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Full Length Research Paper

Effects of *Pseudomonas syringae* Infection on the Stomatal Anatomy and Leaf Morphology in *Lycopersicon esculentum*

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ABSTRACT

In pathogenesis, pathogenic penetration is one of the basic steps in disease development. For foliar bacterial plant pathogens, natural surface openings, such as stomata, and wounds are important entry sites. These surface openings have been considered passive points of entry for plant pathogenic bacteria. *Lycopersicon esculentum* was inoculated with *Pseudomonas syringae* to induce a diseased condition. Subsequently leaf anatomical studies were carried out to observe the penetration ports of the bacterium. Rupture of guard cells, reduced in size of stomata and occurrence of small dark microscopic spots on the stomata are features which serve as an indication of pathogenic penetration. Also morphological growth parameters assessed revealed significant reduction in plant height, leaf length, leaf area and leaf weight in the test plant compared with the control. However, no significant differences were observed in terms of number of leaf and width of the leaf. The results suggest that disruption of stomatal anatomy by the bacterium will translates to low yield and accumulation of impurities such as carbondioxide from the atmosphere.

Keywords: *Pseudomonas syringae,* infection, stomatal anatomy, leaf morphology and *Lycopersicon esculentum*

INTRODUCTION

The phyllosphere of terrestrial plants provides one of the most important niches for microbial inhabitation (Lindow and Brandl, 2003). The phyllosphere comprises both the surface and interior of a leaf. The leaf interior is composed mostly of mesophyll cells, in which the bulk of photosynthate is made; some vascular tissues (veins); and a large, air-filled intercellular space (apoplast) between mesophyll cells. Because of its close proximity to mesophyll cells, the leaf apoplast is thought to contain abundant nutrients, whereas nutrients on the leaf surfaces are much more limited and spatially heterogeneous, mostly leaked out from the apoplast through natural surface openings and wounds. Strict epiphytes complete their life cycles on the surface of plants and generally do not (or are unable to) colonize the leaf apoplast (Beattie and Lindow, 1999).

In contrast, foliar pathogens, such as *Pseudomonas syringae*, can multiply aggressively in the apoplast as pathogenic endophytes, ultimately leading to disease production under favorable conditions (Hiranno and Upper, 1990). Entry into plant tissue is likely a critical first step in the establishment of foliar infection. To gain access to the intercellular spaces and internal leaf tissues, pathogens must cross the surface cuticle and epidermis. Many fungal

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plant pathogens have the ability to directly penetrate the epidermis using cuticle - and cell wall-degrading enzymes, mechanical force, or both (Mendgen, *et al.*, 1996). Bacteria, however, cannot directly penetrate the leaf epidermis and must enter leaf tissues by either natural openings or wounds (Agrios, 2005).

There are a number of natural surface openings such as stomata, hydathodes, necterthodes and lenticels, through which bacterial pathogens have been observed to enter the plant (Mehrotra, 2001). Among natural openings, stomata dominate in number in the aerial part of the plant and therefore represent one of the most important routes for the entry of foliar bacterial pathogens. In addition to natural openings, bacteria can also reach the plant interior through surface wounds caused by insects, mechanical or frost damage, hail, wind-blown sand, rain storm, or damage caused by lateral root formation, breakage of trichomes, or abscission of leaves (Mehrotra, 2001). The ability of coronatine (that is a virulence factor involved in suppressing stomatal defense by some bacterial pathogens) to promote opening of dark-closed stomata may provide a mechanism for bacterial entry into plant leaves at night when most stomata are closed, which may have significant epidemiological implications.

It has been widely assumed that natural surface openings and wounds are passive ports for bacterial entry, where bacteria lack active mechanisms for gaining entry, and plants similarly lack active mechanisms for preventing entry. However, recent evidence suggests that entry of bacteria into leaf tissue through stomata is more complex and dynamic than the simple act of swimming into the leaf through passive openings.

