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HISTOPATHOLOGY OF LEAF SPOT OF SESAME (*SESAMUM INDICUM* L.) CAUSED BY *PSEUDOMONAS SYRINGAE* PV. *SESAMI*

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ABSTRACT

Pseudomonas syringae pv. *sesami*, the causal agent of bacterial leaf spot of sesame is responsible for sesame production constraints during monsoon season in Pakistan. In the present study, mode of infection of causal bacterium was conducted in susceptible genotype to elucidate the process of infection within host tissues. Bacterium was identified as dark blue masses in infected tissues using toluidine blue O. Results showed that pathogen colonized substomatal and intercellular spaces of the spongy parenchyma cells when initial water soaking symptoms developed at 2-3 days after inoculation. Upon water soaking progressed, disruption of mesophyll cells occurred, mesophyll tissues were surrounded by bacterium followed by thinning and disruption of the cell walls. Later, when bacterial cells increased in space previously occupied by mesophyll cells, there were empty spaces without any differentiation of tissues. Bacterium did not occur in vascular bundles (tracheary elements) of leaf and stem, but some phloem tissues of stem sections were found infected. It was concluded that damage chloroplast was might be due to chlorosis or necrosis inducing toxins and toxins played a crucial role in pathogenesis of *Pseudomonas syringae* pv. *sesami* in sesame plant.

Key words: Pathogenesis, leaf spot, sesame, light microscopy.

INTRODUCTION

Sesame (*Sesamum indicum* L.) locally called as til is an important conventional oilseed crop of Pakistan. Pakistan ranks 14th among major sesame producing countries in the world (http://www. thefinancialdaily. com/2009). Pakistan is facing a chronic shortage in edible oil and the situation is getting serious with alarmingly explosion of population. Its indigenous production is below the utilization level and there exists wide gap between production and utilization. Sesame crop is subjected to various abiotic and biotic stresses in all stages of growth. Among biotic stresses, the prominent bacterial pathogen is bacterial leaf spot caused by *Pseudomonas syringae* pv. *sesami* (*Psse*) (Akhtar, 1986). The pathogen is responsible for sesame production constraints during monsoon season in Pakistan (Ahmad, 2004). Despite the shortage of edible oil, no profound efforts have been made on this host pathogen interaction.

Bacterial genera infect plants through stomata and wounds and multiplying in the apoplast of host (Romantschua and Bamford, 1986; Beattie and Lindow, 1996; Boureau *et al*., 2002) and produce virulence factors which contribute to the formation of symptoms. *P. syringae* reported to induce wide variety of symptoms on plants, including blights, leaf spots and galls (Bender, 1999). Many Pathovars including *P. syringae* pv. *syringae* (*Psse*), *P. syringae* pv. *tomato* (*Pst*), *P. syringae* pv. *phaseolicola* (*Psph*) have been widely used as model organisms to study bacterial pathogens in plants (Hirano and Upper, 2000). The infection of host plants by *P. syringae* involved entry through natural openings, bacterial multiplication in apoplast, water and nutrient deficiency (Bender and Scholz-Schroeder, 2004). *P. syringae* pathovars produce a large number of protein and non-protein virulence factors that have direct or indirect affect in plant cells or protect the plants from defense responses (Bender, 1999).

Many species of the genus *Pseudomonas* produce various phytotoxic compounds (Bender and Scholz-Schroeder, 2004), which on susceptible plants induce many symptoms including chlorotic and necrotic. Phytotoxins inhibit enzymes surrounding the host cells and suppress some host defense and provide mechanism for movement and multiplication of the pathogen in the host (Brooks *et al*., 2004; 2005). Coronatine (COR) cause chlorosis symptoms and act as virulence factor in some pathovars of *P. syringae* (Couch *et al.*, 2004). COR contributes to bacterial multiplication and lesion formation in numerous host plants, including *ryegrass, soybeans, tomato* and *Arabidopsis thaliana* (Brooks *et al*., 2004, 2005; Budde and Ullrich, 2000).

