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Induce resistance in tomato plant against *Alternaria solani* by *Bacillus subtilis* strain 30B-B6

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Abstract

Early blight (EB) caused by the necrotrophic fungus *Alternaria solani* is a devastating plant disease of tomato crops, currently mainly controlled by synthetic chemical fungicides. In the framework of sustainable agriculture, a promising new control strategy is the induction of plant defense responses by elicitors. These last decades, numerous researches on new elicitors have emerged, and recent findings have reported a promising strain of *Bacillus subtilis*, 30B-B6, as a potential elicitor of tomato plants.

The present study investigates the systemic induction of defense response in tomato (*Solanum lycopersicum* var. Moneymaker) against *A. solani* strain MBC9282, using *B. subtilis* 30B-B6 as an elicitor. Trials were conducted under controlled greenhouse conditions. Results revealed that root application of the eliciting bacterium 6 hours before the fungal leaf inoculation significantly increased tomato plant protection by 83% against EB compared to control plants. Disease protection was assumed to be due to the induction of resistance in the host plant since experimental protocols were designed in order to avoid any direct contact between the biocontrol agent and the pathogen.

A second bioassay with the bacterial supernatant (filtered 30B-B6 suspension) highlighted an inability of the metabolite suspension to trigger tomato resistance against *A. solani*, when the bacterium was not present.

A third bioassay was performed to evaluate the expression of tomato defense-related genes by RT-qPCR on four treatment groups: (1) bacterial elicitation, (2) fungal inoculation, (3) both bacterial elicitation and fungal inoculation and (4) control plants. Gene expression levels were assessed separately on tomato's lower-older leaves and upper-younger leaves. The fungal inoculations were performed on the lower leaves, while the upper ones served for a systemicity assessment. The target genes analyzed were assigned to specific plant metabolic pathways related to the induced systemic resistance (ISR) and/or the systemic acquired resistance (SAR): the SAR marker *PR1* gene, the ISR markers *LOX* and *PI-1* genes, the *NPR1* gene linked to the crosstalk between both signaling pathways and *PR2-b* gene, responsive to salicylic acid and/or jasmonic acid. The fungal inoculation of non-elicited plants led to a systemic significant increase in the *PR2-b* gene expression compared to non-inoculated plants, at 6 hours post inoculation (hpi). No activation of *PR1* gene was detected in plants challenged by the pathogen. The 30B-B6 application triggered significant increases in *LOX* and *PI-1* gene expression in tomato upper leaves, respectively at 6 hpi and 9hpi, compared to the other treatments. However, when both the bacterium and the fungus were present, only the *PR2-b* gene expression was up regulated in the systemic leaves compared to uninoculated plants, at 24 hpi. Our results indicate that the 30B-B6 root application enhances tomato resistance to EB, deriving from an induction of defense genes, but the signaling pathways involved remain equivocal. The promising features of 30B-B6 highlighted in the present work make it a potential biological agent in the control of tomato crops against EB and encourage its practical use in agriculture.

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Chapter 1

Introduction

Plants are constantly challenged by a wide range of organisms, including bacteria, viruses, fungi, oomycetes, insects and nematodes. In this hostile environment, plants must continuously adapt their defenses to survive. Besides constitutive defenses and structural barriers, plants have evolved a complex molecular network to prevent from biotic and abiotic stresses. This is the plant immune system, based on pattern-recognition receptors (PRRs) mainly localized in the plasma membrane that detect conserved molecular patterns of external agents, referred to as elicitors. Inside the cells, similar recognition occurs using the polymorphic nucleotide binding – leucine rich repeat (NB-LRR) proteins, mainly encoded by R genes [Jones and Dangl, 2006]. These perceptions lead to changes in the plant metabolism driving by phytohormones, essentially jasmonic acid (JA), ethylene (ET) and salicylic acid (SA), allowing the set of plant defenses against potential invaders. The stimulation of the host immune system following a pathogen intrusion is usually referred to as systemic acquired resistance (SAR). Furthermore, some beneficial microbes are also able to induce a resistance in the host plant, generally through another mechanism involving other signaling pathways, referred to as induced systemic resistance (ISR). These two mechanisms both lead to a systemic immunity through specific long distance signaling pathways.

Alternaria solani is a necrotrophic fungus causing diseases on solanaceae, including the early blight (EB) of tomato and potato leaves, which leads to important crop losses. Currently, the management of EB depends mainly on the use of fungicides such as quinone outside inhibitor (QoI) and copper-based compounds. The increased concerns of searching alternative methods to control diseases and reduce the farmer dependency on chemical products have led to the emergence of many promising biological pesticides, such as beneficial and antagonistic microbes that can promote plant growth and protect the plant against biotic and abiotic stresses. Among these potential bioagents, members of the Bacillus family constitute an important source, due to their broadspectrum antagonistic activity against pathogens and their ubiquitous presence in the soil. *Bacillus subtilis* strain 30B-B6 has recently been reported by Caulier and his team as a promising bacterium in the protection against many potato diseases, including the EB [Caulier et al., 2018]. The bacterium also demonstrated a high ability of protecting tomato, *Solanum lycopersicum*, against the EB. A recent bioassay indicated that this bacterium could stimulate the plant immune system.

This work aims at confirming the role of *B. subtilis* 30B-B6 in the induction of tomato resistance against EB and studying the molecular mechanisms in the induced systemic immunity. To reach those objectives, bioassays were conducted and the dynamic of gene expression profiles of tomato plants treated by 30B-B6 and/or inoculated by *A. solani* was analysed, compare to healthy and untreated control tomatoes. Several specific genes have been selected, according to their function in the plant defense response and their involvement in one signaling pathway or another. The analyses of those gene expression levels were performed through reverse transcription quantitative PCR (RT-qPCR), an accurate method allowing the quantification of the amount of specific mRNA transcripts in a sample. The expression profile was monitored at specific time points following the bacterial elicitation and/or fungal inoculation of tomatoes.

The present work is structured as follows. In a second chapter, the important features of *S. lycopersicum*, *A. solani* and *B. subtilis* 30B-B6 are given, as well as current knowledge on mechanisms of plant immunity. Chapter 3 exposes the material and methods used in the experimental part of this work. Subsequently, experiment results are presented in chapter 4 and are discussed in chapter 5. Finally, this work ends with a conclusion and opens on further perspectives.

Chapter 2

State of the art

This state of the art introduces the main actors of this work and set the major concepts of plant immunity. First, an introduction and general characteristics of both agents involved in the pathosystem *Solanum lycopersicum / Alternaria solani* are developed. Then, the epidemiology of early blight (EB) disease triggered by leaf infection by *A.solani* and the compounds implied in the pathogenicity are exposed. The subsequent part is dedicated to the introduction to the basis of the plant immune system and the common plant defense responses deployed by plants. A specific section on the induced resistance is then presented, insisting on the induced systemic resistance (ISR), which is the key concept of the present study. The last part of this literature review introduces to *Bacillus subtilis* 30B-B6, a tomato elicitor against diverse pathogens, including *A. solani*.

2.1 *Solanum lycopersicum*

2.1.1 Taxonomy and history

The cultivated tomato, named as *Solanum lycopersicum* by Linnaeus in 1753 (formerly *Lycopersicon esculentum*), belongs to the *Solanaceae* family. The species is native to Andrean region of South America, but has been first domesticated in Mexico in 1544 [Jones, 2007] [Goffau et al., 2005]. It has been introduced in Europe by the Spanish in the sixteenth century [Razdan, 2006]. Initially grown as ornamental plants, it was thought to be a poison and was produced for its edible fruits only much later. Its cultivation has become more popular since mid-nineteenth century because of its varied climatic adaptability and its high nutritional value [Roopa, 2012]. Nowadays, it is the world's second most consumed vegetable, just after potato [Adhikari et al., 2017]. Tomato fruits are consumed in several ways: fresh, mixed in other food items or processed, with major products being canned tomatoes, ketchup, juices, sauces, paste, soups, powder, etc. [Adhikari et al., 2017] [Razdan, 2006].

Tomato fruits constitute a rich source of nutrients with interesting health benefit. A tomato contains 95% of water, 4% carbohydrates and less than 1% each of fat and protein (Figure appendix B.1.). It provides important amount of vitamin A and C as well as compounds with antioxidant properties, such as the lycopene[1] [Adhikari et al., 2017]

[1]Red carotenoid pigment, whose chemical formula is $C_{40}H_{56}$, found in tomatoes and other red fruits and vegetables, such as carrots, watermelons, cherries, etc. It is assumed that lycopene participates to the human protection against cancer and heart disease

[Blancard, 2009]. Because of its wide use and high nutritional values, this vegetable is more and more consumed, thereby requiring always an enhanced production.

2.1.2 Botanical description

Tomato is a herbaceous perennial, although it is often annual in temperate regions due to its intolerance to the cold temperatures. It is a dicot with indeterminate growth, continuously producing three nodes between each inflorescence. There exist some determinate varieties with terminal inflorescence, breeding for their ease in mechanical harvest. Wild types are usually cross-pollinated, although selection leads to self-pollinated varieties [Jones, 2007]. Thus, domestic cultivars are much more self-compatible, with a pistil that extends far less out of the flowers than wild type varieties. Wild tomato species have tiny fruits of various colors, mainly inedible except some varieties. The main varieties that have been bred for consumption are the following:

- *Solanum lycopersicum var. esculentum*, tomato
- *Solanum pimpinellifolium*, currant tomato
- *Solanum lycopersicum var. cerasiforme*, cherry tomato

However, there exist many more varieties, over 10 000 have been identified until now. Tomato cultivation is encountered in all parts of the world as it tolerates a wide range of temperature, with an optimum for growth between 18 and 23 °C [Goffau et al., 2005]. It is a highly drought intolerant plant. A brief morphological description of tomato plants is presented below.

Vegetative system

Tomato plants have strong, pivoting root producing a high density of lateral and adventitious roots. The growth is first monopodial and turns in sympodial after 4- to 5 leaves formed. The solid stem is highly hairy and pubescent, allowing the vining process of the plant. The alternate leaves are odd-pinnately lobed, up to 25 cm long, with 5 to 9 leaflets, each one measures up to 8 cm long (see figure 2.1). The leaflets are densely hairy and have a serrated margin. Both the stem's and leaves hairs are either simple or glandular, producing essential oil giving the plant its typical smell [Goffau et al., 2005], [Britannica, 2018].

Reproductive system

The yellow flowers are bisexual and measure about 2 cm in diameter with 5 petals on the corolla. They are clustered in a cyme of 3 to 12 flowers. There are 6 stamens whose anthers are fused at the top, encapsulating the style. Bees and bumbles are the main tomato pollinators. The fruit developed from the fertilized ovary is a berry which displays a wide range of size, shape and color. They can measure 1 to 10 cm in diameter, with varying shape from spherical to oval and elongate, red color as well as yellow, green and purple. The fruit contains hollow spaces full of seeds and moisture, called locular cavities. The seeds are numerous, pear shape, hairy, measuring 3-5 x 2-4 mm and have an epigeous germination (see figure 2.1) [Goffau et al., 2005], [Britannica, 2018].

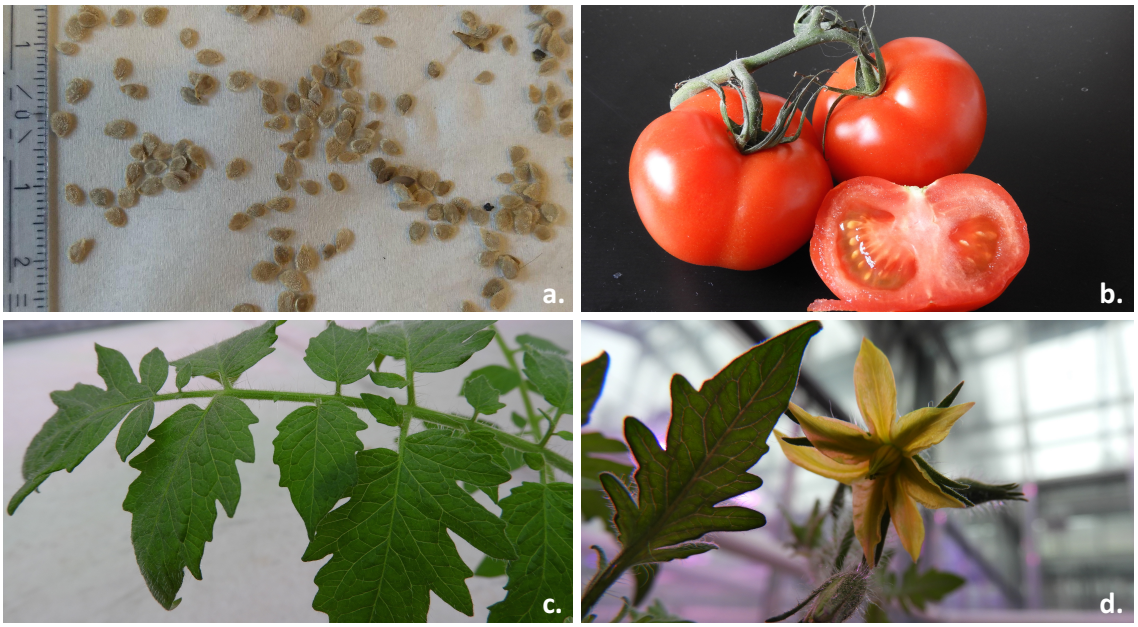

Figure 2.1: Picture of tomato organs: a. Seeds. b.Fruits. c. Leaves. d. Flowers.

2.1.3 Cultivation

Production

The world tomato production reached 177 million tons in 2016 and the average yield was about 37 t/ha, but it depends a lot of the region producers [FAOSTAT, 2016]. Among them, Belgium accounted for 0,15% of this production, which stood for 259,5 thousand tons. The record was held by Asia, which provided 60,1% of the tomato world production in 2016. The figure 2.2 shows the 10 bigger tomato producers in the world in 2016 [FAOSTAT, 2016]. A general increase in tomato production and consumption has been observed since the beginning of the last century [Razdan, 2006]. Consequently, a high production of tomatoes is required to fulfill this demand.

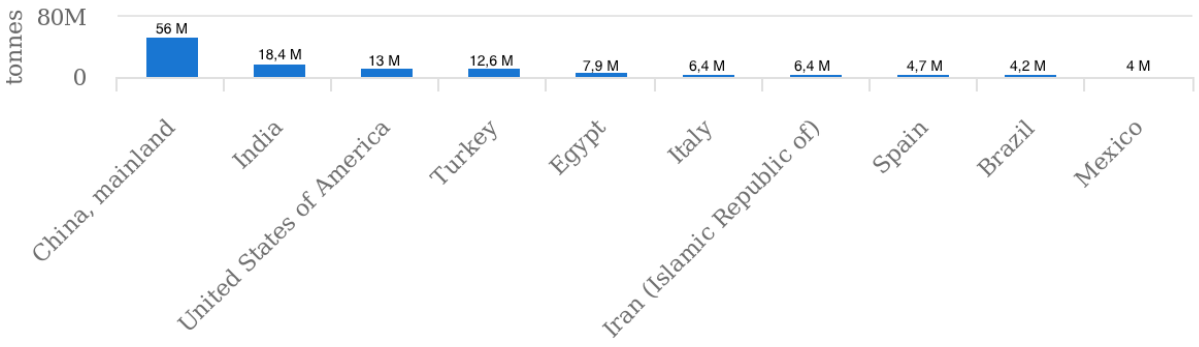

Figure 2.2: Production of tomatoes in 2016: world top ten producers [FAOSTAT, 2016]

Diseases and pests

The genetic diversity of cultivated tomato is quite narrow due to its intensive selection through centuries of domestication. This homogeneity obviously has led to a higher

susceptibility to multiple pathogens (fungi, chromista, bacteria, virus), insects and nematodes. This is the current reality of tomato commercial production, which is threatened by many biotic stresses that sometimes lead to catastrophic yield reductions [Jones, 2007]. Among the most damaging tomato pathogens, the early and late blight, caused by *Alternaria solani* and *Phytophthora infestans* represent an important part. Other fungal diseases also affect tomato crops: anthracnose (*Colletotrichum coccodes*, gray mold (*Botrytis cinerea*), fusarium wilt (*Fusarium spp.*), etc. Several pathogenic bacteria can also triggered severe disease on tomato, such as *Clavibacter michiganensis*, agent of bacterial ring rot, *Pectobacterium carotovorum*, responsible of the aerial stem rot of the tomato, etc. Finally, the most important viruses encountered on tomato are the *Potato leaf roll virus* (PLRV), *Potato Virus X* (PVX), *Potato Virus Y* (PVY), *Tomato Mosaic Vurus* (ToMV). Nematodes, such as *Meloidogyne spp.* and insect like *Bactericera cockerelli* also are significant pests of tomato crops. All of these diseases and pests affect all *Solanaceae*, including potato crops, which are economically very important [Blancard, 2009] citeAdhikari2017.

Thus, an important challenge is to find solutions to keep a high production of tomato while limiting the environmental impact of intensive use or over chemical pesticide applications. Actually, chemical pesticide applications allow the repression of many pests and diseases and lead to quick and efficient results. However, they are very costly and lead to a heavy dependence of farmers, which thus requires a reasonable and limited use of this chemical approach. Moreover, although chemical pesticides are generally very efficient in controlling pests and diseases, an abusive use leads to the emergence of resistance and an inefficiency of some products [Blancard, 2009], [Jones, 2007]. Therefore, alternative methods are required for the management of crop biotic attacks. The integrated pest management (IPM) provides such a service, by controlling pests and diseases through the keeping of the undesirables at a certain level which is not economically damaging. IPM combines a variety of measures such as the use of resistant or tolerant cultivars, the respect of appropriated cultural practices, involving long rotations or mixed cultivation, and a moderated application of pesticides, focusing predominantly on bio-pesticides [Jones, 2007].

Resistant cultivars are a key approach in preserving crops from pests and microorganism attacks. Unlimited resources of resistance are found in wild type species, and breeding for resistance is mainly based on hybridation between commercial cultivars and heirloom plants [Adhikari et al., 2017]. Another major aspect for sustainable control of diseases is the application of bio-pesticides, which includes the so-called elicitors of defenses, that stimulate plant defenses and/or growth.

2.2 *Alternaria solani*

2.2.1 Taxonomy, morphology and general characteristics of *Alternaria spp.*

Alternaria belongs to the domain *Eukaryota*, kingdom *Fungi*, phylum *Ascomycota*, class *Dothideomycetes* and order *Pleosporales* [Agrios, 2005], [Lawrence et al., 2013]. It is an ubiquitous fungal genus that includes saprophytic, endophytic and pathogenic species. It

joins with a wide diversity of substrates, including plants, agricultural products, animals and soil. Some species of this genus caused serious diseases on plants while growing and in storage, resulting in major losses on several crops [Chaerani et al., 2007], [Woudenberg et al., 2013]. Pathogen species of this genus are predominantly necrotrophic agents. *Alternaria* was first described by Nees in 1816, based on *A. tenius* which was later renamed by Keissler in 1912 as *A. alternata* [Elliot, 1916], [Simmons, 1967]. Hyphal cells are multinucleate and dark-colored due to melanin, a pigment that provides protection against adverse environmental conditions, including resistance against hydrolytic enzymes of other microbes [Chaerani and Voorrips, 2006], [Adhikari et al., 2017], [Woudenberg et al., 2013].

The heterokaryosis[2] could explain the important genetic variation within *Alternaria* genus. This occurence of multiple genetically different nuclei in a same cell is probably the result of hyphal anastomosis. This high genetic variability provides the fungus the ability to react quickly to changing environments. This characteristic may facilitate the development of resistances against fungicides as well as the bypass of resistance genes in host plants [Pasche et al., 2004]. This source of variety through genetic mixing is interesting in *Alternaria* as no sexual stage is known in the genus, except for one sexual lineage, *A. infectoria* [Woudenberg et al., 2013]. However, scientific community claims some ascomycete fungi to be the equivalent teleomorph stage of several *Alternaria* species, for instance, *Pleospora solani* would be the teleomorph stage of *Alternaira solani* [Adhikari et al., 2017], [Chaerani and Voorrips, 2006].

2.2.2 Specific features of *Alternaria solani*

A. solani is a species of big interest in the *Alternaria* genus due to its implication in many diseases on *Solanaceae* plants. It belongs to the section *Porri*, which contains the largest number of species of all *Alternaria* sections, with 82 species. They are characterized by medium to large conidia and long beaks. Among them, a lot of plant pathogen species are encountered including *A. solani*, the causal agent of EB disease of several *Solanaceae*, including tomato and potato [Woudenberg et al., 2014].

A. solani was first described by Ellis and G. Martin in 1882 who named it *Macrosporium solani*. This name was then changed by Sorauer in 1896 as *Alternaria solani*, the current official name. The classification and synonyms of the fungus is given in table 2.1 [Woudenberg et al., 2014], [Mycobank, 2018].

Conidia of *A. solani* are usually single, occasionally in short chains, with an oblong or ellipsoidal body, with beaked apical cells which are commonly the same length as the body. They measure 150 - 300 x 15 - 19 μm, with 9-11 transverse septa and none or a few longitudinal septa. Conidiophores arising singly or in small groups, septate, pale brown, thick-walled, up to 110 x 6-10 μm (see figure 2.3). Colonies are grewish brown to black and hairy [Agrios, 2005], [CMI, 1975].

A. solani is a common pathogenic fungus of potato, tomato and other Solanaceae crops and has also been detected on a wide range of other species [Chaerani and Voorrips, 2006], [CMI, 1975]. It is an air borne and soil inhabiting pathogen [Roopa, 2012]. Another closely related species, *A. tomatophila*, has been recognized in 2000 as a widely distributed

[2]A cell is heterokaryotic when it is multinucleate with genetically different nuclei. It can occur during sexual reproduction, or by the fusion of two genetically different cells, or through hyphal anastomosis.

Table 2.1: Old name, synonyms and classification of *Alternaria solani* Sorauer, Z. Pflanzenkrankh. Pflanzen-schutz (1896). From [Woudenberg et al., 2014] and Mycobank.

Old name	Synonyms	Classification
	Alternaria americana Sawada, Rep. Dept. Agric. Gov. Res. Inst. Formosa (1931)	
Macrosporium solani (Ellis and G. Martin), The American naturalist (1883)	*Alternaria porri f. sp. solani* (Ellis and G. Martin) Neerg, Danish species of Alternaria and Stemphylium (1945)	*Fungi, Asomycota, Pezizomycotina, Dothideomycetes, Pleosporomycetidae, Pleosporales, Pleosporaceae, Alternaria*
	Alternaria solani (Ellis and G. Martin) L.R. Jones and Grout, Vermont Agric. Exp. Sta. Annual Rep. (1899) (illegitimate name, Art. 53.1)	

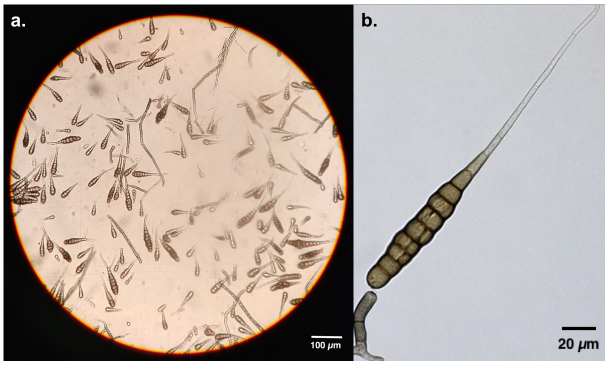

Figure 2.3: a. Conidia and conidiophores of *Alternaria solani* observed at the optical microscope at the 400 times magnifying. b. Focus on one conidia and its conidiophore. From [Woudenberg et al., 2014].

causal agent of EB of tomato [Simmons, 2000], like *A. solani*. More recently, the taxonomy of *Alternaria* has been revised with the inclusion of a new species in the genus, *A. linearіae*, that includes *A. solani* and *A. tomatophila* [Bessadat et al., 2017]. This last one includes large-spored isolates specific to Solanaceae, Cucurbitaceae and Scrophulariaceae. They distinguish from small spore forms characteristic to *A. alternata* and *A. arborescens*.

The diversity among isolates of *A. solani* is considerable, despite this fungus possesses only an asexual reproduction. As explained above, it is probably due to its heterokaryotic cells and natural mutations [Adhikari et al., 2017]. Because of this high genetic variability, *A. solani* has a strong adaptability to survive in a hostile environment and developes a lot of resistances against fungicides. Moreover, the fungus can also easily overcome the existing genetic resistance of the host, leading to many susceptible cultivars to EB disease [Pasche et al., 2004], [Adhikari et al., 2017]. However, no race specific resistance (R gene resistance type, explained in section 2.3.1) seems to be involved in the interaction host R proteins and pathogen avirulence genes, as no races are known for *A. solani* [CMI, 1975], [Gill, 1999], [Adhikari et al., 2017]. The compounds imply in the plant pathogen interaction are described in the next section. Even if no races have been described for the

fungus, a wide variation in pathogenicity of isolates is observed [CMI, 1975].

2.2.3 Compounds imply during *A. solani* infection

Fungal pathogens produce numerous enzymes, toxins and other secondary metabolites to help the infection of the host plant. Eleven toxins have been identified in filtrate exudates of *A. solani* [Van der Waals et al., 2001]. Among these, alternaric acid and solanapyrone A, B and C are responsible for the typical necrotic symptoms of EB. Alternaric acid is probably the main secondary metabolite produced by the fungi and is non-host specific toxin [Van der Waals et al., 2001]. The toxin is already present in dormant spore and is released and produced in high quantities during germination. It has been observed that when sprayed alone on tomato leaves, alternaric acid is not phytotoxic, but enhances the infection process when coupled with spore suspension [Chaerani and Voorrips, 2006], [Langsdorf et al., 1990]. Concerning solanapyrone, mutant tomato plants unable to produce this substance, did not induced change in virulence compared to wild types, demonstrating the non-pathogenicity of it, despite its strong phytotoxicity [Kim et al., 2015].

During the infection process, host plant detects the fungus and sets up a series of responses to prevent fungal progression. Those include production of secondary metabolites, such as chitinase, β-1,3-glucanase, peroxidase (POX), phenylalanine ammonialyase (*PAL*) and other phenolic compounds (tanin, flavonol and phenol) [Nicholson and Hammerschmidt, 1992], [Adhikari et al., 2017]. They are recognized as pathogenesis related (PR) proteins by the plant, which partly leads to the set of defense responses against fungal attack [Adhikari et al., 2017]. Their mechanisms of action are further described in section 3.5.3 (paragraph "enzymatic productions"). In EB-resistant cultivars, all those compounds are highly produced in stems and leaves compared to susceptible plants [Adhikari et al., 2017]. The constitutive expression of those PR-proteins that possess inhibitor activities has been associated with non-host resistance [Nicholson and Hammerschmidt, 1992].

Results of all studies on *A. solani* highlight the fact that host resistances against this pathogen are not R gene type resistance (pathogen recognition), but rather based on defense response process. EB resistance is governed by multiple genes, and until now no races with specific avr genes have been discovered. It is thus not a gene for gene relationship, and no major resistant genes have been discovered. This assumption is confirmed by QTLs mapping, which shows a high correlation between QTLs of defense response genes and phenotypical disease resistance [Gill, 1999], [Adhikari et al., 2017].

Further research needs to be conducted to discover additional sources of resistance in order to develop resistant cultivars with high yielding potential and early maturity [Adhikari et al., 2017].

2.2.4 Early blight of tomato

Generality and distribution

Early blight (EB) of tomato, caused by *A. solani* and, in a less extend, by *A. tomatophila*, is a severe disease of tomato crops in warm and humid regions, and in semiarid areas where frequent wet nights occur. EB reduces the photosynthetic surface of leaves and may induce complete defoliation of the plant in some rare extreme cases [Chaerani et al.,

2007], [Adhikari et al., 2017]. The responsible fungus, *A. solani*, is also the causal agent of many other diseases including collar rot disease on basal stems of seedlings, stem lesions of adult plants, and fruit rot diseases on fruits [Chaerani and Voorrips, 2006]. Infections of leaves, causing the EB disease, are the most destructive of these diseases, as it can completely destroy the host foliage [Chaerani and Voorrips, 2006], [Foolad et al., 2008]. Annual economic yield losses due to EB have been estimated at 20% and can reach up to 79% on one field [Chaerani and Voorrips, 2006], [Adhikari et al., 2017].

Geographical distribution of EB extends to all parts of the world where Solanaceae are grown, but the pathogen is especially prevalent in temperate and tropical areas. The disease constitutes a real threat during very wet periods [Van der Waals et al., 2001], [Blancard, 2009].

