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**Effects and modes of action of COS-OGA based
elicitors against late and early blight on *Solanaceae***

Aurélie Clinckemaillie

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Promoter :	Prof. Anne Legrève	(UCL/AGRO/ELIM)
President :	Prof. Jacques Mahillon	(UCL/AGRO/ELIM)
Members of the jury :	Prof. Claude Bragard	(UCL/AGRO/ELIM)
	Prof. Xavier Draye	(UCL/AGRO/ELIA)
	Dr. Jean-Pierre Goffart	(CRA-W)
	Prof. Pierre Van Cutsem	(UNamur/URBV)

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Abstract

The development of alternatives to the use of chemical pesticides constitutes the current challenge of the control of plant diseases. The stimulation of plant immune system using plant defense elicitor constitutes an interesting approach. COS-OGA is a novel plant defense elicitor combining fungal-derived chitooligosaccharides (COS) and plant cell wall-derived oligogalacturonides (OGA). A first formulation of this product, FytoSave®, is now registered in Europe to control powdery mildew in grapevine and cucurbit. Another composition of COS-OGA, FytoSol, is under development by Fytofend SA to enlarge the action range of this elicitor.

This thesis aims at studying the potential use of the elicitor COS-OGA to protect *Solanaceae* against *Phytophthora infestans* and *Alternaria solani*. Bioassays were performed to evaluate the efficacy of two formulations of the elicitor, FytoSave® and FytoSol, and to understand their modes of action. Preventive sprayings of both formulations were shown to reduce early and late blight of potato and tomato. However, the levels of protection differed according to the formulation of the elicitor and the plant-pathogen interactions, in particular the types (species and strain) of pathogens and their lifestyle, the genotypes of plant species and the environmental conditions. FytoSol is more efficient against the hemibiotrophic and necrotrophic pathogens than FytoSave®. The modes of action of both formulations were investigated by quantification of defense gene expression and enzymatic activities. Our work confirms that FytoSave® activate a defense response through the salicylic pathway in tomato in interaction with *A. solani* and in potato in interaction with *P. infestans*. The protective effect of FytoSol was higher but, despite a significant augmentation of endogenous SA in tomato treated leaves, the molecular pathways induced by this elicitor is yet not so clear. The results of this thesis demonstrated that plant defense elicitors interact differently with plant depending on the plant-pathogen interactions. Further studies using whole transcriptome shotgun sequencing and proteomics are required to better understand the modes of action of plant defense elicitors. These information are important to optimize protective effect of elicitors in order to reinforce the interest to their use for the integrated control and encourage their practical use in agriculture.

Abbreviations

α-LA	α -linoleic acid
ABA	Abscisic acid
ANOVA	Analyze of variance
AUDPC	Area Under the Disease Progress Curve
Avr	Avirulence
BABA	β -amino-butyric acid
Bp	Base pairs
BRCA2A	Breast Cancer 2A
BSA	Bovine Serum Albumin
BTH	Benzodithiazole
cDNA	complementary DNA
Ch	Chorismate
COI1	Coronatine Intensive 1
COS	Chitooligosaccharides
Cq	Cycle of quantification
CRA-W	Centre de Recherche Agronomique Wallon
dai	days after inoculation
DAMP	Damage associated molecular pattern
DGDG	Digalactosyldiacylglycerol
DNA	Deoxyribonucleic Acid
EC	European Community
EDS	Enhance Disease Resistance
ERF	Ethylene Response Factor
ET	Ethylene
ETI	Effector Triggered Immunity
ETS	Effector Triggered Susceptibility
EU	European Union
Flg22	Flagelline 22
G3P	Glycerol 3-phosphate
GA	Gibberellin
GFG	Gene For Gene
GK	Glycerol Kinase
HDA4	Histone Deacetylase 6
HKG	Housekeeping Gene
Hpi	Hours post inoculation
HPLC	High Performance Liquid Chromatography
HR	Hypersensitive Response
ICS	Isochromate Synthase
INA	2,6-dichloro-isonicotinic acid
IPM	Integrated pest management
ISR	Induced Systemic Resistance
JA	Jasmonic Acid
JA-Ile	Jasmonyl-Isoleucine
JAZ	Jasmonate ZIP
LAR	Local Acquired Resistance

Abbreviation

LRR	Leucine Rich Repeat
MAMP	Microbial Associated Molecular Pattern
MAPK	Mitogen-Activated Protein Kinases
MATE	Multidrug and Toxin Extrusion
Mb	Millions of base pairs
MeJA	Methyl Jasmonate
MGDG	Monogalactosyldiacylglycerol
NCBI	National Center for Biotechnology Information
NB-LRR	Nucleotide Binding – Leucine Reach-Repeat
NDR1	Non-race specific Disease Resistance 1
NIM1	Non-Inducible Immunity 1
NINJA	Novel Interactor of JAZ
NPR1	Non-expressor of PR genes 1
NRQ	Normalized Relative Quantity
oAA	ortho-Anisic Acid
OGA	Oligogalacturonide
OPDA	12-Oxo-Phytodienoic acid
p	phosphorylate protein
PAD	Phytoalexin Deficient
PAL	Phenylalanine Ammonia Lyase
PAMPs	Pathogen Associated Molecular Patterns
PCD	Program of Cell Death
PCR	Polymerase Chain Reaction
PDF1.2	Plant Defensin 1.2
PGPF	Plant Growth Promoting Fungi
PGPR	Plant Growth Promoting Rhizobacteria
Phe	Phenylalanine
PLRV	Potato Leaf Roll Virus
Ppm	Part per million (mg/L)
PR	Pathogenesis-Related
PRLs	Pathogenesis-Related Like
PRR	Pattern Recognition Receptor
PTI	PAMPs Triggered Immunity
qRT-PCR	Quantitative Real-Time reverse transcription PCR
RAD51	RAS Associated with Diabetes 51
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species
SA	Salicylic Acid
SAR	Systemic Acquired Resistance
SNI1	Suppressor of NPR1 Inducible 1
SSN	Suppressor of SnI1
TGA	Transcription Factor
TIR	Toll-Interleukin-1 Receptor
TMV	Tobacco Mosaic Virus
TNV	Tomato Necrotic Virus
TRX	Thioredoxins
TTSS	Type Three Secretion Systems
Ub	Ubiquitinated Protein
VSP2	Vegetative Storage Protein 2

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General introduction

1. *Research Context*

Agriculture has changed noticeably, especially since the end of the Second World War. In response to human population growth and urbanization, agricultural practices have been intensified to meet the growing food demand. Food productivity soared due to new technologies, mechanization, increased chemical use, specialization and government policies that favored maximizing production (Agricultural Sustainable Institute, 2017). Although these changes have had many positive effects and reduced many risks in farming, they also lead to environmental degradation. Most crops required an extensive use of repeated pesticide treatments to keep them healthy but they are costly and have environmental side effects (Cooke et al., 2011).

During the past two decades, a growing movement for sustainable agriculture has emerged. This movement is defined as the management and utilization of the agricultural ecosystem in a way that maintains its biological diversity, productivity, regeneration capacity, vitality and ability to function (Van Cauwenbergh et al., 2007). The implementation of sustainable agriculture has become a principal challenge for agricultural research, practice and policy. In the field of plant protection, the directive 2009/128/EC of the European Parliament and of the Council of 21 October 2009 established a framework for Community action to achieve a sustainable use of pesticides by reducing the risks and impacts of pesticide use on human health and on the environment. This directive forced member states to establish national action plans based on integrated pest management reducing the dependence on pesticides for plant protection. The regulation 1107/2009/EC concerned the placing of plant protection products on the market and repealing Council Directives 79/117/EEC and 91/414/EEC. This regulation ensures a high level of human, animal and environmental protection and provides clearer rules to make the approval process for plant protection products more effective.

The new pesticide legislation scenario that covers all EU member states creates an opportunity to build a common Integrated Pest Management (IPM) framework in agriculture that is based on a sustainable approach (Lamichhane et al., 2015). IPM is a system approach that integrates all available preventive, mechanical, physical and biological control methods combined with a possible limited recourse to conventional pesticides. Over the last decade, increased efforts in research and policy development have yielded useful knowledge and alternatives to chemical pesticides that can help to reduce pesticide use in agriculture in Europe (Lamichhane et al., 2015).

This PhD thesis has been performed based on aforementioned framework. The research was achieved as a part of a collaborative project between the laboratory of phytopathology of the Université catholique de Louvain (UCL) and the unit of research in plant cellular and molecular biology at the University of Namur (UNamur), granted by the Walloon region (Direction générale opérationnelle de l'Agriculture, des ressources naturelles et de l'Environnement, project D31-1339). This project aimed at studying modes of action and the possibility to use COS-OGA, a new elicitor of natural defense, in integrated pest management programs in potato. COS-OGA is a new active ingredient from biological origin. It was approved in 2015 for use as plant protection product under European regulation 1107/2009 EC (European commission, 2015) against powdery mildews in cucurbit and *Solanaceae* and allowed to organic farming since 2016 (European commission, EGTOP, 2016) It combines fungal-derived chitooligosaccharides (COS) and plant cell wall-derived oligogalacturonides (OGA) which stimulate the plant immunity. These characteristics make it an interesting candidate within the framework of IPM. This PhD thesis focuses on the challenges associated to the use of COS-OGA in plant protection.

2. Objectives and outline of the thesis

Beside the side effects of pesticides on environment and humans, the use of pesticides by farmers for the control of plant diseases and pests offers considerable advantages. They help farmers in preventing crop damage by avoiding infection or infestation by killing harmful pests and pathogens. Furthermore, they often act rapidly and effectively. The use of plant defense elicitors constitutes a distinct control strategy which depends on the response of the plant to the interaction with an elicitor and can result in an elevated plant defense level limiting crop damage. Elicitors are molecules that stimulate plant defense mechanisms and act thus on the plants, and eventually indirectly on pathogens or pests. This indirect effect of the elicitors deserves in-depth studies to characterize their modes of action and limiting factors associated to plant responses. The main question treated in this thesis concerns the potential use of the elicitor COS-OGA to protect *Solanaceae* against diseases caused by fungi or oomycetes.

To address this question, assays were performed to evaluate the efficacy of two formulations of the elicitor COS-OGA against early and late blight of potato and tomato and to understand their modes of action. These diseases are caused by two major pathogens, *Alternaria solani* and *Phytophthora infestans* that differ in their types of parasitism. Three hypotheses are assumed. First, we hypothesize that the efficacy of the elicitor is influenced by its formulation, by the pathogen and its type of parasitism. Secondly, and based on the first set of hypothesis, we assume that plant responses which rely on the elicitor's mode of action may differ. The major impact of factors like climatic conditions, host genotype and pathogen strain on the interaction between plant, pathogen and elicitor constitutes our last hypothesis.

To test these hypotheses, bioassays were conducted under controlled conditions using two formulations of COS-OGA (FytoSave® and FytoSol) and distinct pathosystems¹. Potato (*Solanum tuberosum*) was the first plant species used as host plant because potato crop protection was the focus of the research project. Afterwards, tomato (*Solanum lycopersicum*) was used as plant model because, as a *Solanaceae*, it is

¹ An ecological subsystem defined by the phenomenon of parasitism

close to potato and therefore affected by similar pathogens. Tomato is also a widely-used plant model system for studying the complex network of hormone pathways involved in host defense response. Many mutants or transgenic plants that are altered in hormonal metabolism or signaling are characterized and available. Furthermore, the whole genome of tomato is known and tomato grows more rapidly than potato and it is easy to grow (no tubers). Two distinct pathogens, *Phytophthora infestans* and *Alternaria solani*, were also successively used in our experiments to compare the effects of COS-OGA on both hemibiotrophic and necrotrophic pathogens².

The following dissertation is composed of 6 chapters. **Chapter 1** gives a literature review on important topics addressed in this thesis, including the disease triangle, the characteristics of the plant species and pathogens studied in this work and the methods used to control the diseases caused by these agents. Current knowledge on mechanisms of plant defenses are also introduced because they play a major role in the interaction plant-parasite. The COS-OGA elicitor is described in the last part of this chapter. The second part of the thesis (chapters 2, 3 and 4) focuses on the experimental work of our research. The different chapters were conducted as independent studies and do not include the same pathosystems. **Chapter 2** focuses on the effect and mode of action of FytoSave®, against *P. infestans*, a hemibiotrophic pathogen on potato. Studying the potential effects of factors as potato cultivars and *P. infestans* strains on efficacy of FytoSol is presented in **chapter 3**. The protective effect and mode of action of FytoSave® and FytoSol against *A. solani*, a necrotrophic pathogen on tomato is addressed in **chapter 4**. In **chapter 5**, the hypotheses questioned above are confronted with the results and conclusions that were gathered during this research and the research project.

Finally, a general conclusion is drawn in **chapter 6**, including the new insights and the perspectives gained through this thesis.

² This was encouraged by the research and thesis committees to differentiate from Geraldine Van Aubel's thesis work on the *S. tuberosum*-*P. infestans* pathosystem.

Chapter 1

Literature review

This literature review focuses first on an introduction of the ‘disease triangle’, a basic concept in plant pathology illustrating well the importance of the major components of a disease and of their interactions to set the strategy of plant disease control. This conceptual model is also used to predict epidemiological outcomes in plant health, both in local and global communities (Scholthof, 2007). An overview of the major components of the diseases referred to in this work is then provided. The second part of this review concerns the evolution of pest management strategies in crop protection and the potential role of plant defense elicitors. Current knowledge on plant immune system is given in order to help understanding modes of action of plant defense elicitors. The last part of this review introduce to the COS-OGA elicitor.

1. The disease triangle: a fundamental principle in plant pathology and disease control

The disease triangle (Figure 1.1) is one of the first concepts encountered in an introductory plant pathology course. This triangle illustrates one of the paradigms in plant pathology; that is, the existence of a disease caused by a biotic agent absolutely requires simultaneously the interaction of a susceptible host, a virulent pathogen and an environment favorable for disease development (Francl, 2001). Each of the three components can display considerable variation and as one component changes it can affect the disease severity. For example, the pathogen may be of a less virulent race, some plants become more resistant with age or environmental conditions may be too dry for the pathogen to infect the plant.

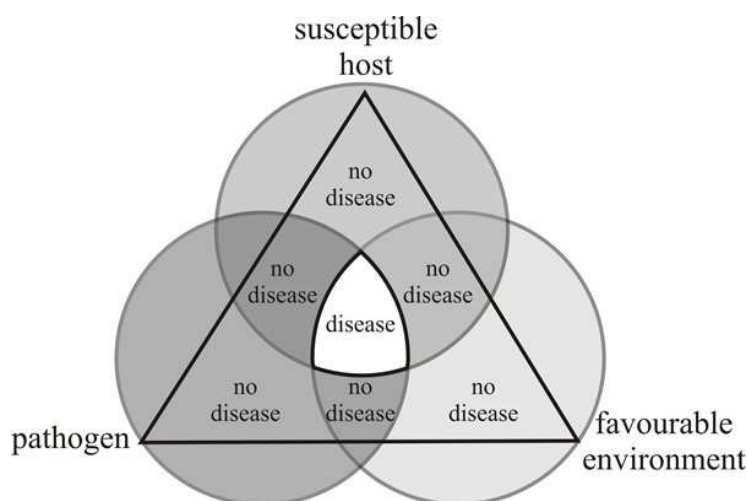


Figure 1.1 : The disease triangle illustrating the phenomenon of plant disease as the interior space of a triangle with the three essential factors (susceptible host, favorable environment for disease, and pathogen) at the vertices. Variation in the ‘strength’ of the contributions of these factors to the relationship (‘strength’ is indicated by the size of the circles) will quantitatively alter the severity of the disease, which will be shown by a change in the area of the central disease envelope (Moore et al., 2011).

Some plant pathologists have suggested elaborating the disease triangle by adding additional parameters, such as human activities, pathogen vectors and time (Franci, 2001). Human activity, in agriculture, contributes widely to the disease triangle because it impacts all three factors aforementioned. This activity can profoundly affect the occurrence and severity of plant diseases. Disease vectors are not essential to all plant disease even though they play a critical role in vector-borne diseases. They are only worth including in those special cases, when it connects the host and pathogen vertices; this arrangement emphasizes the dependence of the pathogen on its vector. The last additional parameter, time, is an essential dimension. A disease does not occur instantaneously. Time is required for the pathogen to infect the plant and to produce characteristic lesions as well as to produce sporulation. A prolonged period of time can also elapse between infection of the plant and symptom expression (Moore et al., 2011).

The disease triangle illustrates quite clearly that three components are required simultaneously to cause a disease and that an array of factors or

conditions can hamper a disease. This thesis aims at understanding how the elicitor COS-OGA can interfere with the other components of the disease triangle and activate plant defense depending on the host, the pathogen and the environment. The following sections detail the actors of the disease triangle that were used throughout this thesis. The ways used to control these diseases are also described.

1.1. Hosts

In the disease triangle, the host is the plant itself. Some plant species can be infected by many pathogens, others only suffer from particular ones. All plants have a range of susceptibilities to a range of diseases. Our work focuses on two species from the genus *Solanum*. This is a large and diverse genus of flowering plants, which includes two important food crops of the highest economic importance, potato (*Solanum tuberosum*) and tomato (*Solanum lycopersicum*). Tomato has also several characteristics that makes it an ideal plant model for plant research. These two species are briefly presented in this section.

1.1.1. Potato

Potato (*Solanum tuberosum*) is an annual herbaceous, dicotyledonous plant originating and domesticated in the Andean mountains of South America. It was first reported in Europe before the end of the sixteenth century. This annual crop is grown worldwide and belongs to the most important food crops in the

family (Singh and Kaur, 2016). In Europe, 1.6 million hectares of potatoes are grown and Belgian potato production represents less than 5% of this area (Eurostat, 2014). The potato is the world's number one non-grain food crop and the fourth main food crop grown in the world after maize, rice and wheat (Cunnington, 2008). It is also an important source of vitamin belonging to the B and C groups and it supplies a variety of dietary nutrients like potassium, magnesium and phosphorus (Prokop & Albert, 2008).

Potato plants produce flowers and berries that contain 100-400 true potato seeds. These true potato seeds (TPS) are also called "botanical potato seed" to differentiate them from "seed potatoes" which are genetically identical clones produced in large numbers by planting pieces

of a potato stem, tuber or through tissue culture. Propagation from TPS is common in potato breeding programs. In contrast, in most commercial production, potatoes are vegetatively propagated from seed potatoes (Stevenson et al., 2001).

Potato is a particularly valuable and important crop in Europe but current farming practices come at a high environmental and financial cost. A huge amount of fungicides are used to protect the potato from potato late blight. The use of varieties with resistance to late blight would be a sustainable and environmentally friendly way of cultivating potatoes. Several resistant potato varieties have been developed but the ones currently available suffer of defects, such as taste, color, shape and process ability, making them unappealing to consumers and the processing industry. As a result, these varieties are hardly cultivated. For this reason, the majority of the potato production area is covered by susceptible varieties. For example, potato cv. 'Bintje', sensitive to the late blight, accounts for more than half of the Belgian potato (Landbouwcentrum Aardappelen, 2016).

The genome of the tuber crop potato was known since 2011 (The Potato Genome Sequencing Consortium, 2001). Common cultivated potato varieties are autotetraploid ($2n = 4x = 48$), highly heterozygous, with a basic chromosome number of 12. There are also cultivated species at the diploid ($2n = 2x = 24$) to pentaploid ($2n = 5x = 60$) levels. The triploid and pentaploid cultivated species are grown only on highland plateaus and slopes of the Andes, but diploid cultivated species are grown more widely and also used for breeding tetraploid varieties. Wild and cultivated potato genetic resources provide a variety of reproductive and genetic features associated with species differentiation and breeding applications (Watanabe, 2015).

1.1.2. Tomato

Tomato (*Solanum lycopersicum* L.) is a dicotyledonous species. It originated in western South America, and domestication is thought to have occurred in Central America (Kimura and Sinha, 2008). The Spanish conquistadors most likely sent the first seeds to Spain in the early 1500s. The fruit gained little attention in Spain, but soon traveled

to Italy, a country that embraced tomatoes with great passion. In Europe, the production is estimated at 16.8 million tons of tomatoes in 2014, of which approximately two thirds come from Italy and Spain (Eurostat, 2014). In Belgium, 500 ha tomato are grown under glasshouse in Flanders (De Blaiser, 2016).

In addition to its worldwide cultivation and economic importance, tomato has several characteristics that make it an ideal dicotyledonous model to investigate various physiological phenomena, because of a relatively compact genome (950 Mb) combined with a marker-saturated genetic linkage map, rich germplasm collections and highly efficient transformation protocols (Carvalho et al., 2011). The tomato genome of the cv. 'Heinz' 1706 was sequenced in 2012 and makes of the tomato a very popular alternative system for plant research (The tomato consortium, 2012). The genome of *S. lycopersicum* cv. 'Moneymaker', used in this thesis, was also sequenced thanks to the reference genome of the *S. lycopersicum* Heinz 1706 (The 100 Tomato Genome Sequencing Consortium, 2014).

1.2. Pathogens

Potato and tomato plants are host of many pathogenic organisms including fungi, bacteria, viruses, phytoplasmas and nematodes. Fungi and Chromista are important groups of potato and tomato pathogens. Early and late blight, caused by *Alternaria solani* and *Phytophthora infestans*, respectively, are the most important diseases caused by them, but other fungal diseases also affect these plant species: anthracnose caused by *Colletotrichum coccodes*, fusarium wilt caused by several *Fusarium spp.*, gray mold caused by *Botrytis cinerea*. Several plant pathogenic bacteria can also severely affect tomatoes and potatoes during the growing season and the storage. For example, *Clavibacter michiganensis* is the agent of bacterial ring rot, *Pectobacterium carotovorum* is the agent of the aerial stem rot or tuber soft rot and *Ralstonia solanacearum* is the agent of the brown rot. Similarly, some viruses and viroids are extremely important pathogens of potato and tomato plants. Most of them can be readily transmitted in symptomless seed tubers from generation to generation, but each virus and viroid is also specifically transmitted by another mode, such as by insect or nematode vectors or by mechanical means. *Potato leaf roll virus*

(PLRV), *Potato Virus X* (PVX), *Potato virus Y* (PVY), *Tomato mosaic virus* (ToMV) or *Potato yellow mosaic virus* (PYVV), *Potato spindle tuber viroid* (PSTVd) are some examples. Nematodes like *Meloidogyne* spp. or insects like *bactericera cockerelli* are also important pests that damage plants. They also transmit diseases like the bacterium '*Candidatus Liberibacter solanacearum*' the putative causal agent of zebra chip disease of potato transmitted by *B. cockerelli* (Stevenson et al., 2001).

As mentioned above, fungi and oomycete organisms are an important group of potato and tomato pathogens. Among them, first and foremost *Phytophthora infestans* but also *Alternaria solani*, the causal agents of late and early blight, respectively, caused serious diseases. As our research focuses on pathosystems associated to these pathogens, we described the two diseases caused by them in the following sections.

1.2.1.Late blight

Potato late blight is the most famous and probably the most important disease of *Solanaceae*. It is caused by the oomycete *P. infestans*. The Oomycetes are a large group of terrestrial and aquatic eukaryotic organisms. Although they superficially resemble fungi in mycelial growth and mode of nutrition, molecular studies and distinct morphological characteristics place them in the kingdom Chromalveolata (phylum Heterokontophyta) with brown and golden algae, and diatoms (Heffer Link et al., 2012). *P. infestans* is well known for its causal involvement in the Irish potato famine after introduction of the HERB-1 strain to Ireland from the Americas, which totally destroyed staple crop during 1845 and 1846. This led to the death of one million people and the displacement of another million refugees (Nowicki et al., 2011). Potato late blight is still among the worst crop diseases of the world despite much research efforts over the years (Fry, 2008). Even using resistant potato varieties and effective fungicides, global losses caused by this disease (costs of control and damage) are estimated to 4.8 billion euros per year (Haverkort et al., 2008).

The life cycle of the causal agent and its high virulence are associated to the severity of the disease. *P. infestans* can reproduce both sexually and asexually as presented in the Figure 1.2.

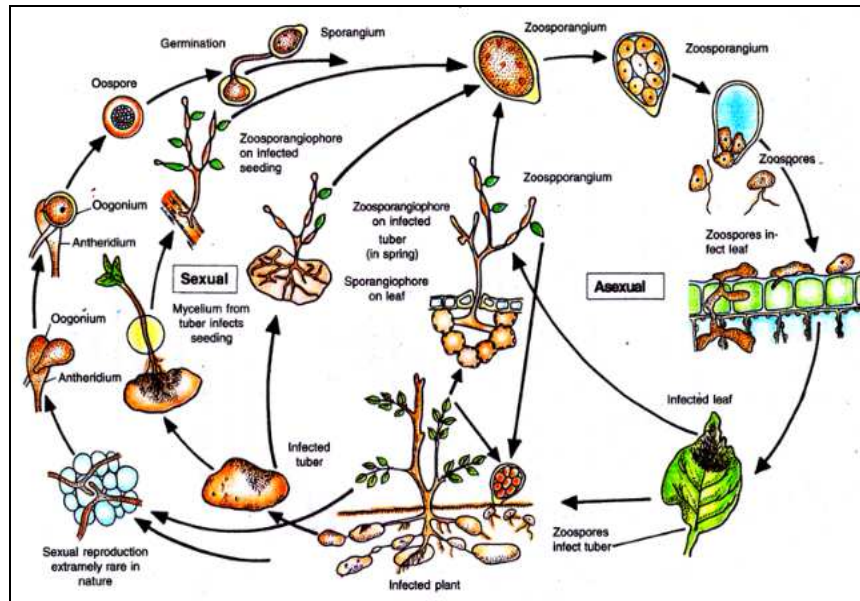


Figure 1.2 : Life cycle of *P. infestans* (Agrios, 2005)

Asexual reproduction is the major contributor to epidemics of the late blight with an entire cycle realized in three or seven days depending on the environmental conditions. This rapid cycle allows several cycles of infection to occur within a growing season. In the asexual form, *P. infestans* produces, on sporangiophores, sporangia containing biflagellate zoospores inside. This type of reproduction facilitates rapid population growth and serves as the major vehicle driving epidemics during the season. As long as *P. infestans* remains exclusively asexual in an area, there are two primary mechanisms by which the pathogen can occur in a field: via plant debris surviving in soil from the previous year and via aerial transport of sporangia from outside sources (Aylor et al., 2001). In response to temperature, sporangia germinate forming a germ tube at 18°C-24 °C or release zoospores at 8°C-18°C and infect leaves, stems, and potato tubers (Goodwin et al., 1998; Vleeshouwers et al., 2000; Judelson & Blanco 2005). Encysted zoospores lose their flagella

and produce germ tube to develop appressoria for epidermis penetration (Judelson & Blanco, 2005). After penetration, the pathogen then establishes a biotrophic interaction with the host plant and forms haustoria from which nutrients are taken up. This biotrophic phase is followed by a necrotrophic phase (Vleeshouwers et al., 2000; Avrova et al., 2007). Necrotrophism leads to formation of lesions on infected tissue. Mycelium emerges through the stomatal openings within 3 to 5 days and produce new sporangia for a secondary infection (Kamoun et al., 1998).

A sexual reproduction can also be involved in the life cycle of *P. infestans*, but is less frequent than asexual reproduction. *P. infestans* is a heterothallic organism with two known mating types, A1 and A2 (Brasier, 1992). In close proximity, both the mating types A1 and A2 can induce their reproductive structures called antheridia and oogonia which, after fusion, can produce dormant oospores. These oospores can survive for long periods in plant debris or soil. Germinating oospores produce sporangium under favorable climatic conditions and establish a new infection cycle.

Unlike most plant pathogens, *P. infestans* can infect the distinct parts of plants: foliage, stems, fruits or tubers. Symptoms are first small, dark and circular to irregularly shaped lesions on stems and leaves (Figure 1.3). These usually appear in the lower leaves where microclimate is more humid. During cool and wet weather conditions, lesions enlarge rapidly and become water soaked. In tomato plants, infection of green or ripe fruit produces large, irregularly shaped brown blotches. Infected fruits rapidly deteriorate into foul-smelling masses. With the progression of disease, production of sporangia gets started. Detached sporangia fall into leaves, stems or soils and start to infect leaves, stems, fruits or tubers in potato plants. Tubers in the early infection get slightly brown or produce purple blotches on the skin and later tuber decays rapidly before harvest.

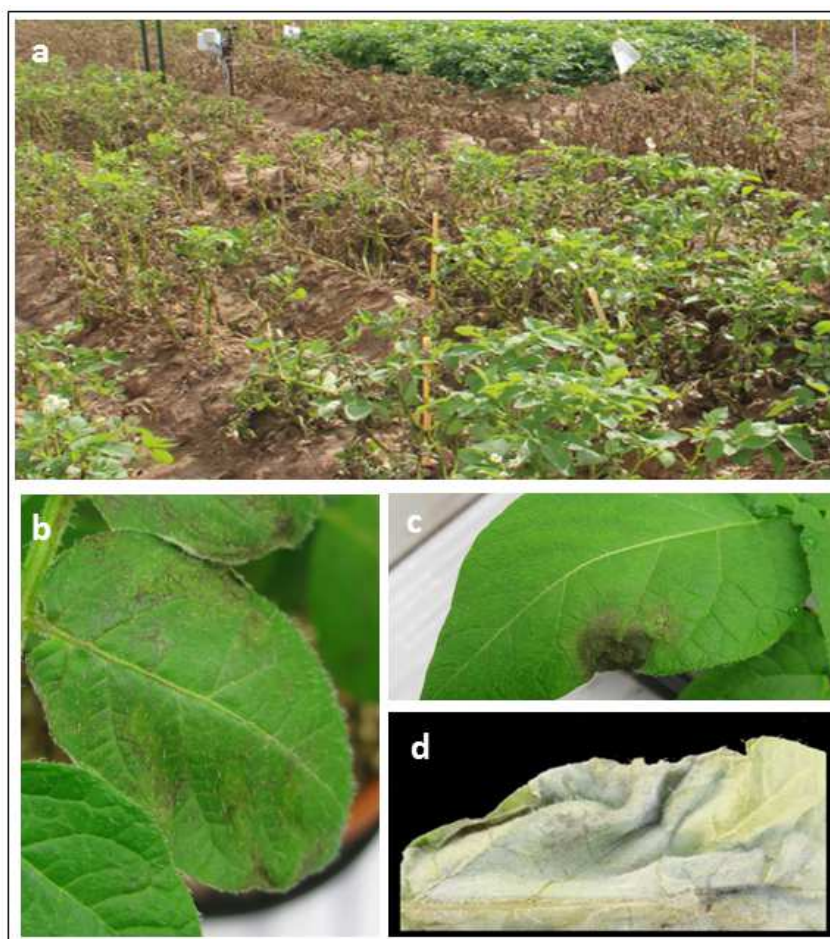


Figure 1.3: Symptoms of potato late blight. **a.** Infection of *Phytophthora infestans* on potatoes cv. 'Challenger' (photo: Gil Colau, UCL). **b.** Foliar symptoms on potatoes cv. ' Bintje': first small, dark and irregularly lesions. **c.** Foliar symptoms on potatoes cv. ' Bintje': large lesions which become water soaked. **d.** Sporulation on potato leaves cv. ' Bintje' (photo: Alain Decroës, UCL).

