Biochemical and molecular background of the combination of rhizosphere bacteria from Ethiopia and silicon application to induce resistance in tomato (*Solanum lycopersicum*) against bacterial wilt caused by *Ralstonia solanacearum*

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Dedicated to my father the late

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SUMMARY

Bacterial wilt caused by *Ralstonia solanacearum* is one of the most destructive and economically important diseases of tomato worldwide. Control of *R. solanacearum* has proven to be a very difficult task not only due to its broad distribution and wide host range, but also the limited means of protection measures available. Therefore, use of biotic and abiotic elicitors such as antagonistic rhizobacteria and silicon, respectively, is a possible control strategy. In line with this, 150 strains of rhizobacteria were isolated from Ethiopian soil and screened for *in vitro* antibiosis. Thirteen strains inhibited the growth of *R. solanacearum* and identified as *Pseudomonas spp*., *Bacillus spp*. and *Serratia marcescens*. These strains were further characterized for their plant growth promoting traits. Five strains were selected for *ad planta* tests based on *in vitro* antibiosis results and of the five, *B. cereus* BC1AW and *P. putida* PP3WT reduced bacterial wilt incidence, number of *R. solanacearum* in mid-stems and increased dry weight tomato plants.

The second part of the study focused on the induction of systemic resistance and activities of defence related enzymes such as peroxidase (POD) phenylalanine ammonia lyase (PAL) and lipoxygenase (LOX) elicited by application of elicitor alone or in combination. Application of silicon and rhizobacteria reduced bacterial wilt, bacterial populations in the mid-stems and increased dry shoot weight of the tomato plants indicating the priming effect of each elicitor. However, the combined application of the elicitors did not. In addition non-significant increases of POD and PAL activity were observed in the individual treatments of each elicitor upon

inoculation with *R. solanacearum* .The activity of LOX, however, was decreased in the pathogen inoculated and silicon amended treatment, but increased in the rhizobacteria treatment. During dual application of both elicitors, the activity of POD and PAL, LOX dropped significantly.

In the transcriptome analysis of Si-rhizobacteria mediated gene expression profiling, after inoculation of *Ralstonia solanacearum* we found regulation of 174 genes of which 113 were up-regulated and 61 down-regulated. Here, Si regulated more defence related genes than *B. pumilis*. However, during the simultaneous application of the two elicitors antagonistic interaction occurred between ethylene-jasmonate and salicylate pathways which are elicited by rhizobacteria and silicon, respectively. Therefore, separate application of silicon and rhizobacteria strain is best alternative for the induction of systemic resistance that will switch on defence arsenal of the plant against *R. solanacearum* where Si being the best inducer and controlling agent against the pathogen.

Keywords: Lipoxygenase, peroxidase, phenylalanine ammonia lyase, rhizobacteria, transcriptome

ZUSAMMENFASSUNG

Die durch *Ralstonia solanacearum* verursachte bakterielle Welke ist eine der zerstörerischsten Krankheiten bei Kulturpflanzen wie z. B. der Tomate. Es hat sich herausgestellt, dass die Bekämpfung von *R. solanacearum* sehr schwierig ist, bedingt zum einen durch die weite Verbreitung und das breite Wirtsspektrum, zum anderen durch die begrenzten Bekämpfungsmöglichkeiten. Daher ist der Einsatz von biotischen und abiotischen Elicitoren wie z. B. antagonistischen Rhizobakterien oder Silizium eine mögliche Bekämpfungsstrategie. In diesem Rahmen haben wir 150 Rhizobakterien-Stämme aus Äthiopien isoliert und auf in-vitro-Antibiose gescreent. Die dreizehn Stämme, die das Wachstum von *R. solanacearum* hemmten, wurden als *Pseudomonas* spp., *Bacillus* spp. und *Serratia marcescens* identifiziert. Diese wurden in Hinblick auf wachstumsfördernde Eigenschaften für Pflanzen weiter charakterisiert. Basierend auf *in-vitro*-Tests wurden fünf Stämme für *ad-planta*-Tests gewählt. Die Stämme *B. cereus* BC1AW und *P. putida* PP3WT verminderten das Auftreten von bakterieller Welke und die Anzahl von *R. solanacearum* im Mittelstängel und führten zu einem erhöhten Trockengewicht der Tomatenpflanzen.

Der zweite Teil der Arbeit war auf die Induktion von systemischer Resistenz und die Aktivität von zur pflanzlichen Abwehr gehörenden Enzymen, wie Peroxidase (POD), Phenylalanin-Ammoniak-Lyase (PAL) und Lipoxygenase (LOX), die durch die Applikation der Elicitoren allein oder in Kombination ausgelöst wurde, ausgerichtet Die getrennte Applikation von Silizium und Rhizobakterien verminderte die bakterielle Welke und Bakterienpopulationen im Mittelstängel, und erhöhten das Trockengewicht

der Tomatenpflanzensprosse, was die Sensitivierung ("priming"-Effekt) durch die einzelnen Elicitoren zeigte. Bei der gleichzeitigen Anwendung der Elicitoren wurde dies jedoch nicht nachgewiesen. Zusätzlich wurde ein nicht-signifikanter Anstieg der Aktivität von POD und PAL bei den einzelnen Behandlungen mit Elicitoren nach Inokulation mit *R. solanacearum* beobachtet. Bei der Behandlung mit Silizium nahm die Aktivität von LOX ab, und bei der Behandlung mit Rhizobakterien zu. Bei der gleichzeitigen Applikation beider Elicitoren nahm die Aktivität von POD, PAL und LOX signifikant ab.

Bei der Transkriptom-Analyse von durch Silizium-Rhizobakterien vermittelter Genexpression nach Inokulation mit *R. solanacearum*, haben wir 174 Gene gefunden von denen 113 heraufreguliert und 61 herabreguliert wurden. Hierbei regulierte Si mehr Gene als *B. pumilis*, die mit der pflanzlichen Abwehr in Verbindung stehen. Jedoch trat bei der gleichzeitigen Applikation der beiden Elicitoren eine antagonistische Wechselwirkung zwischen den Stoffwechselwegen von Ethylen-Jasmonat bzw. Salicylat auf, was durch Rhizobakterien beziehungsweise Silizium ausgelöst wurde. Daher ist eine getrennte Applikation von Silizium und Rhizobakterienstämmen die beste Alternative für die Induktion von systemischer Resistenz, die die pflanzlichen Abwehrmaßnahmen gegen *R. solanacearum* aktiviert, wobei Si der beste Induktor und Bekämpfungsmittel gegen das pathogen ist.

Schlagworte: Lipoxygenase, Peroxidase, Phenylalanin-Ammoniak-Lyase, Rhizobakterien, Transkriptom,

ABBREVATIONS

A: Antagonist
ACCO: 1-aminocyclopropane-1-carboxylate oxidase
AHL: Acyl homoserine lactone
ANOVA: Analysis of variance
ASM: Acibenzolar-*S*-methyl
AUDPC: Area under disease progress curve
BABA: ß-aminobutyric acid
BSA: Bovine serum albumin
BTH: Benzothiadiazole
C4-AHL: N-butynol homoserine lactone
C6-HSL: N-hexanoyl-L-homoserine lactone
CAS: Chrome Azurole S
CFU: Colony-forming units
Cy3: Cyanine-3-Tyramide
Cy5: Cyanine-5-Tyramide
DNA: Deoxy ribonucleic acid
DPI: Days post inoculation
ET: Ethylene
GC-FAME: Gas chromatography - Fatty Acid Methyl Ester
HCN: Hydrogen cyanide
IAA: Indoleacetic acid
ISR: Induced systemic resistance
JA: Jasmonic acid
KB: King's B
KK2: Tomato genotype King Kong 2

LB: Luria-Bertani

LOX: Lipoxygenase

LPS: Lipopolysaccahride

MAMPs: Microbe-associated molecular patterns

MAPK: Mitogen-activated protein kinase

NGA: Nutrient glucose agar

NPR1: Non-expressor of PR genes 1

OD: Optical density

PAL: Phenylalaninie ammonia lyase

PAMPs: Pathogen-associated molecular patterns

PBS: Phosphate buffered solution

PGPR: Plant growth promoting rhizobacteria

PMT: Photomultiplier tube

POD: Peroxidase

PPO: Polyphenol oxidase

PRs: Pathogenicity related proteins

PUFAs: Polyunsaturated fatty acids

QSI: Quorum sensing inhibition

ROS: Reactive oxygen species

Rs: *Ralstonia solanacearum*

SA: Salicylic acid

SAR: Systemic acquired resistance

SAS: Statistical analysis system

Si: Silicon

SiIR: Silicon-induced resistance

TTC: Triphenyl tetrazolium chloride

VOCs: Volatile organic compounds

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Chapter 1

General Introduction

1.1 The phytopathogen: *Ralstonia solanacearum*

Ralstonia solanacearum [synonyms: *Pseudomonas solanacearum*] is the casual agent of bacterial wilt disease in many plants (Smith, 1896, Hayward, 1995, Yabuuchi et al., 1995). It is a Gram-negative, strictly aerobic rod bacterium (0.5-0.7 × 1.5-2.0 µm in diameter) classified in the-subdivision of the Proteobacteria (Kersters et al., 1996). Formerly, *R. solanacearum* was a member of the *Pseudomonas* rRNA homology group II that includes many other non-fluorescent pseudomonads (Palleroni et al., 1973). In a taxonomic study based on 16S rRNA sequences, DNA-DNA homologies, fatty acid analysis, and other phenotypic characteristics, *Pseudomonas solanacearum* was categorized into a new genus *Burkholderia* (Yabuuchi et al., 1992). Subsequent study of this genus revealed that *Burkholderia solanacearum* was sufficiently distinct from other members of the genus to warrant assignment to the newly proposed genus *Ralstonia* (Yabuuchi et al., 1995).

The species *R. solanacearum* is responsible for causing the devastating disease in tropical, subtropical and some relatively warm temperate regions of the world where the environmental condition is optimal for the survival of the pathogen (Hayward, 1991). Recently, the geographical spectrum has extended to more temperate countries in Europe and North America as a result of dissemination of strains adapted to cooler environmental conditions (Genin and Boucher, 2004).The host range of *R. solanacearum* is unusually wide for a plant pathogen, including over 450 host species in 54 botanical families (Wicker et al., 2007). Some of its economically important hosts are tomato, potato, tobacco, banana/plantain, cowpea, peanut, cashew, papaya, and olive. There are also weed and asymptomatic hosts that may

play a role in the survival and persistence of *R. solanacearum* (Hayward, 1994; Granada and Sequeira, 1983; Moffett and Hayward, 1980). In tomato, the disease may lead to yield loss of 75-100% (Kishun, 1987; Nirmila et al., 2002).

Ralstonia solanacearum is a highly heterogeneous bacterial species. Based on host range, the species is divided into five races (Buddenhagen et al., 1962; He et al., 1983; Pegg and Moffet, 1971) and according to the ability of species to metabolize three sugar alcohols and three disaccharides into six biovars (Hayward 1964, 1991, 1994; He et al., 1983). Both classifications lack an exact concordance with the genetic background of the complex members. Therefore, molecular-based assessment of the genetic diversity of *R. solanacearum* employing restriction fragment length polymorphism analysis resulted in two clusters of strains as divisions 1 Asiaticum and 2 Americanum (Cook et al., 1989; Cook and Sequeira, 1994). Recently, a phylogenetically meaningful classification scheme was developed based on DNA sequence analysis (Fegan and Prior, 2005; Fegan and Prior, 2006). This scheme divides the complex species into four phylotypes that broadly reflect the ancestral relationships and geographical origins of the strains. Accordingly, phylotype I, II, III and IV strains are originated in Asia, America, Africa, and Indonesia, respectively.

The phylotypes are further subdivided into sequevars based on the sequence of the endoglucanase (*egl*) gene (Fegan and Prior, 2005; Fegan and Prior, 2006). *R. solanacearum R3bv2* strains belong to phylotype II and sequevars 1 and 2 (Fegan and Prior, 2005). This phylotyping scheme proposed by Fegan and Prior (2005) is consistent with the former phenotypic and molecular typing schemes and adds

valuable information about the geographical origin and in some cases the pathogenicity of strains.

R. solanacearum invades the plant through wounded roots or at sites of secondary root emergence, although aerial transmission by insects has also been reported for certain strains. After entering the plant, the bacteria proceed to the xylem vessels and spread rapidly to aerial parts of the plants through the vascular system. After 5-6 days, *R. solanacearum* cells can be readily detected throughout the stem (Saile et al., 1997; McGarvey et al., 1999). At this stage plants begin to show an extensive wilting, probably from reduced sap flow caused by the presence of large amounts of *R. solanacearum* cells and their exopolysaccharide (EPS I) slime in xylem vessels (Schell, 2000). The cell wall degrading enzymes such as pectin methyl esterase (Pme) and polygalcturonases (PehA, PehB, PehC), proteases and glucanses (Schell, 2000) generate low-molecular weight products for the assimilation of the bacterium and enhance aggressiveness of the pathogen (Gonzalez and Allen, 2003). Plants rapidly collapse and die with further degradation of vessels and surrounding tissues resulting in return of *R. solanacearum* cells back to a saprophytic life in the soil, awaiting a new host (Kelman and Sequeira, 1965).

Control of *R. solanacearum* has proven to be a very difficult task not only due to its broad distribution and wide host range, but also the limited means of protection measures available (Genin and Boucher, 2004). The use of soil fumigants or antibiotics is environmentally destructive, expensive, and largely ineffective against bacterial wilt (Saddler, 2005). The main control strategy has been the use of resistant varieties. However, such resistance is liable to breakage at an ambient temperature

by virulent and highly polymorphic strains of the pathogen and also in presence of root-knot nematodes (Prior et al., 1994; Wang and Lin, 2005). Alternatively, the use of biotic and abiotic elicitors such as antagonistic rhizobacteria and silicon, respectively, proved to be an alternative to control the wilt disease. Application of antagonistic rhizobacteria as biocontrol agents and soil amendments has been used to enhance host plant resistance (Anith et al*.,* 2004). Previous studies indicated the control of bacterial wilt using various species of antagonistic rhizobacteria (Lemessa and Zeller 2007; Kurabachew et al., 2007). The rhizobacterial species *B. subtilis, P. macerans, S. marcescens, B. pumilis* and *P. fluorescens* (Aliye et al, 2008), and *Bacillus sp.* and *Pseudomonas sp.* (Ramesh et al, 2009) were reported to reduce bacterial wilt under *in vitro* and *in vivo* conditions. Recently, Silicon (Si) amendment has been reported to significantly reduce bacterial wilt incidence in tomato (Dannon and Wydra, 2004; Wydra et al*.,* 2005; Diogo and Wydra, 2007). Houng, (2006) reported on the biochemical and phenotypic response due to biotic and abiotic elicitors on tomato against bacterial wilt. But to date there is no report on the effect of single and combined application of both biotic and abiotic elicitors on the different defense enzyme activities and on gene profiling expression after *R. solanacearum* inoculation in the primed tomato plant. Thus, in the present study antagonistic rhizobacteria from tomato and potato fields of Ethiopia were characterized and evaluated for their biocontrol potential, and, induction of systemic resistance by antagonist and silicon was studied by analyzing the common defense enzymes and differentially regulated genes in response to elicitor application.

1.2 The rhizosphere

The term “rhizosphere" was coined by Hiltner in 1904, defined as a volume of soil surrounding plant roots, much richer in the diverse community of microorganisms than the surrounding soil. The rhizosphere is a habitat where several biologically important processes and interactions take place which are driven by root exudates (Lugtenberg et al., 2001; Walker et al., 2003). Within this community of competing and interacting microbes, a whole range of parasitic and beneficial microorganism (plant growth promoting rhizobacteria) is found that either cause disease or enhance plant performance, respectively.

1.2.1 Plant growth promoting rhizobacteria

Plant growth-promoting rhizobacteria (PGPR) are a class of soil-borne microbes with beneficial effects on plant performance. They enhance plant growth and yield by fixing atmospheric nitrogen (Hong et al., 1991), solubilizing minerals such as phosphorus (Whitelaw, 2000), producing plant growth regulators hormones (Beyeler et al., 1999), producing siderophores that sequester iron (Glick, 1995), decreasing heavy metal toxicity (Burd et al., 1998), promoting mycorrhizal function (Garbaye, 1994) and regulating ethylene production in roots (Glick, 1995). They also promote plant growth by enhancing the plant’s photosynthetic capacity (Zhang et al., 2008) and by increasing tolerance to abiotic stress (Yang et al., 2009). Furthermore, they can reduce the activity of soil-borne pathogens in disease-suppressive soils (Weller et al., 2002; Duff et al., 2003) and provide the first line of defense for the plant against pathogen (Mazzola, 1998) and also insect herbivory (Van Oosten et al.,

2008). The disease suppressive activity of PGPR is exerted either directly by hampering growth and development of soil-borne pathogens through competition for nutrients or secretion of antibiotics in the rhizosphere (Bakker et al., 2007; Kamilova et al., 2008), siderophore-mediated competition for iron, and production of lytic enzymes (Van Loon and Bakker, 2003), or indirectly by eliciting a plant-mediated systemic resistance response (Kloepper et al., 2004; Van Wees et al., 2008). In addition they are known to control the development and persistence of plant bacterial pathogens through inactivation or suppression of the quorum sensing regulatory mechanism by a phenomenon known as quorum sensing inhibition (quenching) (Zhang, 2003).

1.3 Systemically induced disease resistance

1.3.1 Systemic acquired resistance

During evolution plants have developed sophisticated defensive strategies to perceive pathogen attack and to translate this perception into an appropriate adaptive response. In response to microbial attack, plants activate a complex series of responses that lead to the local and systemic induction of a broad spectrum of antimicrobial defenses (Hammond-Kosack and Jones, 1996). Local infection by a necrotizing pathogen leads to a HR, and the enhanced state of resistance extends systemically into the uninfected plant parts. This long-lasting and broad-spectrum induced disease resistance is referred to as systemic acquired resistance (SAR) (Ross, 1961; Durrant and Dong, 2004). The induction of SAR is accompanied by local and systemic accumulation of endogenous levels of the plant hormone salicylic

acid (SA), followed by the coordinate activation of a specific set of pathogenesis-related (PR) genes, many of which encode PR proteins with antimicrobial activity (Van Loon et al., 2006). The importance of the accumulation of SA for the expression of SAR was demonstrated by using transgenic NahG plants. These plants express the bacterial salicylate hydroxylase *nahG* gene which converts SA into catechol, a product that does not induce systemic resistance and makes the plant incapable of accumulating SA or PRs and unable developing SAR in response to necrotizing pathogens (Gaffney et al., 1993). Therefore, transgenic NahG plants do not show a SA mediated response indicating the central role of this phytohormone in SAR (Ryals et al., 1996).

Transduction of the SA signal into *PR* gene expression requires the regulatory protein nonexpressor of PR Genes1 (NPR1) (Dong, 2004). Mutant *npr1* plants can accumulate normal levels of SA after pathogen attack, but are incapable of transducing the accumulated SA into response leading to *PR* gene expression and SAR.

1.3.2 Induced systemic resistance

Plant growth-promoting rhizobacteria (PGPR) are among the various groups of plant-associated microorganisms that can elicit plant defense (Van Loon and Glick, 2004). Systemic resistance triggered by beneficial microorganisms confers a broad-spectrum resistance that is effective against different types of plant pathogens such as viruses, bacteria, and even insect herbivores (Van Wees et al., 2008). Among the induced systemic resistance (ISR) inducing PGPR documented to date are many

non-pathogenic *Pseudomonas spp*. and *Bacillus spp*. (Kloepper et al., 2004; Van Loon and Bakker, 2006).

Induction of a plant-mediated ISR response starts with the recognition of the beneficial microorganism. In the plant-microbe interaction, both the pathogenic and beneficial microorganisms are specifically recognized by the plant through conserved microbial cell surface components, collectively called pathogen or microbe-associated molecular patterns PAMPs or MAMPs, respectively (Schwessinger and Zipfel, 2008; Van der Ent., et al., 2009).

Interaction of a PAMP with the corresponding pattern recognition receptor of the plant activates a primary defense response that is called PAMP-triggered immunity (PTI) (Jones and Dangl, 2006; Schwessinger and Zipfel, 2008). On a similar way , in PGPR a diversity of MAMPs such as flagellin, LPS, Fe^{3+-}chelating siderophores, antibiotics, biosurfactants, and even volatile organic compounds (VOCs) are known to elicit ISR (Iavicoli et al., 2003; Raaijmakers et al., 2006; Ryu et al., 2004; Bakker et al., 2007).

In rhizobacteria mediated ISR, the signal transduction is mediated by the phytohormone jasmonic acid (JA) in concert with ethylene (ET) path way (Van Loon and Bakker, 2006). Different studies indicated that treatment of the roots with ISR-inducing WCS417r bacteria failed to trigger ISR in JA-insensitive *jar1* plants or ET-insensitive *etr1* plants. This indicates the pivotal role of JA and ET-pathways in the establishment of ISR (Figure 1) (Pieterse et al., 2000).