Because bacteria are necessary to reproducibly and uniformly induce disease in tomato leaves by the surface inoculation method (Chisholm, *et al.*, 2006). In nature, however, epiphytic populations of bacteria are expected to be highly variable, even on the same leaf, due to tremendous spatial heterogeneity of nutrient availability and/or leaf surface topology (Lindow and Brandl, 2003). The total epiphytic bacterial population on the leaf surface can be very high (Kinkel, *et al.*, 2000). Even when the total number of bacteria is low on a leaf, bacterial concentrations at specific sites can be high, especially in aggregates (Lindow and Brandl, 2003) which could contribute to the observed discrete infection sites or lesions on the same leaf in the field (in contrast to the uniform infection of entire leaves in the laboratory). It will be interesting to determine how the sizes and locations of these bacterial communities affect stomatal defense and whether aggregation of bacteria near stomata promotes stomatal movement by exposing stomata to high concentrations of compounds that promote opening or closure.

Meanwhile our intention is on foliar disease of *Lycopersicon esculentum* Mill caused by *Pseudomonas syringae pv tomato* Van Hall. Tomato is a savory, typically red, edible fruit. It originates from South America; it is widely grown, often in greenhouses in cooler climates. Tomato belongs to the Solanaceae (night shade) family. The plants typically grow to 1-3 metres (3-10ft) in height and have a weak stem that often sprawls over the ground and vines over other plants. It is a perennial plant in its native habitat, although often grown outdoors in the temperate climates as annual plants. The leaves are 10-25centimetres (4-10 in) long, odd pinnate, with 5-9 leaflets on petioles (Acquaah, 2002) each leaflet up to 8 centimetres (3 in)

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long, with a serrated margin; both the stem and leaves are densely glandular- hairy. The flowers, appearing on the apical meristem, have the anthers fused along the edges, forming a column surrounding the pistils. Flowers in domestic cultivars tend to be self-fertilizing. The flowers are 1-2cm (0.4-0.8 in) across, yellow, with five pointed lobes on the corolla; they are borne in a cyme of 3-12 together. Tomato fruit is classified as a berry. As a true fruit, it develops from the ovary of the plant after fertilization, its flesh comprising the pericarp walls. The fruit contains hollow spaces full of seeds and moisture, called locular cavities (Olorode, 1984). These vary, among cultivated species, according to type. Some smaller varieties have two cavities, globe-shaped varieties typically have three to five, beefsteak tomatoes have a great number of smaller cavities, while paste tomatoes have very few, very small cavities. The seeds need to come from a mature fruit, and be dried and fermented before germination. Tomato plants are vines, initially decumbent, typically growing six feet or more above the ground if supported, although erect bush varieties have been bred, generally three feet tall or shorter. Indeterminate types are tender perennials, dying annually in temperate climates (they are originally native to tropical highlands). Determinate types are annual in climates. Tomato is very important vegetable used for variety of purposes such as soup, medicine.

In this study, we elucidate the possibility of natural openings on leaf i.e. stomata serving as entry points for the foliar pathogen *Pseudomonas syringae pv tomato* Van Hall infection in *Lycopersicon esculentum* Mill, and subsequent effects on the stomata and other epidermal features.

MATERIALS AND METHODS

Study material

Tomato seeds were collected at National Horticulture Research Institute (NIHORT), Ibadan and air dried. Dried seeds were planted in 6 different plastic pots. The pots were divided into 2 groups consisting of 3 pots for experimental and 3 pots for control plants. Duration of experiments was 6 weeks.

Culturing of the pathogen

The slant of *P. syringae* was obtained from stock culture at the Microbiology Department, Faculty of Science, University of Ilorin, Ilorin, Nigeria. The slant was subcultured using nutrient agar media. Nutrient agar was poured into a Petri-dish; after it solidified it was streaked with the pathogenic organism, *P. syringae* and incubated for 24 hours. The growth of the bacterium was seen after 24 hours of incubation.

Washing of the bacterium into distilled water

Distilled water was collected from Chemistry Department, Faculty of Science, University of Ilorin, Ilorin, Nigeria. The bacterial growth was then washed into the distilled water in the conical flask, and placed on the shaker for proper mixing of the content. This procedure was carried out every other day.

Irrigation of the plants

Irrigation was carried out using Cooksey's method (1988) with modification. The distilled water containing bacterium, *P. syringae* was used to irrigate the experimental plant everyday in the morning and in the evening before symptoms begins to show. The control plants were also irrigated with ordinary water everyday.

