Identification of elicitors inducing resistance in wheat against *Zymoseptoria tritici* and characterization of the subsequent triggered defense-signaling pathways

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ABSTRACT

The implementation of biocontrol products in integrated pest management strategies is a major challenge today in the transition to sustainable and environment-friendly agro-ecosystems. In particular, the use of natural elicitors, also called plant resistance inducers, represents an interesting alternative to conventional fungicides. Elicitors are natural immune-stimulating compounds which offer the advantage to indirectly target a broad spectrum of pathogens by enhancing the defensive state of the plant. Yet today, wheat is one of the most cultivated crops in the European Union and still requires fungicide protection every year for the control of a harmful disease: *Septoria tritici* Blotch (STB), caused by the fungal pathogen *Zymoseptoria tritici*. At a time when few elicitor products are available on the market for the sustainable management of crop diseases, the objective of this thesis project was to screen and identify innovative elicitors able to preventively protect wheat against the STB disease. Greenhouse trials successfully demonstrated the ability of λ-carrageenan, cytosine-phosphate-guanine oligodesoxynucleotide motifs (CpG-ODN), *Spirulina platensis*, glycine betaine and ergosterol to protect wheat by up to 70 % against the pathogen *Z. tritici*. These results are promising as previous research has indeed demonstrated the elicitor properties of these five compounds on other plant species and/or animals. Besides, no direct anti-fungal activity was recorded during *in vitro* experiments towards the disease. The risk of resistance development of the pathogen to these potential elicitors can thus be considered as low. Furthermore, the defense mechanisms of wheat were successfully demonstrated to be significantly induced following treatment with each of these formulated compounds. The relative expression of 23 plant defense genes was analyzed by qRT-PCR at 1, 2 and 3 days after plant treatment. Defense mechanisms involving the two hormones salicylic acid (SA) and jasmonic acid (JA) were triggered in treated wheat. These hormones play a key role in the transduction of defense signals throughout the plant. In addition, the protection efficacy of the two preferential candidates (λ-carrageenan and Spirulina) was investigated in the field during two successive years. Numerous parameters, among which environmental conditions, plant developmental stage, plant genotype and disease pressure, can indeed cause a variability of elicitor protection efficacy under practical conditions. Unfortunately, important contrasts in disease pressures and extreme weather conditions did not allow confirming the elicitor potential of the corresponding treatments on field. Finally, the potential effect of the formulation on the eliciting activity was characterized in order to rule out the possibility of interference by the selected adjuvants. Additional greenhouse experiments showed that a water solution containing only the adjuvants was as efficient to protect wheat against STB as plants treated with formulated or non-formulated λ-carrageenan. These last results highlighted the necessity of developing an appropriate formulation at an early stage before elicitor screening. Overall, the findings of this research study could open the way to the development of innovating biocontrol products based on λ-carrageenan for the sustainable protection of wheat against *Zymoseptoria tritici*.

RÉSUMÉ

L'intégration de produits de biocontrôle dans les stratégies de lutte intégrée contre les ennemis des cultures représente aujourd'hui un enjeu majeur pour développer des agro-écosystèmes durables et respectueux de l'environnement. En particulier, l'utilisation d'éliciteurs naturels, aussi appelés stimulateurs de défense des plantes, représente une alternative intéressante aux fongicides conventionnels. Les éliciteurs sont des molécules capables d'assurer un contrôle indirect des ennemis des cultures en induisant la résistance des plantes à un large spectre de pathogènes. Pourtant, le blé représente actuellement une des céréales les plus cultivées dans l'Union Européenne et requiert chaque année l'utilisation de fongicides chimiques pour contrôler une maladie fortement nuisible au rendement: la septoriose, causée par le champignon pathogène *Zymoseptoria tritici*. A l'heure actuelle, un faible nombre de produits éliciteurs sont disponibles sur le marché pour contribuer à une protection durable des plantes céréalières. Dans ce contexte, l'objectif de ce travail de thèse était de cribler et d'identifier des éliciteurs des défenses naturelles du blé innovants et permettant de protéger cette céréale majeure contre la maladie de la septoriose à titre préventif. Des essais en serre ont permis de montrer avec succès que le λ-carrageenan, les motifs cytosine-phosphate-guanine oligodesoxynucleotide (CpG-ODN), *Spirulina platensis*, la glycine betaine et l'ergosterol avaient chacun la capacité de protéger la plante de blé jusqu'à 70 % contre *Z. tritici*. Ces résultats sont prometteurs étant donné que des études antérieures ont démontré les propriétés élicitrices de ces composés sur d'autres espèces de plantes et/ou sur des animaux. De plus, aucune activité fongicide directe vis-à-vis de *Z. tritici* n'a été observée lors de tests en laboratoire. Le risque d'apparition de résistance du champignon pathogène à l'encontre de ces éliciteurs potentiels peut donc être considérée comme acceptable. En outre, la stimulation des défenses du blé suite à l'application de chacun des composés formulés a été démontrée avec succès. L'expression relative de 23 gènes de défense du blé a en effet été analysée par qRT-PCR à 1, 2 et 3 jours après traitement. Des mécanismes de défense impliquant les deux hormones acide salicylique (SA) et acide jasmonique (JA) ont été induits dans les plantes de blé traitées. Ces deux protéines jouent un rôle majeur dans la tranduction des signaux de défense au sein des plantes. Par ailleurs, l'éfficacité de protection des deux candidats préférentiels (λ-carrageenan and spiruline) a été étudiée au champ sur 2 ans. De nombreux facteurs tels que les conditions environnementales, le stade de développement de la plante et son génotype, ainsi que la pression parasitaire peuvent en effet entrainer une variabilité de l'efficacité de protection des éliciteurs appliqués au champ. Toutefois, des conditions climatiques extrêmes et des pressions parasitaires très contrastées n'ont pas permis de confirmer le maintien de l'éfficacité de protection des deux composés en conditions pratiques. Enfin, l'effet de la formulation a été évalué afin de confirmer l'absence d'interférence des adjuvants utilisés. Des essais supplémentaires en serre ont ainsi démontré que les adjuvants appliqués seuls protégeaient le blé aussi efficacement que des traitements à base de λ-carrageenan formulé ou non. Ces derniers résultats soulignent l'importance de mettre au point une formulation appropriée au plus tôt avant le criblage d'éliciteurs. Dans l'ensemble, les résultats de ce projet de recherche pourraient ouvrir la voie au développement de produits de biocontrôle innovants à base de λ-carrageenan pour une protection durable du blé contre *Zymoseptoria tritici*.

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ABBREVIATIONS

ABA	Abscisic Acid
ANOVA	Analysis of Variance
APX	Ascorbate peroxidase
ASM	Acibenzolar-S-methyl
BABA	β-Aminobutyric Acid
Bion	Bion®50WG
BR	Brassinosteroids
CAT	Catalase
CHS	Chalcone Synthase
CK	Cytokinin
CpG-ODN	Cytosine-phosphate-Guanine Oligodeoxynucleotide motifs
DAMP	Damage-Associated Molecular Pattern
DMI	Demthylation Inhibitor
DPI	Days Post Inoculation
EPS	Exopolysaccharide
ET	Ethylene
ETI	Effector-Triggered Immunity
ETS	Effector-Triggered Susceptibility
GA	Gibberellin
GB	Glycine Betaine
GPX	Gaiacol peroxidase
GR	Glutathione reductase
GSH	Glutathione
HMGR	Hydroxymethyl Glutarate-CoA Reductase
HPT	Hours Post Treatment
HR	Hypersensitive Reaction
IPM	Integrated Pest Management

ABBREVIATIONS

ISR	Induced Systemic Resistance
JA	Jasmonic Acid
JA-Ile	Jasmonoyl-Isoleucine
LOX	Lipoxygenase
LPS	Lipopolysaccharide
LRR	Leucine-Rich Repeats
MAMP	Mircobial-Associated Molecular Pattern
MANOVA	Multivariate Analysis of Variance
MAPK	Mitogen-Activated Protein Kinase
MeJA	Methyl Jasmonate
NHR	Non-Host Resistance
NO	Nitric Oxide
NPR1	Non-expressor of pathogenesis-related proteins 1
OGA	Oligogalacturonide
OXO	Oxalate oxidase
PAL	Phenylalanine Ammonia Lyase
PAMP	Pathogen-Associated Molecular Pattern
PCA	Principal Component Analysis
PCD	Programmed Cell Death
PDA	Potato Dextrose Agar
PGPF	Plant Growth-Promoting Fungi
PGPR	Plant Growth-Promoting Rhizobacteria
POX	Peroxidase
PTI	PAMP-Triggered Immunity
PR	Pathogenesis-related
PUFA	Hydroperoxide Fatty Acids
QoI	Quinone Outside Inhibitor

ABBREVIATIONS

qPCR	quantitative Polymerase Chain Reaction
qPFD	RT-qPCR-based low-density microarray
QTL	Quantitative Trait Loci
RLP	Receptor-Like Protein
RLK	Receptor-Like Kinase
RNA	Ribonucleic Acid
RNS	Reactive Nitrogen Species
ROS	Reactive Oxygen Species
RT	Reverse-Transcription
SA	Salicylic Acid
SAR	Systemic Acquired Resistance
SDHI	Succinate Dehydrogenase Inhibitor
SOD	Superoxide dismutase
Spirulina	*Spirulina platensis*
STB	Septoria tritici Blotch
TF	Transcription Factor
TLP	Thaumatin-Like Protein
VOC	Volatile Organic Compound

PREAMBLE

This PhD thesis was conducted at the **'Integrated and Urban Plant Pathology'** laboratory – Gembloux Agro-BioTech – Université de Liège, as a four-year research project (2013-2017) funded by the **AgricultureifLife research platform**.

AgricultureIsLife was launched in Gembloux as a means to promote projects looking at the agroecosystem as a whole and to stimulate the development of tools, techniques and knowledge which would improve the performances of agricultural practices in the prospect of sustainable agroecosystems in a near-future. To that extent, 4 research axes were created and entitled as follows: (1) Performance of non-conventional agroecostems; (2) Optimizing crop residue management in agroecosystems; (3) New tools to increase sustainability of agroecosystems; (4) Valorization of agroecosystem products.

The present doctoral thesis is part of the AgricultureifLife research axis 3. This research project aimed to stimulate preventively the expression of the wheat systemic defense mechanisms using elicitors. More precisely, **the objective was to identify innovative eliciting agents to protect wheat, a major European crop, against a harmful disease; namely *Septoria tritici* Blotch.** With the support of complementary research units of Gembloux Agro Bio-Tech, INRA Angers, ISA Lille and Arvalis-institut du végétale, this project was structured as follows: (1) the screening of a large number of molecules from different origins and structures, (2) the characterization of the subsequent defense-signaling pathways triggered in treated wheat, (3) the determination of the potential effect of formulation on the eliciting activity of the two most effective molecules, and (4) finally the confirmation of the practical efficacy of the two most effective molecules in the field. The experimentations were led under laboratory, greenhouse and field conditions.

I carried out this work at the Integrated and Urban Plant Pathology unit as a member of the **Biological Control research team** and under the supervision of Pr. M.Haissam Jijakli. This unit has been working for over 25 years on the research and development of biopesticides. It is also committed to the development of sustainable and alternative control methods to counter plant pathogens.

The Plant pathology unit has built-up considerable knowledge in the field of biocontrol, from the screening and identification of biological control agents (BCAs) to the large-scale testing of formulated products. The Biological control team works on the development of alternative crop protection methods based for instance on essential oils and elicitors of plant defenses. This PhD thesis actively participated in the work of this research team as a means to screen and develop innovative elicitors for wheat protection.

PREAMBLE

My manuscript is organized as follows: (i) a general introduction in which are successively presented the current context of elicitor use in agriculture, current knowledge regarding plant induced resistance and an in-depth overview of the various defense signaling pathways triggered in the plant in response to an elicitor; (ii) the research questions and objectives of this thesis; (iii) a chapter entitled 'strategic choices' which presents the pathosystem case study and the selected elicitor candidates; (iv) a 'Results' chapter in which the results of laboratory, greenhouse screening, biomolecular experimentations and field trials are reported; (v) a last chapter dedicated to the conclusions and perspectives of this PhD work. A list of the different articles published in scientific and peer-reveiwed journals is provided at the very end of this manuscript.

1

BIBLIOGRAPHICAL INTRODUCTION

1. What is at stake today for elicitors as biocontrol tools?

Parts of this chapter were published in the following article:

The decline in natural resources and the environmental damage inflicted by current agricultural practices have become major limitations in conventional agriculture. Against this background, agroecology offers an important scientific approach that takes into account the current societal concerns linked to agriculture, economy and, in particular, the environment. By using ecological principles, it aims at studying and designing agricultural systems based on the interactions of their main biophysical, technical and socioeconomic components (European Commission, 2012).

Research is now strongly focused on the use of agroecological principles to minimize potentially harmful chemical inputs and manage ecological relationships and agro-biodiversity. The past decade has seen the emergence of technological tools developed to promote sustainable agroecosystems. Pest management researchers have made major advances in the development of efficient biocontrol methods to protect plants against biotic stresses. Biocontrol refers to any method, product or organism using natural mechanisms in the context of integrated crop protection against bioaggressors (Herth, 2011). The biocontrol market represents currently 3 - 4 % of the global pest control market. It is rapidly gaining ground with an estimated annual growth of 15 to 20 % as biocontrol products are being used on a wider variety of crops. Besides, the biocontrol market is estimated to reach as far as $2,871.6 Million by 2020 (IndustryARC, 2016).

Biocontrol products include macro- and microorganisms, natural substances of plant, mineral or animal origin, and chemical mediators. Elicitors (also called plant resistance inducers) are considered to be the most promising biocontrol tools in agriculture as they induce plant resistance to various diseases. Several products are already on the market.

This chapter gives an overview of the present status of elicitors as biocontrol tools in agricultural production. We then address the question of how and why future strategies should increase the use of elicitors by highlighting both their limitations and their potential for contributing to sustainable and agroecological agriculture.

1. Elicitor as biocontrol tools in conventional agriculture

The development of new green technologies has led to greater research focus on biocontrol tools (Boller & Felix, 2009). In the late 1970s, it was discovered that plants have inducible defense mechanisms that are activated by infection and could potentially provide protection against a broad spectrum of pathogens (Schwessinger & Ronald, 2012). This resistance is triggered by the plant when it senses 'non-self' molecules released during the attack, known as general elicitors.

The term 'elicitor' refers to all the signal molecules that are perceived and that induce a defensive reaction in the plant. They therefore play a key role in plant-pathogen interactions (Vallad & Goodman, 2004). Induced resistance has long been recognized as a valuable approach in disease control strategies because it offers the promise of durable, broad-spectrum disease control using the plant's own resistance (Walters et al., 2014b).

The elicitor products currently in the marketplace are used mainly in integrated pest management (IPM) strategies as complementary tools to help reduce chemical inputs. Until now and depending on their efficiency, elicitors are usually applied alone or in combination with other fungicides, once or several times in a crop cycle (Walters et al., 2013). Additional information on currently commercialized elicitor products is given in Table 1.

Table 1. Examples of commercialized products with elicitor properties (Le Mire et al., 2016)

Products	Product origin	Crop	Disease Target	Manufacturor
Vacciplant®	Laminarin extract from the brown algae *Laminaria digitata*	Apple orchards, tomato, lettuce, cucumber, strawberry, grapevine	Powdery mildew, Downy mildew	Laboratoire Goëmar, FRANCE
Actigard®/Bion ® /Blockade®	Acibenzolar-S-méthyl	Wheat, Tomato	Powdery mildew, Bacterial diseases	Syngenta Crop Protection, USA
Elexa®4 PDB	Chitosan	Grapevine, tomato, potato, cucumber, field crops	Botrytis grey mould (*Botrytis cinerea*), Powdery mildew, Downy mildew	Plant Defense Boosters Inc., USA

Armour-Zen®	Chitosan	Grapevine, ornamentals	Botrytis grey mould (*Botrytis cinerea*), White rot (*Sclerotinia sclerotiorum*)	Botry-Zen 2010 Ltd, NEW ZEALAND
Chitoplant®		Tomato,	Powdery mildew	ChiProGmbH, GERMANY
Harp-N-Tek®	Harpin protein from the bacteria *Erwinia amylovara*	Apple and pear orchards, grapevine, tomato	Apple and Pear scab, Downy mildew	Plant Health Care Inc., USA
Milsana®	Ethanolic leaf extract from the giant Knotweed *Reynoutria sachalinensis*	Cucumber, Strawberry, Tomato, Wheat	Powdery mildew	KHH BioScience, USA ; BIOFA AG, GERMANY
Stifenia®	FEN 560 (Fenugrec)	Grapevine	Powdery mildew	S.O.F.T, FRANCE
Helena Prophyt®	Potassium phosphite	Field crops, vineyards, orchards	Downy mildew, Purple blotch (*Alternaria* spp.), Brown rot (*Monilia fructicola*)	Helena Chemical Company, USA
Aliette®WG	Fosetyl-Al	Ornamental trees and bushes, Strawberry	Downy Mildew	Bayer Crop Science, GERMANY

Two well-known elicitor products are the algae extract laminarin, and benzo-(1, 2, 3)-thiadiazole-7-carbothioic acid S-methyl ester (BTH, also known as BION® from Syngenta Europe) (Sobhy et al., 2012). BION®, however, is a chemical elicitor with a structural analogy to the plant hormone salicylic acid. Herth (2011) defines biocontrol products as « agents or products which use natural mechanisms in the frame of an integrated pest management (IPM). This includes macro-organisms (insects, nematodes), micro-organisms (bacteria, fungi, viruses), chemical mediators (pheromones, kairomones), and natural substances of plant, animal or mineral origin. ».

Some elicitor products are also suitable for organic farming, which has surged in popularity in recent years (Dayan et al., 2009). Organic practices prohibit the use of synthetic chemical products and therefore have to address important disease pressures because of the lack of pesticide applications. For acceptance in organic agriculture, the elicitor compounds should occur in nature and should not be derived from genetically modified organisms (García-Mier et al., 2013). For example, laminarin is considered to be suitable for organic farming, and plant protection products that contain it are registered in Belgium, Greece, France, the Netherlands, the UK and Germany (Commission Implementing Regulation, 2011).

There has been an intense hunt for elicitors since the discovery of interesting ones that could be used for agricultural purposes. Three categories of elicitors have been determined so far: pathogen-associated molecular patterns (PAMPs) emitted specifically from pathogenic organisms or parasites; microbe-associated molecular patterns (MAMPs), overall released by beneficial/non-pathogenic microorganisms such as yeasts, and plant growth-promoting rhizobacteria or plant growth-promoting fungi; endogenous molecular patterns related to injured plant tissue (DAMPs, for Damage- or Danger-associated molecular patterns) emitted from the plant itself (Henry et al., 2012).

These danger-signaling compounds are essential components of entire classes of microorganisms and have been shown to protect a variety of plants against a broad spectrum of pathogens (Schwessinger & Ronald, 2012). Non-microbial elicitors have also been identified, originating from an array of organic sources, such as algae extracts, crustacean shells and minerals, as well as from chemical synthesis (Henry et al., 2012). The various signaling pathways triggered by these three elicitor categories are described in Figure 1.
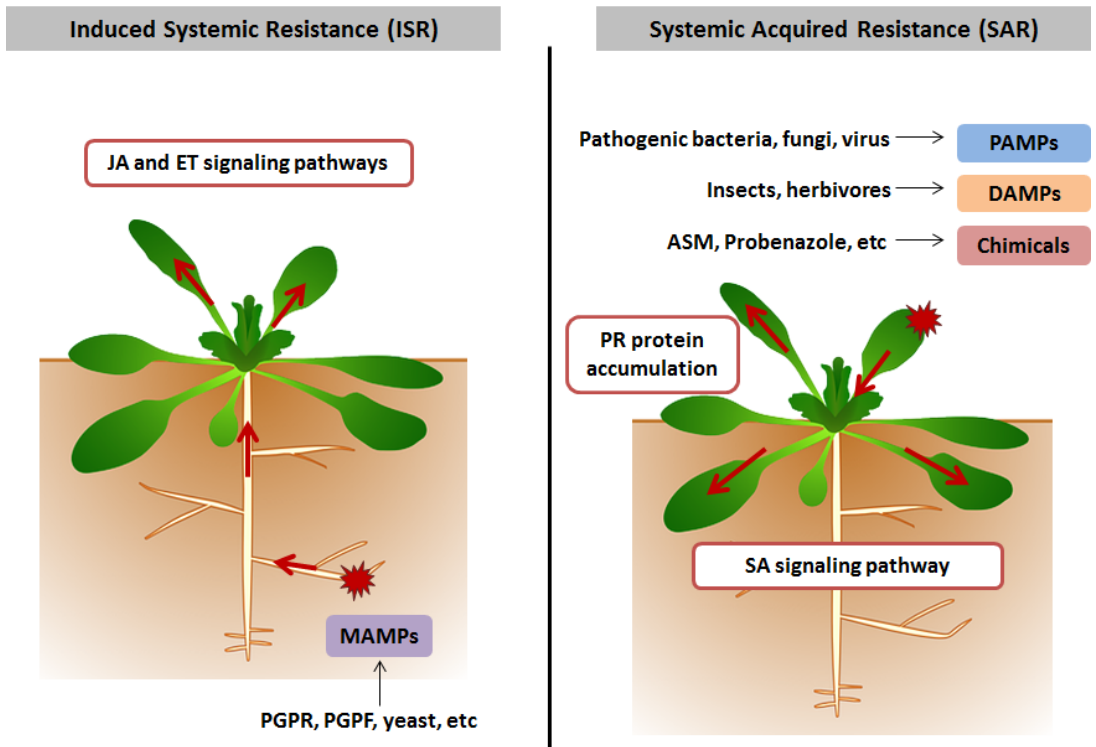
Figure 1. Elicitors and the plant signaling pathways for induced resistance.
Different elicitor categories have been characterized. Elicitors can belong to Microbe-associated molecular patterns (MAMPs) emitted from non-pathogenic microorganisms such as plant growth- promoting rhizobacteria and fungi (PGPR, PGPF) or yeasts, and triggering induced systemic resistance in the host plant (ISR) through jasmonic acid (JA) and ethylene (ET) signaling pathways; Damage-associated molecular patterns (DAMPs) resulting from plant cell degradation after wounding by insects or herbivores; Pathogen-associated molecular patterns (PAMPs) emitted from various pathogens. Chemicals such as Acibenzolar-S-methyl (ASM) and Probenazole, along with DAMPS and PAMPs, trigger a systemic acquired resistance in the plant (SAR) through the salicylic acid (SA) signaling pathway, as well as the accumulation of pathogen-related (PR) proteins. The various signaling pathways cited here are non-exhaustive, and other types of resistance can occur in the plant. Excellent details are provided in the work of Jones & Dangl (2006).
(Source: Le Mire et al., 2016)

Most of the organic elicitors that have been characterized include fungal chitin, bacterial flagellin, lipopolysaccharides (LPS), oligogalacturonides (OGAs), ergosterol, siderophores, surfactin and fengycin cyclic lipopeptides, and volatile organic compounds (VOCs).

Elicitor perception activates the plant's immune system which is characterized by a cascade of events with a complex spatial and temporal regulation. This includes a local burst of reactive oxygen species (ROS), ion fluxes across the plasma membrane, and the production of phytoalexins and pathogenesis-related (PR) proteins (Henry, 2013). At the scale of the whole plant, elicitor perception triggers specific signal transduction pathways involving one or several key regulators, and resulting in one of two possible forms of induced resistance: systemic acquired resistance (SAR) against biotrophic and hemibiotrophic pathogens, combined with a characteristic accumulation of PR proteins; or induced systemic resistance (ISR) against necrotrophic pathogens, chewing herbivores and phloem-feeding insects (Henry et al., 2012; Wasternack & Hause, 2013). Depending on the plant and the elicitor, a set period of time is required for systemic resistance to be embedded.

In addition, some elicitors can trigger a process called priming, which prepares the plant for a faster and stronger resistance only when a subsequent pathogen attack occurs (Walters et al., 2014b).

Priming is more cost-effective than elicitation because the energy cost of induced resistance in the plant is optimized (Beckers & Conrath, 2007). Although the molecular mechanisms behind priming remain poorly understood, some natural and synthetic compounds have demonstrated good priming-inducing activity in laboratory and field conditions, such as the nonprotein β-aminobutyric acid (BABA) (Walters et al., 2014b). The diversity of defense mechanisms and signaling pathways involved in induced resistance underline the potential use of elicitors in plant protection. Since the first discovery of elicitors about 20 years ago, research on these specific compounds and their mode of action has considerably increased our understanding of the plant immune system and opens the way to the development of new tools for disease management strategies (Schwessinger & Ronald, 2012). More details on the diversity of the elicitors identified up to now and their mode(s) of action in the plant are provided in the next chapter devoted to plant innate immunity.

2. Implementation challenges in agricultural practices

The literature supports the implementation of biocontrol tools in agroecological practices, with clear demonstrations of their potential to reduce chemical inputs, save energy and provide farmers with new opportunities for disease control (Mejía-Teniente et al., 2010; Chandler et al., 2011; Wezel et al., 2014). The agroecological use of these tools will obviously require a shift in conventional practices from total reliance on pesticides to the integrated management of biotic stresses (Vallad & Goodman, 2004; Wezel et al., 2014). However, biocontrol products are not yet used as routine tools in agriculture.

We describe here the drawbacks restricting the widespread use of elicitors in agriculture, and what is being developed to enhance their use, and thus make an important contribution to the agroecological and sustainable management of cultivated ecosystems.

2.1. Elicitor screening

The screening of suitable elicitors for specific crops, growth conditions and pathogens is critical if the efficacy of these products in the field is to be guaranteed.

Elicitor screening is usually done under controlled conditions initially, before being done in the field. Screening protocols are adapted to a targeted disease and to the plant to be protected. Different plant genotypes showing various levels of susceptibility to the disease can be used, as well as one or several infectious strains of the pathogen. The amount and positioning of the elicitors need to be optimized, as does the mode of application, the number of treatments and the plant development stage (Walters et al., 2013). The next step is to investigate the signaling pathways involved in the elicitation process using various methods, such as biochemical studies measuring the amount of plant defense-related compounds (plant hormones, phytoalexins, enzymatic activity, ROS) or molecular biology studies measuring the expression of genes associated with plant defense mechanisms (Walters et al., 2014b). The final step involves investigating the influence of various environmental parameters (temperature, relative humidity, luminosity) for subsequent field trials.

In the present study, we took into account the overall recommendations for elicitor screening by performing experiments on elicitor candidates under semi-controlled conditions in the greenhouse at first, and then in the field, on a wheat cultivar susceptible to the targeted *Septoria tritici* Blotch disease. A single strain of *Z. tritici* isolated from Northern France was used for greenhouse trials. Besides, the defense signaling pathways triggered in the wheat plant were studied through biomolecular trials using qRT-PCR to monitor the relative expression of defense-related genes in the plant. Further details are provided in the 'Thesis objectives' and 'Results' sections.

2.2. Formulation and application methods

The formulation and application method are probably among the most critical parameters determining the efficiency of biocontrol products. The formulation must maintain an effective biocontrol capacity and be easy to use (Walters et al., 2014b). Commercialized elicitor products are usually applied as a topical spray, once or several times in the season, to complement fungicide treatments (Walters et al., 2014b).

Worrall et al. (2012) demonstrated that seeds are also receptive to plant elicitors such as jasmonic acid or β-aminobutyric acid (BABA), thereby triggering long-lasting protection against a wide spectrum of pathogens. Seed treatments using elicitors represent a promising technique in pest management for sustainable agriculture, but more research is needed to understand the benefits and costs of such application method. Soil drench applications of elicitors have also recently been reported to achieve good results (Walters al., 2014b).

2.3. Farmers and the use of alternative methods

Farmers do not always greet the suggestion of using alternative methods with much enthusiasm, especially those on small-scale farms or in developing countries (Gozzo & Faoro, 2013). They tend not to adopt innovative crop protection strategies unless their success is guaranteed. The highest number of farmers currently using bio-based products, which include plant biostimulants and biopesticides, is in North America, representing 40 % of the biocontrol market, compared with 25 % in Europe, 20 % in Asia, 10 % in South America and 5 % in the rest of world (Cox & Wong, 2013).

The main reason for farmer skepticism about these alternative methods relates to their variable efficacy in the field compared with conventional chemical inputs (Moser et al., 2008; Arora et al., 2010; Walters et al., 2013). Many studies have shown that these products can have a variable field performance, in contrast to the promising results obtained in the laboratory or in greenhouse conditions (Gozzo & Faoro, 2013). There are several reasons for this inconsistency in practical conditions. The performance of elicitors depends greatly on field environmental conditions (temperature, relative humidity, disease pressure), crop systems (plant genotype, nutritional requirements, physiological state) and the formulation (Walters et al., 2014b).

Farmers' decisions on whether or not to adopt new methods often depend on how much they want to change their agricultural practices. Total reliance on new strategies can be challenging. The benefits of these strategies have to be clearly demonstrated through educational programs that focus on field data (*e.g.* pest/disease identification, timing of infestation, crops) (Rodriguez-Saona & Stelinski, 2009). This includes detailed knowledge about agronomic parameters and designing adapted crop management techniques, with the appropriate biocontrol product applied at the right time and frequency, in combination with other control methods and on responsive cultivars (Walters et al., 2013; Bashan et al., 2014).

Henceforth, tools need to be designed that meet farmers' demands by ensuring: optimal crop yield with lower input costs; compatibility between the applied products and soil conditions, farming machines and equipment; and good shelf life and long-term survival during storage (Bashan et al., 2014). The integration of elicitor biocontrol products into agricultural practices depends on their economic relevance compared with conventional practices (Rodriguez-Saona & Stelinski, 2009; Walters et al., 2014b). Currently, apart from open field applications, biocontrol techniques are widely and efficiently used in the pre- and post-harvest treatments of specific product lines, such as horticultural and ornamental crops (*e.g.* cucumber, lettuce, cyclamen, roses) (Darras, 2012).

Further research is needed on improving the understanding of which field conditions are most suitable to the use of a specific biocontrol product (Bhattacharyya & Jha, 2012; Walters et al., 2013). Scientists are aware of the stakes involved here and many partnerships have been launched. In France, an integrated network called Elicitra was created in 2011 with the aim of promoting the strategy of plant induced resistance by elicitors through research, training and development

(Bazinet, 2012). This network includes partners from public research bodies, technical institutes, crop industries and universities. Partners in the network include Arvalis-Plant Institute and the French National Institute for Agricultural Research (INRA).

2.4. Regulatory framework

A large number of biocontrol products, that have long been known and have been patented for agricultural pest management, are still not available commercially in the EU, unlike the situation in other countries in the world (Dayan et al., 2009).

Many products that encourage plant protection have not been registered and there is a lack of fit-for-purpose regulatory procedures in the EU because of the time and costs of registration (Walters et al., 2014b). The approval of any Plant Protection Product (PPP) requires the registration of the active material on a list validated by the EU (European Parliament, 2009b). Each substance has to comply with strict criteria, listed in Annex IV of Regulation 1095/2007, in order to be considered safe (Commission Implementing Regulation, 2011).

The current strategy of the EU in sustaining the development of new biocontrol methods in agriculture is implemented *via* various legislative procedures. Regulation 1107/2009 aims to harmonize the overall procedures authorizing plant protection products in the EU market. It also facilitates approval of natural substances (Article 23), thereby simplifying the regulation procedures for natural preparations with low risk. The EU has recently proposed granting the first approvals for agrochemicals in a new category entitled 'basic substances'. In addition, Directive 2009/128/EC regulates the sustainable use of pesticides in Europe: « Member States shall take all necessary measures to promote low pesticide-input pest management, giving wherever possible priority to non-chemical methods, so that professional users of pesticides switch to practices and products with the lowest risk to human health and the environment among those available for the same pest problem » (European Parliament, 2009a).

In 2008, France announced its Ecophyto2018 plan, which aims to reduce pesticide use by 50 % by 2018, mainly through the identification and development of bioactive compounds able to stimulate plant immunity (Information Réglementaire Ecophyto 2018, 2011). The reduction of conventional inputs is also planned in other European countries, including Belgium (Belgium NAPAN, 2013), Germany (Germany NAPAN, 2013) and the UK (UK NAPAN, 2013). The promise of strong growth in the biocontrol market in a near future has also led major agrochemical companies to invest in these green technologies. All stakeholders in the agricultural sector, including agricultural distributors and plant breeders, could play an important role in promoting the use of biocontrol products.

3. Conclusion and perspectives

Strong efforts are being made to improve attitudes in the farming community and in society in general, towards the use of alternative methods to chemical inputs. It is widely agreed that elicitors should not be used as stand-alone methods in agroecological management, but integrated into disease control strategies to

complement chemical inputs and contribute to a reduction in their dosage amounts and application frequency.

Although elicitors have been widely endorsed for their advantages, farmers and growers are still not completely confident about using them, mainly because of their fluctuating field performance (Beckers & Conrath, 2007).

Farmers need more information on how to use these tools in their agricultural practices. Regulators, investors, growers and consumers also need to be well informed about the advantages of these alternative methods and their potential in promoting sustainable agriculture.

Further research is needed to better understand the environmental parameters affecting the efficiency of these products, particularly for field crops. Special attention should also be given to the formulation and the potential interactions of these products with the plant environment. Multidisciplinary research groups, such as the AgricultureIsLife platform (Gembloux Agro-BioTech, Université de Liège, Belgium), should address the question of how best to use these tools, given current practices, by studying the issues that still need to be overcome (*e.g.* screening methodology, formulation, environmental impact).

Many challenges remain before biocontrol products can be widely and successfully used on a commercial basis, but the intensive efforts in research and the legislative area, as well as in enhancing society's awareness of these products, will increase their credibility and acceptance (Wezel et al., 2014). Agricultural practices using these tools need to be adapted (*e.g.* using cultivars specifically chosen for the appropriate responses) (Walters et al., 2014b).

In Europe, the long-term objective to be pesticide free is already leading to changes in crop management practices and represents a major driver in the use of biocontrol products. Within the context of climate change, increasing environmental concerns and population increase, these alternative methods offer important potential tools for achieving sustainable food production.

2. Plant innate immunity: generalities and definitions

Plants must constantly face multiple stresses in their living environment, may they be abiotic (*e.g.* drought, salinity, UV radiation, extreme temperatures or heavy metals) or biotic (*e.g.* insects, vertebrates, bacteria, viruses or fungi). However, they lack an adaptive immune system comparable to animals in order to survive in such challenging conditions. Instead, plants have evolved an incredibly wide array of structural, chemical and protein-based defense mechanisms which are either constitutive or induced and which represent the plant innate immune system (Jones & Dangl, 2006). Each plant cell possesses such innate immunity and can send defense signals throughout the plant when infected.

1. Constitutive and induced defense mechanisms

1.1 Constitutive resistance

Constitutive resistances (passive or continuous defenses) mainly rely on the plant structure as a shield against external stresses: the rigid plant cell wall, waxy epidermal cuticles and bark represent true barriers which also confer plant strength and rigidity (Figure 2) (Reina-Pinto & Yephremov, 2009)
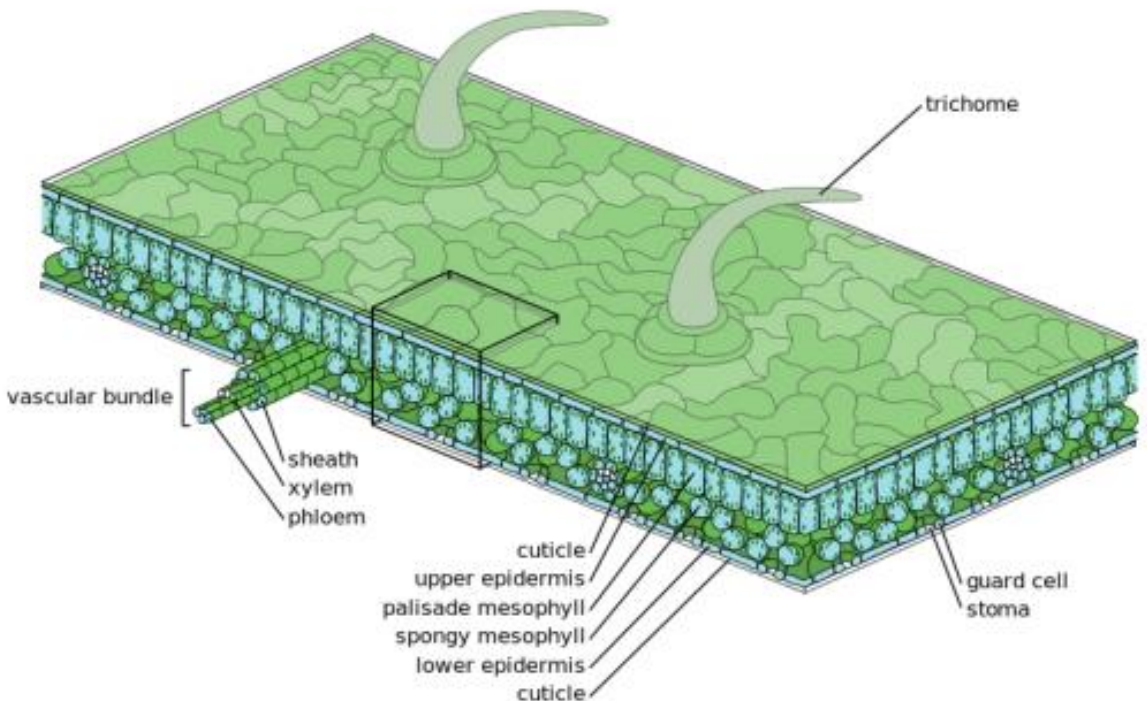
Figure 2. Internal leaf anatomy. (Source: https://en.wikipedia.org/wiki/Leaf)

The plant cuticle is composed of cutin and wax, two hydrophobic components located on the outer side of the epidermal cell wall. It prevents plant water losses and represents the first uneasy-to-cross physical barrier against pathogens. The plant cell wall represents the second major obstacle that pathogens must face on their way to plant infection. The cell wall is mainly composed of cellulose and pectins. Trichomes ("leaf hairs"), thorns and spines also contribute to constitutive resistance. In addition, some plants continuously produce secondary metabolites (precursors) and antimicrobial enzymes, such as catechol (a phenolic compound produced by onions), tannins, capsaicin (active component of red chili peppers), or the toxic cyanogenic glycoside amygdaline (produced in plants of the Rosaceae family) (Mysore & Ryu, 2004). Triterpenoids, phenylpropanoids and flavonoids can also be found in waxes at the cuticle surface (Reina-Pinto & Yephremov, 2009).

1.2. Induced resistance

When constitutive defenses of the plant are bypassed by a pathogen, an induced resistance (active defenses) can be triggered. Such resistance can be defined as a **"state of enhanced defensive capacity"** (Vallad & Goodman, 2004). Plants and animals have evolved the ability to perceive endogenous or microbially-derived compounds and respond *via* strong defense mechanisms (Schwessinger & Ronald, 2012). All signals perceived by plant cells and inducing a defensive reaction are called **elicitors**. These conserved molecules can be emitted by pathogenic or non-pathogenic microorganisms, or by the plant itself in response to wounding (Henry et al., 2012).

The first studies reporting the existence of plant elicitors and the corresponding induced resistance fall back in the early 1970s (Schwessinger & Ronald, 2012). Since then, intensive research has been carried out to identify these remarkable molecules, their receptors in the plant, and their exact mode of action. A large panel of elicitors have been identified and have been grouped in 3 major categories (Henry et al., 2012):

▪ *Pathogenic-associated molecular patterns (PAMPs)*

Elicitors emitted by plant pathogens are considered as PAMPs. These elicitors are generally essential structural components of whole classes of pathogens or consist of secreted enzymes and proteins originally located in the cytoplasm (Mejía-Teniente et al., 2010; Henry et al., 2012). For instance, flagellin (major component of bacterial flagella) induces systemic resistance in Arabidopsis against *Pseudomonas syringae* (Meziane et al., 2005). Similarly, lipopolysaccharides (LPS), which are major components of the cell wall of Gram negative bacteria, were reported to induce systemic resistance of plants against various diseases (Leeman et al., 1995). Other examples of PAMPs include chitin from fungal cell walls and its derivative chitosan (El Hadrami et al., 2010). Moreover, some elicitors such as Pep-13 and xylanase are only perceived by a narrow range of plant species, whereas other elicitors such as chitin and flagellin can be perceived by numerous species of host plants (Henry et al., 2012; Schwessinger & Ronald, 2012).

The plant cells perceive PAMPs through transmembrane pattern recognition receptors (PRRs) consisting of receptor-like proteins (RLPs) or receptor like kinases (RLKs). For instance, *Phytophtora infestans* emits structurally conserved and extracellular proteins named elicitins which are recognized by potato RLPs and trigger a general and quantitative resistance (Du et al., 2015). Fungal chitin is recognized by a chitin elicitor receptor kinase (CERK1) receptor in Arabidopsis and rice (Miya et al., 2007). The RLKs present an extracellular ligand-binding domain with leucine rich-repeats (LRR), a single transmembrane domain and an intracellular serine/threonine kinase-signaling domain (Henry et al., 2012). These RLKs bind to elicitors at their C-terminal end in the apoplast, and bind to kinases at their N-terminal end in the cytoplasm (Kushalappa et al., 2016). On the other hand, RLPs lack intracellular kinase domains (Kushalappa et al., 2016). It is noteworthy that only a few PRRs have been characterized up to now, compared to the broad array of structurally diverse PAMPs that have been identified.

▪ *Microbial associated molecular patterns (MAMPs)*

Non-pathogenic microorganisms can also emit elicitors, referred to as MAMPs. Numerous studies have reported that PGPRs (Plant Growth-Promoting Rhizobacteria) and PGPFs (Plant Growth-Promoting Fungi) are able to produce specific compounds at the root level which spread systemically within the plant and trigger defense mechanisms, thus increasing the defensive capacity of the plant to subsequent pathogenic attacks.

The use of PGPRs to promote both plant growth and induced resistance to various diseases has propelled these rhizobacteria at the very top of the list of interesting biocontrol tools. Various compounds retaining elicitor properties have been identified and isolated from selected strains of nonpathogenic PGPR such as *Pseudomonas*, *Serratia* and *Bacillus* (Ongena & Jacques, 2008; Henry, 2013). These MAMPs include LPS (Leeman et al., 1995), antibiotics such as 2,4-diacetylphloroglucinol (DAPG) (Iavicoli et al., 2003), cyclic lipopetides with biosurfactant properties such as surfactin, mycosubtilin and iturin (Ongena & Jacques, 2008; Jourdan et al., 2009), exopolysaccharides (EPS) (Ortmann et al., 2006), N-alkylated benzylamines (NABD) (Ongena et al., 2005), siderophores such as pyocyanin (De Vleesschauwer et al., 2006) or volatile compounds such as 2,4-butanediol (Ryu et al., 2004).

Compared to PAMPs, less information is available on these molecules, including their mode of perception by the plant (Schwessinger & Ronald, 2012). Recently, it was suggested that cyclic lipopeptides like surfactin are recognized by plants cells *via* a lipid-driven process at plasma membrane level (Jourdan et al., 2009; Henry et al., 2011).

▪ *Damage-associated molecular patterns (DAMPs)*

Plants can also detect a pathogen or a herbivore attack through the recognition of endogenous compounds. These so-called DAMPs can be components of structural

barriers or other macromolecules which were released in the apoplast under the action of pathogenic lytic enzymes or by the plant itself following cell damage (Henry et al., 2012; Choi & Klessig, 2016). To date, few DAMPs have been successfully identified. The best characterized compounds belong to the class of polypeptides and peptides which are produced from precursor proteins (Choi & Klessig, 2016). For instance, systemin is a well-known polypeptide DAMP which is released as a wound signal in the apoplast of damaged tomato leaves and induces systemic resistance in the plant *via* the jasmonic acid-dependent signaling pathway (Ryan & Pearce, 2003).

In addition, two polypeptide hormones and a 23 amino acid peptide (*At*Pep1) were shown to act as DAMPs in tobacco and Arabidopsis plants respectively (Pearce et al., 2001; Huffaker et al., 2006). Moreover, oligogalacturonides (OGs) are cell wall fragments that can be released mechanically or by pathogen lytic enzymes and induce systemic resistance upon recognition by a wall-associated kinase 1 receptor (WAK1) (Choi & Klessig, 2016). It appears that DAMPs are perceived similarly to PAMPs by high-affinity plasma membrane receptors. For instance, AtPep1 is recognized in Arabidopsis by the PEPR1 receptor, and systemin interacts with the SR160 cell-surface receptor, a 160-kDa transmembrane protein with an extracellular leucine-rich domain and an intracellular receptor kinase domain (Ryan & Pearce, 2003; Huffaker et al., 2006).

It is now clear that plants are surrounded by numerous potential elicitors, may they be recognized as PAMPS, MAMPs or DAMPs. However, beyond these three categories, elicitors can have incredibly diverse origins. They may be minerals (phosphite, potassium phosphonate), synthesized chemicals with functional analogy to natural elicitors (BTH, Probenazole, silicon), or derived from plants (extracts of *Hedera helix*, *Reynoutria sachalinensis* or *Solidago canadensis*), from algae (laminarin, carrageenans, ulvans), from microorganisms (chitin, harpin, ergosterol, lipopeptides) or from animals (cholic acid) (Lyon, 2014).

2. *Quantitative and qualitative plant resistance*

Although plants continuously interact with microorganisms, the development of a plant disease actually remains a special occurrence (Uma et al., 2011). Most plant species generally possess a durable and pathogen non-specific protection, also called **"non-host resistance"** (NHR) (Uma et al., 2011). It is effective against a wide range of pathogens. The plant-pathogen interaction is considered **"incompatible"** and the pathogen is unable to infect the plant. The amplitude of plant defenses towards a pathogen can fluctuate considerably, and was represented as a zigzag model by Jones & Dangl in 2006 (Figure 3).
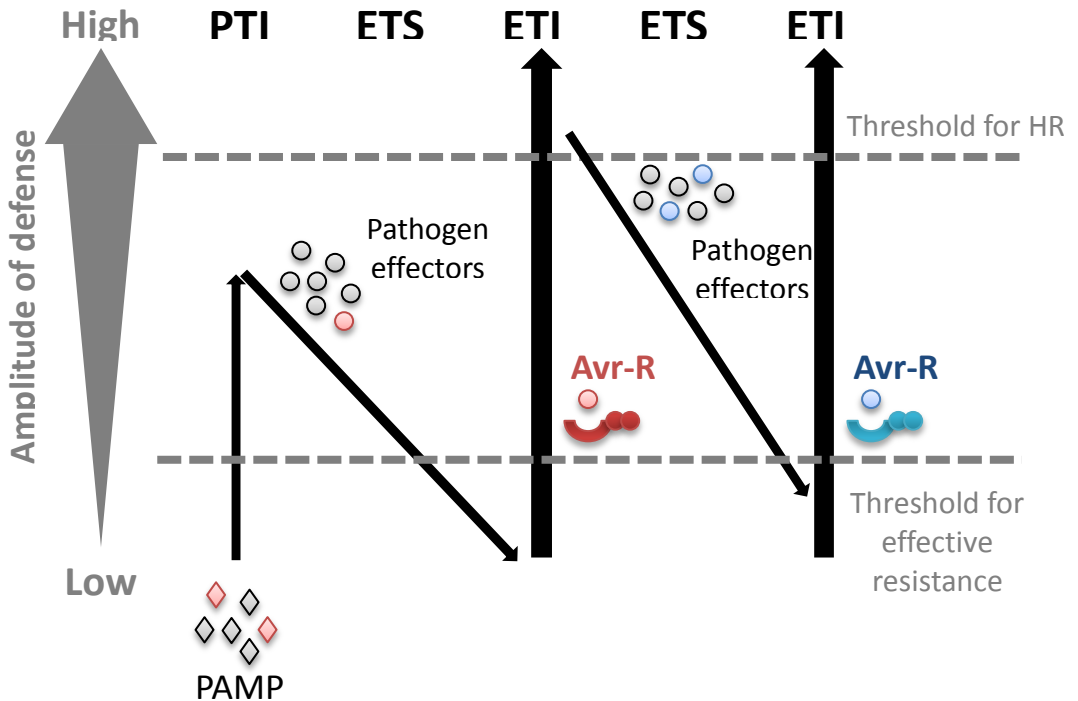
Figure 3. Zigzag model illustrating the amplitude of plant defenses triggered upon recognition of non-self molecules. In a first step, plants detect pathogen-associated molecular patterns (PAMPs) via PRRs leading to PAMP-triggered immunity (PTI). In a second step, pathogens succeed to infect the plant by delivering effectors to counter PTI, resulting in effector-triggered susceptibility (ETS). In a third step, an effector (in red) is recognized by a protein in a "gene-for-gene interaction" (Avr-R), thus activating a stronger version of PTI consisting of effector-triggered immunity (ETI). In a fourth step, rapidly evolving pathogens have gained new effectors (in blue) and can counter ETI. (Source: Jones and Dangl, 2006)

The most common NHR, also called **Type-I NHR**, does not lead to any visible symptoms since the pathogen didn't succeed in overcoming the plant constitutive defenses (physical barriers) or induced defenses (phytoalexins, secondary metabolites) (Mysore & Ryu, 2004). Type-I NHR can be considered as a general and polygenic plant resistance, characteristic of **PAMP Triggered Immunity (PTI),** and involving QTLs (Quantitative Trait Loci) (Jones & Dangl, 2006). PTI is triggered by the recognition of extracellular and general elicitors (PAMPs/MAMPs) by transmembrane pattern recognition receptors (PRRs) (Uma et al., 2011). Such innate immunity also goes under various names: **basal, partial, horizontal or quantitative resistance** (Kushalappa et al., 2016).

Owing its genetic determinism, and as its names suggest, such partial resistance is less likely to exert a major selective pressure on pathogen populations. The defense reaction triggered in the plant may be strong enough to stop pathogen infection.

However, some pathogens can still overcome this basal resistance by emitting specific elicitors (effectors) such as detoxifying enzymes. These effectors can be produced in the extracellular matrix or in the host cell and act in favor of pathogen development by inhibiting the plant defense signaling pathways or the synthesis of defense compounds. The plant loses its primary innate immunity and is then in a state of **Effector Triggered Susceptibility (ETS)** (Jones & Dangl, 2006). The disease spreads, and the plant-pathogen interaction is referred to as **"compatible"**.

At this stage, the cat-and-mouse game or arms race between plants and pathogens takes on its full meaning since the plant may still stop the infection.

A **Type-II NHR** may take place if the plant is able to recognize the pathogen effectors and produce complementary proteins. The **Effector Triggered immunity (ETI)**, or secondary innate immunity, relies on the indirect or direct interaction of products of a single plant resistance (*R*) gene with the products (effectors) of a single pathogen avirulence (*Avr*) gene (Flor, 1971; Jones & Dangl, 2006). Such **"gene-for-gene" interaction** is mono or oligogenic. The products of the R gene are generally intracellular NB-LRR proteins presenting characteristic nucleotide-binding (NB) and leucine-rich repeats (LRR) domains (Jones & Dangl, 2006). Plant ETI is an amplified version of PTI defenses which culminates to a fast hypersensitive (HR) reaction in infected cells, eventually leading to their apoptosis. ETI also goes by many names: **total, qualitative or vertical resistance** (Kushalappa et al., 2016).

The induction of a type-I or type-II resistance depends on the plant and the pathogen species altogether. As a matter of fact, a given plant can show a type-I NHR against a pathogen and a type-II NHR against another (Mysore & Ryu, 2004). On the one hand, total resistance is specific to a given plant cultivar towards a given pathogen species. Such resistance is controlled by a small number of genes (monogenic or oligogenic control), and is thus easier to bypass by quickly evolving pathogens (Flor, 1971). On the other hand, partial resistance represents an interesting alternative for sustainable plant protection strategies by being controlled by multiple genes. The risk that plant resistance might be circumvented by pathogens is significantly reduced. Primary innate immunity is therefore considered crucial today for the development of biocontrol products in the frame of Integrated Pest Management (IPM). Research is intensively dedicated today to the identification of general elicitors able to induce plant defense mechanisms by triggering such primary innate immunity. Inducing partial resistance by recognition of general elicitors can besides occur in a plant towards a large spectrum of diseases (Figure 4).
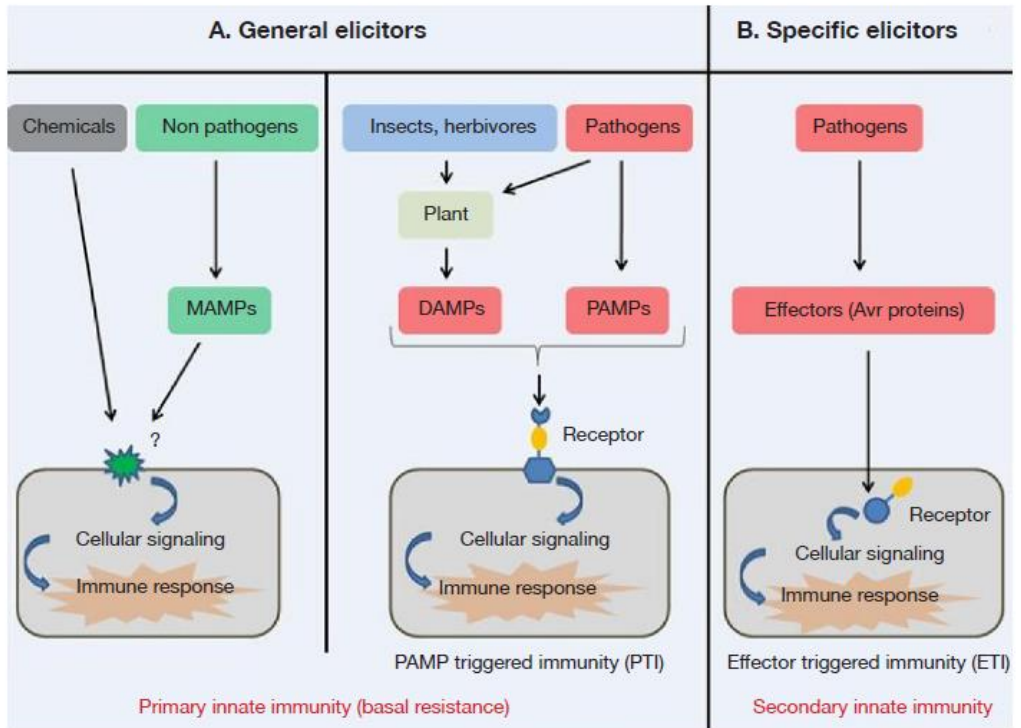
Figure 4. General and specific elicitors are involved in different types of resistance. General elicitors such as Pathogen-associated molecular patterns (PAMPs) released by pathogens, microbial-associated molecular patterns (MAMPs) released by non-pathogenic microorganisms, and damage-associated molecular patterns (DAMPs) released by the plant itself, are involved in Primary innate immunity (PTI). The recognition process by the plant is assumed to depend upon high-affinity receptors, although only a few have been identified up to now. Specific elicitors (or effectors) are released by specialized pathogens and are only perceived by plant cultivars which carry the corresponding disease resistance gene. A "gene-for-gene interaction" takes place which leads to secondary innate immunity (ETI). (Source: Henry et al., 2012).

