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PhD thesis in bioscience engineering

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Mechanisms underlying the protective effect of the biocontrol agent *Bacillus subtilis* 30B-B6 in *Solanaceae*

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« Pour les uns le sommet, pour les autres le début de l’approche »

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Abstract

Bacillus spp. offer a promising and sustainable alternative in crop protection. Both their direct antagonism towards pathogens as well as their indirect action by either eliciting systemic resistance or stimulating plant growth is mostly explained by the numerous bioactive compounds they produce.

Our objective was to evaluate and understand how the *B. subtilis* 30B-B6 can control early and late blight diseases on *Solanaceae*.

Recent work reported the interest of 30B-B6 against potato late blight caused by *Phytophthora infestans*, an hemibiotrophic pathogen. This research aims to deepen knowledge on the effective biocontrol potential of this strain by performing further field trials and investigating its activity against potato early blight caused by the necrotrophic pathogen, *Alternaria solani*. Field experiments have proved that 30B-B6 can contribute to reducing the potato late blight pressure. Greenhouse experiments on tomato revealed that 30B-B6 also reduced (up to 76%) the disease pressure caused by *A. solani*, showing that 30B-B6 exhibits effective antagonistic properties against hemibiotrophic and necrotrophic pathogens. Bioassays performed on tomato plants also revealed the that the application of the bacterium on the root substrate led to reduce the disease severity to the same level as the foliar treatment. *B. subtilis* 30B-B6 is able to control the pathogen by direct antagonism and by stimulation of the plant defenses. This work gives a first insight into the molecular pathways involved in the induction of systemic resistance by 30B-B6 in tomato. We used RT-qPCR and salicylic acid (SA) quantification to understand the action of 30B-B6 on plant defenses. We showed that

the genes *PR2b* and *WRKY1*, coding for a glucosidase and a transcription factor involved in the expression of SA dependent genes, are overexpressed in plants displaying resistance to the pathogen after a treatment with the bacterium. The higher content of SA in these plants supports the involvement of SA in response to treatment with B. *subtilis* 30B-B6. In order to test the bioactive compounds produced by 30B-B6, comparative bioassays were performed. We investigated the potential implication of lipopeptides (LPs) and siderophore produced by 30B-B6 in the induction of the systemic resistance. LPs and siderophores partially purified from culture in G-A medium significantly reduced symptoms of early blight (protection around 53%). This thesis shows that the stimulation of tomato systemic defenses by the bacterium against a necrotrophic fungus involved the SA-pathway. While resistance to necrotrophic pathogens is generally described as independent of the SA-pathway. Taken together, this thesis reveals how *B. subtilis* 30B-B6 acts via direct antagonism and by stimulating the plant host defense mechanisms. The interest of this bacterium as a biocontrol agent is thus confirmed.

Scientific communications

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Paper in collaboration

Caulier S, Gillis A, **Colau G**, Licciardi F, Liépin M, Desoignies N, Modrie P, Legrève A, Mahillon J, Bragard C (**2018**) Versatile antagonistic activities of soil-borne *Bacillus* spp. and *Pseudomonas* spp. against *Phytophthora infestans* and other potato pathogens. Front Microbiol 9, 1-15.

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Oral presentations

Colau G, Caulier S, Bragard C, Mahillon J, Legrève A (**2019**) *Bacillus subtilis* 30B-B6, a promising bacterium for the biocontrol of early and late blight. EuroBlight Workshop (York, England, from 12/05/2019 to 15/05/2019, York).

Colau G, Caulier S, Gillis A, Licciardi F, Liépin M, Desoignies N, Modrie P, Bragard C, Mahillon J, Legrève A (**2018**) Characterization of the biocontrol of *Phytophthora infestans* and *Alternaria solani* by bacteria isolated from Belgian agroecosystems. XV Meeting of the Working Group Biological and Integrated Control of Plant Pathogens, Biocontrol products: from lab testing to product development (Lleida, Spain, from 23/04/2018 to 26/04/2018).

Posters

Colau G, Licciardi F, Caulier S, Liépin M, Gillis A, Mahillon J, Bragard C, Legrève A (**2016**) Biological control of potato diseases: control of *Phytophthora infestans* and *Alternaria solani* by indigenous Belgian bacteria. XIV Meeting of the IOBC-WPRS Working Group Biological Control of Fungal and Bacterial Plant Pathogens (Berlin, Germany, from 12/09/2016 to 15/09/2016).

Caulier S, Gillis A, **Colau G**, Licciardi F, Liépin M, Desoignies N, Modrie P, Legrève A, Mahillon J, Bragard C (**2018**) Antagonistic soil-borne *Bacillus* spp. and *Pseudomonas* spp. bacteria against late blight and other potato pathogens: from the laboratory to the field. 10th World Potato Congress and XXVIII ALAP Congress (Cuzco, Peru).

Caulier S, Gillis A, **Colau G**, Licciardi F, Liépin M, Desoignies N, Modrie P, Legrève A, Mahillon J, Bragard C (**2018**) Naturally occurring soil-borne *Bacillus* spp. and *Pseudomonas* spp. with versatile antagonistic activities against *Phytophthora infestans* and other potato pathogens. International Plant Growth Promoting Rhizobacteria Workshop (Victoria, Canada, from 17/06/2018 to 22/06/2018).

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List of abbreviations

ABA	abscisic acid
Ac	acetylation
ACC	1-aminocyclopropane-1 carboxylic acid
ACO	ACC oxidase
ACS	ACC synthase
AOC	allene oxide cyclase
AOS	allene oxide synthase
APX	ascorbate peroxidase
ATP	adenosine triphosphate
Aux	auxins
AzA	azelaic acid
AZI1	azelaic acid induced 1 protein
BA2H	benzoic acid-2-hydroxylate
BCAs	biocontrol agents
BTB	Broad-complex, tramtrack and bric à brac domain
CAT	catalase
CC-NB-LRR	coiled-coil nucleotide-binding-leucine reach repeat receptor
CKs	cytokinins
COI1	coronatine insensitive 1 protein
CTR1	constitutive triple response 1 protein
CWDE	cell wall degrading enzymes
DA	diterpenoid dehydroabietinal
DAMPS	damage-associated molecular patterns
DIR1	defective in induced resistance 1 protein
EB	early blight
EF-Tu	elongation factor Tu
EHM	extrahaustorial membrane
EHMX	extrahaustorial matrix
EIL1	EIN3-like 1
EIN2	ethylene-insensitive 2 protein
EIN3	ethylene insensitive 3 protein
EPS	exopolysaccharides
ERF1	ethylene response factor 1
ERS	ethylene response sensor
ET	ethylene
ETI	effector-triggered immunity
ETR	ethylene response protein
ETS	effector triggered susceptibility
FMO1	flavin-dependent monooxygenase 1 protein
FRO2	ferric reduction oxidase 2
G-A	Grimm-Allen medium
G3P	glycerol-3-phosphate
Gas	gibberellins
GSNO	S-nitrosoglutathione enzymes
HDA6	histone deacetylase 6
hpi	hours *post* inoculation
HR	hypersensitive response

HSTs	Host specific toxins
IAA	indole-3-acetic acid
ICS	isochorismate synthase
INF1	elicitin infestans-1
IP	invasion pattern
IPL	isochorismate pyruvate lyase
IPM	integrated pest management
IPRs	invasion pattern receptors
IPTRs	IP-triggered responses
IRT1	Fe^{2+} transport protein 1
ISR	induced systemic resistance
JA	jasmonic acid
JA-Ile	jasmonoyl-isoleucine
JAR1	JA conjugate synthase 1
JAZ	jasmonate ZIM-domain protein
JMT	JA carboxyl methyltransferase
LOX	lipoxygenase
LPs	lipopeptides
LRRs	leucine-rich repeats
MAMPs	microbe-associated molecular patterns
MAPK	mitogen-activated protein kinase
MAPKKK	mitogen-activated protein kinase kinase kinase
Me	methylation
MeJA	methyl jasmonate
MES	methyl esterase
MeSA	methyl salicylate
NB-LRR	nucleotide-binding–leucine-rich repeat
nHSTs	Non-host specific toxins
NIM1	noninducible immunity 1 protein
NIMIN-2	NIM-interacting 2 protein
NINJA	novel interactor of JAZ protein
NLS	Nuclear localization sequence
NRP	non ribosomal peptides
NPR1	nonexpressor of *PR* genes 1 protein
OGs	oligogalacturonides
ORA59	octadenoic-responsive Arabidopsis 59
OPDA	12-oxo-phytodienoic acid
PAL	phenylalanine ammonia lyase
PAD4	phytoalexin-deficient 4 protein
PAMPS	pathogen-associated molecular patterns
PDF1.2	plant defensin 1.2
PGs	polygalacturonases
PGPF	plant growth–promoting fungi
PGPR	plant growth–promoting rhizobacteria
Pip	pipecolic acid
PK	polyketides
POD	peroxidase
POZ	Poxvirus and zinc finger domain
PPO	polyphenol oxidase

PR-gene	pathogenesis-related gene
PRRs	pattern recognition receptors
PTI	PAMPs-triggered immunity
RBPG1	responsiveness to polygalacturonases 1
ROS	reactive oxygen species
RLKs	receptor-like kinases
RLPs	receptor-like proteins
SA	salicylic acid
SABP2	SA-binding protein 2
SAG	salicylic acid O-β-glucoside
SAGT	SA glucosyl transferase
SAMT	SA methyl transferase
SAR	systemic acquired resistance
SNE1	suppressor of necrosis 1
SNI1	suppressor of NPR1, inducible 1 protein
SOD	superoxide dismutase
TF	transcription factor
TGA	TF which binds the sequence TGACG of the gene promoters
TIR-NB-LRR	toll-interleukin 1 nucleotide-binding-leucine reach repeat receptor
TPL	topless protein
TRX	thioredoxin enzymes
VCs	volatile compounds
VOCs	volatile organic compounds
VSP2	vegetative-storage-protein 2
WAK1	wall-associated kinase 1

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General introduction and research outline

Plants colonized the earth long before the first anthropoids. Flowering plants or angiosperms appeared about 150 million years ago while the first anthropoids appeared about 45 million years ago (Boyce and Lee, 2017; Brunet and Jaeger, 2017; Morris *et al.,* 2018; Donoghue, 2019). One of the peculiarities of plants is that they take root in their substrate where they grow and have to adapt to their biotic and abiotic environment (Pereira, 2016; Crang *et al.,* 2018). Due to their attachment to the substrate, they are more susceptible to enemies and depend on water and nutrient content of the substrate. Plants are photoautotrophic, using the energy provided by the photons of sunlight to fix carbon dioxide and synthesize their food (Crang *et al.*, 2018).

Since the beginnings of agriculture 12,000 years ago, human has selected and cultivated plant species for food (Howden *et al.,* 2007; McDonald and Stukenbrock, 2016). The growing population has led to an intensification of agriculture to ensure sufficient yields, and cultural practices have evolved during the human history (Pimentel, 1996; Tubiello *et al.*, 2007). From the start of Green Revolution (late 1950s), agriculture has become highly dependent on plant protection products, mineral fertilizers, plant breeding and the use of less sensitive varieties (Imfeld and Vuilleumier, 2012; Kim *et al.,* 2017; Lamichhane *et al.*, 2018). At that moment, the majority of agro-ecosystems planted are monocultures (Mueller *et al.*, 2005; McDonald and Stukenbrock, 2016). The natural endemic plant species were replaced by genetically uniform but broadly adapted and high-yielding cultivars. This leads to fields cultivated with single clone or population of near clones with poor genetic diversity

(McDonald and Stukenbrock, 2016). The genetic uniformity of host populations and the increased planting density in agro-ecosystems compared with natural ecosystems make pathogen transmission easier (Thrall and Burdon, 2003). To manage these pathogens, chemical pesticides are sprayed on crops, and their massive use has led to a range of problems in agriculture, for the environment and for the human health (Pimentel, 1996; Lamichhane *et al.*, 2015). Examples of these problems include contamination of soils and water, deleterious effects on non-target organisms (Gill and Garg, 2014). In addition to this, the evolution of pesticide resistance among pest populations is becoming more and more of a concern. Although chemical pesticides were used initially to benefit human life through increase in agricultural productivity and by controlling infectious diseases, their negative side effects have overweight the benefits associated with their use (Gill and Garg, 2014). To implement new strategies, it is necessary to understand the interactions between plants and their wider environment.

Plants have developed multiple mutualistic interactions with different organisms for their development and reproduction such as insects, birds, mycorrhizae and growth-promoting rhizobacteria (Tscharntke and Brandl, 2004; Potts *et al.,* 2010; Nadeem *et al.*, 2014; Krauss *et al.*, 2017; Nath and Meena, 2018). Plant-microbe interactions in the rhizosphere are actually the determinants of plant health, productivity and soil fertility (Souza *et al.,* 2015). Indeed, in the soil, there are a lot of beneficial microorganisms which can protect plants either directly by suppressing pathogens or indirectly by stimulating the plant development and growth (Sansinenea, 2019). Among these beneficial

microbes, the *Bacillus* genus is one of the most common bacterial genera present in the soils and their members are good plant colonizers (Mandic-Mulec *et al.,* 2015). Bacterial inoculants can thus contribute to increasing agronomic efficiency by reducing production costs and environmental pollution, once the use of chemical fertilizers can be reduced or eliminated if the inoculants are efficient (Souza *et al.,* 2015). In this context, studies must be performed to increase our knowledge of the modes of action and the effects of particular strains of bacteria identified as potential candidates of biocontrol agents in order to improve their efficacy, to understand the determinant factors and avoid possible side effects.

Bacillus spp. are widely used in crop protection and present several advantages compared to chemical pesticides such as lower toxicity and persistency in the environment, higher specificity, and have multiple modes of action to dam pathogens (Borriss, 2011; Cawoy *et al.,* 2011). Moreover, *Bacillus* spp. are also associated with the improvement of plant nutrition and modulation of plant growth (Kloepper *et al.,* 2004).

In 2012, with a view to valorizing the microbial potential of soil for the biocontrol of pathogens of *Solanaceae*, the WACOBI (WAllonie COntrol BIologique) project was initiated and funded by the "Service public de Wallonie – DGO3". The main purpose of this six-year research project was to provide a bacterial strain or a consortium of bacterial strains isolated from Belgian samples that would be effective against pathogens of potatoes such as *Alternaria solani, Phytophthora infestans, Rhizoctonia solani, Pectobacterium carotovorum* and *Fusarium solani*. An *in vitro* screening of the antagonistic activity of

bacterial strains isolated from Belgian substrates (*e.g.* field soils, manure, potato plants) was carried out (Caulier *et al.,* 2018). A collection of 2,826 bacterial strains, mainly from *Pseudomonas* and *Bacillus* genera, was created. Among them, the strain 30B-B6 of *B. subtilis* was extracted from a 13-24 cm horizon in a crop field in Waremme (Belgium). A screening for direct antagonism properties was performed on the bacterial collection. Sixty strains of *Bacillus* and *Pseudomonas* exhibit a high potential for biocontrol of fungal and chromista pathogens, including *Phytophthora infestans* and *Alternaria solani*. In particular, the strain 30B-B6 shows valuable properties for biocontrol of plant pathogens: PCR analyses revealed that the strain is positive to seven out of the 23 tested genes known for their involvement in the synthesis of antimicrobial compounds (Caulier *et al.*, 2018). Besides, 30B-B6 has proteolytic and cellulolytic activities and is able to produce siderophores. In addition, analyses have also revealed that this strain does not synthesize any of the major toxins (*e.g.* cereulide, cytotoxin K1 and K2) that constitute a risk to human health (Caulier *et al.,* 2018). Direct confrontation tests on solid media revealed that the strain 30B-B6 inhibits the growth of *P. infestans* and *A. solani* by 80% and 50% respectively. The foliar application of the strain also provides protection to *Solanum tuberosum* around 70% against both pathogens under controlled conditions in the greenhouse. Based on the results of direct antagonism against *P. infestans* on potato plants in the greenhouse, seven bacterial strains (*B. amyloliquefaciens* 17A-B3, *B. subtilis* 30B-B6, *B. mycoides* 15A-B2, *B. pumilus* 30B-B2, *Pseudomonas protegens* 44R-P8,

P. putida 42R-P6 and *P. brenneri* 43R-P1) were evaluated in the control of *Phytophthora infestans* in field condition on potato crop.

This PhD thesis was initiated as part of the WACOBI project. The objective of my thesis is to evaluate the potential of *B. subtilis* 30B-B6 to control pathogens of *Solanaceae*, and to improve our knowledge of the underlying mechanisms involved in the biocontrol.

Structure of the manuscript

The following manuscript is divided into five chapters. **Chapter 1** provides a general description of pest management and biocontrol in agriculture. An important section of this chapter presents the state of knowledge on plant defense mechanisms. Both specific and non-specific defenses are described. Then, the mechanisms involved in systemic defense pathways are detailed, as well as the interactions between them. Another section of the chapter presents the bacterial genus *Bacillus* and its use in biocontrol. The mechanisms involved in the biocontrol of phytopathogenic agents by PGPR bacteria, and in particular those of *Bacillus* spp., are presented. Finally, the protagonists of the two pathosystems: *S. lycopersicum*/*tuberosum* – *A. solani*/*P. infestans* are described. **Chapter 2** presents the results of two field trials (2016 and 2017) on potato crop with bacteria of the genera *Pseudomonas* and *Bacillus* (including 30B-B6) to control potato late blight. **Chapter 3** investigates the potential of 30B-B6 as PGPR and its capacities to manage the early blight disease on tomato under controlled conditions. It also gives a first insight into the molecular pathways involved in the resistance of tomato elicited by 30B-B6 against *A. solani*. C**hapter 4** study the prospective implication of LPs and siderophores produced by the strain 30B-B6 in

the induction of systemic resistance of tomato against early blight. **Chapter 5** summarizes the major findings and discusses the potential applications of this research and suggests some perspectives for new research.

Chapter 1.

State of the art

This literature review describes the evolution of pest management in agriculture, the current context and the challenges associated with it. Then a wide overview of the plant defense mechanisms is presented. The following section also presents the *Bacillus* genus and its interest in biocontrol. Finally, the pathosystems *Solanum lycopersicum* - *Solanum tuberosum* - *Alternaria solani* - *Phytophthora infestans* are described.

1.1 Pest management in agriculture and emergence of biocontrol

In nature, plants are confronted with a complex and changing environment and constantly deal with a wide variety of pests and pathogens with different lifestyles and infection strategies. They cannot escape, so they have developed a complex defense system, which will be described further in this manuscript (Mengiste, 2012; Pieterse *et al*., 2012; Fu and Dong, 2013; Pieterse *et al*., 2014). Domestication of certain plant species (mainly cereals) by humans more than 10,000 years ago and agriculture, defined as the use of lands to produce food and other resources useful to humans, have altered the biodiversity at the local and global level and deeply changed the ecosystems (Howden *et al.,* 2007; Ross-Ibarra *et al.*, 2007; Geiger *et al.*, 2010; Power, 2010). In response to population growth, humans have intensified agriculture, notably through the crop fertilization and use of agrochemicals (Tubiello *et al.*, 2007). This marked the beginning of the Green Revolution, which started in the late 1950s, and was characterized by an important increase in the use of fertilizers in crop production (Pimentel, 1996). Since then, agriculture has become highly dependent on plant protection products to ensure sufficient yields (Imfeld and Vuilleumier, 2012). Indeed, agriculture is the largest consumer of chemical pesticides, nearly 85% of the world production, to control pests and biotic diseases (Kim *et al.*, 2017). For example, the average use of pesticides, from 2007 to 2014 in Belgium, is estimated at 21.18 kg of active ingredients ha^{-1} $year^{-1}$ on the potato crop and 22.49 of active ingredients ha^{-1} on tomatoes in greenhouses (Sonia and Deuninck, 2016; Comité regional PHYTO, 2020). Fungicides accounted for 16.55 kg of active

ingredients ha^{-1} $year^{-1}$ in tomato crops (Sonia and Deuninck, 2016). Fungicides are classified mainly into two groups: protectant and penetrant fungicides (Leroux, 2003; Ivic, 2010; Dias, 2012). The protectant fungicides, also called contact fungicides, are active on the plant surfaces and do not penetrate into the plant tissues. They take preventive action by killing or inhibiting fungi or fungal spore germination before the mycelium can grow and develop within the plant tissues. They lose their activity after being washed off the plant (Agrios, 2005; Dias, 2012). These fungicides are generally inexpensive and do not contribute to the development of resistance in pathogen populations (Leroux, 2003). Resistance is defined as a heritable modification in the sensitivity of a pest population which is reflected in the repeated failure of a product to achieve the expected level of control when used according to the recommendation for that pest species (Hawkins *et al.,* 2019). The penetrant fungicides are absorbed by the plant and carried by translocation to the site of infection. Thus, they are also called systemic fungicides (Agrios, 2005; Dias, 2012). They kill the fungus after the mycelium has penetrated the parenchyma of the plant tissues, stopping the dispersal or infection within the plant (Leroux, 2003; Agrios, 2005). Hence, they can be used as protectants, eradicants or both (Dias, 2012). They also have a very specific site of action in the target fungus, and their intensive use can lead to the appearance of resistance in pathogen populations (Leroux, 2003; Chandrashekara *et al.,* 2012; Gill and Garg, 2014).

In parallel with the use of pesticides since the 1950s, yields have been increased through plant breeding (*e.g.* short growth duration, grain

yield, abiotic stress tolerance) and the use of more pest-resistant varieties (Khush, 2001; Crossa *et al.,* 2017; Lamichhane *et al.*, 2018). However, the massive use of chemical pesticides has led to a range of problems in agriculture, for the environment and for the human health, such as contamination of soils and water, deleterious effects on non-target organisms. Moreover, the exposure with some pesticides can be associated with various diseases such as cancer, hypersensitivity, hormonal perturbation but also with birth defects (Kim *et al.,* 2017). In addition to this, the evolution of pesticide resistance among pest populations is becoming more and more of a concern (Pimentel, 1996; Lamichhane *et al.*, 2015).

Taking all this into account, in the 1990s, a new approach to farming and in particular pest management has emerged. The directive 2009/128/EC of the European Parliament and of the Council of 21 October 2009 has established a framework for Community action to achieve a sustainable use of pesticides (Lamichhane *et al.*, 2015). This approach, called integrated pest management (IPM), is more based on a rational and sustainable use of pesticides rather than their prohibition (Ehler, 2006). IPM is defined as careful consideration of all available plant protection methods and subsequent integration of appropriate measures that discourage the development of pest populations and keep the use of plant protection products and other forms of intervention to levels that are economically and ecologically justified and reduce or minimize risks to human health and the environment (European Commission, 2009). IPM emphasizes different methods, such as cultural practices (*e.g.* rotations, intercropping, false seedbed), plant breeding or biological control rather than the use of chemicals,

to limit the establishment and development of pests (Ehler, 2006; Lamichhane *et al.*, 2015).

The biological control, or biocontrol, in agriculture can be defined as the use of natural organisms or their by-products that bring a pest below a break-even point for growers in IPM strategy (Pal and Gardener, 2006; Gill and Garg, 2014; Seiber *et al.*, 2014; Hinarejos *et al.*, 2016). Biopesticides (the contraction of "biological pesticide") include a wide range of products such as plant extracts, micro- or macro-organisms and pheromones (Copping and Menn, 2000; Kumar and Singh, 2015; Mishra *et al.*, 2015). As defined by the European regulation, a biopesticide differs from a biostimulant. A biostimulant is "a product stimulating plant nutrition processes independently of the product's nutrient content, with the sole aim of improving one or more of the following characteristics of the plant or the plant rhizosphere: nutrients use efficiency, tolerance to abiotic stress, quality traits, and availability of confined nutrients in soil or rhizosphere" (Council of the European Union, European Parliament, 25/06/2019). The market share of biopesticides is about 3.5% of the global pesticides market (Glare *et al.*, 2012). In 2019 in the European Union, 54 strains of microorganisms or biocontrol agents (BCAs) are registered in the "EU Pesticide Database" to control plant diseases (Andrivon *et al.*, 2019). Among all the BCAs available on the market, bacteria belonging to the *Pseudomonas* and *Bacillus* genera are widely represented (Mishra *et al.*, 2015; Salomon *et al.*, 2017). They present numerous advantages compared to chemical pesticides such as lower toxicity and persistency in the environment, higher specificity, and have multiple modes of action to dam pathogens (O'Brien *et al.*, 2009;

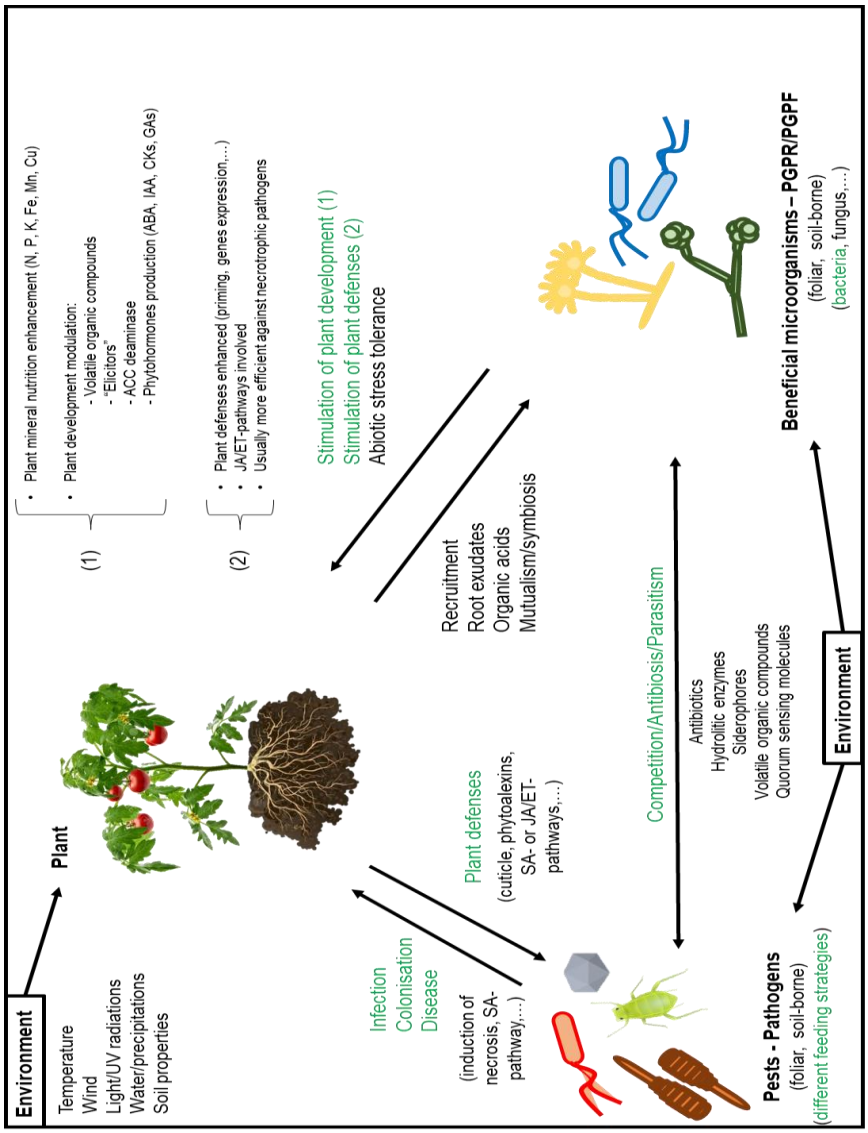
Borriss, 2011; Gerbore *et al.*, 2013; Andrivon *et al.*, 2019). However, more studies on the impact of long-term use of BCAs in the fields will have to be done to prevent their possible harmful effects on agroecosystems including the indigenous microflora (Butt *et al.*, 2001; Gerbore *et al.*, 2013). One of the main impediments to the development of BCA-based products is the economical/commercial aspects (Butt *et al.*, 2001; Bailey *et al.*, 2010). In general, BCAs are more expensive to produce than chemical pesticides. There are also costs to adapt the infrastructure of the end users (*e.g.* fermentor, agricultural machinery) to store and use BCAs (Whipps and Lumsden, 2001). Most of the time, biopesticides are used for ornementals and vegetables in greenhouses, and to a lesser extent in field crops (Fravel, 2005). As an example, in Belgium, Serenade ASO® (active substance *B. subtilis* QST713) is mainly authorized on fruit trees (such as pear tree, apple tree, apricot tree), vegetables (including tomato), aromatic plants in greenhouse but not on cereals or potato (Fytoweb, 2020).

Finally, the scientific community faces several challenges such as improving the screening methods to select efficient BCAs in the field conditions, the understanding of the complex relationship between the BCAs, the plant and the environment (Whipps and Lumsden, 2001; Babendreier, 2008; Gerbore *et al.*, 2013). Moreover, improving the efficacy of BCAs under field conditions will also require to elucidate the mechanisms involved in the antagonism between BCAs and the pathogens and factors that influence this antagonism. It is also important to understand how the plant defend itself against the pests and pathogens, and how BCAs can interfere with these plant defense pathways.

1.2 Plant defenses

This section provides an overview of plant interactions with pathogens or beneficial microorganisms which are summarized in **Fig. 1**. When a plant is confronted with a microorganism potentially pathogenic, two situations are possible (Agrios, 2005; Nürnberger and Lipka, 2005). The most common is called “non-host resistance” and referred to a heterologous plant-microorganism interaction meaning that there is no compatibility between the plant and the microorganism due to basic plant resistance (Agrios, 2005; Ham *et al.*, 2007). The second one, called homologous plant-microorganism interaction, occurs when a pathogen has the capacity to colonize the host and suppress its defenses (Jones and Dangl, 2006).

Fig. 1: Overview of the interactions between plant, pathogens and beneficial microorganisms. Each of the protagonists interacts with the others directly or indirectly. Beneficial microorganisms can directly inhibit the growth of pathogens by competition, antibiosis and/or parasitism, or indirectly by stimulating the development and the defenses of the plants. Similarly, the plant can directly defend itself against the pathogens or take advantage of beneficial microorganisms that will inhibit the development of pathogens. All these interactions are subject to environmental conditions. The green text indicates what will be studied in more detail in this thesis.



1.2.1 Non-specific defenses

As plants take root in the substrate where they grow, they cannot escape the pathogens or pests (Vos *et al.,* 2013). Plants have developed different levels of protection to face adverse environmental conditions both biotic and abiotic stresses (Bigeard *et al.,* 2015; Köhl *et al.,* 2019). First of all, plants are protected by physical barriers, including cuticle, waxy layer and cell wall, and chemical barriers such as phytoanticipins and phytoalexins (Dixon, 2001; Bigeard *et al.,* 2015; Conrath *et al.,* 2015; Hacquard *et al.,* 2017). Most of the pathogens are not able to overcome this first line of defense and the phenomenon is called "non-host resistance". The few pathogens that are able to skip these constitutive barriers are then confronted with the plant immune system in the true sense of the word which is developed in the following section (Spoel and Dong, 2012; Choi and Klessig, 2016).

1.2.2 Specific defenses: local defenses

Activation of the immune system requires the ability of the plant to distinguish its own compounds from those that are retained in pathogens. These non-self compounds, called microbe- or pathogen-associated molecular patterns (MAMPs/PAMPs), are recognized by surface-localized pattern recognition receptors (PRRs) leading to the activation of PAMPs-triggered immunity (PTI) (**Fig. 2**) (Macho and Zipfel, 2014; Andolfo and Ercolano, 2015; Hacquard *et al.,* 2017). PRRs can be receptor-like kinases (RLKs) or receptor-like proteins (RLPs). RLKs possess an ectodomain involved in ligand binding, a single-pass transmembrane domain and an intracellular kinase domain. The structure of RLPs is quite similar but the intracellular

kinase domain is missing and must interact with additional components for the activation of cytoplasmic response outputs (Nürnberger and Kemmerling, 2006; Mueller and Felix, 2012; Macho and Zipfel, 2014; Trdá *et al.,* 2015). PRR ectodomains can contain leucine-rich repeats (LRRs), lysine motives, lectin motives, or epidermal growth factor-like domains (Nürnberger and Kemmerling, 2006; Zipfel, 2014). LRR-type PRRs recognize proteins or peptides, as bacterial flagellin and bacterial elongation factor Tu (EF-Tu), while PRRs containing other domains bind to carbohydrate-containing molecules, such as fungal chitin, bacterial peptidoglycans, extracellular ATP, or plant-cell-wall-derived oligogalacturonides (Mueller and Felix, 2012; Macho and Zipfel, 2014; Zipfel, 2014).

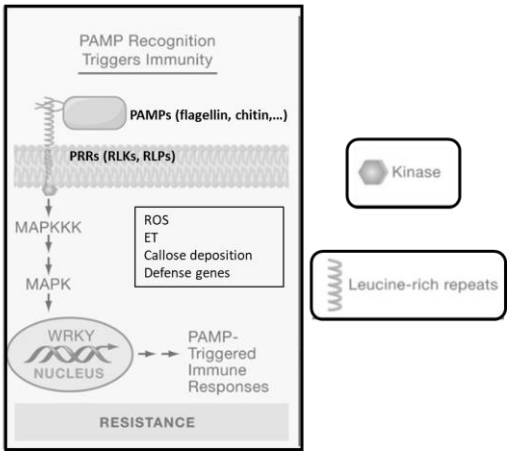


Fig. 2: Activation of PAMPs-triggered immunity. Recognition of MAMPs/PAMPs (such as bacterial flagellin) by surface-localized pattern recognition receptors, PRRs (RLPs, RLKs) promptly triggers basal immunity (PAMP-triggered immune responses), which requires signaling through MAP kinase cascades and transcriptional reprograming mediated by plant WRKY transcription factors. The PAMPs-triggered immunity responses include production of reactive oxygen species (ROS), enhanced biosynthesis of ethylene (ET), deposition of callose and the activation of the defense gene expression (adapted from Chisholm *et al.*, 2006). Legend: MAPK, mitogen-activated protein kinase; MAPKKK, mitogen-activated protein kinase kinase kinase; WRKYs, transcription factors and key regulators of many processes in plant; RLK, receptor-like kinase; RLP, receptor-like proteins; ET, ethylene; ROS, reactive oxygen species.

MAMPs/PAMPs are molecules often conserved among taxa, such as bacterial flagellin, lipopolysaccharides, EF-Tu and fungal chitin (Choi and Klessig, 2016). Plants also perceive endogenous signals due to damages caused by invaders. Damage-associated molecular patterns (DAMPs) are equally recognized by PRRs (Henry *et al.,* 2012; De Lorenzo *et al.,* 2018).

