SALMONELLA INFECTION ON ARABIDOPSIS SEEDLINGS REQUIRES BOTH HOST AND PATHOGEN FACTORS

A Thesis

by

YULAN ZHANG

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

December 2010

Major Subject: Plant Pathology

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Approved by:

Chair of Committee,	Libo Shan
Committee Members,	Mike Kolomiets
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ABSTRACT

Salmonella Infection on Arabidopsis Seedlings Requires Both Host and Pathogen Factors.

(December 2010)

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Chair of Advisory Committee: Dr. Libo Shan

Human enteric pathogen Salmonella contaminates raw produce and triggers significant economic loss and illness. Under a natural environment, Salmonella resides in soil and enters the interior of plants without causing disease or eliciting symbiotic growth. Upon being consumed by humans, complex virulence mechanisms are elicited by the specific intestine conditions, such as high temperature and humidity and lead to profound infection. The lack of effective prevention and drug treatment are largely attributed to the unclear mechanistic understanding on Salmonella association with environmental media, and in vivo host and pathogen factors required for persistent infection. We have explored the potential of deploying the model plant organism *Arabidopsis thaliana* to tackle this fundamental yet clinically challenging question, as *Arabidopsis* possesses many advantages as a model system, including enriched genomic resources, powerful genetic tools, low maintenance cost and a large collection of individual gene deletion mutants. Our preliminary data demonstrated Arabidopsis seedlings under liquid culture conditions mimicking the intestine environment were infected and killed by salmonella within 2 days upon inoculation. The Arabidopsis system possesses well-developed genetic information and the resources to study host factors required for infection on very short time scales, thus complementing traditional animal genetic studies. We aim to define the pathogen factors

required for this infection. By merging the fields of extremely powerful Arabidopsis genetics and bacterial genetics/genomics, we hope to provide insight into possible new paradigms for addressing salmonella-mediated food born infection

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NOMENCLATURE

SPI	Salmonella Pathogenicity Island
ABA	Abscisic Acid

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1. INTRODUCTION

Salmonella is a genus of Gram-negative, facultative, and rod-shaped bacterium, the cause of zoonosis: salmonellosis and typhoid (Todar, 2008).They are also the major cause of foodborne diseases all over the world. Although in most Salmonella-induced food-poisoning cases, people heal after a very hard time, including fever, abdomen pain, diarrhea, nausea and vomit, young kids and the old may have a severer situation. Death happens sometimes (WHO).

Usually Salmonella-caused food poisonings are upon consumption of contaminated animal products e.g. contaminated eggs. While vegetable sourced Salmonella contamination does happen. From September 2008 to April 2009, an outbreak of Salmonellosis from contaminated peanut butter spread multiple states in the U.S. More than 500 people were infected in this outbreak (CDC, 2009) . In vegetable-sourced Salmonella infection cases, bacteria is often considered from feces or other animal material polluted irrigation water, water for processing deli vegetable. However, in recent year people found that Salmonella may also parasite on plants and show endopathogenic life style. (Brandl 2006; Schikora, Carreri et al. 2008)

It has been shown that Salmonella enters the plant and survive in the interior part of plants (Ref), which pose challenge for fresh produce processing and sanitation. It remains enigmatic that how Salmonella overcome the plant innate immune system and survive as an endophyte. We observed Salmonella killing Arabidopsis seedlings in laboratory conditions. To further test the mechanisms of Salmonella-Arabidopsis interactions, we examined possible factors required by infection, turned out that Salmonella infection on Arabidopsis

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This thesis follows the style of Molecular Plant-Microbe Interactions.

required both host and pathogen factors.