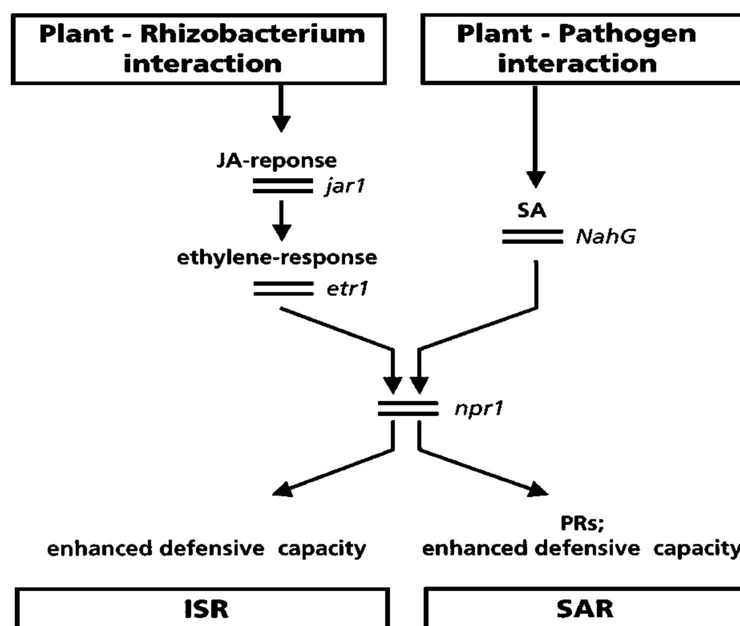


Fig. 1:1 Schematic representation of the signal transduction pathways leading to ISR and SAR, including the putative positions of different mutants as postulated by Pieterse et al. (1998) and Ton et al. (2002).

Unlike SAR where resistance induction is followed by production of SA, in ISR colonization of the roots by ISR-inducing PGPR is often not associated with an increase in the production of jasmonate (JA) and ethylene (ET) (Pieterse et al., 2000). Hence, ISR seems to be based on increased sensitivity rather than on increased production of these hormones. Thus, the transcriptional changes that occur in the systemic tissue upon colonization of the root by beneficial microbes are relatively weak compared to the massive transcriptional reprogramming that occurs upon pathogen attack (Fu et al., 2007). Consequently, upon pathogen inoculation ISR-expressing plants display an accelerated defense response (Verhagen et al., 2004; Van Wees et al., 2008). This PGPR-mediated sensitization of the tissue for enhanced defense expression is called 'priming' which is characterized by rapid and effective

activation of cellular defense against the attacker, pathogen or insect (Conrath et al., 2006; Frost et al., 2008).

1.4 Silicon

1.4.1 The role of silicon in plant biology

Silicon is the second most abundant element in the lithosphere following oxygen and comprises approximately 28% of the earth crust (Epstein, 1994). Though, Si is not recognized as an essential element for the majority of plants, the beneficial roles of this element in growth, development, yield and plant resistance to biotic stress (disease and pest) and abiotic stress (metal toxicity, nutrient imbalance, salt stress, extreme temperature, radiation and drought), improvement of mechanical properties (stature, soil penetration by roots, exposure of leaves to light, resistance to lodging) have been verified in a wide variety of plant species (Ma, 2004; Hattori et al., 2005). These properties are due to the deposition of amorphous silica ($SiO_2.nH_2O$), and also bioactivity of monosilicic acid. Accordingly, plants are classified into three classes based on their Si-accumulation nature: high accumulators (10-15% Si in dry weight) including wetland grasses and rice; intermediate (1-3% Si in dry weight) including cucumber, and non-accumulators (<1% Si in dry weight) including dicots such as tomato (Jones and Handreck, 1967).

1.4.2 The role of silicon in plant disease resistance

Disease resistance induced by Si has been observed in many plant species including rice, cucumber and wheat. Si enhances rice (Si-accumulator) resistance to many

diseases such as blast, sheath blight, brown spot leaf scad and stem rot (Datnoff et al., 1997; Rodrigues et al., 2003; Fauteux et al., 2005; Cai et al., 2008). Si also increases plant resistance to powdery mildew in wheat, barley, cucumber and Arabidopsis (Fauteux et al., 2005, 2006; Ma and Yamaji, 2006). Recently, Si has been shown to induce resistance in tomato against bacterial wilt caused by *R. solanacearum* (Dannon and Wydra, 2004; Diogo and Wydra, 2007; Schacht et al., 2010).

Si was suggested to activate plant defense mechanisms, but the physiological and molecular mechanisms underlying the Si induced resistance phenomenon are poorly understood. Several studies using Si-accumulator plants, however, attempted to interpret the role of Si in plant resistance, which was mainly attributed to mechanical barriers and induction of resistance components (Fauteux et al*.,* 2005). This resistance could be explained based on cell silicification, the polymerization of silicic acid into silica gel SiO_2H_2O, whereby silicon together with lignin contributes to the rigidification of cell walls in leaves and xylem vessels (Ma et al., 2001) which mechanically restrict the ingress and/or penetration of pathogens (Bélanger et al., 1995; Datnoff et al., 2001).

Silicon induces defense responses similar to SAR. Different studies showed that Si-treatment increased the activity of the common protective enzymes i.e., peroxidase (POD), polyphenol oxidase (PPO) and phenylalanine ammonia lyase (PAL) in leaves of rice (Cai et al., 2008), wheat (Yang et al., 2003), and cucumber (Liang et al., 2005). These enzymes played a pivotal role in regulating the production and accumulation

of antifungal compounds such as phenolic metabolism product (lignin), phytoalexins and pathogenesis-related proteins in plants. Si application can induce the production of antifungal compounds after the penetration of pathogens (Liang et al., 2005; Rémus-Borel et al., 2005). Furthermore, Si treatment resulted in the increase of flavonoid phytoalexin in cucumber plants infected by powdery mildew (*Podosphaera xanthii*) (Fawe et al., 1998).

Si acted as a signal in triggering plant defense mechanisms similar to SAR (Fauteux et al., 2005; Cai et al., 2009). If Si is involved in the signaling events leading to the enhancement of the host resistance, it should also influence the systemic signals. The signals are transmitted to the cell nucleus, where the signal is translated into expression of the defense-related genes, through the activation of specific kinase/phosphatase cascades. In other words, the gene expression is modulated by activating defense-regulating transcription factors, or deactivating inhibitors of defense response (Fauteux et al., 2005). Si can also bind to hydroxyl groups of proteins strategically involved in signal transduction; or it can interfere with cationic co-factors of enzymes influencing pathogenesis-related events. Therefore, Si interacts with several key components of plant stress signaling systems leading to induced resistance.

1.5 Defense related enzymes

1.5.1 Lipoxygenase

Lipoxygenases (LOX-linoleate: oxygen oxidoreductase, EC 1.13.11.12) constitute a large gene family of nonheme iron containing fatty acid dioxygenases, which are

ubiquitous in plants and animals (Brash, 1999). LOX catalyze the regio- and stereo-specific dioxygenation of polyunsaturated fatty acids (PUFAs) containing a cis cis-1, 4-pentadiene system (Feussner and Kuhn. 2000), as in linoleic acid (LA-18:2), α-linolenic acid (ALA-18:3), and arachidonic acid (AA-20:4). These enzymes are predominantly located in the cytoplasm, but they are also associated with vacuoles, mitochondria, chloroplasts, microsomal membranes, plasmalemma (Prescott and John, 1996), and lipid-bodies (Feussner and Kindl, 1994).

When plant tissues are attacked or injured by insects, pathogens or mechanical wounding, lipid degrading enzymes are activated (Narvaez-Vasquez et al., 1999) providing the necessary polyunsaturated fatty acid substrates for LOX. The products of LOX i.e. the fatty acid peroxides are highly reactive and further metabolized to biologically active compounds. These include jasmonic acid and traumatin, which evoke a variety of cellular responses (Rosahl, 1996; Staswick et al., 1998; Vijayan et al., 1998), and highly reactive aldehydes with anti-microbial activity (Hamberg and Gardner, 1992).

1.5.2 Phenylalanine ammonia lyase

Phenylalanine ammonia-lyase (PAL; EC 4.3.1.5) is an important enzyme of the plant secondary metabolism (Croteau et al., 2000). It resides at a metabolically important position, linking the secondary metabolism to primary metabolism. PAL activity may be induced by elicitors present in cell walls or culture filtrates of both phytopathogenic and non-pathogenic micro-organisms, and by structurally unrelated abiotic elicitors and mechanical damage (Keen and Dawson, 1992). PAL catalyzes the deamination

of phenylalanine to produce trans-cinnamic acid, which is converted to p-coumaric acid by an oxidative reaction catalyzed by a cytochrome P450 enzyme, C4H. PAL is the first enzyme activated in this pathway and it regulates the production of precursors for lignin biosynthesis and other phenolic protectants in plant cells (Hahlbrock and Scheel, 1989).

1.5.3 Peroxidase

Peroxidases (EC 1.11.1.7.) are heme enzymes that are implicated in a large number of physiological processes in plants. They are located mainly in the cell walls, in vacuoles, in transport organelles and on membrane bound ribosomes (Gaspar et al., 1982). The activities of peroxidases are associated with resistance elicited by PGPR strains (Ramamoorthy et al., 2001) as well as resistance induced by pathogens and chemicals (Hammerschmidt and Nicholson, 1998; Stadnik and Buchenauer, 2000). Hence, the increase of peroxidase activity is used as a biochemical marker of induced resistance (Ozlem and Gray, 2003). These enzymes play an integral role in cell wall biosynthesis and lignification, which is a structural barrier to pathogens (Kärkönen et al., 2002).

Chapter 2

Characterization of plant growth promoting rhizobacteria and their potential as bioprotectant against tomato bacterial wilt caused by *Ralstonia solanacearum*

Abstract

Bacterial wilt caused by *Ralstonia solanacearum* is one of the most destructive bacterial diseases of economically important crops such as tomato. To develop a biological control strategy against the pathogen, 150 strains of rhizobacteria isolated from Ethiopia were screened for *in vitro* antibiosis. Thirteen strains identified as *Pseudomonas spp.* (PS1AW, PS2WT), *P. putida* (PP1WT, PP2SS, PP3WT, PP4AM, PP5WO), *P. veronii* (PV6BA), *Serratia marcescens* (SM1BA) and *Bacillus cereus* (BC1AW, BC2BA, BC3AW, BC4SS) by fatty acid methyl ester analyses and biochemical methods, effectively inhibited the growth of *R. solanacearum in vitro*. The rhizobacterial strains were further characterized for their plant growth promoting traits resulting in eleven strains producing siderophores, nine strains solubilising inorganic phosphate, all strains producing indole acetic acid and one strain producing hydrogen cyanide. Only *P. putida* (PP3WT) produced the quorum sensing molecule acyl homoserine lactone (AHL) and showed quorum sensing inhibition (QSI) which was depicted by the lack of pigment production by an indicator strain in a qualitative bioassay. Based on the *in vitro* screening, BC1AW, BC2BA, BC3AW, BC4SS and PP3WT were selected for *ad planta* tests under standardized conditions. Strains BC1AW and PP3WT significantly reduced bacterial wilt incidence in tomato genotype King Kong 2 (moderately resistant) by 46.8% and 44.7%, respectively, and in genotype L390 (susceptible) by 33.6% and 30% respectively, in pot experiment. While in split root experiment wilt incidence was reduced by 48.7%, 43.2% and 25.7%, 20.1% in King Kong 2 and L390 genotypes, respectively, indicating induction of systemic resistance. Shoot dry weight increased in rhizobacteria treated plants

compared to the untreated control, and reduced the number of *R. solanacearum* in mid-stems of both tomato genotypes. Hence, BC1AW and PP3WT are suggested as promising strains for further testing their effectiveness under field conditions

Keywords: Acyl-homoserine lactone, hydrogen cyanide, induced resistance, *R. solanacearum*, rhizobacteria, siderophore

2. 1 INTRODUCTION

Bacterial wilt caused by *Ralstonia solanacearum* (Yabuuchi et al., 1995) is one of the most devastating bacterial diseases in the tropics, subtropics, and warm temperature regions of the world. The pathogen is a Gram-negative soil-borne β-Proteobacterium with an extensive host range of over 450 plants species (Prior et al., 1998). In many parts of the world, this disease is a primary constraint to crop production.Tomato (*Solanum lycopersicum*) is one of the economically important host plants among other crops such as potato, banana, egg plant and ginger (Hayward, 1995; Denny, 2006).

Ralstonia solanacearum is a complex species subdivided into races based on host range and geographic distributions. Physiological and genetic characterization resulted in the formation of biovars and divisions (Hayward, 1964; Genin and Boucher, 2002). Recently, phylogenetically a more meaningful system classified *R. solanacearum* into four phylotypes according to geographic origin (Fegan and Prior, 2005).The bacterium generally enters the host plant through wounded roots or natural openings at lateral root emergence points, colonizes the root cortex, and subsequently invades the developing xylem vessels (Vasse et al., 1995). Once established in the xylem, the pathogen spreads rapidly resulting in browning of the xylem, foliar epinasty, wilting and death (Buddenhagen and Kelman, 1964). The common control measures employed against bacterial wilt, such as the use of resistant varieties, crop sanitation and crop rotation, and other cultural practices have limited success. Breeding for resistance is unreliable since promising genotypes lack

stability and durability (Boucher et al., 1992). The high variability of strains of *R. solanacearum* and its wide host range combined with the influence of environmental factors on host–pathogen interactions (Hayward, 1995) often limits the expression of resistance to specific geographic regions. As a result, no universal control measures exist which are effective across the wide host range of the pathogen (Cook et al., 1989). Therefore, the use of plant growth promoting rhizobacteria (PGPR) which induce systemic resistance and parallely act directly as biocontrol agent is suggested as a promising strategy to reduce the damage inflicted by the pathogen.

Plant growth promoting rhizobacteria which are antagonistic to pathogens provide the first line of defense for the plant against pathogen attacks (Mazzola, 1998). Previous studies indicated the control of bacterial wilt using various species of antagonistic rhizobacteria (Lemessa and Zeller 2007; Kurabachew et al., 2007). The rhizobacterial species *B. subtilis, P. macerans, S. marcescens, B. pumilis* and *P. fluorescens* (Aliye et al, 2008), and *Bacillus sp.* and *Pseudomonas sp.* (Ramesh et al, 2009) were reported to reduce bacterial wilt under *in vitro* and *in vivo* conditions. Moreover, Ciampi-Panno et al. (1989) proved the use of antagonistic microbes in the control of *R. solanacearum* under field conditions. They enhanced plant growth directly by fixing atmospheric nitrogen, solubilizing minerals such as phosphorus, producing plant growth regulators (hormones), and indirectly through production of siderophores that sequester iron (Glick, 1995; Persello-Cartieaux et al., 2003).Therefore, in this study antagonistic rhizobacteria from tomato and potato fields of Ethiopia were characterized and evaluated for their direct biocontrol potential and capability to induce systemic resistance in tomato genotypes.

2. 2 MATERIALS AND METHODS

2. 2.1 Isolation of antagonistic bacteria

A total of 150 bacterial strains were collected from the rhizosphere of tomato and potato plants in Ethiopia. The fluorescent pseudomonads were isolated following the method of Vlassak et al. (1992). One gram of each soil sample was shaken (2 h, 200 rpm) in 100 mL of phosphate buffered saline (PBS). Then, serial dilutions of each suspension were plated on King's B agar (KB) medium: 20 g/L Bactopeptone, 1.5 g /L K_2HPO_4, 1.5 g/L Mg $SO_4 . 7H_2O$, 10 mL/L glycerol, 15 g/L agar. After incubation at 28°C for 24 h, colonies were selected for further studies. *Bacillus* species were isolated based on the resistance of their endospores to elevated temperatures (Földes et al., 2000). Soil suspensions (2 g of rhizosphere soil in 100 mL of water) were placed in a water bath at 80°C for 10-15 min. Serial dilutions were spread on Nutrient Glucose Agar (NGA) medium: 3 g/L beef extract, 5 g/L peptone, 2.5 g/L glucose, 15 g/L agar and incubated at 28°C for 48 h. Distinct colonies were preserved for further characterization studies.

2. 2.2 *In vitro* screening for antagonistic activity

Antagonistic activity of the rhizosphere bacteria against the virulent *R. solanacearum* strain To-udk2 (race 1, biovar 3) obtained from Thailand (N.Thaveechai, Kasetsart University, Bangkok) was tested by the dual culture assay method on KB-medium. The KB-medium was inoculated with *R. solanacearum* by evenly spreading 100 µL of the suspensions of ~10^8 colony-forming units per millilitre (CFU/mL). Sterile paper

discs of 6 mm diameter were immersed in the suspension of 2.6×10^8 CFU/mL of each test antagonist and placed at the centre of the pathogen inoculated plate. A water soaked sterile paper disc was used as a control. Plates were kept at 28°C and the inhibition-zone around the disc was measured after 3 days. The trial was done three times with four replicates.

2.2.3 Identification of antagonist strains

Strains that inhibited the growth of *R. solanacearum* strain To-udk2 under *in vitro* conditions were identified by gas chromatographic, fatty acid methyl ester (GC-FAME) analyses (Sasser, 2001). Furthermore, isolates were characterized based on cultural, morphological and biochemical tests as described in Bergey's Manual of Determinative Bacteriology.

2.2.4 Screening of isolates for quorum sensing and quorum sensing inhibition activity

Bacterial strains were screened for production of the quorum sensing signal acyl-homoserine lactone (AHL) following the method of Moons et al. (2006). The mutant *Chromobacterium violaceum* CV026 which is unable to produce its own but able to respond to AHL provided by other organisms acts as a biosensor strain. AHL production was detected in a cross-feeding assay by stabbing the selected antagonistic isolates onto LB agar plates seeded with *C. violaceum* CV026. After an incubation of 24 h at 28°C, production of purple pigment was evaluated as positive for the test.

Similarly, quorum sensing inhibition (QSI) activities of strains were determined by adapting the method of McLean et al. (2004). The test strains were streaked in the centre of a plate with NGA-medium and incubated overnight at 28°C. Then, each plate was overlaid by 5 mL LB soft agar medium composed of 5 g/L yeast extract, 10 g/L tryptone, 10 g/L sodium-chloride, 7.5 g/L agar, cooled to 45°C, containing 10^6 CFU/mL of the wild type indicator organism *C. violaceum* ATCC12472. A positive QSI result was indicated by lack of pigmentation of the indicator organism in the vicinity of the test organism.

2.2.5 Characterization of plant growth promoting traits

Siderophore production of strains was determined by the Chrome Azurole S (CAS) method of Schwyn and Neilands (1987). The production of siderophores was indicated by a change in colour of the medium from blue to orange. Phosphate solubilizing activity of strains was evaluated on Sperber medium: 0.5 g/L yeast extract, 0.1 g/L $CaCl_2$, 0.25 g/L $MgSO_4.7H_2O$, 2.5 g/L $Ca_3(PO_4)_2$,10 g/L glucose, 15 g/L agar (Sperber, 1958). The medium was spot inoculated with 7 µL of inocula and incubated at 28°C for 7 days. The development of a clear zone around the bacteria was taken as an index of phosphate solubilization. It was computed as the ratio of total diameter (colony + halo zone) to colony diameter (Edi-Premono et al., 1996). Test strains were inoculated in nutrient broth containing 2.5 g/L $Ca_3\ (PO_4)_2$ and incubated at 27°C in a shaker incubator for seven days. Each day the change in pH in the broth culture was determined with a pH meter.

The strains were screened for the production of hydrogen cyanide (HCN) using NGA-medium amended with 4.4 g/L glycine following the methods described in Lorck (1948). A Whatman filter paper No.1 soaked in 2% sodium carbonate in 0.5% picric acid solution was placed at the top of the plate. Plates were sealed with Parafilm and incubated at 28ºC for four days. Development of orange to red colour indicated HCN production.

The production of indole acetic acid (IAA) by the strains was determined following the method of Bric et al. (1991). The 48 h old test bacterial culture was inoculated in nutrient broth supplied with 3 mM tryptophan and incubated at 28°C for 48 h. Bacterial cells were centrifuged at 3,000 rpm for 30 min. Two mL of the supernatant were mixed with 100 µL of *ortho*-phosphoric acid and 4 mL of Solawaski's reagent (50 mL 35% perchloric acid; 1 mL 0.5M $FeCl_3$) and incubated for 30 min. Development of pink colour indicates IAA production. The pink colour was quantified at 535 nm in a spectrophotometer (Beckmann DU 640, USA). The concentration of IAA produced by strains was determined using a standard curve prepared from pure IAA.The trials were done three times with four replicates.

2.2.6 *Ad planta*

2.2.6.1 Planting material and bacterial inoculum preparation

Tomato genotypes King Kong 2 (KK2) and L390, moderately resistant and susceptible to bacterial wilt, respectively, were obtained from the Genetic Resources and Seeds Unit of the Asian Vegetable Research and Development Centre (AVRDC,

Taiwan). A suspension of a fresh re-isolate of *R. solanacearum* strain To-udk2 was streaked on NGA agar medium and incubated for 48 h at 28°C. Bacterial colonies were harvested with distilled water and the inoculum was prepared by adjusting the concentration of bacterial cells to an optical density of 0.06 at 620nm wave length, corresponding to about 7.8×10^7 CFU/mL. The suspensions of the selected PGPR strains (BC1AW, BC2BA, BC3AW, and BC4SS and PP3WT) were prepared with an optical density of 0.2 at 620nm, corresponding to about 2.6×10^8 CFU/mL.

