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PATHOGENESIS OF *PSEUDOMONAS SYRINGAE* PV. *SESAMI* ASSOCIATED WITH SESAME (*SESAMUM INDICUM* L.) BACTERIAL LEAF SPOT

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Abstract

Sesame bacterial leaf spot caused by *Pseudomonas syringae* pv. *sesami* was observed to cause severe symptoms on sesame crop during the monsoon season in barani areas of Pakistan. Histological study was conducted to elucidate the mode of infection of the causal bacterium by incubating leaves or discs in bacterial suspension and by infiltration methods. Infected leaves were cleared in lactophenol: ethanol solution. Periodic histology of cleared inoculated discs from infected leaves showed that bacterial cells resided and multiplied in depressions and around trichome bases for 24 h before penetration through stomata and trichome basal cells. When pin-point-sized spots first appeared at 3 days after inoculation, chloroplast membrane was damaged at this stage. With the early appearance of small necrotic spot symptoms at 4 days after inoculation, the bacterium was detected in parenchymatous tissues and apparently moved from parenchymatous tissues to transverse vascular systems. This systematic infection process in sesame indicated the involvement of any secondary metabolite/s. With the appearance of typical leaf spot symptoms comprised of brown lesions 5 to 6 days after inoculation, no further histopathological changes were observed using infected cleared disc technique. Bacterial infection was confirmed in tolerant and susceptible genotypes by both methods and it was shown that mode of infection in two genotypes was same but infection was delayed in tolerant genotype.

Introduction

Sesame (*Sesamum indicum* L.) is an important oilseed crop of Pakistan. Bacterial leaf spot caused by *Pseudomonas syringae* pv. *sesami* (*Pss*), is a serious disease on sesame (*Sesamum indicum* L.) in barani areas of Pakistan. The disease caused by *Pss* was recorded from Pakistan by Mirza & Akhtar (1987). They identified the symptoms of bacterial leaf spot as light brown angular spots with dark purple margin appearing in the leaf veins. Defoliation and death of plant may occur in severe leaf and stem infections. Severity of bacterial blight is related to soil moisture (30-40%) and relative humidity (75-85%). A study on disease assessment was also monitored that showed 75.6% contribution by pathogen in the development of disease severity in sesame (Bashir *et al.*, 2007).

Pseudomonas and other Gram-ve bacterial genera infect plants through natural openings such as stomata and wounds, multiplying in the intercellular space outside of the plant cell wall (Romantschuk & Bamford, 1986; Beattie & Lindow, 1994; Wilson *et al*., 1999; Boureau *et al*., 2002) and produce virulence factors which contribute to the formation of symptoms. Bashan *et al.,* (1981) studied infection and symptom development in tomato leaves inoculated with *P. syringae* pv. *tomoto* and found that stomata and bases of trichomes acted as primary infection sites.

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P. syringae is a host-specific pathogen with more than 50 pathovars and is capable of infecting aerial parts of the host plant. It is reported to induce wide variety of symptoms on plants, including blights, leaf spots and galls (Bender *et al*., 1999). Many pathovars including *P. syringae* pv. *syringae* (*Pss*), *P. syringae* pv. *tomato* (*Pst*), *P. syringae* pv. *phaseolicola* (*Psph*) have been widely used as model organisms to study bacterial pathogens in plants (Hirano & Upper, 2000). The infection of host plants by *P. syringae* is a complex process involving epiphytic colonization of plant surfaces, entry into host tissue, then bacterial colonization of the intercellular space between plant cells, water and nutrient acquisition (Alfano & Collmer, 1999).

P. syringae pathovars produce a large number of protein and non-protein virulence factors that are directly or indirectly toxic to plant cells or protect them from plant defenses. They have been considered to be virulence factors, since their production results in increased disease severity (Bender *et al*., 1999). Several species of the genus *Pseudomonas* produce various phytotoxic compounds (Bender *et al*., 1999) which on susceptible plants cause a range of symptoms including leaf spots, chlorosis, necrosis, blight and galls. Many strains of *P. syringae* pv. *syringae* are known to produce cyclic lipodepsinonapeptides (LDPs) as secondary metabolites, which are thought to be plant virulence factors and antifungal agents. They affect plant plasmalemma activity, protoplast permeability, vacuoles, plasma membrane vesicles, the chloroplast membrane and mitochondria (Di Giorgio *et al*., 1996). Extra cellular polysaccharides like alginate may protect the bacterium from the oxidative stress and promote disease colonization (Keith *et al*., 2003). 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 (Finaly & Falkow, 1997). Tabtoxin causes chlorosis (Turner, 1986) by accumulating ammonia in sufficient concentrations around the infected cells. Phaseolotoxin produced by *Psph* besides causing chlorosis in bean, is necessary for systemic invasion of the plant by the pathogen (Patil *et al*., 1974).