3. Induced resistance upon elicitor recognition: a complex spatio-temporal process

Great advancements were realized over the past years to investigate the mechanisms underlying elicitor perception by plants. Induced resistance comprises early defense responses, followed by the expression of a set of defense genes, the spreading of defense signals throughout the plant and finally several metabolic modifications, including the production of secondary metabolites and cell wall reinforcement.

1. Early defense responses

1.1 Ion fluxes across the plasma membrane

Upon elicitor recognition, a set of early defense responses are triggered in the plant cell (Figure 5).
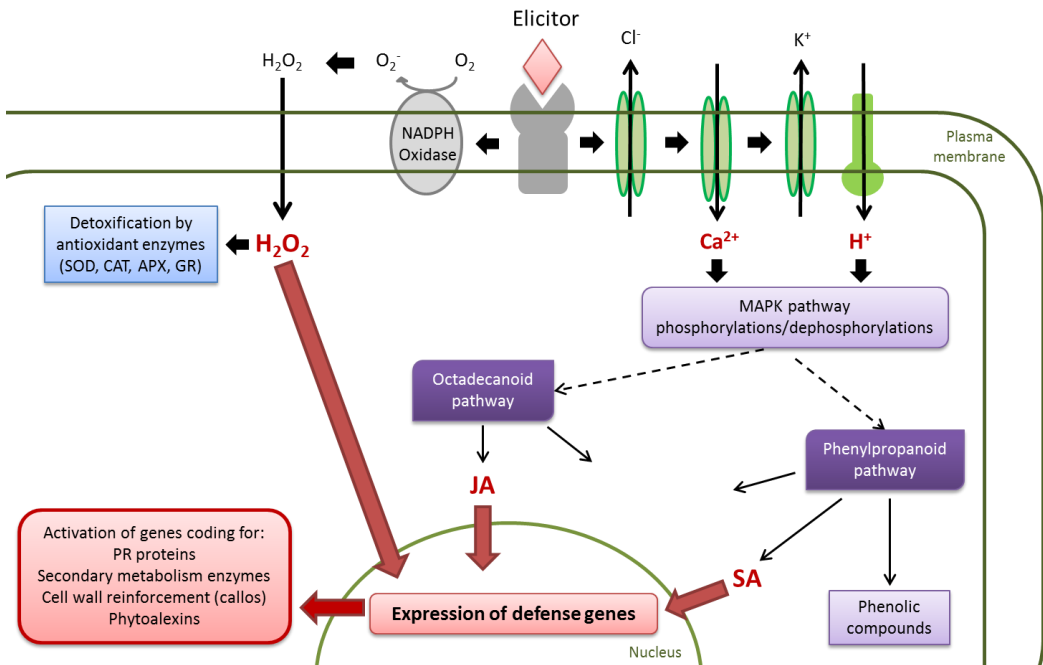
Figure 5. Schematic representation of the defense reactions induced during the interaction of a plant with an elicitor. Recognition of an elicitor induces the production of a panoply of signal molecules (Ca^{2+}, H^+, H_2O_2, hormones SA, JA) which activate various defense signaling pathways (purple flags) and the expression of defense genes. SA: salicylic acid; JA: jasmonic acid; SOD: superoxide dismutase; CAT: catalase; APX: ascorbate peroxidase; GR: glutathione reductase. (Source: Saubeau, 2014)

After barely 2 to 5 minutes following elicitation, an important influx of H^+ and Ca^{2+}, and an efflux of Cl_2 and K^+ take place at the plasma membrane level, thereby

causing membrane depolarization (Muthamilarasan et al., 2013). The influx of H^+ protons leads to an acidification of the cytoplasm, and calcium was reported to play a major role in induced resistance by mediating the plant cell oxidative burst, the production of the signal hormone salicylic acid (SA) and stomatal closure (Muthamilarasan et al., 2013). The increase of calcium concentration in the cytoplasm is perceived by sensor proteins such as calcium-dependent protein kinases (CDPKs) which in turn play a crucial role in mediating the subsequent activation of PTI in the corresponding plant cells (Muthamilarasan et al., 2013).

1.2 Oxidative and nitrosactive burst

Together with important ion fluxes, a set of reactive oxygen species (ROS) and reactive nitrogen species (RNS) are produced (Lehmann et al., 2015). The ROS include different forms of partially reduced or excited forms of oxygen molecules such as superoxide anion ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), a hydroxyl radical ($OH^{\bullet}$) and a singlet oxygen ($^1O_2$) (Figure 6).
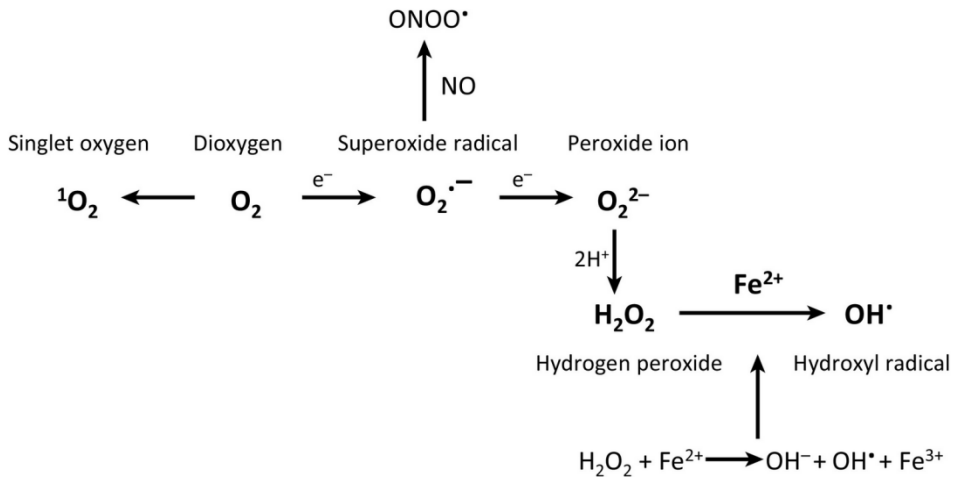
Figure 6. Simplified version of the various reactions using oxygen to produce reactive oxygen species. (Source: Mittler, 2017)

Multiple studies have demonstrated that ROS have a dual role in plants: they can act as toxic molecules that accumulate under abiotic and biotic stress, and they are also important signaling regulators in cells. They indeed mediate cell wall reinforcement by oxidative cross-linking of glycoproteins, regulate plant signaling pathways such as SA synthesis and the activation of MAPK cascades, and mediate plant developmental processes (Lehmann et al., 2015). DNA, RNA, proteins and lipids thus represent the main targets of ROS during the oxidative stress (Mittler, 2017).

Actually, ROS are regularly produced by plant cells in various compartments at non-toxic levels, and their concentration only increases drastically in response to a stress (Künstler, 2015). The toxicity of ROS is dependent on the availability of free iron in the form of Fe^{2+}, due to its role in the Fenton reaction leading to the

formation of hydroxyl radicals ($OH^{\bullet}$) (Mittler, 2017). Given their potential toxicity during an oxidative stress, the production of ROS in the plant cell is tightly regulated both spatially and temporally at specific cellular compartments by antioxidant enzymes such as peroxidase (POX), oxalate oxidase (OXO), catalase (CAT) and superoxide dismutase (SOD) (Figure 7) (Halliwell, 2006; Saubeau, 2014; Kärkönen & Kuchitsu, 2015; Mittler, 2017).
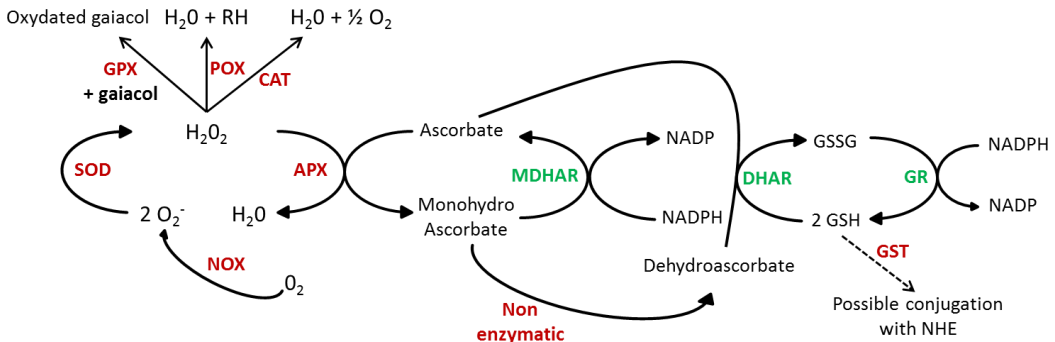
Figure 7. Schematic representation of the enzymatic and non-enzymatic antioxidant system of plants. The NADPH oxidase (NOX) leads to the production of reactive oxygen species. The enzymes superoxide dismutase (SOD), catalase (CAT), peroxidase (POX), gaiacol peroxidase (GPX) and ascorbate peroxidase (APX) scavenge the ROS. Non-enzymatic scavenging of ROS is also realized by ascorbate, and oxidized (GSH) or reduced (GSSG) glutathione. The enzymes responsible for the ascorbate-glutathione cycle are monodehydroascorbate reductase (MDHR), dehydroascorbate reductase (DHR) and glutathione reductase (GR). NHE: 4-hydroxy-2-nonenal. (Source: Saubeau, 2014)

SODs are metalloenzymes located in the stroma of chloroplasts or attached to the thylakoid, near the photosystem I (PSI) where O_2^- is mainly produced. Three different types of SODs have been identified, depending on the heavy metal located in the active site of the protein: copper-zinc (CuZn), iron-containing (Fe) or manganese (Mn)-SODs (Künstler, 2015). These enzymes are the only antioxidants which scavenge superoxide by converting it to H_2O_2.

Catalases are haem-containing tetramer proteins which dismutate two H_2O_2 molecules to water and O_2 (Halliwell, 2006). They are mainly located in plant peroxisomes where they scavenge hydrogen peroxide produced by photorespiration or by the β-oxidation of fatty lipids (Künstler, 2015). Three CAT genes are known to encode for catalase in Arabidopsis and in Angiosperm species in general, and the CAT3 gene in particular is expressed in vascular tissues and leaves (Künstler, 2015).

Peroxidases (POX) are enzymes which reduce H_2O_2 using a co-substrate. For instance, Ascorbate peroxidase (APX) is a class I peroxidase which remains the most versatile antioxidant molecule by scavenging all ROS types. This enzyme predominantly scavenges H_2O_2 by exclusively using two ascorbate (AsA) molecules as electron donors. This antioxidant is either located in the cytoplasm, attached to the thylakoid membrane or soluble in the stroma similarly to SODs (Künstler, 2015).

Glutathione peroxidase (GPX) on the other hand uses glutathione (GSH) as an electron donor to reduce H_2O_2, lipid peroxides and organic peroxides.

Finally, ascorbate, glutathione, tocopherols, carotenoids and polyphenols are nonenzymatic antioxidants that can directly scavenge ROS without any enzyme activity (Künstler,, 2015).

To summarize, ROS are produced in various plant cell compartments, have various modes of actions and involve several scavenging systems. Mittler (2017) provided a good summary of the above points, as illustrated in Table 1.

Table 2. Mode of action, migration distance, production sites and scavenging systems of ROS in plant and animal cells. (Source: Mittler, 2017)

ROS	$t_{1/2}$	Migration distance	Mode of action	Production site	Scavenging systems
Superoxide ($O^{\bullet-}_2$)	1–4 µs	30 nm	Reacts with Fe–S proteins Dismutates to H_2O_2	Apoplast (RBOHs), chloroplasts, mitochondria, peroxisomes, electron transfer chains	SOD, flavonoids, ascorbate…
Hydroxyl radical (OH•)	1 ns	1 nm	Extremely reactive with all biomolecules including DNA, RNA, lipids, and proteins	Iron and H_2O_2 (Fenton reaction)	Flavonoids, proline, sugars, ascorbate,…
Hydrogen peroxide (H_2O_2)	>1 ms	>1 µm	Reacts with proteins by attacking cysteine and methionine residues. Reacts with heme proteins. Reacts with DNA.	Peroxisomes, chloroplasts, mitochondria, cytosol, apoplast	APX, CAT, GPX, PER, PRX, ascorbate, glutathione,…
Singlet oxygen ($^1O_2$)	1–4 µs	30 nm	Oxidizes lipids, proteins (Trp, His, Tyr, Met, and Cys residues), and G residues of DNA	Membranes, chloroplasts, nuclei	Carotenoids and α-tocopherol

Concerning RNS, Nitric oxide (NO) was shown to play a crucial role in plant development and defense responses, together with ROS (Figure 8) (Scheler et al., 2013; Künstler, 2015). As a matter of fact, both NO and ROS play a major role in the triggering of programmed cell death (PCD), characteristic of the plant hypersensitive reaction (HR) which occurs during an attack or during leaf senescence.
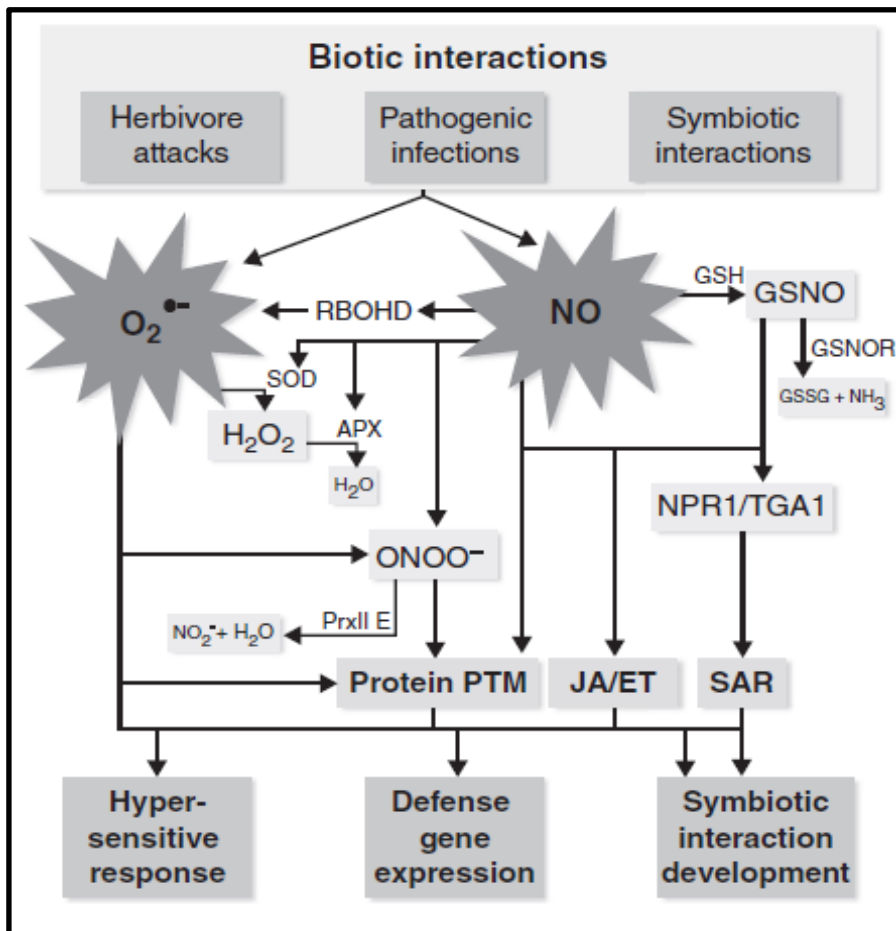
Figure 8. Schematic representation of the signaling interplay between nitric oxide (NO) and reactive oxygen species (ROS). (Source: Scheler et al., 2013).

The fine tuning of NO and ROS concentrations is crucial to correctly protect the plant against a pathogenic infection. Moreover, NO and ROS were reported to modulate together systemic acquired resistance by affecting the functions of the NPR1 (Non-expressor of pathogenesis-related gene 1) protein (Scheler et al., 2013). The oligomeric state of NPR1 indeed depends on its S-nitrosylation by NO, while its transport into the nucleus depends on the concentration of ROS in the cytoplasm (Scheler et al., 2013). The crosstalk between NO and ROS signaling pathways is also visible when NO reacts with superoxide to form a lipid-, protein- and DNA-damaging compound, peroxynitrite (ONOO⁻) (Scheler et al., 2013). It is noteworthy that the sources of nitric oxide still remain unclear (Muthamilarasan et al., 2013; Scheler et al., 2013). It was suggested that NO is synthesized in peroxisomes by two major enzymatic pathways: a nitric oxide synthase (NOS) may convert L-arginine

and L-citrulline to NO, and a nitrate reductase (NR) may reduce nitrite to NO (Foyer & Noctor, 2003; Künstler, 2015).

Moreover, NO and O_2^- are produced after an attack by pathogens or herbivores, or during symbiotic interactions. Both can directly mediate post-translational modifications of proteins (PTM) or react together to produce peroxynitrite ($ONOO^-$). Nitric oxide can react with glutathione (GSH) to produce S-nitrosoglutathione (GSNO) which is a natural NO reservoir. Nitric oxide is also a key regulator of systemic acquired resistance (SAR) *via* the regulation of the NPR1/TGA1 system. Finally, NO is involved in jasmonic acid (JA) and ethylene (ET) signaling pathways. To be noted that both antioxidant enzymes SOD and APX are sensitive to NO donors.

1.3 MAPK cascades

Between the moment a plant membrane receptor recognizes an elicitor to the triggering of local and systemic defense responses, a cascade of signals takes place in the elicited plant cells. This includes a succession of MAPK (Mitogen-Activated Protein Kinases) phosphorylations and dephosphorylations (Figure 9).
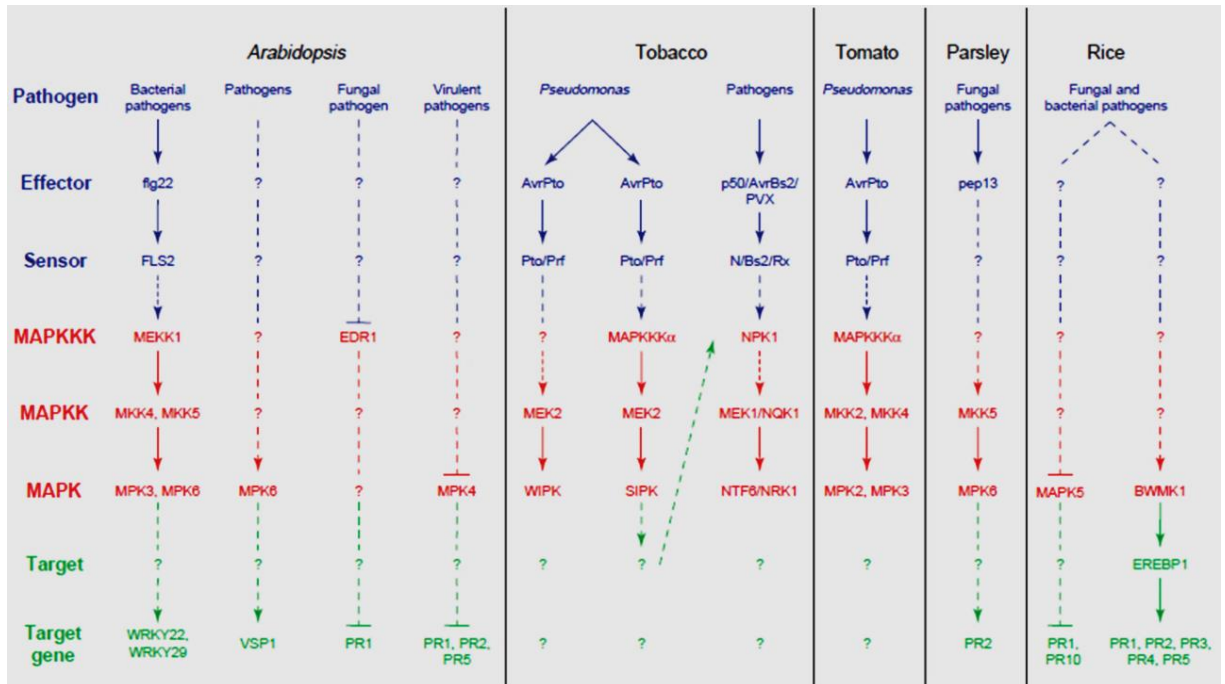
Figure 9. Model describing the MAPK signalization leading to the activation of defense responses in Arabidopsis, tobacco, tomato, parsley and rice (Source: Nakagami et al., 2005).

MAPKs belong to a large family of serine/threonine protein kinases involved in plant cell proliferation, differentiation, defense responses, hormone perception and danger signal transduction (Nakagami et al., 2005).

About 20 different MAPKs have been identified in the Arabidopsis genome, and similar numbers are likely in other plants (Zhang & Klessig, 2001).

Three major protein kinases are functionally interlinked in the MAPK pathway and represent the MAPKKK-MAPKK-MAPK module (Hirt, 2000). Upstream MAPKKKs are activated by receptors either through direct physical interaction and/or by phosphorylation (Nakagami et al., 2005). In turn, MAPKKKs activate MAPKKs by phosphorylating two conserved serine/threonine residues. Similarly, MAPKKs phosphorylate the threonine and tyrosine residues of downstream MAPKs. Activated cytoplasmic MAPKs may then phosphorylate specific enzymes (lipases, protein kinases, etc.) or cytoskeleton-associated proteins (Nakagami et al., 2005). They may also be translocated into the nucleus where they phosphorylate specific transcription factors, thereby indirectly activating the expression of a set of genes (Hirt, 2000). Consequently, different signals are triggered in a plant cell depending on the induced MAPK pathway. Moreover, by controlling the biosynthesis of phytoalexins and the expression of defense genes, it is clear that MAPK cascades are crucial in plant defenses (Bi & Zhou, 2017).

Such involvement of MAPK pathways in plant defense responses to pathogens was demonstrated by several studies. For instance, tobacco leaves treated with the harpin elicitor extracted from *Erwinia amylovora* induced the activation of various protein kinases (Zhang & Klessig, 2001). Similarly, the interaction of parsley with the fungal pathogen *Phytophtora sojae* resulted in the activation of a specific MAPK involved in the transfer of dangers signals in plant cells (Hirt, 2000). In Arabidopsis plants, once the well-known elicitor flagellin (emitted from bacterial pathogens) is recognized by the corresponding plant receptor FLS2, a specific MAPK module is activated (MEKK1-MKK4/MKK5-MPK3/MPK6) that leads to the downstream activation of *WRKY22* and *WRKY29* defense genes (Asai et al., 2002).

It appears that some effectors emitted by pathogenic bacteria and filamentous phytopathogens may be able to inhibit MAPK cascades directly or by targeting upstream components such as receptor kinases (FLS2, EFR etc.) (Bi & Zhou, 2017).

1.4 Transcription factors

The triggering of early plant defense responses eventually leads an activated MAP kinase to be translocated in the nucleus where it phosphorylates specific transcription factors, thereby regulating the transcription of specific defense genes. The stimulation of plant defense mechanisms requires a fine spatio-temporal regulation of the expression of stress-related genes. Transcription factors thus play a crucial role in plant resistance to pathogens. Besides, a large part of the plant genome is dedicated to transcription. For instance, the genome of the model plant Arabidopsis codes for more than 1500 transcription factors (Singh et al., 2002). They generally belong to large gene families and can be structurally classified by their DNA-binding domains (Jakoby et al., 2002).

Three major Transcription factors (TFs) families have been thoroughly studied up to now, and are unique to plants: ethylene-responsive-element-binding factors (ERF), basic-domain leucine-zipper (bZIP) and WRKY proteins (Singh et al., 2002). In Arabidopsis, it is estimated that there are 124 ERF, 75 bZIP, and about 74 WRKY

proteins (Singh et al., 2002). These transcription factors play major roles in every plant biological process. The bZIP transcription factors (named AtbZIP) of Arabidopsis were shown to regulate pathogen defenses, stress signaling, seed maturation and flower development (Jakoby et al., 2002). Another TF family, the MYB proteins, are linked to stress responses to ultra-violet light, wounding and pathogen attacks (Singh et al., 2002).

Experiments of overexpression or knock-down of WRKY gene expression demonstrated that WRKY factors are key regulators and central components of plant primary innate immunity (PTI) and effector-triggered immunity (ETI) (Rushton et al., 2010). The WRKY proteins regulate for instance the expression of regulatory genes such as receptor protein kinases and NPR1. It was also reported that several WRKY proteins are MAP kinase targets (Popescu et al., 2009). Finally, and as previously mentioned, elicitor treatment with flg22 triggers the expression of 9 distinct WRKY proteins in Arabidopsis, among which *WRKY22* and *WRKY29* activated by MAPK, MPK3 and MPK6 (Asai et al., 2002).

2. Defense signaling throughout the plant

A few hours after elicitor recognition, danger signals are transmitted to neighboring cells and throughout the plant. Such signal transduction is operated by specific plant hormones which play a key role in the induction of local and systemic resistance: salicylic acid (SA), jasmonic acid (JA) and ethylene (ET) (Verhage et al., 2010).

2.1. Salicylic acid

The plant hormone salicylic acid (SA, 2-hydroxy benzoic acid) is a phenolic compound generally involved in plant defense responses to biotrophic and hemibiotrophic pathogens by inducing systemic acquired resistance (SAR) (Dempsey et al., 2011). For instance, an increase of SA in wheat heads infected by *Fusarium graminearum* was correlated with induced resistance against the pathogen and an increased expression of the *pr1* gene. This hormone is also involved in plant defense against abiotic stresses (*e.g.* drought, heavy metals, osmotic stress) and multiple plant physiological processes (*e.g.* photosynthesis, plant growth and respiration, seed germination, flowering, etc) (Vlot et al., 2009).

The biosynthesis of SA in the elicited plant cells may follow two pathways: the isochorismate and/or the phenylalanine pathway respectively, both of which originate from chorismate (Figure 10) (Shah, 2003; Vlot et al., 2009; Dempsey et al., 2011).
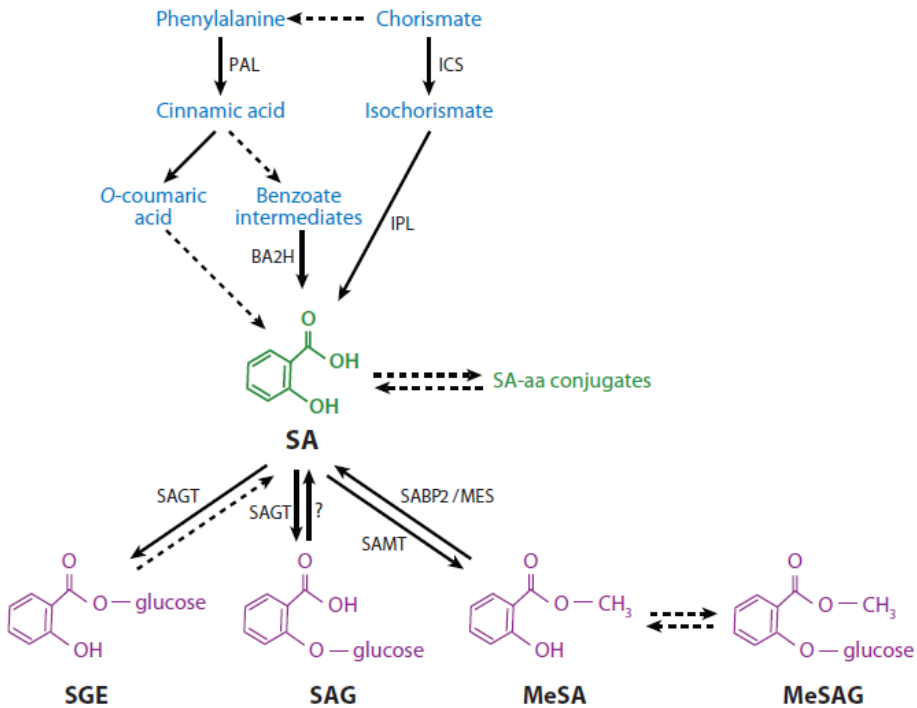
Figure 10. Simplified schematic of the pathways leading to SA biosynthesis and metabolism. Abbreviations: Phenylalanine ammonia lyase (PAL); isochorismate synthase (ICS); isochorismate pyruvate lyase (IPL); benzoic acid-2-hydroxylase (BA2H); salicylic acid (SA); SA glucosyltransferase (SAGT); amino acid (aa); SA methyltransferase (SAMT); SA-binding protein 2 (SABP2); methyl esterase (MES); salicyloyl glucose ester (SGE); SA *O-β*-glucoside (SAG); methyl salicylate (MeSA); methyl salicylate *O-β*-glucoside (MeSAG). (Source: Vlot et al., 2009)

For instance, the recognition of a PAMP or a DAMP by plant cell receptors triggers early defense responses, among which an increase of cytoplasmic Ca^{2+}. The amount of calcium in the cytoplasm is perceived by calmodulins. In turn, the calmodulin-binding protein CBP60g may activate the enzyme isochorismate synthase (ICS) involved in the isochorismate pathway (Vidhyasekaran, 2015).

An alternative SA biosynthesis route involves nitric oxide (NO) which induces the conversion of phenylalanine to *trans*-cinnamic acid by enhancing the activity of the phenylalanine ammonia lyase (PAL) enzyme (Shah, 2003; Vidhyasekaran, 2015). In addition, the simultaneous accumulation of hydrogen peroxide (H_20_2) triggers an increase of intracellular benzoic acid which is then converted to SA by the benzoic acid-2-hydrolase (BA2H) enzyme (Vidhyasekaran, 2015).

Several regulatory proteins are crucially involved in the upstream production of SA. For instance, in Arabidopsis plants, the *enhanced disease susceptibility 1* (EDS1) and *phytoalexin deficient 4* (PAD4) proteins were shown to transduce ROS-redox signals leading to SA production. The EDS4 protein activates SA biosynthesis, while EDS5 (a homolog of flavonoid transporting proteins) is involved in the transport of phenolic precursors of SA. Finally, the *SA induction deficient 2* (SID2) protein is involved in SA biosynthesis through the isochorismate pathway (Glazebrook, 2005; Shah, 2003; Vidhyasekaran, 2015).

The endogenous accumulation of SA or its homologs in plant cells activates a series of biochemical and metabolic changes leading to defense signaling. The various bioactive SA conjugates obtained by glycosylation, methylation or amino acid conjugation can induce SAR similarly to SA (Loake & Grant, 2007). This includes methyl salicylate (MeSA), dehydroabietinal, pipecolic acid and azelaic acid (Vidhyasekaran, 2015).

The *Non-expressor of pathogenesis-related proteins 1* (NPR1) protein is the main regulator of defense responses downstream of SA production (Shah, 2003). If SA (or a homolog) doesn't accumulate in response to an attack, the NPR1 proteins usually form an oligomer which remains in the cytoplasm. Such NPR1 oligomerization through intermolecular disulfide bonds is mediated by nitric oxide (NO): activated S-nitrosoglutathione (GSNO) S-nitrosylates the NPR1 protein at *Cys*156 (Vidhyasekaran, 2015).

In the case of SA accumulation, it appears that modifications of cell redox and the direct binding of NPR1 to SA induce a conformational change of the protein and its reduction to a simple monomer which is then translocated in the nucleus (Figure 11) (Van Loon & Van Strien, 1999; Verhage et al., 2010; Vidhyasekaran, 2015).
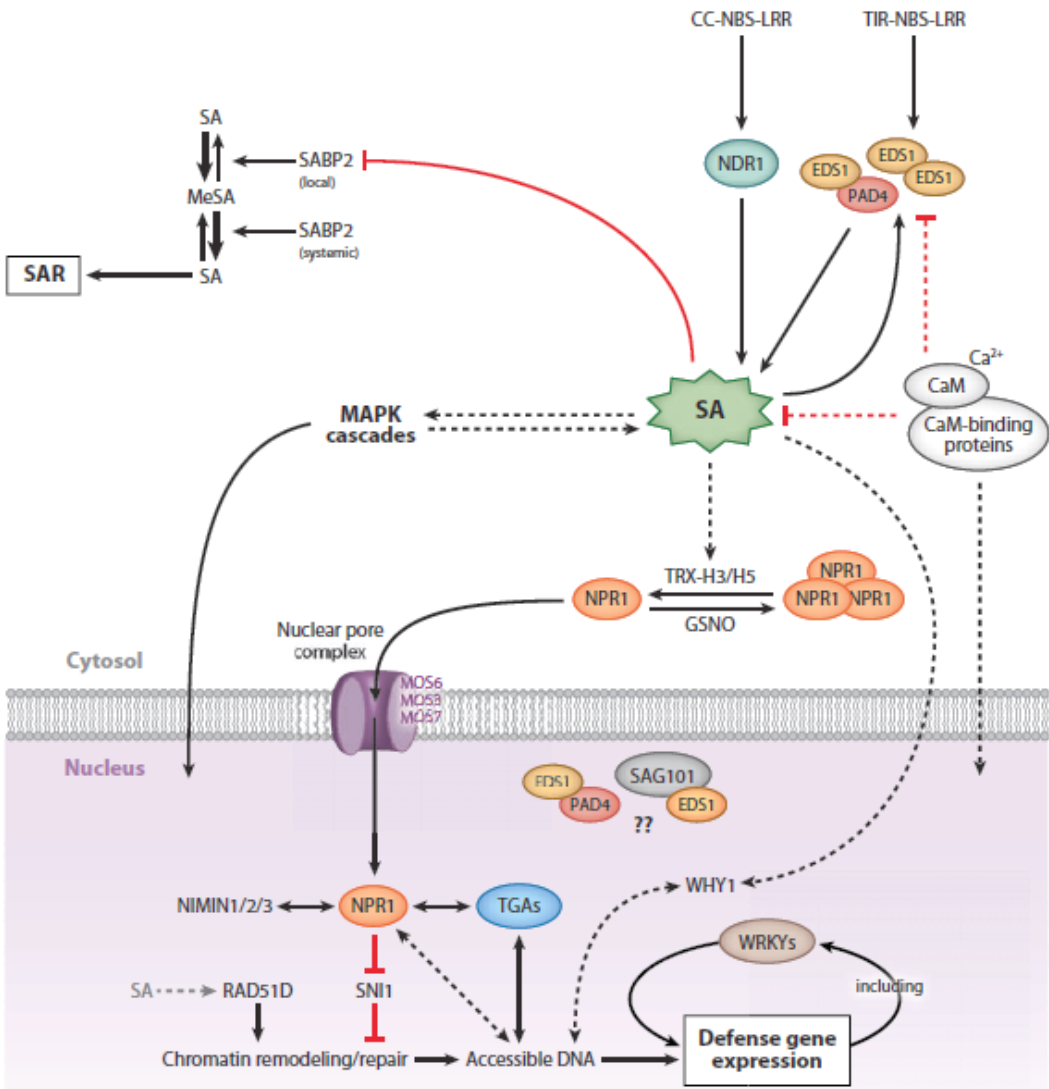
Figure 11. Schematic overview of SA signaling leading to plant systemic acquired resistance. (Source: Vlot et al., 2009).

Studies dedicated to SA-defense signaling demonstrated that it can also be mediated by an NPR1-independent pathway in some plant-pathogen interactions, although NPR1-dependent SA-signaling is better understood (Shah, 2003).

Overall, the induction of SAR in plants is characterized by SA-dependent signaling and the production of specific PR-proteins such as PR-1, PR-2 and PR-5 (Van Loon & Van Strien, 1999; Verhage et al., 2010).

2.2. Jasmonic acid (JA)

JA biosynthesis has been thoroughly studied and is actually better understood than SA biosynthesis. It occurs through the octadecanoid pathway (Figure 12).
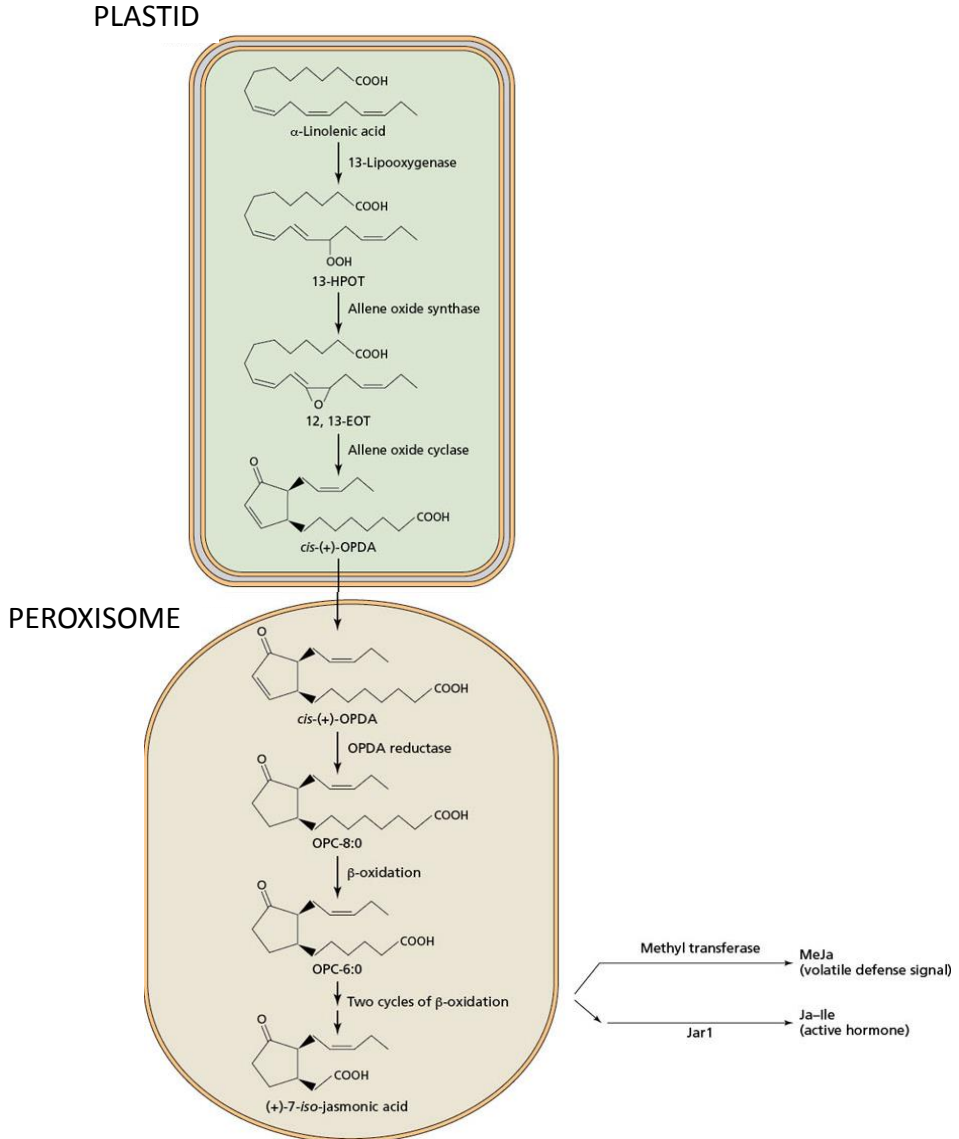
Figure 12. Biosynthesis of jasmonates from α-linolenic fatty acid (octadecanoid pathway). Abbreviations: (13S)-hydroperoxyoctadecatrienoic acid (13-HPOT); (13S)-12,13-epoxy-octadecatrienoic acid (12,13-EOT); *cis*-(+)-12-oxophytodienoic acid (OPDA); (13S)-12,13-epoxy-octadecatrienoic acid (12,13-EOT); 12-oxophytoenoic acid (OPC-8); jasmonic acid-amido transferase 1 (JAR1); methyl jasmonate (MeJA); jasmonoyl-isoleucine (JA-Ile). (Source: Taiz et al., 2014)

The perception of a pathogen attack activates phospholipase proteins which in turn release unsaturated fatty acids. The substrate of JA consists of an α-linolenic fatty acid (C18:3) released from galactolipids localized in the chloroplast membrane (Wasternack & Hause, 2013). The oxygenation of free α-linolenic acid represents the first step of JA biosynthesis: oxygen is inserted in the C-13 position by a lipoxygenase (13-LOX) enzyme, thereby leading to the formation of hydroxyperoxide fatty acids (PUFAs). In Arabidopsis, 4 LOX genes encode for lipoxygenase enzymes (Vidhyasekaran, 2015). In turn, PUFAs are modified into 12-oxo-phytodienoic acid (OPDA) by the successive action of 2 enzymes: an allene oxide synthase (AOS) and an allene oxide cyclase (AOC). The resulting OPDA is transported into cell peroxisomes were it is reduced by the enzyme OPR3, followed by 3 rounds of β-oxidations leading to the final formation of jasmonic acid. Several transformations of JA by enzymes (*e.g.* methylation, decarboxylation, etc.), lead to the formation of multiple derivatives with distinct biological activities, among which methyl jasmonate (MeJA), *cis*-jasmone, tuberonic acid, cucurbic acid, and jasmonoyl-isoleucine (JA-Ile) (Browse, 2009). MeJA is active in transplant and systemic signaling, and both MeJA and JA-Ile are responsible for the activation of defense gene expression (Browse, 2009).

JA- and ET-dependent defense signaling is characteristic of an Induced Systemic Resistance (ISR) in plants in response to necrotrophic pathogens and non-pathogenic microorganisms (Browse, 2009; Van der Ent et al., 2009). JA also plays a regulator function in plant growth and development processes such as senescence and reproductive development (Browse, 2009). Studies on Arabidospsis plants with mutations of the JA-signaling genes *jar1*, *jin1*, *eds8* and *coi1* resulted in the absence of ISR usually triggered by *Pseudomonas fluorescens* WCS417r (Pieterse et al., 1998).

In addition, the plant growth-promoting rhizobacteria (PGPR) *Pseudomonas putida* BTP1 was reported to induce ISR in various plant species, including in cucumber against *Pythium aphanidermatum*, and in bean and tomato against *Botrytis cinerea* (Ongena et al., 2000; Ongena et al., 2004; Akram et al., 2008).

In tomato plants, this PGPR induced plant resistance *via* an increase of the transcription level of two LOX isoforms genes (TomLoxD and TomLoxF), a higher linolenic acid-consuming LOX activity, and a rapid accumulation of antifungal oxylipins against *Botrytis cinerea* (Mariutto, 2013). Similarly, the PGPR *Pseudomonas fluorescens* WCS374r triggered ISR in rice against the pathogen *Magnaporthe oryzae* through a JA/ET-mediated signaling pathway (De Vleesschauwer et al., 2008).

MAPK cascades were once again shown to be involved in the regulation of JA biosynthesis: in wounded tomato leaves, the release of the systemin DAMP activates MPK1, MPK2 and MPK3 which are involved in the expression of JA biosynthesis genes (Wasternack & Hause, 2013).

In addition, the expression of JA-dependent defense genes is under a COI1-dependent transcriptional control (Figure 13).
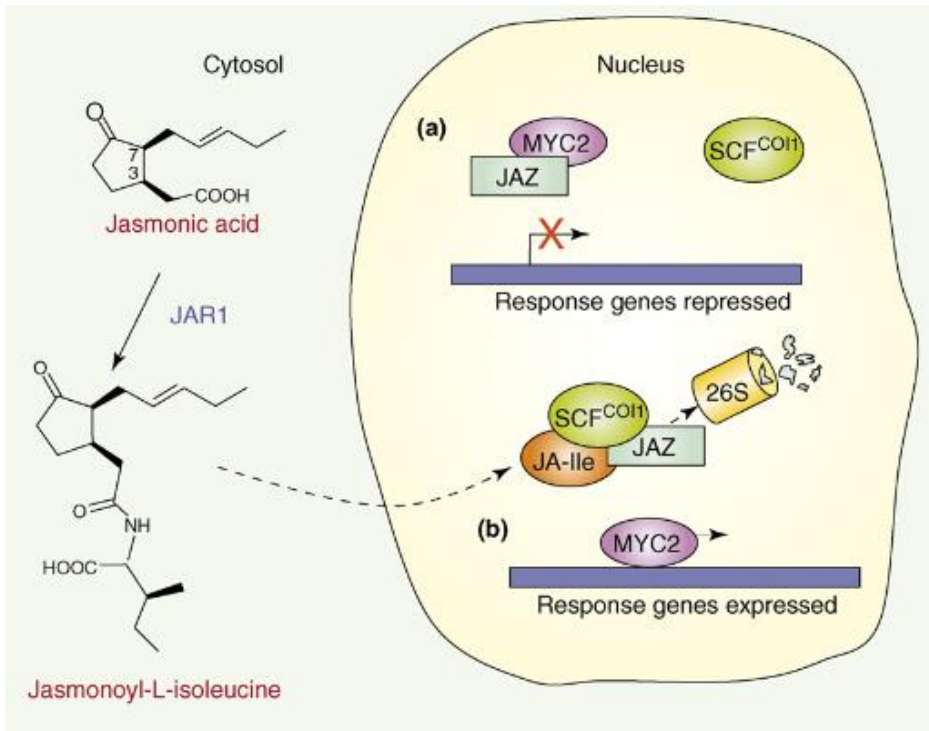
Figure 13. Schematic of COI1-JAZ jasmonate signaling in Arabidopsis. Jasmonoyl-isoleucine (JA-Ile) promotes the interaction of SKP1, Cullin, F-box protein E3 ubiquitin ligase (SCF^{COI1}) with JAZ transcriptional repressors, leading to their degradation by the 26S proteasome. The MYC2 transcription factor is then free to regulate the expression of JA-related defense genes. (Source: Staswick, 2008).

The fixation of JA-Ile to COI1 enables the proteasomal degradation of the negative-regulating JAZ proteins (Jazmonate zim domain proteins). The transcription factors MYC2 and ERF1 are then activated and trigger the expression of JA-dependent genes. However, these 2 TFs regulate a different set of defense genes and are mutually inhibitory. For instance, the genes *pdf1.2* and *pr-4* are induced during a synergistic cross-talk between JA and ET in response to a necrotrophic pathogen *via* activation of ERF1 (Lorenzo et al., 2004). On the other hand, the transcription factor MYC2 is only involved in JA-dependent signaling in response to wounding by insects, and activates the expression of genes such as *vsp2* and *lox3* (Lorenzo et al., 2004; Wasternack & Hause, 2013).

To be noted that wheat plants preventively treated with either HSA (Heptanoyl salicylic acid) or Milsana (*Reynoutria sachalinensis* plant extract) showed an increased activity of LOX associated with induced resistance against the biotrophic pathogen *Blumeria graminis* (Randoux et al., 2006).

In the case of wheat infection by the hemibiotrophic fungi *Zymoseptoria tritici*, an early induction of *lox* gene transcription occurred in both susceptible and resistant cultivars, reaching a peak at 3 hours after inoculation before quickly decreasing (Ray et al., 2003). It is suggested that the LOX enzyme promotes *Z. tritici* early infection and development by favoring the synthesis of pathogen virulence factors. Hence, the quick inhibition of LOX activity in resistant wheat cultivars could contribute to delay fungal colonization (Ray et al., 2003).

2.3. Ethylene (ET)

Ethylene (C_2H_4) is a volatile hormone produced in plants tissues. It regulates both growth dynamics and various development processes such as seed germination, seedling growth, leaf, root, stem and flower development, fruit ripening, and organ senescence and abscission (Yoo et al., 2009).

The precursor of ethylene is L-methionine (L-Met) which is converted to S-adenosylmethionine (S-AdoMet) by the enzyme S-AdoMet synthase (SAM synthase) (Figure 14) (Wang et al., 2002).

In turn, S-AdoMet is converted to ACC by an ACC synthase (ACCS). The activation of ACCS requires its phosphorylation which is regulated by the ETO1 protein. Finally, ACC is oxidized into ethylene, cyanide and CO_2 by the enzyme ACC oxidase (ACCO).

The binding of ET to specific receptors triggers the transduction of defense signals. In Arabidopsis, a family of 5 membrane-bound receptors was identified in the endoplasmic reticulum: Ethylene response 1 (ETR1), ETR2, Ethylene Insensitive 4 (EIN4), Ethylene sensitivity 1 (ERS1), and ERS2 (Vidhyasekaran, 2015).

These receptors act as negative regulators in the ET signaling pathway. CTR1 is a key regulator of ET-dependent defense responses acting just downstream of the receptors and inhibiting the ethylene signaling pathway in absence of ET (Huang et al., 2003; Yoo et al., 2009). Under normal plant growth conditions, ethylene levels are usually low and the active receptors remain associated to CTR1. In reaction to a stress, ET is produced and is linked to the corresponding receptors. CTR1 is then inactive and released from the membrane of the endoplasmic reticulum. The proteins EIN2 and EIN3 are produced downstream of ET biosynthesis, and both EIN3 and EIL1 mediate the expression of defense genes coding for the transcription factors ERF1, FLS2 and SID2. These TFs are responsible in turn for the activation of ET-dependent defense genes (Vidhyasekaran, 2015).
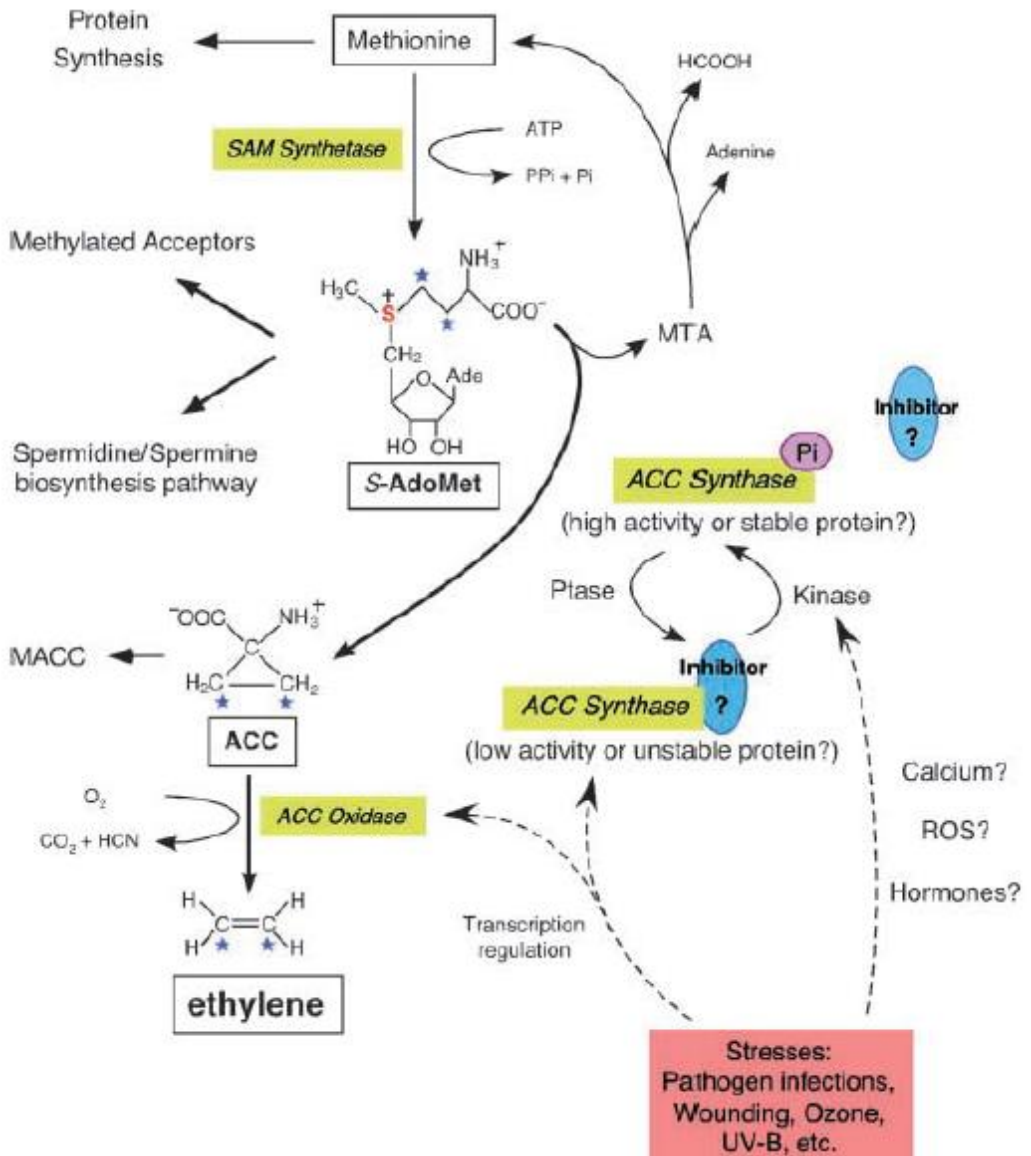
Figure 14. Biosynthetic pathway of ethylene. Ethylene is synthesized from methionine which is transformed into S-Adomet by the S-AdoMet synthetase. ACC is the immediate precursor of ethylene. MTA is the by-product generated along with ACC production by ACC synthase. Recycling of MTA back to methionine is able to maintain a constant concentration of cellular methionine. ACC oxidase catalyses the last step of ethylene synthesis using ACC as substrate. The transcriptional regulations of ACC synthase and ACC oxidase are indicated by dashed arrows. (Source: Wang et al., 2002)

ET synthesis is regulated by several hormones such as JA, auxin, gibberellin (GA), cytokinin (CK) and brassinosteroids (BR) (Figure 15). It is also involved in stress responses and was shown to induce resistance (ISR) in numerous plant species, such as Arabidopsis against the fungal pathogen *Botrytis cinerea*, or rice against the blast pathogen *Magnaporthe oryzae* (Vidhyasekaran, 2015).
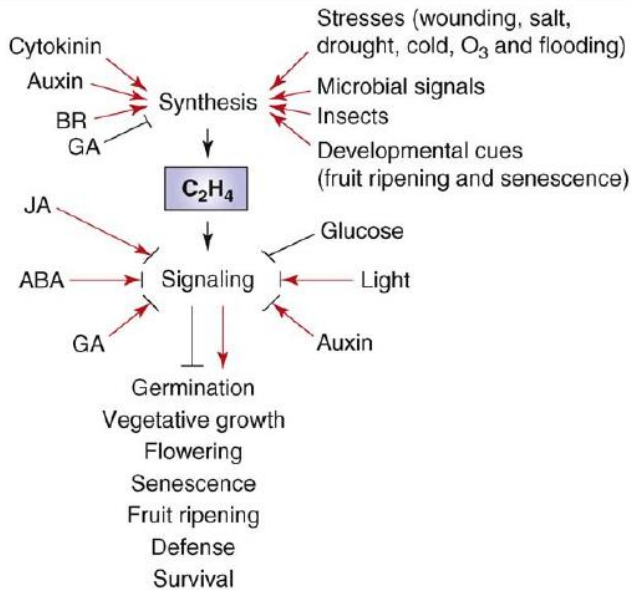
Figure 15. Ethylene regulatory network. Ethylene (C2H4) biosynthesis and signaling are regulated by multiple hormones, plant development processes and environmental stresses. Arrows indicate activation and T arrows indicate inhibitions. Abbreviations: ABA: Abscissic acid; BR: brassinosteroid; GA: gibberellin; JA: jasmonic acid. (Source: Yoo et al., 2009)

2.4. Hormone conversations

Plant growth and defense are regulated by a complex network of cross-communicating signaling pathways, also called hormone cross-talk (Walters & Heil, 2007; Pieterse et al., 2009). The three hormones salicylic acid (SA), jasmonic acid (JA) and ethylene (ET) regularly interact synergistically and/or antagonistically (Verhage et al., 2010).