2.2.6.2 Plant growth conditions and inoculation

The seeds were sown in the greenhouse (20°C, 14 h photoperiod per day, 30 K lux and 70% RH). The roots of four-week-old tomato seedlings of each genotype were immersed in each bacterial suspension of 2.6×10^8 CFU/mL for 60 min and transplanted to individual pots with approximately 300 g of soil (Fruhstorfer Erde, type P: 150 mg/L N, 150 mg/L P_2O_5, and 250 mg/L K_2O). Potted seedlings were transferred to a climate chamber (30/28°C day/night temperature, 14 h photoperiod, 30 K lux, and 80% RH). Additionally, 20mL of each bacterial suspension were poured onto the substrate of each pot. Seedlings immersed in tap water were used as a negative control. After two days, each plant was artificially wounded and inoculated with *R. solanacearum* by pouring 25 mL of bacterial suspension per pot at the base of the plant to obtain a final inoculum concentration of approximately 10^7 CFU/g of soil followed by watering up to soil field capacity.

2.2.6.3 Quantification of *R. solanacearum* in tomato stems

The bacterial multiplication in mid-stems of tomato plants was determined 5 days post inoculation (dpi). Approximately 3 cm long, parts of the lower stem were collected from three plants. Each stem sample was weighed, surface sterilized for 15 s in 70% ethanol, rinsed and macerated in 2 mL sterile water. After 20 min the macerate was filtered through cotton wool and pelleted by centrifugation (7000 x g, 10°C for 10 min). The pellet was re-suspended in 1mL sterile water and serially diluted 10 fold at least four times. Then 100 µL of the respective dilutions were distributed evenly in two replicates on triphenyl tetrazolium chloride (TTC) medium: 20 g Bacto peptone, 5 g glucose, 1 g casamino acids, 15 g Bacto agar and 1 L H_2O; after autoclaving, 10 mL of filter-sterilized 0.5% (w/v) 2, 3, 5-TTC (SERVA, Germany) solution as a redox indicator was mixed with sterile medium before pouring into Petri plates and incubated for 48 h at 28°C. Typical colonies of *R. solanacearum* that appeared large, elevated and fluidal with red centers were counted to calculate the bacterial population as colony-forming units per gram of fresh weight (CFU/g). Each treatment consisted of thirteen plants and the trial was repeated three times.

2.2.6.4 Monitoring and evaluation of disease symptoms

The typical symptoms of bacterial wilt were monitored daily in disease severity scores from 0 to 5, with 0 = no wilt symptoms, 1 = one leaf wilted, 2 = two leaves wilted, 3 = three leaves wilted, 4 = wilting of all leaves without tip and 5 = wilting of the whole plant, plant death. The symptoms were evaluated for four weeks starting the day of first symptom appearance.

Wilt incidence was calculated as the percentage of dead plants (disease score 5) at the evaluation date to the total number of plants in the treatment. Additionally, disease severity was calculated as the mean of disease scores at the evaluation date. The area under disease progress curve (AUDPC) for each plant in each treatment and experiment was calculated on the basis of disease severity and wilt incidence using the trapezoid integration of the disease progress curve over time following the equation (Jeger and Viljanen-Rollinson, 2001):

$$AUDPC = \sum [(x_i + x_{i-1})/2] (t_i - t_{i-1})$$

with x_i and x_{i-1} are disease severity or wilt incidence at time $t_i - t_{i-1}$, respectively, and t_i and t_{i-1} are consecutive evaluation dates, with $t_i - t_{i-1}$ equal to 1.

At the end of the experiment plant fresh weight, and dry weight obtained by drying the sample at 80°C for 48 h, were measured for all plants.

2.2.6.5 Split-root test

The seedlings of the two genotypes and the bacterial inocula were prepared as described above. The root system of four week old tomato plants was split into two equal parts by cutting the lower few centimetres of the stem vertically. Two connected pots each in a separate plastic bag were filled with about 200 g of substrate. Each half of the root system was planted in a separate pot and transferred to a climate chamber. Each half of the plant was artificially wounded by stabbing a scalpel into the soil and inoculated sequentially with 40 mL and 20 mL antagonistic strains and *R. solanacearum* suspensions, respectively, as described above after four and six days

of transplanting. Each plant was monitored and disease symptom development was recorded. Quantification of the pathogen population in mid-stems was performed as described above. Each treatment consisted of thirteen plants and the trial was repeated three times.

2.2.7 Statistical analysis

The Statistical Analysis System (SAS For Windows,1999-2001,SAS Institute Carry, NC, USA.) program was used for analysis of variance (ANOVA) according to Tukey test at α = 5% for means separation. Data of bacterial numbers were log-transformed.

2.3 RESULTS

2.3.1 *In vitro* antagonistic activity of strains against *R. solanacearum*

A total of 150 rhizobacterial strains were screened in an *in vitro* dual culture assay. Thirteen strains inhibited the growth of the pathogen with inhibition zones from 5.4 mm to 21.5 mm (Table 2.1). Strains of *B. cereus* (BC1AW, BC2BA, BC3AW, BC4SS) and strain *P. putida* PP3WT with the largest growth inhibition zone (mean inhibition diameter > 11mm) were selected for *ad planta* studies under standardised conditions with tomato genotypes King Kong 2 and L390.

Table 2.1: Antagonistic bacterial strains identified by FAME technique, place of collection, soil type, host plant and diameter of inhibition zone in dual culture test

Strains	Bacterial species	Location	Soil type	Plant rhizosphere	Mean diameter of IZ (mm)
PS2WT	*Pseudomonas spp.*	Wondogenet	Sandy loam	Potato	5.4 f
PS1AW	*Pseudomonas spp.*	Awassa	Sandy loam	Potato	4.7 f
PP4AM	*P. putida*	Ambo	Loam	Potato	6.6 def
PP2SS	*P. putida*	Shashamane	Sandy loam	Tomato	8.2 de
PP5WO	*P. putida*	Wolayta	Sandy loam	Potato	8.8 d
PP3WT	*P. putida*	Wondogenet	Sandy loam	Tomato	20.2 b
PP1WT	*P. putida*	Wondogenet	Sandy loam	Potato	8.9 d
PV6BA	*P. veronii*	Bako	Loam	Potato	6.1 ef
SM1BA	*S. marcescens*	Bako	Loam	Tomato	5.2 f
BC1AW	*B. cereus*	Awassa	Sandy loam	Potato	16.9 c
BC3AW	*B. cereus*	Awassa	Sandy loam	Potato	17.7 bc
BC2BA	*B. cereus*	Bako	Sandy loam	Potato	21.5 a
BC4SS	*B. cereus*	Shashamane	Sandy loam	Potato	18.8 bc

Locations in Ethiopia, IZ = Inhibition Zone

Means within a column followed by the same letters are not significantly different according to Tukey test at $\alpha = 5\%$.

2.3.2 Identification of rhizobacteria

Strains were identified by FAME (fatty acid methyl ester analysis) as *Bacillus cereus* (BC1AW, BC2BA, BC3AW, and BC4SS), *Pseudomonas species* (PS1AW, PS2WT), *P. putida* (PP1WT, PP2SS, PP3WT, PP4AM, and PP5WO), *P. veronii* (PV6BA) and *Serratia marcescens* (SM1BA) (Table 2.1). Additional biochemical characterization indicated that *Pseudomonas species* and *S. marcescens* were Gram-negative and oxidase and catalase positive.

Pseudomonas species produced yellow-green diffusible pigment on King's B medium, except *P. putida*, formed levan from sucrose and were unable to hydrolyze starch. All tested rhizobacteria strains grew in a broth containing 1%, 3%, and 3.5% NaCl and liquefied gelatine except *P. putida*. All *Bacillus* species hydrolyzed starch.

2.3.3 Quorum sensing and quorum sensing inhibition

Among tested strains only *Pseudomonas putida* PP3WT produced the blue pigment violacein on LB agar medium at the point of contact between this strain and the mutant biosensor strain *C. violaceum* CV026. It also inhibited production of pigment by the wild type indicator strain *C. violaceum* ATCC12472 in the quorum sensing inhibition (QSI) test (Table 2.2).

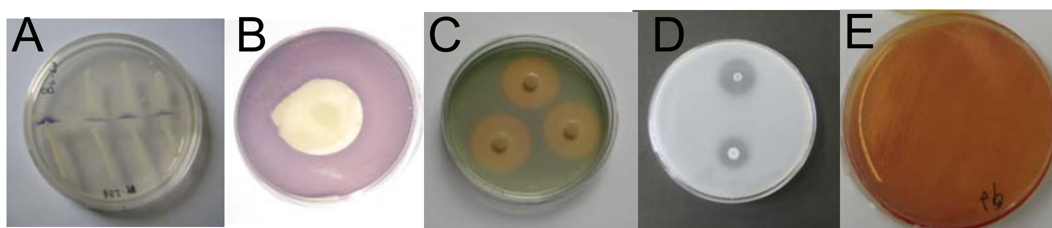


Fig. 2.1: (A) AHL production by PP3WT on LB medium, (B) quorum sensing inhibition by PP3WT on LB medium seeded with *C. violaceum* ATCC12472, (C) siderophore production, (D) phosphate solubilzation on Sperber medium supplemented with 2.5 g $Ca_3 (PO_4)_2$ (E) HCN production by PP2SS on KB agar medium supplemented with glycine (4.4 gL^{-1}).

Table 2.2: Siderophore and hydrogen cyanide production, quorum sensing and quorum sensing inhibition activity of antagonistic rhizobacteria

Strains	Bacterial species	Siderophore	HCN production	Quorum sensing	Quorum sensing inhibition
PS2WT	*Pseudomonas spp.*	+	-	-	-
PS1AW	*Pseudomonas spp.*	++	-	-	-
PP4AM	*P. putida*	++	-	-	-
PP2SS	*P. putida*	++	+	-	-
PP5WO	*P. putida*	++	-	-	-
PP3WT	*P. putida*	+++	-	+	+
PP1WT	*P. putida*	++	-	-	-
PV6BA	*P. veronii*	+++	-	-	-
SM1BA	*S. marcescens*	+	-	-	-
BC1AW	*B. cereus*	-	-	-	-
BC3AW	*B. cereus*	±	-	-	-
BC2BA	*B. cereus*	±	-	-	-
BC4SS	*B. cereus*	-	-	-	-

Key: + = Positive, **-** = Negative; ± = Intermediate, ++ = Strong producer, +++ = Very strong producer; representative results of three separate assays are shown

2.3.4 Plant growth promoting traits

All rhizobacterial strains except *Bacillus species* BC1AW and BC4SS were able to scavenge the ferric form of iron from the ferric-blue dye complex shown by medium colour change from blue to orange indicating siderophore production. Except the four *Bacillus species,* all strains produced a halo on Sperber medium indicating their capability to solubilize the inorganic phosphate with the highest solublization index of 5.1 by *P. putida* PP3WT (Table 2.3). The pH of nutrient broth amended with $Ca_3(PO_4)_2$ significantly dropped to 3.5 by test strain *P. veronii* (PV6BA). All the strains produced indole acetic acid, with *P. putida* PP4AM and *P. putida* PP1WT showing the highest and lowest production with values of 5.6µg/mL and 2.8µg/mL, respectively. And only *P. putida* PP2SS produced cyanide on NGA-medium amended with glycine.

Table 2.3: Indole acetic acid production and phosphate solubilization capacity of antagonistic rhizobacteria

Strains	Bacterial species	IAA production (µg/mL)	Phosphate solubilization capacity	
			pH value	Solubilization index
PS2WT	*Pseudomonas spp*	5.3 ± 0.7 a	4.2 ± 0.8 de	2.1± 0.3 bcd
PS1AW	*Pseudomonas spp*	3.4 ± 1.0 ab	4.3 ± 0.1 cde	2.5 ± 0.4 bc
PP4AM	*P. putida*	5.6 ± 1.1 a	4.1 ± 0.2 de	2.1 ± 0.5 bcd
PP2SS	*P. putida*	3.7 ± 0.9 ab	4.2 ± 0.3 cde	1.8 ± 0.3 cd
PP5WO	*P. putida*	4.0 ± 0.6 ab	4.5 ± 0.7 bcde	2.9 ± 0.6 b
PP3WT	*P. putida*	5.4 ± 1.1 a	4.2 ± 0.5 de	5.1 ± 0.6 a
PP1WT	*P. putida*	2.8 ± 0.8 ab	3.9 ± 0.3 de	1.6 ± 0.4 cd
PV6BA	*P. veronii*	3.9 ± 0.5 ab	3.5 ± 0.03 e	2.9 ± 0.6 b
SM1BA	*S. marcescens*	4.4 ± 1.0 ab	4.7 ± 0.8 abcd	1.3 ± 0.2 d
BC1AW	*B. cereus*	3.9 ± 0.4 ab	5.8 ± 0.3 a	0 e
BC3AW	*B. cereus*	3.9 ± 0.5 ab	5.4 ± 0.2 abc	0 e
BC2BA	*B. cereus*	4.6 ± 0.87ab	5.3 ± 0.14 abc	0 e
BC4SS	*B. cereus*	4.3 ± 0.6 ab	5.5 ± 0.3 ab	0 e

Means of three reptead trials ± SE. Means followed by the same letter within a column are not significantly different according to Tukey test at α = 5%.

Phosphate solubilization index was computed as the ratio of total diameter (colony + halo zone) to colony diameter (Edi-Premono et al., 1996).

2.3.5 *Ad planta* experiment

Based on the *in vitro* inhibition assay five strains were selected for *ad planta* experiments with the tomato genotypes King kong-2 (KK-2) and L390, moderately resistant and susceptible, respectively. In the pot experiment strains *B. cereus* BC1AW and *P. putida* PP3WT significantly reduced bacterial wilt incidence expressed as area under disease progress curve (AUDiPC) in tomato genotypes King Kong 2 by 46.8% and 44.7%, respectively, and in L390 by 33.6% and 30%, respectively, while in split root experiment they reduced AUDiPC by 48.7% and 43.2%, and 25.7% and 20.1% in King Kong 2 and L390, respectively.

Similarly strains *B. cereus* BC1AW and *P. putida* PP3WT reduced bacterial wilt severity expressed as area under disease progress curve (AUDsPC) in tomato genotypes King Kong 2 by 24.2% and 20.4%, respectively, and in L390 by 17.5% and 14.1% respectively, in pot experiments. In the split root test experiment they reduced AUDsPC by 20.3% and 18.3% and 15% and 12.9%, in King Kong 2 and L390, respectively, compared to the untreated *R.solanacearum* infected control (Fig. 2.3).

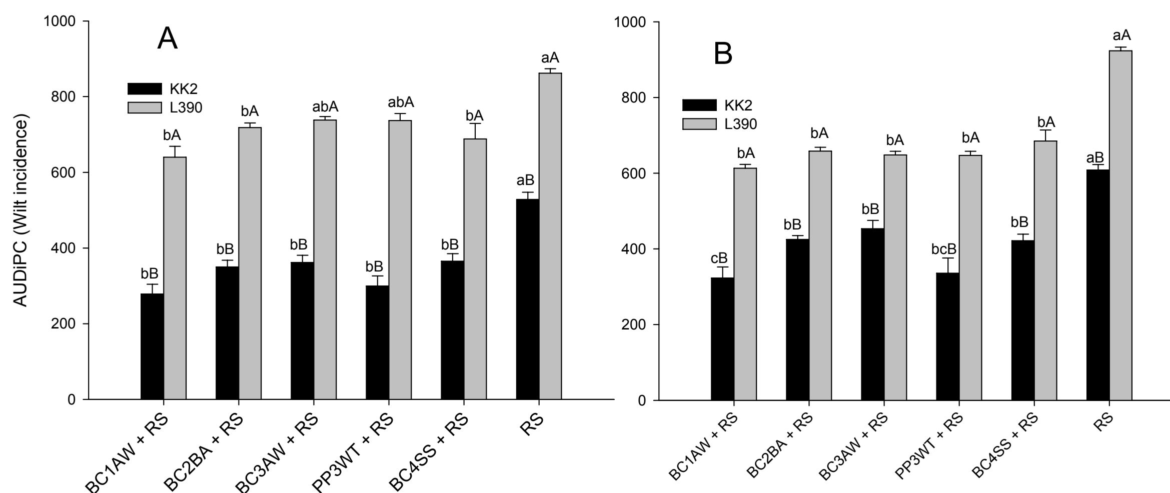


Fig. 2.2: Wilt incidence expressed as area under disease progress curve (AUDiPC) in tomato genotypes King Kong 2 (moderately resistant) and L390 (susceptible) over four weeks after treatment with bacterial antagonists and inoculated with *R. solanacearum* strain To-udk2 in (a) split-root experiments, (b) pot experiments. Disease incidence is the percentage of dead plants at each evaluation date.

Means of three repeated trials ± SE. Bars with the same letters are not significantly different. Small letters refer to comparison between treatments for the same genotype, while capital letters refer to the comparison between genotypes for the same treatment. Tukey test at α= 5% probability.

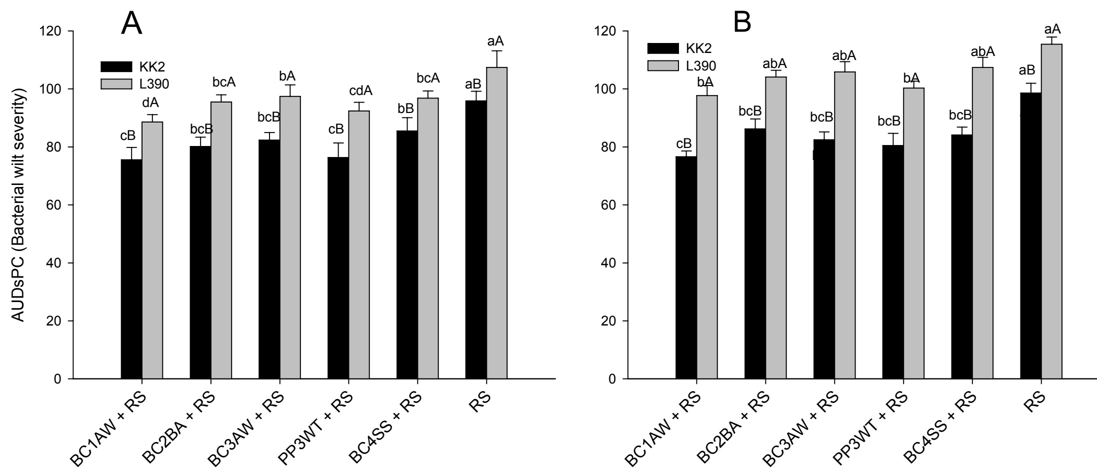


Fig. 2.3: Bacterial wilt severity expressed as area under disease progress curve (AUDsPC) in tomato genotypes King Kong 2 (moderately resistant) and L390 (susceptible) over four weeks after treatment with bacterial antagonists and inoculated with *R. solanacearum* strain To-udk2 in (a) split-root experiments, (b) pot experiments.

Disease severity was defined as the average of disease classes of all plants of treatment at an evaluation date.

Means of three repeated trials ± SE. Bars with the same letters are not significantly different. Small letters refer to comparison between treatments for the same genotype, while capital letters refer to the comparison between genotypes for the same treatment. Tukey test at α= 5% probability.

Bacterial numbers in the mid-stems of King Kong 2 and L390 were significantly reduced by strains BC1AW and PP3WT in pot and split root experiments five days after inoculation (Fig. 2.4). Shoot dry weight was increased by 58.3% and 50%, respectively in King Kong 2 and by 42.8% and 46.7% respectively, in L390 genotypes in pot experiment compared to the antagonist untreated *R. solanaceaum*

inoculated control and by 75% and 62.5% in KK2 and 57.1% and 50% in L390 non-pathogen inoculated, healthy control.

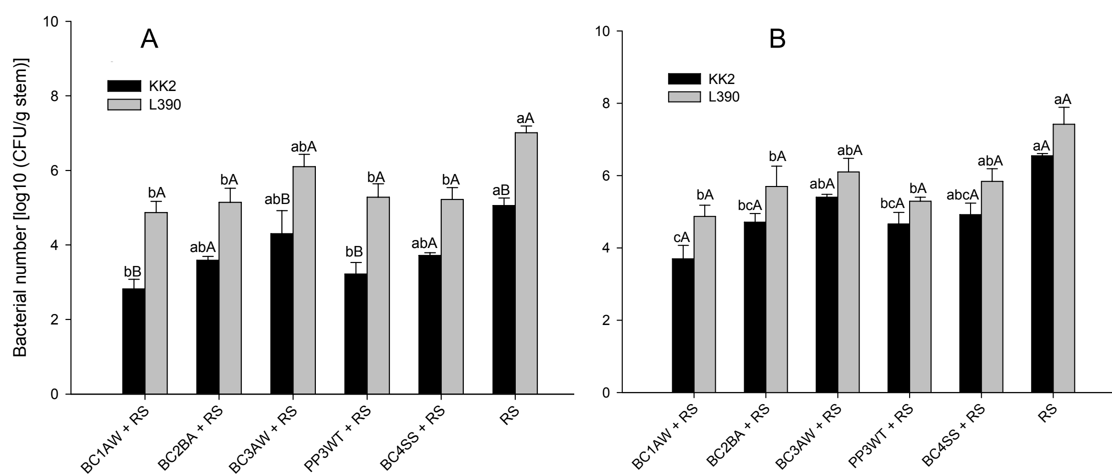


Fig. 2.4: Bacterial numbers in mid-stems of tomato genotypes King Kong 2 (moderately resistant) and L390 (susceptible) four weeks after treatment with bacterial antagonists and inoculated with *R. solanacearum* strain To-udk2 in (a) split-root) & (b) pot experiment.

