ENVIRONMENTAL REGULATION OF STOMATE-BASED DEFENSE AGAINST BACTERIAL INFECTION IN ARABIDOPSIS

by

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ABSTRACT

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Stomata are natural openings in the plant epidermis responsible for gas (O2 and CO2) exchange between plant interior and environment. They are formed by a pair of guard cells, which are able to close the stomatal pore in response to a number of external factors including light intensity, carbon dioxide concentration, and relative humidity. The stomatal pore is also the main route for pathogen entry into leaves, a crucial step for disease development. Recent studies have unveiled that closure of the pore is effective in preventing bacterial disease in Arabidopsis plants and the successful plant pathogen *Pseudomonas syringae* pv. tomato (*Pst*) DC3000 is able to re-open stomata by producing the phytotoxin coronatine. A major unanswered question is: “how do stomata respond to combined effect of biotic and abiotic stresses?” I found that coronatine can re-open dark-closed stomata as early as four hours post-incubation with purified coronatine or the coronatine producing *Pst* DC3000. Same trend did not hold for the coronatine deficient mutants, *Pst* DC3118 and *Pst* DB29. I also have

evidence that high relative humidity (RH; 95 ± 5%) reduces bacterium-triggered stomatal closure. The same effect was not observed under low RH (60 ± 5%). Taken together, these results suggest that guard cells prioritize their response when exposed to multiple stimuli. Understanding this process should help elucidating the effectiveness of stomatal-based defense in nature where plant experiences constant influx of external stimuli and also implementing additional measures to control disease outbreaks in the field.

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CHAPTER 1

GENERAL INTODUCTION

Arabidopsis thaliana is a small flowering plant which has been long used as a model organism due to its small genome, short life cycle, and tractability for genetic manipulation. In fact, its genome was the first to be sequenced among plants (The Arabidopsis Genome Initiative, 2000). Arabidopsis is a member of the mustard (Brassicaceae) family, which includes cultivated species such as cabbage and radish. Arabidopsis is not of major agronomic significance, but it offers important advantages for basic research in genetics and molecular biology. In addition, Arabidopsis has been established as a model plant to study plant-pathogen interactions (Rensink *et al*. 2004).

The plant pathogenic bacterium *Pseudomonas syringae* is also a model organism to study plant-pathogen interactions. It is a rod shaped gram negative bacterium with polar flagella. It has been long known to infect a variety of plants and it exists as over 50 pathovars depending on the host range. *P. syringae* pv. *tomato* DC3000 (*Pst* DC3000) is pathogenic on tomato (Cuppels 1986), edible *Brassica* spp., (collard, turnip) (Elizabeth & Bender 2007, Zhao *et al.* 2000) and Arabidopsis (Whalen *et al.* 1991), and is extensively used for studying plant-microbe interactions (Buell *et al.* 2003). Under unfavorable conditions in nature, it can survive as epiphyte in the phyllosphere for an extended period of time; however this bacterium needs to penetrate plant tissue to be fully pathogenic. How the bacterium transitions from epiphytic life style to an endophytic parasite is not well understood but this transition has been found to be associated with high humidity and rainfall (Hirano & Upper, 2000, Melotto *et al*. 2008). Unlike fungi, bacteria are incapable of directly penetrating the plant surface. Thus they have to find wounds or natural openings such as stomata and hydathodes to enter the leaf interior. Bacterial entry into plant tissue has beginning to be elucidated (Melotto *et al.* 2008).

Stomata are the natural opening found on the leaf surfaces of plants. They are formed by a pair of specialized epidermal cells known as guard cells. Guard cells can change their shapes depending on

turgidity; hence they regulate the opening and closing of the stomatal pore. When the guard cells are turgid, stomata open whereas they are flaccid, stomata close. Stomata play an important role in respiration, photosynthesis, and transpiration. Stomatal regulation is affected by various environmental factors such as light, humidity, CO_2 concentration, among others (Schroeder *et al*. 2001, Fan *et al*. 2004, Zelitch 1969).

Previous concept of stomata being the passive pores on the leaf surface which allow unrestricted entry for microbial pathogen entry has been recently challenged (Melotto *et al*. 2006). These authors found that stomata actively close when approached by microbial pathogens or exposed to conserved molecules found on the surface of bacterial cells, also known as pathogen associated molecular patterns (PAMPs) such as flg22 (Melotto *et al*. 2006). Hence, it has been established by several lines of evidence that stomata can close in response to pathogen/PAMPs as part of the plant innate immunity against pathogens. In counter action, some pathogens are capable of re-opening the stomata some time after initial infection. The phytotoxin responsible for this action is coronatine (COR). Pathogens that produce COR have been found to successfully re-open stomata after initial closing (Melotto *et al*. 2006). In *Pst* DC3000, COR functions as a virulence factor in tomato, Arabidopsis, and Brassica spp. (Brooks *et al*. 2004, Elizabeth & Bender 2007, Penaloza-Vazquez *et al.* 2000) and is required for lesion formation and bacterial multiplication in tomato and Arabidopsis (Brooks *et al.* 2004, Mittal & Davis, 1995, Penaloza-Vazquez *et al.* 2000).

As stomata are strongly regulated by environmental factors, it would be interesting to see if any relation exists between environmental factors and successful bacterial infection. The main focus of this research is to examine *Pseudomonas syringae* pv. *tomato* infection of *Arabidopsis thaliana* under a range of relative humidity and light/dark treatments.

Understanding the effects of varying environmental conditions on the effectiveness of stomatal defense may lead to new understanding of outbreaks of bacterial disease and contamination. Bacterial speck disease is affected by weather and other environmental factors; e.g. rain increases disease severity (Goode & Sasser 1980). It is interesting to note that severe outbreaks of bacterial disease in the field are often associated with periods of heavy rain or high humidity. It is possible that, under these

environmental conditions, stomatal defenses are partially compromised owing to the plant's need to prioritize stomatal responses to other stimuli, therefore allowing more bacteria to enter the leaf tissue to promote infection (Melotto *et al*. 2006). Interestingly, to ensure infection in the laboratory, researchers commonly expose plants to very high humidity for an extended period after surface inoculation.

High humidity has been found to significantly promote the infection of Arabidopsis plants by *cor* mutant bacteria (Melotto and He, unpublished results). Is stomatal defense compromised under high humidity? This question was addressed in this research. Likewise, light has a major influence on stomatal movement. In the dark, most stomata are closed. Interestingly, Mino *et al*. (1987) found that COR re-opens dark-induced stomata closure in broad bean and Italian ryegrass. Thus, COR may provide a mechanism for bacterial entry into plant leaves at night when most stomata are closed, which could have significant epidemiological implications in foliar bacterial infection. In this study, this phenomenon was studied in Arabidopsis plants and extended to bacterial infection.

Environmental factors play an important role in stomatal regulation. Study of effect of environmental factors on a laboratory model Plant-microbial interaction can have similar implication in nature. These studies will provide valuable insight on whether environmental factors affect pathogen attacks on plants and thereby enabling us to find better ways to develop control measures to protect crop plants.

1.1 Objectives

The primary goal of this research was to examine how environmental factors affect stomate-based defense against the bacterial *Pseudomonas syringae* pv. *tomato*. The specific objectives were:

1.1.1 Objective 1

To develop an easy, reliable, and reproducible assay to assess stomatal response to stimuli in whole leaves

1.1.2 Objective 2

To determine whether coronatine promote the re-opening of dark-closed stomata

1.1.3 Objective 3

To assess the effect of relative humidity on bacterium and PAMP-triggered stomatal closure

CHAPTER 2

DEVELOPMENT OF AN EASY, RELIABLE, AND REPRODUCIBLE ASSAY TO ASSESS STOMATAL RESPONSE TO STIMULI IN WHOLE LEAVES

2.1 Abstract

The use of epidermal peels to study stomatal physiology is well established. This method has been extensively used to study various functional attributes of stomata. Stomata respond to a number of external stimuli like light, humidity, carbon dioxide, and pathogens. Hence, an ideal method for assessing the effect of a particular stimulus on them should present minimal interference to stomatal physiology and mimic the natural environment of the plant as much as possible. Use of epidermal peels is not best suited for plant biologists who study the role of stomata response to biotic stress caused by pathogens for a number of reasons. Firstly, leaves are damaged while collecting peels. It has been found that guard cells, which form stomatal pore, require the support from neighboring cells to properly maintain the stomata. Secondly, it requires technical expertise to collect a peel itself. Thirdly, isolated peels are prone to dehydration. Hence, it is difficult to monitor the same sample over time. Due to these limitations, it has become necessary to find alternatives for stomatal assays. In this study, I explored four alternative methods for stomatal assay aiming to develop a method that reduces sample manipulation and it is easily reproducible and reliable across laboratories. Comparison of all the methods indicated that propidium iodide (PI) staining of whole leaves is the most suited for stomatal assays. This method allowed for a quick and efficient way to visualize stomata in whole leaves. Moreover, PI staining is stable in the leaf over extended period of time and does not alter stomatal aperture. Hence, this method allowed for easy assessment of stomata in the same sample for a longer time with minimal manipulations. In addition this method should facilitate studies to understand the physiology and functions of stomata in response to live microbes.

2.2 Introduction

Stomata are the natural openings found on the leaf surfaces of plants. They are formed by a pair of specialized epidermal cells known as guard cells. Guard cells can change their shapes depending on their turgidity regulating the opening and closing of the stomatal pore. Changes in the width of the stomatal aperture reflect the cumulative effect of many physiological responses by the leaf to environmental cues. Recent studies have found that many plant pathogens can trigger stomatal response (Melotto *et al.* 2006, Gudesblat *et al*. 2009). Typically, stomata close in response to bacteria and conserved molecules in the bacterial cell surface collectively known as PAMPs (pathogen associated molecular patterns), thereby minimizing chances of pathogens to enter the plant interior. Measurements of the degree of stomatal opening on a leaf surface provide a convenient visual indication of stomatal response to external stimuli. The width of stomatal aperture is an easily measured indicator of stomatal function (Gorton *et al.* 1989). Because stomata movement is tightly regulated by a number of many external factors, the slightest variation on experimental conditions affects the results. An ideal method for these assays should cause minimal interference to the leaf physiology and mimic natural environment in which plants are attacked by pathogens.

Stomatal assay using epidermal peels of leaves is quite common practice for electrophysiology experiments. For instance, Hosy *et al.* (2003) used epidermal peels of Arabidopsis to study involvement of potassium channels in regulating stomatal movement and plant transpiration. This technique has been adapted to study stomatal response to bacterial pathogens (Melotto *et al.* 2006). In their study, the epidermal peels were treated with live bacteria and stomatal response was examined over time. However maintaining favorable conditions to maintain both plant epidermal peels and bacterial cells alive for the duration of the experiment has been challenging. For instance, leaf epidermis can be kept alive and healthy with MES buffer (10 mM KCl, 25 mM MES-KOH, pH 6.15) for electrophysiological experiments of guard cells. However, this buffer is not appropriate for bacterial cells. On the other hand, bacterial cells can be kept alive in water which is not proper to maintain epidermal peels for a long period of time. When an epidermal peel floats on water, the cells in the peel that are exposed to air dry within 4 hours limiting the timing to conduct the experiment. It has also been found that epidermal peels require neighboring

cells to properly regulate stomatal aperture and hence, variation in stomatal aperture is significant in epidermal peels as compared to whole leaves (Allegre *et al*. 2006). An ideal method for assessing the effect of a particular stimulus on guard cells should present minimal interference to stomatal physiology and to the natural environment of the plant as much as possible.

The objective in this chapter was to explore different methods to assess stomatal response to live bacteria, in which leaf wounding and manipulation is greatly minimized aiming to provide an easily reproducible and reliable stomatal assay. Methods studied here were: 1) imprinting of whole leaf 2) Measuring stomatal conductance, 3) green fluorescent protein (GFP) labeling of the Arabidopsis plasma membrane and 4) whole leaves staining with propidium iodide (PI). The common feature in all of these methods is the use of whole leaves. Use of whole leaves allow for the observation of the same live leaf sample over extended periods of time using conditions that closely mimic the natural conditions under which plants are attacked by pathogens. Imaging of intact leaf tissue under the microscope can be challenging; the uneven topography and thickness of the leaf makes it difficult to visualize cells under the microscope. This problem can be solved by removing the mid vein of the leaf so it lays flat on the slide and using confocal microscopy.

2.3 Methods

2.3.1 Growing Arabidopsis

Soil mix containing growing soil medium ((Redi-earth plug and seedling mix, Sun Gro, Bellevue, WA), fine vermiculite, and perlite in 1:1:1(v:v:v) was prepared. Three-inch plastic pots were filled with this mix and soaked overnight in water. The pots were topped off with thin layer of vermiculite and covered with mesh. Columbia (Col-0) (ABRC stock Center, Ohio) seeds were suspended in 0.1% agarose solution and sowed on 4 corners and the center of each pot. The pots were placed in plastic trays, watered, and kept at 4^{o}C for 2 days. This cold treatment helps synchronize the germination of the seeds. The flats were then transferred to a growth chamber with the following conditions set: 22^{o}C, 12 hours of 100µmol/mm^2 /sec light, and 65±5% relative humidity. Gnatrol (Valent Professional Products, Walnut Creek, CA) was added to the flats to prevent fungus gnat larval infestation. Flats were watered as

needed. Plants became ready for use in about 4-6 weeks when they had numerous young fully expanded leaves and no bolting had occurred.

