

Stem Histopathology of Sesame Seedlings Infected with *Alternaria alternata*

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Abstract

In the present study, histopathology of three varieties of sesame TS 3, TS 5 and SG 27 infected with *Alternaria alternata* was carried out to understand the mechanism of fungal infection and penetration in sesame plant as well as to determine the histological manifestation in sesame cells by light microscopy. Fungus was identified in infected tissues as a dark bluish black with toluidine blue O staining. Light microscopic examination of sesame stem showed that the fungus was present in epidermis, hypodermis and cortical parenchyma tissue as the symptoms became visible by naked eye ten days after inoculation (DAI). As the disease progress, the fungus moved from cortical parenchyma to vascular bundle, xylem and phloem. Later on, it completely overlapped the vascular bundle and entered in pith. When necrotic lesion appeared, fungus was present abundantly in epidermis, hypodermis, cortical parenchyma, vascular bundles and in pith. Due to its excessive growth and complete overlapping of cells, disorganization or destruction of cells of sesame took place. It was concluded that the *Alternaria alternata* was not a tissue limited pathogen instead of this it spread in to all tissues of stem from epidermis to pith.

Keywords

Sesame, *Alternaria alternata*, Pathogen, Fungus, Light microscopy

1. Introduction

Sesame (*Sesamum indicum* L.) is the most important and oldest oilseed crop, commonly known as till that belongs to the family Pedaliaceae [1]. Globally sesame production is 4.04 million tons annually with an average productivity which is about 5.1 quintals per hectare [2] in an area of 7.54 million hectares [3]. Pakistan is at 14th position among the major sesame producing countries in the world having a total production of 31,000 tons in an

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area of 76,000 hectares and yielding only 402 kilogram per hectare [4]. Seeds possess a wide variety of bioactive compounds [5] which exhibit a wide range of therapeutic values, including antioxidative [6], anti-inflammatory [7] cholesterol lowering [8] anticancer [9] antihypertensive [10] and neuroprotective properties [11]. Sesame oil is twelfth largest vegetable oil worldwide [12] [13]. Sesamoline and sesamine are present in sesame oil which are used as an insecticide [14], having cholesterol lowering activities [15], strong antioxidant activity and lower the blood pressure [16]. The world especially Pakistan is facing shortage of edible sesame oil due to the various infectious plant pathogens which act as a major damaging factor to this crop [17]. About 72 fungi, 7 bacteria, 1 mycoplasma, 38 species of pest and 29 species of insects are recorded on sesame crop [18] from them; the most destructive pathogen is *Alternaria*. *Alternaria* ranked at 10th position among all the fungal genera on the basis of their host plant interaction [19]. *Alternaria* cause the most important disease on sesame that reduces the yield of the crop. It is the spot disease [20] which is caused by the *Alternaria sesami* [21] and *Alternaria alternata* [22]. No prodigious efforts have been done on this host plant interaction despite of shortage of edible oil.

Previous studies show that these fungus genera infect the plants through the infection site, enter in epidermal region and move towards intercellular spaces of cortical parenchyma which result in the formation of symptoms on plant surface including blight and spot [23]-[26]. Later, it spreads in all layers of plant tissue including vascular bundle and develops a large necrotic area [27]. Differentiations are not occur in plant tissue and in severe cases destruction of host cells are reported [28]. This pathogen also produces several toxins to plant which have a direct effect on cells organelles, destruction of plasma membrane, vesiculation and micro vacuole formation occur. Accumulation of plastoglobuli in chromoplast also occur [29]. Previous studies do not indicate the histopathology of sesame seedlings infected with *Alternaria* spp. Currently this disease has become more devastating and is posing great threat to sesame production in all over the world specially Pakistan. The main objectives of this study are to understand the mechanism of fungal infection and penetration in infected sesame plant as well as to determine the histological changes in sesame cells infected with *Alternaria alternata*.