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Pathogenecity Test

The pathogenecity test of *P. syringae* was carried out using the method adopted by Gavrilovic *et al.*, (2012) and Wokoma (2008) with modifications.

Measurement of morphological features

On weekly basis, plant height, leaf weight, and leaf number were determined. These measurements were subjected to Analysis of Variance (ANOVA) and Dunca Multiple Range Test (DMRT).

Frequency of stomatal complex types

Prepared slides of macerated cuticles from the leaves of the species were observed using 35 fields of view at x40 objectives as quadrats. The number of subsidiary cells per stoma were noted, and recorded to determine the frequency of the different stomatal complex types present in each specimen. Frequency of each complex type was expressed as percentage occurrence of such complex type based on all occurrences (Obiremi and Oladele, 2001). The terminologies for naming stomatal complex types followed those of Dilcher (1974).

Stomatal index

The *SI* was determined as follows: $SI = S/E + S \times 100$

Where S = numbers of stomatal per square millimeter

E = number of ordinary epidermal cells per square millimeter

Stomatal density

The stomatal density was determined as number of stomata per square millimeter.

Stomatal size and epidermal cell size

The mean stomatal size was determined as product of length and breadth of guard cells using eye piece micrometre. The size of the epidermal cell was also determined from the length and breadth.

RESULTS

Disease symptoms on leaf

On leaves, symptoms were localized as black specks, not more than 2mm in diameter, which were surrounded by a yellow halo. Speck lesions caused distortion of the leaf, as the infection restricted the expansion of leaf tissue. Lesions were concentrated near leaf edges, and leaf margin burn, and where infection was many lesions coalesce, and entire leaflets died. Severely infected seedlings became stunted. The characteristic black spot were sub circular and were observed on the adaxial surfaces extending to the abaxial surfaces of the leaf. The spots were also found irregularly on the leaf surface. The leaf size, leaf height, leaf weight and leaf number were reduced in diseased plants (Table 1; Figs. 3 and 4). Though there was an increase in height of the diseased plants but it was not as prominent as in the health ones.

Table 1: Morphological characters for control and experimental plants

Treatments	Plant height (cm)	Number of leaves	Length of leaf (cm)	Width of leaf (cm)	Area of leaf (cm^2)	Weight of leaf (g)
control	13.40a	3.30a	2.70a	3.68a	11.08 a	0.45a
Experimental	8.20b	3.10a	2.05b	2.22a	4.69b	0.17b

Means with same letters along the columns are not significantly different at $p<0.05$

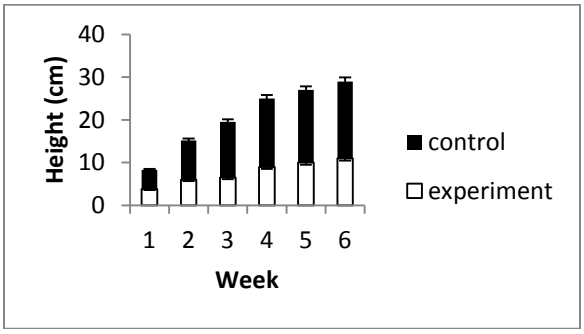

Figure 3: Plant height for both control and experimental plant.

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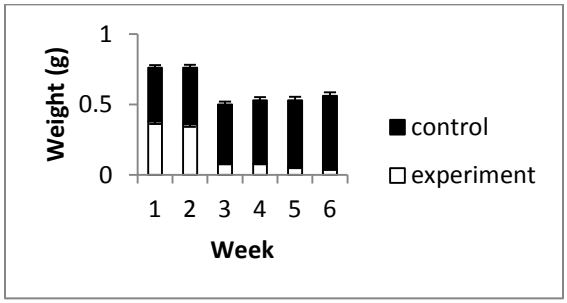

Figure 4: Weight of leaves for both control and experimental plant

Response of leaf epidermal features

Stomatal complex types present in tomato are paracytic and pericytic and occurred on both leaf surfaces (Table 2; Figs. 1 and 2). Stomatal features such as density, index and size revealed some variations in diseased and healthy plants. Stomatal density and index are lower in diseased plants than in healthy ones. Stomata were also relatively larger in healthy plants than in diseased ones. The epidermal cell was irregular on both the adaxial and abaxial surfaces with trichomes moderately distributed on both surfaces. The epidermal cell sizes for the experimental plant ranges from 40.0µm to 55.2µm on the adaxial surfaces with a mean size of 47.6 while on the abaxial surfaces ranges from 42.2µm to 59.0µm with a mean size of 50.6 (Table 2). Sparsely distributed trichomes were also found on both surfaces of the control and experimental plants.