MAMPs/DAMPs recognition by PRRs triggers basic and widespread responses in plants (Ludwig *et al.,* 2005; Desender *et al.,* 2007; Jourdan *et al.,* 2008; Boller and Felix, 2009). These early responses include the increasing of ion fluxes across the plasma membrane, the activation of mitogen-activated protein kinase (MAPK), the production of reactive oxygen species (ROS), the enhanced biosynthesis of ethylene, the deposition of callose and the activation of the defense gene expression (Chisholm *et al.,* 2006; Mueller and Felix, 2012). One of the first measurable changes that occur in plant cells within two minutes after MAMPs/DAMPs recognition is the alkalinization of the extracellular environment (Boller and Felix, 2009; Mueller and Felix, 2012; Shen *et al.,* 2017). Under normal physiological conditions, the plant cell plasma membrane is polarized and the extracellular pH is maintained around pH5. The stress perception triggers the opening of ion channels in the plasma membrane resulting in an influx of H^+ and Ca^{2+} ions to the cytoplasm coupled with an efflux of K^+, Cl^- and $NO3^-$ and leads to the membrane depolarization. The proton influx increases the extracellular pH and the Ca^{2+} cations also play the role of a messenger which can activate calcium-dependent proteins (Zebelo and Maffei, 2016; Shen *et al.,* 2017; Kaur *et al.,* 2019). Another early response to MAMPs/DAMPs,

with a lag phase of about two minutes, is the oxidative burst (Kombrink and Schmelzer, 2001; Noctor *et al.,* 2018). The oxidative burst is characterized by the generation of ROS, such as superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and hydroperoxyl radical (HO_2.) in the apoplast and a decrease of the ROS detoxifying enzyme activity initiated by salicylic acid and nitrous oxide (Kombrink and Schmelzer, 2001; Apel and Hirt, 2004; Baxter *et al.,* 2014; del Rio, 2015; Stael *et al.,* 2015; Noctor *et al.,* 2018). The oxidative burst can be recorded by H_2O_2-dependent luminescence of luminol (Kim *et al.,* 2019). During plant-pathogen interaction, ROS production frequently precedes a rapid and localized necrosis of plant cells at the site of infection, called "hypersensitive response" (HR) (Kombrink and Schmelzer, 2001; Apel and Hirt, 2004; Desender *et al.,* 2007). The HR is the first macroscopically visible symptom proving that the plant has reacted to the pathogen infection (Kombrink and Schmelzer, 2001). However, the simultaneous production of ROS and the down-regulation of ROS scavenging mechanisms are required to initiate HR (Kombrink and Schmelzer, 2001; Desender *et al.,* 2007; Stael *et al.,* 2015). The oxidative burst encompasses two phases: 1) a very rapid and unspecific accumulation of H_2O_2 also triggered by abiotic stresses, and 2) a prolonged production of H_2O_2 notably associated with HR (Apel and Hirt, 2004; Stael *et al.,* 2015). The ROS accumulation in the apoplast also leads to an increase of the cross-linking within the plant cell wall and renders these walls more resistant to degrading enzymes from pathogens (Mueller and Felix, 2012; Stael *et al.,* 2015).

Some pathogens have acquired the capacity to bypass the PRRs defense system by using proteins, called effectors, and cause "effector triggered susceptibility" (ETS) (Lo Presti *et al.*, 2015; Mine *et al.*, 2018). Although secreted effectors are involved in suppressing PTI, intracellular plant proteins, nucleotide-binding–leucine-rich repeat (NB-LRR) receptors can mediate recognition of these pathogen effectors, resulting in "effector-triggered immunity" (ETI) (**Fig. 3**) (Macho and Zipfel, 2014, Hacquard *et al.*, 2017). Most of the time, ETI is also associated with HR at the site of infection, but it is not an absolute rule (Andolfo and Ercolano, 2015; Hacquard *et al.*, 2017).

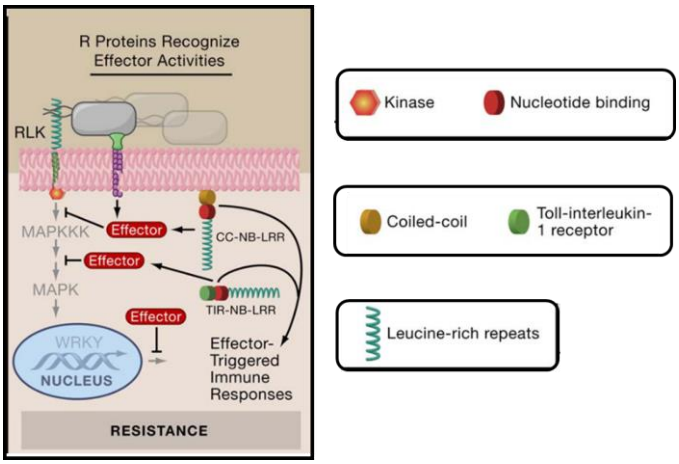


Fig. 3: Activation of effector-triggered immune responses. Perception of pathogen effectors by intracellular receptors (represented by CC-NB-LRR and TIR-NB-LRR; see text) and activation of effectors-triggered immune responses (adapted from Chisholm *et al.*, 2006). Legend: MAPK, mitogen-activated protein kinase; MAPKKK, mitogen-activated protein kinase kinase kinase; WRKYs, transcription factors and key regulators of many processes in plant; RLK, receptor-like kinase; CC-NB-LRR, coiled-coil nucleotide-binding-leucine reach repeat receptor; TIR-NB-LRR, toll-interleukin 1 nucleotide-binding-leucine reach repeat receptor.

Effector perception by NB-LRRs is highly specific and can be either direct (with the receptor binding the effector) or indirect (involving accessory proteins). The NB-LRR proteins encompass mainly two subclasses, one with a toll-interleukin 1 receptor (TIR) domain and

called TNLs, and the other with a coiled-coil (CC) domain and called CNLs (**Fig. 3**) (Cui *et al.,* 2015; Mine *et al.,* 2018). CNLs are found in dicot and monocot plants, while TNLs are restricted to dicots (Cui *et al.,* 2015). These NB-LRRs proteins are encoded by *R*-genes while effectors recognized by NB-LRR proteins are encoded by pathogen *Avr*-genes (Lo Presti *et al.,* 2015; van Wersch and Li, 2019). Although direct interactions between Avr-protein and R-protein exist, effector recognition and the following activation of R-proteins are often indirect (**Fig. 4**). The figure 4 presents the different models of interactions between R-proteins and effectors (van Wersch and Li, 2019).

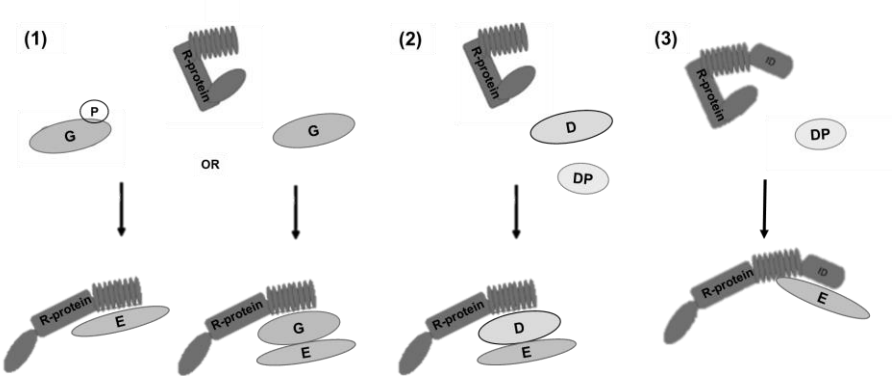


Fig. 4: Simplified models of interactions between plant R-protein and pathogen effector (adapted from van Wersch and Li., 2019). Note that effectors (E) do not necessarily stay bound to their targets and NLRs may not interact directly with their modified guardee (G). Activation of NLRs is likely to require the conversion of bound ADP to ATP and oligomerization. **(1)** The guard model. A guardee (G) is an effector target that is monitored (guarded) by an R-protein. The R-protein is activated by the modified guardee (*e.g.* by phosphorylation, P) or by the interaction between the guardee and the pathogen effector (E). **(2)** The Decoy model. A decoy (D) is a protein which is the direct target of pathogen effector but does not have any immunity-related function. The R-protein guards a decoy protein, mimicking a defense-related protein (DP), activated by the interaction between the decoy and the pathogen effector. The decoy avoid that DP becomes the effector target. It has been hypothesized that decoys evolved to protect key host immune regulators, which share sequence similarity with the decoy. **(3)** The integrated decoy model. The R-protein is activated when one its part (called integrated decoy domain; ID) is modified by the pathogen effector. The decoy and the integrated decoy domain share common characteristics and sequence homology.

The coevolution between pathogen effectors and plant NB-LRR proteins led to the so-called "zig-zag" model (**Fig. 5**) (Jones and Dangl, 2006). This model encompasses four phases. The first one leads to the activation of PTI. Some pathogens are able to interfere with PTI by producing effectors and cause ETS, which is the second phase. The third phase is the recognition of these effectors by intracellular plant proteins resulting in ETI. In the fourth phase, the pathogens which not possess these effectors, are selected in the pathogen populations because they will not be recognized by the intracellular plant proteins. In parallel, selection favors new plant NB-LRR alleles that can recognize one of the newly acquired effectors, resulting again in plant resistance.

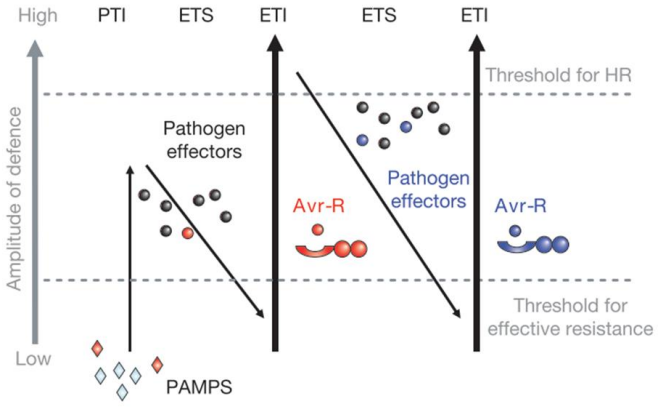


Fig. 5: The "zig-zag" model proposed by Jones and Dangl (2006). The ultimate amplitude of disease resistance or susceptibility is proportional to [PTI – ETS + ETI]. In phase 1, plants detect microbial/pathogen-associated molecular patterns (MAMPs/PAMPs, red diamonds) via PRRs to trigger PAMP-triggered immunity (PTI). In phase 2, successful pathogens deliver effectors that interfere with PTI, or otherwise enable pathogen nutrition and dispersal, resulting in effector-triggered susceptibility (ETS). In phase 3, one effector (red circle) is recognized by NB-LRR protein, activating effector-triggered immunity (ETI), an amplified version of PTI that often passes a threshold for induction of hypersensitive cell death (HR). In phase 4, pathogen strains are selected that have lost the red effector, and perhaps gained new effectors through horizontal gene flow (in blue) - these can help pathogens to suppress ETI. Selection favors new plant NB-LRR alleles that can recognize one of the newly acquired effectors, resulting again in ETI. Legend: Avr-R, proteins from plants.

However, the PTI/ETI model or "zig-zag" model, has some limitations (Pritchard and Birch, 2014; Andolfo and Ercolano, 2015). In particular, for necrotrophic pathogens (kill the host cells and use the nutrients released from dead host cells) because HR is favorable for their development and change the classical interpretation of induced cell death in ETI from "immunity" to "susceptibility" (Andolfo and Ercolano, 2015). In addition, the "zig-zag" model also fails to integrate a potential role of the plant defense mechanisms for the recognition of beneficial microbes (Pritchard and Birch, 2014; Hacquard *et al.*, 2017).

Cook *et al.* (2015) proposed another model called "Invasion Model" that takes into account the limitations of the "zig-zag" model (**Fig. 6**). They discuss an alternative view of plant innate immunity as a system that evolves to detect invasion. The host receptors, called invasion pattern receptors (IPRs), recognize either an externally encoded or modified-self ligand that indicates invasion, called invasion pattern (IP). IPs can include MAMPs, PAMPs, DAMPs or any other molecules potentially detected by IPR. Moreover, the IP-triggered responses (IPTRs) do not automatically imply the activation of immunity. Indeed, the IPTR(s) resulting from the perception of one or more IPs can lead to two situations: the end of symbiosis or continued symbiosis. These two outcomes are mediated by three mechanisms defined from the perspective of the invader: i) failure to suppress IPTR, ii) suppression of IPTR, or iii) manipulation of IPTR. Invaders may use effectors to influence the outcome of the IPTR. The use of effectors or the act of continued symbiosis can induce the production of new IPs that can in turn be recognized by IPRs, triggering

continued plant responses, which can again result in a continuation or not of the interaction. The responses mediated by IPRs are not defined in absolute terms in order to (i) accommodate the range of responses that plant immune receptors can trigger, (ii) reflect that multiple, synergistic, and/or antagonistic responses can be triggered simultaneously that collectively determine the outcome of the symbiosis, and (iii) because the way in which plants ultimately stop symbiosis is not well understood and not necessarily the same for all types of invaders.

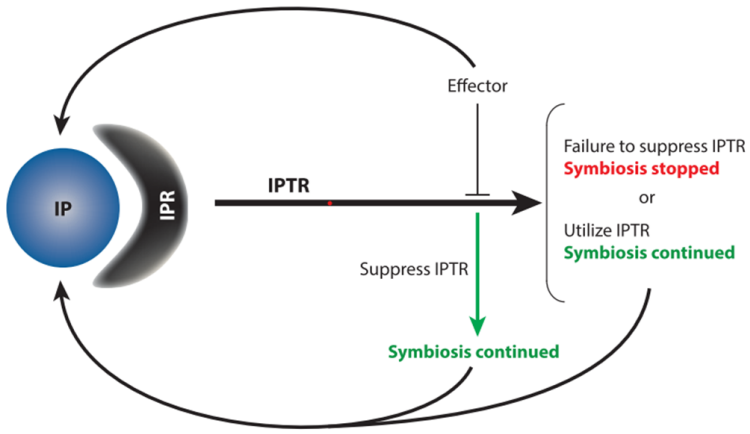


Fig. 6: The Invasion Model proposed by Cook *et al.* (2015) to describe an attempted plant-invader symbiosis. Upon attempted symbiosis, invasion patterns (IPs) from invaders are perceived by plant IP receptors (IPRs), inducing an IP-triggered response (IPTR). Invaders may produce effectors to influence the interaction, but if it does not work, the symbiosis stops. Potentially, the IPTR may be suppressed (*e.g.* by biotrophs) or manipulated (*e.g.* by necrotrophs) to continue symbiosis. Continued symbiosis and effector usage may generate host-perceivable IPs, leading to continued IPTR. Collectively, multiple recognition events and invader strategies influence the IPTR and eventually result in termination or continuation of symbiosis.

1.2.3 Specific defenses: systemic resistance in plants

In addition to induce local defense responses (in the immediate surroundings of wounding or sites of pathogen invasion), a microbial attack can trigger a systemic defense response. “Systemic” resistance

is a general term for an induced state of resistance in plants triggered by biological or chemical inducers, which protects the whole plant, and not only the infected tissues against future attacks by invaders or pests. In the literature, two forms of systemic resistance, called systemic acquired resistance (SAR) and induced systemic resistance (ISR) have been described. SAR is mainly induced by pathogens, whereas ISR is activated by beneficial microorganisms (Vlot *et al.*, 2009; Hacquard *et al.,* 2017). These systemic responses are the consequences of the local perception of MAMPs/DAMPs or other danger signals at the site of infection or colonization.

1.2.3.1 Systemic acquired resistance

The onset of PTI and ETI at the infection site often triggers a systemic state of resistance in all plant tissues (Bostock, 2005; Spoel and Dong 2012; Gao *et al.,* 2015). The establishment of this induced systemic resistance state involves long-distance signals that propagate an enhanced defensive capacity in undamaged parts of the plant (Dempsey and Klessig, 2012; Shah and Zeier, 2013). This systemic resistance induced by pathogens is called systemic acquired resistance (SAR) and confers enhanced resistance against a broad spectrum of pathogens especially against biotrophes (Jones and Dangl, 2006; Pieterse *et al.,* 2014). Biotrophes grow thanks to nutrients present in living host cells as opposed to necrotrophes which kill the host cells and use the nutrients released from dead host cells. SAR can also be triggered by chemical compounds or abiotic stresses (Vlot *et al.,* 2009; Spoel and Dong, 2012). The vascular tissues act as a conduit for the translocation of factors and facilitate long-distance intra-plant communication (Shah and Zeier, 2013; Rodrìguez-Celma *et al.*, 2016;

Yang *et al.,* 2020). SAR is often associated with increased salicylic acid (SA) levels in plant tissues and with the activation of pathogenesis-related (*PR*) genes which encode PR proteins with antimicrobial properties (Bostock, 2005; Vlot *et al.,* 2009; Jain and Khurana, 2018).

SA, a phenolic compound, can be synthesized by two different pathways from chorismate, an intermediate compound of plant phenylpropanoid pathway (**Fig. 7**). The first one is the phenylalanine ammonia lyase (PAL)-mediated phenylalanine pathway, and the second one is the isochorismate synthase (ICS)-mediated isochorismate pathway (Vlot *et al.,* 2009; An and Mou, 2011; Ding and Ding, 2020). Although the ICS pathway is responsible for the majority of SA synthesis in UV-treated or pathogen-infected *A. thaliana* and *Nicotiana benthamiana* plants, evidence for an important role of PAL activity for pathogen-induced SA formation has been repeatedly found in multiple plant species (An and Mou, 2011). The major part of the SA synthesized by the plant is converted into SA O-β-glucoside (SAG). SAG is mainly stored into the vacuole as a pool of inactive form of SA (Vlot *et al.,* 2009). The methyl salicylate (MeSA), a volatile form of SA-compound, is highly suspected of being one of the mobile SAR signals (An and Mou, 2011).

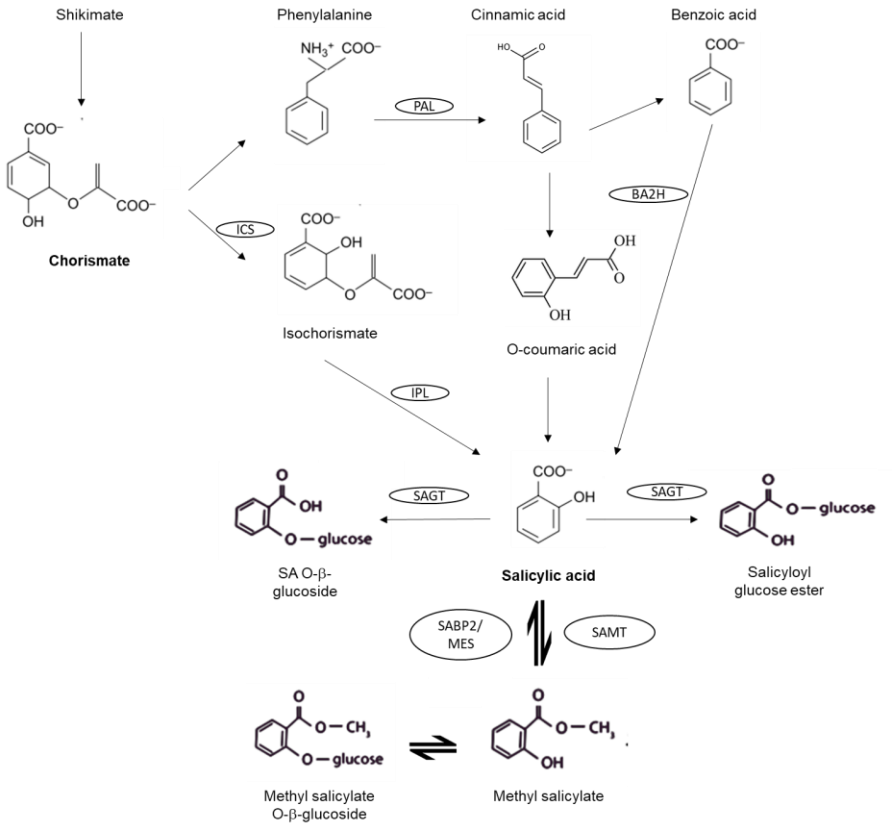


Fig. 7: Schematic representation of the salicylic acid biosynthesis pathway in plant (adapted from Shah, 2003; Vlot *et al.*, 2009). Abbreviations: PAL, phenylalanine ammonia lyase; ICS, isochorismate synthase; IPL, isochorismate pyruvate lyase; BA2H, benzoic acid-2-hydroxylase; SAGT, SA glucosyltransferase; SAMT, SA methyltransferase; SABP2, SA-binding protein 2; MES, methyl esterase.

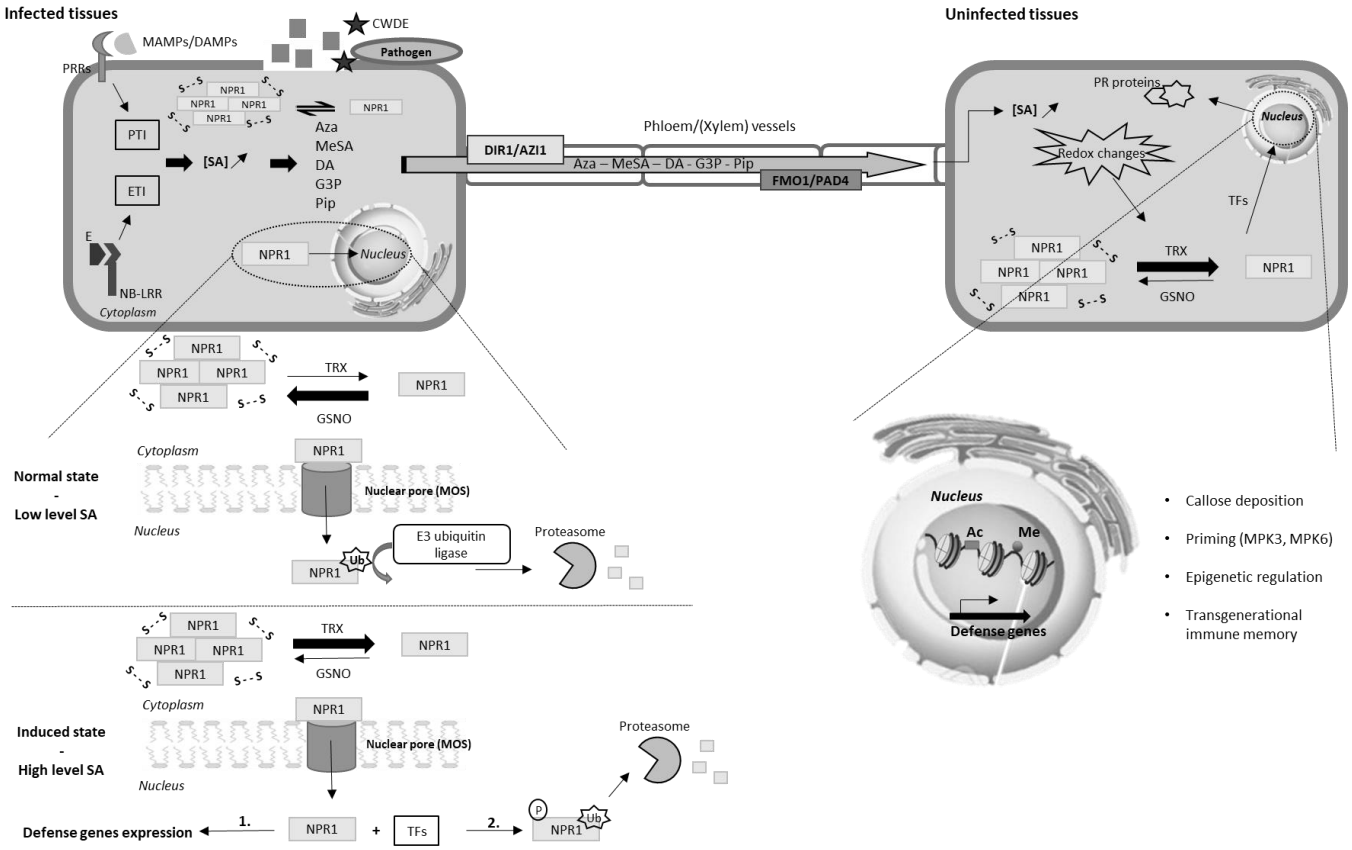
The translocation by the vascular tissues of the SAR signal, from the infected tissues to distal organs requires the lipid-transfer protein "defective in induced resistance 1" (DIR1) and/or "azelaic acid induced 1" (AZI1) protein (**Fig. 8**). Even if SA accumulation in the phloem has been proved, grafting experiments with tobacco showed that SA itself is not the translocated SAR signal (Vernooij *et al.*, 1994). Several metabolites are potentially involved in the long-distance SAR signaling, such as MeSA, the diterpenoid dehydroabietinal (DA), a glycerol-3-phosphate (G3P)-dependent

factor, azelaic acid (AzA) and pipecolic acid (Pip) (Durrant and Dong, 2004; Dempsey and Klessig, 2012; Shah *et al.,* 2014). In the distal non-infected tissues, the onset of SAR is depending of "flavin-dependent monooxygenase 1" (FMO1) and "phytoalexin-deficient 4" (PAD4) probably to amplify the signal from infected tissues (Misina and Zeier, 2006; Shah *et al.*, 2014). The incoming of messengers from infected tissues leads to an accumulation of SA and the synthesis of PR proteins, rendering uninfected cells prepared for further pathogen attack (Spoel and Dong, 2012; Fu and Dong, 2013).

SAR also involves priming mechanisms (Conrath *et al.,* 20002; Jung *et al.,* 2009). Priming is a state defined as "an enhanced sensitivity and responsiveness to stress that results from a prior experience and leads to increased resistance and/or abiotic stress tolerance" (Conrath, 2011; Mauch-Mani *et al.*, 2017). This state is characterized by a faster and/or a stronger activation of the plant defenses in response to biotic or abiotic stress (Conrath, 2011; Conrath *et al.,* 2015; Rodrìguez *et al.,* 2018). Beckers *et al.* (2009) showed that plant treated with benzothiadiazole (a synthetic analogue of SA) induced gradually the expression of MAPK3 and MAPK6, inactive forms of mitogen-activated protein kinase (MAPK). After pathogen infection, the enhanced levels of these latent signaling components are activated, resulting in potentiated *PR1* gene expression and the development of systemic immunity. Priming is also associated with chromatin modifications in the promoters of WRKY transcription factor genes that regulate SA-dependent defenses, thereby facilitating potentiated expression of these defense-regulatory genes upon pathogen infection (Jaskiewicz *et al.,* 2011; Spoel and Dong, 2012; Fu and Dong, 2013;

Pieterse *et al.,* 2014). Epigenetic regulation of pathogen- and β-aminobutyric acid -induced priming for SA-dependent defenses was shown to be inherited by the next generation via chromatin remodeling or DNA methylation (Akimoto *et al.,* 2007; Luna *et al.,* 2012; Fu and Dong, 2013; Pastor *et al.,* 2013; Zhu *et al.,* 2016). Plants seem to have the capacity to memorize a stressful situation and subsequently immunize not only themselves but also next generations against future attacks.

Fig. 8 (next page): Overview of the mechanisms and molecular components involved in response to a pathogen, and in the activation of systemic acquired resistance (SAR). Abbreviations: PRRs, pattern recognition receptors; NB-LRR, nucleotide-binding–leucine-rich repeat; MAMPs, microbial-associated molecular patterns; DAMPs, damage-associated molecular patterns; CWDE, cell wall degrading enzymes; PTI, pathogen-triggered immunity; ETI, effector-triggered immunity; Aza, azelaic acid; MeSA, methyl salicylate; DA, diterpenoid dehydroabietinal; G3P, glycerol-3-phosphate-dependent factor; Pip, pipecolic acid; SA, salicylic acid; NPR1, nonexpressor of *PR* genes 1 protein; E, pathogen effector; DIR1, defective in induced resistance 1 protein; AZI1, azelaic acid induced 1 protein; TRX, thioredoxin enzymes; GSNO, S-nitrosoglutathione enzymes; TF, transcription factors; Ac, acetylation; Me, methylation; FMO1, flavin-dependent monooxygenase 1 protein; PAD4, phytoalexin-deficient 4 protein.


Infected tissues
MAMPs/DAMPs
CWDE
Pathogen
PRRs
PTI
ETI
[SA]
Aza
MeSA
DA
G3P
Pip
E
NB-LRR
Cytoplasm
NPR1
Nucleus
DIR1/AZI1
Phloem/(Xylem) vessels
Aza – MeSA – DA - G3P - Pip
FMO1/PAD4
Uninfected tissues
PR proteins
Redox changes
TFs
TRX
GSNO
Normal state
-
Low level SA
Nuclear pore (MOS)
E3 ubiquitin ligase
Proteasome
Ub
Induced state
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High level SA
Defense genes expression
1.
2.
P
Ac
Me
Defense genes
Callose deposition
Priming (MPK3, MPK6)
Epigenetic regulation
Transgenerational immune memory

SAR signaling downstream of SA is controlled by the redox-regulated protein "Nonexpressor of *PR* genes 1" (NPR1), also called "noninducible immunity 1" protein (NIM1) (**Fig. 9a**) (Pieterse *et al.*, 2012; Fu and Dong, 2013). In non-induced cells, NPR1 is sequestered in cytoplasm as oligomers via disulfide bonds. The few NPR1 monomers which are translocated to the nucleus are ubiquitinylated and degraded by the proteasome to avoid the continuous activation of defense genes (Spoel *et al.*, 2009; Fu and Dong, 2013). Increasing SA concentration in cells leads to a redox change facilitating the monomerization of NPR1 via the thioredoxin enzymes (TRX) (Pieterse *et al.*, 2012; Fu and Dong, 2013). NPR1 monomers are then translocated into the nucleus and interact with transcription factors (*e.g.* TGAs, WRKYs), regulating the defense gene expression (Durrant and Dong, 2004; Pieterse *et al.*, 2014; Shah *et al.*, 2014; Backer *et al.*, 2019). Proteins such as NIM-interacting 2 (NIMIN-2), suppressor of NPR1, inducible 1 (SNI1), NPR3, and NPR4 seem to be negative regulators which fine-tune NPR1-dependent gene expression (Mosher *et al.*, 2006; Backer *et al.*, 2019).

In 2012, two different models for the regulation of NPR1 by NPR3 and NPR4 were published (Fu et *al.*, 2012; Wu *et al.*, 2012; Ding and Ding, 2020). In the model proposed by Fu *et al.* (2012) SA does not bind directly to NPR1, but rather, binds to NPR3 and NPR4. NPR3 and NPR4 function as E3 ubiquitin ligases to mediate ubiquitination of NPR1 and its subsequent degradation. Moreover, the model postulates that the turnover of NPR1 is required for the activation of NPR1-dependent promoters. The model of Wu and colleagues (2012) proposes that NPR1 binds SA with high affinity. The C-terminal

domain of NPR1 is required for the activation of defense genes in response to SA. The bond between SA and the C-terminal domain of NPR1 releases it from auto-inhibition by the N-terminal BTB/POZ domain and enables the C-terminal domain to function as a co-activator with TGA transcription factors. In this model, the regulation of NPR1 activity is not mediated by protein degradation, and there was no requirement for the NPR3 and NPR4 proteins to achieve full activation of defense gene expression.

These two conflicting models could not be fully reconciled by the existing data (Innes, 2018). Ding *et al.* (2018) established that the protein degradation model of Fu *et al.* (2012) was likely incorrect, while the NPR1 activation model of Wu *et al.* (2012) was incomplete. Ding *et al.* (2018) showed that NPR3 and NPR4 are transcriptional co-repressors for SA-responsive genes (**Fig. 9b**). They found a dominant negative mutation in *NPR4* that constitutively suppresses the immune responses. This mutation leads to a substitution of a glutamine (Q) for an arginine (R) at position 419 within the C-terminal domain of NPR4. The generation of the same mutation in *NPR3* also suppresses the immune response. This finding suggests that NPR3/ NPR4 functions are, at least partly, redundant. Moreover, the R419Q mutation in *NPR4* nearly eliminated SA binding, indicating that NPR4 functions a transcriptional co-repressor in the absence of SA, and that SA binding eliminates this co-repressor activity. Even if the co-repressor activity of NPR4 is suppressed by SA binding, it does not prevent association with TGAs, nor association with defense gene promoters (Ding *et al.,* 2018). Thus, SA leads to a release of the

defense gene promoters from repression by NPR3 and NPR4 by blocking the repressive activity directly of their C-terminal domains.

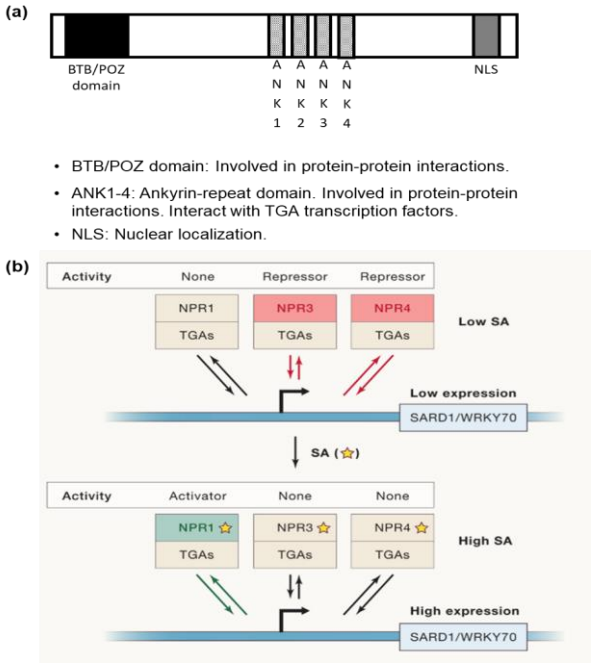


Fig. 9: **(a)** Schematic representation of the NPR1 protein. **(b)** Model for the interactions between NPR1 and NPR3/NPR4 paralogs and the TGA transcription factors (from Innes, 2018). Independently of salicylic acid (SA) presence, NPR1/NPR3/NPR4 are associated with TGAs transcription factors to form complexes. These complexes compete to bind SA-dependent promoters (represented by bi-directional arrows) and this competition is independent of the SA presence. The concentration of SA impacts the activity of the complex. SA binding to NPR1 enhances the transcription (green arrows), whereas SA binding to NPR3/NPR4 prevents their transcriptional repression activity (red arrows). It means that the higher the SA concentration, the higher the expression of SA-inducible genes and the ability for plant cells to fine-tune the transcriptional response in lower SA. Black arrows: weak interactions compared to the interactions represented by the red and green arrows.

Downstream of SA signaling several SA-inducible defense genes are involved and can be classified in two groups: those directly induced within 30 minutes of SA treatment (such as *Glutathione S transferase 6* and *Immediate early-induced glucosyltransferase*) and those induced later (Durrant and Dong, 2004; Vlot *et al.*, 2009). Among the second group, *PR*-genes are the most studied (Vlot *et al.*,

2009; Backer *et al.,* 2019). The transcriptional activity of these genes is notably regulated (positively or negatively) by members of the TGAs and WRKYs transcription factors (Durrant and Dong, 2004; Eulgem and Somssich, 2007; Vlot *et al.,* 2009; Pieterse *et al.,* 2012; Pieterse *et al.,* 2014; Backer *et al.,* 2019).