The name of Salmonella came after American veterinary pathologist Daniel Elmer Salmon. In a project he led in 1885, a rod shaped, gram-negative bacterium was separated and identified as the pathogen of swine typhoid (Cornell U.). The genus of Salmonella is a member of the family Enterobacteriaceae, their primary habitat is animal intestine tract. Up to 2000 serovars of Salmonella has been identified by now, among them *Salmonella enterica* serovar *typhi*(S. typhi) and *Salmonella enterica* serovar *typhimurium* (*S. typhimurium*) are most famous for they are the pathogen of typhoid and Salmonellosis (Giannella RA. Salmonella. In: Barron's Medical Microbiology (Barron, 1996)), both diseases attack human beings, Salmonellosis is actually zoonosis. Bacteria enter human intestine tract with contaminated foods, then they penetrate intestinal epithelium cells, replicate in lymph, release endotoxin and new bacteria, cause disease. (Todar, 2008) Symptoms of Salmonella infections include intestine symptoms like diarrhea, vomit, belly ache; the bacteria are also able to spread in system via blood. For most people Salmonella infections are just hard times, however, for infants, young kids, old people and immuno-compromised patients, the infections can be server or even fatal.

Salmonella infections are not rare, in contrast, they are one of the most frequently reported foodborne illness (WHO) Each year Center of Disease Control received approximately 40,000 laboratory confirmed Salmonella infections, mostly by serovar Thyphimurium(CDC, USA). Salmonella transport from individual to individual by feces contaminated materials. Usually Salmonella caused food poisoning outbreak are from contaminated animal sourced food, such as eggs, vegetable food sourced are relatively rare, pathogens are thought to be from contaminated irrigation water, etc. From 2005-2006, a

multistate outbreak of Salmonella infections attacked United States, in this outbreak the food source was raw tomato and the bacteria was believed to from animal feces contaminated water wound the tomato field(CDC, USA)

Although cases were relatively less, people still noticed mechanism of vegetable borne food poisoning. Salmonella was on the list of most concerned. People started to notice that host plants may support Salmonella growth. Schikora and Carreri showed lesions by Salmonella in Arbidopsis leaves 14 days past inoculation (Schikora, Carreri et al. 2008)

Due to the importance of food safety, Salmonella and its story with plants are of great significance. For once Salmonella is confirmed to be able to infect plants; it may set up new conceptions about food hygiene. Also it may bring change in understanding, procedures, and standards for food industry.

2. SALMONELLA KILLS ARABIDOPSIS SEEDLINGS IN LIQUID MEDIUM

2.1. Salmonella kills Arabidopsis seedlings in liquid medium

Bacterial endophytes stay inside plants without hurting them. And Salmonella are among one of them. We wish to test if this endophyte situation would be twisted in certain condition, e.g. liquid medium generated seedlings. Seedlings are weaker, and liquid medium generated seedlings are in better conditions for bacteria growth.

We generated Arabidopsis thaliana ecotype Columbia seedlings in 1ml of 1/2MS+0.5% sucrose medium in tissue culture plates, under a photoperiod of 16hr light/8hr dark. At day 12, we inoculated the seedlings by dropping bacteria suspension into new 1ml of 1/2MS+0.5% sucrose medium, to a final concentration of OD600=0.5; then used the inoculated medium to substitute the old batch, and co-culture for 48hr. We used Pseudomonas syringae pv tomato DC3000 (Pst DC3000), a known Arabidopsis pathogen; E. coli, a known bacteria that was non-pathogenic to Arabidopsis; and H2O as positive and negative controls. After 48 hrs, Pst DC3000 and Salmonella inoculated seedlings turned yellow, the leaves became tender, while E. coli group almost green and H2O group green (Fig. 2.1 and Fig. 2.2).