1.2.2. Early blight

Early blight, caused by *Alternaria solani* (Ellis & Martin) Sorauer, is well known in tomato production to be economically the most important disease. Worldwide, this pathogen is also one of the most important foliar pathogens of potato, generating yield losses of up to 30% depending on location, cropping season and stage of potato maturity (Olanya et al., 2009). In recent years, early blight infections have been detected with increasing frequency in various potato-growing areas of Europe (Runno-Paurson et al., 2015; Edin & Andersson, 2014). In tomato, crop losses of up to 79% due to early blight were reported from Canada, India, USA, and Nigeria (Gwary & Nahunnaro, 1998; Sherf & MacNab, 1986; Datarm & Mayee, 1981; Basu, 1974). During storage, *A. solani* can cause dry rot of potato tubers and also reduce storage length, both of which diminish the quantity and quality of marketable tubers (Olanya et al., 2009).

A. solani is a mitosporic fungus which belongs to the ascomycete phylum in the class Dothideomycetes and order Pleosporales (Lawrence, 2013). As a necrotrophic pathogen, it extracts nutrients from death cells killed prior or during colonization. To help cell necrosis and leakage of nutrients, such pathogens produce phytotoxins. In the case of *A. solani*, the most toxic compound produced is alternaric acid, a host specific toxin essential for the pathogenicity and virulence of the pathogen (Langdsorf et al., 1990).

A. solani has no known sexual stage and the genetic variation among strains can be explained by hyphal anastomosis (Stall & Alexander 1957; Stall 1958). The fungus survives between successive crop seasons as mycelium or conidia on decaying plant debris or as latent infection in seeds (Rotem, 1994) (Figure 1.4). Under free moisture or near-saturated humidity at a wide range of temperatures (8-32°C), conidia of *A. solani*, can germinate to produce one or more germ tubes which develop appressoria for leaf epidermis penetration or enter through stomata or wounds by hyphal growth (Simmons et al., 2007). After penetration, the pathogen uses necrotrophic nutrition facilitated by enzymes (cellulases, pectin methyl galacturonase) that degrade the host cell wall and by toxins that kill host cells (Rotem 1994). Lesions become visible into two

or three days after infection, and spore production occurs onto three or five days later. The relatively short disease cycle of the fungus allows for polycyclic infection (Sherf & MacNab 1986; Chaerani & Voorrips, 2006).

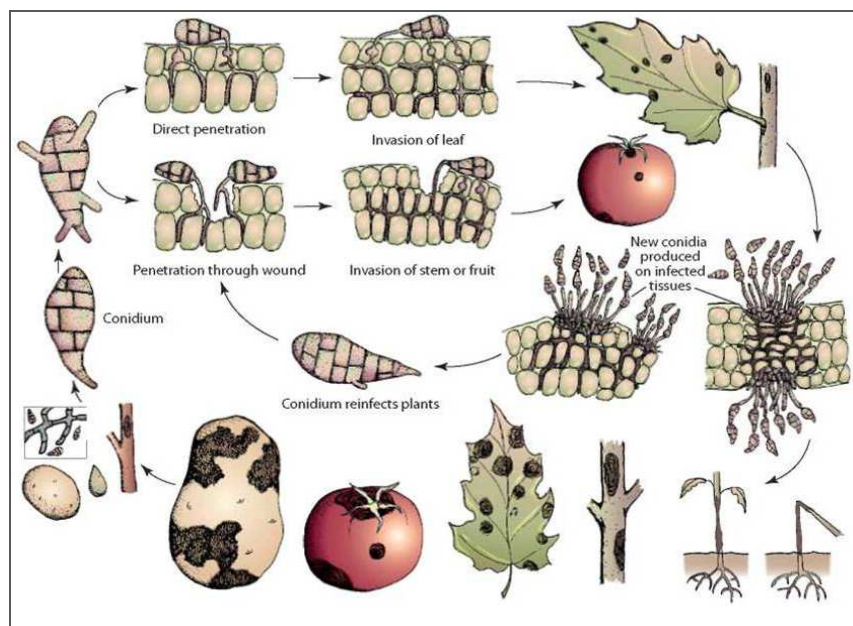


Figure 1.4 : Life cycle of *A. solani* (Agrios, 2005)

Early blight is principally a disease of aging plant tissues. Lesions first appear on older and lower leaves with small, brownish to black lesions (Figure 1.5). Leaf spots enlarge in diameter in a characteristically concentric fashion. The area around the spot may become yellow and dry out. On stems, spots are gaunt with no clear contours. On tomato plants, the fungus occasionally attacks fruit at the stem end, causing large, sunken areas with concentric rings and a black, velvety appearance. On potato, tuber lesions are dry, dark and pressed into the tuber surface, with the underlying flesh turning dry, leathery and brown. During storage, tuber lesions may enlarge and tubers may become shriveled (Kemmitt, 2002).

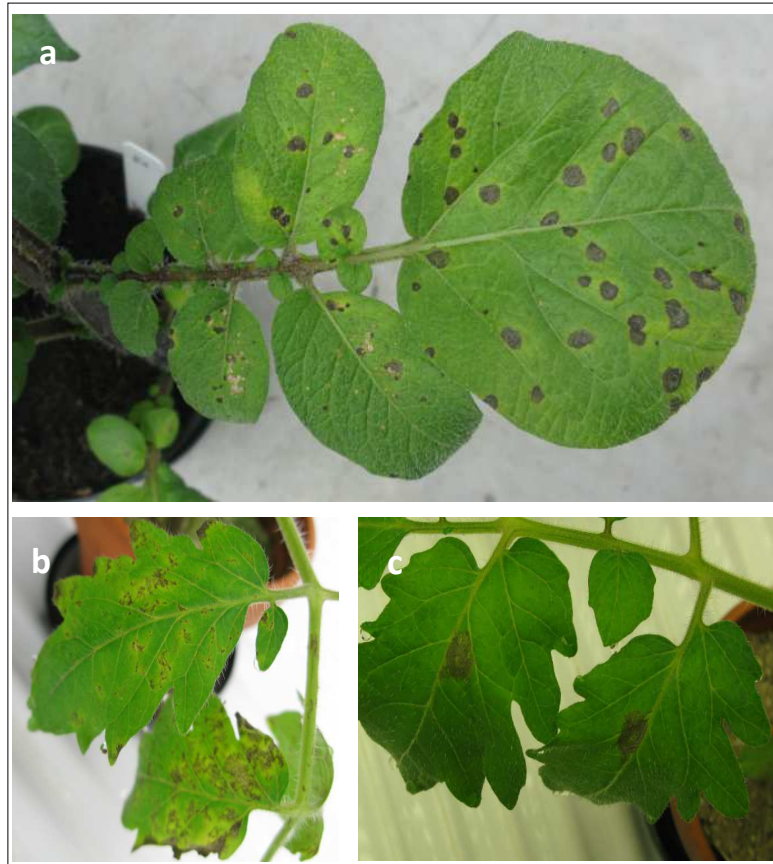


Figure 1.5: Symptoms of early blight a. Foliar symptoms on potatoes cv. 'Markies' (photo: Alain Decroës, UCL). b. Foliar symptoms: beginning of the infection with small, brownish lesions of *A. solani* on tomato leaves cv. 'Moneymaker'. c. Foliar symptoms on tomato leaves cv. 'Moneymaker'.

1.3.Environment: abiotic and biotic factors

The outbreak of a disease requires not only the interaction between a virulent pathogen and a susceptible plant but also specific ecological conditions. Favorable environmental conditions include all abiotic factors, such as moisture (e.g. air humidity, rainfall and irrigation), temperature, sunlight, wind, nutrition and soil quality, as well as biotic factors, such as beneficial microorganisms and/or predators that might interfere with the pathogen or the plant (Legrève and Duveiller, 2010).

In the case of potato late blight, the disease development is favored by moderate temperatures (15°-25°C) and wet conditions. It can develop in very warm daytime temperatures (ca. 35°C) if conditions are extremely wet and night temperatures are moderate (15°-20°C) (Fry, 1998). For tomato early blight, the minimum temperature for infection can be as low as 10 °C, the maximum > 35 °C, and the optimum between 20 °C and 30 °C. Rainy weather or heavy dew (90% humidity or greater) also favored disease development (Van der Waals et al., 2001).

2. Disease control: How to break the disease triangle in a sustainable way?

As explained above, the occurrence of a disease caused by a biological agent absolutely requires the interaction of a susceptible host with a virulent pathogen under environmental conditions favorable for disease development. This basic concept of ‘disease triangle’ in plant pathology is also well adapted to illustrate how to prevent a disease. The mechanisms that contribute to pathogenesis can all be thought of as modifying the disease triangle by reducing or eliminating one of the corners of the triangle or by interfering in the interaction between triangle’s components (Moore et al., 2011).

In the actual agricultural systems, diseases are mainly controlled by repeated fungicide treatments, using fungicides with different modes of action. Up to one application a week is required to prevent or control late blight under favorable conditions. For early blight, the application of foliar fungicides is not necessary in plants at the vegetative stage. Accordingly, spraying should commence at the first sign of disease or at the row closure. The frequency of subsequent sprays should be determined according to the cultivar (Warton and Wood, 2013). The control of these diseases is first based on the prevention of primary infection using contact fungicides (Euroblight, 2017) which prevent spores from germinating and infecting the potato plant if applied prior to spore release. However, once the infection has occurred and the fungus has penetrated the leaf, it will no longer control the disease. Systemic fungicides are absorbed into the plant and are translocated. As a consequence, they can be curative. A whole range of curative fungicides are registered in Europe to control late and early blight (Euroblight,

2017). Each fungicide has its own mode of action, efficacies and other characteristics. However, resistance to some group of curative fungicides has been already reported (Warton and Wood, 2013). Furthermore, fungicide treatments are costly and do have environmental side effects (Cooke et al., 2011).

Environmental policies and an increased public concern related to the environmental effects of fungicides, encourage the use of other prophylactic methods to control diseases. The tightening of European environmental policies led to the implementation novel EU directives and regulations. These aim at harmonizing regulation between member states and, especially, to decrease environmental contamination as well as to increase safety of plant production products for farmers and consumers (Hillocks, 2012). A new legislative framework on pesticides has been adopted by the European Parliament and the Council: Regulation (EC) No 1107/2009 of the European Parliament and of the Council of 21 October 2009 concerning the placing of plant protection products on the market and repealing Council Directives 79/117/EEC and 91/414/EEC. This Regulation aims at ensuring a high level of human, animal and environmental protection as well as to provide clearer rules to make the approval process for plant protection products more effective (Hillocks, 2012; Skevas et al., 2013; Villaverde et al., 2014). It has been estimated that the implementation of the new Regulation would lead to the removal of 20% to 50% of the available active ingredients, even if part of it is seen as essential for plant protection (Hillocks, 2012; Lamicchane et al., 2015). The fungicide availability is now declining as a result of this new legislation and the evolution of resistance in pest populations. There is therefore an urgent requirement for alternative tactics to make crop protection more sustainable. Many experts promote Integrated Pest Management (IPM) as the best way forward, and the EU has placed IPM centrally within its 2009 Sustainable Use Directive on pesticides (European Parliament, 2010).

IPM means the careful consideration of all available pest control techniques and subsequent integration of appropriate measures that discourage the development of pest populations and keep pesticides and other interventions to levels that are economically justified and reduce or

minimize risks to human health and the environment. IPM emphasizes the growth of a healthy crop with the least possible disruption to agro-ecosystems and encourages natural pest control mechanisms. It is an ecosystem approach to crop production and protection that combines different management strategies and practices to grow healthy crops and minimize the use of pesticides (FAO, 2017). In this approach, careful monitoring of pests and their natural enemies are taken into account (Bajwa and Kogan, 2002; Flint and van den Bosch, 1981). The aim is not to eradicate pest populations but rather to manage them below levels that cause economic damage (Chandler et al., 2011).

The control strategies include the use of crop cultivars bred with total or partial pest resistance. Potato breeding for late blight resistance was one of the earliest practices to reduce the incidence of disease by means of genetic improvement (Bengtsson, 2013). In potato breeding, two varietal resistances to late blight were used (Hossard et al., 2010): the quantitative resistance and the race-specific R gene breeding or "gene-for-gene" resistance. The quantitative resistance based on polygenic determinism, is thought to be much more persistent. The race-specific resistance states that a single resistance (R) gene product in the plant is required for recognition of a single protein encoded by a pathogen avirulence (Avr) gene. The recognition of such an Avr factor results in the activation of plant defenses that culminate in a hypersensitive response (HR) and local cell death. Initially *Solanum demissum* was used as source of resistance. More than 11 R-genes were found in this species. The last years, many new resistance sources have been found in several wild species. Isolation of R-genes from these sources is more and more possible through map based cloning and allele mining. Therefore, the use of combinations of R genes with different spectra must be pursued to increase durability of resistance and thereby providing food security under no or little fungicide application. Cisgenesis is a promising approach that introduces native genes from the crops own gene pool using GM technology, thereby retaining favorable characteristics of established varieties (Jo et al., 2014).

Control strategies amongst IPM also include good agricultural practices, such as crop rotation, intercropping or under sowing, physical methods, such as mechanical weeders and decision support system (DSS). A thorough knowledge of disease epidemiology makes it possible

for predictive models to forecast infection or disease occurrence (Madden et al., 2007). Decision support tools inform farmers when it is economically beneficial to apply pesticides. These include the calculation of economic action thresholds, phenological models that forecast the timing of pest activity and basic pest scouting. These tools can be used to move away pesticide application from routine calendar spraying to a supervised or targeted program (Chandler et al., 2011). Across Europe, there is a variety of decision-support systems aiming at ensuring efficient spraying against late blight on potato, such as a Guntz-Divoux-based model in Belgium, Guntz-Divoux/Milsol in France, NegFry in Denmark, PhytoPre+2000 in Switzerland, PLANT-Plus and ProPhy in The Netherlands and Simphyt in Germany (Schepers, 2004; Wander et al., 2006; Small et al., 2013). Even if fungicide choice and application timing is driven by decision support systems, a complete growing season usually requires 10 to 20 spraying to achieve an efficient control of late blight (Haesaert et al., 2015).

Other strategies exist within IPM like the use of biological control with natural enemies, including predatory insects and mites, parasitoids, parasites and microbial pathogens used against invertebrate pests, microbial antagonists of plant pathogens and microbial pathogens of weeds (Chandler et al., 2011). Natural or synthetic products, such as semiochemicals, biocidal plant extracts or elicitors are also a group of crop protection tools used in IPM (Chandler et al., 2011). The elicitation of plant defense by natural and synthetic compounds called elicitors is a novel strategy based on the induction of similar defense responses in plants, similarly to a pathogen infection (Thakur & Sohal, 2013). Some chemical inducers of disease resistance like benzothiadiazol (BTH) or β -aminobutyric acid (BABA) have already been tested for controlling the severity of late blight disease in potato. Results show varying efficiency depending on the product tested, the potato cultivar and the *P. infestans* strain (Sharma et al., 2010). Their use in IPM agricultural practices could minimize the application of chemical pesticides and contribute to the development of sustainable agriculture. The mode of action of these compounds is however still sometimes unknown. Their application requirements also differ from chemical products. A better understanding is necessary for them to be broadly used in agriculture.

3. Plant immune system: current concepts and mechanisms

This section focuses on the description of knowledge in the plant immune system. As a prerequisite of this PhD thesis, the defense mechanisms resulting from the plant-pathogen interaction and contrasting according to the lifestyle of the pathogen are also presented.

3.1. The zig-zag and co-evolutionary models

Despite plants being fixed to the ground and interacting with a variety of plant pathogens, they show a resistance to the majority of pathogen. This suggests an innate immune system allowing the plant to escape from pathogen infection. Plants, unlike mammals, lack mobile defender cells and a somatic adaptive immune system. Instead, they rely on the innate immunity of each cell and on systemic signals emanating from infection sites (Jones and Dangl 2006). In recent years, many research studies allowed to better understand the mechanisms involved in plant defense and are summarized hereafter.

It is usually recognized that the first layer of disease prevention in plant consists of a passive physical barrier composed of the cuticle and cell walls which prevent the entry of pathogens. The second resistance layer is an active mechanism called hypersensitive response (HR) (Freeman and Beattie, 2008). It corresponds to a rapid cell death in the local region surrounding the infection. Both responses belong to an “incompatible plant-pathogen interaction”. In this situation, the pathogen is called “avirulent” and the plant host is defined as “resistant”. To the opposite, when a “virulent” pathogen interacts with a “susceptible” plant host, a “compatible interaction” can occur and cause disease.

The perception of a pathogen by a plant takes place at two locations: in apoplast and inside the cell (Win et al., 2012). In 2006, Jones and Dangl (2006), proposed a simple coevolutionary model of plant-pathogen interactions, called the ‘zig-zag’ model, which encompasses two branches of the plant immune system (Jones and Dangl, 2006). Although this model is now questioned (Pritchard and Birch, 2014) by recent studies, the clear schematic representation of the plant immune

system in this model constitutes a good basis to explain the successive and distinct responses of a plant to a pathogen. Each step is illustrated in the figure 1.6.

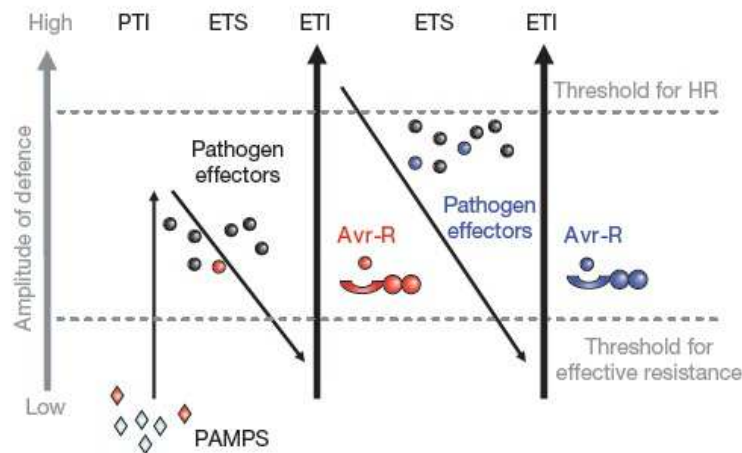


Figure 1.6: A **zigzag model illustrates the quantitative output of the plant immune system (Jones & Dangl, 2006).**

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 (indicated in red) is recognized by an 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.

The first phase in the plant-pathogen interaction conducts to the **PAMP-triggered immunity (PTI)** (figure 1.6; Figure 1.7, step 1). It relies on the recognition by the cell surface pattern-recognition receptor (PRRs) of conserved microbial signatures termed pathogen-associated (or microbe-associated) molecular patterns (PAMPs/MAMPs).

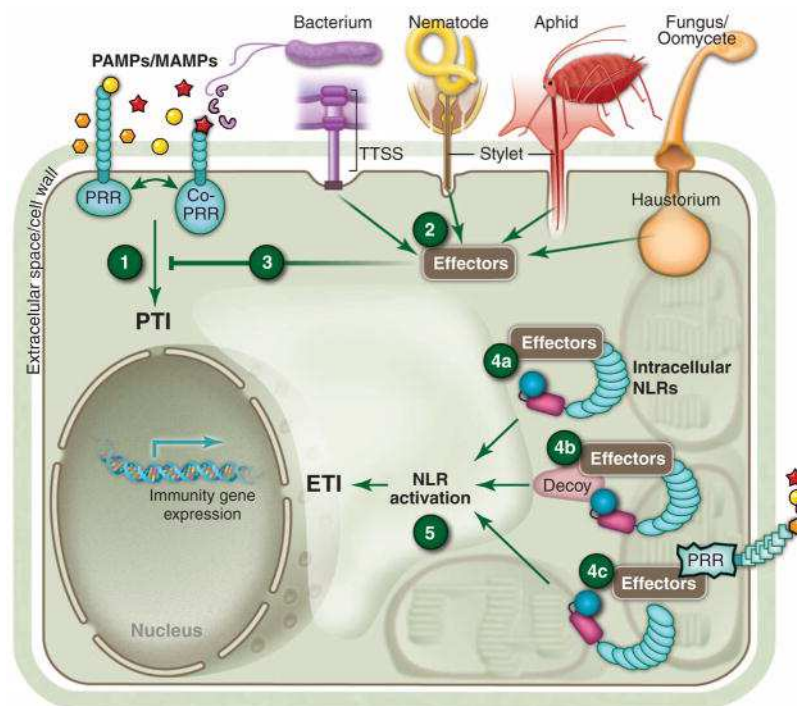


Figure 1.7: **Schematic of the plant immune system (Dangl et al., 2013)**

Pathogens of all lifestyle classes (color coded and labeled) express PAMPs and MAMPs as they colonize plants (shapes are color coded to the pathogens). Plants perceive these via extracellular PRRs and initiate PRR-mediated immunity (PTI; step 1). Pathogens deliver virulence effectors to both the plant cell apoplast to block PAMP/MAMP perception (not shown) and to the plant cell interior (step 2). These effectors are addressed to specific subcellular locations where they can suppress PTI and facilitate virulence (step 3). Intracellular NLR receptors can sense effectors in three principal ways: first, by direct receptor ligand interaction (step 4a); second, by sensing effector-mediated alteration in a decoy protein that structurally mimics an effector target, but has no other function in the plant cell (step 4b); and third, by sensing effector-mediated alteration of a host virulence target, like the cytosolic domain of a PRR (step 4c). It is not yet clear whether each of these activation modes proceeds by the same molecular mechanism, nor is it clear how, or where, each results in NLR-dependent effector-triggered immunity (ETI).

PAMPs/MAMPs are defined as invariant epitopes within molecules that are fundamental to the pathogen's fitness, widely distributed amongst different microbes, absent in the host and recognized by a wide array of potential hosts (Schwessinger & Zipfel, 2008). Various PAMPs

have been identified, such as the bacterial flagellin (flg22), harpin, elongation factor (EF-Tu), peptidoglycan, lipopolysaccharide, cold shock protein and fungal chitin, necrosis-inducing protein, transglutaminase, cryptogein and elicitor (Boller and Felix, 2009; Pel and Pieterse, 2012). In addition to sensing pathogen invasion by means of PAMPs or MAMPs, plants are also able to sense infectious-self or modified-self via damage (or danger)-associated molecular patterns (DAMPs), like plant cell wall fragments or peptides derived from cleaved and degraded products, including oligogalacturonides, prosystemin, hydroxyproline-rich systemins, proPeps and phytosulphokines (Yamaguchi & Huffaker, 2011; Ferrari et al., 2013; Wu et al., 2014). In plants, DAMPs are cell-derived and initiate and perpetuate immunity in response to trauma, ischemia, and tissue damage, either in the absence or presence of pathogenic infection (Tang et al., 2012). They induce a set of basal responses such as indirect and direct antimicrobial effects (cell wall strengthening and anti-microbial agents) and also serve as signals (prime defense responses) (Heil & Land, 2014).

Plants perceive PAMPs, MAMPs or DAMPs through Pattern recognition receptors (PRRs) localized on the plasma membrane (Figure 1.7.). PRRs are the sentinels of the apoplast, dedicated to PAMP sensing. PRRs comprise a ligand-binding ectodomain and are separated between two classes: the receptor-like kinases (RLKs) or receptor-like proteins (RLPs). RLKs are composed of an ectodomain potentially involved in ligand binding, a single-pass transmembrane domain, and an intracellular kinase domain. RLPs have a similar structural organization but lack the intracellular kinase domain (Macho and Zipfel, 2014). PRR ectodomains can contain leucine-rich repeats (LRRs), lysine motifs (LysMs), lectin motifs, or epidermal growth factor (EGF)-like domains. LRR-type PRRs bind to proteins or peptides, such as bacterial flagellin like the 22 amino-acids sequence originating from flagellin (Flg22), bacterial elongation factor EF-Tu, or endogenous peptides (Pep) (Chinchilla et al., 2006; Macho and Zipfel, 2014; Sun et al., 2013; Yamaguchi et al., 2006; Zipfel et al., 2006). PRRs containing other domains are involved in the recognition of carbohydrate-containing molecules, such as fungal chitin, bacterial peptidoglycans (PGN), extracellular adenosinetri-phosphate (ATP), or plant-cell-wall-derived oligogalacturonides (OGA) (Couto and Zipfel, 2016; Macho and Zipfel,

2014; Pel and Pieterse, 2012). After recognition of their specific ligands, PRRs trigger a range of universal molecular, physiological and pathogenesis-related responses including ion fluxes across the plasma membrane, increased intracellular Ca^{2+} concentration, production of reactive oxygen species (ROS), mitogen-activated protein kinase (MAPK) cascades, protein phosphorylation, cell wall reinforcement, receptor endocytosis and protein–protein interaction and rapid induction of defense genes, largely regulated by WRKY transcription factor (Nicaise et al., 2009; Tena et al., 2011). All these events trigger PAMP–triggered immunity (PTI) also called basal immunity or horizontal resistance.

In response to this basal plant immunity, some successful pathogens have evolved and deployed molecules called effectors that contribute to pathogen virulence and especially can interfere with PTI (Figure 1.7, step 2 and 3). This results in **effector-triggered susceptibility (ETS)** which is the second phase of the zigzag model (Figure 1.6). All pathogenic microbes are expected to encode effectors that suppress PAMP-triggered defenses. Up to now, the best characterized effectors come from the phytopathogenic bacteria. Six types of secretion systems are known in Gram-negative bacteria (Tseng et al., 2009) of which the type III secretion system (T3SS) appears to be the most important for virulence. By the T3SS, plant pathogenic bacteria inject multiple effectors into the host plant, suppressing PAMP defense responses. Without a functional T3SS, they only induce the primary defense response and are not pathogenic (de Wit et al., 2007). In recent years, research on fungal and oomycete effectors has largely increased the knowledge of how these effectors function within the plant cell to promote disease. Effectors from fungi and oomycetes have been shown in the host cell and in the apoplastic space between adjacent cells (Giraldo & Valent, 2013). Within the host cell, effectors interact with numerous plant components to alter plant metabolism, signaling pathways and gene transcription to manipulate the plant defense response. For example, effectors Pslsc1 from *Phytophthora sojae* and Vdlsc1 from *Verticillium dahliae* are required for full virulence of both pathogens and reduce the amount of salicylic acid when expressed within plant cells (Liu et al., 2014). Effectors can also be released in the apoplastic space where glucanases and chitinases are produced by host plants to disrupt the pathogen cell wall and stop the pathogen from its

progression. These enzymes also release MAMPs that are recognized by plant PRRs and conduct to the production of phytoalexins, PR proteins, ROS, and lignins (Sanchez-Vallet et al., 2014). Effectors from filamentous pathogens that function in the apoplast were shown to play a central role in blocking these plant responses in a variety of ways (Sharpee & Dean, 2016). For example, chitin, a well-characterized MAMP, is competed by effectors like Slp1 from *Magnaporthe oryzae* (Chen et al., 2014; Mentlak et al., 2012) or Ecp6 from *Cladosporium fulvum* (de Jonge et al., 2010) with the chitin-elicitor binding protein (CEBiP) and chitin elicitor receptor kinase 1 (CERK1). These two receptors are well characterized PRR proteins that mediate PTI (Kaku et al., 2006; Miya et al., 2007). Other effectors than those located within the host plant cell or apoplast also exist but their location within the plant cell are ambiguous or unknown. CfTom1 from *C. fulvum* is a glycosyl hydrolase effector (GH10) that interacts with and detoxifies the steroidal glycoalkaloid α -tomatine from tomato, which constitutes a chemical barrier against a wide range of fungal and bacterial pathogens (Ökmen et al., 2013). Although α -tomatine was shown to be present in both the plant cellular vacuole and within the apoplast during *C. fulvum* infection, the exact location where CfTom1 operates to detoxify α -tomatine is currently unknown (Ökmen et al., 2013, Sharpee & Dean, 2016).

Unlike animals, plants do not have an adaptive immune system to eliminate pathogens that have entered their intercellular spaces and vascular systems. Nevertheless, plants have developed a secondary defense response. This is triggered upon specific recognition of an effector termed pathogen avirulence (*avr*) protein by specific plant disease resistant (*R*) protein (Chisholm et al., 2006; Jones & Dangl, 2006). This relationship is a gene-for-gene (GFG) interaction (Jones & Dangl, 2006). After activation, a local defense response induces a hypersensitive response (HR, a localized programmed cell death response) or hypersensitivity followed by an incompatibility interaction. This plant defense response is called '**effector-triggered immunity**' (**ETI**) and is the third phase of the zigzag model (figure 1.6; Figure 1.7, step5) (Jones & Dangl, 2006). R-proteins are encoded by plant R-genes. Different classes of R-genes exist but the two major classes are the NB-LRR genes that produce intracellular proteins which contain a nucleotide binding site (NB) and leucine-rich repeats (LRRs) (McHale et al., 2006)

and the cell surface PRR (Song et al., 1995). The protein products of the PRRs contain extracellular, juxtamembrane, transmembrane and intracellular non-RD kinase domains (conserved arginine (R) located adjacent to the key catalytic aspartate (D) also called RD motif (Dardick and Ronald, 2006). Analysis of the fully sequenced model plant *Arabidopsis thaliana* ecotype Columbia (Col-0) has revealed approximately 149 genes that encode NB-LRR proteins (Meyers et al., 2003). Within the NB-LRR class of R-genes, two subclasses exist (McHale et al., 2006). The first subclass has an amino-terminal Toll-Interleukin-1 receptor homology region (TIR). The other one contains a leucine zipper region at its amino terminal. Specific NB-LRR proteins are activated by specific pathogen effectors. This can be via direct interaction, as receptor and ligand, respectively (Figure 1.7, step 4a). Alternatively, an effector can modify its host cellular target (or a molecular decoy of that target), and a specific NLR associated with the target or decoy can be activated through the modification (Figure 1.7, steps 4b and 4c). NLR activation coordinates effector-triggered immunity, a rapid and high-amplitude reboot of effector-suppressed, PRR-dependent output that limits pathogen proliferation (Figure 1.7, step 5) (Dangl et al., 2013). Pathogen effectors from diverse kingdoms are recognized by NB-LRR proteins and activate similar defense responses which are effective against obligate biotrophs or hemibiotrophs (Glazebrook, 2005). The GFG concept suggests a direct and specific interaction between pathogen *avr* gene loci and alleles of the corresponding plant disease R-locus and avirulence (Avr)-protein. However, in the majority of cases, recognition of an Avr protein by its corresponding R protein may be indirect. This “guard hypothesis” implies that R proteins indirectly recognize pathogen effectors by monitoring the integrity of host cellular targets of effector action (Jones & Dangl, 2006). GFG resistance to necrotrophic fungi is rare in plants (Wang et al., 2014).