2.3.2 Bacterial Culture

Pseudomonas syringae was streaked from glycerol stock on modified low-salt Luria Bertani (LB) medium (10g/L tryptone, 5g/L yeast extract, 5g/L NaCl, pH=7.0, and 1.5% agar). Plates were incubated for 24 hours at 28^{o}C. Fresh bacterial culture was used to prepare inoculum and cultures were always started from glycerol stocks as bacterium becomes less virulent after sub-culturing.

Liquid culture of *P. syringae* was prepared in an Erlenmeyer flask and incubated overnight at 28^{0}C with vigorous shaking until it reaches optical density (OD) at 600nm between 0.8 and 1. Cells were harvested by centrifugation at 1360 Xg for 10 minutes and resuspended in distilled water so that the final O.D is 0.2. This O.D corresponds to 10^{8} colony forming units (CFU)/ml (Katagiri *et al.* 2002).

2.3.3 Dip Inoculation

Wild type strain, *P. syringae* pv. *tomato* strain DC3000 (*Pst* DC3000), and coronatine mutant strain, *P. syringae* pv. *tomato* strain DB29 (*Pst* DB29) were used for this infection. Bacterial inoculum was prepared as described in item 2.3.2. *Pst* DC3000 was selected by growing in100µg/ml rifampicin and *Pst* DB29 can be selected by growing in 100µg/ml rifampicin, 25µg/ml kanamycin and 25µg/ml spectinomycin. To each inoculum, 0.03% silwet L-77 (Lehle seeds Co., Round Rock, TX) was added and stirred for 10 minutes to homogenize the mixture. Plants were inverted into the inoculum, dipped for 2-3 seconds and removed. Inoculated plants were immediately transferred to the following conditions: 100 µmol/mm^{2}/sec light, 25^{o}C, and 65±5% relative humidity. Plants were incubated in the same conditions for 3 days.

2.3.4 Stomatal Assay Using Epidermal Peels

Lower epidermis was peeled from 4-5 week old, fully expanded leaf and floated on 300µl of water or *Pst* DC3000 bacterial suspensions. Leaf cuticle was in contact with the solution. The peels were incubated for 3 hours under 100µmol/mm^{2} /sec light, 22^{o}C, and 65±5% relative humidity. Peels were observed under microscope (see item 2.3.9) and aperture width of 50-60 stomata were measured using the microscope software. Statistical analysis was done as described in item 2.3.10.

2.3.5 Imprinting Of Whole Leaves

A thin layer of nail polish was applied on the surface of the glass slide. Leaves from light-treated Arabidopsis plants were placed on top of the nail polish with upper epidermis facing up. Imprint of the leaf was made on the nail polish surface by gently pressing the leaf on the thin layer of nail polish. The leaf was removed and imprint was allowed to dry. The imprints were observed under light microscope (Leica DM LB2; Leica Microsystems, Bannockburn, IL). The stomatal aperture of the leaf imprint was measured using software Q color3 (Olympus).

2.3.6 Stomatal Conductance Measurements

2.3.6.1 Stomatal Assay Of Light And Dark Treated Plants

Plants were incubated under 100 µmol/mm^2/sec light and 100 µmol/mm^2/sec light at 22^oC, and 65±5% relative humidity for 3 hours. A standard hand-held leaf porometer (Decagon Devices Inc., Pullman, WA) was used to measure the stomatal conductance of leaves following incubation. Intact leaf, which is still attached to the plant, was placed in the porometer chamber so that the conductance was recorded with the sensor and generated in display panel (Figure 2.1). Readings from each leaf were taken in triplicates and represented as mean ± standard error.

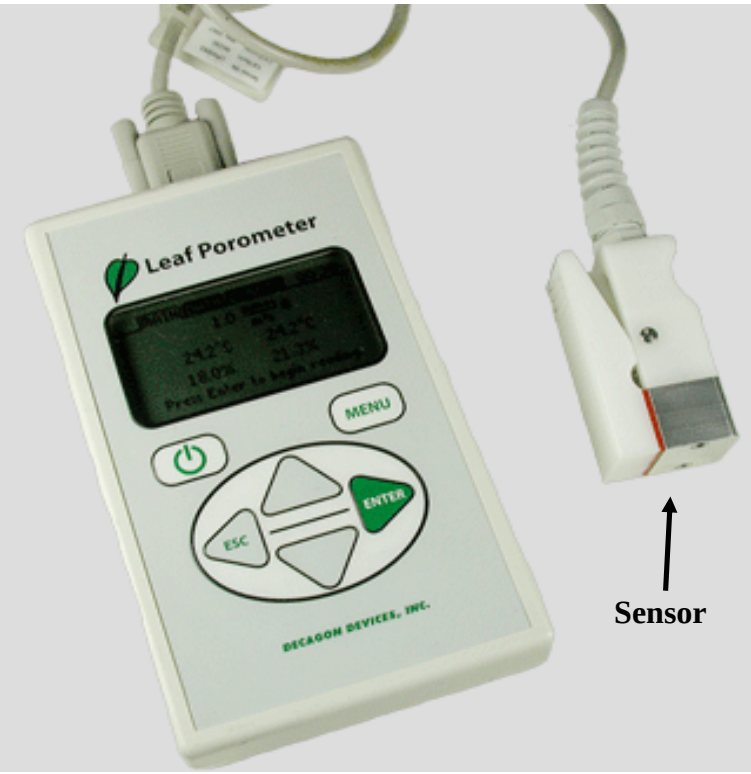


Figure 2.1 Leaf porometer. Intact leaf is places between the clamps of the sensor and clamps are closed shut. The stomatal conductance is generated in the display panel. Similar procedure was done three times with each leaf sample and results are presented as the mean (n=3) ± standard error.

2.3.6.2 Stomatal Assay Of Dip Inoculated Plants

Plants were acclimated to 100 µmol/mm^2/sec light, 25^{o}C, and 65±5% relative humidity for 12 hours. Stomatal conductance of the leaves was measured. Plants were, then, dip inoculated with *Pst* DC3000 and *Pst* DB29 as described in item 2.3.3 and incubated under same conditions for 24 hours. Stomatal assay was done using a porometer prior to infection and at the intervals of 2 hours and 20 hours post infection. The reading were taken in replicates and represented as mean ± standard error.

2.3.7 GFP labeling of Plasma Membrane

We obtained a plasmid carrying the *AtPIP2A* gene (At3g53420), which encodes for a plasma membrane aquaporin, fused with *gfp* gene (clone: pm-gb; stock: CD3-1004, Arabidopsis Biological Resource Center, ABRC, Ohio State University, http://abrc.osu.edu/). The plasmid also carries the *bar* gene which confers resistance to the herbicide glufosinate, in plants (Nelson *et al*. 2007). This gene

construct has been transiently expressed in Arabidopsis allowing for high resolution live cell imaging by Gardner *et al*. (2008). In this study, I created stable transformant plant lines of the above construct.

2.3.7.1 Transformation Of *Agrobacterium tumefaciens* by Tri-Parental Mating

Agrobacterium tumefaciens strain C58C1, helper *Escherichia coli* strain HB101, and *E. coli* (pm-gb-CD3-1004) cells were grown overnight in low-salt LB broth with appropriate antibiotic selection (*A. tumefaciens*: 100µg/ml rifampicin, 10µg/ml tetracycline; *E. coli* HB101 and pm-gb-CD3-1004: 50µg/ml Kanamycin). The cultures were allowed to grow until the cell concentration reached the O.D of 0.6 at 600nm. Bacterial cultures (100 µl of each culture) were mixed and the mixture was spotted on a low-salt LB plate without antibiotic selection. Negative control was also spotted on a plate in which *E. coli* HB101 was replaced by 100 µl of water. The plates were incubated at 28^0C overnight. Colonies from the plate was transferred to a low salt LB plate with 100µg/ml rifampicin, 10µg/ml tetracycline, and 50µg/ml kanamycin and incubated at 28^0C for 24 hours. Only transformed *A. tumefaciens* grew in this selection medium. Bacterial transformation was confirmed by polymerase chain reaction (PCR) using aquaporin gene specific primers (Forward: ATGGCAAAGGATGTGGAAGCCG; Reverse: TTAGACGTTGGCAGCACTTCTG).

2.3.7.2. Transformation of Arabidopsis by Floral Dip

Arabidopsis plants were transformed according to the floral-dip method (Cough and Bent, 1998). Briefly, Arabidopsis plants were grown until they are flowering. Flowers were dipped into *A. tumefaciens* strain C58C1 carrying the binary plasmid pm-gb-CD3-1004 was grown in low-salt LB with antibiotic selection at 28^0C up to the O.D of 0.8 at 600nm. The cells were harvested by centrifugation at 3,184 Xg for 20 minutes and resuspended in 5% sucrose solution with 0.02% L-77 silwet. Plants were laid flat in trays and immediately covered with plastic dome for 16 to 24 hours to maintain high humidity and let grow normally as described in item 2.3.1. T_1 seeds were harvested from these plants and sown on moist soil mixture (as described in 2.3.1) like a lawn. Ten days after germinations, seedlings were sprayed with 0.06% Finale (Bayer Cropscience LP., RTP, NC) which is the brand name for commercially available glufosinate. Plant survivors were transferred to pots and allowed to grow as described in item 1.1. Transformation of the plants was further confirmed by observing GFP expression in the plasma

membrane of leaves using a fluorescent LSM 510 Meta microscope, excitation of 475nm and emission of 510nm.

2.3.8 Stomatal Assay Using Propidium Iodide Staining Of Whole Leaves

Plants were incubated under light intensity of 100µmol/mm^2 /sec and 22^oC. At the beginning of the incubation, 3 leaves were plucked from the plants and were immersed in 20µM propidium iodide (PI) for 5 minutes. Leaves were then, rinsed briefly with distilled water. Each leaf was mounted on a microscope slide with lower surface facing down. Cover slip was not used to mount the slides. The mid vein was removed from the leaf so that it lays flat on the slide. The lower surface of the leaves was observed using a microscope to detect the fluorescence of PI. This chemical has an excitation and emission wavelengths of 543nm and 620nm, respectively. Images of stomata over time were captured using the same leaf samples using LSCM. Following imaging, the leaf samples were incubated under light intensity of 100µmol/mm^2 /sec and 22^oC and were placed under a plastic dome to prevent leaves from wilting. Same leaf samples are imaged for 4 hours at an interval of 1hour.

2.3.9 Microscopy

Laser scanning confocal microscope (LSM 510 Meta, Carl Zeiss Inc., Thornwood, NY) was used to observe the leaf surface. Images were taken using the various capacities of the microscope, *i.e.* bright field, fluorescence, and confocal images, according to the stomatal assay method used. Images were saved for measuring the width of the stomatal aperture at later time using the LSM browser.

2.3.10 Statistical analysis

Stomatal aperture width of at least 60 stomata was measured for each treatment at each time point using LSM image browser. Average and standard error was calculated for the stomatal aperture width and statistical significance of the results was calculated using 2-tailed, paired wise Student's t-test.

2.4 Results And Discussion

2.4.1 Epidermal Peel

Figure 2.2 shows the micrograph of epidermal peel, following 3 hours incubation with water under 100µmol/mm^2 /sec light, 22^oC, and 65±5% relative humidity. As shown, the peel appears wrinkled and most stomata appear to be closed (indicated by arrows). This is a common observation when conducting

experiments with peels. Therefore, it is necessary to seek alternative methods which minimize these problems.

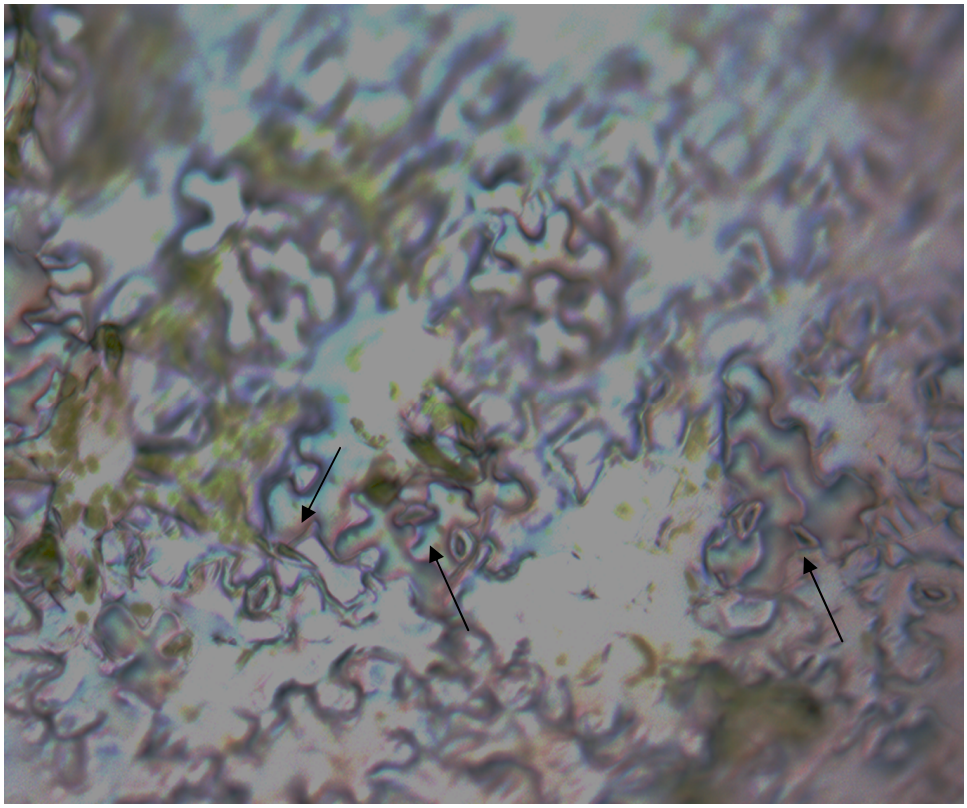


Figure 2.2 Micrograph of epidermal peel under 20x of bright field microscope. The peel was imaged 3hrs after it was made. The arrows points to areas where peel appears dried and wrinkled. Stomata appear adversely affected by this.

