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Criblage, efficacité et modes d'action de composés de biocontrôle microbiens sur le pathosystème blé-*Zymoseptoria tritici*

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RÉSUMÉ

La septoriose, causée par le champignon hémibiotrophe *Zymoseptoria tritici*, est l'une des maladies les plus préjudiciables sur les cultures de blé, responsable de pertes de rendement pouvant atteindre 50%. La lutte contre cette maladie repose essentiellement sur l'utilisation des fongicides conventionnels et, dans une moindre mesure, sur le recours aux ressources variétales. Toutefois, en raison des résistances développées par l'agent pathogène vis-à-vis de ces leviers et d'une demande sociale croissante pour limiter l'utilisation des intrants chimiques en agriculture, des alternatives agroécologiques, comme le biocontrôle, doivent être développées. L'objectif de ce présent projet de thèse se situe dans ce contexte et vise à identifier des composés de biocontrôle efficaces sur le pathosystème blé-*Z. tritici* et à caractériser leurs modes d'action. Tout d'abord, un criblage *in vitro* d'un panel de 181 composés microbiens (extraits fongiques ou bactériens, rhamnolipides, lipopeptides, etc.) a été réalisé pour évaluer leur activité antifongique directe vis-à-vis de l'agent pathogène. Les composés les plus actifs ont ensuite été sélectionnés pour vérifier leur efficacité de protection *in planta*. Une étude de relation structure-activité a été menée avec 19 rhamnolipides (RL) et a révélé que ceux présentant des liaisons éthers ou esters, avec une chaîne carbonée hydrophobe de 12 carbones, sont les plus efficaces pour toutes les activités biologiques testées (activité biocide, élévation des défenses du blé et efficacité de protection). Parmi eux, le Rh-Est-C12 s'est montré le plus prometteur, avec une efficacité de protection de 78,9%. Le potentiel de ce RL, ainsi que de la mycosubtiline, un lipopeptide efficace pour protéger le blé contre *Z. tritici*, pour stimuler les défenses du blé, a été étudié à l'aide d'une approche combinée de transcriptomique et de métabolomique. Le RL n'a pas déclenché de modifications majeures de la physiologie foliaire de blé ; celui-ci protégerait donc l'hôte principalement *via* son activité directe vis-à-vis du champignon, alors que la mycosubtiline semble présenter un mode d'action double, basé sur une activité antifongique directe et la potentialisation des défenses du blé. Cette induction des défenses pourrait résulter de l'interaction de la mycosubtiline avec les membranes cytoplasmiques du blé, conduisant à une accumulation de flavonoïdes dans les feuilles, mais aussi à d'autres réponses dépendantes de la voie de l'acide abscissique. Ces travaux ont, par ailleurs, permis de mettre en évidence de nouveaux mécanismes dans les réactions de défense du blé vis-à-vis de *Z. tritici*. Enfin, deux nouvelles souches isolées de *Bacillus velezensis* ont présenté une activité antifongique prometteuse contre l'agent pathogène, résultant probablement de la production de lipopeptides, comme la bacillomycine D, dont l'activité sur *Z. tritici* mériterait d'être explorée.

Mots clés : Blé, *Zymoseptoria tritici*, biocontrôle, biofongicides, induction de défenses, bioprotection.

ABSTRACT

Septoria tritici blotch, caused by the hemibiotrophic fungus *Zymoseptoria tritici*, is one of the most severe diseases on wheat, responsible for yield losses up to 50%. Currently, strategies used to control this disease rely mainly on the use of chemical fungicides, and to a lower extent, on the use of partially resistant cultivars. Nevertheless, because of the pathogen resistances and an increasing social demand to reduce conventional pesticides in agriculture, new agroecological alternative crop protection methods, like biocontrol, must be developed. The main purpose of this thesis project was, therefore, to identify new effective biocontrol compounds on the wheat-*Z. tritici* pathosystem and to decipher their modes of action. An *in vitro* screening of a set of 181 microbial compounds (fungal and bacterial extracts, rhamnolipids, lipopeptides, *etc.*) was performed to assess their antifungal activity towards the pathogenic agent. The most active compounds were selected for further experiments, such as verifying their protection activity *in planta*. The ability of 19 rhamnolipids (RLs) to control the pathogen was explored and a structure-activity relationship study revealed that bioinspired RLs with ether or ester links and a 12 carbon hydrophobic fatty acid tail were the most effective for all tested biological activities (biocide activity, wheat defense elicitation and protection efficacy). Among them, Rh-Est-C12 was the most promising compound, with a protection efficacy of 78.9%. The potential of Rh-Est-C12 as well as mycosubtilin, a lipopeptide also effective to protect wheat against *Z. tritici*, to induce host defenses, was investigated using a combined transcriptomic and metabolomic approach. The RL did not trigger any major disorganization of the wheat leaf physiology, hence it would protect the host mainly through its direct activity towards *Z. tritici*, while mycosubtilin would display a dual mode of action, antifungal effect and plant defense priming. Mycosubtilin could prime wheat by interacting with its plasma membranes leading to flavonoid accumulation and other abscisic acid dependent responses. Besides, new insights into wheat defense mechanisms towards *Z. tritici* were reported. Finally, two *Bacillus velezensis* strains, isolated from wheat phyllosphere, displayed promising antifungal effect towards the pathogen. A characterization of their metabolites was performed and the putative role of bacillomycin D as a potent *Z. tritici* antifungal lipopeptide was suggested.

Key-words : wheat, *Zymoseptoria tritici*, biocontrol, bio-fungicides, defense induction, bioprotection

AVANT-PROPOS

Le laboratoire d'accueil dans lequel j'ai réalisé mon projet de thèse fait partie de l'Unité Mixte de Recherche transfrontalière (UMR-t) INRAE 1158 BioEcoAgro qui fédère plusieurs acteurs académiques de la région transfrontalière franco-belge travaillant dans le domaine de l'ingénierie biologique appliquée à l'agriculture, la biotechnologie, l'agro-alimentaire et l'environnement. Ce laboratoire multi-site est composé de l'Université de Lille, l'Université de Liège, l'Université de Picardie Jules Verne, l'INRAE (tutelles principales) ainsi que de l'Université du Littoral-Côte d'Opale, l'Université d'Artois et Junia (tutelles associées). Les thématiques de recherche de cet UMR-t s'articulent autour de trois pôles, neuf équipes, ainsi que de 4 axes transversaux (**Figure i**). Plus précisément, mon projet a été conduit sur le site ISA-Junia dans le cadre de l'équipe 5 « Métabolites secondaires d'origine végétale » et dans l'axe transversal « Biocontrôle ».

Certains travaux ont été réalisés en collaboration avec des partenaires internes et externes à l'UMR-t BioEcoAgro (Université de Lille, Université de Liège, Université du Littoral-Côte d'Opale, Université d'Artois, Université de Gand, INRAE de Colmar, Université Catholique de Louvain, Université de Reims Champagne-Ardenne, INRA de Kenitra).

Cette thèse a été réalisée du 1^{er} novembre 2017 au 3 juin 2021, avec une interruption des travaux de mars à juin 2020, en raison des conditions sanitaires liées à la pandémie de la Covid-19.

