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# Biological control of leaf pathogens of tomato plants by *Bacillus subtilis* (strain FZB24): antagonistic effects and induced plant resistance

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*Dedicated to my beloved bleeding national SYRIA*

# Abstract

*Bacillus subtilis* reisolated from the biological control agents FZB24® and Phytovit® has shown promising results against several pathogens causing important foliar tomato diseases (late blight, early blight, powdery mildew, and leaf mold) with higher activity when applied prior pathogen infection. Since most previous studies focused primarily on the degree of disease reduction, further investigations on the mechanisms contributed to disease suppression and enhancement of plant resistance are attractive properties explored further and in more detail in the current study at microbial, histological, and molecular levels. This will help to optimize the application strategies of *B. subtilis* as a biological control agent or their metabolites as biopesticides.

Application of *B. subtilis* cells and their excreted metabolites resulted in a significant reduction in disease severity of tested pathogens. In spite of *B. subtilis* cells significantly reduced late blight severity on the entire plant by 44%, but when they applied merely on the lower leaves they showed no systemic protection on the upper leaves. Using qRT-PCR, cells showed as well no induction in the expression of *PR1a* gene, which is an indicator of SAR. In addition, no changes in other responses of plant defense were observed demonstrating the antagonistic effect of bacterial cells and non-involvement in plant resistance.

Metabolites formed by *B. subtilis* strains FZB24 and Phytovit inhibited the development of diseases and the pathogen better than the bacteria itself revealing their important role as effective substances in disease suppression. This was in favor of metabolites produced by FZB24 strain harvested 72 hours of culturing. The highest destructive effect of metabolites proved to be against *Phytophthora infestans* restricting its developmental structures and decreasing its biomass in leaf tissue by 83% and resulted in more than 70% reduction in late blight severity. They strongly inhibited the inter- and intracellular growth of *P. infestans* and resulted in superficial horizontal colonization of *P. infestans* with no progress in deeper tissue layers, besides to reduce the formation of haustoria, which are responsible for pathogen establishment. Moreover, metabolite application on the lower leaves resulted on the upper leaves in systemic protection associated with *PR1a* gene activation at 12 hpi.

The susceptible tomato plants (cv. Money Maker) could not limit the colonization by *P. infestans* that effects on the essential activities of the plant cells changing host metabolism and activating the basal immunity after 12 hours of inoculation. All those responses were proved to be insufficient to limit *P. infestans* growth because infection resulted in more than 80% disease severity 6 days after inoculation. However, the number of differentially expressed genes after pathogen inoculation investigated using microarray analysis were reduced by 50% in metabolite-treated plants after 12 hours of inoculation. Therefore, such reduction in plant responses reflect less susceptibility, which depends on modified patterens of gene responses during the attempts of the pathogen to establish the infection structure. In addition, other changes in plant responses were exclusively upregulated after metabolite application involved in hormone signaling and photosynth esis function, besides to suppression in stress responses.

Systemic protection achieved by *B. subtilis* metabolites was correlated to certain changes in gene expression under the influence of this type of resistance inducer affecting on the ability of the pathogen to form the haustoria, which is necessary for development of the pathogen and disease establishment. That indicates haustoria provide ideal targets for late blight control.

# Kurzfassung

*Bacillus subtilis,* isoliert aus den biologischen Pflanzenschutzpräparaten FZB24® and Phytovit®, zeigte an Tomaten vielversprechende Wirkungen gegenüber verschiedenen Blattkrankheiten - Braunfäule, Dürrfleckenkrankheit, Echtem Mehltau und Samtfleckenkrankheit- insbesondere wenn die Applikation vor der Infektion mit den Pathogen erfolgte. Während erste Untersuchungen sich vor allem auf das Ausmaß möglicher Befallsreduktionen konzentrierten, wurden im weiteren mit Hilfe von mikrobiologischen, histologischen und molekularbiologischen Methoden die Mechanismen, die die Entwicklung der Krankheiten verhindern und die Resistenz der Pflanzen bedingen können, detailiert untersucht. Dies sollte dazu beitragen, die Applikationsstrategien für *B. subtilis* als biologisches Pflanzenschutzpräparat oder dessen Metaboliten als Biopestizid zu optimieren.

Die Applikation von Zellen von *B. subtilis* oder deren ausgeschiedene Metaboliten führten zu signifikanten Verminderungen des Befalls mit *Phytophthora infestans*, *Alternaria solani*, *Oidium neolycopersicum* und *Cladosporium fulvum*. Die Befallsintensität mit *P. infestans* der gesamten Pflanze verminderte sich um 45%, wenn Zellen des Bakteriums appliziert wurden, allerdings bewirkten die Behandlung der unteren Blätter der Pflanzen keinen systemischen Schutz höher inserierter Blätter. Mit Hilfe von qRT-PCR wurde nachgewiesen, dass es in diesen Pflanzen nicht zur gesteigerten Expression des Gens *PR1a* kam, das als Indikator von systemisch induzierter Resistenz (SAR) angesehen wird. Verminderungen des Befalls werden auf antagonistische Effekte zurückgeführt, da auch keine weiteren anderen pflanzlichen Abwehrreaktionen beobachtet wurden. Die Metaboliten, gebildet von den *B. subtilis* Stämmen FZB24 and Phytovit, hemmten die Entwicklung der Krankheiten und der verschiedenen Pathogene effektiver als die Bakterien selber. Die beste Wirksamkeit zeigten die Metaboliten, die von dem Stamm FZB24 nach 72-stündiger Kulturzeit produziert wurden. Sie verminderten die Entwicklung der Infektionsstrukturen von *P. infestans*, was zu einer Reduktion der Pathogenbiomasse im Pflanzengewebe von 83% und zu einer Befallreduktion von mehr als 70% führte. Es wurde ein stark eingeschränktes inter- und intrazelluläres Myzelwachstum, vor allem in die tieferen Gewebeschichten, und eine verringerte Ausbildung von Haustorien, die verantwortlich sind für die erfolgreiche Etablierung des Pathogens, beobachtet. Darüber hinaus führte die Applikation der Metaboliten in höher inserierenden Blättern zu systemisch induziertem Schutz, der assoziiert war mit einer gesteigerten Expression des Gens *PR1a* 12 Stunden nach Inokulation. Die hochanfällige Tomatensorte 'Money Maker' war nicht in der Lage, die Besiedlung durch *P. infestans* zu verhindern, so dass 6 Tage nach Inokulation die Pflanzen eine Befallsintensität von mehr als 80% aufwiesen. Dies ging mit tiefgreifenden Veränderungen der Genexpression der infizierten Pflanzen gegenüber nicht befallenen Pflanzen bereits zu einem sehr frühen Zeitpunkt der Pathogenese einher. Betroffen waren Gene, die in primäre wie auch sekundäre Stoffwechselaktivitäten involviert sind, wie auch in die Aktivierung basaler Abwehrreaktionen 12 Stunden nach Inokulation.

Mit Hilfe von Microarry-Analysen wurde in mit Metaboliten von *B. subtilis* FZB24 behandelten Pflanzen 12 Stunden nach Inokulation mit *P. infestans* eine um circa 50% verminderte differentielle Expression von Genen gegenüber unbehandelten Pflanzen nachgewiesen. Diese Reduktion der pflanzlichen Reaktionen spiegelt die geringere Anfälligkeit wider, die auf einem veränderten Muster der Genexpression während der Etablierungsversuche des Pathogen beruht. Darüber hinaus waren nach Behandlung mit den Metaboliten in infizierten Pflanzen Gene, die an Phytohormon-Signalling und Photosynthese beteiligt sind, exklusiv verstärkt exprimiert.

Der systemische Schutz, der durch die Metaboliten von *B. subtilis* ausgelöst wurde und verbunden war mit Veränderungen der Genexpression, beeinflusste die Fähigkeit des Pathogens, Haustorien zu bilden, die damit ein wichtiges Ziel für die Kontrolle des Pathogens darstellen.

# List of abbreviations

| | |
|---|---|
| **ACC. No** | Gene bank accession number |
| **ATP** | Adenosine triphosphate |
| **BLAST** | Basic local alignment search |
| **cDNA** | Complementary deoxy ribonucleic acid |
| **cRNA** | Complementary ribonucleic acid |
| **DBI** | Day before inoculation |
| **DEGs** | Differentially expressed genes |
| **DEPC** | Diethylpyrocarbonate |
| **DMSO** | Dimethyl sulfoxide |
| **DNase** | Deoxyribonuclease |
| **dNTP** | Deoxynucleotide triphosphate |
| **DPI** | Day post inoculation |
| **DTCS** | Dye terminator cycle sequencing |
| ***E. coli*** | *Escherichia coli* |
| **EDTA** | Ethylenediaminetetraacetic acid |
| **ESTs** | Expressed sequence tags |
| **FDR** | False discovery rate |
| **GCRMA** | Guanine cytokine multi array |
| **GTP** | Guanosine triphosphate |
| **HPI** | Hours post inoculation |
| **IPTG** | Isopropyl β-D-1-thiogalactopyranoside |
| **IVT** | In vitro transcription |
| **TFGD** | Tomato Functional Genomics Database |
| **LIMMA** | Linear models for microarray data |
| **NAOAc** | Sodium oxaloacetic acid |
| **NCBI** | National center for biotechnological information |
| **RIN** | Ribonucleic acid integrity number |
| **RNase** | Ribonuclease |
| **rpm** | Rotation per minute |
| **SAS** | Statistical Analysis System |
| **SDS** | Sodium dodecyl sulfate / Sequence detection system |
| **SGM** | Synthesis growth medium |
| **SSC** | Sodium chloride sodium citrate |
| **TAE** | Tris acetate ethylendiamin tetra acetat |
| **TE** | Tris-ethylendiamin-tetra acetat |
| **UTP** | Uracil triphosphate |
| **X-gal** | 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside |

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# 1 INTRODUCTION

Plant diseases cause severe crop losses and make agriculture highly dependent on adequate disease control. Managing and controlling plant diseases efficiently is important for crop growers, environmentalists, legislators, policy maker and implementers. Disease management strategies primarily depend on sanitary practices and well-timed pesticides applications. Many plant diseases heavily depends on agrochemicals and mainly relies on fungicides. These fungicides can prevent infection but not all have curative activity; therefore the interval between sprayings is usually short. In addition to the appearances of more aggressive isolates, and isolates that are no longer inhibited by chemical protectants, hence, the burden on the environment is high. Subsequently, plant pathogens are responsible for large amounts of chemical fungicides applied annually exacerbating control strategies (Deahl *et al.,* 1993; Fry *et al.,* 1993; Niederhauser, 1993). To cope with these problems and due to the increase of public concern about adverse effects of agrochemicals on food safety and environment, there is need to stimulate the search for control strategies that are more durable and preferably based on natural products. Therefore, alternative approaches that can be incorporated into integrated pest management of plant diseases are needed.

Biological control agents, which include effective microorganisms and microbial products, and organic fertilizers, have been attracting attention as alternatives to chemical agents (Fravel, 2005). Many species of *Bacillus* including *B. cereus*, *B. subtilis*, *B. mycoides* are known to suppress several pathogens belonging to the genera *Rhizoctonia, Sclerotinia, Fusarium, Gaeummanomyces, Pythium* and *Phytophthora* (Cook and Baker, 1983; McKnight, 1993; Fiddaman and Rossall, 1994). Several strains of *B. subtilis* have been reported that have potential for biological control of several plant diseases. For example *B. subtilis* strains 5PVB, B94 and RC-2 against *Botrytis elliptica*, a pathogen of lily grey mould, *Rhizoctonia* seedling disease on soybeans, and *Colletotrichum dematium*, mulberry anthracnose fungus, respectively (Bonmatin *et al.,* 2003; Mukherjee *et al.,* 2005; Stein, 2005). Since the *B. subtilis* group is considered as safe and have "generally recognized as safe" status (Emmert and Handelsman, 1999), *B. subtilis* have been developed as commercially available biological control agents such as FZB24® and Phytovit® against soil borne diseases. The use of bacteria strain FZB24

has been successfully applied to control plant diseases. *B. subtilis* strain FZB24 is able to reduce the *Fusarium* wilt infection on ornamentals (Grosch *et al.,* 1999) and showed distinctly less attack by *P. infestans* and by *Botrytis cinerea* on tomato by up to 50% reduction in disease severity (Kilian *et al.,* 2000). *B. subtilis* strain B2g from Phytovit® is able to suppress soil-borne pathogens e.g. *Pythium ultimum, Rhizoctonia solani* in the rhizosphere of plants.

Tomato (*Solanum lycopersicum* L.) or (*Lycopersicon esculentum* Mill.) is one of the most widely grown vegetable food crops in the world, second only to the potato with world production about 152.9 million ton ($74.1 billion) according to FAOSTAT Database (2009). Tomato plant is attacked from many serious diseases under greenhouse and field conditions. Several important diseases of tomato reduce crop yield and the most devastating plant pathogens are fungi and oomycetes (Agrios, 2005). For example, the early blight disease caused by *Alternaria solani* can be severely damaged incurring a loss of 50 to 80% on tomato susceptible hybrids (Mathur and Shekhawat, 1986). Other important diseases are powdery mildew and leaf mold (Panthee and Chen, 2010). The powdery mildew caused by *Oidium neolycopersici* is one of the principal main foliar tomato diseases in greenhouse conditions (Bardin *et al.,* 2008) and affecting tomato in commercial organic production fields. Powdery mildew damage is increased when plants are stressed due to heavy fruit load or insufficient water. While, leaf mould caused by the fungus *Cladosporium fulvum* (syn. *Fulvia fulva*), which is in the absence of control measures large portions of the leaves can be killed resulting in significant yield reduction (Smith *et al.,* 1969), is one of the most destructive foliar diseases of tomato grown under humid conditions.

The destructive late blight disease caused by *Phytophthora infestans*, awaits the tomato where it is cultivated in moist, cool, rainy, and humid environments. This plant pathogen is one of the most notorious and devastating organisms in recent human history, being responsible for the terrible Irish potato (*Solanum tuberosum*) famine in the 1840s, and it is arguably the most important pathogen of potatoes and tomatoes worldwide. The pathogen can cause up to 100% yield losses. And, although this pathogen (Erwin and Ribeiro, 1996; Govers and Latijnhouwers, 2004) has been intensively studied by scientists now for close to 150 years, it still continues to cause

upwards of $7 billion in annual agricultural losses around the globe causing threaten to food security worldwide.

The devastating economic impact of late blight disease intensified the related pathology and genetics research. There is, however, an insufficient number of potato and tomato cultivars with late blight resistance, resulting in continued expensive as well as the hazardous and increasingly ineffective use of chemicals for disease control. In an era when both host plants and *P. infestans* genomes are sequenced and considerable genomic information is available, it is not unexpected that a more sustainable solution to controlling late blight is on the horizon. Many of the crucial steps involved in late blight defense response in host plants have been elucidated through the use of modern cytological and molecular biology techniques. Also, genetic and biochemical studies have revealed differences between oomycetes and pathogenic fungi, which has led to more selective use of chemicals for late blight control. Furthermore, the discovery of *P. infestans* two mating types and the resultant generation of more aggressive lineages by sexual recombination stresses the need for an integrated and sustainable approach to late blight control. These measures would include the use of cultural practices, selective fungicide applications, and genetic resistance. Taking into consideration that many important plant diseases are caused by oomycetes, there is a high demand for novel agents that specifically target oomycetes; especially that environmental friendly control of plant disease is an imperative need for agriculture in the 21st century (Emmert and Handelsman 1999).

To control late blight biologically, several antagonistic agents have been tested for their activity against *P. infestans*, including nonpathogenic *P. cryptogea* (Stromberg and Brishammar, 1991) and other endophytic microorganisms such as *Cellulomonas flavigena*, *Candida* sp., and *Cryptococcus* sp. (Lourenço Júnior *et al*., 2006). Although some effective fungal antagonists were identified, bacterial antagonists have shown by far the most promising results to date. Bacteria with antagonistic activities against *P. infestans* are mainly found in the genera of *Pseudomonas* and *Bacillus* (Sanchez, 1998; Yan *et al*., 2002; Daayf *et al*., 2003; Kloepper *et al*., 2004).

Over decades, cyclic lipopeptides (CLPs) produced by *Pseudomonas* and *Bacillus* species have received considerable attention for their activity against a range of microorganisms, including mycoplasmas, trypanosomes, bacteria, fungi, viruses and Oomycetes (Nybroe and Sørensen, 2004; Raaijmakers *et al*., 2006). Lipopeptide production was demonstrated for *Bacillus* populations growing on roots, leaves and fruits (Asaka and Shoda, 1996; Bais *et al.,* 2004; Toure´ *et al.*, 2004; Ongena *et al.,* 2007; Romero *et al.,* 2007). The members of the *Bacillus* genus are often considered microbial factories for the production of a vast array of biologically active molecules potentially inhibitory for phytopathogen growth. Their ability to form spores also makes these bacteria some of the best candidates for developing efficient biopesticide products from a technological point of view. Loeffler (1990) found that the lipopeptides formed by *B. subtilis* are released into the medium only at the time of endogenous spore formation during the stationary phase of the culture. However, Lin *et al.* (1998) and Koumoutsi *et al.* (2004) showed that in artificial media cells in the transition from exponential phase to stationary phase mostly produce surfactins, which is a very powerful biosurfactant, while fengycin synthesis is delayed to early stationary phase, and iturins, exhibiting powerful antifungal activities, accumulate later. These three substances consist of amino acids and fatty acids as side chains and thus are easily biodegradable in soil in sharp contrast with persistent chemical pesticides. These three families of *Bacillus* lipopeptides are known to act in a synergistic manner as suggested by several studies on surfactin and iturin (Maget-Dana *et al.,* 1992), surfactin and fengycin (Ongena *et al.,* 2007) and iturin and fengycin (Koumoutsi *et al.,* 2004; Romero *et al.,* 2007). Therefore, it is speculated that the mixed production of these substances and the cooperative function against plant pathogens are the main reasons why *B. subtilis* has a wid broad suppressive spectrum against various plant pathogens.

Since numerous studies have shown the potential of the iturin family as alternative antifangal agents. Leclère *et al*. (2005) revealed that LPs are important determinants of biocontrol activity, when he found that overproduction of mycosubtilin, which is a member of iturin family, by *B. subtilis* strain BBG100 had significant antagonistic properties against phytopathogenic fungi, such as *Pythium aphanidermatum* on tomato seedlings. In addition, *B. subtilis* strain FZB24 produces iturin-like lipopeptides such as

those described by Krebs *et al.* (1996). Noteworthy, iturin production seems to be restricted to *B. subtilis* (Bonmatin *et al.,* 2003) and *B. amyloliquefaciens* (Koumoutsi *et al.,* 2004).

Interestingly, recent advances show that these LPs can act not only as 'antagonists' or 'killers' by inhibiting phytopathogen growth but also as 'spreaders' by facilitating root colonization and as 'immuno-stimulators' by reinforcing host resistance potential. Recent investigations direct attention on the fact that these lipopeptides have a key role in the beneficial interaction of *Bacillus* species with plants by stimulating host defense mechanisms (Ongena and Jacques, 2008).

Although activity and effects of *B. subtilis* strain FZB24 in soil application have been reported, the underlying effects and mechanisms of action of its foliar applications against pathogens causing diseases on plant foliage are not fully understood, in addition to the relatively few studies of *B. subtilis* effects against late blight disease. Also, the little available information and the deficiency in such knowledge often hinder attempts to optimize the biological activity by employing tailored application strategies. Better understanding of the interactions between antagonistic agents and plant pathogens is needed to optimize methods of application.

The life cycle of the heterothallic hemibiotrophic oomycete *P. infestans* (Mont.) de Bary differentiates into many cell types involved in sexual and asexual reproduction, propagule dispersal, spore germination, host penetration, and biotrophic or necrotrophic phases of infection. Germination becomes possible once sporangia detached from sporangiophores encounter liquid. While, indirect germination predominates in the absence of nutrients and at cool temperatures, typically below 12°C (Ribeiro, 1983), the direct germination is favoured by higher temperatures and nutrients. Germination takes about one hour and involves the cleavage of sporangial cytoplasm into multiple zoospores displaying several tactic behaviours (Deacon and Donaldson, 1993; Hill, 1998) until encystment occurs in response to chemical or physical stimulation (Griffith *et al.,* 1988). Cysts subsequently elaborate a germ tube that swells to form appressorium for host epidermal cell penetration.

After breaching the plant cuticle and cell wall, an intracellular, biotrophic infection vesicle is produced in the epidermal cell. Afterwards, the pathogen grows well intercellularly and then intracellularly (Coffey and Wilson 1983). The hyphae grow intercellularly into the mesophyll cell layers and produce haustoria, as new host cells are encountered and well establishment of the biotrophic phase of interaction. During the first hours of the interaction with potato, the first cells involved in the interaction die and host cells remain apparently unaffected by *P. infestans*, but within three to five days, the dead cells at the initial penetration site produce characteristic macroscopic symptoms. While necrotic lesions develop even in highly compatible interactions between potato and *P. infestans*, an extended period of biotrophy occurs during the interaction between tomato and certain isolates of *P. infestans* (Berg 1926; Vega-Sanchez *et al.,* 2000). This interaction results in rapid growth of the pathogen and can lead to severe epidemics.

Macro- and microscopic observations have provided a fairly complete phenotypic description of this hemibiotrophic interaction, but there have been relatively few studies of gene expression during the compatible interaction (Dellagi *et al.,* 2000; Beyer *et al.,* 2001). Upon pathogen infection, once extracellular pathogen-associated molecular patterns (PAMPs) are recognised by plant transmembrane pattern recognition receptors (PRRs), basal defense responses in the host plant are activated (Nürnberger *et al.,* 2004; Zipfel and Felix, 2005).

The terminal step in the defense-signaling cascade is the activation of defense genes, called pathogenesis-related (PR) genes that encode PR-proteins, which are highly correlated with acquired resistance (Ward *et al.,* 1991; Uknes *et al.,* 1992). Systemic acquired resistance (SAR) is one of the most widely studied mechanisms resulting in a defense response against a broad spectrum of pathogens throughout the plant (Ryals *et al.,* 1994; Sticher *et al.,* 1997). Since, SA-dependent pathways (SAR) seem to be involved in defense mechanisms against biotrophic pathogens and lead to hypersensitive response (HR) and/or local resistance (Durrant *et al.,* 2004), SAR was exhibited in tomato plants against late blight disease in studies accomplished by Cohen *et al*. (1994) and Stierl *et al.* (1997) and exhibited as well as a result of inoculating the lower leaves of tomato with *P. infestans* (Heller and Gessler, 1986) or with tobacco

necrosis virus (TNV) (Anfoka and Buchenauer, 1997). Therefore, expression level of *PR1a* gene, which have been frequently used as marker genes for SAR in many plant species, such as tobacco, *Arabidopsis*, and rice (Ward *et al.,* 1991; Friedrich *et al.,* 1996; van Loon and van Strien, 1999; Agrawal *et al.,* 2001), was followed to determine if its induction is correlated with the systemic protection achieved by *B. subtilis* cells or metabolites applied prior *P. infestans* inoculation.

*Phytophthora* species, like many pathogens, secrete effector proteins (Catanzariti *et al*., 2006; Kamoun, 2006; Whisson *et al*., 2007) that alter host physiology and facilitate colonization. Part of *P. infestans* success is accounted for by its biological lifestyle and remarkable capacity to rapidly adapt to overcome the resistance in plants (McDonald and Linde, 2002). The pathogen has developed mechanisms to overcome detection by release effectors into plant cells, which interfere with signaling cascades and thereby abolish basal defense response in susceptible host. As part of these mechanisms, genes have to be temporally and spatially regulated. Several previous studies focusing on potato genes regulated during colonization by *P. infestans* demonstrated that the attack of *P. infestans* leads to transcriptional activation of various genes (Zhu *et al.,* 1995; Avrova *et al.,* 1999; Beyer *et al.,* 2001; Collinge and Boller, 2001; Restrepo *et al.,* 2005; Tian *et al.,* 2006). Herein, to explore the molecular features of plant susceptibility to infection caused by *P. infestans*, changes in the tomato transcriptome at the stage of haustorium formation involved in establishment of the pathogen, were examined. Since, the molecular characteristics of host cell responses at this particular infection step are not well understood, knowledge of the early host cell alterations generated in response to attack by this virulent pathogen might lead to a better understanding of the molecular processes involved in tomato infection, as well as potentially contributing to the development of biotechnological strategies for the fight against this disease by identifying the process involved in pathogen inhibition as a result of applying *B. subtilis* cells and metabolites.

**Hypothesis**

Since, most studies of the biological control agent *B. subtilis* have focused primarily on the degree of disease reduction, in the current study further investigations were carried

out on the mechanisms of suppression have not been as extensively investigated, hypothesizing the involvement of bacterial cells and metabolites in elevation of host resistance to suppress late blight disease in addition to their direct effect. Therefore, the present study, which shows the various effects produced by *B. subtilis* and their secreted metabolites on pathogen and disease development and the proposed mechanisms for those effects as well as the interactions between the antagonist, the plant, and the pathogen, is to answer the following questions in order to optimize the application strategies:

- Is foliar application able to induce protection in tomato plants or inhibit the foliar pathogens?
- What is the mode of action of the cells and metabolites?
- Does the protection of the plants depend on alterations in gene expression?

# 2 MATERIALS AND METHODS

## 2.1 Plants

Tomato plants (*Lycopersicum esculentum* Mill.) of the highly susceptible cv. Money Maker were used for all experiments.

## 2.2 Bacteria

Two commercial bacterial biological control agents Phytovit® and FZB24® (PROPHYTA Biologischer Pflanzenschutz GmbH, FZB Biotechnik GmbH, Germany) were used to determinate their effects against different pathogens of tomato plants. *Bacillus subtilis* B2g strain Phytovit with the concentration of $1.25 \times 10^{10}$ viable endospores per gram and *Bacillus subtilis* strain FZB24 consisting of $5 \times 10^{10}$ endospores per gram were the two tested strains.

## 2.3 Pathogens

| | |
|---|---|
| *Phytophthora infestans* (Mont.) de Bary | late blight |
| *Alternaria solani* (Ellis et Martin) Sorauer | early blight |
| *Oidium neolycopersici* Cooke et Masse | powdery mildew |
| *Cladosporium fulvum* Cooke | leaf mold |

## 2.4 Chemicals, kits, and biological materials

| Chemicals or biological materials | Manufacturer/Supplier |
|---|---|
| 10x PCR buffer | Promega, WI, USA |
| 2-Mercaptoethanol | Sigma-Aldrich Chemie GmbH, Munich, Germany |
| 2x rapid ligation buffer | Promega, WI, USA |
| 5x First-Stand buffer | Invitrogen Life Technologies, Karlsruhe, Germany |
| Acetic acid | Roth, Karlsruhe, Germany |

| | |
|---|---|
| Agar-Agar | Roth, Karlsruhe, Germany |
| Agarose | Sigma-Aldrich Chemie GmbH, Munich, Germany |
| Ampicillin | Roth , Karlsruhe, Germany |
| Bromophenol blue | Roth, Karlsruhe, Germany |
| Calcium chloride | Sigma-Aldrich Chemie GmbH, Munich, Germany |
| Chloroform | Roth , Karlsruhe, Germany |
| Dimethyl sulfoxide (DMSO) | Roth , Karlsruhe, Germany |
| DNase I, EDTA | Invitrogen, Karlsruhe, Germany |
| dNTPs | Roth , Karlsruhe, Germany |
| DTT | Invitrogen Life Technologies, Karlsruhe, Germany |
| Dye terminator cycle sequencing (DTCS) | Beckman Coulter, Krefeld, Germany |
| *E. coli* competent cells | Stratagene, Amsterdam, The Neatherlands |
| Ethanol | Roth, Karlsruhe, Germany |
| Ethidium bromide | Roth, Karlsruhe, Germany |
| Ethylenediaminetetra acetic acid (EDTA) | Roth , Karlsruhe, Germany |
| Eukaryotic poly-A RNA control kit | Affymetrix, CA, USA |
| ExoSAP-IT | USB, Ohio, USA |
| FZB24® | FZB Biotechnik GmbH, Germany |
| GenElute™ plasmid mini prep kit | Sigma-Aldrich, St.Lous, MO, USA |
| Glycogen for sequencing | Beckman Coulter, Krefeld, Germany |
| High-Capacity cDNA Reverse Transcription Kits | Applied biosystems, CA, USA |
| Hydrochloric acid | Roth, Karlsruhe, Germany |
| Isopropyl -D-thiogalactoside (IPTG) | Roth, Karlsruhe, Germany |
| iTaq SYBR Green Supermix with ROX | Bio-Rad laboratories, Munich, Germany |
| Leadder 100 pb | Promega, WI, USA |
| Magnesium chloride | Sigma-Aldrich Chemie GmbH, Munich, |

| | |
|---|---|
| | Germany |
| MEGAscript® T7 Kit | Applied Biosystems, CA, USA |
| Mineral oil | Sigma-Aldrich Chemie GmbH, Munich, Germany |
| NucleoSpin® 8 RNA Kit | Machery-Nagel GmbH & Co. KG, Düren, Germany |
| NucleoSpin® RNA purification Kit | Machery-Nagel GmbH & Co. KG, Düren, Germany |
| Oligonucleotide primers | MWG Biotech, Eberberg, Germany |
| Penicillin | Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany |
| Pepton | Roth , Karlsruhe, Germany |
| pGEM®-T vector | Promega, WI, USA |
| Phytovit® | PROPHYTA Biologischer pflanzenschutz GmbH, Germany |
| Potassium chloride | Sigma-Aldrich Chemie GmbH, Munich, Germany |
| Potato dextrose agar | Merck, Darmstadt, Germany |
| Primers | Biomers.net GmbH, Ulm, Germany |
| QIAquick PCR™ Purification Kit | Qiagen, Hilden, Germany |
| Random primer | Promega, WI, USA |
| Ribo-nuclease inhibitor (RNasin) | Promega, WI, USA |
| RNA 6000 Nano LabChip® Kit | Agilent Technologies Inc, CA, USA |
| RNeasy plant mini kit | Qiagen, Hilden, Germany |
| RQ1 RNase-free Dnase | Promega, Madison, WI, USA |
| Sample loading solution (SLS) | Beckman Coulter, Krefeld, Germany |
| Sodium acetate | Roth , Karlsruhe, Germany |
| Sodium chloride | Roth , Karlsruhe, Germany |
| Sodium dodecyl sulfate (SDS) | Sigma-Aldrich Inc, MO, USA |
| Sodium pyruvate | Sigma-Aldrich Inc, MO, USA |
| Superscript II reverse transcriptase | Invitrogen, CA, USA |
| SYBR® Green Jump start™ | Sigma-Aldrich Chemie GmbH, |

| | |
|---|---|
| Taq Ready Mix™ | Steinheim, Germany |
| T4 DNA ligase | Promega, WI, USA |
| Taq DNA polymerase | Sigma-Aldrich Inc, MO, USA |
| Tomato juice agar | EDEKA bio, Germany |
| Tris | Roth ,Karlsruhe, Germany |
| X-Gal (5-bromo-4-chloro-3-indolylbeta-Dgalactopyranoside) | Roth, Karlsruhe, Germany |
| Yeast extract | Roth, Karlsruhe, Germany |

## 2.5 Media, buffers, and reagents

### 2.5.1 Culture media

The following media were used for isolation and in vitro tests. The stated recipes are per liter of distilled water. The culture media were autoclaved at 121°C for 20 minutes at 1 bar pressure allowed to cool to about 55°C and dispensed into 9 cm diameter disposable petri dishes.

#### 2.5.1.1 Growth media for culturing of pathogens

Potato dextrose agar (PDA, Merck, Darmstadt, Germany)

| | |
|---|---|
| Potato dextrose agar | 39 g |
| Aqua. dest. $H_2O$ | 1000 mL |

Tomato juice agar (TA, EDEKA bio, Germany)

| | |
|---|---|
| Potato dextrose broth (Difco™, France) | 12.8 g |
| Agar | 21.3 g |
| CaCO3 | 3 g |
| Tomato juice | 200 mL |
| Aqua. dest. $H_2O$ | 800 mL |

In case of contamination, the following ingredients were used:

| | |
|---|---|
| Ampicillin | 50 mg |
| Ensofloxacin | 20 mg |

| | |
|---|---|
| Rifampicin | 50 mg |

**2.5.1.2 Growth media for culturing of bacteria**

Synthetic Growth Medium (SGM) (Schlegel, 1976)

| | |
|---|---|
| $Na_2HPO_4.2\ H_2O$ | 0.50 g |
| $NH_4Cl$ | 0.60 g |
| $KH_2PO_4$ | 0.29 g |
| NaCl | 0.10 g |
| $MgSO_4.7\ H_2O$ | 0.20 g |
| $CaCO_3$ | 0.022 g |
| D(+)-Sucrose | 5 g |
| Yeast extracts | 0.5 g |
| Iron citrate solution (3.8 m M) | 5 mL |
| Trace element solution | 1 mL |
| Aqua dest. $H_2O$ | 1000 mL |

To solidify the medium 20 g of agar was added to it. The PH value of SGM medium was adjusted to 7.8 with NaOH 3 M before autoclaving with the help of pH meter.

Ingredients of trace element solutions

| | |
|---|---|
| $ZnSO_4.7\ H_2O$ | 0.10 g |
| $MnCl_2.4\ H_2O$ | 0.03 g |
| $H_3BO_3$ | 0.30 g |
| $COCl_2.6\ H_2O$ | 0.20 g |
| $CuSO_4.5\ H_2O$ | 0.015 g |
| $NiCl_2.6\ H_2O$ | 0.02 g |
| $Na_2MoO_4.2\ H_2O$ | 0.03 g |
| Aqua dest. $H_2O$ | 1000 mL |

**2.5.1.3 Growth media for cloning**

| | | |
|---|---|---|
| LB-agar | Sodium chloride | 8.0 g |
| | Peptone | 8.0 g |
| | Yeast extract | 4.0 g |

| | | |
|---|---|---|
| | Agar-Agar | 12.0 g |
| | Sodium hydroxide (40 mg/ml) | 480.0 µl |
| | ddH2O added to | 800.0 ml |
| LB-broth | Sodium chloride | 8.0 g |
| | Peptone | 8.0 g |
| | Yeast extract | 4.0 g |
| | Sodium hydroxide (40 mg/ml) | 480.0 µl |
| | ddH2O added to | 800.0 ml |

### 2.5.2 Buffers and reagents

All solutions used in these investigations were prepared with deionized Millipore water (ddH2O) and pH was adjusted with sodium hydroxide (NaOH) or hydrochloric acid (HCl). During this experiment, the following reagents and media formulation were used.

| | | |
|---|---|---|
| DEPC-treated water | Diethylpyrocarbonate | 1 ml |
| | added to water | 1000 ml |
| TAE (50x) buffer, pH 8.0 | Tris | 242.0 mg |
| | Acetic acid | 57.1 ml |
| | EDTA (0.5 M) | 100.0 ml |
| | ddH2O added to | 1000.0 ml |
| X-gal | X-gal | 50.0 mg |
| | N, N'-dimethylformamide | 1.0 ml |
| Agarose loading buffer | Bromophenol blue | 0.0625 g |
| | Xylencyanol | 0.0625 g |
| | Glycerol | 7.5 ml |
| | ddH2O added to | 25 ml |
| IPTG solution | IPTG | 1.2 g |
| | ddH2O added to | 10.0 µl |
| 3M Sodium Acetate, pH 5.2 | Sodium Acetate | 123.1 g |
| | ddH2O added to | 500 ml |
| 1M EDTA, pH 8.0 | EDTA | 37.3 g |

| | | |
|---|---|---|
| | ddH2O added to | 1000 ml |
| Phenol Chloroform | Phenol : Chloroform | 1 : 1 (v/v) |
| SDS solution | Sodium dodecylsulfat in ddH2O | 10% (w/v) |

## 2.6 Equipments

| Equipment | Manufacturer |
|---|---|
| ABI PRISM® 7000 SDS | Applied Bio systems, Foster city, USA |
| Affymetrix®GeneChip Fluidics Station 450 | Affymetrix, CA, USA |
| Affymetrix®GeneChip Hybridization oven 640 | Affymetrix, CA, USA |
| Affymetrix®GeneChip™3000 scanner | Affymetrix, CA, USA |
| Agilent 2100 bioanalyzer | Agilent Technologies , CA, USA |
| Centrifuge Z 200 | Hermle, Wehing |
| CEQ$^{TM}$ 8000 Genetic Analysis system | BeckmanCoulter,Krefeld, Germany |
| Electrophoresis (for agarose gels) | BioRad, Munich, Germany |
| GeneChip® Tomato Genome Array | Affymetrix, CA, USA |
| Incubator | Heraeus, Hanau, Germany |
| Inverted fluorescence microscope DM IRB | Leica Microsystems, Wetzlar, Germany |
| Leica Stereomicroscope SMZ 16 F | Leica Microsystems, Wetzlar, Germany |
| Lyovac GT2 freeze dryer lyophilizer | Leybold Heraeus, Cologne, Germany |
| Millipore apparatus | Millipore corporation, USA |
| My Cycler Thermal cycler | Bio-RadLaboratories, CA, USA |
| Nanodrop 8000 Spectrophotometer | Thermo Fisher Scientific, Wilmington, DE, USA |
| pH meter | Kohermann, Germany |
| Power supply PAC 3000 | Biorad, Munich, Germany |
| Rigid thin wall 96 X 0.2 ml skirted microplates for real-time PCR | STARLAB GmbH, Ahrensburg, Germany |
| Savant SpeedVac® | TeleChem International, Sunnyvale, USA |
| Shaker (Certomat) | Braun Biotech, Melsungen, Germany |
| SHKE6000-8CE refrigerated Stackable | Thermoscinentific, IWA, USA |

| | |
|---|---|
| Shaker | |
| Thermal incubator | Memmert, Schwabach, Germany |
| Thermalshake Gerhardt | John Morris scientific, Melbourne; Australia |
| Tuttnauer autoclave | Connections unlimited, Wettenberg, Germany |
| Universal centrifuge Z233 MK | Hermle Labortechnik, Wehingen, Germany |

## 2.7 Programs (soft wares) and statistical packages used

| Programs (soft wares) and statistical packages | Source of the programs (soft wares) and statistical packages |
|---|---|
| GeneChip® Operating System | Affymetrix, CA, USA |
| R statistical computing and graphics software | http://www.r-project.org/ |
| Bioconductor packages<br>Library (affy), Library (marray)<br>Library (GCRMA), Library (LIMMA)<br>Library (sma), Library (anotate)<br>Library (gostats), Library (Go)<br>Library (qualityMetrix), Library (gplots) | http://www.bioconductor.org/ |
| SAS (version 9.2) | SAS Institute Inc., NC, USA |
| Tomato Functional Genomics Database (TFGD) | (Fei *et al.,* 2011) |
| Mapman (ver. 3.5.1) | (Thimm *et al.,* 2004) |
| Entrez Gene | http://www.ncbi.nlm.nih.gov/sites/entrez?db=gene |
| EndNote X4 | Thomoson |
| Primer 3 (version 4) | http://frodo.wi.mit.edu/primer3/ |
| BLAST program A265 | http://blast.ncbi.nlm.nih.gov/Blast.cgi |
| Prism for windows (ver.5.0) | GraphPad software, Inc. |

## 2.8 Plant cultivation

Tomato seeds were cultivated in a tray filled with Klassmann® potting substrate (Klassmann-Deilmann, Geeste, Germany). Two weeks after germination the seedlings were transferred to 11 cm diameter plastic pots (one plant per pot). Seedlings were grown on greenhouse benches at 18 to 24°C and 16 h light photoperiod for 4-6 weeks.