Means of three repeated trials ± SE. Bars with the same letters are not significantly different. Small letters refer to comparison between treatments for the same genotype, while capital letters refer to the comparison between genotypes for the same treatment. Tukey test at 5% probability.

Table 2.4: Dry weight of aerial parts of 2 month-old tomato plants of genotypes King Kong 2 (moderately resistant) and L390 (susceptible) treated with bacterial antagonistic rhizobacteria, four weeks after inoculation with *R. solanacearum* strain To-udk2 in pot experiments

	Genotypes	
	KK-2	**L390**
Treatment	dry weight [g]	dry weight [g]
Antagonist only		
BC1AW	5.6 ± 1.1 a	4.4 ± 1.0 a
BC2BA	4.9 ± 1.5 ab	4.1 ± 1.7 a
BC3AW	4.8 ± 0.4 ab	3.9 ± 0.5 a
PP3WT	5.2 ± 1.0 ab	4.2 ± 1.6 a
BC4SS	4.6 ± 0.5 bc	3.8 ± 0.8 a
Antagonist + pathogen		
BC1AW+ Rs	3.8 ± 0.8 cd	2.3 ± 1.6 bc
BC2BA+ Rs	3.4 ± 0.5 d	2.1 ± 1.1 bc
BC3AW+ Rs	3.5 ± 1.1 d	2.14 ± 0.5 bc
PP3WT + Rs	3.6 ± 0.9 d	2.20 ± 0.8bc
BC4SS +Rs	3.3 ± 1.0 d	2.04 ± 0.3 bc
Controls		
Pathogen	2.4 ± 0.5 e	1.54 ± 0.8 c
Distilled water	3.2 ± 1.5 de	2.8 ± 1.0 b

Means from three repeated trials ± SE. Means followed by same letters are not significantly different according to Tukey test at 5%.

2.4 DISCUSSION

In vitro study and identification of rhizobacteria

Beneficial rhizobacteria are known to exert an array of mechanisms to inhibit the growth of target pathogens (Compant et al., 2005). The antagonistic bacteria tested under *in vitro* conditions against *R. solanacearum* on KB-medium showed different levels of antagonism with *Bacillus spp.* BC1AW, BC2BA, BC3AW, BC4SS and *Pseudomonas spp* PP3WT showing the highest zone of growth inhibition of 11-20mm. Similarly, Lemessa and Zeller (2007) and Alyie et al.(2008) found growth inhibition of *R. solanacearum* by *Pseudomonas spp.* and *Bacillus spp. Paenibacillus macerans* under *in vitro* condition Therefore, the selected antagonists could have the potential to be used for bio-protection of tomato against this drastic pathogen under field condition.

Results of the *in vitro* antibiosis test suggest that, the inhibitory activity by all strains except *B. cereus* BC1AW and BC4SS, against *R. solanacearum* could be partly explained by production of siderophores. Since KB is an iron deficient medium, it is optimal for siderphore production (Lim and Kim, 1997).Also Chen et al. (2003) reported the type of culture medium had an impact on the inhibitory activity of antagonists by mediating the production of growth inhibiting substances. This was further strengthed by Muleta et al. (2007) who found the growth inhibition of fungal pathogens of coffee on KB medium by a *Pseudomonas* strain through production of siderophores. Though, *B. cereus* strains BC1AW and BC4SS were unable to produce siderophores, they still showed inhibitory activity against the pathogen suggesting the

production of antibiotics. Although there is a general agreement that *in vitro* antibiosis does not necessarily relate to the same effect under *in vivo* conditions (Klopper, 1993), it is a common method in the initial screening of antagonists against bacterial and fungal pathogens.

Our result of FAME identification of rhizobacteria strains is in line with Linu et al. (2009) who identified the phosphate solubilizing bacteria *Gluconobacter spp.* and *Burkholderia spp.* with fatty acid methyl ester profiling technique. The biochemical and physiological characteristics described for *Pseudomonas species*, *P. putida, B. cereus* and *S. marcescens* are in agreement with the reports of Bossis et al. (2000) and Foldes et al. (2000).The utilization of different carbon sources by *Pseudomonas* species indicates their metabolic and ecological diversity, presupposing their success of survival and competency in the environment where they are applied or introduced as a biocontrol agent.

Quroum sensing and quorum sensing inhibition

Quorum sensing is a regulatory mechanism by which diverse microorganism control specific processes in response to population density through release of a signal (McClean et al., 2004). In this experiment only PP3WT produced the purple pigmented quorum sensing signal, acyl-homoserine lactone (AHL), suggesting the capability of the strain to communicate within its vicinity in density dependent manner for a diverse living and ecological activity. This is inline with Elasri et al. (2001) who reported, that AHL production is more common in plant associated *Pseudomonas species* than among soil-borne species. The indicator organism *C. violaceum* CV026

is unable to synthesize its own endogenous N-hexanoyl-L-homoserine lactone (C6-HSL) inducer, but the relevant operons can be induced by the exogenous supply of the appropriate AHL to the mutant bacteria (McClean et al., 1997). Thus, strain CV026 can induce violacein in the presence of AHL compounds with N-acyl side chains from C_4 to C_8 length, but not with AHL compounds with N-acyl side chains from C_{10} to C_{14} (McClean et al., 1997). Accordingly, in our case the biosensor produced the purple pigment violacein, in response to AHL provided by the test strain PP3 WT on the LB agar medium.

In *C. violaceum* ATCC12472 production of the purple pigment violacein is under the control of the QS system. This wild type strain produces and responds to the cognate auto inducer molecules (C6-HSL) and N-butynol homoserine lactone (C4-AHL) (McClean et al., 1997). In the current quorum sensing inhibition (QSI) test no pigment was produced in the vicinity of the test strain, *P. putida* PP3WT indicating the inhibition or disruption of QS-regulated violacein pigment production in *C. violaceum* by production of AHL through the test strain. The QSI detected by the indicator bacteria may function by the competitive binding and inhibition of various AHL molecules other than C6-HSL to the receptor protein CviR, a LuxR homologue in *C. violaceum* ATCC12472 (Blosser and Gray, 2000). This is an important phenomenon as the growth of the bacteria is not affected, and there is no selective pressure for the development of resistant bacteria (Henther and Givskov, 2003). Therefore, the inactivation and suppression of the QS signal might be useful in controlling the development and persistence of plant bacterial pathogens (Zhang, 2003). Thus, ccurrently quorum quenching becomes an attractive approach in plant biotechnology

and disease management as potential strategy for development of an efficient biological control method against a variety of plant pathogens (Czajkowski and Jafra, 2009).

HCN production

Rhizosphere associated bacteria are known to be vital in plant growth promotion and protection against soil borne plant pathogenic organisms (Rajkumar et al., 2005). Among the tested strains only *P. putida* PP2SS produced HCN when grown on glycine supplemented KB-medium, indicating its capability to catabolise glycine (Askeland and Morrison, 1993), a common root exudate available in the rhizosphere as a precursor for HCN synthesis. Though this compound is reported as a potential inhibitor of many enzymes involved in major plant metabolic process (Bakker and Schippers, 1987), it is attracting remarkable attention and wide applications in areas of biocontrol. In line with this, various authors reported that *Pseudomonas spp*. was implicated in suppression of soil-borne fungal diseases (Voisard, et al., 1989), weed seedlings (Kremer and Souissi, 2001) and plant parasitic nematodes (Siddiqui et al., 2006).

Characterization of plant growth promotion traits

Out of thirteen rhizobacterial strains tested, nine used the complex form of phosphate accompanied by a significant decline in the pH of the broth suggesting the production of organic acids by the microorganisms. Such reduction of pH in the broth were also reported by other studies (Rashid et al., 2004; Whitelaw, 2000), who stated that

production of organic or inorganic acids was critical for solubilizing phosphates from the Ca-phosphate complex. Thus, Gram-negative bacteria that produce gluconic acid from the extracellular oxidation of glucose via quinoprotein glucose dehydrogenase, thereby acidifying their medium, solubilize insoluble inorganic phosphates efficiently (Goldstein, 1996).

PGPR promote plant growth indirectly through production of siderophores which scavenge ferric iron from the rhizosphere that make it unavailable to the phytopathogens and render protection to the plant (Glick et al., 1999). In our study eleven rhizobacterial strains produced siderophores on the CAS plate qualifying them as inducers of resistance against the pathogen.

Many plant-associated rhizobacteria produce the plant growth regulator indole-3-acetic acid that enhances plant growth directly (Patten and Glick, 2002). In this study, all thirteen strains produced IAA at different levels. This agrees with Mirza et al. (2001) who reported variable IAA production by PGPR among different species and strains, culture conditions, growth stages and substrate availability. In addition Sarwar and Kremer (1992) reported that isolates from the rhizosphere are more efficient producers than isolates from the bulk soil, supporting our result. Patten and Glick (2002) reported that IAA produced by bacteria promotes root growth directly by stimulating plant cell elongation or cell division or indirectly by influencing bacterial 1-aminocyclopropane-1-carboxylate (ACC) deaminase activity, which is the immediate precursor of the phytohormone ethylene, thereby preventing the production of plant growth-inhibiting levels of ethylene (Penrose et al., 2001).

Ad planta

In our *ad planta* experiments *B. cereus* BC1AW and *P. putida* PP3WT significantly reduced severity and incidence of bacterial wilt and increased the plant biomass in pot and split-root experiments in both genotypes. Similarly, Aliye et al. (2008) and Lemessa and Zeller (2007) reported that application of rhizobacteria such as *B. subtilis, P. macerans* and fluorescent pseudomonads significantly reduced disease symptoms caused by *R. solanacearum* in potato and tomato by 48 to 78.6% and increased the biomass of the plants up to 63%. Ramesh et al. (2009) also reported that bacterization of tomato seedlings with *Pseudomonas* and *Bacillus* strains significantly reduced the incidence of bacterial wilt by 80% and 70%, respectively.

The enhanced plant growth by treatments with BC1AW and PP3WT could be attributed to their production of growth stimulating substances such as indole-3-acetic acid (IAA). Moreover, the production of siderophores by the test strains might give them competitive advantage to make Fe^{+3} unavailable to the pathogen (Bakker and Schippers, 1987). It also triggers induction of systemic resistance which switches on the battery of defence mechanisms of the plant against pathogens (Bakker et al., 2007). Our split root experiments confirmed the induction of systemic resistance in tomato genotypes, since there was no direct contact between antagonist and pathogen. This is proven by the reduction of wilt incidence and number of the pathogen in the mid-stem of tomato. Furthermore, the quorum sensing inhibition caused by PP3WT might play a role in the suppression of bacterial wilt, as it can arrest or stop the virulence activity of the pathogen by imitating the AHLs structure of

the pathogen that block the AHL receptor protein and prevent activation of the target gene expression (Maneifeld et al., 2001).

Since the strains used in this investigation are isolated from potato and tomato rhizosphere, it is supposed that they are well adapted to utilize exudates from their original host plants. Also Bakker and Schippers (1987) reported that success of plant growth promotion by the rhizobacteria largely depends on their timely establishment and persistence throughout the growing season at sites where the pathogen may become active. Furthermore, Bias et al. (2004) explained that most rhizosphere bacteria and fungi are highly dependent on the association with plants which is regulated by the root exudates. Therefore, such interactions would enhance the plant protection and growth promotion rendered by these strains when introduced in to plant microbe interaction.

In conclusion, *P. putida* PP3WT and *B. cereus* BC1AW posses the desirable plant growth promoting traits and had the potential to protect tomato against *R. solanacearum* damage. Therefore, they are suggested to be included as part of an integrated disease management package against bacterial wilt. However, field studies should be performed to confirm their effectiveness under natural conditions.

Chapter 3

Induction of systemic resistance and defence related enzymes after elicitation of resistance by rhizobacteria and silicon against *Ralstonia solanacearum* in tomato (*Solanum lycopersicum*)

Abstract

Induction of Si-rhizobacteria mediated systemic resistance was investigated in tomato genotypes.Each elicitor was tested separately and in combination to elicit active defence responses in tomato against *R. solanacearum*. Application of silicon and rhizobacteria significantly reduced bacterial wilt incidence by 50.7% and 26.7%, respectively, in King Kong 2 (moderately resistant) and by 31.1% and 22.2%, respectively, in L390 (susceptible) genotypes, compared to the pathogen inoculated control. However, the combined application of silicon and rhizobacteria reduced wilt incidence by 16.9% in King Kong 2 and 13.2% in L390. The single application of elicitor also reduced bacterial populations in the mid-stem of tomato, but the dual application of the two elicitors did not. Silicon amendment significantly increased the silicon content in the root of both genotypes but not in the stem. The activity of lipoxygenase (LOX) was significantly decreased in the pathogen inoculated and silicon amended treatment, but increased in the rhizobacteria treatment. In simultaneous application of silicon and rhizobacteria, the activity of peroxidase (POD) and phenylalanine ammonia lyase (PAL), lipoxygenase (LOX) dropped significantly. In contrast non-significant increases of peroxidase (POD) and phenylalanine ammonia lyase (PAL) activity were observed in the individual treatments of silicon and rhizobacteria upon inoculation with *R. solanacearum*.

Key words: Lipoxygenase, peroxidase, phenylalanine ammonia lyase, rhizobacteria

3. 1 INTRODUCTION

Plants have evolved complex and varied defense mechanisms to protect themselves against pathogen attack. These mechanisms may be constitutive or induced but can fail when a plant is infected by a virulent pathogen, as the pathogen avoids triggering resistance reactions or evades the effect of activated defenses (van Loon et al., 1998; Pieterse and van Loon, 1999). Therefore, priming plant with either biotic or abiotic elicitor prior to infection by a pathogen will enhance the level resistance against a pathogen and resulted in reduced disease symptoms (Conrath et al., 2002). Research on induced resistance has highlighted the essential role of some beneficial microorganisms and of natural and/or chemical products in activating expression of the defense genes.

Plant growth-promoting rhizobacteria (PGPR) strains are reported to produce a variety of metabolites which play an important role in elicitation of plant-mediated resistance referred to as induced systemic resistance (ISR) (Van Loon et al., 1998; Pieterse and van Loon, 1999). Induction of such ISR has been demonstrated in beans, carnation, cucumber, radish, tobacco, tomato and the model plant *Arabidopsis thaliana*, and has been effective towards a wide range of pathogens including fungi, bacteria and viruses (Van Loon et al., 1998). Unlike systemic acquired resistance, induced systemic resistance (ISR) is independent of accumulation and activation of the PR genes (Pieterse et al., 1996). The signal transduction which leads to ISR requires the production of jasmonic acid and ethylene (Pieterse et al., 1998). Several studies on rhizobacteria-mediated ISR indicated the role of common defense enzymes such as peroxidase (POD)

(EC1.11.1.7), phenylalanine ammonia lyase (PAL) (EC4.3.1.5) and lipoxygenase (LOX) (EC1.13.11.12) in the induction of systemic resistance (Ramamoorthy et al., 2002; Silva et al., 2004; Jetiyanon, 2007).

Among the different abiotic elicitors silicon is the most abundant element in soil that is known to enhance plant growth, development and resistance to biotic and abiotic stress in different plant species (Epstein, 1994; Ma, 2004; Hattori et al., 2005). It was proposed that silicon plays a role in the formation of mechanical barriers restricting the penetration of pathogens (Datnoff et al., 2001). Similar to commercially available products such as Acibenzolar-*S*-methyl (ASM) and benzothiadiazole (BTH) Si was reported to induce systemic acquired resistance SAR (Oostendrop et al., 2001; Fauteux et al*.,* 2005). Exogenous application of silicon enhanced resistance against bacterial wilt in tomato (Dannon and Wydra, 2004; Diogo and Wydra, 2007), fungal diseases such as sheath blight in rice (Datnoff et al., 2001) and *Pythium* and *Sphaeroteca fuliginea* in cucumber (Samuels et al., 1994; Fawe et al., 2001). Several studies showed that lower disease severity in the Si-treated plants was in line with higher activity of the protective enzymes POD and PAL in leaves of rice (Cai et al., 2008), wheat (Yang et al., 2003), and cucumber (Liang et al., 2005). These enzymes play an important role in regulating the production and accumulation of antifungal compounds such as phenolic metabolism product i.e. lignin, phytoalexins, and pathogenesis-related proteins in plants (Cai et al., 2009). Silicon also enhanced resistance against bacterial wilt in tomato *Ralstonia solanacearum,* (Dannon and Wydra, 2004; Diogo and Wydra, 2007) a soil-borne phytopathogenic bacterium that causes lethal systemic vascular wilt disease in over 450 different plant species, more

than 54 botanical families, including dicotyledones and monocotyledons (Wicker et al., 2007). This pathogen in particular, limits the production of solanaceous crops of economical importance such as tomato, potato, tobacco and eggplants in tropical, subtropical and some warm temperature regions of the world (Hayward, 1991).

Therefore, the present investigation was undertaken to evaluate the effect rhizobacteria strain *B. pumilis* and silicon alone or in combination, on bacterial wilt reduction and to determine the activity of the defense-related enzymes peroxidase, lipoxygenase and phenylalanine ammonia lyase which are known to be involved in the induction of systemic resistance.

3.2 MATERIALS AND METHODS

3.2.1 Planting material and bacterial isolate preparation

Tomato genotypes King Kong 2 (moderately resistant) and L390 (susceptible) to bacterial wilt were obtained from the Genetic Resources and Seeds Unit of the Asian Vegetable Research and Development Centre (AVRDC, Taiwan). A suspension of a fresh re-isolate of *R. solanacearum* strain To-udk2 was streaked on nutrient glucose agar medium (NGA) for 48 h at 28°C. Bacterial colonies were harvested with distilled water and the inoculum was prepared by adjusting the concentration of bacterial cells to an optical density of 0.06 at 620nm wave length, corresponding to about $7.8x10^7$CFU/mL. The suspension of PGPR strain *Bacillus pumilis* (A8) was prepared similarly, but adjusted to an optical density of 0.2 at 620nm, corresponding to about $2.6x10^8$ CFU/mL.

3.2.2 Plant growth conditions and inoculation

The tomato seeds were sown in white peat (Klasmann-Deilmann, Germany) supplemented with 4 g /L $CaCO_3$ (Roth, Germany) for the non-silicon treatment and 4 g/L $CaCO_3$ plus 1g/L Aerosil (Degussa, Germany) for the silicon treatment. Plants were kept under greenhouse conditions (20°C with 14 h light per day at 30 K lux and 70% relative humidity) and watered throughout the whole experiment with a nutrient solution composed of 5 mM $Ca(NO_3)_2$, 1.875 mM K_2SO_4, 1.625 mM $MgSO_4$, 0.5 mM KH_2PO_4, 0.04 mM H_3BO_3, 0.001 mM $ZnSO_4$, 0.001 mM $CuSO_4$, 0.01 mM $MnSO_4$, 0.00025 mM Na_2MoO_4, 0.05 mM NaCl and 0.1 mM Fe-EDTA for the non-silicon treatment, and the same solution containing additionally monosilicic acid at a final concentration of 1.4 mM [$Si(OH)_4$] for silicon treatments. Monosilisic acid was obtained after exchange of potassium silicate solution K_2SiO_2 (VWR, Germany) with cation exchangers (20 mL volume, Biorad Laboratories, Germany) (Hochmuth, 1999).

The roots of four-week-old tomato seedlings of each variety were immersed in bacterial suspension of $2.6x10^8$ CFU/mL for 60 min and transplanted to individual pots with approximately 300 g of white peat. Potted seedlings were transferred to a climate chamber (30/28°C day/night temperature, 14 h photoperiod, 30 K lux, and 80% RH). Twenty millilitres of each bacterial suspension was additionally poured onto the substrate of each pot. Seedlings immersed in tap water were used as negative controls. After two days, each plant was artificially wounded by stabbing with a scalpel onto substrate and inoculated with *R. solanacearum* by pouring 25 mL

of bacterial suspension per pot at the base of the plant to obtain a final inoculum concentration of approximately 10^7 CFU/g of soil, followed by watering up to soil field capacity.