Previous studies on this disease do not indicate histopathology of sesame plants to confirm bacterial multiplication and translocation in leaf tissues. The main purpose of this study was to confirm pathway of infection of the causal bacterium in sesame plant. Currently this disease has become more devastating and is posing great threat to sesame production in Pakistan. Therefore, the

objective of this study was to monitor histological manifestations in inoculated leaves due to infection so that pathogenesis of *Psse* in sesame plants can be elaborated.

MATERIALS AND METHODS

Plants, Bacterial Isolate and Growth of Bacterium: Susceptible genotype GP-9 was used in the present study. Seeds of susceptible genotype were heated at 50 °C for I hr before planting. The trial was conducted in growth chamber under a period of 14 h light at 32°C and 10 h dark at 25°C per day. About 40 days after the sowing, plants at 6-8 leaf stage were used for inoculation. Virulent Isolate of *Psse* (Chakwal-1) was streaked on Nutrient Agar (NA) and incubated at 27°C under alternating light/dark conditions (14h/10h) for 24 hrs.

Artificially Inoculation of Sesame Plants: After incubation, bacterium was collected and suspended in sterile distilled water (SDW). The suspension was maintained to an optical density of 0.3 [10^8 CFU/ml estimated spetrophotometrically at a wavelength of 600 nm. Six-to eight week old intact plants were used for inoculation. Intact leaves were inoculated with bacterial suspensions using a 1 ml disposable hypodermic syringe at a dose of 10^8 CFU/ml on lower side of leaf. Inoculated plants were incubated at 30°C and approximately 80-85% relative humidity under a 14 h light and at 25°C under a 10 h dark period and incubated at room temperature for 2-4 hrs. Control plants were inoculated with Sterilized Distilled Water (SDW) only and were incubated using the same methods employed for inoculated plants. Infected plants were analyzed 0, 2, 4, 6 and 8 days after inoculation (DAI).

Tissue processing for Paraffin Embedding: Small pieces (1 × 3 mm) of leaf tissues were fixed in 4% formaldehyde in 50 mM Phosphate buffer with pH 7.2 at 4°C for 48 h. Then two washes in same buffer at pH 7.2, dehydrated in an ethanol series (30, 50, 70, 90 and 100 percent) each for 1 hr, except 70 percent overnight at 4°C. Clearing of tissue were done in ethanol: xylene series. Finally in pure xylene and placed overnight at room temperature. After overnight clearing, infiltration was done by adding paraffin chips until saturation and placed at 40°C. Paraffin was further added and placed overnight in oven at 60°C (Ruzin, 1999). The specimens were taken from oven and placed in paper boat and melted paraffin was poured over into paper boat. Embedding tissue in paraffin was fixed on block holders and stored at 4°C.

Microtome Sectioning, Slide processing and Staining: Position of blocks in microtome (Leica 2125RT) was like the edge of the razor blade just touched the block. The thickness of the ribbon was 11-15 µm, so that whole section was cut serially. Tissues with ribbon pieces were placed on the slide that was precoated with adhesive and dried (Jense, 1996). Slides were placed on the slide warmer at 40°C for short time. After drying, slides were passed through a series of chemical and stained with 0.5% toluidine blue-O containing 0.5% H_3BO_3 and 2% Na_2CO_3. The sections examined with Nikon 80 i eclipse microscope. Photographs were taken and saved in PC, DCE-2 and Nikon DS camera with bright field optics using 20, 40 and 100 X magnifications. Nikon Images were captured and saved. Tissues without infection were also used as control.

RESULTS AND DISCUSSION

Symptoms Produced by *Psse*: Light brown angular spots with dark purple margin appeared in the interveinal area of leaves. Defoliation and death of plant may occur in severe leaf and stem infections under natural condition (Fig. 1A). Sunken and shiny spots appeared on the capsules. Early capsule infection renders them black and seedless (Fig. 1 B).