2.4.2 Imprinting Of Whole Leaves

Whole leaf imprinting using a nail polish was extremely difficult to reproduce, owing to the fact that thickness of the nail polish coat varied from sample to sample. Several hindrances like nail polish coat drying before imprint could be made or nail polish getting stuck to the leaf while leaf is being removed was noted. Due to these technical difficulties, data obtained from this method has not been presented. Interestingly however, once the imprint is made, stomata were easy to visualize under the microscope. Figure 2.3 shows micrograph of leaf imprint on a microscopic slide, taken under 20x of bright field view of light microscope. Even though it was difficult to demarcate between two guard cells that form the aperture, the stomatal aperture is quite evident. Had this method technically reproducible, the imprints

could have been saved for extended time and could have served as a permanent record for the experiment conducted.

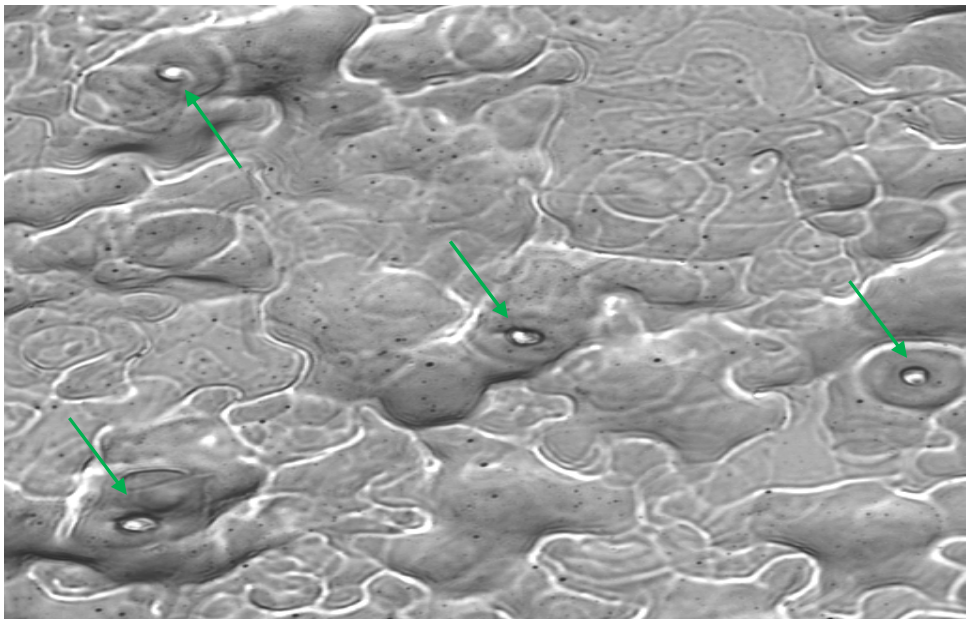


Figure 2.3 Micrograph of imprint of Arabidopsis leaf surface. One field of view of the leaf surface under bright field microscope using the 20x shown here. The stomatal apertures are indicated by green arrows.

2.4.3 Stomatal Conductance Measurements

A simple experiment of light and dark treatment of Arabidopsis was conducted to see if porometer can yield expected results. Plants under light would have most stomata open and dark treated plants would have most stomata closed. Hence, higher stomatal conductance for light treated plants as compared to dark treated plants was expected from this experiment. Figure 2.4 shows the results obtained from 3 hrs of light and dark treatments in Arabidopsis. The results are represented as mean of triplicate readings from the same leaf for a given time point and error bars represent the standard error. However, the results obtained are not statistically significant at p=0.05.

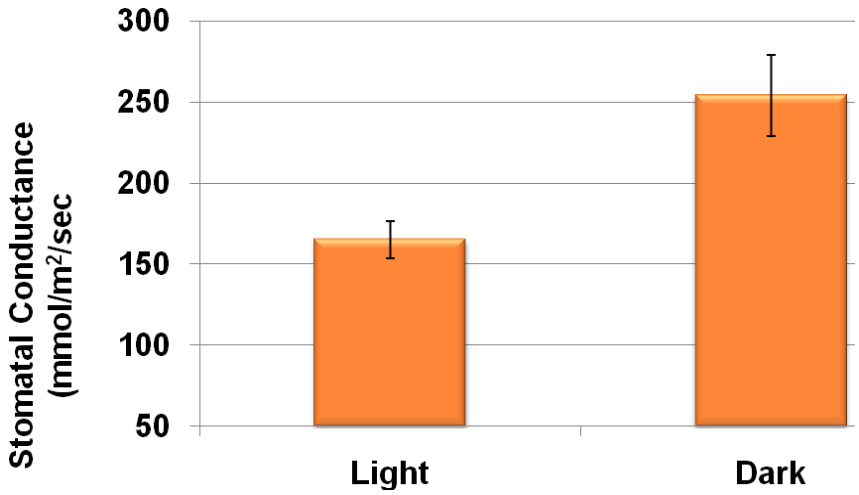


Figure 2.4 Stomatal conductance of plants incubated under 100µmols/mm^2/sec of light and under dark for 3 hrs. Stomatal conductance was taken 3hrs using porometer. Results are shown as the mean (n=3) ± standard error.

Similarly, stomatal conductance was measured for plants infected with *Pst* DC3000 and *Pst* DB29, with an expectation of higher stomatal conductance for *Pst* DC3000 infected leaves as compared to those from *Pst* DB29 infected results. Figure 2.5 shows the results of stomatal conductance done before infection, and 2 hours and 20 hrs post infection. There is no statistical significant difference between *Pst* DC3000 and *Pst* DB29 infected plants for any given time point. Moreover, the porometer readings from the same leaf, taken at the same instant ranged from 400 to 1400 mmol/m$^{2/}$sec**.** The clamps that hold the sensor of porometer were too big for Arabidopsis leaves and could have led to aberrant readings from the same sample. Leaves appeared damaged and water soaked, following the measurement with porometer. This might also have led to the discrepancy in the results. Even though use of porometer is quite common in field studies to determine various physiological aspects of plants such as rates of transpiration, drought resistance, it did not produce consistent result in case of Arabidopsis.

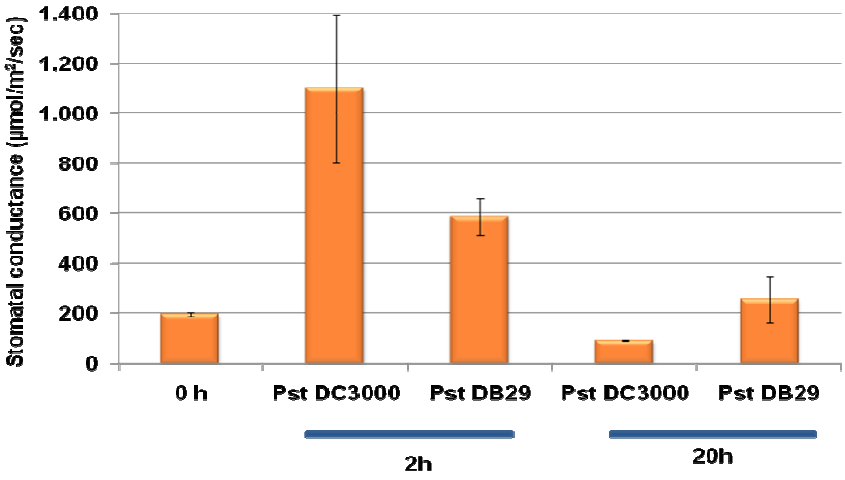
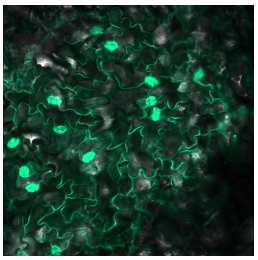


Figure 2.5 Stomatal conductance of plants were infected with *Pst* DC3000 and *Pst* DB29 incubated under 100µmols/mm^2/sec of light and under dark for 20 hrs. Stomatal conductance was taken before infection, 2hrs and 20hrs after using porometer. Results are shown as the mean (n=3) ± standard error.

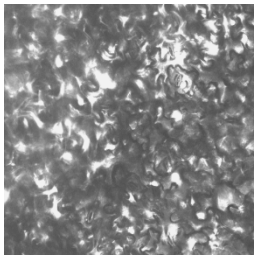
2.4.4 GFP labeling Of Plasma Membrane

Transformation of Arabidopsis plants expressing GFP-labeled plasma membrane has been successfully completed. Out of 11 plants that were resistant to Glufosinate, 4 (36%) were found to be expressing GFP in their plasma membrane. Figure 2.6(a) depicts a micrograph of Arabidopsis expressing GFP in their plasma membrane under fluorescent microscope. As shown, plasma membrane lining in the epidermal cells were very clear and obvious as compared to figure 2.6 (b) where the leaf surface of the wild type plant have been shown under bright field microscope. Single stomate has been focused in figure 2.6(c). However, guard cells had higher expression of GFP than other epidermal cells. Recent findings have shown that expression levels of aquaporin are high in transport cells including guard cells. This might explain the expression of GFP in whole of guard cells and not just in plasma membrane as shown in figure 2.6 Due to this, stomata imaging using this transformant did not improve substantially. Moreover, t has also been found that aquaporin is induced by drought (Lee *et al*. 2009). Hence, this gene cannot be considered a suitable candidate for stomatal assay. Instead a housekeeping gene which has been well characterized of being constitutive and having definite localization in outer boundaries of the cell such as plasma membrane or cell wall might be a better candidate for this purpose. For instance, NADPH:quinine oxidoreductase (NQR) (AT3G02600) is the gene that is involved in phospholipid metabolic process and

is integral to plasma membrane. This protein has phosphatidate phosphatase activity and is expressed in several parts of the plant including leaf (www.Arabidopsis.org). The transformation work has not actually been conducted using the suggested gene. Nonetheless, work represented here indicates the feasibility of this type of method and opens another avenue for easy and reliable method to study stomatal response to live bacteria.



(a)



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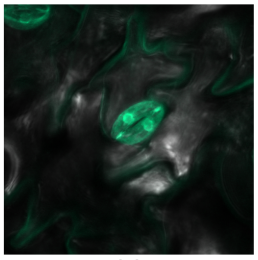


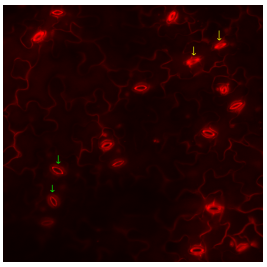
(c)

Figure 2.6 Micrographs of Arabidopsis leaf surface with GFP expression in plasma membrane. a) One field of view of the leaf surface of transformed Arabidopsis under20x of LSCM. Note the GFP expression in the plasma membrane of the epidermal cells as well as guard cells. b) One field of view of the leaf surface of wild type Arabidopsis under 20x of LSCM. c) Single stomate from transformed plant is shown.

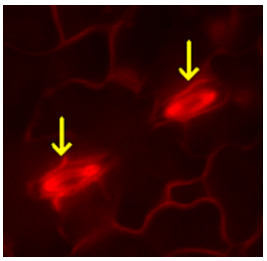
2.4.5 PI Staining Of Whole Leaves

Propidium iodide is fluorescent dye, extensively used for DNA staining in animal cells. In case of plants, entry of PI into the cell is restricted by the presence of cell wall, therefore, it accumulates outside. This accumulation gives a fluorescent outline to the cells and hence, facilitates their imaging under

fluorescence. This feature was exploited here to visualize guard cells in whole leaves of Arabidopsis. Staining duration as short as 5 minutes was sufficient to stain the leaves and guard cell outline became prominent post staining. Figure 2.7(a) shows micrograph of PI stained whole leaf under 20x objective of LSCM. Yellow arrows are pointed to completely close stomata (also shown in b) and green arrows are pointed to wide open stomata (also shown in c). Figure 2.7(d) depicts the micrograph of whole leaf under bright field view of LSCM. Comparison of (a) and (b) in figure 2.7 details the improvement in visualizing stomata in whole leaves due to Pi staining. The stomatal pore is evident in PI stained leaves whereas it is quite difficult to find stomata when not aided by PI. Moreover, staining introduced least manipulation to the leaves, allowing same sample to be assessed repetitively.