Table 3.1: Treatment combinations for tomato genotypes King Kong 2 and L390

No.	Treatments	Designation
T1	Plants without silicon, antagonist and *R. solanacearum*	-Si-A-Rs
T2	Plants without silicon and antagonist, with *R. solanacearum*	-Si-A+Rs
T3	Plants without silicon, with antagonist, without *R. solanacearum*	-Si+A-Rs
T4	Plants without silicon, with antagonist and *R. solanacearum*	-Si+A+Rs
T5	Plants with silicon, without antagonist and *R. solanacearum*	+Si-A-Rs
T6	Plants with silicon, without antagonist, with *R. solanacearum*	+Si-A+Rs
T7	Plants with silicon, with antagonist, without *R. solanacearum*	+Si+A-Rs
T8	Plants with silicon, with antagonist and *R. solanacearum*	+Si+A+Rs

3.2.3 Quantification of *R. solanacearum* in tomato stems

The bacterial multiplication in mid-stems of tomato was determined with selected symptomless plants five days post inoculation (dpi). Approximately 3 cm long stem lower parts were collected from three plants. Each stem sample was measured, surface sterilized for 15 s in 70% ethanol, rinsed and macerated in 2 mL sterile water. After 20 min, the macerate was filtered through cotton wool and pelleted by centrifugation (7000 x g, 10°C for 10 min). The pellet was re-suspended in 1mL sterile water and serially diluted 10 fold at least four times. Then 100 µL of the

respective dilutions were distributed evenly in two replicates on triphenyl tetrazolium chloride (TTC) medium: 20 g Bacto peptone, 5 g glucose, 1 g casamino acids, 15 g Bacto agar and 1 L H_2O; after autoclaving, 10 mL of filter-sterilized 0.5% (w/v) 2, 3, 5-TTC (SERVA, Germany) solution as a redox indicator was mixed with sterile medium before pouring into Petri dishes and incubated for 48 h at 28°C. Colonies of *R. solanacearum* appearing large, elevated and fluidal with red centers due to consumption of TTC dye by the pathogen were counted to calculate bacterial populations as colony-forming units per gram of fresh weight (CFU/g).

3.2.4 Disease symptom evaluation

The typical symptoms of bacterial wilt were monitored daily in disease severity scores from 0 to 5, with 0 = no wilt symptoms, 1 = one leaf wilted, 2 = two leaves wilted, 3 = three leaves wilted, 4 = wilting of all leaves without tip and 5 = wilting of the whole plant, plant death. The symptoms were evaluated for four weeks starting from the day of first symptom appearance.

Wilt incidence was calculated as the percentage of dead plants (disease score 5) at the evaluation date in relation to the total number of plants in the treatment. Additionally, disease severity was calculated as the mean of disease scores at the evaluation date. The areas under disease progress curves (AUDPC) for each plant in each treatment and experiment were calculated on the basis of disease severity and wilt incidence using the trapezoid integration of the disease progress curve over time following equation (Jeger and Viljanen-Rollinson, 2001):

$$AUDPC = \sum [(x_i + x_{i-1})/2] (t_i - t_{i-1})$$

with x_i and x_{i-1} are disease severity or wilt incidence at time $t_i - t_{i-1}$, respectively, and t_i and t_{i-1} are consecutive evaluation dates, with $t_i - t_{i-1}$ equal to 1.

3.2.5 Silicon quantification

Total silicon content in the stems and roots of the same plant sample that was used for bacterial quantification and enzyme assay were determined by spectrophotometry using the method developed by Novozamsky et al. (1984), modified according to Iwasaki et al. (2002). Stem and root samples were dried at 80°C for at least 3 days and grounded in a swing mill (Sartorius, Germany). For each sample 10 mg material was weighed in an Eppendorf tube and digested with 500 µL of a solution composed of 1M HCl and 2.3 M HF in a ratio of 1 : 2, while shaking overnight. After centrifugation step at 10,000 x g for 10 min, 20 µL supernatant was added to 250 µL 3.2% H_3BO_3 and incubated overnight while shaking. Then 250 µL color reagent (1:1 mixture of 0.08 M H_2SO_4 and 20g /L $(NH_4)_6Mo_7O_2.4H_2O$) were added and incubated for 30 min at room temperature. Color development occurred after adding 250 µL of a solution composed of 33 g/L tartaric acid and 0.25 mL of 4 g/L ascorbic acid. Samples were measured in micro cuvettes at 811 nm by spectrophotometry (Beckmann DU 640, USA) against a blank containing 20 µL HCl and 2.3 M HF (1:2) without plant material. A series of standards with Si concentrations ranging from 0 to 100 ppm was prepared and the silicon content of the samples (mg/g dry weight) was calculated using regression equation of the standard absorbance values

3.2.6 Enzyme assays

Plant sample of genotypes L390 and King Kong 2 sampled 5 dpi and subsequently frozen at -20°C were used for enzyme assay. Frozen plant samples were macerated in 10 mM sodium phosphate buffer (pH 6.0) 1: 10 (w/v) for 50 s and subsequently centrifuged at 16,600 x g for 15 min at 4°C. Supernatants were used as plant extract for enzyme assays and total protein quantification. The experiments were carried out in triplicate in three independent sets of experiments.

3.2.6.1 Guaiacol peroxidase activity

The guaiacol peroxidase (POD) activity was measured as described in Fecht-Christoffers et al. (2003) with modifications. The reaction mixture contained 850µL 20 mM guaiacol (Sigma, Germany) as substrate in 10 mM sodium phosphate buffer (pH 6.0), 50 µL enzyme extract. The reaction was started by adding 100 µL 0.3% H_2O_2 (Merk, Germany) to the reaction mixture. The formation of tetraguaiacol resulting in a linear change of absorbance at 470 nm was monitored for 2 min with a spectrophotometer (Beckmann DU640, USA). A mixture containing substrate and enzyme extract served as blank for each sample. The activity was calculated from the extinction coefficient of 26.6 $mM^{-1}cm^{-1}$ for guaiacol.

3.2.6.2 Lipoxygenase activity

An increase in Lipoxygenase (LOX) activity was measured following the method described by Axelrod et al. (1981) based on the increase in absorbance at 234nm resulting from the conjugated double bound system in the hydroperoxide produced from the substrate, linoleic acid (10mM sodium linoleate, pH 9.0). The reaction

mixture consisted of 1mL of 50mM sodium phosphate buffer, pH 6.0, 20 µL substrate, and 10 µL plants extract. Absorbance readings were made spectrophotometrically for three minutes at room temperature. Mixture containing substrate and buffer was used as blank for each sample. The activity was calculated from the extinction coefficient of 25 $mM^{-1}cm^{-1}$.

3.2.6.3 Phenylalanine amonia-lyase activity

Phenylalanine amonia-lyase (PAL) activity was determined spectrophotometrically as described by (Peltonen and Karajalainen, 1995). The reaction mixture contained 500 µL plant extract and 2500 µL of a 0.2% L-phenylalanine solution in 50mM Tris-HCl (pH 8.5). The reference cuvette contained 500 µL extraction buffer and 2500 µL of the 0.2% D-phenylalanine solution. The reaction mixture was incubated in a 40°C heated metal plate for 1 h and absorbance at 290nm was measured in 30 min interval.

3.2.6.4 Total protein content

Total protein contents were determined according to Bradford (1976) with bovine serum albumin (BSA) (Sigma, Germany) as standard. A volume of 50 µL plant extract was incubated with 1450 µL Bradford reagent (100 mg Coomassie brilliant blue G250 50 mL ethanol absolute, and 100 mL ortho-phosphoric acid and 850 mL demineralised H_2O) and incubated for 20 min at room temperature. Samples were measured spectrophotometrically at 595 nm (Beckmann DU 640, USA) against a blank containing Bradford reagent and 50 µL demineralised H_2O. A standard series

was prepared in a range of 0 to 1000 µg bovine serum albumin (BSA) per mL rising in 100 µg steps, and 50 µL of each standard was incubated with Bradford reagent in triplicates. Total protein concentration was calculated by using regression equation of the standard concentrations and the corresponding absorbance values.

Enzyme activity was calculated from the change in absorbance:

$$\text{Activity} = \Delta OD/\text{Min} \times Vt/Vs \times 1/\varepsilon d \times F = \mu\ \text{mol}*\text{min}^{-1}*\text{ml}^{-1}$$

with: ΔOD = change of absorbance per minute, Vt = total volume of the assay (mL), Vs = volume of enzyme extract of sample (mL), ε = extinction coefficient; d = diameter of the cuvette used in the assay, equal to 1, and F = dilution factor. The specific activity represents the moles converted per unit time per unit mass of enzyme (enzyme activity / actual mass of protein present). Specific activity of peroxidase was calculated as:

Specific activity = Enzyme activity/ total protein content

3.2.7 Statistical analysis

Statistical analysis of the data was conducted using ANOVA procedure of the SAS software version 8.1 (SAS; USA). Mean separation was done according to Tukey's test at α = 5% probability level. Data of bacterial numbers were log-transformed.

3.3 RESULTS

3.3.1 Disease symptom development

First wilt symptoms were observed two dpi in L390 genotype and four to five days after inoculation in King Kong 2 (Fig. 3.1 and Fig 3.2). Application of silicon significantly reduced disease severity and wilt incidence in genotype King Kong 2 by 23.9% and 50.7%, respectively, treatment with rhizobacteria by 14.7% and 26.7%, respectively. Similarly, in genotype L390 a reduction of disease severity and wilt incidence by 17.5% and 31.1%, respectively, due to silicon amendment and by 12.7% and 22.2% after rhizobacteria treatment, respectively, was found. However, the combined application of silicon and rhizobacteria reduced wilt incidence by 16.9% and in 13.2% in King Kong 2 and L390, respectively (Table 3.2).

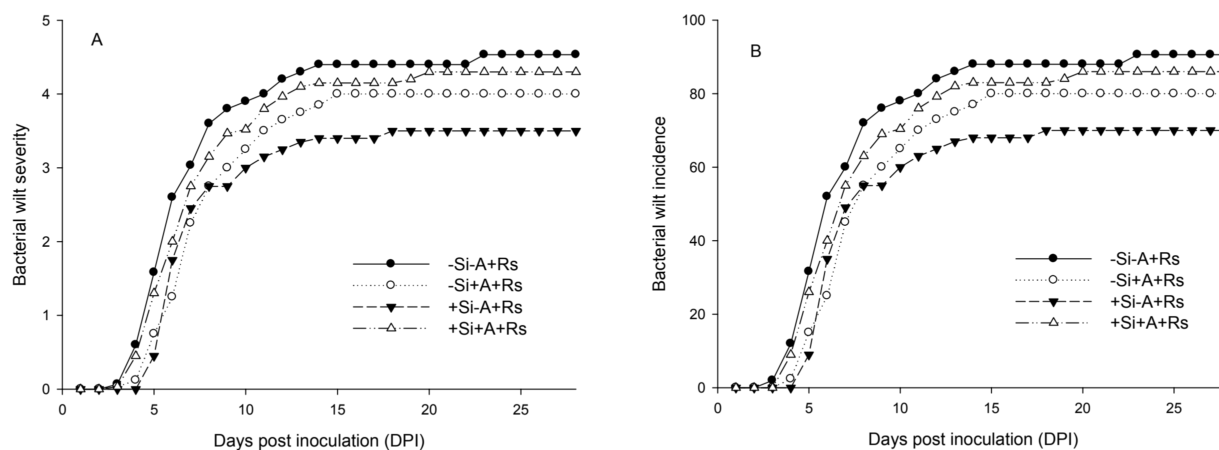


Fig. 3.1: Development of bacterial wilt severity (A) and incidence (B) of tomato genotype King Kong 2: -Si-A+Rs: without silicon and antagonist with *R. solanacearum;* -Si+A+Rs: without silicon, with antagonist and *R. solanacearum;* +Si-A+Rs: with silicon, without antagonist, with *R. solanacearum;* +Si+A+Rs: with silicon, with antagonist and *R. solanacearum* over four weeks.

Data are means of three individual experiments with ten plants per treatment. Bacterial wilt severity was evaluated according to the scale: 0 = no leaf wilted, 1 = one leaf wilted, 2 = two leaves wilted, 3 = three leaves wilted, 4 = whole plant wilted except the top, 5 = dead plant. Disease incidence is the percentage of dead plants at each evaluation date.

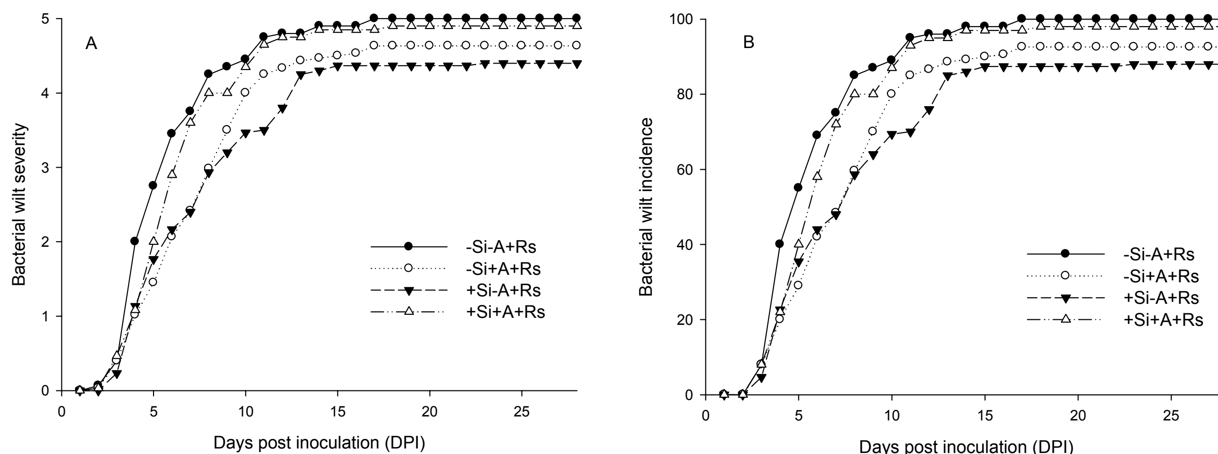


Fig. 3.2: Development of bacterial wilt severity (A) and incidence (B) of tomato genotype L390 -Si-A+Rs: without silicon and antagonist with *R. solanacearum;* -Si+A+Rs: without silicon, with antagonist and *R. solanacearum;* +Si-A+Rs: with silicon, without antagonist, with *R. solanacearum;* +Si+A+Rs: with silicon, with antagonist and *R. solanacearum* over four weeks.

Data are means of three individual experiments with ten plants per treatment. Bacterial wilt severity was evaluated according to the scale: 0 = no leaf wilted, 1 = one leaf wilted, 2 = two leaves wilted, 3 = three leaves wilted, 4 = whole plant wilted except the top, 5 = dead plant. Disease incidence is the percentage of dead plants at each evaluation date.

Table 3.2: Effect of silicon and rhizobacteria treatment on disease severity and disease incidence in tomato genotypes L390 and King Kong 2 inoculated with *R. solanacearum* strain ToUdk2

	AUDPC			
	Disease severity		Wilt incidence	
Treatments	L390	King Kong 2	L390	King Kong 2
+Si-A+Rs	59.0 ± 11.1 bA	47.8 ± 14.0 cB	620.1 ± 31.8 cA	353.3 ± 38.4 cB
+Si+A+Rs	68.7 ± 12.0 abA	58.8 ± 16.2 aB	781.7 ± 68.1 bA	595 ± 29 abB
-Si+A+Rs	62.4 ± 20.2 abA	53.5 ± 13.2 bB	700.5 ± 26.8 bcA	525 ± 19 bB
-Si-A+Rs	71.5 ± 15.2 aA	62.8 ± 16.2 aB	900.5 ± 73.8 aA	716.7 ± 33.7 aB

Data are means ± SE of three independent trails with three plants per treatments. AUDPC calculated based on bacterial wilt severity and disease incidence. Small letters vertically refer to comparison with in the same genotype and capital letters horizontally to comparison between genotypes for the same treatment. Means followed by same letters are not significantly different according to Tukey test at α = 5% probability level.

3.3.2 Bacterial quantification

The application of silicon reduced the bacterial population in mid-stems significantly by 16.9% and non-significantly by 3.2% in genotypes KK2 and L390, respectively while treatment with rhizobacteria reduced the pathogen by 5.4% and 1.8% in KK2 and L390, respectively at five days post inoculation. The combined application of both elicitors resulted in reduction of bacterial numbers in the mid-stems of King Kong 2 and L390 by 2.7% and 1.4%, respectively, which is lower than its individual effect (Table 3.3).

Table 3.3: Bacterial numbers in tomato mid-stems inoculated with *R. solanacearum* strain ToUdk2 as affected by silicon and rhizobacteria treatments at 5 days post inoculation

	Bacterial population (Log CFU/g FW)	
	Genotypes	
Treatments	L390	KK2
+Si-A+Rs	6.9 ± 0.27 aA	5.54 ± 0.29 bB
+Si+A+Rs	7.02 ± 0.33 aA	6.49 ± 0.13 aA
-Si+A+Rs	6.99 ± 0.07 aA	6.31 ± 0.10 aA
-Si-A+Rs	7.12 ± 0.35 aA	6.67 ± 0.10 aA

Data are means ± SE of three independent trails with three plants per treatments. Small letters vertically refer to comparison with in the same genotype and capital letters horizontally to comparison with in genotypes for the same treatment. Means followed by same letters are not significantly different according to Tukey test at α = 5% probability level.

3.3.3 Silicon quantification

Silicon amendment significantly increased the Si content in the root but not in the stem of both genotypes at 5 dpi (Table 3.4) Comparing silicon content in stems, plants supplemented with silicon showed a slight but non-significant increase in silicon concentration in inoculated (+Si-A+Rs;+Si+A+Rs) and non-inoculated treatments (+Si-A-Rs, Si+A-Rs) at 5 dpi in both genotypes. Treatment with rhizobacteria and inoculation of *R. solanacearum* did not influence the distribution and accumulation of silicon in the tomato genotypes.A difference between genotypes in their ability to accumulate silicon was not found.

Table 3.4: Silicon content (mg/g dry matter) in stems and roots of tomato genotypes L390 and King Kong 2 in healthy plants or plants inoculated with *R. solanacearum* strain ToUdk2, amended with or without silicon and rhizobacterium *B. pumilis* 5 days post inoculation

Treatments	Silicon content at 5 dpi (mg/g DW)			
	L390		KK-2	
	Stem	Root	Stem	Root
-Si-A-Rs	0.33 ± 0.03 aA	0.41 ± 0.02 aA	0.25 ± 0.02 aA	0.24 ± 0.01 aA
-Si-A+Rs	0.30 ± 0.03 aA	0.34 ± 0.03 aA	0.27 ± 0.02 aA	0.28 ± 0.02 aA
-Si+A-Rs	0.34 ± 0.02 aA	0.44 ± 0.01 aA	0.29 ± 0.02 aA	0.25 ± 0.02 aA
-Si+A+Rs	0.28 ± 0.02 aA	0.30 ± 0.01 aA	0.31 ± 0.01 aA	0.31 ± 0.02 aA
+Si-A-Rs	0.42 ± 0.05 aB	1.04 ± 0.01 bA	0.44 ± 0.03 aB	0.98 ± 0.02 bA
+Si-A+Rs	0.48 ± 0.02 aB	1.16 ± 0.03 bA	0.47 ± 0.02 aB	1.05 ± 0.04 bA
+Si+A-Rs	0.36 ± 0.02 aB	0.99 ± 0.06 bA	0.41 ± 0.02 aB	1.16 ± 0.08 bA
+Si+A+Rs	0.52 ± 0.04 aB	1.23 ± 0.02 bA	0.43 ± 0.01 aB	1.11 ± 0.07 bA

Data are means of three plants per treatment of three independent trails ± SE. Small letters refer to the comparison of treatments for the same genotype. Capital letters refer to the comparison of the same treatment across genotype. Means followed by same letters are not significantly different according to Tukey test at α = 5% probability level.

3.3.4 Effect of rhizobacteria and silicon on a plant growth

The measured shoot dry weight was used as as an indicator of the plant growth at four weeks post inoculation. In general, reduction of shoots dry weight occurred after inoculation with *R.solanacearum*, while a slight increase was observed when Si was amended to plants inoculated with the pathogen. Plant growth promoting effects of the biotic and abiotic elicitors were observed in non-pathogen infected plants of both tomato genotypes. Individual application of silicon and rhizobacteria increased shoot dry weight compared to the control. Combined application of the elicitors resulted in a reduction of shoot dry weight in both genotypes compared to single treatments with either silicon or rhizobacteria. Reduction in plant biomass was observed in pathogen inoculated plant while a slight increment in plant biomass was observed when a pathogen inoculated plant was treated with Si or rhizobacteria.

Table 3.5: Shoot dry weight of tomato genotypes L390 and King Kong 2 inoculated with *R. solanacearum* strain ToUdk2 in single and combined treatment with silicon and rhizobacteria four weeks after inoculation

	Shoot dry weight [g]	
	Genotypes	
Treatments	L390	KK-2
-Si-A-Rs	4.3 ± 0.36 bB	6.9 ± 0.12 cdA
-Si-A+Rs	1.8 ± 0.15 eB	4.5 ± 0.06 fA
-Si+A-Rs	5.0 ± 0.05 aB	8.1 ± 0.04 abA
-Si+A+Rs	3.2 ± 0.26 cdB	6.6 ± 0.07 deA
+Si-A-Rs	5.5 ± 0.35 aB	8.3 ± 0.17 aA
+Si-A+Rs	3.9 ± 0.10 bcB	7.5 ± 0.05 bcA
+Si+A-Rs	2.8 ± 0.06 cdB	6.4 ± 0.2 deA
+Si+A+Rs	2.5 ± 0.48 deB	6.1 ± 0.23 eA

Data are means ± SE of three independent trails. Small letters vertically refer to comparison with in the same genotype and capital letters horizontally to comparison between genotypes for the same treatment. Means followed by same letters are not significantly different according to Tukey test at α = 5% probability level.