In the zigzag model (Figure 1.6), the last step is reached when the pathogen has gained new effectors that can help it to suppress the ETI. This results then again in a compatible interaction and disease development. In response, selection of plants with modified NB-LRR genes that can recognize these effectors would result in a new ETI. Two extreme NB-LRR evolutions exist. Some NB-LRR genes are not prone to duplication, and are evolving relatively slowly. Their products are

perhaps stably associated with a host protein whose integrity they monitor, retarding diversification. Others are evolving more rapidly and may interact directly with rapidly evolving effectors (Kuang et al., 2004; Van der Hoorn et al., 2002).

3.1. The limit of the current model: is it time to move on?

The zigzag model of the plant immune response was introduced by Jones and Dangl in 2006 and is essentially an expository model to clarify the principles of plant-pathogen interaction. However, as all models, it is a simplification of the reality and has limitations. It is therefore not a quantitative or predictable framework for the direct study of plant-microbe interaction (Pritchard and Birch, 2014).

The original zigzag model applies well to biotrophic plant-pathogen interactions, but is less applicable to processes involving necrotrophs. In contrast with biotrophs, necrotrophic pathogens do not develop complex interactions but promote necrosis to ensure nutrition by releasing toxins. These toxins can also manipulate plant immunity. For example, necrotrophic fungi from the order Pleosporales (such as *Alternaria*, *Cochliobolus* and *Pyrenophora* spp.) produce host-specific toxins (HSTs), which direct host pathways towards programmed cell death and exclusively benefits the fungus. These HST help the pathogen to subverted ETI, rendering necrotrophic pathogen hyper virulent (Keller et al., 2016).

The time scale and the ordering of the events in the zigzag model are also very simplified. Most of the molecular processes described in this model occur essentially at a population level and over longer time-scales. Furthermore, effectors appear to completely repress the PTI. Plant innate immunity is more an integrative and essentially stochastic process combining the detection and action of effectors, MAMPs and damage-associated signals. This interpretation casts doubt on the validity of a conceptual division between PTI and ETI, when considered at a molecular or system level in the plant (Cook et al., 2015; Pritchard and Birch, 2014; Thomma et al., 2011).

3.2. Systemic immunity

Defense implemented by plants is not only limited to local responses. It is also associated with the transport of signals throughout the plant resulting in a broad-spectrum disease resistance against secondary infections. This induced resistance can be defined as a “state of enhanced defensive capacity” (Van Loon et al., 1998). Systemic acquired resistance (SAR) and induced systemic resistance (ISR) are two forms of induced resistance (Figure 1.8). They are described below.

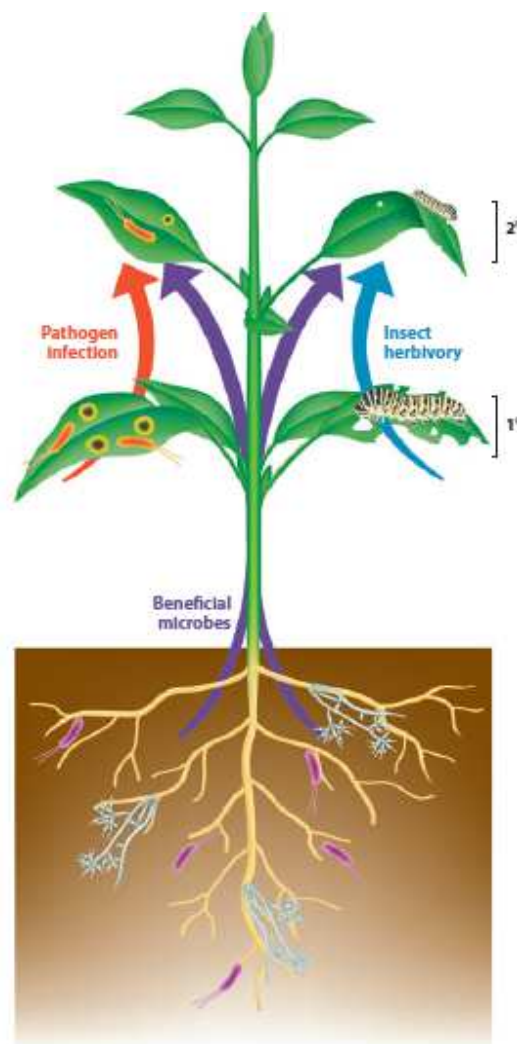


Figure 1.8: (previous page) **Schematic representation of biologically induced resistance triggered by pathogen infection (red arrow), insect herbivory (blue arrow), and colonization of the roots by beneficial microbes (purple arrows) (Pieterse et al., 2014).**

Induced resistance involves long-distance signals that are transported through the vasculature or as airborne signals, and systemically propagate an enhanced defensive capacity against a broad spectrum of attackers in still healthy plant parts. Consequently, secondary (2°) pathogen infections or herbivore infestations of induced plant tissues cause significantly less damage than those in primary (1°) infected or infested tissues.

3.2.1. Systemic acquired resistance

Systemic acquired resistance (SAR) refers to an induced immune mechanism in plants, which is triggered by a pathogen attack. It is a conserved mechanism amongst diverse plants and confers long lasting resistance to unrelated pathogens (Gao et al., 2015). An avirulent pathogen causing local programmed cell death can induce SAR through generation of mobile signals, which can protect the other part of the plant from secondary infection (Fu and Dong, 2013). Several studies have shown that the establishment of SAR involves the generation and transport of signals via phloem to the uninfected distal tissues (Guedes et al., 1980; Tuzun & Kuc, 1985, Gao et al., 2015). SAR can be induced by both PTI and ETI and is associated with local and often systemic accumulation of SA (Pieterse et al., 2014). The essential signal of SAR is SA accumulation and several components of the SA pathway including the methylated derivative of SA (methyl SA, MeSA) (Park et al., 2007). However, other SAR inducers have also been proposed. This section reviews the biosynthesis and the role of SA in SAR and discusses the other SAR inducers and their possible cross-talk with SA.

The phytohormone SA plays a central role in a number of physiological responses, including plant defense against pathogens. SA biosynthesis is triggered during PTI and ETI upon recognition of PAMPs or pathogen effectors (Pieterse et al, 2012). Generally, the SA pathway is typically effective against biotrophic pathogens and some phloem-feeding insects (Glazebrook, 2005). SA is a phenolic compound that can be synthesized from the primary metabolite chorismate (Ch) via the shikimic acid pathway, which forms two distinct sub-branches enzymatic pathways: the first one involving phenylalanine ammonia-lyase (PAL)

and the second the isochromistate synthase (ICS/SID2) (Garcion & Metraux 2006) (Figure 1.9).

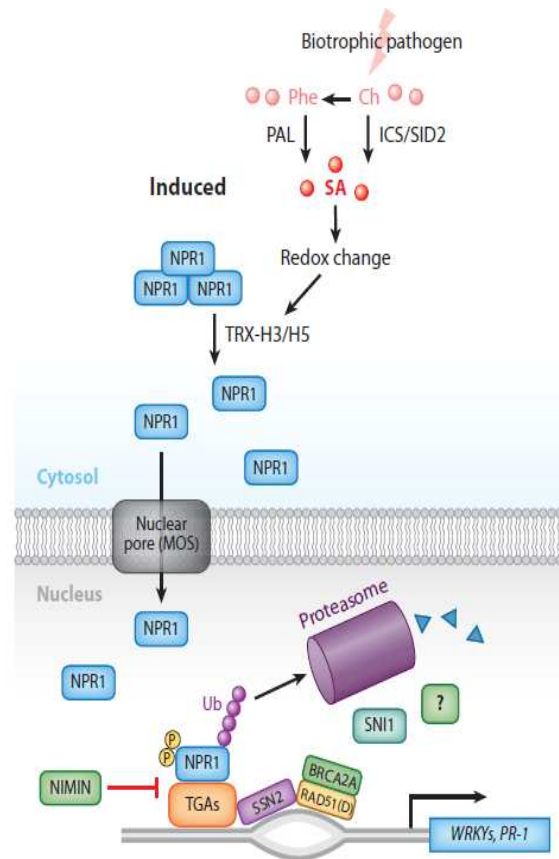


Figure 1.9 : **Schematic overview of the salicylic acid (SA) signaling pathways (Pieterse et al, 2012).**

Solid arrows indicate established activities or accumulation of compounds and red inhibition lines and crosses indicate repression of transcription. Abbreviations: BRCA2A, Breast Cancer2A; Ch, chorismate; ICS/SID2, isochromistate synthase; NIMIN, NIM1-Interacting; 1 NPR1, Non expresser of PR-gene 1; P, phosphorylated protein; Phe, phenylalanine; RAD51, Ras associated with diabete51; SA, Salicylic acid; SNI1, Suppressor of npr1 Indicible 1; SSN2, Suppressor of SNI1; TRX-H3 /H5, thioredoxins; TGAs, TGA transcription factors; Ub, ubiquitinated protein.

It is currently unclear which factors govern the specific recruitment of PAL or ICS pathway for SA biosynthesis. SA is synthesized in chloroplast and exported to the cytosol by Enhanced Disease

Susceptibility 5 (EDS5), a member of Multidrug and Toxin Extrusion (MATE) transporter family, located in the chloroplast envelope (Serrano et al., 2013). Other proteins also participate to SA accumulation like EDS1, Phytoalexin Deficient 4 (PAD4), and Non-race-specific Disease Resistance 1 (NDR1) (Gao et al., 2015). Studies on *Arabidopsis* ICS1 and PAL mutants showed the importance of SA in SAR. ICS1 and PAL mutants are unable to induce SAR (Wang et al., 2014; Huang et al., 2010) suggesting that SA synthesized by both PAL and ICS pathway is critical for induction and/or establishment of SAR (Gao et al., 2015). *NagH* transgenic plants, which express bacterial salicylate hydroxylase (Vernooij et al., 1994) that catalyzes the conversion of SA to catechol, are also unable to induce SAR, suggesting the importance of SA in SAR.

An important part of synthesized SA is converted and stored as SA-derivatives after glycosylation, methylation or amino acid conjugation. SA-derivatives include SA 2-*O*- β -D-glucose (SAG), SA Glucose Ester (SGE), Methyl SA (MeSA) (Dempsey et al., 2011). MeSA is volatile and phloem mobile and must be converted in SA in distal tissue infection. The biosynthesis of MeSA is catalysed by SA methyltransferases and the reconversion of MeSA into SA is due to a methyl esterase (Koo et al., 2007). Studies have reported that the ability to convert SA into MeSA and reconvert MeSA back to SA is critical for SAR (Gao et al., 2015). With the exception of MeSA, the role of the others SA-derivatives in SAR remains unclear.

SAR signaling downstream of SA is controlled by the redox-regulated protein Non-expressor of *PR*-genes 1 (NPR1), which upon activation by SA acts as a transcriptional coactivator of a large set of *PR*-genes (Pieterse et al., 2014; Pieterse et al., 2012). Under uninduced conditions in *Arabidopsis*, NPR1 is sequestered in the cytoplasm as an inactive oligomer formed by intermolecular disulfide bonding (Mou et al., 2003). Reducing conditions resulting from accumulation of SA cause dissociation of the NPR1 oligomer by the activity of thioredoxins TRX-H3 /H5 (Tada et al., 2008) into active monomers. The monomeric form of NPR1 is translocated into the nucleus via nuclear pore proteins, such as Modifier Of *Sni1* (MOS) (Mou et al., 2003; Tada et al., 2008). Nuclear localization of NPR1 facilitates its interaction with members of the TGA transcription factor subclass of the basic leucine zipper (bZIP),

a family of transcription factors (Fan & Dong, 2002). This in turn enhances binding of the TGA transcription factors to promoter elements of NPR1-dependent target genes such as PR-proteins (Wang et al., 2006; Wang et al., 2011). During this process, NPR1 becomes phosphorylated and subsequently ubiquitinated by an E3 ubiquitin ligase with high affinity for phosphorylated NPR1, after which it is targeted for degradation by the proteasome. This clearance of phosphorylated NPR1 from the target gene promoter is required for full induction of the SA-responsive target genes, probably because it allows new NPR1 monomers to reinitiate the transcription cycle (Spoel et al. 2009; Pieterse et al, 2012) (Figure 1.9). Recently, the NPR1 paralogs NPR3 and NPR4 were identified as SA receptors that bind to SA with different affinity (Fu et al., 2012) (Figure 1.10). NPR3 and NPR4 were shown to function as adaptors of the CULLIN 3 (CUL3) ubiquitin E3 ligase to mediate NPR1 degradation, thereby regulating NPR1 stability and activity. It was proposed that the differential affinity for SA causes NPR3 to mediate degradation of NPR1 at high SA levels, resulting in local programmed cell death during ETI. At lower levels of SA, such as during PTI or in distal SAR-expressing tissues, NPR4 stabilizes NPR1. That results in the activation of PR-gene expression (Pieterse et al., 2014). Thus, NPR1-mediated signaling is active only at intermediate SA levels (Seyffert and Tsuda, 2014)

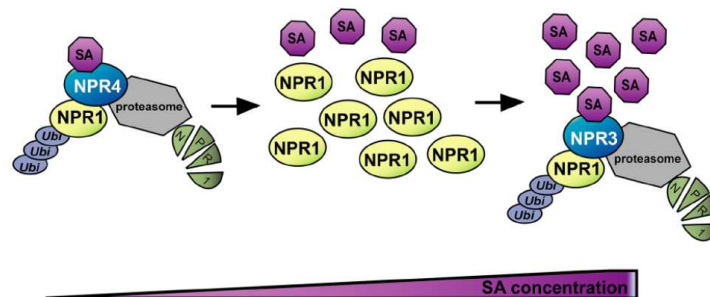


Figure 1.10 : Model of SA perception by its receptors NPR1, NPR3, NPR4 (Seyffert and Tsuda, 2014).

NPR1 accumulation is regulated by SA through the SA receptors NPR3 and NPR4. Pathogen infection triggers SA accumulation. In the case of low SA, the SA-receptor NPR4 triggers NPR1 degradation through the 26S proteasome. When SA levels are intermediate, NPR1 protein accumulates. High SA-concentrations trigger the SA receptor NPR3-mediated NPR1 degradation. Thus, only intermediate levels of SA achieve NPR1 accumulation thereby activating SA-mediated transcriptional reprogramming.

Several negative regulators of SA- and NPR1-regulated genes exist such as the NIM1-Interacting 1 (NIMIN1) and Suppressor of NPR1 Inducible 1 (SNI1) in *Arabidopsis thaliana* (Song et al. 2011). NIMIN1 inhibits promoter activity of defense genes likely by targeting TGA transcription factors (Weigel et al. 2005). SNI1 exerts its negative effect through association with defense gene promoters (Song et al. 2011). On the *PR1* gene promoter, interaction of NPR1 with TGA transcription factors helps recruit Suppressor of *sni1* 2 (SSN2) to counteract SNI1 repression (Li et al. 1999, Song et al. 2011). A complex of RAS-associated with diabetes 51 (RAD51) and Breast Cancer 2A (BRCA2A), both involved in double stranded break repair via homologous recombination (Doutriaux et al., 1998; Siaud et al., 2004), is also recruited to the *PR-1* promoter, which together with SSN2 and RAD51D positively regulates plant immune gene expression, likely through a direct effect on DNA accessibility (Song et al. 2011, Wang et al. 2010, Pieterse et al, 2012).

Other SAR inducers exist and involve free radical nitric oxide (NO) and reactive oxygen species (ROS) (El Shetehy et al., 2015). Both NO and ROS function in a concentration-dependent manner because they can confer SAR only when present at an optimal concentration (Wang et al., 2014). Free radicals are thought to participate in SAR by mediating the oxidation of carbon (C) 18 unsaturated fatty acids (FAs) containing a double bond on C9 present on monogalactosyldiacylglycerol (MGDG) and digalactosyldiacylglycerol (DGDG) lipids (Figure 1.11). This results in the formation of 9-oxo nonanoic acid (ONA), which is converted to the di-carboxylic acid azelaic acid (AzA) by the addition of a carboxylic group. AzA triggers biosynthesis of Glycerol-3-phosphate (G3P) via upregulation of genes encoding G3P biosynthetic enzymes. G3P operates in a feedback loop with lipid transfer proteins DIR1 and AZI1 to regulate SAR (Yu et al., 2013). Cross talks exist between SA and NO pathway in SAR. For example, NO-mediated S-nitrosylation of NPR1 (Lindermayr et al., 2010) and EDS1 regulated levels of SA and AzA (Witteck et al., 2014). These results suggest that the interlinked SA- and NO-pathways might allow to a fine regulation of the optimal onset of SAR (Gao et al., 2015).

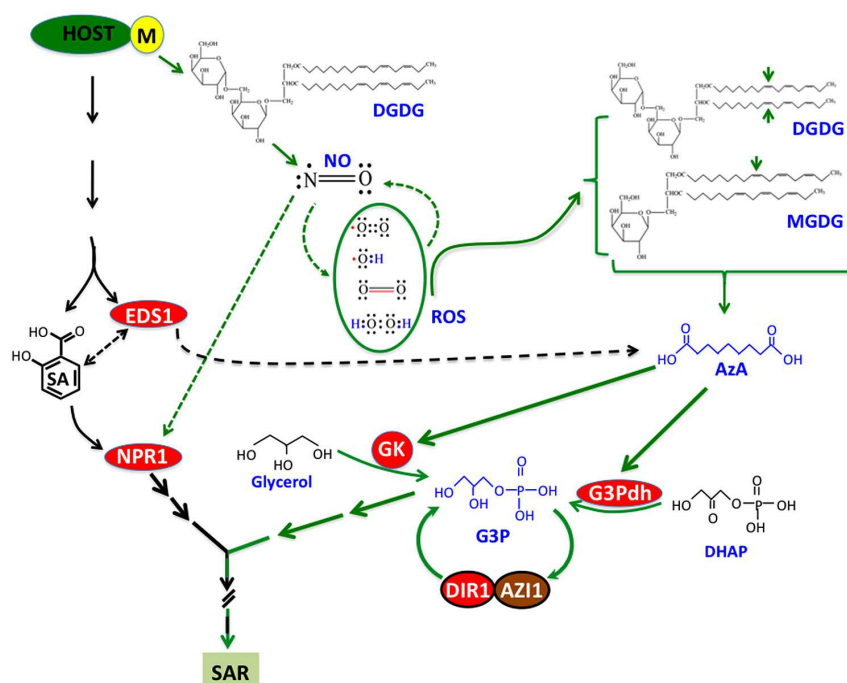


Figure 1.11 : A simplified model showing pathways, chemicals, and proteins involved in SAR (Gao et al., 2015).

Infection with avirulent (avr) pathogen induces accumulation of SA and nitricoxide (NO) in a digalactosyldiacylglycerol (DGDG)-dependent manner. NO operates in a feedback loop with reactive oxygen species (ROS), which catalyze oxidation of C18 unsaturated fatty acids (FA) present on monogalactosyldiacylglycerol (MGDG) and DGDG lipids. Oxidation of C18 FAs at C9 carbon (indicated by the arrows) generates azelaicacid (AzA), which triggers biosynthesis of glycerol-3-phosphate (G3P) via upregulation of genes encoding G3P biosynthetic enzymes (glycerol kinase, GK and G3P dehydrogenase, G3Pdh). G3P-mediated signaling is dependent on DIR1 and AZI1, which interact with each other and require G3P for the stability of their respective transcripts. Conversely, DIR1 and AZI1 are also required for G3P biosynthesis, suggesting that G3P and DIR1/AZI1 regulate SAR via a feedback loop. In the SA branch, EDS1 regulates both SA and AzA levels. NPR1 is a key downstream component in the SA branch which is nitrosylated by NO (Gao et al., 2015).

The transcription activator NPR1 induces the production of pathogenesis-related (PR) proteins. The term PR proteins was originally used to indicate “those 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” (Van Loon & Van Strien; 1999 et Van Loon et al., 2006). However, this proposed definition caused confusion in the past as the term PR proteins was often used to designate all microbe-induced proteins, including enzymes like phenylalanine ammonia-lyase which are constitutively present but also increase during most infections. These proteins also detected in healthy tissues were named pathogenesis related like (PRLs) proteins (Van Loon et al., 1994). PR proteins are now defined as “all microbe-induced proteins and their homologues to the extent that enzymes which are generally present constitutively and only increased during most infections” (Van Loon et al., 2006). Plant pathogenesis related (PR) proteins are generally induced by various types of pathogens such as viruses, bacteria, and fungi. Some of these proteins are also expressed in response to some chemicals that act in a similar way as pathogen infection. However, some of the PR proteins are constitutively expressed in some organs or during certain developmental stages. They are regarded as PR-like proteins because of their sequence homology. However, some PR-like proteins are found to be strongly induced by infections and hence they are also designated mostly as PR proteins (Sinha et al., 2014).

The functions of the seventeen families of PR proteins are presented in the table 1.1. PR proteins exert a broad range of biological actions: chitinases which degrade hyphal walls, permatins, and proteins facilitating the permeabilization of membranes or enzyme inhibitors. A lot of PR proteins remain poorly known. Studies with transgenic plants expressing PR proteins are helpful to understand the mechanism of action of these proteins (Van Loon et al., 2006).

Table 1.1: PR proteins families, adapted from Sinha et al. (2014).

Family	Proteins	Functions
PR-1	PR-1 a, PR-1 b, and PR-1 c	Antifungal
PR-2	β -1,3-Glucanases	Cleaves β -1,3-glucans
PR-3	Chitinase types I, II, IV, V, VI, and VII	Endochitinase
PR-4	Chitinase types I and II	Antifungal and chitinase
PR-5	Thaumatin-like proteins	Antifungal
PR-6	Tomato proteinase inhibitor I	Proteinase inhibitor
PR-7	Tomato endoproteinase P	Endoproteinase
PR-8	Cucumber chitinase	Chitinase III
PR-9	Tobacco lignin-forming peroxidase	Peroxidase
PR-10	Parsley “PR-1” Bet v 1, Mal d 1, Api g 1, and Dau c 1	Ribonuclease-like
PR-11	Tobacco chitinase type V	Chitinase
PR-12	Radish Rs-AFP3	Defensin
PR-13	<i>Arabidopsis</i> THI2.1	Thionin
PR-14	Lipid transfer proteins	Shuttling of phospholipids and fatty acids
PR-15	Barley OxOa	Oxalate oxidase
PR-16	Barley OxOLP	Oxalate-like oxidase
PR-17	Tobacco PRp27	Unknown

3.2.2. Induced systemic resistance

In contrast with SAR, the second form of induced resistance, called induced systemic resistance (ISR), is triggered by non-pathogenic but plant growth-promoting microorganisms, including fungi (PGRFs) or rhizobacteria (PGRPs). Phenotypically, ISR is similar to SAR with a lower disease severity on elicited plants. However, the molecular mechanisms are different. ISR requires responsiveness to both jasmonic acid (JA) and ethylene (ET) pathways (Van Wees et al., 2008) and the defense response, controlled by NPR1 protein, is different. In ISR, the defense response is called priming (Conrath, 2006). Priming consists in an induced state, non-perceptible in healthy conditions, which allows the plant to develop more rapidly its defense mechanisms when a pathogen attacks. The system of priming allows a good protection of the plant with minimal costs: nothing is produced before the arrival of the pathogen (Vos et al., 2013).

JA and its active derivative jasmonyl-isoleucine (JA-Ile) play an important role in the regulation of defense against necrotrophic pathogens and chewing herbivores (Pieterse et al., 2014). Furthermore, jasmonates, which include biological active intermediates in the pathways for JA biosynthesis and the biologically derivatives of JA, affect many plant processes as fruit ripening, production of viable pollen, root growth or tendril coiling.

JA biosynthesis begins via the oxylipin pathway with the release of α -linolenic acid (α -LA) from membrane lipids in chloroplast and its conversion to 12-oxo-phytodienoic acid (OPDA). OPDA migrates into peroxisome and undergoes a reduction and three rounds of oxidation to form JA. JA itself can be further metabolized into active or inactive derivatives. Methyl JA (MeJA) is a volatile compound that is potentially responsible for interplant communication. JA conjugated, via the JA conjugate synthase JAR1, with amino acid isoleucine (Ile) results in JA-Ile, which is currently the only known JA derivative needed for JA signaling (Katsir et al., 2008). Under uninduced conditions, jasmonate ZIM (JAZ) proteins bind to transcriptional regulators like histone deacetylase 6 (HDA6) or the complex novel interactor of JAZ (NINJA)/Corepressor Topless (TPL) and prevent untimely activation of

JA pathway. However, in the presence of JA or its bioactive derivatives, the physical interaction between JAZ proteins and transcriptional factor are broken and JAZ interact with SCF^{COI1} complex composed by the F-box protein Coronatine intensive 1 (COI1). Binding of JA-Ile to COI1 leads to ubiquitinylation and subsequent degradation of JAZ repressor proteins through the proteasomes and activation of a large number of JA-responsive genes (Chini et al., 2007) (figure 1.12).

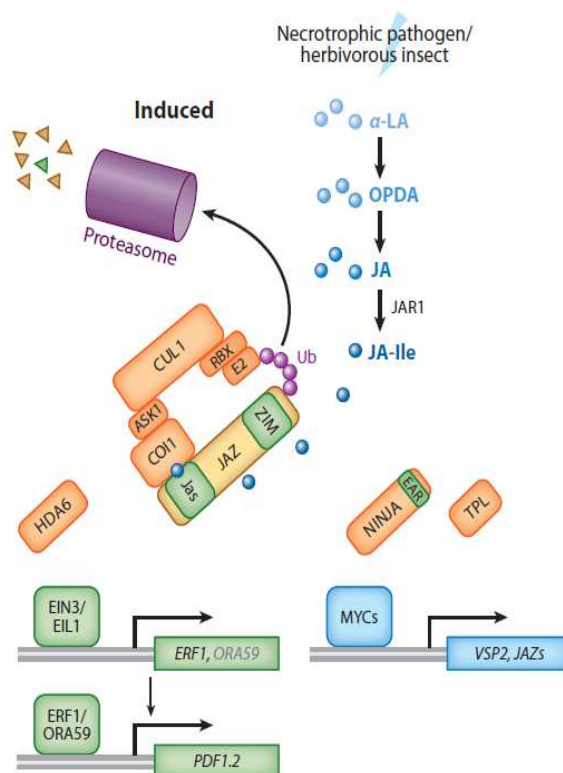


Figure 1.12 : **Schematic overview of the jasmonic acid (JA) signaling pathways (Pieterse et al, 2012).**

Solid arrows indicate established activities or accumulation of compounds. ORA59 (grey text) is a hypothesis target of EIN3/EIL1. Abbreviation: α -LA, α -linolenic acid; EIN3, Ethylene insensitive 3; EIL1, EIN3-like1; ERF, Ethylene Response Factor; JA-Ile, jasmonyl-isoleucine; JAR1, JA conjugate synthase; JAZ, jasmonate ZIM proteins, HDA6, Histone deacetylase 6; MYC-type transcription factors; NINJA, novel interactor of JAZ; OPDA, 12- oxo-phytodienoic acid; ORA59, octadenoic-responsive *Arabidopsis* 59; PDF1.2, plant defensin 1.2; SCFCOI1 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, Corepressor Topless; Ub, ubiquitine; VSP2, Vegetative-storage-protein 2.

Ethylene (ET) is a gaseous hormone which has S-adenosyl-methionine and 1-aminocyclopropane-1-carboxylic acid as biosynthesis precursors (Wang et al., 2002). This plant hormone is involved in many aspects of the plant life cycle, including seed germination, root hair development, root nodulation, flower senescence, abscission, and fruit ripening (Johnson & Ecker, 1998). Ethylene is also a major constituent of defense signals in plants and functions as a modulator of plant immunity (Von Dahl & Baldwin, 2007).

ET gas is perceived by a family of ET-associated receptors (ETR/ERS) localized in membranes (Sakai et al. 1998) (Figure 1.13). In the absence of an ethylene signal, ethylene receptors activate a Raf-like kinase, constitutive expression of *PR* gene 1 (*CTR1*) that negatively regulates the downstream ethylene response pathway, possibly through a MAP-kinase cascade. Binding of ethylene inactivates receptors, resulting in deactivation of *CTR1*, which allows several positive regulators (*EIN2*, *EIN5*, *EIN6* and the transcription factors *EIN3* and *EIL1*) to function. *EIN2* positively signals downstream to the *EIN3* family of transcription factors located in the nucleus. *EIN3* binds to the promoter of *ERF1* gene and activates its transcription in an ethylene-dependent manner. *ERF1* is also involved in JA-mediated gene regulation transcription factors. *ERF1* and other ethylene-response-element-binding proteins (EREBPs) can interact with the GCC box in the promoter of target genes and activate downstream ethylene responses including *PDF1.2* and *HookLess1* (*HLS1*). These genes encode effector proteins that are needed to execute a wide variety of ethylene responses, from disease resistance to differential cell growth (Wang et al., 2002)

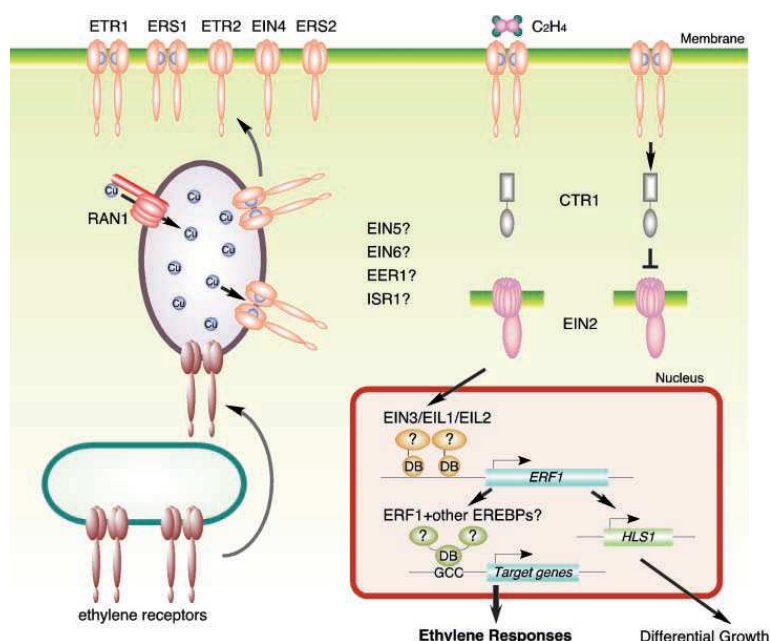


Figure 1.13 : **Model of Ethylene Signal transduction Pathway (Wang et al, 2002).**

There are five ethylene receptors in *Arabidopsis*, ETR1, ETR2, EIN4, ERS1, and ERS2. Ethylene binding occurs at the N-terminal transmembrane domain of the receptors, and a copper co-factor is required for the binding. RAN1, a copper transporter, is involved in delivery of copper to the ethylene receptor. In the absence of an ethylene signal, ethylene receptors activate a Raf-like kinase, CTR1, and CTR1 in turn negatively regulates the downstream ethylene response pathway, possibly through a MAP-kinase cascade. Binding of ethylene inactivates the receptors, resulting in deactivation of CTR1, which allows Ethylene insensitive 2 (EIN2) to function as a positive regulator of the ethylene pathway. EIN2 positively signals downstream to the EIN3 family of transcription factors located in the nucleus. EIN3 binds to the promoter of Ethylene response factor 1 (*ERF1*) gene and activates its transcription in an ethylene-dependent manner. Transcription factors ERF1 and other ethylene-response-element-binding proteins (EREBPs) can interact with the GCC box in the promoter of target genes and activate downstream ethylene responses.