Table 2: Stomatal characters and epidermal cells size in both control and experimental plants

Plant	Leaf surface	Stomatal complex type	Frequency (%)	Stomatal density (mm^2)	Stomatal index (%)	Stomatal size (µm)	Epidermal cell size (µm)
Control	Abaxial	Pericytic	26.38	161.18a	29.87a	457.14a	55.05a
		Paracytic	27.47				60.65a
	Adaxial	Pericytic	21.98	138.15b	29.16a	285.25b	50.24a
		Paracytic	24.18				52.44a
Experimental	Abaxial	Pericytic	23.81	75.65c	17.15b	122.61d	56.00a
		Paracytic	30.95				55.41a
	Adaxial	Pericytic	23.82	62.49d	15.82b	144.96c	51.10a
		Pericytic	21.43				50.28a

Means with same letters along the column are not significantly different at $p<0.05$

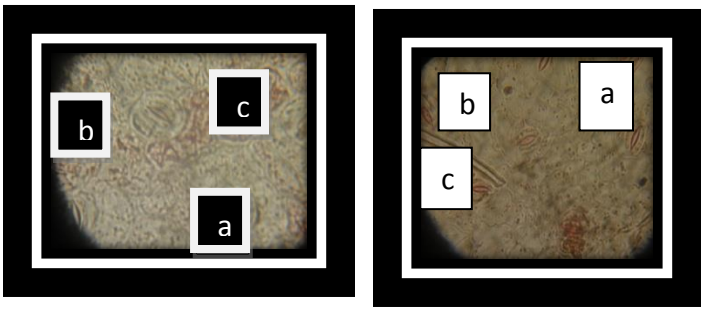

Figure 1: Adaxial (left) and abaxial (right) leaf surfaces of control plant showing paracytic (a) and pericytic (b) stomatal, and trichome (c)

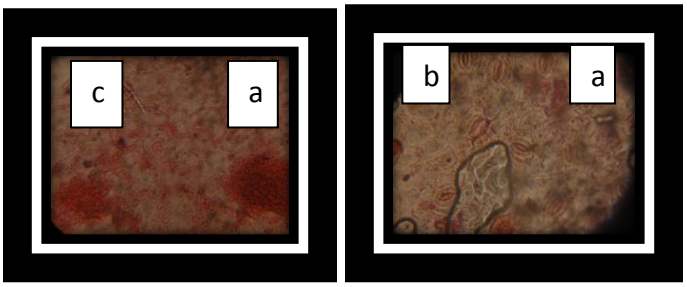

Figure 2: Abaxial (left) and adaxial (right) leaf surface of experimental plant showing paracytic (a) and pericytic (b) stomatal, trichome (c)

DISCUSSION

Significant difference was noticed in the height and weight of both control and experimental plants in the second week of planting. This is probably due to the fact that the experimental plants were infected at this period with the bacterium, *P. syringae*. The disease started manifesting in the third week as the plants showed dark necrotic spot surrounded by yellow haloes. Subsequently the weight and height of the plant reduced drastically as earlier reported by Beattie and Lindow (1995). The experimental plant showed shrinkage of the stomata as the bacteria produces some characteristics that regulate the opening and closing of the stomata (Marre and Fusicoccin, 1979),