3.3.5 Defense related enzymes

In all plants primed with silicon and rhizobacteria but non pathogen inoculated treatment, the activity of common defense related enzymes i.e. POD, PAL and LOX was very low in both genotypes. However, upon inoculation the pathogen a tendency of non-significant increased activity of POD and PAL was observed in silicon and rhizobacteria primed plants compared to the control. The activity of LOX was significantly decreased in silicon amended and pathogen inoculated treatment, but increased in the rhizobacteria treatment. In simultaneous application of silicon and rhizobacteria, the activity of peroxidase (POD) and phenylalanine ammonia lyase (PAL), lipoxygenase (LOX) dropped significantly. The activities of the three enzymes were still higher in the pathogen inoculated treatment for the moderately resistant genotype than the susceptible one.

Table 3.6: Peroxidase (POD) activity in $\mu mol*min^{-1}*gFW^{-1}$ in tomato genotypes L390 and King Kong 2 healthy and inoculated with *R. solanacearum* strain ToUdk2, amended with and without silicon and rhizobacteria at 5 days post inoculation .

Treatments	Enzyme activity ($\mu mol*min^{-1}*gFW^{-1}$)		Specific POD activity (units/mg protein)	
	L390	KK2	L390	KK2
-Si-A-Rs	1.9 ± 0.2 cdB	2.6 ± 0.12 deA	7.3 ± 0.6 abAB	9.5 ± 0.4 abA
-Si-A+Rs	2.9 ± 0.15 abcB	3.9 ± 0.2 abcA	4.1 ± 0.32 cAB	6.5 ± 0.7 cdA
-Si+A-Rs	2.1 ± 0.1 bcdB	3.1 ± 0.29 cdA	8.5 ± 0.78 aA	10.0 ± 0.3 aA
-Si+A+Rs	3.2 ±0.17 abB	4.2 ± 0.16 abA	5.1 ± 0.2 bcA	6.6 ± 0.4 cdA
+Si-A-Rs	1.7 ± 0.29 dB	2.5 ± 0.07 deA	6.4 ± 0.3 bcA	8.5 ± 0.6 abcA
+Si-A+Rs	3.4 ± 0.11 aB	4.3 ± 0.26 aA	4.7 ± 0.34 bcA	5.4 ± 0.6 dA
+Si+A-Rs	1.6 ± 0.10 dB	2.1 ± 0.19 eA	6.9 ± 0.1 abA	7.5 ± 0.4 bcdA
+Si+A+Rs	2.4 ± 0.2 bcdB	3.3 ± 0.13 bcdA	5.7 ± 0.3 bcA	6.8 ± 0.3 cdA

Data are means ± SE of three independent trails. Small letters vertically refer to comparison with in the same genotype and capital letters horizontally to comparison between genotypes for the same treatment. Means followed by same letters are not significantly different according to Tukey test at α = 5% probability level.

Table 3.7: Phenylalanine ammonia lyase (PAL) activity in µmol*min-1*gFW-[1] in tomato genotypes L390 and King Kong 2 healthy and inoculated with *R. solanacearum* strain ToUdk2, amended with and without silicon and rhizobacteria at 5 days post inoculation .

Treatments	Enzyme activity (µmol*min^{-1}*gFW-^{1})		Specific activity (units/mg protein)	
	L390	KK2	L390	KK2
-Si-A-Rs	2.4 ± 0.1 cdA	3.5 ± 0.34 dA	8. 5 ± 0.4 abAB	12.4 ± 1.0 aA
-Si-A+Rs	3.2 ± 0.3 abcB	5.6 ± 0.22 abA	5.3 ± 0.5 cA	7.3 ± 0.4 defA
-Si+A-Rs	2.0 ± 0.15 dA	2.8 ± 0.21 dA	7.6 ± 0.4 abcA	10.2 ± 0.3 abcA
-Si+A+Rs	3.4 ± 0.12 abB	6.7 ± 0.5 aA	5.2 ± 0.5 cA	6.7 ± 0.23 efA
+Si-A-Rs	2.2 ± 0.2 dAB	3.6 ± 0.4 cdA	9.6 ± 0.33 aA	9.7 ± 0.2 bcdA
+Si-A+Rs	3.7 ± 0.22 aB	6.4 ± 0.41 aA	4.9 ± 0.25 cA	6.2 ± 0.3 fA
+Si+A-Rs	2.3 ± 0.2 dAB	4.2 ± 0.2 bcdA	10.5 ± 1.2 aA	11.3 ± 0.6 abA
+Si+A+Rs	2.7 ± 0.15 bcdB	5.2 ± 0.3 abcA	6.2 ± 0.45 bcA	8.7 ± 0.5 cdeA

Data are means ± SE of three independent trails. Small letters vertically refer to comparison with in the same genotype and capital letters horizontally to comparison between genotypes for the same treatment. Means followed by same letters are not significantly different according toTukey test α = 5% probability level.

Table 3.8: Lipoxygenase (LOX) activity in µmol*min-1*gFW^{-1} in tomato genotypes L390 and King Kong 2 healthy and inoculated with *R. solanacearum* strain ToUdk2, amended with and without silicon and rhizobacteria at 5 days post inoculation

Treatments	Enzyme activity (µmol*min^{-1}*gFW-^{1})		Specific activity (units/mg protein)	
	L390	KK2	L390	KK2
-Si-A-Rs	0.8 ± 0.1 cdA	1.1 ± 0.16 bA	4.8 ± 0.26 abA	6.8 ± 0.8 abcA
-Si-A+Rs	1.3 ± 0.24 bB	2.5 ± 0.18 aA	2.5 ± 0.35 cA	4.5 ± 0.41 dA
-Si+A-Rs	0.6 ± 0.18dA	0.9 ± 0.21 bA	5.3 ± 0.29 cA	7.2 ± 0.34 abA
-Si+A+Rs	1.4 ± 0.12 aB	2.7 ± 0.15 aA	3.3 ± 0.15 bcA	3.3 ± 0.25 dA
+Si-A-Rs	0.8 ± 0.12 cdA	0.95 ± 0.12 bA	5.7 ± 0.58 aAB	8.1 ± 0.41 aA
+Si-A+Rs	0.8 ± 0.14 cdA	1.1 ± 0.12 bA	3.3 ± 0.36 bcA	5.4 ± 0.69 bcdA
+Si+A-Rs	0.7 ± 0.26 cdA	0.97 ± 0.26 bA	4.5 ± 0.37 abA	6.2 ± 0.17 abcA
+Si+A+Rs	0.98 ± 0.15 bcA	1.3± 0.17 bA	5.1 ± 0.31aAB	7.5 ± 0.43 abA

Data are means ± SE of three independent trails. Small letters vertically refer to comparison with in the same genotype and capital letters horizontally to comparison between genotypes for the same treatment. Means followed by same letters are not significantly different according to Tukey test α = 5% probability level.

3.4 DISCUSSION

Ad Planta

In the *ad planta* experiment application of silicon and/or rhizobacteria significantly reduced wilt symptom development in both genotypes.where silicon gave better protection of the plants against the pathogen Similarly, Dannon and Wydra (2004) found that Si amendment reduced wilt in tomato genotype L390 by 26.8% and in King Kong 2 (KK2) by 56.1%, grown in hydroponic culture, while in tomato plants grown in peat substrate Si reduced wilt by 38.1% and 100% in KK2 and Hawaii 7998 (resistant genotype) respectively (Diogo and Wydra, 2007). The latter authors suggested that Si increases plant tolerance and induces resistance against *R. solanacearum* since infected plants tolerated the existence of the bacteria and continued growing without showing severe symptoms. Furthermore, Schacht et al. (2010) reported that silicon-amendment decreased wilt symptom development in three of four tomato recombinant inbred lines (RILs) differing in their resistance to *R. solanacearum.* With regard to the rhizobacteria, Jetiyanon (2007) and Kurabachew et al. (2007) reported reduction in bacterial wilt disease in tomato and potato through application of *Bacillus strains* (IN937a and IN937b) *P. fluorescens* by 50% and 60%, respectively.

Our results indicated that combined application of silicon and rhizobacteria did not result in an additive effect on the suppression of bacterial wilt disease rather an antagonistic effect was observed. This might be due to the elicitation of different signaling pathways by each elicitor which might interact in an antagonistic manner. Similarly, Huong (2006) reported combined application of silicon and *B. atropheus* did

not reduce bacterial wilt in tomato. Also Ishida et al. (2008) found no synergistic effect on the suppression of bacterial blight caused by *Xanthomonas axonopodis* pv. *malvacearum* in cotton when Acibenzolar-S Methyl (ASM) (Oostendrop et al., 2001) and rhizobacterium *B. cereus* isolate L2-I were applied simultaneously.

The jasmonate (JA) and salicylate (SA) signaling pathways in plants provide resistance to herbivore and pathogen attack. These pathways can interact antagonistically where the salicylate pathway had a stronger effect on the jasmonate pathway (Thaler et al., 2002). Furthermore Thaler et al. (1999) indicated that simultaneous application of JA and ASM in tomato resulted in the attenuation of expression of hallmark biochemical responses compared to a single elicitor. Polyphenol oxidase, a JA responsive protein, had a lower activity in plants elicited with both JA and BTH compared to plants elicited with only JA. Accumulation of *PR-4* mRNA, a SA responsive protein, was reduced in plants elicited by both JA and BTH compared to plants elicited with only BTH. This negative interaction in the biochemical expression of the two pathways compromised the resistance of the plant against the pathogen and herbivore. In addition Niki et al. (1998) also found antagonistic effects of SA and JA on the expression of PR protein genes in wounded mature tobacco leaves.

Regarding bacterial colonization in mid stem, application of Si and rhizobacteria significantly reduced bacterial population in the mid-stems of tomato compared to the pathogen inoculated control in King Kong 2. Though not significant, a trend for lower

bacterial numbers was observed in the Si-amended susceptible genotype, L390. In genotype L390, although the number of pathogen in mid-stems of rhizobacteria treated plants was nearly equal to that of *R. solanacearum* infected control it still reduced the bacterial wilt development. Similarly, Dannon and Wydra (2004) and Diogo and Wydra (2007) reported a significant reduction of the bacterial population in Si-amended tomato cultivated in hydroponic and peat substrate, respectively. This may be due to the quick and efficient Si accumulation in the roots providing a structural barrier for further movement of the bacteria in the stem and also to priming the plant's defense making the plant to respond faster and stronger to bacterial infection. The suppression of wilt development and the bacterial population in mid stems of tomato by the rhizobacteria treatment could be explained by the induction of systemic resistance to the host plant that triggered on defense mechanisms. Systemic resistance has proven to be effective against bacterial, fungal and viral pathogens of different crops (Van Loon et al., 1998). It has been reported that the extent of the protective effect of silicon in tomato against *R. solanacearum* depends on the genetic background of the tomato genotype. Also Diogo and Wydra (2007) reported that silicon-induced resistance was more effective in the moderately resistant genotype than in the susceptible one. Induction of disease resistance by Si was observed in many plant species against diseases, such as in rice against sheath blight and brown spot leaf scad (Rodrigues et al., 2003; Fauteux et al., 2005) and in wheat, barley cucumber and Arabidopsis against powdery mildew (Fauteux et al., 2005; 2006, Ma and Yamaji, 2006).

The silicon quantification result indicated that, the silicon content in the roots was higher than in the stems of both genotypes amended with silicon which is typical for non-silicon accumulator plants. Ma et al. (2001) and Diogo and Wydra (2007) found higher amounts of silicon in the root of silicon-amended tomato plant. According to Ma and Yamaji (2005) the variable accumulation of silicon between plant species is due to difference in Si uptake ability of the roots. In Si-accumulator plant such as rice, transportation of Si from the external solution to the cortical cell is mediated by a transporter, while in non- Si- accumulator plants such as tomato since they lack this transporter, transportation of Si is takes place by diffusion, followed by silification, resulting in high and low Si content in the shoot of rice and tomato, respectively (Mitani and Ma, 2005). The identification of influx gene Low silicon rice 1 (*Lsi1*) (Ma et al., 2006) and efflux gene Low silicon rice 2 (*Lsi2*) (Ma et al., 2007) responsible for active Si uptake in rice, support the unequal distribution of total silicon between organs found in most crops.

Increment of tolerance in response to an application of an elicitor is considered to be a type of induced resistance (Vallad and Goodman, 2004). It is known that induced resistance requires or needs extra costs, e.g, reduction in the plant growth, yield, etc. (Romero et al., 2001). However, in our study we did not observe any significant difference in the dry weight of the shoots as parameter for the plant growth indicating no additional costs due to Si application and resistance induction.

Defense related enzymes

Plant defense mechanisms against pathogens are mediated in part by an array of constitutive and inducible chemical resistance factors (Bennett and Wallsgrove, 1994). In both tomato genotypes, the plant reacted to pathogen inoculation by inducing defense enzymes. Reduction of bacterial wilt development reflected by activation of defense related enzyme by the application of the abiotic and biotic elicitors.

In the present study, application of rhizobacteria non-significantly increased activity of lipoxygenase (LOX) in tomato after challenge inoculation by the pathogen compared to the non-amended pathogen inoculated control. Similarly, Ongena et al. (2004), Silva et al. (2004) and Sailaja et al. (1997) reported the induction of systemic reaction by the increased activity of LOX. The products of lipid membrane peroxidation by LOX contribute to defense reactions by inhibiting pathogen growth and development (Croft et al., 1993), induction of phytoalxein accumulation (Li et al., 1991), as precursors for jasmonic and methyl jasmonate that would be involved in signal transduction of induced disease resistance (Xu et al., 1994). However, in Silicon amended plants the activity of LOX was significantly declined, which might be due to the ameliorative effect of Si on membrane integrity. This result is consistent with Gunes et al. (2007) who reported the decline of LOX activity in spinach grown in Boron toxic soil due to exogenous application of Si that reduce lipid peroxidation.

In the current investigation, inoculation pathogen to silicon and rhizobateria primed tomato plants showed an increased of PAL and POD activities. POD is involved in the biosynthesis of lignin which provides a physical barrier and/or limits the extent of pathogen invasion and spread in the plant (Bruce and West, 1989) while PAL is the first enzyme activated in the phenylpropanoid pathway that regulates the production of precursors for lignin biosynthesis and other phenolic protectants in plant cells (Hahlbrock and Scheel, 1989). Furthermore, increased activity of POD, PAL and LOX were also observed, in plants primed with plant growth promoting rhizobacteria in cucumber (Chen et al., 2000), tomato (Silva et al., 2004) and coconut (Karthikeyan et al., 2006) after inoculation of the pathogen.

In Si-amended and non pathogen inoculated treatments the activity of PAL and POD enzymes were significantly lower than in pathogen inoculated plants, suggesting that the ameliorative effect of silicon manifests only in the presence of the biotic stress. Also Yang et al. (2003) and Cai et al. (2008) reported that Si application alone has no protective effect on plants growing in stress free environment.

In conclusion, this study showed the vital role of the biotic and abiotic elicitors in the induction of defense in tomato against the pathogen. Application of either Si or rhizobacteria alone led to reduction of wilt incidence indicating the induction of systemic resistance. Tomato being a non silicon accumulator plant supports the idea that the protection rendered by its application comes from through induction of systemic resistance rather than its mechanical barrier role for the ingress of the

vascular pathogen. This is also supported by the observed activity of the common defense related enzymes. But, the combined application of the two elicitors resulted in antagonistic interaction rather than additive which was expressed at phenotypic and biochemical level. To elucidate the intricate plant-microbe-Si interaction and better understand the modes of actions each elicitor further molecular analyses are recommended.

Chapter 4

Transcriptome analysis of rhizobacteria-silicon mediated induced systemic resistance in tomato (*Solanum lycopersicum*) against *Ralstonia solanacearum*

Abstract

Transcriptome analysis of tomato (*Solanum lycopersicum*) stem tissue was performed to elucidate silicon and/or rhizobacteria primed gene expression profiling after inoculation of *Ralstonia solanacearum.* A total of 174 genes were differentially regulated, of which 113 were up-regulated and 61 down-regulated. Functional categorization revealed most of the up-regulated genes involved in signal transduction, defense, protein synthesis and metabolism, while a large proportion of down regulated genes were involved in metabolism, photosynthesis, signal transduction, lipid metabolism. Here Si regulated more defense related genes than *B. pumilis*. However, during the simultaneous application of the two elicitors antagonistic interaction occurred manifested in no reduction of bacterial wilt, with genes of the ethylene-jasmonate and salicylate path ways which is elicited by rhizobacteria and silicon, respectively. In this case five genes were down regulated which were up-regulated during separate application of each elicitor. Results suggest separate application of silicon and rhizobacteria strain as best alternative for the induction of systemic resistance that will switch on defense arsenal of the plant against *R.solanacearum.*

Key words: Ethylene, jasmonic acid, priming, *R. solanacearum,* rhizobacteria, signal transduction, silicon, transcriptome

4.1 INTRODUCTION

Plants, being sessile, have evolved a battery of defense response genes to protect themselves from biotic and abiotic stress. Defense may be preformed or induced. Induced plant defenses are regulated by a highly interconnected signaling network in which the plant hormones jasmonic acid (JA), ethylene (ET) and salicylic acid (SA) play central roles (Pozo et al., 2004; Van Loon et al., 2006; Asselbergh et al., 2008). In induced resistance the defense capacity of plants is enhanced biologically by beneficial rhizobacteria, mycorrhizal fungi or chemically by exogenous application of low doses of SA, its functional analog benzothiadiazole (BTH), Acibenzolar-*S*-methyl (ASM), JA or *ß* aminobutyric acid (BABA) and silicon (Dannon and Wydra, 2004; Fauteux et al*.,* 2005; Conrath et al., 2006; Frost et al.,2008). Si is known to induce systemic acquired resistance (SAR) and modulate the defense response of the plant by participating in signal transduction, which leads to the enhancement of host resistance (Fauteux et al*.,* 2005).

Phenotypically, rhizobacteria-mediated induced systemic resistance (ISR) resembles classical pathogen-induced systemic acquired resistance (SAR), in which non-infected parts of locally infected plants develop increased resistance to further infection (Ross, 1961). Although both ISR and SAR are effective against a broad spectrum of pathogens, their signal-transduction pathways are clearly distinct. The onset of SAR is associated with increased levels of salicylic acid (SA), and is characterized by the coordinate activation of a specific set of pathogenesis-related (PR) genes, many of which encode PR proteins with antimicrobial activity (Van Loon

et al., 2006). In such primed plants, defense responses are not induced directly by the priming agent, but are activated in an accelerated manner following perception of biotic or abiotic stress signals, resulting in an enhanced level of resistance against the stressor encountered (Walters et al., 2007).

Silicon-mediated gene expression was studied by Fauteux et al. (2006) in the *Arabidopsis*-powdery mildew system. Their results contradicted the hypothesized role of Si as a fertilizer, whereas the expression of only two genes out of 40,000 genes was regulated by Si in unstressed plants, i.e., without pathogen. However, upon inoculation of *E. cichoracearum* DC to Arabidopsis, Si obviously attenuated the overall down-regulation in gene expression, indicating a role in alleviating the stress imposed by the pathogen. Additionally, Si modulated the expression of some defense-related genes as well as genes involved in different metabolic pathways in plants inoculated with the pathogen.

Similarly, Chain et al. (2009) has also performed a comprehensive transcriptomic analysis of silicon on wheat and found that 47 genes were regulated in the silicon treated control while 699 genes were differentially expressed after the inoculation of *B. graminis f. sp. tritci.* Nickel et al. (2010) and Ghareeb and Wydra (2007) also conducted silicon-induced gene expression profiling in tomato against tomato *R. solanacearum* and found up-regulation of genes that are involved in defense, signal transduction, response to stresses, and metabolism.

Analysis of the transcriptome of ISR-expressing *A. thaliana* leaves after challenge inoculation with the bacterial speck pathogen *P. syringae* pv. *tomato* DC3000 (*Pst* DC3000) revealed 81 genes with amplified expression patterns, indicating that the plants were primed by fluorescent *Pseudomonas spp.* to respond more rapidly and/or more strongly to pathogen attack (Verhagen et al., 2004). Similarly, various studies reported the up-regulation of metabolism, signal transduction, defense and stress related genes in rhizobacteria induced systemic resistance in *A. thaliana* (Wang et al., 2004; Pozo et al., 2008) and in tomato fruit (Jiang et al., 2009) against different plant pathogens. Therefore, the objective of the current study was to unveil by high throughput gene expression profiling induced resistance by silicon and *B. pumilis* in *R. solanacearum* inoculated tomato genotypes using DNA-microarray analysis.

4.2 MATERIALS AND METHODS

4.2.1 Experimental design

RNA was extracted from plant material collected from three independent trials (see chapter three) with the following four different treatment combinations: (i) without silicon and antagonist, with *R. solanacearum* (-Si-A+Rs: control), (ii) without silicon, with antagonist and *R. solanacearum* (-Si+A+Rs), (ii) with silicon, without antagonist, with *R. solanacearum* (+Si-A+Rs), (iv) with silicon, with antagonist and *R. solanacearum* (+Si+A+Rs) arranged in a complete randomized design. Stem tissue from three plants per treatment that was also used for bacterial and silicon quantification and for the enzyme assay five days post inoculation was collected and kept frozen until RNA extraction.