## 2.9 Bacterial culturing and metabolite production

### 2.9.1 Isolation of bacteria from biological control agents

One gram from each product FZB24® and Phytovit® was dissolved in 5 mL of sterile distilled water (SDW) and 100 µL of the suspension was streaked on synthetic growth medium SGM (Schlegel, 1976). The plates were incubated for 3 days under room temperature. Bacterial cells were recovered from the plates using 10 mL sterile distilled water to obtain pure culture for *in vitro* bioassays. The suspension was passed through muslin cloth to get pure solution without any debris. The number of bacterial cells per milliliter was counted using counting chamber (Thoma). For the tests, different concentrations of bacterial suspension (ranging from $10^4$ to $10^7$ cells $mL^{-1}$) were prepared.

### 2.9.2 Production of bacterial metabolites

To harvest the metabolites, the re-isolated bacterial cells were grown in broth medium of SGM (Schlegel, 1976) on a rotary shaker at 130 rpm $min^{-1}$ for 72 hours at 30°C, final $O.D._{480}$ approximately 1.5. Subsequently, the broth was centrifuged at 5000 xg for 15 min at 20°C, filtered through a sterile 0.2 µm nylon filter, and used as metabolites suspensions and called M72. A part of M72 was autoclaved for 20 min at 121°C to verify the stability of effectiveness of the ingredients and was called ($M72^{heated}$). The pellets containing the cells were washed twice and re-suspended in water and shook over one hour and then centrifuged and filtered as previously. The resulting metabolites were called (M1). The obtained pellets again were re-suspended in water and incubated under room conditions for 24 hours. The solution was centrifuged and filtered as previous and was called (M24). The pellet of bacterial cells, which is called in all experiments cell-treatment, was finally re-suspended in water and the concentration was

adjusted to $10^8$-$10^9$ cells $mL^{-1}$. Broth medium without bacteria was used once as a control to be sure that it has no effect on pathogen development.

## 2.10 Culturing of pathogens

*Alternaria solani* and *Cladosporium fulvum* were cultured on potato dextrose agar for 10 days in darkness at 21°C. Spores were harvested by washing the mycelium with sterile distilled water and lightly scraping with spatula to dislodge the spores. The suspension was passed through double-layered cheesecloth and the desired concentration spore $mL^{-1}$ was prepared for the inoculum using a Fuchs-Rosenthal hemocytometer.

*Phythophthora infestans* (Mont.) de Bary was grown on modified tomato juice agar (identical to V8 agar except V8 juice was replaced by tomato juice, Smart *et al.,* 2000) and maintained at 18°C in the dark for 8 days. Sporangia were washed from cultures and the concentration was adjusted as sporangia $mL^{-1}$. To release zoospores, the sporangia suspension was chilled for 2.5 hours at 4°C and incubated for at least 20 min at 20°C before inoculation. *Oidium neolycopersici,* which is an obligate fungus, was maintained on tomato plants in the greenhouse for use in experiments.

## 2.11 Inoculation

The adjustment of inoculum density and the incubation conditions of the various pathogens throughout the experimental periods are listed in table (2.1). In general, the aerial parts of tomato plants were sprayed with the pathogen inoculum using air hand sprayers, unless the experiment was designed for specific purpose. Both the upper and the lower leaf surfaces of tomatoes were inoculated. Powdery mildew caused by *Oidium neolycopersici* was inoculated with spores from infected plants by shaking the diseased leaves gently over them. All tomato plants used in the experiments were incubated under optimal conditions for the pathogen of interest until symptoms developed and disease severity was evaluated. In case of insect infection such as whitefly and thrips, the plants were treated with suitable pesticides.

## 2.12 Measurement of pathogen growth and symptom development parameters

The disease severity parameters were gathered according to the nature, duration and extent of signs and symptoms expression of tested pathosystem. Generally, measurements of parameters described in table (2.2) were supposed to be reflecting identities of each pathosystem (Agrios, 1997). Percentage damaged or necrotic leaf area, which represents disease severity, was defined as visual estimate of the infected leaf areas in relation to the total healthy tissues in a sampling unit (leaflets or leaves) (Kranz, 1974).

**Table 2.1**: Inoculum density of pathogens and incubation conditions utilized during investigations.

| Pathogens | Inoculum density | Incubation conditions |
|---|---|---|
| **Phytophthora infestans* | $1 \times 10^5$ sporangia $mL^{-1}$ | 18°C |
| | | 48 h darkness |
| | | 95 ± 5 % RH |
| **Alternaria solani* | $5 \times 10^4$ spores $mL^{-1}$ | 20 - 25°C |
| | | 24 h darkness |
| | | 90 ± 5 % RH |
| *Cladosporium fulvum* | $5 \times 10^4$ spores $mL^{-1}$ | 20 - 25°C |
| | | 90 ± 5 % RH |
| *Oidium neolycopersici* | 4 infected leaves/plant | 20 - 25°C |
| | | 90 ± 5 % RH |

*After dark incubation period, the plants were maintained under greenhouse conditions (18 - 24°C, 16 h light photoperiod, 60 - 70% RH).

**Table 2.2:** Parameters considered for estimating disease severity in various host pathogen systems

| Pathogens | Disease parameter |
|---|---|
| *Phytophthora infestans* | Necrotic leaf area (%), spore germination (%), germ tube length (µm), formation of appressoria and primary vesicles (%), primary vesicles size (µm$^2$), amount of *P. infestans* DNA pg/mg leaf material |
| *Alternaria solani* | Necrotic leaf area (%), germ tube length (µm), spores germination (%) |
| *Cladosporium fulvum* | Damaged leaf area (%) |
| *Oidium neolycopersici* | White powdery leaf area (%), spore germination (%), germ tube length (µm), formation of appressoria and haustoria (%) |

## 2.13 *In vivo* bioassays with *Bacillus subtilis*

### 2.13.1 Test on antagonistic effect against different diseases

To identify the activity of FZB24® and Phytovit® in a greenhouse screening the recommended application rate (0.3 g L$^{-1}$) as well as 10x higher concentration (3 g L$^{-1}$) were applied on foliar parts of 4-6-week old tomato plants before or after inoculation to assess protective and curative effects. Inoculated tomato plants treated with water were used as control. Disease severity was rated based on percentage of damaged tomato leaf area.

### 2.13.2 Systemic activity of *B. subtilis*

#### 2.13.2.1 Translaminar translocation

To investigate if there is any systemic activity of *Bacillus subtilis* involved in reducing the disease severity, pathogens were inoculated on the same or different sides of leaf surfaces sprayed with bacterial cells or their metabolites. The third to forth leaf of 4 weeks old plants were collected, washed, and four leaves were placed together in plastic chamber under 100% relative humidity. Bacterial cells or metabolites were applied one

day before inoculation with *P. infestans*. In the first case, both *B. subtilis* (cells and metabolites) and pathogen inoculum were applied on the same surface of tomato leaves, either on the upper or lower side. In the other case, *B. subtilis* cells or metabolites was applied on the upper leaf surface and the pathogen inoculum on the lower leaf surface and vice versa. The boxes were then incubated under optimal conditions for *P. infestans*.

#### 2.13.2.2 Apical translocation

*Bacillus subtilis* strain FZB24 cells or metabolites were sprayed on a pair of lower leaves of 6 week-old tomato. Each fully-expanded pair of leaves were incubated separately in plastic bags, and into each of these a manual sprayer was introduced in such way that the *B. subtilis* suspensions could not drop on the pot substrate or touch any of the remaining aerial plant parts. After spraying, the device and the plastic bags were carefully retrieved. One day after application, leaves from treated and untreated plants were inoculated with *P. infestans*. For the control, the lower leaf pairs were sprayed with water.

## 2.14 *In vitro* bioassays with *Bacillus subtilis*

### 2.14.1 Inhibition of mycelial growth

The effect of *B. subtilis* against the pathogens was tested at different application times using different concentrations. Dual culture test was used to determine the application time of the antagonists. Two cylindrical pieces (Ø 9 mm) of agar colonized by the pathogens were placed on two edges of a Petri dish. Bacterial colonies of three-day old culture were streaked between the pathogen disks, one day before, one day after, or at the same time of pathogen culturing. To evaluate the effective concentration of bacterial strains, 250 µL of $10^4$, $10^5$, $10^6$, and $10^7$ cells $mL^{-1}$ of bacterial cells were distributed on culture medium. After one day of incubation in darkness at 21°C, one disk of individual pathogen was placed in the centre of the plates.

### 2.14.2 Inhibition of spore germination

The effect of *B. subtilis* on spore germination was studied according to the method described by Nair and Ellingboe (1962). A drop of each isolate of bacterial cells was

deposited on dried clean glass slides as a film. A drop of the spore suspension of the pathogen was spread over this film. Control treatment was prepared as a film of sterilized distilled water. Percentage of spore germination was determined microscopically using 400 folds magnification.

## 2.15 Microscopical investigations of *Bacillus subtilis* effects on pathogen development

To investigate the effects of *B. subtilis* strains FZB and Phytovit on the development of *Oidium neolycopersici*, *Alternaria solani,* and *Phytophthora infestans* on tomato leaves, light microscopy and different histochemical techniques were used.

### 2.15.1 Light microscopy

The Leitz microscope DMR photomicroscope from Leica Microsystems (Wetzlar, Germany) was used with Nomarski-interference contrast and with UV-excitation for epifluorescence. The filter combinations that were used are given in table (2.3). Images of the observed specimens were photographed with a fitted digital camera and could be observed on a screen. The images were saved using the program "Discus" (Technisches Büro Hilger, Königswinter, Germany).

**Table 2.3:** Filter combinations for the incident fluorescence microscope

| Exciter filter (nm) | Chromatic beam splitter (nm) | Barrier filter (nm) |
|---|---|---|
| BP 340-380 | FT 400 | LP 430 |
| BP 355-425 | FT 455 | LP 460 |

### 2.15.2 Specimen preparation techniques

#### 2.15.2.1 Glass surface

To evaluate the direct effect of *B. subtilis* strain FZB24 on development of *P. infestans* on glass surface, sporangium of *P. infestans* suspension ($10^5$ sporangia $mL^{-1}$) was added on a glass slide over a drop of *B. subtilis* cells or metabolites. The effects on germ tubes elongation were evaluated 6 hours after incubation in darkness at 18°C.

#### 2.15.2.2 Fresh specimen

Leaf samples inoculated with *P. infestans* taken 3 hours post inoculation were used to determine zoospore germination on untreated and leaf surfaces treated with *B. subtilis* strain FZB24.

#### 2.15.2.3 Fixed specimen

Detached tomato leaves treated with *B. subtilis* strains one day before pathogen inoculation were used to determine post-germination and pre-penetration pathogen structures on the leaf surfaces. Circular leaflet samples cut out from infected detached tomato leaves were taken 24 hours after *O. neolycopersici* inoculation, 12 hours after *A. solani* inoculation, and 3, 6, 12 and 24 hours after *P. infestans* inoculation.

In order to observe and describe the pathogen structures inside the leaf tissue, the chlorophyll was first removed and the samples then were stained with various staining solutions. The pathogen structures were fixed either on the leaf surfaces or in leaf components. The leaflets were cleared in saturated chloralhydrate (250 g/100 mL $H_2O$) at room temperature for at least 7 days.

### 2.15.3 Staining techniques

#### 2.15.3.1 Bruzzese and Hasan solution

Different aspects of fungal growth of *O. neolycopersici* on and in tomato leaf tissues were monitored. Such parameter included observations of conidia germination, elongation of germ tubes, and fungal penetration by forming appressoria and haustoria. The tomato leaflets (24 hpi) were fixed, cleared and then stained for 5 minutes in a solution (300 mL 95% ethanol, 150 mL chloroform, 125 mL 90% lactic acid, 450 g chlorohydrate, and 0.6 g aniline blue) according to Bruzzese and Hasan (1983).

The stained samples were mounted on a microscope slide and covered with a cover slip for light microscopic observations under interference contrast.

#### 2.15.3.2 Acid Fuchsin

The development of fungal structures were stained with 0.01% acid Fuchsin acid for 24 h. Proteins in pathogens and damaged plant cells are stained pink. The samples were observed with interference contrast.

#### 2.15.3.3 Diethanol (Uvitex 2B)

To determine the germination rate and to describe the pre-penetration structures of the pathogens on leaves, fresh leaf specimens with *P. infestans* were stained in 10 µl of 0.05% diethanol (w/v) and then covered with a cover slip and observed with the BP340-380/FT 400/LP 430 filter combination. Diethanol binds to polysaccharides with β-glycosidic bonds. The stain does not penetrate the plant cuticle; therefore it stains the cell wall of the pathogen on the plant surface fluorescence under UV-light.

## 2.16 Molecular investigations on quantification of *Phytophthora infestans* biomass in leaf tissue

### 2.16.1 Growth of *P. infestans* depending on inoculum concentration

To monitor the growth of *P. infestans* biomass in leaf tissues, tomato leaves were inoculated with $3 \times 10^2$, $3 \times 10^3$, or $3 \times 10^4$ sporangia $mL^{-1}$ of *P. infestans* to quantify the amount of *P. infestans* DAN as indicator of biomass.

### 2.16.2 Influence of *B. subtilis* strain FZB24 on *P. infestans* biomass throughout the infection course

*Bacillus subtilis* strain FZB24 cells and their metabolites harvested 72 hour after culturing were applied on foliar parts of tomato plants in the greenhouse 24 h before inoculation with *P. infestans* ($10^5$ sporangia $mL^{-1}$). Immediately after inoculation, leaves of half of the plants (detached leaves) were cut and incubated in plastic boxes under the same incubation conditions of individual plants (samples are called attached leaves). Untreated inoculated plants were used as positive control and the non-inoculated plants were a negative control to see if there is any natural infection. The samples were taken 3, 6, 12, 48, 96, and 144 hours after inoculation. For each treatment, four individual plants were maintained and 10 leaflets per plant were taken as one sample.

### 2.16.3 DNA extraction

#### 2.16.3.1 DNA extraction from *P. infestans*

Genomic DNA from *P. infestans* was extracted using the CTAB method (Murray and Thompson, 1980) simplified by Stewart and Via (1993) for preparing standard dilution series for the corresponding target. The CTAB protocol was further modified to obtain high quality DNA.

DNA was extracted from 10 day-old cultures of *P. infestans* grown on TA. The mycelia were collected in 2 milliliter tubes and frozen at -80°C. Mycelia were ground under liquid nitrogen to a fine powder using mortar and pestle and then 100 - 250 mg mycelia powder were transferred to 50 milliliter tubes. DNA was extracted under a fumes chamber.

Ten mL of CTAB-extraction buffer (10 mM Tris, 20 mM EDTA, 0.02 M CTAB, 0.8 M NaCl, 0.03 M N-laurylsarcosine, 0.13 M sorbitol, 1%(w/v) polyvinylpolypyrolidone, pH set to 8.0 with NaOH); 40 µL mercaptoethanol and 50 µL proteinase K (from a stock solution 10 mg $mL^{-1}$), were added to the ground mycelium (approximately 200 mg) in 50 mL plastic centrifugation tube and mixed vigorously. The mixture was incubated at 65°C for 60 min and mixed after every 10 min. Eight hundred µL of the upper phase was transferred to a 2 mL new tube containing 10 µL of RNAase (50 mg $mL^{-1}$) and incubated for 10 min at 65°C, Nine hundred µL of chloroform-isoamyl alcohol (24:1) was added into each tube. The samples were mixed by inverting the tubes and centrifuged for 10 min at 5,000 g at room temperature. The upper phase (600 µL) was transferred into a 2 mL tube and the precipitation step with chloroform-isoamyl alcohol (24:1) was repeated twice to obtain high quality DNA. After the last centrifugation, the aqueous phase was transferred into a 1.5 mL tube containing 500 µL isopropanol, mixed and incubated for 20 min at room temperature and centrifuged for 15 min at 15,000 g at room temperature. The pellet was washed twice with 70% (v/v) ethanol, dried and dissolved in 200 µL TE buffer and incubated at 4°C over night and then in -20°C until use. The quality and quantity of isolated DNA were checked on agarose gel and with a spectrophotometer. A 10-fold dilution series (from 0.9 to 9000 pg µL-1) of purified DNA were used for generating a standard curve in every real-time PCR run.

### 2.16.3.2 DNA extraction from tomato leaves

DNA from non-inoculated and *P. infestans*-inoculated leaves was extracted using the Plant Mini kit Method "Wizard® Magnetic DNA Purification System for Food" (Promega, Mannheim, Germany) following the manufacturer`s protocol. Briefly, the collected leaves were frozen at -80°C and ground under liquid nitrogen to a fine powder of less than 0.1 mm using an ultracentrifugal mill (Retsch, Haan, Germany). Into 2 mL Eppendorf tube, 18-22 mg of ground tomato leaves was weighed and stored at room temperature. Four hundreds microliters of lysis buffer A and 4 µL RNase A were added, the tube was capped and vortexed vigorously. Tow hundreds microliters of lysis buffer B was added, vortexed for 10-15 seconds and incubated for 10 minutes at room temperature (23 ± 2°C) with occasional mixing. Six hundreds microliters of precipitation solution was added and vortexed vigorously. The mixture was spinned for 10 minutes in a microcentrifuge at 13000 rpm. The supernatant was immediately transferred to a new 2 mL tube. The Magnesil® PMPS bottle was shaken by hand to thoroughly re-suspend the Magnesil® PMPS before dispensing in each sample. Fifty micro iters of Magnesil® was added to the supernatant and the tubes vigorously shaken by hand. Approximately 1 mL isopropanol was added; then the tubes were inverted 10-15 times and the samples were incubated for 5 minutes at room temperature with occasional mixing by hand. The tubes were placed on the magnesphere® (magnetic separation stand) and left for 1 minute. The liquid phase was discarded by turning round the tubes and excess liquid dried on paper towels.

The tubes were removed from the stand and 250 µL of lysis buffer B added. The tubes were inverted 2-3 times and placed back in the stand. The Magnesil® was allowed to separate for 1 minute and the liquid phase removed by turning round the tubes by hand. One milliliter of wash solution (70% ethanol) was added to the tubes, which were then placed on the stand. The tubes were turned several times to wash the DNA. The liquid phase was discarded like before. This step was repeated twice for a total of 3 washes. Using a pipette, as much liquid as possible was removed and discarded to remove the rest of the alcohol. The particles were dried for 10 minutes at 65°C, 100 µL of sterile water was added to dilute the DNA, vortexed and incubated for 5 minutes at 65°C. The tubes were placed onto magnetic stand for 1 minute. The liquid was removed without Magnesil® PMPS carefully to a clean tube. The extracted DNA was stored at 4°C for

some days or -22°C for a longer period. The quality and quantity of isolated DNA were checked on agarose gel and with a spectrophotometer.

### 2.16.4 Gel electrophoresis analysis

The agarose gel that was used in this analysis was prepared with 1X Tris-Acetate EDTA Buffer (TAE, AppliChem). For this, 2.5 g agarose (Sigma) was added to 250 mL of TAE buffer and heated for 5 minutes in a microwave (MW800, Continent) at 650 watts. After cooling at approx. 50°C, 2.5 µL of 10 mg $mL^{-1}$ ethidium bromide (AppliChem) was added. This solution was poured into an electrophoresis tray and left for approx. 30 minutes until the gel had solidified. The gel was subsequently transferred to the gel electrophoresis chamber filled with 1X TAE buffer. After transferring all samples to the wells of the gel, electrophoresis was conducted for 20 minutes at 120 Volt. The presence and specificity of DNA bands were observed under BioRad Chemidoc XRS Gel Documentation System (Biorad, München, Germany).

### 2.16.5 SYBR green® real-time PCR reactions

Quantitative PCR was carried out in an ABI Prism® 7000 SDS (Applied Bio systems, Foster city, USA) instrument based on the changes in fluorescence proportional to the increase of the PCR product. SYBR Green, which emits a fluorescent signal upon binding to double stranded DNA, was used as a detector. Fluorescence values were recorded during every cycle representing the amount of product amplified to a point known as threshold cycle (Ct). The higher the initial transcript amount, the sooner accumulated product was detected in the PCR.

The PinfRAS-Forward and PinfRAS-Reverse *P. infestans* primers, (CATTACATTGCTCACATGGCTTTC) and (ATCACGCGGGGACAAATG), respectively, were designed according to Atallah *et al.* (2006). Prior to quantification, preimers concentrations were optimized using different combinations of forward and reverse (0.2, 0.3 and 0.4 µL of 10 pg $\mu L^{-1}$) in presence of low concentration template (DNA) and non-template as control to avoid primer dimer formation. At the end of the run, the dissociation curve was generated to check the absence of the nonspecific amplification and subsequent confirmation by analysis of the PCR products on agarose gel electrophoresis. The primer combination with the lowest threshold cycle and without primer dimer formation was used to perform subsequent PCRs.

Standard curve was generated using a serial dilution (0.9, 9, 90, 900 and 9000 pg) of purified genomic DNA of *P. infestans*. Polymerase chain reactions (PCRs) were carried out in 20 µL reaction volume containing 10 µL SYBR® Green Jump start™ Taq Ready Mix™ (Sigma-Aldrich Chemie, Steinheim, Germany), 0.2 µL Rox as internal reference dye, 0.3 µL of forward primer and 0.4 µL of reverse primer, 2 µL genomic DNA and 7.1 µL sterile Millipore water. PCR reactions were performed in duplicates for standard curves and samples to control the reproducibility of quantitative results. A universal thermal cycling programme (10 sec at 50°C, 10 min at 95°C, 40 cycles of 15 sec at 95°C and 60 sec at 60°C) was used for the quantification. The specificity of amplification was confirmed by generating melting curve at the end of PCR reactions revealing the presence of a single peak for *P. infestans* (Fig. 2.1). The curve was used as control for the specificity of real-time PCR during the quantification. Final quantification of pathogen DNA analysis was performed using the standard curve method (User bulletin of ABI PRISM 7700 SDS, Http://docs.appliedbiosystems.com). The results were reported as the absolute amount of *P. infestans* DNA. The correlation coefficient ($R^2$-value) of the standard curve was at least 0.99 while the slope ranged from –3.1 to – 3.8 (Fig. 2.2).

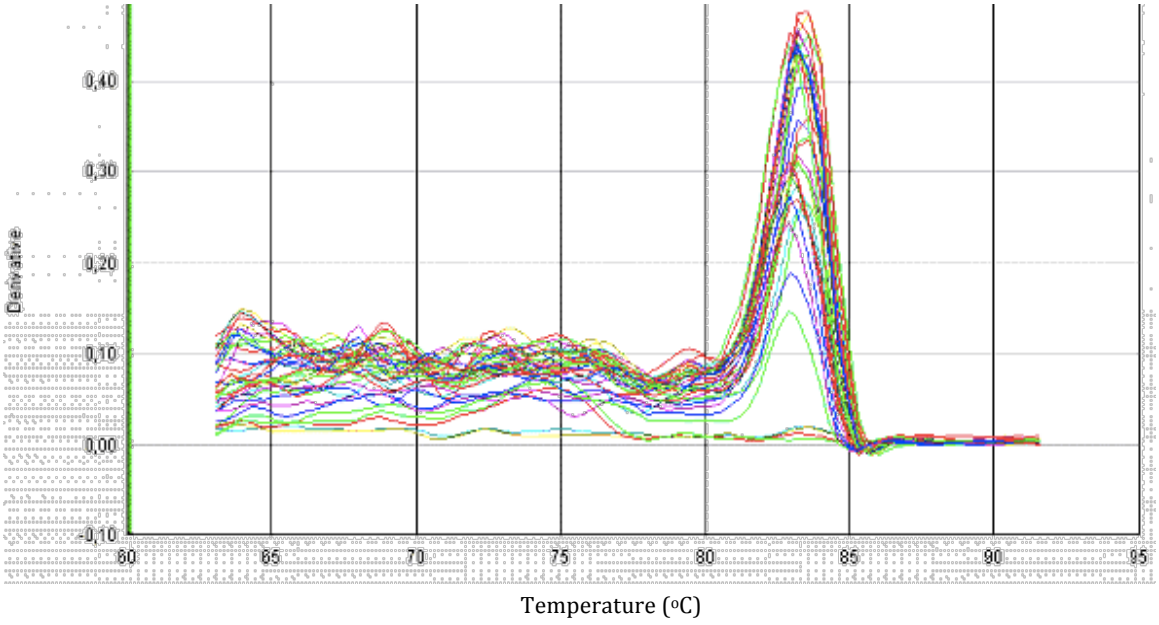


**Figure 2.1:** Dissociation curve (fluorescence derivative versus temperature °C) of specific *Phytophthora infestans* amplicon in tomato leaf matrix. Peaks of amplification plots indicated species-specific amplification in real-time PCR with a mixture of plant and pathogen DNA in different samples.

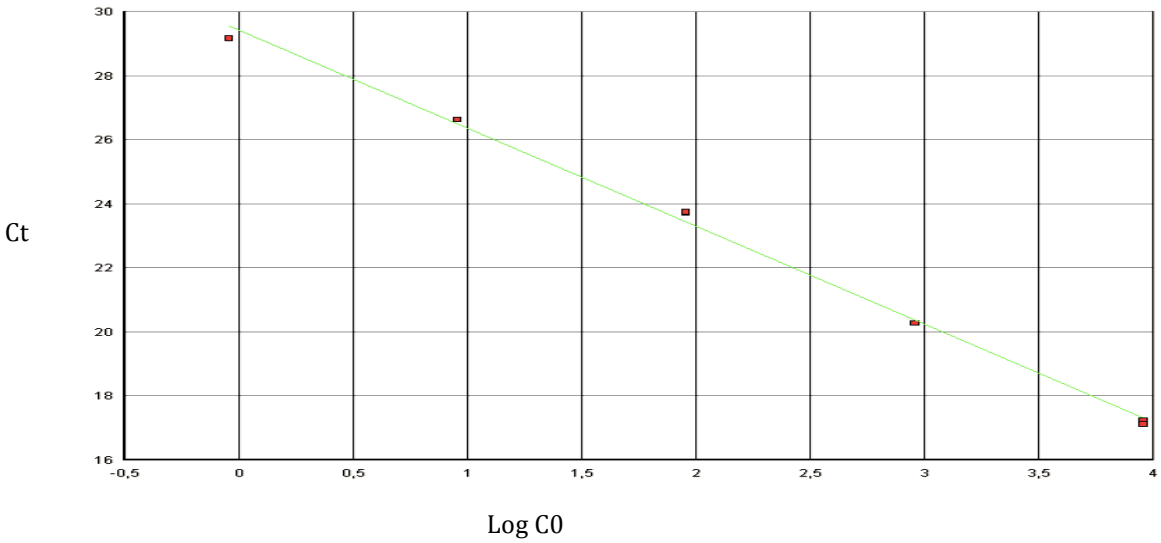


**Figure 2.2:** Calibration curve based on 40 threshold cycles from ten-fold serially diluted DNA in two replications of RT-PCR using SYBR Green® for the quantification of *Phytophthora infestans* DNA. $R^2$ = 0.997, slope = -3.3. (Ct): cycle threshold, (Log C0): log of standard.

## 2.17 Expression profile of *PR1a* gene in leaf tissue

### 2.17.1 Experimental design and tissue collection

*Bacillus subtilis* strain FZB24 cells and metabolites were sprayed on the lower leaves 24 hours before *P. infestans* inoculation on both the untreated upper leaves and the cell- or metabolite-treated lower leaves. Plants were divided into two groups, *P. infestans*-inoculated and non-inoculated plants, each consisted of three subgroups untreated (water-treated), cell-treated, and metabolite-treated plants. The samples were taken from 4 plants for each group. Each sample was taken from a pool of 10 leaflets from the bottom treated and upper induced leaf pairs per plant. In addition to 4 sampling times corresponded to the pathogen development also other sampling times were taken to investigate the influence of *B. subtilis* on the gene expression of lower treated leaves before inoculation (Fig. 2.3). Leaf samples from the bottom as well as from the upper leaves for each individual plant were separately transferred into 15 mL plastic tubes and immediately frozen in liquid nitrogen. Plant material was lypholized using a Lyovac GT2 freeze dryer lyophilizer (Leybold Heraeus, Cologne, Germany) for 24 hours. The frozen dried samples were stored at -80°C until RNA extraction.

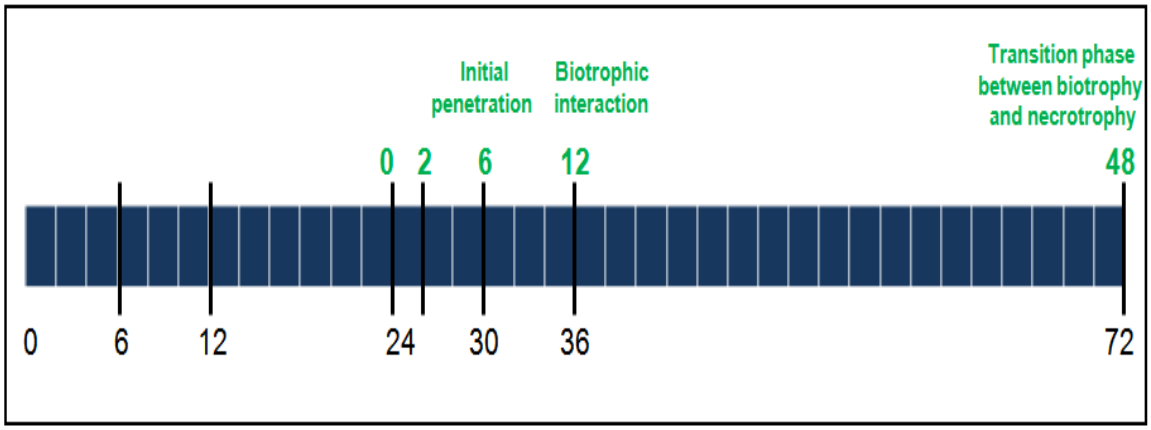


**Figure 2.3:** Sampling times to collect the lower and upper leaves from non-inoculated and *P. infestans*-inoculated plants. Black numbers indicate the time after application of *Bacillus subtilis* strain FZB24 cells or metabolites on the lower leaves. Green numbers indicate the time after *Phytophthora infestans* inoculation correspondung to its development stages.

### 2.17.2 RNA extraction and DNA digestion

Total RNA was isolated from frozen dried tomato leaves, approximately 20 mg, using the NucleoSpin® 8 RNA Isolation Kit (Machery-Nagel GmbH & Co. KG, Düren, Germany). The samples were homogenized with a mortar and pestle in liquid nitrogen and the ground powder was transferred to a polypropylene tube to follow the manufacturer`s protocols. RNA yield and quality were assessed using the Nanodrop 8000 spectrophotometer (Thermo Fisher Scientific Inc, DE, USA) at 260 and 280 nm. RNA integrity was confirmed by agarose gel (1.5% w/v). Prior to subsequent application, genomic DNA contamination of the samples was removed using DNA digestion kit (Invitrogen, Karlsruhe, Germany) according to the manufacturer`s protocol. Then the samples were stored at -80°C.

### 2.17.3 Synthesis of cDNA

One microgram of total RNA were reverse transcribed in 20 µL reaction using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, USA). Nine microliters mixture consisting of 2 µL of 10x RT buffer, 2 µL of 10x random primers, 0.8 µ1 (25 nM) dNTPs, 1 µL MultiScribe reverse transcriptase, and 3.2 nuclease-free water was added to 11 µL RNA sample, then reverse transcription was

run using the following protocol: 25°C for 10 min, 37°C for 120 min, 85°C for 5 min and holding at 4°C. The synthesized cDNA was stored at -20°C for further use.

### 2.17.4 Primer design and gene specific amplification

In the current study, quantitative RT-PCR was used to quantify the *PR1a* gene in leaf tissue depending on different treatments. The primers for the target gene *LePr1a* and the internal control gene *TIP41*, designed based on tomato mRNA sequence deposited in GenBank, were choosen accourding to Aimé *et al.* (2008) and Expósito-Rodríguez *et al.* (2008), respectively. All primers were purchased from biomers.net GmbH (Ulm, Germany). Primer sequences, size of amplified products, annealing temperature and GenBank accession numbers are shown in table (2.4). PCR reaction was carried out for each primer in 20 µL reaction volume using 4 µL of 5x PCR buffer (Sigma-Aldrich), 0.5 µL of dNTPs (50 µM), 0.5 µL of each specific primer (10 pmole forward and reverse), 0.2 µL of Taq polymerase (Sigma-Aldrich) and 12.3 µL Millipore water which finally added to 2 µL of cDNA templates or to 2 µL genomic DNA as positive control and 2 µL of Millipore water as negative control. The thermal cycling program was set as: denaturation at 95°C for 5 min, followed by 40 cycles at 95°C for 30 sec, annealing at the corresponding temperature as shown in table 3 for 30 sec and extension at 72°C for 1 min, final extension step at 72°C for 10 min and then at 4°C forever. Finally, 2 µL of loading buffer were added to the PCR products and loaded on 2 % agarose gel in 1X TAE buffer by staining with ethidium bromide. PCR products were electrophoresed for 30 min at 120 voltages. The presence and specificity of DNA bands were observed using BioRad Chemidoc XRS Gel Documentation System (Biorad, Munich, Germany).

**Table 2.4:** Details of the primers used for quantitative real-time PCR analysis

| Primer | Nucleotide sequence (5`–3`) | Amplicon size | Annealing temp. | Acc.No |
|---|---|---|---|---|
| LEPR1A-F | TCTTGTGAGGCCCAAAATTC | 246 | 56 | AJ011520 |
| LEPR1A-R | ATAGTCTGGCCTCTCGGACA | | | |
| TIP41-F | ATGGAGTTTTTGAGTCTTCTGC | 235 | 52 | AT4G34270 |
| TIP41-R | GCTGCGTTTCTGGCTTAGG | | | |

### 2.17.5 Preparation of plasmid DNA

#### 2.17.5.1 PCR product extraction, ligation, and transformation

The PCR product was purified using QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) and ligated to pGEM®-T easy vectors using pGEM®-T Vector System I ligation kit (Promega, WI, USA), according to the manufacturer`s instructions. Ligation was performed in 6 µL reaction mix containing 3 µL 2X rapid ligation buffer, 0.5 µL pGEM vector (50 ng), 0.5 µL T4 DNA ligase enzyme (3 units $\mu L^{-1}$), and 2 µL of purified PCR product. The reaction was then incubated at 4°C overnight. The ligation reaction was incubated in a thermocycler at 20°C for 2 hours. Transformation was performed by combining 3 µL of each ligation product with 70 µL of competent *E. coli* cells (JM109 strain) in a 15 mL sterile falcon tube. The tubes were gently flicked and placed for 20 min on ice followed by 90 sec at 42°C and immediately returned to ice for 2 min. Afterwards, 650 µL of Luria-Bertani (LB) broth was added to the previous mixture and cultured at 37°C in SHKE6000-8CE refrigerated stackable shaker (Thermoscinentific, IWA, USA) for 90 min with speed of 110 rpm. After 70 min, 20 µL of IPTG and 20 µL of X-gal were added and homogeneously spread with a glass spreader on the LB agar-ampicillin plate (5 µL ampicillin (10 mg $mL^{-1}$) per mL of LB agar medium) and plates were left until the chemicals were absorbed for 20 min under laminar prior to culture transformation. After the incubation period, 300 µL of each transformation culture was transferred to duplicate LB agar/ampicillin/IPTG/X-gal plate and incubated overnight at 37°C till the colonies become visible.

#### 2.17.5.2 Blue/White colony secreening and colony picking

Successful cloning of DNA insert in the pGEM-T Easy vectors was checked based on the activity of β-galactosidase. β-galactosidase is an enzyme produced by *lacZ* gene in pGEM®-T vector which interacts with IPTG to produce a blue colony. On the other hand, when an insert was successfully cloned, *lacZ* is disrupted leading to interrupt the coding sequence of β-galactosidase resulting recombinants in white colony formation. Following this screening, four independent white colonies (assumed to contain inserts) in addition to one blue colony (as control) were picked up and transferred into 30 µL 1X PCR buffer for M13 reaction for further confirmation of transformation and sequencing

(Messing *et al.,* 1981). At the same time, colonies were also cultured in 650 µL LB-broth with ampicillin (5 mg per 100 mL) and incubated at 37°C and 110 rpm on SHKE6000-8CE refrigerated stackable shaker (Thermoscinentific, IWA, USA). The bacterial suspension in the 30 µL 1xPCR was lysed by heating for 15 min at 95°C. The colonies were screened for the insert by performing a PCR reaction using M13 specific primers designed from the promoter region of the vector. 20 µL total reaction volume containing 10 µL of lysate cells as a template, 0.5 µL dNTPs (10 mM), 0.5 µL of each M13 primers (forward: 5′- TTGAAAACGACGGCCAGT-3′, reverse: 5′-CAGGAAACAGCTATGACC-3′), 0.1 µL (0.5 U) Taq polymerase (Sigma) in 1 µL 10X PCR reaction buffer and 7.4 µL water was amplified. M13 PCR reaction was carried out following this protocol: first denaturation at 95°C for 3 min, followed by 35 cycles that repeated at 95°C for 30 sec, 60°C for 30 sec, 70°C for 2 min. M13 PCR reaction was terminated after final extension at 72°C for 10 min. 5 µL of the M13 product mixed with 2 µL loading buffer was loaded to 2% agarose gel stained with ethidium bromide. The colonies that contained PCR fragments (white colonies) were identified depending on the distance travelled by DNA fragment in 2% agarose gel electrophoresis. Clones having insert would have higher molecular weight fragments than blue clones.

The best confirmed samples for the presence of PCR fragment were selected and transferred to 15 mL sterile tube and additional 5 milli Liter LB broth/ampicillin was added. The bacterial suspension was further cultured over night at 37°C to increase numbers and therefore the amount of DNA.

#### 2.17.5.3 Plasmid isolation

The plasmid was isolated using GenElute$^{TM}$ plasmid mini prep kit (Sigma-Aldrich, St.Lous, USA) based on the manufacturer's instructions. Briefly, overnight cultured competent cells were centrifuged at 12000 rpm for 1 min. The supernatant was discarded and the pellets were re-suspended in 200 µL lysis solution. After a short vortex, the solution was removed and again 200 µL lysis solutions were added and mixed by gently inverting the tubes until it became clear and viscous. After incubating at room temperature for 4 min, 350 µL neutralization/binding buffers was added, the cell suspension was centrifuged for 10 minutes at 14000 rpm for 30 sec and the clean

suspension was taken by avoiding the sediment. Then, 500 µL of column preparation solution was added to the GenElute$^{TM}$ Miniprep binding column inserted into a provided 2 mL microcentrifuge tube and centrifuged at 12000 rpm for 30 sec. The cleared lysate was then transferred to the column and centrifuge at 12000 rpm for 1 min. The filtrate was decanted and 750 µL of the diluted wash solution was added to the column followed by centrifugation at 13000 rpm for 1 min. The flow-through liquid was discarded and the column was centrifuged again at maximum speed for 2 minutes to eliminate excess ethanol and make sure that there is no liquid in the column tube. Finally, the spin column was transferred into a fresh collection tube, 30 µL of Millipore water was added to the centre of the spin column membrane and the tubes were incubated for 5 min, and then centrifuged at 12000 rpm for 1 min to elute the plasmid DNA. Again, 20 µl of Millipore water was added and incubated for 5 minutes and centrifuged at high speed for 5 min.