It is generally accepted that SA defense signaling triggers plant resistance in against biotrophic and hemibiotrophic pathogens, thereby inducing systemic acquired resistance (SAR) and the characteristic accumulation of phenolic compounds. On the other hand, JA and ET interact *via* the ERF1 transcription factor to stimulate plant resistance against necrotrophic pathogens, thereby triggering induced systemic resistance (ISR) (Glazebrook, 2005).

During SAR, the activation of NPR1 and WRYK70 proteins induce a repression of JA/ET signaling and promotes SA-dependent defense responses against biotrophic pathogens (Adie et al., 2007).

However, these hormone interactions can be considered as over-simplified since there are cases where ET cooperates with SA, and even induces plant resistance against biotrophic and hemibiotrophic pathogens (Adie et al., 2007). Moreover, ET and JA can act antagonistically in response to wounding and in some plant-insect interactions (Adie et al., 2007).

Not to forget the involvement of other hormones such as auxins, gibberellins, abscisic acid and cytokinins which also interact synergistically or antagonistically with the SA-JA-ET backbone of plant defense signaling pathways in response to biotic and abiotic stress (Robert-Seilaniantz et al., 2011; Atkinson & Urwin, 2012).

Brassinosteroids for instance enhance resistance to biotrophic and hemibiotrophic pathogens and mediate abiotic stress through NPR1 which, as a reminder, is the key regulator of SA signaling. Similarly, cytokinins (CKs) enhance SA responses in the plant through NPR1. On the other hand, auxins inhibit SA biosynthesis and signaling, while abscisic acid (ABA) promotes plant susceptibility to diseases and protects it from abiotic stresses all at once (Robert-Seilaniantz et al., 2011).

Specific proteins known as DELLA proteins were recently reported to play a crucial role in the fine-tuning of defense signaling pathways in the plant (Figure 16). These proteins are at the intersection between numerous phytohormones such as JA, SA, ET and ABA.
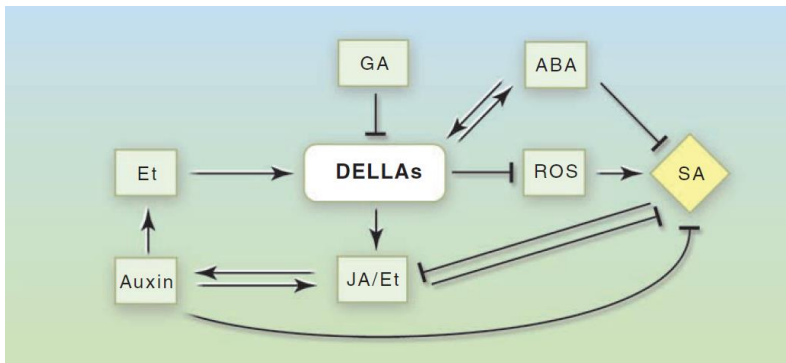
Figure 16. Possible interactions of DELLA proteins with various defense signaling pathways in the plant. JA/Et signaling interacts antagonistically with SA signaling. ABA, auxin and Et interfere with SA signaling and strengthen JA/Et signaling via DELLA stabilization. Finally, DELLAs probably repress SA signaling by attenuating oxidative stress. Abbreviations: JA, jasmonic acid; SA, salicylic acid; Et, Ethylene; GA, gibberellin; ABA, abscisic acid; ROS, reactive oxygen species. (Source: Grant & Jones, 2009)

To conclude, plant disease resistance is regulated by multiple signaling pathways involving intricate hormone crosstalk. Moreover, the allocations costs of induced resistance is finely balanced with the energy devoted to plant growth and development (D. R. Walters et al., 2005; Denancé et al., 2013). The hormone antagonism can thus be considered as an adaptation or a limitation to a resource-limited environment (Thaler et al., 2012). SAR and ISR are complementary types of induced resistance with partly overlapping and partly specific actions against different types of pathogens (van Loon et al., 2006).

3. *Metabolic modifications*

The activation of plant defense genes is followed by the synthesis of an array of compounds contributing to induced resistance. They may be involved in the reinforcement of the plant cell wall, or consist of pathogenesis-related proteins and secondary metabolites.

3.1. Plant cell wall reinforcement

The plant cell wall is a physical barrier contributing to passive resistance against external threats. However, after the perception of a pathogen attack (or an elicitor treatment), the plant cell wall surrounding the site of pathogen detection is actively reinforced and modified through papillae appositions (Figure 17) (Underwood, 2012).
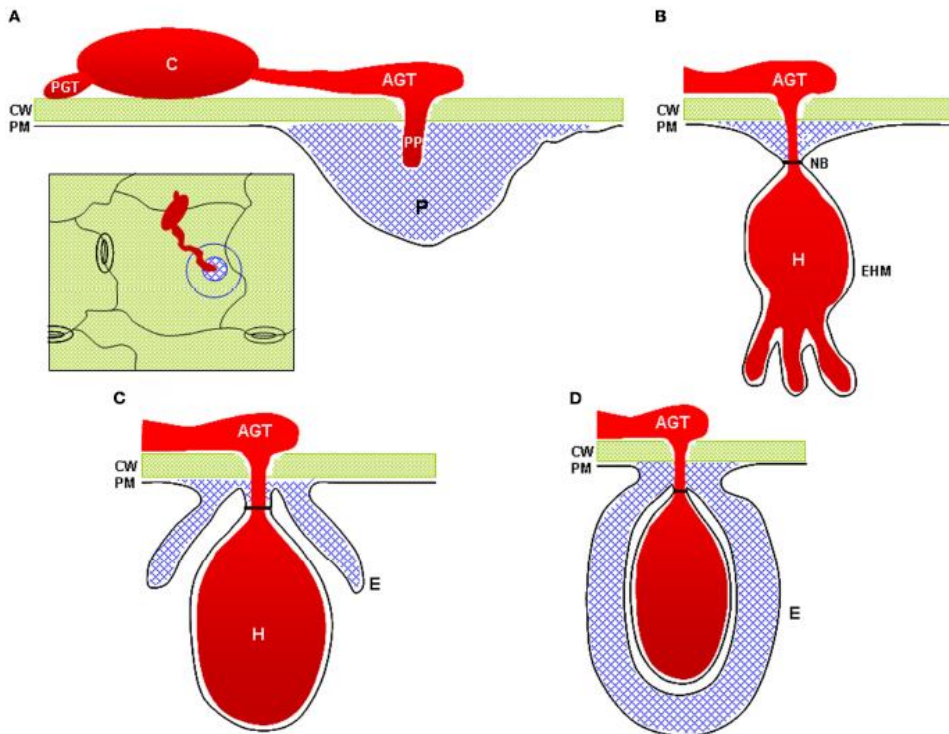
Figure 17. Schematic illustration of the cell-wall associated structures commonly observed at the detection site of fungal pathogens such as powdery mildew. (A) A cell wall apposition (in blue) halted the penetration of a fungal pathogen (in red); (B) A case of successful penetration of the fungal pathogen and formation of haustorial feeding structures. The cell wall appositions form a neck-band around the haustorium; (C) A haustorial encasement (same composition as cell wall appositions) surrounds partially a fungal haustorium; (D) A fully encased haustorium. Abbreviations: CW, cell wall; PM, plasma membrane; C, conidiospores; PGT, primary germ tube; AGT, appressorial germ tube; PP, penetration peg; H, haustorium; EHM, extra-haustorial membrane; NB, haustorial neck-band; P, papilla; E, haustorial encasement. (Source: Underwood, 2012).

Such barrier reinforcement represents a common component of PTI responses and is thought to limit the access of pathogens to the underlying protoplast.

Moreover, papillae are sites which accumulate various antimicrobial compounds (Bednarek, 2012). The biochemical composition of papillae varies between plant species, but the compounds commonly associated to papillae include: callose, phenolic compounds (including lignin), phenolic conjugates (*e.g.* phenolic-polyamines), ROS, peroxidases, cell wall structural proteins (*e.g.* arabinogalactan proteins and hydroxyproline-rich glycoproteins HRGP), and cell wall polymers (*e.g.* pectin, xyloglucans) (Underwood, 2012).

3.2. Pathogenesis-related proteins

The activation of defense genes following elicitor perception in the plant induces the production of a panoply of antimicrobial molecules, among which pathogenesis-related (PR) proteins (Van Loon & Van Strien, 1999).

Accumulation of PR proteins occurs in response to pathogen attack, abiotic stress, hypersensitive response (HR) and systemic acquires resistance (SAR) (Agarwal & Agarwal, 2014). Moreover, they can be secreted in situations other than pathogen infection, notably during specific plant development processes such as leaf senescence and fruit ripening (van Loon et al., 2006).

To be noted that no defense-related proteins have been identified in plants during induced systemic resistance (ISR) in response to growth-promoting rhizobacteria or fungi. During ISR, the JA-responsive genes coding for PR-proteins are only activated upon a subsequent challenge. The activation of these defense genes is then particularly accelerated and strong. Such phenomenon is commonly referred to as priming (van Loon et al., 2006).

PR proteins can be produced both locally near the point of pathogen detection and systemically in the plant in order to protect it against subsequent attacks (Van Loon & Van Strien, 1999). They have been classified into 17 distinct families (ranging from PR-1 to PR-17) depending on their primary structure and biological activity in plants (van Loon et al., 2006).

For instance, the PR-1 family is considered as a marker of SA-dependent defense responses and these proteins typically accumulate during SAR. However, still little is known on their exact biological activity in the plant.

The PR-2 family consists of β-1,3-glucanases with antimicrobial activity by hydrolyzing the β-1,3-glucans composing the cell wall of fungal pathogens.

The families PR-3, PR-4, PR-8 and PR-11 are plant endochitinases which can also act as antimicrobials by hydrolyzing the β-1,4 links of fungal chitin. These chitinases, as well as the PR-6 family of proteinase inhibitors, may also defend the plant against herbivorous insects and nematodes.

The family of PR-5 proteins includes permatins, osmotins and thaumatin-like proteins (TLPs) which permeate fungal plasma membranes. The TLPs are often associated with PR-1 in plant defense against oomycetes.

The family of PR-9 proteins consists of specific peroxidases which scavenge ROS and play a crucial role in cell-wall reinforcement by catalyzing lignification.

The families of PR-15 and PR-16 proteins (oxalate oxidases) are typical of monocotyledonous plants and contribute to oxidative stress by generating hydrogen peroxide.

Finally, PR-17 proteins were recently identified in infected tobacco, wheat and barley, although their mode of action is still under investigation (van Loon et al., 2006).

In Arabidopsis plants, some PR-proteins are specifically synthesized in response to a given defense signaling pathway: SA-dependent defense responses are typically associated with the activation of genes coding for PR-1 proteins, PR-2 β-1,3-glucanases and PR-5 thaumatin-like proteins. On the other hand, JA- and ET-dependent defense responses are associated with the accumulation of PR-3 chitinases, PR-4 hevein-like proteins and PR-12 defensin PDF1.2. Similarly to SA which is used as a marker of SAR, the protein PDF1.2 is commonly used as a marker of JA/ET defense signaling. However, in other plants such as tobacco, proteins belonging to the same PR family can be differentially induced by SA and JA/ET signaling (van Loon et al., 2006). Moreover, it appears that JA and ET induce the production of multiple PR proteins, and their occurrence can be further modulated by abscisic acid. It is therefore clear that the production of PR proteins in plants is influenced by intricate hormone crosstalk, making it difficult to make generalizations concerning the signaling pathways triggered by different plant species against a given pathogen.

In the case of an infection of wheat by *Zymoseptoria tritici*, the genes coding for PR-1, PR-2 and PR-5 are strongly transcribed at 12 hours after infection, especially in resistant cultivars (Ray et al., 2003). It appears that the early induction of PR protein synthesis, such as PR-2 and PR-3 displaying antimicrobial activities is crucial for the wheat plant in order to prevent pathogen infection (Shetty et al., 2009).

3.3 Secondary metabolites

A number of secondary metabolites are produced by the plant prior to an infection or in response to a stress. Based on their chemical structure, plant secondary metabolites can be divided into four major groups: terpenes, phenolics, nitrogen- and sulphur-containing compounds, and oxylipins (Goyal et al., 2012). These various compounds are derived from different signaling pathways: the phenylpropanoid/shikimate pathway, the malonic pathway, the mavalonic acid pathway, the MEP/DOXP (2-*C*-methyl-D-erythritol 4-phosphate/1-deoxy-D-xylulose 5-phosphate) pathway, and the oxylipin pathway (Goyal et al., 2012).

Some compounds are already present prior to an infection. They are preformed antimicrobial metabolites or phytoanticipins, and contribute to constitutive (passive) resistance. They are mildly toxic towards microorganisms and mainly help to put off non-aggressive pathogens (Vermerris & Nicholson, 2006). On the other hand, compounds formed in response to a pathogen attack are produced systemically throughout the plant and/or at the infection site (Vermerris & Nicholson, 2006). In this study, we will focus on the secondary metabolites derived from the phenylpropanoid pathway, the oxylipin pathway and phytoalexins.

▪ *Phenylpropanoid pathway*

The phenolic compounds produced by the phenylpropanoid pathway are derived from phenylalanine through several hydroxylation and methylation steps: the phenylpropanoids are first derived from cinnamic acid produced from L-phenylalanine by the L-phenylalanine ammonia-lyase (PAL) enzyme (Figure 18).
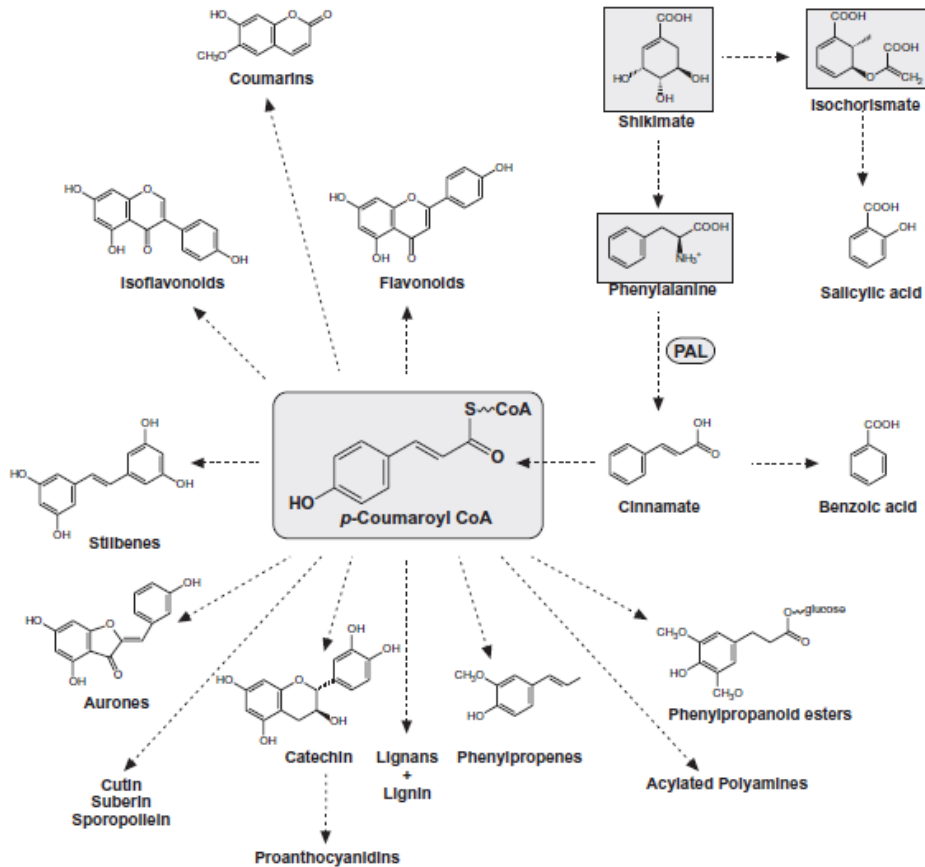
Figure 18. Diversity of phenolic compounds produced in the phenylpropanoid pathway. The metabolites of the shikimate pathway and 4-coumaroyl CoA are shaded in grey. (Source: Vogt, 2009)

The p-coumaroyl-CoA and other hydrocinnamoyl-CoA can lead, *via* the key enzyme chalcone synthase (CHS), to the formation of multiple compounds with antioxidative properties (flavonoids), antimicrobial activities (isoflavonoids) or toxic for herbivores (condensed tannins) (Dao et al., 2011). Cinnamic acids can also be conjugated to other molecules such as esters and phenolamides (Vermerris & Nicholson, 2006).

Phenolics have diverse roles in the plant. They were demonstrated to be involved in the induced resistance of numerous plant species against diverse pathogens (La Camera et al., 2004; Ahuja et al., 2012). They also provide scent/colour/flavour to attract insects, and can act as semiochemicals during the interaction of the plant with beneficial microorganisms (Treutter, 2006).

It is thus noteworthy that both PAL and CHS are key enzymes regulating the production of phenolic compounds in response to a stress. PAL is responsible for the biosynthesis of SA, lignin, phytoalexins and flavonoids, and is thus often associated with resistance in various plant species. However, the involvement of PAL in the induced resistance of wheat against the pathogen *Zymoseptoria tritici* remains controversial. It was reported that only resistant cultivars showed an activation of the gene coding for PAL at 3 and 6 hours after infection with the pathogen (Adhikari et al., 2007). Conversely, another study demonstrated that the expression of the *pal* gene was not induced upon infection by *Z. tritici*, neither in susceptible nor in resistant cultivars (Shetty et al., 2009).

CHS is a key enzyme of the flavonoid/isoflavoid pathway and is involved in SA defense signaling as well (Dao et al., 2011). The expression of the *chs* gene is induced by multiple environmental factors (pathogen infection, UV radiation, drought and cold temperatures) (Treutter, 2006). In barley, the expression of two genes coding for CHS was induced upon infection by *Botrytis cinerea* (Christensen et al., 1998). In wheat infected by *Z. tritici*, the transcription of the *chs* gene was repressed both in compatible and incompatible plant-pathogen interactions (Shetty et al., 2009).

▪ *Phytoalexins*

Some phenolics such as stilbenes, coumarins and isoflavonoids are phytoalexins. They correspond to low-molecular mass secondary metabolites which are synthesized *de novo* in plants only in response to various stresses and which exert an antimicrobial activity against a large spectrum of diseases (Ahuja et al., 2012). Widely distributed among crop species, phytoalexins are considered as biomarkers of plant induced resistance against diseases (Ahuja et al., 2012).

Moreover, the chemical structure of phytoalexins varies between plant families. Camalexin and rapalexin are two phytoalexins which are known to be induced in Arabidopsis in response to an attack, while kauralexin and zealexin are the most recently identified phytoalexins found in maize.

The incredible diversity of phytoalexins was studied notably in plants belonging to the family of Brassicaceae, Fabaceae, Solanaceae, Vitaceae and Poaceae (Ahuja et al., 2012). For instance, 3-deoxyanthocyanidins are a class of phytoalexins found in sorghum, rishitin is found in potato, pisatin is synthesized in pea, and phaseollin is produced in bean plants (Vermerris & Nicholson, 2006). However, no phytoalexins have been identified up to now in wheat (Ors, 2015).

▪ *Oxylipin pathway*

Oxylipins are secondary metabolites produced by the oxidation and further transformation of polyunsaturated fatty acids (PUFAs), mostly linoleic acid (18:2) and linolenic acid (18:3) (Prost et al., 2005).

The structural diversity of oxylipins (Figure 19) is generated by the coordinate action of multiple enzymes, among which lipases, a group of cytochrome P450, α-dioxygenase (α-DOX) and lipoxygenase (LOX) which initiate the first steps of PUFA oxidation, (Blée, 2002; Howe & Schilmiller, 2002).
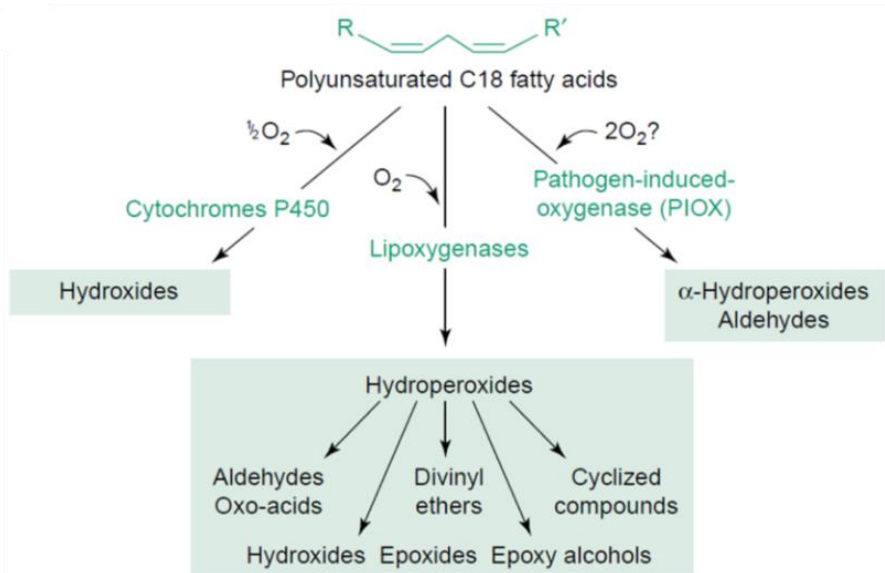
Figure 19. Oxylipin pathway in plants. (Source: Blée, 2002)

The long list of oxilipins includes 9- or 13-hydroxyoctadecadi(tri)enoic acid (9- or 13-HPOD/T), fatty acid kitodienes (KOD) or kitotrienes (KOT), aldehydes, and divinyl ester fatty acids (Prost et al., 2005). These metabolites play a major role in plant defense by acting as antimicrobial compounds in response to pathogen attack (*e.g.* 13-HPOD, 13-HOT, colneleic acid, colnelenic acid, etc) or as defense signaling molecules (*e.g.* JA and MeJA) (Prost et al., 2005).

The major role of oxylipins in plant induced resistance to pathogens was demonstrated by several studies. Notably, an increased accumulation of colneleic and colnelenic acid which are known to inhibit spore germination was reported in potato plants infected by *Phytophtora infestans* (Göbel et al., 2002). Similarly, an overexpression of the 9-LOX in tobacco and MeJA transferase in Arabidopsis was associated with increased resistance to *Phytophotora parasitica* and *Botrytis cinerea* respectively (Seo et al., 2001; Mène-Saffrané et al., 2003).

2

THESIS OBJECTIVES

   This thesis is dedicated to the identification of innovative stimulators of plant defenses as alternative products for the sustainable protection of cultivated crops. This topic is part of the main research subjects of our laboratory by focusing on the biological control of plant diseases. The corresponding case study is the pathosystem wheat-*Zymoseptoria tritici*. The objective of this PhD is to **identify effective and innovative elicitors able to induce the resistance of wheat against the fungal pathogen *Zymoseptoria tritici*, and to characterize the subsequent triggered defense-signaling pathways in the plant.**

   As previously stated in the bibliographical introduction: (i) elicitors are biocontrol products intended for the preventive treatment of cultivated crops against various diseases, in combination with reduced-rate fungicides; (ii) they trigger an array of defense mechanisms in the plant against a broad spectrum of pathogens; (iii) the protection efficacy of elicitors tends to decrease when shifting from the greenhouse to the field.

   As a result, **elicitor screening requires the achievement of an array of experiments** to search and double-check the elicitor properties of a given compound. Working methodically from the lab to the field, undertaking an array of biomolecular, biochemical and greenhouse tests, and finally interacting with other research units dedicated to elicitor screening, is definitely essential.

   On this basis, the research problem examined in this thesis was divided into six sub-questions as follows:

- The first two questions focus on the screening of the elicitor candidates under semi-controlled conditions in order to select two compounds which most protect the wheat plant against the *Septoria tritici* Blotch disease. *In vitro* bioassays were conducted in parallel to rule out the compounds displaying any fungicidal activity:

  Do the elicitor candidates effectively protect winter wheat against the pathogen *Z. tritici* under greenhouse conditions?

  Is such protection efficacy attributable to a direct biocidal effect of the tested compounds towards the fungi?

- The third question focused on the mode(s) of action of the elicitor candidates in the plant, in order to confirm their elicitor properties.

  Do the elicitor candidates trigger characteristic defense signaling pathways in the plant?

- **The fourth questions focused on the potential interference of the adjuvants used in the formulation in the protection of wheat against *Z. tritici*?**

  Do the adjuvants used in the formulation of the tested compounds interfere in the protection of wheat against *Z. tritici*?

- ▪ The fifth question concerns the potential phytotoxicity effect of the formulated elicitor treatments on the plant's health and defenses:

  Do the formulated elicitor treatments have a phytotoxic effect on the plant's growth and development?

- ▪ Finally, the last question focused on the potential variability of their protection efficacy when shifting from greenhouse to field conditions. Two years of field studies were undertaken to answer this question, notably in collaboration with the experimental station of Gembloux Agro Bio Tech and Arvalis-Institut du Vegetal:

  Is the protection efficacy of wheat by λ-carrageenan and *Spirulina* (*Arthrospira*) *platensis* maintained under practical conditions in the field against *Z. tritici*?

Detailed explanations are provided in the following chapter entitled 'Strategic choices' concerning the reasons why the pathosystem wheat-*Z. tritici* was selected as the case study of this work. Similarly, the relevance of the various elicitor candidates selected for this study is presented in detail.

3

STRATEGIC CHOICES

1. The pathosystem wheat-*Zymoseptoria tritici*

1. Wheat, a dominant crop in temperate countries

1.1 A cereal of major economic and social impact

Wheat is one of the 'big three' cereal crops cultivated in the world, along with maize and rice (Shewry, 2009) (Figure 20).
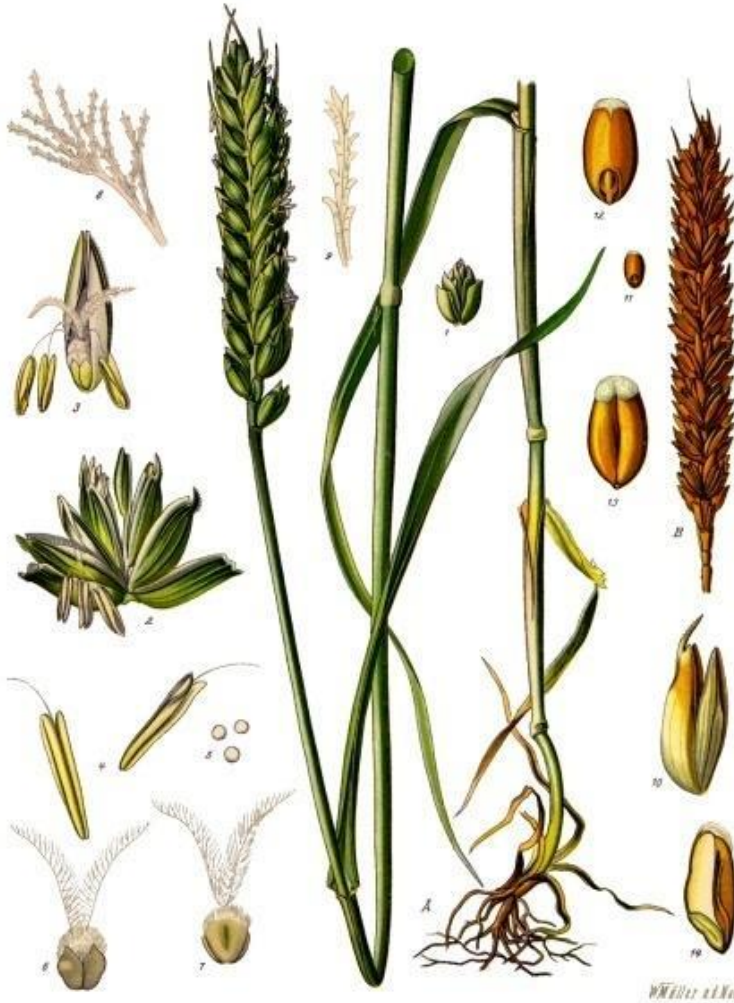
Figure 20. Wheat (Source: Köhler et al., 1883)

In 2015-2016, the global wheat production reached a new record of 734 million tons according to the International Grains Council (IGC), which represents 6 % more than the last five-year average (Satger, 2016). In the European Union (EU), soft wheat harvest reached around 134 million tons, which represents approximately 45 % of the total EU cereal production (European Commission, 2016). Among the various EU state members, France and Germany are the two most important wheat producers by harvesting around 26 % and 17 % of the EU total wheat respectively (Fones & Gurr, 2015).

With over 600 million tons being harvested each year in the world, wheat thus remains a dominant crop in temperate countries for human nutrition and livestock feed. Moreover, a strong rise in wheat consumption is predicted for 2016/2017 due to large supplies and attractive prices, combined to an ever-increasing world population contributing to important food demand (International Grain Council, 2016). The success of wheat relies on its adaptability and high yields, along with the unique properties of doughs formed from wheat flour which allow the processing of a wide range of baking products (bread, biscuits, pasta, noodles, etc) (Shewry, 2009). Such figures and statistics clearly highlight the major economical and societal influence of this crop at a global scale.

It is rightly pointed out by Sébastien Abis in his book entitled "Le blé: géohistoire d'un grain au coeur du pouvoir" (Abis, 2015):

'*Wheat has always been at the core of History and balance of power. Key issue in ancient times, it is linked to sourcing strategies, through trade, but also war, already in a perspective of securing this vital resource. (…) At the 20st century, it plays a central part as a strategic resource for the socio-political and geoeconomical balance, and it becomes an international vector of power for major exportators.*'

1.2 Origin and evolution of wheat

The history of wheat is closely related to the history of human civilization, especially in western countries (Bonjean, 2001; Abis, 2015). The first cultivation of wheat is thought to have occurred about 10,000 years ago in the Fertile Crescent, a region stretching from ancient Mesopotamia (Iraq) to the Levant (Syria, Lebanon, Jordan and Israel) (Bonjean, 2001; Shewry, 2009).

During the 'Neolithic Revolution', it appears that a climatic period of drought and cooling called 'Dryas' led the population to change their hunting and food-gathering habits to settled agriculture. The spread of wheat across Europe, Asia and Africa then followed major trading routes (Shewry, 2009; Abis, 2015).

The earliest forms of cultivated wheat (Figure 21) were the diploid 'einkorn' *Triticum monococcum* spp. *monococcum* (genome AA) and the tetraploid 'emmer' *Triticum turgidum* spp. *dicoccom* (genome AABB).
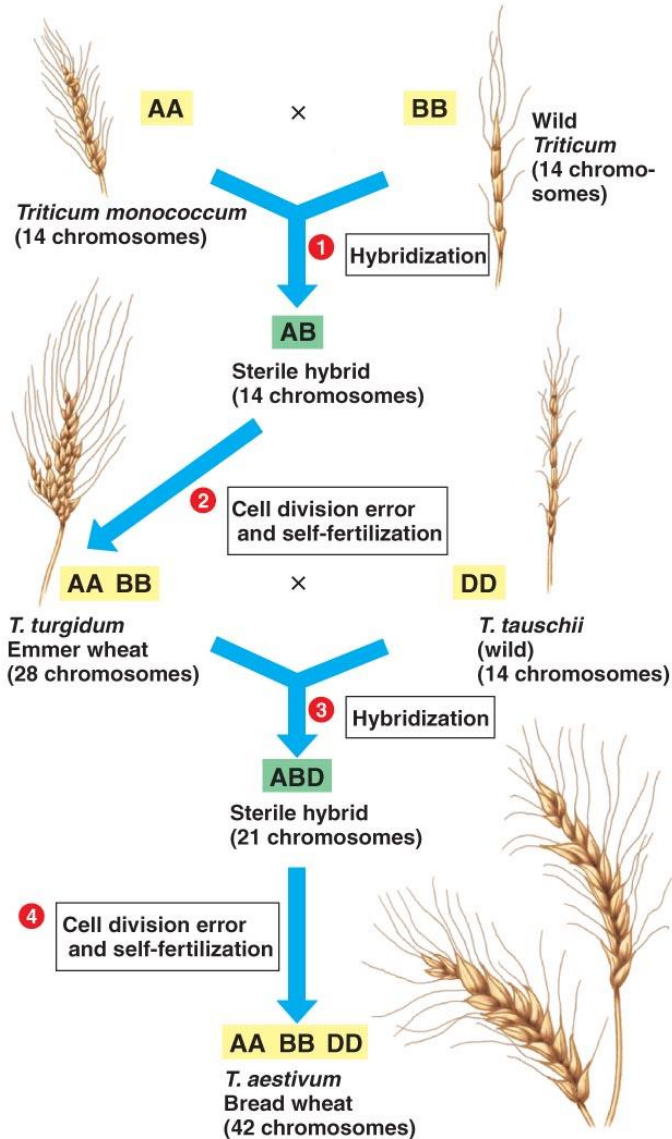
Figure 21. Evolution of the wheat phytobiome leading to the common bread wheat *Triticum aestivum*. (Source: http://broderslab.agsci.colostate.edu/research/)

The discovery of the wild ancestors of einkorn and emmer at the end of the 19th and 20th century confirmed that these landraces were selected by farmers from wild populations based on several characteristics, among which their superior yields.

Since then, wheat domestication has been associated with the selection of various genetic traits such as the loss of spike shattering at maturity and the selection of free-threshing naked grains (Bonjean, 2001).

Scientists discovered only by the end of the 20th century that cultivated hexaploid wheat *Triticum aestivum* had no wild ancestors. Common bread wheat *Triticum aestivum* spp. *aestivum* (genome AABBDD) indeed resulted from spontaneous hybridization of cultivated emmer with the unrelated and diploid wild grass *Triticum taushii* (genome DD), also called *Aegilops taushii*. Farmers probably selected this novel wheat hexaploid.

The *Triticum* genus comprises 6 species and 19 sub-species which are usually divided into 3 categories based on their number of chromosomes:

- **The diploid form** (2n = 14 chromosomes), which includes the species *Triticum monoccocum* spp. *monococcum* (einkorn) and *Triticum urartu*.

- **The tetraploid form** (2n = 28 chromosomes), which includes the species *Triticum turgidum* spp. *dicoccum* (emmer), *Triticum turgidum* spp. *durum* (durum wheat) and *Triticum timopheevi* (Zanduri wheat).

- **The hexaploid form** (2n = 42 chromosomes), which includes *Triticum aestivum* spp. *aestivum* (bread wheat) and *Triticum zhukovsky* (cross of *T.monococcum* and *T.timopheevi*).

Nowadays, the two most cultivated wheat species are bread wheat (*T.aestivum*), which represents 95% of the wheat grown worldwide, and durum wheat (*T.durum*) (Shewry, 2009). The difference between these 2 species relies on the grain composition and their geographical distribution: Bread wheat is used to make baking products and is cultivated in temperate areas, whereas durum wheat is rather used for making pasta and is cultivated in dry Mediterranean regions (Bogard, 2011).

1.3 Wheat development

Wheat is a herbaceous annual, and more precisely a monocotyledonous angiosperm which is grown in countries all over the World. Its genetic diversity indeed allowed the development of over 25,000 different types of wheat plants adapted to various environments (Shewry, 2009). The wheat optimal growth temperature is about 25 °C, with minimum and maximum growth temperatures of 3-4 °C and 30-32 °C respectively (Hatfield & Prueger, 2015). An adequate amount of water is also required during the growing season to ensure optimal production. However, too high humidity can also favor the development of diseases (Buck et al., 2005).

The successive stages of wheat development have been thoroughly described by Zadoks et al. (1974) by using the so-called 'Zadok's scale' with the help of decimal codes (Figure 22).
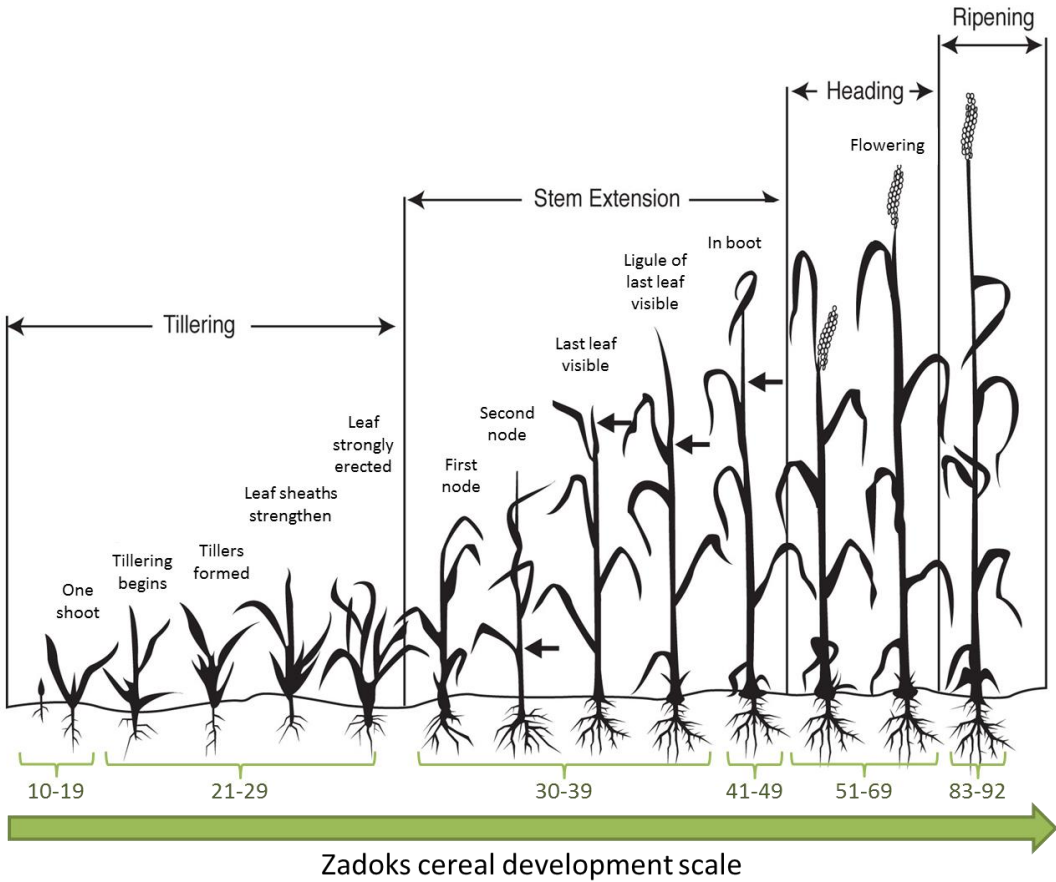
Figure 22. Cereal development scale proposed by Zadoks et al. (1974).

The growing period of the plant starts in autumn with the germination of the wheat grain and finishes in summer with the ripening of new seeds. Wheat development follows 3 main phases:

- Vegetative phase, corresponding to the herbaceous development of the plant with the growth of roots, leaves and stems.
- Reproduction phase, or flowering phase, corresponding to the development of ears and flowers.
- Ripening phase, corresponding to grain filling and ripening.

1.4 Spring vs Winter wheat

Common bread wheat can be classified as hard or soft, and winter or spring. Hard wheat is generally used for hard baked goods (*i.e*., bread), while soft wheat is used for soft baked goods (*i.e*., pastries) (Peña, 2002).

Winter and spring wheat varieties differ in terms of vernalization requirements: winter varieties need to experience a long cold period (0-5 °C) to be able to flower, contrary to spring varieties. The initiation of flowering is indeed a complex process influenced by a number of genetic and environmental factors, including vernalization (Dileo, 2010). As a consequence, spring wheat is generally planted in spring from April through May, rushes to flowering, and is harvested at the end of summer in August and early September. On the other hand, winter wheat is planted in autumn, germinates before the winter season, stays dormant over the winter, and is only harvested the following summer in July and August.

1.5. Integrated disease management of the wheat crop

Wheat is regularly subject to numerous biotic and abiotic stresses which can strongly influence the growth and the yield of the plant. Wheat diseases can affect different plant organs (*e.g*. roots, leaves, flowers, grains), and can be bacterial, viral, fungal, or even parasitic infestations (Mehta, 2014).

In Europe, the most harmful diseases for wheat crops are the following: *Zymoseptoria tritici* (*Septoria tritici* Blotch), *Fusarium graminearum* (Fusarium Head Blight), *Puccinia recondita* (Brown Rust), *Puccinia striiformis* (Yellow Rust), *Stagonospora nodorum* (Stagonospora Nodorum Blotch), and *Blumeria graminis* (Powdery mildew) (Mehta, 2014).

These pathogens can be obligate parasites which infect only living plant tissues, such as rusts and powdery mildew, or facultative parasites which can live in dead plant tissues, such as *Septoria tritici* Blotch and scab.

Nonetheless, wheat yield has increased steadily during the last decades (by about 3 T ha^{-1}) due to the introduction of highly performant cultivars and the considerable improvements in integrated disease/pest management (IPM) practices (Mehta, 2014). The main objective of IPM systems is to ensure economical and sustainable disease control, without relying solely on pesticides. The management of crops in an integrated manner implies the ecofriendly use of multiple tactics to keep the pest levels below an economic threshold (Herzfeld & Sargent, 2012).

All the diseases caused by fungal pathogens which can infect the plant throughout its development are mentioned in Figure 23.
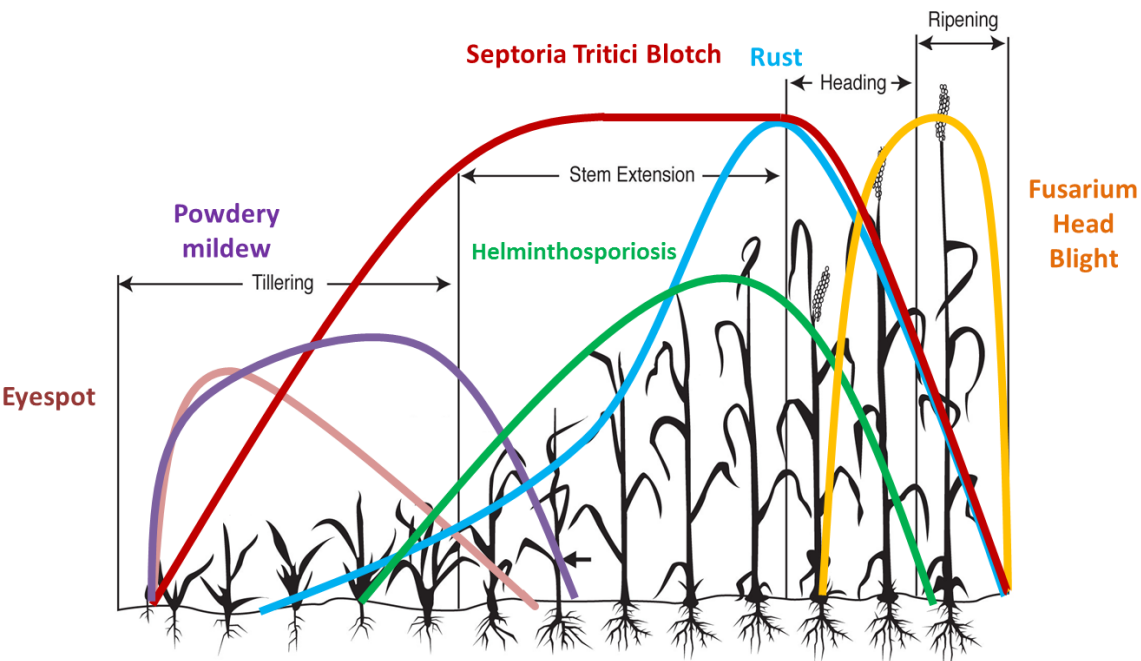
Figure 23. Amplitude of wheat diseases in the field over a growing season. (Source: BASF France Agro https://www.agro.basf.fr/)

- ▪ *Phytosanitary protection*

One of the oldest fungicidal input occurred in 1882 in Bordeaux, when Millardet applied the Bordeaux mixture, a copper fungicide, to protect grapes against downy mildew (Goyal, 2014). Since then, several generations of fungicides have been developed, which include dithiocarbamates, benzimidazoles, organophosphorus compounds and strobilurins.

However, multiple side effects have been observed, including fungicidal residues in the host plant, toxicity, and physiological/ biochemical changes in the host (Goyal, 2014). In addition, some fungal pathogens have developed resistance towards fungicides, especially towards those which have a site-specific mode of action (benzamidines, phenylamines, etc).

One typical example is the use of the antibiotic kasugamycin to control rice blast in the 1960s in Japan: a few years after the introduction of the antibiotic, some resistant strains of *Pyricularia oryzae* were identified in the rice fields where the antibiotic was used regularly and in high doses (Mehta, 2014). Other well-known examples include resistance of *Erwinia amylovora* to streptomycin, or powdery mildew to dimetirimol.

The risk of resistance development is linked to the chemical nature of the fungicide as well as its mode of action (Goyal, 2014). It appears that the use of a combination of systemic and non-systemic fungicides can minimize the emergence of pathogen resistance.

As a consequence, the use of fungicides in agriculture has become increasingly controversial with the growing awareness of the environmental and health hazards associated with these chemical inputs.

Farmers have long used fungicides successfully in the field, but such practices have also led to unbalanced ecosystems, sometimes even eradicating beneficial insects. It is thus mandatory now to take into account several aspects before recommending a fungicide on the market: the adequate dose, the application method, the spraying schedule, the residual effect on the plant and, last but not least, the toxic effects on plants, animals, and human beings (Mehta, 2014). Chemical inputs are now used as a tool in IPM strategies.

A good integrated disease management program nowadays will identify and monitor pest issues, select the adequate pest management tactics, and evaluate the efficiency of the program in terms of profitability and sustainability (Herzfeld & Sargent, 2012).

For wheat cultures, the use of fungicides still remains necessary as totally resistant wheat cultivars unfortunately do not exist. Fungicides are thus mainly used to retard the start of epidemics or to reduce the disease infection rate in order to maintain wheat yield. In order to successfully protect wheat against *Septoria tritici* Blotch, a first fungicide application at the stage 39 (first node) is recommended. Since the upper leaf stages are not protected and since the plant remains exposed to ear diseases, a second fungicide treatment (*e.g.* chlorothalonil or captafol) is usually realized at the stage 59 (heading stage). This last application offers an efficient and long-lasting protection of the plant throughout the grain filling phase (Bodson et al., 2000). When the disease pressure in the field is high, a curative fungicide application (*i.e.* triazoles, or a combination of triazoles and morpholines) can be realized at the flag leaf stage in order to protect the 2 upper leaves of the plant. These leaves indeed contribute most to the plant yield. In all cases, it is necessary to realize constant field monitoring for the spread of diseases in order to identify potential risks and choose the adequate protection strategy (Bodson et al., 2000; Mehta, 2014).

- ***Breeding for resistant varieties***

Plant breeding plays a major role in integrated disease management strategies. Breeding for resistance is the preferential control tool for wheat protection. The main objectives of breeders are to improve both yield performances, the nutrient use efficiency of the plant and its resistance to numerous diseases in order to reduce the use of chemical inputs. The successful breeding and selection methodology of new wheat varieties resistant to diseases is influenced by three factors: (i) the nature and virulence of the targeted pathogen(s), (ii) the diversity and the type of wheat genetic resistance and (iii) the screening methodology to track resistant traits in the environment (Singh & Rajaram, 2002).

Specific resistance protects a wheat cultivar for a short period of time against a few races of a given pathogen. However, such resistance becomes useless as soon as the pathogen evolves to a new race able to attack the cultivar.

On the other hand, non-specific resistance, or generalized resistance, is partly effective against all pathogens.

Breeding programs thus favor the selection of new wheat varieties displaying both specific and non-specific resistance genes against major diseases (Mehta, 2014).

The creation of a new wheat variety remains however a time-consuming, costly, and skilled work which can take around ten years to be achieved. Still, plant breeding has become a crucial tool in IPM strategies ever since an increasing number of countries have set stringent legislation requirements regarding the reduction of phytosanitary inputs in agriculture. Moreover, plant breeding remains an ever-improving and promising technique thanks to the emergence of new technologies (Rajaram, 2001).

- *Alternative protection strategies*

In addition to plant breeding and fungicide applications, several alternative techniques have been developed. Cultural practices represent an important lever as a means to minimize diseases and the use of agrochemicals all together. Common cultural practices include (Mehta, 2014):

- **Adequate plant fertilization:** The physiological and nutritional states of the plant are known to influence the success of its resistance to a disease. High nitrogen amounts can increase the susceptibility of wheat. Recommended rates are about 120-200 kg ha^{-1}.

- **Crop rotations:** The use of different plant species in a crop rotation contributes to breaking the life cycle of diseases specific to a given species. Non-host crops are generally used for subsequent planting. In addition, such rotations maintain plant biodiversity, supplement the soil with essential nutrients, and keep soils covered while reducing weed infestations. For instance, the incidence of STB is generally decreased by a 2-year rotation between wheat crops (Suffert et al., 2011).

- **Management of crop residues:** Crop residues can contain important amounts of pathogen spores which can significantly increase disease levels. Correct disposal of these residues is thus of major importance. In the case of wheat, stubble is usually ground after harvest and subsequently buried in the soil by ploughing (Suffert et al., 2011).

- **Diversification of sowing dates and cultivars:** Disease severity can be considerably reduced by changing sowing dates in order to avoid the usual period of disease infestation. For instance, early sowing of wheat is known to increase disease levels of Septoria tritici Blotch in autumn, resulting in an even higher disease pressure in spring and summer. On the other hand, late sowing can delay the epidemic. Similarly, the use of different cultivars reduces the risks of a disease propagating in the field thanks to their genetic diversity.

- **Crop density:** plant density is another factor which can influence disease severity, and recommended wheat density is about 200-250 plants/m^2 (Bodson et al., 2000).

- **Biocontrol methods for disease control** (Walters et al., 2014b)**:** a number of natural biocontrol agents have been identified and developed in the last decades in order to promote sustainable and integrated disease control. Biocontrol agents and biological products involve plant and/or pathogen natural mechanisms in order to maintain targeted bioagressors below a critical level of biological and economical damage. Four categories of biocontrol 'tools' have been depicted so far: macroorganisms (insects, mites, and nematodes); microorganisms (fungi, bacteria, viruses); natural substances (plant extracts, natural elicitors); semiochemicals (pheromones, kairomones, etc.).

2. Septoria tritici Blotch

2.1 Presentation of *Zymoseptoria tritici*

Septoria tritici Blotch (STB) is a foliar disease of wheat (Figure 24) which is caused by the fungal pathogen *Zymoseptoria tritici* (previously *Mycosphaerella graminicola* teleomorph, *Septoria tritici* anamorph) (Rudd, 2015).

Figure 24. *Septoria tritici* Blotch. (Source: Syngenta, https://www.syngenta.fr/traitements/septoriose-des-feuilles-septoria-tritici)

The first description of *Z. tritici* as the causal pathogen of the STB disease was in 1842 by Desmazieres in France (Ponomarenko et al., 2011). *Zymoseptoria tritici* is an Ascomycete belonging to the class of Dothideomycetes, order of Capnodiales and family of Mycosphaerellacae (NCBI Taxonomy browser). Close relatives of the pathogen include *Zymoseptoria pseudotritici* and *Zymoseptoria ardabiliae* which were discovered when analyzing population genetics of fungi collected on wild grass growing in the surroundings of wheat crops in Iran (Mcdonald et al., 2015). Unsurprisingly, the evolutionary history of STB is strongly related to the history of bread wheat, and both share a common center of diversity in the ancient Fertile Crescent (Mcdonald et al., 2015).

STB is considered as one of the most challenging wheat diseases throughout the world. This serious and persistent pathogen was shown to cause up to 50% of yield losses on susceptible wheat varieties (Eyal, 1987). In the European Union, STB is considered as the most important wheat disease, although rust is also becoming a major problem (Jørgensen et al., 2014; Torriani et al., 2015). Control methods mainly involve the use of fungicides and plant breeding.

Each year, about 70 % of EU fungicides are used to protect wheat crops against STB (Fones & Gurr, 2015). In France, Germany and UK alone, wheat fungicide applications against this disease are worth € 1.2 bn (Torriani et al., 2015).