3.2.3. Interactions between SA and JA and ET defense pathways

In nature, plants are simultaneous or sequentially attacked by multiple enemies with often different strategies and lifestyles. To build an optimal defense, cross-talk between SA, JA and ET pathways allows the plant to divert resources to the most appropriate defense mechanisms

(Pieterse et al., 2012). Antagonistic relationships are most often reported but synergistic SA and JA/ET pathway interactions also occur (Mur et al., 2008). The crossroad between rhizobacterium-derived signal (ISR) conducted by JA/ET and systemic-derived signal (SAR) transduced by SA is NPR1 (Spoel et al., 2003) (Figure 1.14). However, the defense responses that are activated downstream of NPR1 are clearly different. Sequence analysis revealed that *NPR1* contains a functionally ankyrin-repeat domain that might be involved in protein–protein interactions (Cao et al., 1997). Binding of pathway-specific proteins to NPR1 might therefore account for the separation of the SAR and ISR pathways downstream of NPR1. Studies have also shown that ISR and SAR can be activated simultaneously, resulting in an additive level of protection (Van Wees et al., 2000).

ET and JA signaling may also function independently to regulate distinct processes in defense response and interact with SA. SA-JA cross-talk components include the protein kinase MPK4 (Petersen et al., 2000) and in particular the interplay of transcriptional regulators appears to play large roles in SA–JA antagonism. Over-expression of WRKY70, a transcription factor, increased SA-dependent genes expression (*PR1*, *PR2*, and *PR5*) and suppressed JA-dependent defense gene transcription (*COR1* and *VSP1*); with anti-sense WRKY70 plant displaying opposite effects (Mur et al., 2013). Interactions also exist between SA and ET pathways. In enhanced disease resistance 1 (*EDR1*) mutant, ethylene potentiates SA-mediated *PR-1* gene expression. In the absence of constitutive expression of *PR* gene (*CPR5*, *CPR6*), the ethylene pathway can also activate SA-mediated *PR-1* gene expression independent of NPR1 to promote SAR.

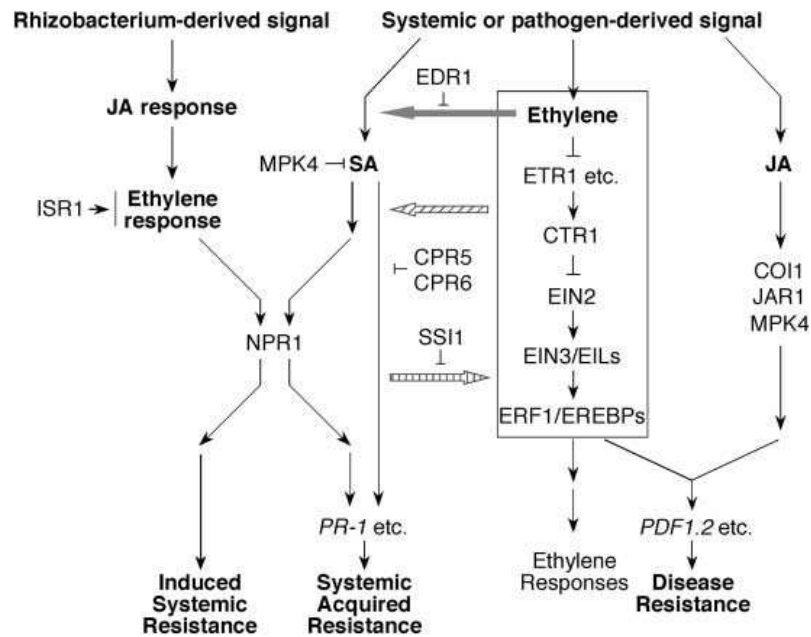


Figure 1.14 : Interactions between Ethylene (ET), Jasmonic acid (JA) and Salicylic acid (SA) in plant disease resistance (Wang et al., 2002).

The ET signal transduction pathway can interact with the JA pathway to co-regulate expression of a subset of defense-related *PR* genes, for example, *PDF1.2*, involved in plant disease resistance. Meanwhile, there are considerable interactions between JA/ET- and SA-dependent pathways in systemic acquired resistance. In *edr1* mutant, ethylene potentiates SA-mediated *PR-1* gene expression. In the absence of *CPR5* and *CPR6*, the ethylene pathway can also activate SA-dependent *PR-1* gene expression independent of *NPR1* to promote systemic acquired resistance. In the *ssi1* mutant, the JA/ethylene-dependent *PDF1.2* gene is constitutively expressed. Moreover, the ethylene pathway is also required for the rhizobacteria-mediated induced systemic resistance, which is independent of SA and pathogenesis-related gene activation. ET signaling acts downstream of the JA pathway but upstream of *NPR1* in ISR activation. Plants that lack *ISR1* fail to develop ISR and display ethylene insensitivity. Arrows indicate positive regulation, and open blocks indicate negative regulation.

3.2.4. The emerging players and their crosstalk with SA, JA and ET defense pathways

The essential defensive roles of primary defense hormones, JA, SA and ET, have been well established. Other phytohormones, such as abscisic acid (ABA), auxins (indole-3-acetic acid [IAA]), cytokinins (CKs), brassinosteroids (BRs), gibberellins (GA), and strigolactones, which are better known for their roles in stress tolerance or plant growth and development, also regulate plant defense, either alone or in conjunction with the primary defense hormones (Torres-Vera et al., 2014).

It is not surprising that pathogens have developed capabilities to manipulate or subvert plant phytohormone signaling pathways for their benefit. Tactics frequently employed by plant pathogens involve hijacking, evading, or disrupting hormone signaling pathways and/or crosstalk. As explained in the following section, the lifestyle of a pathogen often dictates the type of phytohormone intervention strategy adopted.

3.3. Contrasting plant defense mechanisms according to the lifestyles of the pathogens

Plants pathogenic micro-organisms can often be distinguished into three broad classes based on their modes of nutrition. Biotrophs are pathogens that derive their nutriment from living host tissues (Glazebrook et al., 2005). Necrotrophs infect and kill host tissue and extract nutrients from dead host cells. A third group, hemibiotrophic organisms, display both forms of nutriment acquisition shifting between an early biotrophic phase, allowing invasive hyphae to spread throughout the infected plant tissue, to necrotrophy at later stage of disease during which toxins are secreted by the pathogen to induce host cell death (Lo Presti et al., 2015). Fundamental differences exist between biotrophs and necrotrophs in many aspects of their pathogenesis. The infection processes, histology of disease, infection-related morphogenesis, nature of effector proteins, associated host defense responses, and most importantly their nutrient acquisition strategies vary significantly. These

differences account for why one defense strategy may effectively restrict biotrophs and enable necrotrophs (Laluk & Mengiste, 2010).

For the success of pathogenesis including attachment, host recognition, penetration and proliferation, biotrophic fungi develop intimate relationships with their host cells using infection structure regulated by gene expression and complex regulatory pathways (Kahmann & Basse, 2001). These pathogens also secrete limited amounts of cell-wall-degrading enzymes (CWDE) and generally lack toxin production (Mengiste, 2012). Typically, biotrophic fungi produce nutriment-absorbing structures called haustoria within the living plant cell from which nutriments are taken-up (Mendgen & Hahn, 2002). By their feeding activities, they create a nutrient sink at the infection site that causes serious economic losses and threaten global food security (Koeck et al., 2011). For a compatible interaction with the host plant, biotrophic pathogens have to pass the complex multi-layered defense but also suppressed host defense mechanisms. Fungus protection may include fungal chitin shield which protects the fungal cell wall and the chitin fragments from chitinases. Plants secrete beta-1,3-glucanases to damage fungal cell walls but some pathogen produces glucanase inhibitor protein. Other effectors (proteinase inhibitors and phytoalexin detoxifying enzymes) may aid the pathogen's success too (Gebrie, 2016). Once the fungal effector passes the plant defense mechanism the plant will not resist. Subsequently, the plant reduces production of defense signaling molecules like salicylic acid (Mendgen & Hahn, 2002). As discussed above, plants can also respond to pathogen attack by activation of a large number of defense mechanisms. Against biotrophic fungal pathogens, two main strategies are used by plants to restrict the invasion and growth: penetration resistance and gene-for-gene resistance. Plants can strengthen cell wall and membrane to halt spore germination and prevent the formation of the haustorium by penetration resistance. The gene-for-gene resistance applies inside the penetrated epidermal cell and it is associated with the activation of SA-dependent signaling and activation of the systemic acquired resistance (SAR) in distal tissues, which reduces the effects of secondary attacks. Recognition and activation of the incompatible interaction result in several responses to limit the feeding strategy and the propagation of the pathogen to the infection site. That includes the accumulation of reactive oxygen species (ROS) and pathogenesis-related (PR) proteins, the

induction of the hypersensitive response (HR) which confines biotrophs by abolishing nutrient supplies thereby limiting pathogen growth, callose deposition, tissue disorganization, changes in the size and shape of chloroplasts, nuclear and nucleolar degradation, and programmed cell death (PCD) (Alazem & Lin, 2015; Baebler et al., 2014).

Where biotrophs have developed intricate infection strategies aimed at maintaining host viability, necrotrophs are facultative saprophytes that deploy diverse phytotoxic compounds and CWDEs including laccases, proteases, pectinases, and specifically endo-polygalacturonases (endo-PGs) (Kars et al., 2005) to induce cell necrosis and cause leakage of nutrients (Oliver & Ipcho, 2004). Necrotrophs are further divided into two groups. The first one is the host specific necrotrophs group. The pathogens from this group produce host specific toxins (HST) that are essential for their pathogenicity and virulence to their specific host (Mengiste, 2012). For example, *Cochliobolus carbonum* produces HC toxin to cause Northern corn leaf spot disease in maize and *Alternaria alternata* f. sp. *lycopersici* produces AAL toxin to cause stem canker disease in tomato. *Alternaria solani* synthesizes phytotoxic metabolites, especially alternaric acid and zinniol, to cause early blight and collar rot diseases on tomatoes. The second group is composed of the broad-host necrotrophs that produce an extensive array of compounds, enzymes and toxins which alone or in combination likely interfere with common structural and functional features shared among different plant families (Laluk & Mengiste, 2010). The pathogens *Botrytis cinerea*, *Alternaria brassicicola*, *Plectosphaerella cucumerina*, and *Sclerotinia sclerotiorum* are good examples of such broad host range fungal necrotrophs. To cause disease, necrotrophic pathogens actively circumvent or suppress the plant defense weaponry and/or modify the host cellular and physiological environment. Generally, necrotrophs are considered less sophisticated organisms that deploy ‘brute force’ mechanisms to overcome the host. It is now understood that necrotrophic pathogens employed protection of fungal structures, inhibition of elicitor-induced defenses, de-toxification of plant defense compounds and manipulation of specific plant proteins or physiological processes to evade host immune responses. Owing to their defense functions, plant hormones are major targets of pathogen attack and manipulation (Mobius & Hertweck, 2009). *Botrytis cinerea*, for example, produces ET and ABA that may

perturb host response pathways by promoting HR-like symptoms with features of plant programmed cell death (PCD) and activation of the HR that promotes susceptibility to necrotrophs (Govrin & Levine, 2000). Defense against necrotrophic pathogens include constitutive as well as induced physical and chemical barriers. Multiple PAMPs, DAMPs, and effectors from necrotrophic pathogens are perceived by various receptors in the plants. PAMPs of necrotrophic fungal pathogens include constituents of the fungal cell wall such as chitin, chitosan, and endopolygalacturonases which are perceived by pattern recognition receptors (PRRs) of host plants to activate PTI. Following infection by necrotrophic fungal pathogens, plants produce also small signaling molecules and peptides such as DAMPs which trigger defense response. The fungal necrotroph, *B. cinerea*, produces polygalacturonases (PGs), as one of the virulence components recognized by the host plant via two mechanisms. In the first mechanism, these directly function as PAMPs which are recognized by Responsiveness to polygalacturonases 1 (RBPG1), a receptor-like protein to activate defense response against *B. cinerea* (Zhang et al., 2014). Plant polygalacturonase inhibiting proteins (PGIPs) counteract PGs contributing to basal resistance against necrotrophic infection in various species (Ferrari et al., 2003; D'Ovidio et al., 2004). The contribution of PGs to basal resistance is not only by directly blocking cell wall degradation but also by enhancing accumulation of long chain oligogalacturonides (OGs) (D'Ovidio et al., 2004). OGs are fragments released following partial degradation of the primary cell wall capable of activating innate immune responses as damage-associated molecular patterns (DAMPs) (Boller & Felix, 2009). Depending on the degree of chain polymerization, OGs induce oxidative burst, cell wall lignification, phytoalexin accumulation, protease inhibitor expression, and changes in ion fluxes as well as SA, JA, and ET biosynthesis (D'Ovidio et al., 2004). Other cell wall components may also serve as DAMPs as *Phytophthora* cellulose binding lectin (CBEL) protein or plant-derived peptides Pep1 and Pep2. Due to their destructive pathogenesis strategies, DAMP-triggered immune responses may be commonly activated by necrotrophic pathogens and involve an array of defense responses including the accumulation of PR proteins, defensins, antimicrobial compounds and activation of hormone-regulated defenses like SA, JA, ET but also ABA, Auxins and GAs. In addition to PAMPs and DAMPs, fungal necrotrophs produce various effectors, such as pathogenic proteins and toxins, which are sometimes perceived by host

cell receptors to trigger ETI. *RLM3*, A Toll-interleukin receptor domain which has been identified in *Arabidopsis*, is involved in ETI response against necrotrophic fungal pathogens, such as *B. cinerea*, *A. brassicicola*, and *A. brassicae* (Staal et al., 2008). However, the effector that binds with *RLM3* has yet to be identified. In general, the perception of necrotrophic fungal effectors leads to effector-triggered susceptibility (ETS) or HR cell death which favors colonization of host plants by necrotrophs (Pandey et al., 2016).

3.4. Elicitors that induce resistance

As described by Thakur and Sohal (2013), the term ‘elicitor’ was originally exclusively used for molecules capable of inducing the production of phytoalexins. The term is now commonly used for molecules that stimulate any type of plant defense mechanism. Elicitors can be natural substances that come from the pathogen (exogenous elicitors) or released from plants by the pathogen’s action (endogenous elicitors) (Boller, 1995). Elicitors can also be synthetically generated.

Elicitor activity has been revealed for plant-derived cell wall components, proteinaceous pathogen molecules, oomycete-derived elicitor INF1 and non-proteinaceous molecules (Wielsel et al., 2014). Non-pathogenic microorganisms can also be recognized by plants and are classified in three groups: cell surface compounds, metabolites under iron regulation and antibiotics (Ongena and Thonart, 2006). Synthetic elicitors also called chemical inducers or plant health promoter may trigger defense reactions by mimicking interactions of natural elicitors or defense signaling molecules with their respective cognate plant receptors or by interfering with other defense signaling components (Bektas & Eulgem, 2014). First synthetic elicitors, polyacrylic acid derivatives, were identified by Gianinazzi & Kassanis (1974). These molecules were able to activate *PR-1* gene expressions and mediate resistance of tobacco (*Nicotiana tabacum*) against *Tobacco mosaic virus* (TMV) or *Tobacco necrosis virus* (TNV). Since then, many other chemical activators including benzothiadiazole (BTH, chemical analogue of SA), the non-protein amino acid β -aminobutyric acid (BABA) or 2,6-dichloroisonicotinic acid (INA) have been used with various efficacy

depending to the pathogen and the host plant (species and variety) (Thakur & Sohal, 2013).

4. COS-OGA elicitor

COS-OGA is a defense elicitor based on the MAMP/DAMP combination containing chitosan oligomers (COS) and pectin-derived oligogalacturonides (OGA). COS-OGA was developed by the Laboratory of Plant Biology at the University of Namur and is produced by Fytofend SA. It was recently registered at European level as FytoSave® and contains 12.5 g/L COS-OGA as active substance.

Pectin is a structural heteropolysaccharide contained in the primary cell wall of plants where it constitutes a dense molecular network. Acidic pectin (homogalacturonans) binds calcium ions and forms chain dimers called egg-boxes which play important roles in the properties of the pectin network (Cabrera et al., 2010; Grant et al., 1973). Upon invasion, most pathogens secrete enzymes that degrade the wall pectin into oligogalacturonides (OGA). OGAs function as elicitors of plant defense responses (Van Cutsem and Messiean, 1994; Ridley et al., 2001). They can also adopt a calcium-induced egg-box conformation (Figure 1.15a).

Chitin is a long-chain polymer of *N*-acetylglucosamine, and is a characteristic component of the cell walls of fungi and vertebrates like, arthropods (crustaceans and insects), mollusks and cephalopods (Tang et al., 2015). Plants attacked by fungi respond by secreting pathogenesis-related proteins among which several chitinases that break down glycosidic bonds in chitin. Chitosan oligosaccharides (COS) (Figure 1.15a) that result from chitin deacetylation and hydrolysis can elicit defense responses mostly in dicots (Shibuya and Minami, 2001). Chitosan is a cationic polymer that can interact with negatively charged OGA and stabilizing the egg-box conformation of OGA (Figure 1.15) (Cabrera et al., 2010). These molecules, COS and OGA, therefore mimic a plant pathogen interaction as a PAMP (COS) and a DAMP (OGA) (El Gueddari et al., 2002; Van Cutsem and Messiean, 1994). The stabilized complex of calcium, COS and OGA constitutes a warning signal to the plant in response to the simulation of wall degradation due to the presence of a pathogen. This complex has been reported to trigger

defense responses (e.g., alkalinization, potassium efflux, oxidative burst) of *Arabidopsis thaliana* cell suspensions (Cabrera et al., 2010).

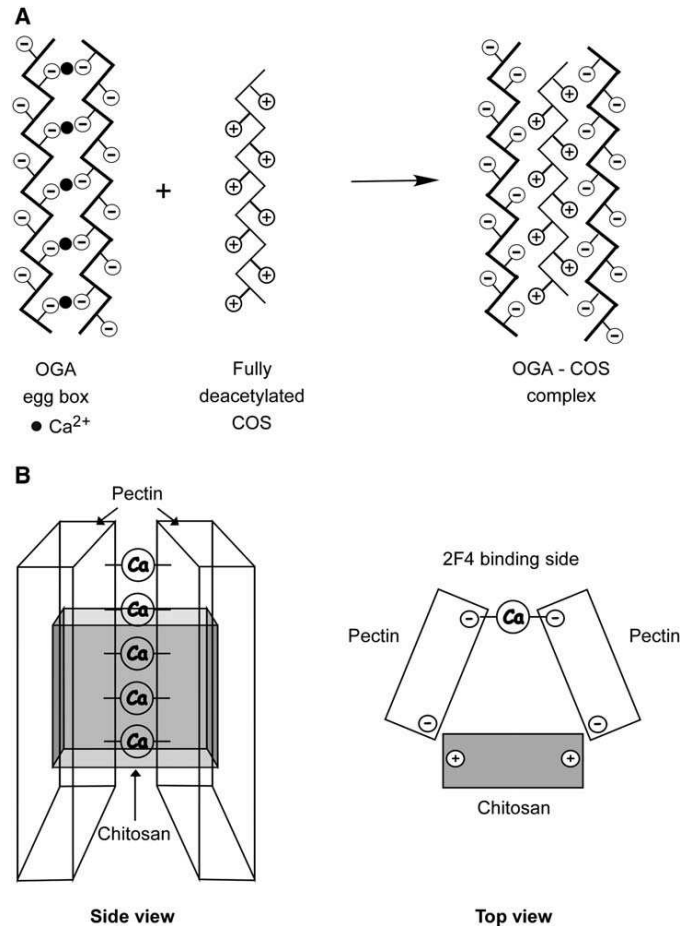


Figure 1.15 : **Model for the interaction of chitosan oligosaccharides with OGA**(Cabrera et al., 2010)

(a) Schematic representation of OGA egg box disruption by fully deacetylated COS. Polycationic COS interact with identically spaced negative charges of OGA, replacing calcium ions and dismantling pectic egg boxes. (b) Schematic representation of OGA egg box stabilization by interaction with partly acetylated COS. Side view (left) represents pectin egg box dimer with at least five calcium ions that bind. A positively charged, partly acetylated chitosan molecule binds external negative charges of calcium-bound pectin while higher COS concentrations could dislodge calcium from the complex. The chitosan molecule being shorter than the OGA, the extremities of the egg box remain accessible. Top view (right) schematically represents the location of charges on the oligomers. The 2F4 antibody could still bind the COS-stabilized egg box dimer on the calcium-binding side of the complex.

When this PhD was started, a first composition comprising COS-OGA was available. In 2015, this composition was registered in Europe as FytoSave® against powdery mildew in *Cucurbitaceae*, *Solanaceae* and grapevines. It has proved to be effective in protecting cucumbers against low to moderate pressure of *Sphaerotheca fuliginea* (72-85% protection compared with the control in terms of severity) and grapevines against *Uncinula necator* (from 96% during the first assessment to 76% at the end of the experiment). These pathogens are biotrophs. The plant defense mechanisms involved in response to the application of this elicitor on tomato leaves seems to be rely on SAR as COS-OGA sprayings induce a cumulative accumulation of SA as well as an overexpression of SA-mediated gene expression (van Aubel et al., 2014). As degree of polymerization of OGA, the degree of OGA methylation, the degree of acetylation and the pattern of acetylation of COS can strongly modify the way oligosaccharides stimulates PTI (Cabrera et al., 2006, Madhuprakash et al., 2015; Ridley et al., 2001), another composition, called FytoSol, was developed by Fytofend SA in 2013. This composition is still under development.

Chapter 2

Studying the effect of FytoSave®, a COS-OGA elicitor, against *Phytophthora infestans*, a hemibiotrophic pathogen on potato

Opening comments

The main objective of this thesis is the evaluation of the potential use of the COS-OGA elicitor to protect *Solanaceae* against diseases caused by fungi or oomycetes. In order to meet this objective, we have firstly studied the effect of COS-OGA on potato late blight. Preliminary assays were performed under controlled conditions in order to standardize the pathosystem *Solanum tuberosum*-*Phytophthora infestans* under controlled conditions: culture media, the quantity of inoculum or the age of plants were optimized to produce moderate to high infection (data not shown). After these preliminary assays, we have tested the potential effect of FytoSave®, the commercial product containing COS-OGA, against potato late blight. This product was shown to be effective against the biotrophic pathogens involved in the powdery mildews on cucurbits and *Solanaceae* (van Aubel et al., 2014). *P. infestans*, the agent of potato late blight, differs from them by its pathogenesis strategy: it is a hemibiotrophic pathogen which displays an early biotrophic phase followed by a necrotrophic phase. The second objective of this study was to decipher, to some extent, the mode of action of this product and the eventual induction of plant defense by a differential gene expression analysis using quantitative PCR assays.

This chapter is adapted from the paper published in Plant Pathology³ and titled **‘The novel elicitor COS-OGA enhances potato resistance to late blight’**

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A. Clinckemaillie^a, A. Decroës^a, G. van Aubel^b, S. Carrola dos Santos^a,
M. E. Renard^a, P. Van Cutsem^b and A. Legrève^a

^a Université catholique de Louvain, Earth and Life Institute, Applied Microbiology, Phytopathology, Croix du Sud 2 box L7.05.03, 1348 Louvain-la-Neuve, Belgium

^b Université de Namur, Unité de Recherche en Biologie végétale, Rue de Bruxelles 61, 5000 Namur, Belgium

Contribution in this paper by authors (initial)

Experimental procedure: AC and AL

Establishment and harvesting of the bioassays: AD, AC, MER and SCdS

In vitro test: AC and MER

Gene profiling: AD, AC, GvA, MER and SCdS

Data analysis, AC and AD

Writing paper: AC

Critical reviews: AL, PVC

1. Abstract

Phytophthora infestans is the causal agent of potato late blight. This pathogen is usually controlled by fungicides, but new European regulations have imposed changes in crop protection management that have led to a search for alternative control measures. The induction of plant defense responses by elicitors is a promising new strategy compatible with sustainable agriculture. This study investigated the effect of eliciting a defense response in potato against *P. infestans* using a formulation of the COS-OGA elicitor⁴ that combines cationic chitosan oligomers (COS) and anionic pectin oligomers (OGA). Trials were conducted under greenhouse conditions to assess the ability of COS-OGA to control *P. infestans*. The results showed that three foliar applications with this elicitor significantly increased potato protection against late blight in controlled conditions. The activation of potato defense genes was also evaluated by RT-qPCR during these trials. Two pathogenesis-related proteins, basic PR-1 and acidic PR-2, were rapidly and significantly up-regulated by the elicitor treatment. Therefore, these

⁴ Formulation used is FytoSave®

results suggest that the COS-OGA elicitor increases the protection of potato against *P. infestans* and that this protection could be explained by an increase in the expression of potato defense genes rather than by biocide activity.

2. Introduction

Late blight in potato (*Solanum tuberosum* L.) and tomato (*Solanum lycopersicum* L.) is caused by *Phytophthora infestans* (Chromista, Oomycota). This polycyclic and hemibiotrophic pathogen is controlled mainly by repeated fungicide treatments, up to one application a week when conditions favor infection, which are costly and do have environmental side effects (Cooke et al., 2011).

Recent advances in the understanding of plant defense mechanisms have opened up a new approach to managing plant diseases in an economic and sustainable way. The innate immunity of plants is a complex of interconnected mechanisms that plants use to resist potential pathogens and herbivore attacks. These mechanisms also take into account the evolution of the plant immune system and the sequential events in pathogenesis in a ‘zigzag model’ (Jones & Dangl, 2006). Common microbial components, such as bacterial or fungal constituents (known as pathogen or microbe-associated molecular patterns, PAMPs or MAMPs; Ausubel, 2005) or molecules released from the plant host by wounding or enzymatic degradation (known as damage-associated molecular patterns, DAMPs; Vorwerk et al., 2004) can be recognized by cell surface pattern-recognition receptors (PRRs) (Boller & Felix, 2009). The recognition of these molecules by a host membrane receptor induces primary non-host immune resistance in the plant that can reduce infection by the pathogen. This response is called PAMP triggered immunity (PTI). However, pathogens have evolved and are able to release effector molecules that they inject directly into the cytoplasm. These effectors interfere with PTI and make possible the nutrition and dispersal of the pathogen. This is known as effector-triggered susceptibility (ETS). As a co-evolutionary response, plants have acquired the ability to recognize the presence and activity of these effectors. This co-evolved plant defense is called effector-triggered immunity (ETI) and induces a hypersensitive response (HR) (Jones & Dangl, 2006). The relationship between host and pathogen has thus

evolved into a gene-for-gene interaction based on the specific recognition of a pathogen protein, called the avirulence (Avr) protein, by a plant protein called the R (resistance) protein (Jones & Dangl, 2006).

In addition to conferring total resistance to the pathogen that is present, the HR induces one of the systemic resistance pathways, known as systemic acquired resistance (SAR), which will be active against future pathogen infections. The induced resistance can be defined as a 'state of enhanced defensive capacity' (van Loon et al., 1998). SAR and induced systemic resistance (ISR) are two forms of induced resistance. SAR is triggered by localized pathogen attacks and uses the salicylic acid (SA) pathway to transduce the signal in the whole plant (Dong, 2001). ISR is triggered by nonpathogenic but plant growth-promoting microorganisms, including fungi (PGPFs) or rhizobacteria (PGPRs). ISR relies on jasmonic acid (JA) and ethylene (ET) to transduce the defense signal in the whole plant (Van Wees et al., 2008). COS-OGA is an elicitor that was recently registered at European level as FytoSave® and combines chitosan oligomers (COS) and pectin-derived oligogalacturonides (OGA) (Cabrera et al., 2010). These molecules mimic a plant pathogen interaction as a PAMP (COS) and a DAMP (OGA) (Van Cutsem & Messiean, 1994; El Gueddari et al., 2002). This complex has been reported to be able to trigger defense responses (e.g. alkalization, potassium efflux, oxidative burst) from *Arabidopsis thaliana* cell suspensions (Cabrera et al., 2010). COS-OGA has been assessed under commercial greenhouse conditions against *Sphaerotheca fuliginea* on cucumbers and in the field against *Uncinula necator* on grapevines. It proved effective in protecting cucumbers against *S. fuliginea* (72–85% protection compared with the control in terms of severity) and grapevines against powdery mildew (from 96% during the first assessment to 76% at the end of the experiment; van Aubel et al., 2014). These results encouraged further studies on the potential use of the COS-OGA elicitor against other fungal diseases.

In this study, bioassays were conducted under controlled conditions in order to evaluate the effect of COS-OGA on the susceptibility of potato plants to *P. infestans*. The activation of selected potato genes was also analysed in order to investigate the impact of elicitation with COS-OGA on plant defenses.

3. Materials and methods

3.1. *Phytophthora infestans* inoculum

Strain 10022 (A2 mating type, race 1.3.4.5.7.10.11; CRA-W, Belgium) was used as inoculum in the assays and was maintained on V8 medium. Hyphal tips were transferred from V8 medium onto rye sucrose agar medium and incubated in the dark at 20 °C for 4 weeks to induce sporulation. In order to ensure pathogenicity, the strain was inoculated onto detached potato leaflets before each *in vivo* experiment. After 10 days of incubation at 20 °C, the potato leaflets were rinsed with sterile demineralized water to remove the sporangia that had been produced. A suspension of 10^4 sporangia per millilitre was prepared and placed in a cool room for 4 h at 4 °C in order to stimulate the release of the zoospores.

3.2. *In vitro* test on oomycidal effect of COS-OGA

The oomycidal effect of COS-OGA was investigated by placing hyphal tips in Petri dishes containing V8 media with or without 62.5 mg L⁻¹ active ingredient of soluble liquid (SL) FytoSave® (Fytosend) and incubated in the dark at 17 °C. This was repeated 12 times and the radial growth of the resulting colonies was measured after 7 days and compared for both treatments.

3.3. *Plant growth, treatments and Phytophthora infestans* inoculation

Plantlets of *S. tuberosum* 'Bintje' (CRA-W, Belgium) were produced *in vitro*, subcultured on MS medium, then delicately removed from the media and transplanted into pots containing a sterilized mixture of compost and vermiculite (1:2 v/v). The plants were irrigated daily with Hoagland's solution and maintained under greenhouse conditions with a photoperiod of 16 h at 20/16 °C (day/night temperature). After 4 or 5 weeks growth (at the five-leaflet stage), they were transferred into two separate greenhouse tunnels (ACD; Serrabris) placed inside the main greenhouse. These tunnels were used to separate inoculated and noninoculated plants and to maintain 90% humidity using humidifiers (CUOGHI type NEB-5000; BEPA - SA). Plants were treated with 0.5% (v/v) of FytoSave®, containing 12.5 g L⁻¹ of the active ingredient (COS-

OGA) or with water (control plants) by spraying the upper and lower leaf surfaces until run-off. This was performed on each plant at 7, 3 and 1 days before inoculation of plants with *P. infestans*. Before and after these treatments, a high relative humidity was maintained in the greenhouse to ensure good adsorption of COS-OGA/water by the leaves. Subsequently, half the plants were inoculated with *P. infestans* by spraying 1 mL of sporangial suspension (10^4 sporangia mL⁻¹) on each leaf of the plant using a sterile glass sprayer (Merck). As a control, the remaining plants were sprayed in the same way with water.