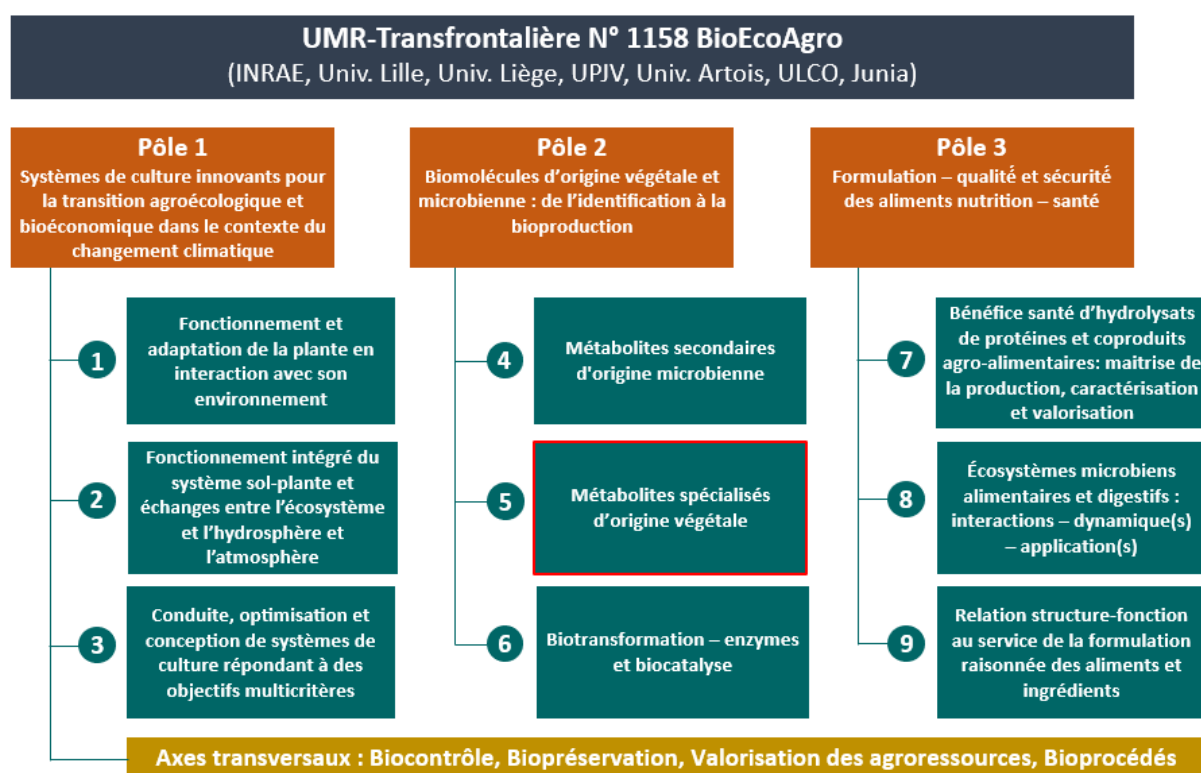


Figure i : Organisation des thématiques de recherche de l'UMR-t 1158 BioEcoAgro (www.bioecoagro.eu).

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LISTE DES ABRÉVIATIONS

4CL	4-coumarate-CoA ligase
ABA	Acide abscissique
ABC	<i>ATP-binding cassette</i>
ACS1	<i>ACC synthase</i>
AIM1	<i>Abnormal inflorescence meristem 1</i>
AMPc	Adénosine monophosphate cyclique
AOC	Allène oxyde cyclase
AOS	Allène oxyde synthase
AQP	<i>Aquaporin</i>
BA2H	<i>Benzoic acid 2-hydroxylase</i>
BR	Brassinostéroïdes
C3'H	<i>4-coumarate 3-hydroxylase</i>
C4H	Cinnamate 4-hydroxylase
CAD	<i>Cinnamyl alcohol dehydrogenase</i>
CAT	Catalase
CCoAOMT	<i>Caffeoyl CoA O-methyltransferase</i>
CCR	<i>Cinnamoyl CoA reductase</i>
CDPK	<i>Calcium dependant protein kinase</i>
CEBiP	<i>Chitin elicitor binding protein</i>
CERK1	<i>Chitin elicitor receptor kinase 1</i>
CI₅₀	Concentration inhibitrice à 50%
CIPK	<i>CBL-interacting protein kinase</i>
CK	Cytokinines
CLP	Lipopeptide cyclique
CM	Chorismate mutase
CMI	Concentration minimale inhibitrice
COMT	<i>Caffeic acid O-methyltransferase</i>
CWDE	<i>Cell wall degrading Enzymes ; Enzymes de dégradation de la paroi cellulaire</i>
DAM	<i>Differentially accumulated metabolite</i>
DAMP	<i>Damage-associated molecular patterns</i>
DEG	<i>Differentially expressed gene</i>
DMAP	<i>N,N-dimethylpyridin-4-amine</i>
DMF	<i>N,N-dimethylformamide</i>
DMI	<i>Demethylation inhibitors</i>
DMSO	<i>Dimethyl sulfoxide</i>
EDS5	<i>Enhanced disease susceptibility 5</i>
EPS1	<i>Enhanced pseudomonas susceptibility 1</i>
ESI	<i>Electrospray ionization</i>
ET	Ethylène
ETI	<i>Effector-triggered immunity</i>
ETS	<i>Effector-triggered susceptibility</i>
F5H	<i>Ferulate-5-hydroxylase</i>
FAO	<i>Food and Agriculture Organization</i>
FC	<i>Fold change</i>
FDR	<i>False discovery rate</i>
FLA15	<i>Fasciclin-like protein 15</i>
GA	Gibbérellines
HCN	Cyanide d'hydrogène

HCT	<i>Quinate/shikimate p-hydroxycinnamoyltransferase</i>
HR	<i>Hypersensitive response</i>
HrBP1-1	<i>Harpin binding protein 1-1</i>
HRGP	<i>Hydroxyproline-rich glycoproteins</i>
HSP	<i>Heat-shock protein</i>
IAA	<i>Indole-3-acetic acid, Acide indole-acétique</i>
IC	<i>Isochorismate</i>
ICS	<i>Isochrosimate synthase</i>
ID50	<i>Half-maximal inhibitory dilution</i>
IGS	<i>Isochorismoyl-glutamate synthase</i>
ISR	<i>Induced systemic resistance</i>
IWGSC	<i>International Wheat Genome Sequencing Consortium</i>
JA	<i>Jasmonic acid, acide jasmonique</i>
JA-Ile	<i>Jasmonoyl-isoleucine</i>
Jpi	<i>Jours post inoculation</i>
KB	<i>King's B</i>
KEGG	<i>Kyoto Encyclopedia of Genes and Genomes</i>
LACs	<i>Laccases</i>
LAR	<i>Local acquired resistance, Résistance locale acquise</i>
LB	<i>Lysogeny broth</i>
LLP	<i>Lipopeptide linéaire</i>
LOX	<i>Lipoxygénase</i>
MALDI-ToF	<i>Matrix assisted laser desorption Ionization-time of flight</i>
MAMP	<i>Microbe-associated molecular patterns</i>
MAPK	<i>Mitogen-activated protein kinases</i>
MDR	<i>Multi-drug resistant</i>
MeJA	<i>Méthyl-jasmonate</i>
MeSA	<i>Méthyl-salicylate</i>
MID	<i>Minimal inhibitory dilution</i>
MYB2	<i>Myeloblastosis 2</i>
NB-LRR	<i>Nucleotide binding leucine-rich repeat</i>
NIP	<i>Necrosis-inducing proteins</i>
NMR	<i>Nuclear magnetic resonance</i>
nsLTP	<i>Non-specific lipid transfert protein</i>
OAD	<i>Outils d'aide à la décision</i>
OPDA	<i>Cis-(+)-12-oxo-phytodienoic acid</i>
OXO	<i>Oxalate oxydase</i>
PAL	<i>Phénylalanine ammonia lyase</i>
PAMP	<i>Pathogen-associated molecular patterns</i>
PBS3	<i>AvrPphB susceptible 3</i>
PCA	<i>Plate count agar</i>
PDA	<i>Potato dextrose agar</i>
PDX1.1	<i>Pyridoxal biosynthesis protein 1.1</i>
POX	<i>Peroxidase ; Peroxydase</i>
PR1	<i>Pathogenesis-related protein 1</i>
PRR	<i>Pattern recognition receptors</i>
PTI	<i>PAMP-triggered immunity</i>
Qol	<i>Quinone outside inhibitors</i>
QTL	<i>Quantitative trait loci</i>
Rh-Est	<i>Rhamnose Ester</i>
Rh-Eth	<i>Rhamnose Ether</i>
Rh-Suc	<i>Rhamnose succinate</i>

RIN	<i>RNA intergity number</i>
RL	<i>Rhamnolipid</i>
ROS	<i>Reactive oxygen species</i> , Espèces réactives de l'oxygène
RT	<i>Retention time</i>
SA	<i>Salicylic acid</i> , Acide salicylique
SAR	<i>Systemic acquired resistance</i> , Résistance systémique acquise
SDHI	<i>Succinate dehydrogenase inhibitors</i>
SDP	Stimulateur de défense des plantes
SL	Strigolactones
SOD	Superoxyde dismutase
SRB	<i>Synthetic rhamnolipid bolaforms</i>
STB	<i>Septoria tritici blotch</i> ; Septoriose du blé
TaRH1	<i>Triticum aestivum RNA helicase 1</i>
TF	<i>Transcription factor</i>
TL4	<i>Thaumatococcus-like protein</i>
TLC	<i>Thin layer chromatography</i>
Tween 20	<i>Polyoxyethylene-sorbitan monolaurate</i>
UE	Union Européenne
UHPLC-MS	<i>Ultra-high performance liquid chromatography-mass</i>
UN	<i>United nations</i> ; Organisation des nations unies
WCOR518	<i>Cold acclimation protein</i>
WZF1	<i>Zinc finger protein</i>

INTRODUCTION GÉNÉRALE

Selon les prévisions de l'Organisation des Nations unies (*United Nations*, UN), la population humaine devrait continuer à croître considérablement dans les décennies à venir, atteignant 9,7 milliards d'individus en 2050 et possiblement 11 milliards vers 2100 (UN, 2019). Dans ce contexte, il est crucial d'arriver à assurer une production suffisante et durable des principales denrées alimentaires. Les céréales regroupent des espèces végétales, appartenant principalement à la famille des graminées, cultivées pour leurs grains. Elles sont utilisées aussi bien pour la subsistance humaine que pour celle des animaux. Parmi toutes les céréales consommées et produites dans le monde, le blé occupe une place essentielle, tout particulièrement en Europe. Ingrédient principal pour de nombreuses sociétés au cours du temps, il a joué, et joue toujours, de nombreux rôles, que ce soit au niveau agronomique, industriel, économique, historique et même géopolitique. Le blé est ainsi l'une des plantes les plus cultivées et consommées dans le monde. Selon les estimations de la *Food and Agriculture Organization* (FAO), la production de blé aurait dépassé les 761 millions de tonnes sur la période 2018-2019 (FAO, 2020). Cette céréale est cependant soumise à des nombreuses contraintes biotiques et abiotiques pouvant impacter significativement son rendement.