Previous studies on this important disease from Pakistan do not indicate histopathology of sesame plants to confirm bacterial multiplication and translocation in leaf tissues. 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 leaf discs so that pathogenesis of *Pss* in sesame can be elaborated.

Materials and Methods

Plants: Tolerant genotype (Gp) 34 and Susceptible Gp-9 were obtained from Oil Seed Programme, NARC, Islamabad. Plants were grown in pots containing sterilized potting mixture consisting of sand, farmyard manure and clay (1:1:2). The trial was conducted in growth chamber under a 14 h light at 32ºC and 10 h of darkness at 28ºC per day. About 40 days after the sowing, plants at 6-8 leaf stage were used for inoculation.

Bacterial Isolates and growth of bacteria: Isolates of *Pss* were streaked on Nutrient Agar (NA) and incubated at 27°C for 48 hrs.

Fig. 1. Sesame pathogenicity assay. A. Healthy plant B. Infected plants incubated in growth chamber.

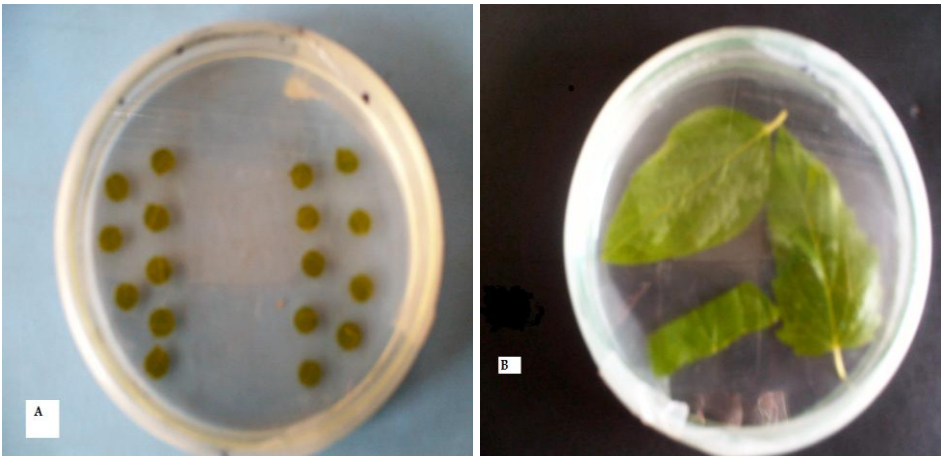

Fig. 2. The discs A, and detached leaves B, were on a 1.5% (w/v) water agar surface with the detached leaves petiole embedded into the agar after immersion in a bacterial suspension.

Sesame pathogenicity assay: After incubation, bacterium was collected using a plastic spatula and suspended in sterile distilled water (SDW). The suspension was adjusted to an optical density of 0.3 [10^8 cfu (colony-forming units)/ml] estimated spetrophotometrically (Thermo spectronic Genesys 20) at a wavelength of 600 nm. Six-to eight week old intact or detached sesame leaves were used for pathogenicity assay (Fig. 1A). Intact leaves were infiltrated with bacterial suspension using a 1 ml disposable hypodermic syringe at a dose of 10^8 CFU/ml. 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 (Fig. 1B). Control plants were inoculated with SDW and were incubated using the same methods employed for inoculated plants. Infected plants were analyzed 0, 2, 4, 6 and 8 days after inoculation that showed early, middle and late stages of symptom

development. Samples of very early symptoms were also harvested 3 days after inoculation. Leaf samples from control plants were also collected at the same intervals for histological study.

In another experiment, whole leaves or 4mm diameter leaf discs were cut from a central portion of leaf from each side of the central vein with a cork borer and immersed in a bacterial suspension adjusted as described above and incubated at room temperature for 2-4 hrs. The detached leaves and discs were subsequently transferred on a 1.5% (w/v) water agar surface with the leaf petiole embedded into the agar (Fig. 2 A&B) (Plotnikova *et al*., 2000).

Light microscopy: For trypan blue staining, infected leaves were first cleared in a solution of lactophenol: ethanol (1:2) for a period of 12 to 24 h with one change of solution. Lactophenol was prepared by adding equal volume of phenol, lactic acid, glycerol and water. Cleared leaves were moved to a fresh lactophenol solution containing 1mg/ml of trypan blue for 10 min before mounting on slides and examined with bright field and photographs were taken with a DCE-2 camera.

Results and Discussions

Symptoms: 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 (Fig. 3A). Leaf spot symptoms were reproduced on sesame seedling via artificial inoculation. In the very early stages of disease development, pin-head sized necrotic spots were formed on the surface of the leaf blade (Fig. 3B). Such dots were seen more clearly by holding the leaves up against a light source. They enlarged and turned brown and produced spot symptoms with purple margin (Fig. 3C).