To confirm the presence of the plasmid DNA, 5 µL of the plasmid DNA with 2 µl loading buffer was loaded on 2% agarose gel stained with ethidium bromide and run in 1X TAE buffer. Concentration and quality of the plasmid was measured by reading the absorbance at 260 and 280 nm using Nanodrop 8000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA). An aliquot of DNA plasmid was subjected to sequence check; the rest was stored at -20°C to be used for setting up the standard curve for real-time PCR.

#### 2.17.5.4 Sequencing

The specificity of gene cloning was further validated by sequencing of M13 PCR product, in spite of identification of recombinants on LB-agar/ampicillin/IPTG/X-gal plate as a result of insertional inactivation of the α-peptide. Only the M13 PCR products from white colonies containing inserts were used as a template for subsequent sequencing. A volume of 5 µL of M13 products was purified by adding 1 µL of ExoSAP-IT (USB, Ohio, USA) then incubated at 37°C for 30 min followed by enzyme inactivation at 80°C for 15 min. The purified DNA product (6 µL) was subsequently used as template for the sequencing PCR which contains 8 µL of Millipore water, 2 µL of 1.6 pmole M13 forward or reverse primer, 4 µL master mix (DTCS). The PCR sequencing reaction was performed for 30 cycles at 96°C for 20 sec, 50°C for 20 sec

and 60°C for 4 min, followed by holding step at 4°C. The stop solution was prepared in a volume of 2.0 µL of 3M NaOAc (pH: 5.2), 2.0 µL of 100 mM EDTA (pH: 8) and 1.0 µL of glycogen (20 mg $mL^{-1}$). The sequencing PCR product was transferred to a 1.5 mL sterile tube and mixed with 5 µL stop solution and homogenized by vortexing. A volume of 60 µL 98 % cold ethanol was added and mixed by vortex and then centrifuged at 14000 rpm for 15 min at 4°C in refrigerated universal centrifuge Z233MK (Hermle Labortechnik, Wehingen, Germany). The supernatant was removed and the pellet washed 2 times with 200 µL 70 % cold ethanol and centrifuged for 5 min at 4°C and left to be dry by the speed vacuum machine for 10 min at 35°C and re-suspended in 40 µL SLS (Sample loading solution). Dried pellet were transferred to the sequencing plate (Beckman Coulter, Krefeld, Germany). After covering the plate with mineral oil and immediately loaded to CEQTM 8000 Genetic Analysis sequencing machine (Beckman Coulter, Krefeld, Germany). The similarity of the sequence result to the original sequence was verified using the NCBI/BLAST search tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

#### 2.17.5.5 Preparation of serial dilution from plasmids

The copy number per microlitre of plasmid DNA was calculated based on the nucleic acid size (size of the pGEM®-T easy vectors (2 kb) + PCR fragment for each gene) and the plasmid concentration (ng $\mu L^{-1}$). The plasmid serial dilution was prepared by converting concentration of plasmid into numbers of molecules using the online tool (http://molbiol.ru/eng/scripts/01_07.html). After selecting the dilution that contains $10^9$ molecules (copies $\mu L^{-1}$), it was then determined in 50 µL volume based on the number of molecules obtained in 1 µL plasmid DNA. Using 5 µL of $10^9$ dilutions and 45 µL Millipore water, the $10^8$ dilutions were prepared. The remaining $10^7$-$10^1$ dilutions were prepared in a similar way. Serial dilutions were then stored at -20°C and a PCR reaction was performed to test whether the serial dilution could be a suitable standard curve for RT-PCR. Afterwards, the plasmid DNA serial dilutions were used as template to generate the standard curve during RT-PCR analysis.

### 2.17.6 Quantitative real-time PCR analysis

After selection of primer concentration as previously described (2.17.4), similar amount of cDNA (2 µl) from the upper and lower leaves were used to compare samples from different treatment groups. Quantitative PCR was performed in 20 µL reaction volume containing iTaq SYBR Green Supermix with ROX (Bio-Rad laboratories, Munich, Germany), cDNA samples, up and down stream primers. Thermal parameters used to amplify the template started with an initial denaturation at 95°C for 3 min followed by 40 cycles of 95°C for 15 sec and 60°C for 45 sec annealing and extension. The specificity of amplification for each gene was evaluated by monitoring the dissociation (melting) curve at the end of the last cycle by collecting the fluorescence data at 60°C and taking measurements every 7 sec until the temperature reached 95°C.

The relative standard curve method was used to determine transcript abundance of the samples using a serial dilution of $10^1$-$10^9$ copy numbers of target plasmid DNA. The data generated was considered for further analysis. The slope and the regression line ($R^2$) of the standard curve were (-3.2 to -3.6) and > 0.99, respectively. The copy numbers of the target genes were normalized against the housekeeping gene *TIP 41*, which expression was not significantly different between the samples to be compared. The results were reported as the relative expression as compared to the calibrator after normalization of the transcript level to the endogenous control.

## 2.18 Microarray analysis of gene expression of tomato leaves

### 2.18.1 Experimental design and tissue collection

In order to get an overview of molecular plant process involved in *P. infestans* – tomato interaction and in reducing late blight disease severity using *B. subtilis* strain FZB24, an experiment was conducted using the design of the previous experiment (*B. subtilis* strain FZB24 cells and metabolites were sprayed on the lower leaves 24 hours befor *P. infestans* inoculation on both the untreated upper and the treated lower leaves.). The plants were divided into two groups, non-inoculated and *P. infestans*-inoculated plants, then each group consisted of three subgroups untreated (water-treated), cell-treated, and metabolite-treated plants. After 12 hours of inoculation, only the upper leaves were collected to perform this analysis. Four replicates of each subgroup were maintained

and each replicate was collected from three plants (10 leaflets of each plant) to prepare a pool of leaf tissue in one plastic tube. Samples were individually transferred into 15 mL plastic tubes and immediately frozen in liquid nitrogen. Plant materials were lypholized using a Lyovac GT2 freeze dryer lyophilizer (Leybold Heraeus, Cologne, Germany) for 24 hours. The Freeze-dried samples were stored at -80°C until RNA extraction.

### 2.18.2 RNA extraction and DNA digestion

Total RNA was extracted from four pools of both non-inoculated and *P. infestans*-inoculated plants at two times, first for RNA amplification and further hybridization on the array and second for validation of array results using quantitative RT-PCR. Total RNA was isolated from freeze-dried tomato leaves, approximately 20 mg, using the NucleoSpin® 8 RNA Isolation Kit (Machery-Nagel GmbH & Co. KG, Düren, Germany). Samples were homogenized with a mortar and pestle in liquid nitrogen. The ground powder was transferred to a polypropylene tube according to manufacturer`s protocols. Prior to subsequent application, genomic DNA contamination was removed by performing DNA digestion using RQ1 RNase-free DNase (Promega, Madison, WI, USA) and then samples were further purified using RNeasy Plant mini kit (Qiagen, Hilden, Germany), following the manufacture's recommendation. RNA yield and quality were assessed using the Nanodrop 8000 spectrophotometer (Thermo Fisher Scientific Inc, DE, USA) at 260 and 280 nm. RNA integrity was confirmed by agarose gel (1.5% w/v) and evaluated using Agilent 2100 bioanalyzer with RNA 6000 Nano LabChip® Kit (Agilent Technologies Inc, CA, USA). The ribosomal RNA ratio (28S to 18S) of the RNA samples was between 1.9 and 2.1 and the RNA integrity number (RIN) was about 7.

### 2.18.3 Biotin labeled cRNA synthesis

For microarray analysis, total RNAs were processed for use on Affymetrix Tomato Genechip arrays as described in the GeneChip® Expression Analysis Technical Manual. Starting material containing 250 ng of total RNA was used in a reverse transcription reaction to generate cDNAs. After amplification the resulting double-stranded cDNA, it

was labeled using a biotinylated nucleotide analog/ribonucleotide mix using GeneChip IVT Labeling Kit (Affymetrix, Inc., Santa Clara, CA, USA). The biotin labeled cRNA was fragmented and analyzed in the Bioanalyzer and the RNA peaks evaluated the success of fragmentation.

### 2.18.4 Affymetrix array hybridization and scanning

A hybridization mixture consisting of fragmented and labeled cRNA (5 µg), 20X eukaryotic hybridization controls (bioB, bioC, bioD, cre), 2X hybridization mix, control oligonucleotide B2 (3 nM), DMSO, and RNAse free water was mixed. The final volume of 200 µL was heated to 99°C for 5 minutes, and then incubated at 45°C for 5 min. The samples were then hybridized to the Affymetrix tomato GeneChip for 16 h. For each group, four biotin-labeled cRNA hybridizations were performed. The arrays were washed and stained using the Fluidics Station 450 and scanned using the GeneChip® scanner 3000 integrated with Affymetrix® Microarray Suite software.

### 2.18.5 Microarray chip description

The GeneChip tomato genome array (Affymetrix) was used in the current experimet. This oligonucleotide array contains over 10,000 *L, esculentum* probe sets monitoring gene expression for over 9,200 *L, esculentum* genes. The GeneChip Tomato Genome array is a169-format, 11-micron array design and contains 11 probe pairs per probe set. A description of the GeneChip tomato genome array is available at the manufacturer's website.

### 2.18.6 Affymetrix array data analysis

The microarray data normalization and background correction was performed using Guanine Cytosine Robust Multi-Array Analysis (GCRMA) according to Vardhanabhuti *et al.* (2006). R software, (www.r-project.org) and bioconductor packages (www.bioconductor.org) were used. During normalization, the CEL files were converted into expression set using GCRMA considering probe sequence and the GC-content background correction. Following this and starting with the probe-level data from a set of GeneChips, the perfect-match values were background corrected,

normalized and finally summarized resulting in a set of expression measures (App. 1). After hybridization, the quality of the arrays was assessed by the absent and present calls of the control probesets. Differentially expressed genes (DEGs) were obtained using Linear Models for Microarray Data Analysis (LIMMA) (Smyth 2005). The DEGs were selected based on $p < 0.05$, fold changes $\geq 2$ and false discovery rate (FDR) $\leq 0.1$. P-values were adjusted using the Benjamini–Hochberg procedure, which controls the false discovery rate (Benjamini and Hochberg 1995). The raw and normalized data from non-inoculated and *P. infestans*-inoculated untreated plants are available at Gene Expression Omnibus (GEO), http://www.ncbi.nlm.nih.gov/geo/ with accession numbers (GSE33177).

Additionally, the DEGs dataset was compared to all available tomato microarray analysis in the genevestigator database (334 experiments) (Hruz *et al.,* 2008). Figure 2.4 shows a portion of the results obtained by genevestigator, which shows the best correlated experiments and genes that were correlated with our study.

### 2.18.7 Pathways and networks analysis

Molecular pathways associated with resulted DEGs, were identified by using Mapman (ver. 3.5.1) (Thimm *et al.,* 2004). Gene Ontology (GO) Slim enrichment and GO annotation of DEGs was done by using the Tomato Functional Genomics Database (TFGD) (Fei *et al.,* 2011). Fisher's exact test was used to calculate a P-value determining the probability that each biological function or canonical pathway assigned to the data set was because by chance alone.

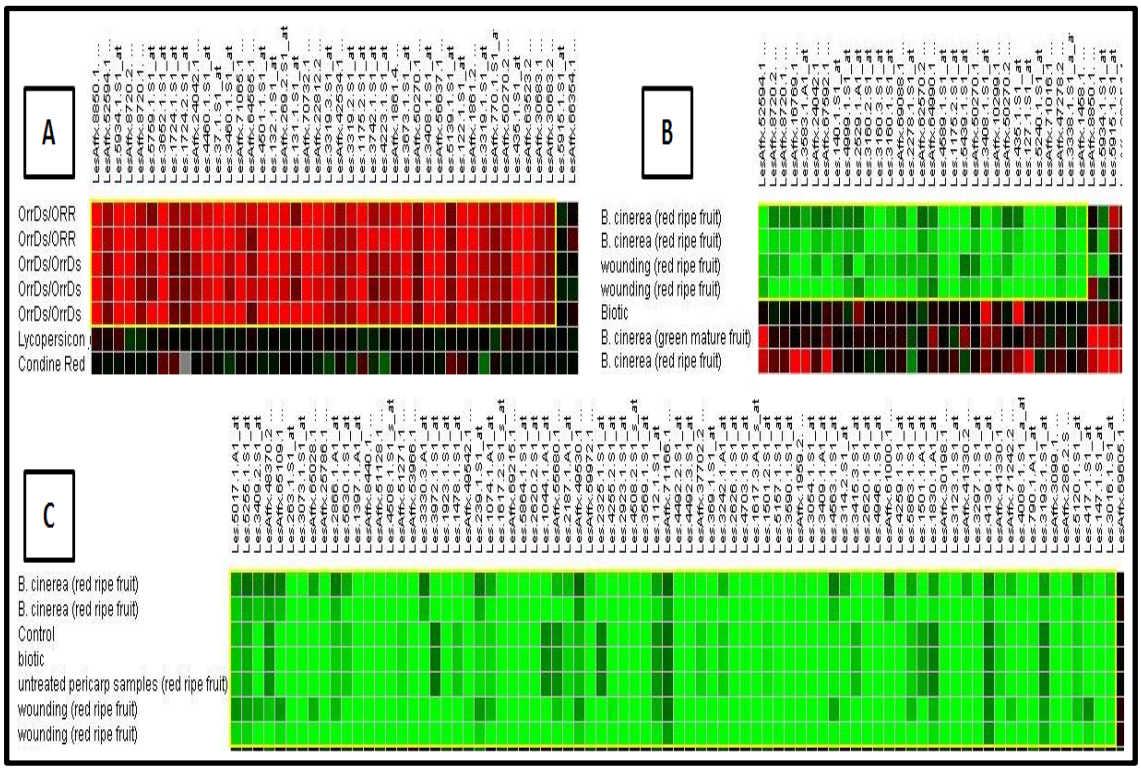


**Figure 2.4:** Differentially expressed genes (DEGs) in tomato responded to biotic and abiotic stress in studies from all 334 available tomato microarray analyses in the genevestigator database found in comparison to current study. DEGs in OrrDs/ORR and OrrDs/OrrDs studies (A) and DEGs in red ripe friut study (B) are upregulated in the current microarray analysis, and DEGs in red ripe friute (wounding, biotic, and infected with *Botrytis cinerea*) studies are down regulated in current microarray analysis. Where: red colours indicates activated genes and green refferes to down regulated genes.

### 2.18.8 Validation of microarray results using quantitative RT-PCR

In the current study, RT-PCR was used to validate some candidate genes differentially expressed in the comparisons of microarray analysis. Sequence specific primers (Tab. 2.5) were designed using 3.0 primer design tool (http://frodo.wi.mit.edu/primer3/). All primers were purchased from biomers.net GmbH (Ulm, Germany) and diluted at 100 pmol stock solution. After confirmation of specific DNA bands for each primer as mentioned in 2.17.4, the amplified PCR product was sequenced to verify the identity of the gene as previously described in details (2.17.5.4). The similarity of the sequence

result to the original sequence was verified using the NCBI /BLAST search tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

The total RNA samples from the same biological replicates used for microarray analysis were used for RT-PCR verification. Total RNase-free DNase-treated and purified RNAs isolated as described previously were reverse transcribed into cDNA template in 20 µL reaction using MultiScribe$^{TM}$ Reverse Transcriptase (High-Capacity cDNA Reverse Transcription Kits, Applied Biosystems) applying the following protocol: 25°C for 10 min, 37°C for 120 min, 85°C for 5 min, and then hold at 4°C. For quantifications, the RT-PCRs were performed in 20 µL reaction volume containing iTaq SYBR Green Supermix with ROX (Bio-Rad laboratories, Munich, Germany), the cDNA samples, the specific forward and reverse primer in ABI PRISM® 7000 sequence detection system instrument (Applied Biosystems). The thermal cycling parameter was set as 95$^{o}$C for 3 min, 40 cycles of 15 sec at 95$^{o}$C and 45 sec at 60$^{o}$C.

The quantification of gene expression was performed using the relative ΔΔCT method, which has been described in Applied Biosystems User Bulletin No. 2 (P/N 4303859), by comparing the data with two internal control genes (GAPDH and TIP41) whose expression was determined to remain constant under different treatment conditions. The data was normalized by geometric mean of two endogenous controls. Where, relative aboundance = $2^{-\Delta\Delta ct}$

Δct = average ct target – average ct endogenous control*

ΔΔct = Δct target – Δct calibrator**

Expression level (fold chang) = $2^{-\Delta\Delta ct}$

* Geometric mean of endogenous controls (GAPDH and TIP 41) was used to normalize each target gene expression 12 hours post inoculation.

** the one with the highest Δct value among the groups was used as a calibrator.

**Table 2.5:** Details of primers used for validation of microarrayy results using quantitative Real Time-PCR analysis

| Gene name | ACC. No. | Primer sequences 5`-3` | bp |
|---|---|---|---|
| Alpha-DOX1 | AY344539 | F: AGGCCAGATCCTATTGACCTT<br>R: CGCCATGAGTCCCACTAATAA | 210 |
| Auxin-regulated dual specificity cytosolic kinase | GU184126 | F: CTTGGAACCAGTCTTGGACAT<br>R: GACTCTCCGTTGCTTAGCATT | 201 |
| chitinase | Z15138 | F: CTGTGCTTCAAGTCAGCAGTG<br>R: CTGTTGTGCTGTCATCCAGAA | 202 |
| Calmodulin-binding protein | B3H796 | F: ACAGATGATGAACGCGAAGAC<br>R: AGGGCCATACATGTTGAAACC | 201 |
| Expansin12 | AF096776 | F: CAAGTGATGGAAGGACACTCA<br>R: ACCACAGCAATAGAGCCAAAC | 209 |
| Expansin | AF059489 | F: GATGGTCGCACTGTTGTTTC<br>R: GACAAAAAGTGCCAAACTGC | 229 |
| GAPDH | U97257 | F: GTTGTGGGTGTCAACGAGAAT<br>R: AGCTCTTCCACCTCTCCAGTC | 210 |
| Hexose transporter protein | AJ010942 | F: TATGATCCCACTTCTCGGTTG<br>R: CCAAATGCACACCTATTTTGTG | 179 |
| Hypothetical LOC543672 | AF308937 | F: TTGTCCACCAACAACAAAGG<br>R: AAGTGTTGTGCAAAGGCAAG | 178 |
| Lipoxygenase | U37840 | F: GACACATTCCCCAGATGAAGA<br>R: CCTGAACTTGGTGCCAATAGT | 210 |
| Pathogenesis-related protein P2 | X58548 | F: CTAGCGTTTACTGCGCTACCT<br>R: TAGCCCAATCCATTAGTGTCC | 244 |
| Peroxidase | X94943 | F: AGATCTTGTCACCCTTGTTGG<br>R: CCGACCATTCCTCAAGTTAGA | 241 |
| Pti5 | U89256 | F: TCGTCCATTACAGTGCATAGG<br>R: ACTGAAACAGAGGCGTTCACT | 231 |
| Subtilisin-like protease | AJ006377 | F: CGTACCACGACGAATAATTGC<br>R: CCCATCTATGGCATCATCGAT | 201 |
| TI P 41 | AT4G34270 | F: ATGGAGTTTTTGAGTCTTCTGC<br>R: GCTGCGTTTCTGGCTTAGG | 235 |
| TSI-1 protein | Y15846 | F: CAAATTTGAAGCTGCTGGAG<br>R: TCTCTCACGTGTGGATCTTTG | 212 |

bp: Amplicon length; F: forwarded primer; R: reverse primer

## 2.19 Statistical analysis

The experiments were conducted under completely randomized design. The mean value of the replicates for each treatment was presented in the results**.**

All data were analyzed using the Statistical Analysis System (SAS) software package version 9.2 (SAS Institute Inc., NC, USA). The parameters were analysed using the General Linear Model of SAS. Mean comparisons were made using Duncan`s Multiple Range test or Tukey`s Honestly Significant Difference test at 5 % of error probability.

Disease severity parameters were gathered as percentage of infected leaf areas and the RNA expression analysis for the studied genes was performed based on the relative standard curve method. Efficacy of bacterial cells and their metabolites against test pathogens *in vitro* and *in vivo* was computed by applying the methods of Abbott (1925).

$$\text{Efficacy (\%)} = \frac{(Ut - Tr)}{Ut} \times 100$$

Whereby; Ut = untreated control

Tr = treated with bacterial cells or metabolites

# 3 RESULTS

## 3.1 Influence of foliar application of bacterial biocontrol agents FZB24® and Phytovit® on different leaf diseases of tomatoes

To identify the activity of the biocontrol agents FZB24® and Phytovit® under greenhouse conditions two concentrations were applied on leaves of tomato plants before and after inoculation of the pathogens. The results showed significant reductions in disease severity when the agents were applied prior to inoculation (protective effect). There were no significant differences between the two application rates in most cases, but the suppression was more pronounced with the high application rate 3 g $L^{-1}$.

Both products reduced severity of late blight disease when applied before and after inoculation of *Phytophthora infestans*. No significant differences between the two application rates were observed. The reduction achieved by FZB24® when applied before inoculation was higher than 80% with the high concentration (3 g $L^{-1}$) and over than 60% for the recommended application rate (0.3 g $L^{-1}$). Phytovit® suppressed disease severity in average about 50% reduction with the exception of spraying the high concentration 3 days before inoculation, which caused 83% reduction compared to the control (Fig. 3.1A).

The efficacy of antagonists to suppress the early blight disease varied in respect to the time and rate of application (Fig. 3.1B). The disease was significantly suppressed by applying FZB24® prior and post *Alternaria solani* inoculation with one exception when the antagonist (0.3 g $L^{-1}$) was sprayed one day post inoculation. The antagonistic effect was more pronounced for the higher concentration prior to inoculation. The highest antagonistic activity achieved by FZB24® (3 g $L^{-1}$) applied one day before inoculation was 85% reduction. Application of Phytovit® resulted in significant reduction of the disease severity by about 50% once the high concentration was used before inoculation.

In figure 3.2A, the data show that *Cladosporium fulvum* attacked tomato plants causing leaf mold disease with 27% leaf damaged area. The antagonists slightly reduced the disease severity either before or after the inoculation. The reduction was significant approximately 60% using the high application rate of both antagonists applied one day prior to inoculation. Applying the products resulted in no significant differences between the application times and between rates.
For *Oidium neolycopersici* (Fig. 3.2B), the results showed no significant difference in the efficacy between the two products against powdery mildew disease. The antagonists significantly suppressed the disease by 50-70% regardless the application time or the concentrations of the products compared to disease level on untreated control plants.

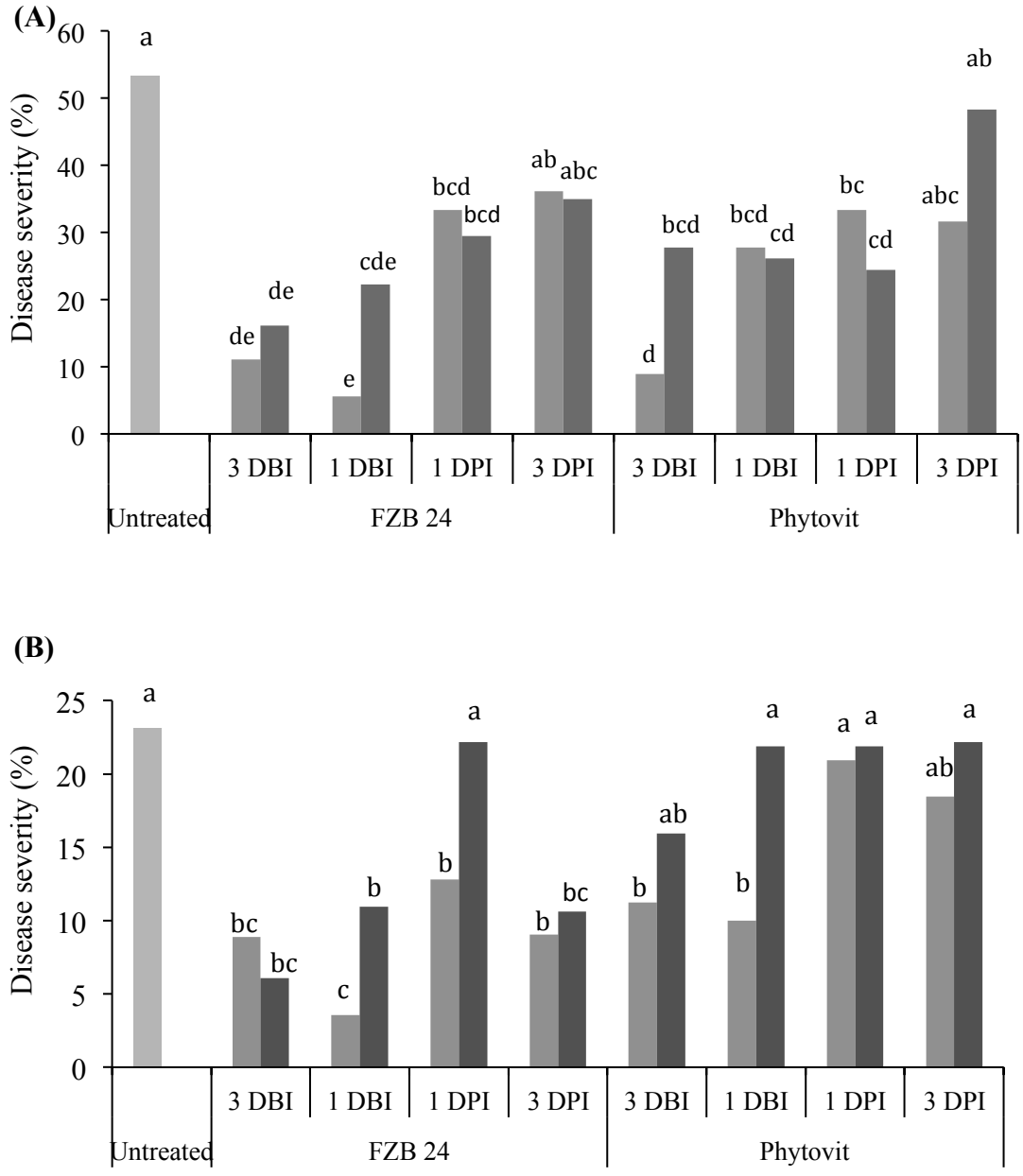


**Figure 3.1:** Influence of foliar application of biocontrol agents FZB24® and Phytovit® on disease severity of late blight (A) and early blight (B) 7 days post inoculation of tomato plants. The products were applied before or post pathogen inoculation. Light and dark gray colors of the columns indicate the concentration of 3 and 0.3 g $L^{-1}$ prepared from the products. (Columns marked with the same letters do not differ statistically using Tukey`s Test at $P \leq 0.05$; n=4)

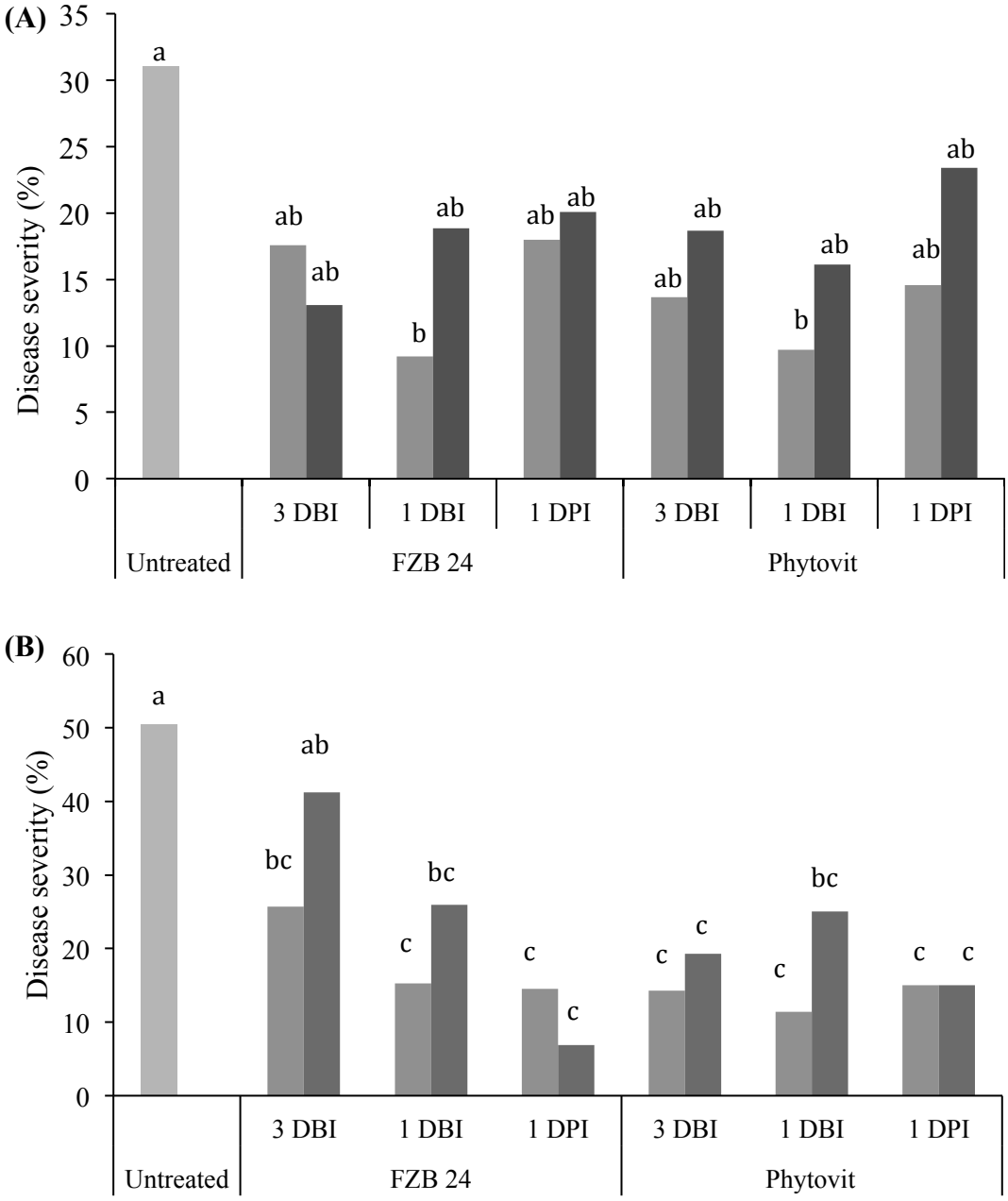


**Figure 3.2:** Influence of foliar application of biocontrol agents FZB24® and Phytovit® on severity of leaf mold (A) and powdery mildew (B) two weeks post inoculation of tomato plants.The products were applied before or post pathogen inoculation. Light and dark gray colors of the columns indicate the concentration of 3 and 0.3 g $L^{-1}$ prepared from the products. (Columns marked by the same letter do not differ statistically using Duncan`s Multiple Range Test at $P \leq 0.05$; n=4)

## 3.2 Influence of isolated bacteria from FZB24® and Phytovit® on growth of different leaf pathogens

### 3.2.1 Influence of application time of *Bacillus subtilis* on myclial growth

To evaluate the efficacy of *B. subtilis* re-isolated cells from the biocontrol agents FZB24® and Phytovit® against several leaf pathogens *in vitro*, dual culture test was used (Fig. 3.3). Bacterial colonies were streaked between two agar pieces colonized with the pathogen one day before or one day after or at the same time of pathogen presence. The results of application time of re-isolated cells from FZB24® and Phytovit® on pathogens growth has been summarized in table (3.1). With *Phytophthora infestans, Alternaria solani* as well in case of *Cladosporium fulvum* applying the bacterial strains at different times resulted in significant reduction of the mycelial growth compared to untreated culture media. The effect was more pronounced in application of bacterial strains before the pathogen culture and reduced after grown the mycelia of the pathogen on the media with no significant difference between the two strains in mycelia growth inhibition.

### 3.2.2 Influence of inoculum density of *B. subtilis* on myclial growth

The affectivity of *B. subtilis* cells re-isolated from the biocontrol agents FZB24® and Phytovit® against the mycelium growth of pathogens was investigated using different concentrations ranged between $10^4$-$10^7$ cells $mL^{-1}$. Generally, the bacteria strongly inhibited the mycelial growth of the pathogens (Tab. 3.2). Increasing high concentrations of bacteria intensified this inhibition. The effect of bacterial strains on the growth of pathogens proved to be highest with *P. infestans* followed by *C. fulvum* and *A. solani*. The effectiveness of the two strains, which is rated as inhibition of pathogen growth, was different. The re-isolated cells from Phytovit® were more effective against *A. solani*. In contrast, the re-isolated cells from FZB24® were more effective against *C. fulvum* (98% inhibition). On the other hand, the two strains had a similar strong effect against *P. infestans* (100% inhibition) (Fig. 3.4)

### 3.2.3 Influence of *B. subtilis* on spore germination of different leaf pathogens

The efficacy of *B. subtilis* re-isolated cells from the biocontrol agents FZB24® and Phytovit® against spore germination of leaf pathogens on glass surfaces was studied according to the method described by Nair and Ellingboe (1962). The results showed

that the inhibitory effect varied according to the pathogen and the strongest effect was against spore germination of *P. infestans* followed by *O. neolycopersici* and *C. fulvum* in descending order (Tab. 3.3). In comparing with the control, the reduction was not significant against *A. solani* and the potential of both bacterial strains was approximately 30% inhibition of spore germination. There were no significant differences observed between the two strains with the exception of *C. fulvum*.

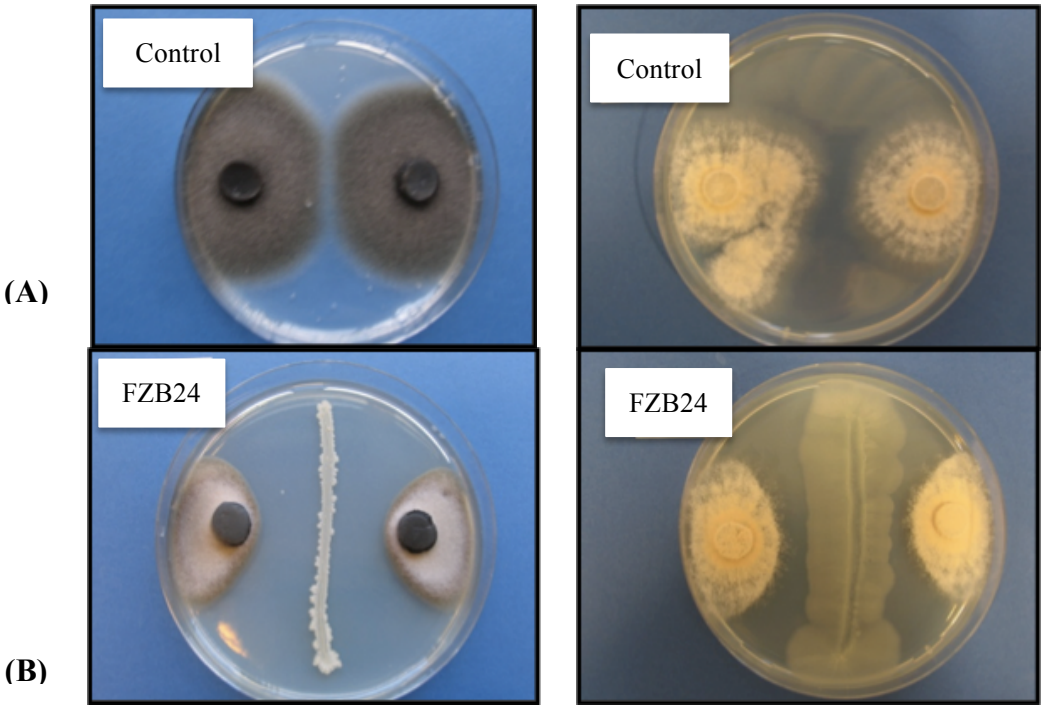


**Figure 3.3:** Mycelia growth of *Alternaria solani* (left) and *Cladosporium fulvum* (right) on untreated PDA medium (A) and on *Bacillus subtilis* strain FZB24-treated PDA medium one day before pathogen disks presence (B), 8 days post culture at 26°C using dual culture test.

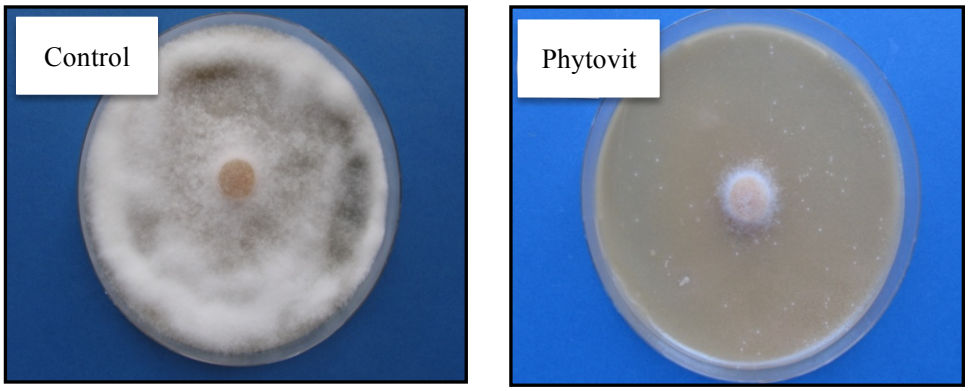


**Figure 3.4:** Mycelia growth of *Phytophthora infestans* on (left) untreated tomato juice agar medium and on (right) *Bacillus subtilis* strain Phytovit-treated medium ($10^6$ cells $mL^{-1}$), 7 days post culture in darkness at 21°C.

**Table 3.1:** Influence of application time of *Bacillus subtilis* re-isolated from FZB24® and Phytovit® on pathogen mycelial growth using dual culture test.

| Application | Application time (day) | Pathogens | | |
|---|---|---|---|---|
| | | *Alternaria solani* ** | *Cladosporium fulvum* | *Phytophthora infestans* |
| Water | - | 2.80 a | 0.30 f | 2.34 a |
| FZB24® | Before* | 0.78 d | 2.46 b | 0.24 d |
| | After | 1.56 b | 1.48 e | 0.70 b |
| | Same | 1.06 c | 1.72 d | 0.48 bc |
| Phytovit® | Before | 0.64 d | 3.10 a | 0.22 d |
| | After | 1.22 c | 2.16 c | 0.50 bc |
| | Same | 1.56 b | 2.24 c | 0.38 cd |

*Application time: placing the bacterial colonies one day before, after, or at the same time of pathogen culture. **For *A. solani*, the distance between colonies was measured, but for other pathogens the linear growth of mycelia was measured**.** (Line for individual pathogen marked with a common letter do not differ statistically using Duncan`s Multiple Range Test at $P \leq 0.05$; n=4).