En Europe de l'Ouest, la maladie foliaire la plus dévastatrice sur la culture de blé est la septoriose, causée par le champignon phytopathogène *Zymoseptoria tritici*, qui peut, selon les conditions, conduire à des pertes de rendement pouvant atteindre les 50% (Fones and Gurr, 2015). Les pertes économiques causées par cette maladie cryptogamique, chaque année, en Europe seulement, sont estimées à 400 millions de dollars, faisant de la septoriose du blé une problématique agronomique et économique majeure au niveau européen comme au niveau mondial (Yemelin *et al.*, 2017). Les principales méthodes employées pour contrôler cet agent phytopathogène au champ reposent majoritairement sur l'utilisation de produits phytopharmaceutiques de synthèse et, dans une moindre mesure, sur la lutte variétale. Cependant, en raison du développement fréquent de phénomènes de résistance aux fongicides chez l'agent pathogène et d'une demande sociale toujours plus croissante pour réduire l'utilisation des intrants d'origine chimique en agriculture, des méthodes alternatives de protection des cultures efficaces doivent être développées. L'une des alternatives possibles pour la gestion des maladies des cultures est le recours au biocontrôle. Celui-ci peut se définir comme un ensemble de méthodes de protection contre les bio-agresseurs des cultures, basées sur l'utilisation d'organismes vivants bénéfiques ou de substances d'origine naturelle (INRAE, 2016).

Dans le cadre de ce projet de thèse, nous avons cherché à identifier de nouveaux composés de biocontrôle efficaces pour protéger le blé vis-à-vis de *Z. tritici* et à caractériser leurs modes d'action. Dans le présent manuscrit, nous débiterons avec un état de l'art présentant les différentes facettes du sujet, puis viendra la description du contexte général dans lequel s'inscrit ce projet de thèse ainsi que les objectifs visés. Les principaux résultats obtenus seront ensuite décrits et discutés sous forme d'articles scientifiques. Enfin, une discussion générale, comprenant les perspectives des travaux, parachèvera ce manuscrit.

ÉTAT DE L'ART

1. LE BLÉ

1.1. Présentation générale et classification

Le blé est une plante angiosperme monocotylédone appartenant à la famille des *Poaceae* ou graminées (**Figure 1**). Cette dernière regroupe un grand nombre d'espèces parmi les plus importantes sur les plans agronomique et économique, comme, outre le blé, le maïs (*Zea mays*), l'orge (*Hordeum vulgare*), le riz (*Oryza sativa*), les millets ou encore le sorgho (*Sorghum bicolor*) qui constituent l'alimentation de base dans plusieurs régions à travers le monde. Dans cette famille, on retrouve le genre *Triticum*, qui comporte les deux espèces de blé les plus cultivées dans le monde, le blé dur et le blé tendre (**Figure 1**). Le blé dur est majoritairement cultivé dans des régions chaudes et sèches comme celles du pourtour méditerranéen (*i.e.* Afrique du nord et Europe du sud) et sert principalement à la production de semoule et de pâtes alimentaires. Le blé tendre, aussi appelé froment, est cultivé dans des régions présentant des climats plus tempérés. Grâce aux propriétés visco-élastiques particulièrement intéressantes de sa farine, dû à la présence des protéines du gluten, il est utilisé majoritairement pour la fabrication du pain. Il peut aussi être utilisé pour l'alimentation animale, la mouture ou la production de biocarburant. Au niveau mondial, la culture du blé tendre représente plus de 90% de la production de blé (Shewry, 2009).

A

Règne	<i>Plantae</i>
Sous-règne	<i>Tracheobionta</i>
Division	<i>Magnoliophyta</i>
Classe	<i>Liliopsida</i>
Sous-classe	<i>Commelinidae</i>
Ordre	<i>Cyperales</i>
Famille	<i>Poaceae</i>
Sous-famille	<i>Pooideae</i>
Tribu	<i>Triticeae</i>
Genre	<i>Triticum</i>

B



Figure 1 : Présentation taxonomique et botanique du blé tendre. (A) Classification taxonomique du blé tendre et dur et (B) planche botanique du blé tendre (d'après Brandt *et al.*, 1883)

1.2. Histoire évolutive du blé

Historiquement, le blé est l'une des premières plantes domestiquées par l'homme il y a environ 10 000 ans lors de l'apparition de l'agriculture dans le Croissant fertile. Le blé dur (*Triticum turgidum* ssp. *durum*) est un organisme tétraploïde possédant 14 paires de chromosomes ($2n=4x=28$; AABB). Il aurait été domestiqué par d'anciens cultivateurs du Moyen-Orient à partir d'une espèce sauvage locale tétraploïde, toujours existante aujourd'hui, appelée *Triticum dicoccoides*. Cette dernière est issue de l'hybridation de deux espèces graminées diploïdes sauvages, *Triticum urartu* ($2n=2x=14$; AA) et *Aegilops speltoides* ($2n=2x=14$; BB), suivie d'un doublement du nombre de chromosomes (**Figure 2**). Ce croisement aurait eu lieu il y a plus de 500 000 ans. Le blé tendre (*Triticum aestivum*), quant à lui,

est un organisme hexaploïde possédant 21 paires de chromosomes ($2n=6x=42$; AABBDD). Cette espèce serait apparue il y environ 9500 ans. Les preuves génétiques et cytologiques accumulées ces dernières décennies suggèrent que ce blé est issu d'une espèce ancestrale hexaploïde appelée *Triticum spelta*. Cette dernière serait apparue à la suite d'une hybridation, suivie encore une fois d'un doublement des chromosomes, entre une espèce ancestrale tétraploïde de blé dur cultivé au Moyen-Orient à cette époque, *Triticum turgidum* ($2n=4x=28$; AABB), et une autre poacée sauvage diploïde, *Triticum tauschii* ($2n=2x=DD$) (**Figure 2**) (Shewry, 2009 ; Snape and Pánková, 2013).

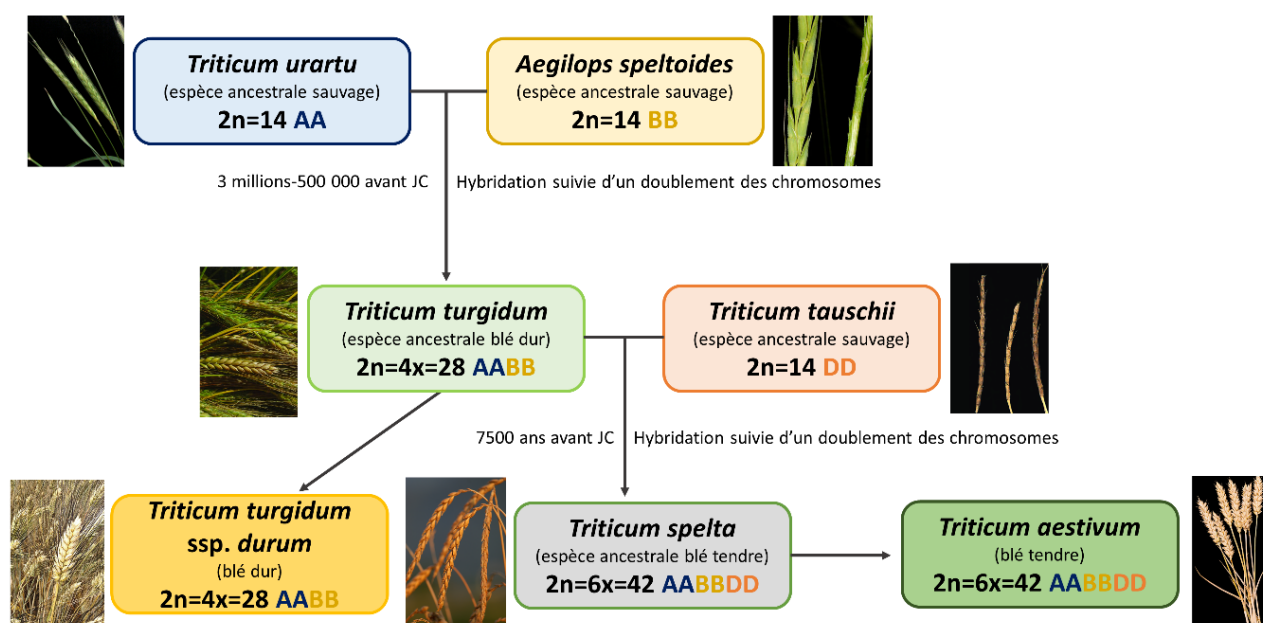


Figure 2 : Histoire évolutive et événements génétiques ayant mené à l'émergence des deux espèces de blé les plus cultivées actuellement, le blé dur (*Triticum turgidum ssp. durum*) et le blé tendre (*Triticum aestivum*). Le blé dur est un organisme tétraploïde (sous-génomes AA et BB). Le blé tendre (sous-génomes AA, BB et DD) est, quant à lui, hexaploïde (modifié d'après Snape and Pánková, 2013).