Pathogenesis-related proteins (PR proteins) are defined as "proteins that are not or only at basal concentrations detectable in healthy tissues, but for which accumulation at the protein level has been demonstrated upon pathological conditions and related situations in at least two or more plant-pathogen combinations" (Sels *et al.,* 2008; Jain and Khurana, 2018). These proteins have a low-molecular weight, stable below pH3, thermostable and highly resistant to proteases (Edreva, 2005; Sels *et al.,* 2008; Fu and Dong, 2013). PR proteins are essentially found in the apoplast, but also in the vacuole (van Loon, 1999). Acidic and basic form of PR proteins have been identified, each of them having both apoplastic and vacuolar localization (Buchel and Linthorst, 1999; Jain and Khurana, 2018). Though PR proteins are most abundant in the leaves, where they can amount to 5-10% of total leaf proteins, they have been detected in almost all plant organs such as stems, roots and flowers (van Loon, 1999). In general, acidic PR proteins are up-regulated by various signaling molecules such as SA and ROS, while basic PR proteins are up-regulated during pathogen infection by gaseous phytohormones ethylene and methyl jasmonate (Yalpani *et al.,* 1991; Xu *et al.,* 1994; Chamnongpol *et al.,* 1998; Sinha *et al.,* 2014). For example, PR2b is involved in the resistance of tomato against *A. solani* and PR1a is induced during infection of potato plants by *P. infestans* (Lawrence *et al.,* 2000; Gallou *et al.,*

2011; Moushib *et al.,* 2013). So far, the PR proteins have been categorized into 17 families, based on their biological activities, molecular mass, isoelectric point and localization (**Table 1)** (Jain and Khurana, 2018).

Table 1: Classification of PR proteins into 17 families with their typical sizes (kDa) (from Sels *et al.,* 2008).

Families	Biological properties	Typical sizes (kDa)
PR1	Antifungal	15
PR2	β-1,3-Glucanase	30
PR3	Chitinase type I, II, IV, V, VI, VII	25-30
PR4	Chitinase type I, II	15-20
PR5	Thaumatin-like	25
PR6	Proteinase inhibitor	8
PR7	Endoproteinase	75
PR8	Chitinase type III	28
PR9	Peroxidase	35
PR10	Ribonuclease-like	17
PR11	Chitinase, type I	40
PR12	Defensin	5
PR13	Thionin	5
PR14	Lipid transfer protein	9
PR15	Oxalate oxidase	20
PR16	Oxalate oxidase-like	20
PR17	Unknown	27

1.2.3.2 Induced systemic resistance by beneficial microorganisms

In the rhizosphere, nonpathogenic microorganisms such as fungi and bacteria can also trigger or stimulate systemic plant defenses and render the plant more resistant to future attacks by pathogen microorganisms (Kloepper *et al.,* 2004; Pieterse *et al.,* 2014; Hacquard *et al.,* 2017). This phenomenon, called induced systemic resistance (ISR), confers a broad-spectrum effectiveness against pathogens (Pieterse *et al.,* 2014; Hacquard *et al.,* 2017). Beneficial microorganisms can modulate the plant immunity to facilitate the

plant colonization or to establish a partnership with the plant (Zamioudis and Pieterse, 2012; Mhlongo *et al.,* 2018). Fungi or bacteria able to stimulate the plant growth and the plant health are referred as plant growth–promoting fungi (PGPF) or rhizobacteria (PGPR) (Kumar *et al.,* 2016; Venturi and Keel, 2016; Olanrewaju *et al.,* 2017).

Most of the time, ISR is activated through a SA-independent way (Bostock, 2005; Gao *et al.,* 2015; Hacquard *et al.,* 2017). The transduction signal of the onset of ISR is mainly mediated by ethylene (ET) and jasmonic acid (JA) (Ongena *et al.,* 2004; Pieterse *et al.,* 2014). Moreover, ISR is rather based on a higher sensitivity to ET and JA than an increase of their biosynthesis. An analysis of the "ISR transcriptome" of *Arabidopsis thaliana* before and after exposure to *Pseudomonas syringae* pv. tomato DC3000 confirms that ISR is associated with potentiated expression of genes regulated by JA and ET, a phenomenon known as priming (Verhagen *et al.,* 2004; Conrath, 2011; Conrath *et al.,* 2015).

Jasmonic acid belongs to jasmonates, a family of oxylipins arising from the enzymatic oxygenation of 18 and 16-carbon triunsaturated fatty acids (Liechti and Farmer, 2002; Browse, 2009; Ahmad *et al.,* 2016). Jasmonates are involved in the regulation of many biological processes such as growth, defense against biotic and abiotic stresses (Dar *et al.,* 2015). Jasmonate biosynthesis begins with the release of α-linolenic acid (18:3)[1] from membrane lipids (**Fig. 10**) (Wasternack, 2007; Browse, 2009). The α-linolenic acid (18:3) is oxygenated by lipoxygenase (LOX) to form 13(S)-hydroperoxylinolenic acid

[1](18:3) means that fatty acid contains 18 carbon atoms and 3 unsaturations.

(13-HPOT), which is then converted to 12-oxo-phytodienoic acid (OPDA) by allene oxide synthase (AOS) and allene oxide cyclase (AOC). JA is synthesized from OPDA through a reduction and three steps of β-oxidation after which it is further catabolized by JA carboxyl methyltransferase (JMT) to form its volatile counterpart MeJA or conjugated to amino acids, as isoleucine via the JA conjugate synthase JAR1, which results in a biologically highly active form of jasmonoyl-isoleucine (JA-Ile) (Liechti and Farmer, 2002; Browse, 2009; Ahmad *et al.*, 2016).

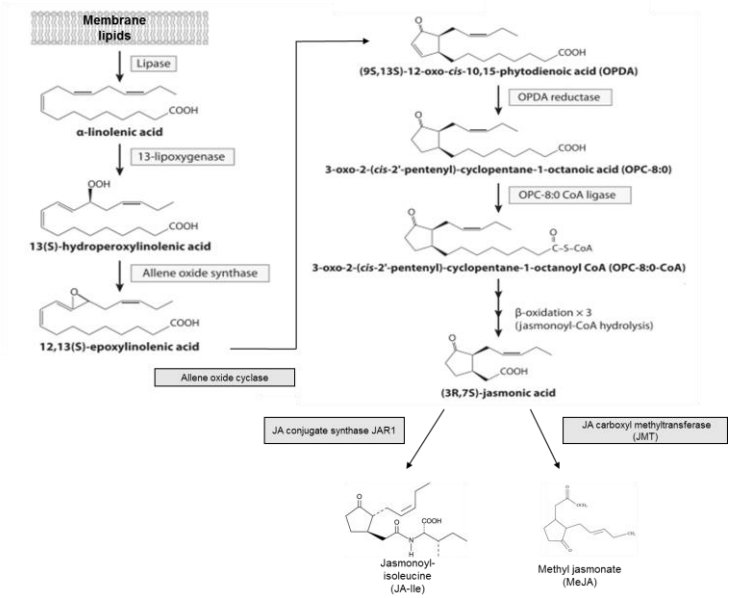


Fig. 10: Schematic representation of the jasmonic acid biosynthesis pathway in plant (adapted from Browse, 2009; Dar *et al.*, 2015).

JA-Ile is perceived by a co-receptor complex consisting of the F-box protein "coronatine insensitive 1" (COI1) and "jasmonate ZIM-domain" (JAZ) proteins (**Fig. 11**) (Pieterse *et al.*, 2012). Perception of JA-Ile by the COI1-JAZ co-receptor leads to ubiquitinylation and subsequent degradation of JAZ proteins by the

proteasome. In uninduced cells, the ZIM domain of most JAZ proteins is linked to transcriptional regulators, such as histone deacetylase 6 (HDA6) or the complex "novel interactor of JAZ" (NINJA)/ "Topless" (TPL) co-repressor, suppressing the positive regulators of JA-mediated defense responses, such as the transcription factors MYC2, 3, and 4 (**Fig. 11**) **(**Pauwels *et al.*, 2010; Pieterse *et al.*, 2014; Kumar *et al.*, 2016). In JA-stimulated cells, the physical interaction between JAZ proteins and transcriptional activators is broken, which results in the activation of a large number of JA-responsive genes (Pauwels *et al.*, 2010; Pieterse *et al.*, 2012; Pieterse *et al.*, 2014; Ahmad *et al.*, 2016).

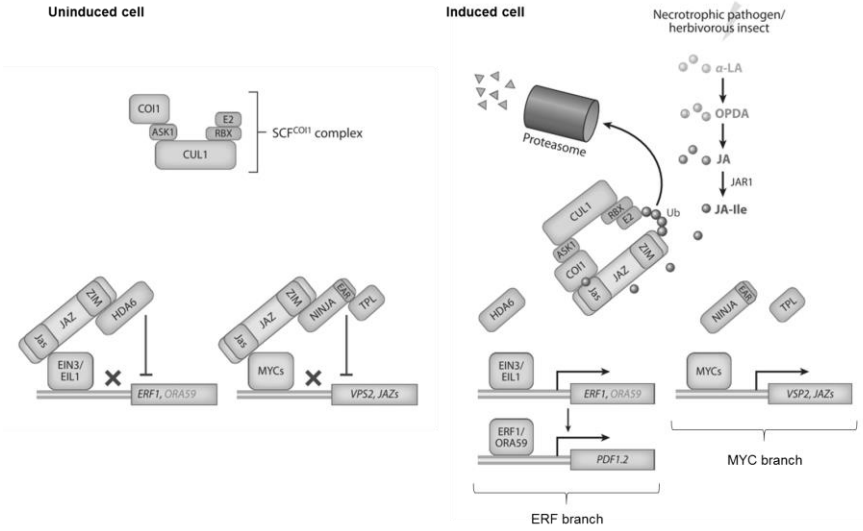


Fig. 11: Schematic overview of the jasmonic acid signaling pathways (from Pieterse *et al.*, 2012). Abbreviations: α-LA, α- linolenic acid; EIN3, ethylene insensitive 3; EIL1, EIN3-like 1; ERF 1, ethylene response factor 1; JA-Ile, jasmonoyl-isoleucine; JAR1, JA conjugate synthase 1; JAZ, jasmonate ZIM proteins; HDA6, histone deacetylase 6; MYC, transcription factors; NINJA, novel interactor of JAZ; OPDA, 12-oxophytodienoic acid; ORA59, octadenoic-responsive *Arabidopsis* 59; PDF1.2, plant defensin 1.2; SCF^{COI1}, complexes composed by a protein coronatine intensive 1 (COI1), a Ring Box protein (RBX), a cullin adaptor (ASK1), a cullin ligase 1 (CUL1) and a ubiquitin-activating enzyme E2; TPL, co-repressor Topless; Ub, ubiquitine; VSP2, vegetative-storage-protein 2.

In *A. thaliana*, two main branches of the JA signaling pathway have been identified: the "MYC branch" and the "ERF branch" (**Fig. 11**) (Pieterse *et al.,* 2012). In brief, the "ERF branch" is associated with enhanced resistance to necrotrophic pathogens and is regulated by "ethylene response factor 1" (ERF1) and "octadenoic-responsive Arabidopsis 59" (ORA59) transcription factors (Lorenzo *et al.* 2003), whereas the "MYC branch" is generally associated with the wound response and defense against insect herbivores, although MYC2 also plays a role in priming for enhanced pathogen defense (Pieterse *et al.*, 2014; Du *et al.*, 2017).

Ethylene (ET) is a gaseous hormone and a major compound involved in plant defense mechanisms (Pieterse *et al.*, 2012; Jain *et al.*, 2016; Hacquard *et al.*, 2017). One of the main steps in ET production by plants is the synthesis of its precursor, the 1-aminocyclopropane-1 carboxylic acid (ACC). ACC, synthesized by an ACC synthase (ACS), is then transformed into ET by the ACC oxidase (ACO) (**Fig. 12**) (Ravanbakhsh *et al.*, 2018).

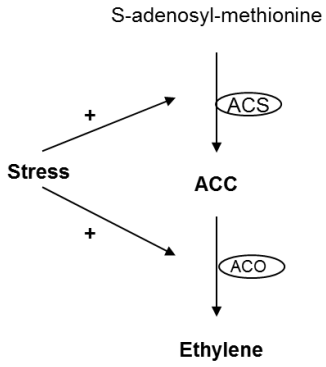


Fig. 12: Schematic representation of the ethylene biosynthesis pathway in plant. Stress enhances the activity of ACS and ACO to favor the production of ethylene. Abbreviations: ACC, 1-aminocyclopropane-1 carboxylic acid; ACS, ACC synthase; ACO, ACC oxidase.

The activation of PTI or ETI induces notably the production of ET (Broekgaarden *et al.,* 2015). ET is perceived by transmembrane-receptor proteins, located in the endoplasmic reticulum, belonging to the ethylene response (ETR) family and to the ethylene response sensor (ERS) (**Fig. 13**) (Wang *et al.,* 2002; Ju and Chang, 2015; Chen *et al.,* 2018). In the absence of ET, the ethylene receptors activate the "constitutive triple response 1" (CTR1) protein, which is a negative regulator of ethylene signaling. The binding of ET inactivates these receptors and thus CTR1. This lifts the inhibition of "ethylene-insensitive 2" (EIN2) protein, a positive regulator of ethylene signaling. Ultimately, the C-terminal end of EIN2 is cleaved and its N-terminal end moves to the nucleus, leading to the stabilization of a specific transcription factor, EIN3, which promotes the expression of ethylene-dependent genes, such as "ethylene response factor" (ERF). Downstream in the ET signaling cascade, ERF transcription factors play a major role in the regulation of defenses (Pieterse *et al.,* 2014; Chen *et al.,* 2018; Ravanbakhsh *et al.,* 2018).

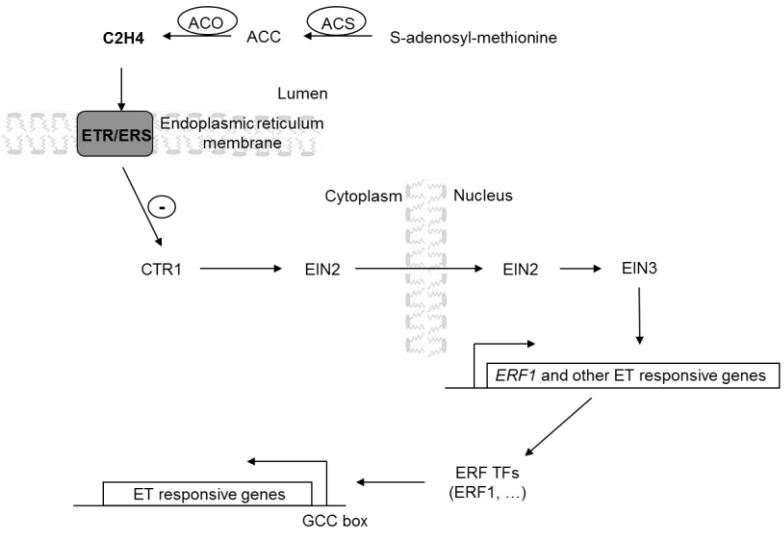
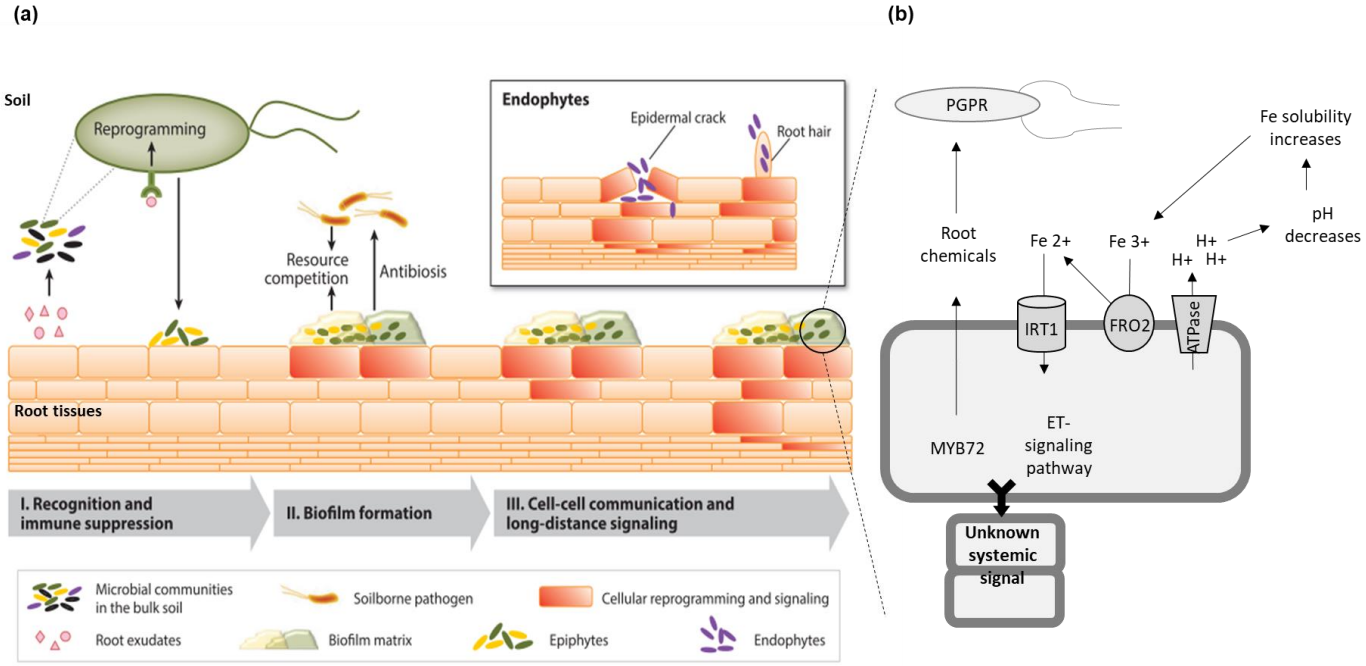


Fig. 13: Schematic overview of the ethylene signaling pathway. Abbreviations: ACC, 1-aminocyclopropane-1-carboxylic acid; ACS, ACC synthase; ACO, ACC oxidase; C_2H_4, ethylene; CTR1, constitutive triple response 1 protein; EIN2, ethylene-insensitive protein 2; EIN3, ethylene-insensitive protein 3; ERF, ethylene response factors.

As already mentioned, to initiate ISR in plant, beneficial microbes (as PGPR and PGPF) have to establish interactions with their host (Zamioudis and Pieterse, 2012; Pieterse *et al.*, 2014; Venturi and Keel, 2016; Hacquard *et al.*, 2017). In response to root exudates, such as sugars and amino acids, PGPR can initiate a chemical communication with the plant (Badri *et al.*, 2009). At the root surface, PGPR epiphytes typically form biofilms in which multicellular communities are enclosed within an extracellular matrix mainly made up of exopolysaccharides (EPS) and mucilage (**Fig. 14a**) (Rudrappa *et al.*, 2008). For example, the biofilm formation is crucial for the colonization of roots by *B. subtilis* strain NCIB 3610 (Beauregard *et al.*, 2013). Within the matrix, bacteria integrate host and self-derived signals and release compounds involved in the onset of ISR. Many inducers of ISR have been identified such as lipopolysaccharides, siderophores, antibiotics, flagellin, N-acyl homoserine lactones,

biosurfactants, volatile organic compounds (Ryu *et al.*, 2004; van Loon and Bakker, 2005; Jourdan *et al.*, 2008; Ongena and Jacques, 2008; Choudhary and Johri, 2009; De Vleesschauwer and Höfte, 2009; Lugtenberg and Kamilova, 2009; Bakker *et al.*, 2013; Venturi and Keel, 2016; Rodrìguez *et al.*, 2018). Several of these determinants have been shown to operate in a redundant way (Bakker *et al.*, 2003). In addition to locally suppressing the plant defenses, PGPR effectors may also function as host-range specificity determinants under the control of plant R-proteins (Yang *et al.*, 2010).

Fig. 14 (right page): **(a)** Schematic steps of the ISR establishment at the root level (Pieterse *et al.*, 2014). (I) Plant produces and secretes organic compounds acting as chemoattractants for the whole microbiome. However, only some bacterial strains specifically recognize and respond to the plant signal. The local immune responses in plant roots are transiently suppressed by PGPR, allowing bacteria to colonize the epidermis. (II) PGPR initiate the formation of biofilm on the root surface. Molecular exchanges start between plant and PGPR to activate processes leading to ISR. PGPR, in addition to stimulating plant defenses, protect the plant from pathogens through direct inhibition mechanisms (antibiotics production or competition for nutrients and space). (III) Early root responses to beneficial microbes are locally expressed in the epidermis and are subsequently communicated to the whole plant. **(b)** MYB72 at the cross between ISR and iron deficiency response in *Arabidopsis thaliana* (adapted from Pieterse *et al.*, 2014; Pieterse *et al.*, 2012). In a soil where iron is poorly available, the roots will extrude protons via ATPase to acidify the soil and promote the solubilization of Fe^{3+}. Fe^{3+} is reduced in Fe^{2+} thanks to the ferric chelate reductase FRO2. Fe^{2+} is then taken from the soil by roots via the iron transporter IRT1. During the root colonization by PGPR, the gene *MYB72* is up-regulated at the same time as the genes *FRO2* and *IRT1*. The transcription factor MYB72 in concert with ethylene-signaling pathway is involved in the production of unknown systemic signal. MYB72 also appears to be involved in the production and/or secretion of chemical compounds by the plant roots that stimulate PGPR activity and hence the ISR. Abbreviations: FRO2, ferric reduction oxidase 2; IRT1, Fe^{2+} transport protein 1.


(a)
Soil
Reprogramming
Resource competition
Antibiosis
Endophytes
Epidermal crack
Root hair
Root tissues
I. Recognition and immune suppression
II. Biofilm formation
III. Cell-cell communication and long-distance signaling
Microbial communities in the bulk soil
Soilborne pathogen
Cellular reprogramming and signaling
Root exudates
Biofilm matrix
Epiphytes
Endophytes
(b)
PGPR
Fe solubility increases
pH decreases
Root chemicals
Fe 2+
Fe 3+
H+
H+ H+
IRT1
FRO2
ATPase
MYB72
ET-signaling pathway
Unknown systemic signal

Using *Arabidopsis* mutant *eir1* (ethylene insensitive root 1), which is insensitive to ET in the roots only, Knoester *et al.* (1999) have shown that ET signaling in the roots is required for the expression of ISR in the leaves and possibly facilitates the generation or the translocation of an unknown systemic signal (**Fig. 14b**). The gene *MYB72* was also identified as one of the genes strongly induced in *A. thaliana* roots in the presence of *Pseudomonas fluorescens* WCS417r, an ISR-inducing bacterium (Segarra *et al.,* 2009). Knockout *myb72* mutants of *A. thaliana* are impaired in their ability to express ISR against different foliar pathogens upon treatment with *P. fluorescens* WCS417r or *P. putida* WCS358r, indicating that this root-specific transcription factor is essential for the onset of ISR (Pieterse *et al.*, 2014). However, the overexpression of *MYB72* in *A. thaliana* does not confer enhanced resistance to foliar pathogens, suggesting that MYB72 acts in concert with one or more other signaling components to initiate ISR (Van der Ent *et al.*, 2008). Moreover, *MYB72* is also strongly expressed in roots under iron-limited conditions, pointing to a link between iron homeostasis and the onset of ISR (Palmer *et al.*, 2013).

The root treatment with inducing-ISR bacteria can prime the expression of genes coding for proteins, such as peroxidase (POD), phenylalanine ammonia-lyase (PAL), lipoxygenase (LOX), polyphenol oxidase (PPO), chitinase and β-1,3-glucanase (PR2). All are involved in plant resistance against invaders (Jain *et al.,* 2016; Oliveira *et al.,* 2016; Battilani *et al.,* 2018). POD expression limits the spreading to the cells of the infection through the establishment of structural barriers such as lignin and suberin deposition or generation

of highly toxic environment to pathogens by massively producing ROS and reactive nitrogen species (Passardi *et al.,* 2015). PAL is a key enzyme in plant defenses which catalyzes the first step of the phenylpropanoid pathway, leading to the production of many metabolites including flavonoids, coumarins, phytoalexins, lignin and also SA (Kim and Hwang, 2014, Zhang and Liu, 2015, Liu *et al.*, 2018). The lignin participates to the reinforcement of plant cell walls (Kurepa *et al.*, 2018). LOX is an enzyme involved in the production of diverse compounds exhibiting signaling functions (*e.g.* JA, traumatin), antimicrobial activity (*e.g.* colneleic acid) or implicated in the development of HR (Dar *et al.,* 2015; Thakur and Udayashankar, 2019). As an example, Ramamoorthy *et al.* (2002) showed a higher expression of genes *PAL* and *LOX* in tomato roots treated with *P. fluorescens* Pf1 and infected with *Fusarium oxysporum* f. sp. *lycopersici* at the foliar level. PPO catalyzes the oxidation of hydroxy phenols present in the plants to their quinone derivatives, which have antimicrobial activity to fight against pathogens (Chunhua *et al.*, 2001). Chitinase and β-1,3-glucanase degrade, respectively chitin and glucan, two major constituents of fungal walls and therefore have a direct inhibitory effect on the growth of fungal pathogens (Li *et al.*, 2019).

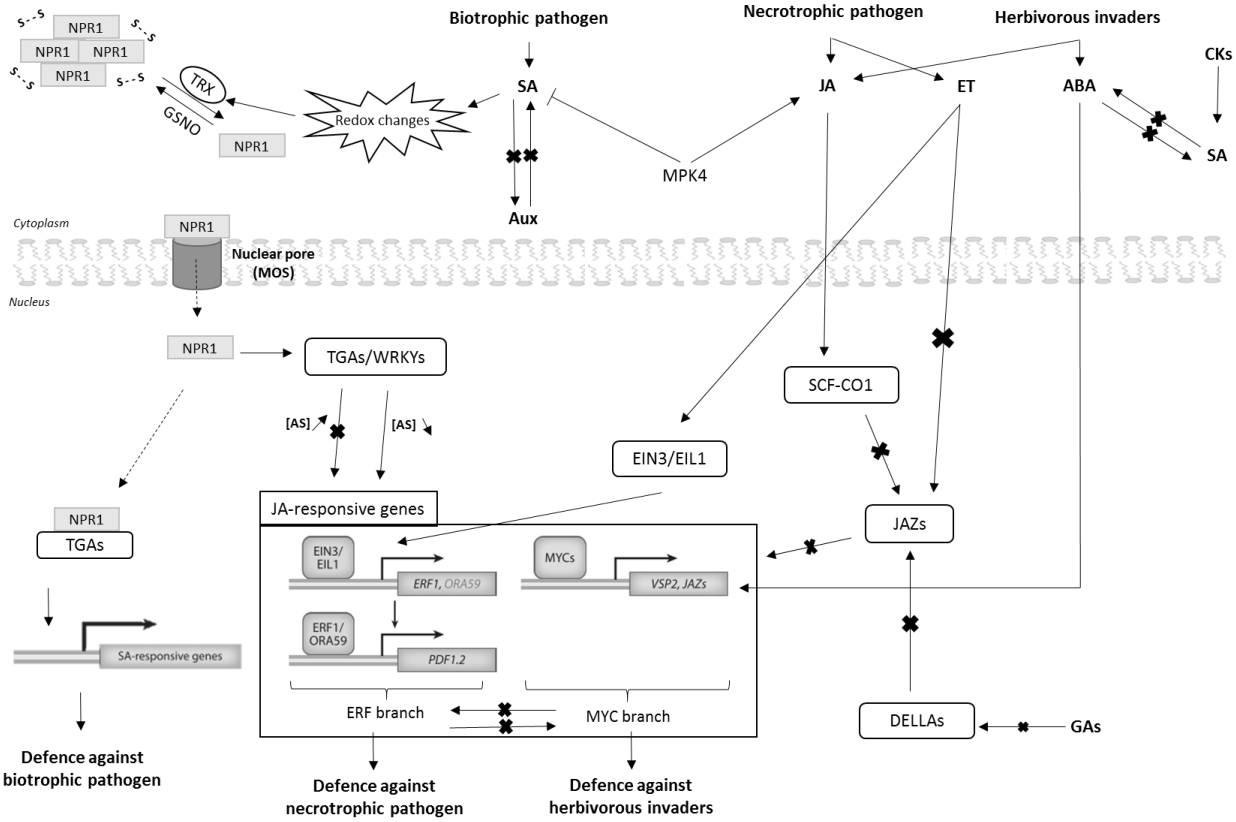
1.2.4 Crosstalk between the different plant defense pathways

Plants are constantly subject to changes in their environment (biotic and abiotic) and must find a balance between growth, reproduction and defense. Many players are involved in this balance, which is finely regulated by the plant. The co-evolution of plants and their pests means that some pests are also able to interfere with these

signaling pathways (Van der Ent and Pieterse, 2012; Crang *et al.*, 2018).

Interactions between SA-signaling and JA-signaling pathways can be either negative, neutral or positive (Kunkel and Brooks, 2002; Mur *et al.*, 2006; Browse, 2009). As an example, low concentrations of JA and SA resulted in synergistic effects on the expression of *PR1* and *PDF1.2* whereas higher concentrations lead to antagonistic effects (Glazebrook, 2005; Mur *et al.*, 2006). The antagonism between SA- and JA-signaling pathways allows plants to prioritize defense against either biotrophic or necrotrophic pathogens (Pieterse *et al.*, 2012; Fu and Dong, 2013). As a reminder, biotrophic pathogens grow thanks to nutrients present in living host cells, while the growth of necrotrophic pathogens is based on the use of nutrients released from dead host cells. Many molecular players are involved in the interaction between SA and JA pathways (**Fig. 15**) (An and Mou, 2011; Pieterse *et al.*, 2012; Fu and Dong, 2013; Pieterse *et al.*, 2014; Broekgaarden *et al.*, 2015; Ahmad *et al.*, 2016).

Fig. 15 (right page): Schematic overview of the crosstalk between plant defenses. JA- and SA-signaling pathways are most of the time antagonists. Necrotrophic pathogens induce JA- and ET-dependent signaling pathways. Herbivorous invaders induce JA- and ABA-dependent signaling pathways. The ET- and ABA-regulated branches of the JA pathway are mutually antagonistic. SA-pathway is positively regulated by gibberellins and cytokinins, while auxins have a negative effect on it. Abbreviations: EIN3, ethylene insensitive 3; EIL1, ethylene insensitive 3-like 1; ERF, ethylene response factor; ORA59, octadenoic-responsive *Arabidopsis* 59; PDF1.2, plant defensin 1.2; VSP2, Vegetative-storage-protein 2; MYCs/WRKYs/TGAs, transcription factors; DELLAs, transcriptional regulators that inhibit the cell proliferation and expansion; SCF-CO1, complex composed notably by the protein coronatine intensive 1 (COI1); JAZ, jasmonate ZIM protein; NPR1, nonexpressor of *PR* genes 1 protein; TRX, thioredoxin; GSNO, S-nitrosoglutathione enzymes; AS, salicylic acid; JA, jasmonic acid; ET, ethylene; ABA, abscisic acid; CKs, cytokinins; GAs, gibberellic acid or gibberellins; Aux, auxins. Solid arrows indicate stimulation; arrows with the black crosses means negative effect; dotted arrows indicate a movement.


NPR1
NPR1
NPR1
NPR1
TRX
GSNO
NPR1
Redox changes
Biotrophic pathogen
SA
Aux
MPK4
Necrotrophic pathogen
JA
ET
Herbivorous invaders
ABA
CKs
SA
Cytoplasm
NPR1
Nuclear pore
(MOS)
Nucleus
NPR1
TGAs/WRKYs
[AS]
[AS]
NPR1
TGAs
SA-responsive genes
Defence against
biotrophic pathogen
JA-responsive genes
EIN3/
EIL1
ERF1, ORA59
MYCs
VSP2, JAZs
ERF1/
ORA59
PDF1.2
ERF branch
MYC branch
Defence against
necrotrophic pathogen
Defence against
herbivorous invaders
EIN3/EIL1
SCF-CO1
JAZs
DELLAs
GAs

Mitogen-activated protein kinases 4 (MPK4) is a negative regulator of SA-pathway and a positive regulator of JA-pathway (Petersen *et al.*, 2000; Meng and Zhang, 2013; Ahmad *et al.*, 2016).

Glutaredoxins and thioredoxins (TRXs) are key enzymes in the regulation of cellular redox and affect the protein activity. The cellular redox buffer glutathione is both influenced by SA- and JA-pathways (Spoel and Loake, 2011). JA decreases the glutathione pool while SA increases both the glutathione pool and the ratio between the reduced and oxidized form. Modulating the redox state of the plant cell seems to be important in the JA/SA crosstalk (Koornneef *et al.*, 2008).

NPR1 is required both for SA- and JA/ET-signaling pathway (Pieterse *et al.*, 2012; Pieterse *et al.*, 2014; Jain *et al.*, 2016). On the one hand, NPR1 is a transcriptional co-activator of the genes induced by SA during SAR (Ding *et al.*, 2018). Its presence is thus required in the nucleus. On the other hand, NPR1 located in the cytosol seems to have a role during the induction of ISR (Spoel *et al.*, 2003). The simultaneous activation of SAR and ISR in *A. thaliana* leads to an additively enhanced defensive capacity (Van Wees *et al.*, 2000). It suggests that the actions of NPR1 in ISR and SAR are not mutually exclusive and do not compete for the same subcellular pool of NPR1. However, the molecular mechanisms involved in the regulation of ISR pathway by NPR1 remain unknown (Pieterse *et al.*, 2014; Jain *et al.*, 2016). Although cytosolic NPR1 seems to be sufficient for SA-mediated suppression of the JA-pathway, nuclear NPR1 regulates several SA-responsive transcriptional factors, such as TGAs, and WRKYs, with a direct or indirect role in suppressing JA-responsive gene expression.

WRKYs transcription factors are part of a large family which is involved in the regulation of SA-dependent genes (Jiang *et al.,* 2017). As an example, WRKY50 and WRKY51 repress the JA-pathway and the use of plant mutants *WRKY50* and *WRKY51* tends to prove that those two transcription factors are essential for SA-mediated suppression of JA signaling (Gao *et al*., 2011). Another WRKY transcription factor, WRKY70, is sufficient but not essential for the suppression of JA-pathway by SA (Li *et al.,* 2004; Li *et al.,* 2006).

TGA transcription factors are major regulators of SA-induced genes (Shearer *et al.,* 2012). TGAs recognize specifically the core motif TGACG in SA-responsive promoters. The promoter of *PDF1.2,* which is a JA- and ET-induced gene, contains a TGACG motif, suggesting that TGAs can interfere with the expression of this gene (Spoel *et al.,* 2003). However, deletion of the TGACG motif from the *PDF1.2* promoter does not affect the JA inducibility nor SA-mediated suppression of the promoter activity (Spoel *et al.,* 2003; Zander *et al.,* 2009). TGAs have probably not a direct role in the SA-JA crosstalk. In the absence of a SA stimulus, TGAs are involved in the activation of JA- and ET-dependent defense genes (Zander *et al.*, 2009).

ET impacts many steps of plant defense pathways and can both positively and negatively affect plant defenses. As an example, in *A. thaliana* ET potentiates SA-responsive *PR1* expression (De Vos *et al.*, 2006). Conversely, the transcription factors EIN3 and EIL1 were implicated in global repression of PAMP-responsive genes in *A. thaliana*, resulting in reduced accumulation of SA (Chen *et al.*, 2009).