Symptoms

The first symptoms of EB appear 2 or 3 days after infection as small, dark, necrotic and concentric lesions, first on the older leaves and subsequently spread upward on the younger ones. The lesions progressively enlarge and are surrounded by a narrow chlorotic halo due to toxins produced by the pathogen (see figure 2.4). In extreme cases, when the polycyclic infections reach high level thanks to favorable conditions, a complete defoliation can occur [Chaerani and Voorrips, 2006], [Foolad et al., 2008]. The defoliation causes a strong decrease of the photosynthesis and exposes fruits directly to the predators and the sun, causing sunscald. This finally leads in poor fruit quality and significant yield losses [Adhikari et al., 2017], [CMI, 1975]

Figure 2.4: Early blight symptoms on tomato leaves 10 days after infection by *A. solani*.

Disease cycle

The life cycle of *A. solani* comprises only the asexual stage, as no sexual stage has been observed for this fungus. The main stages of this cycle are the following:

1. ***Overwintering and survival***
   The pathogen overwinters in soil, plant debris and seeds as conidia or mycelia. Chlamydospores have also been reported as a source of overwintering inoculum for

EB, providing the pathogen a high resistant form to survive during cold conditions [Van der Waals et al., 2001], [Agrios, 2005]. Due to the diversity of reservoirs and the resistance of the melanised spores, it is complicated to eradicate the pathogen once installed in an area. Long rotation are efficient but not sufficient alone to avoid the disease damage, and the combination of multiple control strategies is required to handle the pathogen [Chaerani and Voorrips, 2006], [Foolad et al., 2008].

2. ***Dispersal and primary infection***
   The primary inoculum of the fungus is dispersed in the form of conidia during the spring, through wind and rain drops. These conidia can be deposit on host plant leaves, where they germinate and infect. Spore germination is induced by high humidity conditions (HR close to 100%) and temperatures of 8-32 °C. A germ tube penetrates the host epidermal cells directly through an appressorium or enters through a stomate or wound, thereby causing infection. The infection is facilitated by the production of enzymes (cellulases, pectin methyl galacturonase) that degrades the host cell walls, and secretion of toxins lethal for the host cells, which allow the pathogen to draw nutrients from the dead cells. The incubation periods vary greatly, depending on age and susceptibility of plants [Agrios, 2005], [Chaerani and Voorrips, 2006], [Adhikari et al., 2017].

3. ***Sporulation and secondary infections***
   Sporulation is affected by the state of the host and tends to increase as necrotic lesions expand and photosynthesis slows down [Van der Waals et al., 2001], [Roopa, 2012]. Two or 3 days after infection, lesions appear on the plant. Spores are produced 3 to 5 days after the lesion emergence, depending on environmental conditions. Spore production begins with the formation of conidiophores during wet night, which requires to be followed by a drought period during day light to finally allow the production of conidia during the subsequent wet night. Thus alternation of wet, darkness and light and dry conditions is required for the spore production [Adhikari et al., 2017], [Van der Waals et al., 2001], [Agrios, 2005]. The cycle of the disease is relatively short, allowing multiple infections in a season (see disease cycle in Figure 2.5). Once the primary infection occurs, the disease progression can be very fast and it is hard to restrain it. An epidemic of EB first progresses slowly but then accelerates until all parts of the leaves are touched by necrotic lesions, resulting in a typical sigmoidal aspect of the disease evolution curve [Chaerani and Voorrips, 2006].

Favourable factors

The susceptibility of tomatoes to EB disease is highly correlated with the cultivar, the maturity of the plant, the vigor of plant growth and the nutritional statues. Vigorous plants with high photosynthetic activities and sugar content will be much more resistant to the fungus than weaker plants. It is rather due to the increased sporulation of the fungus than to an increased infection [Van der Waals et al., 2001], [Roopa, 2012]. Climate also plays a major role in the development of EB by influencing plant growth and thus the susceptibility of the host. For instance, cooler temperatures may retard the growth of the plant and short photoperiods may decrease the sugar content in leaves, both conditions that accelerate development of EB. Moisture and free water increase sporulation and

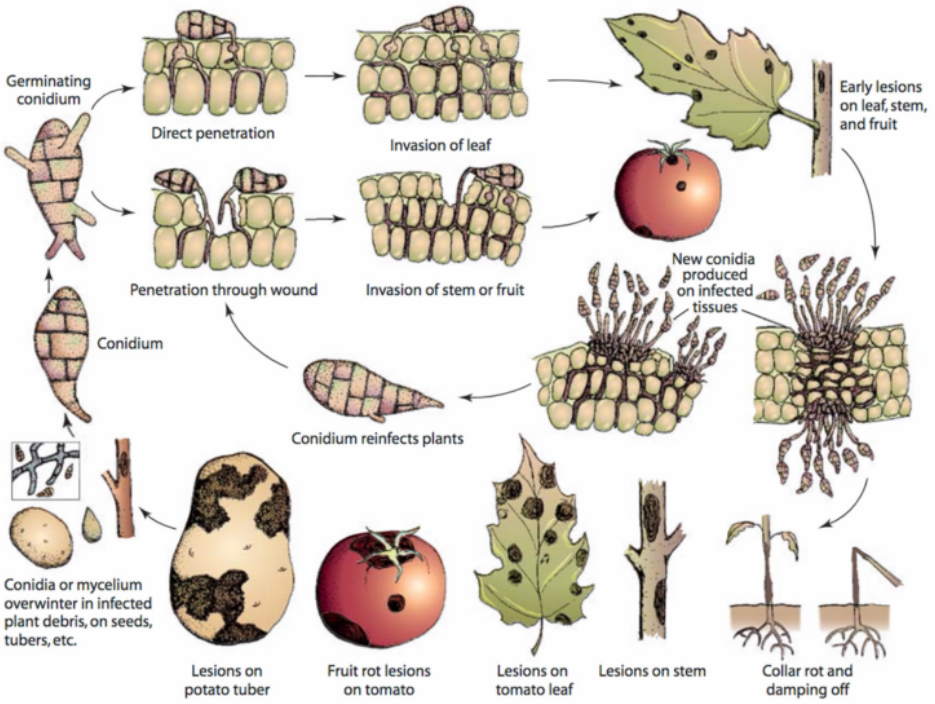

Figure 2.5: Disease cycle of early blight disease in tomato caused by the infectious agent *A. solani* [Agrios, 2005]

facilitate mobility of the fungus, thus resulting in higher infection and disease severity [Roopa, 2012], [Agrios, 2005].

Control measures

Currently, cultural practices and fungicide treatments are the main employed methods to control the EB disease, due to the lack of existing resistant varieties [Adhikari et al., 2017], [Foolad et al., 2008], [Roopa, 2012]. Cultural practices include:

- a 3- to 5- year crop rotation with non-host crops (generally, the best crops for rotation are forage and grain crops, such as maize),
- maintenance of a healthy field and crop vigor,
- plantation of healthy transplants,
- removal of plant debris and infected vines and fruits of the field,
- limitation of the environment wetness by soil-direct irrigation system.

Even if no resistant plant has been yet discovered, planting less susceptible cultivars to EB disease helps to reduce severity.

To control EB disease, cultural practices are not sufficient under high inoculum pressure and favourable environmental conditions, and need to be applied together with fungicide treatments. The authorized chemical treatments in Belgium to fight against the EB of tomatoes are presented in appendix B.2 [Phytoweb, 2018]. Fungicides act either by inhibiting conidia germination or appressoria formation [CMI, 1975]. *A. solani* is sensitive

to quinone outside inhibitor (QoI) and strobilurine. However, resistances are being encountered, as the so-called F129L mutation resulting in a total resistance of QoI [Adhikari et al., 2017], [Roopa, 2012]. Applications must be realised reasonably, respecting an alternation between the active substances and following an interval of 7-10 days between each treatment, in order to limit the emergence of resistances. Fungicides are generally effective in the control of *A. solani*, even if many resistances exist. Moreover, they are costly, unsustainable and not usable in all parts of the world, such as extremely wet areas [Özer and Topkaya, 2011]. Up to date, the best trade-off seems to be the use of les susceptible cultivars, which maintains a low disease impact, and the extension of the intervals between fungicide applications [Foolad et al., 2008].

The use of resistant varieties is still limited in crops due to lack of interesting cultivars with both resistance features against the pathogen and crop features for the production and commercialization. However, studies on resistances encountered on wild species are more and more conducted, allowing a better understanding of genes implied in the resistance [Adhikari et al., 2017]. Some of the wild species that have been identified as potential sources of resistance are *Solanum habrochaites, Solanum pimpinellifolium, Solanulm peruvianum* and *Solanum chilense* [Chaerani et al., 2007], [Upadhyay et al., 2016]. Analyses of gene expression profiling is useful to investigate differential gene expressions in susceptible and resistant tomato varieties against *A. solani*. Numerous genes involved in the PR-protein activations were highly expressed in resistant cultivars, highlighting the key role of those genes in tomato defense against *A. solani* [Upadhyay et al., 2016]. This leads to effective breeding of EB resistance in tomato, with decreased susceptibility of breeding lines against *A. solani* [Adhikari et al., 2017].

An important consideration is arising across the world over the past decades concerning the use of biological agents as control measures for crop diseases. Those microorganisms with beneficial effects on plants are called plant growth promoting rhizobacteria (PGPR) and plant growth promoting fungi (PGPF) and their applications in crops is more and more widespread. They form a beneficial association with plants providing them a lot of services such as facilitating the plant growth or decreasing the pathogen pressure by either stimulating the host defense or by antagonist effects against the undesirables [Jetiyanon and Kloepper, 2002]. The use of PGPR/F as biocontrol agents may help to decrease the global dependence on hazardous chemical agents which threat the agroecosystem stability. In that context of IPM, many researchers have focused their works on the efficiency of diverse PGPR/F in different pathosystems.

2.3 The plant immune system

2.3.1 Resistance and susceptibility in plants

Compatible and incompatible interaction

During their life cycle, plants are often challenged by numerous abiotic and biotic stresses. Among biotic stresses, those triggered by microorganisms (fungi, bacteria and viruses), insects and nematodes have significant economic impacts. In most cases, plants are resistant to most invaders and only a few pathogens / pests are able to cause diseases / symptoms on certain plant species. Those plants are referred to as susceptible plants.

The other ones are resistant, thanks to a dynamic immune system that defends them from some pathogens. Therefore, the result of pathogen challenge is characterized by the ability or not of microorganisms to effectively infect a plant. A successful infection leading to disease results from a compatible interaction between a pathogen and a susceptible host plant, whereas an incompatible interaction results in an inability of a pathogen to infect a resistant plant, because of the successful host defenses. Of course the reality is far more nuanced and there is a continuum of susceptibility and resistance to a given pathogen [Glazebrook, 2005], [Gill et al., 2015].

Host and non-host resistance

During an incompatible interaction, the resistance of the plant can be a host resistance or a non-host resistance, depending on the nature of the plant-pathogen interaction. Host resistance is mostly cultivar specific whereas non-host resistance can occur in all cultivars of a host plant species. Host resistance is generally controlled by single gene, called resistance (R) gene, and is less durable compared with non-host resistance, which appears to be multigenic and more durable [Gill et al., 2015]. The non-host resistance is the common form of plant defense responses against a wide range of potential pathogens. To sum up, when a resistant plant is challenged by a pathogen, it can defend itself by either establishment of physical and/or chemical barriers, which are non-specific resistance (non-host resistance), or by the activation of defense responses following the recognition of specific races of pathogens (host resistance) [Gill et al., 2015], [Heath et al., 2003].

2.3.2 The basis of plant immunity: molecular pattern recognition

Host and non-host resistance differ primarily in the adaptation of a pathogen to a particular species (host) and lack of adaptation to other species (nonhost). However, they both result from the plant immune system [Gill et al., 2015].

The plant immune system is a complex molecular network based on PRRs. They are membrane or cytoplasmic proteins expressed by cells of the innate immune system that recognize microbial compounds of bacteria, fungi and oomycetes called pathogen-associated molecular patterns (PAMPs) or microbe-associated molecular patterns (MAMPs), and thus allow a reaction of the plant to the attack. The recognition can also come from endogenous plant derived signals, called damage-associated molecular patterns (DAMPs), that arise from damages caused by invaders, mainly on the cell wall which releases fragments of oligosaccharides and glycolipids detected by the PRRs. This immunity has been elaborated by plants that have evolved together with pathogens and herbivores in order to withstand them [Pieterse et al., 2014], [Nürnberger and Kemmerling, 2009]. PAMPs and MAMPs are typical conserved structures that are indispensable for the microbe and are constitutively present, such as bacterial lipopolysaccharides and flagellin, or fungal constituents, including ergosterol and chitin [Nürnberger and Kemmerling, 2009] [Baluška et al., 2007]. Upon recognition of these specific molecular patterns by PRRs, plants can develop an array of defense mechanisms to counteract infections by initiating signal transduction cascades. A summary of MAMPs and PAMPs with proving role in the plant immunity stimulation is listed in table 2.2.

The transduction of PAMPs perception into cellular responses is generally permitted by a mitogen-activated protein kinase (MAPK) cascades. After the establishment of the

Table 2.2: Inducers of PAMP- MAMP-Triggered Immunity. From [Baluška et al., 2007].

P(M)AMPs	**Origin**	**Minimal structural motif required for defense activaiton**	**Sensitive plants**
Lipopolysaccharide	Gram-negative bacteria (xanthomonas, Pseudomonas)	Lipid A, lipooligosaccharides	Peeper, tobacco
Peptidoglycan	Gram-positive and Gram-negative bacteria	Muropeptides	*Arabidopsis*, tomato
Flagellin	Gram-negative bacteria	flg22(amino-terminal fragment of flagellin)	*Arabidopsis*, tomato
Elongation factor thermo unstable (EF-Tu)	Gram-negative bacteria	elf18(N-actylated amino-terminal fragment of EF-Tu	*Arabidopsis* and other Brassicaceae
Harpin HrpZ	Gram-negative bacteria (*Pseudomonas, Erwinia*)	Undefined	*Arabidopsis*, cucumber, tobacco, tomato
Cold-shock protein	Gram-positive and Gram-negative bacteria	RNP-1 motif (amino-terminal fragment of the cold-shock protein)	Solanaceae
NEP-like protein (NLP)	Bacteria (*Bacillus* spp.), fungi (*Fusarium* spp.), oomycetes (*Phytophthora* spp., *Pythium* supp.)	Undefined	Dicotyledonous plants
Transglutaminase	Oomycetes (*Phytophthora* spp.)	Pep-13 motif (surface-exposed epitope of the transglutaminase)	Grapevine, *Nicotiana benthamiana*, parsley, potato, tobacco
Cellulose-binding elicitor lectin (CBEL)Z	Oomycetes (*Phytophthora* spp.)	Conserved Cellulose-binding domain	Tobacco, *Arabidopsis*
Lipid-transfer proteins (elicitins)	Oomycetes (*Phytophthora* spp., *Pythium* supp.)	Undefined	Tobacco, turnip
Xylanase	Fungi (*Trichoderma* spp.)	TKLGE pentapetide (surface-exposed epitope of the xylanase)	Tobacco, tomato
Invertase	Yeast	N-mannosylated peptide (fragment of the invertase)	Tomato
β-glucans	Fungi (*Pyricularia oryzae*) Oomycetes (*Phytophthora* spp.) Brown algae	Tetraglucosyl glucitol Branched hepta-β-glucoside Linear oligo-β-glucosides	Rice, tobacoo,Fabaceae
Sulfated fucans	Brown algae	Fucan oligosaccharides	Tobacco
Chitin	All fungi	Chitin oligosaccharides (degree of polymerisation > 3)	*Arabidopsis*, barley, rice, tomato, wheat
Ergosterol	All fungi		Tomato
Cerebrosides A, C	Fungi (*Magnaporthe* spp.)	Sphingoid base	Rice
Oligouronides	Plant cell wall pectins	Oligomers	*Arabidopsis*, tobacco
Cellodextrins	Plant cell wall cellulose	Oligomers	Grapevine
Cutin monomers	Plant cuticle	Dodecan-1-ol	Apple, cucumber, tomato
Sliderophores	*Psuedomonas fluorescens*	Undefined	Tobacco

bond between plant transmembrane PRRs and PAMPs, a MAMP kinase kinase kinase (MAP3K) is activated and leads to the phosphorylation and activation of dowstream MAP2K. Ultimately, this last one phosphorylates a downstream MAPK, resulting in phosphorylation of substrates including transcription factors which alter patterns of gene expression (figure 2.6 a.) [Rasmussen et al., 2012].

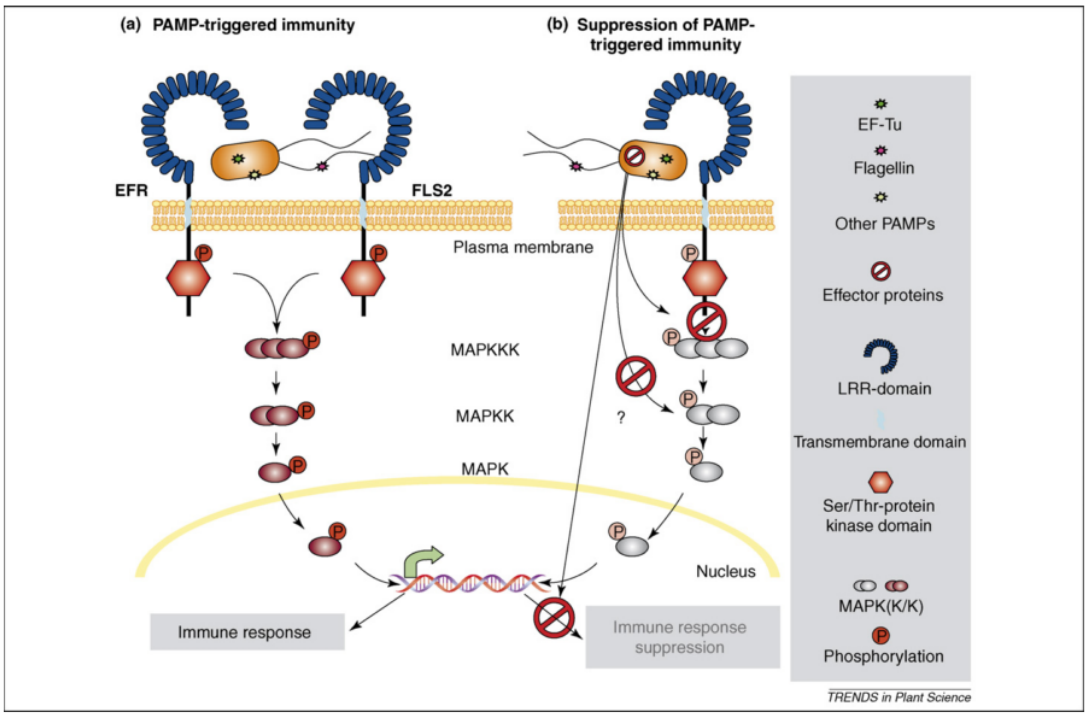

Figure 2.6: Plant immunity (a) induced by the detection of PAMPs and (b) by the inhibition of this recognition by microbial effector proteins. Transduction of the signal from PAMPs detection to all part of the plant to set up defense responses requires MAPK activity. From [Nurnberger and Kemmerling, 2006]

In contrast to MAMPs and PAMPs, DAMPs are not compounds from the exogenous agents, such as beneficial microbes or pathogens, but are disrupt products of the plant cell walls, referred to as "endogenous elicitors", such as cellulose fragments, pectic fragments or cutin monomers. They are able to elicit plant immune response as described for the PAMPs and MAMPs. These plant-derived elicitors are released upon pathogen or insect herbivore attack through lytic activities, and also after abiotic stresses [Baluška et al., 2007]. Consequently, DAMPs act as mediators of cell damages or distresses, and lead to the activation of immunity responses. DAMPs may act in concert with PAMPs, as an infection by pathogens or herbivore attack prompts tissue damages, and thus may constitute a more efficient defense response than either alone [Baluška et al., 2007]. The initial pattern recognition from PAMPs results in the transduction of PRR-ligand-encoded information across the plasma membrane. Subsequently, it initiates a host signaling cascade as above explained, that leads to a first defense, called PAMP-triggered immunity (PTI), which controls most of the invaders [Burketova et al., 2015], [Baluška et al., 2007], [Halim et al., 2006].

The PTI results in a massive transcriptional reprogramming related to defense, which is triggered by the modulation of transcription factors (TF) activities that follow the recognition of PAMPs [Burketova et al., 2015]. It leads to the activation of multiple

defense related genes that encode for different enzymes and stress-specific metabolites [Newman et al., 2013]. Responses activated by PTI receptors include the generation of reactive oxygen species (ROS), extracellular alkalinisation, and protein phosphorylation [Rasmussen et al., 2012].

2.3.3 Zigzag model and gene-for-gene resistance

Some pathogens have evolved to bypass the PTI by secreting effector molecules, referred to as avirulence (avr) factors, that suppress PTI signaling or prevent the host detection, resulting in effector-triggered susceptibility (ETS). Those avr factors are product of pathogen-encoded avr genes, and often constitute elicitors of the plant immune system. Studies revealed that those avr genes actively participate to the virulence of pathogen on plant missing a corresponding R gene. Therefore, those avr factors were initially virulence determinants, until plants evolved R proteins to detect these molecules. The ETS often targets specific compounds involved in the cascade reactions during the transduction signals of PTI, such as MAPK (figure 2.6 b.) [Rasmussen et al., 2012]. In turn, plants have evolved to recognize these avr proteins based on a second line of defense through resistance receptor proteins, encoded by R genes. This counter-reaction is called effector-triggered immunity (ETI), an amplified version of PTI. ETI often results in a programmed cell death, referred to as hypersensitive reaction (HR), at the site of infection, preventing the progression of biotrophic pathogens thereof. It is clear that the HR is much less efficient against necrotrophic pathogens which feed from killed cells [van Loon, 2009]. Besides this local defense, a resistance is induced in distal tissues through long distance signal leading to an enhance defensive capacity in the entire plant. Hence, the onset of PTI and ETI results in the activation of an immune response that is first manifested locally, in the infested zone, but may also occur systemically in the non-infected tissues via molecular signaling [Cawoy et al., 2014]. This systemic resistance is called SAR [Pieterse et al., 2014], [Burketova et al., 2015]. The set of ETI, through recognition of pathogen avr proteins by corresponding plant R proteins is the basis of classical gene-for-gene resistance, and it is usually specific to a particular cultivar against a particular pathogen race (host resistance). Most R proteins contain a nucleotide-binding site and leucine rich repeats (NBS-LRRs), which function as receptor PAMPs, but not highly conserved PAMPs, such as LPS or flagellin (innate immunity) (figure 2.6). They also bind with the pathogen avirulence factors. Instead PRRs transmembrane recognition, the NB-LRR detection occurs inside the cell [Pieterse et al., 2014], [Baluška et al., 2007], [Jones and Dangl, 2006].

The co-evolution pathogen avr proteins and plant R proteins leads to a continuous change of those effectors in order to bypass the resistance of the other. In that sense, pathogens can once again develop new effectors that would help to suppress the ETI established, which will in turn trigger the emergence of new adapted R proteins. This scheme is illustrated in figure 2.7, and is called the "zigzag model" [Jones and Dangl, 2006]. This model is largely used to understand host-pathogen interactions. However, as mentioned by some scientists [Pritchard and Birch, 2014], although this model helps to clarify principles it remains simplistic and limited. It is clear that the R gene-mediated resistance results from the host-pathogens evolution over long periods [Halim et al., 2006] [van Loon, 2009].

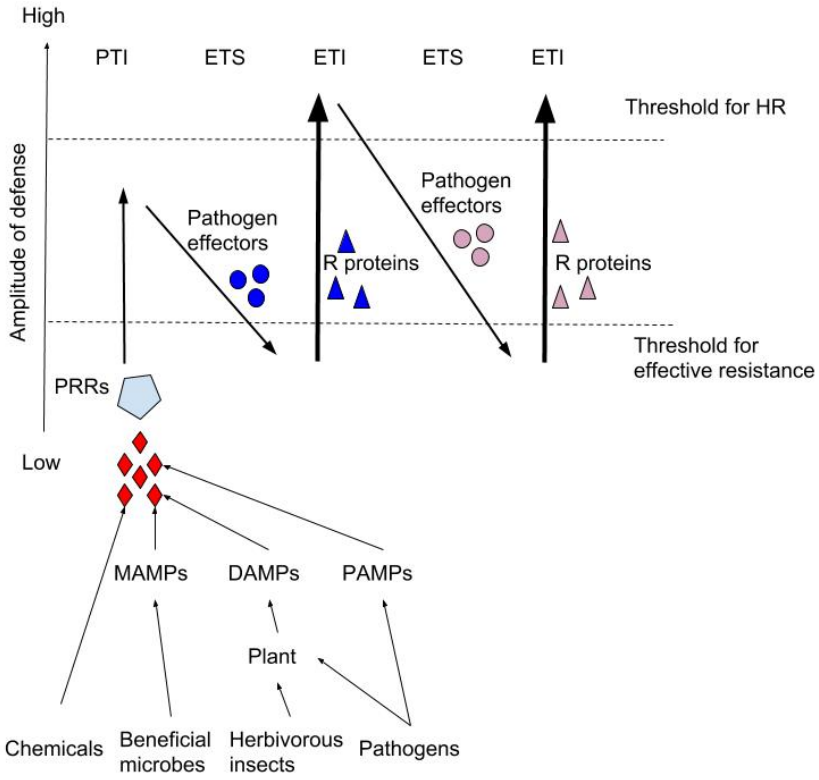

Figure 2.7: A first immunity called PAMPs triggered immunity (PTI) is set up by the plant through the PRRs recognition of intruder specific molecular patterns. Similar immunity can be triggered by DAMPs after pathogen or herbivore attacks, and be stimulated by MAMPs or chemical elicitors. Pathogen effectors can bypass PTI, resulting in effector-triggered susceptibility (ETS). In turn, the plant can detect these effectors through elaboration of resistance genes, and sets up a second immunity, called effector-triggered immunity (ETI), an amplification version of PTI. It is driven by the constant co-evolution between plants and their pathogens, which leads to this zigzag scheme. Inspired from [Jones and Dangl, 2006]

2.3.4 Common plant defense responses

The plant immune system leads to an enhanced plant resistance level after detection of external agents through the expression of an integrated set of defenses in systemic tissues that halts the pathogen. A lot of defense responses depending on the plant or the microbe has been discovered.

Oxidative burst

Plant cells often respond to elicitors or microbial pathogens with an oxidative burst, during which reactive oxygen species (ROS) are produced. This type of reaction is one of the earliest host responses to pathogen attacks. ROS are typically H_2O_2 and superoxide radicals, and lead to cellular damages for both the plant and the intruder. Moreover, hydrogen peroxide also contributes to lignin formation through its oxidative potential, thereby reinforcing cell wall. This helps to limit microbial expansion, preserving the uninfected tissues [Heath, 2000], [Cohn et al., 2003]. Another feature of these molecules is its capacity to induce a variety of defense-related genes. Indeed, exogenous application of

H_2O_2 to mutant tobacco plants unable to produce catalase appears to activate PR gene expression [Chamnongpol et al., 1998].

Production of antimicrobial compounds

One of the most significant change in protein composition occurring after pathogen intrusion is the accumulation of PR proteins. Several PR-proteins display antimicrobial properties. For instance, *PR-1* and *PR-5* family members have lytic activities towards fungal plasma membrane. Some PR genes encode for chitinase and β-1,3-glucanase that degrade cell walls. In addition to their direct antibiotic activities, PR-proteins also demonstrate the ability to stimulate the host plant defense response. For instance, small oligosaccharides resulting from their degrading action on cell walls can act as elicitors. Therefore, PR-proteins are important compounds in plant protection against pathogen attacks by either directly attacking the invading microbes or by amplifying plant defense responses. For this reason, following the detection of an exogenous agent in a host, they are encountered as much in the site of infection as in all part of the plant, where their production have been systematically induced [Gill et al., 2015], [Ellis et al., 2003].

Another class of compounds highly produced after pathogen attacks are phytoalexins, low molecular weight antimicrobial compounds. They accumulate around the site of pathogen penetration and their production is highly activated under a wide variety of elicitors, including several released fragments from fungal cell wall degradation [Gill et al., 2015] [Heath et al., 2003].

Some of those secondary metabolites appear to be preformed in host cells and are activated when pathogen attacks. This kind of preformed defenses constitutes a major component of non-host resistance. They are either expressed constitutively, and often accumulated in plant vacuole, or are induced by general elicitors of beneficial microbes or pathogens, M/PAMPs. For instance, some antimicrobial compounds require the action of plant hydrolytic enzymes, specifically produced during an infection, to display their activity. Under action of those enzymes, the stored secondary metabolites change from inactive conjugate to active one [Heath et al., 2003].

Secondary signaling molecules

In addition to direct antibiosis, secondary signaling molecules are generated and seem to be implied in long distance signaling of plant immunity. Several phytohormones have been identified as key regulators of defense related gene expressions: SA, JA and ET. Their mechanisms of action are further explained in the following section. They take part in the upstream regulation of some important signaling pathways resulting in inducible immunity of plants.