(a)



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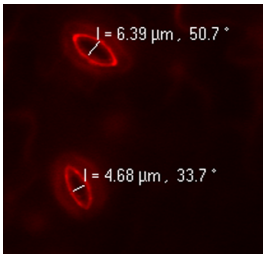
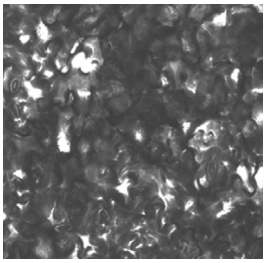


(c)



(d)

Figure 2.7 Micrographs of Arabidopsis leaf surface stained with propidium iodide. a) One field of view of the leaf surface under laser scanning confocal microscope (LSCM) using the 20x objective. Note a range of stomatal pore opening. Yellow arrows are pointed to completely close stomata (also shown in b) and green arrows are pointed to wide open stomata (also shown in c). b) Completely close stomata identified by the shape of and the opening between the guard cells. The aperture width is considered to be 0µm. c) Open stomata showing the aperture width in µm. Measurements were taken by using the LSCM browser based on a straight line drawn across the widest area of the stomatal pore. d) LSCM bright field view of an Arabidopsis leaf surface. Note that stomatal aperture is not as evident when compared to fluorescent view aided by propidium iodide staining (a-c)

To ensure that PI did not introduce any alteration in stomatal aperture and also to test the stability of PI in stained leaves, stomatal assay was conducted on PI stained leaves, incubated under 100µmol/mm^2 /sec light over a period of 4 hours. Stomata were assessed at an interval of 1 hour. Figure 2.8 shows the mean stomatal aperture ((n=50-70) ± standard error) of same leaf sample over the period of 4 hours. Statistical significance was detected with two-tailed Student's t-test. The bars shown here are not statistically different. This shows that PI staining does not interfere with the stomatal function as most stomata remained open during this time as expected. Hence, the method allows easy assessment of stomata in the same sample for a longer time with minimal manipulations.

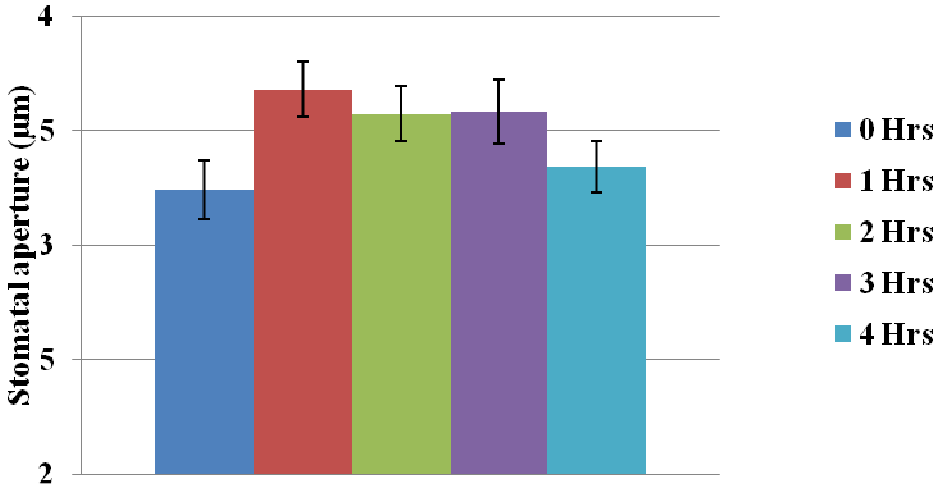


Figure 2.8 Stability of PI in stained leaves under light. Leaves were stained with 20µM PI and incubated under 100µmols/mm^2/sec of light for 4 hrs. Images were taken at an interval of 1hr using LSCM. Results are shown as the mean (n=50-70) ± standard error. No significant statistical differences were detected among time points.

2.5 Conclusion

Overall conclusion that can be drawn from exploring all this techniques is that PI staining is best suited for studying plant-bacteria interactions and was adopted for all future experiments because this method presented minimal manipulations to the leaf samples, therefore same samples could be used for an extended period of time. The staining procedure as well as the visualization under the microscope was straight forward. The stomatal outline became evident after staining. Although results presented here were done using the model system Arabidopsis/*Pseudomonas syringae*, the intact leaf stomatal assay

can be potentially performed with any plant-bacterium combination. The protocol described here can easily be modified to fit growth requirements of other plants and bacterial pathogens. The overall principle and procedure of the method remains the same. In addition, this method may be beneficial to researchers who wish to study functional output of guard cells not only to live microbes, but also to other stimuli and chemical agents under conditions that maintain the leaf's natural environment.

Table 2.1 Comparison of alternative methods for stomatal assay

Method	Natural Environment	Same sample over time	No Adverse Effect	Easy and Quick	Reliable and reproducible
Epidermal Peel	✗	✗	✗	✓	✓
Nail Polish Imprint	✓	✗	✗	✓	✗
GFP Labeled PM	✓	✓	N/A	✓	✓
Leaf Porometer	✓	✗	✗	✓	✗
PI Staining	✓	✓	✓	✓	✓

CHAPTER 3

CORONATINE IS EFFECTIVE IN RE-OPENING DARK INDUCED STOMATAL CLOSURE

3.1 Abstract

Coronatine is a non-host specific phytotoxin produced by several pathovars of the plant pathogenic bacterium *Pseudomonas syringae*. This toxin contributes to disease developments in plants at both early and late stages of infection. A prominent role of coronatine is to facilitate penetration of bacteria into leaves by overcoming stomatal defense. Specifically, coronatine re-opens pathogen-triggered stomatal closure. However, its role in overcoming stomatal closure due to environmental factors such as darkness, low relative humidity is unknown. In this study, I found that coronatine is effective in opening dark-closed stomata after four hours of incubation of Arabidopsis leaves with purified coronatine or the coronatine producing bacterium *Pst* DC3000. Same trend did not hold for the coronatine deficient mutants, However, the plants inoculated in dark with coronatine producing bacteria was unable to produce severe disease as compared to plants inoculated in light. Taken together, these results suggest that coronatine can effectively open stomata in dark and help pathogen to enter in the early stages of infection but disease development and proliferation of bacterial population inside the plant light requires light. Further assessment of plant and bacterial behavior is needed to understand this aspect of plant and pathogen interaction.

3.2 Introduction

Plant phyllosphere is one of the most diverse niches for microbe inhabitation. Numerous bacteria can survive and proliferate on the surface of the plant without causing any harm to the plant. However, for any pathogen to cause a successful infection, they should not only enter the plant but also be able to survive and proliferate inside of the plant. The mode and mechanism of penetration of plant tissue is very critical for any type of infection. Bacterial pathogens rely on natural openings and accidental wounds on the plant surface to penetrate the tissue. Such a natural opening is stomata, the main route through which bacterial pathogens transition from epiphytic to endophytic lifestyles. Until recently stomata were thought

to be passive ports which allowed unrestricted entry of microbes. However, it has been shown recently that stomata are able to respond to pathogen by actively closing the pore. Similar response is seen when plant leaves are exposed to conserved bacterial motifs collectively known as pathogen associated molecular patterns (PAMPs) such as lipopolysaccharides (LPS), bacterial flagellin, and lipoteichoic acid. The molecular mechanisms by which stomata close in response to pathogens and PAMPs is beginning to be elucidated (Baker *et al*. 2010). . Several lines of evidence point to the complexity of this response. As a part of plant immune defenses, bacterium-induced stomata closure requires the FLS2 receptor, production of nitric oxide (NO), salicylic acid (SA) homeostasis, abscisic acid (ABA) signaling components, such as the guard-cell-specific OST1 kinase (Melotto *et al*. 2006), K^+ channel regulation via heterotrimeric G-Protein (Zhang *et al* 2008), and *Mitogen-Activated Protein Kinase3* (*MPK3*; Gudesblat *et al*. 2009). Thus stomatal closure is an integral basal plant defense mechanism to restrict the invasion of pathogenic bacteria into plant tissues

Two bacterial pathogens possess virulence factors which enable them to overcome stomatal defense: *Xanthomonas campestris* pv. *campestris* can synthesize or perceive diffusible signals through the rpf/diffusible signal factor (DSF) system and reverse stomatal closure in Arabidopsis leaves (Gudesblat *et al*. 2009) whereas *Pseudomonas syringae* can produce a phytotoxin called coronatine which overcomes the initial stomatal response and re-opens them. Coronatine also has a significant role in disease production in plants. This phytotoxin is produced by several pathovars of *P. syringae* including tomato, *maculicola, glycinea,* and *atropurpurea* and it induces chlorosis in infected leaves (Brooks *et al*. 2004, Mittal and Davis 2005). It also induces modifications in the plant's physiology such as anthocyanin production, alkaloid accumulation, ethylene emission, tendril coiling, and root inhibition (Bender *et al*1999). Coronatine producing strains of P. syringae have been found to be remarkably more aggressive than ones that are coronatine defective mutants. Comparisons between bacterial population of coronatine producing and coronatine deficient bacteria showed that numbers were significantly higher for coronatine producing bacteria during surface infection. However, when plants were vacuum infiltrated, the populations of coronatine producing strain and coronatine deficient strain was comparable. Surface infection like spray or dip inoculation mimic the natural infection where bacteria have to overcome surface

defenses and enter plants through natural routes. In contrast, vacuum infiltration, bacteria are delivered directly into plant interior. These observations led to explore the role of coronatine in overcoming stomatal defense. In the study done by Melotto and collaborators (2006), they found that stomata in Arabidopsis plants infected with coronatine producing bacteria, *Pst* DC3000 re-opened as early as 3 hours post infection. However, the re-opening was not observed in coronatine deficient mutant strain, *Pst* DC3118. This finding represents the first identification of bacterial virulence factor that suppresses stomatal closure (Baker *et al*. 2010). These results signified involvement of coronatine in re-opening stomata enabling pathogenic bacteria to gain entry to the plant interior, and hence cause more severe disease.

Coronatine is a nonhost specific phytotoxin, and its structure consists of two distinct moieties that function as intermediates in the biosynthetic pathway: (a) the polyketide coronafacic acid (CFA) and (b) ethylcyclopropyl amino acid coronamic acid (CMA). CMA and CFA are synthesized by separate pathways and joined by an amide bond to form coronatine (Bender *et al.* 1999). Coronatine is a structural and functional mimic of plant hormone jasmonic acid (JA). JA is a lipid derived hormone which has regulatory functions in vegetative and reproductive growth, defense responses against abiotic stresses such as ultraviolet light and ozone, insect herbivory, and necrotrophic pathogens (Katsir *et al*. 2008). Coronatine activates JA signaling and induces JA-responsive genes in Arabidopsis-Pseudomonas interaction. It is believed to utilize the antagonism between JA signaling and salicylic acid (SA) signaling which is also a plant hormone actively involved in plant defense (Thomma *et al*. 2001. By up regulating JA signaling, pathogen is able to down regulate SA signaling. Overall effect of coronatine is suppression of plant defense and increase the severity of the disease. Mino *et al.* (1987) discuss that COR activates membrane bound ATPase activity. More recently, the plant protein RIN4 (RPM1 INTERACTING PROTEIN 4) and activation of H^+-ATPase have been found to be necessary for COR to re-open stomata (Liu *et al.* 2009). How exactly COR functions via RIN4 and activation of H^+-ATPase is not yet clear. COI1 (CORONATINE INSENSITIVE1) is another plant protein necessary for COR function in the guard cell is (Melotto *et al*. 2006). In fact, COI1 has shown to be a receptor for COR (Yan *et al.* 2009).

Coronatine is well characterized for its role in overcoming stomatal defense, promoting entry of bacteria through stomata at the initial stages of infection. Figure 3.1 depicts the sequence of events

occurring on plant surface following the infection by P. syringae. A cross-sectional view of leaf epidermis and mesophyll cells showing that stomata, formed by pairs of guard cells (GC), in light-adapted Arabidopsis leaves are mostly fully open is shown in figure 3.1.A. Upon exposure to bacteria, guard cells perceive PAMPs and many stomata close within 1hr. Because not all stomata are closed, bacterial may enter at a basal level (shown in figure 3.1.B). Figure 3.1.C shows virulent plant pathogen *Pst* DC3000 reopening stomata 3hrs after infection by producing diffusible COR, thereby increasing the number of sites for bacterial invasion (Melotto *et al.* 2008).

Role in coronatine in re-opening stomata directed us to investigate whether this toxin is also effective in opening stomata during dark periods. Usually in most plants, stomata open during the day maximizing the CO_2 diffusion into the apoplast for photosynthesis and close during the night minimizing the water loss from the plant. If coronatine can open stomata in dark, *P. syringae* would have advantages over non-cor producing strains by invading plants during the night.