When ET and JA are produced at the same time, such as upon infection by necrotrophic pathogens, ET favors the expression of the ERF branch of the JA-pathway, whereas it antagonizes the MYC branch, resulting in prioritization of the immune signaling network associated with resistance to necrotrophs (Pieterse *et al.*, 2012; Fu and Dong, 2013). The repression of the MYC branch renders plant more susceptible to herbivorous invaders (Verhage *et al.*, 2011). Moreover, ET contributes to immunizing plant tissues to future SA-mediated suppression of the JA pathway (Leon-Reyes *et al.*, 2010). When the ET and JA signaling pathways are activated during or after the onset of the SA response, SA is able to strongly repress the transcription of JA-responsive genes. However, when the ET and JA pathways are fully induced prior to SA induction, the antagonistic effect of SA- on the JA-pathway is completely abolished (Broekgaarden *et al.*, 2015).

Abscisic acid (ABA), in addition to being involved in abiotic stress tolerance (drought and salinity stress), is a negative regulator of SA-pathway (Cao *et al.*, 2011; Trivedi *et al.*, 2016). Plant fine-tunes the balance between ABA-mediated abiotic stress tolerance and SA-mediated biotic stress resistance through ABA-SA crosstalk. A bidirectional antagonism between SA and ABA can occur at multiple points of their signaling pathways (Moeder *et al.*, 2010; Robert-Seilaniantz *et al.*, 2011). When ABA is produced at the same time as JA, it favors the "MYC branch" and thus represses the "ERF branch" of JA-signaling pathway (Pieterse *et al.*, 2012).

Cytokinins (CKs) can interact with TGAs, such as TGA3, and thus positively regulate the SA-responsive genes (Choi *et al.*, 2010; Choi *et al.*, 2011).

The role of auxins (Aux) in modulating JA-defenses is less understood. Several microbes produce auxins or manipulate the auxin-signaling pathway to favor their development (Robert-Seilaniantz *et al.,* 2011; Spaepen, 2015). In particular, biotrophic pathogens interfere with the auxin defense mechanisms because auxin-signaling can repress SA levels in the plant (Chen *et al.,* 2007; Robert-Seilaniantz *et al.,* 2011).

Gibberellins (GAs) control plant growth by regulating the degradation of growth-repressing DELLA proteins (An and Mou, 2011; Sun, 2011). The degradation of DELLA proteins was shown to promote susceptibility to necrotrophs and resistance to biotrophs through modulations of JA- and SA-signaling (Navarro *et al.,* 2008). In the absence of GAs, DELLA proteins inhibit the JAZ activity by bonding with it. This reduces the interactions between JAZ and the MYC transcription factors and leads to the activation of "MYC branch" of JA-signaling (Hou *et al.,* 2010). GAs, by degrading DELLA proteins, disrupt the negative regulation of JAZ activity, which results in SA-signaling and in the resistance against biotrophic pathogens.

All the interactions between each player provide a powerful tool to plants to fine-tune their defenses depending on the type of pathogens encountered. As already mentioned, plants and their pests evolve together which means that the pests have learned to take advantage of this crosstalk. For example, El Oirdi *et al.* (2011) have shown that the fungal pathogen *Botrytis cinerea* manipulates the antagonistic effect between SA- and JA-pathway to facilitate its development in *S. lycopersicum*. The fungus releases an exopolysaccharide which promotes SA accumulation. The increase of the SA concentration

leads to the inhibition of the JA-responsive genes through NPR1. The same principle is used by *A. solani* to colonize *S. lycopersicum* (El Rahman *et al.,* 2012).

1.2.5 Plant immunity according to the pathogen lifestyle

The fungal phytopathogens can be broadly divided in two categories, based on their nutrient acquisition strategies (Glazebrook, 2005; Kliebenstein and Rowe, 2007). The biotrophic pathogens, feeding on living plant tissues and the necrotrophic pathogens, feeding on dead plant tissues. Between these two categories, some fungal pathogens, called hemibiotrophs, are characterized by an initial biotrophic phase followed by a secondary necrotrophic phase in which hyphae kill the host tissues (Perfect and Green, 2001; Lo Presti *et al.,* 2015).

Necrotrophs to induce cell necrosis and the leakage of nutrients release diverse phytotoxic compounds and cell wall–degrading enzymes (CWDE), such as cellulases, xylanases and pectinases (Mengiste, 2012; Kubicek *et al.*, 2014). Based on the type of toxins they produce, necrotrophs can be divided into two groups: host specific and non-host specific (Mengiste, 2012; Meena *et al.*, 2017a). The first group secretes host-specific toxins (HSTs) which are typically active only toward plants that serve as hosts for the pathogen that produce them. The second group produces non-host specific toxins (nHSTs) which have relatively mild phytotoxic effect in comparison with HSTs (Wolpert *et al.,* 2002; Laluk and Mengiste, 2010; Meena *et al.,* 2017a). As an example, *Alternaria* species synthesize HSTs, as AAL-toxins, and nHSTs, as alternariol (Logrieco *et al.,* 2009; Meena *et al.,* 2017a,b). Defenses against necrotrophs include constitutive and induced physical and chemical barriers. The

plant cuticle and the cell wall slow the initiation and the spread of infection while also serving as sources of DAMPs that trigger induced defenses (Antico *et al.*, 2012; Wen, 2013). Resistance against broad host-range necrotrophs, as *A. solani*, is quantitative and required many genes for resistance (Kliebenstein *et al.*, 2005). Chemical defenses include compounds constitutively present in plant cells (phytoanticipins) and compounds specifically produced only in response to infection (phytoalexins). PAMPs of necrotrophs include constituents of the fungal cell wall such as chitin, chitosan and endopolygalacturonases which are perceived by plant PRRs and triggered PTI (Pandey *et al.*, 2016). PRRs, such as the oligogalacturonides receptor wall-associated kinase 1 (WAK1) and chitin receptor containing lysine motives have been identified as important players in the immune response against necrotrophic pathogens (Wan *et al.*, 2008; Brutus *et al.*, 2010). *B. cinerea*, a necrotrophic fungus, produces polygalacturonases (PGs), as one of the virulence components recognized by the host plant through two distinct mechanisms. In the first one, PGs directly act as PAMPs which are recognized by a PRR, called Responsiveness to polygalacturonases 1 (RBPG1) (Zhang *et al.*, 2014). The second one, PGs degrade pectin from the plant cell wall to generate oligogalacturonides (OGs) which are recognized by WAK1 (Niture, 2008; Brutus *et al*., 2010). OGs induce typical PTI-responses, such as oxidative burst, cell wall lignification, phytoalexin accumulation, hormone biosynthesis (Denoux *et al.*, 2008; Galletti *et al.*, 2008). Generally, HR favors the spread of the necrotrophic pathogens (Wen, 2013; Pandey *et al.*, 2016). Plants have evolved an alternate

mechanism of resistance mediated by the activation of JA- and ET-dependent signaling (Antico *et al.,* 2012; Pieterse *et al.,* 2014).

Biotrophs, to successfully infect and maintain alive the cells of the host plant, secrete few CWDE, almost no toxins and have developed specific feeding structures called haustoria (Kubicek *et al.,* 2014; Lo Presti *et al.,* 2015). These structures are formed by enzymatic digestion of the host cell wall and the invagination of the host cell membrane. Haustorium draw water and nutrients from the host cells (Wang *et al.,* 2018b). There are two main strategies that plants use to restrict the infection by a biotrophic pathogen: the reinforcement of physical barriers and the HR-mediated resistance (Glazebrook, 2005; Kliebenstein and Rowe, 2007). Plants strengthen their cell walls and membranes to slow spore germination and prevent the formation of the haustorium. The HR deprives biotrophic pathogens of their food source preventing their development (Salguero-Linares and Coll, 2019). In general, gene-for-gene resistance and the activation of SA-dependent signaling and SAR are important forms of resistance against biotrophs (Pieterse *et al.,* 2014). As SA and JA/ET-pathways tend to be antagonistic, JA/ET signaling is expected to have negative effects on resistance to these pathogens. However, in some cases, JA-signaling may also be effective against biotrophs (Thomma *et al.,* 1998; Antico *et al.,* 2012).

1.3 The genus *Bacillus*

As this PhD focuses on the use of one strain of *B. subtilis* (30B-B6), this section describes the genus *Bacillus* and, in particular, its use in biocontrol. The different mechanisms involved in the stimulation of plant growth and plant defenses are discussed.

Members of the genus *Bacillus* are ubiquitous Gram-positive, rod-shaped bacteria (0.3-2.2 to 1.2-7 µm), most of them are motile with peritrich flagella (Wang, *et al.,* 2007; Piggot, 2009; Alina *et al.,* 2015; Graumann, 2017). These bacteria are chemoheterotrophic, aerobic or facultative anaerobic and produce catalase (Slepecky and Hemplhill, 2006, Alcaraz *et al.,* 2010, Alina *et al.,* 2015). *Bacillus* spp. can form resistant endospores in response to environmental stresses (Earl *et al.,* 2008; Graumann, 2017). They are found in diverse environments ranging from sea water to soil (Mandic-Mulec *et al.,* 2015). Many members of the *Bacillus* genus are saprophytes and participate in biogeochemical cycles such as carbon cycle. Members of the *Bacillus* genus produce a vast array of biologically active molecules, such as antimicrobial compounds and enzymes used in industry (Sansinenea, 2019). Taxonomy of the genus *Bacillus* is not always unanimously accepted and is regularly modified (Fritze, 2004; Fan *et al.,* 2017; Caulier *et al.,* 2019). Two groups can be distinguished based on the evolution of molecular, chemotaxonomic and physiological characteristics: the *B. cereus* group (also called *B. cereus sensu lato*) and the *B. subtilis* group (Maughan and Van der Auwera, 2011; Fan *et al.,* 2017). The *B. cereus* group encompasses six species: *B. cereus sensu stricto*, *B. thuringiensis*, *B. anthracis*, *B. mycoides*, *B. pseudomycoides*, and *B. weihenstephanensis*. Two of them can infect humans, *B. cereus sensu stricto* and *B. anthracis*, causing food poisoning with symptoms of vomiting or diarrhea and anthrax, respectively (Maughan and Van der Auwera, 2011). The *B. subtilis* group includes four closely related species: *B. subtilis, B. amyloliquefaciens, B. licheniformis* and *B. pumilus* (Fan *et al.,*

2017; Caulier *et al.*, 2019). Both groups contain species which strongly interact with plants (Fritze, 2004; Cawoy *et al.*, 2011). Some species such as *B. subtilis*, *B. amyloliquefaciens* and, *B. cereus* have beneficial effects on plant development and have been included in the generic term of plant growth-promoting rhizobacteria, PGPR (Goswami *et al.*, 2016).

1.3.1 *Bacillus* spp. in biocontrol: generalities

Besides the use, since the 1930s, of the well-known *B. thuringiensis* as microbial insecticide, other species of *Bacillus* were used to develop biopesticides in agriculture (**Table 2**) (Roh *et al.*, 2007; Borriss, 2011; Pérez-García *et al.*, 2011; Cawoy *et al.*, 2011). Currently in the EU, 17 products based on different *Bacillus* species are registered and six of them are in the process of being registered (EU pesticides database, 2020). *Bacillus* spp. have additional advantages compared to other microorganisms such as the ability to form endospores highly resistant to desiccation and high temperatures which improve the shelf life of commercial products (Cawoy *et al.*, 2011; Hubbard *et al.*, 2014). The genus *Bacillus* is one of the two most common bacterial genera present in the soils, where they effectively compete with other microorganisms to establish themselves in the plant rhizosphere (Beneduzi and Passaglia, 2011; Mandic-Mulec *et al.*, 2015; Al-Ali *et al.*, 2018; Sansinenea, 2019). Their capacity of facultative anaerobic is especially useful in the rhizosphere because the oxygen level is generally fluctuating and can be low (Cawoy *et al.*, 2011). Moreover, they are very motile and produce a lot of antimicrobial metabolites that interfere with the growth of other microorganisms and make them

good root colonizers (Hubbard *et al.*, 2014; Mandic-Mulec *et al.*, 2015).

Table 2: Examples of *Bacillus*-based products used in agriculture (adapted from Cawoy *et al.*, 2011; Fytoweb, 2020).

Trade Name	*Bacillus* strain	Established activity
Amylo-X®[A]	*B. amyloliquefaciens* subsp. *plantarum D747*	Fungicide
BioYield™	*B. amyloliquefaciens* GB99 + *B. subtilis* GB122	ISR, PGPR
Ballad®	*B. pumilus* QST2808	Fungicide, bactericide, ISR, PGPR
Florbac®[A]	*B. thuringiensis* ssp. *aizawai ABTS-1857*	Insecticide
Kodiak™	*B. subtilis* GB03	Fungicide, PGPR
Pomex	*B. subtilis* CMB26	Fungicide
Ecoguard™	*B. licheniformis* SB3086	Fungicide
Rhizo Vital® 42	*B. amyloliquefaciens* FZB42	Fungicide, PGPR
RhizoPlus®	*B. subtilis* FZB24	Fungicide, PGPR
Serenade®	*B. subtilis* QST713	Fungicide, Bactericide
Sonata®	*B. pumilus* QST2808	Fungicide
Subtilex®	*B. subtilis* MBI600	Fungicide
Taegro®	*B. subtilis iFZB24*	Fungicide
Vault®	*B. subtilis* MBI600 + rhizobia strains	PGPR
Votivo®[A]	*B. firmus* I-1582	Nematicide
Yield Shield®	*B. pumilus* GB34	ISR, PGPR

ISR: Induced systemic resistance; PGPR: plant growth-promoting rhizobacteria; [A], products authorized in Belgium.

1.3.2 Mechanisms involved in biocontrol by PGPR strains: focus on *Bacillus* spp.

In biocontrol, members of the genus *Bacillus*, and generally PGPR bacteria, can act directly on plant pathogens or indirectly by stimulating the plant growth and development (van Loon, 2007; Berg, 2009; Olanrewaju *et al.*, 2017; Salomon *et al.*, 2017; Sansinenea, 2019). Direct mechanisms involve secondary metabolites produced by bacteria. Indirect mechanisms include the improvement of plant nutrition and modulation of plant growth (PGPR activities).

1.3.2.1 Indirect mechanisms: stimulation of plant development by PGPR

Enhancement of plant mineral nutrition

Nitrogen is one of the three most important macronutrients essential for the growth of plants. The bioavailable amount of nitrogen in the soil is quite limited because it can easily lose by different processes such as leaching (Premachandra *et al.*, 2016; Olanrewaju *et al.*, 2017). Some non-symbiotic or free-living strains, such as *B. polymyxa*, *B. macerans* and *B. azotofixans,* can reduce, with the nitrogenase enzyme, gaseous nitrogen to ammonia, making it available to plants (Seldin *et al.,* 1983; Seldin *et al.,* 1984; Mandic-Mulec *et al.,* 2015). Phosphorus, the second most important macronutrient for plant growth, is poorly bioavailable in the soil. It is notably found in the form of phytate (an organic form of phosphate) complexed with cations such as Ca^{2+}, Mg^{2+}, Fe^{3+} and Al^{3+} (Borriss, 2011; Pérez-Garcìa *et al.,* 2011; Mandic-Mulec *et al.,* 2015). Several *Bacillus* species, such as *B. amyloliquefaciens,* produce organic acids and extracellular phytases which are phosphatases that degrade phytate in more bioavailable forms of phosphate (Borriss, 2011; Pérez-Garcìa *et al.,* 2011). Some bacterial strains such as *Acidithiobacillus ferrooxidans*, *B. edaphicus*, *Paenibacillus* spp. and *Pseudomonas* spp. are also known as potassium-solubilizing microorganisms by releasing organic acids that degrade potassium-containing rocks, mainly mycas and orthoclase (Saha *et al.,* 2016a). Iron is an important nutrient required in many metabolic pathways (Aznar *et al.,* 2015). Its soluble form (Fe^{2+}) in the soil is limiting for an optimal plant nutrition. Indeed, the most prevalent form of iron in the soil is the insoluble form (Fe^{3+})

associated notably with oxygen or hydroxide. Plants, fungi or bacteria excrete low molecular mass molecules called siderophores dedicated to chelate notably iron (Premachandra *et al.,* 2016). The bacterial siderophores are known to chelate iron with a higher affinity than those produced by fungi or plants (Andrews *et al.,* 2003; Olanrewaju *et al.*, 2017). The reduced chelated iron (Fe^{2+}) then becomes available for plants (Crowley, 2006; Premachandra *et al.,* 2016). Other elements such as zinc or manganese can be solubilized by *Bacillus* spp. (*e.g. B. mycoides, B. cereus*) and more easily assimilated by plants (Das *et al.,* 2011; Sharma *et al.,* 2011).

Modulation of plant development by the production of hormones

Auxins or indole-3-acetic acid (IAA) are essential phytohormones involved in a plethora of aspects of plant development. IAA stimulates notably the primary root elongation or lateral roots, or increases root hair formation depending on its concentration (Schaller *et al.,* 2015, Brumos *et al.,* 2018). In the plant meristems, auxins regulate cellular mechanisms such as cell division, elongation, and differentiation which ultimately influence the architecture of shoots and roots (Schaller *et al.,* 2015, Brumos *et al.,* 2018). Loper and Schroth (1986) have reported than 80% of the bacteria isolated from the rhizosphere synthesize and release auxins. Members of the *B. subtilis* group have the capacity to produce IAA or derivative compounds (Borriss, 2011). IAA released in the rhizosphere by PGPR changes the pools of auxin in the plant with positive or negative effects on plant and root development depending on the concentration of auxins in plant tissues (Olanrewaju *et al.*, 2017). In *A. thaliana*, *B. subtilis* strain GB03

interferes with the homeostasis of auxins and thus has a plant growth-promoting effect (Zhang *et al.,* 2007).

Cytokinins (CKs) are other phytohormones implicated in the regulation of plant development (Schaller *et al.,* 2015). CKs regulate with other hormones, mainly auxins, cell division and differentiation. CKs also control various processes such as the delay of senescence, the control of shoot and root balance, the transduction of nutritional signals and increased crop productivity (Sakakibar, 2006; Kieber and Schaller, 2018). Many PGPR, including *B. subtilis* strains, produce CKs that influence the plant development (Arkhipova *et al.,* 2005; Sakakibar, 2006; Sansinenea, 2019).

Gibberellins (GAs) are phytohormones and constitute a large family of tetracyclic diterpenoid carboxylic acids, most commonly interfering with plant tissue morphology (Hedden and Sponsel, 2015; Binenbaum *et al.,* 2018; Takehara and Ueguchi-Tanaka, 2018). GAs are implicated in various plant metabolic processes such as seed germination, stem elongation, flowering and fruit development (Hedden and Sponsel, 2015; Olanrewaju *et al.*, 2017). GAs influence the root growth and the abundance of root hairs (Binenbaum *et al.*, 2018; Sansinenea, 2019). PGPR which produce GAs have been found notably in *Azospirillum*, *Rhizobia*, *Azobacter* and *Bacillus* genera (Bottini *et al.*, 2004; Olanrewaju *et al.*, 2017).

Ethylene (ET) is the only gaseous phytohormone and hence implicated notably in plant-to-plant communication. ET is a pleiotropic hormone which regulates at the same time both growth and senescence of leaves, flowering and fruit production depending on its concentration in plant tissues. ET is produced mostly in response to

various biotic and/or abiotic stresses such as pathogen or herbivore attacks, extreme temperatures, salinity, starvation and water deficiency/excess (Iqbal *et al.,* 2017; Dubois *et al*., 2018). At moderate concentrations ET inhibits both root and shoot elongation, whereas at high concentrations it promotes senescence and organ abscission (Berg, 2009; Dubois *et al*., 2018). One of the main steps in ET production by plants is the synthesis of its precursor, the 1-aminocyclopropane-1 carboxylic acid (ACC). ACC, synthesized by an ACC synthase (ACS), is then transformed into ET by the ACC oxidase (ACO) (Ravanbakhsh *et al.,* 2018). Many PGPR belonging to *Pseudomonas, Rhizobium* or *Bacillus* genera produce an ACC deaminase enzyme that converts ACC to α–ketobutyrate and ammonia, and then use it as a source of carbon (Premachandra *et al.*, 2016; Olanrewaju *et al.*, 2017; Ravanbakhsh *et al.*, 2018). By modulating ACC levels and thus ET production, PGPR help plants to deal with conditions of stress (van Loon, 2007; Premachandra *et al.*, 2016).

It is also important to note that there are many regulatory loops connecting these different hormones and that changes in the concentrations of these hormones can impact positively or negatively the regulation of the others (Depuydt and Hardtke, 2011, Schaller *et al.*, 2015, Verma *et al.,* 2016). As an example, the tryptophan (a precursor of IAA) excreted by plant roots can be converted by some PGPR into IAA. This neosynthesized IAA is then absorbed by the plant and acts as a promoter of plant growth and stimulate the production of the ACS by the plant (**Fig. 16**). It results in an increased synthesis of ET in plant tissues. Therefore, PGPR stimulate and at the

same time inhibit the plant growth. However, these PGPR also possess an ACC deaminase which limits the concentration of ACC neosynthesized by the plant allowing bacterial IAA to stimulate plant growth (Gamalero and Glick; 2015, Olanrewaju *et al.*, 2017; Ravanbakhsh *et al.*, 2018).

Some phytopathogenic bacteria can also interfere with the hormonal plant regulation, such as *Agrobacterium tumefaciens.* This bacterium induces the transfer of tumorigenic plasmids, containing genes involved in the synthesis of auxin and CK, into the plant genome. It results in the alteration of plant cell metabolism, resulting in cell proliferation (tumor) and the synthesis of nutritive compounds that provide a selective advantage for the bacterium (Escobar and Dandekar, 2003).

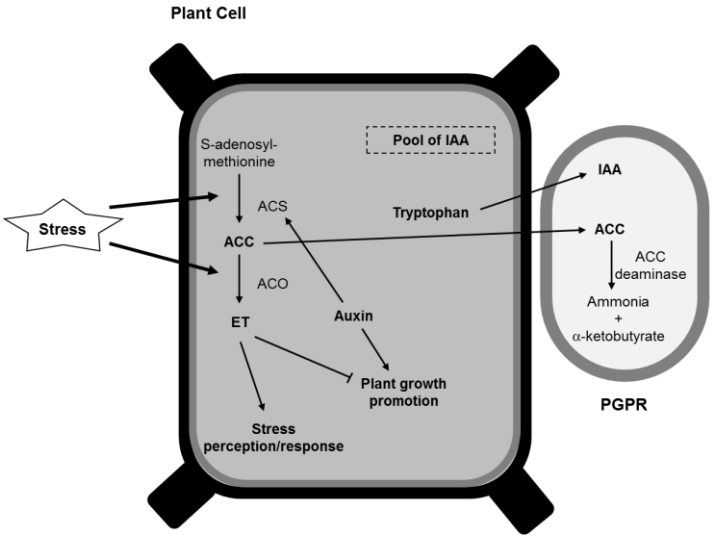


Fig. 16: Schematic representation of the interaction between plant and PGPR possessing an ACC deaminase activity (adapted from Gamalero and Glick, 2015). Abbreviations: ACC, 1-aminocyclopropane-1 carboxylic acid; ACS, ACC synthase; ACO, ACC oxidase; IAA, indole-3-acetic acid; ET, ethylene.

In addition to ET, other volatile organic compounds (VOCs) produced by some PGPR genera such as *Bacillus*, *Pseudomonas*, *Serratia* and

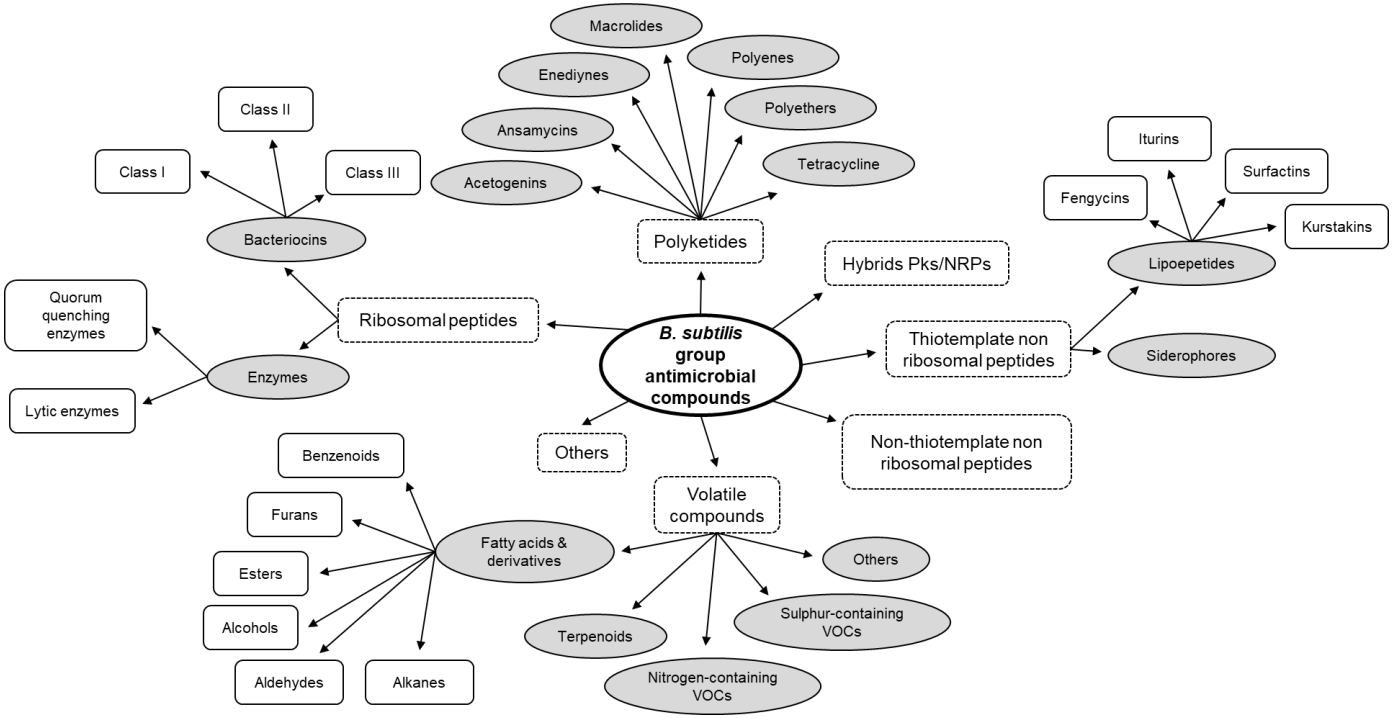
Arthrobacter are involved in the regulation of plant growth and root-system architecture (Ruy *et al.,* 2003; Kanchiswamy *et al.,* 2015; Vejan *et al.,* 2016). *Bacillus* spp. synthesize a wide variety of VOCs including acetoin and 2,3-butanediol, involved in the regulation of plant growth (Choudhary *et al.,* 2008; Vejan *et al.,* 2016; Tahir *et al.,* 2017; Caulier *et al.,* 2019).

1.3.2.2 Direct mechanisms: control of phytopathogens

Production of antimicrobial compounds

PGPRs synthesize a wide diversity of secondary metabolites with antimicrobial activities. These compounds or antibiotics are a heterogeneous group of low molecular weight organic compounds (Raaijmakers *at al.,* 2002; Sansinenea, 2019). About 5 to 10% of the *Bacillus* genome is dedicated to the production of antimicrobial compounds and around 167 antibiotics are produced by *Bacillus* spp. (Earl *et al.,* 2008; Mohkam *et al.,* 2016). They can be classified according to their biosynthetic pathways, biological properties, etc. Caulier and colleagues (2019) reviewed the different antibiotics produced by members of the *B. subtilis* group (**Fig. 17**). In brief, according to their biosynthetic pathways, they can be divided in five different groups: 1) ribosomal peptides, 2) non-ribosomal peptides (NRPs), 3) polyketides (PKs), 4) hybrids between NRPs and PKs, 5) volatile compounds.

Fig. 17 (next page): Antimicrobial molecules classes from the *Bacillus subtilis* group (Caulier *et al.,* 2019). The molecules are divided according to their biosynthetic pathways into five different groups (*i.e.* ribosomal peptides, polyketides, hybrids, non-ribosomal peptides, and volatile compounds). Abbreviations: PKs, Polyketides; NRPs, Non-ribosomal peptides; VOCs, volatile organic compounds.


Macrolides
Polyenes
Enediynes
Polyethers
Ansamycins
Tetracycline
Acetogenins
Class II
Class I
Class III
Bacteriocins
Polyketides
Hybrids Pks/NRPs
Iturins
Surfactins
Fengycins
Kurstakins
Lipoepetides
Quorum quenching enzymes
Ribosomal peptides
B. subtilis group antimicrobial compounds
Thiotemplate non ribosomal peptides
Siderophores
Enzymes
Lytic enzymes
Others
Non-thiotemplate non ribosomal peptides
Benzenoids
Volatile compounds
Furans
Fatty acids & derivatives
Others
Esters
Alcohols
Terpenoids
Sulphur-containing VOCs
Aldehydes
Alkanes
Nitrogen-containing VOCs

Most of the ribosomal peptides are referred to as bacteriocins. They possess a high specificity against closely related bacteria. However, some of them have a wider range of activity (Sumi *et al.,* 2015, Mohkam *et al.,* 2016; Fira *et al.,* 2018; Wang *et al.,* 2018a). Due to their quite specific spectrum, bacteriocins are not the most important compounds to control plant pathogens (Sumi *et al.,* 2015; Fira *et al.,* 2018). Besides bacteriocins, lytic enzymes (*e.g.* lipase, cellulases, glucanases, chitinases and proteases) are another class of molecules ribosomally synthesized with antimicrobial properties (Glick, 2015; Olanrewaju *et al.*, 2017; Shameer and Prasad, 2018; Caulier *et al.*, 2019). Glucanases and chitinases can degrade fungal pathogen cell walls of which chitin and glucan are two major constituents (Chang *et al.*, 2010; Cawoy *et al.,* 2011; Gomaa, 2012). These cell wall degrading enzymes can be involved in parasitism mechanisms set up by some *Bacillus* species (Shafi *et al.,* 2017).

The non-ribosomal peptides, a group of antimicrobial compounds, encompasses mainly lipopeptides and siderophores (Fira *et al.,* 2018; Caulier *et al.,* 2019). Siderophores produced by PGPR can alleviate the proliferation of phytopathogens by binding the Fe^{3+} present in the vicinity of the plant roots. This leads to a lack of iron for the pathogens especially because siderophores produced by the pathogens have a much lower affinity for iron than the PGPR siderophores (Glick, 2015; Zhu and Yang, 2015; Saha *et al.,* 2016b; Olanrewaju *et al.*, 2017). Lipopeptides synthesized by *Bacillus* spp. include the iturin, fengycin, surfactin and kurstakin families. However, *B. subtilis* is not able to produce kurstakin (Béchet *et al.,* 2012; Caulier *et al.,* 2019). Iturins and fengycins have a strong antifungal activity and

iturins have also a partial antibacterial effect. Surfactins exhibit mainly antibacterial activities (Romero *et al.,* 2007; Ongena and Jacques, 2008; Jacques, 2011; Caulier *et al.,* 2019). Their activity is linked to their amphiphilic properties which can destabilize the phospholipid layer of the bacterial cell membrane and thus affect its integrity (Ongena and Jacques, 2008).

So far, only three kinds of polyketides are produced by members of *B. subtilis* group: difficidin, macrolactin and bacillaene (Wang *et al.*, 2018a,c). All these molecules are antibacterial and bacillaene and macrolacin also exhibit antifungal activities (Chen *et al.,* 2006; Caulier *et al.,* 2019).

Like other soil microorganisms, bacteria emit a wide variety of volatile compounds (VCs) both organic and inorganic (Effmert *et al.,* 2012; Audrain *et al.,* 2015). The most common inorganic volatile compounds produced by *Bacillus* spp. are CO_2, CO, H_2, H_2S, HCN, NH_3 and NO (Effmert *et al.,* 2012; Audrain *et al.,* 2015). For example, HCN inhibits the development of aerobic microorganisms by interfering with the cytochrome C oxidases, involved in cellular respiration (Ahmad *et al.,* 2008; Kumar *et al.,* 2015; Paramanandham *et al.,* 2017; Jangir *et al.,* 2018). Depending on their chemical classes, VOCs produced by bacteria can be classified in different categories: fatty acid derivatives (carbohydrates and hydrocarbons), sulfur and nitrogen-containing compounds, terpenoids and metalloid or halogenated containing compounds (Audrain *et al.,* 2015; Caulier *et al*., 2019). According to the database mVOC 2.0, almost three-quarters of the VOCs emitted by *Bacillus* spp. are included in

the following chemical families: fatty acids and derivatives, sulfur- and nitrogen-containing compounds (Lemfack *et al.,* 2018).

Induction of plant defenses by PGPR

As mentioned in the section 1.3.2, PGPR can trigger or stimulate systemic plant defenses, the so-called ISR (Kloepper *et al.*, 2004; Hacquard *et al.,* 2017).

Among the bacteria that elicit ISR, the genera *Pseudomonas* and *Bacillus* are the most studied (Jain *et al.,* 2016). Although many ISR bacterial determinants have been identified so far, only lipopeptides and volatiles from *Bacillus* spp. have been proved to induce ISR in plants (Choudhary and Johri, 2009; De Vleesschauwer and Höfte, 2009). For example, the surfactin and fengycin lipopeptides produced by *B. subtilis* S499 have an ISR protective effect on bean against *Botrytis cinerea* (Ongena *et al.,* 2007). Precise mechanisms are not understood but involved Ca^{2+} flux in cells and are associated with a higher activity of PAL and LOX (Ongena *et al.,* 2007; Jourdan *et al.* 2009). Some volatiles emitted by *Bacillus* spp. such as 3-hydroxy-2-butanone and 2,3-butanediol are also involved in the induction of ISR (Ryu *et al.,* 2004). Ryu *et al.* (2004) showed that *A. thaliana* seedlings exposed to volatiles emitted by *B. subtilis* GB03 and *B. amyloliquefaciens* IN937a are less susceptible to further attacks by the pathogenic bacterial *Pectobacterium carotovorum*. However, the underlying mechanisms are not known (Ryu *et al.,* 2004).

1.4 The hosts

For a pathogen to develop and causes disease, a host plant is needed. The same plant can be affected by several pathogens, and in some cases the host plant - pathogen relationship is very specific. This work focuses mainly on tomato (*Solanum lycopersicum*) and to a less extent on potato (*Solanum tuberosum*). Tomato possesses several characteristics that make it a model plant for research. The following section briefly described these two plant species from the genus *Solanum*.

1.4.1 *Solanum lycopersicum*

Tomato (*S. lycopersicum* L.) is a dicotyledonous species belongs to the *Solanaceae* family. The stem of the tomato is angular, pubescent and thick at the internodes. The alternate leaves are compound, 5 to 7 leaflets with very indented lobes (Naika *et al.,* 2005). The oldest leaves lose their photosynthetic capacity. The leaves are also pubescent and the trichomes contain an essential oil which gives the plant its characteristic smell. The root system is very dense and branched, with a swivel type with a fasciculate tendency (Blancard, 2009). The five-petaled flowers (diameter 2 cm) are yellow, bisexual, pendants and clustered. In general, the plant is self-pollinating, but cross-fertilization can occur. In nature, bees and bumble bees are the main pollinators. Fruits are berries that vary in diameter from 1.5 to 7.5 cm or more. They are usually red, scarlet, or yellow, though green and purple varieties do exist, and they vary in shape from almost spherical to oval and elongate to pear-shaped. Tomato fruit contains 95% of water, 4% of carbohydrates and less than 1% of fat and proteins. It is also an important source of vitamins A and C and of

antioxidant compounds such as lycopene (Adhikari *et al.,* 2017). Each fruit contains at least two cells of small seeds surrounded by jellylike pulp. They are hairy, beige, 3 to 5 mm long and 2 to 4 mm wide.