P. syringae is the causal agent of a wide variety of hosts including apple, beets, beans, olives, peas, tobacco and tomato. This species also produced different kind of symptoms such as bacterial speck, galls, leaf spot and blight. Based on environmental conditions the species produced different kind of symptoms. It infected plants at all stages from vegetative to reproductive stage.

Histological observation of artificially *Psse* inoculated samples: Bacterium in infected tissues was identified as dark blue structures with toluidine blue O. Control did not show any such stained structure treating with same stain. Previous research showed that the *Psse* ingress host through stomata and trichomes (Firdous, 2009). In the present study it was found that after inoculation *Psse* scattered in the substomatal spaces of the spongy mesophyll (dark blue) at 2 DAI as shown by arrow and no obvious changes were observed in the surrounding cells (Fig. 2). As symptoms (water soaking) progressed, bacterial populations were also increased and substomatal as well as intercellular spaces were filled with the pathogen at 2-3 DAI (Fig. 2). Later, when chlorotic symptoms appeared bacterium had multiplied in the intercellular spaces and also damaged the palisade parenchyma at 3-4 DAI (Fig. 2). Previous research reported that inhibition of chlorophyll pigments was due to degradation of enzyme chlophyllase produced in *P. syringae* phytotoxins (Di Giorgio *et al.* (1996). So it was suggested in the present study that chlorosis producing symptoms were due to damaged chloroplast by action of any phytotoxin that induced chlorosis symptoms. Later, masses of bacterium were observed either filled an area occupied by palisade cells. The nearby palisade and spongy parenchyma cells were observed to become

surrounded by bacterium with subsequent thinning and disruption of cell walls. Degradation of cell walls also occurred in plants infected by *Pseudomonas spp i.e.* in *buckthorn*, cell walls were completely degraded at 6 DAI (Temash *et al*., 2002). In oleander plant this degradation was reported early after inoculation (Wilson *et al*., 1999).

Fig. 1. Naturally produced symptoms of *Psse* on leaves A, and pods of sesame B.

At relatively later stages of the infection when chlorosis symptoms disappeared and blackish necrotic spots became elongated at 5-6 DAI, it was shown that the space previously occupied by the mesophyll was empty or having strands of unidentified material, as a results cell destruction, and divided into smaller spaces that were either empty or filled with masses of bacteria (Fig. 3). At the end the necrotic tissues eventually become dehydrated, matted together without bacterium being visible. In the remaining period at 6-7 DAI, enlargement of necrotic tissue was observed with no profound histological changes (date not shown). These findings are different from those of (Bashan *et al.*, 1986) who studied necrotic symptoms developed 100 h after inoculation. Histopathology of stem samples removed at, below, or above the nodes was also investigated at 7-9 DAI. Bacterium did not occur in stem sections cut near the petiole leaf junction (Data not shown). Occasionally the leaf spots symptoms were appeared on veins under natural conditions, so it is thought that the pathogen *Psse*

might caused infection in phloem of vascular elements. However, it was found that *Psse* degraded the cell wall of host tissues such as cortical and pith cell (data not shown).

Present result revealed that rupturing of cell wall was might be due to enzymatic degrading activities by the bacterium. Moreover, it was not found that the bacterium established in the vascular tissues or move into the petiole or stem via the vascular system, but some of the phloem tissues were observed to be infected. The mechanism (s) that prevented spread of the pathogen to the stem tissues was not determined. Thus it had been cleared that *Psse* is not xylem limiting pathogen and apparently did not spread systemically from leaf to stem and hence to other parts of the plant. Another possible reason for the efficient pathogenesis of the leaf spot pathogen is that it produced any secondary metabolite. In fact, many of the members of *Pseudomonas* species are thought to cause diseases by the elaboration of specific chemical compounds, including toxins. Some of these, cause symptoms similar to leaf spot of sesame such as spot, blight and wilt (Bender, 1999).