In the late stage of infection, the lesions elongated very rapidly toward the leaf tips. In severe case, the lesions spread extensively, turned blackish and blight symptoms developed. Such leaves eventually became completely dry (Fig. 3D&E). Hayward & Waterson (1998) reported that *P. syringae* pv. *sesami* produced blackish-brown spots, which extended along whole length of stem. Veins delimit angular spots on leaves.

Histological observation of artificially inoculated samples: Previously published work from Pakistan showed that Gp-9 is highly susceptible to infection by *Pss*, whereas Gp-34 is tolerant. No single genotype was proved resistant against the *Pss* to date in Pakistan. Present study showed that *Pss* entered through trichome within 24 hrs when leaf discs were inoculated at room temperature for 2-4 hrs in a cell suspension and then incubated on the surface of 1.5% (w/v) water agar in closed Petri dishes for 6 days. Inoculated discs showed damaged trichome and blue stained stoma by using trypan blue both from tolerant and susceptible plants (Fig. 4A) as compared to control which did not show any blue stained stoma structure (Fig. 4B) and damaged trichomes (data not shown). These findings are in agreement with those of Schneider & Grogan (1977) and Bashan *et al*.**,** (1981) who reported similar observations and suggested that trichomes serve as a major habitat for survival of resident inoculum and as target sites for infection by the pathogen, especially under dry conditions. Bashan *et al*., (1981) observed microcolonies of the pathogen in substomatal chambers. Same mode of infection also appears to be common among phytopathogenic coryneform bacteria, such as *Clavibacter* spp., and the red stripe bacterium of rice (Nelson & Dickey, 1970).

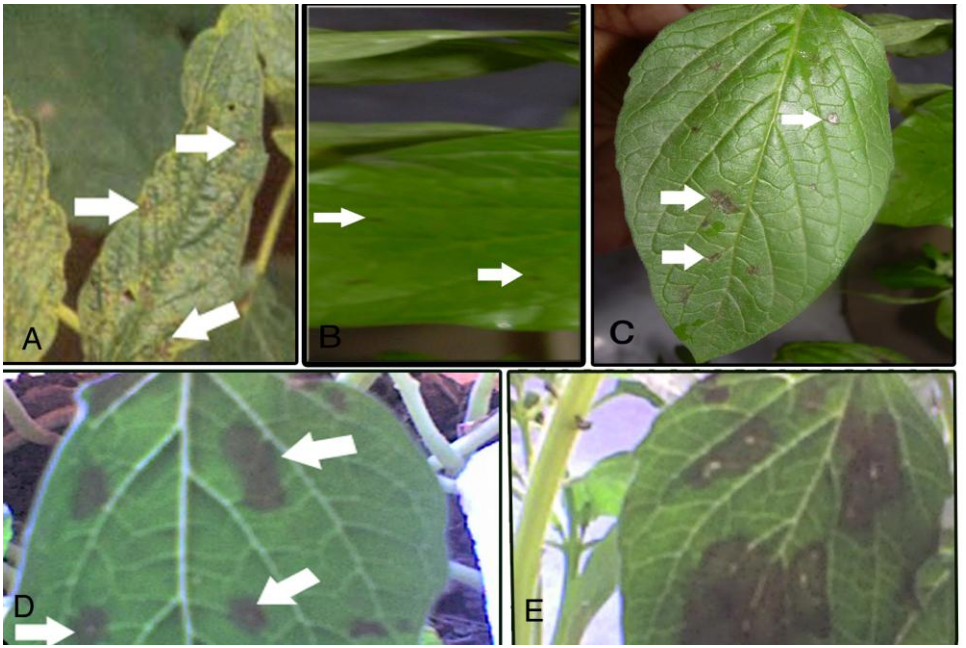

Fig. 3. Symptoms of leaf spot of sesame. A. Typical symptoms of leaf spot composed of light brown angular spots with dark purple margin B. Early symptoms of leaf spot, Pin-point-sized dots on sesame leave (arrows). Dots expand and become small brown and purple spots as the disease progresses. C Lesion enlarged with spots surrounded by purple margins. D&E. In the late stage of infection, the lesions elongated very rapidly toward the leaf tips turned blackish and purplish symptoms developed.

