Available Online at ESci Journals
ESci Journal of Plant Pathology
ISSN: 2305-106X (Online), 2306-1650 (Print)
http://www.escijournals.net/EJPP

HISTOPATHOLOGY OF MACROPHOMINA STEM CANKER DISEASE IN PIGEONPEA (*CAJANUS CAJAN* L.)

[a]Sandeep K. Maurya*, [b]Surinder Kaur, [b]Vijay B. Chauhan
[a] *Directorate of Plant Protection Quarantine and Storage, NH-4 Faridabad, Haryana, India*
[b] *Department of Mycology & Plant Pathology, Institute of Agricultural Sciences, Banaras Hindu University, India.*

A B S T R A C T

Macrophomina phaseolina, causal agent of stem canker disease has recently emerged as an agriculturally important plant pathogen. Macrophomina stem canker disease (MSD), caused by *Macrophomina phaseolina* is a potentially serious disease in pigeonpea that occurs when reaches physiological maturity i.e., during flowering. The fungus incites necrotic lesions on stem and girdles the plant at the base leading to premature flower drop leading to complete witling and finally death of the entire plant. The mechanisms of infection remain to be fully elucidated. The present study investigated histopathology of MSD caused by *M. phaseolina* in pigeonpea seed and seedlings using light microscopy. Pigeonpea variety 'Bahar' was used in this study. Histopathological sections of seed, stem, root, and leaves were prepared and stained with safranin and trypan blue. Histopathology of the infected plant parts showed the presence of intercellular mycelia and microsclerotia in the cortex and vascular tissues. The germ tube colonized the plant with growth of seedlings following seed coat, cotyledon, stem, root and leaves. According to the results, the pathogen can penetrate and invade the seeds within 24 h post inoculation.

Keywords: Histopathology, *Macrophomina phaseolina*, pigeonpea, stem canker.

INTRODUCTION

Pigeonpea (*Cajanus cajan* L.) is an important grain legume crop of rainfed agriculture in the semi-arid tropics. Besides Indian sub-continent, it is widely grown in Eastern Africa and Central America. It is not only an important source of protein, but also plays an important role in atmospheric nitrogen fixation into soil. It is reported that a long duration pigeonpea cropping could fix up to 200 kg N /ha and the residual effect for next crop remains 40 kg N/ha.

Pigeonpea is affected by more than 100 diseases but only few cause economic losses. Recently, *Macrophomina phaseolina* (Tassi) Goid has emerged as one of the important pathogen of different agricultural crops including pigeonpea (Kaur *et al.,* 2012a). *M. phaseolina* is an anamorphic fungus in the ascomycete family Botryosphaeriaceae (Crous *et al.,* 2006). The fungus has a wide geographical distribution from tropics to subtropics ranging from arid to semi-arid climates in Africa, Asia, Europe, and north and South America (Diourte *et al.,* 1995; Wrather *et al.,* 2001). It has a wide host range, infecting about 500 cultivated and wild plant species from more than 100 families around the world (Mihail & Taylor, 1995). Macrophomina is primarily soil and seed-borne fungal pathogen that incites the disease by producing microsclerotia/pycnidia (Pun *et al.,* 1998). *Macrophomina* exhibits high morphological, pathogenic, physiological and genetic variability (Jana *et al.,* 2005; Kaur *et al.,* 2013). Stem canker disease has become one of the most devastating diseases of pigeonpea (*Cajanus cajan* (L.)). The disease incidence and severity of up to 70 and 55% were reported in a survey from regions of eastern Uttar Pradesh in India (Kaur *et al.,* 2012b).

Macrophomina stem canker is a sporadic disease and causes dry root rot, stem canker, and stalk rot or charcoal rot of plant. The symptoms of the disease appear on the stem as the charcoal like appearance which starts from the base and proceeds upward towards the branches. Under conditions of high temperature and water stress, the disease symptoms are more severe (Short *et al*., 1980). Although, disease

* Corresponding Author:
Email: sandeepmr045@gmail.com

symptoms of the disease and mode of infection in *M. phaseolina* are evident, the colonization and pathogenesis have never been studied in pigeonpea. In this context, the study was conducted to analyze the histopathology of the interaction of *M. phaseolina* with the seed coat, cotyledon, seedling and leaf petiole of pigeonpea.