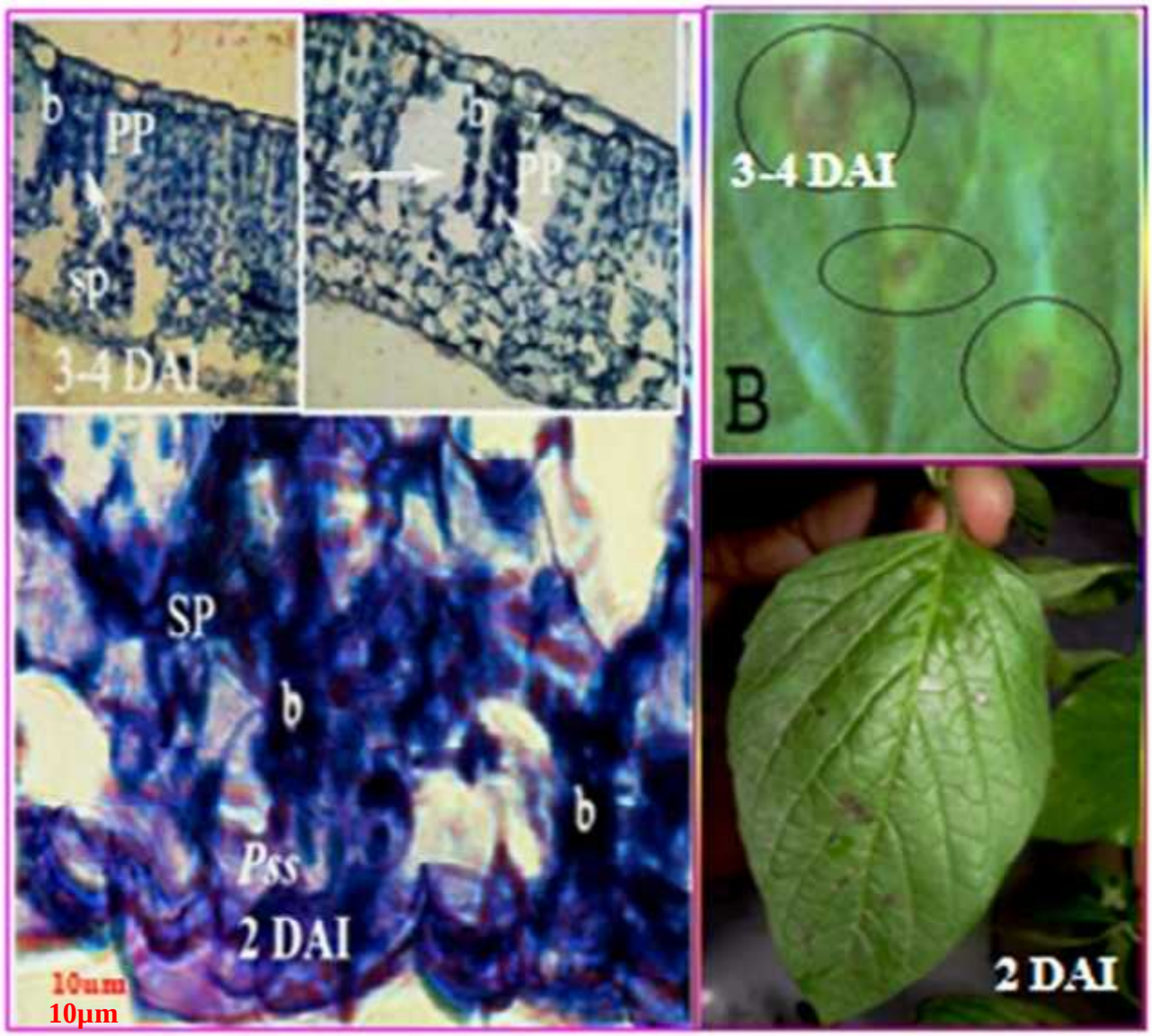

Fig. 2. Histopathology of bacterial leaf spot on sesame leaves through artificial inoculation of plants 2-4 DAI. T.S. of leaf section, where bacteria were found in intercellular spaces of spongy parenchyma (SP) as well as some palisade parenchyma (PP) 2 DAI. Dark blue and ruptured mesophyll tissues on appearance of chlorosis symptoms were appeared 3-4 DAI X100 (X100). Sections also showed damaged mesophyll parenchyma as well as thining and disruption of cell walls X20 (X100). Bar = 10µm.