Priming

The defense response can be established very quickly, through an increased mobilization of the defense molecules at the site of infection, or via an enhanced level of defense-related gene expression. Indeed, upon infection by a pathogen, root colonization by beneficial microbes, or certain chemical treatments, the plant can develop a specific physiological situation which is referred to as “primed state”. It is defined as a sensitization of the whole plant for enhanced defense, characterized by a faster and stronger activation of cellular defenses upon invasion. The plant immune system will be mobilized more robustly, with

a higher PTI reaction than if it was not pre-elicited (figure 2.8). The potentiation of defense responses in primed plants is associated with an enhanced resistance against the challenger, without causing major fitness costs [Conrath, 2009].

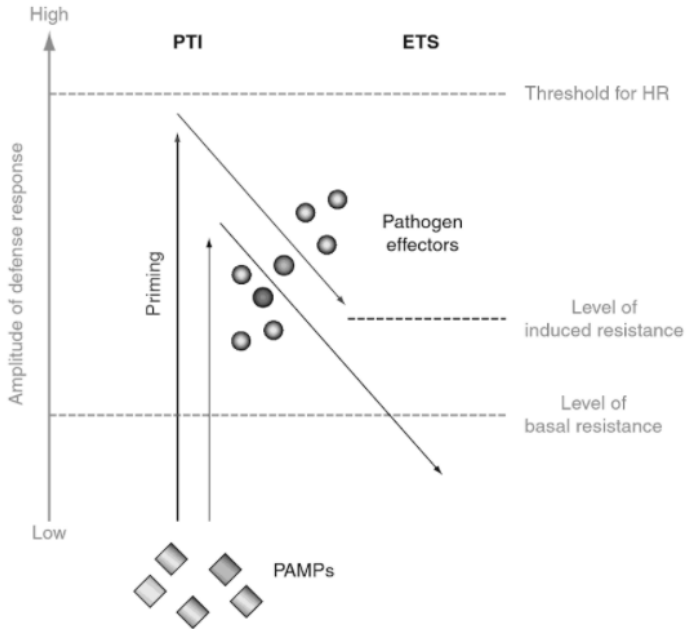

Figure 2.8: After pathogen contact with plants or elicitation with beneficial microbes, plants can develop a higher possibility to protect itself against future attacks with a faster and stronger PTI reaction than if it was based only on constitutive defense. From [van Loon, 2009].

After a pathogen attack, the plant activates its primed state to confront other potential attackers in their hostile environment. The priming is an important process of defense and protection of plants, inducing systemic immunity at a very low cost compared to constitutive defenses. Consequently, the phenomenon appears to be an ecological evolution that prepares a plant to protect itself against further attacks [Burketova et al., 2015], [Pieterse et al., 2014].

Regarding the mechanism of the priming, it remains unclear but it has been suggested that priming includes improvement of pathogen perception and/or amplification of defense response. Observation of primed cells has revealed accumulation and/or posttranslational modifications of defense signaling proteins in a dormant inactive state [Burketova et al., 2015], [Conrath, 2009]. Thereafter, exposure to pathogen will activate or modulate these "dormant" signaling proteins, initiating signal amplification and leading to the potentiation of defense responses and induced resistance. Members of the mitogen-activated protein kinase (MAPK) family of signaling enzymes accumulate upon priming without displaying enzymes activity and thus seem to be responsible for the signal amplification. Future studies will likely reveal more candidates involved in the onset of priming [Conrath, 2009].

The sensitization of the whole plant to biotic stress is provided by an important transcriptome reprogramming of plant in a hostile environment. TF are instruments allowing the plant to modulate specific gene expressions in response to a given stress, particularly after a HR. They stimulate or suppress gene expressions by binding to specific motifs present on target gene promoters. TF can be regulated at the transcriptional level

and/or at the posttranscriptional level. The mechanisms of TF regulation are various, such as (de)phosphorylation, redox, disulfide bond formation ... [Li and Loake, 2008].

Furthermore, it may sometimes be associated with histone modifications, such as acetylation or methylation, at promoter regions of defense genes. This epigenetic DNA modifications are associated with activation of gene expressions. The chromatin remodelling could be inherited and could provide an immune memory of a hostile environment to plant progenies [Burketova et al., 2015].

2.4 Induced resistance

The recognition of pathogens, microbes or insect herbivores by plants through its immune system leads to an enhanced defensive capacity upon a primary infection, which is referred to as induced resistance [Nürnberger and Kemmerling, 2009]. This phenomenon is significant in the process of plant immunity and defense. Generally, the immunity stimulation is expressed systematically in plants and protects them for long periods. Among these induced resistances, three different types are characteristics, according to the nature of the inducer: systemic acquired resistance (SAR), emerges from limited primary infection from a pathogen, herbivore-induced resistance (HIR) following herbivorous insect attack, and ISR which is derived from the colonization of roots or leaves by non-pathogenic organisms [Nürnberger and Kemmerling, 2009]. All these resistances result in an enhanced defense capacity of plants that is manifested by a stronger defense response upon invader challenge [Nürnberger and Kemmerling, 2009].

2.4.1 Interaction plant – pathogens : SAR

When a plant is challenged by a pathogen, it can be protected against a wide range of attackers. The pathogen-induced resistance is generally established in the tissue surrounding the site of primary infection and can be extended in some or all parts of the plant thereafter [Conrath, 2009]. The local type of induced resistance is referred to as localized acquired resistance (LAR) whereas the distant type is called systemic acquired resistance (SAR). Both types are generally associated with accumulation of the so-called pathogenesis related (PR) proteins. These proteins generally display antimicrobial activity through hydrolytic activities on cell walls of infectious agents, contact toxicity, and some are involved in the plant defense signaling, thus contributing to the resistance [Conrath, 2009].

The long-distance signal that goes from the site of primary infection to the remote parts of the plant to induce SAR is SA. Studies on Arabidopsis mutants affected either in the biosynthesis of SA or in SA signaling highlight the key role of the phytohormone in the establishment of SAR [Conrath, 2009]. Consequently, increased level of SA is observed in systemic tissue upon SAR. It is accompanied by the coordinate activation of PR genes, such as *PR-1* which is often used as a marker for SAR [Pieterse et al., 2014]. Several SAR-associated long distance signals have been identified, such as glycerol-3-phosphate [Burketova et al., 2015]. Unsurprisingly, the phytohormone is also required for the hypersensitive reaction of cell death response, which is usually associated with SAR in the early stages of the infection. For this reason, SAR is mainly induced in plant against biotrophic agent, and is less effective against necrotrophic pathogens [Halim et al., 2006].

Concerning the role of SA in the activation of pathogen defense responses on Solanaceae, contradictory models subsist. It has been observed that exogenous applications of SA lead

to enhance PR gene expression but not systematically to enhance level of resistance of the host plant [Halim et al., 2006]. Yu and his team revealed an induction of resistance by exogenous applications of SA only on resistant potato cultivars, but not on the susceptible ones ([Yu et al., 1997]), suggesting that induced resistance by SA requires specific recognition with PAMPs present in resistant cultivars [Halim et al., 2006]. Hence, SA is the phytohormone that orchestrates the SAR signaling in plants, but its mere presence is not sufficient to trigger the induced resistance, it must be linked with the pathogen detection through the plant immune system [Halim et al., 2006].

Even if PR proteins seem to have an important contribution to SAR, it appears that priming is an essential component of SAR. Consequently, a high proportion of the enhanced defensive capacity of plants upon SAR is associated with a primed state in which the plant responds more rapidly and robustly with the activation of defense responses after infection [Conrath, 2009].

2.4.2 Interaction plant - insects : HIR

Herbivorous insects can trigger an induced state of resistance in plants, first locally at the site of infection, and then systematically in all parts of the plant. This form of induced resistance is called herbivore-induced resistance (HIR). HIR signaling is onset upon the detection of plant derived signals (e.g., DAMPS) and elicitors from insect oral secretions at the site of tissue injury, the herbivore-associated molecular patterns (HAMPs) [Pieterse et al., 2014] . The release of HAMPs results in rapid production of oxilipins from membrane lipids. The jasmonate family of oxilipins represents the long-distance signals systemically transmitted after herbivory attacks is the JA [Pieterse et al., 2014], [Cawoy et al., 2014].

As in the case of pathogen attacks, herbivory by insects also induced the direct production of PR proteins. The pathogen-induced pathway relies on SA, whereas the wounding pathway relies on JA as the signaling molecule [Mohankumar et al., 2013]. These phytohormones induce similar responses when they are applied exogenously and crosstalk between both signaling pathways exist. Typically, the PR-proteins derived from the SA-induced pathway are antimicrobial molecules and the ones derived from JA-induced pathway are oxidative and lytic enzymes [Mohankumar et al., 2013].

2.4.3 Interaction plant – beneficial microbes: ISR

Systemic resistance can be induced not only by pathogen infections or herbivore attacks but also by plant-associated microbes, called elicitors [Cohn et al., 2003]. Indeed, some beneficial microbes in the rhizosphere of the plant can stimulate plant growth or protect it against pathogens. This biological activity is exerted either directly through antagonism of pathogens or indirectly by eliciting a plant-mediated immunity response. Those microbes, referred to as PGPR and fungi (PGPF) enhance the plant immune system in a process called induced systemic resistance (ISR). The evidence of their action has been provided by experiments involving PGPR and pathogen kept spatially separated in plants, where ISR was finally activated [Pieterse et al., 2014]. A wide variety of mutualists, such as bacteria and fungi species, are involved in this ISR process and enhance the plant defense against a broad-spectrum of pathogens and insect herbivores [Pieterse et al., 2014]. This defense strengthening is due to the immune response stimulation, similarly to the one following the recognition of PAMPs [Cawoy et al., 2014]. Besides growth promotion

and control of plant pathogens, PGPR can also take part in the fertilization of the soil rhizosphere and the degradation of xenobiotic compounds (rhizoremediation) [Choudhary and Johri, 2009]. The interesting features of PGPR in the biological control is their ability to reduce plant diseases, which make them entities extensively used in agriculture. This capacity is provided by the production of inhibitory allelochemicals by PGPR against soil-borne pathogens, competition for ecological niche or substrate, and/or induction of ISR in host plants against both root and foliar pathogens [Choudhary and Johri, 2009]. There are also some abiotic elicitors that displayed a potential of triggering ISR on plants. This section will first illustrate two important potential features of beneficial microbes: growth promotion and enhancing host defenses through the ISR process. The onset of this process though a wide transcription reprogramming will then be explained, as well as the main change that result; the priming.

Growth promotion

Upon root colonization, some rhizobacteria have the ability to initiate an auxin-dependent root developmental program that results in abundant lateral root formation, increased root hair length and enhanced plant biomass production. The relation between the capacity of PGPR to promote growth and to enhance plant defense is still unclear. Some studies suggest that both effects could function independently [Pieterse et al., 2014]. Still, a lot of beneficial microbes are described as plant growth promoters but not as elicitors of plant defenses and vice-versa.

Enhancing host defense : ISR

Plant systemic immunisation by rhizobacteria is called elicitation. PGPR may act through several mechanisms for disease control, including competition with pathogens for ecological niche or nutrients in the rhizosphere, as well as production of antibiotics with direct inhibitory activity on infectious agents. Another mechanism by which rhizobacteria may control pathogens is the stimulation of the host plant defenses [Cawoy et al., 2014].

As described by van Loon and Glick (2004) in their review, the term ISR refers to the process whereby PGPR treatments of plants elicit host defenses, resulting in reduction in the severity or incidence of diseases caused by pathogens that are spatially separated from the inducing agent [van Loon and Glick, 2004]. This physiological steady state of defense thus results in potentiation of the plant immune system upon environmental stimuli, leading to an enhanced level of resistance against subsequent biotic challenges Choudhary2009.

At first, non-pathogenic microbes mediated ISR was thought to be mechanistically like pathogen-induced SAR. However, obvious differences have been highlighted in the molecular mechanisms between both phenomena, such as the non-accumulation of the PR proteins in ISR, characteristic in the SAR. However, the processes seem to be more complex, and actually other PR-proteins are also involved in the ISR, to a lesser extent. Finally, an independence of the ISR towards SA has been revealed, as ISR was triggered in transgenic Arabidopsis that cannot accumulate SA. Moreover, the difference between rhizobacteria-mediated ISR and pathogen-induced SAR is supported by the divergent between their broad-spectrum effectiveness against enemies [Pieterse et al., 2014]. It has been demonstrated that the local and systemic defense responses that are triggered by non-pathogenic microorganisms are controlled by a signaling network in which the plant hormones JA and ET constitute a key role [Wees and Ent, 2008] (Figure 2.11A).

Thus, rhizobacteria mediated ISR was shown to be effective against attackers that are sensitive to JA/ET dependent defenses, including necrotrophic pathogens and insect herbivores [Pieterse et al., 2014]. Hence, depending on the type of pathogen faced, the signal transduction pathway activated seems to be characteristic. Biotrophic pathogens typically induce defense responses via the SA signaling pathway, whereas JA dependent defense responses are generally activated after infection by necrotrophic pathogens which require host cells death to obtain nutrients [Halim et al., 2006]. Accumulation of JA in elicited plant is observed only after pathogen challenge. It has been highlighted in resistant potato elicited by rhizobacteria and then infected by *P. infestans* [Coquoz et al., 1995]. Resistant cultivars display an increase in JA whereas no accumulation of the phytohormone has been observed in susceptible potato cultivars, suggesting that JA accumulates in beneficial microorganism interactions and in response to PAMP recognition [Coquoz et al., 1995].

The synthesis of JA depends in plants on the content of a-linolenic acid, that transforms into the phytohormone under the actions of some enzymes of the lipoxygenase pathway [Wees and Ent, 2008].

Beneficial microbes produce different MAMPs with immunity-stimulating features, known as elicitors of defenses in many plants [Baluška et al., 2007]. MAMPs are typically conserved molecular structures essential for microbes, mostly extracellular compounds that bind with PRRs, as bacteria stay outside of living plant cells. Bacteria potent inducers of defense associated responses in plants are numerous, such as peptidoglycans, elongation factor, different structural elements of lipopolysaccharides or conserved sequence of flagellin ... [Baluška et al., 2007]. The MAMPs are probably recognize by the plants according to a similar mechanism than PAMPs of the pathogens, unless MAMP-triggered immunity (MTI) does not suppress bacteria as it remains accommodated by the plant. This suggest a high degree of coordination and a continuous molecular dialog between the two protagonists [Wees and Ent, 2008].

Onset of ISR : transcriptome reprogramming

1. ***Root colonization***
   Initiation of ISR generally requires an efficient colonization of root system of host plants by beneficial microbes, even if in some cases, the single presence of volatile organic compounds (VOC) or other secreted metabolites are enought to trigger the stimulation of the host immune system. A molecular dialog is essential to establish a mutualistic association between the host plant and microbes [Lugtenberg and Kamilova, 2009]. It is assumed that PGPR respond to root exudates to established on the root epidermis. Plant roots constantly release a variety of organic compounds that diffuse in the surrounding soil layer, thereby creating a niche favorable for development of very diverse microbial populations. Micro-organisms are attracted through this nutritional sites and use the root exudates for growth and multiplication on the root surface [Choudhary and Johri, 2009], [Pieterse et al., 2014]. Among these rhizosphere communities lie the famous PGPR [Cawoy et al., 2014].

   PGPR epiphytes typically form biofilm of polymeric substances, mainly exopolysaccharides (EPS) and mucilage [Rudrappa et al., 2008]. This biofilm demonstrates a significant role in the root colonization of plants by some epiphyte microorganisms, such as *Bacillus subtilis* [Beauregard et al., 2013]. Within the polysaccharidic

matrix, the bacterium and the plant exchange signals to coordinate production and release of compounds related to growth promotion, nutrition and ISR [Pieterse et al., 2014]. It is also the site where bacteria communicate with each others (quorum sensing) to act in a coordinated manner [Choudhary and Johri, 2009]. Like pathogen, beneficial microbes need to overcome plant immune response to establish and initiate a mutualistic interaction with the host. Molecules and strategies commonly used by pathogens to suppress the plant immune system are also employed by ISR-inducing rhizobacteria. To colonize root, micro-organisms should be able to establish on or in the plant root, to propagate and disperse along the growing root. It implies the local suppression of root immune responses. This ability is a common feature of ISR eliciting beneficial microbes that possibly helps in root colonization [Pieterse et al., 2014], [Choudhary and Johri, 2009].

2. ***Transcription factors***
   Once the mutualistic association plant-microbes is established on the root, signals are being exchanged and a massive change in the gene expression occurs. Studies have revealed that one transcription factors (TF) is required in early signaling steps of ISR, the MYB72 [Verhagen et al., 2004], [Segarra et al., 2009]. Experiments on Arabidopsis myb72 mutants have shown no capacity of developing ISR against both SA controlled and JA controlled pathogens, indicating that MYB72 is essential to establish ISR [Verhagen et al., 2004]. It seems that this TF is linked to the ET response pathway involved in ISR, which is implied in the onset of ISR in the roots [Wees and Ent, 2008]. Indeed, it is assumed that ET signaling is required at the site of elicitation, initiating in the plant the systematically transported ISR signals and translocating its [Segarra et al., 2009]. At the root-microbe interface, MYB72 become highly expressed in root epidermis and cortical cells upon PGPR colonization. Furthermore, MYB72 is specifically induced in roots under iron limited conditions, highlighting the relation between the iron balance and the onset of ISR [Pieterse et al., 2014].

   Another important compound that plays a crucial role in the transcription reprogramming following microbe interactions is Nonexpressor of PR genes-1 (*NPR1*). It is a protein essential for the transcriptional activation of PR-proteins, such as *PR-1*, through SA-mediated signaling pathways. By interacting with the TGA family of TF, NPR1 enhances the capacity of TGA to bind with promoter elements of PR genes and this activates their transcription [Despres et al., 2000], [Li and Loake, 2008]. The mechanism is described in a following section addressing specifically the NPR1 regulator.

   The establishment of primed state is abolished in ISR mutants myb72 and npr1, underlying the essential function of these two TF in priming. (Fig. 2.9) [Wees and Ent, 2008].

Priming in the ISR process

Unlike SA in SAR, the ISR is not linked with a significant increase in phytohormones and there is no accumulation of PR proteins in systemic tissues. It has been demonstrated that the colonization of Arabidopsis roots by PGPR-mediated ISR does not enhance the production of JA and ET but rather stimulates the expression of these hormones responsive genes. After a pathogen attack, ISR expressing plants quickly induced the expression

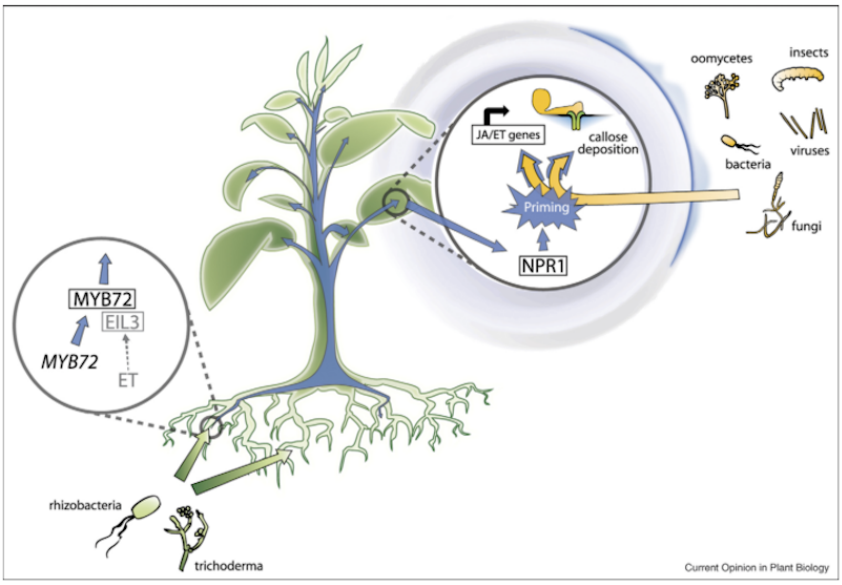

Figure 2.9: Root colonization by beneficial microbes leads to local activation of transcription factor gene MYB72 in the roots. Subsequently MYB72 interacts with the transcription factors EIL3. ET signal is required to translate the signal from the roots into the leaves. The ISR signal transduction cascade requires the transcription factor NPR1. This leads to the onset of ISR and priming by enhancing JA and ET responsive gene expressions related to defense and stimulates callose deposition. Ulterior invasion of pathogens or herbivore will be better managed in ISR-primed plants. From [Wees and Ent, 2008]

of JA/ET regulated genes. This prompt activation of cellular defenses in a plant upon pathogen or insect attack is called priming and results in a higher level of resistance. Priming is the common base of ISR expressing plants, mediated by beneficial microbes. Several studies showed that the transcriptional changes arising from the colonization of the roots by beneficial microbes are relatively slight, especially when compared to the massive transcriptional changes that occur during pathogen attacks. The ISR is therefore best studied in combination with a pathogen to reveal the enhanced transcriptional changes in primed plants that remain undetectable before pathogen or insect attack [Pieterse et al., 2014], [Wees and Ent, 2008].

The molecular mechanisms of priming defense are multiple and varied. As above mentioned, PGPR potentiate the defense-related gene expression through accumulation of signaling proteins and transcriptional regulators after elicitation by beneficial microbes. These molecules are thought to remain inactive in pathogen-free conditions but allow the plant to set its defenses faster upon perception of pathogens [Pieterse et al., 2014].

Apart from transcriptional changing, there are other mechanisms involved in priming, such as enhancing the protection at the site of entry. Plants elicited by rhizobacteria can increase structural barriers to prevent pathogen penetration. For instance, an increase in callose depositions at the site of entry of the pathogen. This phenomenon is regulated by plant hormone abscisic acid. It is specifically useful to fight against pathogens that are insensible to JA/ET – dependent defenses. Some bacteria can also trigger closure of the stomata in response to pathogen attack, and hence prevent it from other pathogens' penetration, in the case of foliar pathogens that invade plants through stomata [Pieterse et al., 2014].

Priming, orchestrated by JA and ET, is the principal phenomenon that drives the ISR.

Hence, it appears that the prime state in a plant induced by beneficial microorganisms, is the predominant pathway of systemic resistance for enhanced defense, rather than on direct activation of defense, through the PR genes [Wees and Ent, 2008].

2.4.4 Similarities and crosstalk between signaling of the SAR and ISR

Both result phenotypically in enhanced level of resistance

These two induced resistance are phenotypically similar in the sense that they both provide enhanced defensive capacity on plants. It is manifested by a faster and stronger defense reaction of plants upon pathogen challenge. However, as explained in sections 2.4.1 and 2.4.3, both forms are mechanistically different and have different inducers. When the induced resistance is triggered by a pathogen or demonstrated to be SA dependent, we refer to SAR. The ISR process is referenced when the induced resistance is triggered by a beneficial microbe or an abiotic elicitors, and demonstrated to be SA independent [Pieterse et al., 2014]. However, sometimes beneficial microbes may also trigger the SAR pathway through the SA regulation, in a similar way to pathogen-induced SAR. Indeed, many rhizobacteria have the capacity to produce SA. Yet, it is mostly not linked with a systemic resistance as it is incorporated in siderophores under iron-limited conditions, instead of being released in the rhizosphere. Thus, SA becomes unavailable for triggering the SAR pathway [Pieterse et al., 2014].

NPR1 regulator

Nonexpressor of PR genes-1 (*NPR1*), also known as noninducible immunity-1 (NIM1) or salicylic-acid insensitive-1 (sai1) [Conrath, 2009] is a key transcriptional factor that regulates the JA and SA-dependent defense response. NPR1 mutants showed an inability to induce PR genes and priming in plants, underlying the essential function of this TF in induced resistance [Li and Loake, 2008], [Wees and Ent, 2008]. NPR1 encodes a redox-regulated protein, which controls the SAR signaling downstream of SA. Once the NPR1 protein is activated by SA, it will act in turn as a transcriptional regulator of the SA that activates the transcription of PR genes. NPR1 works through redox regulation in plant cells. In non-induced cells with low SA content, NPR1 is present in the cytoplasm as oligomer through intermolecular disulfide bonds with cysteine residues. This oligomeric structure prevents the crossing of NPR1 from cytoplasm into the nucleus, thereby inhibiting any interaction with transcriptional promoters related to the expression of PR genes. After pathogen infection or microbe elicitation, SA accumulates in cells, which induces changes in the cellular redox state. Therefore, it leads to the formation of NPR1 monomer forms, facilitating its translocation into the nucleus. It is quickly followed by the activation of targeted genes and NPR1-dependent plant immune responses [Li and Loake, 2008]. In the activated monomeric form in the nucleus, the protein interacts with members of the TGA family of transcription factors which stimulates their DNA binding activity. This allows the TGA factors to bind to PR gene promoters and enhance their expression (Figure 2.10). PR1 is a marker for activated NPR1 [Mohankumar et al., 2013].

At the same time, Wu and coworkers revealed that NPR1 acts as a SA receptor, which leads to a conformational change of the protein that discloses the NPR1 transcriptional activation domain, required for PR gene activation [Wu et al., 2012]. Thus, the activation of defense gene expressions relies partly on the NPR1 in the nucleus. This result highlights

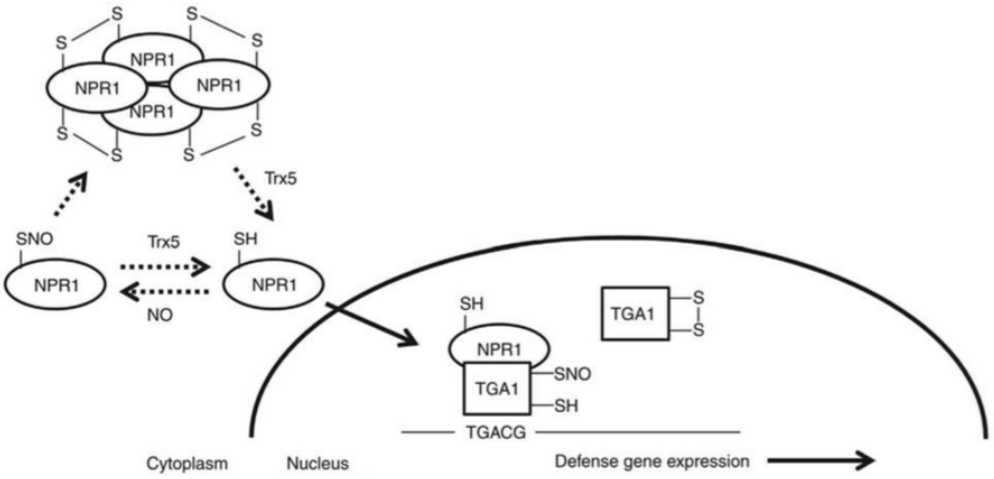

Figure 2.10: In non-induced cell, NPR1 persists in the cytoplasm in an oligomeric form, with intermolecular disulfide bonds between NPR1 monomer that prevent it to cross the membrane into the nucleus. After pathogen challenge, changes in the cellular redox state induce the monomerization of NPR1 which allows its translocation into the nucleus, where it will bind with TGA1, driving defense gene expression. From [Li and Loake, 2008]

the role of NPR1-like proteins as receptors for SA [Pieterse et al., 2014], [Halim et al., 2006]. To summarize, NPR1 has multiple functions, and depending on its localization in the cells it is possible to know whether it is involved in one or another mechanism [Pieterse et al., 2014] :

- redox-sensitive transcriptional regulator of response dependent on SA
- mediator of SA and JA cross talk
- regulator of SAR and ISR.

NPR1: A common regulator of SAR and ISR

NPR1 is not only a co-regulator of SA signaling pathways in SAR, but is also required as regulator of the JA/ET signaling pathways initiated by PGPR through ISR (Figure 2.11). NPR1 is a regulator of both SAR and ISR processes, with different functions depending on the upstream signaling pathway. In the SA dependent SAR, NPR1 is related to a function in the nucleus whereas in JA/ET signaling, it seems to be linked with cytosolic function. Hence, NPR1 has two different functions as well as separated pools, which leads to an additively enhanced defensive capacity of simultaneous activation of SAR and ISR. It occurs without competition between both pathways, as they are not competing for the same subcellular pool of NPR1 [Pieterse et al., 2014]. However, analysis on Arabidopsis revealed an antagonistic effect of SA and JA signaling orchestrated by the regulatory protein NPR1. Nuclear localization of NPR1 (which is essential for SA-dependent plant immune response) is not required for the JA signaling, indicating that crosstalk between SA and JA is modulated through NPR1 in a new pool, the cytosol [Spoel et al., 2003]. Hence, the cytosolic NPR1 is involved in a regulatory action as a modulator of crosstalk between SA- and JA- dependent plant defense responses. Under SA accumulating conditions following pathogen infection, the activated NPR1 moves from the cytosol to the nucleus, where it ultimately leads to the activation of SA responsive PR genes. In the

cytosol, the remaining NPR1 negatively regulates JA-responsive gene expression, possibly by inhibiting positive regulators of JA-responsive genes. The suppression of JA-responsive genes that encode enzymes essential for the JA synthesis, such as LOX2, leads to the inhibition of JA formation [Bell et al., 1995]. Such adaptive strategy is interesting for a plant to counteract microbial agents and herbivorous insects, providing a regulatory potential for the plant to adapt its defense reaction depending on the challenger. [McConn et al., 1997] demonstrate that Arabidopsis plant unable to produce JA were more susceptible to herbivore damage and necrotrophic pathogens. Another experiment conducted by [Felton and Korth, 2000] has revealed that activation of SAR suppresses JA signaling in plants, thus giving the priority to SA-dependent defense against pathogens over JA-dependent resistance to herbivorous insects.