2. Material and Methods

2.1. Fungus Collection, Growth and Slide Preparation

Alternaria alternata (Accession Number: 1200) was collected from the Fungal Culture Bank of Pakistan (FCBP), Institute of Agricultural Sciences, University of the Punjab, Lahore, Pakistan. For the multiplication of fungus, under aseptic conditions fungus was placed on Potato Dextrose Agar (PDA) media. Then incubated at 28°C for seven days for fungal growth. The growth of fungus obtained on PDA media was re cultured. Culturing and sub culturing was continued until the pure culture was obtained. The pure culture of fungus was stored at 4°C and periodically cultured. For slide preparation, Small amount of lacto phenol was placed on glass slide with the help of dropper. With the help of sterilized needle, fungus was placed on slide and covered with coverslip with the care that no air bubble was formed. The slide was observed under microscope and photographs were taken.

2.2. Green House Trial

Three varieties of sesame seeds TS 3, TS 5 and SG 27 were collected from the Oil Seed Program of National Agriculture Research Centre (NARC). Seeds were surface sterilized with 2 percent sodium hypochlorite (NaOCl) for 2 minutes and then rinsed three times with distilled water. Potting mixture contains sand, clay and Farm yard manure with the ratio of 2:1:1. Sesame seeds were sowed in pots with ten replicate of each variety. Each pot contains four to six seeds. Plants were watered regularly. After 40 days of sowing, when the seedlings were at six to eight leaf stage conidial suspension of *Alternaria alternata* was inoculated. For the preparation of conidial suspension, 5 ml of sterilized water were added in 15 days old culture plate of pathogen and conidia were detached with tooth brush and suspension was collected in sterilized beaker. The number of conidia were counted with the help of hemacytometer and adjusted to 2×10^3 conidia per ml [30].

Pathogens were inoculated by spray method. Spray was done in evening and inoculated plants were covered with plastic bags for 24 hours after inoculation and placed under darkness to provide maximum humidity for penetration of fungus into host cells. The plants sprayed with sterilized distilled water were served as control. When artificially inoculated plants showed the typical symptoms of disease, their histopathology was done.

2.3. Histopathology

For histopathological study, following steps were performed [31].

2.3.1. Fixation

Healthy and infected stem section (1 - 2 mm) of TS-3, TS-5 and SG-27 were fixed in 4 percent formaldehyde in 50 mM phosphate buffer with pH is 7.2 at 4 degree centigrade for 48 hours. Then the same buffer was used to wash the sections for 5 minute each.

2.3.2. Dehydration

Sections were dehydrated in ethanol series, 30%, 50%, 70%, 95% and 100% each for one hour at 4°C. The tissue were further dehydrated with ethanol and xylene in ratio of 3:1, 1:1 and 1:3 each for one hour, then tissue were kept in pure xylene overnight at room temperature.

2.3.3. Infiltration

Excessive xylene was removed by adding few paraffin chips until saturation and kept in oven at 40°C. Xylene wax solution was replaced by pure wax by adding more paraffin chips overnight at 60°C [32].

2.3.4. Embedding in Paraffin

Sections were taken from oven and placed in steel mold. The number of sections depends upon the size of the molds. Melted paraffin wax was poured in to steel molds. Tissues were arranged and oriented with the help of needle so that individual pieces can be cut easily from the finished block and placed the boat on the surface of ice water immediately after placing the tissue in melted paraffin until solidified. After solidification the boat was placed in the refrigerator. Embedding tissue in paraffin on block holders and stored at 4°C before staining [32].

