts turned into small round mustard-like sclerotia with exuded water droplets in 10 days (Fig. 2c-e). The production of sclerotia was observed mainly in the periphery of the Petri dish and browning/ tanning was observed upon maturity. Most of the sclerotial bodies were uniform in shape and size and deformations were sometimes observed. Thin-walled hyaline mycelia with septations and proliferation of some branch hyphae from clamps at the right angle were observed microscopically (Fig. 2b). In addition, red arrow indicated formation of anastomosing branches in the culture.

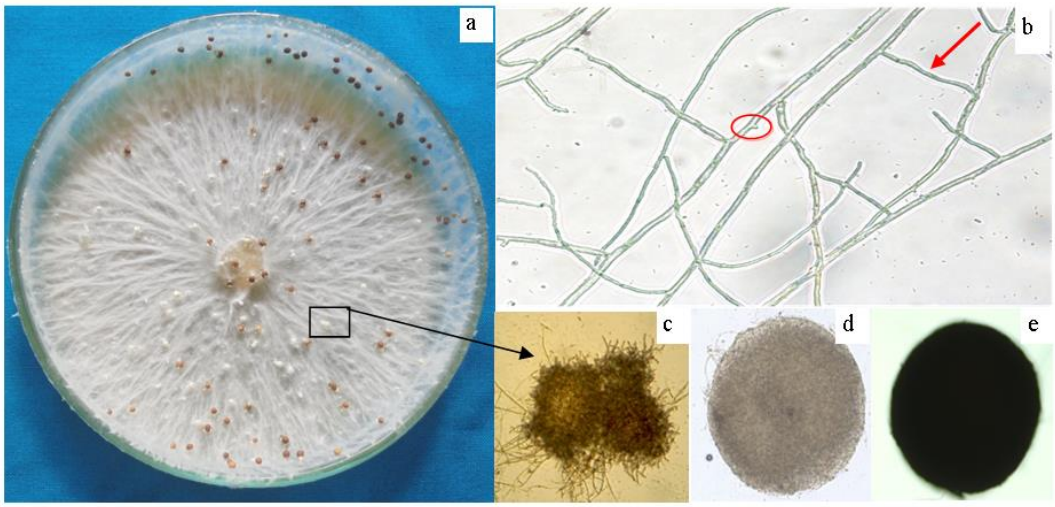

Fig. 2 – *A. rolfsii* macro and micro-morphology. a Colony morphology on PDA. b Thin hyaline mycelia with clamp connections (400x). c Mycelial mass. d Young white tuft. e Mature tanned sclerotia.

Species identity was confirmed by sequencing of the amplified internal transcribed spacer region (ITS). The amplicons obtained (593 bp and 598 bp) showed 99% similarity with sequences from *Athelia rolfsii* in the NCBI database. Based on morphological characteristics and molecular analysis two isolates were authenticated as *A. rolfsii*. The ITS sequences of SR1 and SR2 have been deposited in the NCBI Genbank database (Table 2). The phylogenetic tree of 14 isolates of *A. rolfsii* was constructed using the MEGA X software. From the analysis, unrooted Neighbour-joining (NJ) phylogenetic tree designated the isolates (SR1 and SR2) under different clades of *A. rolfsii*, with the bootstrap values of 99% and 100%, thus confirming their identity (Fig. 3).

Pathogenicity tests

Pathogenicity study was carried out *in-vitro* to visualize the colonization and infestation abilities of *A. rolfsii* in mulberry roots. The pathogen grew faster on sterilized mulberry root bits as

on semi-synthetic media and white strands of mycelia covered 2/3rd of the root bits within 6 days of after inoculation. In the subsequent days, decay of the root tissue was observed, followed by a considerable mass of mycelia turned into sclerotial bodies. In addition, at 20 days after inoculation (DAI), many mature tanned sclerotia were noted (Fig. 4).