Tomato is native to the Andes of South America and it was domesticated in Mexico in 1519 and introduced to Europe in 1544 (Blancard, 2009; Naika *et al.,* 2005). Tomato culture is one of the most common horticultural crops and worldwide distributed. The total world production of tomato is 161.7 million tons with a value of 59 billion dollars (Adhikari *et al.,* 2017). In Belgium, 570 ha of tomato are grown under greenhouse mainly in Flanders (FAOSTAT, 2019).

In addition to being the world's second most consumed vegetable after potato, *S. lycopersicum* is a model plant to study numerous physiological phenomena, notably due to the relatively small size of its genome (~900Mb), distributed across 12 pairs of chromosomes (Sato and Tabata, 2016). In November 2003, the tomato genome-sequencing project was launched by the 'International Solanaceae Genome Project' (Mueller *et al.,* 2005). The tomato variety used for the sequencing project was the 'Heinz 1706' cultivar (Mueller *et al.,* 2005; The Tomato Genome Consortium, 2012). The genome of *S. lycopersicum* variety Moneymaker, used in this work, was also sequenced thanks to the reference genome of the *S. lycopersicum* variety Heinz 1706 (Aflitos *et al.,* 2014).

1.4.2 *Solanum tuberosum*

Potato (*S. tuberosum* L.), belongs to the *Solanaceae* family, is a dicotyledonous species. *Solanum tuberosum* is divided into two subspecies: *andigena* and *tuberosum* (Anjum *et al.,* 2018). The *andigena* is a diploid subspecies which is adapted to short day

conditions and is typically grown in Andes. The *tuberosum* is a tetraploid subspecies now cultivated around the world that later adapted to longer day lengths.

The plant has a rosette or semi-rosette characteristics and in the early stages of growth, the stem is erect. As the season progresses, it becomes proliferate and prostate. The potato has two types of stems: aerial stems on which the leaves are arranged and underground stems, the rhizomes, on which the tubers appear. The leaves are alternate and compound, asymmetrically odd pinnate, with 6-9 pairs of leaflets. The root system is fasciculate and highly branched. The five-petaled flowers (diameter 3 cm) are bisexual, pendants and clustered. In general, the plant is self-pollinating, but cross-fertilization can occur (*e.g.* bees and bumble bees). The fruit of the potato is a small berry. The berries have 2 lodges and can contain approximately 200 to 400 seeds. Some cultivated varieties do not flower, others flower but are sterile (Stevenson *et al.,* 2011; Anjum *et al.,* 2018). The tuber is an enlarged part of an underground stem, which serves as a storage organ of carbohydrates. It carries buds and eyes in the axil of small scale-like leaves. Eyes are gathered near the apical end of the tuber, with a small number near the stolon or the basal end. The number and the distribution of eyes are characteristics of the potato variety (Bradshaw and Ramsay, 2009; Bradeen *et al.*, 2011; Stevenson *et al.,* 2011). Potatoes can be grown from the botanical seeds or propagated vegetatively by planting pieces of tubers, which is the main method of propagation (Bradshaw and Ramsay, 2009; Braden *et al.*, 2011). Fresh tuber contains 77% of water, 20% of carbohydrates (of which about 82% is starch), 1.8% of proteins, the rest is made up of fats, vitamins

(mainly C and A) and minerals (Bradshaw and Ramsay, 2009). Potato yields more calories per unit of surface than any cereal (Rubatzky and Yamaguchi 1997).

Solanum tuberosum is originated from the Andean mountains of South America and introduced in Europe in the 16th century (Bradshaw and Ramsay, 2009; Bradeen *et al.*, 2011). The potato crop is the world's fourth most important food crop after wheat, maize and rice with about 370 million tons produced in 2018 (Axel *et al.*, 2012; Statista, 2020). In Belgium, 100,000 hectares are cultivated with potatoes (Eurostat, 2019).

1.5 The pathogens

Tomato and potato crops are susceptible to a wide range of pests including fungi, viruses, viroids, bacteria, phytoplasma, nematodes, mites and phytophagous insects (Blancard, 2009). All stages of the crop are potentially targeted by pests. One of the most damaging fungal diseases on tomato is early blight. The main enemy of potato crop is late blight caused by an oomycete (Caulier *et al.*, 2018).

1.5.1 *Alternaria solani*

The early blight (EB) disease is caused by the fungus *Alternaria solani* (Ellis and Martin) Sorauer. This pathogen is worldwide distributed but it is most prominent in areas with heavy dew, rainfall and high relative humidity (Agrios, 2005; Adhikari *et al.*, 2017). It affects a wide range of plants in particular from the *Solanaceae* family as tomato, potato and eggplant (Agrios 2005; Woudenberg, 2013). First symptoms appear on the lower and senescent leaves in the form of small dark brown to black spots. When the lesions enlarge, they

develop concentric rings, surrounding by chlorotic yellow halo with a target-like appearance (Agrios 2005; Chaerani and Voorrips, 2006). The fungus can cause the full defoliation of the plant (Chaerani and Voorrips, 2006; Bessadat *et al.,* 2017). Weakened plant tissues, either due to stress, senescence, or wounding, are more susceptible to *Alternaria* infection than healthy tissues (Thomma, 2003). In addition to the vegetative system, EB also affects the fruits. In plantations in the U.S., Australia, Israel, UK, Brazil and India, the losses ranging from 35% to 80% (Basu, 1974; Chaerani and Voorrips, 2006; Bessadat *et al.*, 2017).

Alternaria solani belongs to the *Ascomycota* in the class *Dothideomycetes* and order *Pleosporales* (Woudenberg, 2013). However, the taxonomy of *Alternaria*, especially related to EB, is undergoing revision (Adhikari *et al.*, 2017; Ozkilinc *et al.*, 2018). *Alternaria solani* is a necrotrophic pathogen, meaning that it kills host plant cells by releasing toxins (non-host specific) to derive nutrients from dead cells and infect new healthy tissues (Chaerani and Voorrips, 2006; Adhikari *et al.*, 2017; Meena *et al.,* 2017b). One of the earliest identified non-host specific toxins is alternaric acid, discovered by Brian *et al.* (1952). This toxin causes chlorosis and necrosis by altering the integrity of plasma membranes of plant cells (Van Der Waals *et al.*, 2001). However, alternaric acid does not cause phytotoxicity when sprayed alone on tomato leaves, but it enhances the infection process and the development of necrotic symptoms when added to *A. solani* spore suspension (Van Der Waals *et al.*, 2001; Chaerani and Voorrips, 2006; Patel *et al.*, 2011).

At the moment, no sexual phase has ever been reported but there is a high variability among isolates (Chaerani and Voorrips, 2006; Adhikari *et al.,* 2017). Heterokaryosis could explain this high genetic diversity among the genus *Altenaria* (Stall 1957, 1958; Chaerani and Voorrips, 2006).

Alternaria solani overwinters as mycelium or conidia in the soil, plant debris, seed and alternate hosts (**Fig. 18**; **Fig. 19a**). It constitutes a source for the primary infections. This primary inoculum remains effective for 5 to 8 months (Van de Waals *et al.,* 2001; Adhikari *et al.,* 2017). Hyphal cells are darkly pigmented with melanin which increased their tolerance against adverse environmental conditions (Van de Waals *et al.,* 2001). The dispersion of the primary inoculum occurs with wind, raindrops or insects (Van de Waals *et al.,* 2001). Under free moisture or near-saturated humidity, conidia germinate to produce one or more germ tubes at a wide range of temperature (8°C-32°C). Germ tubes form appressoria and penetrate the epidermic cells directly or through wounds or stomata at temperatures between 10°C and 25°C (Agrios, 2005; Chaerani and Voorrips, 2006; Adhikari *et al.,* 2017). Lesions appear 2 or 3 days after infection followed by the sporulation which occurs at temperatures between 5°C and 30°C, with an optimum at 20°C (**Fig. 19b**) (Agrios, 2005; Chaerani and Voorrips, 2006; Adhikari *et al.,* 2017).

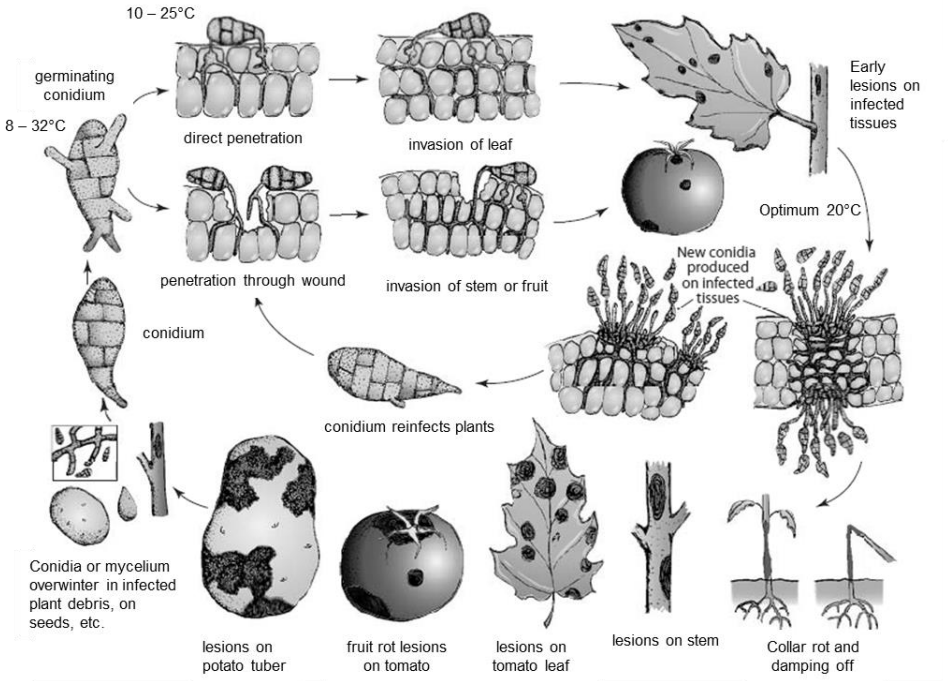


Fig. 18: *Alternaria solani* life cycle (adapted and completed from Agrios, 2005).

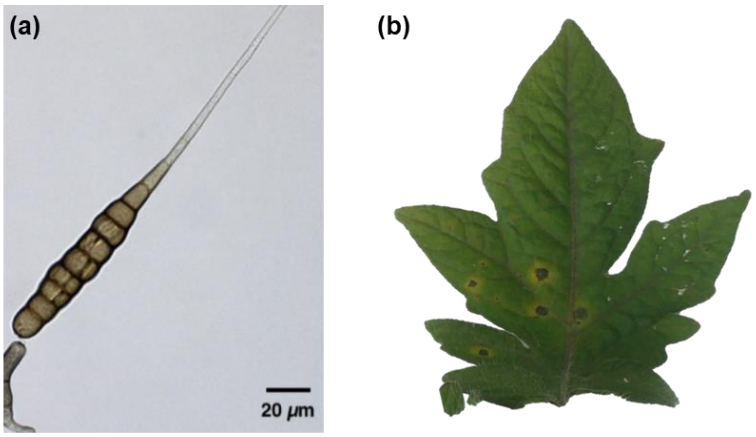


Fig. 19: **(a)** Conidia from *Alternaria solani*. **(b)** Foliar symptoms of *A. solani* on tomato var. Moneymaker. Necrotic lesions are surrounded by chlorotic yellow rings.

Eleven classes of non-host specific toxins (nHSTs) produced by *A. solani* have been identified in culture filtrates so far: zinniol, alternaric acid, tenuazonic acid, alterporiols, altersolanols, alternariol monoethyl ether, alternariol, macrosporin, solanapyrones, zinnolide and altertoxin I (Logrieco *et al.,* 2009; Meena *et al.,* 2017a). nHSTs mainly target basic cellular processes and favor the disease development in plant tissues (Thomma, 2003). As examples, zinniol and alternaric acid disturb membrane permeabilization and tenuazonic acid inhibits the synthesis of proteins (Meronuck *et al.,* 1972; Thuleau *et al.,* 1988, Langsdorf *et al.,* 1991). In addition to nHSTs, *A. solani* produces cell wall-degrading enzymes such as cutinases and pectinases to facilitate the penetration of hyphae into the plant cells and colonize the plant tissues (Kubicek *et al.,* 2014).

El Oirdi *et al.* (2011) showed that *Botrytis cinerea,* a necrotrophic fungus, manipulates the antagonistic effect between SA and JA to promote its development on tomato. This fungus produces an exopolysaccharide which acts as an elicitor of the SA accumulation. In turn, SA suppresses the expression of two JA-dependent genes, *PI-1* and *PI-II*, through NPR1, thereby allowing the fungus to increase the infection in tomato. El Rahman *et al.* (2012) provided evidence that *B. cinerea* and *A. solani*, also use the SA-signaling transcription factor TGA1.a to promote their development in tomato. As for *B. cinerea,* the disease development of *A. solani* on tomato is dependent on NPR1 and is compromised by the expression of *PI-1* and *PI-II*. They suggest that necrotrophs use a common strategy by manipulating a plant signaling pathway to develop their disease.

The main measures to control the early blight disease are: the cultural practices, the use of resistant/tolerant varieties and the application of fungicide treatments. Cultural practices include maintenance of a healthy field and plant vigor, sanitation, removing infected plant tissues, volunteer weeds from the vicinity of the field, crop rotation with non-host plants and reducing the leaf wetness (Agrios, 2005; Adhikari *et al.*, 2017). However, cultural practices alone are not fully sufficient for the control of EB (Van de Waals *et al.*, 2001; Adhikari *et al.*, 2017). The cultivated varieties of tomato are not highly resistant to EB (Adhikari *et al.*, 2017). However, resistant genotypes have been identified within related wild species of tomato such as *Solanum pimpinellifolium* (Chaerani and Voorrips, 2006; Akhtar *et al.*, 2019). Several types of fungicide active substances are used for the control of EB in Belgium (**Table 3**) (Fytoweb, 2020). In addition to problems with the development of resistant strains, due to the use of mono-site fungicides, foliar treatments should also be applied to the lower leaves since they are the most susceptible to infections (Van de Waals *et al.*, 2001; Agrios, 2005; Chaerani and Voorrips, 2006; Adhikari *et al.*, 2017). Moreover, fungicide treatments are not economically feasible in all countries of the world and may not be effective under environmental conditions favorable for the propagation of the disease (Adhikari *et al.*, 2017).

Table 3: Active substances or biocontrol agents approved in Belgium to control *Alternaria solani* (Frac, 2020; Fytoweb, 2020). Abbreviations: CAA, carboxylic acid amides; DMI, demethylation inhibitors; SDHI, succinate dehydrogenase inhibitors; QoI, quinone outside inhibitors; QoIS, quinone outside inhibitors stigmatellin binding type.

Active substance	Group name	Mode of action
Dimetomorph	CAA-fungicide	Inhibition of cellulose synthase
Mandipropamid		
Difenoconazole	DMI-fungicide	Inhibition of C14-demethylase; sterol synthesis
Fluxapyroxad	SDHI-fungicide	Inhibition of succinate dehydrogenase (complex CII); respiration
Azoxystrobin	QoI-fungicide	Inhibition of cytochrome bc1 at QoI site; respiration
Pyraclostrobin		
Ametoctradin	QoIS-fungicide	Inhibition of cytochrome bc1 (complex CIII) at QoI site, stigmatellin binding sub-site; respiration
Fluazinam	-	Uncoupler of oxidative phosphorylation; respiration
Copper hydroxide	Inorganic fungicide	Multi-site activity; respiration and cell energy production
Copper hydroxychloride		
Bacillus amyloliquefaciens FZB24	Microbial	Multiple effects; direct and indirect antagonism

The use of biological control agents has also been documented in several research papers as an alternative method for the management of EB. In addition to the use of *B. amyloliquefaciens* FZB24, Chowdappa *et al.* (2013) observed that the application *B. subtilis* OTPB1 and *Trichoderma harzianum* OTPB3 in the tomato growing substrate increase the systemic resistance in the plant by inducing growth hormones and defense-related enzymes such as peroxidase, polyphenol oxidase and superoxide dismutase. Foliar applications of biocontrol agents such as *Paenibacillus lentimorbus*, *Bacillus* spp. isolated from solanaceous phylloplane and *Pseudomonas flurorescens* limit the disease development on tomato leaves (Khan *et al.*, 2012;

Koley *et al.,* 2015; Pane and Zaccardelli, 2015). Some botanical extracts, such as zimmu leaf extract, possess an activity against *A. solani in vitro* and on tomato (Latha *et al.,* 2009). However, bioproducts are generally less effective than chemical fungicides (Adhikari *et al.,* 2017; Sreennivasulu *et al.,* 2019).

1.5.2 *Phytophthora infestans*

The late blight disease, caused by the oomycete *Phytophthora infestans* Mont. (de Bary) is a major threat to potato crop (Agrios, 2005; Cooke *et al.,* 2011). This oomycete mainly infects members of the genus *Solanum* such as potato, tomato, eggplant and pepper (Erwin and Ribeiro, 1996). The disease is known to be at the origin of the Great Irish Potato Famine in the 1840s (Agrios, 2005; Haas *et al.,* 2009). *Phytophthora infestans* is a hemibiotrohpic plant pathogen and heterothallic with two known mating types, A1 and A2 (Agrios, 2005; Fry, 2008). Hemibiotrophic are pathogens that switch from a biotrophic stage (deriving their nutriments from living host tissues) to a necrotrophic stage (Perfect and Green, 2001; Mendgen and Hahn, 2002). There are differences in the duration of both biotrophic and necrotrophic growth, depending on the interaction between the host and the isolate of *P. infestans* (Zuluaga *et al.,* 2016). The disease can infect all the organs of plants (Agrios, 2005; Blancard, 2009). The first symptoms appear as water-soaked spots, usually on the lower leaves (**Fig. 20**). Shortly after, a white and downy mycelium grows in the lesions on the undersides of the leaves (Agrios, 2005). Lesions quickly spread to all leaves and stems of the plant. In the EU, losses (costs of control and damages) caused by *P. infestans* are estimated to more than 1 billion euros (Haverkort *et al.,* 2008).

Fig. 20: Foliar symtpoms of *Phytophthora infestans* on potato cv. Challenger.

Growth temperatures of *P. infestans* are between 3°C and 25°C with an optimum around 20°C (Blancard, 2009). Temperatures between 18°C and 22°C with a relative humidity close to 100% are the most favorable for sporulation (Agrios, 2005; Blancard, 2009). The pathogen cycle comprises two phases: a sexual and an asexual (**Fig. 21**) (Agrios, 2005; Fry, 2008; Nowicki *et al.,* 2012). The asexual stage is mostly responsible for epidemics (Agrios, 2005). During this stage, sporangia, produced on sporangiophores, are spread out by the wind or raindrops. Sporangia germinate either via a germ tube at temperature between 20°C-25°C, or by releasing biflagellated zoospores at lower temperatures, between 10°C and 15°C (Agrios, 2005; Whisson *et al.,* 2016). Germ tubes from sporangia or zoospores penetrate and infect plant tissues and a few days later new sporangiophores are produced on the mycelium and the cycle starts again (Agrios, 2005; Fry, 2008; Whisson *et al.,* 2016). The entire cycle can occur in three to four days when the climatic conditions are optimal, especially temperature and humidity (Agrios, 2005; Whisson *et al.,* 2016). The sexual stage occurs when both mating types are present and fuse to produce an oospore which germinates into a

sporangium (Fry, 2008). The oospores are survival structures (up to 5 years in the soil) and a source of genetic diversity due to sexual recombination (Agrios, 2005).

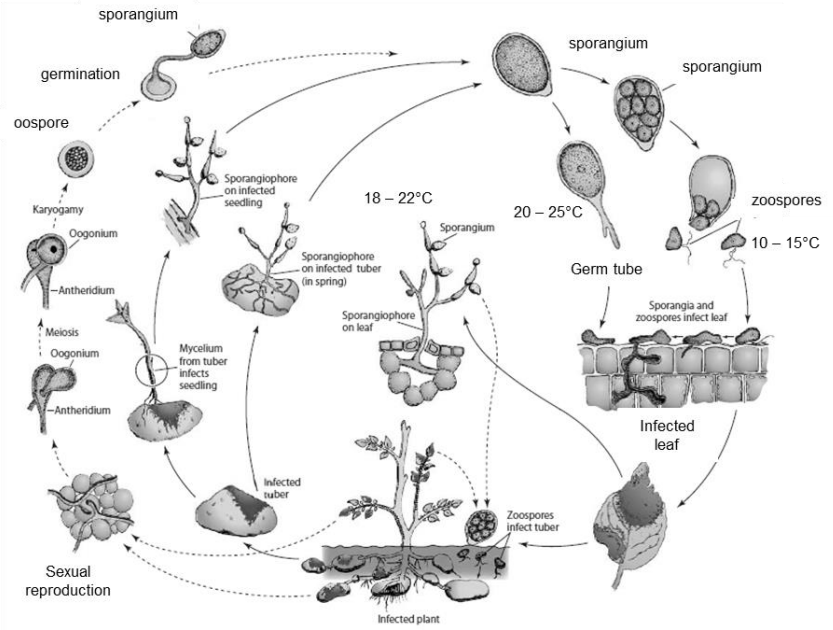


Fig. 21: *Phytophthora infestans* life cycle (adapted and completed from Agrios, 2005).

During the biotrophic stage, *P. infestans* sequentially produces appressoria, primary and secondary hyphae and, finally, haustoria (Zuluaga *et al.*, 2016; Whisson *et al.*, 2016). The following necrotrophic phase, characterized by hyphal ramification and water soaking, correspond to the development of necrosis of the plant tissue (Grenville-Briggs *et al.*, 2005). Appressorium is a terminal swelling at the tips of germ tubes that emerge from spores on the leaf surface (Mendgen *et al.*, 1996). Haustorium is a specialized structure which facilitates the absorption of water and nutrients from the host cells (Lo Presti *et al.*, 2015). This feeding structure is separated from the host cytoplasm through the extrahaustorial membrane (EHM), a newly synthesized plant-derived membrane (**Fig. 22**) (Whisson *et al.*, 2016).

The haustorial interface is demarcated on one side by the EHM and on the other by the pathogen membrane and cell wall that surround the haustorium. These are separated by the extrahaustorial matrix (EHMX) (Whisson *et al.,* 2016; Bozkurt and Kamoun, 2020). *P. infestans* secretes a wide variety of effector proteins, both apoplastic and cytoplasmic, that modulate the plant immunity and enable parasitic infection (Wang *et al.,* 2017; Yin *et al.,* 2017). Within apoplastic effectors secreted by *P. infestans*, elicitin infestin-1 (INF1) causes an HR and cell death, and is highly expressed in later infection stages of potato (Kamoun *et al.,* 1997). In the same way, the Nep-1-like protein PiNPP1.1 acts synergistically with INF1 to induce necrosis late in the infection of tomato (Kanneganti *et al.,* 2006). Among cytoplasmic effectors from *P. infestans*, there is the RXLR family, containing the conserved Arg-any amino acid-Leu-Arg (RXLR) peptide motif that is required for these proteins to be delivered into plant cells (Bozkurt *et al.,* 2012; Anderson *et al.,* 2015; Wang *et al.,* 2019). Around 500 candidates RXLR effector genes from *Phytophthora* species have been predicted using bioinformatic tools (Wang *et al.,* 2019). As an example, the effector RXLR AVR3a, from *P. infestans*, is recognized by the potato resistance protein R3a (Armstrong *et al.,* 2005). AVR3a can suppress the cell death induced by INF1, a PAMP (Bos *et al.,* 2006; Whissom *et al.,* 2016). Another RXLX effector, suppressor of necrosis (SNE1) suppresses necrosis caused by either PiNPP1.1 (Kelley *et al.,* 2010). SNE1, mainly secreted during the biotrophic stage of the tomato – *P. infestans* interaction, was hypothesized to promote biotrophy by suppressing necrosis. However, how the RXLX effectors enter plant cells remains

a mystery. The RXLR domain is dispensable for effector activities inside the host cells and mediates host translocation (Bozkurt *et al.*, 2012; Whissom *et al.*, 2016). The translocation of effectors through the haustorial interface could possibly occur by endocytosis, fusion of extracellular vesicles loaded with effectors, or through active transport facilitated by a pathogen-encoded translocon (Bozkurt and Kamoun, 2020). The RXLR motif undergoes proteolytic cleavage inside the parasite, with mature effectors lacking the motif (Wawra *et al.*, 2017). For most of the RXLR effectors, their biological activities and the underlying molecular mechanisms are unknown (Wang *et al.*, 2019).

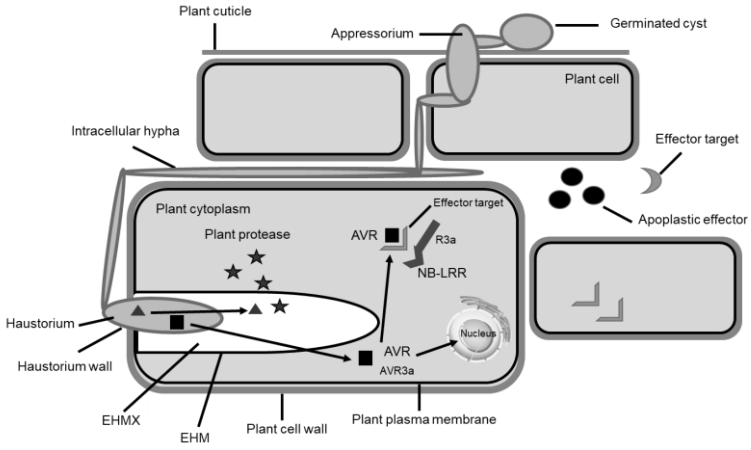


Fig. 22: The plant - *Phytophthora infestans* haurorium interface (adapted from Birch *et al.*, 2006 and Haldar *et al.*, 2006). The potential sites of action of *P. infestans* effector proteins, including AVR proteins, are presented. The plant defense associated proteases (stars) are the target of extracellular protease inhibitor (EPI) proteins (triangles). Cytoplasmic effectors RXLR-motif-containing proteins (squares), such as AVR3a, are secreted inside host cells and which host proteins they target (effector targets) to manipulate host defenses, potentially enabling recognition by NB-LRR proteins, such as Ra3, in the cytoplasm. *Phytophthora infestans* also secretes apoplastic effector (circles) which target apoplastic plant proteins.

The late blight disease can only be managed by a combination of protection methods such as good cultural practices and appropriate use of chemicals (Agrios, 2005; Blancard, 2009). The first step is to

reduce the primary sources of inoculum such as infected plants on dumps or infected volunteer potatoes (Fry 2008; Cooke *et al.*, 2011). The use of less sensitive varieties and disease-free plants are crucial, as well as following the recommendations of forecasting models to apply fungicides at the right time (Agrios, 2005; Fry 2008; Liu *et al.*, 2017). Chemical fungicides used to control late blight encompasses mainly two groups, protectant and systemic fungicides, which are absorbed and distributed through the plant (Agrios, 2005). In most cases fungicides are most effective when applied before the infection occurs. Nevertheless, some fungicides have some curative effect if the plants are treated after the pathogen infection (Ivic, 2010). For example, mandipropamid and dimetomorph applied 24 hours after infection of potato leaflets with *P. infestans* significantly reduced the sporulation of the oomycete. However, no curative effect was observed when these fungicides were applied 48 hours after the infection (Cohen and Gisi, 2007). In Europe, when conditions are favorable to the development of *P. infestans*, the farmers sprayed the crop up to 25 times during the growing season (Hansen *et al.*, 2009). Several types of fungicide active substances are used to control the late blight disease in Belgium (**Table 4**) (Fytoweb, 2020). Decision support systems (DSS) have been developed to make prediction of the disease dynamic based on weather conditions, crop information, pesticide properties and pathogen characteristics (Cooke *et al.*, 2011; Small *et al.*, 2015). DSS integrate these factors to deliver a message to the users. In Europe, different DSS were evaluated in field trials such as PLANT-Plus (in Netherland), Gunts-Divoux/Mileos (France),

Gunts-Divoux/Vigimap (Belgium) (Cooke *et al.*, 2011; CARAH, 2020).

Table 4: Active substances or biocontrol agents approved in Belgium to control *Phytophthora infestans* (Frac, 2020; Fytoweb, 2020). Abbreviations: CAA, carboxylic acid amides; DMI, demethylation inhibitors; OSBI, oxysterol binding protein homologue inhibition; QoI, quinone outside inhibitors; QiI, quinone inside inhibitors; QoIS, quinone outside inhibitors stigmatellin binding type; SDHI, succinate dehydrogenase inhibitors; PA, phenyl amide.

Active substance	Group name	Mode of action
Dimetomorph	CAA-fungicide	Inhibition of cellulose synthase
Mandipropamid		
Valifenalate		
Benthiavalicarb		
Difenoconazole	DMI-fungicide	Inhibition of C14-demethylase; sterol synthesis
Oxathiapiprolin	OSBI-fungicide	Interfere with lipid homeostasis and lipid transfer/storage
Mancozeb	-	Multi-site activity
Zoxamide	Benzamides fungicide	Disruption of β-tubulin assembly in mitosis
Chlorothalonil	Chloronitriles fungicide	Multi-site contact activity
Pyraclostrobin	QoI-fungicide	Inhibition of cytochrome bc1 (ubiquinol oxidase) at Qo site (complex III); respiration
Famoxadone		
Amisulbrom	QiI-fungicide	Inhibition of cytochrome bc1 (ubiquinone reductase) at Qi site (complex III); respiration
Cyazofamid		
Ametoctradin	QoIS-fungicide	Inhibition of cytochrome bc1 (complex CIII) at QoI site, stigmatellin binding sub-site; respiration
Fluazinam	-	Uncoupler of oxidative phosphorylation; respiration
Benalaxyl-M	PA-fungicide	Inhibition of RNA polymerase I; nucleic acids metabolism
Metalaxyl-M		
Copper hydroxide	Inorganic fungicide	Multi-site activity; respiration and cell energy production
Copper oxychloride		
Cymoxanil	Cyanoactamide-oxime fungicide	Unknown
Propamocarb	Carbamate fungicide	Affect cell membrane permeability and lipid synthesis
Bacillus amyloliquefaciens FZB24	Microbial	Multiple effects; direct and indirect antagonism

Research questions

The main objectives of this PhD thesis are i) to evaluate the potential of *B. subtilis* 30B-B6 to control two major foliar pathogens of *Solanaceae*: *P. infestans* on potato (*S. tuberosum*) and *A. solani* on tomato (*S. lycopersicum*), and ii) to improve knowledge on the interactions between *B. subtilis* 30B-B6 and *Solanaceae* by elucidating the modes of action of the bacterium, in particular, the molecular pathways involved in the induced systemic resistance against *A. solani* in tomato. Experiments were conducted in order to address the following questions:

1. Does *B. subtilis* 30B-B6 reduce the late blight disease in field conditions?

2. Is *B. subtilis* 30B-B6 able to reduce the infection on tomato caused by the pathogen *A. solani*?

3. Does *B. subtilis* 30B-B6 stimulate plant defenses and what are the molecular pathways involved?

4. Does *B. subtilis* 30B-B6 promote tomato plant growth?

As this PhD thesis was initiated in the framework of the WACOBI project, we first investigate the efficacy of potential Belgian bacterial strains, including 30B-B6, to manage the late blight disease on potato caused by *Phytophthora infestans*. Those strains have been previously isolated by Caulier *et al.* (2018). Seven bacterial strains from *Bacillus* and *Pseudomonas* genera were evaluated in field trials (2016 and 2017) to control late blight on potato crop. The bacterial cultures from *Bacillus* (*B. amyloliquefaciens* 17A-B3, *B. subtilis* 30B-B6, *B. mycoides* 15A-B2, *B. pumilus* 30B-B2) and *Pseudomonas* (*P. brenneri* 43R-P1, *P. protegens* 44R-P8, *P. putida* 42R-P6) were foliar sprayed once a week during all the growing season, following

the Belgian warning prediction system. Their efficacy was compared to fungicide treatments (used in organic or conventional agriculture). The disease severity was measured during the growing season.

On the other hand, during the WACOBI project, we also showed that the foliar application of a culture of 30B-B6 on potato leaves significantly protected these leaves against *A. solani* under controlled conditions (unpublished results). We therefore decided to decipher the effects of *B. subtilis* strain 30B-B6 on the growth and defenses of *Solanaceae* against the necrotrophic fungus *A. solani* on the model plant *S. lycopersicum*. Bioassays were performed to investigate the potential of 30B-B6 as plant growth-promoting rhizobacterium (PGPR) and its capacities to manage *A. solani* on tomato. The ability of the strain 30B-B6 to enhance the protection of tomato against *A. solani* was evaluated on tomato plants under controlled conditions in greenhouses. Several timings and types of application were tested in order to appraise the effectiveness of the bacterium and the persistence of its activity as well as to hypothesize its modes of action. The bacterial culture was applied on the leaves and/or on the roots of tomato. Based on our results, we hypothesize that 30B-B6 induces a systemic plant defense resistance against *A. solani*.

To understand the molecular pathways (SA-/JA-pathway) involved in the tomato resistance induced by 30B-B6, two different approaches were used: 1) a study of the dynamic of gene expression using quantitative RT-PCR and 2) the quantification of the phytohormone, salicylic acid (SA), in tomato leaves. The capacity of the bacterial culture to influence the expression of some genes (*LOX*, *PR1*, *PR2b*, *PI-1* and *NPR1*) markers of the plant defenses was evaluated at

different times, both in leaves infected or not with *A. solani*. Endogenous SA was also quantified in infected and non-infected tomato leaves collected 1 and 72 hours *post* pathogen inoculation. Indeed, SA and its volatile form (methyl salicylate) are known to be involved in the SAR signaling pathway (Pieterse *et al.,* 2014).

Another question is: which molecules produced by *B. subtilis* 30B-B6 are potentially involved in the induction of systemic resistance in tomato plant infected by *A. solani*? Many bacterial determinants of JA-pathway have been identified and studied (Kloepper *et al.,* 2004; Meziane *et al.,* 2005; Pieterse *et al.,* 2014; Salomon *et al.,* 2017; Caulier *et al.,* 2019; Köhl *et al.,* 2019). Among them, only lipopeptides (LPs) and volatiles from *Bacillus* spp. have been identified to induce JA-pathway (Choudhary and Johri, 2009; De Vleesschauwer and Höfte, 2009). Siderophores produced by *Pseudomonas* spp. have been found to elicit systemic resistance in plants but this could never be demonstrated for siderophores produced by *Bacillus* spp (Audenaert *et al.,* 2002; De Vleesschauwer *et al.,* 2008). Bioassays were performed to study the implication of LPs and siderophores produced by 30B-B6 in the induction of systemic resistance of tomato against *A. solani*. The conditions of culture to favor the production of LPs and siderophores by 30B-B6 were first identified. LPs and siderophores were partially purified by solid-phase extraction. The LPs and siderophores contents of the partially purified extracts were determined by HPLC analysis. Bioassays to evaluate the capacity of those extracts to induce systemic resistance against *A. solani* were conducted on tomato plants under controlled

conditions. The dynamic of the expression of some genes involved in plant defenses was also studied by quantitative RT-PCR.