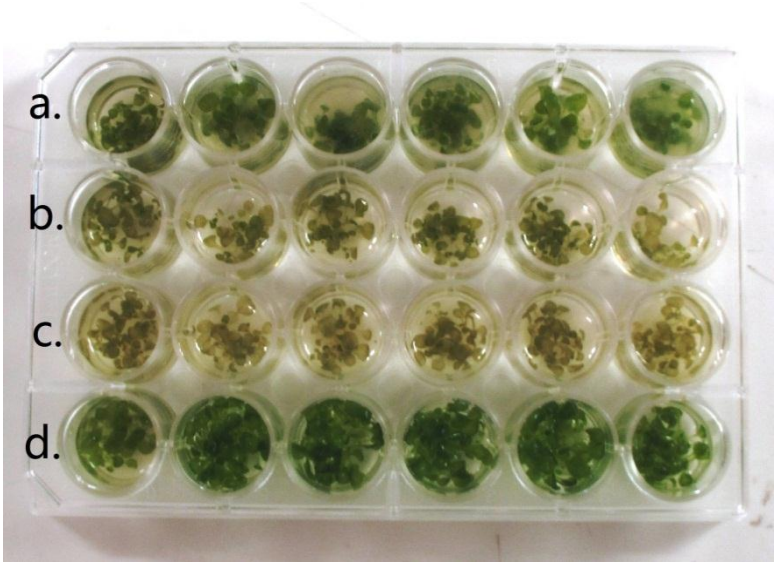


Fig. 2.1 Arabidopsis seedlings survival situation. At 48hrs post inoculation, Pst DC3000 and Salmonella infected Arabidopsis seedlings bleached: a. E.coli inoculation; b. Pst DC3000 inoulation; c. Salmonella; d. H2O

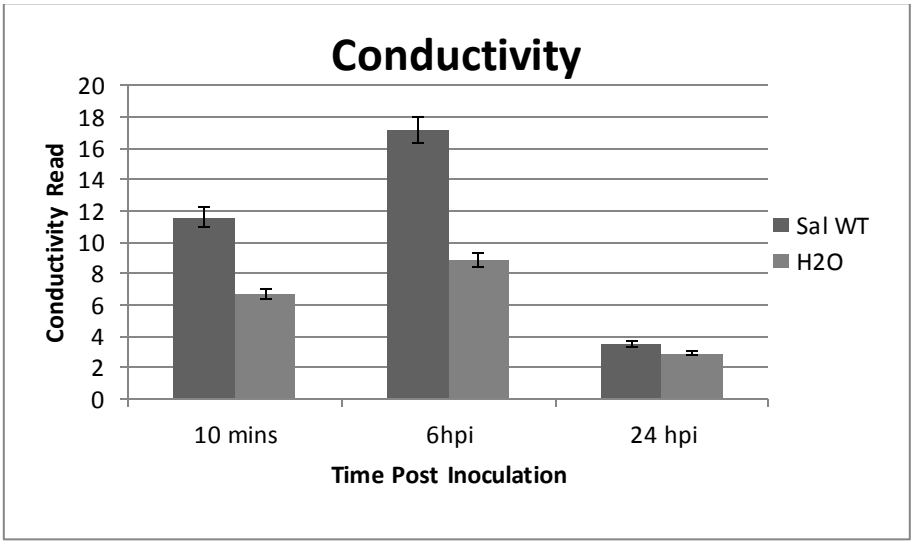


Fig. 2.2 Conductivity raised after Salmonella inoculation.

2.2.Salmonella did not kill Arabidopsis seedlings by released toxins

Salmonella are known to release lipopolysaccharide (LPS), an endotoxin, when infect mammalians. The LPS and the replication of bacteria together hurt the infected mammalian system. When we observed Salmonella brought chlorosis and tenderness to Arabidopsis as fast and server as Pst DC3000, the know plant pathogen, we wished to test possible factors that may involve in this process. The first factor we wished to test was either LPS involved.

We generated Arabidopsis in 1/2MS+0.5% sucrose liquid medium for 12 days. On day 12, we inoculated the seedlings with Salmonella. At 8 hrs post inoculation , we sucked the medium of inoculated wells, filtered half of the medium with microfilter, then drop filtered or not filtered medium to a new well of healthy Arabidopsis seedlings (Fig. 2.3).