3.4. Assessment of disease progress

Disease severity was assessed quantitatively by visual estimation of the percentage of the leaf area covered by symptoms over 7 days using the scale described by James (1971). Disease progress over time was estimated by calculating the area under the disease progress curve (AUDPC). The AUDPC values were calculated using the following equation (Campbell & Madden, 1990):

$$AUDPC = \sum_{i=1}^{n-1} \left[\left(\frac{y_i + y_{i+1}}{2} \right) \times (T_{i+1} - T_i) \right]$$

where y_i is disease severity at the i th observation, T_i is time at the i th observation, and n is the total number of observations.

In addition, the incubation and latency periods, the size of lesions and sporulation were checked daily for 7 days after inoculation using a qualitative scale based on a score between 0 and 5: 0, no symptoms (biotrophic stage); 1, lesions smaller than 0.5 cm (beginning of the necrotrophic stage); 2, lesions larger than 0.5 cm; 3, beginning of sporulation (necrotrophic stage); 4, abundant sporulation covering the entire lesion; 5, coalescence of lesions.

3.5. Gene expression profiling

The expression levels of several genes in the plants were compared at four time points: 0, 15, 48 and 72 h post-inoculation (hpi). The choice of these time points was based on the stages in the infection cycle of this

pathogen (Vleeshouwers et al., 2000; Avrova et al., 2007). The time point 0 h corresponded to inoculation, 15 h to the biotrophic stage, 48 h to the beginning of the necrotrophic stage and 72 h to the necrotrophic stage. At each time point, four plants (biological replicates) for each of the four treatments (described above) were analyzed.

For isolation of RNA, two fully extended leaves were collected from each plant and rapidly ground together into a fine powder in liquid nitrogen. Total RNA was extracted using the RNeasy Plant Mini kit (QIAGEN), following the manufacturer's instructions. DNA was removed from the RNA extracts by DNase digestion using the RQ1 RNase-Free DNase (Promega), following the manufacturer's instructions. The concentration and purity of the total RNA extracts were determined using a NanoDrop ND1000 (Thermo Scientific). The concentration of RNA in extracts was adjusted to $1 \mu\text{g } \mu\text{L}^{-1}$ for each sample before reverse transcription. cDNA was synthesized with an anchored oligo(dT)18 primer, using the Transcriptor First Strand cDNA synthesis kit (Roche) following the manufacturer's instructions.

The expression patterns of four potato defense-related genes, as well as housekeeping genes for normalization of results, were then quantified by real-time reverse transcription quantitative PCR (RT-qPCR). The housekeeping genes were: the elongation factor 1- α (*EF1- α*), glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*; Gallou et al., 2009) and phosphoglycerate kinase (*PGK*) (Ghareeb et al., 2011). The target genes of *S. tuberosum* thought to be involved in plant defense were: genes for the basic pathogenesis related protein 1 (*PR-1*) and acidic pathogenesis-related protein 2 (*PR-2*; Gallou et al., 2009), which are convenient markers for monitoring the defense response (Pieterse et al., 2009); the protease inhibitor 1 (*PI-1*), involved in JA pathway (Fujimoto et al., 2011); and the non-inducible immunity (*NPR1*) gene (van Aubel et al., 2016), implicated in the cross-talk between SAR and ISR (Spoel et al., 2003).

RT-qPCR assays were carried out using SYBR Green chemistry and the primers used for each gene are given in Table 2.1. The reaction components for each sample were 10 μL iQ SYBR Green supermix (Bio-Rad), 0.5 μL of each primer (20 pmol), 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, 1 min at 60 °C and 30 s at 72 °C; and finally, the establishment of the melting curve (55–95 °C with a heating rate of 0.5 °C s⁻¹). Two technical replicates of the qPCR were made for each sample, standard and control.

Standards were obtained by serial dilutions of RNA extracts. Standard curves were generated for all genes in order to examine the linearity of amplification over the dynamic range. The results obtained were treated using CFX MANAGER Software (Bio-Rad) and expressed as normalized relative quantities.

3.6. Data analysis

Data from all the experiments were subjected to analysis of variance (ANOVA) using the SAS ENTERPRISE GUIDE v. 4.1 (SAS Institute Inc.). The treated means were compared using Tukey's HSD test with a confidence interval of 95% ($P < 0.05$).

Table 2.1 List of genes studied with accession number from Sol Genomics Network or NCBI and corresponding primers used for RT-qPCR

Gene	Gene function	Accession no	Primer sequence (5'-3')	References
<i>EF1-α</i>	Elongation factor 1-α	AB061263	ATTGGAAACGGATATGCTCCA TCCTTACCTGAACGCCTGTCA	Gallou et al., 2009
<i>GADPH</i>	Glyceraldehyde 3-phosphate dehydrogenase	AF527779	GGACATTGCTCCAAACGC ATGAGACCCCTCCACAATGC	Gallou et al., 2009
<i>PGK</i>	Phosphoglycerate kinase	SGN-U578082	TCTACAAGGCCCAAGGTTATG GCAGCAAACCTTGTCGCAATC	Ghareeb et al., 2011
<i>PR-1</i>	Basic Pathogenesis Related 1	AJ250136	GGTGCAGGAGAGAACCTT GGTACCATAGTTGTAGTTTGGCT	Gallou et al., 2009
<i>PR-2</i>	pathogenesis related 2	AJ009932	TATCATCAGCAGGGTTGCAAA TCGGGAAAATGCTATTCTAGG	Gallou et al., 2009
<i>NPR1</i>	Non-inducible immunity 1	SGN-U585807	AATCGGCTTAGGGCTCTCTC TGCCTTCTCAGTTGACGCTCT	Van Aubel et al., 2016
<i>PI-1</i>	Protease Inhibitor 1	SGN-U577558	GTGTACCAACAAAGCTTGCTAAAGA GTACAAACAACACCCCAAAATGTTGTC	Fujimoto et al., 2011

4. Results

Oomycidal effect of COS-OGA and its effect on disease severity and progress

The absence of any oomycidal activity of FytoSave® (COS-OGA elicitor) on *P. infestans* was confirmed. In the presence of FytoSave®, the radial growth of colonies reached $93.6 \pm 8.5\%$ of the growth on the control media. A bioassay was conducted under controlled conditions in order to test the effect of FytoSave® on potato infected by potato late blight. Disease progress on potato plants treated by foliar applications with the COS-OGA elicitor before *P. infestans* inoculation was compared with disease progress on plants treated with water (Fig. 2.1).

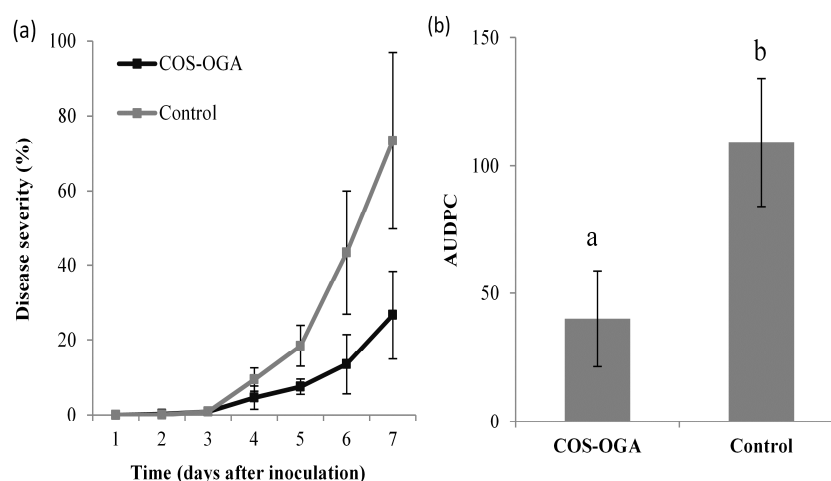


Figure 2.1 Disease severity progress (percentage of leaf area covered with symptoms) (a) and values of area under the disease progress curve (AUDPC) (b) of potato plants elicited with COS-OGA or water (control) and inoculated with *Phytophthora infestans*. No symptoms were observed on non-inoculated plants.

The treatment of plants with FytoSave® resulted in a significant decrease in disease progress. The first symptoms appeared 2 days after inoculation (dai) on both treated and untreated control plants, but disease progress was more rapid on the latter (Fig. 2.1a). By 7 dai, the mean percentage of leaf area covered by symptoms increased to $73 \pm 24\%$ for control plants and $27 \pm 12\%$ for COS-OGA treated plants. The AUDPC values calculated over 7 days differed significantly ($P < 0.05$), depending on the treatment of plants with COS-OGA or water (Fig. 2.1b); the mean AUDPC value reached 109 ± 25 for control plants, which was almost three times greater than the value obtained for COS-OGA-treated plants (40 ± 19). Based on these values, the protection conferred by the COS-OGA elicitor against *P. infestans* was close to 50%.

Qualitative observations of the lesions on the plants revealed that the length of the incubation period remained the same, whether or not plants were treated with COS-OGA, because the lesions appeared at the same time, but sporulation was slightly delayed on COS-OGA-treated plants on day 6 (Fig. 2.2).

A second bioassay was conducted under the same conditions to confirm the above observations. A significant decrease in disease progress on COS-OGA-treated plants was again observed, with a mean percentage leaf area covered by symptoms of $35 \pm 7\%$ for control plants and $22 \pm 2\%$ for COS-OGA-treated plants, at 7 dai. The AUDPC values of potato late blight of non-treated or COS-OGA-treated plants calculated over 7 days were significantly different: 70 ± 10 for non-treated plants and 35 ± 2 for plants treated with COS-OGA. The protection conferred by the COS-OGA elicitor was estimated to be 50%, as in the case of the first bioassay

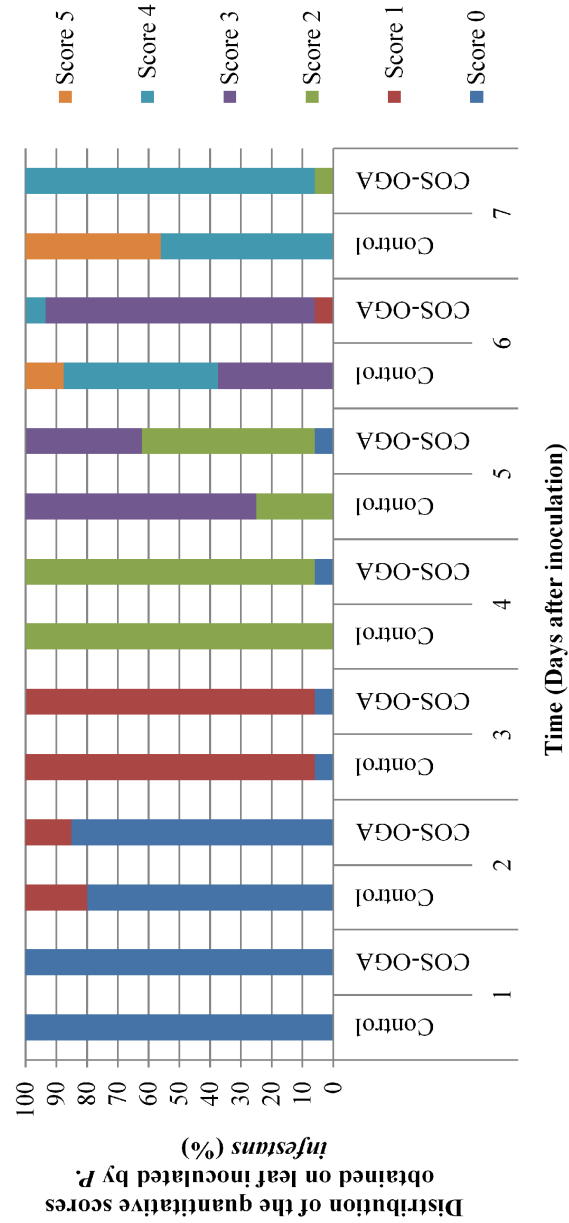


Figure 2.2: Dispersion of qualitative scores of potato late blight lesions on COS-OGA-treated and water-treated plants inoculated with *Phytophthora infestans*. 0, no symptom; 1, lesions smaller than 0.5 cm; , lesions larger than 0.5 cm; 3, beginning of sporulation; 4, abundant sporulation covering the entire lesion; 5, coalescence of the lesions.

Effect of COS-OGA on gene expression in infected and uninfected potato leaves

In order to investigate the impact of treatment with COS-OGA on potato defense gene expression, the expression patterns in potato leaves of four defense related genes (*PR-1*, *PR-2*, *PI-1* and *NPR1*) were quantified using RT-qPCR. Their expressions relative to the control plants (plants sprayed with water and uninoculated) (value = 1) depending on the treatment and over time were compared after normalization using three housekeeping genes (*GADPH*, *PGK* and *EF1- α* ; Fig. 2.3). The expression of *PR-1* and *PR-2*, encoding for basic *PR-1* protein and acidic *PR-2* protein, was significantly up-regulated after COS-OGA treatments, whether inoculated or uninoculated, from the first day after the last treatment (0 hpi) until 72 hpi (Fig. 2.3a,b). For both pathogenesis-related genes, this up-regulation on plants treated with COS-OGA was significantly higher at 0 and 15 hpi in inoculated plants than in uninoculated plants. At 48 hpi, the difference in relative quantities of PR transcripts between COS-OGA treated plants inoculated or uninoculated was no longer significant and at 72 hpi the expression in inoculated plants was even lower than uninoculated. In addition, the *PR-2* transcript levels at 0 hpi and 48 hpi were also significantly higher in plants treated with water and inoculated with *P. infestans* than in plants treated with water alone. The relative quantities of *NPR1* fluctuated less (between 0.5 and 1.5) over the course of the experiment than *PR-1*, *PR-2* and *PI-1* (Fig. 2.3c). Although limited change is difficult to validate by RT-qPCR, significant differences were observed: gene expression was higher than the uninoculated water-treated control at 0 hpi in water-treated inoculated plants, at 15 hpi in COS-OGA-treated inoculated plants, and at 48 hpi in water-treated inoculated plants. In addition, at 72 hpi, a significant decrease in expression was detected for COS-OGA-treated uninoculated plants and for water-treated inoculated plants. For the *PI-1* gene, expression was significantly increased in plants challenged with *P. infestans* and treated with COS-OGA at 0 hpi, and in plants inoculated and/or treated with COS-OGA at 15 hpi (Fig. 2.3d). At 48 hpi, no significant difference was detected between COSOGA-treated and/or inoculated plants compared with the control. However, there was a significant difference between inoculated and uninoculated plants treated with COS-OGA. At 72 hpi, a significant up-regulation of the *PI-1* gene was observed in plants inoculated with *P. infestans* whether they

Effect of COS-OGA against the hemibiotrophic pathogen P. infestans

were treated or untreated with COS-OGA (about 5 and 11 times higher than the control, respectively).

The expression patterns of these genes were also quantified in the plants in the second bioassays. Again, a significantly higher expression of *PR-1* and *PR-2* was observed in plants treated with COS-OGA at all time points. For *PR-1* this up-regulation varied from about 4 to 18 times higher than the control and for *PR-2* it was 5 to 24 times higher. As observed in the first bioassay, the up-regulation of *PR-1* in plants treated with COS-OGA was significantly higher in those that were inoculated than uninoculated at 0 hpi and 15 hpi; again, at 48 and 72 hpi, the difference in relative quantities of *PR-1* transcripts between inoculated or uninoculated plants treated with COS-OGA was no longer significant. However, for *PR-2*, no significant difference was observed between inoculated and uninoculated COS-OGA-treated plants, except at 72 hpi; at this time point, the expression of *PR-2* was significantly higher in the inoculated treated plants. These results for *PR-2* differed from the first bioassay and could be related to the slightly higher severity observed. For *NPR1* and *PI-1* gene transcripts, the same expression patterns as the first bioassay were detected, confirming the initial observations.

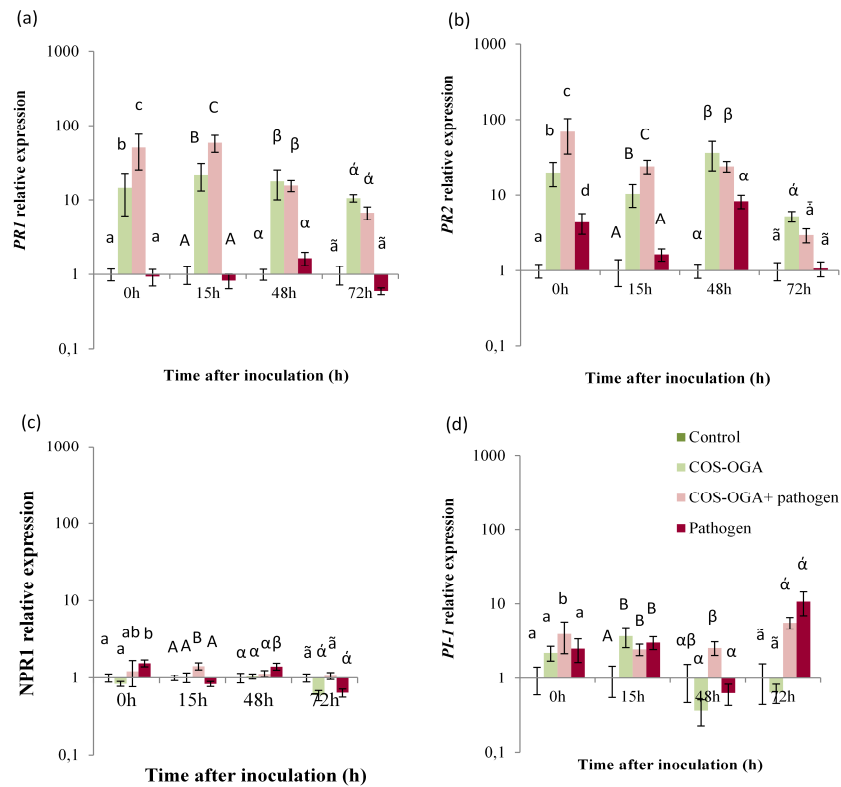


Figure 2.3 Expression relative to control plants over time of four genes in potato leaves treated or untreated with COS-OGA and inoculated or not with *Phytophthora infestans*. The genes investigated were (a) pathogenesis-related 1 (*PR-1*), (b) pathogenesis-related 2 (*PR-2*), (c) non-inducible immunity (*NPR1*) and (d) protease inhibitor 1 (*PI-1*); expression levels were normalized with housekeeping genes *GADPH*, *PGK* and *EF1-α*. The relative expression level of each candidate gene is given in logarithmic form. Results for the same time point with a different letter are significantly different at $P \leq 0.05$ based on a Tukey test. The standard error range is calculated with four biological replicates and takes into account two technical replicates.

5. Discussion

European Union policy is aimed at reducing pesticide use in the short term (Hillocks, 2012). In this context, it is important to find additional approaches for controlling plant diseases. It is now well documented that induced resistance, either local or systemic, caused by biotic or abiotic agents, could confer long-lasting protection against a broad spectrum of plant diseases (Durrant & Dong, 2004; Walters et al., 2013; Li et al., 2016). COS-OGA is a novel agent that stimulates natural plant defenses and has been tested in several European countries (van Aubel et al., 2014). The efficacy of this new elicitor in reducing biotrophic powdery mildew agents has been reported with regard to protecting cucurbits against *S. fuliginea* and grapevines against *U. necator*. Under commercial growing conditions, a reduction in disease severity compared with the untreated control of up to 72% for *S. fuliginea* and 78% for *U. necator* has been reported (van Aubel et al., 2014).

Given the severe losses caused by *P. infestans* on potato and the urgent need to develop control strategies that could reduce the use of chemical pesticides, studies on the potential of using plant defense inducers against this pathogen were needed. Some chemical inducers of disease resistance have already been tested for controlling the severity of late blight disease in potato, with varying efficiency. Examples include benzothiadiazol (BTH) and β -aminobutyric acid (BABA). No protection against *P. infestans* has been reported for BTH (Si-Ammour et al., 2003), but 15–100% protection has been reported for BABA, depending on the potato cultivar and *P. infestans* strain used (Sharma et al., 2010). In the present study, bioassays were conducted under controlled conditions in order to evaluate the effect of COS-OGA on potato plant resistance against potato late blight. The results indicated a significant reduction in disease severity (about 50%) on plants treated with COS-OGA. Visual observations of the progress of lesions over the course of the experiment showed that the treatment of plants with COS-OGA did not inhibit the completion of the pathogen's life cycle, but it did slow down disease progression. This resulted in a reduction in the leaf area covered by symptoms and consequently in reduced sporulation. Although this result does not indicate that the use of COS-OGA should replace synthetic

multisite or curative fungicides, this elicitor could be useful in combination with other control strategies (e.g. plant resistance, fungicides, cultural practices) in integrated pest management programmes based on reduced pesticide use and increased environmental protection. The reduced disease severity associated with those plants treated with COS-OGA suggested that quantitative resistance to *P. infestans* was established. The absence of any direct effect of the COS-OGA elicitor (FytoSave®) at 62.5 mg L⁻¹ of active substance on the in vitro growth of *P. infestans* on V8 medium supported this hypothesis.

In order to confirm the activation of natural defense by COS-OGA, the dynamics of expression of plant defense genes were quantified over time by RT-qPCR assays on the potato leaves. A first interesting result was the significantly higher ($P \leq 0.05$) expression of two pathogenesis-related genes, *PR-1* and *PR-2*, in the plants treated with COS-OGA. *PR-1* codes for the basic PR-1 protein and has long been known to be linked to partial resistance to *P. infestans* in *Solanum* species (Vleeshouwers et al., 2000). *PR-2*, which codes for a β -glucosidase, is often linked to the SA pathway and SA is an important compound required for the basal defense of potato plants against late blight disease (Halim et al., 2007). The PR-1 and PR-2 family proteins also have biochemical and biological properties associated with activity against oomycetes such as *P. infestans* (van Loon et al., 2006). Previous studies revealed that PR-1 protein reduced germination of sporangia in vitro, germ-tube length of *P. infestans* on tomato, and the surface area of leaf discs infected with this oomycete (Niderman et al. 1995; van Loon et al., 2006). A recent study on the biochemical activity and mode of action of PR-1 proteins demonstrates that the inhibitory effect on pathogen growth is caused by the sequestration of sterol from pathogens (Gamir et al., 2017). Sterol-auxotroph pathogens such as the oomycete *Phytophthora* are particularly sensitive to PR-1: they have lost the ability to synthesize their own sterols, which are essential molecules for many cellular functions. They must therefore acquire sterols from host cell membranes. Studies performed on other pathosystems, such as *Eucalyptus*–*Puccinia psidiion* (Boava et al., 2009) and *Poncirus trifoliata*–*Phytophthora parasitica* (Boava et al., 2011), have shown that the PR-2 family has β -1,3-endoglucanase properties and hydrolyses β -1,3-glucans, a major component of the cell wall of oomycetes. The reduction in disease

severity observed on potato plants treated with COS-OGA and inoculated with *P. infestans* could, therefore, be related to an overexpression of *PR-1* and *PR-2* genes. In both experiments, higher expression of *PR-1* transcripts was observed at 0 hpi in plants treated with COS-OGA and inoculated with *P. infestans* than on treated but uninoculated plants. This higher expression at such an early stage may be explained by an interaction between molecules in the inoculum, either from the culture medium or the pathogen itself, with the plant during the short time interval (less than 30 min) between inoculation and harvesting. The expression of *NPR1* was also investigated, as this gene is implicated in the cross-talk between the SAR and ISR pathways. The relative expression of *NPR1* fluctuated less over the course of the experiment than the *PR* genes. This low expression was difficult to validate by RT-qPCR. In addition, it is reported that an up-regulation of the *NPR1* gene is not necessary for activating *NPR1*-dependent defenses (Shah, 2005). In the present study, the expression of the *PI-1* gene was observed to fluctuate, both up and down, throughout the course of the experiment in plants challenged with *P. infestans*, whether treated or untreated with COS-OGA. It was clear that this gene was particularly up-regulated in plants inoculated with *P. infestans* at the necrotrophic switch (48–72 hpi). This is consistent with findings reported in the literature (Beckers and Spoel, 2006). This up-regulation of *PI-1* indicates that the elicitation of plant defense by COS-OGA could also involve, at least partly, the JA-dependent gene. However, in the absence of data on the quantification of JA, this hypothesis cannot be confirmed. Nevertheless, cross-talk between the SA and JA pathways in natural plant defense has been frequently reported (Kunkel and Brooks, 2002), indicating that different metabolic pathways could be involved in the quantitative resistance conferred by COS-OGA. Recent data from a transcriptomic and proteomic study by van Aabel et al. (2016) confirm these results. Further investigation is required to determine the precise role and signaling pathway of COS-OGA during the different life stages of *P. infestans*, as well as the persistence of the effect against this pathogen.

6. Conclusion

The results of this study imply that the application of COS-OGA led to a reduction in late blight severity. This is the first report on the effect of COS-OGA against a hemibiotrophic oomycete fungus. The protection conferred by this elicitor seems to be correlated with defense gene expression, especially the *PR-1* and *PR-2* genes. Whether or not COS-OGA activates the defense of potato against *P. infestans* through modulating the SA-dependent pathway in combination with the JA/ET signaling pathways remains to be determined. These results should encourage further studies to be conducted on the mode of action of COS-OGA and its inclusion in integrated crop protection programmes against potato late blight and other diseases. The use of the elicitor alone would probably be insufficient to prevent potato late blight, especially where there is high disease pressure, but the reduced rate of infection progress in plants treated with COS-OGA could assist integrative disease management strategies when combined with preventive or curative fungicides.

Chapter 3

Influence of pathogen and host genotypes in the response to COS-OGA in the potato late blight pathosystem

Opening comments

In chapter 2, we showed that COS-OGA (FytoSave®, Fytofend SA, Belgium) protects to some extent potato against *Phytophthora infestans*, by stimulation of innate plant defense. A novel COS-OGA based product, FytoSol, was developed by Fytofend SA and further studies performed by G. Van Aubel at UNamur revealed that this product provided a better protection to potato against *P. infestans*. The level of protection obtained under controlled conditions with FytoSol against *P. infestans* on potato cv. Bintje was nearly perfect (van Aubel, 2017). The degree of polymerization of OGA, the degree of OGA methylation, as well as the degree of acetylation and the pattern of acetylation of COS in the novel product could be involved in this phenomenon. As shown in several studies, they strongly modify the way how oligosaccharides stimulate PTI (Cabrera et al., 2006, Madhuprakash et al., 2015; Ridley et al., 2001). In this chapter, we explore and go further into the study on the interaction between COS-OGA and the potato late blight pathosystem by studying the effects of pathogens and host genotypes in the response to the elicitation. As a biological process, plant defenses may be influenced by these factors. Furthermore, in the perspective of the development of an integrated crop management strategy, COS-OGA should be combined with complementary strategies as potato cultivar resistance.

This chapter provides a first insight into the potential influence of factors such as the host cultivar and the pathogen strain on COS-OGA efficacy.

Contribution in this chapter by authors (initial):

A. Clinckemaillie, M. E. Renard^a, H. Verhaggen^a and A. Legrève^a

Experimental procedure: AC and AL

Establishment and assessment of the bioassay: MER and HV

Data analysis, AC

1. Abstract

The development of alternatives to the use of chemical pesticides constitutes the current challenge to control plant diseases. In this context, the induction of plant defenses by using natural or synthetic elicitors is a promising new strategy compatible with sustainable agriculture. The elicitor COS-OGA was shown to be effective against the hemibiotrophic pathogens *Phytophthora infestans* on potato. This study aims at investigating the protective effect of a COS-OGA based product, FytoSol, on ten potato cultivars (*Solanum tuberosum*) against two strains of *P. infestans*. Bioassays were conducted under controlled conditions and the disease severity was assessed during 15 days on plants treated or not with the elicitor. Potato late blight infection remained at very low level during this bioassay, which was performed in greenhouse during the winter. These results reveal that experimental conditions were not optimal. Nevertheless, significant differences of disease severity according to host and pathogen genotypes in response to the COS-OGA treatment were observed, indicating that these factors may influence the defense of plants. This should be confirmed in a further assay.

2. Introduction

The control of potato late blight, caused by *Phytophthora infestans*, currently requires frequent applications of fungicides. However, the emergence of fungicide resistance and pesticides restrictions by EU regulation necessitates the development of alternative strategies. These alternatives to the use of chemical pesticides constitute the current challenge to control plant diseases. In this context, the induction of plant defenses by using natural or synthetic elicitors is a promising new

strategy compatible with sustainable agriculture that could be combined with genetic improvement.

Plants are vulnerable against attacks of many pathogenic microorganisms but are resistant to a multitude of other ones. In the course of evolution, they have developed many different defenses mechanisms against pathogens. In the same time, plant pathogens developed specialized keys to counteract plant defense. Although lacking an immune system comparable to animals, plants have developed a stunning array of structural, chemical, and protein-based defenses designed to detect invading organisms and stop them before they are able to cause extensive damage (Freeman and Beatle, 2008). Research has recently provided new insights on plant defense processes. Plants initially use pattern recognition receptors to recognize, in the apoplast of the plant cell, microorganisms by detecting certain conserved features referred to as microbe- or pathogen-associated molecular patterns (MAMPs or PAMPs) (Schwessinger et al., 2012; Boller and He, 2009). This detection induces pattern-triggered immunity (PTI) which is also known as the basal or early immune response. Induction of PTI induces a mitogen-activated protein kinase (MAPK) cascade that leads to various plant defense mechanisms. Cell wall reinforcement by callose deposition and the production of antimicrobial chemicals including reactive oxygen species (ROS) or antimicrobial proteins and enzymes are some of these mechanisms (Doods and Rathjen, 2010). However, some pathogens can circumvent PTI by delivering proteins called effectors into the plant cell. In turn, plants evolved. They have developed a second layer of immunity mediated by the recognition of these effectors through internal receptors (R proteins) referred to as effector-triggered immunity (ETI). Upon recognition of the effector, these receptors ultimately induce a localized programmed cell death (PCD) called hypersensitive response (HR) that may limit pathogen spread (Doods and Rathjen, 2010). The most common class of these receptors acts inside the cell and typically contain nucleotide-binding site (NBS) and leucine-rich repeat domains (LRR). Co-evolution of plants and their pathogens, illustrated by the zigzag model of Jones and Dangl (2006), leads to an increasing diversity of R proteins, effectors and mechanisms of resistance and susceptibility.