2.3.5. Sectioning and Staining

Excess paraffin was removed around the tissue by trimming with a sharp scalpel. Blocks were placed in rotary microtome (LEICA RM2125RT) in such a position that the edge of the razor blade just touch the block so that whole section cut serially. The microtome handle turned in clockwise direction with a steady, even stroke [33]. 11 - 15 µm thin sections were made with the help of rotary microtome (LEICA RM2125RT), after cutting, with the help of needle the ribbon was removed from the microtome and placed in glass slide. For a short time slide was placed on slide warmer at 40 degree centigrade. After drying, slides were passed through a series of chemical and stained with 0.5% toluidine blue-O containing 0.5% H₃BO₄ and 2% Na₂CO₃. Sections were observed and photographs were taken by light microscope. Photograph was taken by light microscope (NIKON Eclips 80i). Nikon Images were captured in digital sight attached with microscope and automatically saved in computer flash. The experiment was repeated four times to confirm the infection process of pathogens.

3. Results and Discussion

3.1. Colony and Microscopic Examination of *Alternaria alternata*

On PDA media, rapid growth of *Alternaria alternata* was observed (**Figure 1**). Maximum growth of fungus occurred in fifteen days at a temperature of 26°C - 28°C. At earlier stages, the colour of colony was grey whitish but at maturity its colour became brownish black. Fungus forms a compact mass like a carpet. The shape of the colony was circular and margins were smooth, entire or irregular. Fungus grows in all directions equally from the centre without the formation of concentric rings. These results are supported by the [34]-[38].

Microscopic examination of *Alternaria alternata* shows that the mycelium of the fungus was branched, multicelled, septate, dense aerial and golden brown in colour (**Figure 2**). Hyphae were branched, septate, golden brown in colour. Initially the hyphae were thin, later it became thick on maturity. *Alternaria alternata* possess a septate conidia which were produced singly or in short or long chains called conidiophore. Conidiophores was simple, short, straight, branched or unbranched, septate, slightly swollen at the apex, present alone or combine to form group or clusters and golden brown in colour. Conidia was small, multicellular, branched or unbranched, brown in colour, vary in shape and size, may be ovoid, obclavate, and obpyriform or ellipsoidal, smooth walled, broadest near the base and tapered gradually to form conical or cylindrical beak. Conidia possess three to five

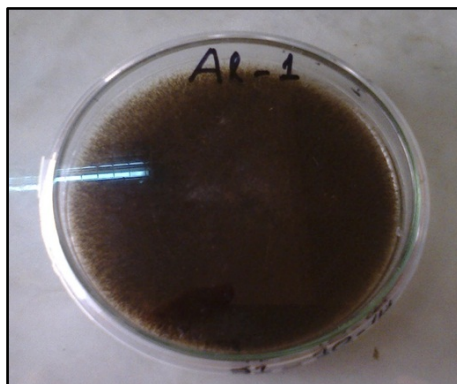


Figure 1. Growth of *Alternaria alternata* on potato dextrose agar (PDA) media.



Figure 2. Microscopic examination of *Alternaria alternata* (a) Mycelium 40X; (b) Hyphae; (c) Conidia.

transverse and one to two longitudinal septa, possess a smooth wall. The length of conidia was 3 - 5 times more than the width. These result are largely resembles with [39]-[43].

3.2. Histology of Uninfected (Control) Stem of Sesame (*Sesamum indicum* L.)

Light microscopic examination of uninfected (control) stem of three varieties TS 3, TS 5 and SG 27 of sesame (*Sesamum indicum* L.) showed the same structure (**Figure 3**). Histologically, the sesame stem consist of non-vascular and vascular tissues. Non vascular tissues consist of trichome, epidermis, hypodermis and cortical parenchyma. Vascular bundles contain xylem and phloem. Epidermis was the outer most single layer of stem consisting of closely packed living cells. Massive growth of trichome was present on the surface of epidermis which was unicellular or multicellular outgrowth of epidermis. Beneath the epidermis, hypodermis was present which consist of two to three layers of cells. Cortex was present next to hypodermis which was multi-layered parenchyma cells. Cells of cortex were separated by intercellular spaces. In discontinuous manner, fiber cells were also present. Vascular tissue contains xylem and phloem. Phloem consists of primary and secondary form, present on the top of xylem. Xylem was inside to the phloem consisting of primary xylem and secondary xylem. Cambium was present between xylem and phloem. The arrangement of vascular tissue was as follow. Primary phloem, secondary phloem, cambium, secondary xylem and the innermost is primary xylem. Pith was composed of parenchyma cells and occupies the centre position; some cells of pith contain food reserves. This result is largely resembled with earlier study reported by [44].