1.3. Implantation du blé en Europe et en Asie

Après les premiers processus de domestication du blé dans le Croissant fertile, la culture de cette céréale a rapidement été introduite en Europe et en Asie *via* différentes routes (**Figure 3**). Avant 4000 ans avant JC, le blé était implanté partout en Europe (Snape and Pánková, 2013). Il s'imposa ensuite comme une denrée essentielle des principales organisations territoriales et culturelles européennes. L'importance de cette denrée dans l'alimentation en fait une ressource majeure ; ainsi, son niveau de production a fortement influencé la stabilité sociale et politique des organisations européennes et ce dès l'antiquité. Durant l'époque romaine antique, il était fréquent que des pénuries de blé conduisent à des périodes de famines et de violence intenses frappant de plein fouet les bases mêmes des régimes politiques. Les régions hautement productrices devinrent des zones stratégiques majeures et furent au cœur de nombreux conflits à travers l'histoire. Ce fut le cas par exemple de l'Egypte au moment de la Rome antique (Evans, 1981).

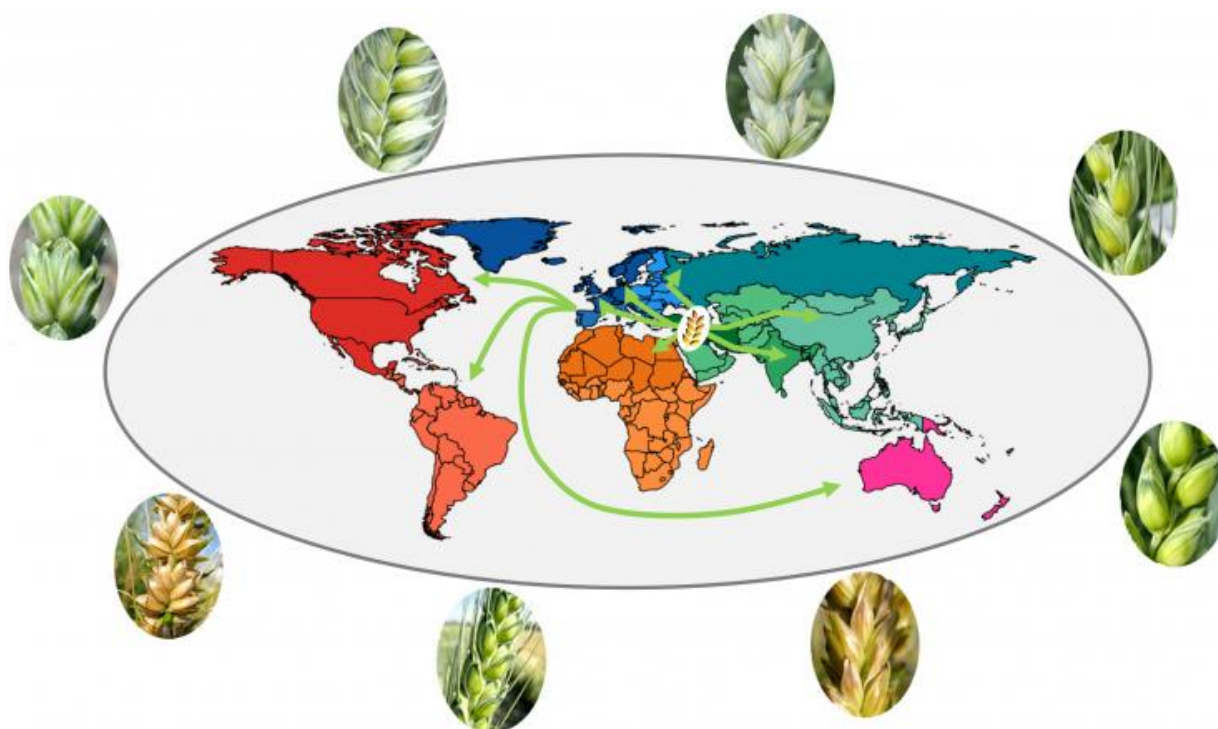


Figure 3 : Expansion de la culture blé depuis sa domestication au Croissant Fertile au Néolithique (d'après INRAE, 2020).

Le génome du blé tendre est l'un des génomes les plus complexes observés pour les organismes végétaux cultivés. Celui-ci est composé de trois sous-génomes différents (AA, BB et DD) du fait des multiples hybridations ancestrales ayant mené à l'hexaploidie de cette espèce (**Figure 2**). La taille particulièrement grande de ce génome ajoute encore à cette complexité. En effet, celui-ci comporte 17 gigabases (17 milliards de paires de base), ce qui correspond à environ 5 fois la taille du génome humain ou à 35 fois celle du riz. Il a fallu plus de 10 ans de travaux au consortium *International Wheat Genome Sequencing Consortium* (IWGSC) pour réaliser le séquençage, l'assemblage et l'annotation complet du génome du blé tendre. Ce travail titanesque s'est terminé récemment, en 2018, et 107 891 gènes ont été identifiés avec un haut degré de confiance, avec une distribution relativement égale entre les différents sous-génomes (AA=35345, BB=35643 et DD=34212 gènes). Les résultats ainsi obtenus pourront être utilisés afin de mieux comprendre la biologie du blé et cibler de nouveaux gènes impliqués dans la régulation de différents caractères d'intérêt agronomique, tels que les résistances aux stress abiotiques et biotiques, le rendement, ou encore les caractéristiques nutritionnelles du grain. Il est à noter que si la taille du génome du blé est exceptionnellement élevé, celui-ci contient plus de 85% de séquences répétées (Consortium IWGSC, 2018).

1.4. Production mondiale de blé

De nos jours, le blé est l'une des cultures les plus produites et consommées dans le monde, avec le maïs et le riz, et constitue l'aliment de base pour plus d'un tiers de la population mondiale. Selon la *Food and Agriculture Organization* (FAO), la production de blé fut de 732 millions de tonnes pour la période 2017-2018 et atteindrait 761 millions de tonnes selon les récentes estimations pour la période 2018-2019 (FAO, 2020). Les cinq principaux producteurs de blé, sont, par ordre décroissant, l'Union

européenne (UE), la Chine, l'Inde, la Russie et les Etats-Unis (**Figure 4**). Au niveau de l'UE, qui enregistra une production de 156 millions de tonnes pour l'année 2019 (FAO, 2020), les deux états-membres produisant le plus de blé sont la France, qui est aussi le premier pays exportateur de blé de l'UE, et l'Allemagne avec des productions respectives d'environ 34 et 20 millions de tonnes pour le blé tendre et 1,8 et 0,1 millions de tonnes pour le blé dur pour la période 2018-2019 (FranceAgriMer, 2020).

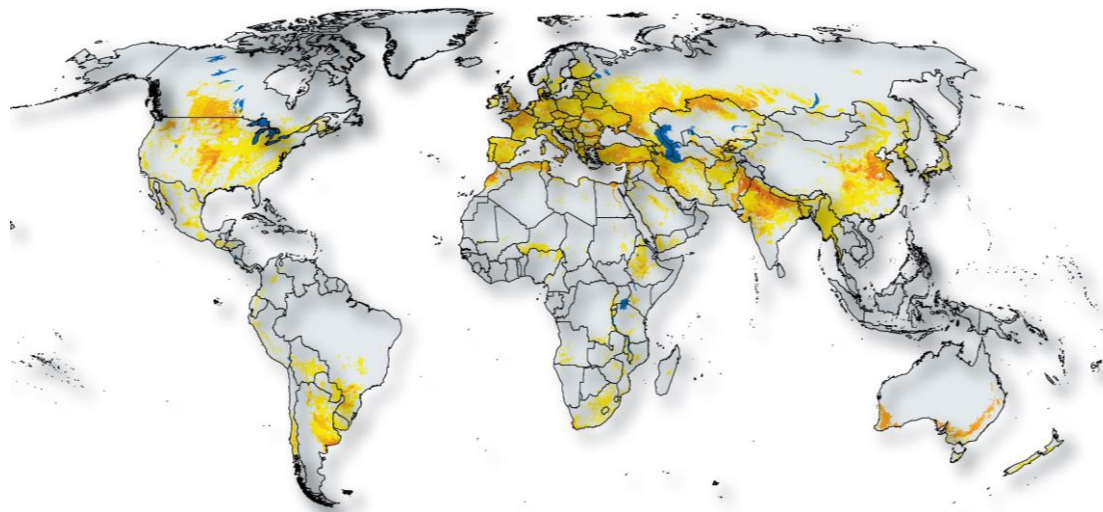


Figure 4 : Principales régions productrices de blé dans le monde en 2014 (modifié d'après You *et al.*, 2014). Les couleurs plus foncées indiquent les zones où le blé est plus cultivé.