2.2 Epidemiology and Life Cycle

The pathogen survives through winter on previous crop wheat residues and starts to infect young wheat plants during spring (Figure 25).
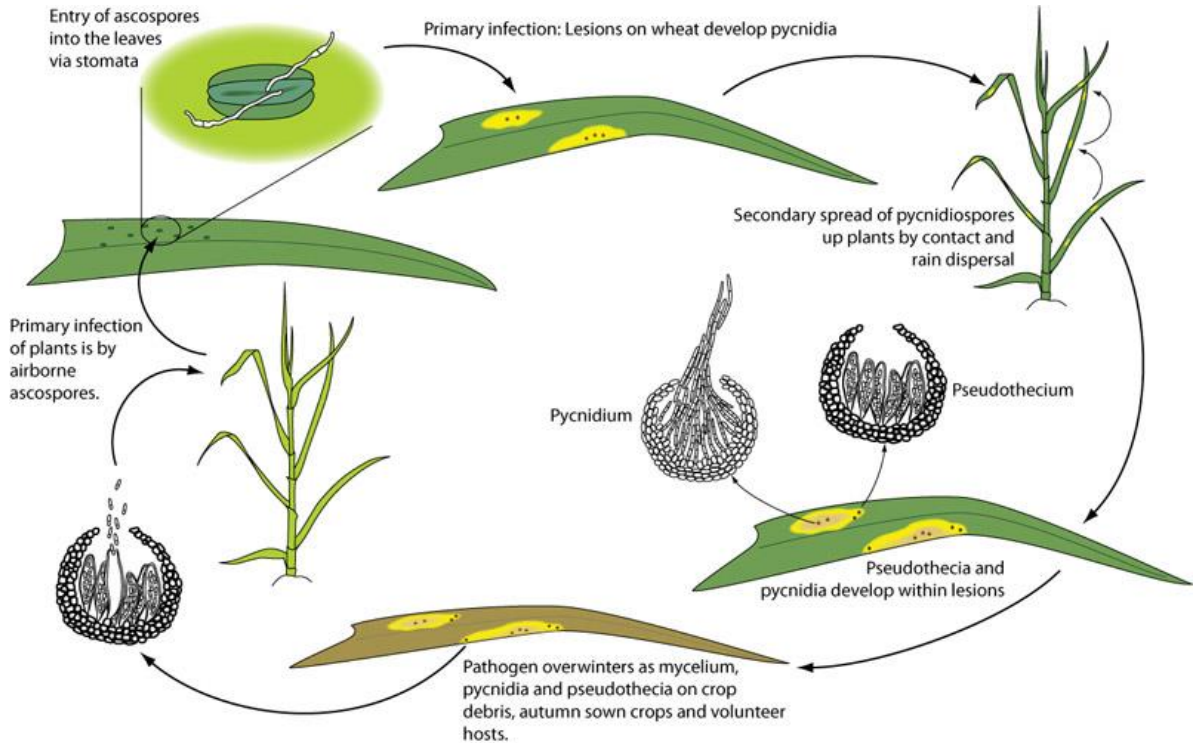
Figure 25. *Septoria tritici* Blotch life cycle. (Source: Ponomarenko et al., 2011)

The primary *Z. tritici* inoculum consists mainly of sexual ascospores released by pseudothecia located in crop debris (Suffert et al., 2011). These spores are the result of the sexual fertilization of two strains of different mating types. Such fertilization is thought to be bipolar and heterothallic (Suffert et al., 2011).

Ascospores are transported by the wind over long distances and eventually land on a host leaf where primary infection begins. Spore germination requires high humidity and moderate temperatures (about 18 °C) for at least 6 to 48 hours in order to form germ tubes and hyphae (Chungu et al., 2001).

The penetration mode of *Z. tritici* in the wheat leaf has often been controversial, and it is now generally admitted that it can occur both through stomata and directly through the cuticle (Palmer & Skinner, 2002; Siah et al., 2010a).

Once inside the leaf, the development of the pathogen remains strictly intercellular and the fungi progressively colonizes the mesophyll tissue. As in the case of

infection, the development of *Z. tritici* in the wheat leaf is favored by high humidity conditions and cool temperatures between 15 and 25 °C (Magboul et al., 1992). Contrary to the infection process which has been extensively studied since the 1990s, the interaction of the pathogen with the plant during its development still arouses considerable controversy due to its peculiar hemibiotrophic lifestyle (Figure 26) (Sánchez-Vallet et al., 2015).
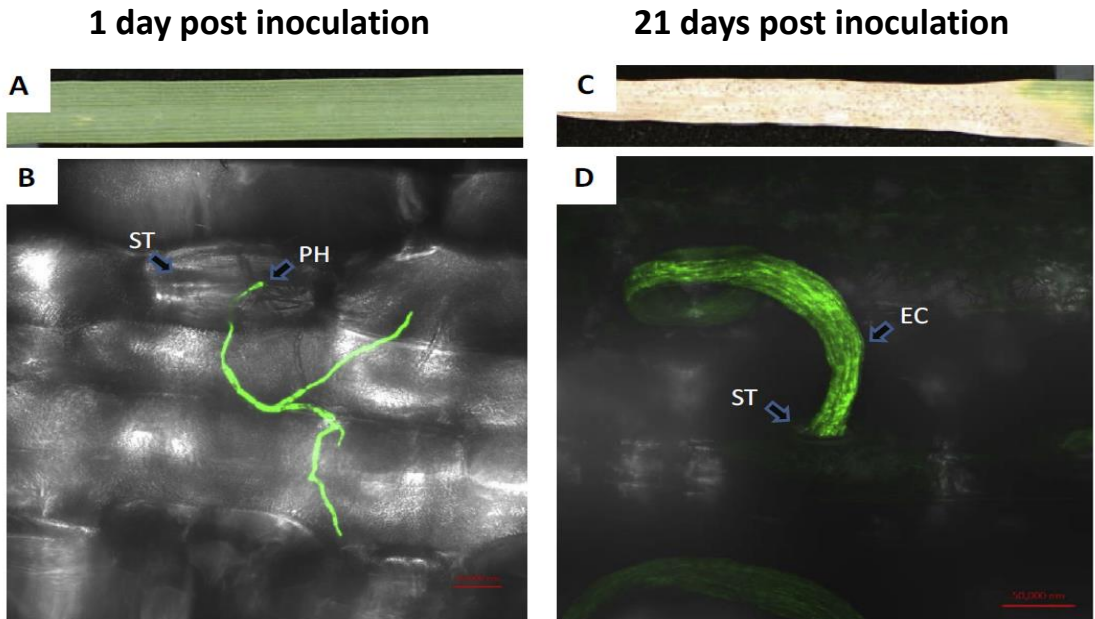
Figure 26. Biotrophic and necrotrophic phase of *Zymoseptoria tritici* development in a plant leaf. (A) Inoculated wheat leaf displaying no symptoms at 1 day post inoculation; (B) Penetrating hyphal (PH) filaments of *Z. tritici* through leaf stomata (ST); (C) Inoculated wheat leaf displaying symptomatic necrosis/chlorosis with pycnidia at 21 days post-inoculation; (D) Extracellular oozing cirrus (EC) releasing new asexual pcynidiospores from leaf stomata. B and D are stereomicroscope images of GFP tagged hyphal filaments. (Source: Rudd, 2015)

- *Biotrophic phase (from 0 to 14 days after inoculation)*

The initial development phase of the pathogen in the wheat leaf is biotrophic, asymptomatic, and lasts for about 9 to 14 days (Figure 27) (Palmer & Skinner, 2002).
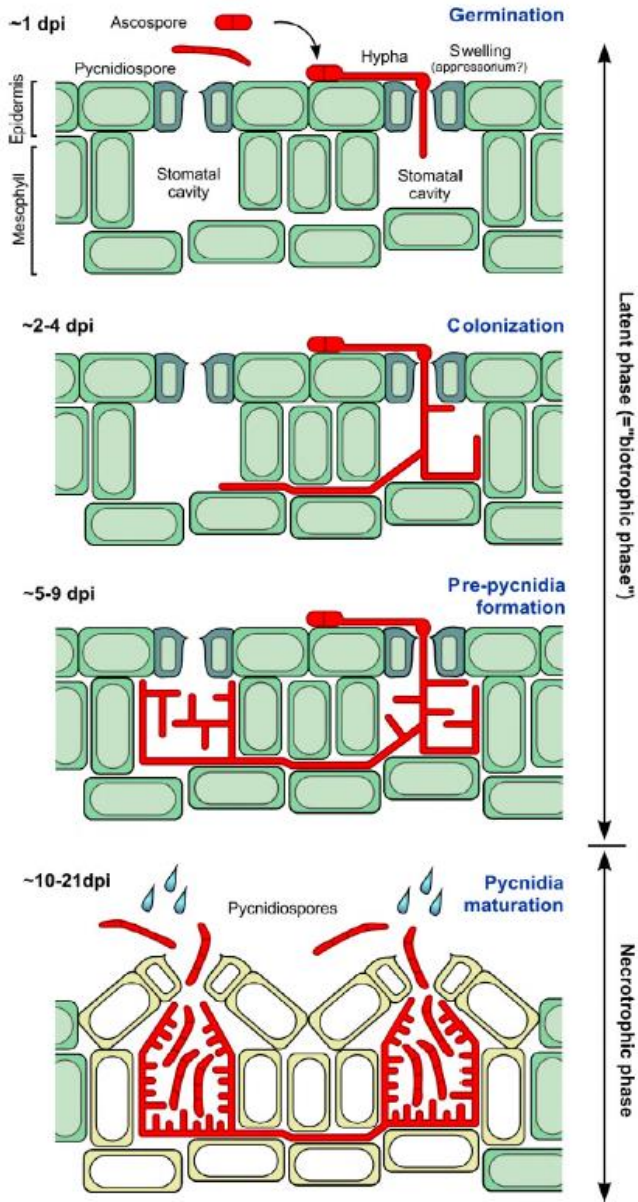
Figure 27. Schematic summary of the plant infection stages of *Z. tritici*. (Source: Steinberg, 2015)

During this period, the fungus grows slowly in the apoplast and closely to the plant cell wall. However, little is known about how the fungus acquires nutrients at that time since no haustoria or any other specialized feeding structures have ever been observed (Mcdonald et al., 2015; Sánchez-Vallet et al., 2015). It is suggested that the germinating spores may possess some stored reserves and/or that some nutrients may be available directly in the apoplast. Recent transcriptomic and metabolic profiling of *Z. tritici* infection of susceptible wheat have indeed revealed that lipids and fatty acids of the fungus were probably used as the main energy sources, as well as some host lipids (Rudd, 2015). Surprisingly, the pathogen doesn't seem to induce any defense reaction from the host plant, thus suggesting that *Z. tritici* may also be able to suppress the elicitation of plant immune responses (Rudd, 2015).

The switch of the fungal lifestyle from biotrophy to necrotrophy occurs only 10 to 14 days after inoculation: the mesophyll collapses, and the first signs of leaf chlorosis and necrosis become visible. Such transition comes together with a considerable reprogramming of the host and pathogen transcriptomes, and with a strong activation of host defense responses (Rudd, 2015). The time after which such transition occurs is influenced by the plant genotype and the virulence of the pathogen (Mcdonald et al., 2015). However, the exact process responsible for triggering the switch to necrotrophy remains unknown, although the involvement of a toxin is suspected (Sánchez-Vallet et al., 2015).

- ▪ *Necrotrophic phase and symptomatology (from 14 to 28 days after inoculation)*

Once in its necrotrophic phase, the fungal biomass increases drastically in the wheat leaf. The fungus is thought to emit a series of soluble toxins in order to speed mesophyll collapse and benefit from the nutrients released by dead leaf cells (Palmer & Skinner, 2002). Symptomatic lesions appear at the wheat leaf surface, first as small yellow blotches, and then as long and narrow necrotic blotches which follow the leaf veins. Within these necrotic areas, some brown-black and round shaped pycnidia appear 14 to 28 days after inoculation. These pycnidia are formed exclusively in substomatal cavities. Under high humidity conditions, the pycnidia exude a gelatinous cirrhi full of asexual conidia (also called pycnidiospores). These spores are then rapidly spread upward towards the canopy by the 'splashing' of water droplets to the upper leaves of a same plant (vertical infection) or to neighboring plants (horizontal infection) (Palmer & Skinner, 2002; Ponomarenko et al., 2011). The dissemination of asexual conidia corresponds to the secondary inoculation of wheat by *Z. tritici*.

Both sexual ascospores and asexual conidia thus contribute to the epidemics of STB. During a growing season, several cycles of sexual and asexual reproduction occur, allowing the disease to spread rapidly in wheat crops and leading to diverse genetic populations of *Z. tritici* (Palmer & Skinner, 2002).

The sexual phase of *Z. tritici* particularly influences genetic mixing through multiple genetic recombinations (Suffert et al., 2011) thus facilitating the bypassing of wheat resistance. It is estimated that 1 m^2 of infected wheat harbours about 70 different strains of *Z. tritici* (Zhan et al., 2001).

Moreover, STB epidemics are complex in the sense that primary inoculum doesn't involve solely ascospores emitted from distant or locally infected wheat debris. Pycnidiospores can indeed represent primary inoculum by remaining on decaying leaves or stubble (Suffert et al., 2011). In addition, primary infection can also originate from alternative hosts of *Z. tritici*. At least 6 grass species have been identified as volunteer plants in the surroundings of wheat cultures (Suffert et al., 2011).

2.3 Disease control of *Septoria tritici* Blotch

Control of STB today mainly relies on intensive fungicide use. Commonly used agrochemicals include (Torriani et al., 2015):

- **Succinate dehydrogenase inhibitors (SDHIs)**. These fungicides interfere with the succinate dehydrogenase molecule (complex II in the mitochondrial respiration chain) which plays a functional part in the tricarboxylic cycle and the mitochondrial electron transport chain. As a result, the production of vital energy for the pathogen is interrupted.

- **Demethylation inhibitors (DMIs)**. These fungicides inhibit a specific enzyme of the pathogen, the C14-demethylase, which plays a major role in sterol production, thus leading to abnormal fungal growth and death. Indeed, sterols such as ergosterol are essential components of fungal membranes and play a crucial role in the development of functional cell walls. DMIs are used in agriculture since their first introduction in the 1970s, and include the group of triazole fungicides such as metconazole, prothioconazole, and epoxiconazole.

Cases of STB resistance have been increasingly reported in a number of Western European countries such as France, Belgium and UK (Amand et al., 2003; Lucas, 2003; Siah et al., 2010b). STB resistance has mainly evolved against site-specific fungicides such as azoxystrobin and other Quinone outside Inhibitors (QoIs), also known as strobilurins (Lucas, 2003). QoIs prevent the pathogen from producing energy by blocking the transfer of electrons in the respiration chain of the fungus, much like SDHI fungicides.

However, since QoIs are no longer effective against STB, and since triazoles already show signs of reduced sensitivity, it appears that SDHIs have become a major lever for the fungicidal control of wheat against this disease (Fraaije et al., 2005; Drummond et al., 2015). Fungicide mixtures of SDHIs, DMIs, and multi-site fungicides such as chlorothalonil are currently considered as most effective against the pathogen and are thought to prevent sensitivity shifts (Drummond et al., 2015; Torriani et al., 2015).

These groups of fungicides are ranked respectively as medium to high, medium, and low risk of favoring *Z. tritici* resistance (Torriani et al., 2015). Systemic triazoles like fluxopyroxad are thus mixed with pyrazole carboxamide SDHIs such as isopyrazam or bixafen as part of an IPM approach (Poole & Arnaudin, 2013; Drummond et al., 2015).

Moreover, in order to avoid further evolutions of *Z. tritici* resistance, extensive research has been dedicated to the effective use of fungicides in cereal crops (Marroni et al., 2006; Poole & Arnaudin, 2013; Drummond et al., 2015). Clearly, the mode of action of the fungicide must be taken into account, as well as its movements within the plant. The time of fungicide treatment and the doses must also be adequately thought through (El Jarroudi et al., 2013).

Breeding for STB-resistant wheat varieties has also become an increasingly popular strategy by contributing to the reduction of fungicide use (Brown, 2012). For breeders, the most important priority is to develop high yielding varieties with a moderate to good field resistance, as requested by farmers (Torriani et al., 2015). Wheat resistance to STB can be categorized into two groups:

▪ **A strong qualitative resistance**, which is mainly controlled by major R-genes according to a gene-for-gene relationship. So far, 21 of these so-called *Stb* genes have been characterized, and the *Stb6* gene in particular was shown to confer increased wheat resistance in the field (Brading et al., 2002; Torriani et al., 2015). However, such specific resistance is unlikely to last and some cases of pathogenic evolution have already been recorded (Brown et al., 2015). Yet, gene « pyramiding » is one interesting approach where several *Stb* genes are combined in a cultivar in order to circumvent *Z. tritici* evolution to virulence (Brading et al., 2002).

▪ **A quantitative resistance**, which is controlled by multiple and non-specific genes with moderate effects on the pathogen. Such resistance was demonstrated to be effective against all *Z. tritici* genotypes (Brown et al., 2015).

Consequently, the use of qualitative STB-resistance in wheat breeding appears less sustainable than the use of quantitative resistance. As a matter of fact, modern selection strategies favor minor or partial QTL genes for partial resistance, and their gradual accumulation in breeding programs these last years is likely responsible for the significant progress in the development of STB-resistant wheat cultivars (Brown et al., 2015; Torriani et al., 2015).

Finally, biocontrol methods have become increasingly popular in response to the EU policy requiring a drastic reduction of pesticide use in the near-future (Walters et al., 2013). Currently, phytopathology research is focused on the identification and the development of biocontrol agents which stimulate the natural defenses of plants against a broad-spectrum of diseases. In the case of wheat, the list of existing elicitors developed to preventively protect the plant against STB is quite short.

Some of the rare registered and commercialized elicitors include BION® (Syngenta), a chemical elicitor containing Acibenzolar-S-methyl (ASM) and which shows a functional analogy to the plant hormone salicylic acid. Bion® was originally registered to control wheat against powdery mildew (Görlach et al., 1996). However, this product was also shown to reduce plant growth and thus plant yield, probably by diverting considerable energy towards the induction of defense mechanisms and to the detriment of plant development (Walters & Heil, 2007).

Iodus40® (formerly Vacciplant®; Goëmar, France) is another registered elicitor of wheat to control powdery mildew and STB. This product contains laminarin, a water-soluble and linear β-1,3-glucan extracted from the brown alga *Laminaria digitata* (Walters et al., 2014b).

This storage polysaccharide was shown to stimulate defense responses of numerous plants such as grapevine, tobacco and maize against a large panel of diseases (Aziz et al., 2003; Klarzynski et al., 2000; Sobhy et al., 2012).

Laminarin induces the synthesis of PR proteins and the accumulation of phytoalexins by activating the salicylic acid (SA) pathway (Ménard et al., 2004). The crucial role of β-1,3-glucans in inducing wheat resistance was highlighted in the study of Shetty et al. (2009) by showing that wheat leaves treated with purified β-1,3-glucans extracted from the cell wall of *Z. tritici* conferred plant resistance to STB. In addition, *Trichoderma* spp. also stimulates wheat defenses against this disease: the coating of wheat seeds with *T. harzianum* efficiently protected the wheat plant (Cordo et al., 2007). Finally, inorganic salts such as chlorides, phosphates, and phosphites can act as good biopesticides of wheat foliar diseases, including STB (Deliopoulos et al., 2010).

3. Disease assessment

STB disease assessment is generally performed on wheat leaves by evaluating the severity and incidence of the pathogen. In greenhouse conditions, disease incidence is measured by assessing the percentage of infected plants out of the total number of plants. Disease severity can be measured visually, in the greenhouse or in the field, by assessing the percentage of foliar necrotic lesions bearing pycnidia (Figure 28).
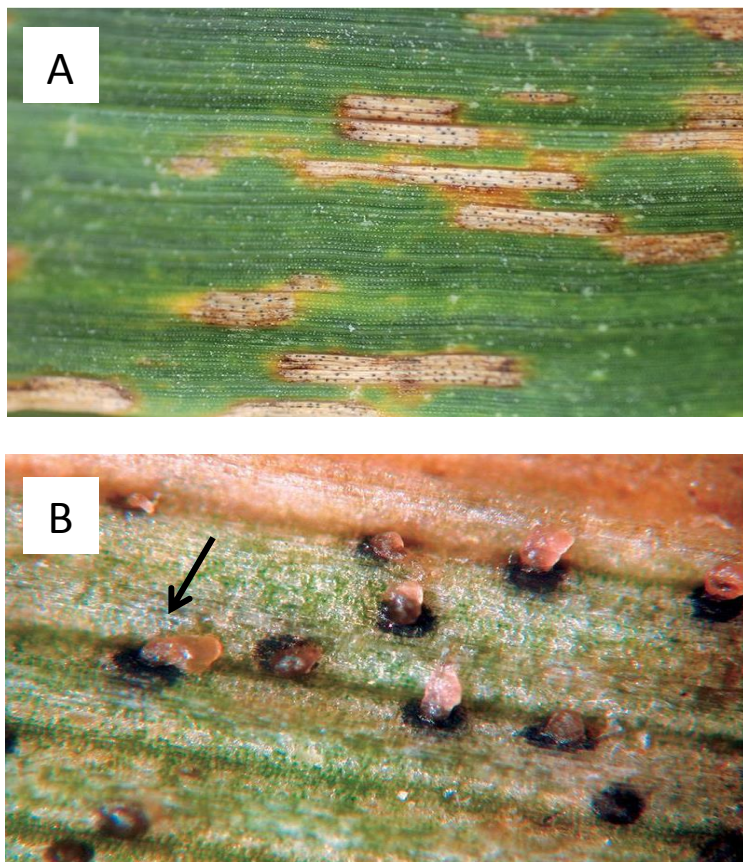
Figure 28. *Zymoseptoria tritici* symptoms at the surface of a wheat leaf: (A) necrosis/chlorosis caused by the fungal pathogen; (B) pycnidia containing asexual pycnidiospores. (Source: Arvalis-Institut du vegetal ; http://www.fiches.arvalis-infos.fr)

2. Elicitor candidates

The elicitor candidates selected for this project have various origins and structures, whether they are purified products, plant extracts, or even algae extracts. Such heterogeneity was sought in order to enhance the probability of identifying a novel elicitor of wheat defenses. Indeed, it is suggested that plants have the ability to recognize a number of structurally distinct elicitor molecules, and most of the elicitors identified up to now share no common chemical structure (Montesano et al., 2003).

More importantly, all the candidates were already proven to have immuno-stimulating properties on plants and/or animals. Those which were already known to stimulate plant natural defenses had never been tested on the pathosystem wheat-*Z. tritici* up to now. Moreover, some compounds had already shown remarkable immuno-stimulating properties on humans and/or animals but were scarcely (or never) tested on plants. We thus decided to take a chance at testing them on wheat. Such decision was encouraged by the fact that multiple elicitors discovered since the 1970s are transkingdom, especially PAMPs such as flagellin, chitin, glucans, lipopolysaccharides or peptidoglycans (Nürnberger & Brunner, 2002). Defense mechanisms of animals and plants show similarities in terms of pathogen recognition and defense signaling pathways (Menezes & Jared, 2002; Nürnberger et al., 2004; Yakushiji et al., 2009). Animals and plants both have the ability to recognize non-self molecules which can be emitted from bacteria, fungi or viruses. The molecular architecture of such recognition process is strikingly similar between animal and plants, and supports the hypothesis of a common evolutionary origin of pathogen defense systems in higher eukaryotes (Nürnberger & Brunner, 2002). PAMPs are sensed by specific pattern recognition receptors, and researchers have confirmed that plants possess an incredible diversity of receptors of conserved microbial signatures (Schwessinger & Ronald, 2012). These receptors share structural and functional similarities with animal Toll-like receptors. Most of the receptors identified up to now in plants are receptor-kinases (RKs) and receptor-like proteins (RLPs) localized in the plasma membrane, or extracellular soluble proteins, or even intracellular kinases (Schwessinger & Ronald, 2012).

Finally, we selected at best compounds which were shown to stimulate the defenses of other monocotyledonous plants such as rice or barley, or even better which were shown to stimulate wheat defenses against diseases similar to STB (hemibiotrophic life cycle, foliar infection, etc). Defense mechanisms indeed differ between monocotyledonous and dicotyledonous plants (Balmer et al., 2013). It is noteworthy to precise that most of the elicitors identified up to now were studied on dicotyledonous plants, and mostly model plants, such as tobacco and tomato.

1. λ-carrageenan

Seaweeds are marine macroalgae which have long been used in human nutrition. They represent indeed an amazing source of bioactive compounds, and particularly complex cell wall and storage polysaccharides (Vera et al., 2011). These polysaccharides can be found in three major seaweed groups and are already commercially exploited: alginates, laminarin and fucans are found in brown algae (Phaeophyta), agarans and carrageenans are found in red algae (Rhodophyta), and ulvans are found in green algae (Chlorophyta) (Campo et al., 2009; Vera et al., 2011). They are also currently used in agriculture as biostimulants of plant growth (Mercier et al., 2001; Khan et al., 2009). In addition to growth-stimulating properties, these algal polysaccharides and their derived oligosaccharides can activate plant defense responses against a broad range of pathogens (Klarzynski & Fritig, 2001; Jaulneau et al., 2011; Vera et al., 2011).

Carrageenan in particular is a family of sulphated, hydrophilic and linear galactans extracted from the cell wall of different species of red seaweed: *Gigartina*, *Chondrus crispus*, *Euchema* and *Hypnea* (Campo et al., 2009). The term carrageenan is derived from the Irish word "*carraigín"* (carrageen) which means "little rock" and designates the red seaweed species *Chondrus crispus*, also known as Irish moss (Figure 29) (Campo et al., 2009).
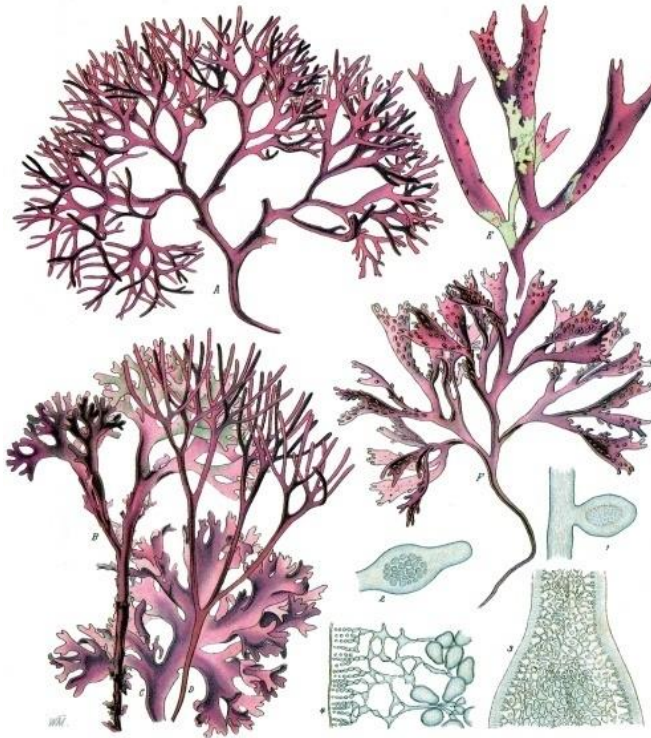
Figure 29. Red seaweed *Chondrus crispus*. (Source: Köhler et al., 1883)

The Irish have used this alga for centuries to treat respiratory problems, and it even became one of the few nutritional sources available during the Great Hunger in 1846-51.

Today, the three most commercialized red seaweed extracts are Iota (τ-), Kappa (К-), and Lambda (λ)-carrageenans. They are widely used in the food industry as additives due to their jellifying and emulsifying properties (McHugh, 2003; Imeson, 2009; Burketova et al., 2015). However, they differ in terms of chemical classification and depending on the seaweed species from which they are extracted (Campo et al., 2009). They are composed of repeated dimers of α-1,4-linked D-galactose (λ-carrageenan) or of 3,6 anhydro-D-galactose residue (К- or τ-carrageenan) with a β-1,3-linked D-galactose residue (Figure 30) (Mercier et al., 2001; Vera et al., 2011).
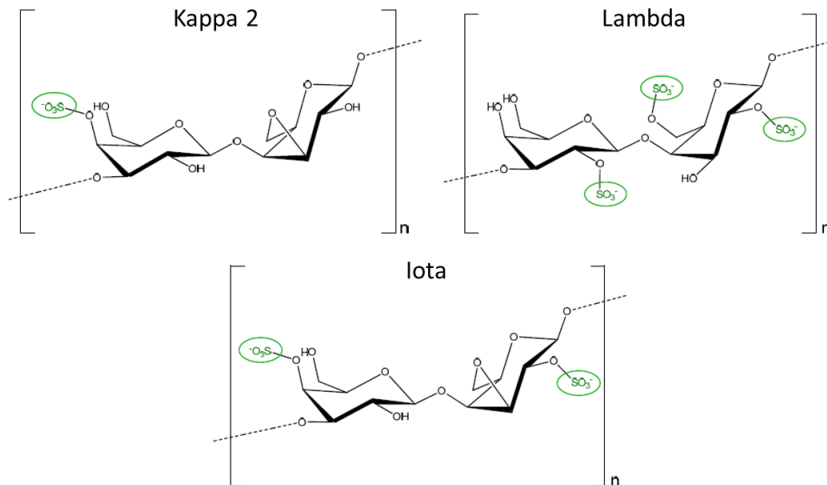
Figure 30. Units of sulphated D-galactose and anhydrogalactose in kappa, iota and lambda carrageenans. (Source: Vera et al., 2011)

The ratio of carrageenan types varies between algae species (Stadnik & de Freitas, 2014). *Kappaphycus alvareizii* and *Hypnea musciformis* are particularly rich in κ-carrageenan, while τ- carrageenan is predominant in *Euchema denticulatum* and λ-carrageenan is abundant in *Gigartina pistillata* (Stadnik & de Freitas, 2014).

One of the first studies demonstrating the eliciting properties of carrageenans goes back to the 1990s, when Patier et al. (1995) showed that κ-carrageenan stimulated defense responses in cells of the plant *Rubus fruticosus* and in protoplasts. In addition, the derivatives oligo-κ-carrageenans displayed even greater eliciting activity than the original polysaccharides. At that time, carrageenans had already been reported to modify immune responses in animal cells (Patier et al., 1995). Since then, multiple evidence of plant induced resistance by these polysaccharides and their derivatives has been published.

In tobacco, carrageenans were proven to be the most efficient in stimulating defense responses compared to the well-known laminarin. Moreover, λ-carrageenan proved to be the most active resistance inducer at doses between 100-1000 µg ml^{-1} (Mercier et al., 2001). The expression of genes encoding sesquiterpene cylase, chitinase and proteinase inhibitors were particularly expressed, and both SA, JA and ET signaling pathways were induced following tobacco leaf infiltration with this remarkable polysaccharide (Mercier et al., 2001). The eliciting potential of carrageenans is thought to be linked to their structure and composition (*e.g.* sulphate content and rhamnose residues) (Mercier et al., 2001; Sangha et al., 2010; Sangha et al., 2011; Burketova et al., 2015). Indeed, λ-carrageenan contains the highest degree of sulfation (41 % of total weight), compared to 20 % and 33 % of sulfate content in κ- and τ-carrageenan respectively and was proven to be the most efficient in stimulating plant defense responses (Mercier et al., 2001; Sangha et al., 2010).

In the model plant *Arabidopsis thaliana*, Sangha et al. (2010) showed that λ-carrageenan was the most active compound stimulating plant defense responses, compared to τ-carrageenan. Foliar treatment with 1 g l^{-1} of λ-carrageenan induced plant resistance against the fungus *Sclerotinia sclerotiorum* and increased the expression of jasmonic acid-related genes: *AOS* (allene oxide synthase), *PDF1.2* (defensin) and *PR3* (chitinases) (Sangha et al., 2010). Similarly, λ-carrageenan effectively protected tomato plants against the tomato chlorotic dwarf viroid (TCDVd), whereas τ- and κ-carrageenan had no effect (Sangha et al., 2015). Tomato leaves sprayed with 1 g l^{-1} of λ-carrageenan showed an upregulation of JA-related gene expression (*e.g. AOS* and *LOX*), suggesting once more the involvement of the JA signaling pathway (Sangha et al., 2015).

Conversely, λ-carrageenan failed to protect Arabidopsis against the insect *Trichopulsia ni*, compared to τ-carrageenan (Sangha et al., 2011). Such result highlights once again that the biological activity of the different types of carrageenan may be linked to their degree of sulphation. This hypothesis is supported by the fact that plant defense mechanisms are known to differ in response to chewing insects and to pathogens. Interestingly, and although plant resistance to chewing insects mainly involves the JA signaling pathway, treatment of Arabidopsis with τ-carrageenan actually induced both SA and JA defense responses (Sangha et al., 2011).

Finally, carrageenan oligosaccharides were also proven to induce plant resistance against a broad spectrum of diseases (Vera et al., 2012). The τ-, κ- and λ-oligocarrageenans applied at 1 g l^{-1} induced long-lasting resistance of tomato plants against the tomato mosaic virus (TMV), the fungal pathogen *Botrytis cinerea*, and the bacteria *Pectobacterium carotovorum* (Vera et al., 2012).

The sulphate content of these derivatives and the pathogen life-style probably had an influence on the eliciting activity. Indeed, λ-oligocarrageenans protected the most efficiently tomato against TMV, whereas τ-and λ-oligocarrageenans equally protected the plant against *Botrytis cinerea*, and plant protection against *P.carotovorum* was similar with all three oligosaccharides (Vera et al., 2012). In addition, these oligosaccharides suppressed disease infections at a systemic scale by increasing the activity of the PAL enzyme and by inducing the accumulation of

phenylpropanoid compounds with antimicrobial properties (Vera et al., 2012). Moreover, oligocarrageenans were proven able to stimulate the growth and photosynthesis of tobacco plants when applied at 1 g l^{-1} once a week (Castro et al., 2012).

To summarize, the local and systemic resistance induced by these elicitors is mediated by different signaling pathways, among which SA and JA either alone or in combination. Still, the exact mode of action of carrageenans and their derivatives is still far from being understood, although the sulphate content of these polysaccharides appears to play a key role in the elicitation of plant defenses. Carrageenans have mostly been studied on dicotyledonous model plants, and there is still a crucial need to investigate their activity on major monocotyledonous plants such as wheat, rice or barley.

2. CpG-ODN

DNA containing Cytosine-phosphate-Guanine oligodeoxynucleotide motifs (CpG ODN) are short and single-stranded DNA molecules which contain a cytosine triphosphate deoxynucleotide ('C') linked to a guanine triphosphate deoxynucleotide ('G') by a phosphodiester (Figure 31).
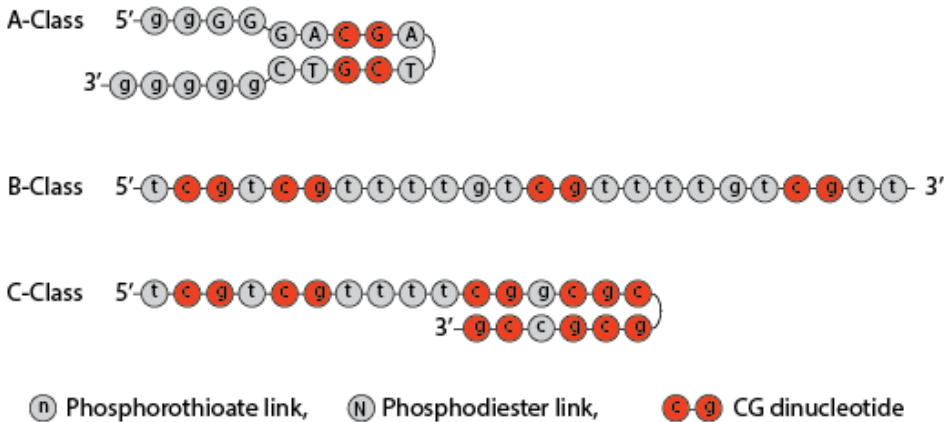
Figure 31. Different classes of CpG-ODN. (Source: InVivoGen, http://www.invivogen.com/tlr9-agonist)

The discovery of the immuno-stimulating properties of CpG-ODN goes back to 1995, when Krieg *et al.* proved that the CpG motif within bacterial DNA induced innate immune responses in B cells (Krieg et al., 1995). In addition, such immune response was only activated if the cytosine residue was unmethylated (Krieg et al., 1995).

CpG ODN is present at a high frequency in the DNA of prokaryotes (bacteria), but is actually rare in eukaryotic DNA (mammals) (Hanagata & Hanagata, 2012). Mammalian cells have evolved specific receptors to recognize such unmethylated DNA emitted from bacteria as a means to identify and eliminate them, especially since mammalian DNA contains only methylated CpG motifs (Hanagata & Hanagata, 2012).

Since the 1990s, multiple research demonstrated that unmethylated CpG ODN acts as a danger signal recognized by pattern recognition receptor (PRR) Toll-Like Receptor 9 (TLR9) in animal antigen-presenting cells and B cells. They induce innate immunity against bacterial, viral and protozoan pathogens (Carrington & Secombes, 2006; Hanagata & Hanagata, 2012). However, CpG ODNs are also rapidly degraded by DNases. Researchers have therefore developed synthetic and DNase-resistant CpG ODNs consisting of a phosphorothioate backbone to replace the oxygen present in the phosphate group of the nucleic acid targeted by DNases (Hanagata & Hanagata, 2012). Such synthetic ODNs expressing unmethylated CpG motifs are able to mimick the immune-stimulatory activity of bacterial DNA (Bode et al., 2011).

Today, synthetic CpG ODNs are commonly used as vaccine adjuvants as they improve the activity of the vaccine against infectious diseases and cancer (Bode et al., 2011). Four classes of synthethic CpG ODNs have been developed up to now, each of which displays specific structural and biological activities (Bode et al., 2011; Hanagata & Hanagata, 2012).

Unmethylated CpG ODN is thus commonly considered as a PAMP which induces defense responses in humans and animals (Covello et al., 2012; Hanagata & Hanagata, 2012). For instance, 31 different types of B-class CpG ODNs were used as vaccine adjuvants in aquaculture and were shown to induce immune responses on various fish species (salmonids, cyprinids, pleuronectiformes), both *in vivo* and *in vitro* (Carrington & Secombes, 2006). Defense responses consisted of macrophage activation, leucocyte proliferation, and stimulation of cytokine expression (Carrington & Secombes, 2006).

On Atlantic salmon, oral administration of CpG-ODN efficiently protected the fish against sea lice (Covello et al., 2012). In comparison, the common elicitor β-glucan failed to protect the salmons. CpG ODN stimulated fish defense responses by inducing inflammatory cytokines at a local and systemic level (Covello et al., 2012). However, the exact mode of action of this immunostimulant in fish remains to be investigated.

Recently, bacterial unmethylated CpG-ODN was also reported to induce defense responses in the plant model Arabidopsis (Yakushiji et al., 2009). This is probably the first time that CpG-ODN is tested on plants. DNA concentrations of 500 µg ml^{-1} induced the accumulation of H_2O_2 and callose depositions. In contrast to mammals where 13 Toll-like Receptors (TLRs) have been identified so far, a limited number of receptors of PAMPs are known in plants (Yakushiji et al., 2009).

Up to now, FLS2, EFR and CERK1 have been identified in Arabidopsis as receptors for flg22, elf26 and chitin (Zipfel et al., 2004; Gómez-Gómez, 2004; Miya et al., 2007). Endocytosis has also been reported as a crucial step in the recognition

of PAMPs by plants (Robatzek et al., 2006). In mammals, CpG ODNs are first translocated into the cytoplasm by endocytosis before being recognized by the TLR9 receptor located on the membrane of endosomes (Yakushiji et al., 2009). The same phenomenon has been proposed in the case of CpG-ODN recognition in Arabidopsis: the immunostimulant might be translocated into endosomes where it is recognized by a specific receptor, leading to plant induced resistance (Yakushiji et al., 2009). The work of Yakushji et al. in 2009 is a first in studying the impact of CpG ODN on plant defense responses. Since then, and to our knowledge, no other studies have been performed to study the elicitor properties of this interesting compound for plant protection.

3. *Spirulina platensis*

Cyanobacteria are at the frontier between the animal and the plant kingdom. They are considered as the first "plants" which appeared on Earth and responsible for the production of vital oxygen by photosynthesis. There are 200 genus and around 1500 species of cyanobacteria identified so far. *Spirulina platensis* (Spirulina) is a one of the best-known cyanobacteria which has been subject to numerous researches (Figure 32).

Figure 32. Microscopic view of Spirulina (Source: Koru, 2012)

It is a photosynthetic and prokaryotic microorganism showing common characteristics with both bacteria and algae. Taxonomically, Spirulina belongs to the division *Cyanobacteria*, class *Cyanophyceae*, order *Nostocales*, family of *Oscillatoriaceae* and genus *Arthrospira* (Lupatini et al., 2017). However, there has often been confusion over the common names "Spirulina" and "Arthrospira".

The term "Spirulina" is the commercial name of a species of dietary cyanobacteria belonging to the *Arthrospira* genus. However, "Spirulina" in English can also

designate other inedible cyanobacteria such as *Spirulina major*, *Spirulina gigantea* or *Spirulina princeps*. In this study, Spirulina will be used to describe the edible cyanobacteria of the *Arthrospira* genus and the *platensis* species.

Spirulina consists of a mobile, multicellular and spiral wrapped filament (hence its name, 'spiralis' in latin) with a characteristic blue-green color. The filament of *Spirulina platensis*, also called trichome, has a characteristic helical shape with a length of 350 μm and a diameter between 6 and 12.45 μm. The spiral turn diameter ranges between 20 to 50 μm (Cruchot, 2008; Lupatini et al., 2017). Spirulina is an autotrophic and aerobic microorganism whose growth is dependent on photosynthesis. Its mode of reproduction is asexual and consists of cross-binary fusion leading to the production of a new filament (Lupatini et al., 2017). Its multiplication rate is particularly quick, and can last only 7 hours under favorable conditions (Cruchot, 2008). The filaments grow spontaneously and form so-called "blooms" at the water surface (Figure 33).
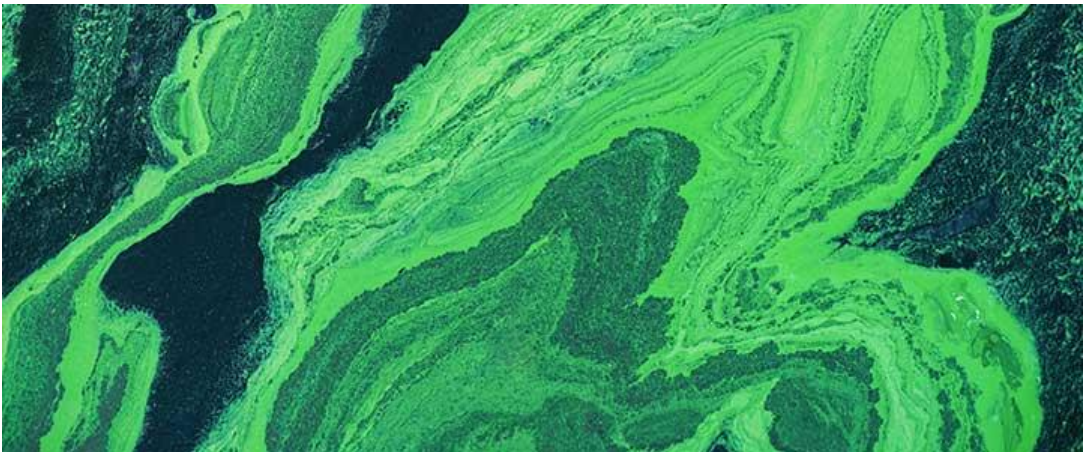
Figure 33. Satellite image of an algal bloom. (Source: LG Sonic, https://www.lgsonic.com/blogs/cyanobacteria/)

This 3.5-billion year old cyanobacteria naturally grows in habitats submitted to extreme conditions, notably small ponds and shallow soda lakes where the fresh-water is alkaline (pH between 8.5 and 11), warm (between 35 and 40 °C) and rich in minerals. These lakes are located in inter-tropical areas, between 35° North latitude and 35° South latitude. African soda-lakes shelter the highest amounts of natural spirulina, notably Ethiopia and Chad. The exclusion of other living beings from such constraining habitats is reinforced by the very presence of Spirulina for several reasons: (i) Spirulina increases the alkalinity of its environment by consuming carbonates and bicarbonates; (ii) its floating and pigmented filaments form a thick screen at the surface of the water which prevents light to pass and thus other algae such as Chlorella to grow; (iii) Spirulina produces a variety of active antibiotics and toxins against an array of bacteria (Cruchot, 2008).

The extreme conditions in which Spirulina grows is probably responsible for its impressive production of a wide array of unique bioactive compounds (secondary metabolites) in order to survive.

The Aztecs called Spirulina "Tecuitlatl" (stone's excrement) and used it as their principal food source in the 16th century in Mexico. The existence of Spirulina was mentioned for the first time in 1492 when Christopher Columbus described in his logbook these small green and dried cakes produced by the Aztecs. Spirulina was mentioned a second time only five centuries later in 1940 by the French physiologist Dangeard during an expedition in Africa. He discovered that the tribe of Kanembous in Chad harvested Spirulina from the banks of their lakes and turned it into sun-dried cakes called "Dihé" (Figure 34) (Lupatini et al., 2017).

Figure 34. People of the Kanembu tribe harvesting spirulina on the edge of lake Chad. (Source: Patrick Fort, Agence France Presse, http://www.lapresse.ca/international/afrique/200912/20/01-932771-la-spiruline-produit-miracle.php)

The first detailed study of the physiology of Spirulina and its growth requirements was only performed in the 1960s, and this remarkable cyanobacteria was finally recognized as a "wonderful future food source" in 1967 by the International Association of Applied Microbiology (Wan et al., 2016a; Lupatini et al., 2017). Today, Spirulina is consumed worldwide as a valuable food supplement due to its high nutritional properties. It is particularly used as a main source of proteins in Africa, notably to prevent malnutrition (Cruchot, 2008). *S. platensis* is indeed one of the richest microbial sources of proteins (460-630 g kg^{-1} dry matter basis), having similar protein levels compared to meat (710-760 g kg^{-1} DMB) and soybeans (around 400 g kg^{-1} DMB) (Lupatini et al., 2017).

Besides traditional harvesting in natural soda lakes, Spirulina is now widely cultured and produced in large outdoor or greenhouse ponds under controlled conditions (Figure 35).

Figure 35. Aerial image of a large-scale Spirulina farm in California. (Source: Earthrise, http://www.whatisspirulina.org/product-review-earthrise-spirulina-natural/)

Another great advantage of *S. platensis* is that it is nontoxic and can be consumed in full without being cooked due to the absence of cellulose in its cell walls (Cruchot, 2008). Numerous research dedicated to the potential toxicity of Spirulina have concluded that it is safe to eat, and it is since long authorized for human consumption by the Food and Drug Administration in Europe, Japan, and in the USA (Lupatini et al., 2017). Recommended daily doses as a dietary complement are comprised between 3 and 5 g (Cruchot, 2008).

The nutrient content of Spirulina depends on the strain of *S. platensis*, but also on its cultivation, yield, drying and conservation method (Wan et al., 2016a). Hence, Spirulina products on the market are not strictly identical in terms of nutritional composition. It is generally composed of proteins (55-70 % of dry weight), polysaccharides (15-25 %), total lipids (5-6 %), nucleic acids (6-13 %) and minerals (2.2-4.8 %) (Wan et al., 2016a). It is particularly rich in essential amino acids, crucial vitamins (*e.g.* β-carotene, vitamin E, vitamin B12) and minerals (*e.g.* Ca, Mg, P, K).

Polyunsaturated fatty acids (PUFAs) represent 1.5-2 % of the total lipid content, and Spirulina notably contains high amounts of γ-linolenic acid (30-35 % of total PUFAs) which is an essential fatty acid particularly rare in food ingredients (Wan et al., 2016a). Spirulina also contains phycobiliproteins (*e.g.* phycocyanin) responsible for its blue-green pigmentation (Lupatini et al., 2017).

The production and commercialization of Spirulina extracts is favored by its large-scale cultivation (Chu et al., 2010; Kepekçi et al., 2013). It has thus gained considerable attention since the last 20 years for the development of pharmaceuticals (it is considered as a nutraceuticals) and as a food supplement with immune-enhancing properties for both humans and animals (Priyadarshani & Rath, 2012).

In aquaculture, notably in Asia, Spirulina is added to granulated food in order, amongst other things, to stimulate the immune system of fish (Cruchot, 2008). Besides its nutritional advantages, Spirulina was indeed shown to stimulate the immune system of humans as-well-as animals (poultry, mammals, and fish) by inducing the production of antibodies and cytokines (Promya & Chitmanat, 2011).

Multiple studies have demonstrated its antitumor and anticancer effects, as-well-as its antibacterial and antiviral properties. The health benefits of Spirulina are largely attributable to its micronutrient content, notably group B vitamins, antioxidant molecules (β-carotene, vitamin E, zinc, and selenium), γ-linolenic acid, and other phenolic compounds. Not to forget the therapeutic activity of complex polysaccharides such as spirulan or phycocynanin (De Jesus Raposo et al., 2013; Wan et al., 2016a). Phycocyanin is a major water-soluble polysaccharide with an antioxidant activity 20 times more efficient than vitamin C (Chu et al., 2010). It also exhibits anti-inflammatory and anticancer properties (Lupatini et al., 2017).

Concerning plant protection, Spirulina culture filtrates have been reported to have *in vitro* fungicidal activities by suppressing the fungal growth of the pathogens *Fusarium oxysporum* and *Rhizoctonia solani* (Tantawy, 2011). Similarly, a phenolic extract of *S. platensis* showed an antifungal effect on the fungal pathogen *Aspergillus flavus* (de Souza et al., 2011). Conversely, culture filtrates of Arthrospira sp. exhibited low *in vitro* fungicidal activity (around 4 % growth inhibition) against *Alternaria porri* (onion purple blotch disease) (Abdel-Hafez et al., 2015). In addition, phycocyanin extracted from *S. platensis* enhanced the accumulation of the secondary metabolites capsaicin and anthocyanin in pepper and carrot cell cultures (Rao et al., 1996). Finally, numerous research strongly supports the potential elicitor properties of cyanobacteria, although these studies mainly refer to cyanobacteria living in the plant environment and their corresponding extracts (Kulik, 1995; Singh, 2014).

In the view of these results, and since *S. platensis* is known to be an excellent source of bioactive compounds, including phenolic compounds such as salicylic acid, it is likely to show interesting elicitor activities in plants. Yet, in the frame of plant protection research,

Spirulina has never been tested as a plant resistance inducer. It could very well be a transkingom elicitor much like CpG-ODN or ergosterol, making it interesting to screen for wheat protection.

4. Glycine betaine

Osmoprotectants are naturally occurring compounds in bacteria, plants, animals and even algae, as a means to resist abiotic stresses such as drought, salinity, UV radiation and heavy metals (McNeil et al., 1999; Wood et al., 2002; Ashraf & Foolad, 2007). Under abiotic stress, they raise the osmotic pressure in the cytoplasm and stabilize folded protein structures and membranes (Wood et al., 2002). Three types of chemical osmoprotectants have been identified so far: betaines, polyols and sugars (*e.g.* mannitol and trehalose) (McNeil et al., 1999).

Betaines are amino acid derivatives with fully methylated nitrogen atoms, which makes them quaternary ammonium compounds (inner salts): they present a permanently positively-charged quaternary ammonium group and a negatively charged carboxyl group (Wood et al., 2002). In plants, betaines are mainly synthetized in the chloroplast stroma through a two-step oxidation of choline (Mäkelä et al., 1996; Huang et al., 2008). The three best-known and naturally occurring betaines in higher plants (Figure 36) are Glycine betaine (GB), Proline betaine (stachydrine) and β-Alanine betaine, but there are also many others (*e.g.* hydroxyproline betaine, pipecolate betaine, hydroxypipecolate betaine, trigonelline) (Wood et al., 2002).
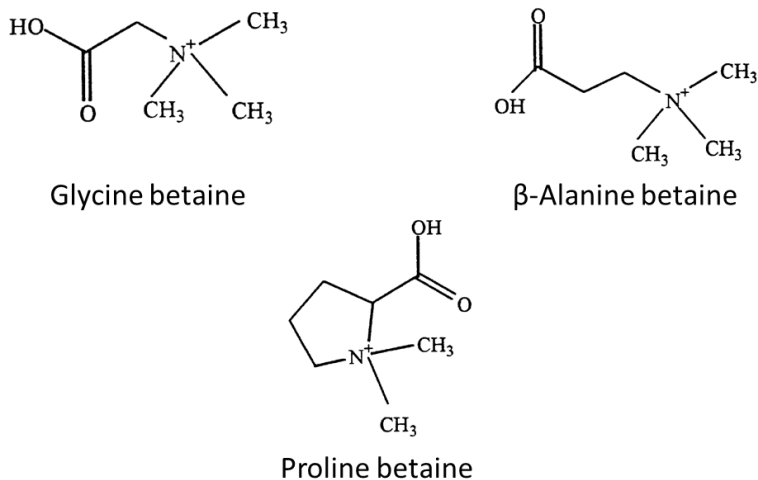
Figure 36. Chemical structure of glycine betaine, β-alanine betaine and proline betaine. (Source: Wood et al., 2002).

The first study demonstrating the protective properties of betaines falls back in 1984 when bacteria were reported to grow faster under high salinity conditions when betaines were supplied to the culture media (Le Rudulier et al., 1984).

In particular, Glycine betaine (N,N,N-trimethylglycine) is a widespread osmoprotectant in flowering plants (beet, wheat, spinach), animals, microorganisms (*Pseudomonas dénitrifiants*, *Propionibacterium shermanii*) and algae (McNeil et al., 1999; Nsimba et al., 2010). This soluble osmolyte is abundant in plant chloroplasts where it plays a crucial role in adjusting and protecting the thylakoid membrane, thereby maintaining the photosynthetic efficiency of the plant (Ashraf & Foolad, 2007). GB is mainly synthesized in chloroplasts from serine *via* choline, ethanolamine and betaine aldehyde dehydrogenase (BADH) (Ashraf & Foolad, 2007). In contrast, certain crop plants such as Arabidopsis, tomato, rice, soybeans and potatoes lack crucial amounts of osmoprotectants (McNeil et al., 1999; Park et al., 2006). Consequently, a great deal of research has been dedicated to the genetic engineering of glycine betaine biosynthesis *via* transgenes in plants which are usually betaine deficient. Exogenous GB applications have also been realized (McNeil et al., 1999; Chen & Murata, 2008).

Overall, studies have demonstrated that GB is a major protectant of plants against abiotic stresses (McNeil et al., 1999; Ashraf & Foolad, 2007; Chen & Murata, 2008; Wang et al., 2010). *In vitro* bioassays demonstrated that cell cultures grew better under osmotic stress when GB was supplied to the culture media (Petronini et al., 1992). In the field, foliar treatment of maize, sorghum or wheat crops with GB significantly reduced yield losses (Agboma et al., 1997).

More recently, GB was reported to induce wheat tolerance to a combination of drought and heat stresses by improving the photosynthesis activity of the plant. It enhanced the cell water status and indirectly eliminated ROS by inducing the activation of antioxidant defense systems, including antioxidative enzymes (Wang et al., 2010).

In tomato plants, GB was also shown to be readily taken up by leaf tissues mainly in the cytoplasm, although tomato does not normally accumulate betaines (Park et al., 2006). Moreover, it induced H_2O_2-mediated antioxidant mechanisms by enhancing the activity of the catalase enzyme and the expression of the catalase gene (*CAT1*) (Park et al., 2006). Another study on the application of radio-labeled GB on the leaves of summer turnips reported that it was translocated to roots within two hours before being spread throughout the plant to various organs (Chen & Murata, 2008). Similar protective properties were also reported in maize and Arabidopsis plants, and it is suggested that tolerance to abiotic stress is enhanced by the high levels of GB translocated from the leaves to the reproductive organs of the plant (Chen & Murata, 2008).