MATERIALS AND METHODS

Mass multiplication of the inoculum, soil preparation and inoculation: Mass multiplication of *M. phaseolina* was done according to Kaur *et al.* (2013) with slight modification. In brief, maize grains + sand (3:1) media was prepared in 500 ml glass bottles. The media was sterilized by autoclaving (15 psi, 121±1 ^{o}C) the bottles for 30 min, cooled and inoculated with mycelium agar disc (5 mm diameter) cut from the margin of an actively growing 7 day old culture of *M. phaseolina* and incubated for 25 days at 30±1 ^{o}C. The incubated bottles were shaken thoroughly every day for uniform and proper colonization of all seeds. Infested maize seeds were powdered finely in grinder which served as the pathogen inoculum. Sand, soil and farm yard manure were mixed properly in the ratio of 4:4:1 and filled in several polythene bags (30 × 20 cm) and clipped with a rubber band. The soil bags were sterilized in autoclave for 30 min at 121 ^{o}C at 15 psi. Earthen pots (18 cm diameter) were selected and were sterilized by dipping in formaldehyde (4%, w/v). Pots were filled with sterilized soil (1.5 kg/ pot) and kept in the glass house. The inoculum (100 g/pot) was thoroughly mixed in the upper 5 cm top soil of each pot 5 days prior to the sowing of pigeonpea seed adopting the method of Mayek-Perez *et al.* (2001). In total, thirty surface sterilized (0.1 %, w/v sodium hypochlorite) pigeonpea seed (variety Bahar) were sown in each pot previously inoculated with the inoculum in three replications.

Collection of diseased samples for histopathology: *M. phaseolina* isolations were attempted from seed coat, cotyledon, seedling and leaf petiole. Five seed were collected at 24 h interval for 16 days after sowing. Seed coat, cotyledon, radical and leaves were surface sterilized in sodium hypochlorite solution (0.1% w/v) for 30 sec., thoroughly washed in three series of distilled water, dried on sterilized blotting paper and placed on different Petri dishes containing 25 ml solidified rose bengal rice agar medium and incubated at 25±1^{o}C.

Histopathological analysis: The samples were fixed in a formalin-acetic-acid-alcohol (Berlin & Miksche, 1976). For fixation, the samples were placed in a vacuum chamber to remove the air bubbles often present within plant tissues. The plant material was subsequently dehydrated through a graded alcohol series (10, 20, 30, 50, 70, 80, 90 and 100 %). Isolation was made at 24 h interval from seed, stem, and root and leaf petiole to confirm the presence of pathogen at different time interval. The plant material was subsequently dehydrated through a graded alcohol series (10, 20, 30, 50, 70, 80, 90 and 100 %) and embedded in molten paraffin wax. Using a Leica RM 2045 rotary microtome, 5-to-7-µm-thick sections were cut and stained. For histological analysis, samples were stained with 1% aqueous safranin O and trypan blue (0.8 mM in PBS). The slides were analysed, and the images were captured with a Leica DC 300F digital camera attached to a Leica DMLD microscope.

RESULT AND DISCUSSION

Macrophomina stem canker disease of pigeonpea caused by *M. phaseolina* is the soil and seed borne disease. Histopathology of disease on seed, seedling stem, and leaf petioles were observed at 24 h interval. The presence of the pathogen in the form of mycelium and microsclerotia was observed microscopically in infected tissues (Table 1).

Seed coat and cotyledon: The inoculated seeds were harvested from experimental pots at 24 h interval. Water soaked brown spot developed on the seed coat (Fig. 1). Symptomatic seeds were separated into the seed coat and cotyledon and inoculated on Rose Bengal rice agar media.

White to grayish fungal growth was observed from the seed coat and cotyledon (Fig 2) after 3rd and 5th day of incubation on media, respectively. Fungal growth from first sample, suggests disease incitation 24 h post inoculation. Mycelial growth from seed coat was relatively slower than cotyledons. Fungus was also isolated from the infected part of plumule after 2-3 days of incubation. Histopathology of infected seed revealed intercellular invasion in cotyledon tissues and the formation of microsclerotia after 4-5 days of inoculation (Fig 3a).

Radical and stem: Isolation was attempted from the seed radicle, emerged three days after sowing. No fungal growth from the radicle was observed up to 3 days of incubation on the isolation media. It showed the absence of infection. Isolation was again attempted, seven days after sowing from the stem.

Table 1. *Macrophomina phaseolina* invasion and disease progression in different parts of the pigeonpea.

DAI*	Seed coat	Cotyledon	Seed / Plumule	Root	Stem	Leaf
1st	1	+	-	-	-	-
2nd	1	+	-	-	-	-
3rd	1	+	+	-	-	-
4th	3	+	+	-	-	-
5th	3	+	+	-	-	-
6th	3	+	+	-	+	-
7th	3	+	+	-	+	-
8th	5	+	+	-	+	-
9th	5	+	+	+	+	-
10th	5	+	+	+	+	-
11th	5	+	+	+	+	-
12th	7	+	+	+	+	-
13th	7	+	+	+	+	-
14th	7	+	+	+	+	-
15th	9	+	+	+	+	-
16th	9	+	+	+	+	-
17th	10	+	+	+	+	-
18th	10	+	+	+	+	-
18th	10	+	+	+	+	-
19th	10	+	+	+	+	-
20th	10	+	+	+	+	-
21st	10	+	+	+	+	+
22nd	10	+	+	+	+	+

*Days after inoculation; **Disease severity.