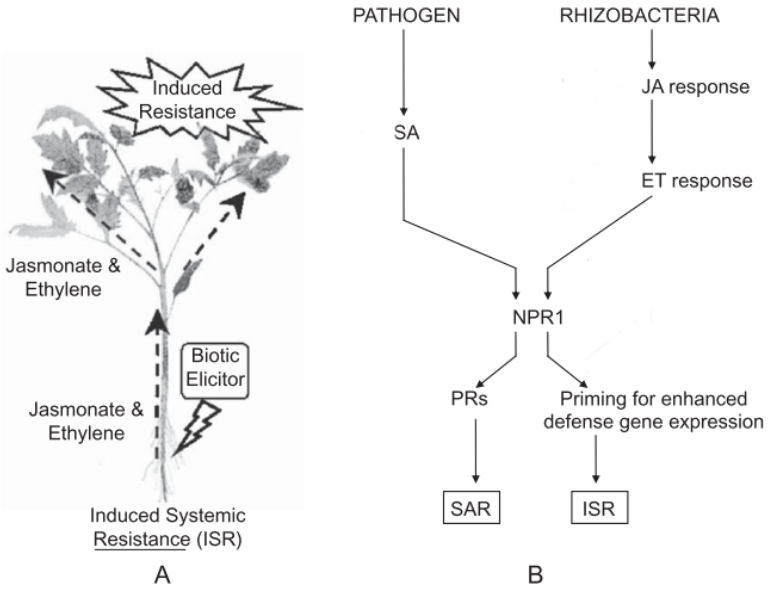

Figure 2.11: Signaling compounds involved in the onset of ISR by biotic elicitor. (A) ISR is triggered by root colonization of PGPR and is dependent on the phytohormones JA and ET and independent of SA. (B) SAR and ISR pathway and their crosstalk through the NPR1 regulator. From [Choudhary and Johri, 2009].

2.5 *Bacillus subtilis*

2.5.1 General characteristics

The genus ***Bacillus***, within the *Firmicutes* phylum, was first named by Ferdinand Cohn in 1872 to defined rod-shaped bacteria, based on the description of two species, *Bacillus subtilis* and *Bacillus anthracis* [Slepecky and Hemphill, 2006]. Later, the description of the genus was modulated to include all endospore-forming, rod-shaped, gram positive, aerobic or facultative anaerobic bacteria. The current phylogeny of the genus is based on comparison of the 16S rRNA/DNA sequences. The over 200 species of the genus are ubiquitous, including both free-living and pathogenic organisms [Fritze, 2004]. The endospores, typically oval or cylindrical, are formed under adverse environmental conditions and allow the bacteria to remain in a dormant state for very long periods, thanks to their high resistance. They are generally oval or cylindrical [Dieter and Dagmar,

1989], [Slepecky and Hemphill, 2006]. As all Gram-positive species, it has a cytoplasmic membrane and a thick cell wall, but no outer membrane. The cell wall is made of multiple layers of peptidoglycans, a polymer of N-acetyl glucosamine linked to N-acetyl muramic acid. They confer the define rod-shaped to the bacteria of the genus, providing the mechanical strength to resist the turgor pressure resulting from the high ion concentration encountered within the cell [Schaechter, 2009], [Aizawa, 2014]. Few species of the genus are able to cause human diseases, specifically two with significant medical impact: *Bacillus anthracis*, which causes anthrax, and *Bacillus cereus*, responsible of foodborne infections. Many species are known to be inducers and growth promotion agents. It includes strains of *B. pumilus, B. mycoides, B. subtilis, B. amyloliquefaciens, B. pasteurii, B. thuringiensis* and *B. cereus* [Ongena et al., 2007], [Kloepper et al., 2004].

Among them, ***B. subtilis*** is the best characterized species and constitutes a model in many researches. It is a non-pathogenic micro-organism commonly found in soil. It has a single circular chromosome with a low GC content [Schaechter, 2009], [Aizawa, 2014]. It was long thought to be strict aerobic organisms, until living species of the genus were found to live in anaerobic conditions, drawing their energy either from fermentation or respiration using nitrate as final electron acceptor. Members of the *B. subtilis* species possess numerous flagella in peritrichous positions, allowing the cells to swim (Figure 2.12a). Chemotaxis reaction triggers special rotation of the flagella which leads to a net movement toward an attractant or away from a repellant [Schaechter, 2009].

When grown on LBA medium, *B. subtilis* forms wrinkled colony embedded in a self-secreted biofilm. The colonies are off-white, more or less circular in shape, slightly elevated with undulate margins. They display a dull, rough and dry texture and are opaque, as illustrated on figure 2.12b.

The range of temperature over which the micro-organism survives and proliferates is from 10 to 55 °C, with an optimum at 42 °C. Above 37 °C, it loses its competence capacity. Actually, in some appropriate conditions, *B. subtilis* have the ability to collect DNA from its surrounding, denominated natural competence. When the compatibility between two strains of the species is sufficient, i.e. they share a lot of common features, then one strain can recombine with the chromosome of the other. Typically, 1-2 % of the donor chromosome ends up in the resultant recombinant strain. This remarkable characteristic is important in the evolution process and has led to the apparition of multiple characters in some strains. The transformation may also occur with foreign DNA from other species, which is useful for the acquisition of interesting genes in a hostile environment [Schaechter, 2009], [Aizawa, 2014].

One typical feature of many strains of *B. subtilis* is their ability to form biofilm, that is an extracellular polysaccharidic and proteic matrix in which bacteria agregate. This biofilm that accommodate multicellular and sessile communities of *B. subtilis* is essential to provide the bacteria with the ability to efficiently colonize the phytosphere and thus to allow phytostimulation [Choudhary and Johri, 2009], [Schaechter, 2009].

The bacterium and derivatives are widely used in commercial products, as later mentioned in table 2.3.

2.5.2 Interaction with the environment

The nutritional demand of *B. subtilis* is relatively low, it requires only essential salts and carbon, nitrogen and phosphorus sources. Nutrients are uptake by a variety of

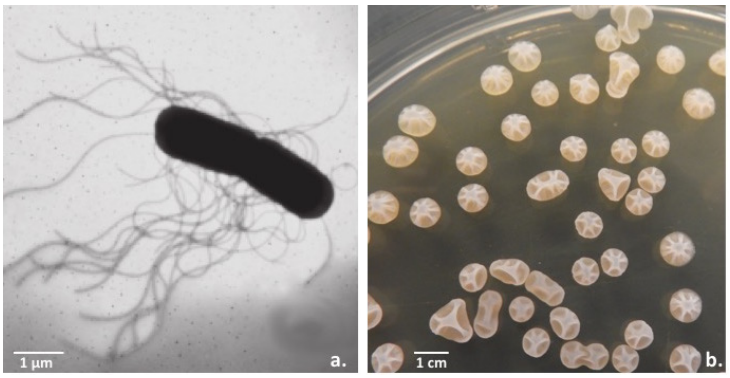

Figure 2.12: a. TEM image of one cell of *Bacillus subtilis* with its numerous peritrichous flagella. From [Schaechter, 2009]. b. Colonies of *Bacillus subtilis* strain 30BB6 grown on LBA medium for 24h at 30 °C.

transport systems through the cytoplasmic membrane, which acts as a permeability barrier [Schaechter, 2009].

Since nutrients can cross the plasmic membrane to deliver resources to the microorganism, chemical and physical signals can also be perceived through transmembrane molecules that receive the signal and transduce it. Consequently, *B. subtilis* is in constant interaction with components of the environment and responds to stimuli to survive and proliferate. There are mainly two classes of signal transduction systems: a sensor kinase and a response regulator [Schaechter, 2009]. Some strains have a well-developed secretory system, synthesizing a wide variety of secondary metabolites which display antibiotic activities [Wang et al., 2015].

If the nutritional demand is not fulfilled, the temperature inadequate, or if some pathogens threaten the bacterial community, *B. subtilis* can undergo a series of responses to help it survive in the hostile environment. These responses include production of a multilayer cell wall structure, lytic enzyme compounds, secretions of peptide antibiotics, development of competence and biofilm formation. Besides, the bacterium initiates the formation of heat-resistant, dormant spores in order to survive in a vegetative form awaiting better conditions [Schaechter, 2009], [Choudhary and Johri, 2009].

When subjected to biotic stress, bacteria can challenge the undesired agent through several mechanisms. Numerous species of the genus *Bacillus* act as PGPR, they possess the capacity to suppress necrotic pathogens and/or promote plant growth, which lead to the improvement of plant health [Cawoy et al., 2014]. This is mediated by three main mechanisms [Choudhary and Johri, 2009] :

- antagonism effects against pathogens,
- promotion of plant growth,
- stimulation of plant defenses.

In an anthropological view, this action of disease control and growth promotion is a real opportunity to increase agrosystem productivity. As matter of fact, species of the genus *Bacillus*, including *B. subtilis*, are commonly used in crops for increasing production, whether by promoting growth or by stimulating the host immune system (table 2.3) [Choudhary and Johri, 2009], [Wang et al., 2015]. A substantial interest lay on the use of mixture strains that deliver plant growth promotion along with disease protection,

Table 2.3: Commercially available *Bacillus*-based products for plant disease biocontrol. From [Choudhary and Johri, 2009].

Product name	**Company**	***Bacillus* component**	**Formulation type**	**Primary target**
Serenade	AgraQuest, Davis, CA	*B. subtlis* QST 713	Wettable powder, aqueousn suspension	Fungi, bacteria on multiple fruit
EcoGuard	Novozymes, Salem, VA	*B. licheniformis* SB3086	Flowable	*Sclerotinia homoeocarpa* on turf
Kodiak	Gustafson, Plano, TX	*B. subtliis* GB03	Wettable powder, flowable	Fungi on cotton, legumes, soybeans
Yield Shield	Gustafson	*B. pumilus* GB34	Wettable powder	Fungi on soybeans
BioYield	Gustafson	*B. amyloliquefaciens* GB99+, *B. subtilis* GB122	Dry flake	Fungi on bedding plants in potting mixes
Subtilex	Beker Underwood, Ames, IA	*B. subtilis* MB1600	Wettable powder	Fungi on cotton, soybeans, legumes
Hi stick, L+Subtiliex	Beker Underwood	*B. subtilis* MB1600 + *Rhizobium*	Flowable	Fungi on soybeans, peanut

thereby providing an increased probability of getting high yield in the field. For instance, the commercial product YieldShield consisting of *B. pumilis* spore preparation, provides the ability to improve both plant growth and protection against pathogens through ISR-inducing PGPR (table 2.3) [Choudhary and Johri, 2009].

2.5.3 Protection of plants against pathogens: antagonisms and elicitation of host defenses

The protecting effect against a lot of pathogens is provided by direct antagonisms and/or elicitation of the host defenses. This protection action depends on three main characters. First of all, a high ecological fitness respecting their ability to colonize roots is essential to efficiently compete for space and nutrients in the rhizosphere. Subsequently, the microorganism has to demonstrate high antagonist activity towards various plant pathogens, which relies on the secretion of highly active antimicrobials compounds. Lastly, it depends on the microbe ability to trigger an immune response in plant tissues leading to a systematical protection [Cawoy et al., 2014].

Antagonism effects

Antagonism towards pathogen populations by *B. subtilis* is well known and is a key biocontrol mechanism. Some strains produce toxins that inhibit growth and/or activity of fungal and nematode pathogens of plants. A wide variety of antimicrobial compounds are synthesized among which lytic enzymes (such as proteases, chitinases and β-1,3-glucanase), (lipo)peptide antibiotics, and other small molecules [Cawoy et al., 2014]. Lytic enzymes produced by bacteria are effective for degrading fungal cell walls and thus limiting plant infections by fungal pathogens. Peptides and lipopeptides are also molecules largely produced and directed against intruders in order to exterminate them. Lipopeptides predom-

inantly produced by *B. subtilis* strains with antibiotic effects are the lipopeptide surfactin, fengycin and iturin A [Akram and Anjum, 2011], [Cawoy et al., 2014]. They constitute the main antimicrobial compounds secreted in relevant amounts under natural condition of growth in the rhizosphere environment [Cawoy et al., 2015]

Antagonism towards pathogens can be expressed by some bacterial strains not only through antibiotic or lytic enzyme productions but also through competition for niche spaces and nutrients with other chemoheterotrophs in the rhizosphere [Choudhary and Johri, 2009].

Elicitation of host defenses

Besides direct antagonism effects, *B. subtilis* may also promote plant health by stimulating the host defenses, through activation of plant resistance pathways. This enhanced level of resistance requires colonization of roots by the bacteria. This steady state of defense is referred to as rhizobacteria-mediated ISR [Choudhary and Johri, 2009] [Zhang et al., 2001].

Protection resulting from ISR elicited by *B. subtilis* has been reported against a wide range of diseases caused either by fungus or viruses, insects, nematodes or bacteria. Generally, *Bacillus spp.* that elicit ISR also elicit plant growth promotion [Zehnder et al., 2001]. Investigations of the signal transduction pathways of elicited plants suggest that *Bacillus spp.* activate JA and ET pathways and the regulatory gene NPR1 but is independent of SA. This is consistent with the assumption that the protection resulting from the plant association rhizobacteria comes from the ISR, that is typically independent of SA and does not result in activation of the *PR1a* gene that encodes production of PR proteins. However, in some cases, ISR by *Bacillus spp.* is dependent on SA and can lead to accumulation of defense genes PR1 in plants. The specific signal transduction pathway activated during ISR by *Bacillus spp.* depends on the strain, the host plant, and less frequently on the pathogen [Kloepper et al., 2004] [Zehnder et al., 2001].

The understanding of specific mechanisms that take part in the set of enhanced level of resistance and the signaling pathway involved is essential for each plant-elicitor association, in order to increase the use and efficiency of some microorganisms as biocontrol agents. One of the key interests is to determine what substances are associated with the host defense following the pathogen challenge and the rhizobacteria-elicited ISR. Furthermore, the analysis of bacterial determinants involved in elicitation is also of great interest [Kloepper et al., 2004], [Zhang et al., 2001].

Mechanisms involved in plant protection

- ***Enzymatic production***
  Induced systemic defense responses in sugar beet elicited by different species of *Bacillus spp.* (*B. subtilis,B. mycoïdes, B. pumilus*) are largely associated with production of PR-proteins such as β-1,3-glucanase, and lytic enzymes, including chitinase and phenylalanine ammonialyase (*PAL*), as well as oxidative enzymes such as peroxidase (POX), polyphenol oxidase (PPO) and superoxide dismutase (SOD) [Chowdappa et al., 2013], [Bargabus et al., 2004], [Kloepper et al., 2004], [Chandrasekaran and Chun, 2016]. These observations highlight the tight link between ISR stimulation and host enzymatic activity. The enhanced level of oxidative enzymes, including POX, PPO and SOD might increase oxidative stress due to an

increased production of H_2O_2. Hydrogen peroxide and other free radicals are toxic to diverse microbial pathogens and are involved in oxidative burst, further explained.

Futhermore, PPO and POX catalyze the oxidation of phenolic compounds which leads to liberation of reactive molecules (quinines, ...) that induce cell death and barriers to secondary infection [Chaerani and Voorrips, 2006], [Akram and Anjum, 2011]. Tomato transgenic plants producing large amount of PPO were highly resistant against some diseases. Complementary experiments revealed that plants challenged with pathogens and bacterial strains produced increased amount of PPO, which may be linked with the increased resistance of those plants compared to a control [Akram and Anjum, 2011]. PPO is systematically over produced in response to some necrotic pathogens, including *A. solani*, in the upper leaves but not the lower ones. This expression coincides with the observation of EB evolution disease on tomato plants, that first have the lower old leaves attacked by the fungus [Chaerani and Voorrips, 2006].

PAL is involved in the phenylpropanoid pathway, and also takes part in the flavonoid production and the lignin biosynthesis. It is a key enzyme of the synthesis of the endogenous signaling molecule SA, which in turn activates the expression of numerous PR genes [Chaerani and Voorrips, 2006]. A rapid increase in PAL activity was observed when tomato plants were challenged by *Fusarium oxysporum* after having being exposed to *B. subtilis* and *B. fortis*, thereby increasing the phenolic contents in the host [Akram and Anjum, 2011]. Those phenols are key compounds in protection of plants against fungal pathogens, by inhibiting their growth.

- ***Oxidative burst***
  The oxidative potential of H_2O_2 also contributes to the formation of lignin during plant-pathogen interactions through PO-mediated cross-linking of proline-rich structural proteins and phytoalexin biosynthesis during oxidative burst and conversion of O-dihydroxyphenols to toxic o-quinones [Chowdappa et al., 2013], [Chaerani and Voorrips, 2006]. The resulting lignin formation and oxidative phenol biosynthesis contribute to the plant protection against pathogens by direct fungal degradation or formation of defense barriers, thanks to changes in the cell structure defense system [Akram and Anjum, 2011]. It may explain experiments realized by Benhamou and his team, who worked on the pathosystem tobacco-*Fusarium oxysporum*. They showed that when elicited by *B. pumilus* strain SE34, the tobacco was significantly less damaged by the fungus. This protection was due to a significant reduction of root colonization by *F. oxysporum*, which may be explained by strenghtening of the epidermal and cortical cell walls [Benhamou et al., 1998].

- ***Lipopeptides***
  Production of antibiotics compounds by *B. subtilis* is common and provides the bacterium a suppressive disease action towards pathogens. Some isolates of Bacillus spp. are among the most efficient microbial biocontrol agents and certain strains have been designed for the market level (table 2.3) [Cawoy et al., 2014]. The antibiotic production is activated when bacteria are competing with pathogens or during the transition state, after the end of exponential growth. An important part of antibiotic compounds that are produced by *Bacillus spp.* concerns lipopeptides, which demonstrates a high damaging activity against fungal pathogens. These cyclic lipopeptides are arranged by non-ribosomal peptide synthetase. Some isolates of

the *B. subtilis* species are able to produce high contents of non-ribosomal lipopeptides [Cawoy et al., 2015], which belong mainly to surfactin, iturin and fengycin families. Lipopeptides might contribute to the ability of the bacterium to efficiently colonize plant roots by acting as wetting agents. Therefore, they increase the mobility of bacterial cells which provides a higher potential to reach new microniches on roots [Cawoy et al., 2014]. Moreover, they positively impact biofilm formation by the bacterium. They also demonstrate strong and specific antibiotic activities allowing direct antagonism against various pathogens. Finally, they have a strong ability to potentiate the immune system, as systemic resistance inducer, specifically for the surfactin [Cawoy et al., 2014]. Among lipopeptides produced by bacteria, surfactin is amply synthesized. It is a lipoheptapeptide that constitutes the most active biosurfactant known. It degrades biological membrane through its role of detergent and has antiviral and antimycoplasma activities [Schaechter, 2009].

- ***MAMPs***
  MAMPs of beneficial microbes that contribute to induce resistance through ISR are multiple. The potent inducers of the host immune response are typically cell surface components of the beneficial microbes, such as flagellin, LPS, etc, or microbial secretions, such as lipopeptides, VOC, specific enzymes, etc. Sometimes, an elicitor can provide specific environmental conditions that will lead to activate ISR within the host. This is the case of siderophores produced by several beneficial microbes, including *Bacillus spp.*, that lead to iron limiting conditions in the rhizosphere, triggering the ISR of the plant. Therefore, there possibly exist multiple MAMPs that trigger immunity in plants during the plant-bacteria-pathogen interaction [Pieterse et al., 2014]. However, still very little is known about the type of elicitors causing ISR produced by *Bacillus* strains.

  - **FLS2** : one of the bacterial determinants of *Bacillus spp.* that is typically implied in the pattern recognition by PRRs during the set of ISR is flagellin. A lot of plants possess flagellin sensing 2 (*FLS2*), which is a PRRs that have a specific binding site for flagellin of bacterium flagella, which is implied in the PTI response. Bacterial pattern recognition through the PRR-FLS2 is greatly assumed to be one of the key inducers of ISR in solanaceae by *B. subtilis* strains [Baluška et al., 2007].

  - **Surfactin**: besides their direct antagonism effects, some lipopeptides also function as MAMPs in triggering the immune response. For instance, the surfactin seems to play an important role in the activation of the host defenses. This has been demonstrated by Cawoy and his team in Gembloux, using overproducing mutants or by applying the pure compound [Cawoy et al., 2014]. The study reveals a high correlation between ISR-triggering activities of the bacterium and its surfactin content. The experiment was conducted using *Bacillus subtilis* strain S499, but results suggest that it can be extended to other Bacillus subtilis strains, and even other closely related species [Cawoy et al., 2014]. Surfactin induces metabolic changes related to defense leading to reduction of disease, such as ion fluxes across the plasma membrane and oxidative burst generating reactive oxygen species (ROS). Cawoy's works demonstrate the importance of surfactin as single compound for plant immunization by *B. subtilis*. In her experiments, Cawoy has revealed that inoculation of tomato leaves with

B. subtilis stimulates the lipoxygenase pathway. Lipoxygenase clusters enzymes that catalysed oxidation of free fatty acids and esterified lipids. Their expression is affected by many factors such as development stage, jasmonic acid, abiotic stress, abscisic acid and pathogen invasion. Farahani and co-workers (2016) have published a study revealing that the expression of LOX appears earlier in an incompatible plant- pathogen interaction than in a compatible one. Moreover, this difference is noted to be more important at the beginning of the infection and disappears after three days. It seems that early induction of LOX plays a crucial role in plant defense against pathogen infection. Some products of LOX metabolism are essential to induce hypersensitive response at the site of infection in an incompatible interaction that limits pathogen growth. It has been approved that LOX mediated lipid oxidation is essential in causing membrane damage during hypersensitive response [Farahani and Taghavi, 2016].

- **2,3-butanediol**: some strains of *B. subtilis* produce volatile organic compounds (VOC) that are suspected to be involved in plant growth promotion and ISR elicitation. This ability has been examined in *Arabidopsis* treated with VOC of *B. subtilis* strain GB03, resulting in a high level of resistance due to the activation of ISR and growth promotion. Ryu and co-workers reported that the exposure of *Arabidopsis* to bacterial volatiles from *B. subtilis* strains significantly reduced the damage from the infection of *Erwinia carotovora* [Ryu et al., 2003]. One key volatile compounds implied in the ISR elicitation iwas the growth promoting 2,3-butanediol. This was confirmed through transgenic *B. subtilis* strains whose 2,3-butanediol emissions were lower than control strains, which displayed less efficiency in protecting *Arabidospis* against pathogen infections, compared to control strains. The exogenous application of 2,3-butanediol was also efficient to trigger ISR in *Arabidospis* [Ryu et al., 2003]. This volatile compound widely produced in the genus *Bacillus* appears to prime defenses of many species, leading to an enhance capacity to mobilize cellular defense responses when plants are faced with herbivore and pathogen challenges. The production of VOC, including 2,3 butanediol, by bacteria appears under low oxygen conditions, thus providing an alternative sink of electron for the NAD+ regeneration in anaerobic environment [Choudhary and Johri, 2009]. It has been demonstrated in several studies that the pathways involved in the elicitation of ISR by volatiles was ET-dependent, but independent of the SA and JA signaling pathway [Choudhary and Johri, 2009], [Kloepper et al., 2004]. The growth promotion effect has been examined in several mutant lines of *Arabidopsis* treated with *B. subtilis* strain GB03, revealing that the cytokinin-signaling and ethylene-signaling pathways play a key role in growth promotion with VOC [Kloepper et al., 2004].

- **Siderophores**: Under conditions of low iron availability, most aerobic and facultative anaerobic microorganisms, including *B. subtilis*, produce low molecular weight Fe^{3+} chelators, called siderophores. This can lead to a competition for iron between bacterial communities and soilborne pathogens, providing to the beneficial microbes with a mode of action to challenge pathogens [Pieterse et al., 2014]. Besides this direct competition, it triggers iron deficiency condi-

tions for the plant that may take a part in the elicitation of resistance. Indeed, studies reveal that iron limited conditions or conditions that distort iron uptake, such as high zinc concentrations, are associated with enhanced level of resistance in plants against pathogens, thereby associating iron homeostasis, or siderophores, with the onset of ISR. Analyses of the genes associated with the iron deficiency response help to understand how the presence of siderophores can impact the resistance response in plants. The gene MYB72 is specifically expressed in roots under iron deficiency. Since this gene is required in the onset of ISR (Figure 2.9), siderophores help the production of chemical signals for the generation or translocation of the ISR long distance signal [Pieterse et al., 2014]. Moreover, the low iron conditions can be associated twith a production of SA by *B. subtilis*, which is the signaling molecule in the SAR. However, as above mentioned, in most cases, this SA production does not contribute to enhanced defense, as it is directly channelled into the production of SA-containing siderophore [Pieterse et al., 2014].

A lot of compounds can induce resistance in plants. The list above is not exhaustive, and there is a wide diversity of MAMPs involved in the ISR, depending on the plant, the elicitor and the type of challenger [Pieterse et al., 2014].

2.5.4 *Bacillus subtilis* strain 30B-B6

Beneficial microbes with ISR-eliciting properties have often been selected from large screening in the root microbiome, looking for members with biological control activities. One of the genera well studied in the set of ISR is *Bacillus*, and particularly the species *Bacillus subtilis*. The strain 30B-B6 (the strain used in this work) has been selected within the collection of 2800 *Bacillus spp.* and *Pseudomonas spp.* isolated from the rhizosphere of potato field in the frame of the WACOBI project [Caulier et al., 2018]. Among them, 52 *Bacillus spp.* and 8 *Pseudomonas spp.* were selected for their antagonist activities against common pathogen agents of the potato, a solanaceae like the tomato. *B. subtilis* 30B-B6 was shown to be antagonistic against *Alternaria solani, Phytophtora infestans, Fusarium solani* and *Rhizoctonia solani* (table 2.4). This strain does not stimulate the growth of potato [Caulier et al., 2018].

Table 2.4: Growth inhibition percentage of different phytopathogen microorganisms through direct antagonism by *Bacillus subtilis* strain 30B-B6. From [Caulier et al., 2018].

Pathogenic microorganisms	**Rank of growth inhibition percentage**
Pectobacterium carotovorum	0 - 30%
Phytophthora infestans	$>$ 70%
Alternaria solani	30 - 50%
Fusarium solani	30 - 50%
Rhizoctonia solani	30 - 50%

Several bio-active metabolites of this strain were mainly lipopeptides, but also lytic enzymes and peptides. The production of bio-active compounds were positively measured for different enzymatic activities, iron chelators and bio-surfactants [Caulier et al., 2018]. No virulence factors for human health were detected for this strain. They all appear in table 2.5.

Table 2.5: Bio-active metabolites and bio-active compound production of the isolate 30B-B6 of *Bacillus subtilis* based on PCR detection of related genes. From [Caulier et al., 2018].

Bio-active metabolites	**Bio-active compound production**
Enzyme: Glucanase	Enzymatic activities: cellulolytic, proteolytic
Lipopeptides: Bacyllomycin D, plipastatin, surfactin	Iron chelators: siderophores
Peptide: Bacilysin	Bio-surfactants: amphiphylic peptides

In planta bioassays revealed that the strain 30B-B6 would also induce systemic resistance on tomato but the nature of this systemic induction still remains unclear. This master thesis aimed at bringing to light the molecular mechanisms of induced resistance in order to find out its nature.

Chapter 3

Material and methods

3.1 Germination, growth and maintenance of plants

Tomato cv. Moneymaker seeds were surface-sterilized in a solution of 2% household bleach solution (1,0% sodium hypochloride) for 5 min and then 3 times rinsed with water milliQ. The seeds were then placed on a moist fanfold paper, in a plastic box hermetically closed in a growth chamber with a 6 hours photoperiod at 22°C during 10 days. The seedlings were then soil potted and the pots were transferred in greenhouse (figure 3.1 a.). Plants were grown during 4 weeks and watered daily under greenhouse conditions at a constant temperature of 24 °C with HR of 60 % and a 16 hours photoperiod, until they reached the five-leaflet stage. Detailed protocol is available in appendix C.1..