Finally, it appears that the effective dose of exogenous GB depends on the plant species (Ashraf & Foolad, 2007). For instance, foliar applications of 0.1, 0.2 and 0.3M of GB increased the shoot growth of apple micro-cuttings up to 70 % (Uosukainen et al., 2000). On the other hand, the supply of a nutrient solution containing 0.1 mM of GB improved the growth of rice plants grown under salt stress (Lutts, 2000).

In cotton fields, application of 1-5 kg ha^{-1} of GB as seed treatments or by foliar spraying induced plant tolerance to drought stress and improved the yield (Ashraf &

Foolad, 2007). In wheat, foliar application of GB at 10 mM counteracted the adverse effects of drought stress (Heshmat et al., 2012).

Moreover, GB is also used for human health: it is notably used for the treatment of homocystinuria by promoting the conversion of homocysteine back to methionine (Nsimba et al., 2010). Homocystinuria corresponds to a cystathionine beta synthase deficiency or CBS deficiency which is an inherited disorder of the metabolism of the amino acid methionine. GB also showed protectant properties against liver lesions, cardiovascular deficiencies, ROS accumulation and lipid peroxidation (Nsimba et al., 2010).

In overall terms, it is clear that much of the existing research has been conducted on GB regarding its protectant properties predominantly against plant abiotic stress. Yet, what about its interests against biotic stress? This matter seems to have rarely been addressed. Still, it was reported that exogenous treatment of winter wheat with GB at 1 mM significantly protected the plant against powdery mildew (Věchet et al., 2005). Similarly, a 3-year field experiment showed that wheat treated with 0.3 M of GB was significantly protected against powdery mildew (Vechet et al., 2009). Another study demonstrated that GB induced both chitinase and β-1,3-glucanase activity in the roots and leaves of sugar beet (Burketová et al., 2003). Moreover, strawberry treatment with Bion or GB induced the production of phenolic compounds, highlighting its potent role as a plant protectant against pathogens (Karjalainen et al., 2002).

5. Ergosterol

Ergosterol (Figure 37) is the principal component of fungal plasma membranes and plays an essential role in membrane stabilization.
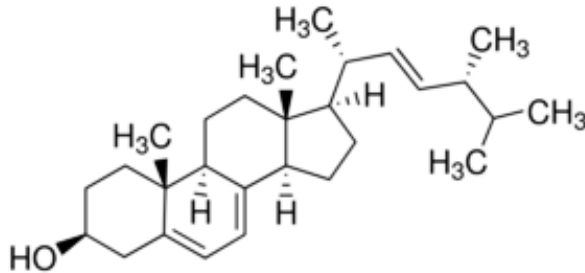
Figure 37. Chemical structure of ergosterol. (Source: Sigma Aldrich)

This sterol has never been found in plants. Besides, it is considered as a general PAMP which is perceived as a non-self molecule by plants and triggers a series of defense responses (Nürnberger et al., 2004). Multiple studies have demonstrated the elicitor properties of ergosterol, even at very low concentrations, on plants such tomato, tobacco, mimosa, and sugar beet (Amborabé *et al.*, 2003; Lochman & Mikes, 2006; Rossard *et al.*, 2010).

For instance, the addition of 10 μM of ergosterol in the culture medium of plant cells induced rapid variations of ion fluxes (including proton H^+ flux) and of transmembrane potential (Amborabé et al., 2003). Such changes in the plasma membrane properties of plant cells are characteristic of an early plant response to an elicitor. In addition, these modifications were dose-dependent (reaching a concentration threshold at 1 μM) (Amborabé et al., 2003).

In tomato and tobacco cells, ergosterol concentrations at the nanomolar range induced early defense responses characterized by the production of ROS, medium alkalinization, ion fluxes across the plasma membrane and phytoalexin production (Granado et al., 1995; Kasparovsky et al., 2003).

Similarly, ergosterol was reported to induce early defense responses in Mimosa plant cells (Rossard et al., 2006). In alfalfa , ergosterol activated specific MAPKs which are known to be involved in elicitor defense signaling (Cardinale et al., 2000). Moreover, in tobacco plants, ergosterol induced the expression of multiple defense-related genes as well as a crosstalk between JA and SA defense signaling pathways (Lochman & Mikes, 2006). Finally, ergosterol was reported to trigger an oxidative burst and JA-dependent defense responses in the leaves of sugar beet (Rossard et al., 2010). Although the signal hormone JA was involved in ergosterol-induced elicitation, it is noteworthy that there was no clear discrimination between the SA and/or JA signaling pathways in this study (Rossard et al., 2010).

It is suggested that plants possess specific ergosterol receptors since the overall defense responses observed up to now were specifically induced by this compound, compared to other tested sterols such as stigmasterol, campesterol or cholesterol (Amborabé et al., 2003; Rossard et al., 2010).

However, another hypothesis also assumes that this sterol induces a perturbation of the plant plasma membrane by forming stable micro domains (lipid rafts) (Xu et al., 2001). To our knowledge, ergosterol has been tested as a resistance inducer of model plants and dicotyledonous plants. There is still no research on the potential elicitor properties of ergosterol in monocots, especially wheat.

4

RESULTS

"No amount of experimentation can ever prove me right;

a single experiment can prove me wrong"

Albert Einstein

1. Screening for elicitors of wheat defenses against *Zymoseptoria tritici*

Results presented in this chapter were published in the following article: Le Mire et al., Under peer-review. **Evaluation of λ-carrageenan, CpG-ODN, glycine beatine, *Spirulina platensis* and ergosterol as elicitors for control of *Zymoseptoria tritici* in wheat.** *Phytopathology* (Accepted with major modifications).

1. Introduction

In 2015-2016, wheat was the most cultivated crop in the world, with a production of up to 734 million tons (Satger, 2016). However, the challenging *Septoria tritici* Blotch (STB) disease keeps threatening both its optimal growth and yield (Torriani et al., 2015). The exceptional weather conditions of 2016 strongly favored high STB disease pressures on wheat crops in France, thereby causing losses of about 25 quintals ha^{-1}, amounting to 36 % of the total yield (Maufras & Maumené, 2016). Warm temperatures during winter combined to important and constant rainfall throughout spring are extremely favorable for the development of multiple diseases in the field (Colart et al., 2016). Plant breeding and fungicides remain the two most effective control methods for crop protection to prevent massive yield losses. However, as previously stated, no wheat cultivar is yet totally resistant to *Z. tritici* while about 70% of EU fungicides are used to protect the crop plant against this pathogen (Palmer & Skinner, 2002; Fraaije et al., 2005; Fones & Gurr, 2015). Besides, most of the fungicides used today are becoming less efficient (Fraaije et al., 2012). Finally, it is well known that the reduction of pesticides used in agricultural practices has become a top priority for multiple countries, including the EU (Dayan et al., 2009; European Commission, 2012). The development and implementation of new tools for Integrated Pest Management (IPM) is thus critical (Le Mire et al., 2016).

In this context, elicitors are considered as promising biocontrol tools in the preventive treatment of plants against various diseases (Lyon et al., 2014). Contrary to fungicides, elicitors indirectly target a pathogen by enhancing plant defenses. The elicitor products currently in the marketplace are implemented in IPM strategies as preventive treatments and are mainly applied as **complementary tools to fungicides** (Walters et al., 2013; Walters et al., 2014a). They contribute to reduce the dosage amounts and application frequencies of chemical inputs.

However, a majority of elicitor screening has been carried out on dicotyledonous plants (*i.e.*, thale-cress, tobacco, tomato, cucumber), and **few elicitors have yet been successfully tested and formulated to protect monocotyledonous plants such as wheat** (Kogel & Langen, 2005; Balmer et al., 2013). The narrow list of elicitor biocontrol products registered for wheat protection on the EU market include: Vacciplant® (Goëmar, France) based on the β-1,3-glucan laminarin extracted from the brown alga *Laminaria digitata* ; BION®50WG (Syngenta, Switzerland) which is a synthetic elicitor with functional analogy to the plant major defense hormone salicylic acid (Leadbeater & Staub, 2014).

Additional biocontrol tools are thus necessary in order to provide growers with a larger panel of crop resistance inducers (Walters et al., 2013).

In the first part of this study, we thus focused on the screening of elicitors of wheat. We selected and tested five different compounds of various origins for their ability to induce resistance of bread wheat against STB under glasshouse conditions: λ-carrageenan, cytosine-phosphate-guanine oligodesoxynucleotide motifs (CpG ODN), *Spirulina (Arthrospira) platensis*, glycine betaine and ergosterol. Each compound was tested at three different concentrations in order to assess both its potential protection efficacy and any dose-dependent effects. All of these elicitor candidates were already proven to have elicitor properties on other plant species and/or animals but have never been tested before as biocontrol tools on the pathosystem wheat-*Z. tritici* (Věchet et al., 2005; Carrington & Secombes, 2006; Lochman & Mikes, 2006; Ongena et al., 2007; Vera et al., 2011; Wu et al., 2016). Further details on their respective origins and elicitor properties, are provided in the "Strategic choices" chapter.

In addition, *in vitro* experiments were conducted to rule out any compound showing a direct biocidal activity towards the fungal pathogen *Z. tritici*. Such bioassays enable to confirm that none of the tested compounds has a fungicidal effect towards the pathogen at the concentrations chosen for glasshouse screening trials, thus increasing chances of identifying an actual elicitor. Indeed, if a given compound had a biocidal activity, then its protection efficacy could be due partly or totally to such biofungicidal properties. There have been cases where compounds such as chitosan were shown to play a dual role as elicitor of plant defenses and as biofungicides against certain pathogens all at once (El Hadrami et al., 2010). Not only do the registration procedures of such compounds as biocontrol tools remain problematic, but the risk of emerging pathogenic resistances can also not be excluded.

2. Materials and methods

2.1 Plant and fungal materials and inoculum production

The experiments were conducted on wheat (*Triticum aestivum* L.) plants of the susceptible cv. Avatar. Plants were grown in the greenhouse under semi-controlled conditions (natural photoperiod supplemented with artificial light if needed, with 20°C±5 according to the sunlight). Seeds were sown in 25 x 15 cm plastic pots filled with loam (10 plants per pot).

The *Z. tritici* strain T01187 (isolated in 2009 from Northern France) was used for plant inoculation and *in vitro* biocide assays. Fungal culture was performed on potato dextrose agar (PDA) medium for eight days at 18 °C with a 12 ∕ 12-h day ∕ night cycle. Inocula were prepared by washing the cultures with 10 mL sterile distilled water and the resulting spore suspension was adjusted to desired concentrations using Malassez cell.

2.2 Elicitor preparation

The sources and characteristics of the five compounds examined in this study are provided in Table 3.

Table 3. Characteristics of the compounds used in the study

Code	Active ingredient	Supplier	Characteristics	General use	Concentrations tested (g l^{-1})		
					C1	C2	C3
A	λ-carrageenan	SIGMA	Linear polysaccharide extracted from red algae	Gelling and emulsifying agent in the food industry	0.1	1	5
B	CpG-ODN	Pr. A. Carpentier, Paris, France	Short single-stranded synthetic DNA molecules (CpG-28)	Human vaccine adjuvant	9.5 x 10^{-5}	9.5 x 10^{-4}	9.5 x 10^{-3}
C	*Spirulina platensis*	Djerbalgue, Tunisia	Dried cyanobacterium	Human dietary supplement	0.3	3	30
D	Glycine Betaine	Ithec, France	Organic osmolyte extracted from beetroot	Protectant of plants against abiotic stress	0.12	1.2	12
E	Ergosterol	SIGMA	Fungal sterol	Stabilization of fungal plasma membranes	0.002	0.08	0.03
BION® 50WG	Acibenzolar-*S*-methyl (50 % w : w)	Syngenta, Europe	Synthetic elicitor	Plant resistance inducer	0.6		
Fungicide Opus®	Epoxiconazole	BASF Agro, France	Triazole fungicide	Broad-spectrum systemic fungicide	0.75 % (v : v)		

The tested concentrations were not the same between each compound. We indeed selected the average effective doses which were generally used in previous studies to demonstrate their respective elicitor potential.

To be noted that the compound CpG-ODN consists of CpG-28 (sequence 5'-TAAACGTTATAACGTTATGACGTCAT- 3') synthesized with a wholly phosphorothioate backbone (Carpentier et al. 2003).

On the other hand, spirulina is a microalgae rich in micronutrients and macronutrients, including polyphenols, flavonoids, phenolic acid, phycocynanin and carotenoids. Therefore, the evaluation of the elicitor properties of spirulina as a whole in this study will not enable to determine for sure at this stage if the eliciting activity is attributable to one or several active substances composing spirulina.

Ergosterol treatments were obtained by dilution of a methanolic stock solution of 2 mg ml^{-1}. The other compounds were all readily soluble in water. Still, λ-carrageenan treatments were heated up to 80 °C for 15 min in order to accelerate the homogenization of the solution. All treatment solutions were freshly prepared before use in distilled water supplemented with 0.1 % (*v/v*) of spreading agent Break-Thru®S240 (polyether trisiloxane, Evonik Industries), and 0.05 % (*v/v*) of solubilizing agent Tween20 (polyoxyethylene-sorbitan monolaurate, Sigma Aldrich). The formulation of elicitor treatments with a wetting agent and a solubilizeing agent was performed in order to maximize the amount of treatment solution in contact with wheat leaves.

Control plants were treated with distilled water alone. In addition, other control plants were treated respectively with 0.75 % (*v/v*) of the epoxiconazole-based fungicide Opus® (BASF Agro, France) or with the synthetic elicitor BION®50WG (Syngenta, Europe) at 0.6 mg.mL^{-1} (recommended doses according to https://ephy.anses.fr)..

2.3 Plant treatment and inoculation

At the three-four leaf stage (third leaf fully expanded – Z13), the plants of each pot were sprayed to run-off with 30 ml of the treatment solutions using a hand spraye (Figure 38).
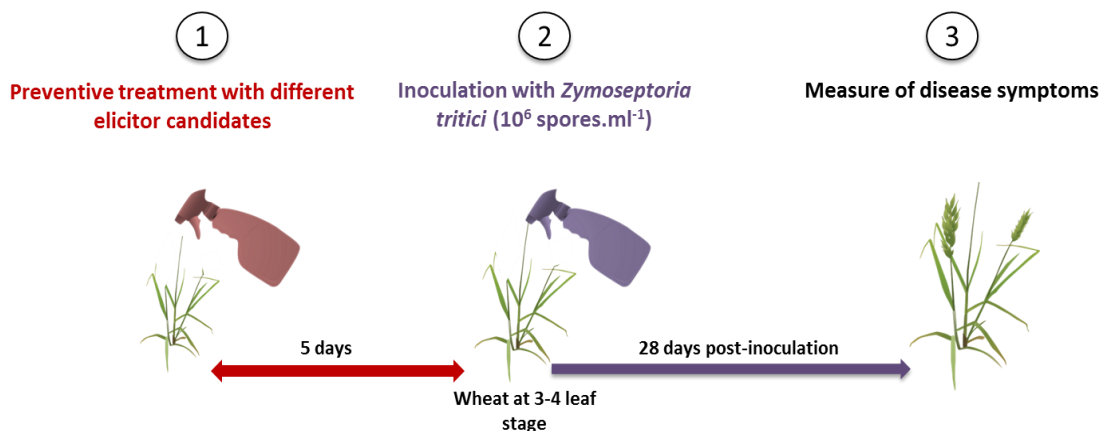
Figure 38. Methodology of greenhouse elicitor screening

Spraying of 30 mL to run-off was realized to ensure that a proper amount of the treatment solution comes in contact with wheat leaves. To be noted that applications carried out later in the field will require higher spraying volumes to maximize leaf coverage.

Each elicitor candidate was tested at three different concentrations (Table 3). Plant inoculation was performed five days after treatment, by spraying the plants of each pot to the limit of run-off with 30 ml of a spore suspension (10^6 spores ml^{-1} in distilled water) amended with 0.05 % (v : v) Tween 20. The positioning of an elicitor treatment is delicate as the triggering kinetic of plant defense mechanisms is complex and depends on the elicitor and on the pathosystem. Current knowledge on elicitors indicates that they are able to induce plant resistance very quickly after application (see chapter 3 – Bibliographical introduction).

In order to reveal at best the potential of the tested compounds, we thus selected a 5 day delay between plant treatment and inoculation, based on the recommendations provided in the "Methodological guide for the evaluation of plant elicitors" (https://www.elicitra.org/vars/fichiers/Livrables/guide_metho_eval_SDP_elicitra_20 13.pdf).

Immediately after inoculation, each pot was covered with a transparent polyethylene bag for three days in order to ensure water-saturated conditions compatible with spore germination. The disease level was scored at 28 days post-inoculation by measuring the percentage of the third leaf area covered with symptomatic lesions (necrosis and chlorosis) bearing pycnidia.

Inoculating wheat with the pathogen *Z. tritici* for the purpose of elicitor screening offers the advantage of evaluating the protection efficacy of potential elicitors compounds against a specific targeted disease (in this case, *Septoria tritici* Blotch). Owning the mode of action of elicitors by triggering a non specific resistance of the plant to a broad spectrum of diseases, it cannot be excluded that the tested compounds may be efficient to indirectly control other plant pathogens. However, in the present case, we focused on STB as it is the principal foliar disease affecting wheat in Europe.

An incomplete block design was carried out due to the large number of elicitor candidates to be tested (Lawal, 2014). Indeed, the lack of space in the greenhouse chambers made it impossible to test the compounds all together during one experiment. Still, each compound was tested in the glasshouse through at least two independent biological experiments, with 40 technical repetitions (plants) during each experiment. Results of combined experiments were analyzed with linear mixed effect models (Gałecki & Burzykowski, 2013). ANOVA and the Tukey multiple comparison procedure at $P = 0.05$ were used to compare the mean disease severity of the plants treated with the different products. Reported values correspond to the average infection levels of the treated plants.

2.4 Biocide bioassays

The potential direct effect of the six elicitor candidates on *Z. tritici* was assessed through *in vitro* bioassays (Figure 39).
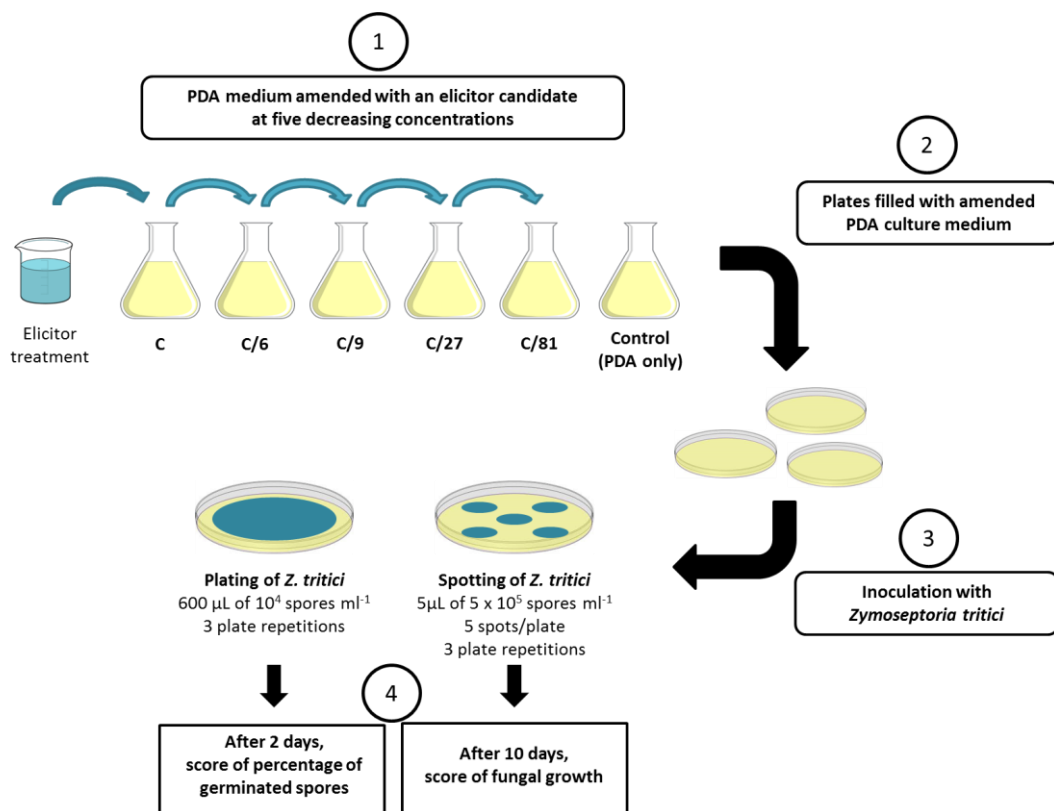
Figure 39. Methodology of *in vitro* bioassays of elicitor biocidal activity towards *Z. tritici*

We thus evaluated the effect of each compound on fungal growth and spore germination. According to the method of Siah et al (2010b), PDA plates were amended with one of the elicitor candidates at different concentrations.

Each compound was first added at the highest concentration 'C' to PDA medium at 30 °C after autoclaving. It corresponds as well to the highest concentration C3 tested in greenhouse trials (Table 3). Successive dilutions were then carried out in order to test five decreasing concentrations (C/6, C/9, C/27, and C/81). The control consisted of plates containing PDA only.

For fungal growth assessment, the plates were subsequently spotted with 5 μL of 5 x 10^5 spores ml^{-1} suspension. Fungal growth was scored by measuring the colony perpendicular diameters of each spot at 10 days after incubation in the dark at 18 °C. Three plates with five spots per plate were used as replicates for each condition, and two independent experiments were carried out for each elicitor candidate.

Regarding spore germination assays, the amended plates were subsequently sprayed with 0.6 mL of 10^4 spores ml^{-1} suspension. The percentage of germinated spores was calculated from 100 spores in each plate using a light microscope (Nikon, Eclipse 80i) after two days of incubation in the dark at 18 °C.

Three plates were used as replicates for each condition, and two independent experiments were also carried out for each elicitor candidate.

Values correspond to the average perpendicular diameter and average spore germination of *Z. tritici* colonies scored on amended PDA media. The comparison of both mean fungal growth and mean spore germination was performed with the Tukey (ANOVA) test at $P = 0.05$.

3. Results

3.1 Protection efficacy against *Z. tritici* in greenhouse conditions

The five candidate elicitors were tested in the glasshouse for their protective efficacy as a preventive treatment of wheat against *Z. tritici*. The mean disease severity assessed during these experiments corresponds to the percentage of symptomatic lesions scored on the third leaf surface of wheat plants.

As a result, a mean disease severity of 23 % was scored on the water control (Figure 40).
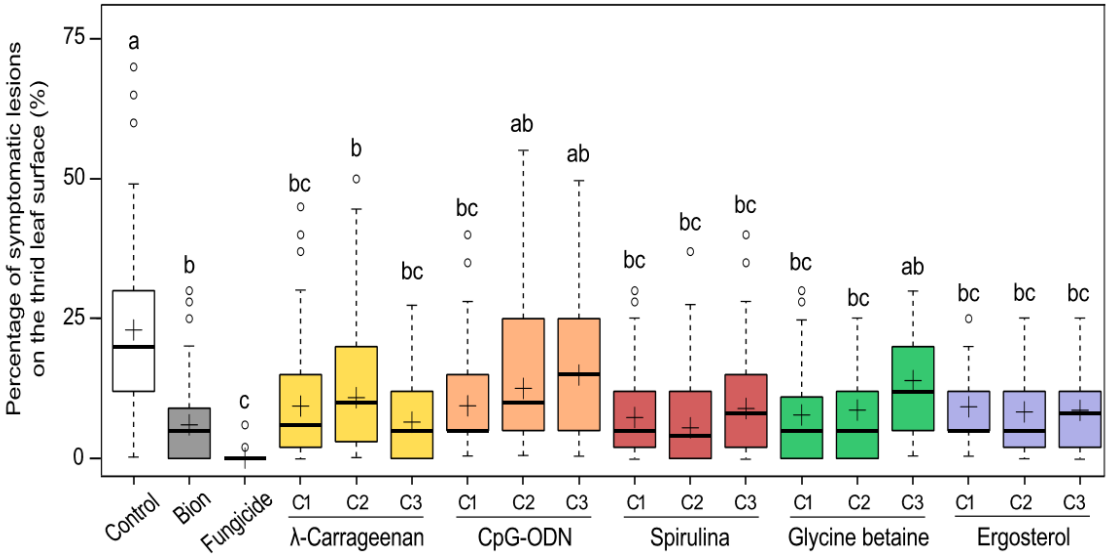
Figure 40. Disease severity of *Septoria tritici* Blotch (STB) on wheat treated with five candidate elicitors tested at three different concentrations under greenhouse conditions. Preventive treatment of wheat with these various compounds significantly reduced *Z. tritici* disease severity compared to the Control. The compounds CpG-ODN and glycine betaine showed no difference with the control at high concentrations. Data are percentages of the third leaf surface of plants exhibiting symptomatic STB lesions (necrosis and/or chlorosis). Medians are represented by black horizontal lines in each box, and means are represented by "+" symbol (n ≥ 80, *e.g.*, incomplete block design with five pots of eight plants and two independent experiments at least per treatment). Boxes tagged with the same letters correspond to means that are not significantly different using the Tukey test at $P = 0.05$. For details on the concentrations of the tested compounds see Table 2.

To be noted that the average disease pressure of *Z. tritici* on control plants (23%) from one greenhouse experiment to another was medium to low. However, it allowed the identification of significant differences in treated plants. Indeed, plants treated with the fungicide epoxiconazole or the elicitor reference Bion showed an average disease severity of 0.1 % and 6 %, respectively. Finally, mean disease severity of wheat plants treated with elicitor candidates ranged from 5 % to 15 %. Overall, plants sprayed with the various treatments showed significantly reduced disease symptoms of *Z. tritici* compared to the control ($P = 0.05$).

However, the disease severity scored on plants treated with CpG-ODN and glycine betaine was not statistically different to that of control plants at the following concentrations: medium concentration C2 for CpG-ODN (9.5×10^{-4} g l^{-1}) and highest concentration C3 for CpG-ODN and glycine betaine (0.0095 g l^{-1} and 12 g l^{-1} respectively). The protection efficacy of a treatment corresponds to the difference of mean disease severity between the control and the treated plants (Figure 41).
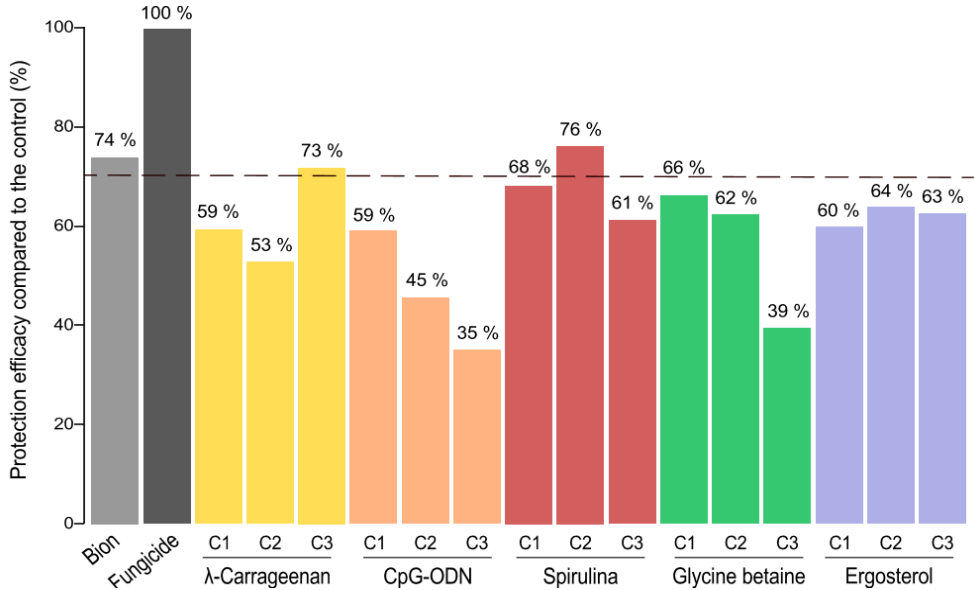
Figure 41. Efficacy of five candidate elicitors tested at three different concentrations to protect wheat against the disease *Septoria tritici* Blotch (STB) under greenhouse conditions compared to the control. Values correspond to the difference between control and treated plants in terms of percentage of mean disease severity scored on the third leaf surface. According to the threshold established at 70 % (black dotted line), λ-carrageenan and *Spirulina platensis* (Spirulina) showed the greatest protection efficacies at concentrations C3 and C2 respectively.

Consequently, the fungicide treatment showed the greatest protection efficacy (100 %), followed by Bion (74 %). In addition, all five elicitor candidates were as effective as the commercial elicitor Bion in protecting wheat against STB disease: Excluding CpG-ODN and glycine betaine at the concentrations which provided similar disease severity to the control, the protection efficacies of the elicitor candidates ranged between 53 % and 76 %. A threshold established at 70 % reveals

that the two elicitor candidates showing the greatest protection efficacies are: λ-carrageenan at concentration C3 (5 g l^{-1}); *Spirulina platensis* at concentration C2 (3 g l^{-1}).

3.2 Direct biocide activity

The eventual biocide effect of the six elicitor candidates was assessed *in vitro* in order to determine if their *in planta* protection efficacy could be linked to a direct antifungal activity (Figure 42).
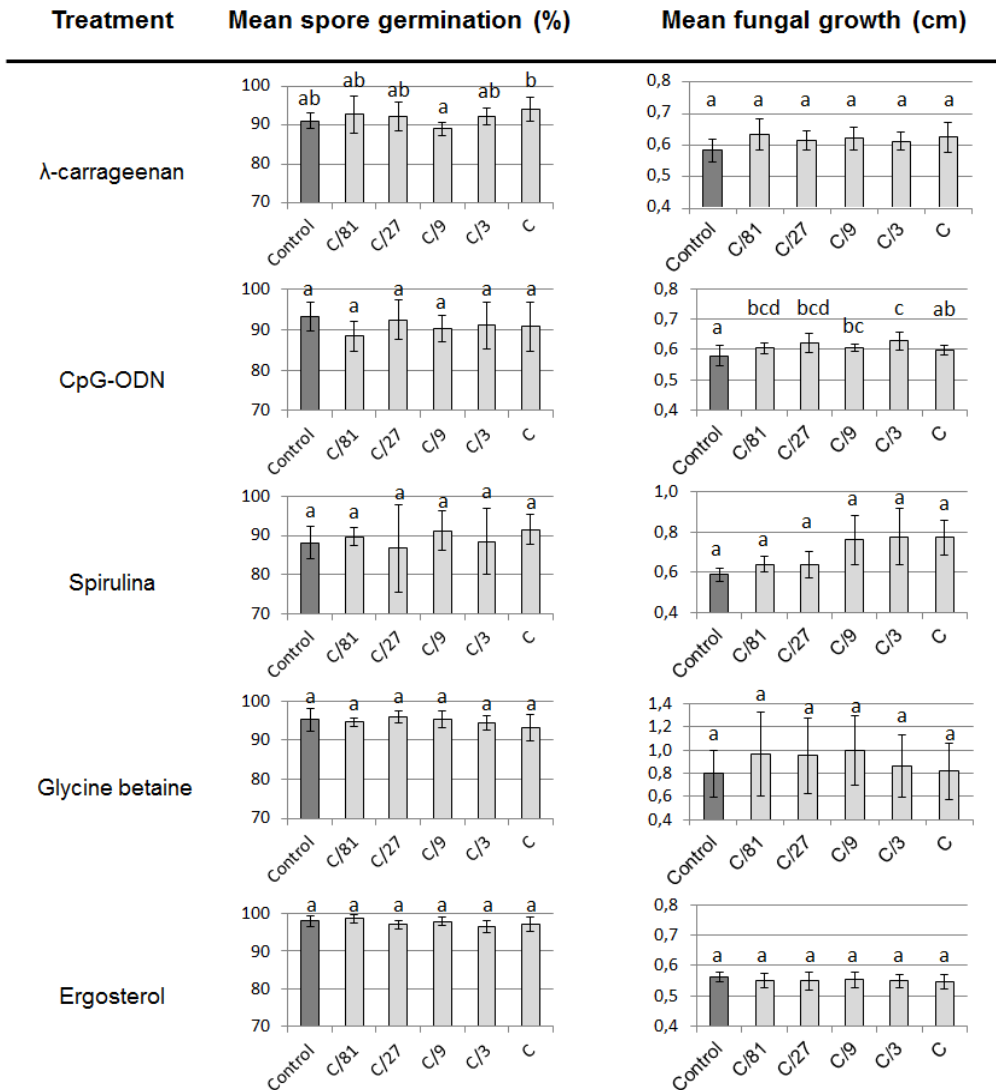
Figure 42. *In vitro* biocidal effect of five candidate elicitors towards *Zymoseptoria tritici*. Values correspond, respectively, to the average percentage of germinated spores and average fungal diameter (cm) of *Z. tritici* scored on amended PDA media. Means tagged with the same letters are not significantly different using the Tukey test at $P = 0.05$.

The means of both spore germination and fungal growth on PDA medium amended with each compound was generally similar to that of the control (PDA alone) (P=0.05). However, the colony diameters were significantly different for culture media amended with CpG-ODN at concentrations lower than 'C' (0.0095 g l^{-1}). The mean fungal growth scored in the corresponding plates was indeed higher (ranging between 0.61 and 0.63 cm) compared to the control (0.58 cm). Still, such results for CpG-ODN do not reveal a biocidal effect of this compound towards the pathogen. Overall, the candidate elicitors had no *in vitro* biocide activity towards *Z. tritici*.

4. Discussion

Greenhouse screening has shown that each of the five compounds, namely λ-carrageenan, CpG-ODN, spirulina, glycine betaine and ergosterol, significantly protected wheat against *Z. tritici* under semi-controlled conditions. The STB disease severity was broadly reduced by up to 70%, thus providing a consistent protection of the wheat plants. Besides, these compounds were as efficient as the commercialized elicitor product Bion. However, we highlighted that high concentrations of applied CpG-ODN and glycine betaine did not effectively protect wheat against the pathogen. Such finding could be related to the fact that elicitor compounds are generally effective within a given concentration range (Trotel-Aziz et al., 2006; Thakur & Sohal, 2013). Moreover, we demonstrated through *in vitro* biocidal assays that none of the compounds behaved as biofungicides at the concentrations used for glasshouse screening. It is therefore likely that these five compounds indeed acted solely as elicitors of wheat defenses.

These findings are in line with previous research demonstrating their elicitor properties. For instance, λ-carrageenan was shown in a number of studies to stimulate the resistance of tobacco, tomato, and thale-cress plants against pathogens such as *Botrytis cinerea* and Tobacco mosaic virus (TMV) (Mercier et al., 2001; Sangha et al., 2010; Vera et al., 2011; Sangha et al., 2015; Shukla et al., 2016). Extracted from the cell wall of red seaweed, λ-carrageenan is a linear polysaccharide which contains the highest degree of sulfation (41 % of total weight) among the three main types of marine carrageenans (Vera et al., 2011). It has been established that the elicitor properties of these seaweed extracts are related to their degree of sulfation, and λ-carrageenan was indeed proven to be the most efficient in stimulating plant defense responses (Mercier et al., 2001; Sangha et al., 2010). Its efficacy to protect wheat against *Z. tritici* could therefore be linked to its high sulfate content, although the involvement of the λ-carrageenan sulphate groups in the recognition process of the compound by the plant still needs to be investigated.

Concerning CpG-ODN, no research has yet reported its ability to protect a crop plant against a fungal pathogen. However, previous studies have demonstrated that CpG-ODN acts as a pathogen-associated molecular pattern (PAMP) in mammalian cells and is recognized by pattern recognition receptor (PRR) Toll-Like Receptor 9 (TLR9) (Carrington & Secombes, 2006). A similar recognition process of CpG-ODN by specific wheat receptors can be assumed. Recently, bacterial CpG-ODN was also reported to induce defense responses in thale-cress (Yakushiji et al., 2009).

However, no studies had yet reported the ability of CpG-ODN to protect a crop plant against a fungal pathogen.

In the case of spirulina, it is a cyanobacterium (also called "blue-green" algae) which has gained considerable attention since the last twenty years in the food industry and for the development of pharmaceuticals (Priyadarshani & Rath, 2012). *Spirulina platensis* was proven to have positive health effects on humans and animals (poultry, mammals, and fish), notably as an immunostimulant promoting the production of antibody and cytokines (Farag et al., 2016; Wan et al., 2016b). Its elicitor properties are probably due to its high content in secondary metabolites such as carotenoids, superoxide dismutase, glycolipids and sulfolipids (Priyadarshani & Rath, 2012). Moreover, its antioxidant properties are attributed to the presence of two phycobiliproteins acting as superoxide radicals: phycocyanin and allophycocynanin (Farag et al., 2016). Most interestingly, and to our knowledge, the transkingdom potential of spirulina in inducing the defense mechanisms of both animals and plants had never been established. In 1995, Kulik had already assumed that cyanobacteria could represent valuable biocontrol agents in agriculture, but since then no studies had yet demonstrated the potential of spirulina as a plant resistance inducer (Kulik, 1995). As it turns out, other elicitors (*e.g.*, lipopolysaccharides, peptidoglycans, flagellin, chitin and glucans), generally PAMPs, are already known to trigger innate immune responses both in plants and in vertebrate organisms (Nürnberger & Brunner, 2002).

Concerning glycine betaine (GB), it is a major organic osmolyte which accumulates in a variety of plant species (sugar beet, barley, wheat, spinach, and sorghum) in response to an abiotic stress such as dehydration (Ashraf & Foolad, 2007). Previous research reported that GB stimulated the defenses of wheat against powdery mildew (Věchet & Šerá, 2015). The fact that this plant osmolyte also plays a role in enhancing plant defenses against diseases opens up a whole new range of biocontrol possibilities.

Finally, ergosterol is the principal component of fungal plasma membranes and is a well-known PAMP (Granado et al., 1995). Its elicitor properties, even at very low concentrations, have mostly been established up to now on dicotyledonous plants such as tomato, tobacco and sugar beet (Amborabé et al., 2003; Lochman & Mikes, 2006; Rossard et al., 2010). Our results confirm that this compound presents a clear benefit for sustainable plant protection extended to crop plants.

5. Conclusion

Overall, the efficacy of these five compounds to protect wheat under semi-controlled conditions opens the way to further studies concerning their potential use as biocontrol tools of wheat crops and in order to promote sustainable agricultural practices. The present screening results are all the more interesting as the tested compounds are already available on the market. Their common use has already required various toxicological tests, making it useful in the event of further investigations (Bode et al., 2011; Marles et al., 2011; European Food Safety Authority, 2013; Weiner, 2014).

For instance, λ-carrageenan is widely used in the food industry as an additive due to its jellifying and emulsifying properties (Weiner, 2014). In addition, spirulina is used as a concentrated and nutritious food supplement (Small, 2011). CpG-ODN is generally used, at low doses, for medical purposes as an adjuvant of vaccines targeting infectious diseases and cancer (Bode et al., 2011). Glycine betaine is an additional product of the sugar beet processing industry, and its osmoprotectant properties make it a commercially important compound with multiple applications in agriculture (to increase plant tolerance to abiotic stresses), medicine and animal husbandry (Mäkelä, 2004; Eklund et al., 2005). Finally, ergosterol is generally extracted from yeast and is of industrial and commercial importance as a precursor of therapeutically useful substances such as vitamin D_2 (Ethiraj, 2013). The next logical step following greenhouse elicitor screening now relies on the understanding of their mode(s) of action in the plant. Biomolecular tests focusing on the expression of wheat defense genes will enable to confirm that these five compounds indeed behave as elicitors of wheat defenses.

2. Which defense signaling pathways are triggered in the plant?

Results presented in this chapter were published in the following article: Le Mire et al., Under peer-review. **Evaluation of λ-carrageenan, CpG-ODN, glycine beatine,** *Spirulina platensis* **and ergosterol as elicitors for control of** *Zymoseptoria tritici* **in wheat.** *Phytopathology* (Accepted with major maodifications).

1. Introduction

Studying the expression of wheat defense genes in treated *versus* untreated plants will provide further evidence of the elicitor potential of the tested compounds and enable to identify which signaling pathways are preferentially triggered in the plant. Indeed, once an elicitor is recognized, a cascade of defense signals is triggered in the host, leading to induced resistance (Muthamilarasan et al., 2013).

As a reminder, the induction of plant resistance against biotic stresses depends on the spread of the primary danger signals salicylic acid (SA), jasmonic acid (JA) and ethylene (ET) (Muthamilarasan et al., 2013). In dicotyledonous plants, SA-dependent defense signaling occurs upon infection by biotrophic or hemibiotrophic pathogens (Glazebrook, 2005). On the other hand, defense responses mediated by JA and ET are triggered upon infection by necrotrophic pathogens and phloem-feeding insects (Adie et al. 2007; Van der Ent et al. 2009). Defense genes can be differentially expressed through these defense signaling pathways. PR1 (marker of SA signaling) and PR5 (thaumatin-like proteins) are systemic acquired resistance (SAR) marker genes which are characteristically induced during SA-dependent defense responses, whereas the synthesis of LOX (lipoxygenase) and PR4 (hevein-like proteins) is generally linked to JA-dependent signaling (Glazebrook, 2005; Van Loon & Van Strien, 1999). Most studies have reported the existence of a mutual antagonism between SA and JA signaling pathways, although synergistic interactions have also been described in thale-cress (Niu et al. 2011; Schenk et al. 2000; Van Pelt et al. 2000). However, most investigations have been carried out on dicotyledonous plants and less is known about SA/JA crosstalk in monocotyledonous plants. More details are provided in the bibliographical introduction. Recently, Ding et al (2016) showed that wheat was able to finely tune its defense responses depending on its pathogenic invader. They reported that SA and JA were able to act synergistically or antagonistically in order to influence the expression of wheat defense genes.

Hence, the main objective of this study was to examine closely the expression of those defense-related genes before and after treatment, and in comparison to a control. The biomolecular tool developed by INRA (a RT-qPCR-based low-density microarray) was used to evaluate the relative expression of a set of twenty-three different genes of wheat which are known to be involved in various defense mechanisms (Brisset & Duge De Bernonville, 2011; Dugé de Bernonville et al., 2014).

We also explored the correlation between the efficacy of each compound in protecting wheat against *Z. tritici* in the greenhouse, and the expression of defense genes in the host plant.

2. Materials and Methods

2.1 Plant material

The experiments were conducted on wheat (*Triticum aestivum* L.) plants of the susceptible cv. Avatar. Seeds were sown in 30 x 20 cm plastic boxes (40 plants per box). Plants were grown in the glasshouse of INRA under semi-controlled conditions (natural photoperiod supplemented with artificial light if needed, with 20 °C ± 5 according to the sunlight).

2.2 Elicitor preparation

Elicitor treatments were prepared the same way as in greenhouse screening trials (see previous chapter). However, each elicitor candidate was tested only at its medium concentration C2 due to space limitations (Table 3).

2.3 Plant treatment

Plants at the three-four leaf stage (Z13) were sprayed to runoff with elicitors or water with the help of an electric airless spray painter. Control plants were treated with distilled water alone. According to the experimental design devoted to the use of the qPFD tool, each treatment was applied on one box of 40 plants (40 repetitions), and two independent biological experiments were performed. In order to test for priming activities, the plants on the half of each box were sprayed one day later with a solution containing 40 nm of hydrogen peroxide (H_2O_2) which mimics a pathogenic attack. Indeed, elicitors can incur fitness costs in plants due to the trade-off between resources allocated for growth and for disease resistance (Heil et al., 2000; Heil, 2002). On the other hand, elicitor priming is characterized by the non-triggering of plant defense mechanisms directly after elicitor recognition. Instead, strong and rapid host defense reactions are activated only upon a subsequent challenge (Van Loon et al. 2006). Such priming phenomenon thus avoids the diversion of essential available resources for growth and the accumulation of toxic sconedary metabolites when the plant is not under disease pressure. In the present case, if a tested compound exerts a priming activity, a strong induction of the expression of defense genes would occur only upon the later application of H_2O_2 which mimics the subsequent presence of a pathogen. As a reactive oxygen species (ROS), H_2O_2 is indeed involved in plant oxidative stress and acts as a key mediator in defense gene activation (Vranov et al., 2002; Halliwell, 2006). Its use to mimic an pathogen infection is justified by the work of Shetty et al. (2007) who found that wheat infected by *Z .tritici* exhibited an important and early accumulation of H_2O_2 in incompatible interactions (Shetty et al., 2007).

2.4 RNA extraction and quantification of gene expression by real-time RT-PCR

The third leaf of five distinct seedlings was sampled at 24 hours after treatment, right before H_2O_2 application on the half of each box.

Similarly, the third leaf of five distinct seedlings was sampled at 48 and 72 hours after treatment on the whole boxes, for plants treated with H_2O_2 or not. All samples were immediately pooled, frozen and stored at - 80 °C until use (Figure 43).
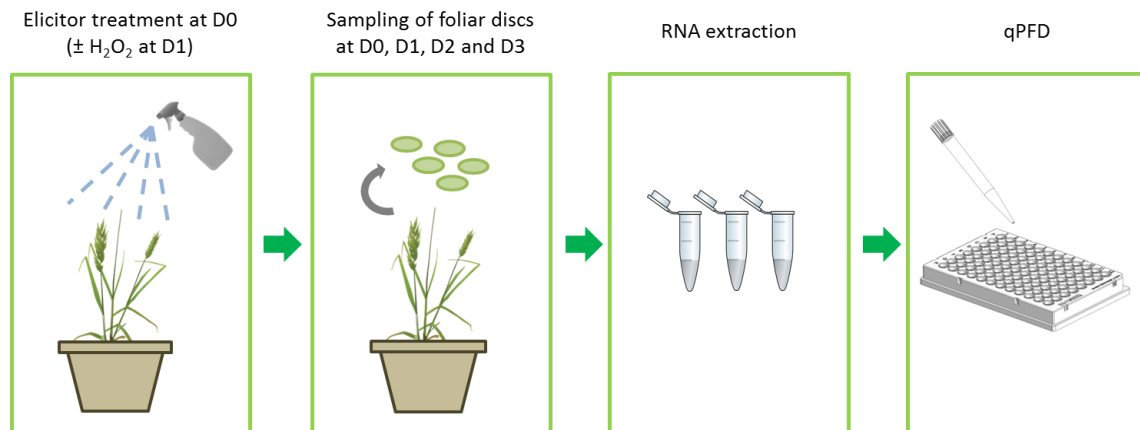
Figure 43. Methodology of defense gene expression investigation. Leaf samplings were realized at day 0 (D0), day 1 (D1), day 2 (D2) and day 3 (D3) after treatment with an elicitor candidate

Total RNA was extracted from around 100 mg of plant tissue using the Nucleospin®RNA Plant Kit (Macherey-Nagel). Reverse-transcription of total RNA was carried out using the M-MLV Reverse Transcriptase (ref M1701, Promega, Madison USA), according to the manufacturer's protocol.

Real-time qPCR was performed with MESA BLUE qPCR MasterMix (ref RT-SY2X-03+WOUFLB, Eurogentec, Liège, Belgique) according to the manufacturer's instructions, and using the biomolecular INRA tool (Patent WO/2011/161388) on a Biorad MyiC detection system (Brisset & Duge De Bernonville, 2011).

The biomolecular study focused on twenty three different genes (Table 4). They are involved in several plant defense mechanisms, including pathogenesis-related (PR) proteins, secondary metabolism, oxidative stress, and signaling pathways (*e.g.*, defense pathways involving salicylic acid, jasmonic acid and ethylene) (Van Loon & Van Strien, 1999; Weber, 2002; Vogt, 2009).

Table 4. List of the defense-related genes studied by qRT-PCR

Metabolic pathway	Gene symbol	Gene name
PR proteins	PR-1	Pathogenesis-related protein 1
	PR-2	Pathogenesis-related protein 2 (glucanases)
	PR-4	Pathogenesis-related protein 4 (hevein-like)
	PR-5	Pathogenesis-related protein 5 (thaumatin-like, osmotin)
	PR-8	Pathogenesis-related protein 8 (class III chitinases)
	PR-14	Pathogenesis-related protein 14 (lipid transfer protein)
	PR-15	Pathogenesis-related protein 15 (oxalate oxidase)
Phenylpropanoid pathway	PAL	Phenylalanine ammonia-lyase
	CHS	Chalcone synthase
	PPO	Polyphenol oxidase
Isoprenoid pathway	HMGR	Hydroxymethyl glutarate-CoA reductase
	FPPS	Farnesyl pyrophosphate synthase
	Far	(E,E)-alpha-farnesene synthase
Oxidative stress	Apox	Ascorbate peroxidase
	GST	Glutathion S-transferase
	POX	Peroxidase
Cell wall reinforcement	CalS	Callose synthase
	CAD	Cinnamyl alcool dehydrogenase
SA pathway	EDS1	Disease resistance protein EDS1
	WRKY	WRKY transcription factor 30
JA pathway	LOX	Lipoxygenase
	JAR	Jasmonate resistant 1
ET pathway	ACCO	1-aminocyclopropene-1-carboxylate oxidase
	EIN3	EIN3-BINDING F BOX protein 1
Housekeeping genes	TubA	Tubulin alpha-1 chain
	Actin	Actin 7
	GAPDH	Glyceraldehyde-3-phosphate dehydrogenase

Relative gene expression was obtained using the $2^{-\Delta\Delta Ct}$ method (Schmittgen & Livak, 2008). Values correspond to the average difference of expression of a given gene between treated and water control plants at each time point (data obtained from the combination of two independent experiments). Three internal reference genes were used for normalization (*e.g.*, TubA, GAPDH, and Actin).

The effect of plant treatment on the wheat defense responses was evaluated by Multivariate ANOVA.

In order to visualize and analyze gene expression, a heatmap representation was performed using dissimilarity distance (1-cor(X, Y)). Moreover, the identification of sets of genes that may be similarly expressed across all conditions within the dataset was realized by clustering gene expression.

Finally, clustering results were confirmed with the help of a Principal Component Analysis (PCA) of the gene expression data.

PCA is a multivariate statistical technique for simplifying complex data sets in which observations are described by several dependent variables (in this study, the twenty three defense-related genes) (Abdi & Williams, 2010).

The statistical programming environment R was used to analyze the data for all experiments and the FactoMineR R package was used for the PCA (Lê et al., 2008; The R Core Team, 2016).

2.5 Correlation between qRT-PCR and glasshouse results

The reliability of the elicitor screening experiments carried out in this study can be strengthened by checking that the protection efficacy of a given compound is correlated to the induction of defense responses in the wheat plant. Such correlation enhances the elicitor potential of the tested compounds, thus increasing their chances of being short-listed for further experiments in practical conditions.

We thus investigated the correlation between the protection efficacy profiles obtained in the glasshouse screening trials and the defense induction levels obtained by real-time qPCR.

The correlation was assessed for each qPCR analysis date and for each treatment. The level of gene expression was estimated with the help of the PCA component score which described the largest variability. The average level of gene expression thus consisted of values of projected individuals (treatments) on the corresponding dimension. Finally, the average protection efficacy values corresponded only to compounds tested at their medium concentration C2 (Table 3) since they were tested at this same concentration in defense gene induction trials.

3. Results

3.1 Induction of plant immune responses

We monitored the expression level of 23 defense-related genes of wheat at 1, 2 and 3 days after treatment with the five compounds (Table 4). A MANOVA test showed that plant treatment had a significant effect on the expression of defense genes (p-value $< 10^{-3}$). For each gene, we then measured the difference of average expression level between treated plants and the water control, and represented it on a heatmap profile (Figure 44).
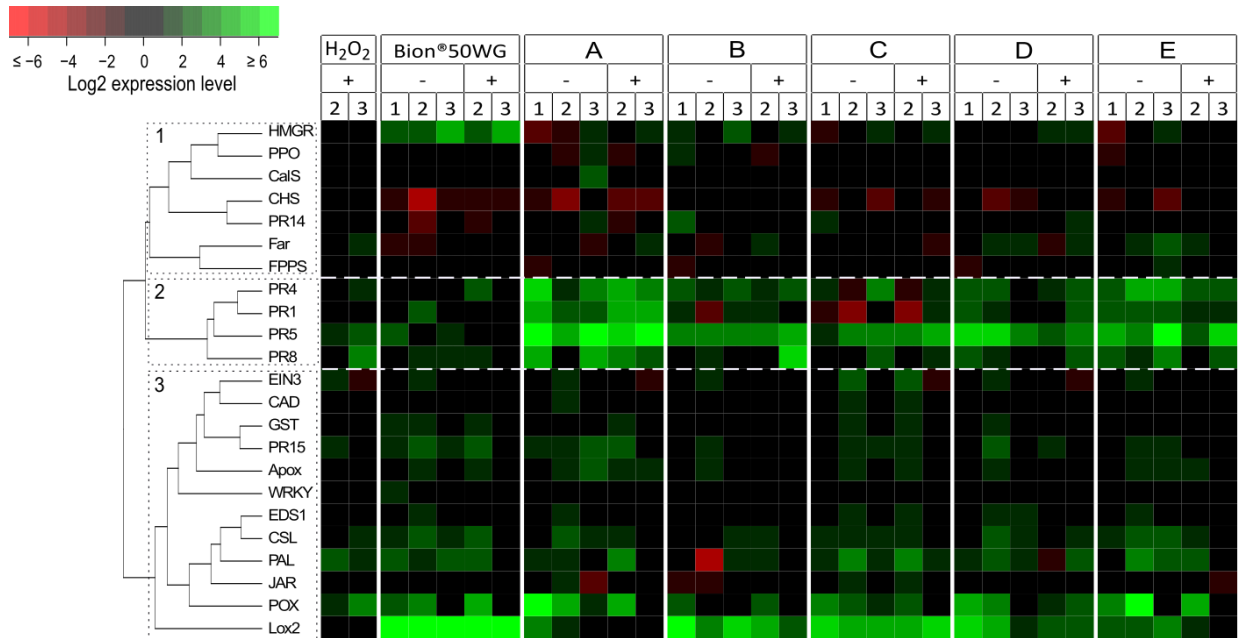
Figure 44. Heatmap profiling of the average expression level of 23 defense-related genes of wheat across all experimental conditions (product, $\pm$ H_2O_2, day post-treatment). Data obtained by the combination of two independent experiments in the greenhouse. For each sampling date, the third leaf of 5 distinct plants were sampled for each modality. For each gene, 2 replicates were realized for RNA-extraction and 3 replicates were realized for qRT-PCR (n = 32). Hierarchical clustering of gene expression highlighted three main patterns. The Log_2 expression levels ($\Delta\Delta Ct$) above or under 0 represent respectively an induction (in green) or a repression (in red) of gene expression in treated plants compared to the water control. Hydrogen peroxide (H_2O_2) was applied (+) or not (-) on plants at 1 day after treatment to mimic a pathogen attack. Gene names are provided in Table 4.