**Table 3.2:** Influence of *Bacillus subtilis* re-isolated from FZB24® and Phytovit® on mycelial growth of different leaf pathogens depending on different concentarations of bacteria (cells $mL^{-1}$) applied one day before placing the pathogen disk.

| Treatment | Cells $mL^{-1}$ | Pathogens | | |
|---|---|---|---|---|
| | | *Alternaria solani* | *Cladosporium fulvum* | *Phytophthora infestans* |
| Water | 0 | 1.90 a | 3.02 a | 3.50 a |
| FZB24® | $10^4$ | 1.83 ab | 0.70 d | 0.50 b |
| | $10^5$ | 1.84 ab | 0.66 d | 0.24 bc |
| | $10^6$ | 1.58 bc | 0.52 e | 0.50 b |
| | $10^7$ | 1.49 cd | 0.06 g | 0.00 c |
| Phytovit® | $10^4$ | 1.80 ab | 1.14 b | 0.01 c |
| | $10^5$ | 1.32 d | 0.90 c | 0.01 c |
| | $10^6$ | 1.26 d | 0.70 d | 0.00 c |
| | $10^7$ | 0.66 e | 0.38 f | 0.00 c |

Column for individual pathogen marked with a common letter do not differ statistically using Duncan`s Multiple Range Test at $P \leq 0.05$; n=4.

**Table 3.3:** Inhibitory effects of *Bacillus subtilis* re-isolated cells from FZB24® and Phytovit® on spore germination of different leaf pathogens on glass surface.

| Pathogens | Germination % | | Reduction (%) | | | |
|---|---|---|---|---|---|---|
| | | | FZB24® | | Phytovit® | |
| *Phytophthora infestans* | 70.4 | a | 69 | b | 66 | b |
| *Alternaria solani* | 78.3 | a | 23 | a | 36 | a |
| *Cladosporium fulvum* | 21.0 | a | 43 | b | 76 | c |
| *Oidium neolycopersici* | 81.9 | a | 59 | b | 51 | b |

Means marked with a common letter for individual pathogen do not differ statistically using Duncan`s Multiple Range Test at $P \leq 0.05$; n=4.

### 3.2.4 Influence of *B. subtilis* on developmental structures of different pathogens on tomato leaf surfaces

The detached leaf assays were carried out to investigate the influence of *B. subtilis* re-isolated cells and metabolites from the biocontrol agents FZB24® and Phytovit® on establishment of three pathogens different in their life cycle and disease development on tomato leaves. Light microscope was used to make the evaluations.

#### 3.2.4.1 *Oidium neolycopersici*

The leaflets samples taken 24 hours post inoculation with *O. neolycopersici*, cleared in saturated chloralhydrate and stained in Bruzzese solution were observed under the interference contrast (Fig. 3.5). Bacterial cells significantly suppressed the fungal development through the whole growth stages; spore germination, appressoria formation and haustoria by more than 50% inhibition with no obvious differences in the inhibitory efficacy between FZB24 and Phytovit strains (Fig. 3.6).

#### 3.2.4.2 *Alternaria solani*

The leaflets samples taken 12 hours post inoculation with *A. solani*, cleared in saturated chloralhydrate, and stained in acid Fuchsin solution were observed under the interference contrast. The fungus produces spores consisting of many cells, which ranged from 2 to 18 (Fig. 3.5). The number of germinated cells per spore has been counted and the germination rate of complete cells number of observed spores was assessed. The bacteria significantly inhibited cell germination by 22.8% for FZB24 and

31.8% for Phytovit with no significant difference between their inhibitory efficacies. The reduction in germ tubes length was pronounced in case of FZB24 by 33.6% (Fig.3.7).

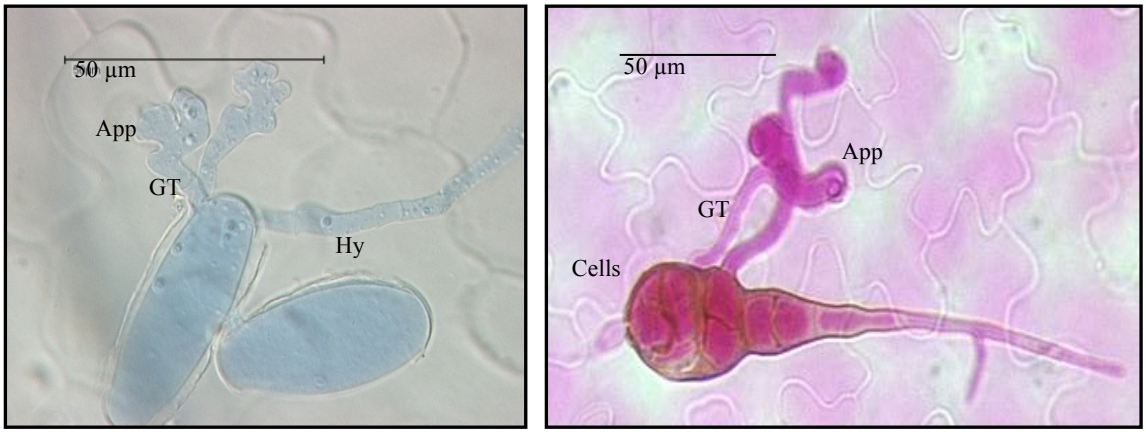


**Figure 3.5:** Infection structures of *Oidium neolycopersici* stained with Bruzzese s-olution on detached leaf surfaces 24 hours post inoculation (left) and the development structures of *Alternaria solani* stained with acid Fuchsin on detached leaf surfaces 12 hours post inoculation (right): germ tube (Gt), appressorium (App), and secondary hyphae (Hy).

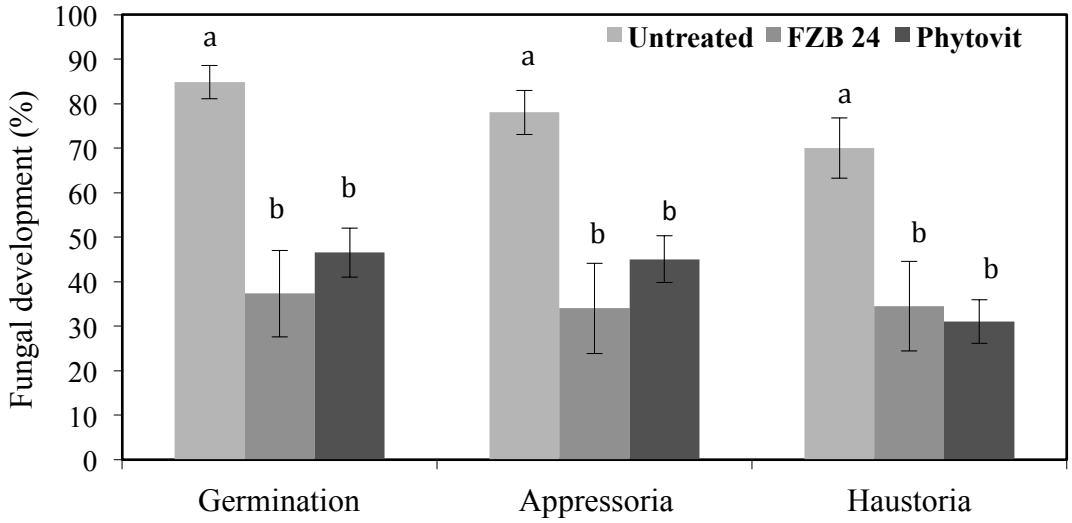


**Figure 3.6:** Influence of *Bacillus subtilis* cells isolated from the biocontrol agents FZB24® and Phytovit® on development of *Oidium neolycopersici* on tomato leaves at 24 hours post inoculation. (Columns for each development stage followed by the same letter do not differ statistically using Tukey`s HSD Test at $P \leq 0.05$; mean± SE; n=100 spores x 10 rep.)

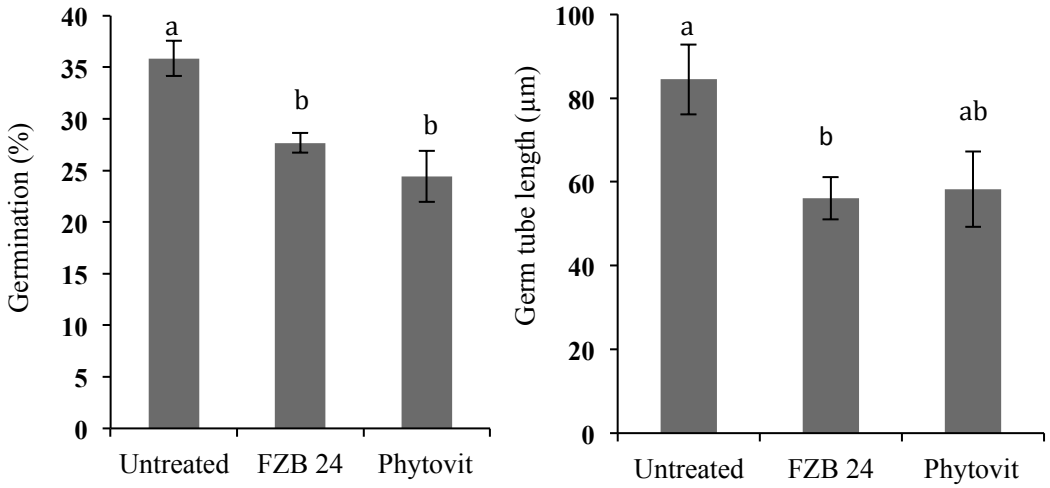


**Figure 3.7:** Influence of *Bacillus subtilis* cells isolated from the biocontrol agents FZB24® and Phytovit® on spore germination of *Alternaria solani* and germ tubes elongation, on detached tomato leaves at 12 hours post inoculation. (Columns followed by the same letter for each parameter do not differ statistically using Tukey`s HSD Test at $P \leq 0.05$; mean±SE; n=60 cells x 6)

#### 3.2.4.3 *Phytophthora infestans*

Because of the highest efficacy of the biological control agents FZB24® and Phytovit® shown from the products in suppression of late blight disease and from the re-isolated bacteria in inhibition mycelium growth and zoospore germination of *P. infestans*. Therefore, it was preferred to do the further investigations on the influences of *Bacillus subtilis* late blight disease and *P. infestans* development in more details giving more concern on potential of systemic activity through plant using the bacterial cells as well as the metabolites secreted in the broth media.

## 3.3 Evaluating the efficacy of metabolites secreted by *Bacillus subtilis* on late blight disease

To investigate the potential effect of metabolites produced by re-isolated bacteria from the biocontrol agents FZB24® and Phytovit® in suppression of late blight disease, cells and secreted metabolites were sprayed on the upper surface of detached tomato leaves 24 hours before *Phytophthora infestans* inoculation on the same surface. The current experiment was repeated three times and the results were homogenized.

Re-isolated cells and the metabolites harvested different times after culturing significantly reduced the disease severity (Fig. 3.8). Metabolites clearly suppressed the disease more than the bacterial cells compared to untreated leaves. The highest reduction (89%) was achieved from metabolites of *B. subtilis* strain FZB24 extracted after 72 hours of culturing (M72). In addition, the autoclaved metabolites (M72 autoclaved), heated at 121°C for 20 min, showed stability to suppress the disease by 70% reuction. The metabolites harvested from re-suspended bacterial cells in water for one hour (M1) and for 24 hours (M24) reduced significantly the disease severity by 36-70% reduction. In case of Phytovit strain, the reduction ranged between 42 and 61%. No significant difference between FZB24 and Phytovit was declared in favor of the FZB24. Moreover, application of culture medium (SGM) used in metabolites production showed no potential activity to reduce the disease severity compared to the control.

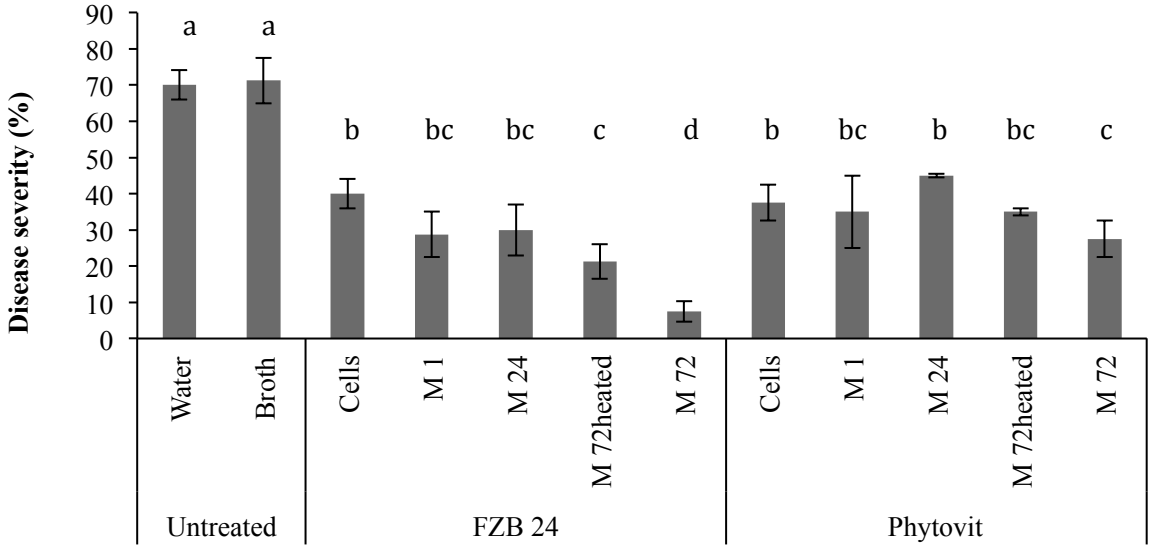


**Figure 3.8:** Influence of bacterial cells re-isolated from the biocontrol agents FZB24® and Phytovit® and their metabolites (M) harvested 1, 24, and 72 hours of culturing on late blight disease severity on detached tomato leaves 6 days post inoculation with *Phytophthora infestans*, which applied 24 hours after treatments spraying. (Columns marked with the same letters for each products in comparison to the untreated do not differ statistically using Tukey`s HSD Test at $P \leq 0.05$; mean ± SE; n=4)

## 3.4 Influence of cells and metabolites from *Bacillus subtilis* strain FZB24 on development of late blight and *Phytophthora infestans*

### 3.4.1 Effects on colonization of leaves

#### 3.4.1.1 Influence on late blight disease development

To investigate the influence of *B. subtilis* strain FZB24 cells and metabolites on progresses of late blight disease symptoms, the diseases severity was evaluated from *P. infestans* inoculated untreated leaves and cell-/metabolite-treated leaves, from both attached leaves (single plant) and detached leaves maintained in plastic boxes under the same conditions.

No symptoms of infection were observed on any of the tomato leaves in the first 24 hours following inoculation. Within several days (2-3), the first cells involved in the interaction died. Three days after inoculation, several small black lesions were seen on surfaces of leaves inoculated with *P. infestans*. By six days after inoculation, severe symptoms were observed on all inoculated attached and detached leaves. The progress of disease was slightly more on detached than attached leaves. The efficacy of protection was higher in attached than in detached leaves with accelerated senescence (Fig. 3.10). Both cells and metabolites were effective in preventing pathogen infection; they inhibited the disease development on tomato leaves and significantly reduced the expansion of existing late blight lesions (Fig. 3.9). More than 80% reduction of disease severity was calculated on inoculated attached treated leaves. However, the potential of treatments to suppress the disease development on detached leaves was about 70% reduction of disease severity. Likewise, no symptoms were observed on non-inoculated tomato plants treated with water.

Untreated leaf | Cell-treated leaf | Metabolite-treated leaf

**Figure 3.9:** Effect of *Bacillus subtilis* cells and metabolites on disease symptoms of late blight on detached tomato leaves 6 days post inoculation with *Phytophthora infestans* ($10^5$ sporangia $mL^{-1}$).

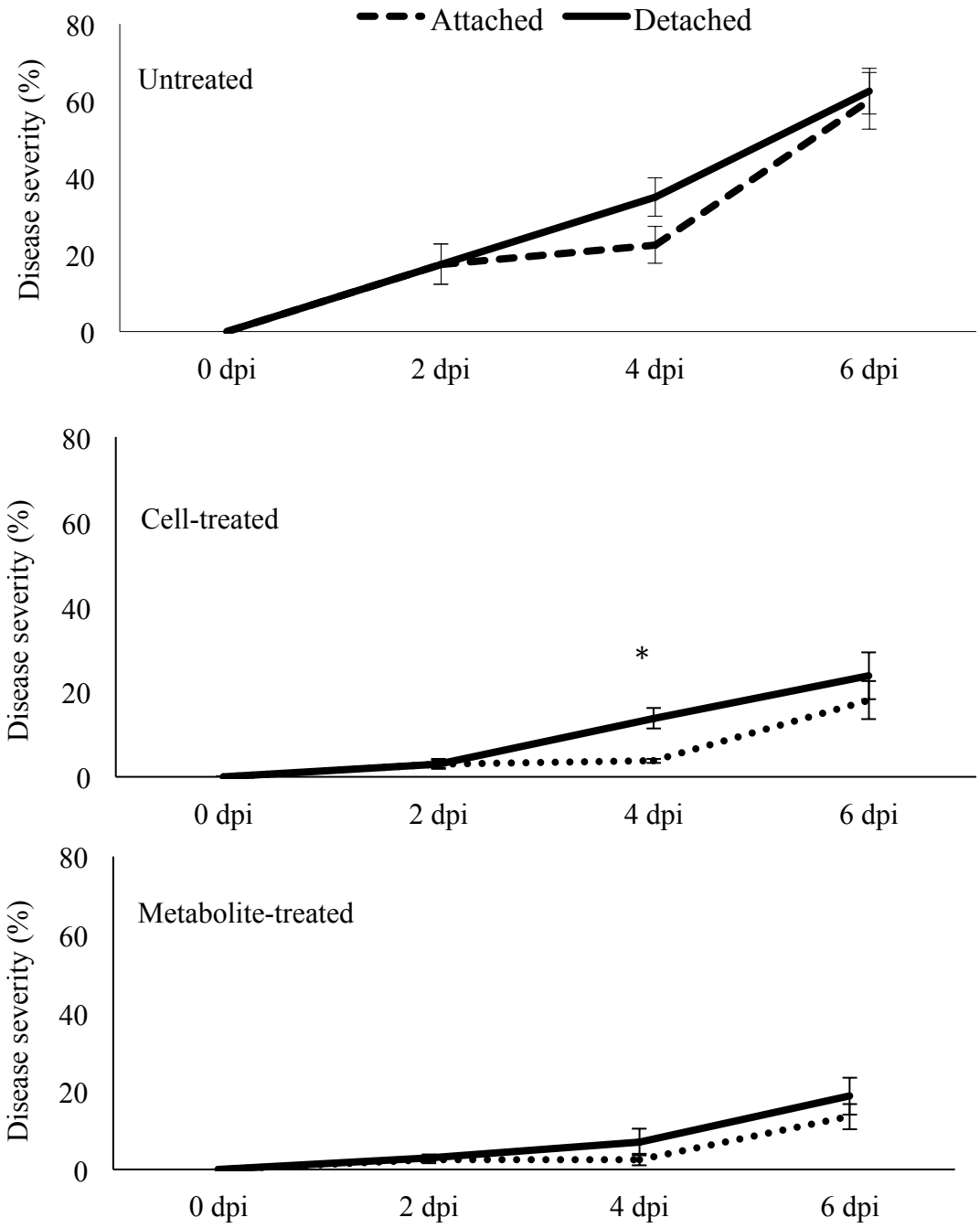


**Figure 3.10:** Influence of *Bacillus subtilis* strain FZB24 on late blight disease on attached and detached tomato leaves. Cells and metabolites were applied 24 hours prior inoculation with *Phytophthora infestans* ($10^5$ sporangia $mL^{-1}$). (Star refers to a significant difference between leaf types at each sampling point using Tukey's HSD Test at $P \leq 0.05$; mean ± SE; n= 4)

### 3.4.1.2 Influences on biomass of *P. infestans* in leaf tissue

#### 3.4.1.2.1 Effect of inoculum density of *P. infestans* on leaf colonization

To investigate the progress of *P. infestans* growth in tissues of detached leaf, DNA content of the pathogen was extracted from leaf samples inoculated with different concentrations of the pathogen 5 days post inoculation.

Biomass of *P. infestans* increased with the concentration of the inoculum. Minor amount was observed in the non-inoculated leaves. No excess in pathogen growth increase was observed in leaf tissue inoculated either with 300 or 3000 sporangia $mL^{-1}$. However, inoculated leaves with 30000 sporangia $mL^{-1}$ resulted in obvious increase in biomass of pathogen by about 11.5 times more than other concentrations (Tab. 3.4).

**Table 3.4:** DNA content of *Phytophthora infestans* in leaf tissue 5 days post inoculation.

| *P. infestans* sporangia $mL^{-1}$ | Amount of *P. infestans* DNA pg $mg^{-1}$ leaf material ± SE |
|---|---|
| Non-inoculated | 00.84 ± 0.11 |
| 300 | 03.81 ± 0.38 |
| 3000 | 03.57 ± 0.25 |
| 30000 | 41.15 ± 1.41 |

#### 3.4.1.2.2 Influence on biomass of *P. infestans* over the time of infection

To evaluate the effect of *B. subtilis* on *P. infestans* biomass in leaf tissue, both cells and the excreted metabolites harvested after 72 hours of culturing were applied on foliar parts of tomato plants 24 hours prior pathogen inoculation. Samples from attached and detached leaves were taken in corresponding to development stages of the infection process. The experiment was performed twice for the most sampling points.

The pathogen biomass was slightly increased during the early infection stage with higher DNA content in the untreated plants in comparison to cell- and metabolite-treated plants and with high content as well in detached leaves than in attached ones (Tab. 3.5). There are differences in growth rate of *P. infestans* between attached and detached

leaves. DNA contents 144 hours post inoculation were 100 times and about 485 times more than 3 hours post inoculation in attached and detached untreated leaves, respectively. That means *P. infestans* colonized detached leaf tissue better than attached leaves and the increase in growth rate was 5 times faster. For treated plants, Data showed that both cells and metabolites reduced pathogen biomass in leaf tissues with no significant difference observed in efficacy between cells and metabolites. The effectiveness to suppress the pathogen growth after 6 days of inoculation were more than 80% in attached leaves compared to about 60% in detached ones for both the cells and metabolites. Interestingly, the effect of cells and metabolites applied on attached leaves showed higher reduction than in detached leaves by 2 fold, which means probability of elevation in treatments efficacy to suppress the pathogen development in the attached leaves. In addition, the approximately similar amounts of pathogen DNA detected at 3 hpi showed no differences in inoculum density applied either on attached or detached treated or untreated leaves. In non-inoculated leaves, the quantification adjusted a small amount of pathogen DNA due to natural infection (data is not shown).

**Table 3.5:** DNA content of pathogen biomass [pg/mg leaf dry weight] in tomato leaf tissues inoculated with $10^5$ sporangia $mL^{-1}$ of *Phytophthora infestans* after 24 hours of *Bacillus subtilis* strain FZB24 cells or metabolites applications.

| Sampling dates | Inoculated-attached leaves | | | Inoculated-detached leaves | | |
|---|---|---|---|---|---|---|
| | Un treated | Cell-treated | Metabolite-treated | Un treated | Cell-treated | Metabolite-treated |
| 3 hpi | 62.1 a | 48.0 a | 58.1 a | 63.1 a | 68.8 a | 51.6 a |
| 6 hpi | 62.8 a | 37.6 a | 28.0 a | 64.0 a | 47.8 a | 54.2 a |
| 12 hpi | 81.6 a | 62.1 a | 61.6 a | 121.1 a | 67.9 a | 79.9 a |
| 24 hpi | 76.5 a | 58.7 a | 54.7 a | 123.4 a | 102.4 a | 73.1 a |
| 48 hpi | 172.9 a | 78.9 a | 84.0 a | 215.6 a | 99.2 a | 98.4 a |
| 96 hpi | 1105.4 a | 247.2 b | 84.1 b | 14007.4 a | 1246.5 b | 778.5 b |
| 144 hpi | 6226.5 a | 1058.6 b | 1126.0 b | 29003.5 a | 17310 a | 11351.1 b |

Data represent means of four measurements and each measurement is a mean of two runs in RT-PCR. (Means followed by the same letters within each line for attached or detached separately are not significantly different at $P \leq 0.05$; mean ± SE; n=4)

### 3.4.1.3 Influence on development structures of *P. infestans*

To gain a better understanding in which way the *B. subtilis* strain FZB24 (cells and their excreted metabolites harvested 72 of culturing) reduces disease severity and which development structure are involved, their influences on early stages of *P. infestans* growth were investigated. Samples taken in concerning the infection course, discolored in saturated chloralhydrate, and stained using acid Fuchsin solution were used to observe the growing stages of *P. infestans* on treated and untreated detached leaves (Fig. 3.11). The experiment was performed twice and the results represent the data of the second once.

The results showed the effect of both cells and metabolites on the pathogen development in the early infection stages before as well as after penetration of the host plant cells (Fig. 3.12). Three hours post inoculation a slight decrease in the germination rate followed by a significant reduction in the pathogen ability to form the appressoria and to penetrate the epidermal cells was observed. Six hours post inoculation the treatments affected on the ability of the pathogen to penetrate the epidermis cells and form a primary vesicles, which was obviously reduced by 23% with a slight effect on the vesicles size. However, metabolites application showed significant decrease in haustoria formation inhibiting the intracellular growth of *P. infestans* by more than 30%, while no influence was observed in cell-treated leaves. Pathogen after penetration the epidermis continued growing intercellularly in the mesophyll at 12 hours post inoculation. While no obvious effects on pathogen growth in palisade mesophyll in treated leaves, both cells and metabolites significantly inhibited the further development in spongy mesophyll at 12 hours post inoculation, followed after 24 hours of inoculation by a strong reduction in the number of infected host cells per infection side by 25% and 45% for cells and metabolites, respectively. Subsequently, the inhibition effect of both cells and metabolites on pathogen growth, which was evident from the first stages of infection, resulted in significant reduction in late blight disease symptoms on tomato leaves in favor of metabolites.

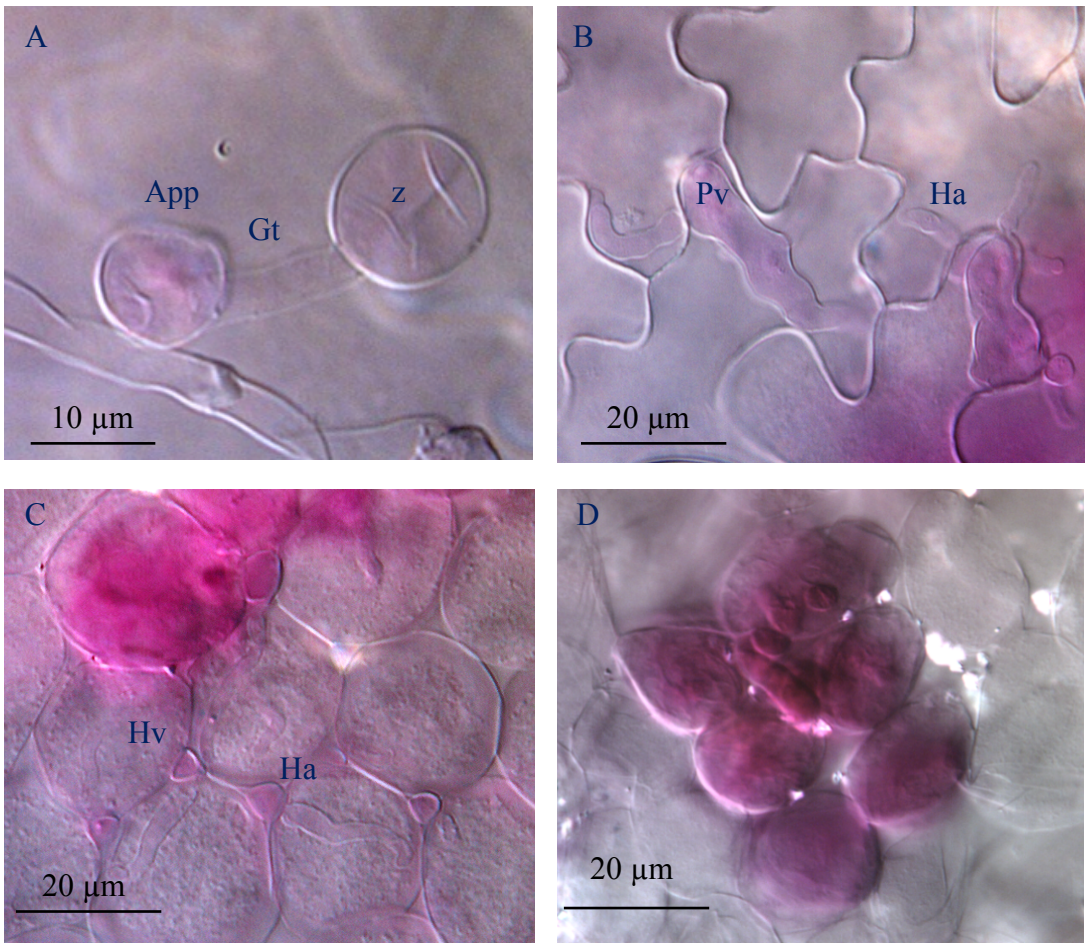


**Figure 3.11:** Developmental structures of *Phytophthora infestans* in the early stages of infection of tomato leaves: zoospore (Z), germ tube (Gt), appressorium (App), primary vesicles (Pv), hyphae (Ha).

A) Zoospore germination, elongation of germ tube and appressorium formation at 3 hours post inoculation

B) Primary vesicle body and haustoria in epidermis at 6 hours post inoculation

C) Intercellular hyphae between the palisade mesophyll cells and haustoria formation at 12 hours post inoculation

D) Pathogen structures inside damaged mesophyll cells at 24 hours post inoculation

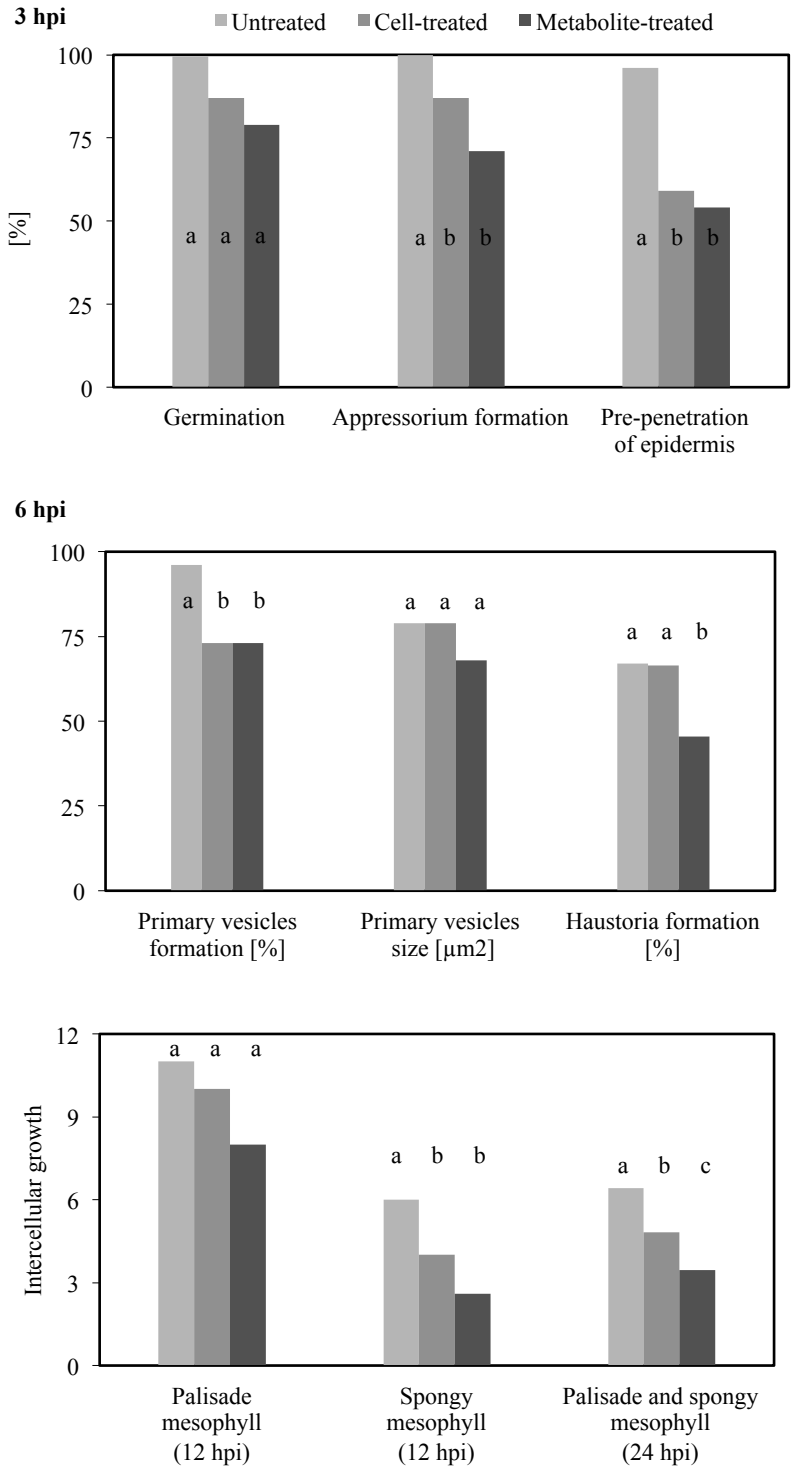


**Figure 3.12:** Influence of *Bacillus subtilis* strain FZB24 cells and metabolites on development of *Phytophthora infestans* structures before and after penetration of tomato leaflets. (Means followed by the same letters for each developmental stages are not significantly different using Tukey‘s HSD Test at $P \leq 0.05$; n=8)

### 3.4.1.3.1 Influence on the germ tube length of *P. infestans* on different surfaces

To investigate the influences of *B. subtilis* strain FZB24 on germ tube elongation of *P. infestans* on different surface models, glass slide and detached tomato leaves were used. For the glass slides a drop of *P. infestans* zoospores was added over a drop of bacteria or metabolites suspensions and for leaf surfaces cells or metabolites were applied prior inoculation with *P. infestans.*

Figure 3.13 illustrated that the average means of germ tube length was 6.3-fold higher on glass surface than on leaf surface in untreated samples. The effect of bacteria and their metabolites on germ tube length of *P. infestans* was three times greater on detached leaves than on glass surfaces compared to the controls. Data showed inhibition of germ tube length by 35% for metabolites and by 20% for bacteria in comparing with the control on glass surface. While on leaf surface, the effects of treatments resulted in increase of the germ tube length by 96% for metabolites and by 65% for the cells. The effect of metabolites was more pronouneced by 1.5-fold than the effet of the bacteria.

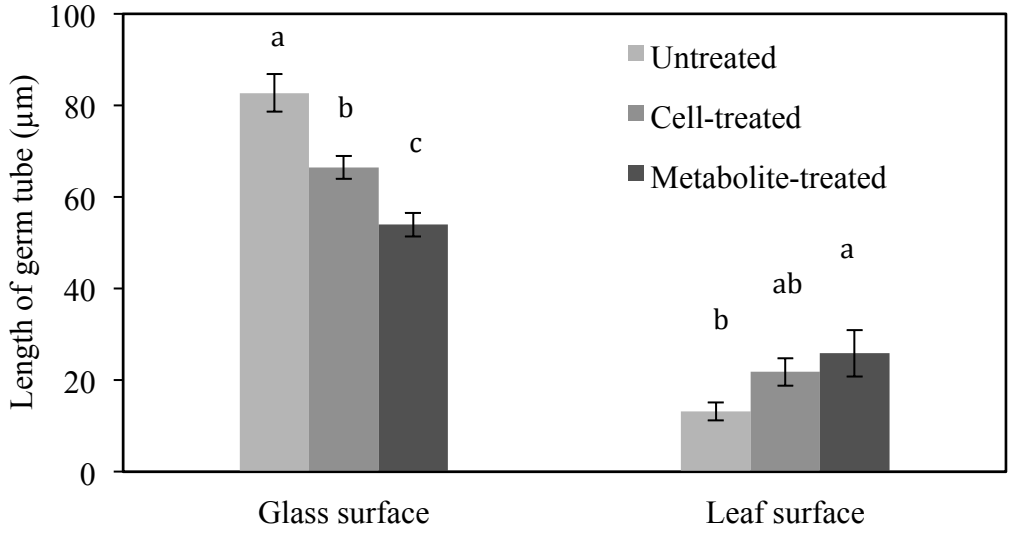


**Figure 3.13:** Effect of *Bacillus subtilis* strain FZB24 cells and metabolites on germ tube length of *Phytophthora infestans* on different surfaces 6 hours post inoculation. (Columns followed by the same letter for each surface do not differ statistically using Tukey‘s HSD Test at $P \leq 0.05$; mean ± SE; n= 50)

### 3.4.2 Systemic activity of *B. subtilis* strain FZB24 in tomato plants

#### 3.4.2.1 Translaminar translocation

To investigate the systemic protection of *B. subtilis strain* FZB24 to suppress late blight disease through leaf tissues, both cells and their metabolites harvested 72 hours after culturing were sprayed 24 hours prior *P. infestans* inoculation. Both *B. subtilis* and *P. infestans* were applied (i) on the same side of leaf surface either the upper or the lower side and (ii) on different sides one on the upper side and the other on the lower side or vice versa. The experiment was repeated three times given the same trend of results.
When *B. subtilis* cells or metabolites and *P. infestans* were sprayed on the same surface, the reduction of disease severity was higher than when they were sprayed on different sides of tomato leaf surface indicating to the direct effect on the pathogen (Fig. 3.14). The results showed reduction in disease severity by 43% for cells, while the potential activity of metabolites was 70.3% on the lower leaf surface and more than 90% on the upper side.
In case of *B. subtilis* cells or metabolites applied on one side and *P. infestans* applied on the other side of leaf surfaces, both cells and metabolites reduced disease severity. Cells when sprayed on the lower surface and *P. infestans* on upper side were more effective than when applied on the opposite sides causing 40% and 16% reduction, respectively. However, metabolites reduced disease severity more than 50 % regardless of the application side.