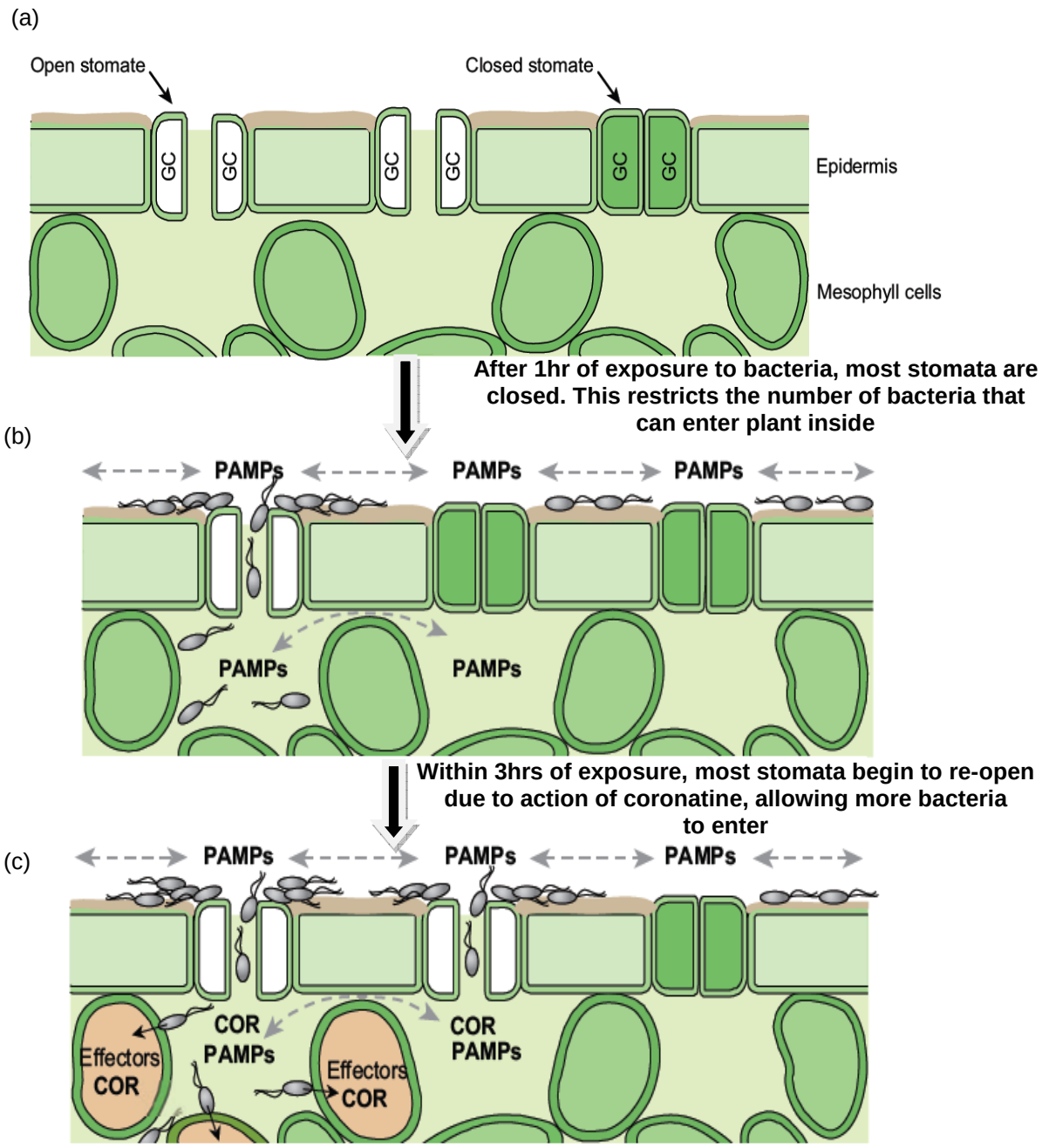


Figure 3.1 A diagram depicting stomata as entry sites for bacterial invasion. (*a*) A cross-sectional view of leaf epidermis and mesophyll cells showing that stomata, formed by pairs of guard cells (GC), in light-adapted *Arabidopsis* leaves are mostly fully open. (*b*) Upon exposure to bacteria, guard cells perceive PAMPs and many stomata close within 1hr. Because not all stomata are closed, bacterial may enter at a basal level. (*c*) In the case of the virulent plant pathogen *Pst* DC3000, 3hrs after infection bacteria produce diffusible COR to reopen closed stomata thereby increasing the number of sites for bacterial invasion. Adapted from Melotto *et al.* 2008

3.3 Methods

3.3.1 Stomatal Assay Using Epidermal Peels

Arabidopsis plants were incubated in dark for 12 hours. Epidermal peels were collected from the leaves as described in item 2.3.4 and treated with 1.5µM purified coronatine from *Pseudomonas syringae* pv. *Glycinea* (Sigma-Aldrich, St. Louis, MO) and incubated in dark. Peels treated with MES buffer was used as negative control. The stomatal assay as conducted before the treatment of peels and 2 hours after the treatment. Images of the peels were captured at each interval using LSCM (see item 2.3.9). Statistical analysis was done as described in item 2.3.10.

3.3.2. Stomatal Assay Using Whole Leaves

Arabidopsis plants were incubated in dark for 12 hours. Leaves were collected from the plants and stained with 20µM Propidium iodide (see item 2.3.8). Leaves were treated with 100µM purified coronatine and incubated in dark for 4 hours. Water was used as negative control. Stomatal assay was conducted at the interval of 2 and 4 hours using LSCM (see item 2.3.9). Similarly, PI stained leaves were treated with bacterial inoculum of *Pst* DC3000, *Pst* DC3118, and *Pst* DB29 and stomatal assay was done in similar fashion (see item 2.3.2 for bacterial inoculum preparation and item 2.3.8 for leaf incubation with bacterial suspension). Statistical analysis was done as described in item 2.3.10.

3.3.3 Inoculation Of Arabidopsis With P. syringae

Arabidopsis plants were inoculated with *P. syringae* in presence and absence of 12hr photoperiod to assess disease symptoms and bacterial population in light and dark.

3.3.3.1 Preparation Of Bacterial Inoculum

Inoculum of *Pst* DC3000 and *Pst* DB29 was prepared as described in item 2.3.2. *Pst DC3118* was prepared the same way with selection of 100µg/ml rifampicin and 25µg/ml kanamycin.

3.3.3.2 Inoculation Of Arabidopsis Plants

Two different approaches of inoculation were followed. One set of inoculation was done in 12hr photoperiod while other set was done in absence of any light. Prior to inoculation, plants for light inoculation were acclimated to 25^0C overnight in 100µmol/mm^2/sec of light. Similarly, plants for dark inoculation were acclimated to 25^0C overnight in 0µmol/mm^2/sec of light. Dip inoculation was done as

described in item 2.3.3. Following inoculation, plants were transferred to same conditions for first 24 hours. Following that, infected plants from both sets of conditions were transferred to 12 hours of 100µmol/mm^2 /sec of light and 25^0C for remaining of the experiment. Disease symptoms were assessed for up to six days and bacterial populations were enumerated 3 and 6 days post infection (dpi) as described in item 3.3.3.3.

3.3.3.3 Enumeration Of Bacterial Population In The Arabidopsis Leaf Apoplast

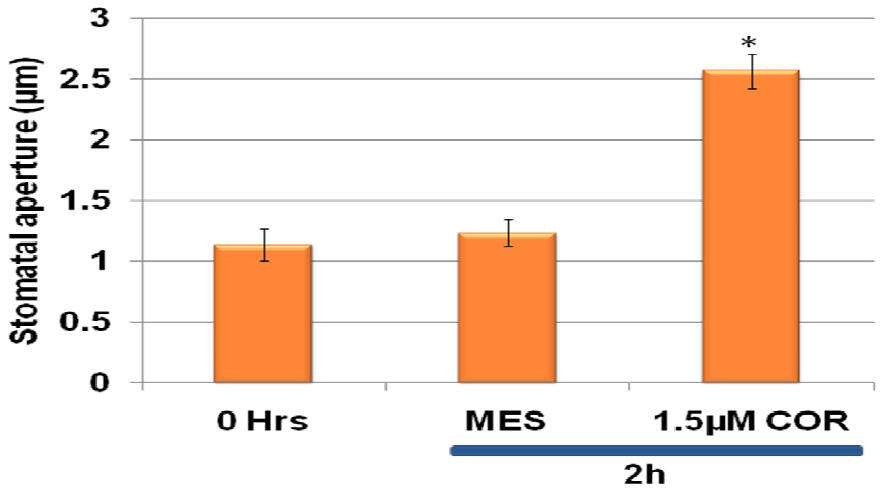
Whole leaves were removed from the host plant and placed in a 70% ethanol solution for 1 minute. The leaves were gently mixed in the solution occasionally. The leaves were then removed, and rinsed in sterile distilled water for 1 minute. The leaves were then removed and blotted dry on paper towels. Leaf disks were excised from leaves with a 0.125 cm^2 cork borer. The leaf disks for a single sample are placed in a 2ml microfuge tube with 100µl sterile distilled water. Typically, 3 samples from each treatment were obtained. The tissue samples were ground with a microfuge tube plastic pestle using a small hand-held electric drill. The samples were thoroughly macerated until pieces of intact leaf tissue were no longer visible. Following grinding of the tissue, the samples were thoroughly vortexed to evenly distribute the bacteria within the water/tissue sample. A 10µl sample was removed and diluted in 90µl sterile distilled water. A serial 1:10 dilution series was created for each sample by repeating this process. The samples were spotted on the low salt LB medium supplemented with the necessary antibiotics to select for the inoculated bacterial strain .Plating can be done by spotting 10µl aliquots of the each dilution of each sample on square plate and allowed to dry onto the surface. The plates were placed at 28℃ for approximately 36 hours and then the colony-forming units (CFU) for each dilution of each sample are counted. Finally, bacterial population per cm^2 of leaf surface was deduced from the numbers obtained from plate.

3.4 Results

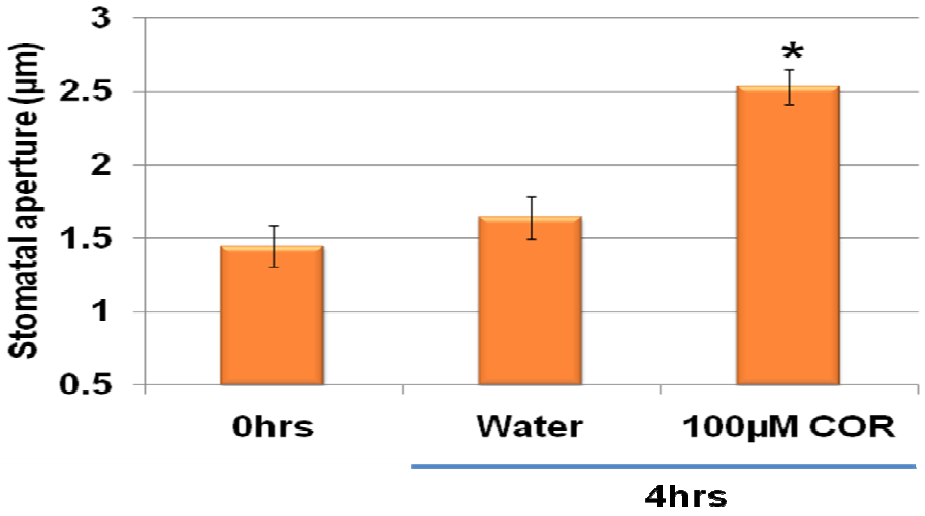
3.4.1 Purified Coronatine Is Effective In Opening Dark-Closed Stomata

Stomatal response to dark is the closure of the pore (Fan *et al.* 2004). Under conditions where plants were exposed to dark 12 hours prior to and during the treatment, I found that coronatine can re-open closed stomata in both epidermal peels as well as whole leaves. Coronatine concentration of 1.5µM

was found to be effective in significantly re-opening stomatal aperture within 2 hours of treatment. The average width of the stomatal aperture increased drastically by 50% in peels incubated with coronatine whereas remained virtually unchanged in peels incubated with MES buffer (Figure 3.2(a)). The change due to coronatine treatment was statistically significant at p=0.05. In case of whole leaves, both duration of incubation as well as concentration of coronatine had to be increased to see a significant change. Minimum of 100µM coronatine and incubation of 4 hours was needed to see significant re-opening. Following these treatments, leaves incubated with coronatine showed an increase of 43% in stomatal aperture which is statistically significant at p=0.05. However, leaves treated with water showed no significant change (Figure 3.2(b)). Similar experiments have been repeated 3 times in the laboratory and each time, similar trend was observed. These results indicate that coronatine can effective re-open dark induced stomatal closure.



(a)



(b)

Figure 3.2 Purified coronatine is effective in re-opening dark-induced stomatal closure a) Stomata aperture in peels incubated with Coronatine and peels in Mes. Epidermal peels incubated with coronatine showed significant increase within 2 hours of incubation (indicated by *) b) Stomatal aperture in intact leaves incubated in Cor and in water. Whole leaves incubated with coronatine showed significant increase within 4 hours of incubation (indicated by *). Results are shown as the mean (n=50-70) ± standard error. Statistical significance was detected with two-tailed Student's t-test.

3.4.2 Coronatine Producing Bacteria (Pst DC3000) Can Successfully Re-open Dark-induced Stomatal Closure

As previous results indicated the effectiveness of purified coronatine in reopening closed stomata, similar treatment was done using coronatine producing *Pst* DC3000 and two coronatine deficient mutants, *Pst* DC3118 and *Pst* DB29. Interestingly, *Pst* DC3000 was able to re-open to closed stomata within 4

hours of incubation in dark with whole leaves. As shown in figure 3.3, *Pst* DC3000 treated leaves showed a significant increase of 52% within 4 hours of incubation which is statistically significant at p=0.05. Same trend did not hold for *Pst* DC3118 and *Pst* DB29, also shown in figure 2.3. Similar experiments have been repeated 3 times and each time, similar trend was observed.