1.5. Cycle de développement du blé

Le blé est une plante annuelle dont le cycle de développement est décrit en plusieurs étapes principales, en allant de la germination du grain (le caryopse, c'est-à-dire le fruit du blé, caractéristique des céréales) à la formation de la génération suivante de grains au sein de l'épi et jusqu'à la sénescence de la plante. Ces étapes sont couramment décrites suivant un code décimal qui se base sur l'échelle de Zadoks *et al.* (1974) (**Figure 5**) (Lancashire *et al.*, 1991). Brièvement, en premier lieu, le cycle débute par la germination du grain, suivie par une phase de développement végétatif de l'individu comprenant des phénomènes d'organogénèse racinaire et foliaire. Lors de cette phase, et lorsque la plante atteint le stade trois feuilles (environ 21 jours après germination), de nombreuses talles (tiges adventices émergeant de la plantule initiale) sont formées. S'en suit une extension importante de la tige et la montaison du blé. La phase reproductrice commence ensuite, lorsque la plante est suffisamment développée, avec les phénomènes d'initiation foliaire, de développement des épis et de production des gamètes. Après fécondation, la phase de formation et maturation des grains a lieu et ce jusqu'à leur déhiscence et sénescence de la plante (**Figure 5**).

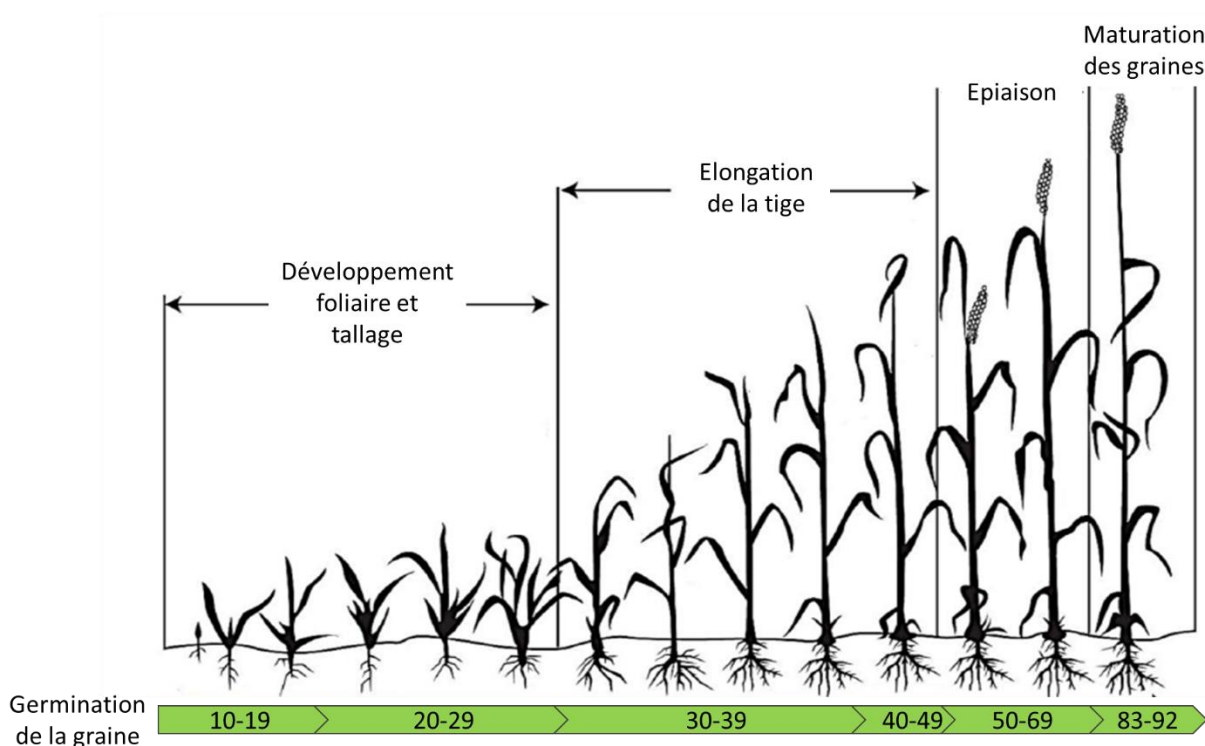


Figure 5 : Principales étapes du développement du blé selon l'échelle de Zadocks *et al.* (1974). Le premier chiffre de cette échelle décimale correspond au stade principal de développement, sous-divisé en étapes plus précises, indiquées par le deuxième chiffre. Stade 0, germination de la graine ; stade 1, développement des feuilles ; stade 2, tallage ; stade 3, élongation de la tige principale ; stade 4, montaison ; stade 5, épiaison ; stade 6, floraison ; stade 7, développement des grains ; stade 8, maturation des grains ; stade 9, sénescence.

1.6. Impact des stress environnementaux sur la culture du blé

Tout au long de son cycle de développement, le blé subit de nombreuses contraintes capables d'affecter sévèrement sa croissance et son rendement. Au niveau abiotique, par exemple, l'une des principales inquiétudes actuelles est l'impact que pourra avoir le réchauffement climatique sur la production du blé. En effet, de nombreuses études ont déjà reporté l'effet négatif de températures élevées (au-dessus de 30 °C) sur le rendement en grain, en particulier lorsque ces épisodes climatiques avaient lieu lors de la phase reproductrice (Girousse *et al.*, 2018). Lobell *et al.* (2011) avaient déjà estimé l'impact de ce stress comme étant responsable chaque année de pertes de rendement d'environ 5,5 % et de pertes nettes de production de 35 millions de tonnes au niveau mondial. Au niveau biotique, les maladies du blé peuvent être causées par un large panel de bio-agresseurs, comme les champignons, les bactéries, les virus et les nématodes. Les maladies les plus fréquentes et causant le plus de dommages au niveau de la culture sont le plus souvent d'origine cryptogamique (Figuerola *et al.*, 2018). En Europe de l'Ouest, la maladie considérée comme la plus menaçante pour la production de blé est la septoriose du blé (*Septoria tritici blotch*, STB) (Fones and Gurr, 2015).

2. ZYMOSEPTORIA TRITICI

2.1. Présentation et classification taxonomique

Zymoseptoria tritici (anciennement forme anamorphe *Septoria tritici* et téléomorphe *Mycosphaerella graminicola*) est un champignon ascomycète appartenant à la classe des *Dothideomycetes* (Quaedvlieg *et al.*, 2011). Plus précisément, il appartient à l'ordre des *Dothideales* et la famille des *Mycosphaerellaceae*. Cette dernière est connue pour contenir un grand nombre d'espèces pathogènes des plantes tel que *Cercospora beticola*, l'agent responsable de la maladie foliaire la plus dévastatrice de la betterave, ou encore *Ramularia collo-cygni*, un agent pathogène très préoccupant sur la culture de l'orge (Groenewald *et al.*, 2007 ; Walters *et al.*, 2008). *Zymoseptoria tritici* est actuellement l'agent principal responsable de la STB, maladie foliaire capable d'affecter le blé tendre comme le blé dur, et considérée comme l'une des principales contraintes actuelles pour la production de cette denrée alimentaire majeure (O'Driscoll *et al.*, 2014). Ce champignon aurait émergé en tant qu'agent phytopathogène spécialisé du blé il y a environ 10 000 ans, lors de la domestication de ce dernier dans le Croissant Fertile (Stukenbrock *et al.*, 2007). Ce parasite se serait ensuite disséminé en accompagnant la migration du blé dans l'Eurasie et l'Afrique d'abord, puis, lors de l'époque des grandes découvertes dans le monde (Banke and McDonald, 2005). La forme anamorphe du champignon a été décrite pour la première fois en 1842 par Desmazières (Shipton *et al.*, 1971) alors que sa forme téléomorphe, moins fréquente et plus complexe à observer, n'a été décrite et rattachée à *Septoria tritici* qu'un siècle plus tard, en 1972, en Nouvelle-Zélande (Sanderson, 1972). Depuis, la forme parfaite de ce champignon a été observée en France et dans plusieurs régions à travers le monde (Halama, 1996 ; Zhan *et al.*, 2003 ; Hassine *et al.*, 2019).