3.3. Symptoms Produced by *A. Alternata* on Sesame Plant

Initial 8 - 10 days of inoculation of pathogen on sesame plant, no visible symptoms or change was appeared on plant. Ten days after infection, minute brown colour spots were appeared at inoculation site which became enlarge later. Initially the colour of spot was brown but later it became darker. At the same time, small lesions surrounded by the yellow halos appeared which enlarge to form large necrotic areas with concentric rings. At initial stage, the shapes of spots were round but later it became irregular. The plants which were used as a control and sprayed with distilled water showed no such symptoms of infection. Same symptoms were recorded

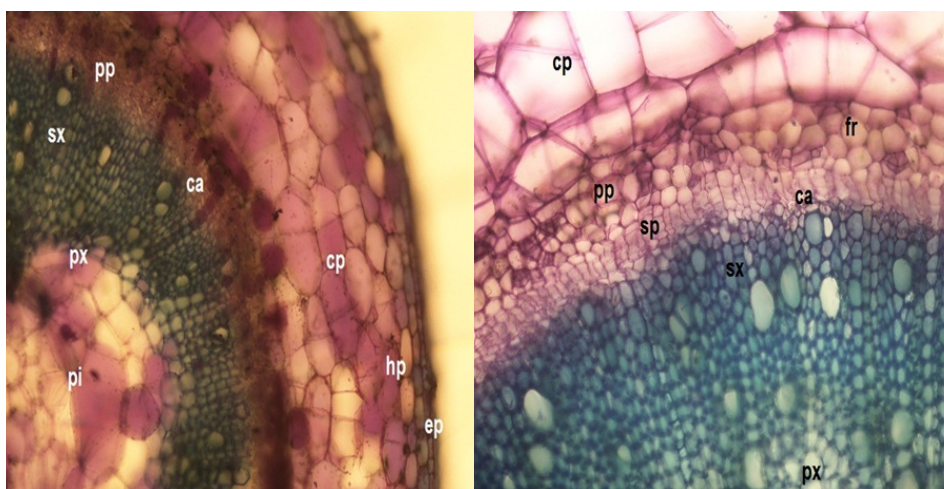


Figure 3. Cross section of uninfected (control) stem of sesame TS 3, TS 5 and SG 27: epidermis (ep), hypodermis (hp), cortical parenchyma (cp), primary phloem (pp), secondary phloem (sp), cambium (ca), secondary xylem (sx), primary xylem (px) and pith (pi).

due to *Alternaria* spp. in tobacco [23], pomegranate [45], soybeans [46], California pistachios [47], cowpea [26] and in citrus fruit [48].