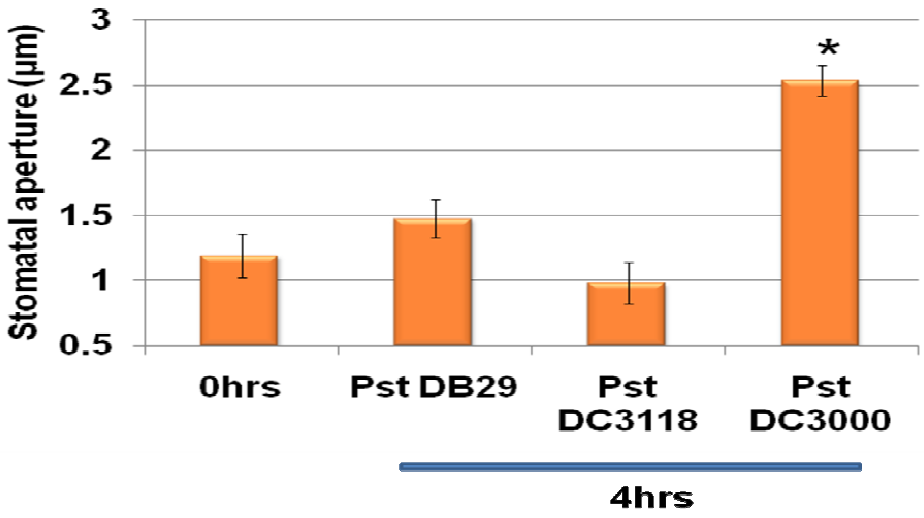
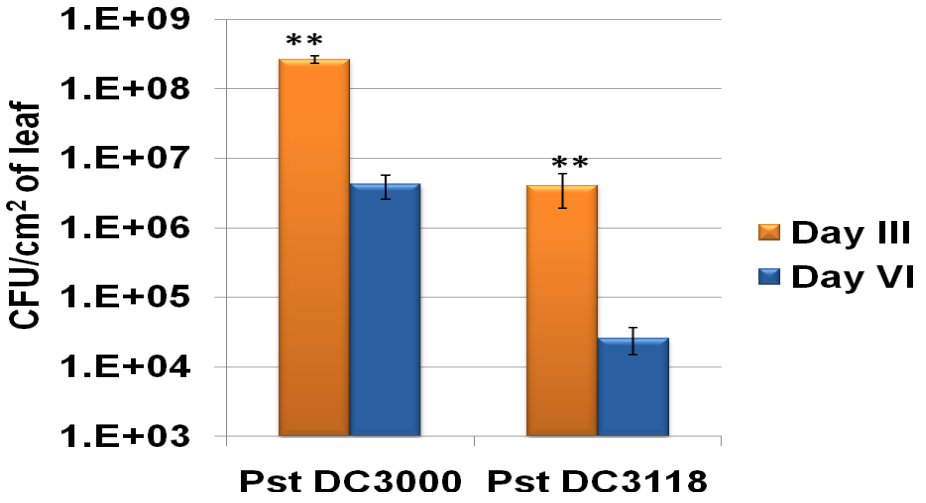


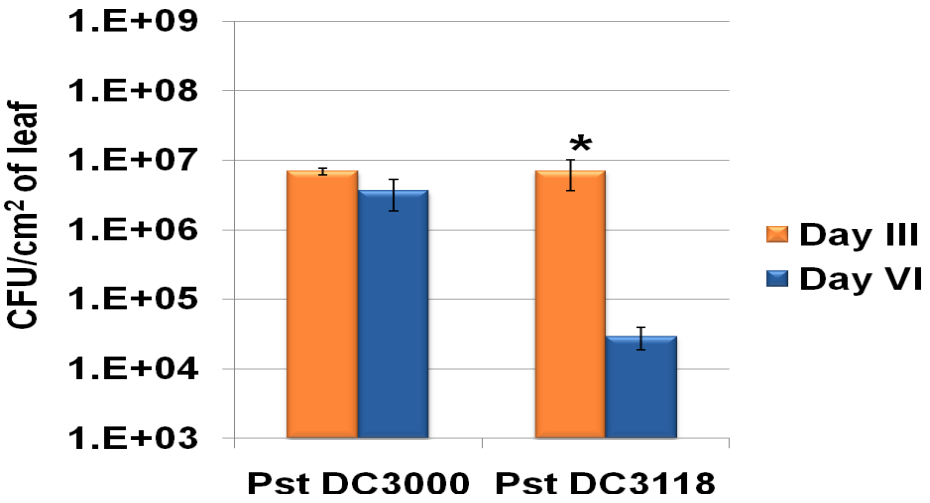
Figure 3.3 *Pst* DC3000 was able re-open dark-induced stomatal closure while *Pst* DC3118 and *Pst* DB29 failed to do so. Stomatal aperture of leaves incubated with *Pst* DC3000 showed significant increase from time zero to 4 hours post incubation (indicated by *). The stomatal aperture of leaves treated with *Pst* DC3118 and *Pst* DB29 did not show significant increase. Results are shown as the mean (n=50-70) ± standard error. Statistical significance was detected with two-tailed Student's t-test.

3.4.3 Bacterial Population In Apoplast Of Dark Inoculated Plants

The inoculation of dark adapted plants with *P. syringae* was also conducted to complement the stomatal response observed when treated with coronatine. Inoculation was also done under normal light conditions as control. The results of this procedure are presented in figure 3.4. I observed that the internal population of *Pst* DC3000, 3dpi in plants infected in dark was unable to attain similar population range as in plants infected in light (compare (a) and (b)). The population is *Pst* DC3118, 3dpi was comparable in both light and dark infected plants. The population of *Pst* DC3000 and *Pst* DC31118 decreased in both light and dark infected plants over time (6dpi).



(a)



(b)

Figure 3.4. Effect of dark in disease production and apoplastic population of *Pst a)* Apoplastic population of *Pst* DC3000 and Pst DC3118 from plants infected under 100µmol/mm^2/sec 3dpi and 5dpi b) Apoplastic population of *Pst* DC3000and *Pst* DC3118 from plants infected under 0µmol/mm^2/sec 3dpi and 5dpi

3.5 Discussion

Recent studies have shown 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. Stomata are the part of plant's innate immunity and can actively close in response to the bacteria. In counter action, bacteria such as *P. syringae* produce coronatine to reopen them. Coronatine is a virulence factor

for plant pathogen, *P. syringae* and have been well characterized to have role in bacterial infection including the penetration of host tissue through stomata. This phytotoxin has also causes several physiological changes in plants like anthocyanin production, alkaloid accumulation, ethylene emission, tendril coiling, and root inhibition in plants (Bender *et al*. 1999). Recently, coronatine has been found to cause reduced expression of photosynthesis-related genes and modulate necrotic cell death by inducing reactive oxygen species in light dependent manner in tomato seedlings (Ishiga *et al.* 2009). Distinctive role of coronatine in re-opening pathogen induced stomatal closure led to explore if it can re-open closed stomata due to dark. Mino *et al.* (1987) have discussed the possible role of coronatine in opening stomata in Italian rye grass. This study has further explored this novel attribute of coronatine in the model plant pathosystem of Arabidopsis and *P. syringae.*

Results have indicated that treatment of dark treated epidermal peels with purified coronatine showed significant re-opening in both epidermal peel as well as whole leaves of Arabidopsis. Coronatine concentration and duration of incubation had to be increased in case of whole leaves as compared to the peels. This could be because of the difference in thickness between peel and leaf. Recent studies have shown that guard cells require back support from mesophyll cells for proper function and in absence of this support, guard cell gain higher plasticity (Allegre *et al*. 2006). Due to this, the stomatal response in peel might be rapid and quick whereas in whole leaves, the response is more controlled and regulated. Similarly, coronatine producing bacteria, *Pst* DC3000 was able to open stomatal in whole leaves within 4hrs while coronatine deficient mutants did not exhibit such trend. This shows that local concentration of coronatine produced by bacteria is sufficient to re-open stomata. Broadly put, this phenomenon could easily occur in natural where plants and pathogens interactions occur.

Melotto and group (2006) have reported the disease symptoms in plants infected with *Pst* DC3000, done under regular conditions of 25^{o}C, 12 hours of 100µmol/mm^{2} /sec light, and 65±5% relative humidity to be most remarkable 3dpi and results obtained here were comparable with published result. However, with inoculations done under dark, disease symptoms became delayed and became visible only on the fifth day. Moreover, the internal population of *Pst* DC3000 was also significant lower in dark inoculated plants as compared to light inoculated plants. Studies done by Ishiga and group (2009) have

shown that induction of ROS due to coronatine is light dependent and necrotic symptoms were more in plants under long daily photoperiod was more than those under dark. Reduced necrosis in dark incubated plants could account for less overall symptoms observed in dark incubated plants. Similar observation has been made by Ramos *et al.* (1987) in tomato infected with bacterial pathogen, *Xanthomonas campestris* pv. *vesicatoria.* The incidence of disease was significantly lower in plants inoculated under dark than for plants inoculated and maintained under light conditions. Taken together, these results suggest that disease incidence as well as severity of the disease is related to stomatal opening at the time of and immediately following the inoculation.

Coronatine has been found to be effective in re-opening stomata in dark and response is quite rapid. However, coronatine was not found to effective in increasing disease production in dark. This suggests that coronatine is effective in dark during early stages of pathogen attack, especially in penetration of the host tissue. Following penetration, effect of coronatine could be less pronounced in absence of light. Inoculation was also done in dark adapted plants and kept in dark for the duration of incubation. All the inoculated plants appeared devastated by the end of 3 days (data not shown) and further conclusions could not be drawn. The *Pst* DC3000 could not attain high populations in dark inoculated plants as compared to the light inoculated ones whereas the population of *Pst* DC31118 was compared under both settings. This indicates that coronatine producing bacteria is hampered in some way under dark. The results shown here are preliminary and further assessments of the bacterial behavior under dark and light conditions needs to studied to confirm this suggestion. In recent years, extensive studies have been done to understand the role of coronatine in bacterial virulence. Even though its role in overcoming stomatal defense is well known, the molecular mechanism by which coronatine does this is unknown. Moreover, how coronatine can open stomata in dark is still a mystery. Coronatine is structural and functional mimic of plant hormone, JA-Ile. This suggests a possible co-evolution of plant and its pathogen. Several lines of evidence support the bacterial pathogens have evolved to exploit the host defense responses (Kunkle and Brooks, 2002). During infection by Pseudomonas syringae, coronatine which is a mimic of JA contributes to the virulence by suppressing SA-mediated host responses (He *et al*. 2004; Reymond and Farmer 1998)

3.6 Conclusion

Coronatine has been found effective in re-opening dark induced stomatal closure as early as 4 hours of treatment. This effect was seen in epidermal peels as well as whole leaves when they were treated with purified coronatine. The coronatine producing bacteria, *Pst* DC3000 was also able to re-open closed stomata in whole leaves while the cor mutants, *Pst* DC3118 and *Pst* DB29 was unable to do so. However, the plants infected with coronatine producing bacteria were unable produce severe disease in dark, pointing towards the requirement of light for disease development and disease severity.

CHAPTER 4

GUARD CELLS PRIOROTIZE THEIR RESPONSE WHEN EXPOSED TO MULTIPLE STIMULI

4.1 Abstract

Relative humidity (RH) is one of the environmental variables that controls stomatal aperture. Severe outbreaks of bacterial disease in crop plants are often associated with periods of heavy rainfall or high humidity. Similar observation has also been made in model pathosystem of Arabidopsis and *P. syringae* and this phenomenon is often exploited to increase the chances of the disease development in laboratory setting. . Interestingly, it was observed that virulence of the *P. syringae* coronatine deficient strain, *Pst* DC3118 increased with high humidity (Melotto and He, unpublished results). Similar results were found in this study where the disease progression in plants infected coronatine deficient bacteria was substantially enhanced in >95% relative humidity as compared to 60% RH. The symptoms in the infected plants correlate with the apoplastic population in infected plants. Under higher humidity, the bacterial population was 100 fold higher than under lower humidity. Similarly, stomatal response to pathogen was severely compromised under higher humidity. This indicates that RH has a role in early stages of the infection where virulence cor^- bacteria is increased due to higher humidity. Taken together, these results indicate that innate response of the plant such as closing of stomata due to pathogen is adversely affected by the environmental factor like relative humidity. This leads to questioning whether environment regulates the plants' innate immunity. As the environment around the plants is constantly changing**,** this aspect of study becomes very appealing and important.

4.2 Introduction

Acquisition of functional stomata together with development of relatively impermeable cuticle and vascular system are thought to be key events in the evolution of higher plants. Stomata can be regarded as structures that tailor plant gas exchange to suit prevailing environmental conditions (Xie *et al*. 2006). As a consequence, stomatal response is regulated by various environmental cues. Relative humidity (RH) is one of the environmental variables that controls stomatal aperture. In general, stomata close in

response to low humidity. This is believed to help prevent the plant from losing excessive water to the dry environment (Lange *et al.* 1971). Recent studies have revealed that RH controls the short term stomatal sensitivity to carbon dioxide in *Vicia faba* (Talbott *et al.* 2003). Similarly, in *Phaseolus vulgaris*, functional stomata seemed to develop when seedlings were transferred from high to low RH environments (Pospisilova 1996). Despite the known role of RH in stomatal response, little is known about molecular and physiological events underlying the stomatal response to RH. Some genes involved in stomatal response to reduced RH such as *OST1* and *ABA2* have been identified that are active components of ABA signaling as well. This has led to believe that some intracellular signaling proteins are shared among guard cell response to different stimuli and guard cell signaling is organized as a complex network (Xie *et al*. 2006).

