Histopathology of neem shoot naturally infected with *Phomopsis azadirachtae* (the die-back of neem pathogen)

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*Phomopsis azadirachtae* Sateesh, Bhat and Devaki, the incitant of die-back of neem is a deuteromycetous fungus which infects the neem trees of all ages and sizes. The chief symptoms of the disease are twig blight, inflorescence blight and fruit rot. In the present study, histopathological studies of die-back infected neem shoots were carried out. Neem shoots showing twig blight symptoms were considered for the study. Healthy neem shoot served as control. The sections showed abundant colonization of inter- and intra-cellular hyphae in bark, cortex, vascular tissues and central pith. The histopathological investigations suggest that the die-back pathogen is heavily colonized in the infected tissues and seems to have greater penetration capacity.

Key words: *Azadirachta indica*, *Phomopsis azadirachtae*, die-back, neem shoots, histopathological studies.

**INTRODUCTION**

The colonization of a fungal pathogen in a host plant tissue can be detected by various techniques such as plating of host explants, tissue clearing, histopathology, fluorescence microscopy and so on (Agrios, 2004; Bermingham et al., 1995; Dhingra and Sinclair, 1995; Sass, 1958). Histopathological studies of infected plant parts offer many advantages while studying the host-pathogen interactions, site of infection, resistance of host and virulence of the pathogen (Knight and Sutherland, 2013; Oh and Hansen, 2007). It helps to know the initial site of infection, penetration, spread of the pathogen in various tissues and its colonization (Pandey et al., 2012; Sarria et al., 2016). Histopathological studies are also carried out to study the invasion mechanism of the pathogen (Knight and Sutherland, 2013; Oh and Hansen, 2007; Pandey et al., 2012).

Neem (*Azadirachta indica* A. Juss. (Meliaceae)) is an important medicinal plant native to India. This tree is presently suffering from a devastating disease called die-back caused by a deuteromycetes fungus *Phomopsis azadirachtae* Sateesh, Bhat and Devaki (Sateesh et al., 1997). The disease is spreading at an alarming rate and in severely affected trees it results in almost 100% loss of fruit and seed production. Neem seed is a highly valuable source of botanical pesticide (Girish and Shankara Bhat, 2008). Further, the infection leads to a significant reduction...
in the green canopy of this evergreen tree. Histopathological investigations of naturally infected neem seeds with *Phomopsis azadirachtae* were carried out to know the colonization and location of the pathogen in the seed tissues (Fathima et al., 2004). However, the literature on histopathology of neem shoot naturally infected with *P. azadirachtae* is lacking. In the present work, histopathological study of the die-back infected neem shoot was carried out with a view to find out the anatomical changes taking place following infection and colonization of the pathogen when compared with healthy tissue to get some knowledge regarding the host parasite relationship.

**MATERIALS AND METHODS**

**Collection of infected neem twigs**

The twigs showing characteristic die-back symptoms were collected from severely infected neem trees from different agroclimatic regions of Karnataka, South India. The twigs were either used immediately or kept in a refrigerator at 4°C and used within 2-3 days.

**Isolation of *P. azadirachtae* from infected neem shoot**

Naturally infected twigs with pycnidial growth on the surface were washed with running tap water for one hour. The twigs were cut into 10-12 cm pieces with middle transition region of healthy and infected portion. Healthy twig explants treated similarly served as control. The healthy and infected twig explants were surface-sterilized separately using sodium hypochlorite solution (with 5% available chlorine) (Sauer and Burroughs, 1986) for 10-15 min, later they were rinsed with sterile distilled water 5-6 times. These twig pieces were placed in square glass chamber that was lined with sterile wet blotter sheets (to maintain high humidity of 80-90%) except on the upper and one lateral side. Glass chambers were incubated at room temperature (26 ± 2°C) near the windows. The cirrhi obtained on infected twigs after incubation was aseptically inoculated on to Potato Dextrose Agar (PDA) Plates and incubated at room temperature (26 ± 2°C).