Chapter 2.

Antagonistic activities of Belgian bacteria against potato late blight in two field trials

The beginning of this PhD thesis was part of the WACOBI project which aimed to identify Belgian bacterial strains for the biocontrol of solanaceous diseases, mainly *P. infestans* and *A. solani*. In this context, we performed two field trials over two growing seasons to evaluate the ability of the strain 30B-B6 and six other bacterial strains (from *Bacillus* and *Pseudomonas* genera) to control late blight on potato crop. Their efficacy was compared to chemical fungicide treatments.

Antagonistic activities of Belgian bacteria against potato late blight in two field trials

Contributions in this chapter

Study conception: Anne Legrève, Jacques Mahillon and Claude Bragard
Experimental procedure and monitoring of field trials: Gil Colau
Harvesting: Gil Colau, Simon Caulier and Florent Licciardi
Data analysis: Gil Colau
Writing chapter: Gil Colau
Manuscript reviewing: Anne Legrève and Claude Bragard

The results from the first field trial (2016) were published by Simon Caulier and colleagues (2018).

Acknowledgments

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Abstract

The use of beneficial bacteria belonging to the genera *Bacillus* and *Pseudomonas* appears as a complementary alternative to the use of chemical pesticides in crop protection. Their activity against plant diseases is mainly related to the biosynthesis of numerous bioactive compounds involved in direct antagonism mechanisms against the pathogens but also in stimulating the plant growth and development. This study aims to investigate the potential of Belgian bacterial strains to manage the late blight disease on potato caused by *Phytophthora infestans*. Over two seasons (2016 and 2017), seven strains of *Bacillus* and *Pseudomonas* were tested in field conditions. The bacterial cultures were foliar sprayed once a week during all the growing season and symptoms of *P. infestans* were monitored at least twice a week. At the end of the 2016 trial, despite the high disease pressure, the strain of *B. subtilis* 30B-B6 led to a significant reduction of the late blight severity (protection index around 22%). During 21 days after the disease outbreak in the field, a reduction in the slope of the disease progression was observed after each application of the strain 30B-B6, despite rainy events. During the season 2017, the climatic conditions delayed the disease outbreak in the field and the protection conferred by the strain 30B-B6 lasted until about a week before the end of the trial. At the end of the season 2017, treatments of plants with the strains of *B. pumilus* 30B-B2 and *P. putida* 42R-P6 significantly reduced the incidence of the disease, with protection indexes of 23 and 16%, respectively. These results demonstrated the potential of some bacterial biocontrol agents in reducing disease

pressure in fields and the interest to combine such type of strategy in an integrated pest management.

2.1 Introduction

Potato late blight disease is one of the most devastating diseases and a major issue for potato industry (Agrios, 2005; Cooke *et al.,* 2011). In particular, temperatures around 20°C and a high humidity level are favorable to the development of *Phytophthora infestans* and fields can be completely destroyed in less than a week (Blancard, 2009; Fry, 2008). The disease is mainly managed by regular applications of preventive foliar treatments with chemicals and by practicing good agricultural methods (Agrios, 2005; Blancard, 2009). As an example, in Belgium, an average of 14 tons of active substances per year of plant protection products, mainly fungicides, were applied on potato crop between 2004 and 2010 (Carrola *et al.,* 2012). Decision support systems (DSS), such as Vigimap and Mileos®, help farmers in the management of fungicidal treatments. DSS can integrate characteristics of the pathogen (*e.g.* lifecycle, resistance to fungicides), weather data, plant varieties and fungicide characteristics and efficacy (Small *et al.,* 2015).

With a view to valorizing the microbial potential of the Belgian soils for the biocontrol of pathogens on *Solanum tuberosum*, a collection of almost 3,000 bacterial strains was created during the WACOBI project (Caulier *et al.,* 2018). *In vitro* screening for direct antagonism properties was performed on the collection to identify the best candidates for biocontrol of solanaceous diseases. The most promising strains were evaluated on potato plants for their ability to control late blight under controlled conditions. Bacterial cultures were sprayed on

potato leaves six hours before the foliar inoculation of *P. infestans*. The results showed that three strains of *Bacillus*, 30B-B6, 15A-B2, 17A-B3 and two strains of *Pseudomonas*, 44R-P8 and 43R-P1 significantly reduced the disease severity with a protection index of up to 75%. The strains 30B-B2 of *B. pumilus* and 42-RP6 of *P. putida* have permitted to obtain a protection index of up to 50% (Caulier *et al.*, 2018). Based on these promising results and on the *in vitro* characterization of these strains, a field trial was set up in 2016 to assess the efficacy of four bacterial strains against potato late blight. Two strains of *Bacillus* spp. (17A-B3, 30B-B6) and two strains of *Pseudomonas* spp. (43R-P1, 44R-P8) were evaluated to control late blight in field conditions. The results of this trial were published by Caulier *et al.* (2018). In 2017, a second field trial was conducted with 3 strains of *Bacillus* (30B-B6, 30B-B2, 15A-B2) and one strain of *Pseudomonas* (42R-P6). The choice of strains was based on the results of the trial in 2016 (the strain 30B-B6 significantly reduced the disease) and on tests carried out *in vitro* and under controlled conditions. In addition, *B. mycoides* strain 15A-B2 has competitive properties due to its mode of growth. The objective of this chapter is to compare and discuss the results obtained in 2016 and 2017.

2.2 Materials and methods

2.2.1 Microorganisms and culture conditions

Seven bacterial strains, *Bacillus amyloliquefaciens* 17A-B3 (MF062580), *B. subtilis* 30B-B6 (MF062631), *B. mycoides* 15A-B2 (MF062604), *B. pumilus* 30B-B2 (MF062620), *Pseudomonas brenneri* 43R-P1 (MF062633), *P. protegens* 44R-P8 (MF062637), *P. putida* 42R-P6 (MF062638) were used in the assays. They are

stored at -80°C in tubes containing 70:30 vol vol^{-1} lysogeny broth (LB):glycerol (LB medium: yeast extract, 10 g l^{-1}; NaCl, 10 g l^{-1}; tryptone, 5 g l^{-1}). The bacterial strains were first grown in solid medium (LB agar, 17 g l^{-1}; 30°C in the darkness) overnight and then in LB for 48 hours at 30°C with constant agitation at 120 rpm. Ten liters of bacterial culture were produced per strain per week.

2.2.2 Plant material and field preparation

Potato tubers of Bintje and Challenger varieties certified free-disease from Condiplant® (Gembloux, Belgium) were used. The two different fields of ten acres, located in Louvain-la-Neuve (Belgium), have been fertilized with a mixture of potassium, phosphorus and nitrogen (NPK: 12-9-22, 1,000 kg ha^{-1}) and ammonia (NH_4: 27%, 300 kg ha^{-1}). Herbicide treatment (Artist®: 2 kg ha^{-1}, Linuron®: 1 l ha^{-1}, Challenge® 2 l ha^{-1}; water 250 l ha^{-1}) has been carried out before the planting of tubers and subsequently weeding has been done mechanically. The fields used in 2016 and 2017 are located in different places (about 500 m apart) and there has been no potato crop on these fields for at least 3 years.

2.2.3 Foliar applications of bacterial cultures and fungicides on potato crop

The field trials were conducted between April 20 (planting) and the end of September (harvesting) for both years. Seven treatments (4 plots/treatment, 48 plants/plot, 15 m^2/plot) were evaluated in a complete randomized block trial: (1) plants treated with *B. amyloliquefaciens* 17A-B3 (in 2016) or *B. pumilus* 30B-B2 (in 2017), (2) plants treated with *B. subtilis* 30B-B6, (3) plants treated with *P. brenneri* 43R-P1 (in 2016) or *B. mycoides* 15A-B2 (in 2017),

(4) plants treated with *P. protegens* 44R-P8 (in 2016) or *P. putida* 42R-P6 (in 2017), (5) plants treated with a conventional fungicide (Revus®, 250 g l^{-1}, 300 l ha^{-1}), (6) plants treated with fungicide authorized in organic farming (Cuprex®, 300 l ha^{-1}) and (7) untreated plants. The variety used was Challenger which is a moderate resistant variety to *P. infestans* infections. Interlines of potato plants Bintje variety (very sensitive to *P. infestans*) have been used to favor infection in the field. Bacterial cultures were foliar sprayed (2.5 l/plot) once a week and fungicides were applied following the Belgian warning prediction system (CARAH, PCA).

2.2.4 Assessment of late blight severity

The disease severity was measured three times a week during the growing season on eight marked plants per plot (for Challenger variety) and 16 marked plants per interline of Bintje. The percentage of the leaf surface displaying symptoms was visually evaluated using a quantitative score (from 0 to 100%; 100% means that the leaf is completely necrotic). At the end of the experiment, the area under the disease progress curve (AUDPC) was calculated (Campbell and Madden, 1990) to compute a normalized protection index (PI) for each treatment (as described by Caulier *et al.*, 2018).

2.2.5 Evaluation of tuber yield and tuber sizes

Potato tubers were harvested on September 22nd in 2016 and on September 20th in 2017, and the total yield (t ha^{-1}) for each plot was recorded. The tubers were divided into 5 classes according to their size (class I, < 28mm; class II, 29 – 34 mm; class III, 35 - 45 mm; class IV, 46 – 55 mm; class V, > 55 mm).

2.2.6 Recording of the meteorological parameters

A weather station (iMetos from Pessl Instruments) was installed in the center of the field for both trials. The data for precipitations (mm), temperatures (°C) and relative humidity (%) were recorded every hour.

2.2.7 Statistical analysis

Data from the assays were statistically treated using the analysis of variance (ANOVA) with the open source software® R (R Core Team, 2020). The Tukey HSD multiple comparison test was used for comparison of the mean values at a significance level of $P < 0.05$.

2.3 Results: field trials against potato late blight

Year 2016

Climate conditions were favorable to potato late blight disease during throughout the growing season (total precipitation for the months of June, July and August, 202.4 mm) (**Fig. 23a**). In June, rainy events (total precipitation for the month of June, 95.6 mm) combined with temperatures around 20°C and high humidity correspond to the first appearance of late blight symptoms within the first 10 days of the symptoms monitoring. The area of primary infection is located in two close plots, one with untreated plants and the other treated with *Pseudomonas* strain 43R-P1 (**Fig. 23b, c**). On average, the months of July (total precipitations, 51.6 mm) and August (total precipitations, 55.2 mm) were drier than June but sufficiently rainy to allow the development of *P. infestans* throughout the season.

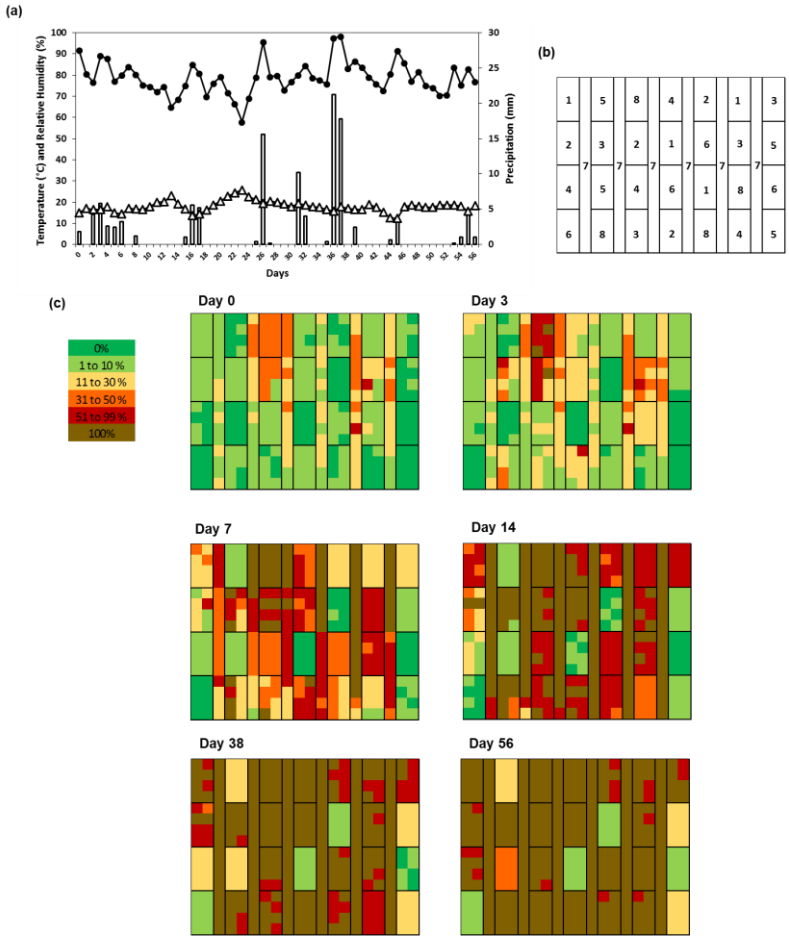

Fig. 23: Meteorological conditions, experimental layout and late blight severity in the field assay located at Louvain-la-Neuve in 2016. **(a)** Evolution of the temperature (Δ), relative humidity (●) and precipitations ([]) during the growing season. Day 0 (June 27th) corresponds to the first day of symptom scoring in the field. The hourly data were recorded by a weather station (iMetos) installed in the center of the field. **(b)** Schematic representation of the field plots. The field is divided into 28 rectangular blocks (5 x 3 m), distributed into 7 lines. Bintjes line spacing is there to favor the development of late blight disease. Numbers 1 to 8 correspond to the different treatments applied: 1, *Pseudomonas protegens* 44R-P8; 2, *P. brenneri* 43R-P1; 3, *Bacillus amyloliquefaciens* 17A-B3; 4, *B. subtilis* 30B-B6; 5, Cuprex®; 6, Revus®; 7, Bintjes untreated; 8, Challenger untreated. **(c)** Representation of the dispersion of the disease over time (from day 0 to day 56). Each plant is represented by a square whose color corresponds to the average percentage of infected leaf area from 0 to 100%. Each block contains 8 plants and each line with Bintjes contains 16 plants marked to monitor the disease development.

Ten days after the beginning of the symptom monitoring, the disease severity reached 90% for potato plants Bintje variety, that were planted in the interlines of the field trial. Untreated plants of Challenger variety reached this level of disease severity after 21 days of scoring. The disease progressed rapidly in the field but significant differences of disease severity between treatments appeared. After ten days, treatments with strains 30B-B6 and 44R-P8 provided a significant level of protection compared to untreated Challenger plants, respectively of 54.46% ± 5.63 and 31.40% ± 3.05 ($F = 45.03$; $df = 6, 217$; $P < 0.001$; Tukey test, $P < 0.05$) (**Fig. 24a**). However, after 21 days, the only bacterial strain that significantly reduced the disease severity was the strain 30B-B6 with a protection index of 38.75% ± 5.95 ($F = 113.7$; $df = 6, 217$; $P < 0.001$; Tukey test, $P < 0.05$) and this percentage fall to 21.84% ± 4.53 at the end of the trial ($F = 329.2$; $df = 6, 217$; $P < 0.001$; Tukey test, $P < 0.05$) (day 56). The two fungicidal treatments, Revus® and Cuprex®, provided the best plant protection throughout all the trial (97.63% ± 0.28 and 82.95% ± 0.80, respectively) (**Fig. 24a**).

The bacterial cultures were foliar sprayed on days 2, 10, 16, 23, 30, 37, 44 and 51 of the trial. During the first 21 days, a reduction in the slope of the disease progression was observed after each application of the strain 30B-B6, despite rainy events (**Fig. 24b**). With the other bacterial treatments, the reduction was less pronounced or absent. After the day 21, due to the high level of infection, no decrease of the slope of the progression was recorded after applications of the bacterial cultures.

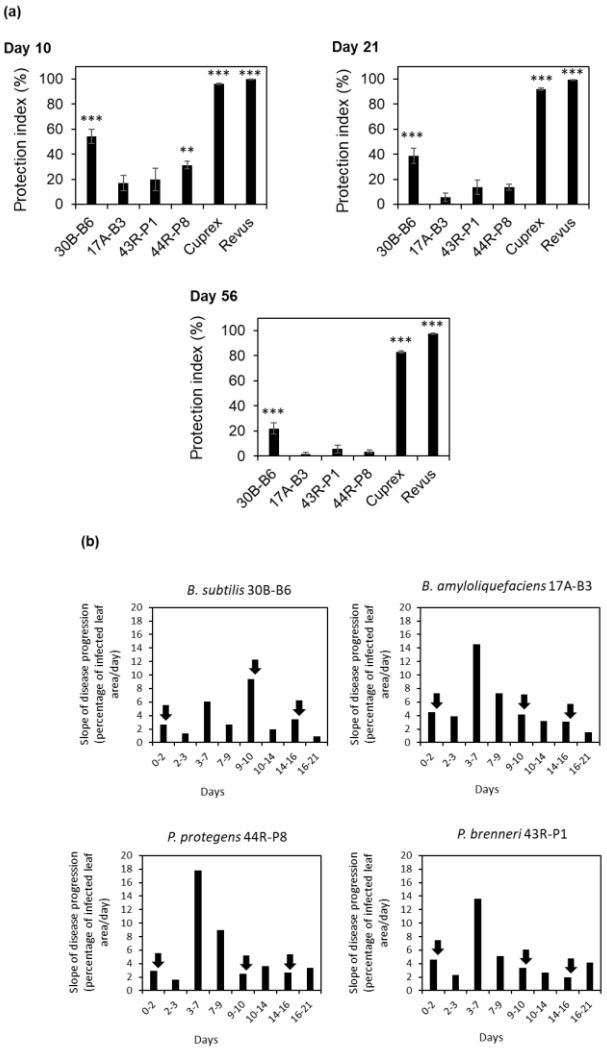

Fig. 24: **(a)** Effect of six treatments against potato late blight in field. Protection index calculated after 10, 21 and 56 days for each treatment. Means ± SE (bars) of four replicates with at least 32 biological replicates per treatment are given. Day 0 (June 27th) corresponds to the first day of symptom scoring in the field. Treatments applied: *Bacillus subtilis* 30B-B6, *B. amyloliquefaciens* 17A-B3, *Pseudomonas brenneri* 43R-P1, *P. protegens* 44R-P8, fungicides Cuprex® and Revus®. Means with stars are significantly different from the control, Challenger plants untreated (***p*-value < 0.01; ****p*-value < 0.001). **(b)** Evolution of the slope of the disease progression calculated between each day of measurement until the day 21. The black arrows indicate the time of foliar applications of the bacterial treatments on days 2, 10 and 16.

At the end of the trial, a total of 1,054 kg of tubers were harvested. There was no significant difference in the yield (min: 9.67 t ha^{-1}; max: 14.69 t ha^{-1}) between bacterial treatments even if plants treated with the strain 30B-B6 produced a little more than the others ($F = 35.48$; $df = 6, 21$; $P < 0.001$; Tukey test, $P < 0.05$) (**Fig. 25**). Plots with plants treated with Revus® or Cuprex® fungicides resulted in a yield of 60 tons per hectare with about 70% of tubers harvested from plots treated with fungicides were class V (>55mm). For plants treated with the bacterial strains, 30% of the tubers harvested were class III (36-45 mm) as for untreated control plants.

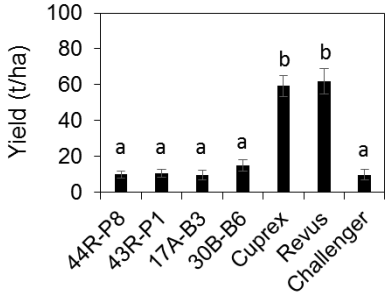

Fig. 25: Tuber yield measured on September 22, for plants treated with bacterial cultures (*Pseudomonas protegens* 44R-P8, *P. brenneri* 43R-P1, *Bacillus amyloliquefaciens* 17A-B3, *B. subtilis* 30B-B6) or fungicides (Cuprex®, Revus®) and untreated (Challenger). Means ± SEM (bars) of four replicates per treatment are given. Means with different letters are significantly different (Tukey test, $P < 0.05$).

Year 2017

Climate conditions were quite favorable to the disease early in the season (total precipitation for the month of June, 55.6 mm) (**Fig. 26a**). Symptoms first appeared on the Bintjes lines (day 19) before spreading to the whole field (day 28) with percentages of infected leaf area ranging from 1 to 10% (**Fig. 26b, c**). Between the 42nd and 49th days, significant rainfall events associated with high humidity and temperatures, favorable to the development of *P. infestans*, caused a

high increase of the disease development in the field. In August 2017 the total of the precipitations (86.8 mm) was higher than in June (55.6 mm) and July (74.4 mm).

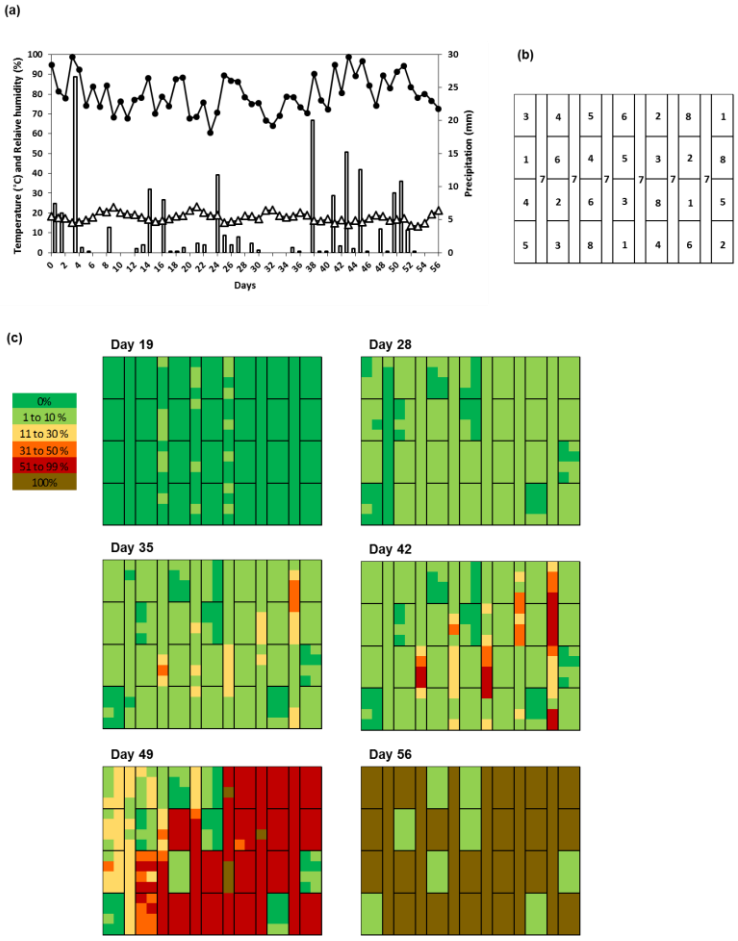

Fig. 26: Meteorological conditions, experimental layout and late blight severity in the field assay located at Louvain-la-Neuve in 2017. **(a)** Evolution of the temperature (Δ), relative humidity (●) and precipitations ([]) during the growing season. Day 0 (June 28th) corresponds to the first day of symptom scoring in the field. The hourly data were recorded by a weather station (iMetos) installed in the center of the field. **(b)** Schematic representation of the field plots. The field is divided into 28 rectangular blocks (5 x 3 m), distributed into 7 lines. Bintjes line spacing is there to favor the development of late blight disease. Numbers 1 to 8 correspond to the different treatments applied: 1, *Bacillus mycoides* 15A-B2; 2, *B. subtilis* 30B-B6; 3, *Pseudomonas putida* 42R-P6; 4, *B. pumilus* 30B-B2; 5, Cuprex®; 6, Revus®; 7, Bintjes untreated; 8, Challenger untreated. **(c)** Representation of the dispersion of the disease over time (from day 0 to day 56). Each plant is represented by a square whose color corresponds to the average percentage of infected leaf area from 0 to 100%. Each block contains 8 plants and each line with Bintjes contains 16 plants marked to monitor the disease development.

Plants untreated from Bintje and Challenger varieties, and plants treated with bacterial cultures reached 90% of infection on day 54 of the trial. After 42 days, treatments with strains 30B-B6, 30B-B2 and 42R-P6 provided a significant level of protection, compared to untreated Challenger plants, respectively of 34.22% ± 5.70, 37.50% ± 6.01 and 24.07% ± 6.81 ($F = 44.59$; $df = 6, 217$; $P < 0.001$; Tukey test, $P < 0.05$) (**Fig. 27a**). However, the significant protective effect conferred by the strain 30B-B6 was reduced and disappeared after 49 days ($F = 82.72$; $df = 6, 217$; $P < 0.001$; Tukey test, $P < 0.05$). At the end of the trial (day 56), a significant reduction of the disease severity was observed for the strains 30B-B2 and 42R-P6 with a protection index of 23.35% ± 3.80 and 16.12% ± 3.72, respectively ($F = 274.9$; $df = 6, 217$; $P < 0.001$; Tukey test, $P < 0.05$). The two fungicidal treatments, Revus® and Cuprex®, provided the best plant protection throughout the season (**Fig. 27a**). The two fungicides achieved an average protection index of around 98% at the end of the growing season.

The applications of the bacterial cultures have been performed in days 0, 7, 14, 21, 28, 35, 42, and 49 of the trial. At the critical time for the spread of the disease (between days 39 and 49 approximately), little or no effect of the sprays on the slope of the disease progression was observed with the exception of day 49 for strains 30B-B6 and 15A-B2 (**Fig. 27b**). After that time, no effect of the bacterial treatments on the slope of the disease progression was observed.

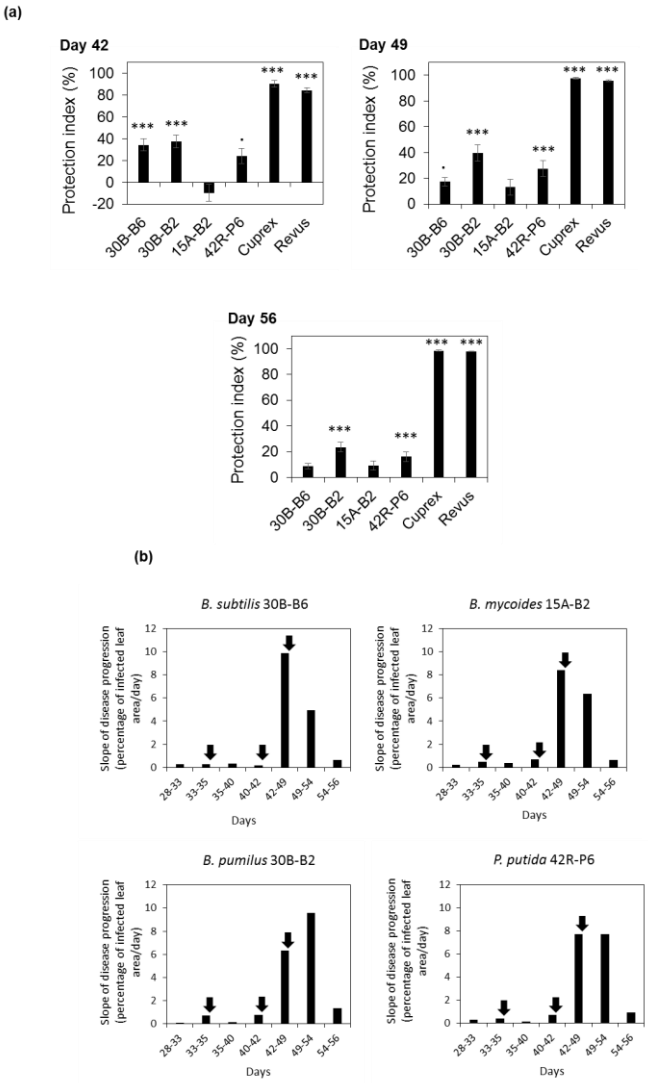

Fig. 27: **(a)** Effect of six treatments against potato late blight in field. Protection index calculated after 42, 49 and 56 days for each treatment. Means ± SE (bars) of four replicates with at least 32 biological replicates per treatment are given. Day 0 corresponds to the first day of symptom scoring in the field. Treatments applied: *Bacillus subtilis* 30B-B6, *B. pumilus* 30B-B2, *B. mycoides* 15A-B2, *Pseudomonas putida* 42R-P6, fungicides Cuprex® and Revus®. Means with stars or a dot are significantly different from the control, Challenger plants untreated (· *p*-value <0.5; ****p*-value < 0.001). **(b)** Evolution of the slope of the disease progression calculated between each day of measurement. The black arrows indicate the time of foliar applications of the bacterial treatments on days 35, 42 and 49.

At the end of the trial, a total of 3,148 kg of tubers were harvested and there was no significant difference between bacterial or fungicide treatments in comparison with Challenger untreated plants ($F = 3.93$; $df = 6, 21$; $P = 0.00865$; Tukey test, $P < 0.05$) (**Fig. 28**). The lowest yield (not statistically significant) observed was in plots treated with strains 30B-B2 and 42R-P6. Regardless of the treatment applied, at least 80% of the potato tubers belonged to class V (>55mm).

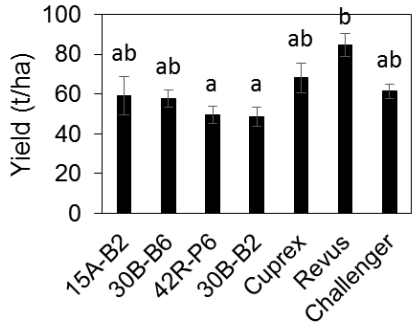

Fig. 28: Tuber yield measured on September 20, for plants treated with bacterial cultures (*Bacillus mycoides* 15A-B2, *Pseudomonas brenneri* 43R-P1, *B. amyloliquefaciens* 17A-B3, *B. subtilis* 30B-B6) or fungicides (Cuprex®, Revus®) and untreated (Challenger). Means ± SEM (bars) of four replicates per treatment are given. Means with different letters are significantly different (Tukey test, $P < 0.05$).

2.4 Discussion

Two field trials were conducted in 2016 and 2017 in Louvain-la-Neuve (Belgium) to evaluate the ability of bacterial strains from Belgian agroecosystems to control the late blight disease on potato crop, in foliar applications. These strains were previously isolated and characterized in 2012 and published by Caulier and colleagues (2018). The strain 30B-B6 of *Bacillus subtilis* (in 2016), and the strains 30B-B2 of *B. pumilus* and 42R-P6 of *Pseudomonas putida* (in 2017) have permitted a significant reduction of the late blight severity on potato plants at the end of the growing season (day

56). Moreover, in both trials, the strain 30B-B6 provided a significant protection of potato foliage against *P. infestans* until at least day 49 of the trial. In the field trials, as bacterial cultures were applied on the foliage, their protective effects were probably linked to direct antagonism against the pathogen. Indeed, characterization studies showed that the tested bacterial strains display antagonistic properties (Caulier *et al.*, 2018). *Bacillus subtilis* 30B-B6 encodes for several antimicrobial compounds, such as glucanase, lipopeptides and bacilysin. This strain also exhibits cellulolytic and proteolytic activities. Glucanase is a glycoside hydrolase that degrades glucan, a polysaccharide notably found in oomycete cell wall (Zevenhuizen and Bartnicki-Garcia, 1969; Adam, 2004). The walls of oomycetes are also made of cellulose, which can be targeted by cellulases of 30B-B6 (Bartnicki-Garcia, 1966; Grenville-Briggs *et al.*, 2008). The mode of action of lipopeptides is the formation of pores in the plasma membrane that interferes with transmembrane ion flows and results in cell death (Bender *et al.*, 1999; Mnif and Ghribi, 2015). Bacilysin is known to exhibit an antibacterial and antifungal properties by interfering with the biosynthesis of several structural macromolecules including peptidoglycans, lipopolysaccharides, glycoproteins and chitin (Milewski, 2002; Wojciechowski *et al.*, 2005; Wang *et al.*, 2018c). Bacilysin is also suspected of having an antagonistic activity against oomycetes (Chung *et al.*, 2008; Caulier, 2019). The activity of *B. pumilus* 30B-B2 in the 2017 trial is probably also explained by this later compound since the bacilysin gene among was identified by PCR by Caulier *et al.* (2018). For *Pseudomonas putida* 42R-P6, we cannot explain the antagonistic activity. Caulier and colleagues (2018) tested

the presence of several genes related to the production of antimicrobial compounds such as phenazin, viscosin, putisolvin, 2,4-diacetylphloroglucinol by PCR assays but none of those genes were identified for *P. putida* 42R-P6. Kruijt *et al.* (2009) reported a zooqporicidal activity of *P. putida* 267 against *Phytophthora capsici* due to a cyclic lipopeptide, identified as a putisolvin-like compound. Such type of compound could participate to the activity of *P. putida* 42R-P6, but we did not yet investigate this point.

The two trials were conducted during the same period of the year, planting took place on April 20, monitoring of the symptoms was carried out until the end of August (around August 22) and tubers were harvested around September 20. For both years, the average climatic conditions (humidity and temperature) were favorable to the development of *P. infestans* as early as June. However, the main difference between the two seasons is the timing of the onset of symptoms of *P. infestans*. In 2016, first symptoms appeared early in the season (end of June) and they evolved steadily until the end. While in 2017, symptoms became visible later in the season (end of July) and their development was favored by heavy rains at the end of the season. This difference can be explained partially by a lower late blight pressure in 2017. The timing of the onset and the intensity of the disease explains the great difference in tuber yields at the end of the two growing seasons. Indeed, we recorded a lower average tuber yield in 2016 (35.73 t ha^{-1}) than in 2017 (65.77 t ha^{-1}).

Although, the variety used was Challenger, a semi resistant to *P. infestans*, the protection obtained with the bacterial strains 30B-B2, 42R-P6 and 30B-B6 are encouraging insofar as the bacterial cultures

have been applied without any formulation. Several factors can improve the performance of the biocontrol agents. Formulation of the products, by the use of adjuvants such as stickers and surfactants, can maximize the contact between the leaf surface and the bacteria and their by-products (Wang and Liu, 2007; Zhao *et al.*, 2017a). The product formulation is also a key element to improve the efficacy, the shelf life, the storage and the delivery of the biocontrol agent (Glare *et al.*, 2012). The culture medium used to grow the bacteria was LB, quite a rich but basic medium. Culture conditions such as media composition, incubation time and temperature, agitation rate, oxygen supply influence the growth characteristics of the bacteria and in particular the antimicrobial compounds they secrete (Gupta *et al.*, 2000; Akpa *et al.*, 2001; Kishore and Pande, 2007; Bartal *et al.*, 2018). Optimizing the culture conditions would make it possible to specifically induce or increase the production of antimicrobial compounds inhibiting the growth of *P. infestans* (Tran, *et al.*, 2007). The adaptation of the timing of bacterial treatments according to climatic conditions and disease pressure could also improve the efficacy of the biocontrol. In the present study, the bacterial cultures were sprayed once a week without taking into account the weather conditions. Rainy events sometimes occurred the day after spraying in 2016 and 2017 which caused the product to run off. Moreover, other parameters such as the aggressiveness of the late blight strains, foliage density, plant age, leaf age and leaf position may condition the effectiveness of the biocontrol agent and the ability of *P. infestans* to infect plants (Shattock, 2002; Visker *et al.*, 2003).