Figure 1. Water soaked brown spots developed on the seed coat of the pigeonpea seed infected with *Macrophomina phaseolina.*

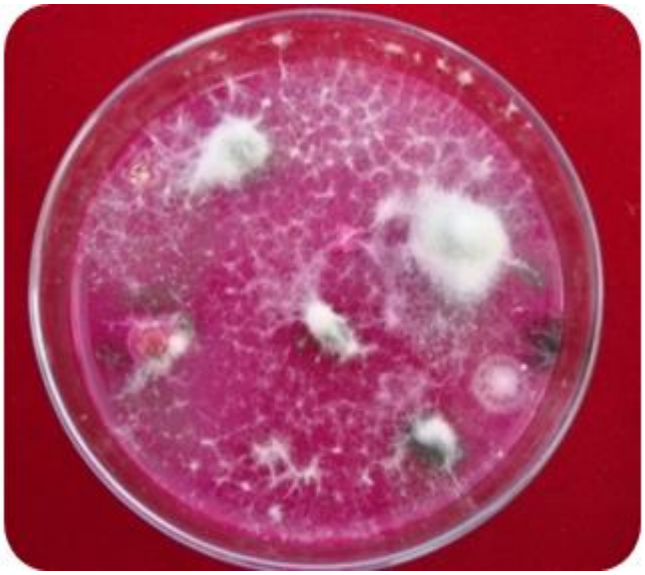

Figure 2. White to grayish mycelial growth of *Macrophomina phaseolina* was observed on pigeonpea seed coat and cotyledon isolated on Rose Bengal medium.

Mycelial growth was evident for the basal part of the stem while no fungal growth was observed from the upper part of the stem. The stem became brown to black in color which progressed in upward, i.e., from base to the upper part of the stem (Fig. 4). The stem girdled completely after 15 days. Histopathological study of the infected stem revealed intercellular mycelia (Fig. 3b & c) in epiblema, cortex and pith of the stem (Kunwar *et al.,* 1985). Inter and intracellular mycelia colonizing the cortex, xylem, and pith tissues and several scattered microsclerotia were evident in the transverse section of the stem (Singh & Singh, 1981).