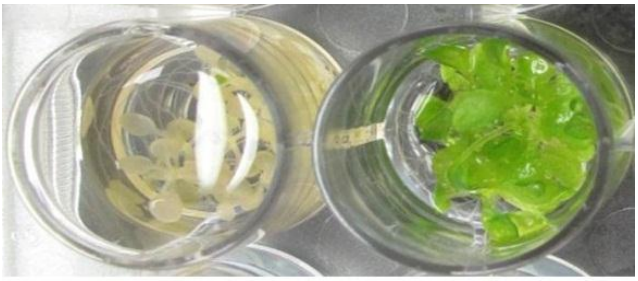
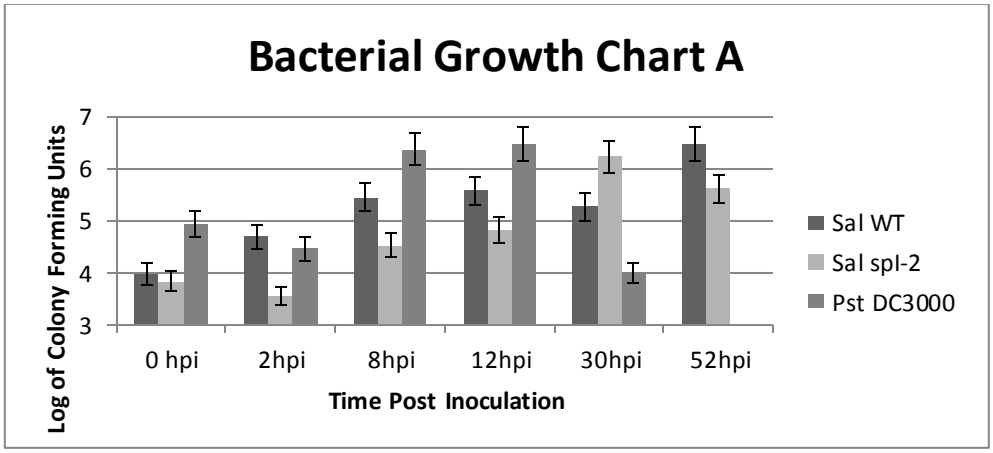


Fig. 2.3 Difference between filtered or non-filtered medium for re-inoculation. 4 days post re-inoculation, the group inoculated with non-filtered medium, plant showed chlorosis and died. The group inoculated with filtered medium remained healthy.

2.3.Salmonella multiplied inside plant

Salmonella do not detach from plant by simply wash or vortex. Hence we soak the seedlings in 70% ethanol for 10 seconds for surface sterile. Then the seedlings were conducted with bacterial count. We ground the seedlings in sterile H2O, diluted the seedlings soup and spread the suspension on LB plates to count the colonies formed by bacteria (Fig. 2.4).



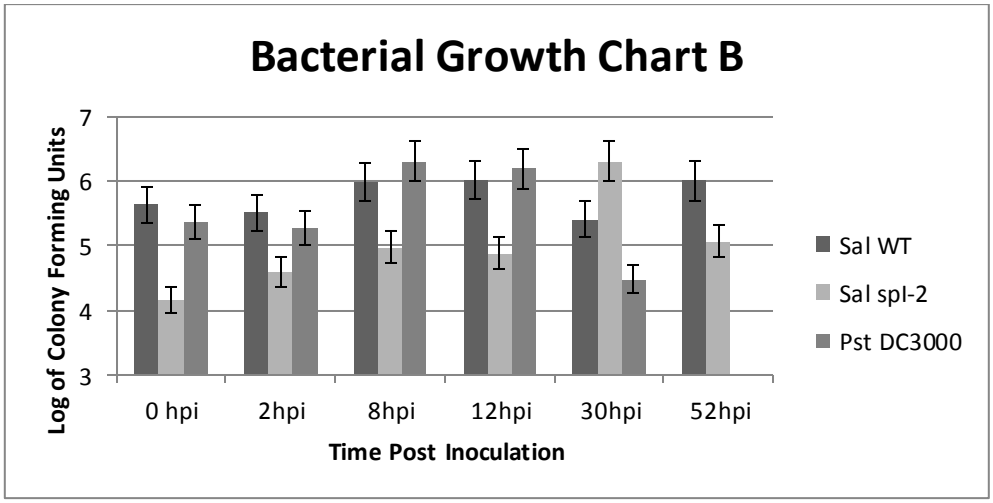


Fig. 2.4 Bacteria growth condition. Bacterial growth chart A：seedlings were surface sterile before count; Bacterial growth chart B: seedlings were not surface sterile before count. The

log of Salmonella wild type colony forming units increased under surface sterile condition; Indicated that bacteria population enlarged a little bit inside the plant tissue.

2.4. Salmonella-Arabidopsis interactions requires living plants

The filter experiment suggested the interaction needed living bacteria, also we thought about if living plants were requested. Thus we designed a leaf soup assay to test the hypothesis. In this experiment, the bacterial growth was not as significant as with living plants (Fig. 2.5).