Three major patterns were highlighted by the hierarchical clustering of genes according to their expression levels.

The first pattern (1) includes genes involved in cell wall reinforcement, in the mevalonate pathway and in the phenylpropanoid pathway. These last two lead respectively to the biosynthesis of isoprenoids and antimicrobial compounds. In particular, we observed that plants treated with the elicitor reference Bion showed a 3-fold upregulation of *HMGR* gene expression (hydroxymethyl glutarate-CoA reductase) across all experimental conditions and a 2-fold downregulation of *CHS* gene expression (chalcone synthase) at day 2 and 3 after treatment, with or without subsequent application of H_2O_2. HMGR and CHS are key regulators of isoprenoid and flavonoid biosynthesis respectively (Antolín-Llovera et al., 2011; Dao et al., 2011). Conversely, plants treated with λ-carrageenan (A) showed a downregulation of these two genes, at day 1 and 2 after treatment, with or without H_2O_2.

The second pattern (2) includes genes coding for PR proteins. A strong upregulation of *PR4* and *PR5* gene expression across all experimental conditions

was observed for plants treated with λ-carrageenan (A), CpG-ODN (B), glycine betaine (D) and ergosterol (E).

These genes code respectively for the synthesis of hevein-like and thaumatin-like proteins displaying antimicrobial activities. *PR1* and *PR8* gene expression were also significantly upregulated by λ-carrageenan and ergosterol (6-fold and 4-fold increase respectively).

PR1 is a well-known marker of salicylic acid-dependent defense responses, while PR8 consists of class III chitinases (Van Loon & Van Strien, 1999). Conversely, spirulina (C) induced a 6-fold downregulation of *PR1* gene expression at day 2 after treatment, with or without H_2O_2 applied afterwards. Interestingly, plants treated with Bion and water-treated plants subsequently sprayed with H_2O_2 showed no difference in PR gene expression compared to the control.

A last pattern (3) includes genes involved in anti-oxidative processes and plant defense-signaling. The expression of *LOX2* (13-lipoxygenase 2) was strongly induced by Bion (10-fold upregulation) and by CpG-ODN, spirulina, GB and ergosterol (between 7- and 9-fold upregulation). Similarly, a 6-fold upregulation of *LOX2* expression was induced by λ-carrageenan but only at day 1 after treatment. It is noteworthy that the lipoxygenase enzyme coded by the *LOX2* gene is involved in jasmonic acid-dependent defense signaling (Wasternack & Hause, 2013). On the other hand, expression of genes *PAL* (phenylalanine ammonia-lyase), *POX* (peroxidase) and *PR15* (oxalate oxidase) were upregulated by Bion and by the other tested compounds, with the exception of CpG-ODN. Such upregulation occurred generally at day 1 and/or day 2 after treatment, with or without H_2O_2 application. The enzyme PAL is involved in the biosynthesis of phenolic compounds, including salicylic acid, whereas peroxidases and oxalate oxidases are antioxidant enzymes (Halliwell, 2006; La Camera et al., 2004). The remaining genes were found to be very weakly influenced by the treatments.

Overall, the tested compounds generally triggered plant defense mechanisms at day 2 after treatment. Besides, no priming activities were observed as the application of H_2O_2 had no supplementary effect to the application of one of the compounds on the expression of wheat defense genes. However, water-treated plants which were subsequently sprayed with H_2O_2 after 24 hours revealed a slight upregulation of *PAL* and *POX* gene expression (4-fold increase), compared to the water control plants which received no H_2O_2. Hydrogen peroxide was thus well perceived as an attack by the plant.

Finally, a Principal Component Analysis (PCA) was performed by using genes as variables and samples (treatments) as individuals in order to confirm clustering results (Figure 45).
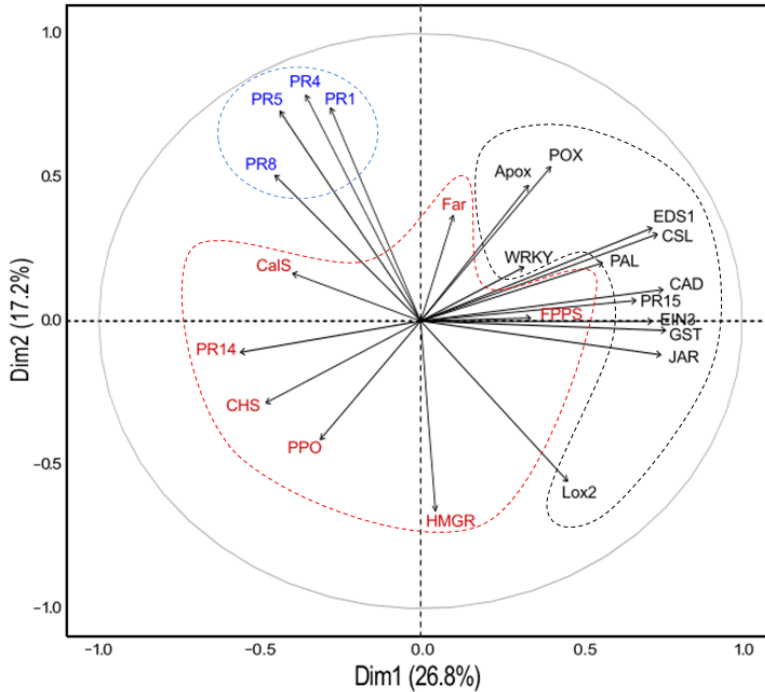
Figure 45. Principal Component Analysis (PCA) of gene expression across all experimental conditions compared to water control (product, $\pm$ H_2O_2, day post-treatment). Three major groups are highlighted in different colors (red, blue and black). The variables correspond to the 23 defense-related genes of wheat examined by heatmap profiling ($n = 32$) and hierarchical clustering. Variables are projected on the first and second principal component scores (Dim1 and Dim2) and were generally significantly correlated to one of the dimensions.

The first two principal components described the largest variability (26.8 % and 17.2 % of initial variation, respectively). These two principal components enabled to separate the samples and cluster the genes into three main groups with strong correlation coefficients ($|r| > 0.5$, *p-value* $< 10^{-3}$). The pathogenesis-related genes were once again clearly separated into a distinct group. Similarly, genes involved in anti-oxidative processes or in plant defense-signaling were also gathered close to one another. In addition, the variables with significant correlation coefficients (*p*-value $< 10^{-3}$) were those which showed strong gene up- and/or down-regulation in the heatmap profile.

An additional PCA was performed by using treatments as variables projected on the dimension 1 and 2, and on dimension 1 and 3 (Figure 46).
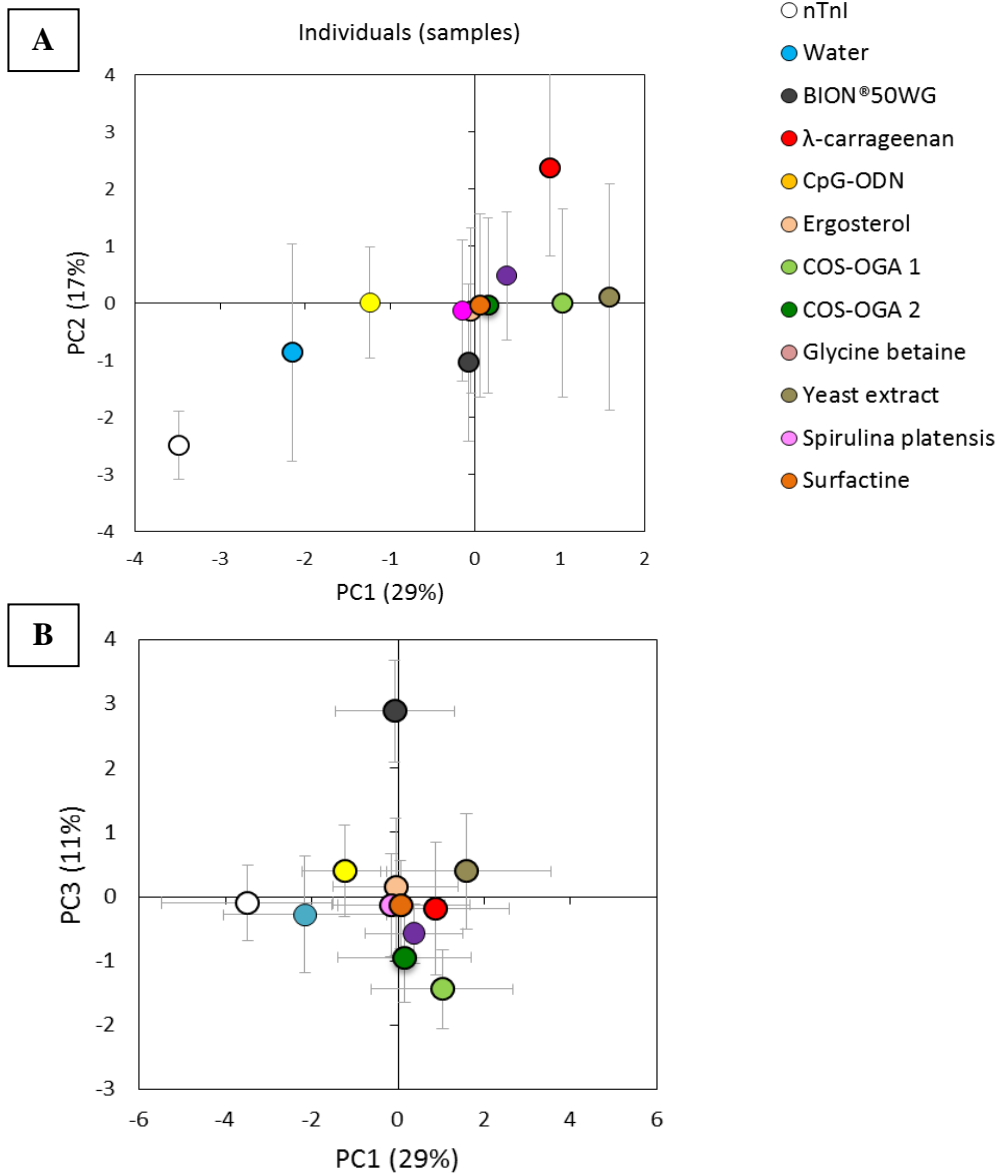
Figure 46. Principal Component Analysis (PCA) of plant treatments. The variables correspond to the 10 initial elicitor treatments applied on wheat for screening and biomolecular studies. Results are compared to a calibrator 'nTnI' (plants which received no treatment at all) and a water control (plants sprayed with water only). Variables are projected: (A) on the first and second principal component scores (PC1 and PC2); (B) on the first and third principal component scores (PC1 and PC3). COS-OGA are chitooligosaccharides provided by Fytofend, Namur; yeast extracts were provided by Ithec, France

This last PCA includes the analysis of gene expression in plants treated with other compounds which were initially comprised in screening trials. Similarly to the PCA illustrated on Figure 45, the three first principal components described the largest variability of the data (29% for PC1, 17% for PC2, and 11% for PC3). The effect of the different treatments on gene expression was analyzed across all experimental conditions. The corresponding PCA results show that plants which received no treatment (white dot) and plants sprayed only with water (blue dot) are clearly separated in terms of gene expression from plants treated with the different compounds. Besides, plants treated with formulated λ-carrageenan (red dot on Figure 46 – A) is separated from the other treated plants in terms of gene expression. The same goes for plants treated with the elicitor control Bion (black dot on Figure 46 – B). Such results are interesting as they illustrate the contrast in terms of gene expression between plants sprayed with the various treatments.

3.2 Correlation between glasshouse protection efficacy and gene induction

For each compound, we investigated the correlation between the level of defense gene expression triggered in the wheat plant through biomolecular tests and the level of protection efficacy conferred against *Z. tritici* through glasshouse experiments. The PCA first component score (Figure 45) described the largest variability (26.8 %) and was used to realize the correlation test (Figure 47).
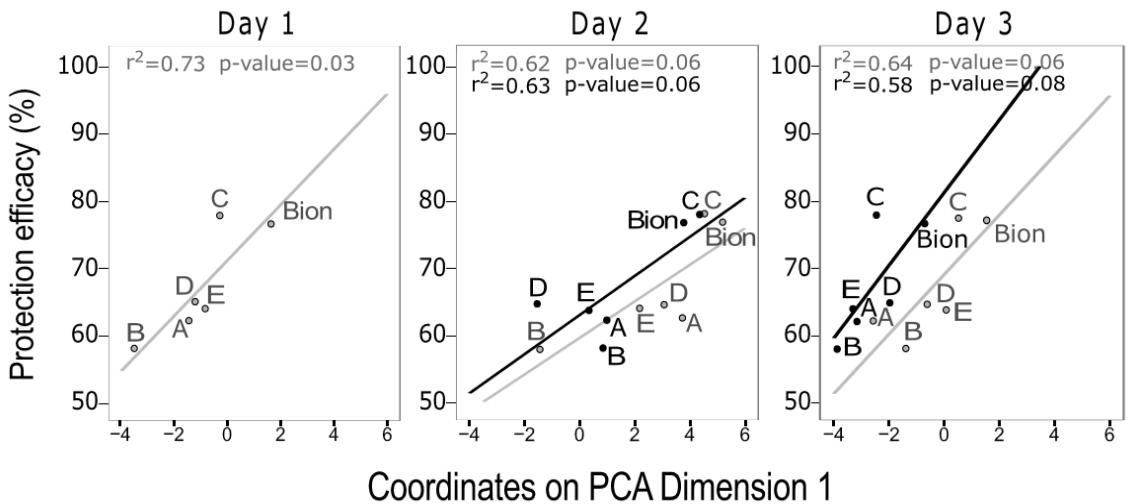
Figure 47. Relationship between the expression of wheat defense genes and the efficacy of five compounds (A-E) in protecting wheat against *Zymoseptoria tritici*. Positive correlations are highlighted and are significant at day 1 after plant treatment. Gene expression was obtained by qRT-PCR while protection efficacy data was a result of glasshouse elicitor screening. Hydrogen peroxide (H_2O_2) applications were realized to test for priming activity: grey, without; black, with. Coordinates on PCA first component score are averaged across biological replicates and were used as a measure of induced gene expression. Correlation coefficients and *p-values* of linear models are indicated in each plot ($n \geq 80$ for screening tests and $n = 32$ for gene expression studies). Compounds were: λ-carrageenan (A); CpG-ODN (B); Spirulina (C); Glycine betaine (D); Ergosterol (E).

The higher the coordinates were on dimension 1, the higher the protective efficacy. Overall, protection efficacy and defense induction were positively correlated at each sampling date for all compounds. The strongest and most significant correlation was obtained for day 1 ($r^2 = 0.73$, *p-value* = 0.03). Bion and spirulina were clearly highlighted as being the most efficient for wheat protection against STB coupled with a significant induction of defense responses. For the two other sampling dates, the correlations were positive but not significant (*p-value* > 0.05). In addition, no difference was visible at day 2 between H_2O_2 untreated and treated leaves ($r^2 = 0.62$, *p-value* = 0.06 and $r^2 = 0.63$, *p-value* = 0.06, respectively), while correlations were slightly stronger for H_2O_2 untreated leaves at day 3 ($r^2 = 0.64$, *p-value* = 0.06).

4. Discussion

4.1 The five compounds triggered multiple defense signaling pathways in wheat

Investigating the immune responses of wheat with the INRA qPFD tool highlighted that every tested compound was indeed perceived by the plant as an elicitor: the expression of genes coding for antimicrobial compounds was upregulated; the expression of genes involved in the synthesis of signal hormones such as salicylic acid (SA) and jasmonic acid (JA) were induced concomitantly and/or at different time scales in treated wheat.

In the present study, JA-dependent signaling was induced in plants treated with λ-carrageenan during the first 24 hours before giving way to SA-dependent defense responses. Previous studies have demonstrated that λ-carrageenan could protect tomato plants and thale-cress by triggering the expression of JA-related genes (Sangha et al., 2010; Sangha et al., 2015). In addition, Vera et al (2011) showed that carrageenans were able to suppress a disease at a systemic scale by triggering SA-related defense responses such as an increased PAL enzymatic activity and the accumulation of phenolic compounds. Besides, Ray et al (2003) reported that infection of susceptible and resistant wheat cultivars by *Z. tritici* induced a strong upregulation of the *LOX* gene expression up to 3 hours after plant infection before quickly decreasing.

On the other hand, SA and JA defense signaling pathways were induced simultaneously in wheat plants treated with GB, CpG-ODN or ergosterol. In dicotyledonous plants, SA and JA are known to interact antagonistically, but it turns out that less is known about these interactions in monocots (Thaler et al., 2012; Balmer et al., 2013). By treating wheat with either λ-carrageenan, glycine betaine or ergosterol, it appears that defense signal hormones are induced concomitantly. These results therefore seem to confirm the existence of intricate hormone crosstalk in plant innate immunity (Balmer et al. 2013; Ding et al. 2016; Lochman and Mikes 2006; Thaler et al. 2012)..

On the other hand, only JA-dependent signaling seems to have been strongly induced in plants treated with spirulina and Bion. The effect of spirulina on plant induced resistance had never been investigated previously. However, numerous

studies have already been dedicated to the elicitor potential of Bion and our results are surprisingly in contradiction with these previous findings. As a chemical elicitor consisting of Acibenzolar-S-methyl, Bion shows functional analogy to the plant hormone SA and was shown to induce a long lasting SAR with a characteristic accumulation of PR1 and PR5 proteins and an increase in PAL and CHS activity (Görlach et al., 1996; Hofgaard et al., 2005). The fact that our results rather suggest the involvement of JA mediated signaling in wheat treated with Bion could be linked to the genotype of the cultivar used in this study (Ors et al., 2017) and/or to the fine tuning of signal hormones in the plant (Ding et al., 2016).

Finally, the potential priming effect of the five compounds was tested by applying H_2O_2 to mimic a subsequent pathogen attack. Hydrogen peroxide is indeed a reactive oxygen species (ROS) involved in plant oxidative stress and acts as a key mediator in defense gene activation (Halliwell, 2006). Shetty et al found that the infection of wheat by *Z .tritici* was associated with an important and early accumulation of H_2O_2 in incompatible interactions (2007). However, our results did not highlight any priming activity. Replacing the application of hydrogen peroxide by an actual pathogen inoculation shortly after plant treatment may however reveal some interesting patterns in defense signaling and potential priming activities. Indeed, the accumulation of ROS in the host plant is only part of the defense reactions triggered upon infection by a pathogen. *Zymoseptoria tritici* for instance emits an array of compounds to facilitate its development in wheat by inhibiting the plant defense responses (*i.e.* cell wall-degrading enzymes such as xylanases cellulases and pectinases, or secondary metabolites such as trichothecenes). Some of these fungal compounds such as chitin act as pathogen-associated molecular patterns (PAMPs) and are recognized by the plant as synonymous of an infection.

Further investigations, including biochemical experiments, would probably help to better understand how the five tested compounds and Bion contributed to induce such defense responses in this major European crop. Research on induced resistance of monocots is slowly emerging (Balmer et al. 2013). Yet, the primary objective of the present study was mainly to rapidly identify interesting elicitor compounds which are effective to protect the wheat plant against STB.

Overall, the qPFD tool thus represents an interesting biomolecular method for thorough elicitor screening. Studying wheat defense responses through the expression of 23 defense genes indeed provided a great deal of information on the defense mechanisms triggered in the plant by an elicitor. However, it is possible that other genes, notably those coding for different defense hormones (*e.g.*, auxin, cytokinins, abscisic acid, gibberellin, brassinosteroids) might also be involved in wheat induced resistance (Robert-Seilaniantz et al., 2011). For instance, glycine betaine is usually known to enhance plant tolerance to abiotic stresses which is largely controlled by abscisic acid (Heshmat et al., 2012). Still, these biomolecular investigations are a useful complement to glasshouse screening trials.

4.2 Protection efficacy is positively correlated with defense gene expression patterns

We studied the potential correlations between the expression of wheat defense genes and the greenhouse protection efficacy conferred by the five compounds against the fungus (Figure 47). Glasshouse screening trials and gene expression studies showed similar and cohesive results. The positive correlation which was identified between these two independent tests strengthens the reliability of this study for elicitor identification. Plants treated with Bion or spirulina were the most protected against STB disease while at the same time inducing significant defense responses in the plant.

Both Bion and spirulina strongly induced JA-dependent signaling in wheat through the qRT-PCR assay.

As a reminder, correlation tests were realized in the present study for compounds tested at a medium concentration C2 (Table 3). Therefore, it would actually be interesting to realize gene expression studies for plants treated with the same compounds at higher concentrations, in order to check if more positive correlations with greenhouse results may be involved. Nevertheless, the potential phytotoxicity effect of the tested compoudsn shall be tested when applied at the selected concentrations.

5. *Conclusion*

We achieved a screening of elicitors of wheat defenses through a succession of experiments: biocidal *in vitro* tests enabled to check for any fungicidal activities; glasshouse experiments allowed to determine the efficacy of a given compound in protecting the plant against a challenging disease; biomolecular tests provided further information on the ability of a compound to trigger defense signaling responses in the plant, thereby confirming or not the existence of elicitor properties.

We therefore demonstrated that λ-carrageenan, CpG-ODN, glycine betaine, spirulina and ergosterol are elicitors of wheat defenses. They were all efficacious in protecting wheat by up to approximately 70 % against *Z. tritici* under semi-controlled conditions and induced both SA- and/or JA-dependent signaling pathways in the plant. Moreover, the results of glasshouse screening trials and biomolecular tests were positively correlated. These findings contribute to extend the narrow list of existing biocontrol tools against *Z. tritici*, which already includes β-1,3-glucans and phosphite-based inducers (Perelló et al., 2009; Deliopoulos et al., 2010). The diverse origins and structure of the tested compounds is a good reminder that plants are able to recognize various types of elicitors in their environment (Henry et al., 2012). Their availability on the market makes them all the more interesting in the prospect of large-scale uses in agriculture.

The qPFD tool was helpful to confirm the elicitor properties of the tested compounds. However, these biomolecular experiments were realized on leaf fragments sampled up to three days after elicitor treatment. Moreover, hydrogen peroxide was used to mimic an attack, but an actual pathogen inoculation may trigger a different set of defense reactions in the plant. Therefore, what about the expression of defense genes up to 5 days after elicitor treatment?

And following pathogen inoculation? It appears mandatory at this stage to study the expression of wheat defense genes in treated *versus* untreated plants inoculated with *Z. tritici* five days after elicitor treatment. Moreover, screening of the five elicitor candidates has been achieved through an array of experiments, and it is now time to select the compounds which showed the most interesting results.

The next logical step to this study is thus: (i) select the two most interesting compounds; (ii) carry out biomolecular experiments on plants treated with these two selected elicitors under conditions similar to that of greenhouse screening trials.

3. Selection of the two most promising elicitor compounds

A list of criteria enabled to select the two most interesting compounds to be used for further investigations (Figure 48).

	Concentration $(g.l^{-1})$	Protection efficacy (%)	Induction of plant defenses	Reliability	Easy formulation	Commercial-scale production	Price $(€\ kg^{-1})$
λ-carrageenan	0,1	59	✔	✔	✔	✔	1 to 4
	1	53					
	5	**73**					
CpG-ODN	9,5 x 10⁻⁵	59	✔	✔	✔	✖	600 € mg⁻¹
	9,5 x 10⁻⁴	45					
	0,0095	35					
Spirulina	0,3	68	✔	✔	✔	✔	16 to 40
	3	**76**					
	30	61					
Glycine betaine	0,12	66	✔	✖	✔	✔	48
	1,2	62					
	12	39					
Ergosterol	0,002	60	✔	✖	✖	✔	6-8 € g⁻¹
	0,08	64					
	0,8	63					

Protection efficacy cotations

0 %	1-20 %	21-40 %	41-60 %	61-80 %	81-100 %

Figure 48. List of criteria to select interesting elicitor compounds. Price sources**:**
λ-carrageenan (https://www.cbi.eu/sites/default/files/market_information/researches/product-factsheet-europe-carrageenan-2015.pdf);
CpG-ODN (Abeomics);
Spirulina (http://www.spiruline-guide.com/);
Glycine betaine (https://www.agrilisa.com/Catalogue/Fiche/catid/892/eid/107/greenstim);
Ergosterol (Sigma-Aldrich, Fisher scientific)

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This list takes into account the results of greenhouse screening and *in vitro* biocidal assays as well as those of biomolecular trials. Above all, the greenhouse screening experiments enabled to highlight the compounds which effectively protected the plant against the fungal pathogen, similarly to the commercial elicitor Bion®, and those which showed no difference with the control when applied on wheat at given concentrations. Therefore, CpG-ODN at concentration C2 and C3 ($9,5 \times 10^{-4}$ and $0,0095$ g l^{-1}) and glycine betaine at concentration C3 (12 g l^{-1}) can be excluded from the list of retained compounds.

Protection efficacy cotations also help to visualize the elicitor candidates which were the most effective in protecting the plant: λ-carrageenan at 5 g l^{-1} and *Spirulina platensis* at 3 g l^{-1} showed a mean protection efficacy of 73 % and 76 % respectively. Moreover, spirulina showed significant and positive correlations between its protection efficacy and its ability to induce wheat defense mechanisms.

In addition, the **variability of elicitor efficiency** is a well-known issue which must be considered. Thus, the reliability of the compound in protecting the plant from one experiment to the other was taken into account. A compound was considered unreliable when showing different results one time in two. As a matter of fact, some elicitor candidates such as glycine betaine and ergosterol failed to ensure a stable protection of wheat against *Z. tritici* when tested several times.

The **easy formulation** of a given compound for plant topical spraying is another important selection criterion. As previously stated, elicitors can have numerous origins and structures, and the candidates selected for this study are no exception. The term "easy formulation" deals with the time spent to prepare an elicitor treatment and the potential requirement of a stock solution. In this particular case, ergosterol can be set aside since this hydrophobic compound requires a methanolic stock solution to be freshly prepared in order to be solubilized homogenously afterwards in water.

Finally, the **market availability** of these compounds is of major importance from a practical point of view. In the event that one of these compounds shows considerable interests as a biocontrol tool in the field, its availability and fair price on the market may enable its quick release as a commercial product for farmers. The price estimations shown in Figure 48 represent an average of the prices found on the various manufacturers' websites. As it turns out, the five compounds are all available on the market but are not all worth the same price. Notably, CpG ODN and ergosterol are the most expensive. On the other hand, λ-carrageenan and spirulina represent the most accessible products by representing large markets in the food industry combined to affordable prices.

In more detail, the leading position of carrageenan in the food industry is due to the increasing demand for processed foods and to its multi-functionality (*e.g.* combination of gelling, thickening, stabilizing properties). The Global carrageenan market was worth about 682 million euros in 2016 and currently represents 13.3 % share of the global food and beverage hydrocolloids market (www.futuremarketinsights.com, 2017). The Asian-Pacific region holds the largest share in the industry: the largest carrageenan producers are the Philippines which provide 77 % of the world's supply, while China is the major exporter to both the USA and Europe. The two major company leaders in the carrageenan industry are DuPont and Cargill located in the USA, while the major manufacturers are based in the Asia-Pacific region (*e.g.* Marcel Carrageenan, Seatech Carrageenan Corp. and FMC Biopolymer).

In the case of spirulina, the global demand for this product is increasing due to its nutritional value and multiple health benefits (www.persistencemarketresearch.com, 2017). Over 128.000 tons of spirulina have been consumed in 2016. The Global spirulina market valued approximately 627 million euros in 2016 and is expected to increase nearly up to 1.79 billion euros by 2026. Among the key manufacturers are Cyanotech Corp., NOW Health Group, DIC Crop., Algene Biotech, Earthrise Nutritionals and All Natural Company. It is estimated that North America will become the world's largest spirulina market in the coming years, followed by Western Europe and Asian-Pacific regions. This trend can be explained by the fact that regulatory bodies of multiples countries have approved the use of spirulina in the production of foods and beverages. Moreover, technological advancements now make it feasible to produce spirulina at a commercial scale and in a cost-effective manner.

In view of all these criteria, λ-carrageenan and spirulina thus stand out clearly as being the most interesting compounds to be selected for further investigations as elicitors of wheat defenses in the prospect of sustainable crop protection.

4. Evaluation of the protection efficacy of λ-carrageenan and Spirulina in the field

1. Introduction

Providing solutions which are adapted to agricultural practices in "real life" is the challenging and crucial task of researchers working in agronomic sciences. Hence, going out of the laboratory to undertake experimentations in the field is essential. In the frame of this thesis, undertaking field trials thus represents a concrete final step to evaluate the elicitor potential of λ-carrageenan and spirulina under practical conditions. Much is indeed at stake when testing potential elicitor compounds in the open field. The preventive elicitor treatment must be applied at the appropriate dose and at the right time before the emergence of the disease, the plant must be in a good physiological state, and the weather conditions must be favorable.

Environmental parameters (*i.e.* temperature, relative humidity) can indeed influence the elicitor efficiency. For instance, drought conditions can cause the plant to prevent water losses by closing its stomata, thereby preventing the elicitor compound to inter.

In addition, the **plant genotype** can affect the expression of induced resistance. For instance, Walters et al (2011a) observed that among a range of spring barley varieties some did not express induced resistance at all in response to treatment with a combination of elicitors (*e.g.* Bion, BABA and *cis*-jasmone).

Moreover, it was suggested that the resistance of plants in the field may be partly induced already due to their constant interaction with the biotic and abiotic environment. **A prior induced state of the plant** must be taken into account. It should not be precluded that the plant ability to further enhance its induced resistance following an elicitor treatment may be restricted. Besides, Walters et al (2011b) demonstrated that a prior infection of *Rhynchosporium secalis* on young barley plants compromised the ability of the crop to effectively respond to elicitors.

Furthermore, induced resistance can incur **allocations costs** to the plant. The triggering of defense mechanisms requires the consumption of resources by diverting energy initially devoted to plant growth and development (Gozzo & Faoro, 2013). For instance, Bion®50WG (Heil et al., 2000) was shown to reduce the growth and seed yield of wheat plants in the absence of pathogens. Van Hulten et al (2006) demonstrated that the direct induction of defenses by an elicitor treatment was likely to be wasteful in the lack of a subsequent disease infection, contrary to priming. So far, previous studies show that plant fitness costs actually depend on an array of factors, among which the elicitor nature, the applied dose, the plant species and variety, the pathosystem, but also the available nutrient soil properties (Gozzo & Faoro, 2013).

Last but not least, the **disease pressure in the field** is known to influence the efficiency of an elicitor. For instance, Walters et al (2011a) reported that the efficiency of elicitor treatment on barley was maintained in the field during years where the disease pressure of *R. secalis* was moderate. On the other hand, such efficiency decreased during years where the disease pressure was low.

A considerable number of factors thus seem to affect the efficacy of induced resistance in the field. Taking into account all these parameters, the objective of the present field trials was to ensure that the two compounds λ-carrageenan and *Spirulina (Arthrospira) platensis* maintain their protection efficacy of winter wheat against *Z. tritici* under practical conditions.The reliability of effectiveness of these compounds will be compared to that of conventional fungicides.

2. Materials and methods

2.1 Wheat grown in the field

The field trials were conducted on the experimental site of Gembloux Agro Bio Tech (Lonzée, Belgium) on winter wheat (*Triticum aestivum* L.) plants of the susceptible cv. Avatar (in 2016) and cv. Edgar (in 2017). During the 2016 season, an additional field trial was conducted at the experimental site of Arvalis - Institut du Végétal (Boigneville, France) on winter wheat of the susceptible cv. Pakito. Technically, the two wheat cultivars Avatar and Pakito show satisfactory STB susceptibility cotation ranks for field trials (5/9 and 4/9 respectively) while Edgar is more resistant to the disease (7/9) (Figure 49). As is customary for winter wheat cultivation, seeds were sown in autumn (late October-early November) and preventive treatments to counter STB were realized in spring.
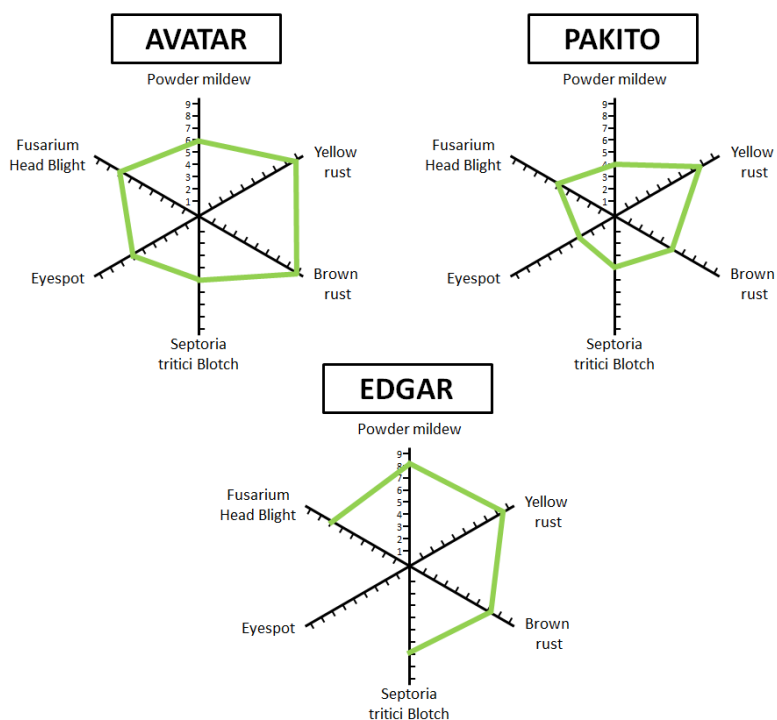
Figure 49. Susceptibility of three different wheat varieties (Avatar, Pakito, Edgar) to diseases. Susceptibility cotations rank from 0 (very susceptible) to 9 (resistant).

2.2 Treatment preparation

The compounds λ-carrageenan and *Spirulina (Arthrospira) platensis* were tested at 5 g l⁻¹ and 3 g l⁻¹ respectively. The elicitor solutions were freshly prepared before use in distilled water supplemented with 0.1 % (*v/v*) of spreading agent Break-Thru®S240 (polyether trisiloxane, Evonik Industries), and 0.05 % (*v/v*) of solubilizing agent Tween 20 (polyoxyethylene-sorbitan monolaurate, Sigma Aldrich). Solutions of λ-carrageenan were heated up to 80 °C for 15 min in order to accelerate the homogenization of the solution.

Control plants received no treatments. Vacciplant® (laminarin; Goëmar, France) was used as an elicitor reference and applied at 0.5 l ha⁻¹ (recommended dose). Laminarin is an oligosaccharin elicitor extracted from the brown algae *Laminaria digitata.* It induces plant natural defenses, including crops, against a broad spectrum of pathogens. The use of Vacciplant® instead of Bion® in the field was due to the fact that this product is currently registered to protect crop plants against powdery mildew and STB, and was already regularly used as an elicitor reference at the experimental site of Arvalis in France. On the other hand, the conventional fungicide Bravo® (chlorothalonil; Syngenta, France) was used as a chemical reference at 0.5 l ha⁻¹ (recommended dose). Chlorothalonil is a broad-spectrum contact chloronitrile fungicide with a non-systemic action. It is commonly used in agriculture, notably to protect wheat against *Zymoseptoria tritici*. As a multi-site inhibitor of fungal enzymes, it prevents spore germination and fungal development.

In addition, an "Adjuvant" treatment and a fungicide combination treatment were respectively tested at the Belgium experimental site. The "adjuvant" treatment enabled to test for a potential formulation effect and consisted of only the adjuvants Tween 20 at 0.05 % (*v/v*) and Break-Thru®S240 at 0.1 % (*v/v*) without any elicitor.

Finally, the fungicide combination treatment consisted of Bravo® applied at 1 l ha⁻¹ together with Opus®Team (triazole mix of epoxiconazole and fenpropimorphe; BASF, France) at 1.5 l ha⁻¹. The plants were then sprayed with Aviator®Xpro (mix of bixafen and prothioconazole; Bayer Crop Science, Belgium) at 1.25 l ha⁻¹. These are all recommended doses.

2.3 Plant treatment

At the first node stage Z31 (Zadoks et al., 1974), each treatment was applied on four distinct plots of wheat of 25 m² in France, and on four plots of 16 m² in Belgium according to a randomized experimental design. The same treatments were realized three more times every 10 days on the corresponding plots till the flag leaf stage (Z39) (Figure 50). Indeed, only one application of an elicitor product is usually recommended for efficacy trials under semi-controlled conditions in the greenhouse (https://www.elicitra.org/vars/fichiers/Livrables/guide_metho_eval_SDP_elicitra_2013.pdf). However, repeated preventive applications can be realized in order to maximize its efficacy in the field. A 10 day delay between treatments was thus selected for field experimentations based on the results of previous research (Obradovic and Jones, 2001; Huang et al., 2012).
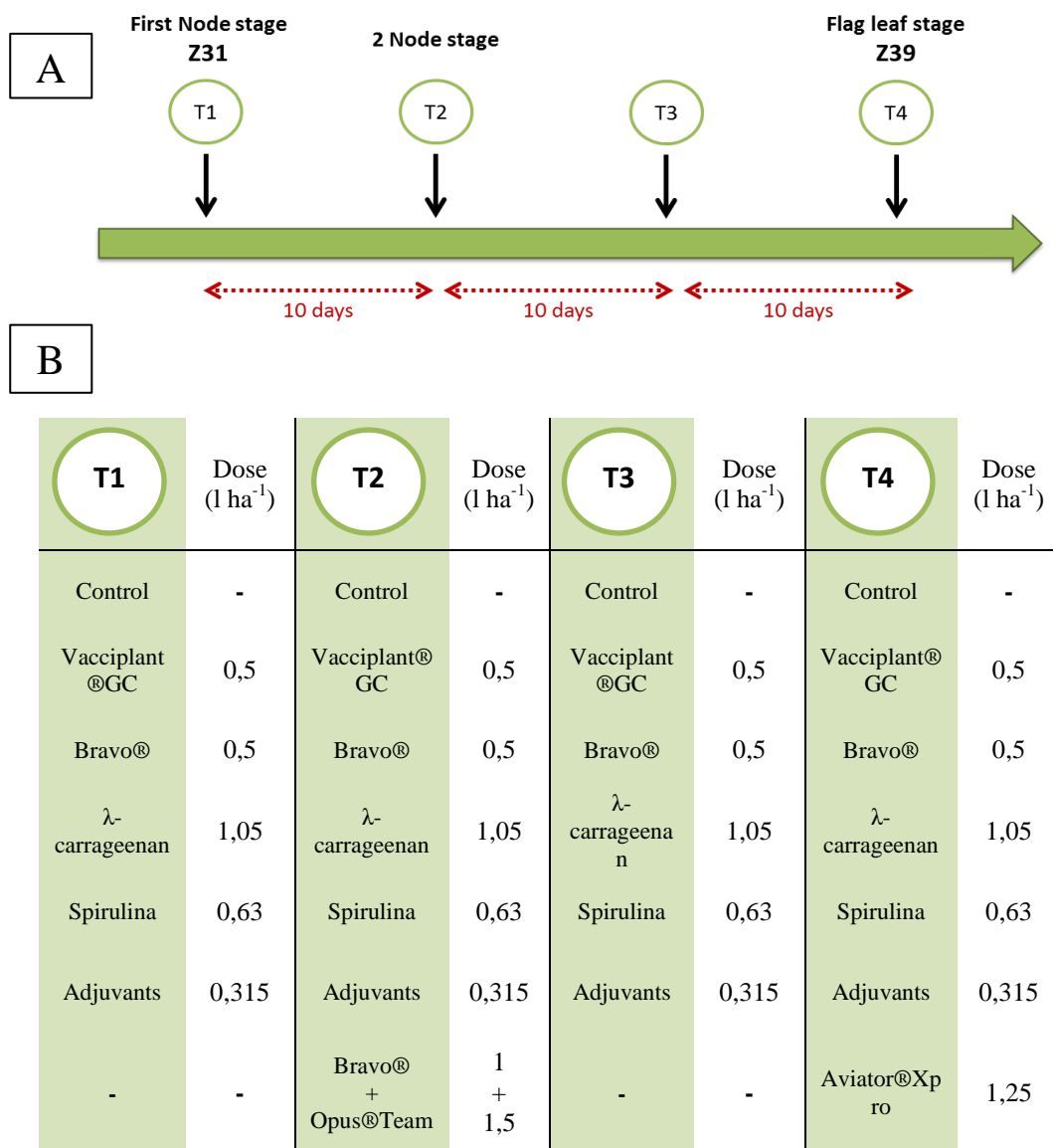
Figure 50. Methodology of field experiments (A) and the different treatments applied on winter wheat (B). The "Adjuvant" treatment and the application of Bravo®+Opus®Team/Aviator® were only realized at the Belgian experimental site of Gembloux Agro Bio Tech.

In Belgium, the combination of Bravo® and Opus®Team was only applied on wheat at the 2 node stage (Z32), and the same plants were subsequently sprayed with Aviator®Xpro at the flag leaf stage.

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The severity of the *Zymoseptoria tritici* disease was scored by measuring the percentage of leaf area covered with symptomatic lesions (necrosis and chlorosis) bearing pycnidia. Moreover, the yield of the wheat plots treated with the various products was measured after harvest.

In France, the disease notations were realized at 25 and 37 days after the last application, on all live leaves of fifteen randomly chosen plants per plot (60 technical repetitions). In Belgium, disease notations were realized at 40, 50 and 80 days after the last application in 2016 (Figure 51 and Figure 52) and at 12, 20, 26 and 33 days after the last application in 2017 (Figure 53), on all live leaves of five randomly chosen plants per plot (20 technical repetitions).

3. Results

In 2016 at the French experimental site of Boigneville (Arvalis), the mean disease severity of *Z. tritici* on control plants reached 95 % at 37 days after the last treatment application (Figure 51).
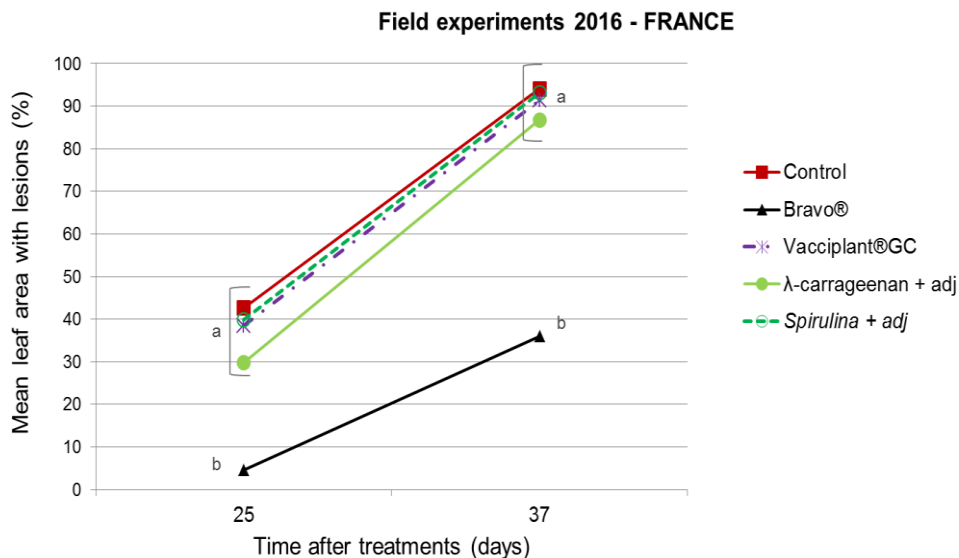
Figure 51. Severity of *Zymoseptoria tritici* infection on winter wheat crops in the field during the 2015-2016 season at the experimental site of Arvalis-Institut du Vegetal (France). The disease pressure of the fungi was drastically important during that season, except for wheat crops which were treated with fungicides (Bravo® alone). Values correspond to the average percentage of symptomatic lesions scored on wheat leaves at various days after the last treatment. Means tagged with the same letters are not significantly different using the Tukey test at $P = 0.05$.

At the same date, the mean disease severity of plants treated with Vacciplant®, λ-carrageenan or Spirulina reached 91 %, 87 % and 93 % respectively. Neither the elicitor reference nor the compounds λ-carrageenan and Spirulina significantly succeeded in protecting wheat against the fungal pathogen ($P = 0.05$).

On the other hand, a mean disease severity of only 36 % was scored at 37 days after the last treatment application on plants treated with Bravo®. The chlorothalonil-based fungicide was thus the only product which significantly protected the crop ($P = 0.05$). Consequently, wheat yield was significantly higher ($P = 0.05$) for fungicide-treated plants (65 quintals ha^{-1}) compared to all the other treatments and the control (47 quintals ha^{-1} on average).

The same year, a few hundred kilometers up North, a similar experiment was taking place at the Belgian experimental site of Lonzée (Gembloux Agro Bio Tech). As it turns out, the *Z. tritici* disease pressure was equally important. The mean disease severity of *Z. tritici* on control plants increased from 28 % to 99 % between 50 and 80 days after the last treatment application (Figure 52).
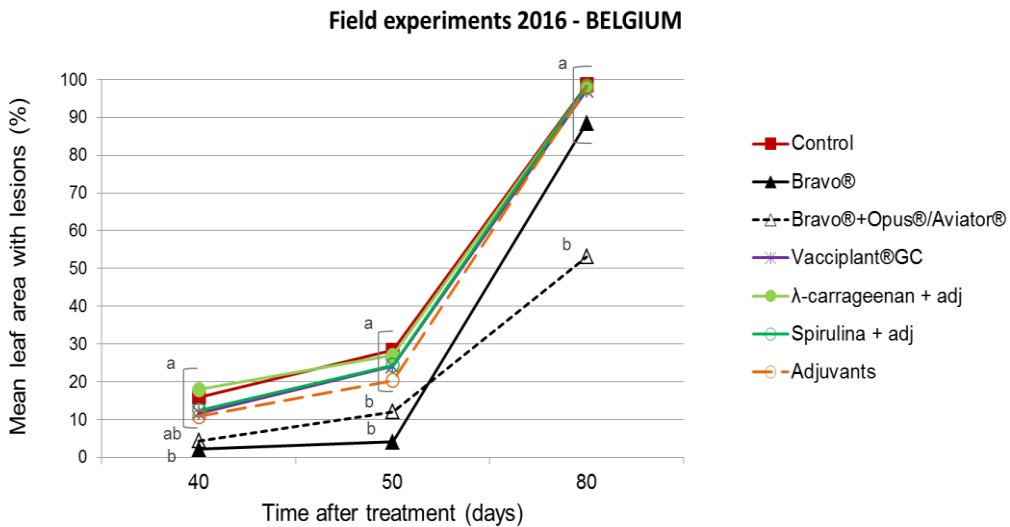
Figure 52. Severity of *Zymoseptoria tritici* infection on winter wheat crops in the field during the 2015-2016 season at the experimental site of Gembloux Agro Bio Tech (Belgium). The disease pressure of the fungi was drastically important during that season, except for wheat crops which were treated with fungicides (Bravo® alone and combined with Opus® and Aviator®). Values correspond to the average percentage of symptomatic lesions scored on wheat leaves at various days after the last treatment. Means tagged with the same letters are not significantly different using the Tukey test at $P = 0.05$.

Similarly, plants treated with Vacciplant®, λ-carrageenan, Spirulina or only the adjuvants showed a mean disease severity of 24 %, 27 %, 24 % and 20 % respectively at 50 days after treatment before drastically increasing to 98 % thirty days later. In the case of plants treated with Bravo®, the mean disease severity affected only 4 % of leaf surface at 50 days after the last treatment before increasing to 88 % at day 80 after the last application of the fungicide. Finally, an increase of mean disease severity from 4 % to 53 % was scored on plants between day 50 and 80 after the last application of the fungicide combination Bravo®+Opus®/Aviator®.

The application of Bravo® alone managed to significantly protect the wheat crop against *Z. tritici* up to 50 days after the last treatment, while the fungicide combination effectively protected the plant over a longer period of time, up to 80 days after the last treatment ($P = 0.05$). On the other hand, plants treated with the remaining products showed a high disease severity comparable to the control. These products thus failed to significantly protect wheat in the field.

Finally, a last field experiment carried out in Belgium during the season 2016-2017 did not allow the evaluation of the protection efficacy of formulated λ-carrageenan or spirulina treatments under practical conditions due to very low STB disease pressures (Figure 53).
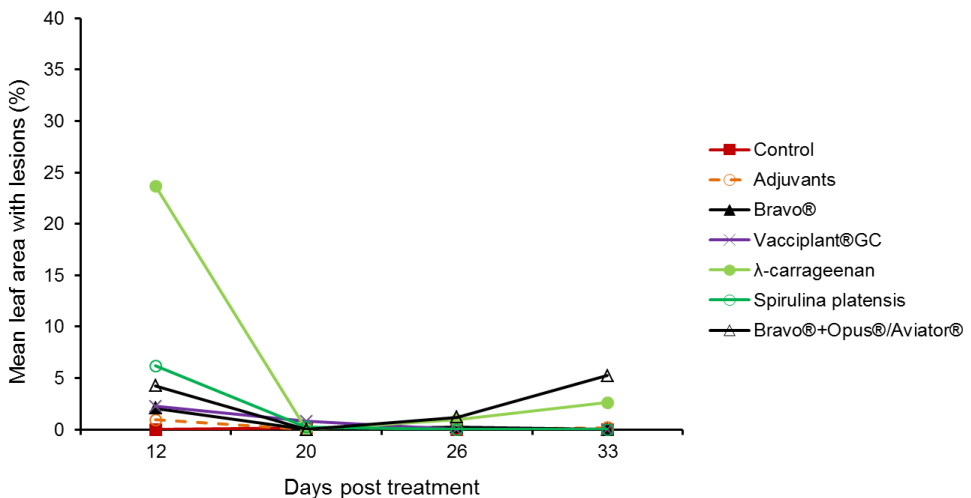
Figure 53. Severity of *Septoria tritici* Blotch infection on winter wheat crops in the field in 2016-2017. Disease pressure of *Z. tritici* was drastically low on all treated crops due to unfavorable weather conditions for the pathogen. On the other hand, rust infection was more considerable. Values correspond to the average percentage of symptomatic lesions scored on wheat leaves at various days after the last treatment.

4. Discussion

The results of the 2016 field trials realized in France and Belgium showed that neither λ-carrageenan nor Spirulina significantly succeeded in protecting wheat against STB. However, the elicitor reference Vacciplant®GC failed as well to protect the crop and only fungicide-treated plots managed to be protected against the disease. On the other hand, the low STB disease pressure during the 2017 field trial made it impossible to conclude for certain whether a given treatment was efficacious or not.

The 2016 field results could be explained by the exceptional weather conditions of that year, which strongly favored high STB disease pressures on wheat crops in France and Belgium. Warm temperatures during winter combined to important and constant rainfall throughout spring were extremely favorable for the development of multiple diseases (Colart et al., 2016).

The winter wheat production in both countries was thus at its lowest since 30 years. Losses of about 25 quintals ha^{-1} were registered, amounting to 36% of the total yield (Maufras & Maumené, 2016). As it turns out, the 2016 harvest season was so poor that France lost its leader position of wheat exporter in Europe (Le Revenu, 2016). Under these circumstances, the use of fungicides actually prevented massive yield losses, thus averting worse scenarios.

Conversely, the 2017 field results obtained in Belgium were the consequence of a very low STB disease pressure. A pervasive water deficit since autumn, coupled with excessive temperatures in spring have made conditions in the field unfavorable for STB development and crop diseases in general. Since the end of May, temperatures have regularly risen above 25 °C and even 30 °C during the grain filling phase. As a consequence, the mean percentage of *Z. tritici* symptoms scored on control wheat leaves was extremely low and the protective effect of biocontrol treatments could not be stated. Plants were mostly infected by brown and yellow rust.

What's more, the 2016 field results highlighted that the use of a combination of fungicides better protected wheat compared to the use of a single contact fungicide like Bravo®. The combination of fungicides actually offered the double advantage to target multiple diseases and to slow down the development of pathogen resistance in the field. The product Opus®Team contains both epoxiconazole (84 g l^{-1}) and fengipropimorph (250 g l^{-1}), while Aviator®Xpro contains bixafen (75 g l^{-1}) and prothioconazole (150 g l^{-1}). Epoxiconazole is a broad-spectrum systemic triazole fungicide commonly used to control diseases such *Septoria tritici* Blotch, brown rust and yellow rust. It is a 14α-demethylase inhibitor (DMI), thereby inhibiting ergosterol biosynthesis and preventing fungal development. Fengipropimorph controls early mildew and rust infection and enhances the uptake of epoxiconazole into the plant leaves. Finally, bixafen is a pyrazole fungicide belonging to the third generation of succinate dehydrogenase inhibitors (SDHIs), while prothioconazole is a broad-spectrum systemic fungicide belonging to the group of DMIs.

5. Conclusion

Numerous parameters, among which environmental conditions, plant developmental stage, plant genotype and disease pressure, can cause a variability of elicitor protection efficacy when shifting from the greenhouse to the field (Ozeretskovskaya & Vasyukova, 2002; Walters et al., 2013). Especially, the high contrast of disease pressures in the field over two subsequent years made it difficult to assess the protective efficacy of λ-carrageenan and spirulina. The gap between greenhouse and field results was clearly noticeable and backs up previous studies (Reglinskli et al., 2007; Gozzo & Faoro, 2013; Walters et al., 2013).

As already stated, elicitors are not to be used as stand-alone treatments in the field (Walters et al., 2013; Le Mire et al., 2016). It would therefore be interesting to repeat the same field experiment another year where STB disease pressure are more satisfactory for experimentation.

Moreover, it would be interesting to test λ-carrageenan and spirulina in half-dose with fungicides in order to have a clearer idea of their potential for IPM strategies. To conclude, it is likely that hot and dry summer followed by mild and wet winter, interspersed with extreme weather conditions, will happen again in the future. Such climatic scenario is a characteristic of climate change and requires some adaptations in terms of agricultural practices and research programs.