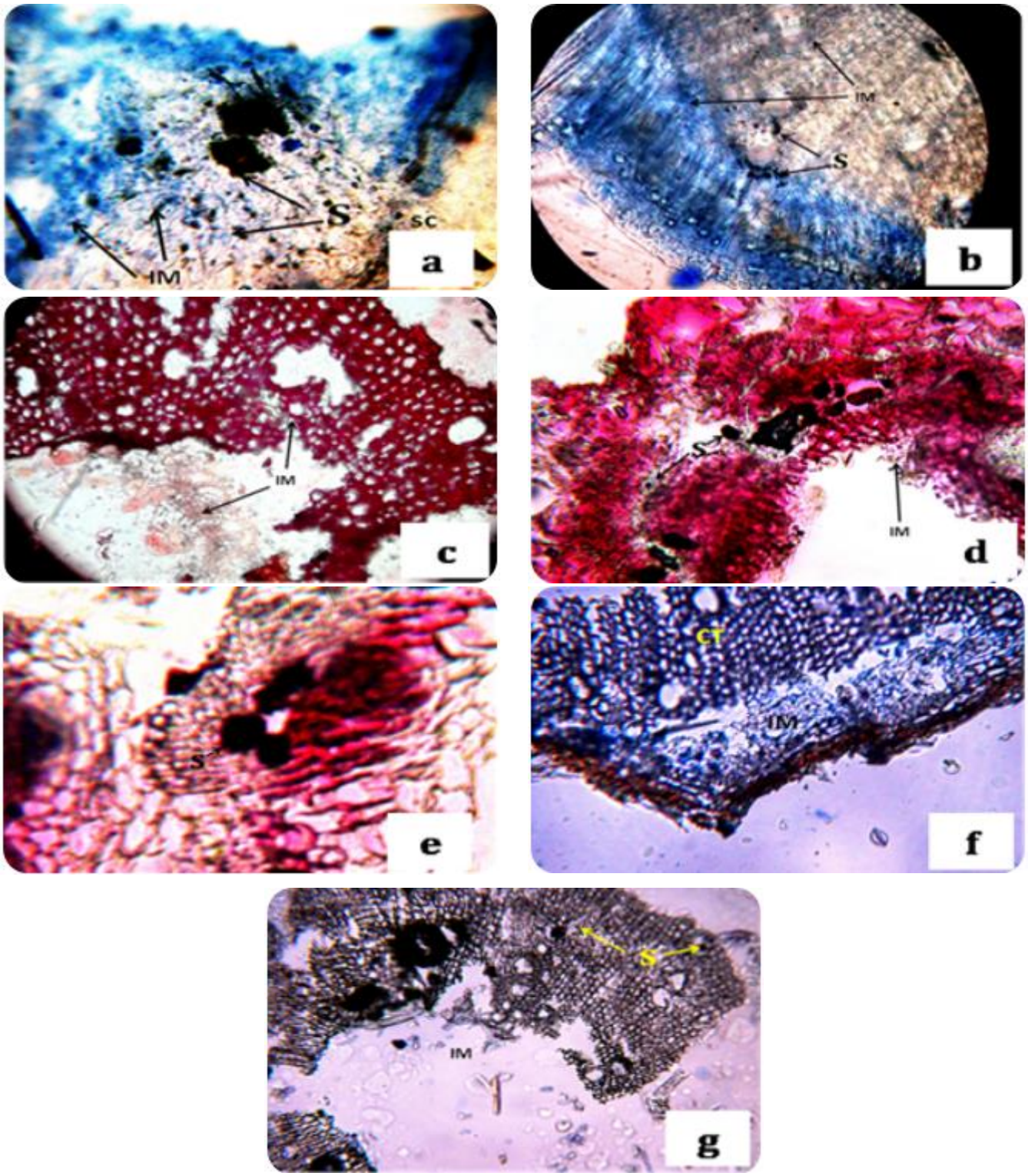

Figure 3 a-g. Histopathology of pigeonpea seedling infected with Macrophomina phaseolina at 10X: a) cotyledon tissues and the formation of microsclerotia after 4-5 days of inoculation; b-c) intercellular mycelia in stem; d-e) fungal growth and mycelial aggregation observed in the cortex; f-g) intercellular fungal growth in the leaf petioles, and microsclerotia in the cortex and epiblema.
(s= microsclerotia, IM= intercellular mycelium, SC= seed coat, CT= cortex tissues).

Root: Histopathological study of the infected root revealed the presence of intercellular hyphae and microsclerotia in the root cortex tissues. Fungal growth on the isolation media 12 days post inoculation suggests pathogen invasion in the root system. Fungal growth after 12 days suggests no fungal invasion during early growth stages of root development. It might be due the presence of higher concentration of the growth hormones during initial days of root growth that inhibits infection by pathogen. All root sections, root tip, nodes and the middle portion exhibited fungal growth. Infected roots exhibited characteristic reddish brown colour and rotting. Numerous microsclerotia were evenly distributed on the primary and the secondary roots (Fig. 4). Intercellular fungal growth and mycelial aggregation observed in the cortex might have plugged the stem cortex tissues which resulted in wilting and death of the plant (Fig. 3d & e). Histopathology of sorghum seedling roots infected by *M. phaseolina* and observed colonization of inter cellular hyphae as well as the formation of microsclerotial bodies inside the cortical tissues, xylem and phloem parenchyma tissues (Karunakar, 1992).

Leaf petiole: Histopathology of the leaf petioles illustrated inter-cellular mycelium and microsclerotia in cortex and pith. Lower leaves exhibited drooping and yellowing symptoms, acropetally and shed prematurely 20-25 days post inoculation (Fig. 5). Pieces of leaf sheath and leaves were plated in the isolation media. Mycelial growth as well pycnidia were observed after three days of incubation. Histopathological study of the leaf petioles revealed intercellular fungal growth and microsclerotia in the cortex and epiblema (Fig. 3f & g). Yellow to brown circular spots on the leaves, stem, and capsule while mycelia and sclerotia were reported in the periphery of the lesion by Singh and Singh (1981).

Figure 4. *Macrophomina phaseolina* infected stem became brown to black in color which progressed in upward, i.e., from base to the upper part of the stem and numerous microsclerotia are distributed on the primary and the secondary roots

Figure 5. Lower leaves exhibited drooping and yellowing symptoms, acropetally and shed prematurely 20-25 days post inoculation.

CONCLUSION

In this histopathological study, we demonstrated infection and colonization of *M. phaseolina* in pigeonpea. It also depicts the invasion of mycelium and presence of the microsclerotia in mycelial aggregates. Infection and disease development enhances understanding approaches for disease management.

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