Pot culture experiment was conducted to confirm the pathogenicity of *A. rolfsii* associated with mulberry root rot disease. Saplings inoculated with *A. rolfsii* inocula (6%) showed little or no sprouting followed by 3% and 1%. A vast number of sclerotial bodies were produced within 15 DAI on the soil surface. Poorly sprouted saplings showed stunted growth and gradual wilting, while plants with a higher inocula concentration (6%) died quickly. The above ground root rot symptoms such as yellowing, withering of leaves and wilting were clearly observed. Roots of the symptomatic mulberry plants were found discoloured and rotten. A reduction in the size and volume of infected roots was also found (Fig. 5). These visually observed root rot symptoms were assessed and presented in Table 3. Re-isolation of the pathogen from the disintegrated roots revealed *A. rolfsii* as that of mother culture and thus confirmed Koch's postulates.

Table 2 Details of *A. rolfsii* isolates obtained during the survey.

Location	Isolate name	Colony morphology	Average sclerotia size (Ø)	NCBI accession number
Harur	SR1	White, branched hyphae. Scattered scelortia production	0.4-2.5mm	MZ216466
Kinathukadavu	SR2	White, branched hyphae. Dense scelortia production in outer edges	0.4-1.9mm	MZ216467

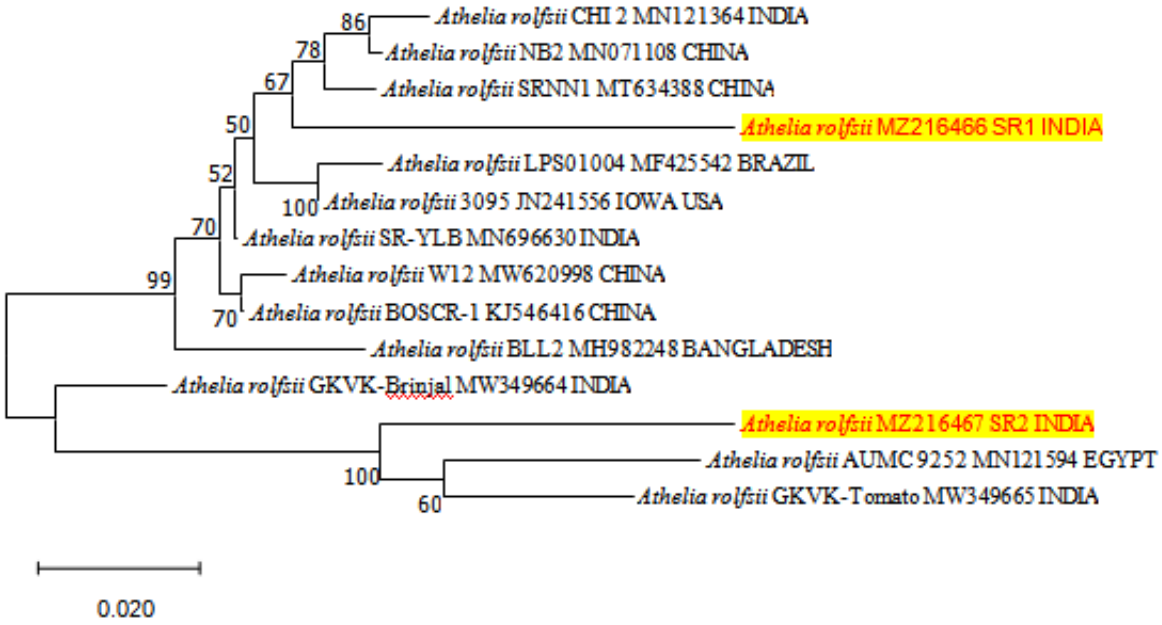

Fig. 3 – Phylogenetic tree constructed with ITS sequences of *A. rolfsii* using Neighbor-Joining method. DNA sequences from NCBI database were aligned using ClustalW. The evolutionary distances were computed using the Kimura-2-parameter method and the rate of variation among the sites was modeled with a gamma distribution. Numbers above the branches indicate bootstrap values and bar indicates the number of nucleotide substitutions per site. The *A. rolfsii* isolates with accession numbers MZ216466 (SR1) and MZ216467 (SR2) identified in this study were highlighted.