Severe outbreaks of bacterial disease in crop plants are often associated with periods of heavy rainfall or high humidity. Similar observation has also been made in model pathosystem of Arabidopsis and *P. syringae* and this phenomenon is often exploited to increase the chances of the disease development in laboratory setting. Usually in a laboratory, plants are incubated under high RH immediately following infection. This has found to promote disease development and severity significantly. Interestingly, it was observed that virulence of the *P. syringae* coronatine deficient strain, *Pst* DC3118 increased with high humidity (Melotto and He, unpublished results). As shown in figure 3.1, inoculation experiments were conducted with *Pst* DC3000 and *Pst* DC3118 under two different air humidity levels (85% and >95%). The increased population of cor mutant, *Pst* DC3118 under higher RH suggest that high humidity could partially compensate for coronatine deficiency and significantly increase the virulence of cor mutant bacteria. This also hints that stomatal response to pathogen is impaired under these conditions. As stomatal response is a part of plants' innate immunity, a question that needs to be addressed is: do environmental conditions such as RH have an effect on plants' innate immunity? Plants have evolved an array of rapid and efficient defense against a wide variety of pathogens including bacteria, fungi, viruses, and nematodes. One of the important weapons is the hypersensitive response (HR). HR response is rapid and localized to the site of infection and characterized by plant tissue death at that site (Cohn *et al*. 2001). Another immune response is triggered when pathogen or microbe associated

molecular patterns (MAMPs) are perceived by the plants and results in basal immune response against the pathogens. If relative humidity affects the innate immunity response of the plants, relative humidity might promote pathogen entry and proliferation in the plant.

One interesting aspect of plant-pathogen interactions is environmental factors which often are the important players in these interactions. Pertaining to the stomatal defense against bacterial pathogens, stomatal behavior needs to be assessed when exposed to multiple stimuli at the same time. These studies will help elucidate what happens in nature where stomata experience a constant influx of multiple stimuli at the same time. Do stomata have a mechanism to prioritize their response to different stimuli? An attempt has been made to address these questions in this research. *Pst* DC3118, a coronatine defective mutant, was used to assess the effect of RH in bacterial-triggered stomatal closure. Use of this bacterium help analyze the role of RH in plant-bacterium interaction in absence of any known virulence factor that triggers stomatal response.

A positive correlation between natural disease outbreaks and periods of heavy rainfall has been documented several times. However, how these environments promote the infection and contamination of plants by pathogens is virtually unknown. Mechanical wounding of plant tissues due to rain might be one way which allows pathogens bypass the stomatal route and gain unprecedented access to plant interior. Splashing of microorganisms during rain may also contribute to the spreading of disease at a higher rate. Another possibility is that stomatal defense is highly compromised during periods of high rainfall. This would allow unrestricted entry of pathogens into plant and consequently produce more severe disease. Understanding the underlying mechanisms of bacterial infections under high RH will help predict outbreaks in fields and might also help employ preventive measures to limit disease outbreaks.

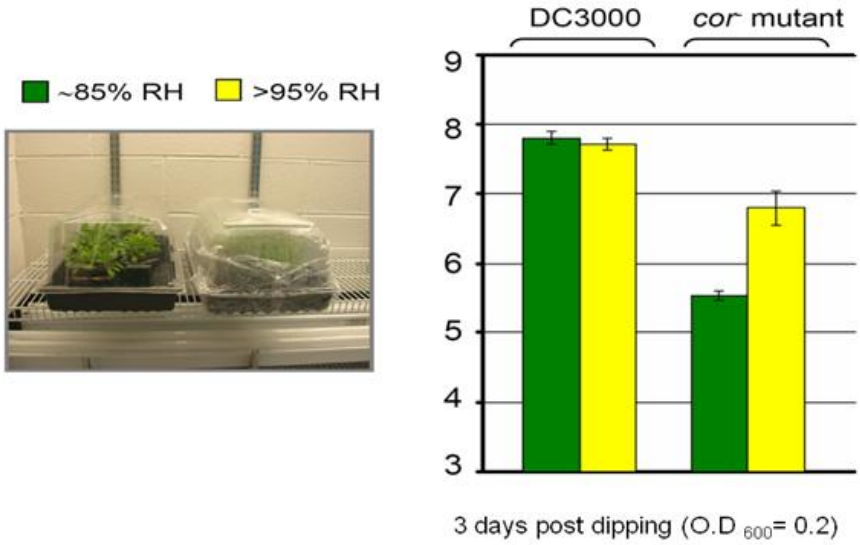


Figure 4.1 Effect of air humidity on the virulence of *cor* mutant bacteria.
Wild-type Col-0 plants were dipped into suspensions (1×10^8 CFU/ml) of *Pst* DC3000, *Pst* DC3118 (*cor* mutant). The inoculated plants were kept under two different relative humidity (RH) conditions (left flat: 85% RH vs. right flat: >95% RH). Bacterial multiplication was assessed 3 days after inoculation (Melotto and He, unpublished results).

4.3 Methods

4.3.1 Stomatal Assay Of Pst DC31118-Infected Plants Under Varying RH

Arabidopsis plants were acclimated under 100µmol/mm^2/sec light, 25^{o}C temperature, and varying RH of 65±5%, 80% and 95±5% for 12 hours. Plants from each RH condition were dip inoculated with *Pst* DC3118 as described in item 2.3.3. A different set of plants were dipped in mock inoculum of water and 0.03% silwet as well. Immediately following infection, plants were incubated under same RH for 4 hours. To assess the stomatal response, 3 leaves were obtained from each plant and stained with PI as described in item 2.3.8. Microscopic observations and statistical analysis was done as described in items 2.3.9 and 2.3.10 respectively.

4.3.2 Disease And Population Assessment Of Pst DC3118 Infected Plants Under Varying RH

Two contrasting humidity conditions were selected to conduct the inoculation procedure. Arabidopsis plants were acclimated under 100µmol/mm^2/sec light, 25^{o}C temperature, and varying RH of 65±5% and 95±5% for 12 hours. Plants from each RH condition were dip inoculated with *Pst* DC3118 as described in item 2.3.3. Immediately following infection, plants were incubated under same RH for 3 days.

Disease symptoms were assessed during the incubation and bacterial population was enumerated on 3dpi as described item 3.3.3.3.

4.4 Results

4.4.1 Effect of RH In Bacterium-triggered Stomatal Closure

Stomatal assay was done with leaves from plants infected under different humidity conditions following 4hrs of incubation. The results have been shown in Figure 4.2. Under 65% humidity, the bacteria treated leaves showed remarkable closure in stomatal aperture when compared with water treated leaves. Statistically, the closure due to bacteria at 60% RH was significant at p=0.01 (indicated by **) in figure 4.2. As humidity is increased to 80%, the stomatal response to bacteria was smaller when compared to water treated plants. The difference is statistically significant at p=0.05 (indicated by * in the figure). At the 95% humidity, the bacteria triggered stomatal closure was negligible.

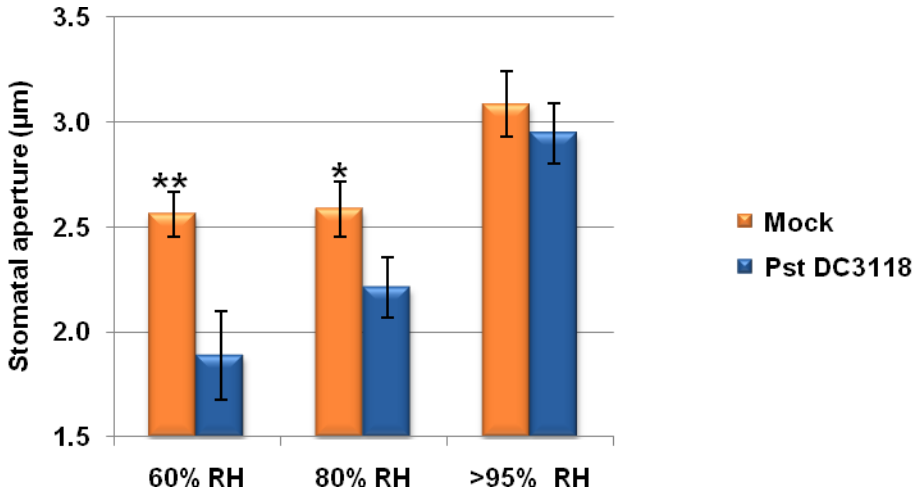
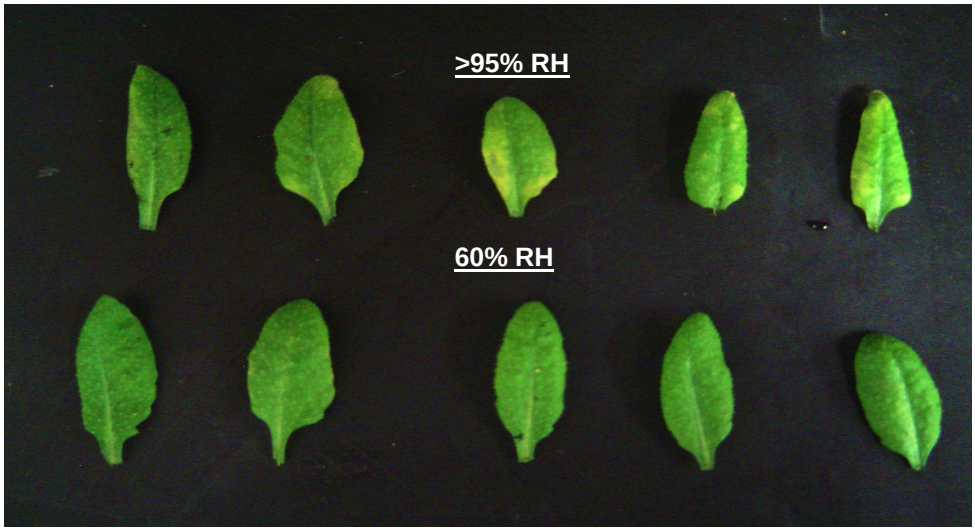


Figure 4.2 Stomatal response to *Pst* DC31118 under varying RH**.** Wild-type Col-0 plants were dipped into suspensions (1 × 10^8 CFU/ml) of *Pst* DC3118 (*cor* ⁻ mutant). The inoculated plants were kept under three different relative humidity (RH) conditions (60%, 80% and >95% RH). Stomatal response was assesses 4 hours after inoculation. Results are shown as the mean (n=50-70) ± standard error. Statistical significance was detected with two-tailed Student's t-test.

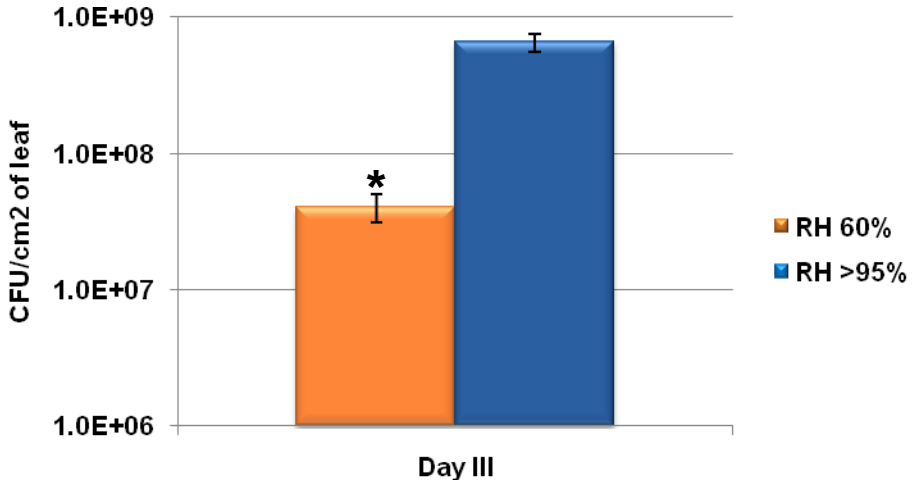
4.4.2 Effect Of RH In Disease Production And Bacterial Population In Arabidopsis

Dip inoculation was conducted with plants acclimated under two contrasting RH of 60% and >95%.Figure 4.3 present the results obtained 3dpi. Figure 4.3(a) shows the disease symptoms in leaves

infected under >95% RH (top) and 60% RH (bottom). The leaves infected under 60% RH were less chlorotic and contained fewer necrotic lesions as compared to leaves infected under >95% RH. These observations are complemented by the bacterial population in the apoplast of the infected leaves (figure 4.3.b). The population of *Pst* DC31118 was significantly higher (~100fold) in plants under higher RH than under lower RH.



(a)



(b)

Figure 4.3 Effect of RH in disease production and population of cor⁻ mutant bacteria a) Disease produced by *Pst* DC3118 under >95% RH (top) and 60% RH (bottom) b0 Population *Pst* DC3118 in plants infected under 60% (orange bars) and >95% RH (Blue Bars) 3 dpi. Statistical significance was detected with two-tailed Student's t-test.

4.5 Discussion

Relative humidity is one of the key regulators of stomatal movements and dictates stomatal opening in under higher humidity and closure under dry conditions. This allows plant to adjust to the environmental conditions. Plant pathogens also have an impact on stomata. Stomata respond to pathogen by actively closing its pore, thereby restricting the entry of harmful pathogens in the plant.