Many examples of gene-for-gene have been described (Brendolise et al., 2017). In the early 20th century, the breeder found resistance genes (R genes) against potato late blight in the hexaploid *S. demissum*, which could directly be crossed with tetraploid *S. tuberosum*. So, this species was considered as the main resistance resource, and 11 R genes (R1-R11) have been integrated into potato cultivars. The durability of resistance associated with particular R proteins remains problematic as the extreme selective pressure they exert against *P. infestans*. Consequently, successful resistance breeding strategies rely on stacking resistance from different origins (gene pyramids) in the same cultivar to reduce the chances of a pathogen evading recognition mediated by a single R protein (Xin et al., 2012). From the pathogen side, allelic diversity of Avr effectors apparently determine the specificity of their recognition by R proteins. We talk about different races of *P. infestans* when e.g. a single amino acid changes in Avr3a and circumvents its recognition by R3a. Such mutations were always associated with the loss of the primary (pathogenic) function of Avr3a in suppressing the plant cell death response (Bos et al., 2009).

More recent studies revealed that plant defense elicitors from biological origin, such as plant-derived cell wall components, proteinaceous pathogen molecules or non-proteinaceous molecules can be used to activate or boost natural disease resistance in non-infected plant tissues (Wielsel et al., 2014). Synthetic elicitors, also called chemical inducers, may also trigger defense reactions. They mimic interactions of natural elicitors or defense signaling molecules with their respective plant receptors or by interfering with other defense signaling components (Bektas and Eulgem, 2015). COS-OGA is a novel plant defense elicitor that was recently registered at European level as FytoSave® against powdery mildew in cucurbits and *Solanaceae*. It combines chitosan oligomers (COS) and pectin-derived oligogalacturonides (OGA) (Cabrera et al., 2010) which are relatively conserved small molecules collectively referred as pathogen associated molecular patterns (PAMPs) and damage associated molecular patterns (DAMPs). The complex of COS and OGA interacts with plasma membrane pattern recognition receptors (PRRs) and stimulate the pathogen triggered immunity (PTI) (Van Cutsem & Messiean, 1994; El Gueddari et al., 2002). The commercial product, Fytosave® is effective

against biotrophic pathogens as Erysiphaceae in *Solanaceae*, Cucurbitaceae and grapevines (van Aubel et al., 2014). In a recent study, we have tested the efficacy of FytoSave® against potato late blight and observed that this elicitor is able to reduce late blight severity in potato cv. 'Bintje'. However, its efficacy was only partial (Clinckemaigne et al., 2017, *cfr* chapter2). A more recent study on the same pathosystem have revealed that another COS-OGA based product, called FytoSol also developed by Fytofend SA, is very effective against *P. infestans* on potato cv. 'Bintje' (van Aubel, 2017).

In the framework of the integrated pest management, different strategies have to be associated to control disease. Disease resistance in plant is likely composed by multiple layers of defenses activating PTI and ETI. Both of PTI and ETI contribute to basal resistance (Zhang et al., 2010). However, the host genotype is known to affect the expression of induced resistance (Martenelli et al., 1993; Hijwegen and Verhaar, 1994; Dann et al., 1998; Resende et al., 2002; Tucci et al., 2011). This expression can vary greatly across a range of varieties. For example, induction of resistance in soybean to *Sclerotinia sclerotiorum* with INA or acibenzolar-S-methyl (ASM) was greatest in susceptible varieties (Dann et al., 1998). In contrast, resistance induction to *Blumeria graminis* was strongest in the most resistant and weakest in the most susceptible barley genotype (Martinelli et al., 1993). Sometimes, there is no clear association between the resistance rating of the variety and its ability to express induced resistance as demonstrated by Walters et al. (2011) in barley against *Rhynchosporium commune* with a combination of resistance elicitors (ASM, BABA and cis-jasmone) and by Sharma et al. (2010) in tomato against *P. infestans* with BABA.

To our knowledge, no studies were conducted on elicitor's effects on resistance amongst potato cultivars. This study aims at evaluating the additional effect of the use of the elicitor FytoSol in combination with several potato genotypes known to differ in their susceptibility to potato late blight. A bioassay was conducted under controlled conditions; ten genotypes of potato and two strains of *P. infestans* with different virulence were tested.

3. Materials and methods

3.1. Inoculum

Two strains of *P. infestans*, 14-009 (A1 mating type, Avr-race 1.3.4.7.10.11, CRA-W, Belgium) and 14-015 (Blu_13-A2 mating type, Avr-race 1.2.3.4.5.6.7.10.11 CRA-W, Belgium), were used as inoculum in the bioassay. They were maintained on V8 medium. Sporulation of the strain, pathogenesis restauration and inoculum preparation were conducted as described in Clinckemaillie et al. (2017) (Cfr Chapter 2).

3.2. Plant growth, treatments with the elicitor and inoculation

Ten potato cultivars (*Solanum tuberosum*) were obtained from CRA-W, Libramont, Belgium. They were chosen for their resistance to *P. infestans*. Cultivars chosen and their resistance rating to *P. infestans* for the growing season 2016 (1 = high susceptible to 9 = less susceptible) were: Bintje (1,8), Tentation (4,3), Campina (5,1), Markies (6,4), Almonda (7,1), Challenger (7,5), Gasoré (7,9), Allians (8,2), Passion (8,6), Cephora (8,6). Before planting, tubers were first treated during 2 hours with gibberellin hormones at the concentration 10 mg L⁻¹ to stimulate sprouting. Thereafter, they were planted into pots containing a sterilized mixture of compost and vermiculite (1/2 v/v). The plants were irrigated daily with water and maintained under greenhouse conditions with a photoperiod of 16h at 20/15°C (day/night temperature). At the four leaves deployed stage, plants were treated with 0.5% (v/v) of FytoSol, containing 12.5 g L⁻¹ of the active ingredient (COS-OGA) or with water (control plants) by spraying the upper and lower leaf surfaces until run-off. This was performed at 7, 3 and 1 days before the inoculation of plants with *P. infestans*. Before and after these treatments, a high relative humidity was maintained in the greenhouse to ensure good adsorption of COS-OGA/water by the leaves. Subsequently, four plants per cultivars and per treatment were inoculated with each strain of *P. infestans* by spraying 1 mL of sporangial suspension (10⁴ sporangia mL⁻¹) on each leaf of the plant using a sterile glass sprayer (Merck). As a control, the remaining plants were sprayed in the same way with water.

3.3. Assessment of disease progress

The disease severity was assessed quantitatively each day by visual estimation of the percentage of the leaf area covered by symptoms from the inoculation to 15 days using the scale described by James (1971). The area under the disease progress curve (AUDPC) was calculated from disease severity as described by Campbell and Madden (1990).

3.4. Data analysis

Data from all the experiments were subject to analysis of variance (ANOVA) using the SAS ENTERPRISE GUIDE v. 4.1 (SAS Institute Inc.). The treated means were compared using Tukey's HSD test with a confidence interval of 95% ($P < 0.05$).

4. Results

This bioassay was performed under controlled conditions in greenhouse during the winter 2016-2017. The potato tubers require a period of dormancy during the winter period to germinate. In this experiment, the end of dormancy was obtained by application of gibberellin hormones to stimulate sprouting. Even with this treatment, the germination of tubers was very slow. The four leaves deployed stage was reached only after five weeks. As shown in figure 3.1, the infection on the susceptible cultivars 'Bintje' by the two strains was very low and delayed in time compared to previous bioassays on this pathosystem realized with another strain (10-022) of *P. infestans*: late blight symptoms appear 7 days after inoculation (dai) on plants inoculated with each strain and previously elicited or not with the elicitor. In previous study, AUDPC calculated over 7 days reached values between 70 and 110 on plants treated with water and inoculated with the strain 10-022 (cfr chapter 2). In own assay, AUDPC calculated over 15 days on plants cv. 'Bintje' treated with water reached values around 6 for the both strains 14-009 and 14-006. On the others cultivars, the first symptoms of potato late blight appeared 2 dai for Cephora, Almonda, Challenger, Campina, Markies and Gasore, and 3 dai for Tentation, Passion et Allians. To analyze the effect of FytoSol on disease development according to the potato cultivar and the strain of *P. infestans* used, the AUDPC was calculated over 15 days for each cultivar and strain (Figure 3.1). Differences in AUDPC values on some cultivars depending on the

Factors affecting the efficacy of COS-OGA

strains, suggest that strains differed in their virulence. AUDPC values on plants treated with water were significantly different for the both strains on the cultivars Markies (33 ± 5 for strain 14-009 and 22 ± 3 for strain 14-015), Challenger (28 ± 4 for strain 14-009 and 17 ± 4 for strain 14-015), Gasore (35 ± 8 for strain 14-009 and 56 ± 7 for strain 14-015) and Cephora (53 ± 10 for strain 14-009 and 38 ± 9 for strain 14-015).

For the cultivars Tentation and Almonda, the treatment of plants with FytoSol resulted in a significant decrease in disease progress with both strains. Based on the values of AUDPC, the protection conferred by the FytoSol elicitor against *P. infestans* was 48.6 % for Gasore with the strain 14-015, 54 % for Tentation and 46 % for Almonda with both strains, 62 % for Cephora and 64.3 % for Challenger inoculated with the strains 14-009.

In this bioassay, the low levels of infection by both strains of *P. infestans*, even on plants treated with water, were not correlated to the field varietal susceptibilities. For example, the disease severity on the susceptible cultivar Bintje was lower than the ones on more resistant cultivars, such as Cephora. These results do not allow analyzing the relationship between the response of the elicitation with COS-OGA and the genotypes of plants.

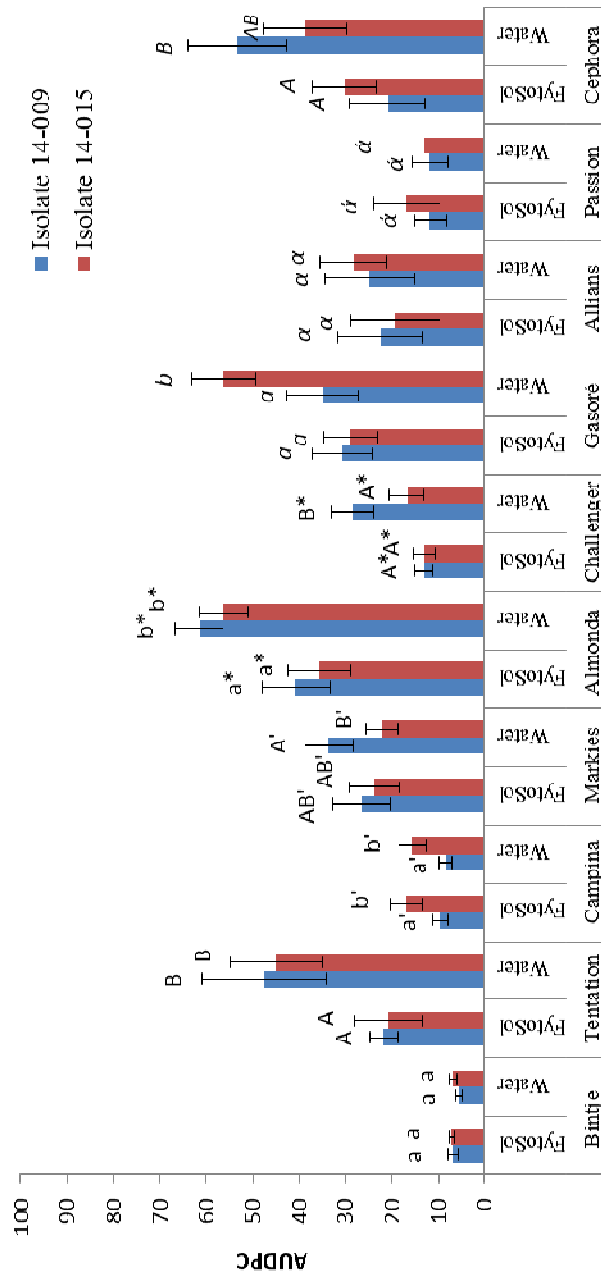


Figure 3.1 Values of area under the disease progress curve (AUDPC) for ten potato varieties. Varieties selected and their resistance rating to *Phytophthora infestans* for the growing season 2016 (1 = high susceptible to 9 = low susceptible) were: Bintje (1,8), Tentation (4,3), Campina (5,1), Markies (6,4), Almonda (7,1), Challenger (7,5), Gasoré (7,9), Allians (8,2), Passion (8,6), Cephora (8,6). Plants were treated with COS-OGA or water (control) and inoculated with two strains of *Phytophthora infestans*: 14009 (Mating type A1) and 14015 (Blue_13). No symptoms were observed on non-inoculated plants. Results for the cultivars with a different letter are significantly different at $P \leq 0.05$ based on a Tukey test. The standard error range is calculated with four biological replicates

5. Discussion

The aim of this experiment was to evaluate the potential additive effect of induced and genetic resistance against *Phytophthora infestans*. In order to be, to some extent, representative to the field infection pressure, two different strains of *P. infestans* have been selected. They were chosen based on their prevalence (14-009) or their particular high virulence in Belgian field (14-015, Blu_13-A2). Ten genotypes with distinct genetic resistance to potato late blight were tested. During this bioassay, the development of plants was slow and the infection was slight. This can be partly explained by the fact that the assay was performed under greenhouse conditions during the winter season and, even with a treatment with gibberellin hormone to break the dormancy of the tubers, the germination of the tubers was very low. After elicitation and inoculation, plants were placed in a greenhouse where the relative humidity was less homogenous and probably not optimal compared to previous assays performed in growth chamber or in another greenhouse. The low disease severity observed in this assay could also be explained by the fact that the strains inoculated differed from the ones used in our previous bioassays. This highlights the difficulty to perform repeatable bioassays and the importance of each factor for the development of a disease. Therefore, the results of this assay must be interpreted with caution and the experimentation should be repeated.

Despite the low level of disease pressure especially on the susceptible cultivar Bintje, some results obtained in this study suggest interesting information. Firstly, the expression of induced resistance conferred by FytoSol against *P. infestans* varied according to the cultivars used. The response of elicitation was not related to the resistance level of the potato varieties. For example, FytoSol reduced significantly *P. infestans* infection on Tentation or Cephora, susceptible and resistant cultivars respectively. Secondly, the response to the elicitation seems to be also affected by the pathogen strain used for challenge inoculation. FytoSol seems to have an effect in combination with the more aggressive strain on certain potato genotypes. It is the case on cultivars Cephora and Challenger with the strain 14-009 and the cultivar Gasore with the strain 14-015. FytoSol had also a significant

effect when the levels of infection were raised (on Temptation and Almondia for both strains). In these experiments, we have highlighted an additive effect of FytoSol, during an interaction plant pathogenic compatible.

The differential resistance induction may be the result of defense mechanisms and responses established by FytoSol applications. Our results show some differences in disease progresses according to the varieties used. Interestingly, there is no relation between the resistance rating of the cultivars and the observed AUDPC. This is valid for both cultivars treated with water or with FytoSol. It could be that the resistance suppressors or the avr gene of the *P. infestans* strains used in the present study interacted differently with the potato varieties and their induced resistance mechanisms.

As previously discussed, results must be interpreted with caution and experimentation will be repeated to confirm results. Implementations could also be done in the experiment design like the addition of FytoSave®, the commercial product containing COS-OGA. A decrease of the number of cultivars can be considered by choosing more contrasted profiles of resistance to late blight. Furthermore, a greater number of *P. infestans* strains with well characterized virulence profile can be suggested in order to broaden the research scope. The use of a mixture of strains as inoculum can also be tested in order to evaluate plant response in the presence of several strains. Sharma *et al.* (2012) reported that BABA performed well when a single pathogen strain was used for inoculation, but less when two or three pathogen strains were mixed.

6. Conclusion

This bioassay is a first investigation to study the effects of host and pathogen genotypes on inducibility resistance in potato against *P. infestans*. The results of this study imply that the application of FytoSol was not always related to the varietal resistance of the potato and it was influenced by the pathogen strain used for inoculation. To confirm these results, the bioassay must be repeated and the research strategy completed in order to obtain right and usable information.

Chapter 4

Protective effect and mode of action of FytoSave® and FytoSol against *Alternaria solani*, a necrotrophic pathogen on tomato

Opening comments

In chapters 2 and 3, we have investigated the effect of COS-OGA based products, on the pathosystem *Solanum tuberosum*-*Phytophthora infestans*. The treatment of plants with FytoSave® induced around 50% reduction of potato late blight severity. This rate of reduction was not sufficient for the management of this disease in field and Fytofend developed a new formulation of the product, FytoSol. Studies performed by van Aubel (2017) in UNamur under controlled conditions revealed that this COS-OGA product was more efficient than FytoSave® against potato late blight, with a protection close to 100%. Despite us not having confirmed this result in chapter 3, further studies reported by van Aubel (2017) corroborate the efficacy of FytoSol against potato late blight. Proteomic and transcriptomic studies performed to understand the mode of action of this elicitor revealed that the metabolic pathways involved in the interaction FytoSol-*S. tuberosum*-*P. infestans* differed from the one observed with FytoSave® with induction of a completely different parallel pathway connected or not to the accumulation of SA or the activation of *PR* genes. These observations, combined to the fact that treatments of plants with FytoSave® are less efficient against late blight compared to powdery mildew and FytoSol is more efficient against *P. infestans* than FytoSave®, indicate that the activity range and mode of action of the elicitor could depend on the plant-pathogen interaction, which is linked to the type of parasitism of the pathogen. In order to test this hypothesis, we decided to evaluate the effects of both compositions of COS-OGA and to analyze the differential expression of defense pathways in a pathosystem caused by a necrotrophic pathogen. We have selected *Solanum lycopersicum*-*Alternaria solani* as a model pathosystem. Tomato is another *Solanaceae*, close to potato but this plant doesn't produce tuber and it grows easily. Its genome is completely annotated and mutants or transgenic plants altered on hormonal signaling

Protective effect of COS-OGA against the necrotrophic pathogen A. solani

pathways are available. The agent of the early blight, *A. solani*, was chosen because it is pathogenic on tomato and potato and necrotrophic. In this experiment, a third elicitor, the well-known benzothiadiazole (BTH), was tested as a control of activation of SAR. This signal is translocated throughout plants and activates SAR signal transduction. The effects of the three elicitors on the protection of tomato against *A. solani* were compared by the assessment of the disease progress on wild tomato and on two tomato mutants deleted on SA- or JA-pathway. Quantification of endogenous salicylic acid and enzymatic activities as well as dynamic of differential defense genes expression help us to understand the mode of action of the tested elicitors and evaluate the relationship between the composition and the activity of the elicitors and the type of parasitism of pathogens.

This chapter will be submitted for publication

A. Clinckemaillie^a, G. van Aubel^b, M. E. Renard^a, H. Verhaggen^a, P. Van Cutsem^b and A. Legrève^a

^a Université catholique de Louvain, Earth and Life Institute, Applied Microbiology, Phytopathology, Croix du Sud 2 box L7.05.03, 1348 Louvain-la-Neuve, Belgium

^b Université de Namur, Unité de Recherche en Biologie végétale, Rue de Bruxelles 61, 5000 Namur, Belgium

Implication in this Chapter:

Experimental procedure: AC and AL

Establishment and harvesting of the bioassays: AC and MER

Conidia *In vitro* test: AC and HV

Gene profiling: AC, GvA, MER and HV

Enzymatic assays: AC, HV

SA quantification: HV, Hélène Dailly (technical supervision) and Emna Gharbi (protocol)

Data analysis, AC

Writing paper: AC

Critical reviews: AL, PVC

1. Abstract

This study investigated the protective effects and the modes of action of two COS-OGA-based products, FytoSave® and FytoSol, against the necrotrophic pathogen *Alternaria solani* on tomato. A third elicitor was introduced in the experiments, the benzothiadiazole (BTH) as a control of activation of the systemic acquired resistance. The efficacy of the three elicitors was compared by quantification of the disease progress on wild tomato plants and two tomato mutants deleted either on SA (*NahG* mutant) or JA (*Def1* mutant) pathways. Endogenous salicylic acid and enzymatic activities as well as dynamic of differential defense genes expression using quantitative RT-PCR were quantified in order to decipher the mode of action of the elicitors. The elicitor FytoSol provided the best protection on wild tomato against *A. solani*. It also provides a significant protection on two tomato mutants *NahG* and *Def1* but the protection was lower than the one observed on wild tomato. These results indicate that each of the SA and JA pathways is not solely responsible of the protection. After the elicitation with BTH that mimic the defense-associated effects of SA, a significant reduction of disease progress was observed on two tomato mutants *NahG* and *Def1*. For FytoSave®, the disease severity was significantly reduced by the treatment on the *Def1* mutant. The absence of protection in the mutant *NahG* demonstrated that the salicylic acid pathway is involved in the protection conferred by FytoSave®. Tomato defense genes such as *PR-1*, *GLU* and *P69b* were generally up-regulated after treatments with the elicitors. Increased activities of β -1.3-glucanase (GLU) and Polyphenol oxidase (PPO) were observed on elicited tomato leaves and only in tomato treated with FytoSol for Peroxydase (POD). Our results reveal that FytoSave® and BTH activate well a defense response of tomato against *A. solani* through the SA pathway as it was observed for other biotrophic and hemibiotrophic pathogens. The protective effect of FytoSol was higher but, despite the significant augmentation of endogenous SA in tomato treated leaves, the molecular pathway induced by this elicitor is not yet so clear. These results suggest that the hormonal regulation induced by FytoSol do not rely on SA only and probably include other pathways.

2. Introduction

Diseases of major crops caused by fungi and oomycete are mainly controlled by repeated fungicide treatments that are costly and do have environmental side effects (Cooke et al., 2011). In this context, the development of complementary and sustainable management practices to conduct integrated pest management (IPM) and decrease the use of chemical pesticides is of prime importance. A promising strategy is the stimulation of plant defense response that conducts to an incompatible interaction with the pathogen by natural or synthetic compounds called elicitors (Thakur and Sohal, 2013). This defense response is stimulated by pathogen or microbe associated patterns, PAMPs or MAMPs, and damage associated patterns, DAMPs which are recognized by cell surface pattern-recognition receptors (PRRs) and lead to the establishment of PAMP triggered immunity (PTI) (Boller & Felix, 2009). The induced resistance spreads into the whole plant and become systemic. This response can be split into systemic acquired resistance (SAR) or induced systemic resistance (ISR) depending on the metabolic pathways associated. SAR is effective against a wide range of pathogens and is mediated by a salicylic acid (SA)-dependent process. ISR can result from the colonization of plant-growth-promoting rhizobacteria (PGPR) or fungi (PGRF) and is mediated by jasmonate (JA) or ethylene (ET) sensitive pathway (Vallad et al., 2004; Walters et al., 2005). Cross-talk between SA-, JA-, and ET-dependent defense pathways can affect each other's signaling, either positively or negatively and provides a finer answer of plant defenses against various pathogens (Pieterse et al., 2012).

The elicitor COS-OGA has been recently registered at European level as FytoSave®. It combines chitosan oligomers (COS) and pectin-derived oligogalacturonides (OGA) (Cabrera et al., 2010). These molecules mimic a plant pathogen interaction as a PAMP (COS) and a DAMP (OGA). The efficacy of this new elicitor in reducing biotrophic powdery mildew agents has been reported with regard to protecting cucurbits against *Sphaerotheca fuliginea* and grapevines against *Uncinula necator*. Mechanisms involved by treatment with FytoSave® likely rely on SAR with an accumulation of SA and an overexpression of SA-mediated genes and proteins (van Aubel et al., 2014). Recent studies

revealed that a treatment with FytoSave® reduced the potato late blight severity up to 50%. The accumulation of *PR-1* and *PR-2* transcripts in elicited potato plants (cv. Bintje) suggests that SA pathway is also involved in the potato response to the inoculation of *P. infestans* (Clinckemaillie et al., 2017; *cfr Chapter 2*).

This study investigates the protective effect and the mode of action of two COS-OGA compositions, FytoSave® and FytoSol, against the necrotrophic pathogen *Alternaria solani*. This pathogen is the causal agent of early blight disease of tomato (*Solanum lycopersicum* L.) and potato (*S. tuberosum*). In recent years, early blight has become more prominent in Northern countries of Europe leading to increased interest for this pathogen (Edin and Anderson, 2014). *A. solani* affects the foliage, stems and fruit of infected plants, causing important losses by a reduction of economic fruit yield (Jones et al., 2014). The plant defense mechanisms induced by elicitation with FytoSave® and FytoSol will be compared to those induced by benzothiadiazole (BTH), another elicitor which has been reported to cause systemic resistance in several crops against foliar diseases (Bokshi et al., 2003; Görlach et al., 1996; Ishii et al., 1999) and which activates SAR signal transduction at a step downstream or at the same site of SA accumulation (Friedrich et al., 1996). The protective effect of FytoSave®, FytoSol and BTH on tomato plants cv. Moneymaker will be compared to two tomato mutants deleted either on SA (*NahG* mutant) or JA (*Def1* mutant). The quantification of endogenous salicylic acid, the evaluation of the expression of tomato defense genes and the assessment of enzymatic activities will help to understand the modes of action of FytoSave® and FytoSol against the necrotrophic pathogen *A. solani*.

3. Materials and methods

3.1. Plant material and growth conditions

Bioassays were conducted on wild tomato cv. Moneymaker. The efficacy of FytoSave®, FytoSol and BTH was also tested on two tomato mutants, deficient in the SA-or JA-signaling pathway involved in the plant defense reactions: tomato transgene *NahG* cv. Moneymaker (Brading et al, 2000) and tomato mutant deficient in Defenseless 1 (*Def1*) gene cv. Castlemart (Howe et al, 1996). Tomato mutant *Def1* is

deficient in a gene product that acts after lipoxygenase conversion of linolenic acid to hydroperoxylinolenic acid but before the conversion of 12-oxo-phytodienoic acid to JA. This function plays a critical role in the octadecanoid signaling pathway and in the defense of plants (Howe et al., 1996). Tomato transgene *NahG* expresses the bacterial gene *NahG*, a gene encoding salicylate hydroxylase that removes endogenous SA by converting it to catechol (Brading et al., 2000). Seeds of each genotype were planted in pots filled with sterilized compost soil. Plants were grown under greenhouse conditions with a 16h photoperiod at constant temperature of 24°C.

3.2. Elicitor application

Three elicitors were used: FytoSave® and FytoSol (Fytosend SA, Isnes, Belgium) with 0.5% (v/v) soluble liquid formulation containing 12.5 g/L COS-OGA and BTH with Bion® WG 50 containing 50% active ingredient (Syngenta) at 10 mg a.i. L⁻¹. The treatment of plants with FytoSave® or FytoSol were applied on four week-old plants three times at 7 days, 3 days and 1 day before T₀ corresponding to the time when some plants were inoculated with *A. solani* (see after). The treatment of plants with BTH was performed in a same way but one time at 3 days before T₀. Non-elicited plants (control plants) were treated at each elicitation time with water. Before and after these treatments, 90% of relative humidity (RH) was maintained in the greenhouse in order to ensure a good absorption of the treatments by the leaves.

3.3. Alternaria solani culture and inoculation

Two isolates of *A. solani*, strains A1 and CRA1 (CRA-W Gembloux, Belgium) were used as inoculum in the assays. The strain A1 was used in all experiments performed in this study. The strain CRA1 was a second strain used for determining the direct effect of COS-OGA on the fungal growth. The strains were conserved on V8 medium under daylight at ambient temperature. To prepare suspension of conidia, the fungus is subcultured on V8 medium for 3 weeks. The day of inoculation, the conidia were harvested from medium in sterile demineralized water by removing the supernatant from the plate and filtered through a meshing 5x5 µm. The concentration of conidia was determined with a Fuchs-

Rosenthal counting chamber and adjusted with sterile demineralized water in order to obtain 45,000 conidia/ml. The suspension was sprayed on the upper surface of four leaves of plants till run off using a sterile glass sprayer (Merck). Sterile demineralized water was sprayed similarly on uninoculated plants. Following the inoculation with *A. solani* or treatment with water, plants were then incubated under controlled-environment chamber for 24h under 90% RH in the dark at 20°C. A 16h photoperiod was restored after 24h with a temperature of 20°C and ambient RH. Each day, during two hours in the early morning the RH was increased to 90% to mimic the nightly dew in nature.

3.4. Disease assessment

In order to investigate the protective effect of the three tested elicitors against *A. solani* (strain A1), the disease severity was assessed quantitatively. Each day, a visual estimation of the percentage of the leaf area covered by symptoms was done from the inoculation to 7 days using the modified scale developing by Ephrath et al. (1989). The area under the disease progress curve (AUDPC) was calculated from disease severity as described by Campbell and Madden (1990).

3.5. Direct effect of COS-OGA on the fungal growth

The direct effect of COS-OGA on the fungal growth of *A. solani* (strains A1 and CRA1) was assessed *in vitro*. Hyphal tips of 5 mm diameter were taken from the periphery of an actively growing V8 sub-culture of *A. solani* and placed face down on Petri dishes containing V8 supplemented with 0, 0.125, 0.625, 1.25, 6.25, 12.5, 62.5, 125 and 625 mg L⁻¹ of FytoSave® and FytoSol. The growth diameters of the colonies were measured daily during seven days of incubation under daylight and ambient temperature.

3.6. Salicylic acid quantification

Endogenous salicylic acid (SA) in wild-type Moneymaker tomato leaves harvested at T₀ and 24h after T₀ in uninoculated plant was assessed for each treatment. Extraction of SA was realized according to Molinari & Loffredo (2006). The procedure was modified as mentioned in Gharbi et al. (2016): 200mg of fresh plant tissue was incubated with 1.8 ml HCl (1mM) under mechanical shaking at 2g. The mixture was

sonicated at 5C° for 1min and centrifuged at 15000g at 5C° for 15 min. The supernatant was collected and added to 2ml of ethyl acetate before vortexing for 1 min and centrifugation at 15000g at 5C° for 15 min. The ethyl acetate fractions were combined and evaporated to dryness on a speedvac at 45°C. The residue was dissolved in 0.5 ml of water/acetonitrile (ACN) (1:1v/v) before SA quantification by high performance liquid chromatography (HPLC) (10 µl of sample was injected). The system consisted of an Agilent 1260 series equipped with an automatic injector and a column (Inertsil ODS-3; 250 × 3.0 mm, 3 µm) oven both thermostated at 30°C. SA was detected by a fluorescence detector at 315 nm emission and 408nm excitation wavelengths. The mobile phase was a water/acetonitrile (ACN) gradient from 10 to 100% ACN and the flow was 1 ml min⁻¹. Quantification of SA was performed by external calibration using SA standards with concentrations from 0 to 10 µM.

3.7. Gene expression profiling

The differential expression patterns of wild-type Moneymaker tomato genes depending from SA (*PR-1*, *GLU*, *P69b*) and JA/ET (*LOXD*, *PR-3* and *PI-1*) signaling pathway were quantified using RT-qPCR. This expression was compared at four time points: T₀ and 24h, 48h and 72h after T₀. At each time point, four leaves per plant and four plants per treatment (described above) were harvested, deep frozen using liquid nitrogen and stored at -80°C until they were processed for enzyme and RNA extractions. Total RNA extraction, the synthesis of cDNA and the quantification by real-time quantitative RT-qPCR were performed as previously described by Clinckemaillie et al. (2017) (*cfr chapter 2*). Primers for three Housekeeping genes (*CAC*, *EF1-α*, *PGK*) and six tomato defense-related genes were retrieved from the literature (table 4.1).