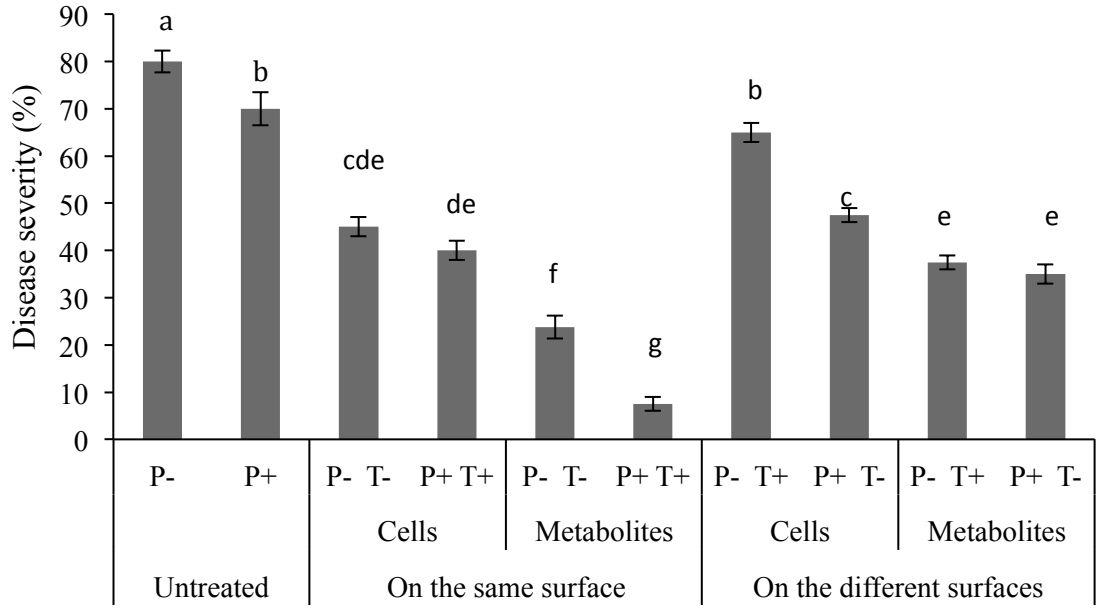


**Figure 3.14:** Influence of *Bacillus subtilis* strain FZB24 applied 24 hours prior inoculation with *Phytophthora infestans* on late blight disease severity on detached tomato leaves 6 days post inoculation: pathogen (P), cells or metabolites treatment (T), spraying on the upper (+) and on the lower (-) leaf surface. (Columns marked with the same letters do not differ statistically using Tukey`s HSD Test at $P \leq 0.05$; mean ± SE; n=4)

### 3.4.2.2 Apical translocation

To investigate the systemic activity of *B. subtilis* strain FZB24 through up tomato plants, the re-isolated cells and metabolites harvested 72 hours after culturing were sprayed on the lower leaf pairs of tomato plants 24 hours prior *P. infestans* inoculation, which was sprayed on the lower and upper leaves of treated and untreated plants. The experiment was repeated three times and the data shown are from the most homogenized one.

Data showed that disease severity was more pronounced on the lower leaves than on the upper ones in untreated and cell-treated plants, while was the opposite for metabolites treated plants without significant difference in disease severity between lower and upper leaves for each treatments (Fig. 3.15). No significant differences in reduction of disease severity between lower and upper leaves in treated plants were observed. Reduction in disease severity was more pronounced after the application of metabolites with significant differences in efficacies between cells and metabolites only on the treated-lower leaves. Results showed that cells reduced disease severity by 40% reduction, which was significant on treated-lower leaves and not on untreated-upper ones. However, metabolites applications resulted in significant reduction on both lower and upper leaves by 57 and 68% reduction, respectively.

Results of evaluating the symptoms development of late blight disease on tomato leaves during pathogenesis illustrate the mean of disease severity from both upper and lower leaves (Fig. 3.16). The disease severity clearly increased by the time from 30% 2 days after inoculation to over 80% 6 days after inoculation on the untreated plants. The disease symptoms appeared in cell- and metabolite-treated plants slowly. Therefore, the results showed a high reduction in disease severity of late blight achieved by *B. subtilis* cells and metabolites in comparison to untreated plants.

Reduction in disease development was more pronounced after the application of metabolites, which reduced the disease severity by 70%, while application of bacterial cells reduced the severity by 44%.

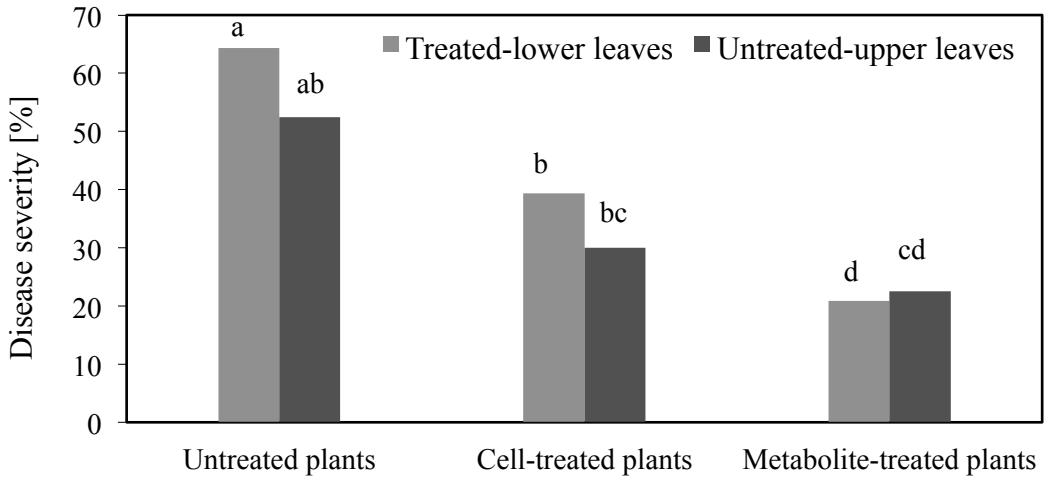


**Figure 3.15:** Apical translocation activity of *Bacillus subtilis* strain FZB24 through the plants against late blight disease 5 days post inoculation. Cells and metabolites were applied 24 hours on the lower leaves prior inoculation with *Phytophthora infestans* ($10^5$ sporangia $mL^{-1}$) on the upper and lower leaves. (Columns followed by the same letter do not differ statistically using Tukey`s HSD Test at $P \leq 0.05$; mean ± SE; n=4)

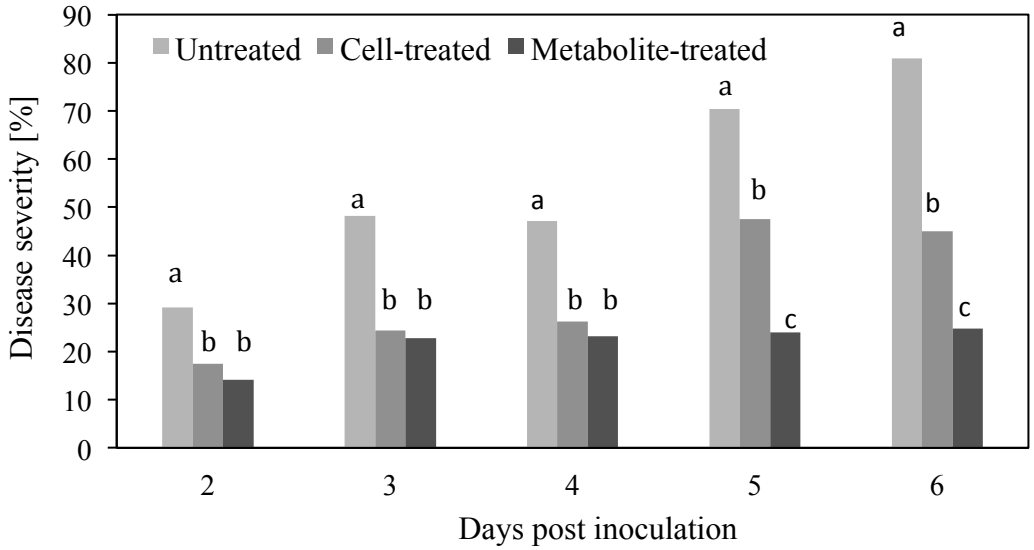


**Figure 3.16:** Influence of *Bacillus subtilis* strain FZB24 on symptom development of late blight disease on tomato leaves. Cells and metabolites were applied 24 hours prior inoculation with *Phytophthora infestans* ($10^5$ sporangia $mL^{-1}$). (Means followed by the same letters at each time point are not significantly different using Tukey‘s HSD Test at $P \leq 0.05$; n =4)

## 3.5 Influence of cells and metabolites of *Bacillus subtilis* strain FZB24 on expression profile of *PR1a* gene in tomato leaves

To gain a better understanding of the mode of action of *B. subtilis* strain FZB24 in suppression of late blight disease, the effects of cells and metabolites harvested 72 hours after culturing on differential expression of *PR1a* gene in both pathogen free leaf tissue and in *Phytophthora infestans* infected leaf tissue were investigated. The plants were divided into two groups, non-inoculated and *P. infestans*-inoculated plants. Cells and metabolites were applied on the lower leaf pairs 24 hours before pathogen inoculation on the upper and lower leaves for each plant. Sampling times are in corresponding with the pathogen development.

### 3.5.1 Expression level of *PR1a* in non-inoculated leaves

To measure the activation time of *PR1a* gene, lower and upper leaves detached from non-inoculated plants different hours after cells and metabolites application were used. There was an increase in the expression level of *PR1a* gene by the time in both untreated and treated plants (Tab. 3.6). This alteration in gene expression slightly and continuously increased in untreated and cell-treated leaves and then more clearly later on. No significant difference in gene activation was observed between untreated and cell-treated plants at all sampling dates. Activation in gene expression was detected starting from 30 hours after application of metabolites in the lower leaves and later by 6 hours in the upper leaves (36 hours post application).

### 3.5.2 Expression level of *PR1a* in *P. infestans*-inoculated leaves

For the lower leaves, alterations in expression level of *PR1a* gene were measured two hours after *P. infestans* inoculation, approx. 24 hours after cell- and metabolite-applications, at the recognition time between pathogen and the host (Fig. 3.17). The alterations in *P. infestans*-inoculated treated leaves were faster than in inoculated untreated ones. A remarkable decrease in the expression level of the gene was observed 6 hours post inoculation in comparison to 2 hours post inoculation. Afterwards, 12 hours post inoculation, there was a significant stimulation observed in metabolite-treated lower leaves in comparison to the untreated plants. Interestingly, highest level of

expression has been found 48 hours post inoculation, the time of transition phase from the biotrophic to the necrotrophic phase, in favour of untreated leaves.

In the upper leaves, the results showed activation in the expression levels of *PR1a* gene in upper leaves of *P. infestans*-inoculated plants with progress of infection (Fig. 3.18). No alterations in expression level of *PR1a* gene between treated and untreated plants were determined 6 hours post inoculation. Twelve hours post inoculation, gene expression level in upper leaves was in metabolite-treated plants significantly higher than in cell-treated plants and as well higher than in untreated plants. A noteworthy activation in the expression levels of *PR1a* gene has been found 48 hours post inoculation regarding the huge number of altered cells in consequence of further infections. This alteration in untreated plants was not significant but higher than in the treated samples.

**Table 3.6:** Time course study of relative expression of *PR1a* gene in upper and lower leaves of non-inoculated plants different hours after application of *Bacillus subtilis* strain FZB24. Both cells and metabolites were applied on the lower leaves and were detached from the plant different hours after application.

| Leaf type | Hours post application | Untreated | | Cell-treated | | Metabolite-treated | |
|---|---|---|---|---|---|---|---|
| Lower leaves | 6 | 109[2] | b | 138 | b | 107 | c |
| | 12 | 68 | b | 162 | b | 270 | c |
| | 24 | 557 | ab | 728 | ab | 1413 | bc |
| | 30 | 276 | b | 698 | b | 2631* | bc |
| | 36 | 2018 | a | 1237 | ab | 3271 | b |
| | 72 | 1971 | a | 5318 | a | 8637* | a |
| Upper leaves | 6 | 20.8 | b | 31.3 | b | 28.8 | b |
| | 12 | 24.6 | b | 25.4 | b | 69.3 | b |
| | 24 | 31.1 | b | 32.3 | b | 55.8 | b |
| | 30 | 61.8 | b | 45.5 | b | 39.1 | b |
| | 36 | 142.9 | b | 155.8 | b | 499.3* | b |
| | 72 | 741.1 | a | 1386 | a | 3404.4* | a |

Means followed by the same letters for each leaf type and separately for each treatment, while means followed by asterisks for each time date on the same line are not significantly different using Duncan's multiple range Test at $P \leq 0.05$; n=4.

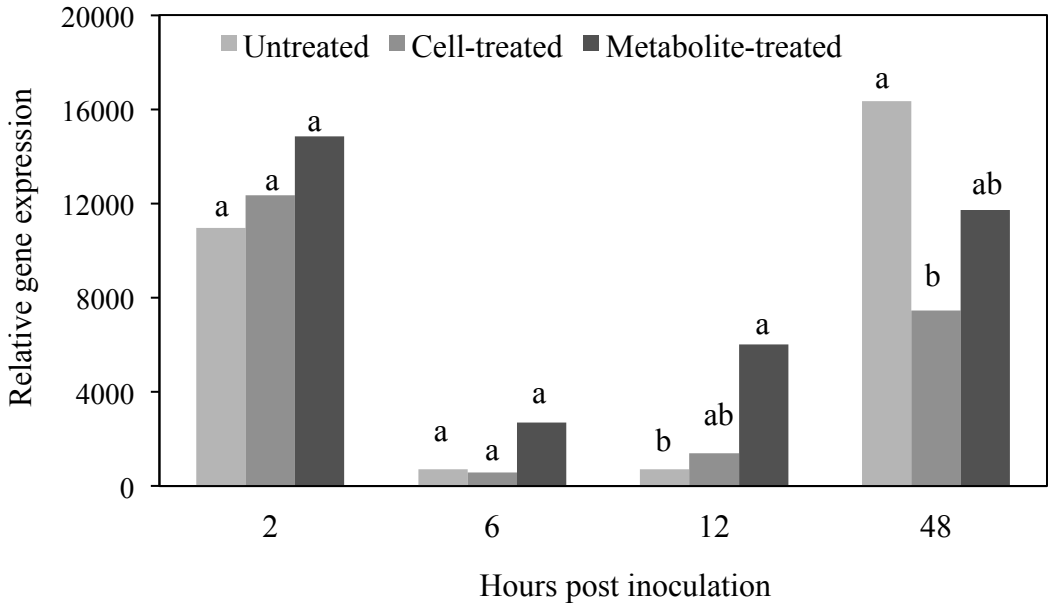


**Figure 3.17:** Relative expression of *PR1a* gene in the lower leaves inoculated with *Phytophthora infestans* after 24 hours of cells and metabolites applications from *Bacillus subtilis* strain FZB24. (Means followed by the same letters for each timing point are not significantly different using Tukey‘s HSD Test at $P \leq 0.05$; n=4)

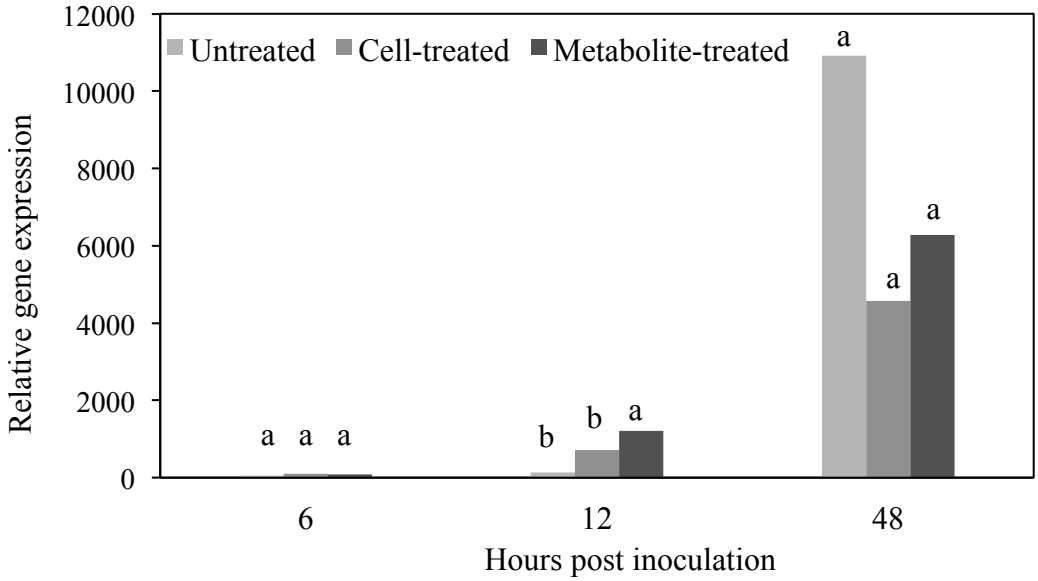


**Figure 3.18:** Relative expression of *PR1a* gene in the upper leaves inoculated with *Phytophthora infestans* after 24 hours of cells and metabolites applications from *Bacillus subtilis* strain FZB24 on the lower leaves of the same plant. (Means followed by the same letters for each timing point are not significantly different using Tukey‘s HSD Test at $P \leq 0.05$; n=4)

## 3.6 Effects of *Bacillus subtilis* strain FZB24 on gene expression of infected leaves with *Phytophthora infestans*

After confirming the effectiveness of *Bacillus subtilis* strain FZB24 in reducing the disease severity of late blight on tomato plants 6 days post inoculation (Fig. 3.16), freeze-dried upper leaves harvested from treated and untreated, non-inoculated and *P. infestans*-inoculated plants 12 hours post inoculation were used to isolate total RNAs, which were further analyzed by hybridizing to the Affymetrix Tomato Genome Array Gene Chip.

Following comparisons were made to investigate plant responses in absence and presence of *P. infestans* either in treated or untreated plants:

| Treatment | Comparison |
|---|---|
| untreated | Inoculated X Non-inoculated |
| Non-inoculated | Cell-treated X Untreated |
| | Metabolite-treated X Untreated |
| | Cell-treated X metabolite- treated |
| Inoculated | Cell-treated X Untreated |
| | Metabolite-treated X Untreated |
| | Cell-treated X metabolite- treated |

### 3.6.1 Host responses towards *P. infestans* infection

Array analysis showed that pathogen infection, 12 hours post inoculation, affected the expression level of a substantial number of genes compared to non-inoculated plants. From the total 682 differentially expressed genes, the expression levels of 429 genes were abundantly upregulated in inoculated leaves in which 75% exhibited 2 to 4 fold change increase and 25% of them exhibited 4.1 to 44.8 fold change increased compared to the non-inoculated plants. On the other hand, the expression level of 253 differentially expressed genes was reduced after infection. From those 96.2% exhibited 2 to 4 and 3.2% showed 4.1 to 10 fold change decreases in inoculated plants compared to the non-inoculated ones (Fig. 3.19).

To analyze the Gene Ontology (GO) annotation of the differentially expressed genes after pathogen infection, the gene ontology (GO) of biological process (Fig. 3.20) and molecular function (Fig. 3.21) was done to help investigating the nature and distribution of the molecular changes after pathogen infection. Different biological processes were found to be involved in the elevated differentially expressed genes (DEGs), which revealed more intensively in the upregulated genes. Analyses indicated that metabolic processes including lipid, carbohydrate, and amino acid; protein turnover process; cell related functions including cell death, cellular growth and development, response to stress and signaling; and the transcription processes were the most significant functions that were targeted to be modulated by infection. Remarkably, the analysis showed down regulation for most genes involved in photosynthesis function (LOC543976, LOC543974, AT3G60370, AT1G77090, LHCA3, LHCA4, LHCB3, LHCB6, PSAO, PSBW, POR2, NDF2, NDF4, NDF6, EGY1), besides to suppression in expression levels of genes associated in regulation, cell wall process, and transcription factors.

#### 3.6.1.1 Functional classification and pathway analysis

Most pathways and metabolism process participated by genes differentially changed after infection are listed in Appendices 1 and 2.

***Genes involved in signaling***

In signaling pathways, 5.4% of DEGs (37 out of 682) were differentially expressed (Fig. 3.22). Noteworthy to mention that all 20 genes involved in calcium signaling were upregulated after pathogen infection, some genes with high expression level such as calmodulin-binding protein (EDA39) and $Ca^{2+}$-mediated signal transduction**.** In addition, several genes involved in MAP kinases signaling, such as mitogen-activated protein kinase 3 (MPK3) were activated.

***Genes involved in hormone responses***

In response to hormone stimulus, 7.9% of DEGs (54 out of 682) were observed (Fig. 3.22). From those 15 genes associated in ethylene signaling pathway, for example, Pti5 and ethylene response factor ERF4 showed high abundance. Besides to 3 genes such as divinyl ether synthase (LeDES), allene oxide synthase (aos), and lipoxygenase (loxD), which are involved in jasmonate synthesis, are activated. Other genes, which are associated with pathogenesis-related proteins, such as EIX receptor 1 (EIX1) were also

changed after infection. In addition, we found auxin signaling related proteins, of which auxin-regulated dual specificity cytosolic kinase (LOC543684) was activated.

***Defense and stress responsive genes***

As stress responses 132 out of 682 genes were classified, which account to 19.4% of total DEGs. From those, 26 genes were engaged in oxidative stress most of them strongly upregulated including peroxidases (cevi16), NADPH-quinone oxidoreductase (NQR), and alpha-DOX1 (LOC543895). In addition, 8.4% of DEGs (57 out of 682), which implicated in defense responses, were changed after infection. From those are chitinase (LOC544149), TSI-1 protein (TSI-1), PR-proteins. The current study showed a large number of proteases during this early stage with high induction in expression level of different genes involved in defense, such as subtilisin-like protease (Sbt4a), which showed the highest level of upregulation, and also the *Phytophthora*-inhibited-protease 1 (PIP1). The analysis showed presence of wide range of genes involved in the ubiquitin-mediated protein degradation and modification pathways, such as ubiquitin-protein ligases (Tab. S2).

***Genes involved in metabolism***

In formation of secondary metabolites 10.8% of DEGs (45 out of 682) are involved (Fig. 3.23), 36 were upregulated and 9 were down regulated. Analyses showed upregulation of many genes involved in phenylpropanoid pathway, for instance, 4-Coumarate: CoA ligase 1 (4CL1) and in chorismate synthesis, such as shikimate kinase precursor (LOC544078). The induction in many genes involved in amino acid synthesis and carbohydrates, such as acid invertase (Wiv-1) and sucrose synthase (sus3), was observed as well (Fig. 3.23 and Fig. 3.24).

***Genes involved in cell wall***

Most genes (29 out of 33) involved in cell wall modification and degradation process such as expansin (LeXEP2), and xyloglucan endotransglycosylase hydrolase (XTH6), were suppressed after infection, while, polygalacturonase inhibiting protein1 (PGIP1) (Fig. 3.22).

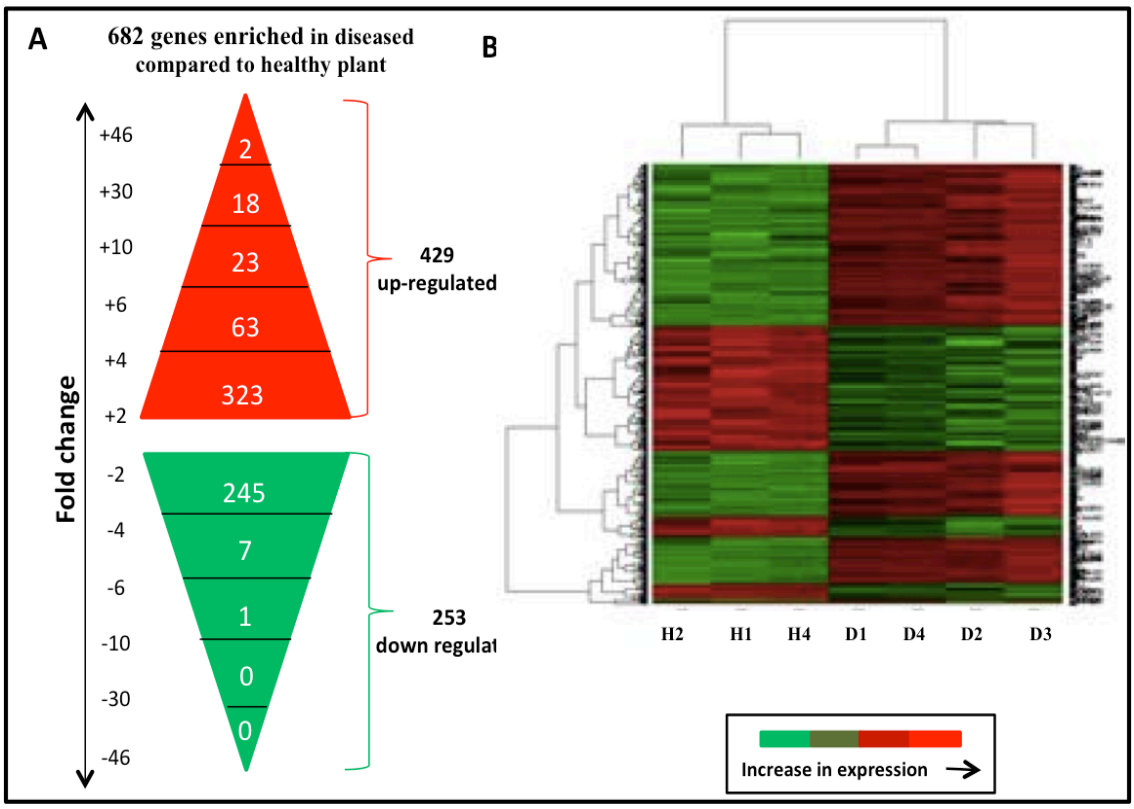


**Figure 3.19:** Differentially expressed genes in tomato leaves inoculated with *Phytophthora infestans* at 12 hours post inoculation compared to non-inoculated plants. (A): The number and fold change distribution of genes changed after inoculation. (B): The heatmap and hierarchical clustering showing the expression pattern of differentially expressed genes in inoculated samples. Labels in the horizontal axis of the heatmap, H1, H2 and H4 represent independent biological replicates in non-inoculated plants and D1, D2, D3 and D4 represent independent biological replicates from infected plants. The horizontal dendrogram indicate the expression pattern of transcripts in non-inoculated and *P. infestans*-inoculated plants. The vertical dendrogram elucidates the similarities or differences of transcript expression within the biological replicates and between the tow groups. Red and green colours in (B) indicate the high and low expression level, respectively.

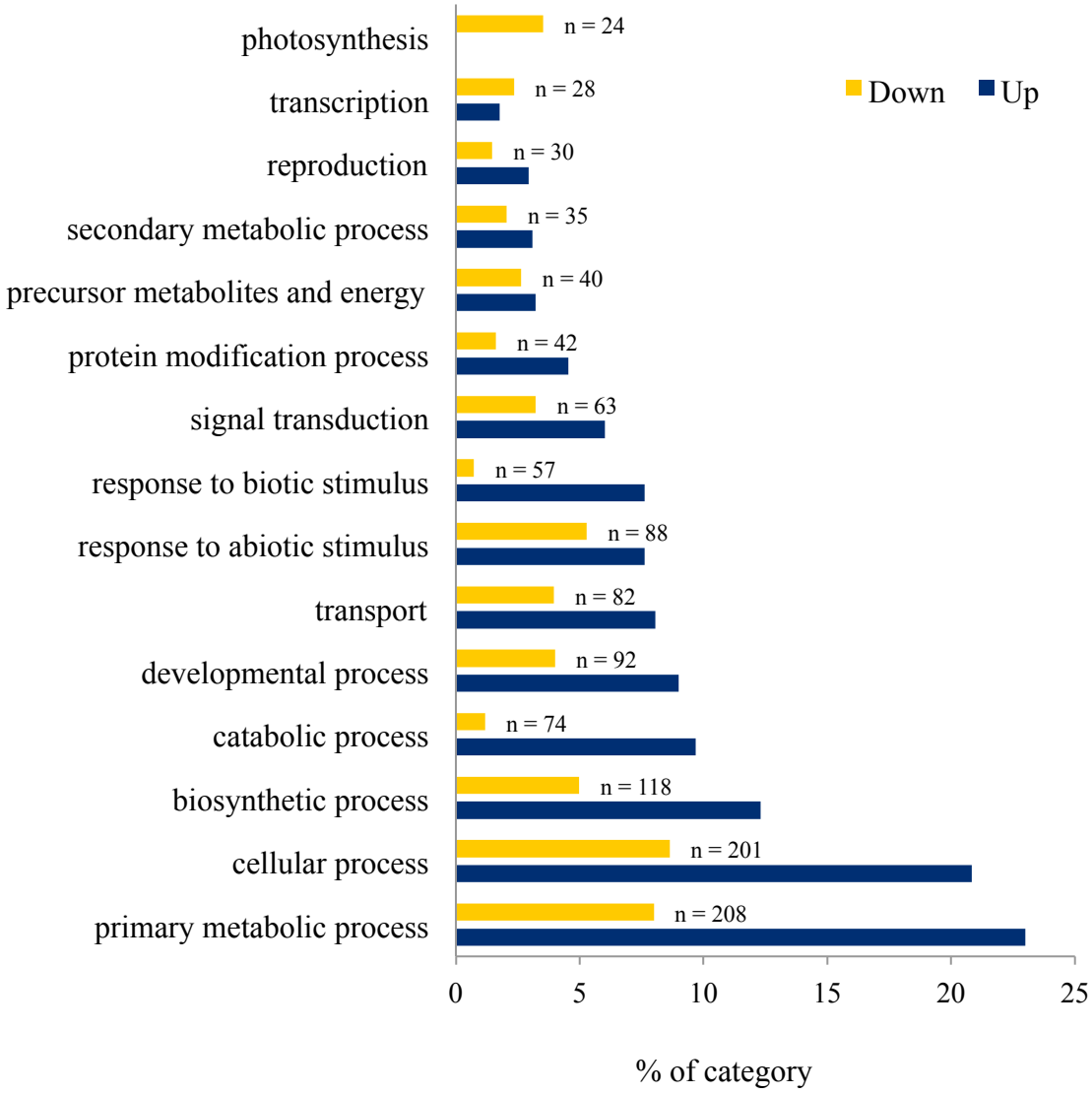


**Figure 3.20:** Distribution of differentially expressed genes, categorized based on their gene ontology annotation, altered in tomato leaves infected by *Phytophthora infestans* 12 hours post inoculation. The vertical axis indicates biological processes and the horizontal axis indicates the percentage of genes in relation to 682 genes changed in *P. infestans*-inoculated plants in comparison to non-inoculated plants. N: number of both up and down regulated genes in each category. (152 of 682 genes are unclassified).

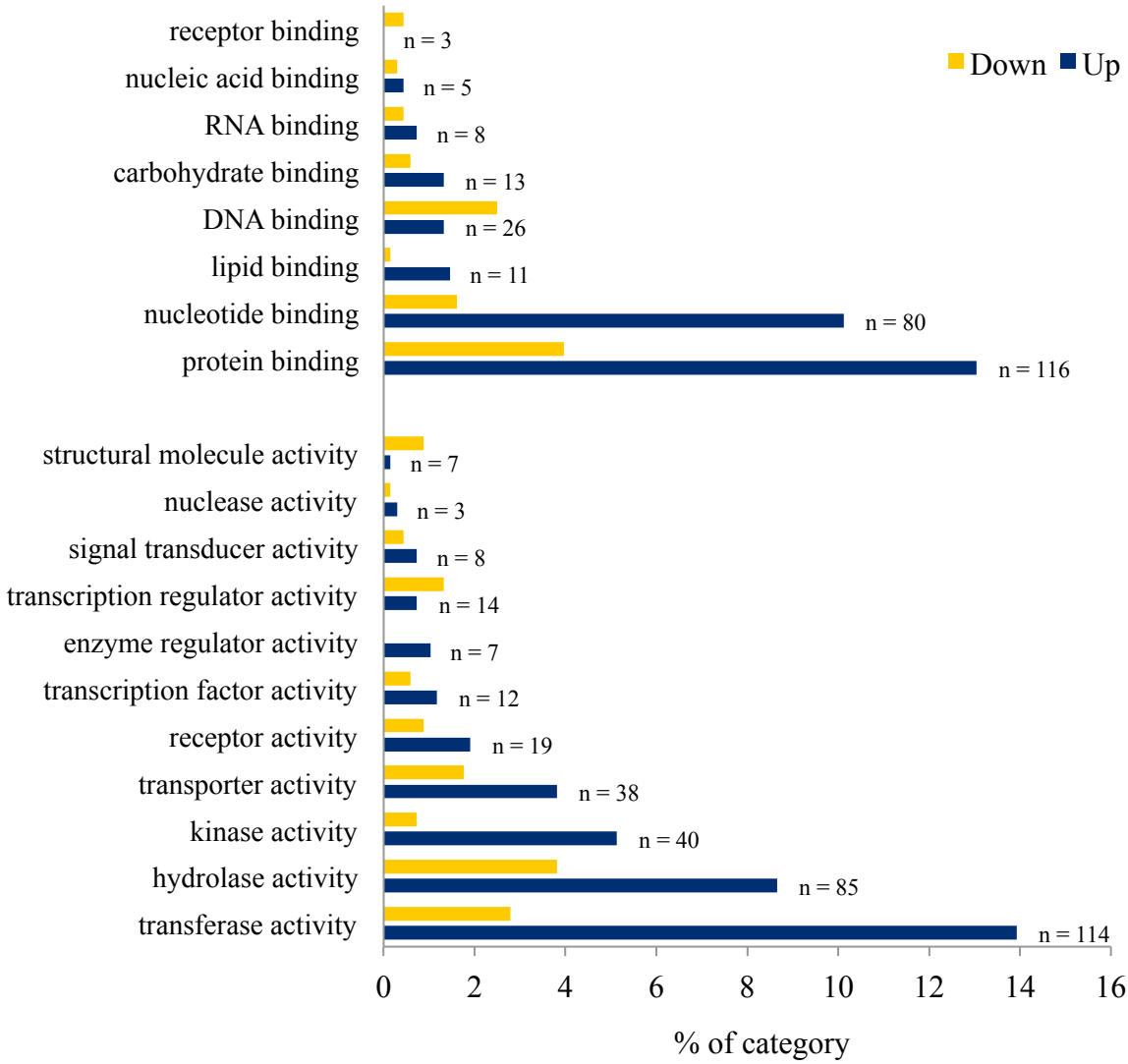


**Figure 3.21:** Distribution of differentially expressed genes, categorized based on their gene ontology annotation, altered in tomato leaves infected by *Phytophthora infestans* 12 hours post inoculation. The vertical axis indicates molecular functions and the horizontal axis indicate the percentage of genes in relation to 682 genes changed in *P. infestans*-inoculated plants in comparison to the non-inoculated ones. N: number of both up and down regulated genes in each category. (152 of 682 genes are unclassified).

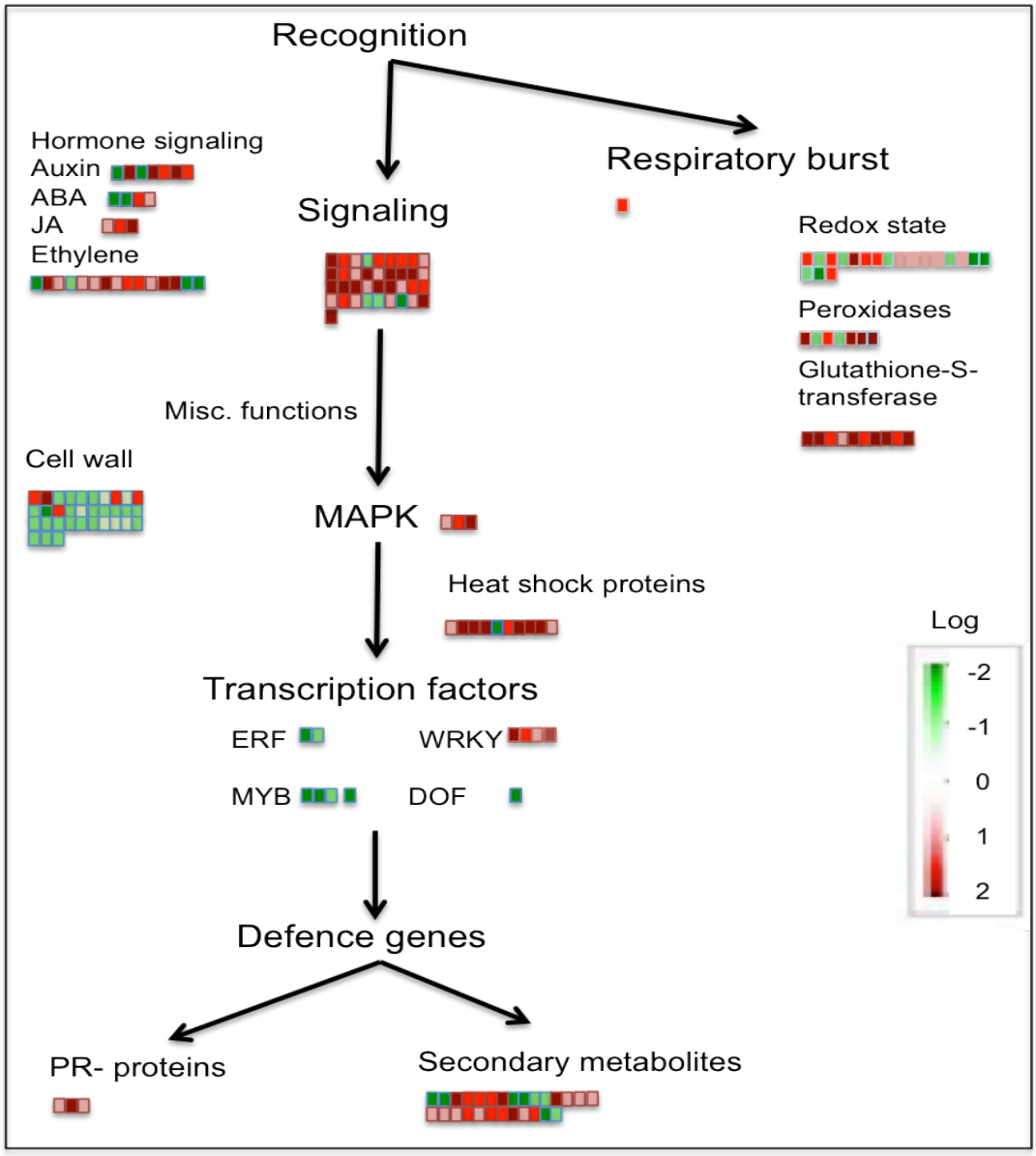


**Figure 3.22:** Functional classification and pathway analyses of differentially expressed genes in tomato leaves infected by *Phytophthora infestans* 12 hours post inoculation. Molecular analyses have done by Mapman (P value < 0.05). Squares are representing upregulated genes (red) and down regulated genes (green) based on their log value.