4.2.2 RNA Extraction

Total RNA used in the microarray experiment was extracted from frozen stem material using the Trizol method. RNA was prepared from three biological replicates that were pooled to reduce the biological noise arising from biological variation. Briefly, the pooled plant material was grinded in liquid nitrogen with mortar and pestle and 100 mg were homogenized with 1mL Trizol (Invitrogen). The homogenate were vortexed briefly and centrifuged at 12,000xg for 10 min at 4°C. The cleared homogenate solution was transferred to a new eppendorf tubes and 200 µL chlofrom was added, briefly vortexed and incubated for 3 min at room temperature, then centrifuged at 12,000xg for 15 min at 4°C. The upper phase of the supernatant was carefully separated and transferred to a new eppendorf tubes. The RNA was precipitated by addition of 500 µL isopropyl alcohol followed by incubation at room temperature for 10 min and centrifugation for 10 min at 12,000xg at 4°C. The supernatant was discarded and the RNA pellet washed by 1mL 75% ethanol followed by centrifugation at 7,500xg for 5 min at 4°C.Then, the RNA pellet was dissolved in RNase free water. The quantity and quality of total RNA was determined by Nano drop ND-1000 spectrophotometer and capillary electrophoresis using an Agilent 2100 Bioanalyzer system (Agilent technology: with RNA 6000 Nano & Pico Lab Chip kit), respectively.

4.2.3 cDNA synthesis and labeling

For further microarray analysis cDNA was synthesized and labeled according to MEN® Micromax TSATM labeling and detection kit (PerkinElmer). Briefly, the mixture of 8 µg total RNA, 2 µL reaction mix (dNTPs), 1 µL biotin nucleotide or fluorescein-nucleotide and 2 µL primer mix [1 µL oligo T (100 µM) and 1 µL random hexamer (100 µM)] was denatured at 65°C for 10 min followed by incubation for 10 s on ice. Reverse transcription was carried out by adding 5 µL 10X reaction buffer and 2 µL AMV RT/RNase inhibitor mix, 3 µL DTT then the mixture was incubated at 42°C for 2 h followed by cooling in ice for 5 min. The reaction was stopped by adding 2.5 µL 0.5 M EDTA (pH 8.0) and 2.5 µL 1 N NaOH, and incubation at 65°C for 30 min followed by cooling in ice for 5 min.

Labeled cDNA was purified with QIAquick PCR purification kit (QIAGEN). The labeled cDNA was mixed with 300 µL PB-buffer and applied to a filter-column, then centrifuged at full speed for 1 min. The flowthrough was discarded and 700 µL 35% guanidinhydrochlorid were added to the filter-column and then centrifuged at full speed for 1 min. The flow through was again discarded and the filter column was dried by centrifuging at full speed for 1 min, then the flowthrough was discarded. The labeled cDNA was eluted twice with 25 µl EB buffer (1:10 diluted). The flowthrough of Cy3- and Cy5-labbled cDNA probes were evaporated to dryness in Speedvac for 1 h in the dark. The dried cDNA probe pellet was re-suspended in 45 µL hybridization buffer (MWG) and incubated at 95°C for 3 min cooled on ice for 2 min. The re-suspended Cy3- and Cy5-labbled cDNA probes were combined and mixed with 10 µL TopBlock (Sigma) making the total volume sample 100 µL.

4.2.4 Hybridization

Before hybridization, the TOM2 microchips were re-hydrated over water at 65°C for 2 min and then treated with UV at 65 mJ for 2 min. The re-suspended cDNA probes were applied to a pre-warmed (65°C) slide, and covered with a clean glass Lifter Slip (Erie Scientific, http://www.eriemicroarray.com). The slides were then sealed with nails polish and maintained at 42°C in a hybridization chamber with gentle shaking at 650 rpm overnight in the dark.

4.2.5 Washing and Fluorescence Detection

The cover was removed and the microchip was washed by gentle agitation in washing buffer 1 (2X SSC, 0.1% SDS) for 5 min, then in washing buffer 2 (1X SSC) for 5 min and then in washing buffer 3 (0.5X SSC) for 5 min. The spotted area was framed with ImmEdgeTM pen and 300 µL TNB-G blocking buffer (0.1 M TrisHCl, 0.15 M NaCl, 0.5% Blocking reagent, 10% Goat serum) were applied and incubated together for 10 min followed by washing in TNT (0.1 M TrisHCl, 0.15 M NaCl, 0.05% Tween 20) buffer for 1 min. The first conjugation step was performed by adding 200 µL anti-Fl-HRP conjugate solutions (2 µL anti-Fl-HRP dissolved in 198 µL TNB-G) and incubation for 10 min followed by washing three times in TNT buffer for 1 min. For the first detection step 250 µL cyanine-3-tyramide (0.75 µL Cy3 dissolved in 249.25 µL amplification diluents) were added and incubated for 15 min followed by washing three times in TNT buffer for 5 min. HRP inactivation was carried out by applying 300 µL HRP inactivation solution (10 µL 3 M NaAc, pH 5.2, 100 µL 35% H_2O_2 and 190 µL ddH_2O) and incubation for 10 min followed by washing three times

in TNT buffer for 1 min. The second conjugation step was performed by adding 200 µL streptavidin-HRP conjugate (2 µL Streptavidin-HRP conjugate dissolved in 198 µL TNB-G) and incubation for 10 min followed by washing three times in TNT buffer for 1 min. For the second detection step 250 µL cyanine-5-tyramide (0.5 µL Cy5 dissolved in 249.5 µL amplification diluents) were added and incubated for 10 min followed by washing three times in 1X SSC buffer for 1 min and once in 0.5X SSC (1:10 diluted with ddH_2O) for 1 min. Finally, the microchip was dried by centrifugation at 560 xg for 2 min.

4.2.6 Data acquisition and data analysis

The microchip was scanned with 8 different laser powers and photomultiplier tube (PMT) settings using the GenePix 4000B scanner and GenePix Pro 6.1 software. The experiment was repeated three times with plant samples from three different experiments. Data from different scans of each chip were extracted by GenePix Pro 6.1 software, normalized and united. The log 2 fold change was calculated from the relative fluorescent intensities, which correspond to the regulation of the gene expression. The regulated genes were identified following these selection criteria i) the changes in the gene expression occurred in the same direction in three microarray analysis ii) statistical significance of gene expression at α= 0.5 using t-test. Significantly regulated genes, annotated by SOL Genomics Network database (Cornell University) and Tomato Expression Database, and then functionally classified using literatures, SOL, TAIR, KEGG and EMBL-EBI databases.

4.3 RESULTS

4.3.1 RNA concentration and quality

RNA extracted by Trizol protocol gave a good quality and a quantity of 1255-3979 ng RNA/μL per plant tissue and purity of OD 260/OD 280 = 2.1. The RNA was further profiled by Agilent 2100 bioanalyzer using RNA 6000 Nano Kit and showed a good pattern of separation.

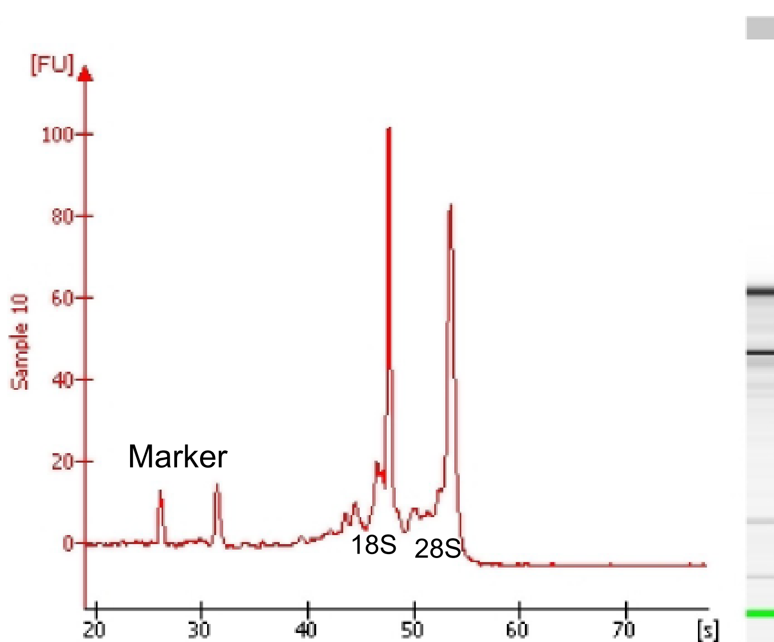


Fig. 4.1: Electropherogram image of total RNA extracted from the stem tissue of tomato plants, analyzed with an Agilent 2100 bioanalyzer using the RNA 6000 Nano Kit.

4.3.2 Gene expression profiling in tomato stem

To obtain a complete picture of gene expression changes triggered by *R.solanacearum* inoculation in rhizobacteria and silicon primed-tomato genotypes, genome wide microarray analysis was performed with tomato stem samples taken five days post inoculation of the pathogen. Accordingly, in response to pathogen inoculation in silicon and rhizobacteria primed tomato genotypes a total of 174 genes were differentially regulated (Table 4.1). Based on annotated gene assignments, genes differentially regulated by application of the elicitors are categorized by function accordingly: transcription factor (7.1% up regulated genes, 9.8% down regulated genes), signal transduction (12.4% up regulated genes, 4.9% down regulated genes), defense related genes (15.9% up regulated genes, 6.6% down regulated genes),protein biosynthesis (6.2% up regulated genes, 8.2% down regulated), energy pathway (4.4% up regulated genes, 8.2% down regulated), lipid metabolism (2.7% up regulated genes, 8.2% down regulated genes),cell wall (6.2% up regulated genes, 3.3% down regulated genes), hormone response (3.5% up regulated genes, 3.3% down regulated genes), photosynthesis (3.5% up regulated, 9.8% down regulated),G-protein (2.7% up regulated), nucleic acid metabolism (4.9% down regulated), unknown function (35.4% up regulated genes, 32.8% down regulated genes).The majority of the up-regulated genes belong to major biological changes induced by silicon followed by a lower number of up-regulated genes by rhizobacteria after pathogen inoculation.

In tomato plants primed by silicon (+Si-A+Rs), a number of defense related genes were up-regulated upon inoculation of the pathogen. Such genes are pathogenesis related protein1 precursor (PR-1), endo-1,3-beta–glucanase-like protein, basic endochitinase, disease resistance protein (NBS-LRR class), hevein-related protein precursor (PR-4), Pathogenesis-related protein, glycosyl hydrolase family 19 (basic endochitinase), leucine rich repeat protein, defensin, disease resistance protein, cytochrome P450, germin like, putative cytochrome P450, peroxidase which are known to be involved in the induction of resistance. Similarly, Si amendment triggered up-regulation of a variety of transcription factors and signal transduction elements such as myb family transcription factor, homeodomain protein containing `homeobox' domain signature, Zip transcription factor ATB2, putative WRKY-type DNA binding protein, zinc finger protein putative, WRKY transcription factor 3 and mitogen-activated protein kinase, transmembrane protein, leucine rich repeat protein family,receptor-related serine/theronine kinase, tyrosine phosphatase, phosphatidylinositol-4-phosphate 5-kinase, MAP3K-like protein kinase, protein phosphatase 2C (PP2C), NADPH oxidase are the common ones which will participate in signal transduction within the plant.

Amendment with *B. pumilis* (-Si+A+Rs) also triggered the expression of defense related genes such as ethylene responsive proteinase inhibitor I precursor, phenylalanine ammonialyase 1, peroxidase, pathogenesis-related protein, a RAS-related GTP binding protein (ARA-1), cytochrome P450. It also up-regulated limited number of transcription and signal transduction signal elements such as: ethylene response factor 1, ethylene responsive element binding factor, WRKY transcription

factor 3, mitogen-activated protein kinase, calcium-dependent protein kinase, calmodulin, calmodulin-binding protein, aminocyclopropane-1-carboxylate oxidase, jasmonate ZIM-domain protein 3 which are known to be involved in ethylene and jasmonate mediated signal transduction .

A number of genes that were up-regulated during the individual application of each elicitor were down regulated in plants primed by simultaneous application of both silicon and *B. pumilis*. For instance defense related genes such as cytochrome P450, putative cytochrome P450, WRKY transcription factor 3, aminocyclopropane-1-carboxylate oxidase, basic endochitinase were down-regulated. In addition a large group of unknown proteins and different categories of genes involved in metabolism, photosynthesis and protein synthesis were down regulated.

Table 4.1: Classification of genes differentially expressed in tomato five days after *R.solanacearum* inoculation in –Si-A+Rs,–Si+A+Rs, +Si-A+Rs, +Si+A+Rs treatments

Functional category	gene number	ratio (tret/conl)
Up regulated		
Transcription factor	8	1.5 - 2.4
Myb family transcription factor, ethylene response factor 1, ethylene responsive element binding factor, homeodomain protein contains 'homeobox' domain signature, Zip transcription factor ATB2, putative WRKY-type DNA binding protein, zinc finger protein putative, WRKY transcription factor 3		
Signal transduction	14	1.4 - 2.2
Mitogen-activated protein kinase, calcium-dependent protein kinase, calmodulin, calmodulin-binding protein, transmembrane protein, leucine rich repeat protein family , receptor-related serine/theronine kinase,tyrosine phosphatase, phosphatidylinositol-4-phosphate 5-kinase, MAP3K-like protein kinase, protein phosphatase 2C (PP2C), NADPH oxidase, aminocyclopropane-1-carboxylate oxidase, jasmonate ZIM- domain protein 3		
Defense related genes	18	2.1- 2.7
Pathogenesis related protein1 precursor (PR-1), endo-1,3-beta–glucanase-like protein, basic endochitinase, disease resistance protein (NBS-LRR class), hevein-related protein precursor (PR-4),pathogenesis-related protein,glycosyl hydrolase family 19 (basic endochitinase), leucine rich repeat protein, defensin ,disease resistance protein, putative cytochrome P450, germin like, ethylene responsive proteinase inhibitor I precursor,phenylalanine ammonialyase 1 , cytochrome P450, RAS-related GTP binding protein (ARA-1),peroxidase,zinc finger protein 5 ZFP5		
Protein biosynthesis	7	1.2 - 1.7
cytosolic cyclophilin (ROC3), ubiquitin family , cyclophilin ROC7, 60S acidic ribosomal protein P0 (RPP0B), symbiosis-related like protein, eukaryotic rpb5 RNA polymerase subunit, 60S ribosomal protein L10A (RPL10aB),		
Energy pathways	5	1.4 - 2.0
mitochondrial aldehyde dehydrogenase (ALDH3) ,GDP-mannose pyrophosphorylase, L-allo-threonine aldolase, cytochrome b561-related, pyruvate		

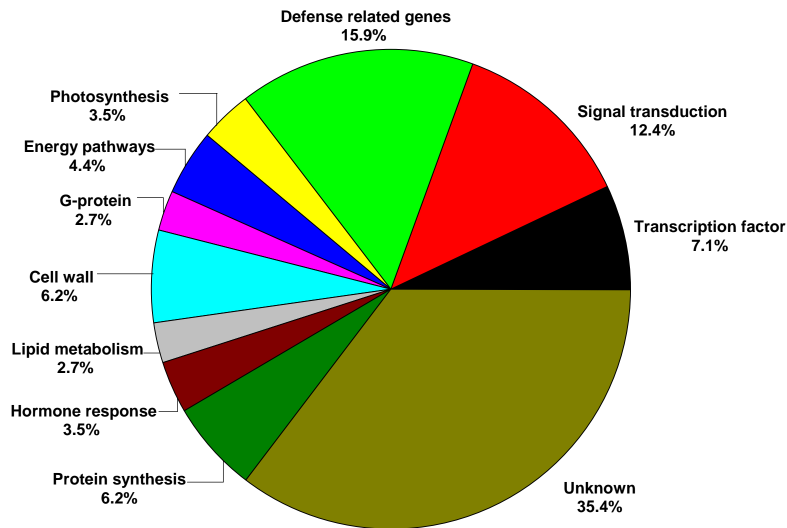
Table 4.1: continued from the previous page

Functional category	gene number	ratio (tret./con)
Photosynthesis	4	1.3 - 1.6
chloroplast nucleoid DNA binding protein, thioredoxin M-type 4, glutathione synthetase (GSH2), chloroplast precursor (TRX-M4)		
G protein	3	1.2 - 1.4
GTP-binding protein, ARFGTPase –activating domain, GTPase –activating protein		
Lipid metabolism	3	1.5 - 1.7
myo-inositol-1-phosphate synthase-related protein, thioesterase family, lipase		
Cell wall	7	1.3 -2.1
extensin, endo-1,4-beta-glucanase, xyloglucan endotransglycosylase, glycosyltransferase family 8, xyloglucan endo-1,4-beta-D-glucanase (EC 3.2.1.-) precursor, alpha-expansin 6 precursor Alpha 1, 4-glycosyltransferase, cellulose		
Hormone response	4	1.4 - 1.8
ethylene-responsive protein, arginine decarboxylase, auxin response factor 8, 2- oxoglutarate dependent dioxygenase		
Unknown function	40	1.5 - 2.2
Down regulated		
Lipid metabolism	5	-1.4 - 2.0
ceramidase family protein, lipase, myo-inositol-1-phosphate synthase-related protein, thioesterase family, lipoic acid synthase		
Photosynthesis	6	-2.3 - 2.6
ribulose bisphosphate carboxylase small chain 2b precursor, plastocyanin, light-harvesting chlorophyll a/b binding protein, protochlorophyllide reductase B, ribulose bisphosphate carboxylase small chain 3b precursor, chlorophyll a-b binding protein 3C-like		
Transcriptional factor	6	-1.4 - 2.2
GATA zinc finger protein, WRKY family transcription factor, myb-related transcription factor LBM1, bZIP transcription factor, PHD finger transcription factor, ethylene-responsive transcription factor		

Table 4.1: continued from the previous page

Functional category	gene number	ratio (tret./con)
Nucleic acid metabolism	3	-2.4 - 2.7
RNA recognition motif (RRM), RNA-binding protein, ADP-ribosylation factor,		
Signal transduction	3	-1.6 - 2.9
1-aminocyclopropane-1-carboxylate oxidase (ACCO), allenoxide,putative protein/phospholipase C		
Defense related genes	4	-2.1 - 2.7
Putative cytochrome P450, polyubiqutin (UBQ4), cytochrome P450, basic endochitinase		
Cell wall	2	-1.5 -2.4
Xyloglucan endotransglucosylase-hydrolase XTH7, pectinesterase		
Hormone response	2	-1.5 - 2.6
Auxin response factor 6, ethylene-responsive protein ETR1		
Protein synthesis	5	-2.2 - 2.7
eukaryotic translation initiation factor 4A-1(eIF4A-1), 40S ribosomal protein S14 (RPS14B), 25S rRNA, rubisco subunit binding-protein ß subunit, deoxyhypusine synthase		
Energy pathways	5	-2.0 - 2.8
short-chain dehydrogenase/reductase, epsilon subunit of mitochondrial F1-ATPase, GCN5-related N-acetyltransferase (GNAT), malate oxidoreductase (NADP-dependent malic enzyme), gamma-VPE (vacuolar processing enzyme)		
Unknown function	20	-2.4 -2.9

A Up-regulated genes



B Down-regulated genes

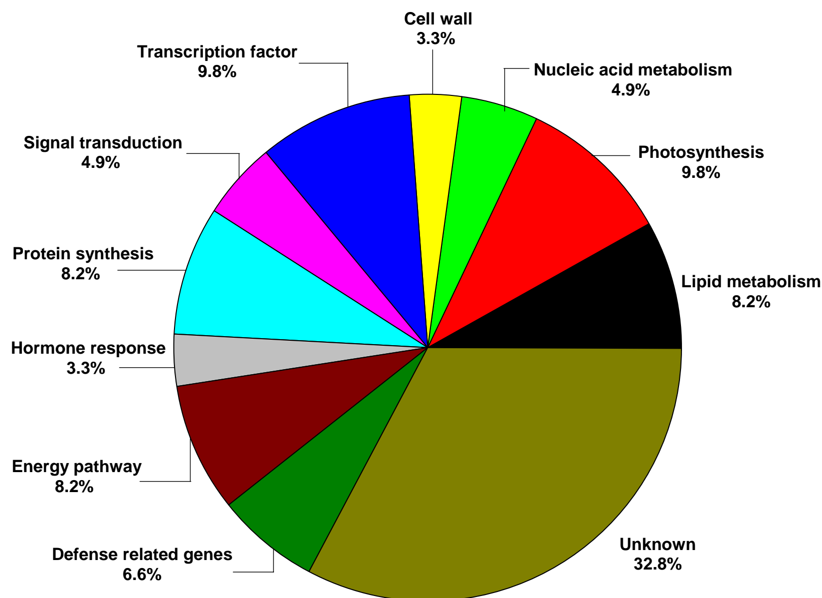


Fig. 4.2: Pie charts showing the number of up-regulated (A) and down-regulated genes (B) in each functional category

4.4 DISCUSSION

Gene expression profiling using microarrays has been recognized as a powerful approach to obtain an overall view on gene expression and physiological processes involved in response to a particular stimulus (Maleck et al., 2000; Schenk et al., 2000). To get molecular insights in the response of tomato after pathogen inoculation in rhizobacteria and silicon-primed tomato genotypes, we analyzed gene expression profiles of tomato plant and found the regulation of a total of 174 genes.