Stomata are now considered a part to plants innate response and believed to have a role in evading pathogen penetration inside the plant. In nature, both of these factors are presented simultaneously to stomata. Hence, the question that arises is: “What is the overall response of stomata to the combined effect of these two contrasting factors?” The results indicate guard cells prioritize their response when expose to the multiple stimuli at the same time. The stomatal response to the bacteria was compromised under high humidity while the response to pathogen under normal humidity was intact. These results were complemented by the disease manifestation in plants. Plants infected under higher RH were clearly more symptomatic as compared to plants infected under normal humidity. The disease development and l population of coronatine mutant strain, *Pst* DC3118 in the apoplast of the plant was comparable to that of *Pst* DC3000. This suggests that high humidity might partially compensate for coronatine deficiency and help bacterial penetration and proliferation inside plant.

Another interesting aspect lies in the innate immune response of the plant under varying environmental conditions. Innate immunity of the plant appears to be compromised under high humidity. Does this mean that environmental cues can impact plants immunity? Plants innate response might be evolving or at least being affected constantly with change in environment.

If the guard cells are prioritizing their response the stimuli, there should be a mechanism that allows this regulation. The molecular players of this regulation are virtually unknown.

Further studies needs to be done to assess the effect other environment factors in the stomatal response to pathogen. This will provide an enhanced picture of what actually happen to the plant when they are in nature and how they response to multiple stimuli at the same time.

4.6 Conclusion

This study uncovers an interesting aspect of host-bacterium interaction in the phyllosphere. In absence any known virulence factors, bacterium relies on the environment conditions to overcome stomatal defense. Suppression of stomatal defense is likely to be a key factor in disease production and bacterial proliferation inside the host plant.

APPENDIX A

INOCULATION OF ARABIDOPSIS WITH CORONATINE MUTANT PATHOGEN, *PST* DB29

INTRODUCTION

Coronatine consists of two distinct moieties that are joined by an amide linkage a) coronafacic acid (CFA) and b) coronamic acid (CMA). *Pst* DB29 was characterized when Brooks *et al*. (2005) screened for Tn5 insertion mutants of *Pst* DC3000 with reduced or altered virulence on *A. thaliana*. The mutant is deficient in synthesizing both CFA and CMA (Brooks *et al.* 2003). Here, the disease production by this mutant is compared with the wild type *Pst* DC3000 through surface inoculation as well as vacuum infiltration. Dip inoculation requires bacteria to overcome the stomatal defense to enter the plant interior whereas vacuum infiltration delivers bacteria directly inside the plant. The results presented here has allowed the comparison of published results for other coronatine mutants with *Pst* DB29 and also helped in characterizing this newer strain of *Pst* in terms of disease production in Arabidopsis.

METHODS

Two methods of inoculation of Arabidopsis plants with wild type strain, *Pst* DC3000 and *Pst* DB29 were used for this study. Two methods of inoculation studied here are 1) dip inoculation 2) vacuum inoculation.

Cell concentration of 10^8/ml was used for dip inoculation whereas 10^6CFU/ml was used for vacuum infiltration. For dip inoculation, 400ml of inoculum is sufficient whereas about 1000ml of inoculum is need for vacuum infiltration. Silwet concentration of 0.03% was added to inoculum for dip inoculation and 0.008% silwet was added to inoculum for vacuum infiltration. The mixtures were homogenized by stirring for 10 minutes. Dip inoculation was done as described in item 2.2.3. During vacuum infiltration, plants were inverted in the inoculum and vacuum pressure of 20mmHg was applied for 1 minute using a vacuum pump. Following infiltration, the leaves of the plants should appear water soaked due to presence of bacterial suspension in leaf apoplast. The plants were left to dry for 3 to 6 hours till they did not look water soaked anymore and then covered with plastic dome till the completion of 3 days.

RESULTS AND DISCUSSION

Arabidopsis plants were infected with *Pst* DC300 and *Pst* DB29 using both surface infection and vacuum infiltration techniques to reiterate the role of coronatine in overcoming stomatal defense. This also allowed comparison of published results with those obtained with another coronatine mutant strain,

Pst DB29. As expected, population of *Pst* DC3000 was 200 fold higher than population of *Pst* DB29 3dpi when dip inoculated (shown in A.1(a)). During dip inoculation, Pseudomonas has to overcome stomatal defense and only coronatine producing strain can effectively do so. However, vacuum infiltration ensures the delivery of bacteria inside the plant, eliminating the stomatal defense faced by the bacteria. In this case, the virulence of the bacteria is not hampered by absence of coronatine. The results of vacuum infiltration of Arabidopsis are shown in figure A.1(b). As indicated by previous results, the bacterial populations of *Pst* DC3000 and *Pst* DB29 are comparable on all three days and are in fact statistically same at p=0.05. The results obtained here agrees with the published results for *Pst* DC31118 (Melotto 2006) and reinforces the role of coronatine in overcoming stomatal defense.

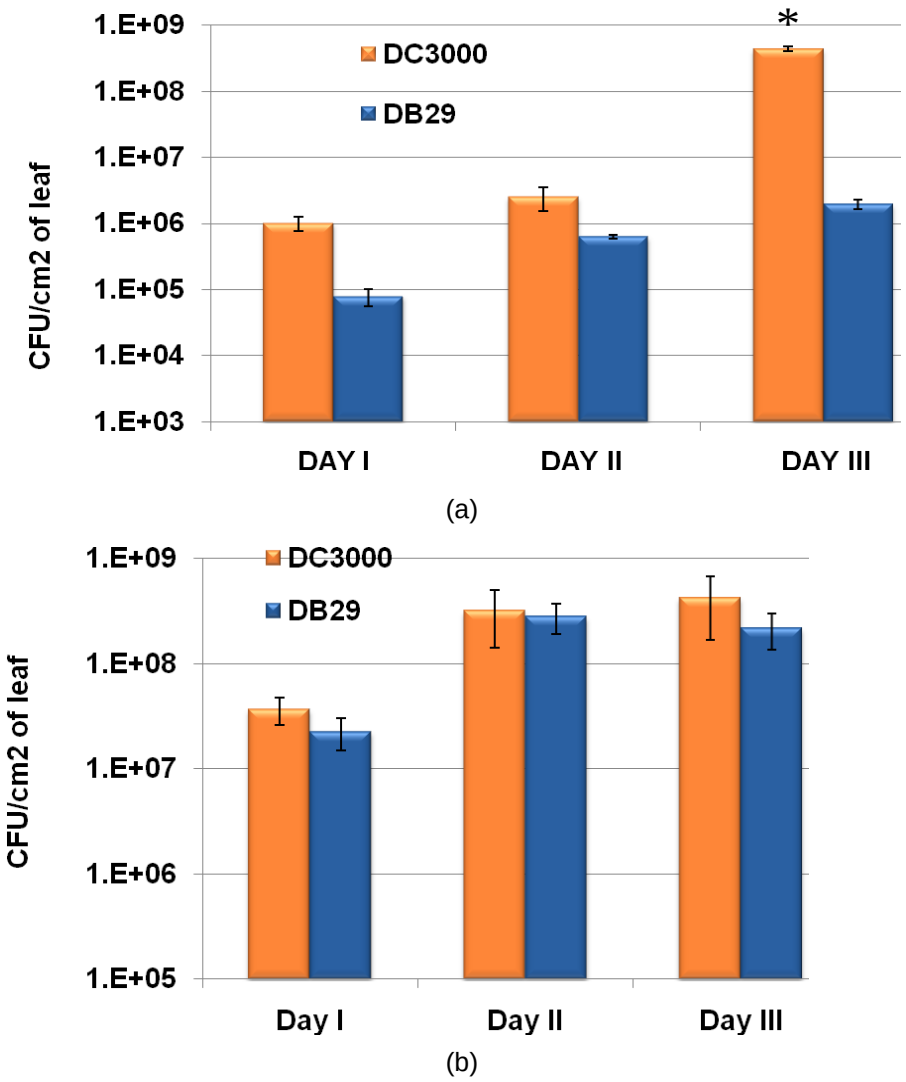


Figure A.1 Inoculation of Arabidopsis with *Pst* DB29 a) Bacterial Enumeration on Days I, II and III after dip inoculation of Arabidopsis with *Pst* DC3000 (orange bars) and *Pst* DB29 (blue bars). Population of *Pst* DC3000 is 200 fold higher than the population of *Pst* DB29 on 3dpi b) Bacterial Enumeration on Days I, II and III after vacuum-infiltrated of *Pst* DC3000 (orange bars) and *Pst* DB29 (orange bars) from infected Arabidopsis. Populations of both strains are statistically the same for same day.

APPENDIX B

DIP INOCULATION OF TGG MUTANTS

INTRODUCTION

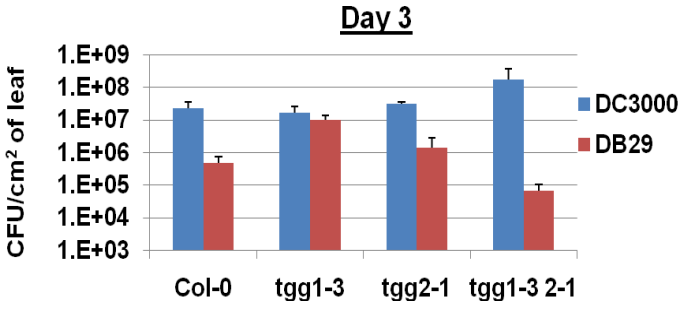
Glucosinolates (1-thio-b-D-glucosides) are the secondary metabolite found in cruciferous plants, including *Arabidopsis thaliana*. These plants also constitutively synthesize and store glucosinolates, whichare converted by endogenous S-glycosyl hydrolases (myrosinases) into compounds that function as insect feeding and/or oviposition stimulants or deterrents. Myrosinases are believed to have a role in plants immune response. The dip inoculation of the transgenic plants deficient in producing these enzymes was conducted to see if these enzymes have a role in stomatal defense.

METHODS

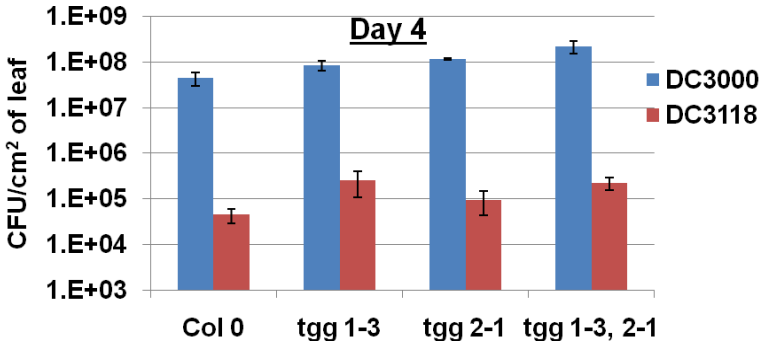
The mutants (tgg 2-1, tgg 1-3, and tgg 2-1, 1-3) were dip inoculated with wildtype bacterium, *Pst* DC3000 and coronatine mutant strain (*Pst* DC3118 or *Pst* DB29). The plants were grown same as wildtype ecotype (described in item 2.3.1). The procedure for inoculated had been described in item 2.3.3. The internal population of the bacteria from the infected plants was enumerated on 3dpi or 4 dpi (see item 3.3.3.3 for procedure). Wildtype ecotype Col0 was used as control.

RESULTS AND DISCUSSION

Disease development and bacterial growth patterns in the infected mutant plants were compared with the Col 0 plants. No significant variation in the disease development pattern was observed for mutant plants. This correlated with the internal population of the bacteria in the infected plants. There was no significant change in the bacterial counts in the mutant plants as compared to wildtype. Figure B.1 represents the bacterial population of the infected plants for all thee infection experiments that was conducted. The population of *Pst* DC3000 was relatively similar for all the instances which showed the variation of 5 fold among experiments. However, there was no statistical significance between the population counts of this bacterium in wildtype or mutant plants. Meanwhile the population of coronatine mutant was varied, and hence no specific conclusions could be drawn from here.



(a)



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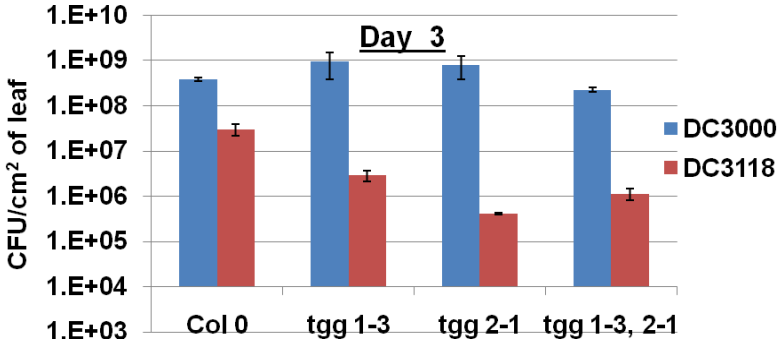

(c)

Figure B.1 Dip inoculation of tgg mutants. Figure (a), (b), and (c) represent the data obtained from 3 different times the inoculation was conducted. The population of *Pst* DC3000 was comparable for all 3 times but the coronatine mutant bacteria did not exhibit any trend.

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BIOGRAPHICAL INFORMATION

Reejana Chitrakar grew up in Nepal. She came to University of Texas at Arlington to pursue her Bachelor's degree in Microbiology. Following her B.S., she worked as a microbiology laboratory technician in Allergan pharmaceutical Inc. for 1.5 years. She returned to UTA as a graduate school in fall 2008 and joined Dr. Melotto's lab as a master student. She worked as graduate teaching assistant in microbiology lab throughout her graduate career. With the completion of this degree, she plans to work as a biology scientist in an industrial setting.