3.4. Histopathology of Infected Stem of Sesame (*Sesamum indicum* L.)

Fungus in infected tissues was stained as dark bluish black with toluidine blue O staining. Control did not show any such structure treated with same stain. In the present study, artificial inoculation of *Alternaria alternata* was done by using spray method. When the symptoms became visible in three varieties TS 3, TS 5 and SG 7 of sesame seedling, their tissues were studied after 10, 15, 20, 25 and 30 days after inoculation to understand the mechanism of fungal infection and penetration in infected sesame plant as well as to determine the histological changes in sesame cells infected with *Alternaria alternata*. 10 days after infection when the symptoms became visible by naked eye as a minute brown spot, microscopic examination of their cross section (CS) showed the presence of fungus in epidermis, hypodermis and in cortical parenchyma cells (**Figure 4**). Previous studies shows that this fungus enters in *Minneola tangelo* through epidermis and penetrate in to the parenchyma tissue [25]. Another study on *Withania somnifera* by [49] revealed the presence of hyphal mat of *Alternaria alternata* on beneath the epidermis, from there; it spread in to the all tissue of plant. 15 days after infection when the spot became larger in size, the CS of three varieties of sesame seedling showed that the fungus entered in vascular bundle from cortical parenchyma tissue (**Figure 5**). Fungus moves through the intercellular spaces of cortex and enter in vascular bundle (phloem and xylem). 20 days after infection, CS of three varieties of sesame seedlings showed that the fungus was present in epidermis, hypodermis, cortical parenchyma, and phloem and xylem tissue (**Figure 6**). Fungus completely overlapped the vascular tissue. Due to excessive replication of fungus with in the plant it completely covers the vascular tissue. These results are very much similar with [50], he reported the shrinkage of vascular bundle with the decrease in the diameter of vessels due to the infection of *A. alternata* on mango bark. 25 days after infection excessive growth of fungus was present in epidermis, hypodermis, cortical parenchyma, vascular bundle and pith. Fungus completely overlapped all the tissues of sesame stem (**Figure 7**). 30 days after infection sesame seedlings showed a complete disorganization of tissue (**Figure 8**). Fungus completely overlapped all the tissue of plant including epidermis, hypodermis, cortical parenchyma, vascular bundle and pith. When fungus growth was most abundant in every tissue it resulted in breakdown of tissue. Previous studies showed that due to fungus host interaction complete disorganization of cells of host plant was recorded without the differentiation of any tissue [27] [28].

4. Conclusions

Present study conducted to determine histopathology of three varieties of sesame TS 3, TS 5 and SG 27 infected with *Alternaria alternata*.

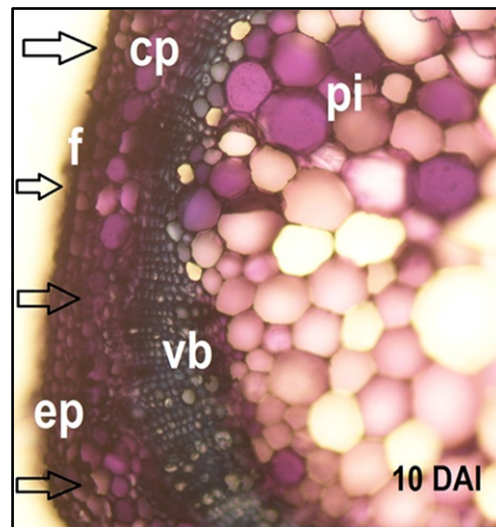


Figure 4. Histopathology of *Alternaria* leaf spot on sesame stem through artificial inoculation of plants; 10 days after infection fungus is visible in epidermis and cortical parenchyma tissue. Fungus (f), epidermis (ep), cortical parenchyma (cp), vascular bundle (vb), pith (pi).

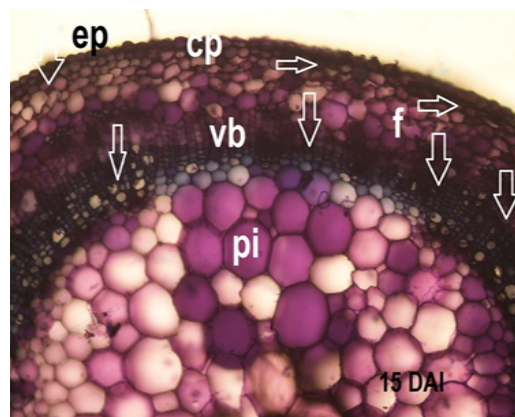


Figure 5. Histopathology of *Alternaria* leaf spot on sesame stems through artificial inoculation of plants; 15 days after infection, fungus moves from cortical parenchyma to vascular bundles. Arrows showing the movement of fungus in vascular bundle. Fungus (f), epidermis (ep), cortical parenchyma (cp), vascular bundle (vb), pith (pi).