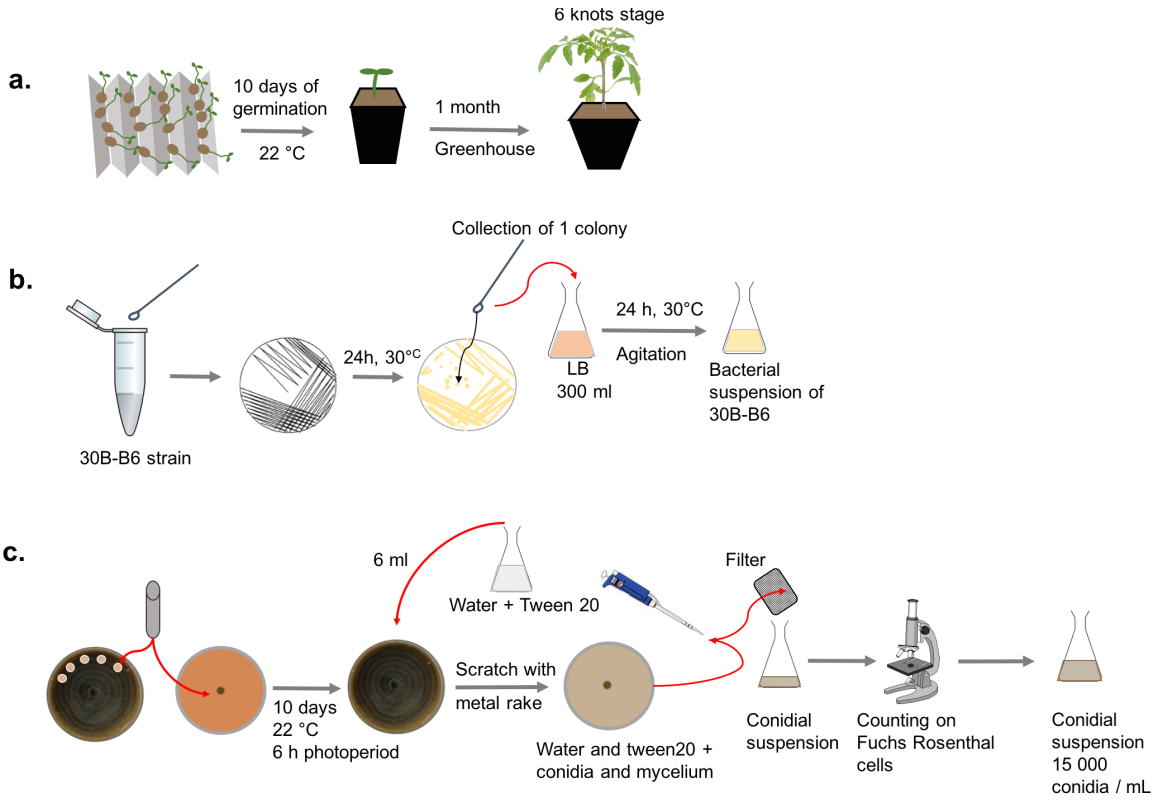

Figure 3.1: Scheme of the material preparation processes: a. seed germination protocol, b. bacterial suspension preparation, c. fungal suspension preparation.

3.2 Treatment and inoculation

3.2.1 Bacterial suspension

Subcultures of the bacterial strain 30B-B6 (GenBank accession Number MF062631), isolated by the Wacobi project were grown on lysogeny broth agar (LBA) (bacto yeast extract, 3 g, bacto tryptone, 1.5 g, NaCl, 3 g, agar, 5.1 g, miliQ, 300 mL) medium in Petri dish [Caulier et al., 2018]. After incubation at 30°C for 24 hours, one isolated colony from LBA was collected and suspended in 300 mL of lysogeny broth (LB) solution, which is then placed in an agitator ($30°^C$, 150 rpm) for 24 hours. The bacterial suspension obtained was then used in further described experiments, for tomato treatments (figure 3.1b.). The detailed protocol and the LB medium preparation are given in appendix C.2. and E.1. respectively. This protocol allows to produce suspensions with similar concentrations of bacteria (calculated in colony forming unit (CFU)) after different preparations. For each suspension prepared, different dilutions (10^{-4} to 10^{-11}) were prepared from the bacterial suspension, and were spread on LB Petri dishes, 3 Petri dishes for each dilution (figure 3.2). After 24 hours at 30°C, the colonies from distinct dilutions are counted and the one with a number of colonies between 15 and 200 was used to assessed the number of CFU in the initial bacterial suspension, using the dilution ratio. The detailed method of CFU calculation is given in appendix D.2. This calculation was carried out through the following equation:

$$CFU = \frac{(Numberofcolony)/(Platevolume)}{Dilution}$$

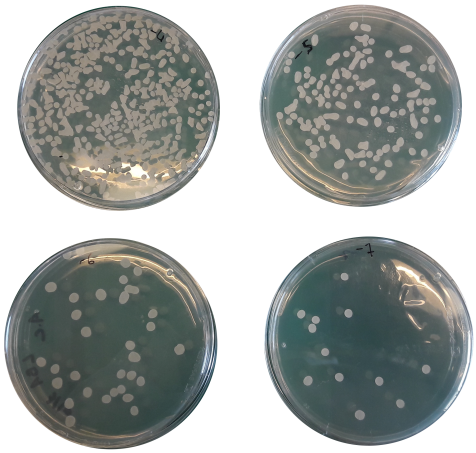

Figure 3.2: Growth of *Bacillus subtilis* 30B-B6 at different dilutions on Petri dishes. The dilutions are, respectively from the top-left corner to the lower-right corner, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}.

The bacterial concentration obtained for all the experiments was about 10^8 CFU/mL.

The application of the supernatant of the bacterial suspension was also performed. This suspension was performed through centrifugation (15 000 rpm, 5 min) and filtration (0.22 μm) of the bacterial suspension.

3.2.2 Fungal growth and conidial suspension

The necrotrophic fungus *Alternaria solani* (MBC928, CRA-w, Belgium) was grown on V8 medium (V8 vegetable juice, 70 mL/L, agar, 5.25 g/L, $CaCO_3$ 0.35 g/L, water miliQ, 280 mL) in Petri dishes, for 10 days in a 16 hours photoperiod (neon lighting) at 22°C (figure 3.3) [Caulier et al., 2018]. Subcultures on V8 agar were performed every two weeks. For the inoculation of 10 plants, about 20 Petri dishes with *A. solani* had to be produced. The collection of conidia was performed through the addition of 7 mL of water and Tween20, a detergent (one drop of Tween20 in 100 mL of distilled water) in a Petri dish, and the use of a metal rake to scratch the medium surface. The suspension was then filtered to eliminate the mycelium surplus. The detailed protocol and the V8 medium preparation are given in appendix C.2. and E.2., respectively. The conidial concentration was determined by counting with a Fuchs-Rosenthal cell under the optical microscope (figure 3.1 c.). The total number of conidia per mL was then estimated through the conversion method exposed in the abacus of the appendix D.1. The final concentration of the suspension was set at 15 000 conidia / mL (the dilution was performed with addition of water + tween20)

Figure 3.3: Culture of *Alternaria solani* on V8, after 10 days of growth.

3.3 Assessment of gene expression by RT-qPCR

3.3.1 Leaf collection and grinding

The leaf collection was carried out by drawing the leaves of each plant with a scalpel blade, disinfected in anios. The collected leaves were then placed in plastic collecting tubes that were directly frozen in liquid nitrogen. The time interval between cutting and freezing was as shortest as possible, in order to avoid perturbations of the expression of gene at the molecular level resulting from the cutting. Those tubes were then stored in freezer at -80°C before RNA extraction.

The frozen leaves were ground using a mortar and pestle partially immersed in liquid nitrogen. Then, 70 to 100 mg of the crushed leaves were transferred in a sterile microtube containing a ceramic ball. Those tubes were then placed in a fastprep (Thermo electron, FastPrep FP120), where they were highly agitated resulting in a fine grinding of the leaf cells.

3.3.2 RNA extraction

The total RNA was purified from samples using the Qiagen RNeasy Plant Mini Kit (figure 3.4). It consists in a cell lysis using buffer with high concentration in guanidine isocyanate, followed by different steps of washing though removal of membrane and intracellular

compounds, and a silica membrane which fixes the RNA purified. A DNase treatment was performed to eliminate any genomic DNA that would have been co-extracted with RNA. Protocol of RNA extraction and DNAse treatment are available in appendix C.3..

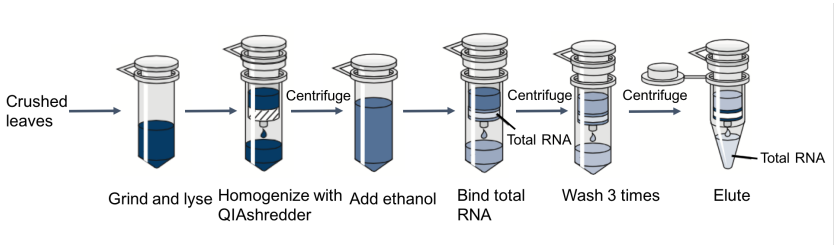

Figure 3.4: RNA purification steps using the RNeasy Plant Mini Kit. From [Qiagen, 2012].

Finally, the quality of the RNA extraction was evaluated through spectrophotometry (Nanodrop ND1000, Thermo Fisher Scientific), by the 260/280 and 260/230 absorbance ratios. Nucleic acid has a typical absorbance at 260 nm, which allows the appreciation to estimate its presence in a preparation relatively to other compounds. The A260/280 value was used to evaluate the percentage of proteins co-extracted with RNA. This value must be superior to 2.0 to consider the RNA sample as relatively free of protein. The 260/230 values assesses the presence of other contaminants such as carbohydrates and phenol, which absorb at 230 nm. A relatively pure preparation has a ratio value between 2.0 - 2.2. An estimation of the RNA concentration in the extract and of the purity was performed using the spectrophotometer NanodropR (raw data exposed in appendix A.1.)

In order to provide a reliable comparison of gene expressions between the samples, all the RNA extracts were adjust to the same concentration of 100 μg RNA per mL.

3.3.3 Synthesis of cDNA

Complementary DNA (cDNA) of each RNA samples was obtained after reverse transcription using M-MLV Reverse Transcriptase (Promega) and Oligo d(T)25, which are going to amplify all the eukaryotic mRNAs in the sample through poly-A-tails fixation. Detailed protocol is available in appendix C.5.

3.3.4 Selection of target genes

Five target genes of *Solanum lycopersicum* that have been selected for their affiliation in one particular pathway of plant defense were (Table 3.1):

- genes related to the SAR; Pathogenesis related 1 song (*PR-1 song*) and Glucan endo-1,3-βglucosidase (*PR2-b*) genes [Song et al., 2011], [Van Aubel et al., 2016],
- genes related to the ISR; the Protease inhibitor 1 (*PI-1*) and Lipoxygenase (*LOX*) genes involved in JA pathway [Fujimoto et al., 2011],
- gene involved in the cross-talk between SAR and ISR; the *NPR1* gene [Van Aubel et al., 2016].

Table 3.1: List of the reference and targeted genes studied with accession number from Sol Genomic Network or NCBI and corresponding primers used for RT-qPCR analysis.

Gene	Gene function	Accession no	Primer sequences (5' - 3')	Amplicon size	References
EF1-α	Elongation factor 1-alpha	AB0611263	CCAGATTGGAAACGGATATGC TCCTTACCTGAACGCCTGTCA	105 bp	[Nicot et al., 2005]
CAC	Clathrin adaptator complex	SGN-U314153	CCTCCGTTGTGATGTAACTGG ATTGGTGGAAAGTAACATCATCG	592 bp	[Expósito-rodríguez et al., 2008]
PGK	Phosphoglycerate kinase	SGN-U578082	TCTACAAGGCCCAAGGTTATG GCAGCAAACTTGTCCGCAATC	148 bp	[Ghareeb et al., 2011]
PR-1 song	Pathogenesis related 1	DQ159948	TGTCCGAGAGGCCAAGCTATAAC AATGAACCACCATCCGTTGTTGC	143 bp	[Song et al., 2011]
PR2-b	Glucan endo-1,3-*β* glucosidase B	SGN-U581016	GCACCTTTGCTCGTTAACATT TTGACGCGATCCATCTTGTA	120 bp	[Van Aubel et al., 2016]
NPR1	Non pathogenesis related protein 1	SGN-U585807	AATCGGCTTAGGGCTCTCTC TGCTTCTTCAGTTGACGCTCT	155 bp	[Van Aubel et al., 2016]
PI-1	Protease inhibitor 1	SGN-U577558	GTGTACCAACAAAGCTTGC-TAAAGA GTACAACAACACCCAAAAT-GTTGTC	155 bp	[Fujimoto et al., 2011]
LOX	Lipoxygenase	NM-001247927	GAGTTCTCCTCATGGT-GTTCGTTTA AGTAGTCTGACACCCAACTT	101 bp	[Bell and Mullet, 1993]

3.3.5 Selection of housekeeping genes

The level of expression of targeted genes has to be normalized using a set of housekeeping genes. For that purpose, reference genes are selected, also called housekeeping genes, that typically encode for molecules that are involved in the primary metabolism and thus whose expression should be stable. The assessment of the level of expression of the genes will be carried out by the housekeeping genes [Vandesompele et al., 2002]. The reference genes used in this work are endogenous genes, encountered in the samples. Three housekeeping genes of tomato have been selected: Elongation factor 1-α ($EF1\alpha$) [Nicot et al., 2005], Clathrin adaptator complex (CAC) [Expósito-rodríguez et al., 2008] and Phosphoglycerate kinase (PGK) [Ghareeb et al., 2011] (Table 3.1). The primers were selected from literature and produced by Eurogentec (Liege). The specificity of the designed primers were verified through electrophoresis gel and the melting curves from RT-qPCR.

3.3.6 Quantification of gene expression by RT-qPCR

The RT-qPCR assay was performed on 96 well plates using SybrGreen as DNA intercalating agent. The SybrGreen is a fluorescent dye that binds to DNA double strand and fluoresces when bound to it and excited by a light source, thus allowing a quantification of the DNA in a well at one moment of the PCR amplification cycles. Each well is filled with 10 µL of iQ SYBR Green supermix (BIORAD), 0,5 µL of each primer (20 µM), 7 µL DEPC-treated water and 2 µL of cDNA. Two technical replicates of each samples were prepared. The amplification cycles were carried out in a CFX 96 Real-Time System (Bio-Rad) respecting the following protocol: 10 min of denaturation at 95°C followed by 40 cycles of 15 s at 95°C, 1 min at 60°C and 30 s at 72°C and a final melting curve from 65 °C to 95 °C, using a heating rate of 0.5 °C per s. The detailed protocol of RT-qPCR is given in appendix C.7. Standards were built by serial 10-fold dilution of RNA extracts and were used to generate standard curves, for all genes. Those standard curves allowed to obtain primer efficiencies for each genes, by plotting the threshold cycle (Ct)[1] values against a log of the standard concentrations. The efficiency of the amplification was evaluated through the standard curves, and similar efficiencies between plates allowed to compare them. The specificity of the amplification is checked through the melting curve to ensure that the amplification was specific. Blanks were loaded in each plates to ensure that no contamination of the products used had occurred.

A standard curve slope of –3.32 indicates a PCR reaction with 100% efficiency. Slopes more negative than –3.32 (ex. –3.9) indicate reactions that are less than 100% efficient. Slopes more positive than –3.32 (ex. –2.5) may indicate sample quality or pipetting problems. Amplification efficiency: the rate at which a PCR amplicon is generated, commonly measured as a percentage value. It is a measure of the overall performance of a RT-PCR assay. The slope of a standard curve is commonly used to estimate the PCR amplification efficiency of a real-time PCR reaction [Biosystems, 2004].

[1] Ct is the cycle number at which the fluorescence generated in a reaction goes beyond the threshold line. This line is set to be above the baseline but sufficiently low to be within the exponential growth region of an amplification curve.

3.3.7 Analysis method

The results obtained were treated using CFX MANAGER Software (BIO-RAD) and expressed as normalized relative quantities. The analysis methodology used was the comparative Ct method, also called the Δ ΔCt method. The CFX MANAGER Software (BIO-RAD) performs the Δ ΔCt calculations, in addition to real-time PCR plate set-up and analysis. The amount of target, normalized to an endogenous reference and relative to a calibrator (for example, the untreated samples), is [Kozera and Rapacz, 2013]:

$$R = \frac{(E_{studiedgene})^{\Delta Ctstudiedgene(control-sample)}}{(E_{controlgene})^{\Delta Ctcontrolgene(control-sample)}}$$

where ΔCt = $Ct_{targetgene}$ - $Ct_{referencegene}$ and E is the amplification efficiency.

3.3.8 Statistical analyses of gene expressions

All of the experiments were conducted in triplicate and results were tabulated as Mean ± standard deviation (SD). Data were analyzed by analysis of variance (ANOVA). Student's t test and the probability values of $p < 0.05$ were considered to be significant.

For each treatment, three replicates were maintained in a completely randomized design. R open source software for MACOS was used for statistical analysis. The data were analyzed with a one- way analysis of variance with the significant differences among means identified by Tukey's multiple range test ($P < 0.05$).

Chapter 4

Results

4.1 Effect of 30B-B6 on disease severity and progress

4.1.1 Bacterial bioassay

The beneficial effect of 30B-B6 as plant protector against EB disease was assessed through a greenhouse experiment under controlled conditions on 10 control plants, which received a bacteria-free LB solution, and 10 plants previously elicited by 30B-B6. All the plants were inoculated by *A. solani* 6 h after the application of the bacterial suspension or the LB solution and the elicitor effect of the bacteria was tested by a daily symptom monitoring. Since 30B-B6 was applied directly on the roots and *A. solani* was sprayed on the leaves, the protective effect due to the bacterium indicates an induced resistance activation, instead of a direct antagonism of pathogens. The first symptoms were visible 3 days after the leaf inoculation - at the first observation time - on control and treated plants. During the course of the trial, the number of leaves affected by EB symptoms did not vary substantially between 30B-B6 treated plants and LB treated plants. However, the disease severity was significantly reduced on the leaves of the plants treated with 30B-B6 compared to control ones. Three weeks after the fungal inoculation, the mean percentage value of the treated plants reached 5,61 $\pm$ 4,24 % whereas this value extended to 38 $\pm$ 27 % for the control plants (figure 4.1 a.). The AUDPC values calculated over 18 days differed significantly for both groups ($p < 0{,}05$). It was estimated at 45 $\pm$ 8 % for the 30B-B6 elicited plants and 259 $\pm$ 18 % for the untreated plants (figure 4.1 b.). The percentage of protection of the 30B-B6 elicitor was of 83 %. The figure 4.2 illustrates this strong decrease of the disease progression in a leaf coming from an elicited plant (figure 4.2 b.) relative to a leaf from a control plant (figure 4.2 a.). The 30B-B6 treated clearly displayed a number and size of necrotic spots inferior to the LB treated leaf, as well as reduced chlorotic halos. Hence, the results were convincing, with a high efficiency of 30B-B6 in protecting tomato host plant against the necrotrophic fungus *A. solani*.

4.1.2 Supernatant bioassay

A second bioassay was conducted under the same conditions except that the supernatant of the bacterial suspension (bacterial suspension filtered to remove bacteria) was used on the treated plants instead of the bacterial suspension itself. This assay was intended to evaluate the protective effect of the supernatant against *A. solani*, and whether the bacteria cells are required in the protection conferred by 30B-B6. As for the first experiment,

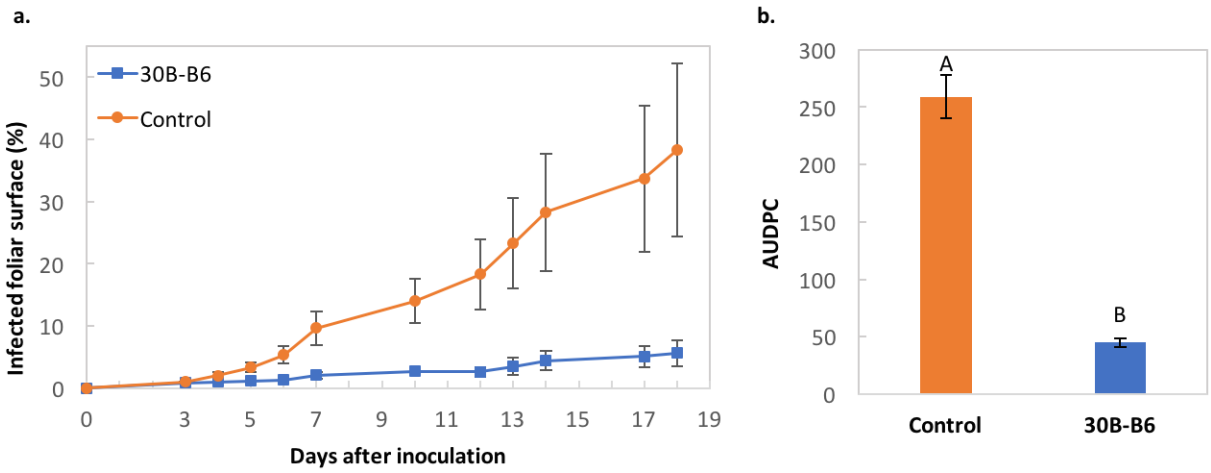

Figure 4.1: a. Percentage of leaf area affected by *A. solani* (disease severity progress) of *S. lycopersicum* elicited by 30B-B6 (n=10) or untreated (control) (n=10), as function of time following the moment of inoculation. b. Area under the disease progress curve (AUDPC) of *S. lycopersicum* elicited by 30B-B6 (n=10) or untreated (control) (n=10). Results indicated with a different letter are significantly different, with a P value ≤ 0.001 based on a Tukey test.

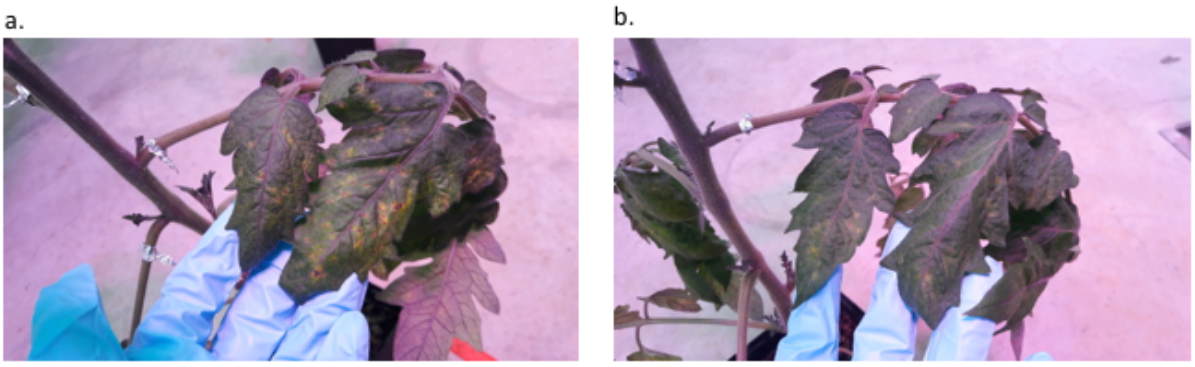

Figure 4.2: Photos of leaf affected by early blight 10 days after *Alternaria solani* infection, a. on a control and untreated plant, b. on a plant elicited by 30B-B6.

the symptoms emerged for both groups of plants, 3 days after the fungal inoculation. The percentages of leaf area covered by the symptoms were similar between the control plants (n=10) and plants treated by the bacterium supernatant (n=10), with a mean value of 27 ± 6 % of covered leaf area, measured 18 days after the inoculation, against $25 \pm 9\%$, respectively (figure 4.3 a.). The AUDPC values were of 193 ± 14 for the untreated plants and 175 ± 18 for the treated ones (figure 4.3 b.). This difference was not significant (Tukey test, $p < 0{,}05$).

The supernatant was not tested simultaneously with the bacterium in a single experiment, but each trial (supernatant vs. control and bacterium vs. control) was repeated 3 times before my master thesis by a phD student in the UCL laboratory of phytopathology (Colau et al., publication under preparation), and the results always were positive for the bacterium and negative for the supernatant, compared to the control.

4.2 Effect of 30B-B6 on gene expression of tomato inoculated or not by EB

4.2.1 Experimental proceeding

An experiment has been conducted to analyze molecular changes associated with bacterial elicitation of tomato inoculated or not by *A. solani*. Three treatments and one LB control have been applied, each on 18 plants (6 time points × 3 repetitions). The treatments were the following :

- T control plants, application of bacteria-free LB solution on the substrate of plants and treatment of leaves (6 hours after) with water + Tween20 without conidia of *A. solani*,
- B (bacteria) plants treated with 30B-B6, and leaves treated with water + Tween20 without pathogen,
- P (pathogen) plants, with application of bacteria-free LB solution and inoculation of a conidial suspension,
- BP (bacteria and pathogen) plants, both inoculated with the conidial suspension and treated with the bacterial suspension.

Leaves of plants were collected individually from separated plants, just before the fungal inoculation, 10 minutes after the inoculation and 3, 6, 9 and 24 hours post inoculation. The two upper younger leaves (knots 5 and 6), that were not inoculated (denominated -), and the lower leaves (knots 3 and 4) inoculated (denominated +) of 3 plants per treatment were collected separately (figure 4.4). The different time points have been chosen in order to analyze any potential changes of defense related gene expression over time.

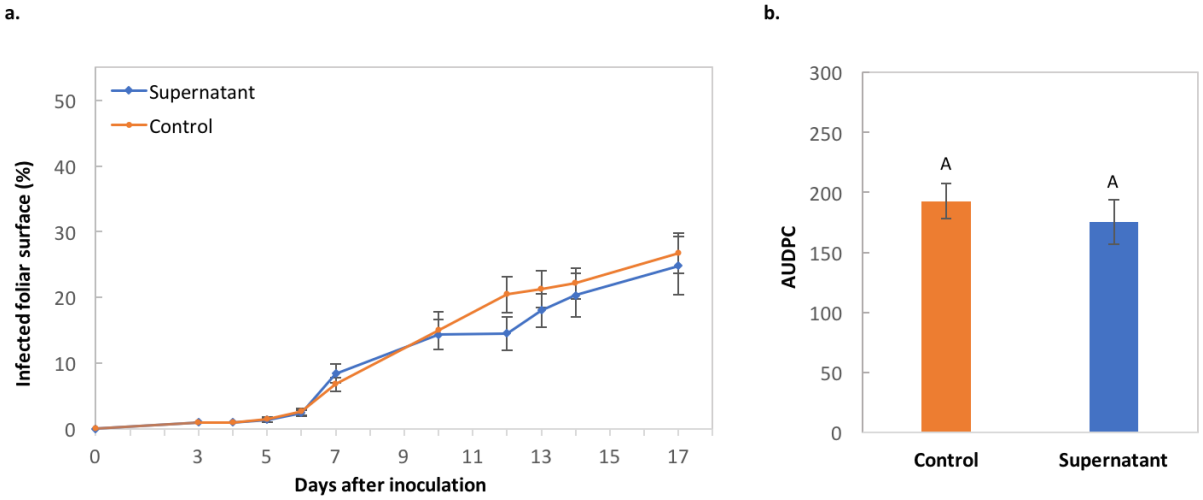

Figure 4.3: a. Percentage of leaf area affected by *A. solani* (disease severity progress) of *S. lycopersicum* treated by the supernatant of a 30B-B6 suspension (n=10) or untreated (control) (n=10), as function of time following the moment of inoculation. b. Area under the disease progress curve (AUDPC) of *S. lycopersicum* treated by the supernatant (n=10) or untreated (control) (n=10). Results indicated with a different letter are significantly different, with a P value ≤ 0.05 based on a Tukey test.