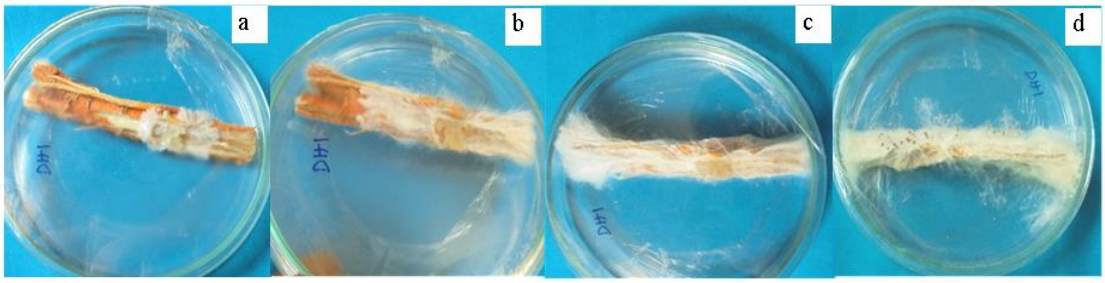

Fig. 4 – *In-vitro* pathogenicity test by detached root cortextechnique. a Growth of *A. rolfsii* on root within 4 DAI. b Colonization 2/3rd portion of root within 6 DAI. c Complete colonization of root within 10 DAI. d Sclerotia production at 20 DAI on disintegrated root tissues.

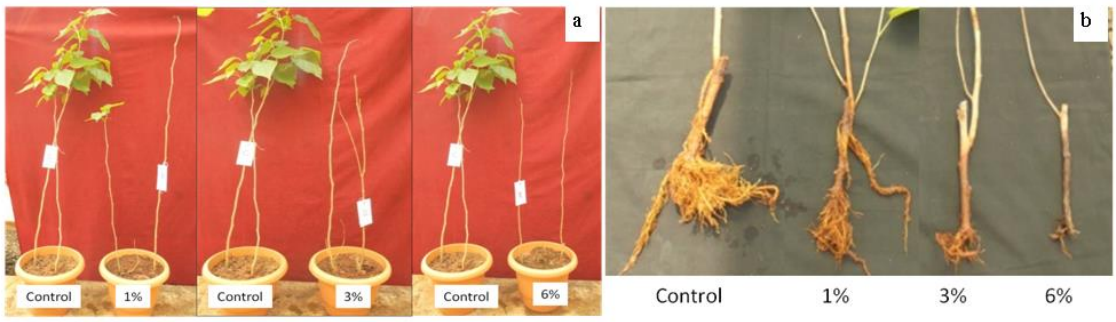

Fig. 5 – *In-vitro* pathogenicity test on mulberry saplings. a Stunted, wilted mulberry saplings at 1%, 3% and 6% inocula. b Mulberry roots rotten, reduced size and volume in *A. rolfsii* inoculated pots (90 DAI).

Table 3 Severity of root rot disease caused by *A. rolfsii* in artificially inoculated mulberry saplings.

***A. rolfsii* inocula concentration (%)**	**Sprouting (%) 10 DAI**	**Wilting (%) 90 DAI**	**Rotting (%) 90 DAI**	**Mortality (%) 90 DAI**	**Disease score**
1	100	22.58	23.91	33.33	Mild
3	100	62.06	60.60	50.00	Severe
6	84	79.31	83.10	83.33	Very severe
Control	100	0.00	0.00	0.00	Healthy

Histopathology

Histopathological observations of the healthy and infected roots confirmed the pathogenicity. Critical examination of the cross-sectional view of uninoculated roots revealed the layers of healthy cells including epidermis (E), cortex (C), endodermis (EM), xylem (X) and phloem (P), while rotten roots, cortex and endodermis disintegrated (Fig. 6). In addition, reduced vascular systems and blocked xylem vessels were marked by the arrow in Fig. 6b.

Discussions

Mulberry is a perennial economic crop which is inimitable for the silk industry. Although various high yielding mulberry cultivars have been commercialized for different locations, the development of a root rot resistant variety is still being researched (Pinto et al. 2018). The polyphagous pathogen *A. rolfsii* has been accounted for rot diseases on a wide hosts' range of 500 plant species (Aycock 1966). It is a necrotrophic soil-borne pathogen that belongs to the phylum Basidiomycota, Agaricomycetes and Atheliaceae. The pathogen attacks almost all parts of the plant and usually multiplies near the ground.