Even if the use of bacterial cultures is not sufficient to fully replace chemical products to control late blight, they can be part of an integrated pest management strategy. Especially in the field trial in 2016, the sprays of *B. subtilis* 30B-B6 in the beginning of the season delayed the progression of the disease. The partial protection conferred by the bacterial strains could be combined with the application of copper. Products based on copper are widely used to control fungal and bacterial diseases, especially in organic farming, due to its preventive wide-spectrum activity (Dagostin *et al.*, 2011; Bangemann *et al.*, 2014; Gopi *et al.*, 2020). However, due to its persistence and toxicity for the environment and humans, the use of copper is restricted in Europe (Dagostin *et al.*, 2011). An alternative could be to combine the bacterial applications with lower doses of copper. La Torre *et al.* (2019) showed that spraying of low copper dosage products, such as MenoRame®, has a preventive activity against late blight and contributes to the reduction of copper released in the soil. Research should be carried out to evaluate the compatibility of the bacteria and copper treatments. However, this would require an entire reformatting of the phytotechnical itinerary, and also considering other parameters such as the choice of the potato varieties, the crop rotations and the fertilization.

Our results show that although it is not always easy to transfer the antagonistic properties of active microorganism strains *in vitro* and under controlled conditions, it is possible to select active strains under field conditions (Glare *et al.*, 2012; Alaux *et al.*, 2018; Coninck *et al.*, 2020). In particular, we showed that *B. subtilis* strain 30B-B6

provided a significant protection of potato against *P. infestans* until at least mid-August during the growing seasons 2016 and 2017.

Chapter 3.

Bacillus subtilis 30B-B6 induces systemic resistance against the fungus *Alternaria solani* in *Solanum lycopersicum*

In the previous chapter we showed that *Bacillus subtilis* 30B-B6 can control a hemibiotrophic pathogen in potatoes in field conditions. Caulier *et al.* (2018) also observed that the strain 30B-B6 exhibits valuable properties for the biocontrol of other *Solanaceae* pathogens. This chapter investigates the potential of *B. subtilis* 30B-B6 to control a necrotrophic pathogen, *Alternaria solani*, on tomato plants. This work also gives a first insight into the molecular pathways involved in the induction of systemic resistance by 30B-B6 in tomato against *A. solani*.

Bacillus subtilis 30B-B6 induces systemic resistance against the fungus *Alternaria solani* in *Solanum lycopersicum*

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Contributions in this chapter

Study conception: GC and AL
Experimental procedure: GC
Greenhouse experiments, harvesting plant material, salicylic extraction: GC
Dosage of salicylic acid: HD
RNA extraction and real-time quantitative RT-PCR: CL (50%), GC (45%) and Alice Cousin (5%)
Data analysis: GC
Writing paper: GC
Manuscript reviewing: AL, SC and Claude Bragard

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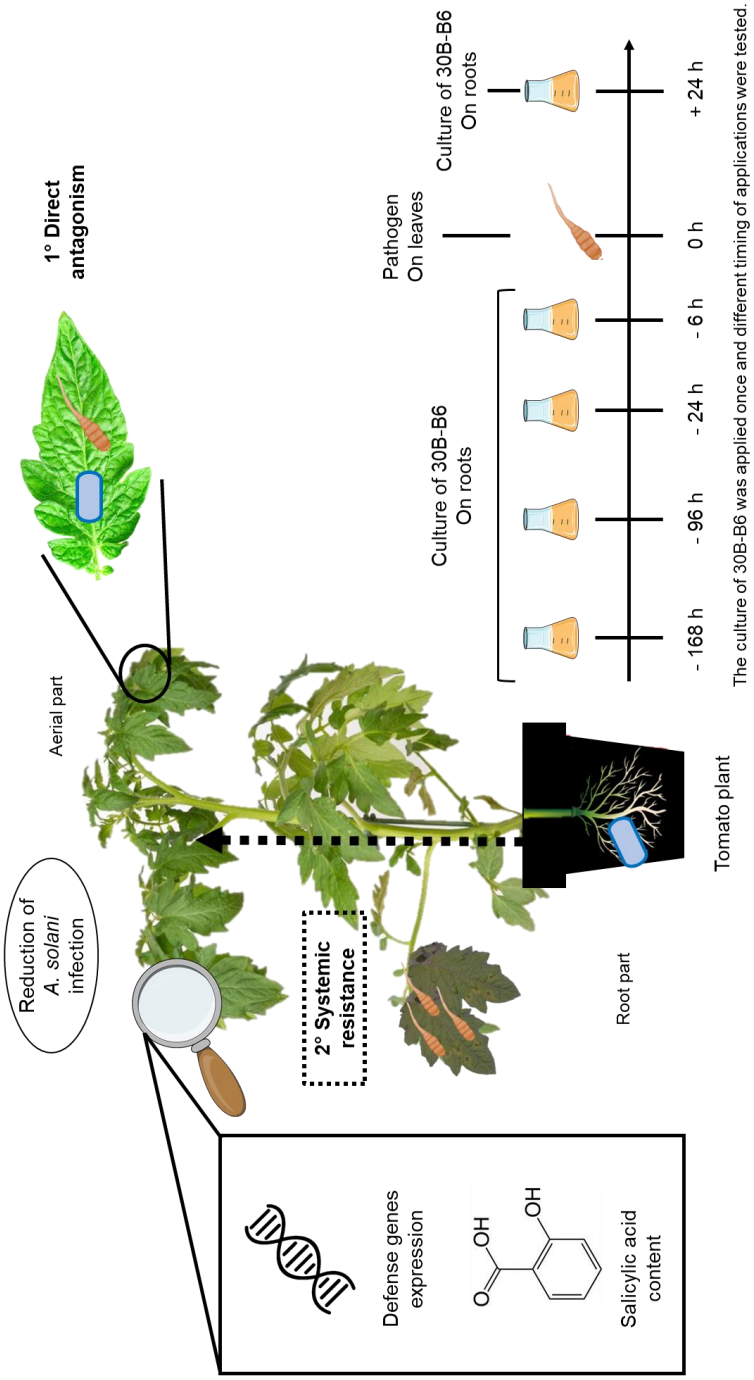
Graphical abstract



Abstract

Bacillus biological control agents offer promising sustainable alternatives in crop protection. This study investigates the potential of *B. subtilis* 30B-B6, previously described (Caulier *et al.*, 2018), to control *Alternaria solani* on tomato. The application of the bacterial culture on the leaves or on the root substrate before the foliar inoculation with *A. solani* lead to a significant reduction of the disease severity (up to 73%). The protection observed on plant whose roots were drenched with the bacterium indicates the induction of a systemic plant defense response since the bacterium and the pathogen remain physically separate. In order to understand the effect of 30B-B6 on plant defenses, the dynamics of known tomato defense gene expression were compared using real-time quantitative RT-PCR assays. The up-regulation of the defense gene *PR2b* in plants pretreated on the root substrate with 30B-B6 and challenged by *A. solani* revealed 30B-B6 to interact with the salicylic acid signaling pathway. This hypothesis is supported by a higher salicylic acid content measured in plants pretreated with 30B-B6 and infected with *A. solani* compared to mock and plants only infected with the pathogen. Further studies on gene expression would help in deciphering 30B-B6-tomato-*A. solani* interaction. This study confirms the interest of 30B-B6 in plant protection by revealing the dual activity of the bacterium exhorting direct antagonism and inducing plant defenses effective against a necrotrophic fungal pathogen.

3.1 Introduction

Plant diseases are a major concern in crop production. Losses due to pests can reach 25% of the world crop production a year (O'Brien, 2017). The management of these diseases represents an important cost for producers. Conventional approaches combine the use of less susceptible varieties, prophylactic measures and chemical pesticides. The latter are increasingly raising questions about their impact on the environment and on human and animal health. This explains a growing interest in the biological control, which is defined in agriculture as the use of natural organisms or their by-products that brings a pest below a break-even point for growers in an integrated pest management strategy (Pal and Gardener, 2006; Seiber *et al.*, 2014; Hinarejos *et al.*, 2016). Biopesticides include a wide range of products, such as plant extracts, micro- or macro-organisms, plant defense elicitors and pheromones (Copping and Menn, 2000; Kumar and Singh, 2015; Mishra *et al.*, 2015). Biocontrol agents present numerous advantages compared to chemical pesticides such as lower toxicity and persistence in the environment, higher specificity, and have multiple modes of action to dam pathogens (Borriss, 2011; Andrivon *et al.*, 2019; Köhl *et al.*, 2019). Rhizobacteria belonging to the *Bacillus* genus appear to be a profitable alternative to protect crops against pathogens (Kloepper *et al.*, 2004; Jain *et al.*, 2016). Indeed, since the 1930s well-known *Bacillus* such as *B. thuringiensis* are used as microbial insecticide, other species of *Bacillus* are used as biopesticides (Roh *et al.*, 2007; Pérez-García *et al.*, 2011). Among them, strains of *B. subtilis* have been formulated in commercially available biofungicides in the European Union (Pérez-García *et al.*,

2011). The biocontrol activity of *Bacillus* spp. is mainly related to the production of various bioactive compounds involved in direct antagonism mechanisms against pathogens (*e.g.* antibiosis and parasitism) but also in indirect mechanisms as eliciting systemic resistance in the plant or promoting plant growth (Choudhary and Johri, 2009; Raaijmakers *et al.*, 2010; Pérez-García *et al.*, 2011; Caulier *et al.*, 2019). Some *B. subtilis* and *B. amyloliquefaciens* are also able to stimulate plant defenses and/or plant growth, enabling them to better resist biotic and abiotic stresses (Ongena *et al.*, 2007; Choudhary and Johri, 2009; Raaijmakers *et al.*, 2010; Pérez-García *et al.*, 2011). Plants have evolved different inducible defense mechanisms against pest or pathogen attacks. The perception of pathogens by plants can trigger a systemic resistance that protects the uninfected parts of the plant from further aggression (Ross, 1961). This phenomenon is often associated with increased salicylic acid (SA) levels in plant tissues and the expression of *PR1*-gene, used as a marker of the SA-pathway (Vernooij *et al.*, 1994; van Loon *et al.*, 2006). SA is a phenolic compound synthesized from chorismate, an intermediate compound of the plant phenylpropanoid pathway (Vlot *et al.*, 2009; An and Mou, 2011; Ding and Ding, 2020). The incoming of messengers from infected tissues leads to an accumulation of SA and the synthesis of PR proteins, rendering uninfected cells prepared for further pathogen attack (Spoel and Dong, 2012; Fu and Dong, 2013). Besides that, the interaction of some beneficial rhizobacteria with plant roots can induce a systemic state of defense in all the plant tissues (Kloepper *et al.*, 1992; Bakker *et al.*, 2013). Most of the time this state is activated independently of SA and is associated with a

higher sensitivity to jasmonic acid and ethylene (Pieterse *et al.*, 2014; Hacquard *et al.*, 2017).

In a previous work, with a view to valorizing the microbial potential of soil for the biocontrol of pathogens of *Solanaceae*, an *in vitro* screening of the antagonistic activity of 2,826 bacterial strains isolated from Belgian substrates was carried out (Caulier *et al.*, 2018). The strain *B. subtilis* 30B-B6 shows interesting features for biocontrol (Caulier *et al.*, 2018). *In vitro* confrontation tests on solid media revealed 30B-B6 to significantly inhibit the growth of *Phytophthora infestans* and *Alternaria solani*. Furthermore, foliar application of 30B-B6 on potato plants resulted in a reduction (around 70%) of late blight severity (Caulier *et al.*, 2018). The present work investigates the activity of *B. subtilis* strain 30B-B6 against the necrotrophic pathogen *A. solani,* and its capacity to promote plant growth and to induce systemic defense in tomato (*Solanum lycopersicum*). Bioassays were performed under greenhouse conditions and the disease progression, the gene expression profiling and the salicylic acid content at distinct time points on plants treated or not with the bacterium and *A. solani* were analyzed.

3.2 Materials and methods

3.2.1 Plant material and growing conditions

Tomato (*Solanum lycopersicum* L.) variety Moneymaker seeds used in the trials were surface-sterilized in a solution of 1.0% sodium hypochlorite for 5 minutes and then 3 times rinsed with sterile water. Then they were placed on a moist fanfold paper, in a plastic box hermetically closed and growth in a chamber with a photoperiod of 16:8 (light/dark; OSRAM L 36 W/840 Lumilux cool white) at 22°C

for 10 days. Subsequently, the seedlings were transplanted individually in plastic pots (11x11x12cm) containing potting soil (DCM®, "clay substrate Nr 9 Fe"), irrigated daily and maintained in a greenhouse with a photoperiod of 16:8 (light/dark) at 24°C with 70% of relative humidity (RH).

3.2.2 Microorganisms and culture conditions

The fungus *Alternaria solani* MBC9282 (isolated from Belgian field; microbial collection of UCLouvain, Louvain-la-Neuve, Belgium) was stored as conidia in water/glycerol solution (70:30 vol vol^{-1}) at -80°C and cultivated on V8 agar (vegetable juice V8, 200 ml l^{-1}; CaCO3, 1 g l^{-1}; agar, 15 g l^{-1}) at 22°C for 10 days with a photoperiod of 16:8 (light/dark) under neon lighting (OSRAM L 36 W/840 Lumilux cool white). Conidia were harvested after 10 days from V8-agar plates that were gently washed with sterile water containing the detergent Tween 20 (0.001% vol vol^{-1}). They were separated from the mycelium by filtration through a 1 mm sterile mesh filter. The conidia concentration was determined with a hemocytometer (Fuchs-Rosenthal counting chamber) and adjusted to 15,000 conidia ml^{-1} with sterile water containing Tween 20 (0.001% vol vol^{-1}).

The bacterium *Bacillus subtilis* strain 30B-B6 (Caulier *et al.*, 2018) was stored at -80°C in conservation tubes containing 70:30 vol vol^{-1} lysogeny broth medium (LB):glycerol (LB medium: yeast extract, 10 g l^{-1}; NaCl, 10 g l^{-1}; tryptone, 5 g l^{-1}). The bacterial strain was first grown on solid medium (LB agar, 17 g l^{-1}) at 30°C overnight and then in LB (300 ml of LB in 500 ml Erlenmeyer flask) at 30°C with constant agitation of 150 rpm until the culture reached the density of 10^8 CFU ml^{-1}. The supernatant of the bacterial culture was obtained by

centrifugation of the culture at 750 g for 10 minutes and sterile filtration through a 0.22 µm membrane of cellulose acetate.

3.2.3 Treatment of plants with bacterial culture or with bacterial culture supernatant

Tomato plants with five to six developed leaves grown in pots were transferred in a greenhouse with a temperature cycle of 22:18°C (day/night) with 70% of RH and a photoperiod of 16:8. In a first experiment, the plants were treated with the bacterial culture applied on the leaves, or on the roots, or both on the roots and the leaves. For the root treatment, the substrate of plants was drenched with 100 ml of bacterial culture (10^8 CFU ml^{-1}). For the leaf treatment, the bacterial culture was spread with a hand sprayer (1 ml/leaf, 3 leaves/plant). The leaves inoculated with the bacterium are the same leaves that were inoculated with the pathogen. Mock treatment consisted in an application of sterile LB medium. In a second set of experiments, plants were treated with the bacterial culture or the bacterial culture supernatant applied on the roots. The root substrate was drenched with 100 ml of bacterial culture (10^8 CFU ml^{-1}) or the bacterial culture supernatant (filtered at 0.22 µm). The root substrate of the controls was treated with sterile LB medium (100 ml/plant).

3.2.4 Inoculation of plants with *Alternaria solani*

A conidia suspension of *A. solani* or a solution of sterile water containing detergent Tween 20 (0.001% vol vol^{-1}; for mock plants) was sprayed on the adaxial face of tomato leaves with a hand sprayer (1 ml/leaf, 2 or 3 lower leaves/plant). In the case of plant inoculation with the pathogen after treatment of the leaves with the bacterium, the same leaves were inoculated with the pathogen. After pathogen

inoculation, plants were placed for 24 hours in a humid chamber at 90% RH to facilitate the infection, and then moved back to the greenhouse.

3.2.5 Evaluation of the effect of 30B-B6 on seed germination and tomato growth

Two experiments were designed to evaluate the effect of 30B-B6 strain on the growth of tomato. A first assay was conducted in vitro. Disinfected tomato seeds (1.0% NaClO) were immersed in a bacterial culture (10^8 CFU ml^{-1}) or in the culture supernatant for 20 minutes at 25°C with agitation. Control seeds were immersed in sterile LB medium. Then the seeds (5 seeds/treatment) were placed in glass test tubes (length 15 cm, diameter 1.5 cm) containing 7 ml of water agar medium (agar, 20 g l^{-1}). The length of the hypocotyl was measured daily for 12 days. The experiment was repeated three times.

A second assay was carried out on tomato plants growing in pots in the greenhouse. The tomato substrate (potting soil) was drenched (100 ml/plant) with a bacterial culture or the culture supernatant at the time of transplanting (cotyledon stage), and at the stage of one-leaf and three-leaf fully developed. The substrate of the control plants was drenched with sterile LB medium (100 ml/plant). At the five-leaf stage, a set of plants was inoculated with *A. solani* (3 leaves/plant, 1 ml/leaf, 15,000 conidia ml^{-1}). Six plants were used for each treatment. The greenhouse was maintained at 24°C with a photoperiod of 16:8 (light/dark) and 70% of RH. The length of the stem was measured daily for 14 days. At the end of the experiment, the dry weight of roots and aerial part of the plants were measured (drying in

an oven for 48 hours at 80°C). The experiment was repeated three times.

3.2.6 Assessment of leaf colonization by *Bacillus subtilis* 30B-B6 after treatment of the root substrate with the bacterium

The possible presence of 30B-B6 on the aerial part of plant growing on the substrate inoculated with the bacterium was checked as follows. Tomato substrate was drenched (100 ml/plant) with a bacterial culture (10^8 CFU ml^{-1}) or sterile LB medium. After 5 and 20 days, samples (1 g fresh weight) of stems and leaves were randomly collected from 3 plants (3 samples/plant) per treatment. Samples were homogenized (vortex, mortar and pestle) in 10 ml of a sterile saline solution (NaCl 8 g l^{-1}) and serial dilutions were plated on LB agar medium. Plates were incubated at 30°C and *Bacillus* colonies were counted 24 hours after incubation.

3.2.7 Assessment of early blight severity

The percentage of the leaf surface displaying early blight symptoms was visually evaluated on the inoculated leaves using a quantitative score (from 0 to 100%; 100% meaning the leaf is completely necrotic). At the end of the experiment, the area under the disease progress curve (AUDPC) was calculated as described by Campbell and Madden (1990) and a normalized protection index (PI) was computed (Caulier *et al.*, 2018):

$$PI\ (\%) = 100 \times [1- (AUDPC_{treatment}/AUDPC_{control)}]$$

3.2.8 Quantification of gene expression

Leaf samples were collected from tomato plants treated or not with 30B-B6 and *A. solani* at successive time points (0, 10 minutes and 3,

6, 9 and 24 hours *post* pathogen inoculation). They were flash frozen in liquid nitrogen, and stored at -80 °C. Total RNA was extracted with the RNeasy Plant Mini Kit (Qiagen) with a DNase treatment step, according to manufacturer instructions. The concentration (≥ 100 ng μl^{-1}) and purity (260/280 nm ratio) of the total RNA extracts were assessed by spectrophotometry (NanoDrop ND1000, Thermo Fisher Scientific). The concentration of RNA was adjusted to 100 ng μl^{-1} for each sample before reverse transcription. The cDNA was synthesized with an anchored oligo(dT)25 primer (Eurogentec®) by reverse transcription, according to the manufacturer instructions. The expression patterns of five tomato defense-related genes were quantified by real-time quantitative RT-PCR. The results were normalized using two housekeeping genes (*PGK* and *EF1*α) after control of their expression stability. Primer pairs used are described in **Table 5**. The specificity of all the primers used for the real-time quantitative RT-PCR was previously confirmed by PCR on samples from untreated tomato plants. To quantify the expression of the target genes, real-time quantitative RT-PCR assays were carried out using SYBR Green technology. The reaction components for each sample were 10 µl Takyon No Rox SYBR (Eurogentec®), 0.5 µl of each primer (20 µM), 7 µl DEPC-treated water and 2 µl cDNA. The amplification reaction was performed in a CFX 96 Real-Time System (Bio-Rad) using the following protocol: denaturation step at 95°C for 10 min; then 40 cycles of 15 s at 95°C, 30 s at 60°C; and the melting curve (55-95°C with a heating rate of 0.5°C s^{-1}). Two technical replicates were made for each sample, standard and control. Standards were obtained by ten-fold serial dilutions of purified RNA extracts

from untreated tomato plants. Standard curves were generated for all genes in order to examine the linearity of the amplification over the dynamic range. The results were treated using CFX MANAGER Software (Bio-Rad) and expressed as normalized relative quantities compared to the housekeeping genes.

Table 5: Characteristics of primers used in this research.

Gene	Accession number	Function	Primer sequence (5'-3')	Reference
Elongation factor 1-alpha (*EF1-a*)[1]	AB061263	Involved in plant architecture	CCAGATTGGAAACGGATATGC TCCTTACCTGAACGCCTGTCA	Nicot *et al.*, 2005
Clathrin adaptor complex (*CAC*)[1]	SGN-U314153	Endocytic pathway	CCTCCGTTGTGATGTAACTGG ATTGGTGGAAAGTAACATCATCG	Expósito-rodíguez *et al.*, 2008
Phosphoglycerate kinase (*PGK*)[1]	SGN-U578082	Catalyze the production of ATP	TCTACAAGGCCCAAGGTTATG GCAGCAAACTTGTCCGCAATC	Ghareeb *et al.*, 2011
Pathogenesis related 1 (*PR1*)[2]	DQ159948	Antifungal	TGTCCGAGAGGCCAAGCTATAAC AATGAACCACCATCCGTTGTTGC	Song *et al.*, 2011
Glucan endo-1,3-β-glucosidase B (*PR2b*)[2]	SGN-U581016	Cleaves β-1,3-glucan	GCACCTTTGCTCGTTAACATT TTGACGCGATCCATCTTGTA	van Aubel *et al.*, 2016
Lipoxygenase (*LOX*)[3]	Y18548	Involved in cell signaling	GAGTTCTCCTCATGGTGTTCGTTTA AGTAGTCTGACACCCAACTT	Gallou *et al.*, 2009
Protease inhibitor (*PI-1*)[3]	SGN-U577558	Serine protease inhibitor	GTGTACCAACAAAGCTTGCTAAAGA GTACAACAACACCCAAAATGTTGTC	Fujimoto *et al.*, 2011
Non-expressor of pathogenesis related gene 1 (*NPR1*)[2,3]	SGN-U585807	Transcriptional co-activator of the defense genes	AATCGGCTTAGGGCTCTCTC TGCTTCTTCAGTTGACGCTCT	van Aubel *et al.*, 2016

[1]Housekeeping gene; [2]Marker genes of SA-pathway; [3]Marker genes of JA-/ET-pathway

3.2.9 Extraction and quantification of salicylic acid in tomato leaves

Tomato leaves were harvested from tomato plants treated or not with 30B-B6 and *A. solani* at two time points (0 and 72 hours post pathogen inoculation). Each leaf was treated individually. The procedure for the extraction of SA was adapted from Verberne *et al.* (2002): 200 mg of fresh plant tissues were sonicated for 5 minutes at 4°C in 800µl of acetonitrile:HCl 0.1M (90:10). An internal standard, 4-hydroxybenzoic acid, was added before the sonication (50µM, 50µl). The mixture was centrifuged at 15,000g and 4°C for 5 minutes. The supernatant was collected. The remaining pellet was extracted twice more with 400µl of acetonitrile (ACN) were done. The supernatants from the three extractions were pooled and 10µl of NaOH (2M) were added before drying the mixture under nitrogen flow at 40°C. Residues were dissolved in 250µl of trichloroacetic acid (5% in water, W/V) and 300µl of HCl (8M) then heated at 80°C for one. Three liquid/liquid extractions were performed with 1ml of a mix of ethyl acetate:cyclohexane (1:1). The organic layers were combined and 60µl of acetate buffer (ammonium acetate 0.2M; pH5.5) was added before drying under nitrogen flow at 40°C. The residue was dissolved in 500µl of H2O/ACN (1:1) before quantification by HPLC. This was done using an Agilent 1200 series (Agilent technologies, Urdorf, Switzerland) equipped with an automatic injector thermostated at 30°C. HPLC separation was performed on a precolumn (C18 Inertsil; 10x4.6 mm) and on a column (C18 Inertsil 3ODS-3; 250×3 mm, 3 µm) at 30°C. Two solvents were used as mobile phase: (A) ACN:H2O (90:10) with 0.1% of formic acid and (B) H2O:ACN (90:10) with 0.1% of formic acid, at a flow rate of

0.4 ml min^{-1}. The gradient profile was as follows: 0 to 5 min, fixed concentrations: 70% A, 30% B; 5 to 15 min, linear gradient to 60% A, 40% B; 15 to 35 min, linear gradient to 20% A, 80% B; 35 to 40 min, fixed concentrations: 20% A, 80% B. After elution of the compounds, the column is cleaned with 100% ACN for 4 min and then re-equilibrated under initial conditions for 4 min. Excitation wavelengths were 252 nm for SA and 315 nm for internal standard (0-5 min). Emission wavelengths were 340 nm for SA and 408 nm for internal standard (5-50 min). Twenty microliters of sample were injected. Quantification of compounds was performed by external calibration using standards with concentrations from 0 to 10 µM.

3.2.10 Statistical analysis

Data from the assays were statistically treated using the analysis of variance (ANOVA) with the open source software® R (R Core Team, 2020). The Tukey HSD multiple comparison test was used for comparison of the mean values at a significance level of $P < 0.05$.

3.3 Results

3.3.1 Effect of 30B-B6 on the tomato seed germination and seedling growth

The daily monitoring data showed that the seeds treated with the bacteria germinated at the same time as the seeds treated with the bacterial culture supernatant or sterile LB medium and the length of the hypocotyl progressed similarly whatever the treatment applied (**Fig. S1a**). Visually, no difference in root appearance, growth and development was observed. Similarly, after 14 days of growth, no significant difference of growth rates and dry weight was observed for

tomato plants after 14 grown in pots in the presence or not of the bacterium or the bacterial culture supernatant with or without the pathogen (**Fig. S1b**).

3.3.2 Reduction of the severity of early blight infection on tomato with 30B-B6

The effect of 30B-B6 against *A. solani* was evaluated in successive bioassays performed under controlled conditions in the greenhouse. First, assays aimed at assessing the effect of 30B-B6 on early blight disease and to appraise the importance of the plant part treated with the bacteria. We compared the disease progression after the application of the bacterial culture or sterile LB medium (as control) on tomato leaves or on tomato root substrate or on tomato leaves and root substrate. The bacterial treatments were carried out six hours (time required for the leaves treated with the bacterial culture to dry) before the foliar inoculation with *A. solani*. After 14 days of monitoring, the average leaf area covered by necrosis reached 27.50% ± 3.31 for plants inoculated with the pathogen but not with 30B-B6 and values of 6.20% ± 1.11, 8.50% ± 1.18 and 9.00% ± 0.51, for *A. solani* infected plants treated with 30B-B6 on leaves, on roots and on roots plus leaves, respectively (**Fig. 29a,b**). Regardless of the plant part treated with the bacterial culture, the disease progressed more slowly than on plants treated (leaves and roots) with sterile LB medium. The protection conferred by the bacteria, whatever the treatment applied, was significant in comparison with plants treated with sterile LB medium ($P < 0.001$) (**Fig. 29a**). However, there was no significant difference between the three bacterial treatments (foliar and/or root applications). A mean protection index (PI) of

70.13% ± 5.19 was conferred by the application of 30B-B6 on tomato against *A. solani*.

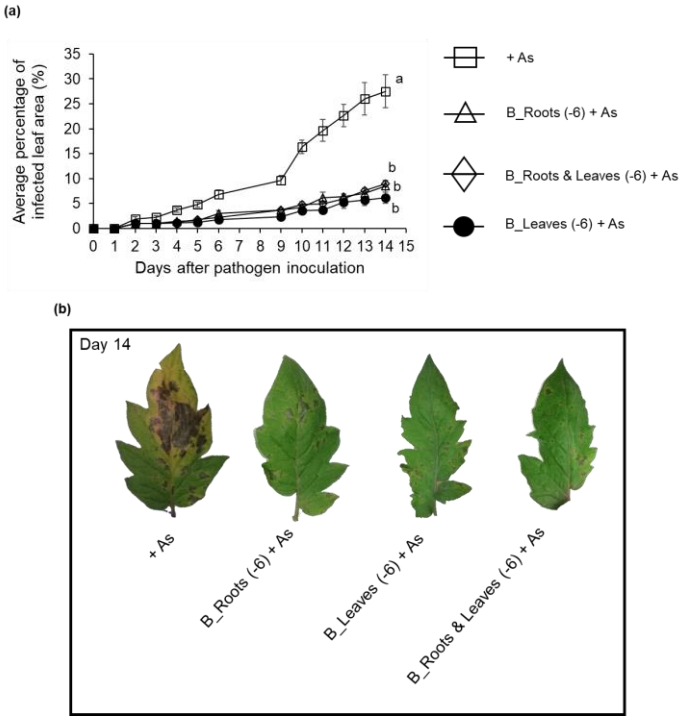


Fig. 29: (a) Evolution of the early blight disease severity on tomato plants var. Moneymaker treated with a bacterial culture of *Bacillus subtilis* 30B-B6 on the leaves (●), on the roots (Δ), on the roots and on the leaves (◊) or treated with sterile LB medium on the leaves and roots (□) and inoculated with *Alternaria solani*. Means ± SD (bars) of one assay with five biological replicates per treatment are given. At 14 days, the area under the disease progress curve (AUDPC) was calculated for each treatment. Curves with different letters have significantly different AUDPC values (Tukey test, $P < 0.05$). **(b)** Pictures of representative tomato leaves infected with *A. solani* taken at day 14 for plants treated or not with the bacteria 30B-B6.

3.3.3 *B. subtilis* 30B-B6 protects tomato plants against *A. solani* by interacting with the roots

In order to confirm that a treatment of roots with 30B-B6 can confer protection against the foliar pathogen *A. solani*, further assays were conducted on tomato plants under controlled conditions. In a first set of three independent experiments, the bacterial culture or sterile LB

medium (as control) was poured on the tomato root substrate six hours before the foliar inoculation with the pathogen. At the end of the three assays (14 days), the severity of early blight disease on plants treated with the bacterial culture was significantly reduced in comparison with plants treated with sterile LB medium ($P < 0.001$) (**Fig. 30a,c**). Fourteen days after infection, the mean leaf surface covered by lesions reached 26.94% ± 2.52 and 5.72% ± 1.41, respectively for the plants treated with sterile LB medium and for the plants treated with 30B-B6 (**Fig. 30a,c**). PI of 75.49% ± 6.01 was conferred by the application of 30B-B6 on the roots. The absence of *Bacillus* type bacteria in Petri dishes inoculated with a solution prepared from leaves and stems of tomato plants grown on the substrate treated with 30B-B6 indicates that the bacterium did not migrate from the potting soil to the aerial parts of the plants during the time of the experiment.

In a second set of three independent experiments, the filtered (0.22 µm filter) culture supernatant of 30B-B6 grown in LB medium (*i.e.* LB medium and molecules produced by the bacteria during the culture) or sterile LB medium (as control) was applied on the tomato roots six hours before the foliar inoculation with *A. solani*. The disease progression curves of plants treated with the bacterial culture supernatant were similar to those of plants treated with sterile LB medium (**Fig. 30b,c**). After 14 days, the mean percentage of infected leaf area rose to 23.28% ± 1.23 for the plants treated with the culture supernatant which is quite similar to that of control plants (24.87% ± 1.61) ($P = 0.345$).

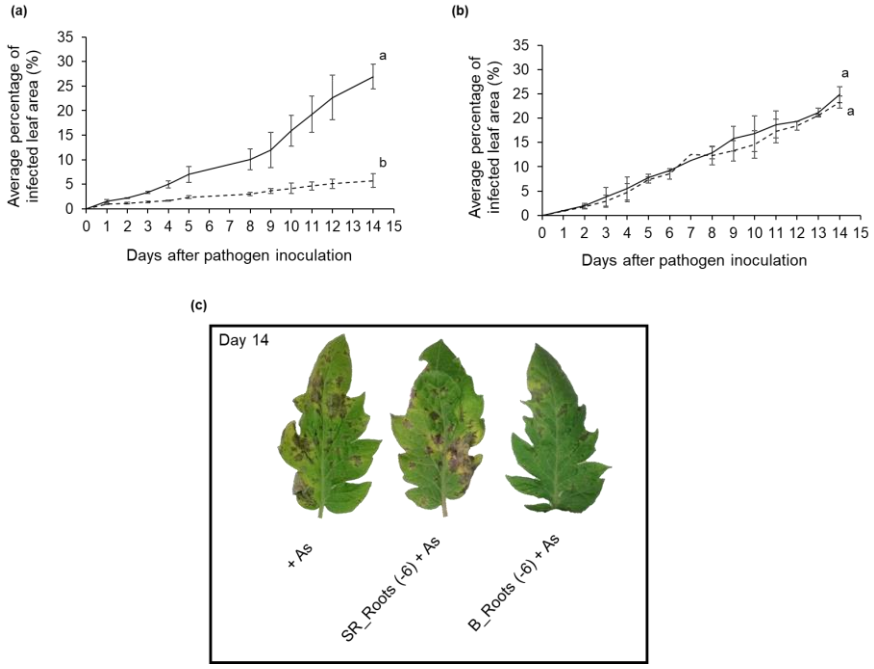


Fig. 30: Evolution of the early blight disease severity on tomato plants with roots treated (- -) or not (—) with a culture of 30B-B6 **(a)**, or with a bacterial culture supernatant **(b)** of 30B-B6 (in LB medium). Means ± SD (bars) of three independent assays with at least five biological replicates per treatment are given. At 14 days, the AUDPC was calculated for each treatment. Curves with different letters have significantly different AUDPC values (Tukey test, $P < 0.05$). **(C)** Pictures of representative tomato leaves infected with *A. solani* taken at day 14 for plants treated or not on the root substrate with a bacterial culture supernatant of 30B-B6 ("SR_Roots (-6) + As") or with a culture of 30B-B6 ("B_Roots (-6) + As").