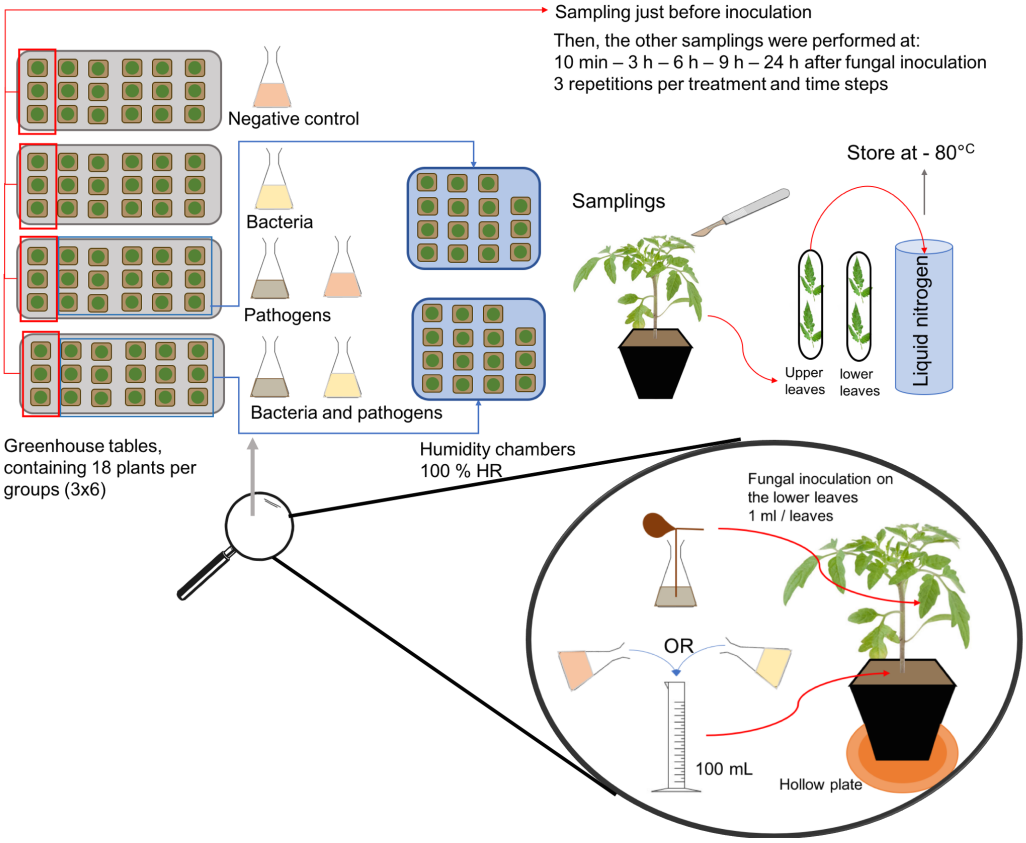

Figure 4.4: Scheme of the experimental layout of the bioassay to evaluate the effect of a treatment of 30B-B6 on the infection by *A. solani* and gene expression pathway

A total of 144 samples (18 plants × 4 groups × 2 leaf positions) were thereby collected for the molecular analyses. In the first step of the RNA extraction, 1 mg of foliar tissues and ceramic balls were added in microtubes, which were strongly mixed using the Fastprep instrument. Since this step led to the breakage of some tubes and losses of samples under the guidance of PhD students from the laboratory, we improved this step by adding lysing buffer (constituted by guanidine salt and DDT) in the microtube containing the ceramic ball. The use of lysis buffer has helped in stabilizing RNA after releasing from leaves and reducing the acceleration of balls in the microtubes. Finally, due to the problem of breaking tubes, we obtained RNA extracts from 141 leaf samples.

4.2.2 Extraction results

The mean RNA concentration obtained from 1 mg of tomato leaves following the extraction was of 611,8 ng/μL, with a great standard deviation of 476,7 ng/μL. Some extract samples did not reach the 100 ng/μL of RNA extracted and have been re-concentrated to obtain the desired final concentration. This step was performed using sodium acetate and ethanol, followed by centrifugation and several step of flushing / drying (see protocol in appendix A.1). The mean purities obtained for each extracted samples were of 2,13 ± 0,050 and 1,95 ± 0,426 for the 260/280 ratio and 260/230 ratio, respectively. The 260/280 ratios generally obtained for the samples are acceptable as its mean is closed and superior to 2, which indicates a poor contamination of the RNA in phenolic and proteic compounds. However, a ratio between 2 and 2,2 is desired for the 260/230 ratio. Slower

values, as generally obtained in our results, indicate the presence of some contaminants as plants carbohydrates or phenolic and guanidinic compounds from the kit. Nanodrop raw data of sample concentrations and purities are given in appendix A.1.

4.2.3 Primers validation

To investigate the signaling pathways that occur in tomato plants following 30B-B6 treatment and *A. solani* inoculation, transcripts of specific signaling pathways responsive genes have been analyzed by RT-qPCR. Five target genes and 3 housekeeping genes were selected, as exposed in the above section material and method. Before loading the samples in the different plates and performed the quantitative PCR, reverse transcription of each sample was required. The primers used for the amplification were designed and selected from diverse works, exposed in table 3.1 of the material and method. The specificity of the primers was checked on PrimerBlast and confirmed with electrophoresis gel, before using them in the RT-qPCR. A Reverse-Transcription PCR (RT-PCR) for each of the tested genes was thereby realized on RNA tomatoes extract, followed by an electrophoresis gel to confirm the specific amplification (figure 4.5). The protocol of the RT and the PCR are given in appendix C.5. and C.6., respectively. The primers were accepted if only a single band was visible on the gel, corresponding to the expected amplicon size.

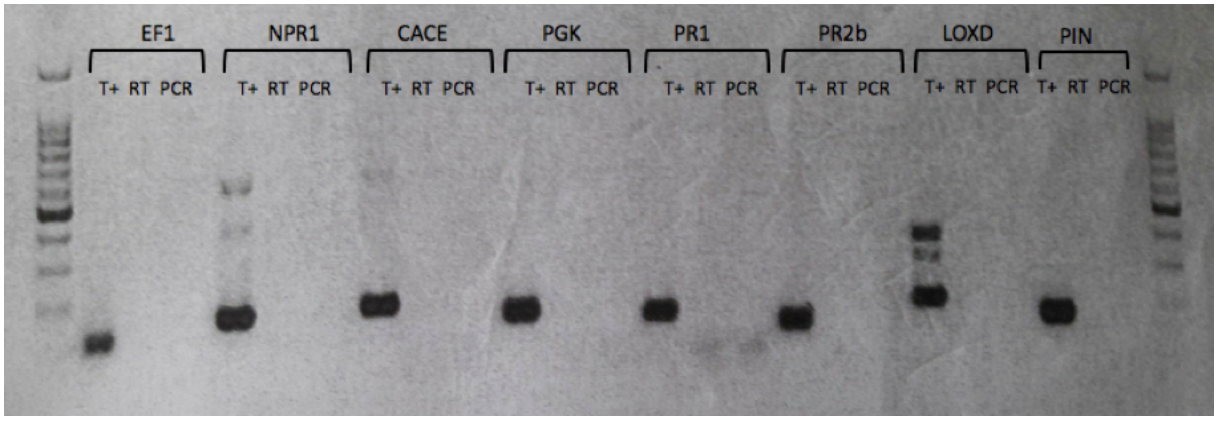

Figure 4.5: Conclusive example of primer tests on RNA samples through RT-PCR revealed on electrophoresis gels. The gene tested here are *EF1α*, *NPR1*, *CAC*, *PGK*, Pathogenesis related 1 (*PR-1*), *PR2-b*, *LOXD*, *PI-1*. Blc RT = Reverse Transcription blanco. Blc PCR = PCR blanco. T+ = Tomato samples containing the expected amplicon.

Several genes presented more than one band in the electrophoresis gel. This could result from diverse causes, such as gDNA contamination or unspecific amplification. To solve gDNA contamination, a DNase treatment of the extract was performed. *CAC* initially revealed two amplicons of different size on the gel, one of 173 bp, which coincides with the expected amplicon, and another around 600 bp, which resulted from a gDNA that remained after the extraction and that was amplified by PCR. A DNase treatment during the extraction step allowed to suppress this double band and only the expected amplicon size of *CAC* remained after this treatment. Unspecific amplification occured for LOXD, as the DNase treatment did not suppress the numerous bands on the gel. To avoid the issue, new primers specific to LOX amplicon of *S. lycopersicum* designed by [Bell and Mullet, 1993], checked on PrimerBlast, were selected. Finally, all primer specificities were confirmed by electrophoresis gel and standard dilution were prepared for

each genes. The melting curve from this standards for all of the tested genes confirmed the specificities of the amplification, with a single peak of melting temperature corresponding to the respective expected amplicons.

The RT-qPCR was performed on 4 plates of 96 wells for each genes, in order to quantify the 141 samples. On each plates, negative control and standard dilutions were loaded. The efficiencies (E) obtain for each amplification of the RT-qPCR between the 4 plates per gene were comparable, between 95% and 100 %. This is calculated based on the slope of the standard curves. Since the efficiencies were closed to 100 %, this indicates a slope of the standard curves closed to -3,2, which confirms that the 10 by 10 dilutions were rigorously realized.

4.2.4 Housekeeping genes stability

The stability of the housekeeping gene expression was previously checked on the software Biorad CFX manager to ensure that they could fulfill their function. The quantification of the level of expression of the three housekeeping genes, *PGK*, *CAC*, *EF1α*, using the RT-qPCR assays revealed, based on their mean stability value and variation coefficient, a stability of the expression for *PGK* and *EF1α* but an instability for *CAC*. The stability values obtain through the Biorad software wihen the three housekeeping genes were selected was not acceptable, since their mean stability values and variation coefficients were superior to the upper thresholds of stable gene expression, which are respectively 1 and 0.5 (CFX96 Instruction Manual, Biorad). The figure 4.6 reports the Cq value of each samples during the course of the RT-qPCR analyses for the three housekeeping genes selected. We notice that *EF1α* and *PGK* have similar expression levels across the treatments, which indicates a stability of their expression. However, the *CAC* gene shows a variation in its expression compared to the two other housekeeping genes, specially under the bacterial treatment (figure 4.6). Variations of the Cq are visible between *EF1a* and *PGK*, but there are more probably linked to experimental factors than the plant treatment. Indeed, the three biological replicates per treatments and time points are placed alongside each others in the figure. If the variations were due to the treatments, we would have expected to see asymmetric variations between *EF1a* and *PGK* per group of 3 plants, as slightly observed with *CAC*. This slight unstability of the CAC gene is confirmed, since the new stability values and variation coefficients for *EF1α* and *PGK* housekeeping genes were in the range of stability, when CAC gene was no longer selected. The new mean stability value obtained for both housekeeping genes was of 1 and the variation coefficients were of 0.4 and 0.33 respectively for *EF1α* and *PGK*. Hence, only *EF1α* and *PGK* housekeeping genes were used to obtain the relative expression of the 5 tested target genes, *PR-1*, *PR2-b*, LOX, *PI-1*, *NPR1*.

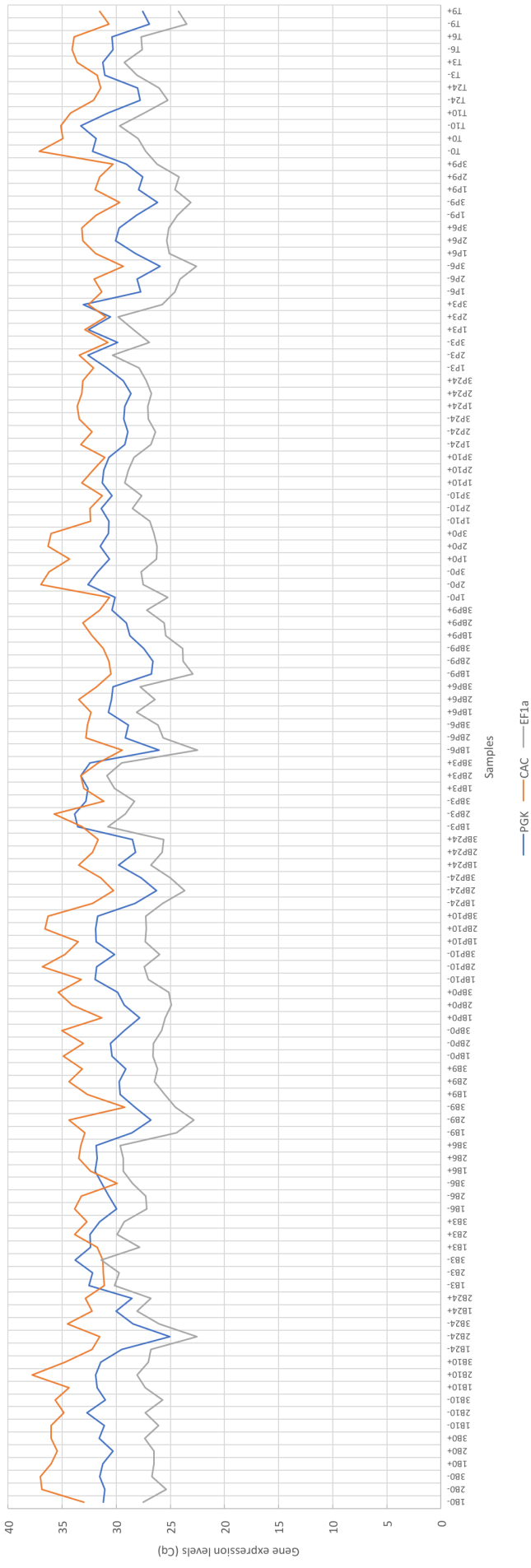

Figure 4.6: Expression profiles of the three housekeeping genes *CAC*, *PGK*, *EF1a* selected. The set of all the plant sample types collected for the analyses of the relative gene expression using RT-qPCR method are exposed in abscissa. The Cq measured in real time by the PCR machine is given for each sample types in ordinate. B = bacterial elicitation; P = Pathogen inoculation; BP = bacterial elicitation + pathogen inoculation; T = untreated control; + = upper-younger systemic leaves; - = lower-older leaves, directly sprayed with the fungal suspension for the P and BP treatments. The first number corresponds to the replicates (3 biological replicates by treatment, time points and leaf position). The second number corresponds to the time points (0 hours, 10 minutes, 3- 6- 9- 24- hours after the fungal inoculation)

Hence, only *EF1α* and *PGK* housekeeping genes were used to obtain the relative expression of the 5 tested target genes, *PR-1*, *PR2-b*, LOX, *PI-1*, *NPR1*.

4.2.5 Gene expression profiles

The progress over time of the relative gene expression levels assessed by RT-qPCR in the lower older leaves (+) and upper younger leaves (-) of four groups of plants is presented in figures 4.7 and 4.8, respectively. The time scale used is defined starting from the moment corresponding to the inoculation of some plants with the pathogen. As a reminder, the application of the bacterial suspension, or bacteria-free LB solution, was carried out 6 hours before the leaf inoculation of *A. solani*.

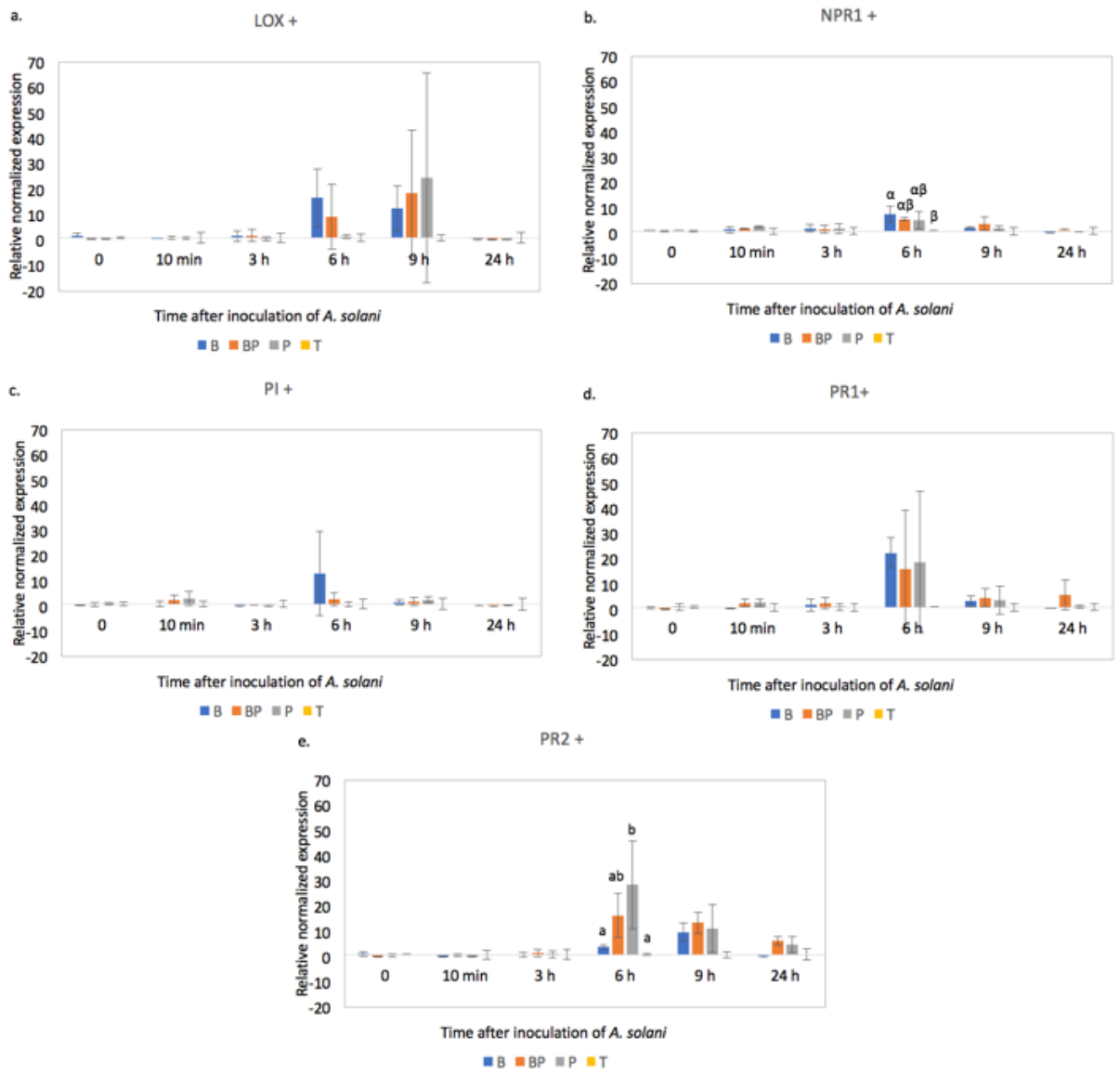

Figure 4.7: Mean relative normalized expression of four defense related genes over time of tomato lower older leaves (+), directly inoculated by the pathogen (for treatments including fungal inoculation), under three treatments relative to a control. These treatments are: one bacterial treatment (B), one bacterial treatment and fungal inoculation (BP), one fungal inoculation (P). The control is constituted of healthy plants without any treatment, its value of relative expression is set at 1. The genes examined were: a. Lipoxygenase (*LOX*), b. Protease inhibitor 1 (*PI-1*), c. Nonexpressor of PR genes-1 (*NPR1*), d. Pathogenesis related 1 (*PR-1*), e. Glucan endo-1,3-βglucosidase (*PR2-b*). The expression of those defense related genes was normalized with two housekeeping genes: Elongation factor 1-α (*EF1α*) and Phosphoglycerate kinase (*PGK*). Results for one time point with a different letter are significantly different, with a P value ≤ 0.05 based on a Tukey test. Results with no letter indicate no significant difference. Error bars represent the standard deviation.

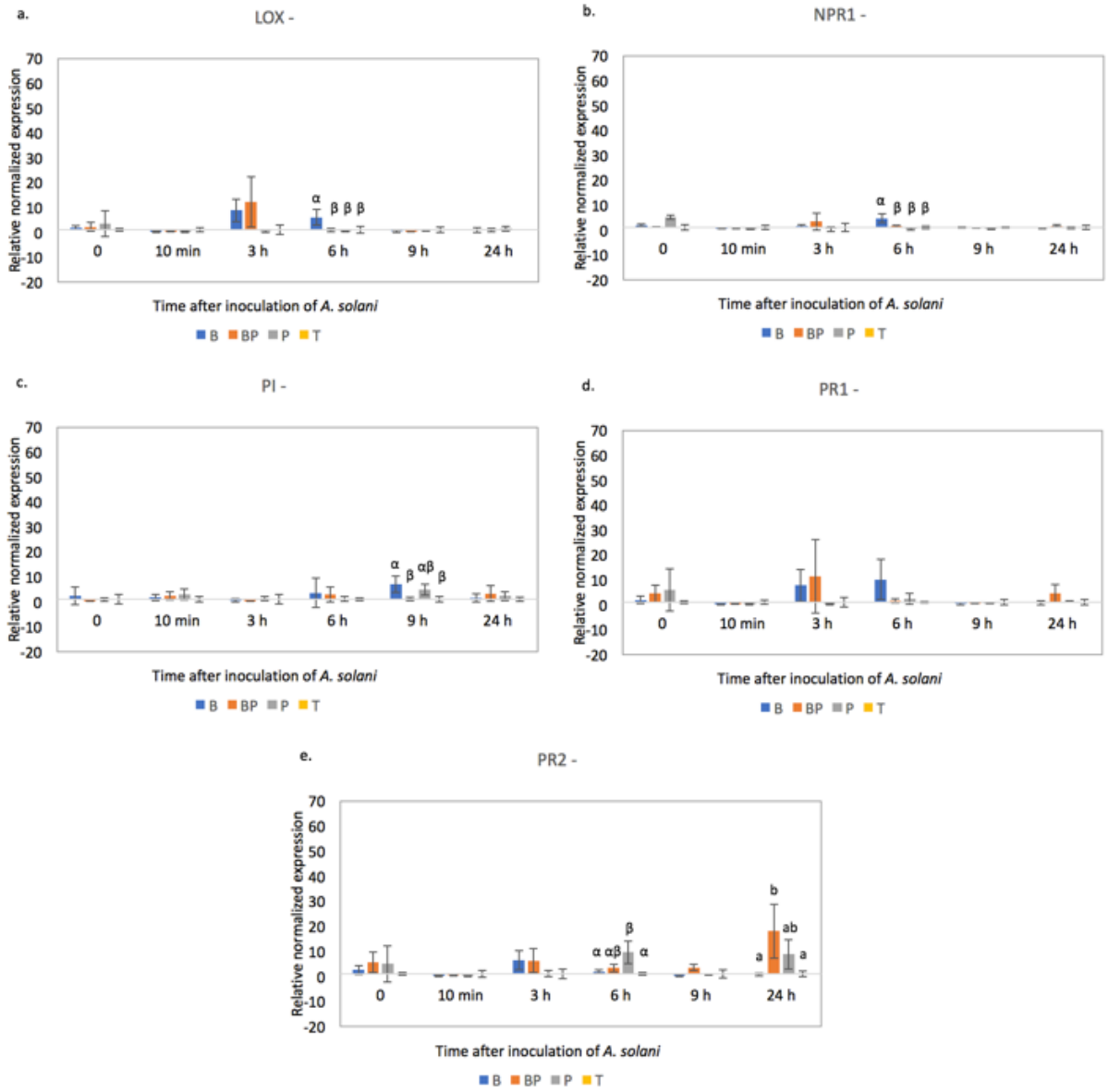

Figure 4.8: Mean relative normalized expression of four defense related genes over time of tomato upper younger leaves, non-directly inoculated with the pathogen (-), under three treatments relative to a control. These treatments are: one bacterial treatment (B), one bacterial treatment and fungal inoculation (BP), one fungal inoculation (P). The control (T) is constituted of healthy plants without any treatment, its value of relative expression is set at 1. The genes examined were: a. Lipoxygenase (*LOX*), b. Protease inhibitor 1 (*PI-1*), c. Nonexpressor of PR genes-1 (*NPR1*), d. Pathogenesis related 1 (*PR-1*), e. Glucan endo-1,3-βglucosidase (*PR2-b*). The expression of those defense related genes was normalized with two housekeeping genes: Elongation factor 1-α (*EF1*α) and Phosphoglycerate kinase (*PGK*). Results for one time point with a different letter are significantly different, with a P value ≤ 0.05 based on a Tukey test. Results with no letter indicate no significant difference. Error bars represent the standard deviation.

The RT-qPCR analyses have revealed some over-expression for both of the JA and ET responsive genes under the treatments with the 30B-B6 elicitor.

The *LOX* gene appeared to be stable over time, except an over expression observed for the elicited and uninfected plants of 6, 15 and 9 fold respectively compared to the treatments T, P and BP, 12 hours after its application, i.e. 6 hours post inoculation (hpi) (4.8a.). This up regulation was encountered only in the harvested upper leaves (-) (figure 4.8a.), but the expression seemed stable in the lower leaves (figure 4.7a.). Furthermore, an increase in *LOX* expression was observed in the lower leaves of inoculated and uninoculated plants compared to the two uninoculated plants at 6 hpi and 9 hpi, but no significant difference was detected.

The *PI-1* gene presented a stable expression over time and treatment for the leaves harvested in the lower part of plants (+) (figure 4.7c.). Few fluctuations of the expression of this gene occurred over the course of the experiments and its expression was kept at a low level, compared to the other genes. At 9 hpi, i.e. 15 hours after the bacterial application, elevated *PI-1* expression of 7 and 5 fold were measured in the upper 30B-B6 treated leaves of uninoculated plants, compared to the control and the BP treatments, respectively (figure 4.8c.). At 24 hpi, this over expression was no longer significant. In the lower leaves, no regulation of the *PI-1* gene was visible, but a high increase of its expression occurred at 6 hpi, with no significant difference because of the standard deviation.

Surprisingly, those two *LOX* and *PI-1* genes related to ISR displayed no significant differences on 30B-B6 treated plants upon pathogen infection, even if an increase in the relative expression of *LOX* was observed for the BP treatment in the upper leaves at 3 hpi.

Concerning the *NPR1* gene involved in the crosstalk between ISR and SAR, an interesting over expression was observed at 6 hpi in plants treated with the elicitor compared to the control, in all part of the plant (upper (-) and lower (+) leaves), as illustrated in figure 4.7b. and 4.8b. The gene was 8 fold up regulated in the lower leaves for the B treatment compared to the control and in the upper leaves the expression for the B treatment was 5 fold the one of the control and 4 fold the ones of the BP and P treatments. In the other conditions, the relative expression of *NPR1* was kept at a low level across treatments and time, with few fluctuations compared to the other genes.

Finally, regarding the genes related to the SA signaling pathway, *PR-1* and *PR2-b*, the expression level of the first one seemed stable (figure 4.7d. and 4.8d.), whereas variations were observed for *PR2-b*. In the lower and older leaves (directly inoculated) at 6 hpi, the level of expression of *PR2-b* was up regulated by 28 and 7 fold after the fungal inoculation in untreated plants, compared to the control and the B treatment, respectively (figure 4.7e.). In the upper younger leaves distant from the inoculation site, an up regulation was also observed at 6 hpi, of 9 and 5 fold compared to the control and B treatment, respectively (figure 4.8e.). Furthermore, *PR2-b* was highly over expressed in the leaves distant from the inoculation site of the BP plants 24 h after the fungal inoculation, compared to the control and the B treatment. Hence, when the tomato was challenged with the pathogen, it triggered a systemic up-regulation of the *PR2-b* gene. This reaction was stronger for the lower leaves, which were directly inoculated with *A. solani* than for the upper leaves. Concerning those upper leaves distant from the inoculation site, the up regulation was much stronger, but delayed, in plants that were previously elicited by

30B-B6. No regulation of *PR2-b* was observed in plants that were unchallenged by the necrotrophic fungus.

Chapter 5

Discussion

5.1 Reduction of early blight disease severity by the *B. subtilis* 30B-B6

B. subtilis strain 30B-B6 has been isolated by Caulier et al. (2018) within the collection of 2800 *Bacillus* spp. and *Pseudomonas* spp. from potato crops in the frame of the WACOBI project [Caulier et al., 2018]. This strain presented a great effectiveness in protecting potatoes against *P. infestans* in control greenhouse trials and field trials. In vitro assays also revealed the antagonistic activity of this strain against 4 other solanaceae pathogens : *A. solani, Fusarium solani, Pectobacterium carotovorum, Rhizoctonia solani*. In addition to the antagonistic activity of 30B-B6, recent experiments under greenhouse conditions performed by Gil Colau, PhD student in the UCL laboratory of phytopathology [Colau et al., publication under preparation], revealed that the bacterium conferred systemic resistance of tomato against EB disease.

The first bioassay conducted in this study aimed at confirming the protective effect of *B. subtilis* 30B-B6 in the pathosystem tomato - *A. solani*. The bacterial suspension was applied on the roots 6 hours before the inoculation of A. solani on leaves. The reduction of the disease severity on plants treated with a bacterial suspension of 30B-B6 onto the roots indicates that the protection is probably due to an induced resistance of the host plant by the contact between the roots of tomato plant and the bacterium. Since the experiment has been designed to avoid any direct contact between the pathogen and the bacterium, the elicitor effect of plant defense by the bacterium is confirmed. We cannot highlight differences between incubation period for treated and control groups, but we can emphazise that the progression of the disease was substantially lower on plants treated with the bacterium. The monitoring of the symptoms delivered a percentage of protection of 83%, highlighting the strong protective potential of the elicitor 30B-B6. The disease progress was markedly slower on plants treated with the bacterium than on untreated plants, resulting in different features. Indeed, the number and the size of necrotic spots as well as the size of the chlorotic halos around infection point were much greater on control plants than on elicited plants. This could result from diverse actions due to the elicitation effect of the bacterium:

- a decrease of the fungal growth (a growth inhibition of the fungal mycelium was demonstrated in petri dishes in direct antagonism [Caulier et al., 2018]),

- an increase of host resistance against fungal infections, a decrease of the toxic substances produced by the fungus, specifically the alternaric acid involved in the production of chlorotic symptoms by *A. solani*,
- an increase of the plant host immune system in degrading those substances.