Table 4.1 Primer sequences used in the gene expression analysis with accession numbers from GenBank database or Sol Genomics Network (SGN). The genes monitored are used as markers for the pathway indicated. Jasmonate (JA), salicylic acid (SA).

Accession number	Target gene	Related pathway	Primer sequence (5'-3')	Reference
AB061263	Elongation factor 1- α (<i>EF1-α</i>)	Housekeeping gene	CCAGATTGGAAACGGGATATGC TCCTTACCTGAACGCCTGTCA	Nicot et al., 2005
SGN-U314153	Clathrin adaptor complex (<i>CAC</i>)	Housekeeping gene	CCTCCGTTGTGATGTAACCTGG ATTGGTGGAAAAGTAACATCAT	Expósito-Rodríguez et al., 2008
SGN-U578082	Phosphoglycerate kinase (<i>PGK</i>)	Housekeeping gene	TCTACAAGGCCCAAGGTTATG GCAGCAAACCTGTCCGCAATC	Ghareeb et al., 2011
DQ159948	pathogenesis related 1 (<i>PR-1</i>)	SA inducible	TGTCCGAGAGGCCAAGCTATAAC ATCCATCGCAGCATAAACAG	Song et al., 2011
Y10149	Subtilisin-like protease b (<i>P69b</i>)	SA inducible	TTATCACAGCAGTAAACACCG CAGATTCTCCTTCAAAATTCCT	van Aubel et al., 2016
SGN-U581016	Glucan endo-1,3- β -glucosidase B (<i>PR-2b</i>)	SA inducible	GCACCTTTGCTCGTTAACATT TTGACGCGATCCATCTTGTA	van Aubel et al., 2016
M80608	β -1, 3-glucanase (<i>GLU</i>)	SA inducible	AGGAACGATGTTAGATGGTTTACT ATCCATCGCAGCATAAACAG	Song et al., 2011
SGN-U268804	Basic endochitinase class II (<i>PR3</i>)	JA inducible	GCGGTGTCATCTCAAAATTCC CTGGGAGAAAGTGAGGACAAT	van Aubel et al., unpublished
U37840	Lipoxygenase (<i>LOXD</i>)	JA inducible	GACTGGTCCAAGTTCACGATCC ATGTGCTGCCAATATAAATGGTTCC	Fujimoto et al., 2011
SGN-U577558	Protease Inhibitor 1 (<i>PI-1</i>)	JA inducible	GTGTACCAACAAAGCTTGCTAAAGA GTACAACAACACCCCAAAATGTTGTC	Fujimoto et al., 2011

3.8. Quantification of enzymatic activities

Enzymatic activities in wild-type Moneymaker tomato were compared at four time points: T₀ and 24h, 48h and 72h after T₀. At each time point, four leaves per plant and four plants per treatment (described above) were harvested and deep frozen using liquid nitrogen. Frozen leaf samples (0.5g fresh weight for polyphenol oxidase (PPO) and peroxidase (POD) or 1g for β -1,3-glucanase (GLU)) were homogenized for 30 min with 5 ml 80 mM sodium acetate (pH 5) with 5mM of reduced glutathione for GLU activity or 3 ml of 50 mM sodium phosphate buffer (pH 7.8) for POD and PPO activities. After centrifugation at 12,000 rpm for 20 min at 4°C, the supernatant (protein extract) was removed, filtered through Wathman n°1 filter paper and used as the source to assay enzymatic activities. For all protein extracts, total protein contents were quantified by the Bradford test (Coomassie Protein assay kit, thermoscientific) by adding 1.5 mL Coomassie reagent to 30 μ l of crude extract and reading absorbance at 595 nm after a reaction time of 10 min at room temperature. Protein quantities were estimated by reference to absorbance obtained for a series of BSA (Bovine albumin standard) concentrations.

Three defense-related enzymes were chosen from the literature for their roles on the defense induction. The peroxidase plays an important role in one of the earliest observable event of plants response: oxidative burst (Wojtaszek, 1997). Polyphenol oxidase catalyzed the oxygen dependent oxidation of phenols to quinines which involved in plants defense against pathogens (Yedidia et al., 2003). Since chitin and glucan are the major components of many fungal pathogens including *A. solani*, an antifungal role as been suggested for these pathogenic-induced hydrolytic enzymes (Lawrence et al., 1996).

β -1,3-glucanase (GLU) activity was determined using azurine-crosslinked pachyman (AZCL-Pachyman) as substrate. This substrate is supplied by Megazyme (Aust.) Pty. Ltd. (Warriewood, NSW, Australia) in a ready to used tablet. The enzyme extraction was performed following the manufacturer's instruction supplied with the tablets. The absorbance of the extract was measured at 590 nm. All extracts were assayed in triplicate. A substrate blank was realized by adding 1,3- β -

gluczyme tablets to 1 ml of extraction buffer. After accounting for background colour, the endo-1,3- β -glucanase activity was determined by using the standard curve prepared by Megazyme (Aust.) Pty. Ltd. from tablets within the same batch. One unit of activity (u) is defined as the amount of enzyme required to release one micromole of reducing sugar equivalents per minute under the defined assay conditions (i.e., 1 milliunit releases one nanomole) and was expressed as units per milligram of protein after taking account of the total protein concentration.

Peroxydase (POD) activity was determined using guaiacol as the substrate (Silva et al., 2004). POD activity measurement was followed at 470 nm every 60s for 5 min on 140 μ l of the protein extracts mixed with 3.29 ml substrate (180 mM guaiacol, 0,1M KH_2PO_4 , pH 5.8) and 5 μ l of H_2O_2 . One unit (u) of peroxidase activity was defined as the change of in 0.01 absorbance units per minute. The specific activity was expressed as units per gram of fresh weight.

Polyphenol oxidase (PPO) activity was determined using catechol as the substrate. PPO activity measurement was followed at 420 nm every 30s for 5 min on 150 μ l protein extracts mixed with 3 mL substrate (catechol 50 mM, 0,1M KH_2PO_4 , pH 6.2 with 0.5 mg/mL of catalase) One unit (u) of PPO activity was defined as the change of in 0.01 absorbance units per minute. The specific activity was expressed as units per gram of fresh weight.

3.9. Data analysis

Data were analyzed with the SAS Enterprise Guide 4.1 (SAS Institute Inc., Cary, USA) program using ANOVA followed by Tukey HDS test (Honestly Significant Different) with a confidence interval of 95% (P-value < 0.05).

4. Results

Direct effect of COS-OGA on the fungal growth

A preliminary assay was conducted to evaluate *in vitro* the direct effect of FytoSave® and FytoSol (COS-OGA elicitors) on the fungal growth. Mycelial plugs of two strains of *A. solani* (A1 and CRA1) were disposed on serial dilutions of both compositions of COS-OGA. No significant difference was observed between the growth of both strains on media with 0 to 125 mg L⁻¹ of the active ingredients COS-OGA, for both compositions. At the highest concentration tested, 625 mg L⁻¹, a decrease of the growth of both strains was observed, for FytoSave® and FytoSol: on FytoSave® amended media, the growth of colonies reached $83.6 \pm 1.1\%$ of the one of the control for the strain A1 and $82.5 \pm 1.2\%$ for the strain CRA1 but, on FytoSol amended media, only $16.5 \pm 1.1\%$ for the strain A1 and $18.7 \pm 1.5\%$ for the strain CRA1. The half maximal effective concentration (EC50) was calculated for FytoSol. EC50 values of 361.22 mg L⁻¹ and 431.41 mg L⁻¹ were calculated for strain A1 and strain CRA1, respectively.

Effect of FytoSave®, FytoSol and BTH treatments on disease severity and progress

In order to investigate the protective effect of FytoSave®, Fytosol and BTH, the severity of early blight (strain A1) infection was assessed on tomato wild-type Moneymaker. The disease severity evolved differently depending on the elicitor used (Figure 4.1a). The first symptoms appeared 2 days after inoculation (dai) whatever the treatment but the disease severity progressed rapidly on plants treated with water. After seven days, the disease severity of untreated plants reached about 15% whereas the severity was approximately 11 % of plants elicited with FytoSave® and BTH but less than 5% for plant tested with FytoSol. Significant differences between the AUDPC calculated on 7 days depending on the treatment allow separating the disease severity of plants treated with FytoSol, the one on plants treated with FytoSave® and BTH and the disease level on water-treated plants. All tested elicitors protected tomato against *A. solani* : around 35% protection for FytoSave® and BTH and 85% for FytoSol.

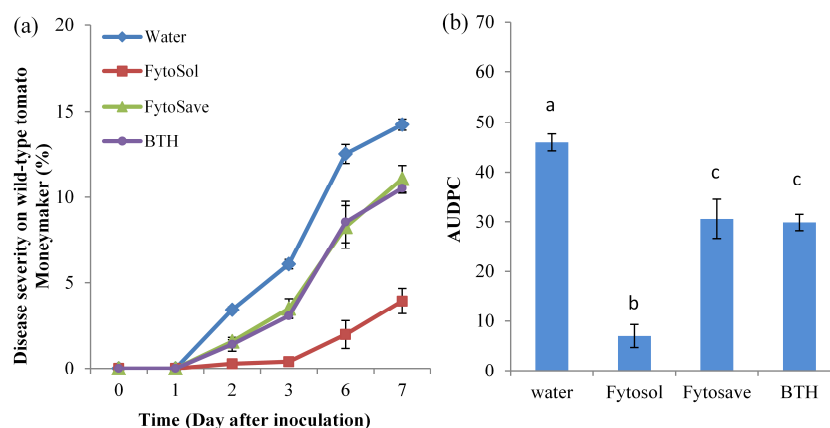


Figure 4.1: Disease severity progress (percentage of leaf area covered with symptoms) (a) and values of area under the disease progress curve (AUDPC) over 7 days (b) of wild-type Moneymaker tomato plants treated with COS-OGA elicitors (FytoSol and FytoSave®), Benzothiadiazole (BTH) or water (control) and inoculated with *Alternaria solani*. No symptoms were observed on non-inoculated plants. Results with a different letter are significantly different at $P \leq 0.05$ based on a Tukey test. The standard error range is calculated from four biological replicates.

A second bioassay was conducted under the same conditions with FytoSol to confirm its high protection against *A. solani*. In this bioassay, two concentrations of conidia were tested: 6,500 and 45,000 conidia/ml. Despite a lower disease progress on plant inoculated with 6,500 conidia/ml, a significant decrease in disease progress on FytoSol-treated plants was again observed for both concentrations of conidia with a mean percentage leaf area covered by symptoms of $3.4 \pm 1.2\%$ with 6,500 conidia/ml and $51.3 \pm 12\%$ with 45,000 conidia/ml for control plants and $0.3 \pm 0.1\%$ with 6,500 conidia/ml and $13 \pm 3\%$ with 45,000 conidia/ml for FytoSol-treated plants, at 7 dai. The AUDPC values of early blight of water- or FytoSol-treated plants calculated over 7 days were significantly different. AUDPC reached 10 ± 4.2 and 1673 ± 790 for untreated- inoculated-plants with 6,500 and 45,000 conidia/ml respectively. For plants treated with FytoSol and inoculated with 6,500 and 45,000 conidia/ml, AUDPC reached respectively 0.8 ± 0.3 and 141 ± 80 . The protection conferred by FytoSol was estimated to be 90% for both conidia concentrations, in the same range as reported here above for the first bioassay.

Protective effect of COS-OGA against the necrotrophic pathogen A. solani

In the same bioassay presented at figure 4.1, the efficacy of FytoSave®, FytoSol and BTH was also tested on tomato mutants in order to highlight the metabolic pathway targeted by the elicitors and specifically evaluate if SA and/or JA signaling pathways are involved in the plant defense reaction. Like on the wild-type Moneymaker (Figure 4.1a), symptoms due to early blight infection appeared two days after inoculation on both mutants, whatever the treatment, but the severity of symptoms evolved differently depending on the elicitor treatment and the mutant (Figure 4.2a). After seven days, a significant reduction of the AUDPC on transgenic *NahG* tomato plants was observed when plants were elicited with FytoSol and BTH compared to plants treated with water (Figure 4.2b). The protection level using BTH stayed stable (around 35%) on transgenic *NahG* tomato plants but it decreased for FytoSol than the one observed on the figure 4.1 on the wild-type Moneymaker. Indeed, the protection conferred by FytoSol against *A. solani* decreased to 50% on transgenic *NahG* plants instead of 85% on tomato wild-type Moneymaker. For tomato *Def1* mutants, a significant reduction of AUDPC appeared between plants treated with water and elicited with FytoSol, FytoSave® and BTH. This reduction was higher with FytoSol (50% of protection against *A. solani*) but it was also less than the reduction observed on the wild-type Moneymaker tomato plants (85% with FytoSol).

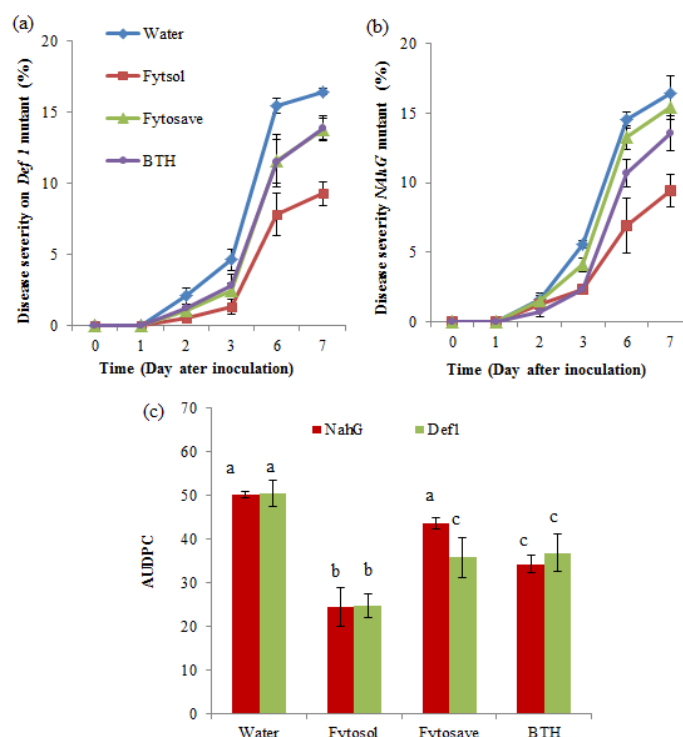


Figure 4.2: Disease severity progress (percentage of leaf area covered with symptoms) (a) and values of area under the disease progress curve (AUDPC) of *NahG* tomato mutant (b) and *Def1* tomato mutant (c) treated with COS-OGA elicitors (FytoSol and FytoSave®), Benzothiadiazole (BTH) or water (control) and inoculated with *Alternaria solani*. Non-mutant control (wild-type money maker tomato) was also used in the same bioassay and presented at figure 4.1. No symptoms were observed on non-inoculated plants. Results with a different letter are significantly different at $P \leq 0.05$ based on a Tukey test. These letters can be compared to the figure 4.1. The standard error range is calculated with four biological replicates.

SA quantification

By its pivotal role in regulation of both local defense and systemic acquired resistance (SAR) activated by PTI and ETI, the quantity of SA in wild-type Money maker tomato leaves was assessed for each treatment one and two days after the last elicitation (dae). SA was significantly higher in plants elicited with FytoSave® and FytoSol (figure 4.3) for both time points. This difference with plants treated with water highlight that SA is produced in response to the elicitation with COS-OGA

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elicitors. This significant increase of SA was not observed in plants treated with BTH despite a slight increase of SA 1dae.

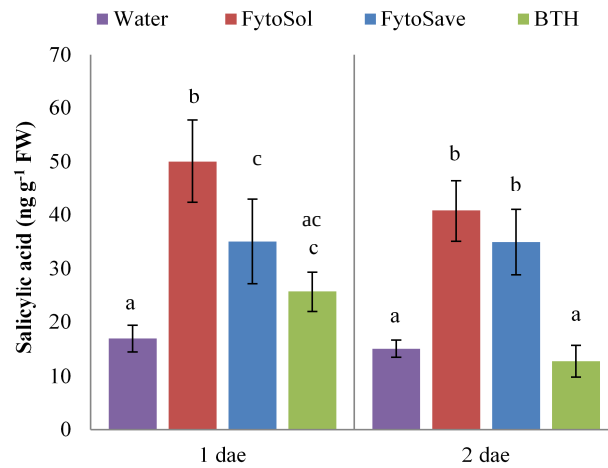


Figure 4.3: Quantification of salicylic acid (SA) content in tomato leaves cv. Moneymaker. Plants were treated with COS-OGA elicitors (FytoSol and FytoSave®), Benzothiadiazole (BTH) or water (control) and harvested one and two day after the last elicitation (dae). Results were expressed as means with standard deviation (n=6). Bars with different letters are statistically different (Tukey test $P < 0.05$).

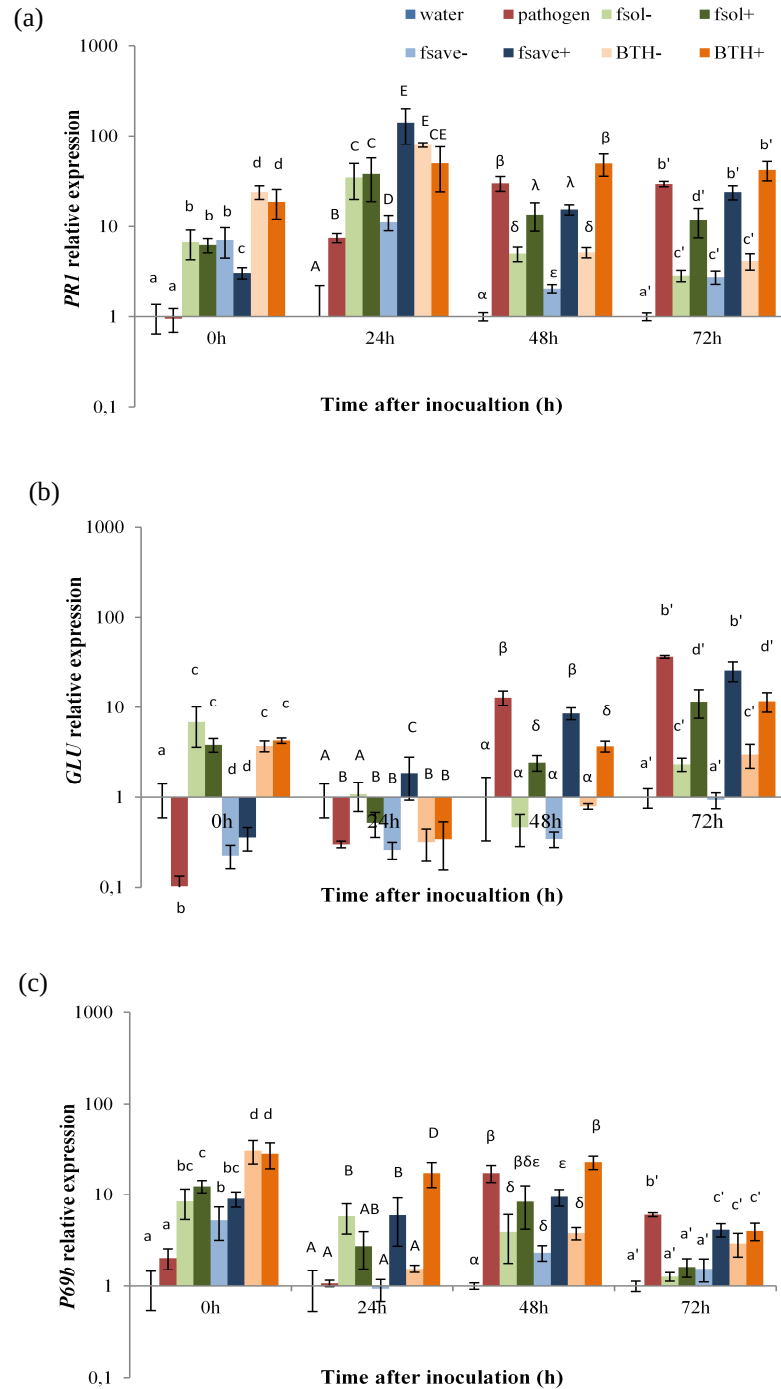
Expression analysis of defense genes

In order to differentiate the metabolic pathways involved in response to treatments with elicitors on tomato defense gene expression, the differential expression patterns of genes depending on SA (*PR-1*, *GLU*, *P69b*) and JA/ET (*LOXD*, *PR-3* and *PI-1*) defense pathways were quantified using RT-qPCR. Their relative expressions compared to the control plants (plants sprayed with water and uninoculated) (value=1) depending on the treatment and over time were compared after normalization using three housekeeping genes, *CAC*, *PGK* and *EF1- α* , previously validated after analysis of their stability and homogeneity.

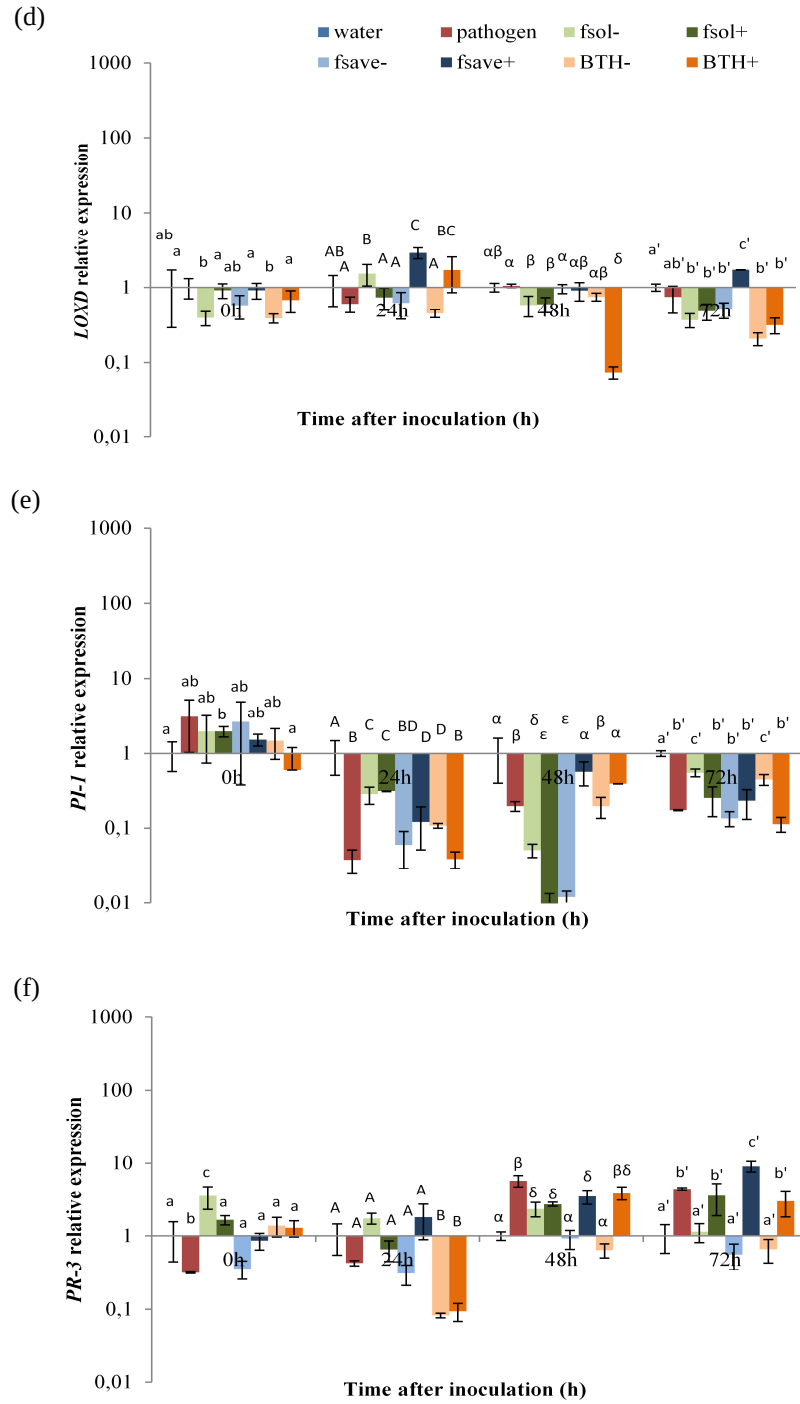
The expression of *PR-1* was significantly up-regulated after treatment with COS-OGA elicitors or BTH in plants, whether inoculated or uninoculated, from 0 hour post inoculation (hpi) until 72 hpi. At 48 hpi, a significant difference in relative quantities of *PR-1* transcripts appeared between inoculated and uninoculated plants treated with elicitors (Fig. 4.4a). The level of *GLU*, coding for a β -1,3-glucanase, also rapidly accumulated in tomato leaves elicited with FytoSol and BTH at 0 hpi but not for FytoSave® treated leaves for which a down-regulation was observed. The up-regulation rapidly decreased at 24 hpi for FytoSol and BTH but an upregulation appeared at that moment in leaves treated with FytoSave® and inoculated. At 48 hpi, the transcript levels of *GLU* were significantly higher in plants inoculated with *A. solani* and elicited than plants uninoculated and elicited whatever the elicitor (Fig. 4.4b). The elicitation of plants with COS-OGA elicitors (FytoSave® and FytoSol) or BTH sharply up-regulated *P69b* gene expression encoding for a subtilisin-like protease b at 0 hpi for all treatments (Fig 4.4c). At 24 hpi, a significant up-regulation of the expression of this gene occurred in plants elicited with FytoSave® or BTH and inoculated with *A. solani*. This up-regulation was generalized in all treatments at 48 hpi but no significant difference was observed at least at 72 hpi for FytoSol and plants treated with FytoSave® and uninoculated. The relative quantities of *LOXD* fluctuated less (between 0.07 and 1.7) over the course of the experiment than *PR-1*, *GLU* and *P69b* (Fig 4.4d). A significant up-regulation of *LOXD* was observed at 24 and 72 hpi in plants treated with FytoSave® and inoculated with *A. solani*, expression. The expression was down regulated in plants treated with BTH at 48 hpi, with FytoSol and BTH (inoculated or not) at 72 hpi, and treated with FytoSave® without *A. solani* at 72 hpi. Non-significant difference of *PI-1* transcripts (Fig 4.4e), encoding for a protease inhibitor 1 protein, is observed at 0 hpi between treatments. After 0 hpi, a significant down regulation appeared for all treatments. A significant up-regulation of *PR-3* encoded for a basic endochitinase class II, was observed only in plants elicited with FytoSol and uninoculated at 0 hpi (fig 4.3f). No more significant difference was observed with this elicitor at 24 hpi but a down regulation was observed for BTH treated plants (inoculated or not). After, an up-regulation of *PR-3* transcript was generally observed in plants inoculated with *A. solani*.

Protective effect of COS-OGA against the necrotrophic pathogen *A. solani*

Genes depending on SA defense pathway



Genes depending on JA defense pathway



Protective effect of COS-OGA against the necrotrophic pathogen A. solani

Figure 4.4 (Two previous pages) Expression relative to control plants over time of six genes in tomato leaves treated or untreated with COS-OGA elicitors (FytoSol or FytoSave®) or Benzothiadiazole (BTH) and inoculated (+) or not (-) with *Alternaria solani*. The genes investigated were (a) pathogenesis-related 1 (*PR-1*), (b) β -1,3-glucanase (*GLU*), (c) Subtilisin-like protease b (*P69b*), (d) Lipoxygenase (*LOXD*), (e) protease inhibitor 1 (*PI-1*) and (f) Basic endochitinase class II (*PR3*); expression levels were normalized with housekeeping genes *CAC*, *PGK* and *EF1- α* . The relative expression level of each candidate gene is given in logarithmic form. Results for the same time point with a different letter are significantly different at $P \leq 0.05$ based on a Tukey test. The standard error range is calculated with four biological replicates and takes into account two technical replicates.

Induction of defense-related enzymes by COS-OGA elicitors and BTH application

Still to differentiate the metabolic pathway and in addition to the expression analysis of defense genes in tomato plants, activities of some enzymes were quantified. A significant increase of β -1,3-glucanase (*GLU*) activity at 0 hpi in all plants elicited with FytoSave®, Fytosol and BTH (Figure 4.5). This activity decreased rapidly at 24 hpi and no more difference between treatments was recorded except for FytoSave® uninoculated-treated plants and BTH inoculated-treated plants. At 48 hpi, the β -1,3-glucanase activity was significantly higher in plants inoculated with *A. solani* for all treatment except FytoSave®. This increase in plants interacting with the pathogen was observed for all treatments at 72 hpi.

Increased peroxidase activity (*POD*) only occurred, at 0 hpi, in plants treated with FytoSol in the presence or not of *A. solani*. This higher activity disappeared at 24 hpi. After, the activity of this enzyme was significantly higher in plants interacting with the pathogen.

Significant higher polyphenol oxidase (*PPO*) activity was observed from 0 to 24 hpi in leaves treated with the three elicitors. After, a higher *PPO* activity was observed in plants interacting with *A. solani* whatever the treatments.