**Killing and fixing**

The procedures used in this study were modification of histopathological procedures described by Johansen (1940) and Sass (1958). The infected and healthy twig explants were fixed separately in Formaldehyde: Acetic acid: 70% Alcohol (FAA 5:5:90 v/v/v) solution for 48 h. One or two transverse incisions were made using a blade on the twig pieces to ensure proper dehydration, infiltration and embedding.

**Softening**

The twig explants were then subjected to lactophenol (Lactic acid: Phenol, 1:1 v/w) treatment for 10-12 days for the softening of the tissues.

**Dehydration**

The fixed twig explants were dehydrated using graded series of Tertiary Butyl Alcohol (TBA) and ethanol starting with 50% TBA and finally in 100% TBA (Figure 1 Flowchart-I).

**Infiltration**

The dehydrated twig explants were placed on the surface of partially solidified paraffin in separate vials covered with a mixture of TBA and paraffin oil. The vials were placed in a hot air oven maintained at 55°C for one hour. The mixture was decanted, replaced with pure melted paraffin three to four times before embedding.

**Embedding**

The twig explants were arranged on small rectangular paper boats or trays to which freshly melted paraffin were added. The paper boats or trays were then placed in cold water for 30 min for rapid cooling of the paraffin. The paraffin blocks were removed from the trays and were stored in a refrigerator before taking microtome sections. The embedded blocks were cut to required size and trimmed to expose one side of the stem tissue and immersed in aqueous solution of Sodium lauryl sulphate for 24 h to further soften the tissues. The paraffin blocks were soaked in 50% glacial acetic acid for 5-7 days (Singh and Singh, 1977).

**Microtomy**

Rotary microtome was used for sectioning the paraffin blocks. Serial microtome sections of thickness 10-15 μm (11 μm) were taken and affixed on to clean microscope slides using egg albumin. The slides were air-dried for one week (Figure 1 Flow chart-II).

**Staining**

The air-dried slides were dipped in xylene for 10 min to remove paraffin. The sections were stained with Schiff’s reagent and fast green and passed through alcohol series. Before mounting, the slides were washed with xylene and finally mounted in DPX mountant. The slides were air-dried for 48 h and observed (Figure 1 Flow chart-III).

**RESULTS**

**Isolation of *P. azadirachtae* from infected neem shoot**

Naturally infected twigs incubated in the glass chambers produced mycelial mat after 3 to 4 days of incubation. The twigs sporulated after seven days of incubation and produced two types of conidia in cream to dark yellow coloured slimy tendrillar cirrhi from large number of pycnidia. Healthy twigs treated similarly did not show any...
Figure 1. Flow charts of the procedures for the preparation of slides for microscopy.

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<td>Deparaffinise sections in xylene-10-15 min</td>
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<td>95% ethyl alcohol 5-10 sec</td>
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<td>Absolute ethyl alcohol 15-30 sec</td>
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<td>Xylene : absolute alcohol (1:1) 5 min</td>
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<td>Xylene (2 changes of 10 min each) Mount in DPX</td>
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fungal growth. The fungal isolates growing on PDA plates were confirmed as *Phomopsis* species by observing the mycelial nature and mycelial pigmentation, conidiogenesis, shape and size of conidia. The isolates produced two types of conidia alpha and beta conidia and were confirmed as *P. azadirachtae* based on Sateesh et al. (1997).

**Histopathology**

The pathogen colonized abundantly almost all the regions of the infected stem. The inter- and intra-cellular hyphae were observed in the bark, cortex, vascular tissue and central pith (Figures 2 to 11). The cells of the bark and cortex colonized by the pathogen were distorted.
vegetative mycelium of the pathogen was composed of septate hyphae with clear cell wall. The hyphae stained reddish brown and the hyphal width ranged between 3.8 and 6.5 µm.

In the stem, hyphae were primarily observed growing through the intercellular spaces of the cortex and intracellularly through the parenchyma of the outer cortex. Numerous tyloses were formed in the vessel elements in advance of the infection (Figures 12 to 14). Hyphae from the infected cortex grew laterally into the

**Figure 4.** Longitudinal section of neem stem naturally infected with *P. azadirachtae* showing distorted cortical cells (450 X).