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therefore the stomata became narrower; longer than wider. The virulence factor involved in suppressing stomatal defense in *P. syringae* is coronatine (Zheng *et.al.* 2012). Coronatine promotes opening of the stomata by the bacteria which produces it. These initial observations indicate that stomata are not merely passive ports for bacterial entry (Mellotte *et al, 2006*). Guard cells could perceive live bacteria including *P. syringae* and close a significant percentage of stomata, suggesting that plants have evolved mechanisms to reduce the entry of bacteria as an integral part of the plant immune system. Since all stomata are not closed in response to bacteria or pathogen-associated molecular pattern, the apparently non-responsive and/or dead open stomata would provide a route for bacterial entry at a basal level. In the case of the virulent plant pathogen *P. syringae pv tomato*, bacteria produce the diffusible virulence factor coronatine to re-open closed stomata, thereby increasing the number of sites for bacterial invasion (Hirano and Upper, 1990). The ability of phytopathogenic bacteria to induce stomatal closure within the first hour of contact with plant tissue suggests that guard cells can sense conserved bacterial molecules. Pathogen-associated molecular pattern are such molecules, and they are best known for their ability to stimulate innate immunity in plants.

Theoretical calculations by Hancock and Huisman (1981) suggest that there is a sufficient amount of nutrition in the apoplast to support high-density growth of bacteria without active transport of sugars from host cells. Numerous bacteria can survive and multiply as epiphytes on a plant surface without causing disease. Saprophytes complete their life cycles on the surface of plants; foliar pathogens eventually enter the plant and multiply in the apoplast as pathogenic endophytes as seen in the tomato plant (Marre and Fusicoccin, 1979). Bacterial penetration into host tissue is a key step for pathogenesis. Bacteria penetrate the plant epidermis and must rely on wound and natural openings unlike fungi that can exert mechanical forces along with aforementioned methods (Romantschuk, 1992). The diseased plants showed that the bacterium came in through the stomata opening. Observation under the microscope showed rupturing and dark microscopic spots on the guard cell surfaces. In this study, the only damaged epidermal cell is the stomata, which may further confirm the penetrability of the pathogen through the stomata. The density of stomata on the upper leaf (where bacteria are most likely to land) is generally and significantly lower than on the lower surface of the leaf as seen in Table 2. Lower stomatal density and index and small stomata compare to those observed in control plants may be responsive actions toward reducing surface areas for penetration of the pathogen especially on the adaxial leaf surface.

The limited entry sites would require a successful bacterial pathogen to adhere and move toward natural openings or wounds. Indeed, adherence and motility have been shown to be important for surface infection by *P. syringae* and other foliar pathogens (Romantschuk, 1992). Also movement toward natural openings and wounds would probably occur only if the leaf surface is wet, which may be one of the reasons why foliar disease outbreaks often follow periods of rain and high humidity (Hirano and Upper, 2000). Thus irrigation of treated plants with *P. syringae* in watery medium might have greater effect which led to virtual death of most of the experimental plants (Bunster *et al*, 1989).

The discovery of stomatal defense and counter-defense in tomato plants is

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likely to open up several new areas for research. Understanding the effects of varying environmental conditions on the effectiveness of stomatal defense would be a particularly important area to enhance our understanding of outbreaks of foliar bacterial disease in the field. Stomata are required to respond to a number of stimuli, including light, humidity, CO_2 concentration, microbes, and the circadian clock (recurring naturally on a twenty-four-hour cycle). How these inputs are prioritized by guard cells and the mechanisms of prioritization are unknown but are interesting and important questions for future study.

It is interesting to note that severe outbreaks of bacterial disease in crop plants are often associated with periods of heavy rain or high humidity. These conditions could create wounds and leaf surface wetness which would be favorable for bacterial movement. It is also possible that, under such environmental conditions, stomatal defense is partially compromised because high humidity promotes stomatal opening, therefore allowing more bacteria to enter the leaf tissue through stomata to promote infection.

Also, the surface structures (e.g. cuticle, trichomes and stomata) in different plant species may differ in sensitivity to surfactant, humidity, and/or mechanical pressure. It would be important to determine how stomata in different plant species are affected by surfactants, humidity, and/or pressure spray in early stages of infection. In the absence of extensive wounding, foliar bacteria may have to invade leaves primarily during the day. Clearly much is to be learned and hopefully future research will provide answers to some of these important questions and contribute to advances and discoveries in the study of bacterial pathogenesis, guard cell signaling, and microbial ecology.

In conclusion, any factor that affects the performance of stomata must affect such important physiological processes like photosynthesis and transpiration, and humidification of the atmosphere. This in turn will lead to low yield and accumulation of impurities such as carbondioxide from the atmosphere.

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