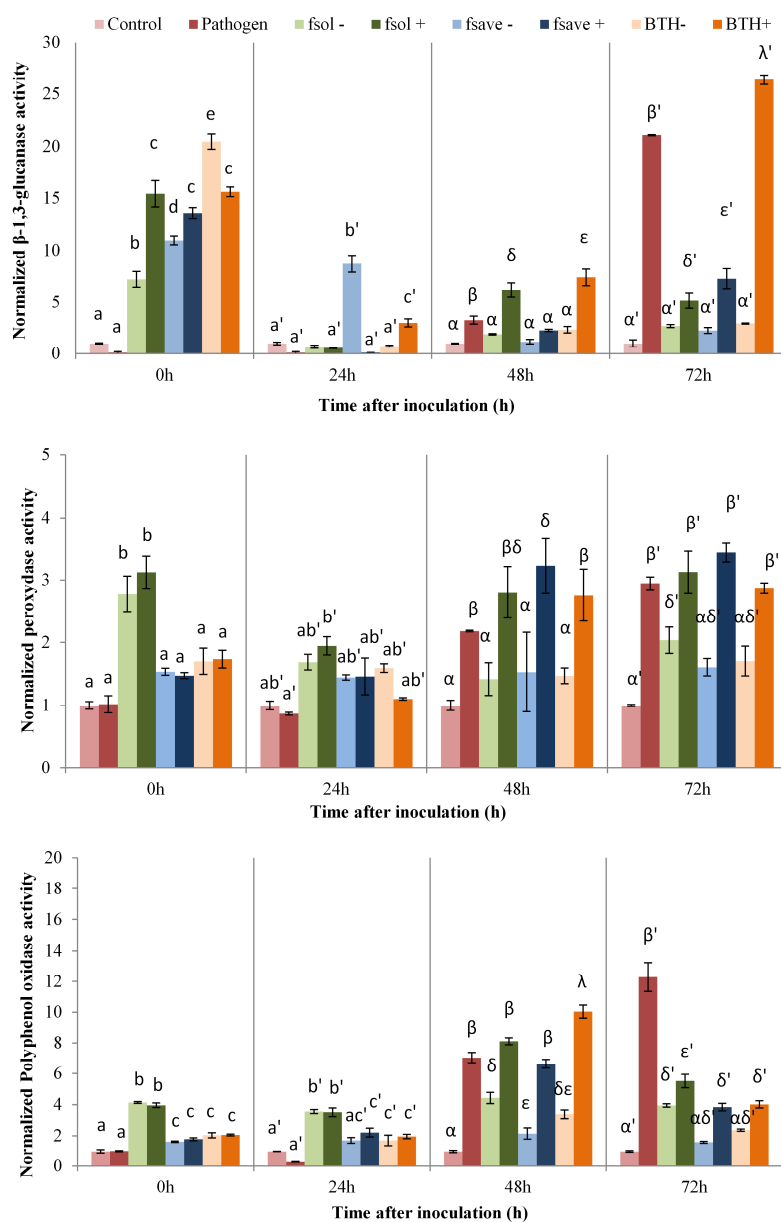


Figure 4.5: Enzymatic activity relative to control plants (treated with water) over time of β -1,3-glucanase, Peroxidase and Polyphenol oxidase in tomato leaves treated or untreated with COS-OGA elicitors (FytoSol and FytoSave®) or Benzothiadiazole (BTH) and inoculated (+) or not (-) with *Alternaria solani*. Results for the same time point with a different letter are significantly different at $P \leq 0.05$ based on a Tukey test. The standard error range is calculated with four biological replicates and takes into account three replicates

5. Discussion

In this study, we investigated the potential use of two synthetic elicitors, FytoSave® and FytoSol, on tomato early blight caused by *A. solani*. Both products contain different chitosan oligomers (COS) and pectin-derived oligogalacturonides (OGA) fractions. These components were associated in order to mimic a PAMP and a DAMP, and induce a plant defense response when they are applied on plants. Bioassays were performed under controlled conditions on wild-type tomato cv. MoneyMaker and on tomato mutants deficient in the SA-or JA-signaling pathway to evaluate the effects of two compositions of COS-OGA elicitors against *A. solani* and to understand their modes of action. Their efficacy was compared with the one provided by BTH (Bion®, Syngenta), well known to mimic the defense-associated effects of SA against a broad spectrum of phytopathogens by SAR activation (Bektas & Eulgem, 2014). Although resistance to necrotrophic pathogens is usually controlled by the plant hormones jasmonic acid and ethylene pathway (Thaler et al., 2012), BTH has repeatedly been reported to be effective against *Botrytis cinerea* in various plants including tomato (Achuo et al. 2004).

In our experiments, a significant reduction in early blight severity on tomato plants treated with the three elicitors was observed. The protection conferred by FytoSol (about 85%) was significantly greater than FytoSave® and BTH (about 35 %). The reduced disease severity on plants elicited with COS-OGA or BTH suggested that these elicitors can induce a defense against *A. solani*, a necrotrophic pathogen. Previous studies reported good protection of COS-OGA (FytoSave®) (respectively 75 and 95%) against the biotrophic pathogens *Sphaerotheca fuliginea* and *Uncinula necator* (van Aubel et al., 2014). The protection by FytoSave® against the hemibiotrophic pathogen *P. infestans* is lower (around 50 %) (Clinckemaillie et al., 2017), but a recent study realized by van Aubel et al. (2017) revealed that FytoSol induce a complete protection in controlled conditions against *P. infestans*. The results obtained with FytoSave® and FytoSol against these various pathogens under controlled conditions show that the application of these elicitors leads to reduction of several diseases. However, the protection conferred largely depends of the composition of

the elicitor tested, the pathogen and its pathogenic strategy. FytoSave® has the strongest protective effect on biotrophic pathogens while FytoSol can control nearly perfectly hemibiotrophic and necrotrophic pathogens.

This differential effective protection depending on the type of pathogens could be explained by different defense metabolic ways. Previous studies showing that chitosan -COS is derived from chitosan, a component of FytoSave® and FytoSol, has a double effect: it acts as antimicrobial abiotic agent (El Hadrami et al., 2010) and it also activates several plant defense mechanisms during host-pathogen interactions. These combined effects led to reduced disease severity due to callose deposition, lignification, synthesis of abscisic acid, phytoalexins, and pathogenesis related proteins as reported in previous studies (Baustista-Baños et al., 2006; Cavalcanti et al., 2007). In our study, no direct effect of 62.5 mg L⁻¹ COS-OGA in FytoSave® or FytoSol (concentration recommended for field application) on the *in vitro* mycelial growth of *A. solani* was observed. This observation supports that the reduction of disease severity in plants treated with the elicitors at 62.5 mg L⁻¹ COS-OGA is an effect of their interaction with plant rather than a biocide activity. However, at the highest tested concentration (625 mg L⁻¹), FytoSave® and FytoSol reduced *in vitro* mycelial growth of *A. solani* with a higher *in vitro* direct toxicity for FytoSol than FytoSave®.

The protective effect of COS-OGA elicitors can be explained by the activation of plant defense mechanisms. Analyze of defense related enzyme activity like PPO, POD and GLU shown that elicitation with the both elicitors conduct to higher activities of these enzymes and thus the activation of plant defense mechanisms. These proteins are often implicated in the induced resistance of plants against pathogen (Baysal et al., 2003; Li and Steffens, 2002; Nagy et al., 2004; Pina and Errea, 2008; Trotel et al., 2006; Song et al., 2011). In order to determine more exactly the plant defense metabolic pathway induced by the tested elicitors, analytical methods were combined to molecular methods. The relative expression of several genes known to be markers of defense signaling pathways in tomato plants depending on the elicitor was quantified in order to understand the defense response in the presence of *A. solani*.

FytoSave® increased the expression of PR-protein like *PR-1*, basic or acidic β -1,3-glucanase (*PR-2*), subtilisin-like protease (*P69b*). Expression of *PR-1* and *PR-2* is generally linked to the SA pathway (Caarls et al., 2015) and *P69b* gene was shown to be induced following SA application (Tornero et al., 1996a; Jorda et al., 1999). However, transcripts specific to ISR and linked to JA and ET like *LoxD*, *PI-1* and *PR-3* (Finiti et al., 2014; Fujimoto et al., 2001) were not regulated. Furthermore, the treatment with FytoSave® induced an increase of the SA level in tomato leaves. The comparison of the effect of FytoSave® of the *NahG* tomato lines and *Def1* mutant, respectively deleted of SA and JA pathway, demonstrated clearly that SA is involved in the FytoSave®-mediated protection of tomato against *A. solani*. The results of this study corroborate results reported in previous studies on the mode of action of FytoSave® on tomato plants (van Aubel et al., 2014) or against *P. infestans* on potato plants (Clinckemaillie et al., 2017).

The mode of action of FytoSol was investigated in a same approach in this study to understand the plant responses in the presence of the necrotrophic pathogen *A. solani* on tomato plants. The accumulation of PR proteins like PR-1 or PR-2 related to the SA pathway and the accumulation of endogenous SA in tomato leaves during the first 24 hours after the elicitation highlight that the SA-defense pathway plays also a role in the defense induced by this elicitor. However, like FytoSave®, transcripts to JA and ET like *LoxD*, *PI-1* and *PR-3* (Finiti et al., 2014; Fujimoto et al., 2001) were not regulated. Furthermore, the significant protection obtained on *NahG* and *Def1* mutants showed that SA and JA pathways don't explain by its own the induction of resistance. The activation of SA and JA pathways was previously reported for other elicitors such as Rhamnolipids, for example in the defense of *Arabidopsis* against the necrotrophic fungus *Botrytis cinerea* (Sanchez et al., 2012). The mode of action of FytoSol seems thus different from one of FytoSave® which can explain why Fytosol has a higher efficacy against the necrotrophic pathogen *A. solani*. Nevertheless, we cannot exclude the participation of others hormones like ET or ABA on this induced resistance in tomato against *A. solani*. Indeed, action and interplay with JA and SA signaling have been already reported after ABA application (Flors et al., 2008) and Song et al. (2011) have suggested that ABA may be an important signal in the activation of

defenses against the necrotrophic pathogens *A. solani*. After analysis of transcripts of the ABA pathway, an accumulation of this hormone was indeed found after elicitation of potato leaves by FytoSol without pathogen (van Aubel et al., 2017). In this study, the JA pathway was also activated with accumulation of transcripts rely to the oxylipins synthesis and an important activation of the ET pathway. SA pathway was lower affected by FytoSol in this study where the time point used (72hpi) differs from those used in this study (0 and 24hpi). It is important to keep in mind that the defense response induced is a dynamic process which involved many gene expressions and vary rapidly over time. The implication of ET, JA and ABA was previously demonstrated against potato late blight after elicitation with β -aminobutyric acid and chitosan in other studies (Atia et al., 2005; Bengtsson et al., 2014; Malerba & Cerana, 2016).

6. Conclusion

The aim of this study was to investigate the effect of repeated treatments of leaves of tomato with FytoSave® and FytoSol against the necrotic fungus *A. solani*. Our results demonstrate that the application of these elicitors conferred a protection on tomato against early blight severity. This effect was demonstrated to be due to the activation of plant defense. The level of protection largely depends of the composition of the tested elicitor, the pathogen and its pathogenic strategy. Gene expression and enzyme activity analysis showed that elicitation by COS-OGA or BTH induced increased expression of PR proteins like *PR-1* and *GLU* (or *PR-2b*). This activation seems to be clearly modulated by SA-dependent pathway for FytoSave®. The higher protection due to the application of FytoSol can be explained by the difference of gene expression and enzymatic activities linked to distinct metabolic pathways. Our results showed that FytoSol activates SA pathway. The protective effect of this elicitor against *A. solani* is not associated to the activation of the SA-signaling pathway, as shown by the protective effect on tomato mutants. The JA pathway was reported to be required for resistance against necrotrophic pathogens (Rahman et al., 2012). Our results corroborate partly results reported by van Aubel (2017) indicating that FytoSol could trigger a completely different parallel pathway connected or not to the accumulation of SA or the activation of *PR* genes. Further investigations are required to determine the precise roles

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and signaling pathways activated by the treatment of plants with FytoSol. Our study suggests that the use of elicitors may be a promising strategy in integrated pest management in sustainable agriculture.

Chapter 5

General discussion

The use of elicitors of plant resistance in crop protection and pest management is still in the early stage of the process but, as suggested before by Stout et al. (2002), this strategy may serve as an important component of a multifaceted, ecologically-based pest management program. Recent studies have shown that the use of elicitors can reduce disease or damage severity caused by insects, fungi, pests or herbivores on various plant varieties (Thakur & Sohal, 2013). Elicitors offer interesting advantages as they reduce environmental hazards and they are not toxic to pathogens. Furthermore, the protection conferred by elicitors is not specific and can potentially act against multiple pathogens (Micheltore et al., 2013). On the other hand, plant defense can be costly, requiring energy and resources that would otherwise be used for growth and development (Walters et al., 2013) and creating a dilemma for the plant: to grow or defend. However, induced plants seem able to compensate for diversion of energy and resources towards defense by increasing photosynthetic rates (Murray & Walters, 1992) and growth rates (Dietrich et al., 2005). Some elicitors act differently and enhance the capacity of the plant to activate immune responses. This sensitization process, which is called priming, can be triggered by elicitors but also by pathogens, beneficial microorganisms or wounding (Conrath, 2006; Beckers & Conrath, 2007; Goellner & Conrath, 2008). This defense priming is postulated to be an adaptive, low-cost defensive measure because defense responses are not, or only slightly, activated by a given priming stimulus (Martinez-Medina et al., 2016). Instead, defense responses are deployed in a faster, stronger, and/ or more sustained manner following the perception of a challenging signal (the triggering stimulus). There is thus a potential for the use of elicitors of plant resistance in the management programs of diseases and pests but in depth knowledge on their effectiveness, required conditions of effectiveness and modes of action of elicitors are of great importance to evaluate their potential to stimulate plant defenses and help the plants against pathogen infection or pest infection.

The main question treated in this thesis concerned the potential use of the elicitor COS-OGA, a PTI inducer, to protect *Solanaceae* against disease caused by fungal and oomycete pathogens. Experiments were performed in order to evaluate the efficacy of this elicitor against *P. infestans* and *A. solani*, to understand the modes of action of two COS-OGA based-products and highlight the conditions that can affect the plant defense response. In this part of the thesis, we will discuss whether the three hypotheses formulated in the beginning of this work are validated or not.

COS-OGA reduces diseases caused by *P. infestans* and *A. solani* but the protection depends on the elicitor composition, the pathogen and its type of parasitism

Firstly, we assumed that the efficacy of COS-OGA is influenced by the composition of the product, the pathogen and its type of parasitism. To answer this question, bioassays were conducted under controlled conditions using two formulations of COS-OGA (FytoSave® and FytoSol) which contain different chitosan oligomers (COS) and pectin-derived oligogalacturonides (OGA) fractions. Previous studies revealed that the degree of polymerization of OGA, the degree of OGA methylation, as well as the degree of acetylation and the pattern of acetylation of COS strongly modified the way how oligosaccharides stimulates PTI (Cabrera et al., 2006, Madhuprakash et al., 2015; Ridley et al., 2001). The protective effect of FytoSave® and FytoSol were tested *in planta* under controlled conditions on the pathosystem *Solanum lycopersicum* (tomato)-*Alternaria solani* to compare the effect of COS-OGA composition. On this pathosystem, both compositions lead to the reduction of the disease severity. However, the efficacy of FytoSave® was lower than those obtained with FytoSol. We have not strictly compared the two COS-OGA elicitors in our assays on the pathosystem *S. tuberosum*-*Phytophthora infestans* but results obtained by Van Aubel (2017) show that FytoSol is more efficient (protection nearby 100%) than FytoSave® (protection around 50%). These results demonstrated that the efficacy of COS-OGA is influenced by the composition of the product.

This efficacy can also vary according to the pathogens and thus its type of parasitism. Our results obtained with FytoSave® and FytoSol showed that treatments of tomato leaves using FytoSave® reduced 30% of the disease severity caused by *A. solani* whereas the protection conferred by FytoSol reached 80%. The protection against the hemibiotrophic pathogen, *P. infestans*, was estimated to 50% for FytoSave® (Clinckemaillie et al., 2017) and nearby 100% for FytoSol (van Aubel, 2017). Previous studies reported that 72-85% protection for FytoSave® on cucumbers against *Sphaerotheca fuliginea* and from 76 to 96% protection on grapevines against *Uncinula necator*. These protection rates depend on the measurement time after treatment and disease pressure (low to moderate) in vineyards and greenhouses (van Aubel et al., 2014). These two latter pathogens are biotrophic; their feeding and infection strategies differ thus from the plant pathogen interaction that involves hemibiotrophs or necrotrophs. All these results suggest that FytoSave® has a strong protective effect on biotrophic pathogens while FytoSol is more efficient against hemibiotrophic and necrotrophic pathogens. These results confirmed that the elicitor composition, the pathogen and its type of parasitism influence COS-OGA efficacy. Some chemical inducers of disease resistance have already been tested for controlling the severity of diseases with varying efficiency. Examples include benzothiadiazol (BTH) and β -aminobutyric acid (BABA) against potato late blight. No protection against *P. infestans* has been reported for BTH (Si-Ammour et al., 2003), but 15–100% protection has been reported for BABA, depending on the potato cultivar and *P. infestans* strain used (Sharma et al., 2010).

Metabolic pathways induced by COS-OGA elicitors

The second hypothesis formulated in this work concerns the plant defense response. We assumed that the plant defense responses differ according to the composition of COS-OGA based product and its mode of action. Since plant defenses are part of metabolic pathways involved in plant-pathogen interaction, we also assumed that the defense responses differ according to the pathogen and its type of parasitism.

Elicitors induced plant resistance through activation of different defense pathways but the molecular mechanisms underlying activation of plant defense responses are complex (Kunkel and Brooks, 2002). This

resistance is also associated with the activation of complex signaling networks where plant hormones play a crucial role. Plant hormones such as SA, JA and ET are considered as the backbone of this network. In this context, our researches on the modes of action of COS-OGA elicitors focused on two main defense pathways, SA and JA/ET related to SAR and ISR, respectively. Other hormones like abscisic acid (ABA), auxins and gibberellins (GA) were reported to be involved in defense signaling networks (Novakova et al., 2014; Pieterse et al., 2012) but they were not taken into account in our experiments. In the plant-pathogen interaction, plant pathogens interact with distinct metabolic pathways depending on their modes of parasitism. Owing to their defense functions, plant hormones are major targets of pathogens attack and manipulation (Mobius and Hertweck, 2009). Information about the mode of action of COS-OGA compositions can help to improve the efficiency or the strategy of use of FytoSave® and FytoSol. In the case of biotrophs that derive their nutriment from living host tissues (Glazebrook et al., 2005), it is easy to imagine that activation of SA signaling leads to HR which could result in resistance. The HR response would deprive such pathogens of a food source. On the other hand, hemibiotrophs and necrotrophs could accommodate increase in SA-related gene expression and endogenous SA accumulation to promote infection. SA has been shown to promote disease development caused by the necrotrophic pathogen *Botrytis cinerea* through NPR1 and the transcription factor TGA1.a, by suppressing the expression of two JA-dependent defense genes, proteinase inhibitors I and II. These data highlight how necrotrophs manipulate the SA-signaling pathway to promote their disease infection (Rahman et al., 2012).

To determine if SA and/or JA/ET- defense pathways are involved in the defense induced by COS-OGA, a comparative study of both oligosaccharide elicitors on potato and tomato defense induction was performed. The treatments of leaves with FytoSave® resulted in an increased expression of PR-protein such as *PR-1*, basic or acidic β -1,3-glucanase (*PR-2*) and subtilisin-like protease (*P69b*) on the pathosystem *S. lycopersicum*-*A. solani*. These results corroborate those obtained in the pathosystem *S. tuberosum*-*P. infestans* with FytoSave®. Furthermore, the treatment with the elicitor induced an increase level of SA in tomato leaves and a higher defense related enzyme activity like

PPO or GLU. The comparison of the effect of FytoSave® of the *NahG* tomato lines and *Def1* mutant, respectively deleted of SA and JA pathway, demonstrated clearly that SA is involved in the mode of action of FytoSave®. These results could explain why FytoSave® is particularly efficient against biotrophic pathogens. SA pathway leads to the induction of the hypersensitive response (HR) which confines biotrophs by abolishing nutrient supplies thereby limiting pathogen growth.

Regarding FytoSol, the accumulation in the pathosystem *S. lycopersicum* – *A. solani* of PR proteins like PR-1 or PR-2 related to the SA pathway and the accumulation of endogenous SA in tomato leaves during the first 24 hours after the elicitation highlight that the SA-defense pathway also plays a role in the defense induced by FytoSol. Furthermore, the significant protection obtained in *NahG* and *Def1* mutants, did not show that either SA pathway or JA pathway are solely responsible for the induction of resistance. Both mutants exhibited lower protection than wild type tomato but non-zero protection, suggesting the contribution of another signaling pathway in the induction of the defense response by FytoSol in tomato against *A. solani*. Previous studies on tomato against *A. solani* reported action and interplay with JA and SA signaling after ABA application (Flors et al., 2008) and Song et al. (2011) suggest that ABA may be an important signal in the activation of defenses against the necrotrophic pathogens *A. solani*. After analysis of transcripts of ABA pathway, an accumulation of this hormone was indeed highlighted after elicitation by FytoSol on potato against *P. infestans* (van Aubel, 2017). In this previous study, JA pathway was also activated with accumulation of transcripts which rely on the oxylipins synthesis. An important activation of the ET pathway was also observed. SA pathway was less affected by FytoSol in this study. Implication of ET, JA and ABA was previously demonstrated against potato late blight after elicitation with β -aminobutyric acid and chitosan (Atia et al., 2005; Bengtsson et al., 2014; Malerba & Cerana, 2016). The use of mutant plants deleted on ET and ABA could confirm this implication.

The mode of action of FytoSol seems therefore different from the one of FytoSave®, with several pathways probably involved. This characteristic could explain why FytoSol is more effective than FytoSave® against hemibiotrophic and necrotrophic pathogens. Our

results combined with the results obtained by van Aubel (2017) demonstrated that the effectiveness of COS-OGA depends on the formulation which can affect the molecular interaction between plant and-pathogen. In the context of plant protection, it is very important to address the right composition against the right pathogen according to its type of parasitism.

Factors affecting the efficacy of COS-OGA

The impact of factors, like climatic conditions, genotype of the host plant and strain of the pathogens, on the interaction between plant pathogen and elicitor was our third hypothesis. The idea here can be illustrated by the disease triangle, where the intensity of the disease (the size of the triangle) could vary depending on the treatment or not with the elicitor (two previous hypotheses) but also depending on the other components of the triangle: genotypes of host, pathogens and the environment.

To demonstrate this assumption, a first challenge in this thesis was to design standardized bioassays to allow the evaluation of protective effect of FytoSave® and FytoSol. In these bioassays performed under phytotrons or greenhouses conditions, we have tried to control parameters such as the relative humidity, the age of plants, the host genotype, the temperature, the strain used, and the concentration of inoculum to obtain a rapid infection. Bioassays were performed by using optimal conditions recommended by the team of Pierre Van Cutsem (UNamur) to enhance plant defense response to the elicitation with COS-OGA. Despite these precautions for a right infection, some parameters are difficult to monitor and vary during the experiment. For example, in greenhouses, it is more difficult to control the relative humidity than in phytotrons. The rate of infection on cv. 'Bintje' with *P. infestans* can easily reach 73 % in phytotrons or in some greenhouses (small compartments) while this rate can be reduced down to only 1 or 2 % when the humidity is low or not strictly controlled. According to this, the elicitor can have different efficiencies. All these factors can also explain why elicitors used in field conditions often show a reduced efficacy when compared to results obtained under laboratory conditions. It is mainly because interactions with the environment affect the outcome of

the induced resistance mechanisms (Aljabal et al., 2015). Defense pathways are a complex metabolic network of cross-talk and feedback mechanisms and therefore affected by many biotic or abiotic environmental factors (Wielsel et al., 2014). Another important parameter modulating plant defense response is the host genotype. Our experiment on potato cultivars but also on tomato mutants revealed that different host genotypes which determine the innate capacity to resist, can show significant differences in the expression of induced resistance to *FytoSol*. Another factor which can affect the induced resistance is the strain used and the density of pathogen inoculum. In this thesis, the density of inoculum was determined from the literature to obtain a good infection. However, this inoculum density was not related to the natural inoculum present in field and can explain why the protection obtained using elicitors in the field under high infection pressure differ from those obtained in laboratory under lower infection.

As mentioned above, to have a successful use of elicitors in agriculture, it is important to understand their effects on plant defense. It is also important to identify plant and environment related factors that can affect the defense responses. Despite all the investigations about induced resistance in plants using elicitors, their use in crop protection is not a common practice, and only a few compounds have been tested or used in agriculture. The protection obtained using elicitors in the field under high infection pressure was often reported to be incomplete (Llorens et al., 2017) For example, although most studies of acibenzolar-S-methyl (ASM) under field conditions report disease control, the level of control ranges from 4-99% (Walters et al., 2005). The efficacy of an elicitor in crop protection relies on the induction of plant resistance and thus on a plant response. It contrasts with conventional pesticides that have a direct toxic effect on the target organisms and therefore a more predictable effect. The use of elicitor in agriculture requires a change in the disease management approach by farmers and in the expectations of a treatment. To promote broader utilization of elicitor in agriculture, it will also be important to specify the required conditions to reach efficacy. Promising results were obtained when several resistance inducers were applied together or with chemical treatments. For instance, in citrus, the application of BABA with ASM resulted in a high and stable reduction of '*Candidatus Liberibacter asiaticus*' (Li et al., 2016). The synergistic effect of resistance inducers, as a preventive compound

or in combination with classical pesticides, can offer a valuable tool for reducing the residues of chemical pesticides. A correct schedule of treatments with alternation between these compounds and classical ones could be useful for both reduction of pest damages and reduction of chemical residues. Moreover, the use of resistance inducers in combination with nutritional compounds like NH_4^+ or the colonization with arbuscular mycorrhiza could be an interesting alternative to preventive and curative treatments. By using these strategies, plants can achieve an enhanced basal resistance that permanently protects themselves against possible biotic but also abiotic stresses (Llorens et al., 2017).

With regards to the use of COS-OGA in agriculture, assays have been performed with FytoSave® in field or commercial greenhouse conditions. Results showed that FytoSave® is effective in preventing protection under low to moderate pressure against powdery mildew (van Aubel et al., 2014). However, results of several assays conducted in fields using FytoSol for the control of potato late blight (G. van Aubel, pers. comm.) were not sufficient to promote the use of this elicitor under high pressure of *P. infestans* on susceptible genotypes. These results highlight the fact that other biotic or abiotic factors can interact in fields and act on the disease triangle, modifying plant response to the elicitation. This confirms the difficulty to use an elicitor under field conditions and the importance to study and specify required conditions to reach efficacy. The combination of COS-OGA and small doses of fungicide can be exploited to perform a better protection in field. In the same way, combination with other resistance inducers can also be investigated. We have also highlighted the possibility to use COS-OGA with different plant genotypes to increase the resistance induced. All these elements demand additional investigations in order to integrate at best COS-OGA into a field treatments scheme.

Chapter 6

Conclusion and perspectives

Plant disease control in the 21st century faces multiple challenges (Walters et al. 2005). Plant pathogens are continuously evolving, leading to the selection of strains able to circumvent host resistance genes. The strains used can also become resistant to fungicides when they are repeatedly used. In addition, the environmental effects of widespread use of chemicals are an increasing concern. In this context, alternative approaches of plant disease control need to be implemented in a sustainable way. Induced resistance exploiting natural defense machinery of plants could be proposed as a promising and a sustainable alternative approach for plant protection. Induced resistance can be defined as an increased expression of natural defense mechanisms of plants against various types of pathogens. It can be activated by avirulent or attenuated pathogenic strains, elicitors of pathogenic origin (glucans, proteins, lipids, etc.), abiotic stress or synthetic elicitors such as 2,6-dichloroisonicotinic acid (INA), β -aminobutyric acid (BABA), benzothiadiazole (BTH), etc. Induced resistance is based on the expression of genetic information present in plants and it is not the result of genome alterations (mutations, introgression of foreign genetic material (Edreva, 2004).

In this thesis, the use of COS-OGA elicitors to control disease on *Solanaceae* was largely investigated. This elicitor stimulates PTI by imitation with chitooligosaccharides, COS (as PAMP) and oligogalacturonides, OGA (as DAMP) of a plant pathogen interaction (Van Cutsem & Messiean, 1994; El Gueddari et al., 2002). Before this thesis, the first data available on COS-OGA were limited to *Arabidopsis thaliana* cell suspension cultures, an excellent laboratory model but far from the whole plant and the field (Cabrera et al., 2010). First assays in fields or in greenhouses against powdery mildew have also been performed with FytoSave® and have given decisive results (Van Aubel et al., 2014). This present work contributes to a deeper understanding of the efficacy and the mechanisms involved after elicitation with COS-OGA elicitors to protect *Solanaceae* against diseases caused by fungi or oomycetes.

To protect plants against pathogenic agents, it is important to have a good understanding of parameters affecting the epidemiology of a disease. As presented in chapter 1, the disease triangle is a conceptual model that shows the interactions between the environment, the host and an infectious agent which lead to a disease. The objective of this thesis was to understand how COS-OGA interacts with the three main actors of this triangle (figure 6.1) –plant, pathogen and the environment- and to highlight the factors determining plant responses.

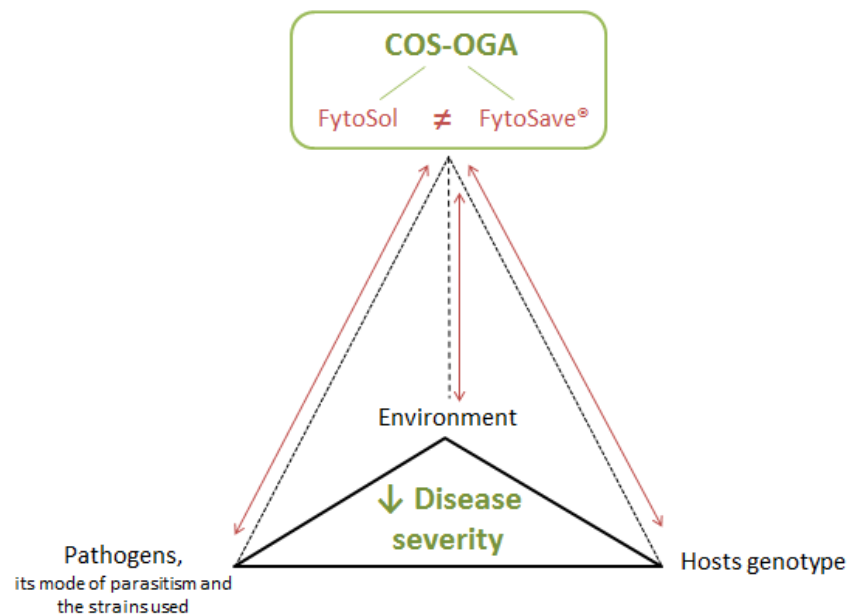


Figure 6.1: Interaction between COS-OGA products and the three main actors of the disease triangle –plant, pathogen and the environment. The application of COS-OGA elicitors on plants leads to reduction of the disease (in green). However, the protection conferred depends of the composition of the elicitor tested; the pathogen, its type of parasitism and the strain used; the host genotype and the environmental conditions (in red).

The application of COS-OGA elicitors on potato or tomato plants leads to reduction of early and late blight. However, the conferred protection largely depends of the composition of the elicitor tested, the pathogen and its pathogenic strategy. FytoSave® is reported to have a strong protective effect on biotrophic pathogens. Our work reveals that the defenses of plants using this product were not sufficient to protect them against potato late blight and that the other formulation of COS-OGA, FytoSol, was more efficient against *P. infestans* and *A. solani*. The hemibiotrophic and necrotrophic character of these pathogens has probably influenced this result. The protection conferred by these elicitors was shown to be correlated with the activation of different defense pathways. FytoSave® induced the SA-dependent pathway while FytoSol induced SA-pathway but also others pathways connected or not to the SA pathway. Results on RNAseq realized by van Aubel (2017) complete these results with an activation of ABA, ET and JA pathways after elicitation with FytoSol.

In this work, we demonstrated that the efficacy of COS-OGA can also be affected by the different, biotic and abiotic, components of the triangle disease, such as host and pathogen genotypes, concentration of inoculum, climatic conditions, ... Further researches are recommended to characterize the range of effectiveness of both COS-OGA elicitors, their mode of action and the requisite conditions to ensure efficient induction of plant defense to increase their place in crop protection.

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