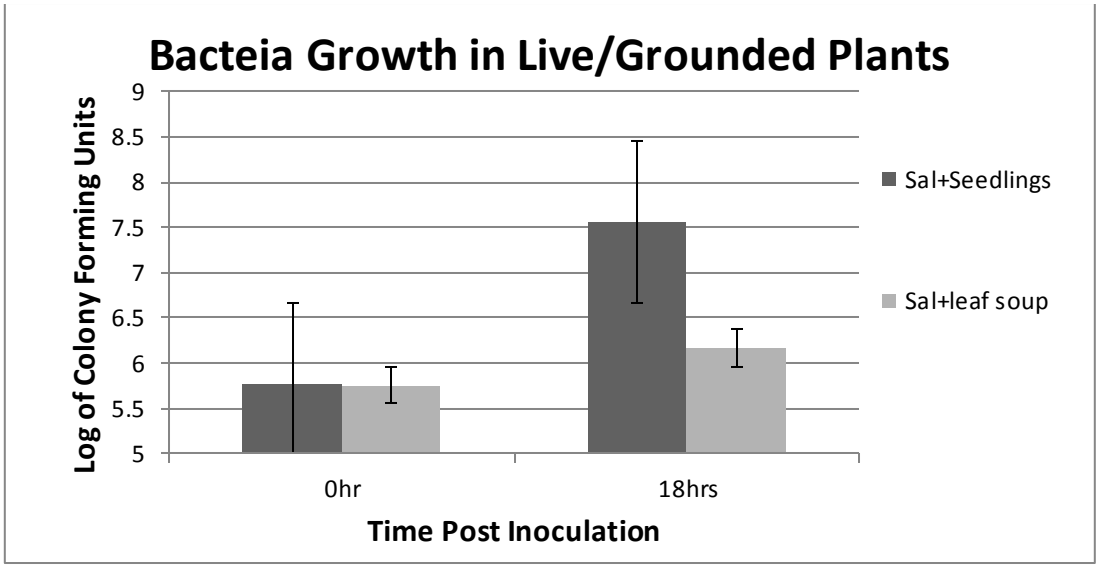


Fig. 2.5 Live Arbidopsis plants required for bacterial multiply. Log of bacterial colony forming units. At inoculation time point 0 and 18 hpi.

2.5. Salmonella associates with Arabidopsis roots

We microscopy examined Arabidopsis seedlings after dropped Salmonella bacterial culture. Under microscopy we saw that the Bacteria associated with Arabidopsis root.

Furthermore, we tested some root-like materials and unattached leaf, turned out bacteria had interest in Arabidopsis roots (Fig. 2.6).