In this regard, inoculation of *R. solanacearum* in tomato primed with silicon induced changes in the expression of defense response genes. Most of the up-regulated defense related genes and transcripts belong to the salicylic acid dependent pathway that leads to induction of systemic acquired resistance (SAR). SAR is induced after local infection of the plant by the pathogen or elicitor accompanied by an increase in the level of endogenous salicylic acid (SA) and subsequent *PR* protein expression (Ross, 1961; Durrant and Dong, 2004). In line with this, in our microarray analysis up-regulation of PR-1 protein, a marker for SAR, was found. PR proteins function either directly on the pathogen through production of antimicrobial substances or indirectly by creating physical barriers to the pathogen infection process or by upstream intrinsic PR signaling (Jiang et al., 2009). Furthermore, PR proteins such as endo-1, 4-beta-glucanase, basic endochitinase and glucan endo-1, 3-beta-glucosidase are known to disrupt the cell wall of fungal/bacterial pathogens (Datta and Muthukrishnan, 1999). All of these events are related to the systemic acquired resistance response of the plant. Therefore, our result indicated induction of SAR

against the vascular pathogen by silicon application which was also depicted by reduction of bacterial wilt severity and incidence in the *ad planta* experiment. These descriptions support the pivotal role of Si in resistance development in tomato. Our result was in line with Nickel et al. (2010) and Ghareeb and Wydra, (2007) who reported the up-regulation of defense related genes in silicon amended tomato plants 72 hours post inoculation of *R. solanacerum.* Similarly Chian et al. (2009) and Fauteux et al. (2006) indicated the silicon-induced regulation of defense related genes in wheat and *Arabidopsis* against *B. graminis f. sp .tritici* and powdery mildew, respectively.

In addition, the increment in peroxidase (POD) activity in our enzyme assay was in line with the microarray result where silicon treatments trigger up-regulation of POD transcript that participates in stress alleviation to reactive oxygen species (ROS).Similarly, Rodrigues et al*.* (2005) reported that Si application results in accumulation of POD transcript in a resistant rice cultivar after inoculation with *M. grisea*, while a susceptible cultivar exhibited higher level and longer time of accumulation. Furthermore Fauteux et al. (2006) indicated up-regulation of peroxidase in *Arabidopsis* plant amended with Si after inoculation with the biotrophic fungus *E. cichoracearum.* ROS function as antimicrobial as well as signaling molecules in activating plant defense gene expression (Khan and Wilson, 1995). In addition, peroxidases play a role in cell wall lignifications which provide a mechanical barrier against pathogen ingress (Kärkönen et al., 2002). Therefore, this enzyme may activate ROS-dependent signal transduction that leads to SAR.

In all the above stated cases inoculation of *R. solanacearum* to the primed plant is decisive. Thus, the effect of Si will only manifest in the presence of the pathogen, as our enzyme assays and *ad planta* results show. Also Fautex et al. (2006) reported that genes related to defense were mostly regulated after pathogen inoculation, including R-genes such as stress related transcription factors, genes involved in signal transduction, the biosynthesis of stress hormones (SA, JA, ethylene), the metabolism of reactive oxygen species, and the biosynthesis of antimicrobial compounds. Furthermore, Ghareeb and Wydra. (2007) observed no changes in gene expression of tomato amended with Si, without *R. solanacearum* inoculation. This suggests that the defense arsenal of primed tomato plant will only be switched on and triggered faster and stronger upon inoculation of the pathogen. In contrary, the presence of the pathogen negatively affected the expression of a number of genes involved in processes such as photosynthesis, energy pathways, protein synthesis, nucleic acid metabolism, lipid metabolism in non-primed plant. Nevertheless, the damage is minimized when the plant is primed with either silicon or rhizobacteria, but not by simultaneous application.

In our experiment inoculation of the pathogen in tomato plants primed with *B. pumilis,* resulted in up-regulation of defense related genes such as peroxidase, PAL and PR proteins, which are common actors in resistance induction. For instance, PAL codes for the first enzyme in the phenylpropanoid pathway, the origin of phenolic compounds, which exhibit defensive activity against pathogens (Piereira et al., 1999). In addition, up-regulation of transcription factors and signal transducing elements

such as myb family transcription factor, ethylene response factor 1, ethylene responsive element binding factor, WRKY transcription factor 3, aminocyclopropane-1-carboxylate oxidase and jasmonate ZIM-domain protein 3 encode genes involved in the downstream ethylene (ET) and jasmonate (JA) signaling of defense related genes. Also Schacht et al. (2010) and Nickel et al. (2010) found thirteen times up regulation of Jasmonate ZIM-domain protein (JAZ). Similarly Wang et al. (2005) reported up regulation of 95 genes involved in signal transduction, stress response, defense and transcription factors after inoculation of the pathogen *P. syringae* pv. *tomato* DC3000 in *Arabidopsis* primed with the endophytic plant growth promoting rhizobacterium *P. fluorescens* FPT9601-T5. Furthermore, Cartieaux et al. (2003) reported transcript modifications of 63 genes in shoots and of nine genes in roots of *Arabidopsis* colonized by the PGPR *P. thivervalensis* MLG45 against *P. syringae* pv. *tomato* (strain DC3000). An increase of defense-related transcripts and a repression of photosynthesis-related transcripts by the colonization were reported as characteristic changes. In addition, Verhagen et al. (2004) also reported an enhanced defense capacity against a broad spectrum of plant pathogens after inoculation of *P. syringae* pv. *tomato* DC 3000 in *A. thaliana* primed by *P. fluorescens* WCS417r. Locally in the roots and leaves *P. fluorescens* WCS417r elicited a substantial change in the expression of 97 and 81 genes, respectively, where the majority of the primed genes were suggested to be regulated by jasmonic or ethylene signaling. Generally, Van Loon et al. (1998) described rhizobacterium-mediated ISR as a broad-spectrum resistance that is triggered by selected strains of nonpathogenic rhizosphere bacteria.

In our gene profiling study we found upregulation the gene for GTP-binding protein which might be a possible consequence of rhizobacteria and/or silicon-mediated plasma membrane ATPase (proton pump) activation that leads to downstream activation of common defense related PR genes. In line with this Wan et al. (2002) reported that the common early events in cellular communication and defense signaling are the transient changes in the ion permeability of the plasma membrane. Upon pathogen recognition, ion channels located in the plasma membrane appear to increase ion fluxes across the membrane and activate downstream defense responses. Similarly Maleck et al. (2000) reported genes encoding ion pumps and channels which are up-regulated by defense-related elicitation. This gene encoding a plasma membrane H^+-ATPase was up-regulated in a constitutive SAR mutant (*cim*) and also in systemic leaves expressing *avrRpt2* after being challenged by *P. syringae pv. tomato* DC3000.

Inaddition we observed the presence of link between signal transduction and downstream elicitation of PR-proteins up-regulation of mitogen-activated kinases (MAP3K and MAPKK) and calcium-dependent kinases (calmodulin and calcium protein kinases) genes after treatment by the rhizobacterial strain. MAPK cascades transfer signals from upstream receptors to downstream cellular effectors, and rapid MAPK activation allows instantaneous modification of down-stream signaling proteins (Krens et al., 2006; Zhang et al., 2006). Also Snedden and Fromm (2001) reported that transient influx of Ca^2+ constitutes an early element of signaling cascades triggering pathogen defense responses in plant cells. Calmodulin proteins bind Ca^{2+}

and are involved in decoding the Ca^{2+} signatures and transducing signals by activating specific targets and pathways. In addition Grant et al. (2000) speculated that downstream responses to Ca^{2+} signaling may be an important component of resistance to Pst since an increase in cytoplasmic calcium in response to Pst (avrRpm1) infection in Col-0 plants was observed. In plants, these cascades have been implicated in typical defense responses, such as production of pathogenesis-related proteins, ROS, ethylene and cell death (Pedley and Martin, 2005). Therefore, our results indicate that MAPK cascades and calcium-dependent kinases (calmodulin and calcium protein kinases) genes play a pivotal role in the induction of resistance against *R. solanacearum*.

In tomato primed by both elicitors (+Si+A+Rs) we found antagonistic interaction between the two pathways induced by each elicitor i.e. rhizobacteria and silicon. In our gene expression profiling study the down-regulation of genes such as cytochrome P450, putative cytochrome P450, WRKY transcription factor 3, aminocyclopropane-1-carboxylate oxidase, and basic endochitinase was observed, while these were up-regulated during the individual application of each elicitor. Thus, the non-additive or antagonistic interactions seem to cancel out the effect of each elicitor which is also supported by our enzyme assay results. Also Ishiad et al. (2008) found an antagonistic effect between Acibenzolar S-Methyl (ASM) and the rhizobacteria isolate L2-I *B. cereus* when applied simultaneously against bacterial blight caused by *Xanthomonas axonopodis* pv. *malvacearum* in cotton where the activities of common defense enzymes such as PAL, POD and ß-1, 3 glucanase

(GLU) were lower compared to ASM alone. Furthermore, Thaler et al. (2002) reported that jasmonate and salicylate signaling pathways interact resulting in antagonism between themselves where the salicylate pathway had a stronger effect on the jasmonate pathway. This statement was further supported by different researchers who indicated that SA interference with JA signaling occurs in three different process: first, before JA synthesis where 13 S-hydroperoxylinolenic acid is converted to 12-oxy-phytodienoic acid by 13 S-hydroperoxide dehydrase (Pena-Cortez et al., 1993; Doarels et al., 1995), second in the conversion of 12-oxy-phytodienoic acid to JA (Engelberth et al., 2001) and after JA synthesis (Doares et al., 1995).These findings suggest that the individual application of each elicitor is an alternative to achieve the induction of systemic resistance in the plant against the target pathogen, as also indicated by our *ad planta* experiments and enzyme assays and gene expression analysis .

Generally, we observed that the protective effect of the elicitors in tomato against *R. solanacearum* varied based on the genotypic background of each tomato genotype. In our gene profiling study, more of the up-regulated defense related genes were found in the moderately resistant genotype King Kong 2 than in the susceptible L390, where the resistance inducing effects of silicon was higher than of *B. pumilis*. Also Dannon and Wydra (2004) reported that silicon may have increased the resistance factors present in genotype King Kong 2 more than in L390 which lacks effective resistance mechanisms.

In conclusion, on the basis of our results the elicitors triggered the regulation of different defense-related genes involved in signal transduction and transcription

factors that increase plant resistance towards *R. solanacearum*. Particularly Si regulated a variety of defense related genes and provides a higher protective role against the pathogen than *B. pumilis* which is also indicated by the *ad planta* experiments and enzyme assay result. This strengthens the hypothesis that silicon alleviates and induces resistance after pathogen inoculation triggering the expression of a variety of defense related genes. However, during the simultaneous application of the two elicitors, a non-additive or antagonistic interaction occurred between the ethylene-jasmonate and salicylate pathways which were elicited by the rhizobacteria and silicon, respectively. Therefore, silicon is suggested as a better alternative for induction of resistance against bacterial wilt than rhizobacteria.

Chapter 5

General Discussion

Over the last decades, various efforts were made to develop an alternative disease management strategy against bacterial wilt caused by *R.solanacearum*. Mostly, the suggested control measures met only limited success due to the wide host range and variability of the pathogen, resulting in breakdown of resistance at an ambient temperature by virulent and highly polymorphic strains of *R. solanacearum* and in nematode infested soil (French and Lindo, 1982; Prior et al., 1994). Furthermore, an increased concern exists on the deleterious effect of chemical pesticides used against this pathogen to the environment and public health (Mazzola, 1998; Mark et al., 2006). These circumstances made the development of effective and pesticide-free biological control strategies against this pathogen necessary. Therefore, antagonistic bacteria (Chapter two) were isolated and tested with the objective to find a potential biocontrol agent that have direct mode of action against the pathogen as well as inducing systemic resistance (ISR) in the plant. To elucidate the molecular reactions of resistance induction by elicitors silicon or rhizobacteria, or their combination silicon-rhizobacteria mediated transcriptomic gene expression profiling was performed (Chapter four). To better understand the physiological background of induced resistance activity of defense related enzymes against *Ralstonia solanacearum* in tomato was analyzed (Chapter three).

Among 150 rhizobacterial strains isolated from Ethiopia 13 strains effectively inhibited the growth of *R. solanacearum in vitro* on KB-agar medium. The thirteen strains were identified as *Pseudomonas spp., P. putida, P.veronii, S. marcescens* and *B. cereus* by fatty acid methyl ester analysis and biochemical methods. These strains represent species of rhizobacteria known for their biocontrol activity. The characterization of

plant growth promoting traits indicated that 11 strains produced siderophores, 9 strains solubilised inorganic phosphate, all produced IAA and only *P. putida* PP2SS produced HCN. The growth inhibition activity of all strains except *B. cereus* BC1AW and BC4SS, against *R. solanacearum* could be explained based on siderophores production. This agrees with result of Muleta et al. (2007) who reported inhibition of fungal pathogens of coffee on KB medium through production of siderophores by a *Pseudomonas strain*. Though, strains BC1AW and BC4SS were unable to produce siderophores, they still showed inhibitory activity against the pathogen which could be due to production of antibiotics. Only *P. putida* PP3WT produced the quorum sensing molecule acyl homoserine lactone (AHL) and showed quorum sensing inhibition (QSI).Under greenhouse conditions, among the five tested strains *B. cereus* BC1AW and *P. putida* PP3WT strains consistently reduced wilt disase, number of *R. solanacearum* in mid-stems in both genotypes. Mechanisms proposed responsible for disease suppression and plant growth promotion by *Bacillus* and *Pseudomonas spp.* are production of siderophores, antibiotics, indole acetic acid, phosphate solubilisation and the induction of systemic resistance which switches on the battery of defense mechanisms of the plant against pathogens (Bakker and Schippers, 1987; Bakker et al., 2007).

Individual application of biotic and/or abiotic elicitor reduced bacterial wilt disease development and bacterial populations in the mid-stems of tomato, while their simultaneous application did not. This phenomenon could be explained by the fact that each elicitor elicits different signaling pathways that might be antagonistic to each other (Thaler et al., 2002). Different types of elicitors are known to induce

different defense signaling mechanism i.e. systemic acquired resistance (SAR) and induced systemic resistance (ISR) mediated by salicylic acid (SA) and by ethylene (ET) and jasmonic acid (JA), respectively (Van Loon et al., 2006; Van Loon et al., 1998).

Regarding silicon content, the highest level of silicon was found in roots of both genotypes than in the stems tomato plant amended with silicon. This result is typical for silicon non-accumulator plants. The activities of peroxidase (POD) and phenylalanine ammonia lyase (PAL) that participate in the biosynthesis of lignin and phenylpropanoid pathway,respectively, increased non-significantly in tomato primed with each elicitor (Hahlbrock and Scheel, 1989).

In contrast the activity of lipoxygenase that catalyzes the peroxidation of lipid membrane was decreased in the pathogen inoculated silicon amended treatment. Products of lipid peroxidations are precursors for jasmonic and methyl jasmonate that could be involved in signal transduction of induced disease resistance. This reduction in LOX activity might be explained by the ameliorative effect of Si on the membrane integrity. Thus, activity LOX showed an increment upon inoculation of *R. solanacearum* into rhizobacteria-primed tomato plant.During simultaneous application of both elicitors, activity of the three common defense related enzymes significantly dropped. This is due to antagonistic cross talk between the two signaling pathways mediated by each inducer.

In the transcriptome analysis of rhizobacteria-silicon mediated gene expression profiling, 174 genes are differentially regulated after inoculation of *R. solanacearum*.

Here inoculation of the pathogen is critical for triggering all the defense battery of the plant in silicon and rhizobacteria amended plants, indicating priming of the plant. This agrees with Conrath et al. (2002) who reported plant cells are sensitized or potentiated to react more rapidly and/ or more strongly to environmental stress upon appropriate stimulation called priming. In primed plants, defense responses are not induced directly by the priming agent, but are activated in an accelerated manner following perception of biotic or abiotic stress signals, resulting in an enhanced level of resistance against the stressor encountered.

Among the total 174 genes were differentially regulated 113 were up-regulated and 61 down-regulated. Based on functional categorization most of the up-regulated genes were involved in signal transduction, defense, transcription factor, protein synthesis, and metabolism with a large proportion of down regulated genes involved in metabolism, photosynthesis, signal transduction and lipid metabolism. Our results have indicated that Si (+Si-A+Rs) regulated the majority of defense related gene following the SA mediated pathway. In line with this Chian et al. (2009) and Fauteux et al., (2006) indicated the silicon-induced regulation of defense related genes in wheat and *Arabidopsis* against *Blumeria graminis f. sp.tritici* and powdery mildew, respectively. Furthermore Ghareeb and Wydra, (2007), Nickel et al. (2010), also reported the up-regulation of defense related genes in silicon amended tomato plants 72 hours post inoculation of, *R. solanacearum.*

Rhizobacteria strain *B. pumilis* (-Si+A+Rs) also triggered the expression of defense related genes such as transcription and signal transduction elements which are known to be involved in ethylene and jasmonate mediated pathways that lead to

resistance induction. Also Wang et al. (2005) and Cartieaux et al. (2003) reported transcript modification after inoculation of the pathogen *P. syringae* pv. *tomato* DC3000 to *Arabidopsis* in priming state with the endophytic PGPR *P. fluorescens* FPT9601-T5 and PGPR *P. thivervalensis* MLG45 , respectively.

However, during the simultaneous application of the two elicitors antagonistic interaction seemed to occur between the ethylene-jasmonate and salicylate pathways, which may have been elicited by the rhizobacteria and silicon, respectively, indicated by *ad planta* and enzyme assay results Ishiad et al. (2008) and Thaler et al. (2002). Here the expression of cytochrome P450, putative cytochrome P450, WRKY transcription factor 3, aminocyclopropane-1-carboxylate oxidase and basic endochitinase were down-regulated, which were up-regulated during separate application of each elicitor. This suggests that application of either silicon or rhizobacteria is the best alternative for the induction of systemic resistance that will switch on the defense arsenal of the plant against *R.solanacearum.* Generally, the results demonstrate the ability of rhizobacteria and silicon to differentially trigger expression of a variety of defense related genes, transcription factors and signal transducing elements, with Si being the stronger inducer.

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II. EDUCATIONAL BACKGROUND

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2002-2005: MSc. degree in Applied Microbiology from Addis Ababa University (AAU), Ethiopia

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2006: Pedagogical training, at Debub University: February, Ethiopia.

2005: International course on research and development in integrated pest Management (IPM), at Volcani Campus, Bet Dagan: June -July, Israel.

IV. WORK EXPERIENCE

2005-2006: Lecturer and research fellow at Hawassa University, Awassa College of Agriculture: Department of Food Science and Post Harvest Technology (FSPT), Ethiopia.

2001-2004: Researcher and Head of the National Rice Research Coordinator at Pawe Agricultural Research Center, Ethiopian Agricultural Research Organization (EARO), Ethiopia.

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1. Kurabachew, H.M and Wydra, K .2010. Characterization of plant growth promoting rhizobacteria and their potential as bioprotectant against tomato bacterial wilt caused by *Ralstonia solanacearum*. (in press).

2. Kurabachew, H.M and Wydra, K. 2010. Defense related enzymes and gene expression after resistance induction by rhizobacteria and silicon against *Ralstonia solanacearum* in tomato (to be submitted).

3. Kurabachew, H.M and Wydra, K. 2009. Biochemical characterization of bacterial antagonists and resistance induction in tomato against bacterial wilt caused by *Ralstonia solanacearum*. Biological control of fungal and bacterial plant pathogens IOBC/wprs Bulletin Vol. 43, pp. 141-144.

4. Kurabachew, H.M., Fasil, A and Yaynu, H. 2007. Evaluation of Ethiopian isolates of *Pseudomonas fluorescens* as biocontrol agent against Potato bacterial wilt caused By *Ralstonia (Pseudomonas) solanacearum. Acta agriculturae Slovenica*, 90 (2), 125-135.

ORAL PRESENTATION

1. Induction of defense related enzymes and gene expression after resistance induction by rhizobacteria and silicon against *Ralstonia solanacearum* in tomato (*Solanum lycopersicum*) at the Tropentag September 14-16, 2010 in ETH Zürich, Switzerland.

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POSTER PRESENTATION

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DECLARATION

I, Henok Kurabachew, declare that this thesis, entitled 'Biochemical and molecular background of the combination of rhizosphere bacteria from Ethiopia and silicon application to induce resiistance in tomato (*Solanum lycopersicum*) against bacterial wilt caused by *Ralstonia solanacearum*' is an original piece of work conducted by myself and has not been submitted for a degree in any other university.

Henok Kurabachew

Hannover, 2011

Gottfried Leibniz Universität Hannover

Erkärung zur Disseratatio

Hierdurch erkläre ich,dass ich meine Dissertation

Sebständig verfassst und die benutzten Hilfsmittel und Quellen sowie gegebenenfalls die zu Hilfeleistungen herangezogenen Institutionen vollständig angegeben habe.

Die Dissertation wurde nicht schon als Masterarbeit,Diplomarbeit oder andere Prüfungsarbeit verwendet.