To evaluate the persistence over time of the protective effect of 30B-B6 against *A. solani*, further bioassays were performed under controlled conditions. In a set of three independent assays, the bacterial culture or sterile LB medium was applied on the tomato root substrate 6, 24, 96 and 168 hours before the plant inoculation with *A. solani*. The strain 30B-B6 reduced significantly the severity of symptoms whatever the time of application ($P < 0.001$). The application of the bacterial culture 168 hours before the infection with the fungus resulted in a protection of 39.20% by comparison with plants treated with sterile LB medium (**Fig. S2**). We have also

appraised the ability of 30B-B6 to control the disease 24 hours after the inoculation with *A. solani*. A significant protective effect (PI 43.00% ± 3.44) ($P < 0.001$) of 30B-B6 applied on the tomato root substrate 24 hours after the infection by *A. solani* was observed (**Fig. S2**). The protective index was slightly lower than the preventive effect (PI 75.49% ± 6.01) obtained when the roots were treated with the bacteria six hours before the inoculation with the pathogen (**Fig. S2**).

3.3.4 Analysis of tomato defense gene expression

To identify which mechanisms are involved in the systemic resistance conferred by the application of 30B-B6 on the root substrate 6 hours before the foliar inoculation of tomato with *A. solani*, the expression of plant defense genes was quantified by RT-PCR separately in two *A. solani* inoculated leaves and in two youngest non-inoculated leaves of plants pretreated or not with 30B-B6 (**Fig. 31a**). Samples were collected at six time points: 0 (just before the inoculation with *A. solani*), 10 minutes, 3, 6, 9 and 24 hours *post* inoculation (hpi) with the pathogen.

In the upper leaves (L1, L2) of plants treated only with 30B-B6, the *LOX* expression 6 hpi was significantly ($P = 0.0096$) up-regulated compared to the mock plants by a factor of 5.0 and the expression of *PI-1* was significantly ($P = 0.0417$) up-regulated 9 hpi by a factor of 7.0 (**Fig. 31b**). No variation in the relative expression of these two genes was observed in the lower leaves (L3, L4) whatever the time and the treatment applied (**Fig. 31b**). In contrast, an up-regulation of *NPR1* was observed 10 minutes *post* inoculation, in the lower leaves (L3, L4) of plants only inoculated with *A. solani* ($P = 0.0315$)

(**Fig. 31b**). Six hpi, an up-regulation of *NPR1* was also observed, in the lower (L3, L4) and in the upper leaves (L1, L2) of plants treated with 30B-B6 in comparison with the mock plants (P = 0.0448 and P = 0.0054, respectively) (**Fig. 31b**). The relative expression of this gene was 7.5- and 4.5-fold up-regulated in the lower leaves (L3, L4) and upper leaves (L1, L2), respectively. The *PR1* expression was not significantly up- or down-regulated, whatever the treatment applied, in the upper leaves (L1, L2) and in the lower leaves (L3, L4) (**Fig. 31b**). The *PR2b* relative expression was significantly higher 6 hpi, both in the upper leaves (L1, L2) (P = 0.0101) (9.8-fold up-regulated) and in the lower leaves (L3, L4) (P = 0.0287) (28.6-fold up-regulated), in plants infected with the pathogen compared to mock plants (**Fig. 31b**). Significant up-regulations of the *PR2b* expression were also observed 9 hpi (P = 0.0966) and 24 hpi (P = 0.0273) in the upper leaves (L1, L2) of plants treated with the bacteria and infected with the pathogen, respectively of 3.5- and 18.1-fold (**Fig. 31b**).

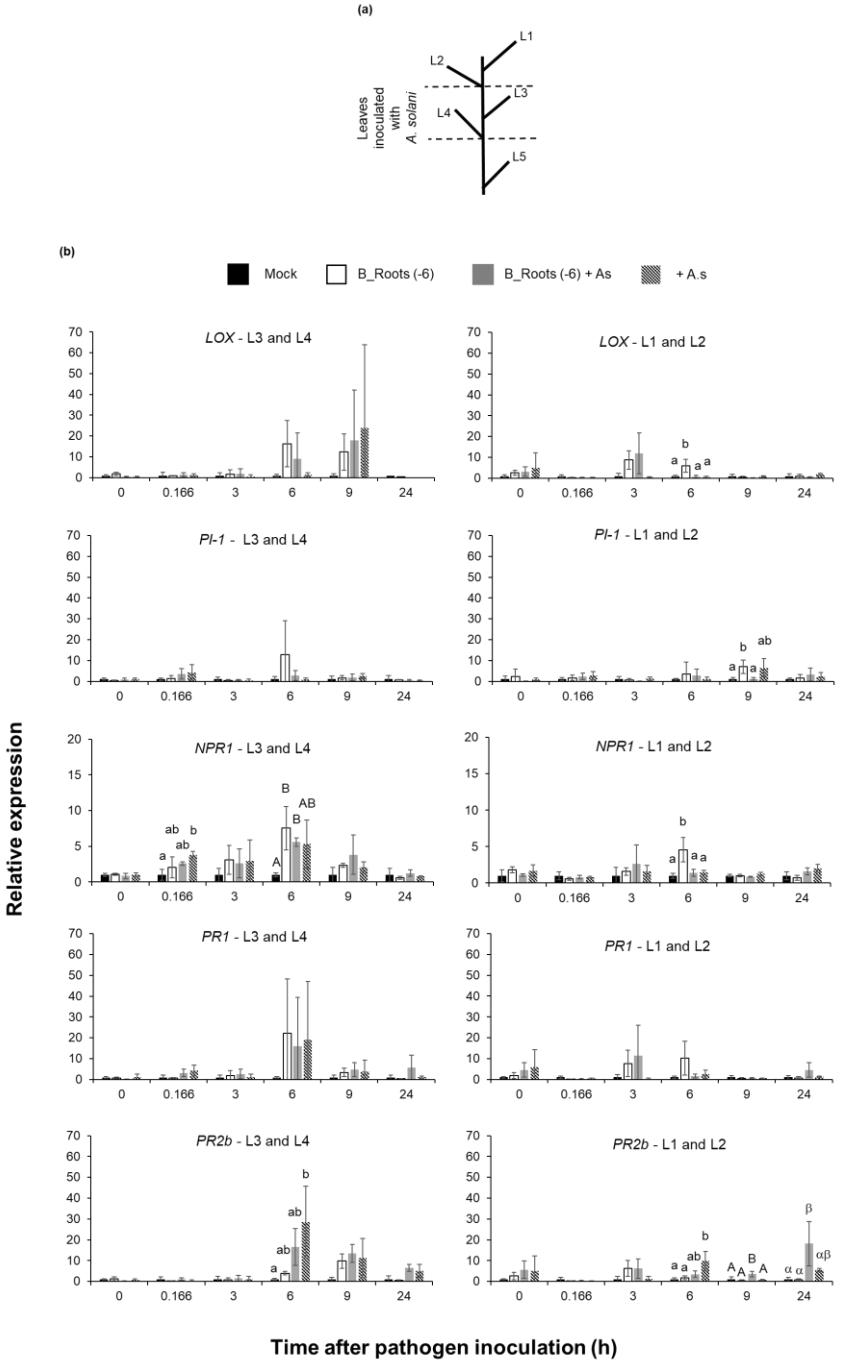

(a)
L1
L2
L3
L4
L5
Leaves inoculated with A. solani
(b)
Mock
B_Roots (-6)
B_Roots (-6) + As
+ A.s
LOX - L3 and L4
LOX - L1 and L2
PI-1 - L3 and L4
PI-1 - L1 and L2
NPR1 - L3 and L4
NPR1 - L1 and L2
PR1 - L3 and L4
PR1 - L1 and L2
PR2b - L3 and L4
PR2b - L1 and L2
Relative expression
Time after pathogen inoculation (h)

Fig. 31 (left page): (a) Schematic representation of tomato plants. The two lower leaves (L3 and L4) between the dotted lines were inoculated with *Alternaria solani* for plants only infected with the pathogen ("+ As") and for plants treated with the bacterium 30B-B6 and inoculated with the pathogen ("B_Roots (-6) + As"). The leaves L3 and L4 were not inoculated with *A. solani* for mock plants and plants only treated with 30B-B6 ("B_Roots (-6)"). **(b)** Expression profiles of selected defense genes in *Solanum lycopersicum* var. Moneymaker in non-inoculated (L1 and L2) and *A. solani* inoculated (L3 and L4) leaves. Leaf samples were collected at 0, 0.166, 3, 6, 9 and 24 hours *post* inoculation. RT-qPCR was carried out with *PGK* and *EF1α* genes as housekeeping genes, and the expression was normalized to mock plant (value = 1). At each time point, for each of the four treatments, the expression of genes was quantified from two upper leaves (L1, L2) and two lower leaves (L3, L4) of three plants, separately. Mock: Plants not treated with the pathogen and the bacterium; B_Roots (-6): treatment of the root substrate with a bacterial culture of 30B-B6; B_Roots (-6) + As: treatment of the root substrate with a bacterial culture of 30B-B6 six hours before the foliar inoculation with *A. solani*; +As: inoculation with *A. solani*. The values represent means ± SD (bars) of three biological replicates, each with two technical replicates. Results for the same time point with a different letter are significantly different (Tukey test, $P < 0.05$).

3.3.5 Salicylic acid involvement in the protection of tomato against *A. solani*

To evaluate the involvement of SA in the induction of the protection conferred by 30B-B6 against *A. solani*, a further bioassay was conducted and the salicylic contents were quantified in plants one and 72 hours after the inoculation of plant with the pathogen (hpi). In this bioassay, the root substrate of tomato plants was treated with either 30B-B6 or LB as described above. Six hours after, each set of plants (30B-B6 or LB) was inoculated or not with *A. solani* on the three lower leaves (L4, L5, L6) (**Fig. S3a**). All the plants inoculated with *A. solani* developed symptoms one day after infection. No symptom was observed on mock plants. From the 5th day, symptoms on plants treated with the bacterium progressed more slowly than on plants treated with sterile LB medium. A global PI of 62.93% ± 7.00 was calculated for plants treated with 30B-B6 (all leaves included). The disease (average AUDPC values) progressed quickly in the oldest pathogen-inoculated leaves, particularly in plants only treated with

A. solani compared to plants treated with 30B-B6 and inoculated with *A. solani* ($P < 0.001$ versus $P < 0.01$) (**Fig. S3b,c**). One hpi, a significant higher average total amount of SA was measured in plants treated with 30B-B6 and non-inoculated with *A. solani* (87.79 ng g^{-1} of fresh weight ± 8.44) compared to mock plants or plants inoculated with *A. solani* pretreated or not with 30B-B6 ($P = 0.0391$) (**Fig. 32**). Seventy-two hpi, plants treated with 30B-B6 and inoculated with *A. solani* exhibited a significantly higher average total amount of SA (88.75 ng g^{-1} of fresh weight ± 6.23) than mock plants and plants only infected with the pathogen ($P = 0.0246$) (**Fig. 32**).

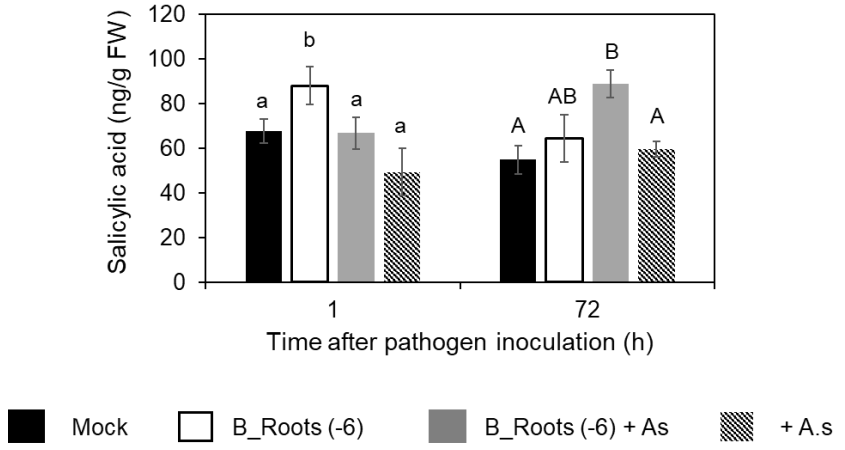


Fig. 32: The average total content of salicylic acid in tomato plants at one hour and 72 hours *post* inoculation with *Alternaria solani*. At each time point and for each of the four treatments, the salicylic content was measured from three upper leaves (L1, L2, L3) and three lower leaves (L4, L5, L6) from four plants, separately. Means ± SD (bars) of four biological replicates are given. Results for the same time point with a different letter are significantly different (Tukey test, $P < 0.05$). Mock: plant not treated; B_Roots (-6): treatment of the root substrate with a bacterial culture of 30B-B6; B_Roots (-6) + As: treatment of the root substrate with a bacterial culture of 30B-B6 six hours before the foliar inoculation with *A. solani*; +As: inoculation with *A. solani.*

4. Discussion

In this study, we investigate the activity of the bacterium 30B-B6 against the necrotrophic fungus *A. solani* on tomato. Bioassays performed under controlled conditions revealed that the application of a culture of 30B-B6 on tomato leaves, on tomato root substrate or on tomato leaves and root substrate before the inoculation with the pathogen significantly reduced the intensity of early blight (average protection index: 70.13%). The significant reduction of early blight severity on tomato plants whose roots or leaves were previously exposed to 30B-B6 confirms the potential of this bacterium to control early blight and highlights the multiple modes of action of this specific strain of *B. subtilis*. Indeed, we observed no significant difference in protection between the application of the bacteria on the leaves or on the root substrate. This indicates that 30B-B6 can control *A. solani* by direct antagonism but also by the stimulation of plant defenses since the bacterium was effective against early blight, a foliar disease of tomato, when applied on the root substrate. In a previous study, the direct antagonism of 30B-B6 on potato plant was evaluated against *P. infestans* but not against *A. solani* (Caulier *et al.*, 2018). The direct antagonism of 30B-B6 may be attributed to several characteristics. Indeed, the genome of the strain 30B-B6 includes several genes involved in the biosynthesis of antimicrobial compounds, such as glucanase, bacilysin and cyclic lipopeptides of the fengycin and surfactin families, which can act against pathogens by distinct mechanisms (Caulier *et al.*, 2018, 2019). The production by 30B-B6 of compounds with antifungal activity may explain the direct antagonism observed on leaves when the pathogen and the

bacteria are in direct contact. In our work, the combination of root and foliar applications of the bacterial culture did not provide better protection against early blight than the individual application on the roots or on the leaves. We hypothesized that the inhibition of the fungal growth and development by direct antagonism of the bacteria was strong enough to maintain the infection below a critical threshold for plants. As plants constantly try to optimize the balance between defense and growth, it seems consistent that they do not invest additional energy for defense when the pathogen is kept below a threshold of harmfulness (Huot *et al.*, 2014). However, we cannot exclude that the bacterium also acted by inducing plant defenses after a leaf application the leaves. Indeed, Lee *et al.* (2017) hypothesized such type of induced resistance to explain why the foliar application of *Pseudozyma churashimaensis* RGJ1 conferred a significant protection against *Xanthomonas axonopodis* pv *vesicatoria* under field conditions, whereas no direct antagonism between strain RGJ1 and *X. axonopodis* pv *vesicatoria* occurred in co-culture assays.

In order to elucidate if the bacterium 30B-B6 is required to trigger the plant immunity, we tested the efficacy of the supernatant of a culture of 30B-B6 in LB medium. However, the culture supernatant provided only a weak protection index. The absence of a significant reduction of disease severity can be due to the absence of the bacterium or low concentration of bioactive metabolites produced by *B. subtilis* 30B-B6 (Gupta *et al.*, 2000; Akpa *et al.*, 2001; Kishore and Pande, 2007; Bartal *et al.*, 2018). In the conditions of our trials, the bacterium was required to induce a systemic resistance in tomato plants against *A. solani*. In a previous study, we reported that the strain 30B-B6 has

the genetic material necessary for the production of compounds such as lipopeptides or siderophores, potentially involved in inducing the systemic defenses of plants (Caulier *et al.*, 2018). Indeed, numerous studies reported the induction of plant resistance by *Bacillus* spp. lipopeptides (Ongena *et al.*, 2005a,b; Ongena *et al.*, 2007). Meziane *et al.* (2005) demonstrated that siderophores produced by *Pseudomonas* are involved in the establishment of ISR, as it is the case of pseudobactin from *P. putida* WCS358 in *S. lycopersicum* cv. Moneymaker against *B. cinerea*. Nevertheless, this could never be demonstrated for siderophores produced by *Bacillus* spp. (Audenaert *et al*., 2002; De Vleesschauwer *et al.*, 2008). Further studies are under progress to identify the active molecules produced by 30B-B6.

In this study, we evaluated the persistence of the protective effect conferred by 30B-B6 against *A. solani* on tomato plants. The root application of 30B-B6 seven days before the foliar inoculation with the pathogen resulted in a significant reduction of the disease severity in comparison with plants treated with sterile LB medium (protection index: 41.04%). The protective activity remains during at least seven days before the inoculation with the pathogen and over 14 days after infection. This suggests the bacterium able to interact and colonize the roots of plants. *Bacillus subtilis* is known to produce biosurfactants, such as mycosubtilin and surfactin, which are involved in the swarming and the production of biofilms (Leclère *et al.*, 2006; Earl *et al.*, 2008). This study also revealed that the application of the bacteria 24 hours after the foliar application of the pathogen resulted in a significant protection (protection index: 43.00%). This shows that the bacterium can induce effective plant defenses even when applied after

the pathogen inoculation. To our knowledge, this is the first report of such a phenomenon using *B. subtilis*.

Various *Bacillus* species, including *B. subtilis*, are plant growth-promoting rhizobacteria (PGPR) and applied as biofertilizer in crop production (Fernando *et al.*, 2005; Borriss, 2011; Pérez-García *et al.*, 2011). For example, *B. subtilis* strain GB03 stimulates the growth, the photosynthetic capacity and improves the salt tolerance of *Arabidopsis thaliana* seedlings (Xie *et al.*, 2009). Mechanisms involved in the growth-promoting activity can include improvement of the bioavailability of the plant nutrients (*e.g.* P, N, Fe), production of phytohormones (*e.g.* auxins, cytokinins, gibberellins) and production of volatile organic compounds such as 2, 3-butanediol and acetoin (Ryu *et al.*, 2003; Zhang *et al.*, 2007; Adesemoye *et al.*, 2010; Ramírez and Kloepper, 2010; Pérez-García *et al.*, 2011; Almoneafy *et al.*, 2012; Bhattacharyya and Jha, 2012). In this study, no PGPR effect of 30B-B6 was evidenced after a treatment of tomato seeds or tomato root substrate. The duration of the assays and the experimental conditions are factors that can explain this result (Mena Violante and Olalde-Portugal, 2007). Plant responses are largely dependent on the type of substrate. Soil properties such as minerals content and pH have a major influence on plant responses (Richardson, 2007; Richardson *et al.*, 2009). We hypothesized that the potting soil used in this research was a relatively rich substrate, which can affect the production of siderophores and other molecules implicated in the growth stimulation of the plant by the bacterium. Moreover, the effects of PGPR on plant growth can depend both on host plant genotype and bacterial strain

(Drogues *et al.*, 2012). Thus, we cannot exclude that the strain 30B-B6 has no PGPR effect whatever the conditions.

As aforementioned, the application of the 30B-B6 bacterial culture on the root substrate of tomato, whose leaves were inoculated with *A. solani*, resulted in a significant reduction of disease severity compared to untreated infected plants. The mean of protection index reaches a value comparable to that obtained by direct antagonism (around 70%). This protection results from the induction of systemic resistance in the host plant since bacterial inducers and pathogen remained located on separate plant organs. Many bacteria of the *Bacillus* genus, including strains of *B. subtilis*, are able to induce systemic resistance in plants (Kloepper *et al.*, 2004; Pieterse *et al.*, 2014). Ongena *et al.* (2005b) reported that *B. subtilis* M4 induces a systemic resistance against *Botrytis cinerea* on *Phaseolus vulgaris*. Seeds were sowed after soaking in a bacterial culture of M4. At 7 days and 14 days after sowing, a bacterial culture of M4 was added as a drench to the roots and a protection level of 36% was observed (Ongena *et al.*, 2005b). The concentration of the bacterial culture applied was similar to that used in our study, but the pathosystem and the number of bacterial applications differ (a single in the case of 30B-B6 and three applications in the case of M4). These defense-inducing bacteria are known to induce SA-independent signaling pathway and not the expression of *PR*-genes (van Loon and Bakker, 2005; Jain and Khurana, 2018). In their study, Ongena *et al.* (2005a) did not detect accumulation of mRNA coding for PR1 proteins in tomato inoculated with *B. subtilis* M4 in comparison with mock plants. Our findings on the relative expression of *PR*-genes are

consistent with this since no significant variation of the *PR1* and *PR2b* expression was observed in plants treated only with the bacteria 30B-B6, at least until 24 hours post inoculation.

The *PR2b* gene, coding for a β-1,3-glucanase, is known to be involved in plant resistance against pathogens (Ferreira *et al.,* 2007; Oliveira *et al.*, 2016). PR2b hydrolyzes glucan, a major constituent of fungal cell walls, and therefore has a direct inhibitory effect on the fungal pathogen growth (Li *et al.*, 2019). Moreover, the glucan hydrolysis releases β-1,3-glucan, a microbe-associated molecular pattern, recognized by plant receptors and triggering plant defenses (Nürnberger and Lipka, 2005). In our trials, as reported by Lawrence *et al.* (2000), we observed, six hours *post* inoculation (hpi), a significant up-regulation of *PR2b* expression in the aerial part of plants only infected with *A. solani* in comparison with mock plants, confirming that this gene is involved in plant response to the pathogen. Contrary to the inactivity of 30B-B6 on the expression of *PR*-genes, our results revealed that expression of *NPR1* was up-regulated 6 hpi in the aerial part of the plant elicited with 30B-B6. Several studies revealed that *NPR1* is required for both SA- and JA-signaling pathways (Spoel *et al.,* 2003). On the one hand, NPR1 is a transcriptional co-activator of the genes induced by SA and its presence is thus required in the nucleus (Ding *et al.,* 2018). On the other hand, NPR1 located in the cytosol seems to have a role during the induction of JA-pathway (Spoel *et al.*, 2003). However, the molecular mechanisms involved in the regulation of JA-pathway by NPR1 remain unknown (Jain *et al.,* 2016). Although cytosolic NPR1 seems to be sufficient for SA mediated suppression of the

JA-pathway, nuclear NPR1 regulates several SA-responsive transcriptional factors, such as TGAs, and WRKYs, with a direct or indirect role in suppressing JA-responsive gene expression. The expression of *NPR1* can be primed by bacteria allowing a higher and/or a faster expression of NPR1-dependent genes in the plant after the infection by a pathogen (Conrath, 2011). In our study, following the up-regulation of *NPR1* (6 hpi) in the plants treated with 30B-B6, we observed a significant up-regulation of *PR2b* 9 and 24 hpi in the upper non-infected leaves of plants treated with 30B-B6 and infected with *A. solani* (lower leaves). This up-regulation demonstrates that this gene is involved in the plant defense against *A. solani* in the presence of the bacteria.

When a plant is infected by a necrotrophic pathogen, the JA-pathway is prioritized at the expense of the SA-pathway (Glazebrook, 2005). In our work, in plants only infected with *A. solani* no significant variation was observed in the expression of the JA-dependent genes, *LOX* and *PI-1* until 24 hours after inoculation. Some necrotrophic pathogens can hijack the antagonistic effect between SA- and JA-signaling through NPR1 proteins to facilitate their development in infected plant. This is reported by El Oirdi *et al.* (2011) in the pathosystem *S. lycopersicum* - *Botrytis cinerea*. Briefly, the fungus produces a polysaccharide which stimulates the SA accumulation in tomato and leads to inactivation of JA-dependent genes such as *PI-1* and *PI-II*. The absence of accumulation of SA in the leaves of plants only infected with *A. solani* in our study does not allow to extend this mechanism in the pathosystem *S. lycopersicum-A. solani*. In contrast to El Oirdi *et al.* (2011), Shinde *et al.* (2018) hypothesized that SA is

involved in the resistance to *A. solani* of *S. arcanum*, a wild tomato species. Seventy-two hpi, they observed that the SA content in resistant accession infected with *A. solani* was significantly higher than in the non-infected plants, but no difference was observed in the susceptible accessions, pointing to the potential involvement of SA in early defense against *A. solani* (Shinde *et al.,* 2018). The variety Moneymaker used in our study is known to be quite susceptible to *A. solani* and we did not observe a higher SA content in plants only infected with *A. solani* in comparison with mock plants which is in line with the findings of Shinde *et al.* (2018). In our trials, plants treated with 30B-B6 and inoculated with *A. solani* displayed significantly fewer symptoms than plants only infected with the pathogen. Moreover, 72 hpi, the SA content in plants pretreated with 30B-B6 and infected with *A. solani* (88.75 ng g^{-1} of fresh weight) was significantly higher in comparison with plants non-treated with 30B-B6 (54.70 – 59.53 ng g^{-1} of fresh weight). These findings show that resistance in tomato to early blight systemically induced resistance in tomato to early blight by *B. subtilis* 30B-B6 may be SA-dependent. The role of SA in induced systemic resistance elicited by plant growth-promoting rhizobacteria is reported by Zhang *et al.* (2002). These authors observed that seedlings of *Nicotiana tabacum* treated with *B. pumilus* strain SE34 and infected with the oomycete *Peronospora tabacina* had a higher SA content (750 ng g^{-1} of fresh leaves) than plants not treated with the bacteria and challenged with the pathogen (450 ng g^{-1} of fresh leaves). *Alternaria solani* and *P. tabacina* differ according to their lifestyles, the first being necrotrophic and the later hemibiotrophic. Plants use different

immune pathways to fight pathogens, the activation of the JA-signaling pathway being mainly required for resistance against necrotrophic pathogens and the activation of SA-signaling pathway to fight biotrophic pathogens. Recent studies demonstrated that SA can antagonize JA signaling and inversely (El Oirdi *et al.,* 2011).

Our results indicate a role of SA in the induction of plant defenses of the susceptible variety of *S. lycopersicum* var. Moneymaker by *B. subtilis* 30B-B6 against the necrotrophic fungus *A. solani*.

Although the stimulation of plant defenses by beneficial rhizobacteria is generally described as independent of the SA-pathway and not associated with the expression of *PR*-genes. Our study revealed that *PR2b* and SA are involved in the systemic resistance of tomato induced by the root application of the *B. subtilis* 30B-B6 against *A. solani*. SA-pathway is a complex phenomenon that involves the interplay of a diverse group of chemicals and associated proteins, including SA (Gao *et al.*, 2015). Next-generation sequencing and quantification of a broad range of phytohormones would help decipher the mechanisms involved in the control of early blight by *B. subtilis* 30B-B6 in tomato plants.

Supplementary data

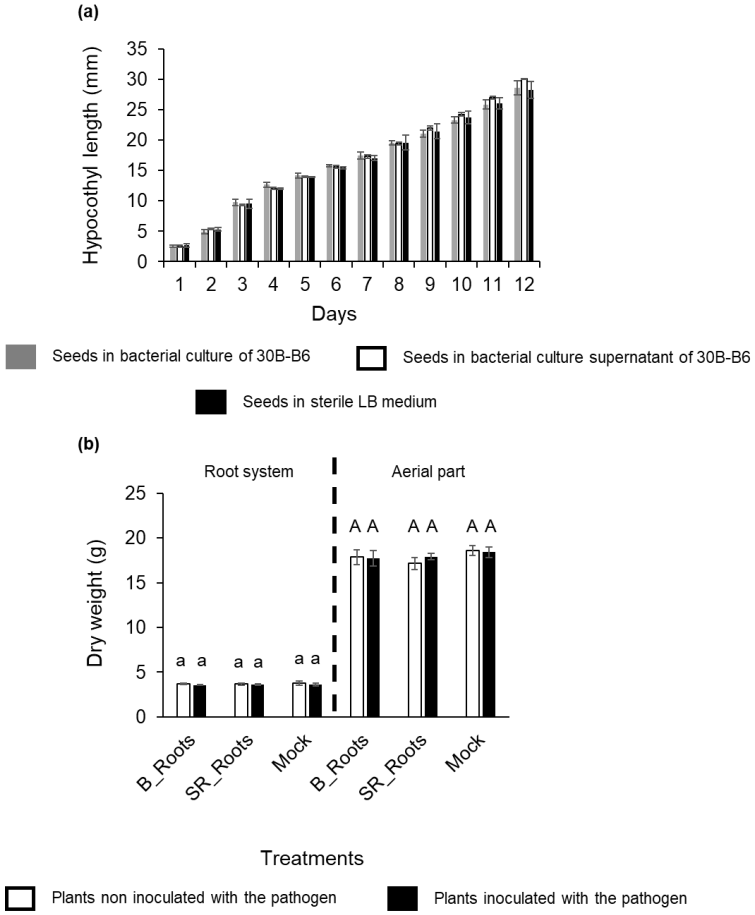


Fig. S1: **(a)** Evolution of the growth of the hypocotyl of tomato seedlings after soaking the tomato seeds in bacterial culture of *Bacillus subtilis* 30B-B6 (gray), in the bacterial culture supernatant (white) of 30B-B6 (grown in LB medium) or in sterile LB medium (black). Means ± SD (bars) of three independent experiments are given (5 plants per treatment and per experiment). **(b)** Average dry weight of the root system and aerial part of tomato plants 14 days after the inoculation (black) or not (white) with *Alternaria solani*. B_Roots: potting soil soaked with bacterial culture of *B. subtilis* 30B-B6 (10^8 CFU ml^{-1}); SR_Roots: potting soil soaked with bacterial culture supernatant; Mock: potting soil soaked with sterile LB medium. Means ± SD (bars) of three independent experiments are given (6 plants per treatment and per experiment). Means with different letters are significantly different (Tukey test, $P < 0.05$). Lower-case letters are used to compare the values obtained for the root system and upper-case letters to compare the values obtained for the aerial part.

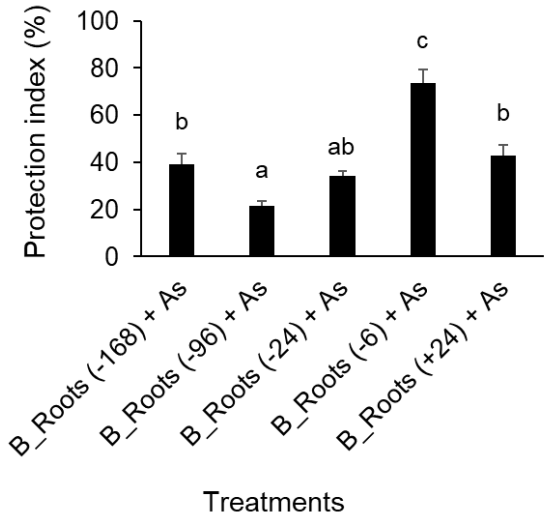


Fig. S2: Normalized protection index over 14 days of plants with roots treated with *Bacillus subtilis* 30B-B6 at five different times, 6, 24, 96 or 168 hours before or 24 hours after *Alternaria solani* inoculation, in comparison with plants treated with sterile LB medium. The bacterial culture was applied on the root substrate. Means ± SD (bars) of the three independent assays with at least five biological replicates per treatment are given. Means with different letters are significantly different (Tukey test, $P < 0.05$). + As: inoculation with *A. solani*; B_Roots: treatment of the root substrate with a bacterial culture of 30B-B6.

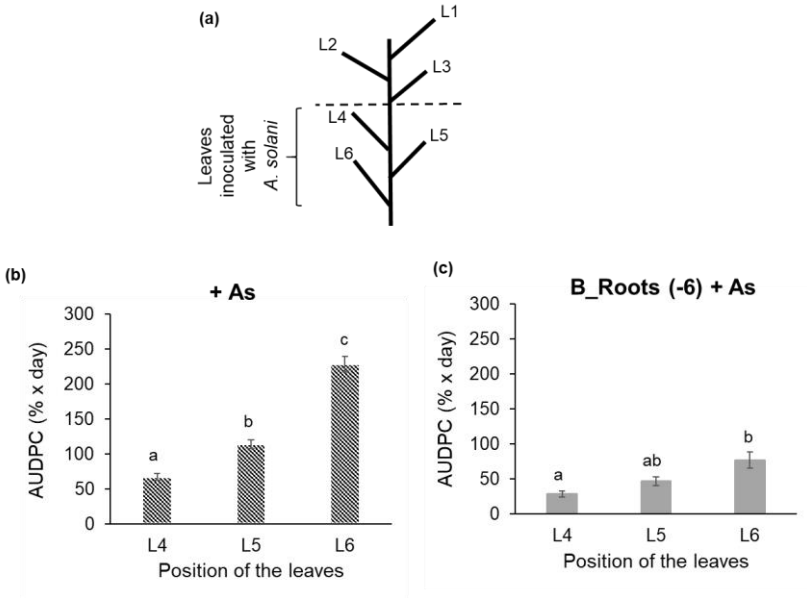


Fig. S3: (a) Schematic representation of tomato plants. The three lower leaves (L4 to L6) below the dotted line were inoculated with *Alternaria solani* whereas the three youngest leaves (L1 to L3) were not inoculated with the pathogen. **(b)** Area under the disease progress curve (AUDPC) over 14 days of the inoculated leaves (L4 to L6) of plants only inoculated with *A. solani*. **(c)** AUDPC over 14 days of the inoculated leaves (L4 to L6) of plants inoculated with 30B-B6 on the root substrate six hours before the foliar inoculation with the pathogen. Means ± SD (bars) with four biological replicates per leaf stage are given. Means with different letters are significantly different (Tukey test, $P < 0.05$).

Chapter 4.

Potential implication of siderophores and lipopeptides produced by *Bacillus subtilis* 30B-B6 in the induction of systemic resistance of tomato against the fungus *Alternaria solani*.

After studying the tomato defense signaling pathways induced by 30B-B6 against *A. solani*, this chapter aimed to investigate which active molecules produced by the bacterium are responsible for the eliciting activity. Caulier and colleagues (2018) have reported that 30B-B6 was able to produce lipopeptides (LPs) and siderophores. The objective of this chapter is to study the implication of these compounds in the induction of systemic resistance of tomato against early blight. Some families of LPs produced by *Bacillus* spp. are well known to induce plant defenses. Similarly, siderophores produced by microorganisms such as *Trichoderma* spp. and *Pseudomonas spp.* have been identified as inducers of systemic resistance. This has never been documented for siderophores produced by *Bacillus* spp.

Potential implication of siderophores and lipopeptides produced by *Bacillus subtilis* 30B-B6 in the induction of systemic resistance of tomato against the fungus *Alternaria solani*.

Contributions in this chapter

Study conception: Gil Colau and Simon Caulier

Greenhouse experiments: Gil Colau (70%), Martin Schoonejans (30%)

Harvesting of plant material: Gil Colau

Extraction of lipopeptides and siderophores: Gil Colau (50%), Charlotte Liénard (50%)

Quantification of lipopeptides and siderophores: Hélène Dailly

RNA extraction and real-time quantitative RT-PCR: Gil Colau (40%), Charlotte Liénard (55%) and Martin Schoonejans (5%)

Data analysis: Gil Colau

Writing chapter: Gil Colau

Manuscript reviewing: Anne Legrève and Claude Bragard

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Chapter 5.

General discussion, conclusion and perspectives

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