5. Evaluation of the potential effect of the formulation on the eliciting activity of λ-carrageenan and Spirulina

1. Introduction

In the same way as fungicides, elicitors are to be formulated appropriately depending on their physical characteristics, the application method, and the plant on which they are to be applied (Wang & Liu, 2007). A formulation is a mixture of active and inert ingredients. In the present case, the active ingredient is the elicitor compound that will induce plant natural defenses, and the inert ingredients (also called adjuvants or co-formulants) are used to dilute the active ingredient or make it safer/easier to handle or even more effective (Mullin et al., 2016). The requirement of a formulation is based on the fact that active ingredients can have different physicochemical properties (*e.g.* molecular size and lipophilicity) and that their foliar uptake greatly depends on plant species. The properties of the leaf indeed play an important role in the penetration of a solution into the plant. Foliar uptake corresponds to the diffusion process of the active ingredient across the leaf epicuticular wax, the cuticle and the plasma membrane of epidermal cells altogether (Wang & Liu, 2007). On the other hand, penetration through the stomata is possible by adding an organosilicone surfactant which enables the aqueous solution to have a surface tension as low as 22 mN m^{-1} (Wang & Liu, 2007). Stomatal uptake thus depends on the plant species and the concentration of silicone surfactants (minimum 0.5 %) used in the formulation. Such uptake presents the dual advantage of being a direct route into the plant and of occurring quickly (within 10 minutes after application).

In the present study, wheat is considered as a difficult-to-wet plant species, thereby representing a challenging target for the efficient application of protective treatments (Massinon & Lebeau, 2013). The objective in this thesis was thus to use standard adjuvants in order to ensure that all the tested elicitor compounds were well solubilized and homogenized in the formulation and that they were well applied on the surface of wheat leaves. Therefore, the adjuvants selected for formulation consisted of the solubilizing agent Tween 20 (polyoxyethylene-sorbitan monolaurate, Sigma Aldrich) and the wetting agent Break-Thru®S240 (polyether trisiloxane, Evonik Industries).

Tween 20 is a non-ionic detergent widely used as an emulsifying agent for the preparation of stable emulsions, while Break-Thru®S240 is a non-ionic trisiloxane surfactant commonly used as a super spreading and super penetrant agent for the formulation of crop protection products. While Tween 20 enables the elicitor formulation to be homogenous, the Break-Thru adjuvant allows a large amount of the active ingredient to enter the plant by lowering drastically the surface tension of the treatment solutions below 25 mN m^{-1}. Aqueous solutions containing this adjuvant thus have a zero contact angle with the leaf surface in contrast to water alone or solutions containing conventional surfactants (Figure 54).

Without surfactant
72 mN m^{-1}
110° angle

Conventional surfactant
31 mN m^{-1}
58° angle

Break-Thru® S240
22 mN m^{-1}
0° angle

A: stomata   B: epidermis   C: intracellular space
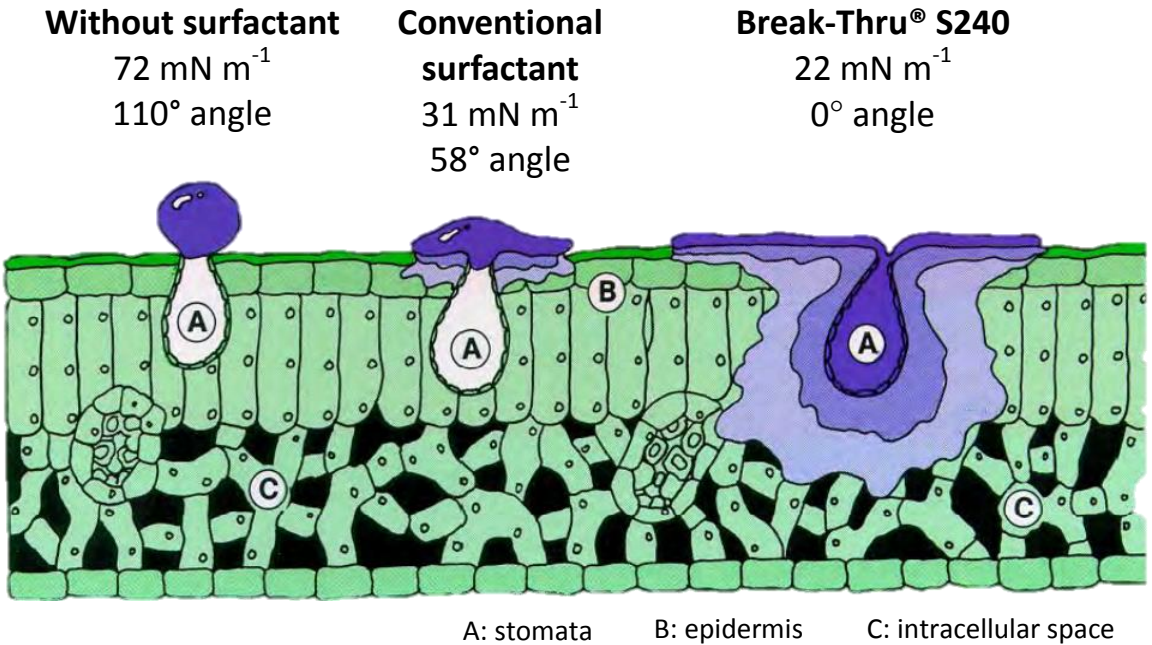
Figure 54. Cross section of a leaf with spray droplets (with and without surfactants) on its surface. (Source: Isagro Italia)

Despite all the benefits provided by the use of adjuvants, it is mandatory to check that these "inert" compounds do not have an effect on the plant or on the targeted pathogen.

The potential influence of Tween 20 and/or Breath-Thru®S240 need to be tested in order to ensure that the protection efficacy of wheat by the different elicitor formulations are actually due to the elicitor compounds themselves.

In the present study, we thus evaluated the ability of formulated and non-formulated solutions of λ-carrageenan and *Spirulina (Arthrospira) platensis* to protect the plant against *Z. tritici* under greenhouse conditions, compared to solutions consisting of adjuvants alone. In other words, we ensured that the adjuvants did not exert any elicitor effect on wheat.

2. Materials and methods

2.1 Plant and fungal materials

The experiments were conducted on wheat (*Triticum aestivum* L.) plants of the susceptible cv. Avatar. Seeds were sown in 25 x 15 cm plastic pots filled with loam (10 plants per pot). Plants were grown in the greenhouse under semi-controlled conditions (natural photoperiod supplemented with artificial light if needed, with 20 °C ± 5 according to the sunlight).

The *Z. tritici* strain T01187 (isolated in 2009 from Northern France) was used for plant inoculation. Fungal culture was performed on potato dextrose agar (PDA) medium for eight days at 18 °C with a 12 ⁄ 12-h day ⁄ night cycle. Inocula were prepared by washing the cultures with 10 mL sterile distilled water and the resulting spore suspension was adjusted to desired concentrations using Malassez cell.

2.2 Treatment preparation

The compounds λ-carrageenan and *Spirulina (Arthrospira) platensis* were tested at 5 g l^{-1} and 3 g l^{-1} respectively. In order to test for potential formulation effects, two different treatment solutions were prepared with each compound: one treatment consisted of λ-carrageenan or Spirulina diluted in distilled water without any formulation; another treatment consisted of λ-carrageenan or Spirulina added to distilled water supplemented with 0.1 % (*v/v*) of spreading agent Break-Thru®S240 (polyether trisiloxane, Evonik Industries) and 0.05 % (*v/v*) of solubilizing agent Tween20 (polyoxyethylene-sorbitan monolaurate, Sigma Aldrich). λ-carrageenan treatments were heated up to 80 °C for 15 min before adding the adjuvants, in order to accelerate the homogenization of the elicitor compound in the solution.

In addition, two additional treatment solutions containing only the adjuvants were prepared. Such treatments will allow evaluating if the protection efficacy of formulated elicitor treatments is potentially related to the effect of adjuvants alone, or even to an additive/synergetic effect of elicitors and adjuvants applied together. One treatment consisted of the adjuvants at the same doses usually used to homogenize the elicitor compounds in water: 0.1 % (*v/v*) of Break-Thru® and 0.05 % (*v/v*) of Tween20. Another treatment was prepared with the same adjuvants but with Break-Thru® at a lower dose: 0.05 % (*v/v*) of Break-Thru® and 0.05 % (*v/v*) of Tween20.

Control plants were treated with distilled water alone. In addition, other control plants were treated respectively with 0.75 % (*v/v*) of the epoxiconazole-based fungicide Opus® (BASF Agro, France) or with the synthetic elicitor BION®50WG (Syngenta, Europe) at 0.6 mg.mL^{-1}.

Finally, it is noteworthy that preliminary *in vitro* biocidal tests were realized according to the method of Siah et al (2010b) to assess the potential fungicidal effect of the adjuvants. Our objective was indeed to formulate properly the elicitors in water by using adjuvants which do not interfere.

Therefore, the goal of such preliminary tests was to ensure that the selected solubilizing agent and wetting agent did not have a direct impact on the pathogen. As it turns out, none of the two adjuvants, whether together or alone, had a direct effect on *Z. tritici* germination and growth when tested *in vitro* at concentrations similar to that used in greenhouse trials (results available in appendix 2).

2.3 Plant treatment and inoculation

Plant treatment and inoculation were realized similarly to greenhouse screening trials. Treatments were realized when the wheat plants reached the three-four leaf stage (third leaf fully expanded – Z13). For each treatment, the plants of five pots were sprayed to run-off with 30 ml of the corresponding solution using a hand sprayer.

Plant inoculation was performed five days after treatment by spraying the plants of each pot to the limit of run-off with 30 ml of a spore suspension (10^6 spores ml^{-1} in distilled water) amended with 0.05 % (*v/v*) Tween 20.

Immediately after inoculation, each pot was covered with a transparent polyethylene bag for three days in order to ensure water-saturated conditions compatible with spore germination.

The disease level was scored at 28 days post-inoculation by measuring the percentage of the third leaf area covered with symptomatic lesions (necrosis and chlorosis) bearing pycnidia. A single experiment was run in the greenhouse, with 40 technical repetitions (plants) per treatment. Results were analyzed with linear mixed effect models (Gałecki & Burzykowski, 2013). ANOVA and the Tukey multiple comparison procedure at $P = 0.05$ were used to compare the mean disease severity of the plants treated with the different products. Reported values correspond to the average infection levels of the treated plants.

3. Results

We evaluated the potential influence of the formulation on the efficacy of λ-carrageenan and spirulina treatments to protect wheat against *Z. tritici*. Such experiment is crucial to check that the protection efficacy is truly attributable to the elicitor compound rather than to the adjuvants alone. By applying adjuvants alone at different concentrations or the two elicitor compounds with and without these adjuvants, we may also be able to detect potential additive/synergetic effects. The mean disease severity corresponds to the mean percentage of *Z. tritici* lesions scored on the surface of wheat leaves which were sprayed with one of the treatments.

As a result, a mean disease severity of 29 % was scored on the water control at 28 days after inoculation (Figure 55).
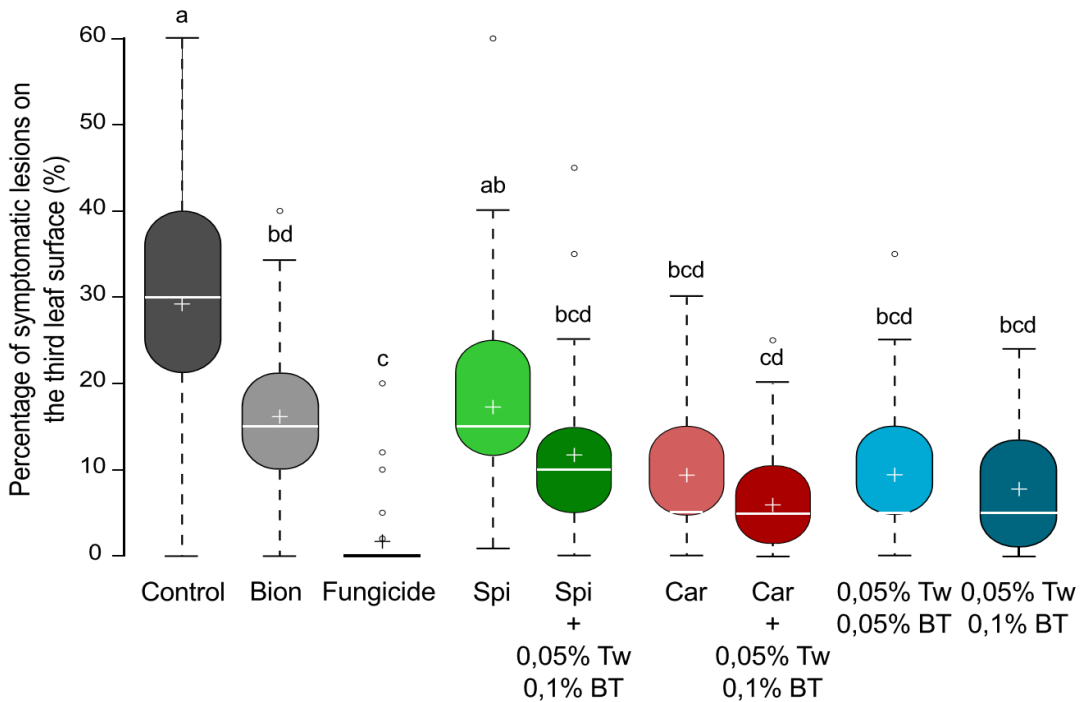
Figure 55. Efficacy of elicitor treatments (formulated or not) to protect wheat against the disease *Septoria tritici* Blotch (STB) under greenhouse conditions. Except for plants sprayed with spirulina alone, all other wheat plants which received a treatment showed significantly reduced *Z. tritici* disease symptoms compared to the Control. Data are the percentages of the third leaf surface of plants exhibiting symptomatic STB lesions (necrosis and/or chlorosis). Medians are represented by white horizontal lines in each box, and means are represented by "+" symbol ($n = 40$, *e.g.*, five pots of eight plants per treatment). Boxes tagged with the same letters correspond to means that are not significantly different using the Tukey test at $P = 0.05$. Treatments corresponded to: adjuvants alone consisting of solubilizing agent Tween20 (Tw) with different concentrations of wetting agent BreakThruS240 (0.05 %Tw + 0.05 %BT ; 0.05 %Tw + 0.1 %BT); elicitors alone consisting of λ-carrageenan (Car) or spirulina (Spi); formulated elicitor λ-carrageenan (Car + 0.05 %Tw + 0.1 %BT) or Spirulina (Spi + 0.05 %Tw + 0.1 %BT).

Plants treated with the fungicide epoxiconazole or the elicitor reference Bion were significantly (*p-value* < 0.01) protected against *Z. tritici*: they showed an average disease severity of 2 % and 16 % respectively. Similarly, plants which were sprayed with a solution of λ-carrageenan formulated or not were significantly protected against the pathogen compared to the control (mean disease severity of 9 % and 6 % respectively). Similarly, treatments consisting of adjuvants alone, whether with 0.05 % or 0.1 % of wetting agent, significantly protected wheat against the disease compared to the control (mean disease severity of 8 % and 9 % respectively). Formulated spirulina also offered a significant protection against the pathogen (12 % mean disease severity).

Conversely, plants treated with spirulina alone showed an average disease severity of 17 % which was not significantly different to the control (*p-value* = 0.06).

Overall, plants sprayed with the various treatments showed a significant reduction of disease symptoms of *Z. tritici* compared to the control ($P = 0.05$). The sole exceptions were plants which were sprayed with spirulina alone.

4. Discussion

The protection efficacy of formulated and unformulated elicitor solutions revealed that λ-carrageenan remains an efficacious plant protector against *Z. tritici* even without the help of adjuvants. On the other hand, spirulina lost its efficacy when applied alone on the plant. Such result raises the question whether the previous greenhouse and biomolecular results obtained for the spirulina formulation were actually attributable to spirulina itself. Results showed that spirulina-treated plants had a disease severity comparable to control plants on one hand, but also comparable to Bion-treated plants on the other hand. It is clear that by undertaking similar experiments of that kind several times in the greenhouse, and with even more plant repetitions, we might at last know for good on which "side" of the balance spirulina lays. For now, we may postulate that an appropriate formulation seems necessary to allow this compound to reach the plant. The most puzzling matter which stands out from the present results is the fact that the application of a combination of adjuvants alone efficaciously protected the plant, similarly to formulated elicitor treatments.

Since preliminary *in vitro* results revealed that these adjuvants do not display any fungicidal activity towards the pathogen, the underlying question would now be: do these adjuvants act as elicitors of wheat defenses? If so, the very use of compounds such as spirulina and λ-carrageenan could be called into question since affordable surfactants available on the market could do the job just as well. The answer to such question would require biomolecular tests in order to check if defense-related genes of wheat are expressed in the plant following treatment with these very adjuvants. And if not an elicitor activity, than what could possibly explain such results? The constitution of a 'security' layer on the surface of wheat leaves which would prevent *Z. tritici* spores to properly germinate?

For now, let's have a step back at the current knowledge regarding the adjuvants used in this study.

BreakThru®S240 (abbreviated from now on to "BT"), is an organo-trisiloxane surfactant which is considered today as one of the most exceptional super spreading agents due to its wetting ability (Radulovic et al., 2009). Extensive research on this type of non-ionic silicone surfactant started in the early 90s. The superiority of trisiloxane surfactants over conventional surfactants such as Triton® X-100 was proven on a number of surfaces with varying degrees of hydrophobicity. However, it appears that the underlying mechanisms of trisiloxane absorption and diffusion are complex and only partially understood (Wang & Liu, 2007; Radulovic et al., 2009). This also seems to apply to the unintended effects of such "inert" ingredient.

A previous field trial realized by Evonik Industries showed that BT could increase the efficacy of the fungicide cyproconazole against brown rust in winter wheat (http://www.break-thru.com/product/break-thru/Documents/alto-cyproconazole_with_break-thru_s_240_in_france.pdf). However, there was no mention of the effect of such adjuvants applied alone on wheat protection. Moreover, recent articles have called for caution concerning the use of trisiloxane surfactants such as BT in agriculture, pointing out the lack of risk mitigation of spray tank mix adjuvants, notably in the USA (Mullin et al., 2016). For instance, these adjuvants can be used as insecticides on their own: three trisiloxanes in aqueous solutions (*i.e.* Silwet L-77, Silwet 408 and Silwet 806) were shown to be toxic to twospotted spider mites (Cowles et al., 2000). In such study, the insecticidal activity of trisiloxane surfactants may have been desired, but what about the detrimental toxicity outcome on non-target species? And to take it a step further, what about their unintended effects on the plant itself? Those spray adjuvants are largely assumed to be biologically inert, although their super-spreading and super-penetrant properties make them likely to readily move across membranes and become systemic in plants and animals (Mullin et al., 2015; Mullin et al., 2016).

Concerning the other adjuvant, Tween 20, it is one of the most frequently used non-ionic surfactants in food (World Health Organization, 1973), flavor, fragrances (Baydar & Baydar, 2005), and cosmetics. This adjuvant presents the advantage of being stable and relatively non-toxic. Still, some studies report that Tween 20 can influence both plant growth and defenses.

The application of high concentrations of Tween 20 at 2 % was shown to cause chlorotic and necrotic foliar lesions as well as a reduction in the rate of plant transpiration and assimilation (Noga et al., 1986). Another study demonstrated that the application of Tween 20 at 0.2 % was toxic to the plant and induced the transcription of the genes *OPR1* (12-oxo-phytodienoate reductase) and *OPR2* (Hunzicker, 2006). It was suggested that the fatty acids present in Tween 20 were responsible for such activation of plant defense genes, notably specific genes related to the flg22 response. Moreover, the detergent nature of Tween 20 applied at high concentrations may alter the plant cell membranes, thereby releasing endogenous plant elicitors.

5. *Conclusion*

Formulation technology is becoming increasing important today in the prospect of sustainable agricultural practices. Innovative formulations are required to guarantee the effective and safe application of protective products on cultivated plants at a time when the introduction of new active ingredients is greatly limited. The goal is indeed to reduce residue levels in the field, reduce the rate of chemical inputs, and ensure the safety of farmers, consumers and more broadly the environment. The choice of the adjuvant in an agrochemical formulation is thus crucial (Castro et al., 2013). Formulations are essential for the preparation and maintenance of the long-term physical stability of an agrochemical, and to enhance its biological performance.

It is clear that the development of biocontrol products which are supposed to be non-toxic and environment-friendly would require the use of safe and sustainable adjuvants for their formulation. New surfactant formulations are thus being developed by suppliers. For instance, natural products such as vegetable oils, lecithin, sugars and amino acid are becoming increasingly popular (Castro et al., 2013). Similarly, new formulation technologies are emerging, such as microemulsions, liposomes and nanoemulsions. Therefore, the use of Tween 20 and Breakthru®S240 as adjuvants in the present elicitor formulations can be debatable, especially in the prospect of elicitor screening. At this stage, we cannot exclude the possibility that BT and Tween 20 somehow contributed to protect wheat against *Z. tritici*. Still, λ-carrageenan showed promising results when applied on its own, and it would thus be interesting to develop an adapted formulation for this compound in order to increase its efficacy on wheat and improve its topical spraying performances in the field.

6. Evaluation of the potential phytotoxicity of formulated λ-carrageenan and Spirulina treatments

1. Introduction

The effect of the formulation was assessed on wheat in an effort to understand whether its protection efficacy was attributable to the tested compounds λ-carrageenan and spirulina or not. In the same vein, we wondered if the elicitor formulation had an influence on the plant's physiology. As previously mentioned in the chapter dedicated to field results, the direct induction of plant defenses following an elicitor treatment can incur allocation costs detrimental to the plant's growth and development (Walters & Heil, 2007). For instance, the chemical elicitor Bion®50WG is composed of Acibenzolar S-methyl which was able to reduce the growth and seed yield of wheat plants in the absence of pathogens (Heil et al., 2000).

The objective of this last study was thus to investigate the influence of formulated λ-carrageenan and spirulina, as well as adjuvants alone, on the growth of wheat plants under greenhouse conditions.

2. Materials and methods

2.1 Plant material

The experiments were conducted on wheat (*Triticum aestivum* L.) plants of the susceptible cv. Avatar.

Seeds were sown in 25 x 15 cm plastic pots filled with loam (10 plants per pot) and plants were grown in the greenhouse under semi-controlled conditions (natural photoperiod supplemented with artificial light if needed, with 20 °C ± 5 according to the sunlight).

2.2 Treatment preparation

The compounds λ-carrageenan and *Spirulina (Arthrospira) platensis* were tested at 5 g l^{-1} and 3 g l^{-1} respectively. Treatments consisted of λ-carrageenan or Spirulina added to distilled water supplemented with 0.1 % (*v/v*) of spreading agent Break-Thru®S240 (polyether trisiloxane, Evonik Industries), and 0.05 % (*v/v*) of solubilizing agent Tween20 (polyoxyethylene-sorbitan monolaurate, Sigma Aldrich).

λ-carrageenan treatments were heated up to 80 °C for 15 min before adding the adjuvants, in order to accelerate the homogenization of the elicitor compound in the solution.

In addition, a supplementary treatment was prepared which contained only the adjuvants: 0.1 % (*v/v*) of Break-Thru® and 0.05 % (*v/v*) of Tween20. Such treatment will allow to evaluate if the adjuvants potentially exert a detrimental effect on wheat growth at the corresponding doses. Control plants were treated with distilled water alone.

2.3 Plant treatment and leaf sampling

Plant treatment was realized similarly to greenhouse screening trials. Treatments were realized when the wheat plants reached the three-four leaf stage (third leaf fully expanded). For each treatment, the plants of nine pots were sprayed to run-off with 30 ml of the corresponding solution using a hand sprayer.

From the first day of treatment till 5 days later, we measured the percentage of the third leaf surface covered with phytotoxic symptoms (necrosis and/or chlorosis). In addition, leaf sampling was realized 1, 2 and 3 weeks after treatment. At each sampling date, and for each treatment, we sampled all the leaves of plants grown in three pots (10 plants per pot). Two independent experiments were run in the greenhouse, with 30 technical repetitions (plants) per treatment. The leaf dry weight (mg) of each plant was measured after drying in the oven at 80 °C during 72 hours. Results were analyzed by ANOVA followed by the Tukey multiple comparison test at $P = 0.05$.

3. Results

None of the treated plants showed phytotoxic symptoms, even after a few days after treatment. On the other hand, we compared the mean leaf dry weight of treated plants with the control from 1 to 3 weeks after treatment application (Figure 56).
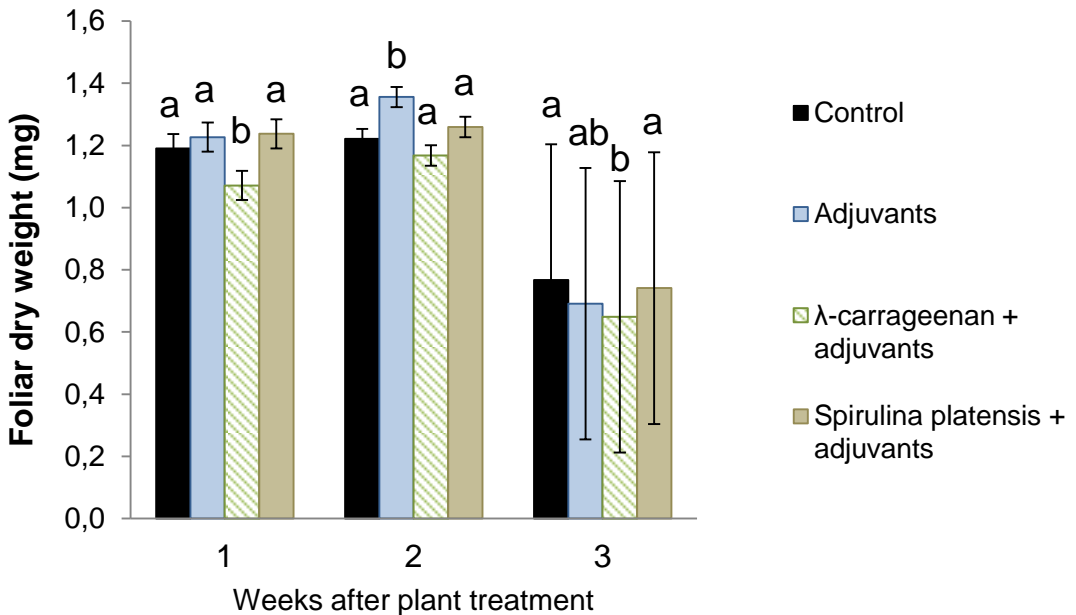
Figure 56. Mean foliar dry weight of wheat plants at 1, 2 and 3 weeks after application of various treatments: formulated elicitors, adjuvants alone, and water only (control). Means tagged with the same letters are not significantly different according to the Tukey test at $P = 0.05$

At 1 and 3 weeks after treatment, the mean foliar dry weight of plants which were sprayed with the λ-carrageenan formulation was significantly (p-$value$ < 0.001) redcued compared to the control: 1.1 and 0.6 mg respectively for the formulation, compared to 1.8 and 0.8 mg respectively for the control. On the other hand, the average leaf dry weight of plants sprayed with the spirulina formulation was always comparable to the control and even slightly greater at 2 weeks after treatment (1.3 mg). Finally, the mean leaf dry weight of plants sprayed with adjuvants (1.4 mg) was significantly (p-$value$ = 0.02) different compared to that of the control (1.2 mg) after 2 weeks. Overall, the λ-carrageenan formulation was the only treatment which appears to have negatively impacted wheat foliar growth.

4. Discussion

From the results, we can clearly see the importance of running phytotoxicity tests since we surprisingly discovered that the wheat leaf biomass was significantly reduced for plants treated with formulated λ-carrageenan compared to the control. It is however unfortunate that treatments consisting of λ-carrageenan or spirulina alone were not tested as it would have provided interesting information. Anyhow, such results are contradictory to the findings of previous studies. Indeed, Bi et al (2011) reported that spray applications of λ-oligocarrageenans resulted in increased plant growth and cell division in tobacco. Similarly, Muñoz et al (2011) demonstrated that the application of λ-oligocarrageenans on tobacco leaves increased the plant height, leaf biomass, chlorophyll content and photosynthetic activity. Furthermore, λ-carrageenan combined to heat stress was shown to stimulate the embryogenesis of broccoli microspores (Lemonnier-Le Penhuizic et al., 2001). Finally, λ-carrageenan and its derived oligosaccharides were reported to stimulate the growth of plants and trees by enhancing carbon and nitrogen assimilation, basal metabolism and cell division (González et al., 2013).

In the present study, the contrasts in mean foliar biomass between control plants and plants treated with formulated λ-carrageenan may be significant but remain small. It would actually be interesting to repeat such experiment over a longer period of time in order to have a better idea of the long term influence of the various treatments on plant growth.

Finally, the objective of such study was to investigate the potential influence of elicitor treatments on the plant's health. Hence, measuring the plant foliar biomass would seem unsatisfactory to fully answer such question. Since the overall aim is to detect if the plant growth and development remain unchanged or not, it would probably have been more meaningful to focus on additional physiological characteristics, such as the foliar photosynthetic activity, the plant developmental stage, or even the root biomass.

5. Conclusion

The induction of plant resistance by an elicitor can go hand in hand with an effect on the plant's growth and development. For instance, chitosan was shown to increase the shoot height and shoot fresh weight of tomato plants while at the same time inducing the plant's defense mechanisms against Ralstonia wilt (Algam et al., 2010). Such "green" effect would have been particularly interesting in the present study in combination to a biocontrol effect, but it apparently wasn't the case. It even appeared to be quite the opposite concerning wheat plants treated with λ-carrageenan.

To get to the bottom of such investigation, it would be good to realize measurements of plant shoot height, shoot fresh weight and photosynthetic activity on a weekly basis after elicitor treatment, and under uncontrolled conditions in the field. The final focus being: the yield.

5

CONCLUSION AND PERSPECTIVES

"Science cannot solve the ultimate mystery of nature.

And that is because, in the last analysis,

We ourselves are part of the mystery that we are trying to solve"

Max Planck

1. Screening for elicitors of wheat against *Z. tritici*: general conclusions

In the prospect of increased agricultural sustainability, the stakes are high in the European Union for the research community as regulatory bodies are requiring a drastic reduction of the use of chemical plant protection products. Intensive research is therefore dedicated today to the identification of innovative elicitors of plant defenses as alternative products to be used in integrated pest management strategies in combination to reduced-rate fungicides. The urgent need for such alternatives concerns major cultivated crops such as wheat. In this framework, the present study was focused on the identification of interesting elicitors to control the challenging *Septoria tritici* Blotch (STB) disease. Overall, the goal was to identify innovative elicitor compounds which could later be interesting to be developed and registered as formulated biocontrol products for the preventive control of STB in wheat crops. Indeed, only few biocontrol products are currently available for that purpose besides the laminarin-based product Vacciplant®GC (Goëmar) or the chemical elicitor product Bion® (Syngenta).

The objective of this study was thus to select compounds which had already showed elicitor properties on other plants or even on animals in the bibliography and evaluate if they also behaved as inducers of wheat resistance under semi-controlled conditions in the greenhouse, and finally under practical conditions in the field. Five potential elicitor compounds were thereby selected for screening trials: λ-carrageenan (algae extract), CpG-ODN (synthetic DNA fragments with CpG motifs), Spirulina (Arthrospira) platensis (dried cyanobacteria), glycine betaine (plant osmolyte) and ergosterol (component of fungal plasma membranes). In addition, the subsequent triggered defense-signaling pathways were investigated in the plant in order to better understand their mode(s) of action.

On the basis of findings obtained throughout this study, a few questions raised in the section "Thesis objectives" (page 65) have been addressed:

- **Do the elicitor candidates effectively protect winter wheat against the pathogen *Z. tritici* under greenhouse conditions?**

  Greenhouse screening trials on the five candidate elicitors revealed that each formulated compound effectively contributed to protect the wheat plant against the pathogen; formulated treatments with λ-carrageenan, *Spirulina* (*Arthrospira*) *platensis*, CpG-ODN, glycine betaine and ergosterol were all efficacious in protecting wheat by up to approximately 70 % against *Z. tritici*.

  Such findings are crucial as they allow the select which candidates are worth keeping for further studies on their elicitor potential (mode of action and treatment positioning) based on their efficiency to protect the plant against the targeted disease. To be noted that during the fist screening experiments in the greenhouse, we had also investigated the protection efficacy of treatments containing only adjuvants diluted in water against

STB. Under conditions of low disease pressure, such "adjuvant" treatments did not control wheat infection by the fungal pathogen.

- **Is such protection efficacy attributable to a direct biocidal effect of the tested compounds towards the fungi?**

  Biocide *in vitro* tests were carried out on each compound to determine their potential direct influence on the targeted pathogen.

  The assessment of *Z. tritici* spore germination and fungal growth confirmed that none of the five tested compounds had a fungicidal effect towards the pathogen at the concentrations intended for glasshouse screening trials. These results strengthen the probability that the protection efficacies recorded in the greenhouse may be attributable to an elicitor activity.

  In addition, we also confirmed that the adjuvants used for the elicitor formulation had no direct fungicidal effect on the fungal pathogen.

- **Do the elicitor candidates trigger characteristic defense-signaling pathways in the plant?**

  The relative expression of 23 defense genes was investigated in treated *versus* untreated plants by qRT-PCR. Defense responses were significantly triggered through salicylic and/or jasmonic acid signaling in treated plants. These two hormones play a key role for the transduction of danger signals throughout the plant. Biomolecular results thereby confirmed that the application of each of the five formulated compounds was perceived by the wheat plant as an elicitor by triggering characteristic defense-signaling pathways. Moreover, the results of glasshouse screening trials and biomolecular tests were positively correlated.

- **Which compounds to select for further investigations?**

  λ-carrageenan and spirulina were selected as preferential elicitor candidates by taking into account the greenhouse and biomolecular results together with other criteria such as the variability of the compound protection efficacy over several tests, its formulation and its availability at a large scale on the market.

- **Are λ-carrageenan and *Spirulina* (*Arthrospira*) *platensis* efficacious in protecting wheat crops against *Z. tritici* under practical conditions in the field?**

  Field trials carried out in Belgium and France in 2016, and in Belgium a year after in 2017, did not allow to answer this question. Indeed, the high contrast of disease pressures in the field over these two subsequent years made it difficult to assess the protective efficacy of the two elicitor candidates. Such results reinforce the need to test plant elicitors under practical conditions at an early stage in research and over several years in order to demonstrate the viability of a biocontrol product.

- **Do the adjuvants used in the formulation interfere in the protection of wheat against *Z. tritici*?**

  During additional greenhouse trials, the protection efficacy of wheat by formulated elicitors, or elicitors and adjuvants alone, revealed surprising results. λ-carrageenan remained effective in protecting the plant against STB when applied on its own, but the super-spreading agent BreakThru®S240 and solubilizing agent Tween 20 contributed as well to protect wheat against the disease. Such adjuvants may thus have behaved as elicitors. These last findings clearly highlight the necessity to carefully develop an appropriate formulation early on in order to test the elicitor properties of a given compound while ruling out the possibility of interference by adjuvants.

- **Do formulated λ-carrageenan and spirulina treatments exert a phytotoxic activity on wheat?**

  Greenhouse trials were finally carried out to study the potential phytotoxicity of treatments consisting of formulated λ-carrageenan or spirulina, or the adjuvants alone. The assessment of the total dry and fresh foliar weight of wheat plants at 1, 2 and 3 weeks after treatment showed that formulated λ-carrageenan has a detrimental effect on total leaf biomass. These results are in contradiction with the findings of previous research which rather demonstrated a growth-promoting effect of this red algae polysaccharide on plants.

2. Perspectives of future research

Several conditions must be met to guarantee the successful implementation of an elicitor in agricultural practices. The first of which is to bring proof that the elicitor product is efficient and competitive. The first motivation of a farmer in using a given product to protect his crops is indeed the actual efficiency of the product in controlling the targeted disease. Elicitors are no exception and must compete with other alternative products. In addition, guarantees must be provided concerning of the product security supply as well as its optimal conditioning and storage. The product must also be easy to use at the appropriate dose, and advice must be provided to the farmers about the optimal time and frequency of application in the field. Decision-support tools must be adapted to the use of elicitor biocontrol products and made available for farmers to decide when to protect their crops preventively against a given disease. Finally, elicitors are often efficient on given plant species and cultivars, and the corresponding information must be provided to growers.

Taking all this into consideration, λ-carrageenan stand out as being particularly eligible for further investigations as we have demonstrated its efficiency in controlling the targeted STB disease on wheat in the greenhouse. Besides, its large scale use as a food additive should guarantee secure supply for agricultural purposes

on crops. However, a few additional experiments would be useful in order to better understand the mode of action of this compound and optimize its positioning in the field. A few specific issues remain to be adressed:

- Do the adjuvants BreakThruS240 and Tween 20 act as elicitors of wheat defenses by inducing the expression of defense genes?
- How to develop an appropriate formulation for λ-carrageenan?
- How long does an application of λ-carrageenan actively induce wheat defense mechanisms?
- What occurs in the plant at the proteomic and metabolomic level from a few seconds to a few hours after treatment with the compound?
- Does λ-carrageenan remain effective to induce defense-signaling pathways in different wheat genotypes?
- Do environmental factors such as temperature and humidity have an effect on the protection efficacy and elicitor potential of the tested compound?
- Does λ-carrageenan remain effective to protect wheat in the field?

1. Deciphering the mode of action of BreakThruS240 and Tween 20 on wheat

The last greenhouse trials revealed that treatment with only the adjuvants BreakThruS240 and Tween 20 effectively protected wheat under conditions of medium STB disease pressure. Such results make it difficult to rule out the potential elicitor activity of these adjuvants and raise the question concerning the actual elicitor potential of the tested compounds. It would therefore be interesting to check with the qPFD tool if wheat plants treated with a water solution containing only the two adjuvants BreakThruS240 and Tween 20 show a significant induction of their defense mechanisms *via* the upregulation/downregulation of defense gene expression. Similarly, it would be useful to investigate the effect of the tested compounds alone (with no formulation) as well as the effect of the tested compounds formulated with "inert" adjuvants on the expression of wheat defense genes. Such investigations would allow to confirm or not the elicitor potential of the tested compounds and/or the adjuvants BreakThruS240 and Tween 20.

2. Improving the formulation for λ-carrageenan

Assuming that future research would be dedicated to the elicitor properties of λ-carrageenan on wheat, it would be useful to develop an appropriate formulation. Indeed, using adjuvants which do not exert an eliciting activity is crucial and was clearly highlighted in the present study. Moreover, λ-carrageenan is a natural hydrocolloid (water-soluble gum) which is used as a thickener in dairy products. Considering its hydrophilic and anionic properties, λ-carrageenan typically forms highly viscous aqueous solutions in cold water, and an appropriate formulation must thus be developed to ensure the proper application of the solution on the leaves of cultivated wheat. The viscosity of this polysaccharide depends on its concentration, the temperature of the solution and the presence of other solutes (Campo et al.,

2009). For instance, an increase of the concentration of λ-carrageenan allows major interactions between the linear chains, while the presence of salts decreases the viscosity by reducing the electrostatic repulsion among the sulphate groups (Campo et al., 2009). Besides, λ-carrageenan being a water-soluble polysaccharide, it is difficult to disperse in water due to the formation of a film layer around each carrageenan particle. The use of high speed mixers could be used in order to break up the lumps of λ-carrageenan formed in water. Finally, recent research has highlighted the interests of gamma irradiated carrageenan (ICA) in agriculture for foliar spraying (Singh et al., 2017). Gamma irradiation of these polysaccharides indeed results in oligomers with lower molecular weight.

3. *Characterization of defense-signaling pathways: ROS, proteomics and metabolomics*

A significant part of this project was dedicated to the study of defense signaling pathways induced in the wheat plant in response to the different elicitor treatments. The importance of such investigation for elicitor screening cannot be emphasized enough as it allows to confirm the elicitor potential of the tested compounds. Technological innovation is continuously providing Science with new and powerful tools for a better understanding of the plant itself and its interaction with the environment. The innovative INRA biomolecular tool that we used in the present case is a concrete example. Using this tool, we clearly noticed that complex networks of defense signaling pathways operate together to protect the plant at best against a stress. Plants treated with formulated λ-carrageenan showed an upregulation of both genes involved in SA- and JA-related defense responses during the first hours after elicitor treatment before giving way to SA-dependent signaling. Such positive interaction may be related to the samping time or to the plant cultivar. There is today growing evidence of a positive crosstalk between salicylic acid- and jasmonic acid-defense signaling during plant induced resistance.

However, the interpretation of biomolecular results concerning the expression of wheat defense genes remains tricky. As Ding et al (2017) pointed out SA/JA responsive genes have been well characterized in dicotyledonous plants but much less is known concerning hormone crosstalk and the involvement of specific genes in wheat induced resistance. Some genes such as PR1 which were previously regarded as typical markers of a given signaling pathway appear to be induced by both signal hormones in wheat. Although it is clear that research on induced resistance of monocots is emerging, it appears that current knowledge on wheat defense responses to elicitors is still limited (Balmer et al., 2013).

Further investigations at the biochemical and cytological level for instance are thus required to better demonstrate and understand the elicitor activity of the tested compounds, notably λ-carrageenan, on wheat. Several techniques exist today to investigate the induction of plant resistance at the metabolomic, proteomic and transcriptomic level. For instance, a list of techniques is available in the "Methodological guide for the evaluation of the mode of action of elicitors" developed by the French scientific partnership 'ELICITRA' (https://elicitra.org).

Two research approaches are possible: a holistic approach allows the evaluation of the global plant defense responses, while a targeted approach consists in evaluating the biosynthesis of given molecules or the expression of specific genes. Considering the array of defense mechanisms triggered in plants from a few second to several hours/days after elicitor recognition, monitoring the synthesis of numerous defense-related compounds over time can be useful to characterize the exact mode of action of λ-carrageenan.

Notably, reactive oxygen species (ROS) are produced within a few seconds after elicitor recognition as important modulators of the plant primary innate immunity. Following wheat treatment with λ-carrageenan, the assessment of ROS concentrations in foliar tissues could be quickly determined by colorimetric dosage with a spectrophotometer in order to detect early plant defense responses (Machinandiarena et al., 2012). An accumulation of hydrogen peroxide (H_2O_2) and superoxide anion (O_2^-) can indeed provide a first clue regarding the recognition of an elicitor by the plant.

In addition, it would be interesting to evaluate the biosynthesis of defense-related proteins such as salicylic acid, jasmonic acid, antimicrobial compounds such as phytoalexins (phenols, camalexin, stilbenes, isoflavonoids, etc), or PR proteins (PAL, POX, LOX, Chitinases, etc). Such monitoring during a time-course experiment would indeed provide useful information concerning the preferential defense-signaling pathways triggered in treated plants. To that end, metabolomic analysis can be carried out both qualitatively or quantitatively by liquid or gas chromatography (LC/MS or GC/MS) (Klarzynski et al., 2000; Massoud et al., 2012). The activity of defense-related enzymes can also be monitored by spectrophotometry or by enzyme-linked immunoabsorbent assays (ELISA). Besides, characterization of global protein biosynthesis can carried out by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) or by High-performance liquid chromatography with tandem mass spectrophotometric detection (HPLC-MS/MS) (Shah at al., 2012).

Finally, cell wall modifications could be evaluated in treated wheat leaves by monitoring callose appositions and the concentration of polyphenols and flavonoids with the help of a fluorescence or confocal microscope (Vicedo et al., 2009; Machinandiarena et al., 2012).

4. Optimizing the positioning of elicitor treatments

As mentioned previously, the induction of plant defense mechanisms occurs in a matter of seconds and can last several hours or days. However, the kinetics of induced resistance varies depending on the recognized elicitor and the studied pathosystem. In practice, elicitor treatments must be applied several times as their persistence to induce plant resistance is limited. Consequently, some elicitor products such as Fytosave® (Fytofend, Belgium) based on chito-oligosaccharides must be applied maximum 5 times in the field every 7 days to effectively protect vegetables such as tomato or cucumber preventively against the powdery mildew

disease, and maximum 8 times every 8-10 days in the case of grapevine (http://www.fytofend.com/fr/produit/belgique/fytosave).

In the prospect of applying λ-carrageenan in the field to preventively protect wheat against STB, greenhouse experiments could be carried out to characterize the optimal positioning of the elicitor treatment before STB infection. To that end, modulating the delay between the application of the product and the inoculation of the fungal pathogen would allow determining the optimal time delay for formulated λ-carrageenan to successfully protect the plant. Moreover, a time-course experiment on the expression of wheat defense genes and the activity of defense-related enzymes (*i.e.* PAL and LOX) would provide useful information concerning the persistence of wheat induced resistance following treatment with λ-carrageenan in order to optimize the number of applications required to protect the plant against *Z. tritici* in practice.

5. *Resistance induction and wheat genotypes*

The choice of the plant cultivar to be used for elicitor screening is crucial as it was demonstrated that the efficacy of an elicitor may vary depending on the genotype of the plant host (Walters et al., 2011b; Ors, 2015). Indeed, Walters et al (2011b) reported that an elicitor combination applied in the field on five different barley cultivars successfully reduced the severity of Rhyncosporum, although in varying proportions depending on the cultivar. Similarly, Ors (2015) showed the existence of an interaction between wheat genotypes and the tested elicitors.

In the present study, we carried out elicitor screening on the susceptible wheat cultivar 'Avatar'. However, it would be useful to investigate in the future if the elicitor potential of the tested compounds varies depending on the wheat genotypes, notably cultivars which are more resistant to *Zymoseptoria tritici*.

6. *Evaluation of the effect of abiotic factors on the elicitor potential*

Since various environmental parameters are thought to have an effet on the efficiency of elicitors to induce plant resistance, it could be interesting to study under semi-controlled conditions in the greenhouse the effect of drought, light, wounds, and water or nutritional stress on the elicitor potential of formulated λ-carrageenan. Various intensities of stress can be evaluated, from acute stress on a short period of time to chronic stress repeated over time (https://elicitra.org/vars/fichiers/Livrables/guide_metho_eval_SDP_elicitra_2013.pdf).

7. *Evaluation of elicitor protection efficacy in the field*

Field trials over at least 2 years should finally be carried out to evaluate the variability of the protection efficiency of formulated λ-carrageenan in controlling STB.

Field applications must take into account the results achieved concerning the optimal positioning and application frequency of the elicitor treatment, the receptive wheat genotype and the optimal environmental conditions for foliar spraying.

In the prospect of using an elicitor product in combination to reduced-rate fungicides, it would also be beneficial to apply formulated λ-carrageenan preventively on winter wheat plants at T1 (first node stage – Z31) before the appearance of STB followed by a fungicide treatment at T3-T4 (3rd node stage – Flag leaf stage), or apply formulated λ-carrageenan with half a dose of fungicide at the same time at T1 followed by a second fungicide application at T3-T4..

Overall, moving from the lab to the greenhouse, we actually demonstrated that each of the five formulated compounds had an interesting elicitor effect on the wheat plant: the expression of genes involved in induced resistance was triggered and a consistent protection efficacy of up to 70 % was observed against STB. However, the lack of an "adjuvant control" during biomolecular trials prevents us to clarify for sure whether the upregulation of defense gene expression in treated wheat may be attributable to the tested compounds, to the adjuvants, or to the combination of both. On the other hand, it was clearly demonstrated that the algae extract λ-carrageenan can effectively protect the wheat plant on its own. At this stage, λ-carrageenan thus represents the most suitable candidate as a potential biocontrol product to be used in wheat protection. The interests of using algae extracts in agriculture is not a novelty as these compounds keep revealing incredible properties (Vera et al., 2011; Popescu, 2013). A few investigations are to be carried out before λ-carrageenan can be added to the waiting list of biocontrol products for registration; notably the development of an appropriate formulation, experiments on the positioning and the frequency of the elicitor treatment, the effect of environmental parameters, and finally its protection efficacy in the field.

To date, no winter wheat cultivars are totally resistant to *Z. tritici*, while this fungal pathogen is increasingly resistant to major fungicides such as quinone outside inhibitors (QoI) and demethylation inhibitors (DMI) (Heick et al., 2017). The need for alternative plant production products is thus crucial for the sustainability of agricultural practices. In parallel to research studies on elicitors, the regulatory advantages for biocontrol products are just starting to be implemented. In Europe, the legislation is slowly putting biocontrol at the forefront in IPM strategies. Besides, the 15th of February 2017, the European Parliament adopted the resolution calling for faster access to the European market for low-risk pesticides (European Parliament, 2017). Before the end of 2018, a specific legislative proposal amending Regulation (EC) No 1107/2009 by enabling the fast-track evaluation, approval and authorization procedure for low-risk pesticides of biological origin is expected (IBMA, 2017).

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7

APPENDIX

APPENDIX 1

Reported data was obtained out of one single experiment. POX activity was measured according to (Randoux et al., 2010) with some modifications. From storage at – 80 °C, 150 mg of leaf fragments were homogenized in 3 ml of an ice-cold buffer solution consisting of 100 mM potassium phosphate (pH 7.0) with 9 mM octylthioglucopyranoside (OTG) and 2 % polyvinylpolypyrrolidone (PVPP). After homogenization for 30 min at 4 °C, the mixture was centrifuged at 12,000 x g for 15 min. The supernatant was used as crude enzyme extract and was kept at 4 °C.

The POX activity was assayed by spectrophotometry: The reaction mixture consisted of 500 μM 2,2′-azino-bis 3-ethylbenzothiazoline-6-sulfonic acid (ABTS) (electron donor), 250 μM hydrogen peroxide (H_2O_2) in 25 mM acetate buffer (pH 4.4), and the suitable volume of crude enzyme extract. The formation of the radical cation was monitored at 412 nm ($\varepsilon = 32,400\ M^{-1}cm^{-1}$).The formation of the radical cation was monitored at 412 nm ($\varepsilon = 32,400\ M^{-1}\ cm^{-1}$). Total protein concentration was determined at 595 nm using bovine serum albumin as a standard (Bradford, 1976).

Plant treatment with adjuvants only or λ-carrageenan + adjuvants (elicitor formulation) appear to increase POX activity in wheat leaves compared to the control and other treatments.
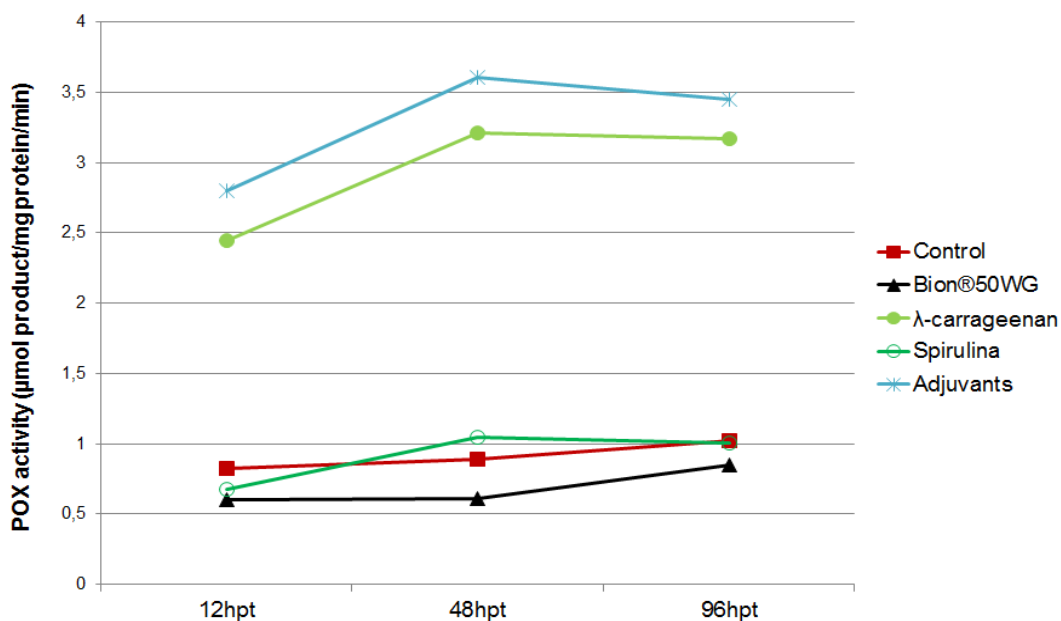
Figure 57. Peroxidase (POX) activity measured on treated plants at 12, 48 and 96 hours post-treatment (hpt).
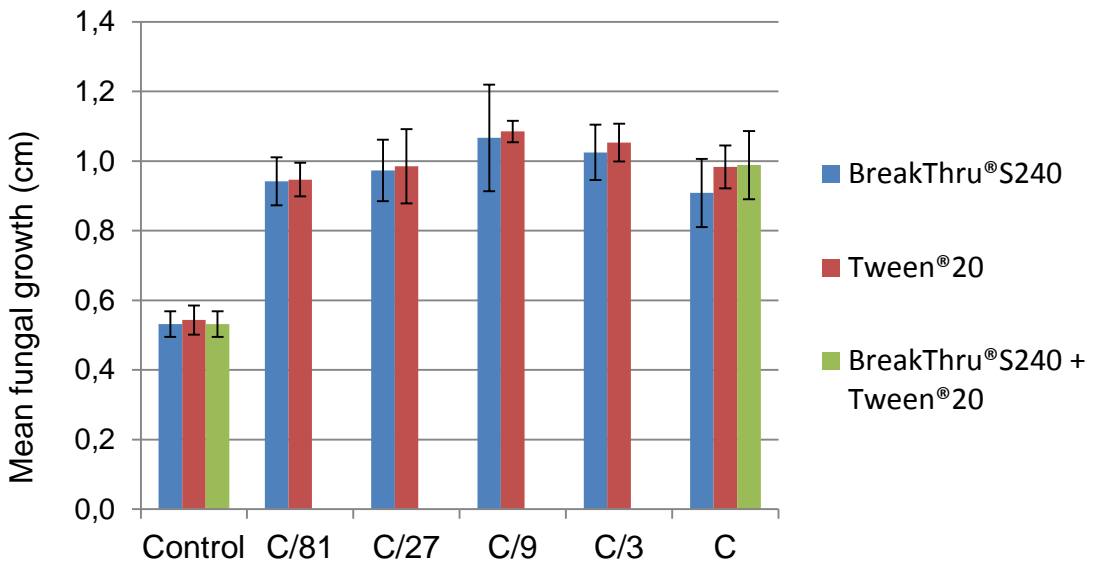
Figure 58. *In vitro* biocidal effect of the adjuvants BreakThru®S240 and Tween20 on the growth of *Zymoseptoria tritici*. Values correspond to the average fungal diameter (cm) of *Z. tritici* scored on amended PDA media. The highest concentration 'C' of each compound amended to the culture medium corresponded to: BreakThru®S40 at 0.1 %; Tween®20 at 0.05 %; BreakThru®S40 + Tween®20 (0.1 % and 0.05 % respectively). None of the compounds had a negative *in vitro* effect on *Z. tritici* growth.