Since the area affected by EB was reduced after a treatment with 30B-B6, we hypothesize that the sporulation of *A. solani* can also be limited.

The second bioassay performed in this study aimed at assessing the effect of the treatment of plants with the supernatant of a 30B-B6 culture in order to evaluate if the presence of the bacterium is required to induce the natural defense of tomato plants or if some metabolites produced by the bacterium in the LB medium are sufficient. A supernatant solution containing only the bacterium metabolites and an effect of this solution would have supported the role of these molecules in the protective effect. In our bioassay, no significant effect of the supernatant in the protection of tomatoes against *A. solani* was observed, showing that the supernatant alone prepared in the LB medium was not sufficient in the stimulation of the host. This indicates that compounds of the bacterial cell wall and/or direct interaction between the bacterium and the cell roots are essential, either for the direct detection from the host or for the bacterial detection of host compounds that would lead to secretion of metabolites involved in the host detection. Thus, maybe metabolites required for the beneficial interaction tomato – 30B-B6 were not yet produced in the supernatant of the LB medium after an incubation of 24 hours with 30B-B6, but were when 30B-B6 is getting in contact with the rhizosphere. Several studies support the inefficiency of the *B. subtilis* supernatant in stimulating the host immune system [Cawoy et al., 2014]. Cawoy and co workers demonstrated that the surfactin production of *B. subtilis* was highly dependent on its association with the host cell roots, and consequently the potential of the elicitor to prime the host cells was truly affected, or absent, when the supernatant was used instead of the bacterium. From data in the literature, several modes of action can be hypothesized to explain the protective effect of 30B-B6; Surfactin, a cyclic lipopeptide, has been proved to play a crucial role in the ability of the bacterium to efficiently colonize the rhizosphere and to induce a systemic resistance in the host plant [Ongena et al., 2007]. The production of this compounds is highly stimulated in the vecinity of plant roots [Chowdhury et al., 2015]. It is also known that this bio-surfactant, as lipopeptides from iturin and fengycin families produced by *B. subtilis* strains, exhibits direct antifungal and anti oomycetal activity [Ongena et al., 2000]. Their fungi toxicity is mediated by mechanisms involving pore formation in the plasmic membrane [Ongena and Jacques, 2008]. The strain used in the present study is known to secrete surfactin [Caulier et al., 2018]. A certain threshold of surfactin in the rhizosphere has been reported as necessary to trigger ISR in tomatoes by *B. amyloliquefaciens* QST713 against *Botrytis cinerea*, and its concentration was correlated with the stimulation of defense pathways [Cawoy et al., 2014]. Surfactin stimulates biofilm formation, which in turn increases cell colonization efficiency as well as enhances local concentrations of metabolites, including surfactin, thus leading to a positive feedback of the surfacing production [Chen et al., 2012]. The biofilm is also the site of production of many metabolites in high concentrations, which all constitute potential elicitors [Chowdhury et al., 2015]. Several studies showed that the bacterial biofilm formation is triggered through recognition of specific molecules coming from the polysaccharides root cell wall, which act as an environmental cue and serve as carbon source to produce the extracellular

matrix [Beauregard et al., 2013], [Chowdhury et al., 2015]. The interaction plant - bacterium is required by bacteria to efficiently secrete biofilm, area where surfactin and other bioactive metabolites will be highly secreted. Considering the positif feedback in surfactin production above mentionned, the root contact of the bacterium is, in some extent, required to trigger the synergistic production of surfactin. This could explain the inability of the supernatant to stimulate the host defenses, since the surfactin concentration did not reach the threshold [Cawoy et al., 2014]. Considering the above hypothesis, Cawoy and coworkers have used a supernatant solution coming from Bacillus suspension that were grown under biofilm forming conditions, in order to get closer to the natural context. The results were still not conclusive using the supernatant solution as elicitor [Cawoy et al., 2014]. This support the necessity of some host molecules signals in triggering the biofilm formation and high production of surfactin.

Another interesting report supports that the supernatant suspension did not succeed in triggering tomato immunity [Belal et al., 2013]. In this study, the authors demonstrated that the quantity of metabolites in the culture medium of *B. subtilis* spp. was the highest at the stationary phase, following the exponential growth phase, after 4 to 6 days of cultivation. Since in our experiment, the supernatant was prepared from a 1 day growth solution, it is possible that the metabolite concentration in our supernatant was thereby not at its maximum. This could justify a too low concentration of the eliciting molecules that could not be detected by the host, thereby preventing any stimulation of the host immune system.

Caulier et al. (2018) have detected some metabolites produced by 30B-B6 that could serve in the recognition and stimulation of the potato immune system. Those are are the non-ribosomally cyclic lipopeptides bacillomycin and surfactin as well as iron chelating siderophores. Siderophores are known to be involved in indirect antagonist effects such as stimulation of plant defenses and/or growth (de Boer et al. 2003, Verbon et al. 2017, Yu et al. 2011). Bacillibactin siderophore were identified by Yu and co-workers (2011) as key compounds in ISR-triggering activity by *B. subtilis* CAS15 on pepper plants, a solanaceae, challenged by Fusarium wilt. It is yet worth noted that siderophores are generally known for their antagonistic activities through efficient competition for iron-uptake, thanks to their high and specific affinities, making it unavailable for pathogens [Kloepper et al., 1980]. Their role in the host stimulation remains unclear, and some authors suggested that siderophores contribute to disease suppression but they are not acting alone but complementary to other compounds [Haas and Defago, 2005], [Caulier et al., 2018].

Dipeptide Bacylisin have also been detected in 30B-B6 secretion by Caulier et al. (2018), but it is more probably involved in direct antagonism than in stimulation of the plant immunity. This dipeptide demonstrates a high potential in inhibiting fungal mannoprotein and chitin biosynthesis, which constitues essential fungal compounds [Milewski et al., 1986].

Other studies also pointed out the role of VOC, in addition to the nonvolatile metabolites, in the stimulation of plant ISR by Gram-positive endospore-forming bacteria, such as *B. subtilis* 30B-B6. [Ryu et al., 2003] pointed out the implication in the stimulation of plant immunity by some VOCs (e.g., acetoin, 2,3-butandiol) produced by some bacteria (*B. subtilis* GB03, *B. amyliloquefacien* IN937) in the rhizosphere of *Arabidopsis thaliana*. The eliciting properties of VOCs produced by beneficial bacteria is nowadays

well known [Ryu et al., 2004]. Colonization of the rhizosphere by the bacterium is required to this high VOC concentration encountered in the phytosphere [Chowdhury et al., 2015]. Assuming that VOCs are involved in the 30B-B6 elicitation on tomato plants, the non-eliciting observed in our work using the supernatant becomes explainable. However, during this present work, no chemical analysis of the bacterial volatile emissions has been carried out, and no data on the VOC production of 30B-B6 are available.

5.2 Effect of 30B-B6 on gene expression of tomato inoculated or not by EB

It is well known that the plant gene expression profiles can be affected through many interactions with the environment and other organisms and the analyses of changes in the gene expressions could provide information on the kind of interactions that occurs. Pattern recognition receptors (PRRs), which are receptor kinases mainly anchored in the plasma membrane, detect microbe-associated molecular patterns (MAMPs), referred to as elicitors. This leads to changes in plant hormone levels, essentially SA, JA and ET, triggering a coordinated response in the whole plant against potential invaders. These events result in the set of a PAMP-triggered immunity (PTI) [Pieterse et al., 2014], [Nürnberger and Kemmerling, 2009]. One of the objectives of this master thesis was to decipher the metabolic pathways involved in the immunity of tomatoes induced by 30B-B6 against the EB disease, triggered by the necrotrophic fungus *A. solani*, by studying the dynamic of gene expression profile using RT-qPCR.

5.2.1 Effects of *A. solani* on the induction of tomato gene expression

Plants adapt to the lifestyle of the pathogen intruders in order to adjust their response immunity, specially to the pathogen feeding's way. The SA pathway, leading to a hypersensitive response, is generally involved in the protection against biotrophic agents, whereas the JA/ET pathways is preferred against necrotrophic agents, such as *A. solani* [Song et al., 2015]. It is worth to note that SA pathway is the distant signal involved in the set of SAR, following a pathogen challenge, and leads to the activation of many pathogenesis related proteins involved in antagonism against fungus, as observed with the *PR2-b* proteins that degrades fungus cell wall [Gill et al., 2015], [Ellis et al., 2003]. Hence, the activation of both SA and JA/ET signaling pathways could be expected. It has frequently been observed in other studies; [Niu et al., 2011] revealed that the PGPR *B. cereus* strain AR156 induces a systemic resistance in *Arabidopsis thaliana* against bacterial infection by simultaneously activating SA- and JA/ET- dependent signaling pathways. It has also been demonstrated against necrotrophic phytopathogens, through colonization of *Arabidopsis* roots by *Trichoderma atroviride* [Salas-Marina et al., 2011].

Our analysis of the gene expression profiles of tomatoes inoculated with the pathogen displayed a high activation of the *PR2-b* genes, encoding a β-1,3-endoglucanase. This enzyme is directly involved in plant defense against phytopathogenic fungi by hydrolyzing their cell walls. The degraded cell wall of fungi leads to a higher susceptibility to cell lysis, and the released β-1,3-glucan have been proven to be implied in elicitation of plant defenses [Somssich and Hahlbrock, 1998], [Mohammadi and Karr, 2002] . The over

expression of the *PR2-b* gene seems to be a natural defense of tomato plants against EB disease. This activation was systemic 6 hours after the infection by *A. solani* in untreated plants, and thus led to a higher resistance of the whole plant against the necrotrophic fungus, compared to the two non-inoculated groups (control and bacterial). Afterward, a strong up regulation of *PR2-b* was detected in the leaves distant from the infection site of the fungus, in plants that were previously elicited by the 30B-B6, at 24 hpi. This reaction was stronger than at 6 hpi, in the upper younger leaves. We hypothesize that the beneficial bacterium stimulates the host defense of the younger leaves by a higher and long lasting transcript of the *PR2-b* gene. It could also be explained by the systemicity of the signal following the pathogen intrusion, as an increase in *PR2-b* expression was also detectable, but was unsignificant, at 24 hpi in untreated but infected plants.

Consequently, under the pathogen infection, *PR2-b* was the only gene among the tested ones that presented an increased expression. Surprisingly, the other pathogenesis related gene, *PR1* encoding for PR-1 protein has not been activated. This last gene is often used as a marker for salicylic acid-mediated response and has been considered as one of the protein most abundantly produced by plants during defense responses [Breen et al., 2017]. Besides, Clinckemaillie has reported in a work on the same pathosystem *S. lycopersicum* - *A. solani* a significant increase of the *PR1* gene 24 h following the inoculation, which reached its maximum at 48 and 72 hpi [Clinckemaillie, 2017]. She thereby confirmed the activation of SA signaling on tomato plants following the inoculation by the necrotrophic fungus *A. solani*. Perhaps a similar activation would have been observed in our work if the gene expression analyses were conducted at belated time points after 24 hpi. Moreover, Clinckemaillie has inoculated tomato leaves by spraying 1 ml of a *A. solani* conidia suspension concentrated at 45 000 conidia/mL, whereas our suspension was concentrated at 15 000 conidia/mL. This difference could explain the non-significant increase in the *PR1* expression level in our works, whereas Clinckemaillie did. Then, although it seems unusual that any up regulation for this SA responsive gene has been observed following the fungal inoculation in our work, early selected time points and low conidial concentration may be involved. However, other studies support our results by revealing no *PR1* gene activation following fungal inoculation and by questioning the involvement of *PR1* gene against fungus [Gamir, 2016]. Hence, Gamir and colleagues demonstrated the efficiency of PR1-proteins in inhibiting the oomycete *Phytophthora brassicae* on tomato, but not on the fungus *Aspergillus niger* and *Botrytis cinerea*. This could explain the activation of *PR2-b* but not of *PR1* obtained in our analysis. Furthermore, even if no significant difference was observed for the relative *PR1* expression under pathogen infection compared to the uninfected plants in our experiment, an increase of its expression was observed in the upper and younger leaves. The strong variations measured between repetitions has led to an insignificant increase, that could be reduced by adding more biological replicates, leading thereby to more significance in our results.

Finally, as above exposed, plants are able to fine tune their defense responses depending on the type of pathogen encountered. Plants are more likely to activate JA and ET defense responses following a necrotrophic infection, as the SA pathway is known to contribute to plant hypersensitive cell death reaction which benefits necrotrophic pathogens [Alvarez, 2000]. This supports the non activation of the *PR1* gene in our results. Nonetheless, some authors have reported a specific SA responsive genes activation under necrotrophic infections [Clinckemaillie, 2017], [El Rahman et al., 2012], [El Oirdi and Bouarab, 2000].

Rahman and his team demonstrated that *A. solani* activates the SA signaling pathway in tomato plants to promote their disease development, by suppressing JA-dependent defense genes, which play a key role in plant resistances against necrotrophic pathogens [El Rahman et al., 2012]. Indeed, SA suppressive action against JA pathway have been widely reported [Spoel et al., 2003], [Felton and Korth, 2000]. Hence, whether SA signaling pathway is activated or not under necrotrophic pathogens remains unclear and opposite results are encountered in the literature.

The *NPR1* gene, that encodes a non-pathogenesis related protein 1, is involved in the crosstalk between SA and JA. The protein is also a SA receptor and has been revealed as suppressor of JA pathway by SA, to prioritize SA over JA, as above mentionned [Spoel et al., 2003], [Bell et al., 1995], [Felton and Korth, 2000]. In our RT-qPCR analyses, non significant increase of the expression of *NPR1* following the pathogen inoculation in both treated and untreated plants was observed. Our results do not support the observation of [El Rahman et al., 2012], who measured a high activation of SA signaling pathway dependent on the *NPR1* gene in tomato plants infected by *A. solani*. The key role of *NPR1* receptor in the perception of SA by plants has been well documented, but some authors suggested that a slight expression level of this *NPR1* gene could be enought for the SAR induction [Van Aubel et al., 2016], [Wu et al., 2012]. Moreover, studies have showed that there are some SA dependent signaling pathways that do not depend on *NPR1* [Janda and Ruelland, 2015]. Consequently, the non over expression of *NPR1* in plants challenged by the pathogen in our study is not enough to conclude on the non implication of the SA signaling pathway.

In contrast to literature observations on the JA/ET activation of plant defenses against necrotrophic pathogens [Song et al., 2015], no up regulation of the genes related to those hormones, *LOX* and *PI-1*, has been detected in our study. However, even if *PR2-b*, that encodes a β-1,3-endoglucanase, has been showed to be generally linked to the SA pathway, some authors revealed that the β-1,3-endoglucanase is strongly activated by JA and ET phytohormones and is independent to SA. Furthermore, an accumulation of this enzyme was associated in another study with ISR in tomato against *A. solani* [Lawrence et al., 2000]. These authors also reported that higher levels of the protein were obtained in EB tomato resistant cultivars compared to susceptible ones [Lawrence et al., 2000]. Thus, even if PR genes are frequently used as marker of the SAR, they can also closely be linked to the JA pathway and play a key role in the ISR [Lawrence et al., 2000], [Lawrence et al., 2015]). Our results also object the ones of [El Rahman et al., 2012] that found a down regulation of *PI-1* gene under *A. solani* infection in tomato plants. They suggest that the SA signaling pathway activated in tomatoes under *A. solani* infection triggered a silencing of the JA responsive genes. A downregulation of *PI-1* was also observed in Clinckemaillie's work 24 hour after the inoculation of *A. solani* on tomatoes, and persisted until 3 days after [Clinckemaillie, 2017]. In our results, the JA responsive genes (*PI-1* and *LOX*) displayed a stable expression in infected plants and similar to the control.

5.2.2 Effects of *B. subtilis* 30B-B6 on the induction of tomato gene expression

The treatment of *B. subtilis* 30B-B6 on tomato plants has demonstrated a high potential to stimulate the immune system, resulting in an enhanced level of resistance of the

host plant against *A. solani*. This kind of elicitation using *B. subtilis* has already been demonstrated for many pathosystems, and involved generally the activation of the ISR of the host plants [Kloepper et al., 2004], [Akram and Anjum, 2014]. Hence, a similar outcome is expected here, by detecting a higher stimulation of the JA/ET responsive genes. Furthermore, necrotrophic pathogens are suspected to enhance the JA signaling pathways in plants as defense response [Song et al., 2011], but our results have not highlighted that. We expected a synergic enhanced expression of the JA signaling pathway for plants pre-treated with the beneficial microbes and inoculated with the fungus, based on those previous studies. On the contrary, other authors reported a specific SA signaling pathway activation in plants following necrotrophic pathogen infection and demonstrated that it is a strategy developed by those pathogens to promote their development in plants, though the suppression of JA/ET pathways and the hypersensitive reaction. Hence, whether JA responsives genes will be activated or suppressed by 30B-B6 following the fungal inoculation cannot be predicted.

The JA responsive genes used in this work were *LOX* and *PI-1*, respectively encoding for a lipoxygenase and proteinase inhibitor. Their expression was increased under the 30B-B6 treatment in healthy plants but not in plants infected by *A. solani*. Some authors reported an increase of the *LOX* expression under surfactin application. *LOX* is related to the oxylipin pathway, which oxidates unsaturated fatty acids into reactive hydroperoxides, which are further converted into several defense metabolites (Shah 2005). Hence, this is consistent with the up-regulation observed in the present work under 30B-B6 treatment, as surfactin is suspected to be a key factor implied in the early set of host defenses induced by 30B-B6. The activation of the proteinase inhibitors *PI-1* in the present study confirms the regulation through JA pathway [Choudhary and Johri, 2009]. However, the non activation of those genes following *A. solani* infection in elicited plant is surprising. Indeed, several studies proved the importance of JA responsive genes in the elicitation by *B. subtilis* against pathogens, as well as the role of this phytohormone in the set of plants defense against necrotrophic pathogens. A synergistic effect of both 30B-B6 and *A. solani* on the expression of the JA responsive genes was thus expected. In our study, the elicitor did activate the gene linked to the JA signaling pathway which may lead to the potentiation of its immune system, but this stimulation did not occur when the 30B-B6 treated plants were inoculated by *A. solani*. Our results do not allow to confirm that the JA phytohormone is involved in the signaling pathways responsible of the higher level of resistance in 30B-B6 tomato against EB disease. This could be explained by a counter impact of *A. solani* on the bacterium, that tends to bypass the expression of the genes activated when the 30B-B6 was present alone. It has already been observed that SA can promote its signaling pathway by downregulated the JA signaling pathway, through the *NPR1* protein [Bell et al., 1995], [Felton and Korth, 2000]. However, in our experiments, the *NPR1* gene was up regulated under the bacterial treatment but not when the elicited plants were inoculated, nor on plants inoculated with *A. solani* without a previous treatment with the bacteria. Thus, this result does not support the above hypothesis. Another hypothesis is a suppressive action of the pathogen against some defense related gene expression that were activated by the elicitor alone. The expression dynamic induced by the bacterium and the pathogen could be different and antagonistic, resulting in the silencing of one of them. Indeed, it is well documented that SA- and JA- dependent pathways are antagonistic [Spoel et al., 2003], [Devoto and Turner, 2003]. The expression dynamic induced by the bacterium and the pathogen could be different

and antagonistic, resulting in the silencing of one of them. Indeed, it is well documented that SA- and JA- dependent pathways are antagonistic [Spoel et al., 2003], [Devoto and Turner, 2003].

30B-B6 was expected to trigger the ISR in the host, as it conferred a systemic protection of tomatoes against EB and any antagonism effects were possible due to the separated application from the fungal inoculation. Hence, no regulation of the pathogenesis related genes (*PR1* and *PR2-b*) was expected in elicited plants [Pieterse et al., 2014]. Indeed, those genes were not up regulated in the present study in plant elicited by the bacterium when no pathogen attack occurred. However, a strong up regulation of *PR2-b* genes was observed in treated plants 24 hours after the inoculation of *A. solani* in leaves distant from the site of infection. This indicates that *PR2-b* gene has been stimulated by a long distant signal in the plant, preparing the upper leaves from further infection. *PR2-b* is generally reported as a SAR marker, whereas an ISR reaction was expected following the bacterial elicitation. As above explained, some authors have yet linked the activation of *PR2-b* gene to a JA/ET response in the ISR process [Lawrence et al., 2000]. As this gene was also activated in LB treated plants infected by EB, but at a lower level than in elicited plants, and as it remained inactivated in the non-inoculated elicited plants, this could point toward a priming of *PR2-b* under 30B-B6 treatment. However, the priming did not seem to trigger a faster expression of this gene, but rather a stronger and long lasting transcript of the *PR2-b* gene in the younger leaves. The expression of this gene could also have been amplified earlier but at another time point than those tested. The β-1,3-glucanase properties of the protein, resulting in the hydrolyzes of β-1,3-glucans of the fungus cell wall, could therefore be the key mechanism of the disease severity reduction of 30B-B6 elicited tomato.

Several studies on *B. subtilis* sp. reported that the expression of defense-related enzymes are activated together with oxidative enzymes. Chandrasekaran and coworkers demonstrated an over expression of β-1,3-glucanase, together with PAL enzymes in the leaves of tomato affected by the bacterial spot disease [Chandrasekaran and Chun, 2016]. This mechanism may occur in the pathosystem tomato - 30B-B6 - *A. solani*, but no genes related to the phenyl propanoid pathway was tested in our study. The enzyme *PAL* plays a key role in the production of defensive compounds (lignins, phytoalexins, flavonoids, ...) through the phenylpropanoid metabolism [Kloepper et al., 2004], [Chowdappa et al., 2013]. Further analyses of this gene expression profile under 30B-B6 elicitation could be interesting.

Since no conclusive result on the selected genes was obtained in our experiment on the activation of specific signaling pathways following pathogen attack in elicited tomatoes, other untested pathways may be involved in the priming of tomato against EB disease. As above mentioned, the phenylpropanoid pathways could constitute a key mechanism in the elicitation. Some authors also reported an elicitation of plant through ET dependent pathway by VOCs produced by beneficial microbes [Ryu et al., 2004], [Ryu et al., 2003]. Such elicitation has been observed through volatiles, and [Ryu et al., 2003] demonstrated that the signaling pathway activated by VOC is dependent on ET, and independent of the SA, *NPR1* and JA signaling pathways. As mentioned in the first part of the discussion, VOCs produced by 30B-B6 have not been analysed, but they can take a part in the elicitation of tomato plants by 30B-B6. However, no gene tested was specifically

dependent on ET. *LOX* and *PI-1* genes are responsive to JA, even if some studies linked their expression also to the ET pathway [Choudhary and Johri, 2009]. ET has been showed to activate members of the pathogenesis related gene super family, including *PR2-b*, whose expression was up regulated in elicited plant following the fungi attack [Choudhary and Johri, 2009]. However, *PR2-b* has also be linked either to JA or SA pathways [Lawrence et al., 2000]. Hence, a more specific analyses of ET responsive genes could provide complementary information.

It is very difficult to draw conclusions in the induction of plant defense by 30B-B6 on tomato infected by EB diseases based on the present work. Few significance differences of gene expression occurred across the four treatments, and several unexpected results were obtained, such as the non up regulation of the SA responsive gene *PR1* in inoculated plants, the non down regulation of JA/ET responsive genes when both the bacterium and fungus were applied and the non involvement of the *NPR1* gene. In this section, we highlight several concerns about the experimental process, in order to improve further experiments and achieve a better comprehension on the 30B-B6 mode of action.

High variations were obtained between biological replicates and can be reduce as follows. The RT-qPCR for each samples must be performed based on a single RT product. It will provide a single source for each samples across the RT-qPCR performed for each gene. However, in the present study, contamination issues have compelled the use of different RT products. Another step that could have altered the precision of the analyses was the RNA extraction. As a reminder, problems were encountered during this step because of the tissue ripping without buffer. This has forced us to freeze and unfreeze some leaf samples, which could alter its RNA content. In addition, this buffer enables a stabilization of the released RNA, which thus ensures that no degradation of the initial amount of RNA in the sample occurs. Finally, the standard deviations of the biological replicates can be reduced by adding more than 3 replicates. This last point obviously presents some logistic issues.

In order to ensure the effective action of the bacterial elicitation and fungal infection of the plants of the trials, a greenhouse control is essential. The bacterial treatment and fungal infection effectivenesses have been proved many time before the quantification assays, but it would have been preferable to confirm once again the macroscopic effects of the quantitative experiment. For this purpose, maintaining additional plants which have received the same treatments than those used in the quantitative analysis is a great control method, through a symptom monitoring.

To ensure the effective detection of the variation of gene expression across treatments and time, the use of positive control is advised. For instance, we could have used in this study an additional group of plants with benzothiadiazole (BTH) treatment. BTH is an elicitor which causes systemic resistance in several plants against many diseases. Its effect have been widely studied and demonstrated a high ability to induce SAR signaling pathway [Bokshi et al., 2003], [Friedrich et al., 2003].

Chapter 6

Conclusion and perspectives

B. subtilis strain 30B-B6 was selected from a recent collection of potential biocontrol agent for its direct antagonism against 5 major Solanaceae pathogens (*Alternaria solani, Fusarium solani, Pectobacterium carotovorum, P. infestans, Rhizoctonia solani*), and its ability to reduce potato late blight symptoms on plants [Caulier et al., 2018]. Further studies performed in planta by Gil Colau, a PhD student of the UCL phytopathology laboratory, revealed that the application of a suspension of 30B-B6 to tomato roots conduct to a decrease of the infection of tomato leaves by *Alternaria solani*, the causal agent of tomato early blight. The first bioassay of this study confirms the protective effect of 30B-B6 against the necrotrophic fungus *A. solani*, with a protection index of 83% compared to untreated control plants under greenhouse conditions. These results and the fact that the bacterium was kept spatially separated and not in direct contact with the pathogen indicate that 30B-B6 would stimulate the plant immune system through the induction of a systemic resistance.

In a second bioassay, bacterial supernatant was tested (filtered solution of 30B-B6), to investigate whether the presence of the bacterium is required or not for the protective effect. No significant protection against EB disease was observed in tomatoes treated by the supernatant, compared to untreated control plants. We conclude that an interaction 30B-B6– tomato roots is required to induce the plant systemic resistance and/or to induce the production of bacterial specific metabolites stimulating the plant immunity. This could be partly explained by the favorable environment provided by plant roots, allowing the bacterial biofilm formation where a high concentration of metabolites is produced [Chen et al., 2012], [Chowdhury et al., 2015]. Severalmetabolites, the lipopeptides surfactin and bacillomycin, the peptide bacilysin and iron chelating siderophores, are produced by 30B-B6 and could be responsible for the elicitation [Caulier et al., 2018]. Some VOC (*e.g.* acetoin and 2,3-butanediol) are also reported in the ISR activation mediated by *B. subtilis* strain GB03 on tomatoes and could be involved for 30B-B6, but no characterization of VOCs productions by 30B-B6 has been yet realized [Ryu et al., 2004].

The quantification of the defense related genes expression was performed on tomato under different treatments to better understand the metabolic pathway involved in the plant resistance induction by 30B-B6, against *A. solani*. The treatments were as follows. Non elicited plants inoculated with *A. solani*, 30B-B6 plants uninoculated by the fungus and both treated and inoculated plants, in comparison to control, in order to distinguish effects from both microorganisms. The pathogen treatment of non elicited plants revealed

a systemic gene expression activation of *PR2-b* after 6 hours following the inoculation. The SA-related gene *PR1* demonstrated a stable expression, whereas an up regulation indicating a SAR reaction was expected, as it has been observed in Clinckemaillie's work on the same pathosystem [Clinckemaillie, 2017]. It was also unexpected to obtain a stable expression of the JA- and ET- related gene expression, for the pathogen treatment. Indeed, several authors have reported a high expression of genes related to the JA/ET pathways following necrotrophic pathogen infection [Song et al., 2015], [Fujimoto et al., 2011]. We could thereby have expected an upregulation of those genes. In contrast, other authors have observed a down regulation of those JA/ET responsive genes following necrotrophic pathogen infection, due to an evolutional strategy of those pathogens to promote their infection in the host plant through the activation of SA signaling pathway [El Rahman et al., 2012], [Clinckemaillie, 2017]. This JA/ET responsive genes silencing was highlighted to be highly dependent on the *NPR1* gene [Spoel et al., 2003], [El Rahman et al., 2012]. In our results, the *NPR1* expression was stable, which is thereby consistent with the non-decrease expression of *PI-1* and *LOX* genes observed. Therefore, neither the literature reviews nor the results obtained in our experiments, under our defined trial processes, allowed to conclude on the signaling pathways activated following *A. solani* infection on tomato plants.