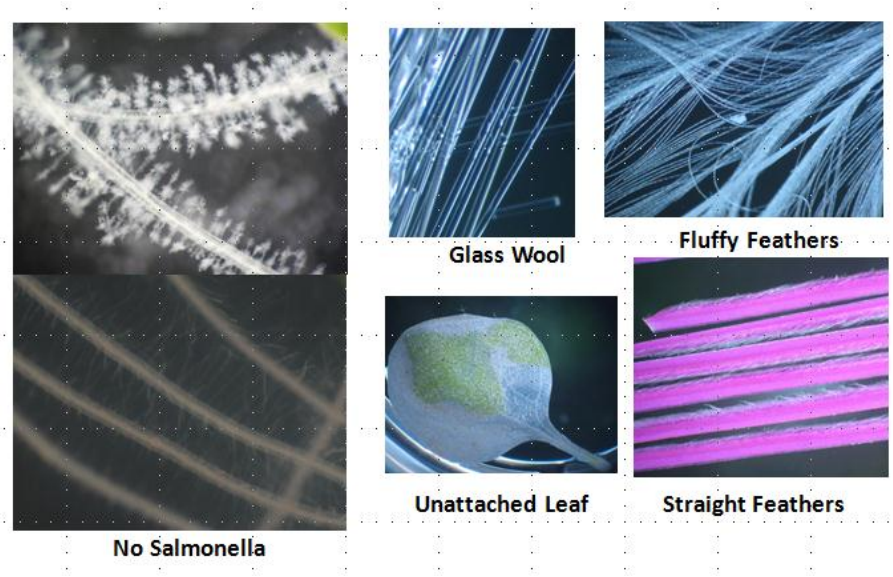


Fig. 2.6 Salmonella associated with Arabidopsis roots

2.6. Salmonella infection on Arabidopsis preferred different carbon sources

We inoculated Salmonella in medium with different carbon sources. In this experiment, the Arabidopsis seedlings were grown in 1 ml of 1/2MS+0.5% sucrose meduium. The carbon source change happened at the inoculation time. Before inoculation, we transferred the seedlings into mediums with different carbon sources. We saw that the seedling in oligose as carbon source mediums bleached out faster than glycerol or mannitol carbon source medium; indicated that Salmonella infection prefers an environment with oligose. Among different oligose carbon sources the difference of bleaching speed was not very

obvious. However, different kind of oligoses did not make much difference in bleaching speed (Fig. 2.7).

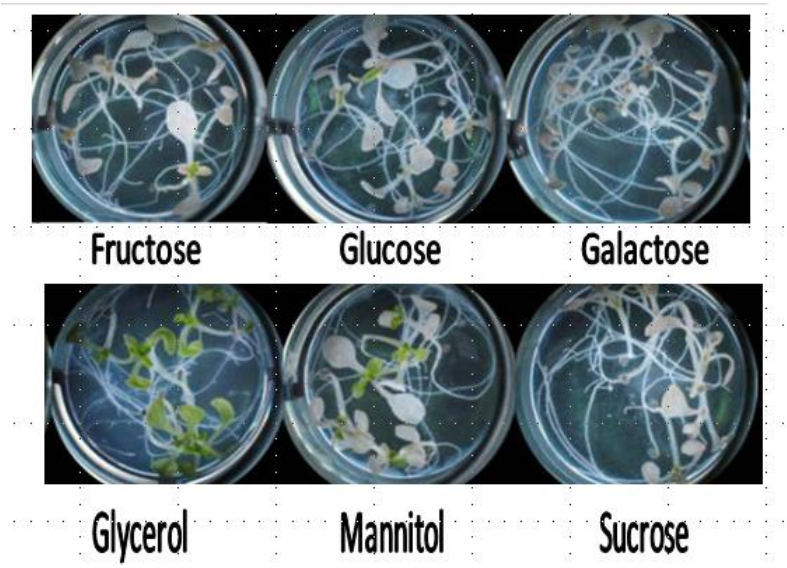


Fig. 2.7 Salmonella infection on Arabidopsis preferred oligose carbon sources

3. SALMONELLA INFECTION ARABIDOPSIS REQUIRES HOST FACTORS

3.1.Mutant screening of Arabidopsis

We screened Arabidopsis mutants by inoculate Arabidopsis seedlings with Salmonella. The more susceptive or resistant mutants were recorded, indicating missing gene traits may involve in the Salmonella-Arabidopsis interactions (Table 2.1).

Table 3.1. List of screened susceptive or resistant mutants. R stands for resistant; S stands for susceptive.

Mutant	phenotype	Mutant	phenotype	Mutant	phenotype
eds1 A	S	etr1 A	R	det2-1 A	R
eds1 B	S	etr1 B	R	det2-1 B	R
det1-1 A	S	dnd1 A	R	Xanthiac A	R
det1-1 B	S	dnd1 B	R	Xanthiac B	R
ein2 A	S	dnd2 A	R	N. Benthamiana A	R
ein2 B	S	dnd2 B	R	N. Benthamiana B	R
nho1 A	S	pmr4 A	R	TW32, tobacco A	R
nho1 B	S	pmr4 B	R	TW32, tobacco B	R
fls2-24 A	S	bri1-119 (CS399) A	R	TW30, tobacco, petuniaA	R
fls2-24 B	S	bri1-119 (CS399) B	R	TW30, tobacco, petuniaB	R