2.2. Importance économique de la septoriose du blé

La septoriose du blé a été rapportée dans toutes les régions du monde où le blé est cultivé. Il s'agit d'une maladie fongique exclusivement foliaire qui limite grandement le potentiel photosynthétique du blé, car il engendre des lésions (chloroses et nécroses) sur les feuilles (**Figure 6**), des modifications dans le métabolisme primaire de son hôte et prélève à la plante ses ressources, incluant ses sucres et acides aminés (Shtienberg, 1992 ; Shetty *et al.*, 2007). Cela entraîne des pertes de rendement qui se caractérisent notamment par une diminution du poids et de la qualité des grains (McKendry *et al.*, 1995)

Dans certaines régions présentant des conditions agro-environnementales propices au développement de la maladie, celle-ci entraîne des pertes de rendement sévères pouvant atteindre les 50 % (Eyal *et al.*, 1987). C'est le cas, tout particulièrement, pour une partie de l'Europe de l'Ouest, caractérisée par Bouma (2005) de « zone maritime », où règne un climat humide particulièrement favorable à la croissance et à la propagation de *Z. tritici*. Dans cette zone, les pays les plus affectés par septoriose sont la France, l'Allemagne et le Royaume-Uni, soit les principaux pays producteurs de blé de l'Union Européenne (UE). Les pertes annuelles engendrées par cette maladie sont estimées à 400 millions de dollars pour l'UE et 70 % des fongicides qui y sont utilisés annuellement ciblent uniquement ce champignon (Fones and Gurr, 2015 ; Yemelin *et al.*, 2017).



Figure 6 : Symptômes de la septoriose du blé causés par le champignon ascomycète *Zymoseptoria tritici*. (A) Effet de la maladie sur un champ d'une variété de blé sensible (d'après Rothamsted Research, Harpenden, UK). (B) Observation de feuilles de blé infestées où sont apparentes les lésions nécrotiques et chlorotiques typiques de la maladie. Les zones nécrosées présentent des ponctuations noires, les pycnides, qui sont issues du mode de multiplication asexuée du champignon (d'après Juina UMR-t 1158 BioEcoAgro).

En France, les pertes de rendement causées annuellement par *Z. tritici* ont été en moyenne de 16,8 quintaux par hectare (q/ha) sur la décennie 2002-2012. La nuisibilité de la maladie diffère grandement au niveau régional. Ainsi, elle est généralement beaucoup plus importante dans le Nord-Ouest du pays. Les pertes moyennes de rendement ont, par exemple, été de 19,2 q/ha dans le Nord-Pas-de-Calais (Hauts-de-France) et ont atteint jusqu'à 24,1 q/ha en Bretagne. Dans d'autres régions, la nuisibilité a été bien moindre, comme en Auvergne, où les pertes ont été de 7,9 q/ha en moyenne (**Figure 7**) (Arvalis-Institut du végétal, 2013a).

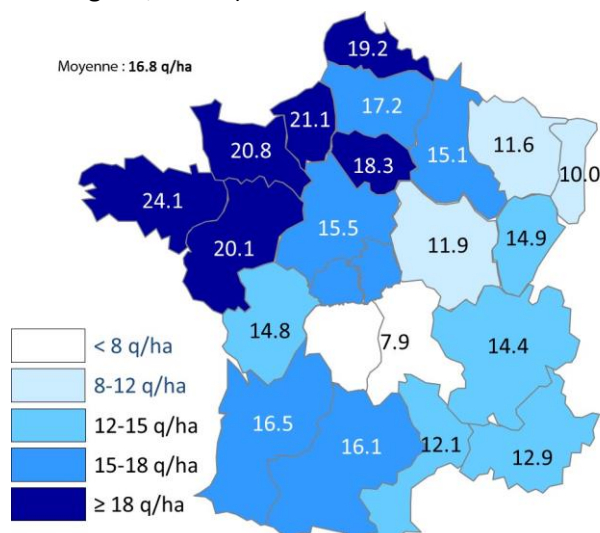


Figure 7 : Pertes de rendement annuel moyen causées par *Zymoseptoria tritici* en France métropolitaine sur la période 2002-2012. Les pertes de rendement sont indiquées en quintaux par hectares (q/ha) selon le découpage régional d'avant 2016 (d'après Arvalis-Institut du végétal, 2013a).

La sévérité de la maladie peut également grandement varier d'une année à l'autre. Différents facteurs interviennent dans cette variabilité, comme la quantité d'inoculum présent en début de saison, l'humidité, la température ou encore le stade de croissance du blé lors de l'infection (Holmes and Colhoun, 1974 ; Chungu *et al.*, 2001). En raison de son impact agronomique et économique majeur, ainsi que pour toutes les caractéristiques biologiques particulières qu'il présente, *Z. tritici* a été classé parmi le top 10 des espèces de champignons phytopathogènes modèles (Dean *et al.*, 2012).

2.3. Cycle épidémique

L'épidémie de septoriose commence le plus généralement en automne dès la levée du blé. L'inoculum responsable des contaminations du blé peut se présenter soit sous forme d'ascospores (**Figure 8**), issues de la reproduction sexuée du champignon, soit sous forme de pycnidiospores (**Figure 9**), issues de la multiplication asexuée.

2.3.1. Les ascospores, forme principale des infections primaires

Les ascospores sont considérées comme majoritairement responsables des premières infections au champ, jouant ainsi le rôle d'inoculum dit primaire. Elles peuvent provenir des résidus de culture de blé de l'année précédente, de repousses spontanées de la culture ou être produites sur d'autres espèces herbacées servant d'hôtes alternatifs pour le pathogène (Wenham, 1959 ; Shaw and Royle, 1989 ; Suffert *et al.*, 2011). Certaines graminées adventices extrêmement communes, notamment en Europe, peuvent ainsi jouer le rôle de réservoir d'inoculum, c'est le cas, des rays-grass (*Lolium perenne*, *Lolium multiflorum*), du vulpin des prés (*Alopecurus pratensis*), du pâturin annuel (*Poa annua*) ou encore du seigle (*Secale cereale*) (Williams and Jones, 1973 ; Ao and Griffiths, 1976; Haghdel and Banihashemi, 2005)

Les ascospores bicellulaires de *Z. tritici* résultent obligatoirement du croisement entre des souches présentant des *mating types* compatibles, MAT1-1 et MAT1-2 (Halama, 1996 ; Kema *et al.*, 1996). Ce mode de reproduction assure une diversité génétique élevée des populations sur le terrain et favorise les flux de gènes ainsi que l'apparition rapide de résistance aux fongicides (Zhan *et al.*, 2003 ; Zhan *et al.*, 2006). Les ascospores sont abritées dans des structures spécialisées du champignon, appelées asques, qui sont eux-mêmes contenus dans des fructifications appelées périthèces ou pseudothèces (**Figure 8**). Ces fructifications, localisées au niveau sub-épidermique des feuilles, présentent une paroi épaisse à double couche et sont de couleur brun foncé. Chaque pseudothèce peut contenir environ 26 asques qui abritent chacun 8 ascospores, les ascospores ainsi produites lors de l'épidémie sont transportées par le vent sur de très longues distances, pouvant dépasser les 100 km (Eriksen and Munk, 2003). Comme elles participent également à la propagation de la maladie pendant la saison, elles sont donc aussi responsables de contaminations dites secondaires (Eriksen and Munk, 2003).

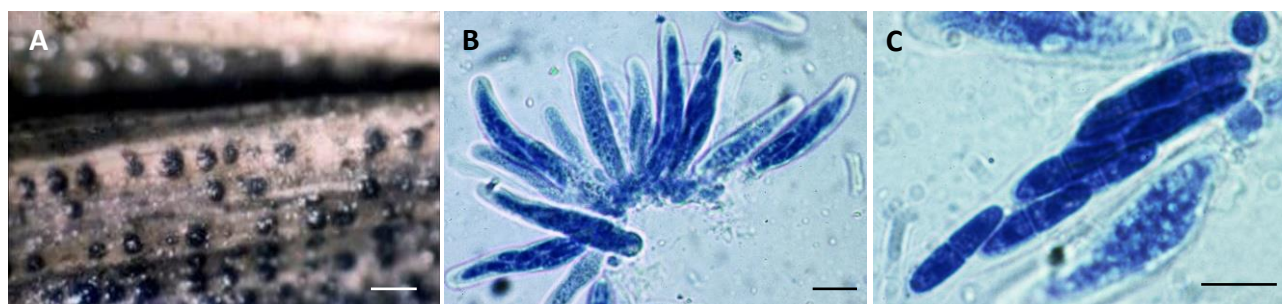


Figure 8 : Structures issues de la reproduction sexuée de *Zymoseptoria tritici*. (A) Observation de périthèces à la loupe binoculaire, barre d'échelle = 1 mm. (B) Asques contenant 8 ascospores observés au microscope optique. (C) Ascospores bicellulaires de *Z. tritici* observées au microscope optique. Barre d'échelle = 10 µm pour (B) et (C) (d'après Halama, 1996).