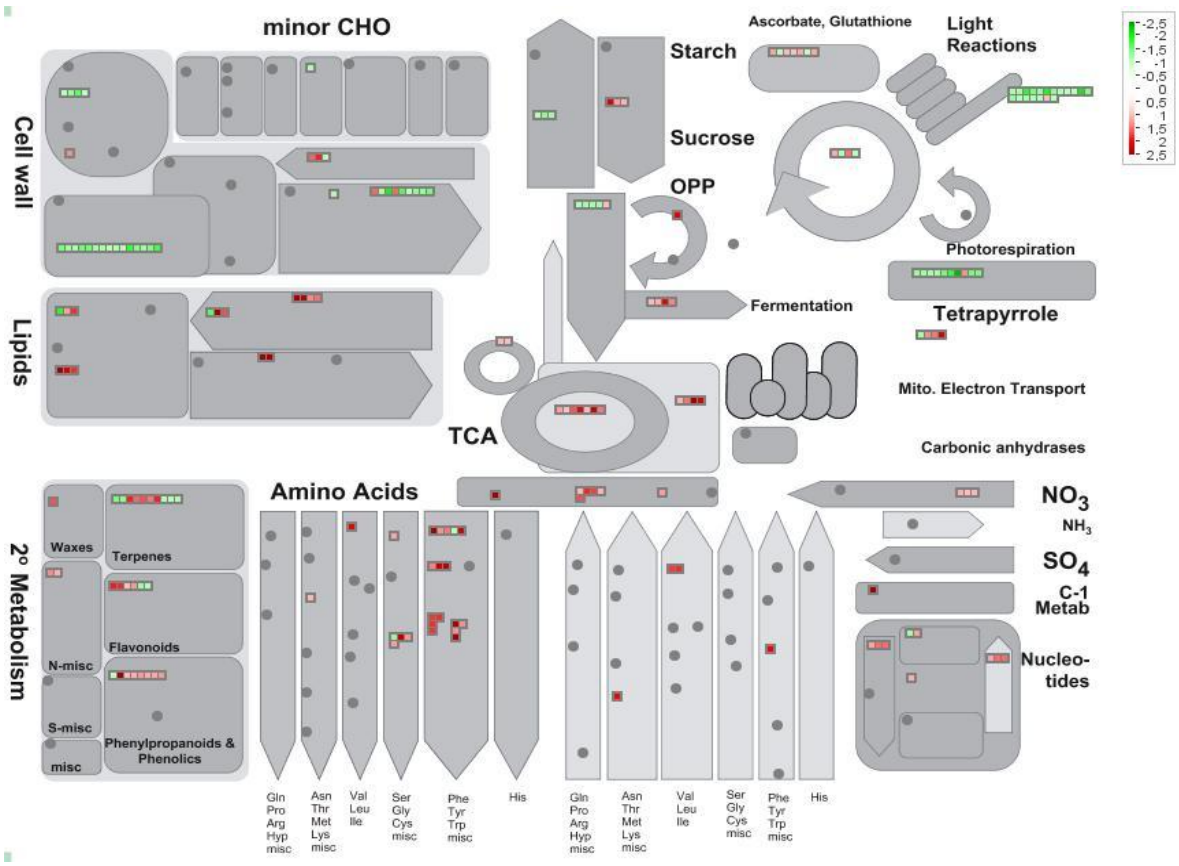


**Figure 3.23:** Overview of functional classification of metabolism changes involved in differentially expressed genes in tomato leaves infected by *Phytophthora infestans* 12 hours post inoculation. Molecular analyses have done by Mapman ($P < 0.05$). Squares are representing upregulated genes (red) and down regulated genes (green) based on their log value.

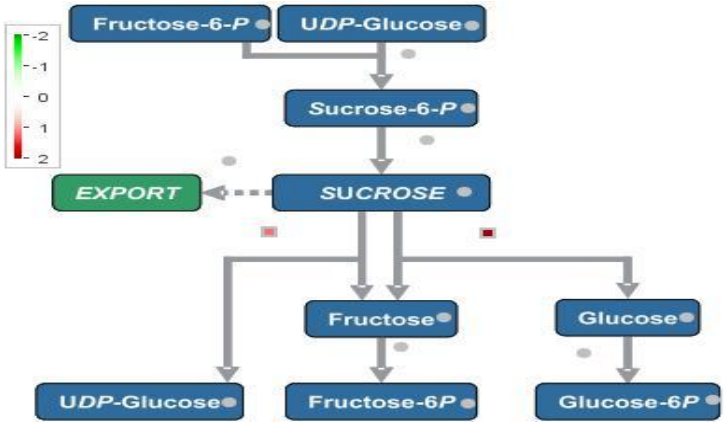


**Figure 3.24:** Differentially expressed genes involved in carbohydrate pathway in tomato host after 12 hours of *Phytophthora infestans* inoculation. Each gene is represented in one red square indicates upregulation in gene expression in *P. infestans*-inoculated plants in comparison to non-inoculated plants. Molecular analyses have done by Mapman ($P < 0.05$).

### 3.6.2 Effects of *B. subtilis* on host responses

#### 3.6.2.1 Response in non-inoculated plants

Spraying of *B. subtilis* strain FZB24 cells and metabolites on the lower leaves of non-inoculated plants resulted in 33 and 8 genes, respectively, differentially expressed in the upper leaves compared to the upper leaves in non-inoculated untreated plants.

In cell-treated plants, expression levels of 33 genes, exhibited 2.3 to 12.5 fold change were changed. Five genes were activated, for example, late elongated hypocotyl (LHY) is the only gene functionally annotated to tomato, which is involved in transcription. However, 28 genes were down regulated and from those that functionally annotated to tomato or highly identical to *Arabidopsis* are alternaria stem canker resistance protein (Asc), chlorophyll A-B binding-early light-inducible protein (ELIP1), lipoxygenase (loxD), and UDP-apiose/xylose synthase (AXS2), which are involved in metabolism; and xyloglycan endo-transglycosylase (tXET-B2), late embryogenesis (Lea)-like protein (LOC544157), and ethylene-responsive late embryogenesis-like protein (ER5), which are involved in response to abiotic stimuli. Two other genes namely regulator of gene silencing (LOC543942) are involved in response to external stimuli as a plant-pathogen interaction (organism-specific biosystem).

However other 7 genes were differentially expressed after metabolites application, exhibited approx. 2 fold changes, while not changed after cells application compared to non-inoculated untreated plants. Three genes were down regulated; one of them is ATP synthase protein I (AT2G31040), which is involved in metabolic process and transport, while the other two are weakly or moderately identical to *Arabidopsis*. Meanwhile, the analysis revealed 5 DEGs activated after metabolites application and they are RNA polymerase sigma factor (SIGE), protein late elongated hypocotyl (LHY), and Constans 1 (CO1) are involved in transcription and regulation of transcription; Elongation factor 1-alpha (LOC544055), which is involved in RNA transport pathway; and ligase family protein (AT3G48990), which is involved in metabolic process.

Interestingly LHY is the only gene, which was activated in both cells and metabolites treatments when compared to the non-inoculated untreated plants. While all other down regulated genes after cells application were not changed after metabolites application in comparison to untreated plants.

### 3.6.2.2 Response in *P. infestans*-inoculated plants

#### 3.6.2.2.1 Gene expression after cells application

The analysis showed that between 682 DEGs in *P. infestans*-inoculated untreated plants compared to non-inoculated untreated plants and 656 DEGs in inoculated cell-treated plants compared to non-inoculated untreated plants there are 489 common genes differentially expressed in both, almost more than 86% of genes are changed regardless cells application (Fig. 3.25A). However, a minor number (24) of DEGs are identified in comparison done between inoculated cell-treated plants and inoculated untreated plants. These 24 DEGs exhibited 2 to 5.6 fold change and might indicate the bacterial cell function. One gene LeEXP2, involved in development and endogenous abiotic stimuli was activated. However, all other 23 down regulated genes, for example LeCBF1, LoxD, ER5, THM16, and LOC543942, are mostly involved in stress responses and transcription (Fig. 3.25B).

#### 3.6.2.2.2 Gene expression after metabolites application

The results revealed 299 common genes differentially expressed between 345 DEGs in metabolite-treated plants and 682 DEGs in infected untreated plants when compared to non-inoculated untreated plants (Fig. 3.26). These 299 DEGs are mostly involved in infection because they changed after infection regardless application of metabolites. However, further analysis done to find out the exclusive genes after infection between untreated and treated plants declared that 79 DEGs, which exhibited 2 to 4 fold change, were identified in metabolite-treated plants in comparison to untreated plants indicating their potential importance for suppression of infection. 15 DEGs activated after metabolites application are mostly involved in hormone metabolism, photosynthesis, and cell wall modification (Tab. 3.7). However, among the other 64 down regulated genes, 30.4% are genes involved in stress responses, 13.9% in metabolic process, 12.7% in catabolic process, and 7.6% in biosynthetic process. 18% correspond to genes involved in transcription, 11.4% in signal transduction, 3.8% in secondary metabolic process were most of them classified as involved in defense and 7.6% are correspond to development process (cell growth, cell death) (Fig. 3.27). Data in Figure 30B illustrate

the molecular functions involved in both up and down regulated genes in metabolite-treated plants reflecting high number of genes involved in binding and transcription.

Noteworthy to mention that in these 79 genes differentially expressed after metabolites application (Fig. 3.28), there are 22 genes common with genes differentially expressed after cells application in inoculated plants compared to inoculated untreated plants and 20 genes commons with genes differentially expressed after cells application in non-inoculated plants compared to non-inoculated untreated. In addition, analysis resulted in 14 common genes differentially expressed after cells applications between both non-inoculated plants compared to non-inoculated untreated plants (33 DEGs) and in inoculated plants compared to inoculated untreated (24 DEGs). From those 14 common down regulated genes are regulator of gene silencing (LOC543942), Calmodulin-related protein, putative (AT1G76640), auxin-regulated protein (LOC543701), zinc finger AN1 domain-containing stress-associated protein 12 (PMZ), lipoxygenase (loxD), late embryogenesis (Lea)-like protein (LOC544157), and ethylene-responsive late embryogenesis-like protein (ER5). Besides to 6 other common genes, which are weakly similar to *Arabidopsis.*

However, still some genes down regulated only after metabolites application are involved in transcription, protein modification process, transport and amino acid metabolism from those: arogenate dehydratase 6 (ADT6), E3 ubiquitin-protein ligase (PUB23), xyloglucan-specific fungal endoglucanase inhibitor protein precursor (ACI25), ethylene-responsive transcription factor (ERF017), plant U-box 29 (PUB29), phospholipaseA 2A (PLA2A), Pti5 (LOC544042), xyloglucan endotransglucosylase-hydrolase XTH3 (XTH3), embryo sac development arrest 39 (EDA39), auxin-regulated dual specificity cytosolic kinase (LOC543684).

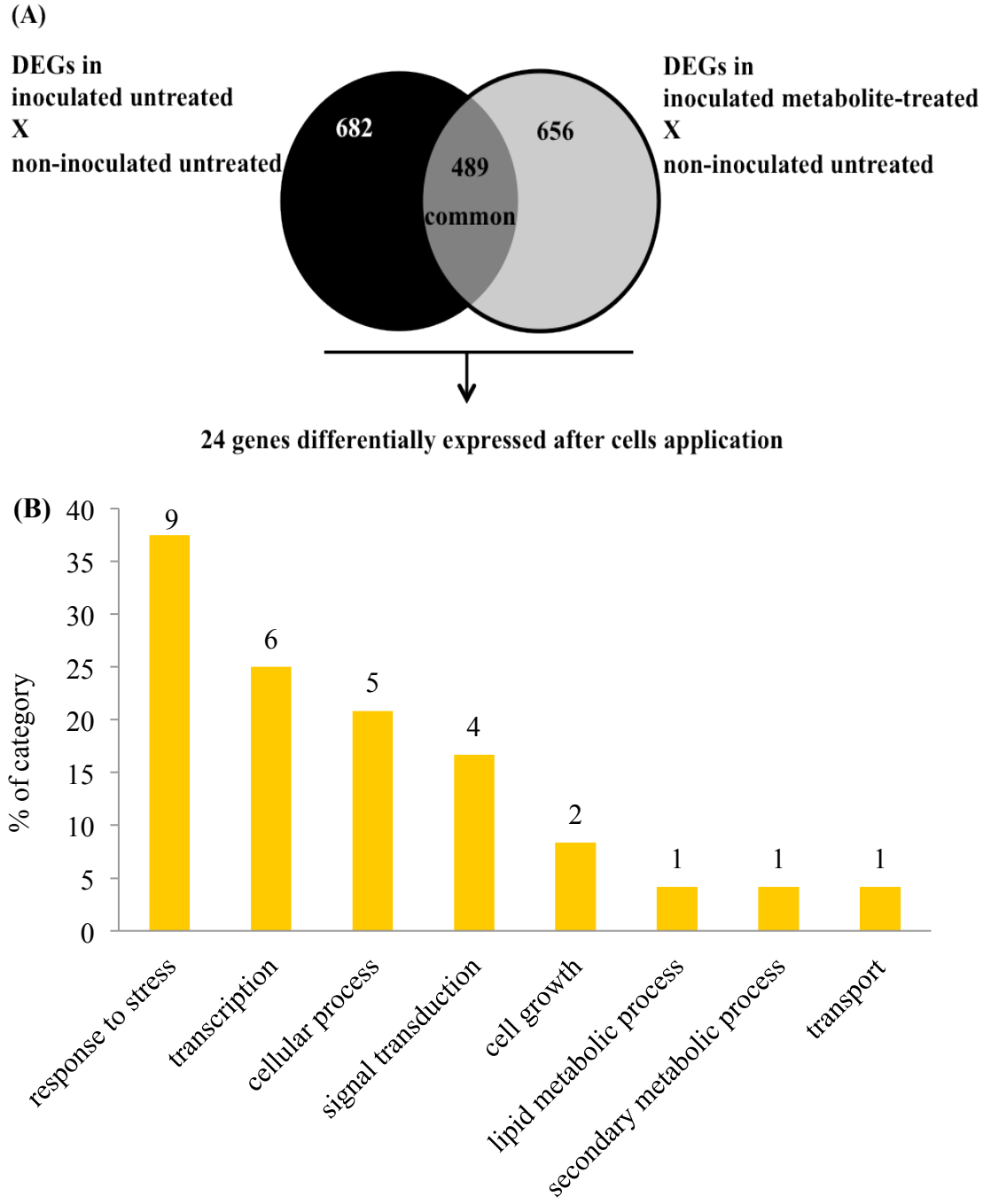


**Figure 3.25:** (A) Venn diagram displaying the number of differentially expressed genes in cell-treated and untreated plants 12 hours after inoculation with *Phytophthora infestans* compared to non-inoculated untreated plants. (B) Biological process of 23 down regulated genes in *P. infestans*-inoculated cell-treated plants compared to *P. infestans*-inoculated untreated plants 12 hours after *Phytophthora infestans* inoculation. (3 genes are unclassified)

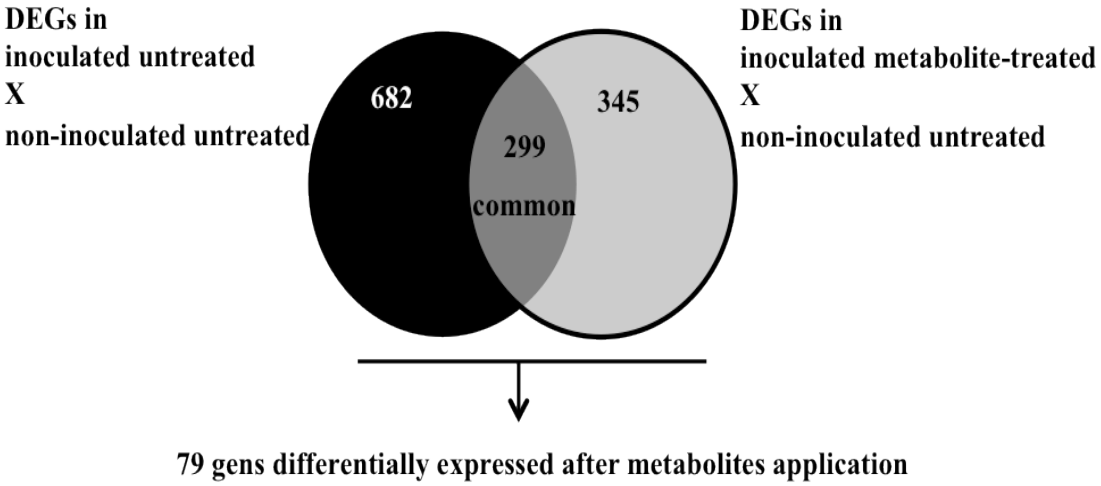


**Figure 3.26:** Venn diagram displaying the number of differentially expressed genes in metabolite-treated and untreated plants 12 hours after inoculation with *Phytophthora infestans* compared to non-inoculated untreated plants.

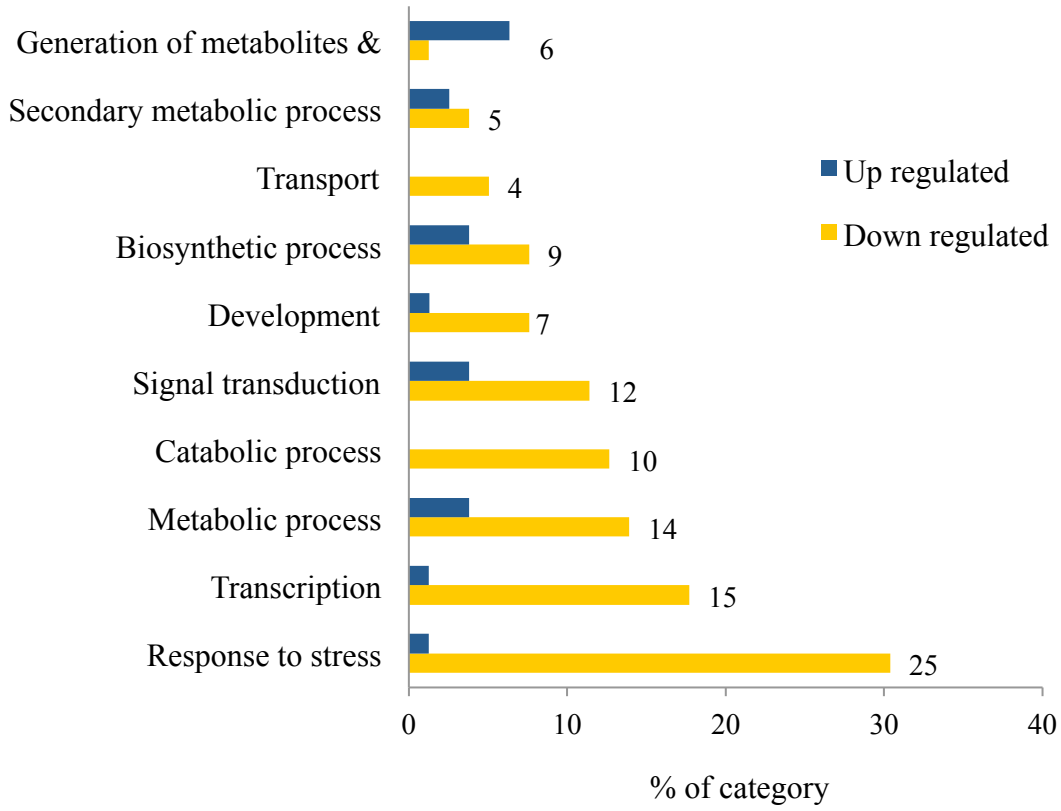


**Figure 3.27:** Function classification of differentially expressed genes in metabolite-treated plants compared to untreated plants 12 hours after *Phytophthora infestans* inoculation. (16 genes are unclassified)

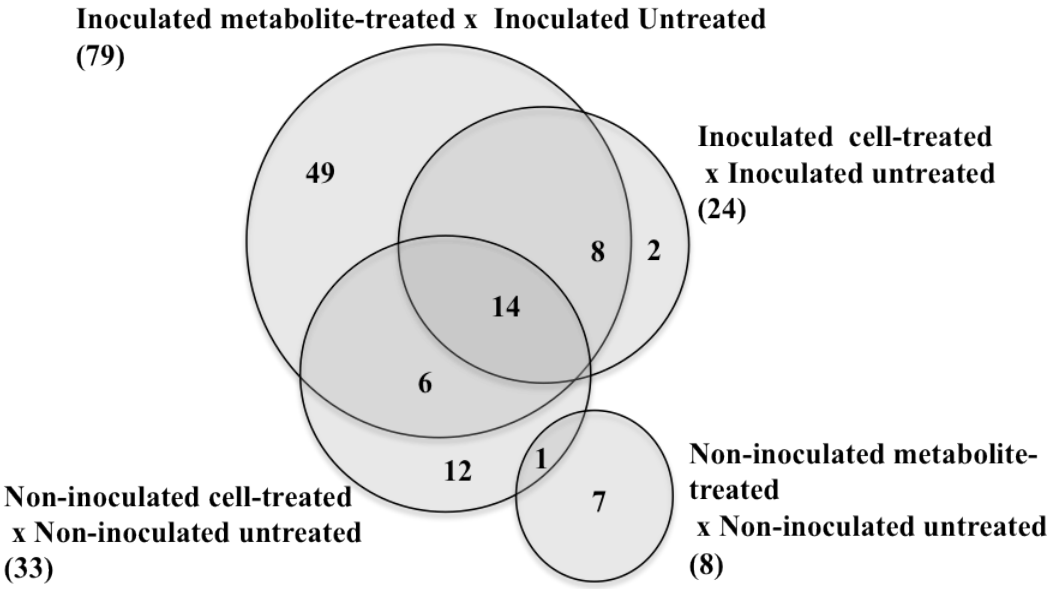


**Figure 3.28:** Venn diagram displaying the number of genes differentially expressed after cells or metabolites applications in both non-inoculated and *Phytophthora infestans*-inoculated plants compared to untreated plants.

**Table 3.7:** Differentially expressed genes enriched in *Phytophthora infestans*-inoculated metabolite-treated plants compared to untreated plants 12 hours post inoculation.

| Affy.ProbeSet | Gene title | Gene symbol | Biological process | Molecular function | Foldchang | ID%* |
|---|---|---|---|---|---|---|
| Les.5146.1.S1_at | Auxin-responsive family protein | AT1G75590 | response to auxin stimulus | auxin mediated signaling pathway | 2,3 | w/56.4 |
| LesAffx.71035.1.S1_at | Auxin-responsive protein, putative | AT5G18030 | response to auxin stimulus | auxin mediated signaling pathway | 2,4 | m/61.0 |
| Les.64.1.S1_at | Gibberellin 20-oxidase-1 | 20ox-1 | gibberellin 20-oxidase activity | iron ion binding, oxidoreductase activity | 2,5 | i |
| LesAffx.29797.1.S1_at | Flavonol sulfotransferase-like protein | SOT12 | brassinosteroid metabolic process, defense response, response to salysilic acid stimuli | sulfotransferase activity, brassinosteroid sulfotransferase activity | 2,6 | w/53.6 |
| Les.1850.1.A1_at | Fatty acid desaturase 5 | FAD5 | photoinhibition, unsaturated fatty acid biosynthetic process | monogalactosyldiacylglycerol desaturase activity, oxidoreductase activity | 2,0 | m/81.6 |
| Les.147.1.S1_at | Chlorophyll a/b-binding protein precursor | LOC543976 | photosynthesis, light harvesting, protein-chromophore linkage | magnesium ion binding, chlorophyll binding, metal ion binding | 2,3 | i |
| Les.3297.1.S1_at | Chlorophyll a-b binding protein4 | LHCA4 | photosynthesis | chlorophyll binding | 2,0 | h/90.0 |
| Les.5850.1.S1_at | Protochlorophyllide oxidoreductase B | PORB | chlorophyll biosynthetic process, response to ethylene stimulus | oxidoreductase activity, protochlorophyllide reductase activity | 2,3 | h/88.5 |
| Les.3016.1.S1_at | Light-harvest chlorophyll B-binding protein 3 | LHCB3 | photosynthesis, light harvesting | structural molecule activity | 2,2 | h/95.5 |
| Les.1603.1.A1_at | Light-harvesting complex I chlorophyll a/b binding protein3 | LHCA3 | photosynthesis, light harvesting | chlorophyll binding | 2,0 | h/90.8 |
| LesAffx.62334.1.S1_at | Paclobutrazol resistance 1 | PRE1 | regulation of transcription, DNA-dependent | DNA binding, transcription factor | 2,0 | h/90.0 |
| Les.3733.1.S1_at | Expansin | LeEXP2 | plant-type cell wall organization | structural constituent of cell wall | 3,0 | i |
| Les.4304.1.S1_at | Expansin12 | Exp12 | plant-type cell wall organization | structural constituent of cell wall | 2,0 | i |

*Similarity or identity of functional classification to *Arabidopsis*: (w) weakly similar; (m) moderately similar; (h) highly similar; (i) identical from tomato sequences dataset.

### 3.6.3 Validation of microarray data using quantitative RT-PCR

A total of 14 genes differentially expressed after infection compared to non-inoculated plants were selected. Hence, the RT-PCR revealed that all 14 genes followed a similar trend to microarray results, despite one gene, namely expansin (LeEXP2) showed no significant difference in RT-PCR analysis when compared to non-inoculated plants (Tab. 3.8), indicating that both results are fitting to each other.

In other comparisons between metabolite-/cell-treated, either from inoculated or non-inoculated plants, and the non-inoculated untreated plants, we found that all selected genes for validation exhibited the same trend like microarray results (Tab. 3.9), but some genes, namely, LOC543684, XTH3, and ER5 showed no significant difference.

**Table 3.8:** Validation of 14 differentially expressed genes in infected tomato leaves by *Phytophthora infestans* 12 hours post inoculation compared to non-inoculated plants using quantitative real-time PCR.

| Gene title | Gene symbol | Microarray results | | RT-PCR results | |
|---|---|---|---|---|---|
| | | FC | P value | FC | P value |
| Subtilisin-like protease | sbt4a | 44.8 | 0.038 | 119.5 | < 0.001 |
| Alpha-DOX1 | LOC543895 | 26.6 | 0.021 | 17 | < 0.001 |
| Hexose transporter protein | LOC543728 | 16.7 | 0.014 | 18.2 | < 0.001 |
| Pti5 | LOC544042 | 16.7 | 0.013 | 9.8 | < 0.001 |
| Embryo sac development arrest 39 | EDA93 | 9 | 0.004 | 6.1 | < 0.001 |
| Pathogenesis-related protein P2 | PR-P2 | 7 | 0.003 | 7.7 | < 0.001 |
| Chitinase | LOC544146 | 6.1 | 0.002 | 7.1 | < 0.001 |
| Auxin-regulated dual specificity cytosolic kinase | LOC543684 | 5.5 | 0.001 | 2.5 | 0.015 |
| TSI-1 protein | TSI-1 | 4.5 | < 0.001 | 12 | < 0.001 |
| Peroxidase | cevi16 | 4.2 | < 0.001 | 2.8 | < 0.001 |
| Lipoxygenase | loxD | 2.5 | < 0.001 | 1.9 | 0.048 |
| Expansin | EXPA5 | -2.3 | < 0.001 | -1.5 | 0.037 |
| Hypothetical protein | LOC543672 | -2.5 | < 0.001 | -2.5 | 0.038 |
| Expansin | LeEXP2 | -3.3 | < 0.001 | -4 | 0.057 |

*P* value ≤ 0.05 considered as significant, positive and negative values indicate genes changed after infection.

**Table 3.9:** Validation of microarray results between treated inoculated / non-inoculated and non-inoculated untreated plants 12 hours post inoculation with *Phytophthora infestans* using quantitative real-time PCR

| Comparisons | Gene title | Gene symbol | Microarray results | | RT-PCR results | |
|---|---|---|---|---|---|---|
| | | | FC | P value | FC | P value |
| Inoculated metabolite -treated x non-inoculated untreated | Subtilisin-like protease | sbt4a | 29.6 | < 0.001 | 139.3 | < 0.001 |
| | Alpha-DOX1 | LOC543895 | 14.6 | < 0.001 | 16.1 | < 0.001 |
| | Hexose transporter protein | LOC543728 | 12.8 | < 0.001 | 17.3 | < 0.001 |
| | Pti5 | LOC544042 | 7.7 | < 0.001 | 5.8 | < 0.001 |
| | Pathogenesis-related protein P2 | PR-P2 | 6.2 | < 0.001 | 6.6 | < 0.002 |
| | Chitinase | LOC544146 | 4.3 | < 0.001 | 6.4 | < 0.002 |
| | TSI-1 protein | TSI-1 | 4.3 | < 0.001 | 13.5 | < 0.001 |
| | Embryo sac developmen arrest 39 | EDA39 | 4.2 | < 0.001 | 3.5 | < 0.001 |
| | Peroxidase | cevi16 | 2.6 | < 0.001 | 3.6 | < 0.002 |
| | Auxin-regulated dual specificity cytosolic kinase | LOC543684 | 2.2 | 0.002 | 1.1 | 0.674 |
| | Xyloglucan endotrans glucosylase-hydrolase | XTH3 | -2.2 | 0.004 | -1.1 | 0.745 |
| | Ethylene-responsive late embryogenesis-like protein | ER5 | -4 | < 0.001 | -2.4 | 0.054 |
| | Regulator of gene silencing | LOC543942 | -6.4 | < 0.001 | -5.0 | < 0.002 |
| Inoculated (cell-treated x non-untreated) | Alpha-DOX1 | LOC543895 | 25.2 | < 0.001 | 14.8 | < 0.001 |
| | TSI-1 protein | TSI-1 | 2.2 | < 0.001 | 11.0 | < 0.001 |
| | Pathogenesis-related protein P2 | PR-P2 | 5.9 | < 0.001 | 9.5 | < 0.001 |
| | Chitinase | LOC544146 | 4.7 | < 0.001 | 6.3 | < 0.001 |
| | Peroxidase | cevi16 | 4.6 | < 0.001 | 3.3 | 0.001 |
| | Auxin-regulated dual specificity cytosolic kinase | LOC543684 | 3 | < 0.001 | 1.5 | 0.324 |
| | Ethylene-responsive late embryogenesis-like protein | ER5 | -4.2 | < 0.001 | -0.4 | 0.028 |
| | Xyloglucan endotrans glucosylase-hydrolase | XTH3 | -2.3 | < 0.001 | -0.7 | 0.454 |
| Non-inoculated (metabolite-treated x untreated) | EF 1-alpha | LOC544055 | 2.2 | < 0.005 | 3.6 | < 0.001 |
| Non-inoculated treated (metabolites x cells) | Lipoxygenase | loxD | 2.8 | < 0.001 | 2.1 | 0.002 |
| | Regulator of gene silencing | LOC543942 | 2.5 | < 0.001 | 2.5 | 0.003 |
| | WRKY33 | WRKY33 | 2.3 | < 0.002 | 2.7 | 0.004 |

*P* value ≤ 0.05 considered as significant, positive and negative values indicate genes changed after infection.

# 4 DISCUSSION

Most studies have focused primarily on the degree of disease reduction using *Bacillus subtilis* in biological control. Hence, for deeper understanding the mechanisms of disease suppression, which have not been as investigated extensively, the current study was carried out using different analyses at microbial, histological, and molecular levels. The bacteria and their excreted metabolites exhibited biocontrol activity against several pathogens causing important foliar tomato diseases. Their direct activity and involvement in enhanced plant defense are attractive properties should be explored further and in more detail.

Both biological control agents FZB24® and Phytovit® were effective in suppression of diseases caused by different pathogens demonstrating the broad spectrum of the biocontrol agents. The presented data was in agreement with results of other workers who investigated the potential of microorganisms for control of the pathogens (Saikia and Azad 1999; El-Sheikh *et al.,* 2002; Daayf *et al.,* 2003). In order to take different modes of action, e.g. curative or protective effects, into account, the antagonists were applied before or after the pathogens inoculation. Generally, better effects were obtained when applications were made before inoculation. This observation corresponds to results found by El-Sheikh *et al.* (2002), who stated that protective treatments with antagonistic *Bacillus* spp. were more effective than curative treatments to control *Phytophthora infestans* on potato.

*B. subtilis* strains FZB24 and Phytovit showed high antagonistic effects on mycelia growth and spore germination. The effect of both strains on the growth of pathogens proved to be highest with *P. infestans* followed by *Cladosporium fulvum* and *Alternaria solani* in descending order. In addition, they showed inhibitory effects against pathogens development structures at the early stages of infection reducing the pathogen ability to infect the plant tissues.

Since the highest destructive effect of bacteria revealed against *P. infestans* growth and late blight development, further investigations were curried out against this pathogen to find out the mechanism involved in plant protection. Metabolites produced by *B. subtilis* in synthetic growth medium exhibited strong suppression in disease severity of

late blight more than the bacteria itself. This showed its important role as effective substances in disease suppression. The metabolites were harvested after 1, 24 and 72 hours of incubation revealing the ability of *B. subtilis* to start secreting the effective substances from the first hours of culturing with increase by the time. In addition, the metabolites showed stability to suppress the disease by heating at 121°C for 20 min. Such stability in the effectiveness plays important role at the commercial level. In many cases, metabolites with antimicrobial properties have been reported to play an important role in the efficacy of microbial antagonists (Filippov and Kuznetsova, 1994; Horvath *et al.,* 1995). The activity of metabolites secreted by FZB24 strain was more pronounce than thos produced by Phytovit strain. The results strongly indicate that metabolites secreted by *B. subtilis* strain FZB24 are the effective ingredients for controlling *P. infestans*.

Therefore, other investigations were carried out using *B. subtilis* strain FZB24 cells and metabolites against *P. infestans* growth and disease development. The applied cells and metabolites were highly inhibitory to pre-infection stages of *P. infestans* reducing the ability of the pathogen to establish itself. That is in consistent with previous results shown by Marrone (2002) who found that lipopeptides produced by *B. subtilis* QST 713 stopped spores of plant pathogens from germinating, disrupted germ tube and mycelia growth and inhibited attachment of the plant pathogen to the leaf surface.

In spite of the strong inhibition of spore germination on glass surface, results showed no effect on zoospore germination on plant leaf surface, which could related to removing the unstable zoospores and counting only the germinated encyct. However, both cells and metabolites have apparent effects on germ tube development and on forming of appressorium and vesicles. It supposed that host plant stimulated the pathogen to develop and form appressoria shortly after germination to start adhearing to the plant in order to infect it as soon as possible and increased frequency of appressorium formation was supposed to be accompanied by shorter germ tubes (Oyarzún *et al.,* 2004). Meanwhile, no signal is produced to form appressorium on glass surface, hence, the pathogen growes slowly and aimless elongating the germ tube to find out its target. *B. subtilis* cells and metabolites behaved differentially on different treated-surfaces to prevent the pathogen growth. Application of *B. subtilis* cells and metabolites resulted in reduction of germ tube length on the glass surface as a mechanism to inhibit directly the

pathogen growth as soon as possible, while, there was an observed increase in its length on the leaf surface to delay the appressorium formation and, subsequently, delay the infection. Other workers previously noted that germ tubes were longer on resistant materials (Lapwood, 1968; Bignell, 1975).

Although no differences were observed in the efficacy between cells and metabolites in suppression of early development of *P. infestans* structures, metabolites application showed less colonization of spongy mesophyll and resulted in more effectiveness in inhibition of the intercellular growth by the time. That is in agreement with Jeun and Buchenauer (2001) who showed that in the leaves of tomato plants expressing SAR most pathogen hyphae remained in the palisade parenchyma layer at 72 h after the challenge inoculation, whereas mycelium developed in the entire leaves of control plants at the same incubation time.

Taking in highly consideration, *P. infestans* following infection engage in an intimate relation with plant cells and grow intracellularly by forming haustoria, which invaginate the host membrane, as new host cells are encountered and well establishment of the biotrophic phase of interaction. Avrova *et al.* (2008) confirmed that haustoria contain specific membrane proteins required for pathogenicity and revealed that formation of biotrophic structures (infection vesicles and haustoria) is essential to successful host colonization by *P. infestans*. Haustoria have been implicated as well as a site of effector production and secretion (Whisson *et al.,* 2007). Hence, haustoria serve dual roles; one was described as nutrient uptake from the host and in addition the other role in delivering effectors to the host. Noteworthy, results showed that metabolites inhibited the haustoria formation. However, it remains unclear whether the protective effect is due to direct activity or if it also indirectly depends on producing planta metabolites playing a role in such suppression of pathogen development.

From another point of view, although, the macro- and microscopic observations to estimate the efficacy of control agents on infection process are providing a fairly complete phenotypic description of this hemibiotrophic interaction, visual inspection misses asymptomatic infections. Therefore, monitoring the pathogen colonization in host tissue provides accurate measurements of the pathogen biomass and that helps in studying the effects on the disease cycle. That is as well considered in recent studies

from prior strategies for controlling late blight. Results showed that there was apparent increase in DNA amount of *P. infestans* with the concentration, which reveal its influence on pathogen development and subsequently the importance of inoculum density in disease epidemic. In current study, pathogen abundance can be reliably quantified from the very beginning of the infection time course and even in tissues exhibiting a low degree of infection. Although the first visible symptoms appeared in plants after the second to the third day of infection, quantification was possible at earlier times in totally asymptomatic plants (as soon as 3 hours post inoculation).

In the biotrophic phase of infection, the increase of pathogen biomass slowly progressed and then followed by a great increase in the necrotrophic phase. The results of *P. infestans* quantification were compared to assessments of percentage of damaged leaf area. There were no obvious differences between the two types of assays. The symptoms progression of the pathogen on a regular basis advanced with more severity on detached leaves. The DNA content detected in detached leaves was double the amount in attached leaves. That might be according to some physiological changes in the single plants or to some stress in the separated detached leaves. The bacterial suspensions either cells or metabolites were effective in preventing pathogen infection; they inhibited the pathogen biomass development in the tissue of the tomato leaves and significantly reduced the expansion of existing late blight lesions.

The accumulation of pathogen DNA was rapid in untreated plants and the pathogen could invade and colonize the host so fast, which is due to the pathogen's ability for quick establishment in plant tissue. Although no differences detected in *P. infestans* DNA between treated and untreated plants during the biotrophic phase (from 3 to 48 hpi), from then on, pathogen biomass sharply increased in untreated plants, while, varied slightly in treated ones. The results showed high reduction in pathogen biomass by applying *B. subtilis* cells and metabolites compare to the untreated leaves in favour of metabolites application. Both cells and metabolites were more effective in inhibition pathogen growth in attached leaves than on the detached ones. That means there is an elevation in treatments efficacy to suppress the pathogen development. Such higher effectiveness in complete plants may be due to active plant defense against pathogen attack by generating a signal in plant stimulated by treatment applications. It is therefore

likely that the activity of these antimicrobial substances is mainly based on effects on the metabolites of the host, similar to induced resistance.

Hence, besides the direct effects of applied bacterial cells and metabolites, it is worth to investigate their involvement in elevation of host resistance to suppress late blight disease. As has been shown by Stenzel *et al.* (1985) who found extracts from *B. subtilis* induced resistance in barley and wheat against powdery mildew.

*P.* infestans sprayed on the lower detached leaf surface (abaxial) resulted in higher disease severity than when they were sprayed on the upper leaf surface (adaxial). This might due to the higher number of stomata on the lower leaf surfaces. In addition to the direct activity of cells and metabolites in reducing the diseases severity when were applied on the same surface with the pathogen either on the adaxial or the abaxial surface. Cells and metabolites showed a translaminar activity through leaf tissue layers when treated leaves were inoculated with pathogens on the leaf surface which was opposite to the treated surface. When Cells were applied on the lower surface and *P. infestans* on the upper side, cells application showed more effectiveness than when they were applied on the opposite sides causing 40% and 16% reduction, respectively. However, metabolites were effective providing more than 50% reduction in disease severity regardless of the application side.

Localized application of cells and metabolites on the lower leaves of tomato plants resulted in significant reduction of disease severity on the same leaves. However, in addtion to the translaminar activity of metabolites they showed apical translocated activity resulted in systemic protection on the upper leaves when the metabolites were applied on the lower leaves. Therefore, it has been hypothesized that a signal is generated and mobilized apically in the plants to enhance disease suppression on the upper leaves. Taking in consideration, the authors emphasized that even when an antagonist is not present at site of exposure, an antimicrobial substance could be synthesized by the biocontrol agent and transported through the plant, inhibiting the pathogen directly. Therefore, it would preffer to distinguish which resistance from other mechanisms is involved in the systemic protection. Previous results come in parallel with this study, for example, studies done by Anfoka and Buchenauer (1997) showed disease suppression in the upper leaves of tomato plants after pre-inoculation of lower

leaves with the tobacco necrosis virus (TNV) demonstrating SAR against late blight. The results are in agreement also with Cohen (1994) after pre-treatment with DL-3-aminobutyric acid (3-ABA). Since, *PR1a* gene is requently used as marker for systemic acquired resistance (SAR) in many plant species (Durrant and Dong, 2004; Qiu *et al.,* 2007), therefore the expression levels of *PR1a* gene was followed during *P. infestans*-tomato compatible interaction in treated lower and induced upper leaves of both inoculated and non-inoculated plants.