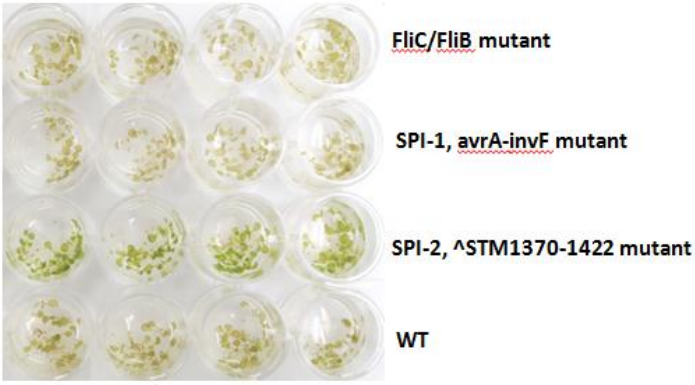
3.2. Ecotype screening of Arabidopsis

We also tried to screen through Arbidopsis ecotypes, see if any ecotypes behave differently from Columbia. We screened more than 100 ecotypes, significant difference from Columbia was not observed

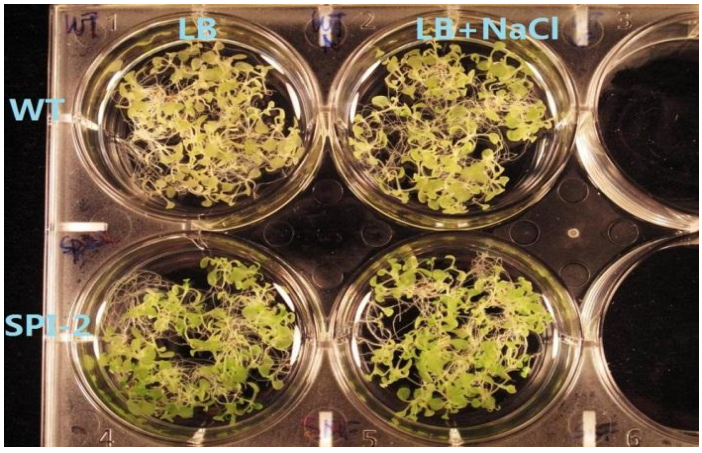
4. SALMONELLA INFECTION ON ARABIDOPSIS REQUIRES PATHOGEN FACTORS

4.1.spI-1 does not play a role, while spI-2 is required

Sp-I and SpI-2 are two major gene islands that confer pathogenicity to Salmonella. SpI-1 codes for type-III secretion system, while SpI-2 is responsible for intra cellular infections.We tested spI-1 and spI-2 mutant Salmonella strains in Arabidopsis, spI-1 did not play a role in infection. While spI-2 is required for Salmonella infection (Fig. 4.1).



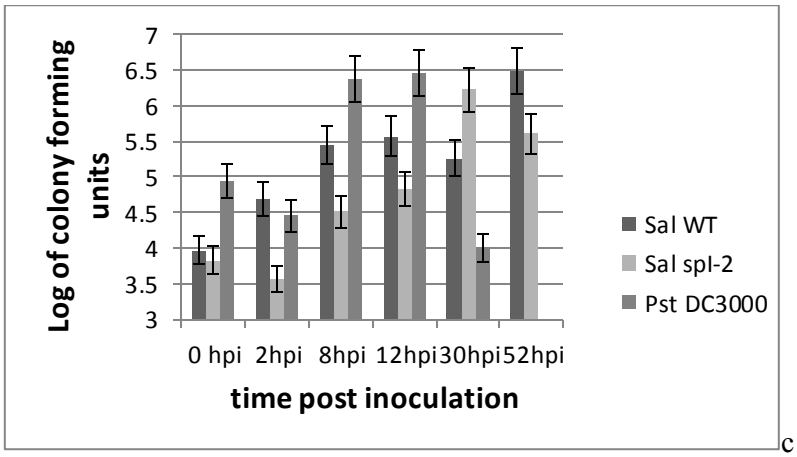
a.



b.

Fig. 4.1 SPI-1 does not play a role in infection while SPI-2 does. (a). spI-2 mutant Salmonella strain inoculated Arabidopsis seedlings kept green. (b). SPI-1 genes are only induced under condition of 0.3M NaCl. In this case we used Sp-1 inducing medium to grow

WT and spI-2 strains, then inoculated them to Arabidopsis seedlings. SPI-1 induction did not rescue loss of spI-2 pathogenicity. (c). Salmonella population enlarge inside plants requires spI-2 existence.



c.