After getting inside through stomata, bacteria entered the chloroplast membrane and damaged it, showed damaged structure after 3 days of inoculation in susceptible plants as shown by arrow (Fig. 4C) but delayed by 24 hrs in tolerant plants (data not shown). The present result is in accordance with Di Giorgio *et al.*, (1996) who showed that LDPs affect plant plasmalemma activity, protoplast permeability, vacuoles, plasma membrane vesicles and the chloroplast membrane and mitochondria. At relatively later stages of the infection the movement of bacterium from chloroplast membrane to leaf veins was clearly observed after 4 days of inoculation in susceptible plants (Fig. 4D) but again in tolerant the reaction was delayed by 24 hrs (data not shown). During the rest of the monitoring period from the 5th to 6th day, radial increase in necrotic tissue was observed with no additional distinctive histological changes. These findings are different from those of Bashan *et al.*, (1981) who reported necrosis to occur 100 h after inoculation.

We also followed systemic spread of pathogen infection at 30°C in intact plants after inoculation of plant leaves with hypodermic syringe at a dose of approximately 10^8 cfu/ml. By 24 h post-infection (hpi), the bacterium enters through trichomes and stomata. In a similar manner to the experiment described in the preceding paragraph, by 72 hpi the bacterium infected the chloroplast membrane and showed blue stained damage structure. By 96 hpi the bacterium spread along the major sesame leaf veins and resulted in death of the tissues by 120 hpi.

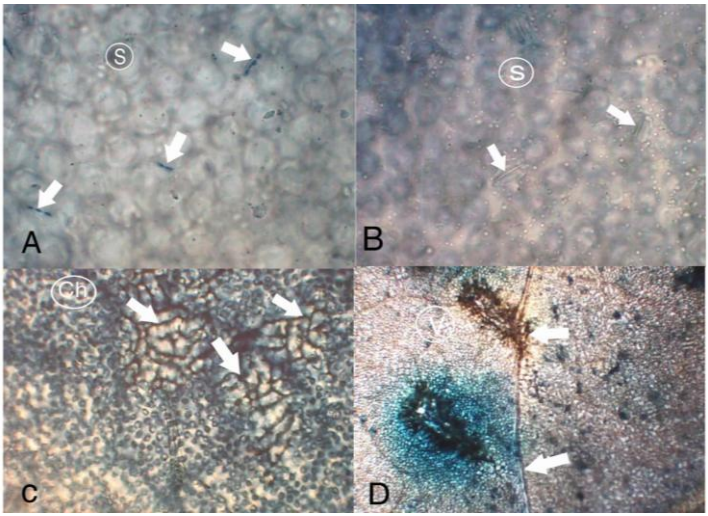

Fig. 4. Infection of leaf spot on sesame leaves (susceptible genotype) after artificial inoculation of plants. Infected discs were cleared and examined under light mcroscope. A. Bacterium entered through trichomes and stomatal cavities shown by arrow (Stain stoma) while B. showed no stained structure and C. At 3 day bacterium attack chloroplast membrane as shown by arrow. D. After 72 hpi bacterium moved from membrane to veins. (S. Stomata, Ch. Chloroplast, V. Vein).

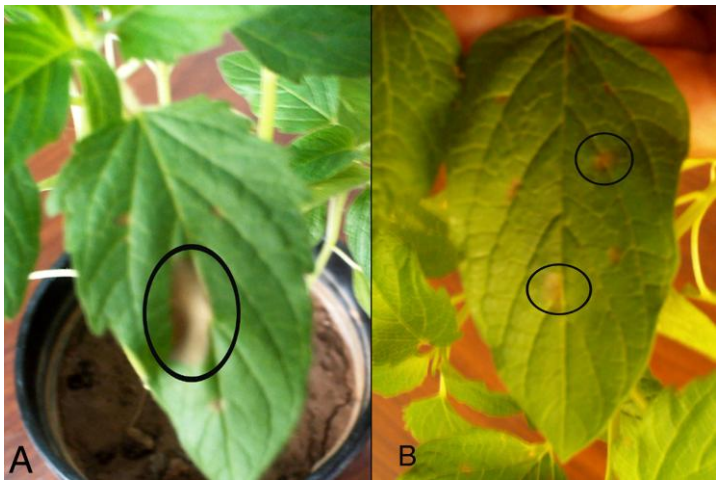

Fig. 5 A&B. Necrotic and toxicity symptoms showing on the leaflets of an excised sesame leaf inoculated with a culture filtrate of *Pseudomonas syringae* pv. *sesami.*

One possible reason for the efficient pathogenesis of the leaf spot pathogen is that it enters chloroplast membrane and vascular tissues of sesame leaves and produces a 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 (Bender *et al*., 1999). Some of these, cause symptoms similar to leaf spot of sesame such as spot, blight and wilt (Vidaver, 1982).

Bioassay tests on culture filtrates of the bacterial pathogen demonstrated the involvement of a translocatable necrosis producing toxin in the infected leaves (Fig. 5 A&B). Necrosis producing toxin (S) by *P. syringae* pv. *syringaeos* reported by Bender *et al.,* (1999) as lipodepsinonapeptide toxins, syringomycin and syringopeptin.

Acknowledgements

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