2.3.2. Les pycnidiospores, forme principale des infections secondaires au niveau local

Les pycnidiospores, spores issues de la multiplication asexuée du champignon, peuvent aussi jouer le rôle d'inoculum primaire même si elles sont considérées moins importantes dans ce processus que les ascospores. Elles sont contenues dans des structures globuleuses, de couleur brune, les pycnides (**Figure 9**). Les pycnidiospores sont produites dans une matrice mucilagineuse appelée cirrhe, qui leur donne la capacité de rester viable durant de longues périodes de conditions défavorables, comme la faible humidité et les températures extrêmes (Gough and Lee, 1985).

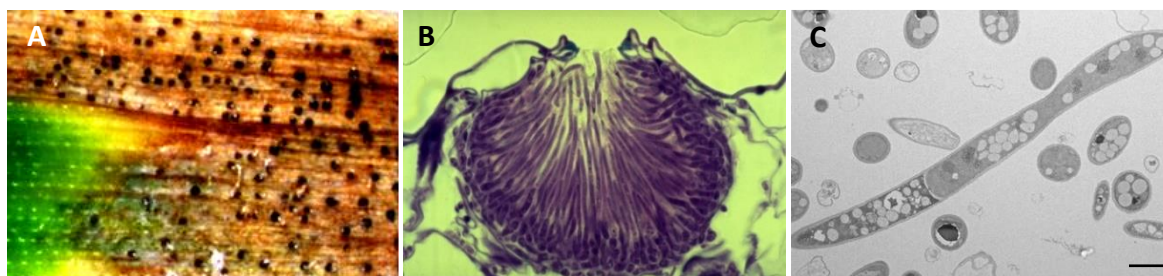


Figure 9 : Structures issues de la multiplication asexuée de *Zymoseptoria tritici*. (A) Lésions causées par le champignon sur une feuille de blé où sont observables les pycnides matures exsudant le cirrhe qui contient les pycnidiospores (d'après Junia UMR-t 1158 BioEcoAgro). (B) Coupe transversale d'une pycnide mature observée au microscope optique contenant un grand nombre de pycnidiospores (d'après Kema et al., 1996). (C) Pycnidiospore cultivée en milieu liquide yeast extract sucrose broth observée au microscope électronique à transmission. Barre d'échelle = 2 µm (d'après Francisco et al., 2019).

Les pycnidiospores sont surtout impliquées dans la propagation de la maladie pendant l'épidémie et à un niveau local. A l'issue des premières infections, des pycnides se développent sur les premiers étages foliaires. Sous l'effet des éclaboussures de gouttelettes de pluie, les pycnidiospores contenues dans le cirrhe se dispersent et ensuite contaminent les étages foliaires supérieurs et les plantes adjacentes ; on parle alors du phénomène de *rain-splashing* (**Figure 10**) (Shaw, 1987). Après la récolte du blé, les spores de *Z. tritici* (pycnidiospores et ascospores) pourront contaminer les hôtes alternatifs, repousses de blé ou persister dans les résidus de culture, ce qui donnera naissance à l'inoculum primaire de la saison suivante (**Figure 10**) (Suffert et al., 2011). Par ailleurs, les populations de *Z. tritici* sont caractérisées par une très grande diversité génétique. En effet, à l'aide de marquages génétiques, Siah et al. (2018) ont estimé à 50 millions le nombre d'haplotypes différents présents dans un seul hectare de blé. Cette taille de population conséquente rend la maladie particulièrement difficile à contrôler.

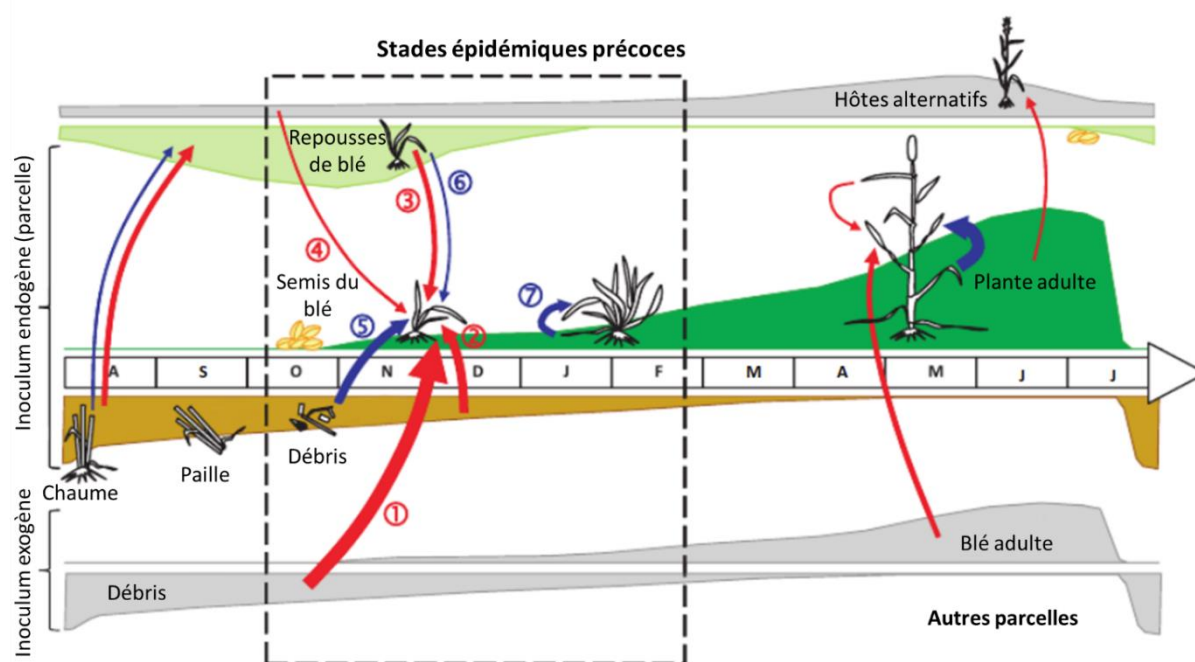


Figure 10 : Représentation schématique de la dynamique épidémique annuelle de la septoriose du blé. Les flèches rouges représentent les contaminations causées par les ascospores et les bleues celles causées par les pycnidiospores. Les ascospores sont disséminées par le vent et peuvent contaminer les cultures de blé sur de longues distances ; les pycnidiospores sont disséminées par *rain-splashing* sur de courtes distances. L'épaisseur des flèches est proportionnelle à l'impact supposé du mécanisme impliqué. Les stades épidémiques précoces principaux sont décrits plus précisément, avec 1 : contaminations par des ascospores provenant de débris de cultures de blé situés à longue distance ; 2 : ascospores provenant de débris de culture de blé proches ; 3 : ascospores provenant de repousses spontanées du blé ; 4 : ascospores provenant d'hôtes alternatifs ; 5 : pycnidiospores provenant de débris de blé ; 6 : pycnidiospores provenant de repousses de blé ; 7 : pycnidiospores provenant de feuilles basales infectées (d'après Suffert *et al.*, 2011).

2.4. Processus infectieux

Zymoseptoria tritici est un agent pathogène hémibiotrophe ; son cycle de développement se compose d'une phase biotrophe suivie d'une phase nécrotrophe.

2.4.1. Germination et pénétration

L'infection du champignon débute par la germination des spores (ascospores et/ou pycnidiospores) environ 12h après contact avec la feuille du blé. Cette étape est fortement dépendante du niveau d'humidité ambiante. Selon Ponomarenko *et al.* (2011), au moins 20h d'humidité relative élevée sont nécessaires pour la réussite de l'infection. Après germination, les tubes germinatifs deviendront des hyphes qui pourront ensuite s'insinuer dans les tissus foliaires (**Figure 11**) (Eyal, 1987 ; Duncan and Howard, 2000). Le mode de pénétration de *Z. tritici* a été le sujet de nombreux débats au sein de la communauté scientifique. Celle-ci a généralement lieu entre 24 et 48h après contamination. Néanmoins, Fones *et al.* (2017) ont récemment montré que la croissance épiphyte des hyphes du champignon pouvait durer plusieurs jours avant pénétration. Il a été rapporté que le champignon était capable de rentrer dans les tissus de l'hôte soit, *via* les stomates, soit, plus rarement, directement à travers l'épiderme de la feuille, généralement au niveau des parois anticlinales (Kema *et al.*, 1996 ; Duncan and Howard, 2000 ; Shetty *et al.*, 2003 ; Siah *et al.*, 2010). Certains auteurs suggèrent que le champignon serait capable de reconnaître et de croître en direction des stomates

grâce à un signal thigmotropique encore inconnu (Duncan and Howard, 2000 ; Cousin *et al.*, 2006). Cette hypothèse est toutefois loin d'être consensuelle, et de nombreux auteurs considèrent que la croissance des hyphes sur la surface des feuilles se déroule de manière aléatoire (Fones *et al.*, 2017). Contrairement à d'autres champignons phytopathogènes, comme *Magnaporthe oryzae*, un agent pathogène majeur du riz, *Z. tritici* ne forme pas d'appressorium lors de cette étape de pénétration foliaire. L'absence de cette structure spécialisée est corrélée avec l'absence de plusieurs gènes impliqués dans sa formation dans le génome de *Z. tritici* (Goodwin *et al.*, 2011). Toutefois, plusieurs équipes ont rapporté la formation de pseudo-appressoria, sous forme de renflements, localisés au niveau des zones de pénétration (**Figure 11**) (Kema *et al.*, 1996 ; Duncan and Howard, 2000 ; Siah *et al.*, 2010).