**Figure 5.** Transverse section of healthy neem stem showing vascular tissue (450 X).
xylem and phloem tissues. A substance often observed blocking the vessel elements stained pinkish when stained with Schiff’s reagent leading to vascular plugging (Figure 15). A large number of hyphae from the colonized vascular tissue grew intracellularly into the pith. Colonization was more intense in vascular region.
Figure 8. Transverse section of neem stem infected with *P. azadirachtae* showing pith cells with intracellular hyphae of the fungus (450 X).

Figure 9. Longitudinal section of healthy neem stem showing pith cells (450 X).

compared to cortex region. There were less hyphae and no cell wall degradation in pith region. Cytoplasm of infected cells appeared grainy. The fungus did not affect cell size, shape or packing as these traits were similar to
control except in areas where cell walls had been degraded. The fungus affected cell size and shape in cortex region by degrading cell wall.

**DISCUSSION**

Pathogens penetrate their host plants by enzymatic or
mechanical means (Deeks et al., 2002). The initial penetration and invasion processes of a plant host by a fungal pathogen ensure successful colonization of host tissue in absence of a plant resistant response (Curry et al., 2002). Cell wall weakening along with tissue maceration facilitates inter or intracellular invasion of host...
Figure 14. Longitudinal section of neem stem infected with *P. azadirachtae* showing tyloses in vascular tissue (450 X).

Figure 15. Longitudinal section of neem stem infected with *P. azadirachtae* showing vascular plugging in the xylem vessels (450 X).

tissues by the pathogen (Deeks et al., 2002). Cell wall degradation was evident in the present study. The cytoplasm of infected cells appeared grainy and this may be due to the addition of degraded cell wall material that had not yet been reduced to monomers by the fungi (Deeks et al., 2002).
The histopathological studies revealed that the pathogen is deep-seated in the stem tissue. The distortion of the bark and cortical cells could be due to the production of secondary metabolites from the hyphae of the pathogen, such a distortion was also observed in soybean seeds colonized by *Phomopsis* species (Krishnamurthy and Raveesha, 1996; Singh and Sinclair, 1986). The drying up of the terminal branches could be attributed to the location of the pathogen in the vascular tissue. Although hyphae of *P. azadirachtae* were often observed in the vascular tissue and pith, these regions were not invaded until the cortex has been completely colonized. This suggests the spread of hyphae along the cortex through the intercellular spaces and intracellularly in the parenchyma of the outer cortex. Similar type of spread is seen during infection by *Phomopsis vaccinii* (Daykin and Milholland, 1990).

Hyphae were confined to the intracellular spaces in the vascular tissues but are inter and intracellular in cortex and pith cells. The xylem and phloem were affected as the fungus invaded the vascular bundle. The fungus moved intercellularly through the vascular tissue via the xylem parenchyma. This is similar to the observations made by Milholland et al. (1981), in burley tobacco stems systemically infected with *Peronospora tabacina*. As the fungus progressed, the phloem, cambium and surrounding parenchyma cells eventually collapsed and died. Generalized disruption of all phloem cells and cell shape loss associated with hypertrophy and hyperplasia was reported in purple passionfruit plants (*Passiflora edulis* Sims) infected by *Fusarium oxysporum* and *F. solani* (Ortiz et al., 2014). Extensive proliferation of parenchyma cells associated with small necrotic areas in the vascular tissue resulted in the disruption and separation of the xylem elements. Similar findings were made by Curry et al. (2002) in *Colletotrichum*. The fungus after penetration and invasion successfully colonized the host tissue expressing the disease symptoms.

The present study revealed that vast numbers of tyloses were formed due to infection by *P. azadirachtae*. Abundant tyloses were also observed in stems of blue berry infected with *Phomopsis vaccinii* (Daykin and Milholland, 1990). Tyloses together with vascular plugging due to the accumulation of gums and hyphae, may contribute to the die-back symptoms in the distal parts of the stem.

**CONFLICT OF INTERESTS**

The authors have not declared any conflict of interests.

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**REFERENCES**