Fig.4.1 Continued.

4.2.Stomata does not involve in Salmonella infection on Arabidopsis

In many cases of plant infections by microbes, the stomata play a role as natural path for the invaders. Some bacteria, e.g. Pseudomonas syringae pv tomato DC3000, even secrets effectors to keep the stomata open. Stomata closure can be induced by ABA within 5 minutes. Thus we pretreated the Arabidopsis with ABA, and then inoculated the seedlings with Salmonella; see if stomata serve as a natural path for the bacteria in our case, too (Fig. 4.2).

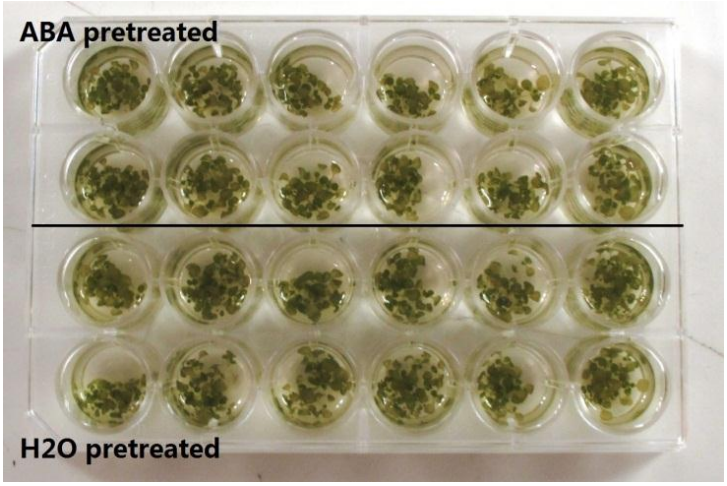


Fig. 4.2. Stomata does not play a role in infection. 48 hrs post inoculation of Salmonella, ABA pretreated or not, the seedlings did not show significant difference, indicated that stomata did not play a role in the infectio

5. SUMMARY AND CONCLUSIONS

Salmonella has long been considered as plant endophyte, means the bacteria only stays in the plant, while not causing any symptoms or diseases (A.Leonardo Iniguez, 2005). However under laboratory conditions we observed Salmonella killing Arabidopsis. In this artificial condition the bacterial population is large, and the plant seedlings, due to the grown environment, may differ from soil crops. This may explain why we can observe killing happened under artificial conditions. This event also suggests that other than just staying in the plant, the bacteria may have ability to do something to the plant. There's necessity of further studying the mechanisms of Salmonella endophytes plants.

We also observed bacteria multiplied inside the plant. Yet bacteria counting only tells the there's more and more bacteria. While the source of the bacteria remains unknown: do they replicate inside the plant, as they did in mammalian system? Or the growing number of bacteria just indicates bacteria rushing into the plant? In either or both ways we get more bacteria inside the plants as time pasts, the phenomenon is interesting.

Another interesting phenomenon in our experiments is that the Salmonella seems to be able of recognizing and swarming to the plant roots. Associating with another phenomenon, that the bacteria kills faster in medium with oligose as carbon source, Salmonella associates with roots might because of some rhizosphere secretion. In mammalian system, the Salmonella is attracted by intestine epithelia cells. The potential relationship between these two kinds of host attraction is interesting, and may help understanding Salmonella pathology on mammalians.

When we look into possible pathogen factors that may involve in the infection, we found that spI-1, a major player in mammalian infections, seems to rest in infection of plants.

While spI-2, another major player in mammalian infections and contributes to bacteria intracellular replication in such cases, shows significance in plant infection. This indicates that infection of Salmonella in plants may hire a different mechanism than in mammalian infections. An even bolder hypothesis is that in the disease cycle of mammalian Salmonella diseases, plant may have a more important role than the bacteria carrier.

We study the Salmonella interaction with Arabidopsis and found some interesting phenomena. These phenomena, more or less, relates to Salmonella infection process in and its pathogenicity to mammalians. We with the future work would focus on eliciting the mechanisms of Samonella infection on Aradbidopsis and hope the work would contribute to better understanding of the most often happened bacteria-related food-borne disease in this world.

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