Hardham and Blackman (2010) found that plant cells are able to recognise the presence of *Phytophthora* spores on their surface and to detect the force exerted by oomycetes hyphae attempting to penetrate the plant epidermis, thus triggering some components of the basal defense response. Two hours post inoculation a substantial and rapid increase in *PR1a* gene expression was observed in response to pathogen recognition in both treated and untreated leaves. It is considered that this induction is as part of basal resistance that operates in susceptible plants, since the untreated plants respond to *P. infestans* inoculation by the same level of gene induction compared to treated plants. Such high induction in gene expression 2 hpi was followed by a remarkable decrease in *PR1a* in both untreated and treated plants 6 hpi. *P. infestans* after successful penetration of the epidermal cells and forming the vesicles produces a wide array of effector proteins facilitating basal resistance suppression and host plants colonization (Dou *et al.,* 2008 and Bos *et al.,* 2009). *P. infestans* has apparently evolved mechanisms to suppress plant defense and to establish the compatibility by releasing these effectors into the host cytoplasm, where they target host proteins to suppress basal defense and to redirect host physiology in support of the pathogen (Espinosa, 2004; Chisholm, 2006).

Subsequently, intercellular hyphae grow into the mesophyll cell layers, producing haustoria as new host cells are encountered and well establishment of the biotrophic phase of interaction. Analysis showed high stimulation of the *PR1a* expression in both upper and lower inoculated leaves of metabolite-treated plants 12 hpi in comparison to untreated and cell-treated inoculated plants. Consequently, the high induction of *PR1a* gene in metabolite-treated plants 12 hpi stimulates SAR resistance targeting the biotrophic stages of *P. infestans* infection and leads to control of infection.

Moreover, high induction in *PR1a* expression 48 hpi was found in untreated and treated plants. That may related to the increased number of further attacked cells under continuous growth of pathogen and subsequently more responding host cells to the presence of the pathogen. Current results are consistent with Rose *et al.* (2005) who found that upon *P. infestans* infection 72 hpi, *PR1a* gene is clearly induced in a susceptible potato cultivar. In spite of no significant difference in *PR1a* alterations after infection between treated and untreated plants, the expression in untreated plants was higher than in treated samples that might be because of suppression of pathogen in treated plants and no need more for further alteration since the pathogen is previously restricted. As has been found by Smart (2003) that the kinetics of the response is consistent with a concept that the induction of *PR* genes may be more accurately described as "pathogen response" genes rather than "defense response" genes. A view has been developed that *PR* genes are rather related to the severity of symptom expression than to resistance (Camacho Henriquez and Sänger, 1982; Naderi and Berger, 1997). They speculated that the induction of *PR* genes accompanying the symptom expression could also have a protective role of "last barrier".

From the point that PRs can be synthesized by plant during development and being *PR* genes induced by a wide array of environmental/external factors, there was an increase in the expression levels of *PR1a* in non-inoculated plants by the time. That comes in agreement with findings of Fraser (1981) who was the first to report the formation of a set of PRs in leaves of non-inoculated tobacco plants as they reached the flowering and senescing stage, and by Hanfrey *et al.* (1996) as well for senescing *Brassica napus* leaves. In addition, old lower leaves showed slightly higher expression of *PR1a* than in upper new leaves, suggesting their involvement in development regulation, as have been shown by Xie *et al.* (2011).

Determination of the necessary time for induction of defense responses after cells and metabolites application was a point of interest. The analysis resulted in significant induction in *PR1a* expression after metabolites treatment following a lag period of 30 and 36 hours of metabolites application in the treated lower leaves and in the induced upper leaves of non-inoculated plants, respectively. These alterations show an elevation in plant activation over the metabolites existence compared to untreated plants and a

continuous capability of applied substances to fast the defense responses in the plant after infection.

Another interesting aspect was the correlation between pathogen colonization, developmental structures, and the induction of *PR1a* gene. In terms of pathogen ability to infect the plant, it clearly seemed that the pathogen in untreated plants had a better capacity not only to infect the plant sooner but also to spread quicker during the time course of infection. While the mycelia continued developing in the entire leaves of control plants, pathogen hyphae remained in the palisade parenchyma layer since it was inhibited obviously in spongy mesophyll metabolite-treated leaves. In addition, in leaf cells of control plants haustoria grew extensively whereas haustoria formation in leaf cells of metabolite-treated plants was restricted. Thus, the systemic protection in metabolite-treated leaves associated with *PR1a* induction was correlated positively with reduction of haustoria formation and mycelia growth in leaf tissue. The restriction of pathogen development in plants expressing SAR can therefore be explained in part by the inability of the pathogen to form functional haustoria. Moreover, no inhibition in pathogen biomass in the biotrophic phase followed by strong inhibition in necrotrophic phase reflect differences in treatments mechanisms between biotrophic and necrotrophic phases suggest either a temporal sequence of systemic colonization by the pathogen in the biotrophic phase or a differential response of the plant defense mechanisms.

Hence the important of understanding the earliest molecular events involved in infection susceptibility/resistance to develop strategies for disease control, further analysis was done to analyse the transcriptional response of tomato plants to *P. infestans* using Affymetrix tomato genome array at the stage of inter-/intra-cellular hyphae growth and haustoria formation. The changes in gene expression that occur during the compatible interaction between tomato and *P. infestans* would reflect the interplay between, on one hand, the attempt of the plant to counteract pathogen invasion and, on the other hand, the activity of pathogen determinants that facilitate the establishment of disease. The analysis resulted in 682 of 9254 transcripts on the array differentially expressed when compared with non-inoculated leaf tissue. The majority of differentially expressed genes were upregulated. That is in consistent with the results observed by Moy *et al.,* (2004), who found that the large number of differentially

expressed genes were upregulated during a compatible interaction with *P. sojae* at this time point in soybean.

The recognition of extracellular pathogen-associated molecular patterns (PAMPs) by transmembrane pattern recognition receptors (PRRs) activates basal defense responses in host plant upon pathogen infection (Nürnberger *et al.,* 2004; Zipfel and Felix, 2005). These defense responses have a profound effect on plant cellular activities including production of reactive oxygen species, activation of mitogen-activated protein kinase cascades, and induction of pathogenesis-related genes (Asai *et al.,* 2002; Nürnberger *et al.,* 2004; Zipfel *et al.,* 2004, 2006).

Molecular crosstalk between tomato plants and *P. infestans* showed a multitude of signal exchange. Previous studies mentioned involvement of inducing many transcription factors and hormones in defense signaling by pathogen infection (Chen *et al.,* 2002; Mysore *et al.,* 2002). In the current analyses, results showed activation in a set of 20 genes involved in calcium signaling and regulation, which play an essential physiological role in the plant cell response to abiotic and biotic stresses (Dangl *et al.*, 1996; Sanders *et al.*, 2002; Hetherington & Brownlee, 2004). $Ca^{2+}$ elevation increases the amount of $Ca^{2+}$ bound to calmodulin (CaM). $Ca^{2+}$/CaM regulates the synthesis of downstream signaling components (nitric oxide (NO) and hydrogen peroxide ($H_2O_2$)) that are essential for the development of the hypersensitive response (HR), which is a type of programmed cell death (PCD) in plant scarifying a few cells immediately surrounding the attacked cell, thereby restricting pathogen growth (Vorwerk *et al.,* 2007; Yuan and Lin, 2008). The increase in $Ca^{2+}$ elevation also results in the binding of $Ca^{2+}$-dependent protein kinase (CDPK), which can activate $H_2O_2$ production through regulation of NADPH oxidase activity. Subsequently, CaM, CDPK, NO and $H_2O_2$ play pivotal roles in activating the expression of pathogen defense genes.

High induction in expression levels of genes involved in jasmonic acid biosynthesis such as divinyl ether synthase (leDES) was observed as well, however, its upregulation is not related to resistance in *P. infestans*-potato pathosystem (Fauconnier *et al.,* 2008). Different genes involved in hormone metabolism synthesis or degradation of ethylene were altered. Some are upregulated such as Pti5 (LOC544042), which involved in transcriptional activation of pathogenesis-related genes (Gu *et al.,* 2002; Balaji *et al.,*

2008). While, other genes from ethylene response factors (ERF), such as ERF4, were down regulated after infection. The increased activity of various signaling pathways and defense responses is associated with differential expression of many families of transcription factors during plant pathogenesis (Eulgem, 2006). For example, upregulation in all genes involved in WRKY family was observed and comes in consistent with results of Balaji *et al.* (2008). In addition, other set of genes involved in MYB transcription family, which described to play a role in the defense response as well (Kranz *et al.,* 1998), were all down regulated. This data suggests that interplay of a broad spectrum of transcription factors possibly regulates multiple signaling cascades during pathogen infection.

Tomato plants exhibited complex defense responses to pathogen infection and results showed alteration in 57 genes such as peroxidases involved in generating the reactive oxygen species (ROS), which are important metabolic events in the execution of HR (Scharte *et al.,* 2005; Essmann *et al.,* 2008). In addition, genes encoding pathogen-inducible antimicrobial enzymes, such as proteases degrading microbial cell walls, were upregulated. From those, PR P69B subtilisin-like Ser protease (*Sbt4a*), which scored the highest level of upregulation within current dataset, was confirmed recently by Vartapetian *et al.* (2011) its involvement in regulation of PCD. Sbt4a was inhibited by EPI1 and EPI0 the inhibitory molecules produced by *Phytophthora,* suggesting a novel type of defense counterdefense mechanism between plants and *Phytophthora* (Tian *et al.,* 2004; 2005). In addition, *Phytophthora*-inhibited-protease 1 (*PIP1*) gene, which is a PR protein induced during infection by *P. infestans,* was upregulated. Previous study done by Tian *et al.* (2007) showed that *PIP1* gene was inhibited by *P. infestans* cystatin-like protease inhibitor (Epic2B) secreted during infection. That suggests the interplay between host proteases of diverse catalytic families and pathogen inhibitors during interactions as a general defense-counterdefense process of strategies for survival (Stahl and Bishop, 2000).

Upregulation in expression levels of several genes related to PCD after *P. infestans* infection is matching with the results obtained by Avrova *et al.* (2004) in *P. infestans*-potato pathosystem. Since previous studies done by Freytag *et al.* (1994) and Vleeshouwers *et al.* (2000) indicated that some epidermal cells undergo a HR in the first 24 hours of compatible interaction of *P. infestans* with potato cells, hence, the rapid

hypersensitive response at the initial infection site is considered to be the key features of active defense. The timing of HR induction and the number of HR responding cells is suggested to have a correlation between resistance level of potato cultivar and HR effectiveness. These findings are in corresponding with work done by Stierl *et al.* (1999) and Smart *et al.* (2003). Subsequently, two factors the timing and frequency of localized accumulation of all features of HR play an essential role in preventions of *P. infestans* growth and the interaction coming.

Under the line of pathogen attack, interfering with plant metabolism is another anticipated activity of oomycete pathogens during the biotrophic interactions. One can envision that effectors not only act on plant defense pathways, but also interfere with host metabolic pathways or transporters, redirecting nutrients and changing host metabolism. Upregulation in several genes involved in amino acid biosynthesis at this early stage of infection was observed, such as aspartate aminotransferase, cysteine synthase, and arginine decarboxylase. Grenville-Briggs *et al.* (2005) found activation in amino acid biosynthesis genes in *P. infestans*-potato pathosystem and clear increases in free amino acid levels in the apoplast and in contrast he found down regulation in genes involved in amino acid biosynthesis in *P. infestans*, speculating that the pathogen may also obtain these amino acids from the host. That might therefore be exploited as potential fungus or oomycete chemical control targets.

Interestingly, the current tomato-*P. infestans* pathosystem was carried in dark under high relative humidity. Hence, several factors play important role in the hypersensitive response. Montillet *et al.* (2005) found that the HR can be initiated under dark conditions but light-generated ROS are required for full lesion development in several pathosystems (Rustérucci *et al.,* 2001; Mateo *et al.,* 2004; Montillet *et al.,* 2005). In addition, the stomatal closure, which is a common response to stress that causes a drop in gas exchange, enhanced photorespiration (Fryer *et al.*, 2003; Mateo *et al.,* 2004). The environmental factor such as high humidity is available under current pathosystem. The stall stomatal closures have been shown to slow the HR and inhibit resistance (Jambunathan *et al.,* 2001; Yoshioka *et al.,* 2001). This suggested that photorespiration and restriction of HR cell death are functionally linked (Mateo *et al.,* 2004). The current results highlights down regulation of several genes associated with photosynthesis, which was found to be down regulated as well in the compatible interaction between

soybean and *P. sojae* (Moy *et al.,* 2004). Requirements of pathogen infection to decrease expression of those genes are proposed as a plant strategy to switch off photosynthesis and other assimilatory metabolisms in favour of respiration process. A decrease in the photosynthetic rate may protect the photosynthetic apparatus against oxidative damage or may be a consequence of oxidative damage (Niyogi 2000; Blokhina *et al.*, 2003).

The reduction in photosynthetic metabolism in conjunction with increased cellular demands initiates the transition from source status to sink status in infected tissue. This transition is accompanied by an increase in expression of extracellular cell wall invertase gene (Wiv-1), which cleaves sucrose into glucose and fructose. That comes in consistent with Roitsch *et al.* (2003) and increase its activity after infection in susceptible interactions was shown in other studies (Fotopoulos *et al.,* 2003; Greenshields *et al.,* 2004; Essmann *et al.,* 2008). In addition, there was increase in expression of hexose transporters genes, which transport these hexoses into the cell to fulfill the energy and carbon requirements for the resistance response as have been shown previously by Truernit *et al.,* (1996). Since, pathogens also possess extracellular sucrolytic enzymes and preferentially utilize hexose rather than sucrose (Voegele *et al.*, 2001). Therefore, it is not always clear in susceptible interactions whether invertase activity is of pathogen origin as have been shown by Jobic *et al.* (2007) or an aspect of plant defense against the invading pathogen. Especially that the carbohydrate increase is believed to be a metabolic signal that induces the expression of defense-related genes and repression of photosynthesis (Ehness *et al.,* 1997; Sinha *et al.,* 2002; Roitsch *et al.,* 2003; Berger *et al.* 2004; Kocal *et al.,* 2008).

However, in response to infection, the strong demand to obtain carbon will likely shuttle amino acids into energy-generating pathways such as the tricarboxylic acid (TCA) cycle. Study resulted in stimulation in the respiration pathways, which were stimulated during the resistance response in early study current by Smedegaard-Petersen and Stolen (1981), the mitochondrial TCA cycle and mitochondrial electron transport (Fernie *et al.,* 2004). However, the glycolysis pathway was down regulated, which was metioned by Bolton *et al.* (2008) that flux through it is an important aspect of the resistance response.

If primary metabolism is reconfigured to support the increased demands of the resistance response, an intriguing question is what responses are the major consumers of primary metabolism output. Phenylpropanoid pathway products, which most genes involved in were upregulated, represent a major flow of carbon from primary metabolism into secondary metabolism (Bolton *et al.,* 2008). The shikimic acid pathway, often upregulated in response to pathogen infection, is driven by phosphoenolpyruvate (PEP) from glycolysis and provides chorismate as an end product and various intermediates involved in plant defense (Weaver and Herrmann 1997).

Noteworthy, a set of 33 genes involved in cell wall modification and degradation process was down regulated. Such reduction shown by Cantu *et al*. (2008) to reduce wall disassembly might be a strategy by the plant to reinforce the cell wall and prevent further infection by the pathogen. However, a paucity of plant cell-wall degrading enzymes is may be in line with the biotrophic lifestyle; the enzymes are likely used to soften or weaken the plant cell walls to facilitate the cell to cell passage of hyphae, rather to facilitate the utilization of cell-wall derived carbohydrates (Spanu and Kämper, 2010).

The induction of the wide array of described defense mechanisms involves a massive redistribution of energy toward the defense response and associated with multiple metabolic processes. However, nutrients available for pathogen metabolism during the current susceptible interaction may be a consequence of host cell leakage during colonization or pathogen manipulation of the plant to provide nutrients rather than a reflection of an active host defense response (Bolton, 2009). It is expected that gene expression changes occurring at the time of inter- and intracellular hyphe growth and haustoria formation reflect the establishment of susceptibility, especially that the activation of basal immunity was proved to be insufficient to limit pathogen growth because the pathogen caused more than 80% disease severity 6 days after inoculation.

However, application of *B. subtilis* strain FZB24 cells and metabolites showed high inhibitory effects on the early stages of *P. infestans* development with obvious differences between cells and metabolites efficacies on intracellular and intercellular progress of pathogen growth in leaf tissue. That consequently resulted in difference in reduction rate of disease severity by 44% and 70% for cells and metabolites,

respectively. In additioin, the analysis showed systemic protection in disease severity on the upper leaves when the lower leaves were treated with metabolites, while no significant suppression was observed when cells were applied. Further determination of the changes in gene expression taking place in tomato plants, in absence and presence of *P. infestans* by genome array analysis after cells and metabolites applications at the time of pathogen establishment at 12 hpi was of great significance in understanding their suppressive effect.

Analysis resulted in a close number of genes differentially changed after inoculation in both untreated and cell-treated plants compared to non-inoculated untreated ones, 682 and 656 DEGs, respectively. However, after metabolites application the number of differentially expressed genes was 345 genes, almost 50% less than those altered in untreated inoculated plants that may reveal the reduction in plant susceptibility towards invader. Remarkable, the largest number of those genes differentially expressed after inoculation was altered in the same direction in all three treatments (untreated, cell-treated, and metabolite-treated) compared to non-inoculated plants. That reflects their involvement in infection because they changed after infection regardless application of cells or metabolites.

Interestingly, some other genes exclusively changed after cells and metabolites applications in inoculated plants compared to untreated inoculated ones, 24 and 79 DEGs, respectively. Most genes changed after cells application, which down regulated, changed as well after metabolites application. That supports previous results that *B. subtilis* could secret metabolites very early but may be in insufficient amount to suppress the disease systemically. Pronounced functions involved in those down regulated genes are stress responses and transcription, such as regulator of gene silencing (LOC543942), zinc finger AN1 domain-containing stress-associated protein 12 (PMZ), and ethylene-responsive late embryogenesis-like protein (ER5). Down regulation in those functions is an attempt to reduce stress responses in order to survive the plant and balance conditions to promote plant growth, especially, there is increasing evidence that plants have a memory of encountered stress situations that allow them to better adapt to changing conditions.

In point of systemic protection achieved by metabolites application, the analysis resulted in some exclusive genes altered only after metabolites application. Results showed down regulation in protein modification process such as E3 ubiquitin-protein ligase (PUB23) and plant U-box 29 (PUB29). Plant U-box proteins are implicated in self-incompatibility (Stone *et al.,* 2003), hormone regulation (Amador *et al.,* 2001), and abiotic stress (Yan *et al.,* 2003). In addition to down regulation in genes involved in RNA-regulation of transcription such as Pti5 (LOC544042), which was highly upregulated in untreated inoculated plants and showed less activation in metabolite-treated inoculated plants.

In parallel to those down regulated genes after application of metabolites secreted by *B. subtilis*, a set of genes involved in hormone metabolism (auxin, gibbrellin) and photosystem II were upregulated. Previous study showed modulation of some plant regulatory mechanisms through the production of hormones or other compounds that influence plant development (Frankenberger and Arshad 1995). Many studies direct attention to involvement of some hormones such as auxin and gibberellic acid (GA) in plant defense signaling pathways but their role in plant defense is less well studied. However, Martínez Noël *et al.* (2001) supported a putative role of auxins in the potato-*P. infestans* pathosystem as natural defense for pathogen spread and disease development. Gibberellic acid promotes plant growth by regulating the degradation of growth-repressing DELLA proteins, which control plant immune responses by modulating SA and JA dependent defense responses (Navarro *et al.,* 2008).

Plant growth and defense are regulated by a network of interconnecting signaling pathways. The involvement of different plant growth regulators in plant immunity suggests that the control of plant growth, development and defense is interconnected in a complex network of cross-communicating hormone signaling pathways. Early work in tomato (*Solanum lycopersicum*) and *Arabidopsis* demonstrated that SA and its acetylated derivative aspirin are strong antagonists of the JA signaling pathway (Doherty *et al.,* 1988). Other study showed as well that JA and ET signaling can act synergistically (Penninckx *et al.,* 1998). However, Koornneef *et al*. (2008) demonstrated that timing and sequence of initiation of SA and JA signaling are important for the outcome of the SA-JA signal interaction. Further interpretation of such results and suggestions can support the findings out from the current study. From

one hand, activation in genes involved in jasmonate and ethelyne pathways was observed in the compatible interaction in untreated plants 12 hours post inoculation, the expected time for pathogen establishment. However, in metabolite-treated plants, the analyses resulted in activation of *PR1a* gene, which is not existence on the tomato genome array, in induced upper leaves when metabolites were applied on the lower leaves 12 hpi, besides to activation in other hormone signaling (auxin and gibberellin). Crosstalk between hormonal signaling pathways provides the plant with such a powerful regulatory potential and may allow the plant to tailor its defense response to the invaders encountered (Reymond *et al.,* 1998; Kunkel *et al*., 2002; Bostock *et al*., 2005; Pieterse *et al*., 2007). The current pathosystem showing regulatory potential of such a network might reflect the plants ability to quickly adapt to their biotic and as well as the abiotic environment under metabolite-induced state.

Although, it is still not clear if the enhanced photosynthesis is an reason to the plant's attempt to fuel defense responses to limit pathogen growth (Berger et al., 2007), or fungal manipulation of the plant to co-opt resources for pathogen nutrition. Current analysis revealed activation in genes involved in photosynthesis in metabolite-induced plants compared to their down regulation in the compatible interaction tomato-*P. infestans*. That comes in agreement with Bolton *et al.* (2009) who assumed that rates of photosynthesis could increase to supply the carbon skeletons, energy, and reducing equivalents required to support the plant defense.

Plant growth and defense are regulated by a network of interconnecting signaling pathways. The involvement of different regulators in plant immunity suggests that the control of plant growth, plant development and defense against pathogen is interconnected in cross-communicating hormone signaling pathways.

The reduction in haustoria formation and the superficial colonization of leaf tissue layers by *P. infestans* at the time of pathogen establishment in the biotrophic phase of the compatible interaction was associated with *PR1a* gene activation in metabolite-treated plants. Metabolite application resulted as well in lower susceptibility in plants towards *P. infestans* by reducing the plant responses necessary for pathogen development and induced the resistance by activation in responses involved in hormone metabolism and photosynthesis function, besides to down regulation in stress responses.

That revealed the enhancement in the ability of plants treated with metabolites to inhibit *P. infestans* development.

# 5 SUMMARY

Most previous studies of the biological control agents *Bacillus subtilis* strains FZB24 and Phytovit and their secreted metabolites focused primarily on the degree of disease reduction. Therefore, further investigations on the mechanisms involved in disease suppression, such as the elevation in host resistance in addition to their direct effect, will help to optimize the application strategies to control plant disaeses. *B. subtilis* cells and metabolites exhibited inhibitory effects against several pathogens (*Alternaria solani, Oidium neolycopersici*, *Cladosporium fulvum*, and *Phytophthora infestans*) causing important foliar tomato diseases. Their direct activity and involvement in enhanced plant defense are attractive properties explored further and in more detail in the current study at microbial, histological, and molecular levels.

- Both biocontrol agents FZB24® and Phytovit® were effective in suppression of diseases caused by *A. solani, O. neolycopersici*, *C. fulvum*, and *P. infestans*; demonstrating the broad spectrum of the biocontrol agents. The protective treatments with antagonistic *B. subtilis* were more effective than curative treatments to control tomato diseases.
- *B. subtilis* strains FZB24 and Phytovit showed high antagonistic effects on mycelia growth and spore germination in addition to the inhibitory effects against pathogens development structures at the early stages of infection reducing the pathogen ability to infect the plant tissues.
- *B. subtilis* start to secret the effective substances from the first hours of culturing with increase by the time showing stability in the effectiveness to suppress the disease when the metabolites were heated at 121°C for 20 min that plays important role at the commercial level and metabolites produced by *B. subtilis* later after 72 hours of culturing in synthetic growth medium exhibited strong suppression in disease severity of late blight more than the bacteria itself revealing its important role as effective substances in disease suppression in favor of metabolites produced by FZB24 strain.
- The highest destructive effect of bacteria and secreted metabolites proved to be against *P. infestans* growth and late blight development.

- The accumulation of *P. infestans* DNA, which was determined by quantitative real-time PCR, was rapid in untreated plants and the pathogen could invade and colonize the host so fast, which is due to the pathogen's ability for quick establishment in plant tissue.
- Application of *B. subtilis* cells or metabolites showed no inhibition of *P. infestans* biomass in the biotrophic phase followed by strong reduction in DNA content of *P. infestans* in the necrotrophic phase reflecting involvement of different mechanisms of disease suppression between biotrophic and necrotrophic phases.
- Metabolites were more effective in preventing *P. infestans* biomass development in leaf tissue of tomato plants than cells application, with higher effectiveness on attached leaves than on detached ones directing the attention to an elevation in treatments efficacy in complete plants, which may be due to active plant defense against *P. infestans* attack.
- While the mycelium continued developing in the entire leaves of control plants, *P. infestans* hyphae remained in the palisade parenchyma layer since it was inhibited obviously in spongy mesophyll metabolite-treated leaves. In addition, in leaf cells of control plants haustoria grew extensively whereas haustoria formation in leaf cells of metabolite-treated plants was restricted that resulted in less colonization of leaf tissue by the time.
- Localized application of metabolites on the lower leaves of tomato plant resulted in systemic protection on the upper leaves, providing that a signal is generated and mobilized apically in the plants to enhance disease suppression on the upper leaves.
- Systemic protection in metabolite-treated leaves was associated with the activation in *PR1a* gene expression, which is a SAR marker, and was correlated positively with reduction of haustoria formation and mycelia growth in leaf tissue 12 hours post inoculation. Consequently, metabolites target the biotrophic stages of *P. infestans* infection and lead to control of infection.
- The analysis resulted in significant induction in *PR1a* expression after metabolites treatment following a lag period of 30 and 36 hours of metabolites application in the treated lower leaves and in the induced upper leaves of non-

inoculated untreated plants, respectively. These alterations showed an elevation in plant activation over the metabolites existence compared to untreated plants.

- Analysing the Affymetrix tomato genome array data resulted in changes in the expression level of a substantial number of 682 genes from over 9200 genes of the tomato genome after infection when compared to non-inoculated plants. From these 682 differentially expressed genes, 429 genes were upregulated and 253 genes were reduced after infection.
- Several genes were identified as up-regulated, including genes involved in plant defense responses (e.g., Pti5, subtilisin-like protease (the most highly induced gene), *Phytophthora*-inhibited-protease 1, peroxidase), metabolic pathways (e.g., transferase family protein), signaling (e.g., calcium-dependent protein kinase, calmodulin-binding proteins, mevalonate diphosphate decarboxylase), transcriptional regulators (e.g., WRKY transcription factors), and stress response (e.g., alpha-DOX1, heat shock protein), among others. Down-regulated genes were identified with genes involved in photosynthesis and in regulation of the cell wall modification and degradation process (e.g., expansin and pectate lyase family protein).
- It is expected that gene expression changes occurring at the time of inter- and intracellular hyphae growth and haustoria formation reflect the establishment of susceptibility, especially that the activation of basal immunity was proved to be insufficient to limit *P. infestans* growth because the *P. infestans* caused more than 80% disease severity 6 days after inoculation.
- More than 70% of genes altered in *Bacillus subtilis* strain FZB24 cell-treated infected plants were changed in the same trend and with the same fold change to those genes changed in untreated infected plants reflecting their involvement in infection regardless of cells application.
- Metabolites application resulted in reduction by 50% in the number of differentially expressed genes compared to those altered in untreated infected plants. This may reveal the reduction in plant susceptibility towards *P. infestans*.
- Analysis resulted in some other genes changed exclusively after cells and metabolites applications. The down regulated genes after cells application,

which are involved in stress responses and transcription, were down regulated as well after metabolites application.

- In point of systemic protection achieved by metabolites application, the analysis resulted in some exclusive upregulated genes involved in hormone metabolism (auxin, gibbrellin) and photosystem II.

In conclusion, application of *B. subtilis* cells and metabolites resulted in a significant reduction in disease severity of different pathogens. But, bacterial cells applied on the lower leaves showed on the upper leaves no systemic protection as well as no induction in *PR1a* gene expression or other plant defense responses. This demonstrates the antagonistic effect of bacterial cells and they are not involved in plant resistance. However, metabolites formed by *B. subtilis* strongly inhibited the inter- and intracellular growth of *P. infestans* especially the formation of haustoria, which are responsible for infection establishment. Since *P. infestans* rapidly colonized the entire leaf tissue horizontally and vertically, metabolites showed no progress of pathogen development in deeper tissue layers. This can be explained as a superficial horizontal colonization of *P. infestans* after metabolite application. Systemic protectin achieved by metabolites at the time of *P. infestans* establishment in the biotrophic phase of interaction was associated with *PR1a* gene activation. Changes in plant responses after infection investigated using microarray analysis were reduced by 50% after metabolite application. Such reduction in the number of differentially expressed genes after infection in metabolite-treated plants compared to those altered in untreated plants revealed that plants showed less susceptibility towards *P. infestans* by inducing higher resistance. In addition, other changes in plant responses were exclusively upregulated after metabolite application involved in hormone signaling and photosynthesis function, besides to suppression in stress responses.

Plants treated with *Bacillus subtilis* metabolites showed lower susceptibility towards *P. infestans* by an induced resistance. This was correlated with changes within the plants affecting on the ability to form haustoria, which is necessary for development of the pathogen. That indicates haustoria provide ideal targets for late blight control.

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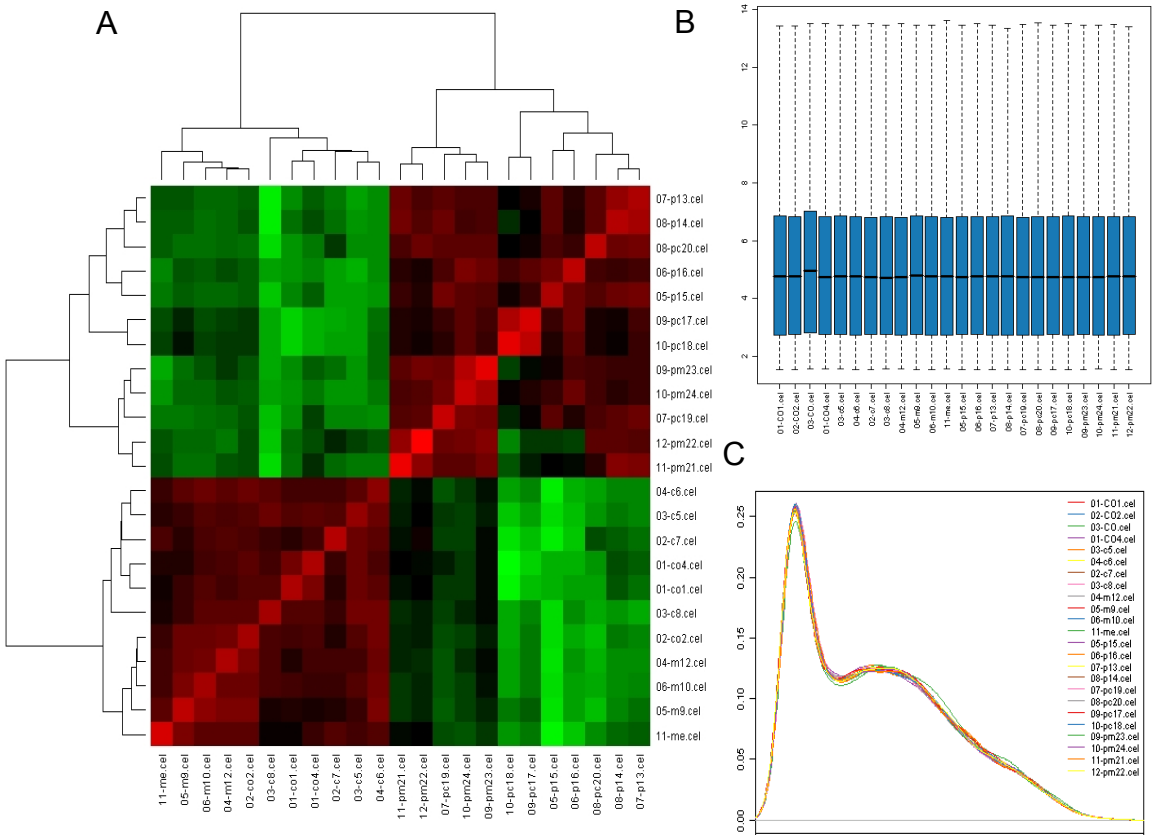
# 7 APPENDICES

**Appendix 1:** The intensity and uniformities between arrays after normalization. A) Pearson correlation of the arrays within and between biological replicates. The higher and lower correlations are represented in red and green colours represent, respectively. B) Box-plots indicating the distribution of probe intensity across all arrays and each box corresponds to one array. The boxes have similar size and Y position (median). C) Smooth histograms represent the density estimates of the data showing similarities in shapes and ranges of the arrays.