APPENDIX 3

Table 5. Details on field trials 2015-2016 – Belgium

Localization	Lonzée - GxABT	
Previous crop	Beetroot	
Variety	AVATAR	
Sowing	12 - oct	250 seeds/m^2
Fertilization	23 - march	60 uN
	12 – apr	60 uN
	12 - may	75 uN
Weeding	04 - apr	Pacifica (300g) + Capri (250) + huile (1L)
Regulator	14 - apr	Meteor (2L)
Fungicide	see Field Trial Protocol – Appendix 6	
Insecticide	26 - may	Karate Zeon (50 mL/ha)
Harvest	08 - aug	
Trial design	Randomized complete block	
Soil texture	Loam soil	
Number of replicates	5	
Plot size (m^2)	16	
Number of observations/replicate	3	
Sprayer	Automatic sprayer	
General conditions at application	Favorable (Little wind; no rain)	

APPENDIX 4

Table 6. Details on field trials 2015-2016 - France

Localization	Boigneville- Arvalis	
Previous crop	Faba bean	
Variety	PAKITO	
Sowing	20 - oct	280 seeds/m^2
Fungicide	see Field Trial Protocol Appendix 7	
Harvest	26 - july	
Trial design	Randomized complete block	
Soil texture	Clay - limestone soil	
Number of replicates	3	
Plot size (m^2)	12	
Number of observations/replicate	4	
Sprayer	Automatic sprayer	
Nozzle type /Brand	Low pressure air injector /LECHLER	
Pressure (bar)	2.8	
General conditions at application	Favorable (Little wind; no rain)	

APPENDIX 5

Table 7. Field trial protocol and yield results 2015-2016 - Belgium

Object n°	19 - april		02 - may		11 - may		19 - may				Yield (kg/ha)	hL weight	Humidity
	T1 (1 Node – Z31)		T2 (2 Nodes – Z32)		T3 (3 Nodes - Z33)		T4 (Flag leaf stage)						
	Treatment	Dose (L/ha)	Treatment	Dose (L/ha)	Treatment	Dose (L/ha)	Treatment	Dose (L/ha)					
1	Control	-	Control	-	Control	-	Control	-			6120	68.4	14.6
2	Vacciplant GC	0.5	Vacciplant GC	0.5	Vacciplant GC	0.5	Vacciplant GC	0.5			6337	69.1	14.6
3	BRAVO	0.5	BRAVO	0.5	BRAVO	0.5	BRAVO	0.5			7671	71.8	14.8
4	λ-carrageenan	1.05	λ-carrageenan	1.05	λ-carrageenan	1.05	λ-carrageenan	1.05			6335	69.1	14.7
	Tween 20	0.105	Tween 20	0.105	Tween 20	0.105	Tween 20	0.105					
	BTS240	0.21	BTS240	0.21	BTS240	0.21	BTS240	0.21					
5	Spirulina	0.63	Spirulina	0.63	Spirulina	0.63	Spirulina	0.63			6202	68.9	14.6
	Tween 20	0.105	Tween 20	0.105	Tween 20	0.105	Tween 20	0.105					
	BTS240	0.21	BTS240	0.21	BTS240	0.21	BTS240	0.21					
6	Tween 20	0.105	Tween 20	0.105	Tween 20	0.105	Tween 20	0.105			6415	69.1	14.6
	BTS240	0.21	BTS240	0.21	BTS240	0.21	BTS240	0.21					
					11 – may (2 nodes)				07 - june (Heading)				
7					BRAVO	1			Aviator Xpro	1.25	9334	74.9	15.1
					Opus Team	1.5							

Table 8. Field trial protocol and yield results 2015-2016 - France

Object n°	05 - april		14 - april		28 - april		11 - may		Yield (kg/ha)
	T1 (1Node – Z31)		T2 (2 Nodes – Z32)		T3 (3 Nodes - Z33)		T4 (Flag leaf stage)		
	Treatment	Dose (L/ha)	Treatment	Dose (L/ha)	Treatment	Dose (L/ha)	Treatment	Dose (L/ha)	
1	Control	-	Control	-	Control	-	Control	-	4560
2	Vacciplant GC	0.5	Vacciplant GC	0.5	Vacciplant GC	0.5	Vacciplant GC	0.5	4540
3	BRAVO	0.5	BRAVO	0.5	BRAVO	0.5	BRAVO	0.5	6450
4	λ-carrageenan	1.05	λ-carrageenan	1.05	λ-carrageenan	1.05	λ-carrageenan	1.05	4780
	Tween 20	0.105	Tween 20	0.105	Tween 20	0.105	Tween 20	0.105	
	BTS240	0.21	BTS240	0.21	BTS240	0.21	BTS240	0.21	
5	Spirulina	0.63	Spirulina	0.63	Spirulina	0.63	Spirulina	0.63	4850
	Tween 20	0.105	Tween 20	0.105	Tween 20	0.105	Tween 20	0.105	
	BTS240	0.21	BTS240	0.21	BTS240	0.21	BTS240	0.21	

SEPTORIA LEAF BLOTCH OF CEREALS (Leaf symptoms)

Key No. 1.6.1
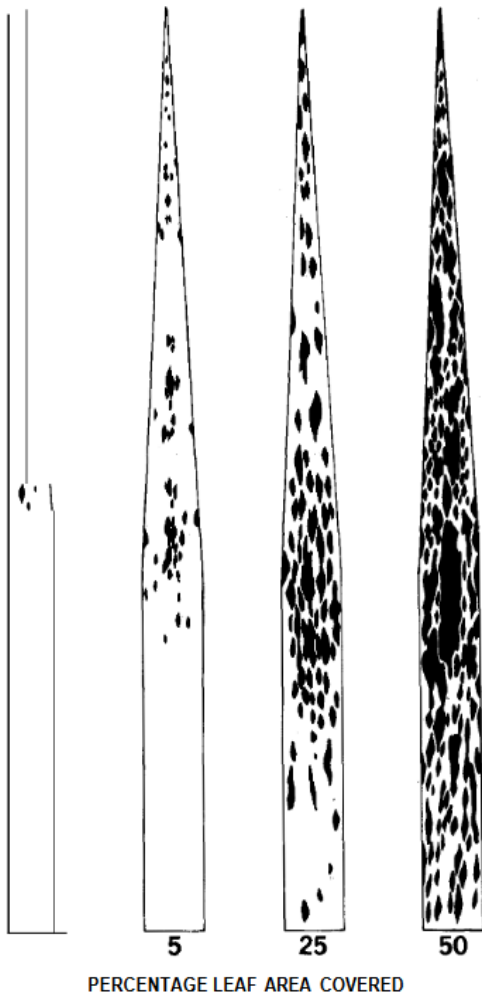
Use for:
Glume blotch of wheat *(Septoria nodorum* Berk.)
Speckled leaf blotch of wheat *(Septoria tritici* Rob. ex Desm.)
Leaf blotch of wheat *(Septoria avenae* Frank f. sp. *triticea* T. Johnson)
Leaf blotch and black stem of oats *(Septoria avenae* Frank f. sp. *avenae)*
Speckled leaf blotch of barley *(Septoria passerinii* Sacc.)

Procedure:
Select a random sample of fertile tillers.

Growth stages:
Assess at growth stages 10.5 and either 11.1 or 11.2 or both. The key can also be used for recording disease at earlier growth stages, but the growth stage and leaf position (top leaf = leaf 1) should be carefully noted, so that valid comparisons can be made between crops.

Assessing severity:
Assess percentage leaf (lamina) area affected by disease on individual top leaves.

Reference.
15

5   25   50

PERCENTAGE LEAF AREA COVERED

Figure 59. Illustrated assessment key for *Septoria tritici* Blotch. (Source: James, 1971)
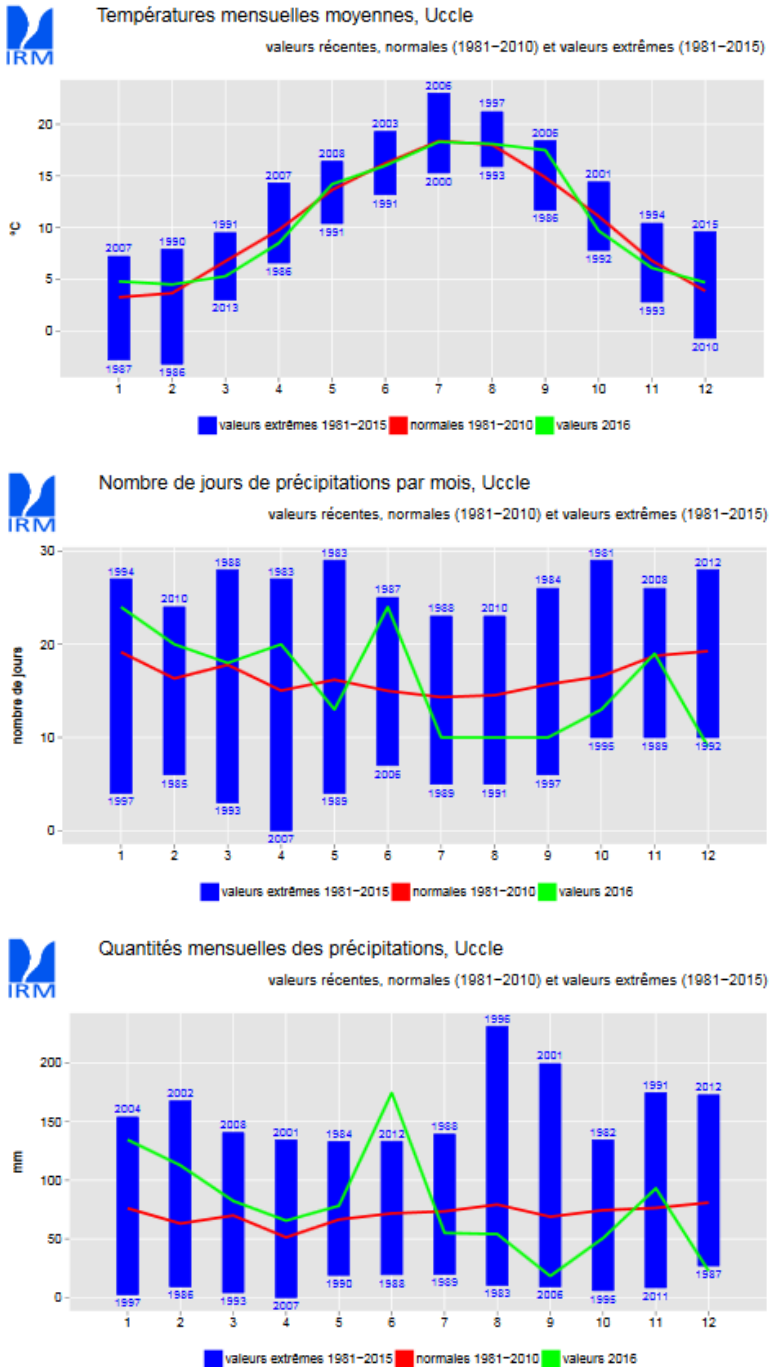
Figure 60. Comparaison of meteorological data of 2016 with monthly values since 1981 in Belgium. (Source : IRM)

The special case of surfactin

Surfactin was part of the candidates which were initially selected for elicitor properties. However, this compound was not retained as a preferential candidate due to its limited production and the time spent to obtain a homogeneous formulation. However, its interesting elicitor properties on wheat against *Z. tritici* do deserve some consideration. A specific article has thus been dedicated to the work realized on this specific compound:

Work published in th following article : Geraldine Le Mire, Ali Siah, Marie-Noëlle Brisset, Matthieu Gaucher, Magali Deleu and M. Haissam Jijakli, 2018. **Surfactin protects wheat against *Zymoseptoria tritici* and activates both salicylic acid- and jasmonic acid-dependent defense responses**. *Agriculture*, **8**(1), 11; Special issue Sustainable Crop Production Intensification. doi:10.3390/agriculture8010011

1. Introduction

Biocontrol is an alternative plant protection method which promotes sustainable agricultural practices and contributes to reducing chemical inputs. Elicitors, in particular, are promising biocontrol tools which are currently the subject of intensive research within the framework of integrated pest management (IPM) strategies (Mejía-Teniente et al., 2010; Walters et al., 2014b; Le Mire et al., 2016). They correspond to natural molecules, generally emitted by pathogens or beneficial microorganisms, which induce a non-specific resistance of the plant against a broad spectrum of diseases (Schwessinger & Ronald, 2012; Thakur & Sohal, 2013). Using elicitors as complements to fungicide applications thus offers the dual advantage of reducing the amount and application frequency of chemical inputs in the field, and of implementing sustainable plant protection methods in agricultural practices (Walters et al., 2013). Among the numerous natural elicitors which have been identified up to now, major attention is today focused on surfactin. This cyclic lipopeptide consists of heptapeptides interlinked with a β-amino fatty acid chain of varying length to form a cyclic lactone ring structure (Henry et al., 2011; Ongena & Jacques, 2008). In contrast to elicitor compounds secreted by pathogens (pathogenic-associated molecular patterns or PAMPs), surfactin is a microbe-associated molecular pattern (MAMP) which is generally produced by plant growth-promoting rhizobacteria (PGPR) belonging to specific Bacillus strains (Ongena & Jacques, 2008; Jacques, 2011; Henry, 2013; Cawoy et al., 2014). Surfactin is a powerful biosurfactant which has mostly been studied for its antagonistic and cytotoxic activity against multiple pathogens (Ongena & Jacques, 2008; Raaijmakers et al., 2010). However, research carried out in the past decade has also demonstrated that surfactin can act as an elicitor, by triggering an induced systemic resistance (ISR) of plants such as tomato, tobacco, bean, and beet against various diseases (Henry et al., 2011; Ongena et al., 2007; Jourdan et al., 2009; Desoignies et al., 2013).

Jourdan et al. (2009) reported, for instance, that the application of surfactin at micromolar concentrations on tobacco cell suspensions led to the induction of early

defense responses (ion fluxes across the plasma membrane and the production of reactive oxygen species) coupled with the activation of defense-related enzymes phenylalanine ammonia lyase (PAL) and lipoxygenase (LOX), and the production of the plant defense hormone salicylic acid (Jourdan et al., 2009). It must, however, be noted that little research has been carried out so far concerning the potential of elicitors, such as surfactin, to induce resistance of major monocotyledonous plants (Balmer et al., 2013).

In the present study, we investigated the elicitor potential of surfactin to protect winter wheat against the *Septoria tritici* blotch (STB) disease. Wheat is indeed one of the most cultivated crops in the world, with up to 734 million tons produced in 2015–2016 (Fones & Gurr, 2015; Satger, 2016). The STB disease caused by the fungal pathogen *Zymoseptoria tritici* (teleomorph: *Mycosphaerella graminicola*) represents a persistent threat each year to wheat crops all over Europe (Fones & Gurr, 2015; Torriani et al., 2015). For instance, particularly strong STB pressures inflicted drastic yield losses during the 2016 season in Northern France: losses reached around 2.5 tons per hectare, which amounts to 36% of the total yield (Maufras & Maumené, 2016). Furthermore, there are, so far, no wheat cultivars which are totally resistant to *Z. tritici*, and only the use of conventional fungicides can prevent massive yield losses (Palmer & Skinner, 2002; Fraaije et al., 2005). The development of new and efficient biocontrol tools for wheat protection is thus critical (Le Mire et al., 2016).

In this study, we evaluated, in three steps, the biocontrol potential of surfactin to induce wheat resistance against *Z. tritici*: (i) we first investigated the efficacy of surfactin in protecting wheat against STB under glasshouse conditions. Three different concentrations of surfactin were tested in order to identify possible dose-dependent effects; (ii) the potential biocidal activity of surfactin directly against the pathogen was assessed through *in vitro* sensitivity bioassays. Such assays enabled us to check whether surfactin behaved as a fungicide and/or as an elicitor at the concentrations tested during greenhouse trials; (iii) biomolecular tests were finally carried out to provide further evidence as to the elicitor potential of surfactin to induce wheat defenses. The recognition of an elicitor by the plant triggers a cascade of defense mechanisms leading to induced resistance. We thus investigated the expression of 23 defense genes of wheat in treated versus untreated plants by using an innovative biomolecular tool developed by INRA (Brisset & Duge De Bernonville, 2011; Dugé de Bernonville et al., 2014). Such tests provided useful information regarding the defense signaling pathways preferentially triggered in the plant by surfactin.

2. Materials and Methods

2.1. Plant and fungal materials

Experiments were conducted on wheat (*Triticum aestivum* L.) of the susceptible cv. Avatar.

Elicitor screening and biomolecular experiments were carried out independently. For screening experiments, seeds were sown in 25×15 cm plastic pots (10 plants per pot). For the investigation of plant signaling pathways, seeds were sown in 30×40 cm boxes (40 plants per box). In both cases, wheat was grown under greenhouse semi-controlled conditions (natural photoperiod supplemented with artificial light if needed, with 20 °C ± 5 according to the sunlight).

The *Z. tritici* strain T01187 (isolated in 2009 from Northern France) was used for plant inoculation during screening trials and during in vitro sensitivity bioassays. Fungal culture was performed on potato dextrose agar (PDA) medium for eight days at 18 °C with a 12/12 h day–night cycle.

2.2. Screening trials

▪ Treatment preparation

Surfactin consisted of a mixture of homologues (95% purity) obtained from the *Bacillus amyloliquefaciens* S499 strain and purified by solid phase extraction. A methanolic stock solution was prepared at 10 mg mL^{-1}, and surfactin was tested at three different concentrations: 0.001, 0.01, and 0.1 mg mL^{-1}, respectively. Treatment solutions were freshly prepared before use in distilled water supplemented with 0.1 % (*v/v*) of spreading agent Break-Thru®S240 (polyether trisiloxane, Evonik Industries, Marl, Germany), and 0.05 % (*v/v*) of solubilizing agent Tween 20 (polyoxyethylene-sorbitan monolaurate, Sigma Aldrich, Saint Louis, MO, USA). Control plants were treated with distilled water only. In addition, the synthetic elicitor BION®50WG consisting of acibenzolar-S methyl (Syngenta, Guyancourt, France) was used as an elicitor reference at 0.6 mg mL^{-1}.

▪ Plant treatment, inoculation, and infection level assessment

At the 3–4 leaf stage (third leaf fully expanded), the plants of each pot were sprayed to runoff with 30 mL of one of the treatment solutions using a hand sprayer. Plant inoculation was performed 5 days after treatment. Inocula were prepared by washing the *Z. tritici* cultures with 10 mL of sterile distilled water, and the resulting spore suspension was adjusted to the desired concentration using a Bürker cell. Inoculation was performed by spraying the plants of each pot to runoff with 30 mL of a spore suspension (10^6 spores mL^{-1} of distilled water) amended with 0.05% (*v/v*) of Tween20 (Sigma-Aldrich). Immediately after inoculation, each pot was covered with a transparent polyethylene bag for 3 days, in order to ensure water-saturated conditions suitable for spore germination. The disease level was scored at 28 days post-inoculation by measuring the percentage of the third leaf area covered with symptomatic lesions (necrosis and chlorosis) bearing pycnidia. Values correspond to the average infection levels scored on the third leaf of plants treated with water, surfactin or Bion. Linear mixed-effects model analysis was realized, and the Tukey multiple comparison procedure at $p = 0.05$ was used to compare the mean disease severity of the treated plants. Two independent biological experiments were performed with 40 technical repetitions (40 plants) for each condition.

APPENDIX 9

2.3. In vitro sensitivity bioassay

The potential direct effect of surfactin on *Z. tritici* fungal growth was assessed through *in vitro* bioassays. Such experiments enabled us to confirm that surfactin did not exert a fungicidal effect against the fungal pathogen, rather than an elicitor activity on wheat, at the concentrations tested during greenhouse screening trials. PDA plates were amended with different concentrations of surfactin, according to the method of Siah et al. (2010b). Surfactin was first added at the highest concentration (0.1 mg mL^{-1}) to PDA medium at 30 °C after autoclaving. It corresponds as well to the highest concentration tested in greenhouse trials.

Successive dilutions were then carried out in order to test five decreasing concentrations (0.02 mg mL^{-1}, 0.01 mg mL^{-1}, 0.004 mg mL^{-1}, and 0.001 mg mL^{-1}). The control consisted of plates containing PDA only. The plates were subsequently spotted with 5 μL of 5×10^5 spores mL^{-1} suspension. Fungal growth was scored by measuring the colony perpendicular diameters of each spot at 10 days after incubation in the dark at 18 °C. Values correspond to the average diameter of *Z. tritici* colonies scored on amended PDA media. The comparison of mean fungal growth was performed with the Tukey (ANOVA) test at $p = 0.05$. Three plates with five spots per plate were used as replicates for each condition, and two independent experiments were carried out.

2.4. Determination of defense gene induction in wheat

▪ Plant treatment

We investigated the defense signaling pathways that were potentially triggered in wheat following treatment with surfactin. For this experiment, treatments were prepared similarly to screening trials, although in this case, surfactin was tested only at the average concentration of 0.01 mg mL^{-1}, due to space limitations. Plants at the 3–4 leaf stage were either sprayed to runoff with surfactin (0.01 mg mL^{-1}), Bion (0.6 mg mL^{-1}), or water using an electric sprayer. Each treatment was applied on one box of 40 wheat plants (40 repetitions). One day after plant treatment, potential priming activities were also tested by applying a water solution containing 40 nm of hydrogen peroxide (H_2O_2) on the plants on each half of the box. Elicitor priming is a phenomenon whereby plant natural defenses are only activated when a subsequent challenge occurs, and not directly after elicitor recognition (Van Hulten et al., 2006). The exact molecular mechanisms involved in priming are still poorly understood. However, fitness benefits have been observed on primed plants in the field under high disease pressures as the energy of the plant remains devoted to its development until a biotic stress actually occurs (Walters & Heil, 2007; Walters et al., 2009). In the present case, we used H_2O_2 to mimic a biotic stress comparable to a *Z. tritici* infection, as described by Dugé de Bernonville et al. (Dugé de Bernonville et al., 2014). Hydrogen peroxide is indeed a reactive oxygen species (ROS) which acts as a central player in the transduction of stress signals in the plant (Shetty et al., 2007; Kuźniak & Urbanek, 2000). In the event that surfactin and/or the elicitor control Bion exert a priming activity, the expression of defense genes in the plant would be strongly induced in wheat after the application of H_2O_2.

- **RNA extraction and quantification of gene expression by quantitative RT-PCR**

For each condition (*e.g.*, water only, Bion or surfactin), the third leaf of five distinct seedlings was sampled at day 1 after plant treatment, right before H_2O_2 application on the half of each box. Similarly, the third leaf of five distinct seedlings was sampled at day 2 and day 3 after treatment on the whole boxes, for plants which received H_2O_2 or were untreated. All samples were immediately pooled, frozen, and stored at −80 °C until use. Total RNA was extracted from 100 mg of plant tissue using the Nucleospin®RNA Plant Kit (Macherey-Nagel, Düren, Germany).

Reverse-transcription of total RNA was carried out using the M-MLV Reverse Transcriptase (ref M1701, Promega, Madison, WI, USA), according to the manufacturer's protocol. Real-time qPCR was performed with MESA BLUE qPCR MasterMix (ref RT-SY2X-03 + WOUFLB, Eurogentec, Liège, Belguim) according the manufacturer's instructions, using the biomolecular tool described by Brisset and Dugé de Bernonville (2011), on a Biorad MyiC detection system. The qRT-PCR bioassay focused on twenty-three different genes involved in various wheat defense mechanisms. These include pathogenesis-related (PR) proteins, oxidative stress, and defense signaling pathways (*e.g.*, salicylic acid, jasmonic acid, and ethylene) (Weber, 2002; Vogt, 2009; Verhage et al., 2010; Wasternack & Hause, 2013). Relative changes in defense gene expression of treated plants were compared to the relative expression of the same genes in water control plants by using the $2^{-\Delta\Delta Ct}$ method described by Schmittgen & Livak (Schmittgen & Livak, 2008). Three internal reference genes were used for normalization (*e.g.*, TubA, GAPDH, and actin). Relative defense gene expression was calculated for each time point. The gene expression levels were obtained from two independent biological experiments, with three technical replicates.

The effect of plant treatment on wheat defense responses was evaluated by multivariate ANOVA. In order to visualize and analyze gene expression, a heatmap representation was performed using dissimilarity distance (1-cor(X, Y)). Moreover, the identification of sets of genes that may be similarly expressed across all conditions within the dataset (relationship discovery) was realized by hierarchical clustering of gene expression. Hierarchical clustering analysis is a stepwise algorithm which merges two gene variables at each step, the two of which have the least dissimilarity distance. Such distance between clusters of genes was defined using the complete linkage method (using the "hclust" function in the R statistical software). In addition, the "pvclust" package in R was used to calculate the probability values (*p-values*) for each cluster using bootstrap resampling techniques. Gene clusters which were not significantly supported by the data were rejected with a significance level of 0.05.

3. Results

3.1. Screening and Biocidal results

The efficacy of surfactin to protect winter wheat against *Z. tritici* was assessed through greenhouse trials (Figure 1).

Control plants were infected on up to 20 % of their third leaf surface by the pathogen. On the other hand, symptomatic lesions occurred only on 6 % to 8 % of the leaf surface of plants treated with surfactin, regardless of its concentration. Finally, plants treated with Bion had barely 6% of their leaf surface covered with lesions. The disease severity was significantly lower ($p = 0.05$) on plants treated with Bion or with surfactin when compared to control plants. Hence, wheat was similarly protected by surfactin and the elicitor control Bion, with a protection efficacy of up to 70 % and 69 %, respectively.
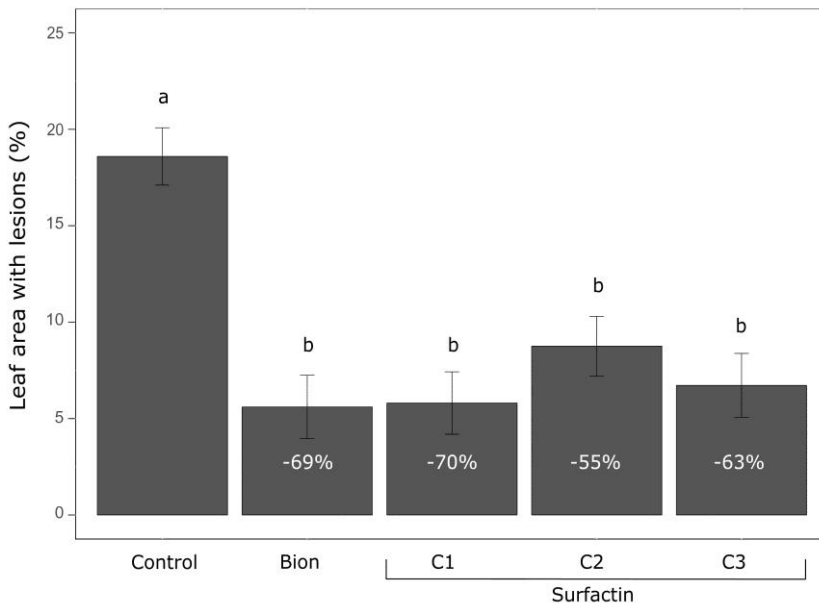

Figure 1. Mean disease severity of *Zymoseptoria tritici* on treated wheat plants. Data corresponds to the average percentage of the third leaf surface of wheat plants exhibiting symptomatic lesions (necrosis and/or chlorosis) bearing pycnidia. Plants were treated at the 3–4 leaf stage and five days before inoculation with water (Control), surfactin (Surfactin), or Bion[®]50WG (Bion, Syngenta Europe). Surfactin was applied at three different concentrations: 0.001 mg mL^{-1} (C1), 0.01 mg mL^{-1} (C2) and 0.1 mg mL^{-1} (C3). Bion was used as an elicitor reference and applied at 0.6 mg mL^{-1}. The protection efficacy of each treatment compared to water treated plants is represented in white inside the bars and corresponds to the percentage of reduction of disease severity. Bars tagged with the same letters correspond to means that are not significantly different using the Tukey test at $p = 0.05$ ($n \geq 40$, e.g., 5 pots of 8 plants per treatment $\times$ 2 independent experiments).

The potential direct effect of surfactin on *Z. tritici* was studied through *in vitro* sensitivity bioassays (Figure 2).

Such experiments are a first indication to understand if the protective efficacy of surfactin assessed during greenhouse trials was potentially due to a direct fungicidal effect against the pathogen.
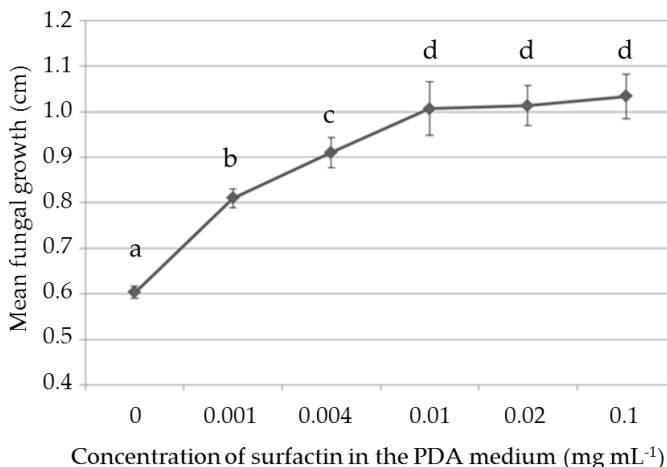
Figure 2. Biocidal effect of surfactin on the in vitro fungal growth of *Zymoseptoria tritici*. Potato dextrose agar (PDA) medium was amended with five decreasing concentrations of surfactin: 0.1 mg mL^{-1}, 0.02 mg mL^{-1}, 0.01 mg mL^{-1}, 0.004 mg mL^{-1}, and 0.001 mg mL^{-1}. The control corresponds to PDA medium without surfactin (0 mg mL^{-1}). Means tagged with the same letters are not significantly different using the Tukey test at $p = 0.05$

The highest concentration of surfactin amended to the PDA media (0.1 mg mL^{-1}) corresponds to the highest concentration of surfactin tested during greenhouse trials. The mean fungal growth of *Z. tritici* was 0.6 cm on control plates containing PDA medium only. On the other hand, the mean diameter of fungal spots significantly increased from 0.8 to 1 cm when *Z. tritici* was grown on PDA amended with increasing concentrations of surfactin ($p = 0.05$) (Figures 2 and 3). It thus appears that surfactin amended to fungal culture media has a positive effect on the in vitro growth of *Z. tritici*.

0 mg mL^{-1} 0.001 mg mL^{-1} 0.004 mg mL^{-1} 0.01 mg mL^{-1} 0.02 mg mL^{-1} 0.1 mg mL^{-1}
(Control)
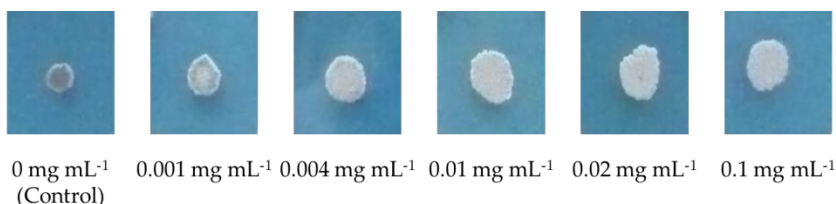
Figure 3. Illustration of *Zymoseptoria tritici* fungal growth on PDA medium amended with surfactin at six concentrations: 0.1 mg mL^{-1}, 0.02 mg mL^{-1}, 0.01 mg mL^{-1}, 0.004 mg mL^{-1}, 0.001 mg mL^{-1}, and 0 mg mL^{-1} (Control).

3.2. Induction of defense responses

The expression level of 23 defense-related genes of wheat was monitored 1, 2, and 3 days after treatment with either Bion or surfactin. The treatments applied on wheat plants had a significant effect on the expression of defense genes (MANOVA, p-value < 0.05). For each gene, the average expression level measured in treated plants was compared to the water control (which received no H_2O_2) and represented on a heatmap profile (Figure 4). The average expression level of genes for water-treated plants which received hydrogen peroxide (labelled "+H_2O_2") after 1 day was similarly compared to the water control. Hierarchical clustering of genes according to their expression levels revealed five gene clusters which were significantly supported by the data (p-value ≤ 0.05).
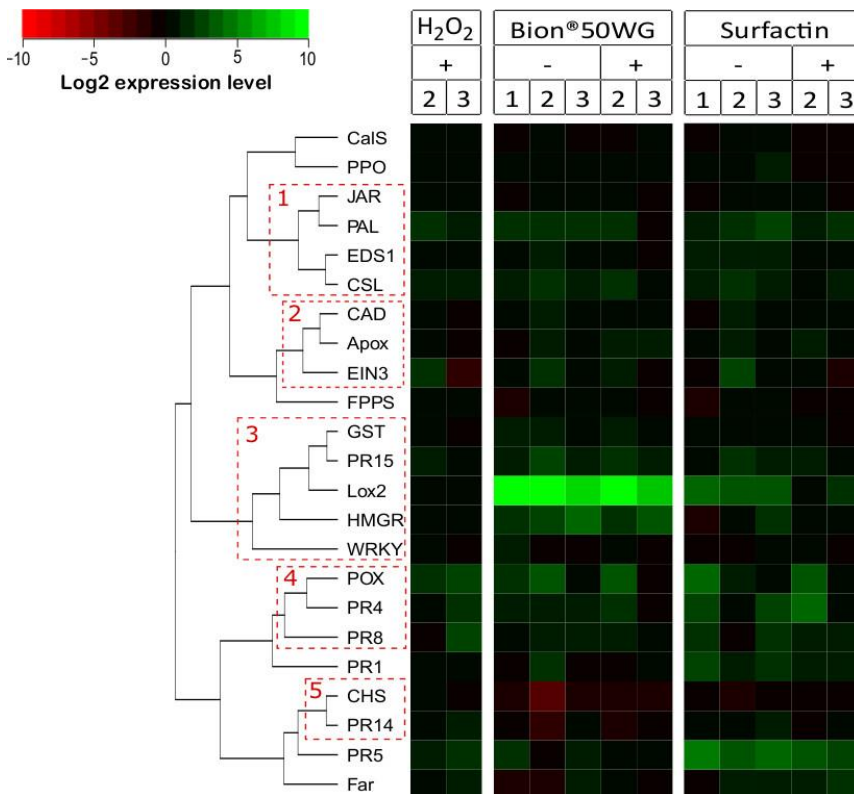
Figure 4. Heatmap profiling across all experimental conditions (product, ±H_2O_2, day post-treatment) with hierarchical clustering of 23 defense-related genes of wheat [23]: *Apox*, ascorbate peroxidase; *CalS*, callose synthase; *CHS*, chalcone synthase; *CAD*, cinnamyl-alcohol dehydrogenase; *CSL*, cysteine sulfoxide; *EIN3*, EIN3-binding F box protein; *EDS1*, enhanced disease susceptibility 1; *Far*, (*E,E*)-α-farnesene synthase; *FPPS*, farnesyl pyrophosphate synthase; *GST*, glutathione *S*-transferase; *HMGR*, hydroxymethyl glutarate-CoA reductase; *JAR*, jasmonate resistant 1; *Lox2*, 13-lipoxygenase 2; *PAL*, phenylalanine ammonia-lyase; *PR*, pathogenesis-related protein; *PPO*, polyphenol oxidase; *POX*, peroxidase; *WRKY*, WRKY transcription factor 30.

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The first cluster (1) includes gene *EDS1* (enhanced disease susceptibility 1) involved in the production of the defense hormone salicylic acid (SA), *JAR* (jasmonate resistant 1) involved in jasmonic acid (JA)-related defense signaling, *PAL* (phenylalanine ammonia lyase) involved in the phenylpropanoid pathway, and *CSL* (cysteine sulfoxide) involved in antioxidative stress. The expression level of genes *EDS1* and *JAR* was similar between the control and the treated plants. On the other hand, Bion and surfactin induced about a 3-fold upregulation of *PAL* and *CSL* gene expression compared to the control, whether the plants were later sprayed with H_2O_2 or not. A similar 2- to 3-fold upregulation of *PAL* and *CSL* occurred for "+H_2O_2" plants which were treated only with water before being sprayed with H_2O_2 one day later.

The second gene cluster (2) includes *EIN3* (EIN3-binding F box protein) involved in ethylene (ET)-related defense signaling, *CAD* (cinnamyl-alcohol dehydrogenase) involved in cell wall reinforcement, and *Apox* (ascorbate peroxidase) involved in antioxidative stress. Wheat plants treated with Bion or surfactin showed a significant upregulation (3- to 4-fold increase) of the expression level of these three genes at day 2 after treatment. On the other hand, "+H_2O_2" plants showed no difference with the control, except for a 4-fold upregulation of *EIN3* expression level at day 2.

A third cluster (3) includes the *WRKY* transcription factor 30 gene involved in defense signaling, *HMGR* (hydroxymethyl glutarate-CoA reductase) involved in the mevalonate pathway leading to biosynthesis of terpenoid defense compounds, *LOX2* (Lipoxygenase 2) involved in the octadecanoid pathway leading to the biosynthesis of the defense hormone JA, and genes *PR15* (pathogenesis-related protein 15) and *GST* (glutathione *S*-transferase) which are involved in antioxidative stress. Both Bion and surfactin induced a significant upregulation of *HMGR*, *LOX2*, and *PR15* gene expression in wheat from day 1 to day 3 after treatment. Such upregulation occurred whether the corresponding plants later received H_2O_2 or not. Notably, the upregulation of *LOX2* gene expression was particularly strong: 10-fold increase for wheat treated with Bion and about 8-fold increase for plants treated with surfactin. On the other hand, "+H_2O_2" plants showed no difference with the control. Another cluster (4) includes genes *PR4* and *PR8* which both code for antimicrobial chitinases, and gene *POX* (peroxidase) involved in antioxidative stress. The expression level of these three genes was significantly upregulated by 5- to 6-fold in plants treated with Bion or with surfactin compared to the control. Finally, a last cluster (5) includes gene *PR14* coding for a lipid-transfer protein and gene *CHS* (chalcone synthase) involved in the flavonoid/isoflavid pathway and SA-related defense signaling. In particular, Bion induced a strong 7-fold downregulation of *CHS* (chalcone synthase) gene expression up to three days after treatment, while surfactin induced a 4-fold downregulation of *CHS* only at day 2. On the other hand, "+H_2O_2" water-treated plants showed no difference with the control.

Overall, treatment of wheat with either Bion or surfactin induced a significant upregulation of the expression level of several genes involved in key defense mechanisms, notably genes involved in SA- and JA-related signaling pathways, oxidative stress, and cell wall reinforcement. Moreover, the application of H_2O_2 on water-treated plants was successful in being recognized as an attack by the plant by inducing the expression of defense genes, such as *PAL* and *POX*.

On the other hand, the application of H_2O_2 on plants treated with Bion or surfactin showed no difference in terms of defense gene expression compared to treated plants which received no hydrogen peroxide, thus suggesting that nor Bion nor surfactin exerted a priming activity.

4. Discussion

Previous studies have demonstrated that pure surfactin extracted from strains of non-pathogenic *Bacillus* could significantly protect thale-cress, bean, tomato and tobacco plants against the fungal pathogen *Botrytis cinerea* (Henry, 2013; Ongena et al., 2007; Jourdan et al., 2009). Surfactin was also proven to efficaciously protect sugar beet against the virus *Polymyxa betae* (Desoignies et al., 2013) and strawberry plants against *Colletotrichum gloesporioides* (Yamamoto et al., 2015). More recently, Mejri et al. (2017) reported that surfactin extracted from the *Bacillus subtilis* strain BBG131 and applied at 0.1 mg mL^{-1} on the susceptible wheat cultivar "Alixan" could efficaciously protect the plant by up to 35% against *Z. tritici* (Mejri et al., 2017). Our results are thus in accordance with previous research, as we demonstrated that surfactin applied at low doses (e.g., 0.001, 0.01, and 0.1 mg mL^{-1}) efficaciously protected wheat by up to 70% against *Z. tritici*. Moreover, surfactin was as efficacious as the synthetic elicitor control Bion. In the present study, such high protection efficacy of surfactin could be linked to the mixture of homologues extracted from the *B. amyloliquefaciens* strain S499 and/or to the wheat cultivar "Avatar" that was used for greenhouse trials. The efficacy of a given elicitor can indeed be cultivar-dependent (Walters et al., 2011a; Ors et al., 2013), and the elicitor activity of surfactin was proven to rely on specific structural traits such as the length of the fatty acid (Henry et al., 2011).

In addition to greenhouse trials, we showed that surfactin had no direct *in vitro* biocidal effect against the pathogen at the concentrations tested in the greenhouse. Rather, it appears that high concentrations of surfactin promoted the *in vitro* growth of *Z. tritici*. This lipopeptide is indeed a powerful amphiphilic biosurfactant involved in bacterial mobility and in the formation of biofilms, pellicles, and fruiting bodies of *Bacillus* (Ongena & Jacques, 2008; Jacques, 2011). It is therefore likely that the surface tension of PDA media containing surfactin was lowered, thereby allowing a better spreading of the inoculum droplets on the plates during inoculation. The reduction of the surface tension was likely enhanced by increasing concentrations of surfactin in the PDA media. Then, such increased fungal growth might probably be due to the physicochemical properties of surfactin, rather than to a growth-promoting effect. These results are once again in accordance with previous studies. Indeed, Mejri et al. (2017) demonstrated the lack of direct antifungal activity of surfactin against the pathogen *Z. tritici* in both in vitro and in planta bioassays. Actually, the fungitoxic effects of surfactin have never been reported (Raaijmakers et al., 2010; Ongena et al., 2007) except in the work of Tendulkar et al. (2007) on rice. They showed that surfactin extracted from *Bacillus licheniformis* BC98 exhibited an in vitro direct fungicidal activity against the rice blast disease *Magnaporthe grisea* (Tendulkar et al., 2007). Taken together, the findings of greenhouse trials and *in vitro* biocidal assays of the present study confirm that surfactin likely protects wheat against *Z. tritici* by inducing plant resistance.

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Going one step further, our biomolecular tests on wheat immune responses confirmed that surfactin was indeed perceived by the plant as an elicitor. Surfactin stimulated wheat defense mechanisms by inducing the expression of various defense genes coding for antimicrobial compounds, regulators of oxidative stress, and enzymes involved in defense signaling (Wiesel et al., 2014). The induction of plant resistance by an elicitor is indeed characterized by a complex spatio-temporal network of metabolic modifications. Early events, such as protein phosphorylation, ion fluxes across the plasma membrane, and a burst of ROS, occur in a matter of seconds after elicitor recognition by plant receptors (Mejía-Teniente et al., 2010; Klarzynski & Fritig, 2001). Proteins such as POX and PR15 are set to work, in order to control the oxidative burst (Kärkönen & Kuchitsu, 2015). After a few hours, defense genes involved in the biosynthesis of phytohormones and antimicrobial compounds are activated (Mejía-Teniente et al., 2010). The hormones SA, JA, and ET are considered as the three crucial primary signals which regulate plant defenses against biotic stress (Verhage et al., 2010; Gozzo & Faoro, 2013). Finally, physical and biochemical changes, such as cell wall reinforcement through callose apposition and PR protein biosynthesis, occur several hours to several days after elicitor recognition (La Camera et al., 2004). Previous research has shown that plant resistance against biotrophic and hemi-biotrophic pathogens is generally regulated by SA, while resistance against necrotrophic pathogens and chewing insects is regulated by JA and ET (Verhage et al., 2010; Glazebrook, 2005). Depending on the triggered signaling pathway, a different set of genes encoding PR proteins are expressed (La Camera et al., 2004; Van Loon & Van Strien, 1999). Induced resistance depending on SA, also called systemic acquired resistance (SAR), involves the marker protein PR1 and the enzymes PAL and CHS (Vogt, 2009; Ors, 2015). Conversely, JA-dependent defense responses induced by MAMPS generally lead to rhizobacteria-mediated induced systemic resistance (ISR), and go hand in hand with the expression of the genes *LOX2* and *PR4* (Verhage et al., 2010; Van Loon & Van Strien, 1999). The LOX enzyme catalyzes the deoxygenation of polyunsaturated fatty acids, leading to the downstream biosynthesis of JA (Weber, 2002). However, most studies on SA/JA crosstalk and the corresponding responsive genes have been carried out on dicotyledonous plants, and less is known concerning monocotyledonous plants. Still, it appears that similar hormone interactions may be involved in cereals. Indeed, a recent work carried out by Ding et al. (2016) showed that SA and JA were able to act antagonistically or synergistically on the expression of wheat defense genes (Ding et al., 2016). They also reported that gene *PR5* was specifically induced by SA in the plant, while *LOX2* was specifically induced by JA, and that gene *PR1* could actually be induced simultaneously by both hormones.

Based on that knowledge, our biomolecular findings suggest that surfactin induced both SA- and JA-dependent defense responses in wheat, as it triggered a significant upregulation of the expression level of genes *PR5* and *LOX2*. Interestingly, surfactin produced by the antagonistic strain *Bacillus subtilis* UMAF6639 was also shown to protect melon plants against powdery mildew, by similarly inducing both SA and JA defense signaling pathways, along with the production of ROS and the reinforcement of the plant cell wall (Garcia-Gutierrez et al., 2013). The simultaneous induction of SA- and JA-dependent defense responses by some

eliciters has also been reported in previous studies on dicotyledonous plants (Tjamos et al., 2005; Niu et al., 2011; Niu et al., 2012). However, in model plants, the elicitor potential of surfactin has been associated with JA-dependent responses. For instance, it was proven to stimulate the activity of the LOX enzyme and the synthesis of numerous active secondary metabolites in tomato plants (Ongena et al., 2007), and the activity of both PAL and LOX enzymes in tobacco cells (Jourdan et al., 2009; Ongena et al., 2004).

It is clear that plant-induced resistance involves intricate hormonal crosstalk, including in wheat, and there is no established boundary between SAR and ISR in plants (Van Loon & Van Strien, 1999; Conrath et al., 2011). Other hormones which were not investigated by qRT-PCR might also be involved (*e.g.*, auxin, abscisic acid, cytokinin, gibberellin) (Denancé et al., 2013; Dolferus, 2014). A better insight into the mode of action of surfactin to induce wheat defense mechanisms would require some additional biochemical experiments on the activity of key defense enzymes. However, the primary objective of this study was to confirm the elicitor potential of surfactin for sustainably protecting wheat. Interestingly, Henry et al. (2011) suggested that, depending on the specific features of the plant plasma membrane (e.g., organization and composition of the lipid bilayer), surfactin could be perceived at the plant cell surface by interacting with the lipids at the plasma membrane level (Henry et al., 2011). This mode of perception can be considered unusual, since most identified eliciters, such as flagellin or chitin, are known to be recognized by high affinity protein receptors (Henry et al., 2011; Balmer et al., 2013).

Concerning Bion, our findings suggest that this synthetic elicitor induced both SA and JA defense signaling pathways in wheat, with JA signaling clearly outweighing SA signaling up to 3 days after plant treatment. Such results are in contrast with previous studies, as Bion has been reported, up to now, to induce solely SA-dependent defense responses in plants (Görlach et al., 1996; Vallad & Goodman, 2004; Hofgaard et al., 2005). As a chemically synthesized elicitor consisting of acibenzolar-*S*-methyl, Bion shows indeed a functional analogy to the plant hormone SA, and is thus well known to trigger SA-responsive genes, notably robust SAR markers, such as *PR1*, *PR2*, and *PR5* (Walters et al., 2013; Verhage et al., 2010; Hofgaard et al., 2005). Our results might be explained by the complex hormonal crosstalk involved in wheat defense signaling. Finally, concerning the investigation of potential priming activities, we demonstrated that the application of H_2O_2 following plant treatment with either surfactin or Bion exerted no additional effect on the expression of wheat defense genes. Plants which are primed following elicitor perception activate faster and stronger defense responses upon a second pathogen challenge, rather than directly inducing their defense mechanisms (Conrath et al., 2011; Beckers & Conrath, 2007). It thus appears that neither Bion nor surfactin had a priming effect on wheat defenses. However, it would be interesting to carry out a similar experiment by replacing H_2O_2 with a real pathogen attack (*e.g.*, an actual inoculation of *Z. tritici*), and to investigate the induction of wheat defenses over more sampling times (*e.g.*, at 6, 12, and 96 h after treatment, for example).

5. Conclusions

This study provides further insight into the remarkable elicitor properties of surfactin by demonstrating its ability to efficaciously protect wheat by up to 70% against the fungal pathogen *Z. tritici*. The stimulation of wheat defense mechanisms appears to involve both SA and JA defense signaling pathways. Research on induced resistance in monocots remains elusive, and is still an emerging field (Balmer et al., 2013; Kogel & Langen, 2005). Both monocots and dicots have undergone evolutionary adaptations which may involve the triggering of distinct sets of defense gene expression after elicitor recognition (Balmer et al., 2013). It is noteworthy that previous studies on the elicitor potential of surfactin have mostly been dedicated to the protection of dicot plants (Ongena et al., 2007; Jourdan et al., 2009; Desoignies et al., 2013). Further research is thus still needed to understand the exact modes of action of surfactin to induce wheat resistance. Besides, field trials are now required to confirm the reliability of this lipopeptide elicitor in efficaciously protecting wheat crops. Several environmental parameters, such as the weather and disease pressure, are indeed known to influence the efficacy of an elicitor in the open field (Walters et al., 2013; Ozeretskovskaya & Vasyukova, 2002). These results open the way towards the development of novel surfactin-based biocontrol tools for wheat protection, in order to enhance the sustainability of current agricultural practices. Moreover, the elicitor potential of surfactin for other cultivated monocots, such as barley and rice, and against others diseases, deserves to be explored.

8

PUBLISHED ARTICLES

&

CONGRESS PRESENTATIONS

PUBLISHED ARTICLES

Le Mire et al., 2016. Review Implementing biostimulants and biocontrol strategies in the agroecological management of cultivated ecosystems. *Biotechnol. Agron. Société Environ.*, **20**(S1), 299–313. http://hdl.handle.net/2268/188662

Le Mire et al., 2018. Surfactin protects wheat against Zymoseptoria tritici and activates both salicylic acid- and jasmonic acid-dependent defense responses. *Agriculture*, **8** (11). doi: 10.3390/agriculture8010011

Le Mire et al., UNDER PEER-REVIEW. Evaluation of λ-carrageenan, CpG-ODN, glycine beatine, *Spirulina platensis* and ergosterol as elicitors for control of *Zymoseptoria tritici* in wheat. *Phytopathology*.

ORAL COMMUNICATIONS AND/OR POSTERS

Oral communication – Natural products and Biocontrol 2016 - Perpignan, France. http://hdl.handle.net/2268/208562
"New elicitors to protect winter wheat against Zymoseptoria tritici?"

Poster – 9th International Symposium on Septoria Diseases of Cereals – Paris, France. http://hdl.handle.net/2268/196695
"New elicitors as biocontrol tools to protect wheat against Septoria tritici Blotch"

Oral communication – 68th International Symposium of Crop Protection – Ghent, Belgium. http://hdl.handle.net/2268/197209
"New elicitors to protect wheat against the fungus Zymoseptoria tritici: biocontrol tools for IPM strategies"

Oral communication – XVIII International Plant Protection Congress. Berlin, Germany. http://hdl.handle.net/2268/187791
"Elicitor screening to protect wheat against Zymoseptoria tritici"

Poster – 5eme Conférence Internationale sur les Méthodes Alternatives de Protection des Plantes - Lille France. http://hdl.handle.net/2268/180429
"Elicitor screening to protect winter wheat against Zymoseptoria tritici"

Oral communication – Natural products and Biocontrol 2014 - Perpignan, France. http://hdl.handle.net/2268/173199
"Elicitor screening to protect winter wheat against *Septoria tritici* Blotch: preliminary results"

Poster – Journée SFR Condorcet (Institut LaSalle de Beauvais) – Beauvais, France. http://hdl.handle.net/2268/154193
"Development of formulated elicitors to control bioaggressors of wheat"

Identification of elicitors inducing resistance in wheat against Zymoseptoria tritici and characterization of the subsequent defense-triggered pathways

The implementation of biocontrol products in integrated pest management strategies is a major challenge today in the transition to sustainable and environment-friendly agro-ecosystems. In particular, the use of natural elicitors, also called plant resistance inducers, represents an interesting alternative to conventional fungicides. Elicitors are natural immune-stimulating compounds which offer the advantage to indirectly target a broad spectrum of pathogens by enhancing the defensive state of the plant. Yet today, wheat is one of the most cultivated crops in the European Union and still requires fungicide protection every year for the control of a harmful disease: *Septoria tritici* Blotch (STB), caused by the fungal pathogen *Zymoseptoria tritici*. At a time when few elicitor products are available on the market for the sustainable management of crop diseases, the objective of this thesis project was to screen and identify innovative elicitors able to preventively protect wheat against the STB disease. Greenhouse trials successfully demonstrated the ability of λ-carrageenan, cytosine-phosphate-guanine oligodesoxynucleotide motifs (CpG-ODN), *Spirulina platensis*, glycine betaine and ergosterol to protect wheat by up to 70 % against the pathogen *Z. tritici*. These results are promising as previous research has indeed demonstrated the elicitor properties of these five compounds on other plant species and/or animals. Besides, no direct anti-fungal activity was recorded during *in vitro* experiments towards the disease. The risk of resistance development of the pathogen to these potential elicitors can thus be considered as low. Furthermore, the defense mechanisms of wheat were successfully demonstrated to be significantly induced following treatment with each of these formulated compounds. The relative expression of 23 plant defense genes was analyzed by qRT-PCR at 1, 2 and 3 days after plant treatment. Defense mechanisms involving the two hormones salicylic acid (SA) and jasmonic acid (JA) were triggered in treated wheat. These hormones play a key role in the transduction of defense signals throughout the plant. In addition, the protection efficacy of the two preferential candidates (λ-carrageenan and Spirulina) was investigated in the field during two successive years. Numerous parameters, among which environmental conditions, plant developmental stage, plant genotype and disease pressure, can indeed cause a variability of elicitor protection efficacy under practical conditions. Unfortunately, important contrasts in disease pressures and extreme weather conditions did not allow confirming the elicitor potential of the corresponding treatments on field. Finally, the potential effect of the formulation on the eliciting activity was characterized in order to rule out the possibility of interference by the selected adjuvants. Additional greenhouse experiments showed that a water solution containing only the adjuvants was as efficient to protect wheat against STB as plants treated with formulated or non-formulated λ-carrageenan. These last results highlighted the necessity of developing an appropriate formulation at an early stage before elicitor screening. Overall, the findings of this research study open the way to the development of new and interesting biocontrol products based on λ-carrageenan for sustainable wheat protection against *Zymoseptoria tritici*.