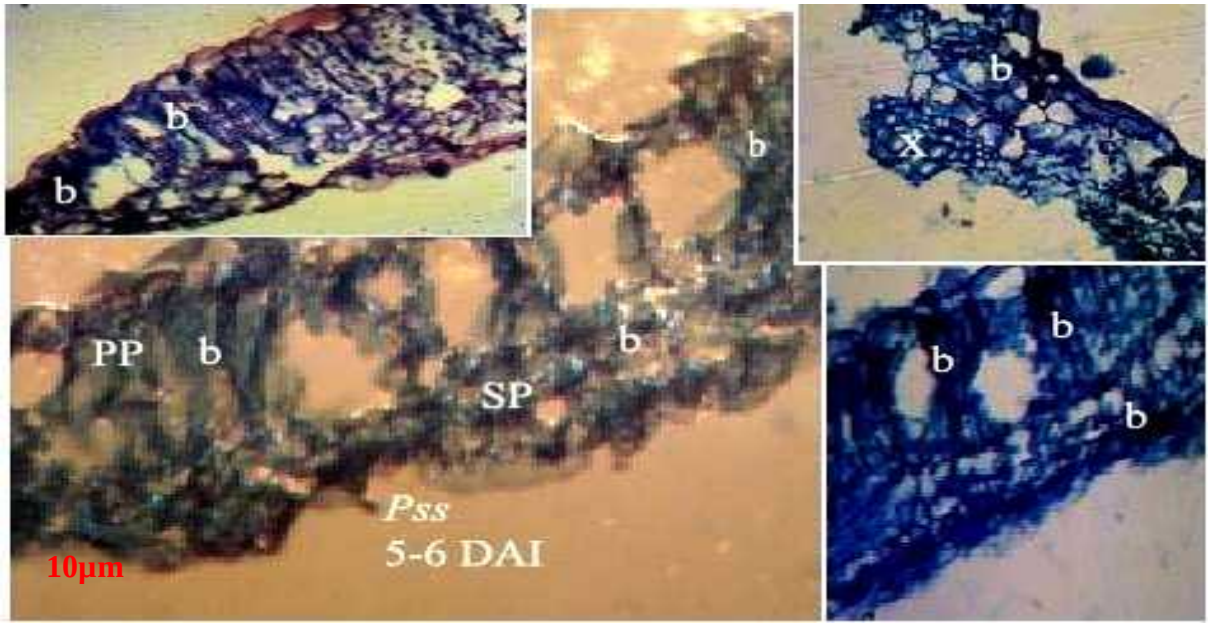

Fig. 3. Histopathology of bacterial leaf spot on sesame leaves through artificial inoculation of plants 5-6 DAI. T.S. of leaf section, where complete destruction of cells without any differentiation of tissues appeared X 20 (X100). Bar = 10µm.

Conclusions: *P. syringae pv. sesami* is not xylem limited pathogen and did not systemically transfer from inoculated petiole to leaf but infect some phloem tissues. It was concluded that leaf spots symptoms were appeared due to infected mesophyll tissues. In the present study it was found that the mesophyll cells were disintegrated and lysigenous cavities were filled with the masses of bacterium at the end of chlorosis. Moreover damaged chloroplast membrane might be due to the action of chlorosis producing toxins. Degradation of chlorophyll in sesame plant cells caused chlorosis, so chlorosis producing toxin can contribute significantly to pathogen virulence, presumably by inhibiting photosynthesis. Based on present finding future efforts can be targeted towards the actual mechanism involved in pathogenesis of *Pseudomonas syringae* pv. *sesam*i in sesame.

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