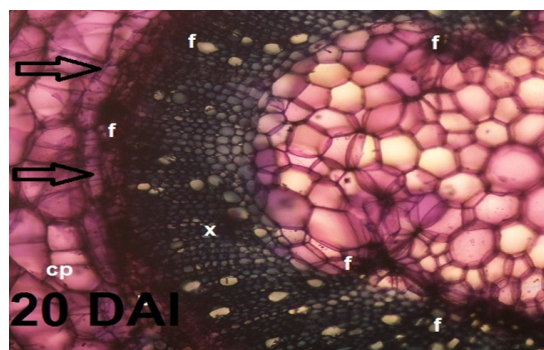


Figure 6. Histopathology of *Alternaria* leaf spot on sesame stem through artificial inoculation of plants; 20 days after infection, fungus completely overlap the vascular bundle. Fungus (f), cortical parenchyma (cp), xylem (x).

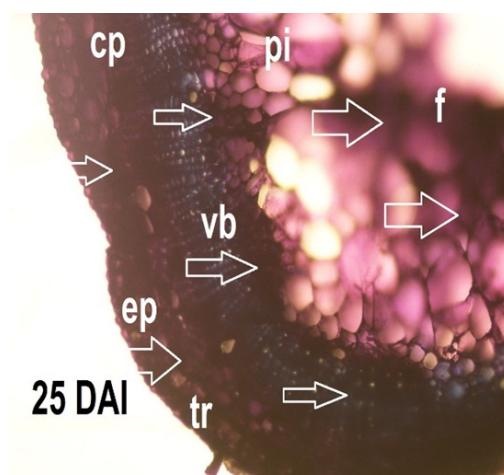


Figure 7. Histopathology of *Alternaria* leaf spot on sesame stem through artificial inoculation of plants; 25 days after infection, fungus is visible in every layer of stem. Fungus (f), trichomes (tr), epidermis (ep), cortical parenchyma (cp), vascular bundle (vb), pith (pi).

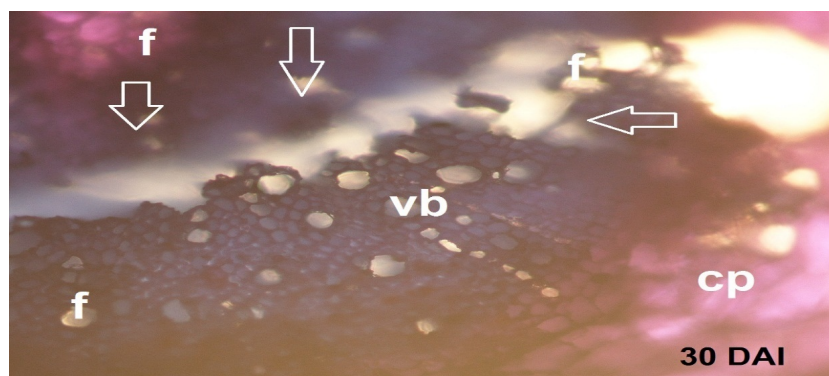


Figure 8. Histopathology of *Alternaria* leaf spot on sesame stems through artificial inoculation of plants; 30 days after infection Destruction of host tissue takes place. Fungus (f) cortical parenchyma (cp), vascular bundle (vb).

Light microscopic examination of cross sections of three varieties TS 3, TS 5 and SG 27 of sesame stem showed that fungus (which is stained as a dark bluish black with toluidine blue O) was not a limited pathogen, it travelled in every layer of stem.

Initially fungus was present in epidermis, hypodermis and cortical parenchyma. From the intercellular spaces of cortical parenchyma, it spread in to xylem, phloem and pith as the disease progress. Later on it completely overlapped the cells of sesame stem and disorganization of cells took place. sesame stem were broken down due to the excessive growth and replication of fungus.

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