**Appendix 2:** Expression profile of differentially expressed genes in tomato leaves inoculated with *Phytophthora infestans* 12 hours post inoculation. Molecular network analyses to find out the associated pathways have done by Mapman (P value <5%).

| ProbeSet ID | Gene title | Gene symbol | Fold change | *Similarity | E value |
|---|---|---|---|---|---|
| **Signaling** | **signaling.in sugar and nutrient physiology** | | | | |
| LesAffx.344.3.S1_at | photoassimilate-responsive protein-related | AT3G54040 | 10.3 | w | 3.00E-44 |
| Les.1175.2.S1_at | photoassimilate-responsive protein, putative | AT5G52390 | 2.9 | m | 1.00E-12 |
| | **signaling.receptor kinase** | | | | |
| LesAffx.59625.1.S1_at | 33 kDa secretory protein-related | AT5G48540 | 4.1 | m | 1.00E-37 |
| LesAffx.50533.1.S1_at | cysteine-rich RLK10 | CRK10 | 2.6 | h | 8.00E-11 |
| Les.1334.1.A1_at | PR5-like receptor kinase | PR5K | 2.5 | m | _ |
| LesAffx.70335.1.S1_at | protein kinase, putative | AT3G57700 | 2.5 | w | 1.00E-33 |
| Les.2137.1.S1_at | EIX receptor 1 | Eix1 | 2.4 | i | 7.00E-77 |
| LesAffx.65273.1.S1_at | protein kinase family protein | AT1G16670 | 2.4 | m | 3.00E-62 |
| Les.1297.1.S1_at | chitin elicitor receptor kinase 1 | CERK1 | 2.3 | h | 1.00E-52 |
| LesAffx.46815.2.S1_at | leucine-rich repeat family protein | AT3G20820 | -2 | m | 4.00E-93 |
| | **signaling.calcium** | | | | |
| LesAffx.69808.1.S1_at | calmodulin-binding protein | EDA39 | 9 | h | 5.00E-120 |
| LesAffx.3635.2.A1_at | calmodulin-binding family protein | . | 8.2 | h | 2.00E-16 |
| LesAffx.16164.1.S1_at | calcium-binding EF hand family protein | . | 5.2 | w | 6.00E-21 |
| Les.1997.1.S1_at | clareticulin 3 | CRT3 | 5.1 | h | 2.00E-34 |
| Les.1997.3.A1_at | clareticulin 3 | CRT3 | 5.1 | h | 1.00E-10 |
| LesAffx.66814.1.S1_at | calmodulin binding | AT1G73805 | 4.6 | m | 1.00E-12 |
| LesAffx.3635.1.S1_at | calmodulin-binding family protein | . | 4.4 | h | 4.00E-98 |
| Les.4651.1.S1_at | calnexin-like protein | LeCNX61.0 | 4.2 | i-h | 4.00E-183 |
| Les.1997.2.S1_at | clareticulin 3 | CRT3 | 3.7 | m | 5.00E-24 |
| LesAffx.70732.1.S1_at | calmodulin-related protein, putative | AT3G50770 | 3.3 | w | 2.00E-40 |
| Les.3334.1.S1_at | calcium-dependent protein kinase 28 | CPK28 | 2.7 | m | _ |
| LesAffx.15921.1.S1_at | lipase class 3 family protein / calmodulin-binding heat-shock protein, putative | AT5G37710 | 2.6 | h | 6.00E-46 |
| Les.1360.2.A1_at | calcium-dependent protein kinase 1 | ATCDPK1 | 2.6 | h | 3.00E-17 |
| LesAffx.9367.1.S1_at | Ca2+-binding protein 1 | ATCP1 | 2.5 | w | 7.00E-30 |
| LesAffx.47666.1.S1_at | C2 domain-containing protein | AT4G34150 | 2.3 | w | 9.00E-32 |
| LesAffx.30900.1.S1_at | calcium-dependent protein kinase 19 | CPK19 | 2.3 | h | 1.00E-53 |
| LesAffx.25303.1.S1_at | calmodulin-binding protein | AT2G15760 | 2.3 | w | 1.00E-10 |
| Les.923.1.S1_at | calcium-dependent protein kinase CDPK1 | LOC543689 | 2.1 | i-h | 2.00E-214 |

## Appendix 2: CONT.

| | | | | | |
|---|---|---|---|---|---|
| Les.783.1.S1_at | calmodulin-binding protein | AT5G57580 | 2.1 | h | 2.00E-159 |
| Les.3416.1.S1_at | calreticulin 2 (CRT2) | AT1G09210 | 2 | h | 4.00E-169 |
| | **signaling.G-proteins** | | | | |
| Les.176.1.S1_at | small GTP-binding protein | LeRab1A | 2.1 | i-m | 7.00E-103 |
| Les.4749.1.S1_at | rac GTPase activating protein, putative | AT2G46710 | -2.1 | m | 3.00E-83 |
| Les.5316.1.S1_at | GTP binding / RNA binding | AT5G66470 | -2.1 | h | 3.00E-143 |
| Les.1179.1.A1_at | Kinase partner protein-like | ATROPGEF7 /ROPGEF7 | -2.7 | h | _ |
| | **signaling-MAP.kinases** | | | | |
| LesAffx.16424.1.S1_s_at | mitogen-activated protein kinase 3 | MPK3 | 4 | i-h | 8.00E-24 |
| Les.4316.1.S1_at | mitogen-activated protein kinase 3 | MPK3 | 3.2 | i-h | 6.00E-144 |
| Les.5060.1.S1_at | *Arabidopsis thaliana* map kinase 4 | ATMPK4 | 2.1 | h | 3.00E-179 |
| **Hormone signaling** | **JA synthesis** | | | | |
| Les.129.1.S1_at | divinyl ether synthase | LeDES | 9.2 | i-m | 2.00E-123 |
| Les.13.1.S1_at | allene oxide synthase | aos | 3.4 | i-h | 3.00E-187 |
| Les.3632.1.S1_at | lipoxygenase | loxD | 2.5 | i-ni | 1.00E-210 |
| | **Abscisic acid** | | | | |
| LesAffx.63074.1.S1_at | GRAM domain-containing protein / ABA-responsive protein-related | AT5G23370 | 3.7 | w | 5.00E-42 |
| Les.4807.1.S1_at | GRAM domain-containing protein / ABA-responsive protein-related | AT5G13200 | 2.3 | m | 8.00E-98 |
| Les.112.1.S1_at | zeaxanthin epoxidase | LOC544162 | -2.3 | i-h | 2.00E-251 |
| | **Ethylene synthesis/signal transduction** | | | | |
| Les.3575.1.S1_at | Pti5 | LOC544042 | 16.7 | i-w | 3.00E-23 |
| LesAffx.3059.1.S1_at | ethylene response factor | . | 14.9 | w | 8.00E-16 |
| Les.132.1.S1_at | ethylene-forming enzyme | LOC544285 | 3.7 | i-w | 1.00E-117 |
| Les.2560.1.S1_at | ethylene-forming enzyme | LOC544052 | 3.7 | i-m | 7.00E-131 |
| LesAffx.63189.1.S1_at | oxidoreductase, 2OG-Fe(II) oxygenase family protein | AT4G25300 | 3.1 | m | 7.00E-23 |
| LesAffx.1861.4.S1_at | 2-oxoglutarate-dependent dioxygenase, putative | AT1G06620 | 2.8 | m | 2.00E-21 |
| LesAffx.1861.2.S1_at | 2-oxoglutarate-dependent dioxygenase, putative | AT1G06620 | 2.4 | m | 4.00E-41 |
| Les.3769.1.S1_at | 1-aminocyclopropane-1-carboxylate synthase | ACS6 | 2.2 | i-h | 5.00E-177 |
| Les.1841.1.S1_at | 1-aminocyclopropane-1-carboxylate synthase | LE-ACS1A | 2.2 | i-h | 7.00E-95 |
| LesAffx.29801.1.S1_at | oxidoreductase, 2OG-Fe(II) oxygenase family protein | DMR6 | 2.2 | m | 4.00E-27 |
| LesAffx.9824.1.S1_at | oxidoreductase, 2OG-Fe(II) oxygenase family protein | AT2G36690 | 2 | m | 3.00E-46 |
| Les.5864.1.S1_at | 2-oxoglutarate-dependent dioxygenase homolog | GAD2 /// LOC544002 | -2.2 | m | 1.00E-52 |
| Les.4753.1.S1_at | 2-oxoacid-dependent oxidase, putative | AT3G50210 | -2.4 | m | 4.00E-133 |

## Appendix 2: CONT.

| | | | | | |
|---|---|---|---|---|---|
| Les.2341.1.A1_at | GCN5-related N-acetyltransferase (GNAT) family protein | . | -2.5 | w | _ |
| Les.4139.1.S1_at | ethylene response factor 4 | ERF4 | -2.8 | i-w | 7.00E-34 |
| | **Biotic stress.PR-proteins** | | | | |
| LesAffx.16769.1.S1_at | disease resistance-responsive protein-related | AT2G21100 | 13.8 | w | 7.00E-21 |
| Les.2137.1.S1_at | EIX receptor 1 | Eix1 | 2.4 | i-m | 7.00E-77 |
| Les.3506.1.S1_at | verticillium wilt disease resistance protein Ve2 | Ve2 | 2.2 | i-m | 8.00E-103 |
| **Secondary metabolism** | **Wax** | | | | |
| LesAffx.60722.1.S1_at | *Arabidopsis thaliana* sterol O-acyltransferase 1 | AtSAT1 | 3.2 | m | 3.00E-27 |
| | **N misc.alkaloid-like** | | | | |
| LesAffx.50270.1.S1_at | strictosidine synthase family protein | AT3G51441 | 2.6 | m | 3.00E-29 |
| LesAffx.50270.2.S1_at | strictosidine synthase family protein | AT3G51440 | 2.2 | w | 4.00E-26 |
| | **Phenylpropanoids-lignin biosynthesis** | | | | |
| LesAffx.62617.1.S1_at | transferase family protein | AT5G07850 | 7.1 | h | 6.00E-67 |
| Les.3741.1.S1_at | Eli3 protein | Eli3 | 2.4 | i-m | 5.00E-97 |
| Les.281.1.S1_at | 4-coumarate :COA ligase 1 | 4CL1 | 2.4 | h | 4.00E-81 |
| Les.1097.1.A1_at | 4-coumarate :COA ligase 2 | 4CL2 | 2.3 | w | _ |
| Les.281.3.S1_at | 4-coumarate :COA ligase 1 | 4CL1 | 2.3 | h | 2.00E-17 |
| LesAffx.47885.1.S1_at | OPC-8:0 COA LIGASE1 | OPCL1 | 2.2 | h | 3.00E-92 |
| Les.4271.2.S1_at | PHE ammonialyase 1 | PAL1 | 2.1 | ni | 2.00E-133 |
| Les.5068.1.S1_at | Nicotinamidase 2 | NIC2 | -2 | m | 6.00E-74 |
| | **Isoprenoids.carotenoids** | | | | |
| Les.72.1.S1_at | farnesyl pyrophosphate synthase | FPS1 | 3.7 | i-h | 1.00E-155 |
| LesAffx.66096.2.S1_at | Mevalonate kinase | MK | 3.2 | m | 3.00E-14 |
| Les.4735.1.S1_at | 3-hydroxy-3-methylglutaryl coenzyme A synthase | HMGS | 3 | i-m | 1.00E-213 |
| Les.2490.1.S1_at | mevalonate disphosphate decarboxylase | MDC | 2.8 | i-w | 2.00E-185 |
| Les.3771.1.S1_at | lycopene epsilon-cyclase | CrtL-e-1 | -2.1 | i-h | 4.00E-213 |
| Les.1785.1.A1_at | amine oxidase family | AT1G57770 | -2.1 | m | 8.00E-90 |
| Les.3958.1.S1_at | violaxanthin de-epoxidase | LOC543696 | -2.2 | i-h | 3.00E-71 |
| Les.3510.1.S1_at | 1-D-deoxyxylulose 5-phosphate synthase | dxs | -2.6 | i-ni | 0 |
| Les.3123.1.S1_at | geranylgeranyl reductase | AT1G74470 | -2.8 | h | 2.00E-70 |
| | **Flavonoids** | | | | |
| Les.1664.1.S1_at | 2-oxoglutarate dehydrogenase E1 component, putative | AT3G55410 | 3.8 | ni | 0 |
| Les.2278.1.S1_at | Flavanone 3-hydroxylase | F3H | 3.7 | h | 3.00E-167 |
| Les.842.1.S1_at | GT72B1 | GT72B1 | 2.5 | w | 5.00E-25 |
| Les.842.2.S1_a_at | GT72B1 | GT72B1 | 2 | w | 1.00E-12 |
| Les.5864.1.S1_at | 2-oxoglutarate-dependent dioxygenase homolog | GAD2 /// LOC544002 | -2.2 | m | 1.00E-52 |
| Les.4753.1.S1_at | 2-oxoacid-dependent oxidase | AT3G50210 | -2.4 | m | 4.00E-133 |

## Appendix 2: CONT.

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|---|---|---|---|---|---|
| **amino acid metabolism** | **Tyrosine synthesis** | | | | |
| LesAffx.66354.1.S1_at | prephenate dehydrogenase family protein | AT1G15710 | 24.6 | m | 7.00E-39 |
| Les.5555.1.S1_at | prephenate dehydrogenase family protein | AT1G15710 | 2.5 | m | 5.00E-121 |
| | **Chorismate synthesis** | | | | |
| Les.3039.1.S1_at | phospho-2-dehydro-3-deoxyheptonate aldolase | LOC544153 | 7 | i-h | 4.00E-236 |
| Les.3672.1.S1_at | 5-enolpyruvylshikimate-3-phosphate synthase | LOC543977 | 4.8 | i-h | 8.00E-211 |
| Les.53.1.S1_at | shikimate kinase precursor | LOC544078 | 2.8 | i-m | 4.00E-83 |
| Les.5092.1.S1_at | Maternal effect embryo arrest 32 | MEE32 | 2.5 | m | 8.00E-75 |
| Les.5122.1.S1_at | shikimate kinase-related | AT2G35500 | -2.1 | m | 2.00E-114 |
| | **Phenylalanine synthesis** | | | | |
| LesAffx.10955.1.S1_at | arogenate dehydratase 6 | ADT6 | 3.6 | h | 1.00E-42 |
| LesAffx.10955.3.S1_at | arogenate dehydratase 6 | ADT6 | 3.5 | h | 3.00E-30 |
| | **Sucrose.degradation** | | | | |
| Les.3460.1.S1_at | acid invertase | Wiv-1 | 4.4 | i-h | 7.00E-151 |
| Les.157.1.S1_at | sucrose synthase | sus3 | 2.3 | i-h | 2.00E-277 |
| LesAffx.53904.1.S1_at | hypothetical protein | AT5G40510 | 2.1 | m | 6.00E-70 |
| Les.1617.2.S1_s_at | fructose-1,6-bisphosphatase, putative | AT1G43670 | -2.1 | h | 2.00E-42 |
| Les.4946.1.S1_at | fructose-1,6-bisphosphatase, putative | AT1G43670 | -2.6 | h | 2.00E-165 |
| Les.1617.3.A1_s_at | fructose-1,6-bisphosphatase, putative | AT1G43670 | -2.4 | h | _ |
| **Enzyme family** | **Misc.peroxidase** | | | | |
| LesAffx.57363.1.S1_at | anionic peroxidase, putative | AT1G14550 | 5.8 | m | 6.00E-44 |
| Les.4999.1.S1_at | peroxidase 21 (PER21) (P21) (PRXR5) | AT2G37130 | 5.5 | m | 6.00E-110 |
| LesAffx.71606.1.S1_s_at | peroxidase 21 (PER21) (P21) (PRXR5) | AT2G37130 | 5.2 | w | 2.00E-13 |
| Les.2832.1.S1_at | peroxidase | cevi16 | 4.2 | i-m | 3.00E-108 |
| LesAffx.60831.1.S1_at | peroxidase, putative | AT4G26010 | 2 | m | 3.00E-19 |
| Les.4492.2.S1_at | Light harvesting complex PSII subunit6 | LHCB6 | -2.3 | m | 1.00E-112 |
| Les.4492.3.S1_at | Light harvesting complex PSII subunit6 | LHCB6 | -2.3 | m | 2.00E-28 |
| | **Misc.cytochrome P450** | | | | |
| LesAffx.8720.2.S1_at | CYP76C5 | CYP76C5 | 27.7 | m | 2.00E-37 |
| Les.4880.1.S1_at | Transparent testa7 | TT7 | 8.4 | m | 8.00E-109 |
| LesAffx.9038.3.S1_at | cytochrome P450, putative | AT1G66540 | 6.5 | m | 2.00E-19 |
| LesAffx.22491.2.A1_at | cytochrome P450 | CYP705A25 | 5.8 | m | 1.00E-11 |
| Les.2988.1.S1_at | Sucrose transporter | LeSUT1 | 4.8 | i-h | 2.00E-226 |
| LesAffx.5912.1.A1_at | Ferulic acid 5-hydroxylase1 | FAH1 | 4.7 | m | 4.00E-39 |
| LesAffx.5912.1.S1_at | Ferulic acid 5-hydroxylase1 | FAH1 | 3.5 | m | 4.00E-39 |
| LesAffx.3698.3.S1_at | *Arabidopsis* P450 reductase2 | ATR2 | 3 | h | 1.00E-128 |

## Appendix 2: CONT.

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|---|---|---|---|---|---|
| LesAffx.63244.1.S1_at | CYP76C3 | CYP76C3 | 2.3 | w | 2.00E-16 |
| LesAffx.9038.1.S1_at | cytochrome P450 probable ent-kaurenoic acid oxidase | CYP88A9 | -2.1 | m | 7.00E-66 |
| LesAffx.30937.1.S1_at | CYP71B9 | CYP71B9 | -2.4 | w | 3.00E-14 |
| **Cell wall** | **Precursor synthesis** | | | | |
| LesAffx.9043.1.S1_at | UDP-glucuronic acid decarboxylase2 | UXS2 | 3.7 | h | 4.00E-107 |
| LesAffx.9043.2.S1_at | UDP-glucuronic acid decarboxylase2 | UXS2 | 3 | h | 7.00E-13 |
| Les.500.2.A1_s_at | Phosphomannomutase | LOC778245 | -2.2 | i-m | _ |
| | **Modification** | | | | |
| Les.4968.1.S1_s_at | ETAG-A3 | LOC543511 | -2.1 | i-m | 2.00E-105 |
| Les.3537.1.S1_at | ETAG-A3 | LOC543511 | -2.1 | i-m | 4.00E-130 |
| Les.3972.1.S1_at | expansin9 | exp9 | -2.1 | i-m | 4.00E-126 |
| Les.141.1.S1_at | expansin A4 | EXPA4 | -2.1 | i-m | 1.00E-124 |
| Les.3569.1.S1_at | expansin A3 | EXPA3 | -2.3 | i-m | 2.00E-115 |
| Les.369.1.S1_at | expansin | EXPA5 | -2.3 | i-m | 6.00E-94 |
| Les.2688.1.S1_at | expansin11 | exp11 | -2.3 | i-m | 6.00E-110 |
| Les.3590.1.S1_at | endo-xyloglucan transferase | ext | -2.4 | i-m | 5.00E-70 |
| Les.4529.1.S1_at | xyloglucan endotransglucosylase-hydrolase XTH7 | XTH7 | -2.7 | i-m | 5.00E-135 |
| Les.4304.1.S1_at | expansin12 | exp12 | -2.7 | i-m | 4.00E-80 |
| Les.4008.2.S1_at | xyloglucan endotransglycosylase/hydrolase 16 protein | XTH16 | -2.7 | i-m | 3.00E-40 |
| Les.4008.1.S1_at | xyloglucan endotransglycosylase/hydrolase 16 protein | XTH16 | -2.9 | i-m | 1.00E-25 |
| Les.4008.1.S1_a_at | xyloglucan endotransglycosylase/hydrolase 16 protein | XTH16 | -2.9 | i-m | 1.00E-25 |
| Les.3733.1.S1_at | expansin | LeEXP2 | -3.3 | i-m | 3.00E-88 |
| Les.4522.1.S1_at | xyloglucan endotransglucosylase-hydrolase XTH6 | XTH6 | -3.5 | i-m | 2.00E-102 |
| | **Degradation** | | | | |
| Les.178.1.S1_at | polygalacturonase inhibiting protein1 | PGIP1 | 3 | m | 6.00E-53 |
| Les.62.1.S1_at | polygalacturonase | PGcat | 2.9 | i-m | 1.00E-80 |
| Les.263.1.S1_at | polygalacturonase isoenzyme 1 beta subunit | LOC543991 | -2 | i-h | 3.00E-148 |
| Les.2187.1.A1_at | (1-4)-beta-mannan endohydrolase, putative | AT5G66460 | -2.2 | h | _ |
| Les.5579.1.S1_at | pectate lyase family protein | AT4G13710 | -2.3 | h | 1.00E-168 |
| Les.4707.1.S1_at | pectate lyase family protein | AT4G24780 | -2.5 | h | 8.00E-198 |
| Les.2298.2.A1_a_at | polygalacturonase (pectinase) family protein | AT3G16850 | -2.6 | w | _ |
| LesAffx.62070.1.S1_at | pectate lyase family protein | AT1G67750 | -2.8 | h | 9.00E-106 |
| LesAffx.59336.1.S1_at | BURP domain-containing protein | AT1G49320 | -3.2 | m | 1.00E-12 |

## Appendix 2: CONT.

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|---|---|---|---|---|---|
| Les.2014.1.A1_at | pectate lyase family protein | AT1G67750 | -4 | h | _ |
| | **Cell wall proteins** | | | | |
| Les.4739.1.S1_at | UDP-glucose:protein transglucosylase-like protein SlUPTG1 | LOC543938 | 2.6 | i-m | 1.00E-108 |
| Les.3409.2.S1_at | Arabinogalactan protein 8 | FLA8 | -2 | m | 4.00E-48 |
| Les.3330.3.A1_at | . | AT4G12730 | -2.1 | m | 1.00E-15 |
| Les.3409.1.A1_at | Arabinogalactan protein 8 | FLA8 | -2.5 | m | _ |
| LesAffx.57251.1.S1_at | . | AT1G03870 | -3 | w | 2.00E-18 |
| **TCA** | **Mitochondrial electron transport / ATP synthesis** | | | | |
| les.4222.1.s1_at | alternative oxidase 1b | LOC543825 | 4.5 | i-m | 2.00E-116 |
| les.4223.1.s1_at | alternative oxidase 1a | LOC543824 | 2.8 | i-m | 6.00E-133 |
| les.4993.1.s1_at | alternative NAD(P)H dahydrogenase 1 | NDA1 | 2.5 | m | 4.00E-104 |
| les.1857.1.a1_at | alternative NAD(P)H dahydrogenase 1 | NDA1 | -2.3 | w | _ |
| | **Mitochondrial_e-transport matabolic transporters** | | | | |
| les.4912.1.s1_at | Peroxisomal adenine nucleotide carrier1 | PNC1 | 3.5 | m | 6.00E-105 |
| les.3691.1.s1_at | UCP protein | UCP | 3.2 | i-m | 5.00E-138 |
| lesaffx.67116.1.s1_at | About de souffle | BOU | 2.7 | w | 3.00E-56 |
| lesaffx.59668.1.s1_at | About de souffle | BOU | 2.7 | m | 1.00E-39 |
| lesaffx.68360.2.s1_at | mitochondrial substrate carrier family protein | AT5G15640 | 2.4 | m | 4.00E-23 |
| lesaffx.68360.1.s1_at | mitochondrial substrate carrier family protein | AT5G15640 | 2.1 | m | 3.00E-37 |
| | **TCA PEP** | | | | |
| les.2323.1.s1_at | pyruvate kinase, putative | AT3G52990 | 2 | h | 1.00E-224 |
| | **TCA / org. transformation** | | | | |
| les.2817.1.s1_at | ACLA-2 | ACLA-2 | 7.6 | h | 2.00E-43 |
| les.3311.3.s1_at | isocitrate dehydrogenase, putative / NADP+ isocitrate dehydrogenase, putative | AT1G65930 | 4.8 | h | 6.00E-73 |
| les.2578.1.s1_at | ACLB-1 | ACLB-1 | 4.6 | m | 0 |
| les.3311.2.s1_at | isocitrate dehydrogenase, putative / NADP+ isocitrate dehydrogenase, putative | AT1G65930 | 4.3 | h | 4.00E-82 |
| les.3311.1.s1_at | isocitrate dehydrogenase, putative / NADP+ isocitrate dehydrogenase, putative | AT1G65930 | 3.2 | h | _ |
| les.4025.1.s1_at | succinyl-CoA ligase alpha 1 subunit | SCOA | 2.8 | i-h | 2.00E-160 |
| les.2817.2.s1_at | ACLA-2 | ACLA-2 | 2.8 | h | 7.00E-07 |
| les.5115.1.s1_at | 2-oxoacid dehydrogenase family protein | AT4G26910 | 2.4 | h | 9.00E-71 |
| les.3773.1.s1_at | cytosolic NADP-malic enzyme | LeME2 | 2.1 | i-h | 2.00E-267 |
| les.461.1.s1_at | NADP-dependent isocitrate dehydrogenase-like protein | LOC544137 | 2 | i-h | 8.00E-76 |
| lesaffx.23253.1.s1_at | ATCS | ATCS | 2 | h | 1.00E-22 |

## Appendix 2: CONT.

| Regulation | WRKY | | | | |
|---|---|---|---|---|---|
| lesaffx.36712.1.s1_at | WRKY53 | WRKY53 | 4.5 | m | 1.00E-54 |
| lesaffx.735.1.s1_at | WRKY33 | WRKY33 | 3.2 | m | 3.00E-32 |
| lesaffx.43341.1.s1_at | WRKY70 | WRKY70 | 2.5 | w | 1.00E-26 |
| lesaffx.9910.1.s1_at | WRKY | LOC100191120 | 2 | i-m | 9.00E-49 |
| | **Protein.degradation** | | | | |
| Les.127.1.s1_at | Subtilisin-like protease | Sbt4a | 44.8 | i-h | 7.00E-137 |
| lesaffx.11941.1.s1_at | Phytophthora-inhibited protease 1 | pip1 | 5.1 | i-m | 6.00E-42 |
| les.4712.1.s1_at | zinc finger (C3HC4-type RING finger) family protein | AT1G49850.1 | 3.5 | w | 3.00E-07 |
| lesaffx.63935.1.s1_at | matrixin family protein | AT1G24140 | 3.2 | m | 1.00E-18 |
| lesaffx.22812.2.s1_at | . | AT3G14250.1 | 3.1 | w | 5.00E-20 |
| les.3675.1.s1_at | metacaspase 1 | MCA1 | 2.6 | i-h | 2.00E-147 |
| les.3155.1.s1_at | Ubiquitin-conjugating enzyme 28 | UBC28 | 2.5 | w | 1.00E-75 |
| lesaffx.42561.1.s1_at | Membrane-anchored ubiquitin-fold protein2 precursor | MUB2 | 2.4 | w | 1.00E-24 |
| lesaffx.66215.1.s1_at | nucellin protein, putative | AT1G77480 | 2.3 | m | 4.00E-22 |
| les.2055.1.s1_at | SKP1/ASK1 interacting protein2 | SKIP2 | 2.3 | h | 8.00E-162 |
| lesaffx.33402.1.a1_at | Metacaspase 1 | AMC1 | 2.2 | h | 4.00E-38 |
| lesaffx.10495.1.s1_at | heat shock protein 70, putative / HSP70, putative | AT4G16660 | 2.2 | h | 8.00E-34 |
| les.5240.1.s1_at | subtilase family protein | AT2G05920.1 | 2.2 | h | 9.00E-100 |
| lesaffx.71026.2.s1_at | zinc finger (C3HC4-type RING finger) family protein | AT3G47550 | 2.2 | m | 2.00E-16 |
| lesaffx.30683.2.s1_at | zinc finger (C3HC4-type RING finger) family protein | AT3G53690.1 | 2.1 | m | 8.00E-35 |
| lesaffx.30683.1.s1_at | . | AT3G14250.1 | 2.1 | m | 3.00E-14 |
| lesaffx.20391.1.s1_at | kelch repeat-containing F-box family protein | AT1G23390 | 2.1 | m | 8.00E-14 |
| les.5228.1.a1_at | Ethylene-dependent gravitropsim and yellow green1 | EGY1 | -2 | h | 5.00E-189 |
| lesaffx.5583.1.s1_at | CAAX amino terminal protease family protein | AT3G26085.1 | -2.1 | m | 5.00E-24 |
| les.4563.1.s1_at | ATP-dependent protease La (LON) domain-containing protein | AT1G35340 | -2.5 | m | 1.00E-40 |
| les.1830.1.a1_at | . | . | -2.7 | w | _ |
| les.2026.2.a1_at | Ring finger protein with Membrane Anchor 1 | RMA1 | -3.7 | w | _ |
| les.2026.1.s1_at | Ring finger protein with Membrane Anchor 1 | RMA1 | -3.8 | w | 6.00E-25 |
| | **Protein.postranslational modification** | | | | |
| les.3502.1.s1_at | auxin-regulated dual specificity cytosolic kinase | LOC543684 | 5.5 | i-h | 6.00E-144 |
| les.1806.1.s1_at | protein kinase, putative | AT2G17220.1 | 3.1 | w | 2.00E-14 |

**Appendix 2: CONT.**

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|---|---|---|---|---|---|
| lesaffx.10313.1.a1_at | protein kinase, putative | AT2G17220 | 3 | h | 6.00E-47 |
| lesaffx.12647.1.s1_at | protein kinase family protein | AT5G55560.1 | 2.8 | w | 1.00E-24 |
| lesaffx.63980.1.s1_at | protein tyrosine phosphatase | AT3G02800 | 2.8 | m | 5.00E-22 |
| les.1806.2.a1_at | . | . | 2.6 | w | _ |
| lesaffx.70335.1.s1_at | protein kinase, putative | AT3G57700.1 | 2.5 | w | 1.00E-33 |
| lesaffx.344.12.s1_at | protein phosphatase 2C, putative / PP2C, putative | AT1G34750 | 2.5 | m | 2.00E-24 |
| lesaffx.70568.1.s1_at | NAD kinase 1 | NADK1 | 2.5 | h | 4.00E-109 |
| les.1235.1.a1_at | protein phosphatase 2C, putative / PP2C, putative | AT1G34750 | 2.5 | m | _ |
| les.5215.1.s1_at | kelch repeat-containing serine/threonine phosphoesterase family protein | AT4G03080.1 | 2.4 | ni | 0 |
| lesaffx.5860.1.s1_at | protein phosphatase 2C/ PP2C, putative | AT4G28400 | 2.3 | m | 2.00E-21 |
| les.1297.1.s1_at | Chitin elicitor kinase 1 | CERK1 | 2.3 | h | 1.00E-52 |
| les.3539.1.s1_at | phosphoenolpyruvate carboxylase kinase | LOC543633 | 2.1 | i-m | 2.00E-59 |
| les.5948.1.s1_at | MAPKK | LeMKK2 | 2.1 | i-m | 4.00E-127 |
| les.2855.1.s1_at | MAP kinase kinase | mek1 | 2.1 | i-m | 1.00E-67 |
| les.738.1.s1_at | Protein kinase 2B | APK2B | 2.1 | h | 9.00E-163 |
| lesaffx.344.7.s1_at | CBL-interacting protein kinase23 | CIPK23 | 2 | h | 9.00E-36 |
| lesaffx.64831.1.s1_at | Serine/threonine protein phosphatase 2A regulatory subunit B', putative | PP2A-AT1G13460 | 2 | h | 6.00E-56 |
| lesaffx.58097.1.s1_at | protein kinase, putative | AT2G07180.1 | 2 | m | 9.00E-28 |
| lesaffx.10444.1.s1_at | Protein phosphatase 2CA | PP2CA | -2.3 | m | 6.00E-38 |
| lesaffx.36086.1.s1_at | tyrosine specific protein phosphatase family protein | . | -2.7 | w | 1.00E-22 |

*Similarity or identity to *Arabidopsis*: (w) weakly similar; (m) moderately similar; (h) highly similar; (ni) nearly identical; (i) identical from tomato sequences dataset.

Background colours of fold change values ranged from strong red to green regarding to the expression level of genes.

**Appendix 3:** Expression profile of differentially expressed genes involved in defense responses in tomato leaves infected by *Phytophthora infestans* 12 hours post inoculation. Molecular network analyses have done by Mapman and TFGD (P value <5%).

| Probeset ID | Gene title | Gene symbol | Fold change | *ID (%) | E value |
|---|---|---|---|---|---|
| Les.3575.1.S1_at | Pti5 | LOC544042 | 16.7 | . | 3.00E-23 |
| LesAffx.57572.1.S1_at | Patatin-like protein 5 | PLP5 | 13.5 | 56.4 | 3.00E-59 |
| LesAffx.62349.1.S1_at | NADPH:quinone oxidoreductase | NQR | 11.6 | 68.7 | 7.00E-30 |
| Les.3652.1.S1_at | glucan endo-1,3-beta-D-glucosidase | tomQ`b | 11.5 | . | 2.00E-94 |
| Les.3583.1.A1_at | TSI-1 protein | TSI-1 | 10 | . | 6.00E-99 |
| Les.129.1.S1_at | divinyl ether synthase | LeDES | 9.2 | . | 2.00E-123 |
| Les.37.1.S1_at | chitinase | LOC544146 | 6.1 | . | 5.00E-61 |
| LesAffx.1959.1.S1_at | Glutathione S-transferase PHI 8 | ATGSTF8 | 6 | 64.1 | 1.00E-71 |
| Les.3493.1.S1_at | phospholipase PLDb1 | PLDb1 | 5.8 | . | 0 |
| LesAffx.69659.1.S1_at | ATEP3 | ATEP | 5.6 | 72.3 | 1.00E-102 |
| Les.2747.2.S1_at | Peroxisomal 3-ketoacyl-COA thiolase3 | PKT3 | 5.5 | 83.5 | 5.00E-48 |
| Les.4999.1.S1_at | peroxidase 21 (PER21) (P21) (PRXR5) | AT2G371330 | 5.5 | 74.3 | 6.00E-110 |
| LesAffx.3002.1.S1_at | Glutathione S-transferase TAU 8 | ATGSTU8 | 5.4 | 52.8 | 6.00E-19 |
| Les.2747.1.S1_at | Peroxisomal 3-ketoacyl-COA thiolase3 | PKT3 | 5.3 | 83.5 | 3.00E-11 |
| Les.4924.1.S1_at | Peptide transporter 3 | PTR3 | 5.3 | 73.5 | 4.00E-223 |
| LesAffx.36712.1.S1_at | WRKY53 | WRKY53 | 4.5 | 52.8 | 1.00E-54 |
| Les.4496.1.S1_at | TSI-1 protein | TSI-1 | 4.5 | . | 6.00E-06 |
| Les.2832.1.S1_at | peroxidase | cevi16 | 4.2 | . | 3.00E-108 |
| LesAffx.71065.1.S1_at | pathogenesis-related family protein | AT1G787800 | 4.1 | 53.4 | 7.00E-22 |
| LesAffx.16424.1.S1_s_at | mitogen-activated protein kinase3 | MPK3 | 4 | . | 8.00E-24 |
| Les.5077.1.S1_at | necrotic spotted lesions 1 | NSL1 | 3.9 | 63.9 | 3.00E-213 |
| Les.3140.3.S1_at | Peroxisomal 3-ketoacyl-COA thiolase3 | PKT3 | 3.6 | 83.5 | 2.00E-36 |
| Les.3140.2.S1_at | Peroxisomal 3-ketoacyl-COA thiolase3 | PKT3 | 3.4 | 83.5 | 3.00E-27 |
| Les.131.1.S1_at | hypothetical LOC543813 | LOC543813 | 3.4 | . | 6.00E-63 |
| Les.13.1.S1_at | allene oxide synthase | aos | 3.4 | . | 3.00E-187 |
| LesAffx.735.1.S1_at | WRKY33 | WRKY33 | 3.2 | 61.8 | 3.00E-32 |
| Les.4316.1.S1_at | mitogen-activated protein kinase3 | MPK3 | 3.2 | . | 6.00E-144 |
| Les.178.1.S1_at | Polygalacturonase inhibiting protein1 | PGIP1 | 3 | 67.6 | 6.00E-53 |
| Les.321.1.S1_at | molecular chaperone Hsp90-1 | LeHsp90-1 | 3 | . | 0 |
| LesAffx.42534.1.S1_at | Lipid phosphate phsphatase 3s | LPP3 | 2.9 | 66.7 | 2.00E-23 |
| Les.3673.1.S1_at | beta-1,3-glucanase | LOC543986 | 2.7 | . | 8.00E-64 |

**Appendix 3: CONT.**

| | | | | | |
|---|---|---|---|---|---|
| LesAffx.43341.1.S1_at | WRKY70 | WRKY70 | 2.5 | 55.5 | 1.00E-26 |
| LesAffx.3554.1.A1_at | Patatin-like protein4 | PLP4 | 2.5 | . | 2.00E-43 |
| Les.3632.1.S1_at | Lipoxygenase | loxD | 2.5 | . | 1.00E-210 |
| Les.122.1.S1_at | Chitinase | LOC544149 | 2.5 | . | 4.00E-46 |
| Les.2137.1.S1_at | EIX receptor1 | Eix1 | 2.4 | . | 7.00E-77 |
| Les.123.1.S1_at | hypothetical LOC543814 | LOC543814 | 2.3 | . | 3.00E-57 |
| Les.3506.1.S1_at | verticillium wilt disease resistance protein Ve2 | Ve2 | 2.2 | . | 8.00E-103 |
| LesAffx.1959.3.S1_at | GSTF7 | GSTF7 | 2.2 | 64.1 | 2.00E-10 |
| Les.3769.1.S1_at | 1-aminocyclopropane-1-carboxylate synthase | ACS6 | 2.2 | . | 5.00E-177 |
| Les.2874.2.S1_a_at | signal recognition particle receptor alpha subunit family protein | AT4G30600 | 2.1 | 79.5 | 1.00E-47 |
| LesAffx.50082.1.A1_at | Peptide transporter1 | PTR1 | 2.1 | 74.9 | 2.00E-81 |
| Les.5948.1.S1_at | MAPKK | LeMKK2 | 2.1 | . | 4.00E-127 |
| Les.2855.1.S1_at | MAP kinase kinase | mek1 | 2.1 | . | 1.00E-67 |
| Les.5060.1.S1_at | MAP Kinase4 | ATMPK4 | 2.1 | 83.1 | 3.00E-179 |
| LesAffx.9910.1.S1_at | WRKY | LOC100191120 | 2 | . | 9.00E-49 |
| LesAffx.69997.2.S1_at | . | AT2G16230 | 2 | 76.2 | 1.00E-23 |
| LesAffx.46815.2.S1_at | DNA-damage repair/toleration100 | DRT100 | -2 | 63 | 4.00E-93 |
| LesAffx.69215.1.S1_at | leucine-rich repeat family protein | AT3G20820 | -2.2 | 70.6 | 2.00E-74 |
| Les.5864.1.S1_at | 2-oxoglutarate-dependent dioxygenase homolog /// hypothetical LOC544002 | GAD2 /// LOC544002 | -2.2 | . | 1.00E-52 |
| LesAffx.14776.1.S1_at | MLP-like protein 28 | MLP28 | -2.2 | 51.3 | 4.00E-36 |
| Les.4255.2.S1_at | hypothetical protein | AT4G23890 | -2.2 | 69.5 | _ |
| LesAffx.30937.1.S1_at | CYP71B9 | CYP71B9 | -2.4 | . | 3.00E-14 |
| LesAffx.1959.2.S1_at | myb family transcription factor | AT1G19000 | -2.4 | 63.9 | 7.00E-29 |
| Les.4139.1.S1_at | ethylene response factor 4 | ERF4 | -2.8 | . | 7.00E-34 |
| LesAffx.6688.1.S1_at | Indole-3-acetate beta-D-glucosyltransferase | IAGLU | -3.2 | 58 | 6.00E-71 |
| Les.417.1.S1_at | gibberellin-regulated family protein | AT5G59845 | -3.6 | 65.2 | 1E-26 |

* Similarity or identity to *Arabidopsis* thaliana given as percentage

Background colours of fold change values ranged from strong red to green regarding to the expression level of genes.

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# CURRICULUM VITAE

## 1. Personal information

## 2. Education

1997 – 2002: Bachelor of Agriculture. Department of Plant Protection, Faculty of Agriculture, Damascus University, Syria.

2002 – 2003: Diploma of Agriculture. Department of Plants Protection, Faculty of Agriculture, Damascus University, Syria.

2006 – 2012: Ph.D student. Institute of Crop Science and Resource Conservation. INRES-Phytomedicine. University of Bonn, Germany.

## 3. Work experience

2003 – 2004: Work at the General Corporation for Scientific and Agricultural Researches (GCSAR)-Plants protection section- field researcher. Meanwhile, I participated in two researches (restricting Nematode of beans in the middles coastal areas and Fighting Nematode with Tricoderma Fungi)

Feb – present 2012: Scientific assistant, Agricultural Faculty, INRES-Phytomedicine, University of Bonn, Germany

**4. Training courses**

1th October 2004 – 28th May 2005: German course (ZD, B1) at Goethe institute, Damascus, Syria

April – September 2006: German course (DSH, B2) University of Bonn, Germany.

17th – 20th April 2007: Data analyzing by SPSS software at Hochschulrechenzentrum, University of Bonn, Germany

3rd December 2008: Diploma of English. Wall Street Institute, Bonn, Germany

4th – 6th December 2009: Learning Intercultural Competence in Windeck-Rosbach by the Bonn Interdisciplinary Graduate school for Development Research (BiGS-DR) of ZEF (Center for Development Research, University of Bonn) funded by DAAD (German Academic Exchange Service).

1th – 4th March 2011: (40 Hours) Active ingredients and plants: uptake, translocation, and biological responses (BIGS-TB-32) at Theodor Brinkmann Graduate School, University of Bonn, Germany

28th March – 1th April 2011: "In silico experimentation" (BIGS-TB-45) at Theodor Brinkmann Graduate School, Agricultural Faculty, University of Bonn, Germany

**5. Abstracts / poster or oral presentation**

Muna Sultan, Heinz-Wilhelm Dehne, Ulrike Steiner. "Investigations on the efficacy of *Bacillus* spp. strains on suppression of tomato diseases (Untersuchungen zur Wirksamkeit von *Bacillus*-Arten gegen Krankheitserreger an Tomatenpflanzen)." In: 56. Deutsche Pflanzenschutztagung "Pflanzenproduktion im Wandel - Wandel im Pflanzenschutz?" Kiel, Germany, 22-25 September, 2008 (Poster).

Muna Sultan, Heinz-Wilhelm Dehne, Ulrike Steiner. The Efficacy of *Bacillus amyloliquefaciens* on Late Blight Development and Biomass of *Phytophthora infestans* in Tomato Leaf Tissue. In: "Biophysical and Socio-economic Frame Conditions for the Sustainable Management of

Natural Resources" Tropentag, Hamburg, Germany, 6 - 8 October, 2009 (Poster). Journal of Plant Diseases and Protection (2010). 117(2): 80-87.

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Muna Sultan, Heinz-Wilhelm Dehne, Ulrike Steiner. Histological Studies on the Efficacy of *Bacillus amyloliquefaciens* on Early Developmental Stages of *Phytophthora infestans* on Tomato Leaves. In: "World Food System - A Contribution from Europe" Tropentag, Zurich, Germany, 14-16 September, 2010 (Poster).

Muna Sultan, Dessie Salilew Wondim, Dawit Tesfaye, Karl Schellander, Abd El Naser El Ashry, Florian M. W. Grundler, Heinz-Wilhelm Dehne, Ulrike Steiner. "Microarray Analysis of Gene Expression Induced by *Bacillus subtilis* in Tomato Leaves infected with *Phytophthora infestans*." In: "Development on the margin" Tropentag, Bonn, Germany, 5 - 7 October, 2011 (Oral).

Muna Sultan, Abd El Naser El Ashry, Heinz-Wilhelm Dehne, Ulrike Steiner. "Gene Expression Profile of Tomato Leaves Induced by *Phytophthora infestans*" In: Tagung der DPG-Arbeitskreise Mykologie und Wirt-Parasit-Beziehungen, Stuttgart-Hohenheim, Germany, 22 - 23 March, 2012 (Oral).

**6. Conference attendance**

25th – 26th March 2010: Tagung der DPG-Arbeitskreise Mycologie und Wirt-Parasit Beziehungen, Konstanz, Germany

3rd – 5th November 2010: Next Generation Sequencing Symposium, Max Planck Institute for Plant Breeding Research, Cologne, Germany

24th May 2011: 63rd International Symposium on Crop Protection, Ghent, Belgium