The 30B-B6 treatment in uninfected plants conducted to an activation of systemic defense response linked to the JA and ET signaling pathways, markers of ISR. Indeed, an up regulation of *PI-1* and *LOX* genes have been observed in the upper younger leaves for the bacterial treatment, respectively 15 hours (9 hpi) and 12 hours (6 hpi) after 30B-B6 root application. This pointed toward a stimulation of JA/ET-related defense by the elicitor, that is consistent with literature reviews on other *B. subtilis* elicitors [Kloepper et al., 2004], [Akram and Anjum, 2014]. However, when challenged by a pathogen, plants pre-treated by 30B-B6 do not showed any up/down regulation of the ISR responsive genes, under the time of the experiment. This is unexpected in consideration of several scientific reports [Song et al., 2015], [Spoel et al., 2003], [El Rahman et al., 2012]. Regarding reports that showed an activation of the JA/ET metabolic pathway in plants under necrotrophic pathogen infection, a synergistic activation of the JA/ET pathway was expected under the 30B-B6 elicitation and *A. solani* inoculation. On the contrary, reports that revealed a SA regulation of defense response in plants challenged by *A. solani* observed a suppressive action of the JA/ET related genes by the SA pathway [Song et al., 2015], [Spoel et al., 2003], [El Rahman et al., 2012]. Our results revealed a stable expression of those genes, which is consistent with the non-over expression of either JA/ET or SA responsive genes that we observed in untreated plants inoculated by *A. solani*. A strong up regulation of *PR2-b* genes was observed on elicited plnats 24 hours after the inoculation of *A. solani* in the leaves distant from the site of infection. This gene encodes for an endo-β-1,3-glucosidase, which demonstrates fungi toxicity by degrading fungus cell walls. In addition to the direct antagonism effect of this enzyme, an elicitation of the plant immune system have been reported through the detection by the plants of some compounds released from the degraded fungus cell walls, such as β-1,3-glucans [Mohammadi and Karr, 2002]. This pathogenesis related gene have been linked to the SAR by many authors [Gupta et al., 2012], [Mohammadi and Karr, 2002], [Somssich and Hahlbrock, 1998], but some studies also attribute the activation of this gene to the JA/ET pathways [Lawrence et al., 2000]. Hence, our result do not allow us to conclude on the signaling pathway activated in the elicitation of tomatoes by 30B-B6 against *A. solani*. However, we can hypothesize

that the reduction in disease severity observed on tomato plants elicited by 30B-B6 and inoculated by A. solani is partly due to the over expression of the *PR2-b* gene. Considering this hypothesis, it would be more probable that the long distant signal that stimulated *PR2-b* gene, preparing the upper leaves from prompt infections, is the JA and/or ET phytohormones, rather than the SA.

Several perspectives can emerge from this study to provide other information on the mechanisms induced by 30B-B6. Since the time points selected in our work do not provide a lot of significant differences in gene expression profiles, delayed the moment of gene expression analysis could reveal more differences. Indeed, many authors have reported significant differences in defense related gene expression in similar pathosystem 24 hours after the inoculation until 3 days after [Clinckemaillie, 2017]. Furthermore, exploring other pathways in the selected target genes could be interesting. VOCs has been showed to stimulate the plant immune system through the ET signaling pathway, independently of the JA and SA pathways [Choudhary and Johri, 2009], [Kloepper et al., 2004]. VOCs production have not been explored for 30B-B6, but other *B. subtilis* strains were shown to activate ISR through those compounds [Ryu et al., 2004]. Hence, an analysis of 30B-B6 VOCs production is also an interesting perspective. The phenylpropanoid pathway could also be promising to investigate, as Chandrasekaran have demonstrated the stimulation of tomatoes immune system affected by bacterial soft rot through the activation of PAL enzymes, responsive to this pathway [Chandrasekaran and Chun, 2016].

Our greenhouse findings on the elicitation effect of 30B-B6 in the pathosystem *A. solani* – tomatoes (Colau, publication under preparation), as well as Caulier report on the direct antagonism activities of this strain against 5 important Solanaceae pathogens and on the elicitation of potatoes against late blight in field [Caulier et al., 2018], make 30B-B6 a promising biocontrol agent of solanaceae crops. Potential human virulence factors of *Bacillus* were tested for this strain and the results were negative for all them, ensuring the non toxicity for human health [Caulier et al., 2018]. Since 30B-B6 is a soil borne naturally present organism, it can be stably established in mutualistic association with Solanaceae. Considering these attracting features of *B. subtilis* 30B-B6, further studies on this strain are essential to better understand its mode of action and the factors allowing it to express its antagonistic capacity before getting commercialized.

Appendix A

Additional results

A.1 RNA concentration and purities of the samples

Samples	260/280	260/230	Concentration ng / µl
1	2,16	2,26	1261,9
2	2,12	1,35	219,2
3	2,11	2,09	362,5
4	2,18	2,2	1100
5	2,08	1,48	452,8
6	2,15	2,31	1576,8
7	2,15	2,39	1409
8	2,1	1,26	135,5
9	2,14	2,18	1708,8
10	2,14	1,46	194,6
11	2,16	2,17	1258,2
12	2,14	2,17	243,9
13	2,13	2,13	219
14	2,24	2,14	1000
15	2,12	2,37	1861,3
16	2,15	2,43	1196,8
17	2,13	2,38	312,8
18	2,16	2,34	589,5
19	2,13	0,96	358,1
20	2,14	1,44	926,8
21	2,16	1,03	550,5
22	2,15	0,87	333,9
23	2,14	1,89	1236,2
24	2,14	2,27	568,7
25	2,12	2,12	1521
26	2,1	1,38	365,1
27	2,14	2,33	1156
28	2,1	1,27	367,8
29	2,14	2,34	1119,2
30	2,13	1,6	252,5
31	2,16	2,25	1456,1
32	2,19	2,01	669,7
33	2,18	2,25	1137,2
34	2,13	1,95	194,8
35	2,18	2,1	770,5
36	2,12	1,47	114,6
37	2,19	1,95	558,9
38	2,12	1,5	229,4
39	2,16	2,14	995
40	2,12	2,29	347,5
41	2,16	2,26	1311,5
42	2,11	1,09	157,7
43	2,13	2,05	1056,9
44	2,11	1,95	156,9
45	2,16	1,78	816,2
46	1,92	1,68	30,9
47	2,15	1,95	661
48	2,12	1,56	329
49	2,15	1,8	754,1
50	2,07	1,8	370,1
51	2,14	2,13	1263,6
52	2,11	1,61	227,3
53	2,15	2,14	1028
54	2,1	1,04	136,3
55	2,16	2,04	897,8
56	2,11	1,78	290,4
57	2,16	1,88	621,1
58	2,11	1,7	237,6
59	2,15	2,05	704,2
60	2,15	2,23	764,8
61	2,13	1,7	963
62	2,11	1,87	129
63	2,13	1,66	1054
64	2,12	1,29	283,8
65	2,12	2,16	1692,6
66	2,13	1,6	148
67	2,07	1,69	339,8
68	2,11	2,12	269,9
69	2,14	2,07	608,6
70	1,92	1,68	30,9
71	2,1	1,77	963,3
72	2,21	1,02	118,5
73	2,1	2,01	367,8
74	2,12	2,09	258,2
75	2,14	2,4	1991,3
76	2,15	1,36	92,2
77	2,18	2,31	987,2
78	2,17	2,21	683,8
79	2,13	2,43	1450,7
80	2,12	1,88	276,4
81	2,15	2,18	1287,9
82	2	1,99	45,1
83	2,17	1,89	615,3
84	2,16	2,22	219,6
85	2,14	2,33	1662,7
86	2,15	2,36	785,6
87	2,1	2,43	2372,3
88	2,16	2,11	253,1
89	2,15	2,35	1031,8
90	2,14	2,07	557,9
91	2,13	0,26	168
92	2,1	1,06	358,8
93	2,07	1,16	440,9
94	2,15	1,29	536,6
95	2,13	1,58	731,3
96	2,13	2,17	171,8
97	2,15	2,37	1502,7
98	2,13	2,13	162,5
99	2,37	2,16	843,7
100	2,16	0,43	45,6
101	2,15	2,3	1116,2
102	2,13	2,16	160,7
103	2,08	2,27	442,4
104	2,13	1,78	125
105	2,12	1,29	320,1
106	1,94	1,77	11,9
107	2,12	2,38	331
108	2,13	2,29	341,7
109	2,14	2,15	532,9
110	2,09	2,26	436,4
111	1,99	2,01	62,8
112	2,17	2,4	685
113	2,09	1,65	36,1
114	2,11	2,13	286,5
115	2,1	2,08	417,6
116	2,14	2,14	731,9
117	2,15	2,08	114,2
118	2,11	1,92	179,3
119	2,1	2,25	376,3
120	2,09	2,34	470,8
121	2,14	1,64	74,1
122	2,17	2,15	723,7
123	2,12	2,16	358,5
124	2,13	1,97	355,1
125	2,2	2,31	501
126	2,06	2,35	464,6
127	2,2	2,28	515,4
128	2,19	2,12	780,3
129	2,12	2,31	306,9
130	2,2	2,29	570,4
131	2,16	1,98	217,2
132	2,19	2,33	898,2
133	2,11	2,39	425
134	2,18	2,33	974,2
135	2,1	2,19	450,9
136	2,11	2,35	423,5
137	2,15	2,28	288,9
138	2,19	2,16	668,1
139	2,14	2	214,3
140	2,11	2,31	257,7
141	2,18	2,2	963,8

Figure A.1: Total of raw data obtained using Nanodrop ND1000 (Thermo Fisher Scientific), of RNA concentration and purities for the 141 samples used for the molecular gene expression analysis

Appendix B

Additional figures of the literature review

B.1 Chemical composition of tomato fruits

Table B.1: Chemical composition of tomato fruits. Values given for 100 g of tomato. From [Razdan, 2006]

Constituents	**Units**
Moisture	95 %
Food energy	22 kcal
Protein	1 g
Fats	0,2 g
Carbohydrates	4,7 g
Fiber	0,5 g
Calcium	13 mg
Phosphorus	27 mg
Sodium	3 mg
Magnesium	17,7 mg
Potassium	244 mg

Iron	0,5 mg
Zinc	0,2 mg
Copper	0,01 mg
Vitamin A	0,27 mg retinol and 0,54 mg βcarotene
Vitamin E	0,4 mg αtocopherol
Vitamin C	23 mg ascorbic acid
Vitamin B6	0,10 mg pyridoxine
Thiamin	0,06 mg
Riboflavin	0,04 mg
Niacin	0,7 mg
Panthothenic acid	0,33 mg
Folacin	39 µg
Biotin	4 µg

B.2 Authorized fungicides on tomato against EB

Table B.2: List of authorized fungicides to fight against the early blight of tomato in indoor cultivation caused by *Alternaria solani*. (*) indicates that the product can be applied in indoor cultivation as well as outoor cultivation. The percentage is expressed in terms of copper. Legend for the number of accession: The first letter indicate the type of users allowed to apply the product: P for professional and G for non professional (garden). The second letter represent the country where the formulation is allowed: B for Belgium and P for another member state that accept the product through parallel authorisation. From [Phytoweb, 2018].

Commercial product	**Accession number**	**Composition**
Amistar	8898 P/B	250 g/l azoxystrobine
Amistar	1018 P/P	250 g/l azoxystrobine
Cuperit	32P/B	50% copper oxycloride
Cuprex 50%	924 P/B	50% copper oxycloride
Cuprex 50% WG	8782 P/B	50% copper oxycloride
Curvata	8922 P/B	50% copper oxycloride
Hydro Super 25 WG	9778 P/B	25% copper hydroxide
Hydro WG	9272 P/B	40% copper hydroxide
KO-Plus 40	9607 P/B	40% copper hydroxide
Koperhydroxide WG	8825 P/B	40% copper hydroxide
Naturen Bordeaux mixture	7216 G/B	20 % copper sulphate (*)
Ortiva	9326 P/B	250 g/l azoxystrobine
Zakeo 250 SC	10716 P/B	250 g/l azoxystrobine

Appendix C

Detailed protocols

C.1 Tomato seed germination

This protocol is used for seed disinfection and germination of about 40 commercial tomato plants (*Solanum lycopersicum* var. Moneymaker).

1. Seed disinfection :
   (a) Agitate the seeds in a solution of bleach (< 5% bleaching agent) and sterile milliQ water (100 mL milliQ water and 2 mL of bleach) for 5 minutes using a bar magnet,
   (b) remove water with paper towel,
   (c) transfer the seeds in an erlen with 100 mL of water milliQ and agitate for 2 minutes using a bar magnet,
   (d) repeat 3 times step c).
2. Seed germination :
   (a) Place the seed in fanfold chart in a box (about 5 seeds per row) using a clean tweezers (dipped in alcohol and heated over a flame)
   (b) slightly humidify the fanfold chart with sterile milliQ water (about 60 mL) using a pipette,
   (c) seal the box using sticky paper,
   (d) place the box at 22°C for 10 days
   (e) transplant the seedlings in pots in greenhouse control condition (24 °C, HR of 60 %, 16 hours' photoperiod).

C.2 Root application of 30B-B6 and leaves inoculation of *A. solani*

This protocol detailed the *B. subtilis* 30B-B6 application on tomato roots and the leaf inoculation of *A. solani* MBC928.

1. Bacterial application :
   (a) Spread the 30B-B6 strain on petri dishes with LBA and place at 30°C for 24 hours (strain collection from the Wacobi project is conserved at -80°C),
   (b) transfer one isolated colony of 30B-B6 from the LBA medium in an erlen of 300 mL of LB and agitate at 150 rpm for 24 hours at 30°C,
   (c) estimate the bacterial concentration of the 24 hours bacterial LB suspension using the CFU calculation method
   (d) apply 100 mL of the 24 hours LB suspension on each tomato pots (5-leaflet stage), and put saucers below the pots to receive the percolating suspension.
2. Fungal inoculation :
   (a) Grow *Alternaria solani* on V8 medium in Petri dishes for 10 days in a 16 hours' photoperiod (neon lighting) at 22°C. For the inoculation of 20 tomato plants, conidial suspension from 20 petri dishes is required,
   (b) add 7 mL of Tween water (one drop of tween 20 in 100 mL of sterile water) in a 10 days *A. solani* V8 Petri dish,
   (c) scratch the mycelium using a metal rake,
   (d) recover the mixed mycelium - Tween water using a pipette and transfer it in an other *A. solani* V8 Petri dish,
   (e) repeat step 3 and 4 for 4 *A. solani* V8 Petri dish,
   (f) filter the suspension to remove the mycelium and transfer the remaining conidial suspension in a falcon,
   (g) estimate the conidial concentration by counting the spores using Fuch-Rosenthall cells, the final concentration is set to 15000 conidia/mL, by adding tween 20 water,
   (h) six hours after the bacterial application in tomato pots, sprayed 1 mL of the conidial suspension per leaves on 3 leaves per plant
   (i) place the plants in humidity chambers, with a 90% HR for 24 hours in the obscurity.

C.3 RNA extraction using RNeasy plant mini kit form Qiagen

RNA extraction using RNeasy plant mini kit form Qiagen allows the purification up to 100 µg of RNA longer than 200 bases, constituted mainly by mRNA. A maximum amount of 100 mg plant material or 1 x 107 cells can generally be processed. The procedure is exposed below.

Things to do before starting :

1. Prepare DNase I stock solution before using the RNase-Free DNase Set for the first time. Dissolve the lyophilized DNase I (1500 Kunitz units) in 550 µl of the RNase-free water provided. To avoid loss of DNase I, do not open the vial. Inject RNase-free water into the vial using an RNase-free needle and syringe. Mix gently by inverting the vial. Do not vortex.

2. Add 20 µl of DDT in 600 µl of RLT buffer.

3. Add 4 ethanol volumes (96-100%) to the RPE buffer.

1. Determine the amount of plant material by weighting tissue. Do not use more than 100 mg.

2. Immediately place the weighed tissue in liquid nitrogen, and grind thoroughly with a mortar and pestle. Decant tissue powder and liquid nitrogen into an RNase-free 2 ml microcentrifuge tube. Proceed immediately to step 3.

3. Add 450 µl Buffer RLT to a maximum of 100 mg tissue powder. Vortex vigorously.

4. Transfer the lysate to a QIAshredder spin column (lilac) placed in a 2 ml collection tube, and centrifuge for 2 min at full speed. Carefully transfer the supernatant of the flow-through to a new microcentrifuge tube (not supplied) without disturbing the cell-debris pellet in the collection tube. Use only this supernatant in subsequent steps.

5. Add 0.5 volume of ethanol (96–100%) to the cleared lysate, and mix immediately by pipetting. Do not centrifuge. Proceed immediately to step 6.

6. Transfer the sample (usually 650 µl), including any precipitate that may have formed, to an RNeasy spin column (pink) placed in a 2 ml collection tube (supplied). Close the lid gently, and centrifuge for 15 s at 10,000 rpm. Discard the flow-through.

7. Add 350 µl Buffer RW1 to the RNeasy spin column. Close the lid gently, and centrifuge for 15 s at 10,000 rpm to wash the spin column membrane. Discard the flow-through

8. Add the DNase I incubation mix (80 µl) directly to the RNeasy spin column membrane, and place on the benchtop (20–30°C) for 15 min

9. Add 350 µl Buffer RW1 to the RNeasy spin column. Close the lid gently, and centrifuge for 15 s at 10,000 rpm. Discard the flow-through. Continue with the first Buffer RPE wash step in the relevant protocol.

10. Add 500 µl Buffer RPE to the RNeasy spin column. Close the lid gently, and centrifuge for 15 s at 10,000 rpm to wash the spin column membrane. Discard the flow-through.

11. Add 500 µl Buffer RPE to the RNeasy spin column. Close the lid gently, and centrifuge for 2 min at 10,000 rpm to wash the spin column membrane.

12. Optional: Place the RNeasy spin column in a new 2 ml collection tube (supplied), and discard the old collection tube with the flow-through. Close the lid gently, and centrifuge at full speed for 1 min.

13. Place the RNeasy spin column in a new 1.5 ml collection tube (supplied). Add 30–50 µl RNase-free water directly to the spin column membrane. Close the lid gently, and centrifuge for 1 min at 10,000 rpm to elute the RNA.

14. If the expected RNA yield is > 30 µg, repeat step 13 using another 30–50 µl RNase-free water, or using the eluate from step 11 (if high RNA concentration is required). Reuse the collection tube from step 13.

C.4 Re-concentration of RNA

1. Add 1/10 volumes of NaOAc 3 M and 3 volumes of absolute ethanol in a 1.5 ml collection tube containing the RNA to re-concentrate.
2. Place the tube at -70°C for 30 minutes.
3. Centrifuge 30 minutes at 4°C (13 000 rpm).
4. Remove carefully the float and add 1 ml of absolute ethanol 70%.
5. Mix vigorously.
6. Centrifugate (13 000 rpm) for 15 minutes and remove the float.
7. Dry the lower part of the tube under vacuum in the Speed Vac machine for 15 minutes.
8. Dissolve the concentrated RNA in the lower part of the tube with 20 µl of RNAse free water
9. Let stand 10 minutes at 18°C and then 30 minutes at 4°C.
10. Stock at -80°C.

C.5 Reverse Transcription

The reverse transcriptions were done by adding, for each μl of RNA sample:

1. 1 μl of OligoDT(25)
2. 8.5 μl of DEPC water (diethylpyrocarbonate)

The resulting mix is incubate at 65°C for 10min and then a second mix is added:

1. 4 μl of M-MLV RT buffer (Promega)
2. 2 μl of dNTP (deoxynucleoside triphosphate)(20nmol) (Promega)
3. 0.25 μl of M-MLV Reverse Transcriptase (Promega)
4. 3.25 μl of DEPC water

The final solution is incubate at 42°C for 1 hour.

C.6 Polymerase Chain Reaction

The protocols and polymerisation cycle temperatures used in the PCR are followed:
The following mix is added to 2.5 μl of RT product (DNA sample):

1. 13.125 μl of DEPC water
2. 2.5 μl of $MgCl_2$ 7.5mM
3. 10 μl of 5X Green GoTaq Reaction Buffer (Promega)
4. 1.5 μl of dNTP
5. 0.5 μl of primer R/F - 20mM
6. 0.125 μl of GoTaq polymerase (Promega)

Place the above solution in thermocycler, adjusted as follows :

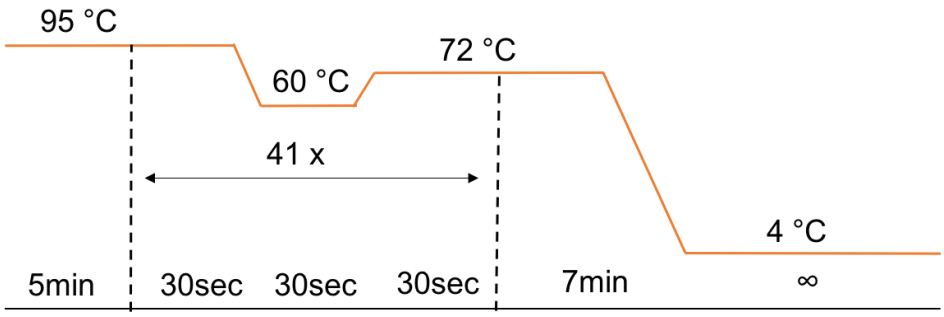

Figure C.1: Schematic representation of the temperatures cycles used for the GoTaq PCR.

C.7 Reverse Transcription Quantitative PCR

The quantitative PCR were done by adding the following mix to 2 μl of DNA:

1. 10 μl of Takyon SYBR Green (Eurogentec)
2. 0.5 μl of primer R/F (20 µM)
3. 7 μl of DEPC water

Seal the plate with plastic film (provided by Biorad) and centrifuge 3 minutes at 4°C (3700 rpm). Place in the qPCR Biorad, adjusted as follows:

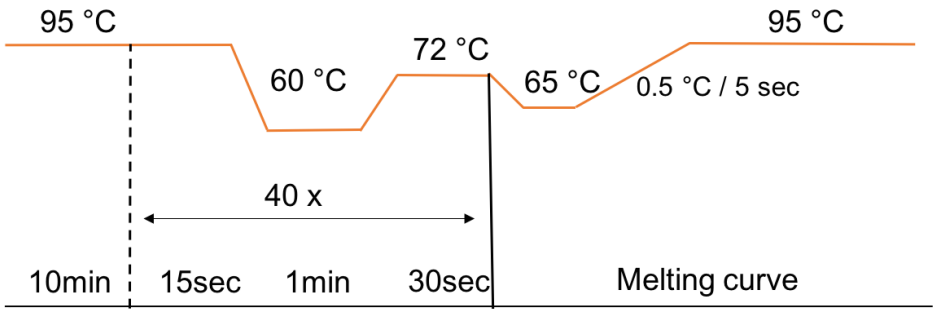

Figure C.2: Schematic representation of the temperatures cycles used for the qPCR. Melt curves were measured from 65°C to 95°using an incrementation of 0.5°C.

Appendix D

Calculation proceedings

D.1 Abacus of Fuch-Rosenthall cell use

Counting Fuch-Rosenthall cells (figure D.1) serve to determine the number of particles per volume unit of liquid. It has been used to determine the concentration of the conidial suspension of *Alternaria solani*. The counting is performed through the optical microscope, and then the conidial concentration is assessed using the below abacus (table D.1).

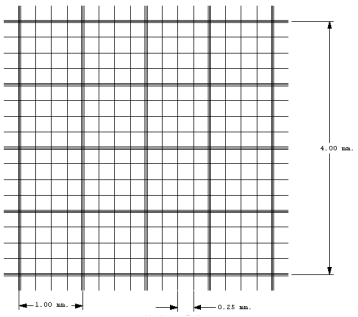

Figure D.1: Illustration of the Fuch Rosenthall grid that constitute the cell

The volume of the Fuch-Rosenthall cell is $125\times10^{-7}\times$N ml. N represents the number of grid unit where the conidial was counted with the optical microscope.

Table D.1: Abacus for the use of the Fuch-Rosenthall cell]

N	**Travel volume (ml)**	**Multiplication factor to express the number of particles in 1 ml of the considered suspension**
16 (1 square)	2×10^{-4}	5000
32 (2 square)	4×10^{-4}	2500
64 (4 squares)	8×10^{-4}	1250
80 (5 squares)	1×10^{-3}	1000
160 (10 squares)	2×10^{-3}	500
256 (16 squares)	3.2×10^{-3}	312.5

D.2 Colony forming unit

This calculation method is used to assess a bacterial concentration of a suspension. In this work, it is employed to estimates the bacterial concentration of *Bacillus subtilis* 30B-B6 LB suspension.

1. Make a series of Eppendorf to 10^{-1} to 10^{-7} with 900 µl LB-medium in each tubes.
2. Sample 100 µl from the bacterial suspension (mother suspension, non-diluted) and put it in the first tube (noted 10^{-1}. Don't forget to shake before sampling.
3. Do the same with the others tubes (10^{-2} to 10^{-7}. Dilution from ten to ten.
4. Pick up 100 µl of dilutions from 10^{-4} to 10^{-7} and spread it on Petri dish (LBA medium). Don't forget to shake before sampling. Do three Petri dishes per dilution.
5. Seal the Petri dishes for 24 hours at 30°C.
6. Count the number of colony for each dilution.

To calculate the number of colony forming unit, use:

$$CFU = \frac{(Numberofcolony)/(Platevolume)}{Dilution}$$

To be usable, the number of counted colonies has to be between 15 and 300 colonies and the ratio between dilutions has to be conserved.

Appendix E

Media

E.1 LBA

Table E.1: Composition of the LBA medium used for the growth of *Bacillus subtilis* 30B-B6

Compounds	**Quantities**
Bacto Yeast Extract	3 g
Bacto Tryptone	1,5 g
NaCl	3 g
Agar	5,1 g
Eau milliQ	300 mL

E.2 V8

Table E.2: Composition of the V8 medium used for the growth of *Alternaria solani*

Compounds	**Quantities**
$CaCO_3$	0,35 g
Agar	5,25 g
V8	70 mL
Eau milliQ	280 mL

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Induced resistance in tomato plant against Alternaria solani by Bacillus subtilis strain 30B-B6
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Résumé

Early blight (EB) caused by the necrotrophic fungus *Alternaria solani* is a devastating plant disease of tomato crops, mainly controlled by synthetic chemical fungicides. In the framework of sustainable agriculture, a promising new control strategy is the induction of plant defence responses by elicitors. These last decades, numerous researches on new elicitors have emerged, and recent findings have reported a promising strain of Bacillus subtilis, 30B-B6, as a potential elicitor of tomato plants.

The present study investigates the systemic induction of defence response in tomato (*Solanum lycopersicum* var. Moneymaker) against *A. solani* using 30B-B6 strain as an elicitor. Trials were conducted under controlled greenhouse conditions. Results revealed that root application of the eliciting bacterium 6 hours before the fungal leaf inoculation significantly increased tomato plant protection by 83% against EB compared to control plants. Disease protection was assumed to be due to the induction of resistance in the host plant since experimental protocols were designed so as to avoid any direct contact between the biocontrol agent and the pathogen.

A second bioassay using the bacterial supernatant highlighted the inability of the metabolite suspension to trigger tomato resistance against *A. solani*, when the bacterium was not present.

A third bioassay was performed to evaluate the expression of tomato defense-related genes by RT-qPCR on four treatment groups: (1) bacterial elicitation, (2) fungal inoculation, (3) both bacterial elicitation and fungal inoculation and (4) control plants. Gene expression levels were assessed separately on tomato lower leaves, while the upper ones served for a systemicity assessment. The target genes analysed were assigned to specific plant metabolic pathways related to the induced systemic resistance (ISR) and/or the systemic acquired resistance (SAR): the SAR marker *PR1* gene, the ISR markers *LOX* and *PI-1* genes, the *NPR1* gene linked to the crosstalk between both pathways and *PR2-b* gene, responsive to salicylic acid and/or jasmonic acid. The fungal inoculation of non-elicited plants led to a systemic significant increase in *PR2-b* gene expression compared to non-inoculated plants, at 6 hours post inoculation (hpi). No activation of *PR1* gene was detected in plants challenged by the pathogen. The 30B-B6 application triggered significant increases in *LOX* and *PI-1* gene expression in tomato upper leaves, respectively at 6 and 9 hpi, compared to the other treatments. However, when both the bacterium and the fungus were present, only the *PR2-b* gene expression was up-regulated in the systemic leaves compared to uninoculated plants, at 24 hpi.

Our results indicate that the 30B-B6 root application enhances tomato resistance to EB, deriving from an induction of defense genes, but the signaling pathways involved remain equivocal. The promising features of 30B-B6 highlighted in the present work make it a potential biological agent in the control of tomato crops against EB and encourage its practical use in agriculture.

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