Morphological characterization of the *A. rolfsii* isolates including white wavy mycelia, mustard-like sclerotia production, clamp regions and formation of anastoming branches

corroborated with earlier reports (Higgins 1922, Mohan et al. 2000). Pathogenicity studies revealed the ability of *A. rolfsii* causing root rot disease as like other pathogens associated with mulberry root rot (Mala et al. 2013). The results revealed that the severity of the disease is directly proportional to the pathogen inocula concentration.

The pathogen was previously reported as the causal agent of stem rot/ collar rot in mulberry cuttings (Siddaramaiah & Patil 1984, Singh 1992) which produced disease/ rot symptoms above and below the collar region resulting 60% wilting of sprouted cuttings in nursery. In the present study, symptoms were prominent in root portions of aged mulberry plants. The significant effects of previous crop, weather and soil parameters were notable in root rot disease incidence and severity. In root rot, the typical infection is underground and wilt symptoms do not appear until later stages, making losses difficult to deal with.

Disintegration of vascular system and cellular damage in cortex were observed in infected roots (Mayek-Pérez et al. 2002) found similar to this study and confirmed the pathogenicity of *A.rolfsii*. Root rot in various crops like beetroot, carrot, stevia, chilli, mungbean, sunflower, maize and so on was incited by *A. rolfsii*. Similarly, the invasion of *A.rolfsii* in established mulberry fields is a menace for sericulture farmers. This pathogen could spread unwieldy under conditions of high relative humidity and temperature conditions (Kwon et al. 2011). The occurrence of the pathogen *A. rolfsii* is minor at present however due to integrated cultivation system followed in many farms, it may become severe in near future. Thus, the present study aids the scientific community in developing effective management to surmount the root rot disease and defend farmers from losses.

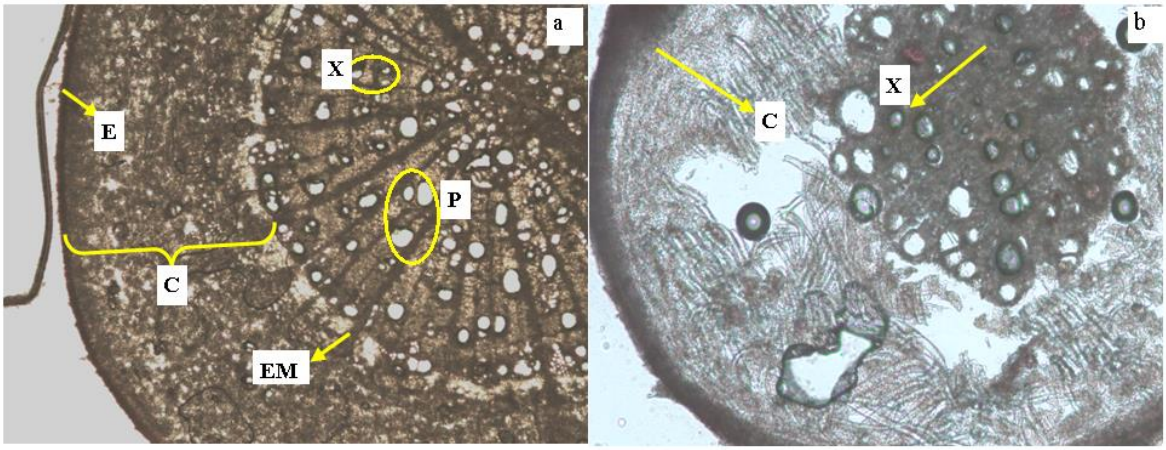

Fig. 6 – Mulberry root histopathology– Cross section. a Healthy root (Epidermis (E), Cortex (C), Endodermis (EM), Xylem (X) and Phloem (P)). b *A. rolfsii* infected root with disintegrated cortex (C) and blocked xylem vessels (X) marked by arrow.

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Disclosure Statements

The manuscript is approved by all the co-authors and I assure that the submitted manuscript or any part of it has not been under consideration or published elsewhere. All the authors declared that there is no conflict of interest.

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