2.4.2. Phase biotrophe

Après pénétration, le champignon colonise les cavités stomatiques sans causer de symptômes apparents sur les feuilles. Cette période est appelée phase latente ou biotrophe et peut durer, selon la variété du blé, la souche de *Z. tritici* et les conditions environnementales, entre 6 et 36 jours au champ et entre 9 et 14 jours au laboratoire (Steinberg, 2015). Durant cette phase, *Z. tritici*, contrairement à d'autres champignons phytopathogènes, ne produit pas de structure spécialisée dans la nutrition (haustorium). Rudd *et al.* (2015) ont révélé que la source d'énergie principale du champignon provenait majoritairement de ses propres réserves lipidiques. Le pathogène est toutefois aussi capable d'utiliser les ressources de la plante, notamment les sucres présents dans l'apoplasme, mais aussi les composés issus de dégradation de la cuticule du blé, comme le suggère la surexpression de gènes codant pour des lipases et des cutinases par *Z. tritici* durant cette phase (Rohel *et al.*, 2001 ; Palma-Guerrero *et al.*, 2016). Pendant cette période, tout en évitant les défenses de la plante, les hyphes croissent et colonisent l'espace, ce qui aboutit à la formation des pré-pycnides (**Figure 11**) (Duncan and Howard, 2000).

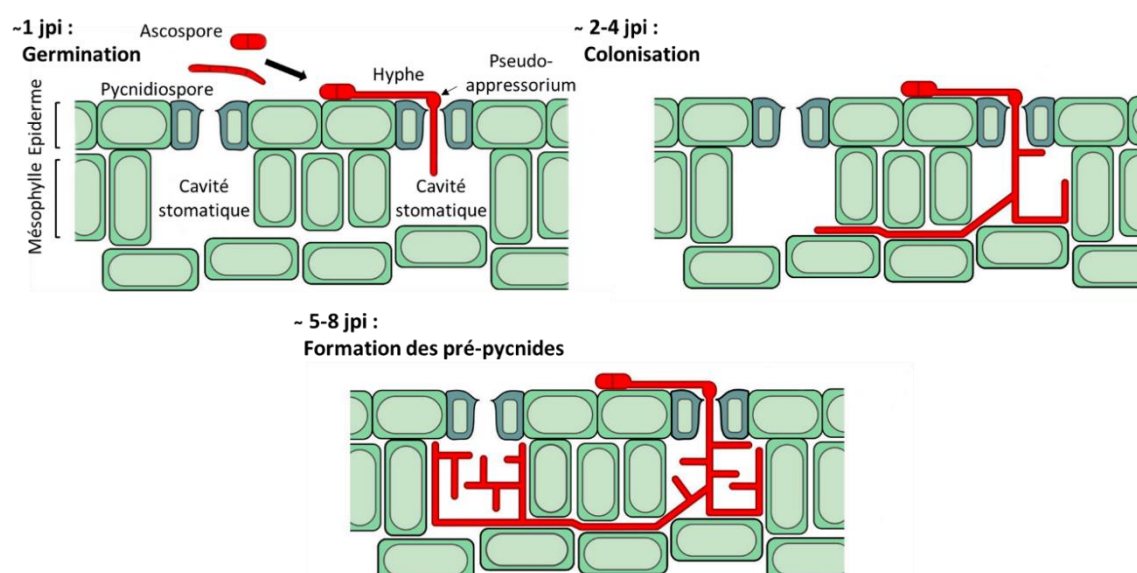


Figure 11 : Représentation schématique du développement de *Z. tritici* sur feuille de blé lors de la phase biotrophe. Après germination, les spores du champignon produisent des hyphes qui pourront infecter les tissus foliaires, soit directement *via* la cuticule, soit *via* les stomates. Le champignon se développe jusqu'à la formation de pré-pycnides. Aucun symptôme d'infection n'est apparent durant cette phase sur la feuille. Jpi : jours post inoculation (d'après Steinberg, 2015).

2.4.3. Phase nécrotrophe

Environ deux semaines après contamination, *Z. tritici* entre dans sa phase nécrotrophe qui se traduit notamment par l'apparition des premiers symptômes sur la feuille, des lésions nécrotiques et chlorotiques, ainsi que par la maturation des pycnides (Kema *et al.*, 1996 ; Steinberg, 2015) (**Figure 12**). Durant cette phase, le champignon libère différentes molécules, comme des protéines effectrices riches en cystéines ou des protéines nécrosantes, qui entraîneront la mort des cellules du mésophylle du blé (Rudd *et al.*, 2015). Parmi les composés impliqués dans le processus d'apoptose, les enzymes de dégradation de la paroi cellulaire du blé (*cell wall degrading enzymes*, CWDE) jouent un rôle fondamental (Brunner *et al.*, 2013). Ainsi, Siah *et al.* (2010) ont pu mettre en évidence, lors de la phase nécrotrophe, une forte corrélation entre l'activité de l'endo- β -1,4-xylanase, une CWDE, et le développement de la maladie ($r = 0,94$), pour 26 souches de *Z. tritici*. La mort des cellules entraîne la libération, dans l'apoplasme, de sucres et d'acides aminés libres, utilisés par le champignon pour augmenter grandement sa biomasse (Keon *et al.*, 2007 ; Shetty *et al.*, 2007). En parallèle, les pycnides matures contenant des pycnidiospores sont formées et ces dernières pourront alors infecter d'autres plantes de blé lorsque les conditions seront favorables (Shaw, 1987).

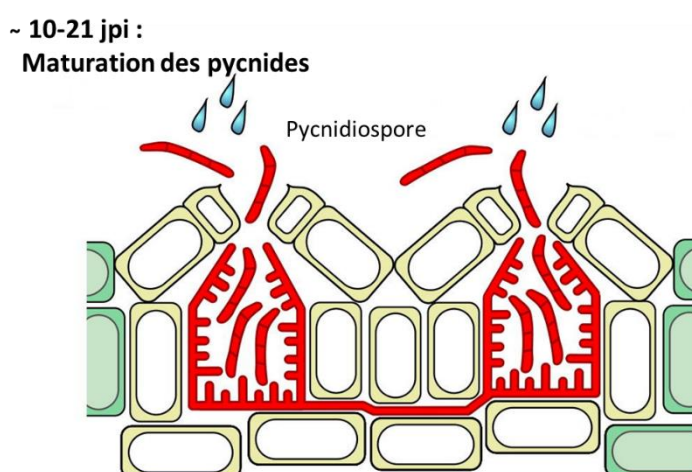


Figure 12 : Représentation schématique du développement de *Z. tritici* sur feuille de blé lors de la phase nécrotrophe. Lors de cette phase, le champignon produit des toxines qui initieront l'apoptose des cellules foliaires. Les ressources libérées sont utilisées par le pathogène comme source d'énergie. En parallèle, les pycnides se développent puis, sous l'effet des gouttelettes de pluie, les pycnides libèrent les pycnidiospores qui pourront contaminer d'autres feuilles ou plantes de blé. Jpi : Jours post inoculation (d'après Steinberg, 2015).

2.5. Organisation génomique

Zymoseptoria tritici est le premier champignon appartenant à la classe des *Dothideomycetes* dont le génome a été séquencé, en utilisant la souche de référence IPO323 isolée aux Pays-Bas. Les séquences des génomes nucléaire et mitochondrial de cette souche sont disponibles dans la base de données EnsemblFungi (Kema and van Silfhout, 1997 ; Goodwin *et al.*, 2011 ; Flicek *et al.*, 2014). La taille de son génome nucléaire assemblé est de 39,69 Mb, réparti sur 21 chromosomes qui présentent une grande variété de longueur, allant de 0,39 à 6,09 Mb (Mehrabi *et al.*, 2007). Parmi les 21 chromosomes, treize ont été décrits comme indispensables à la survie et au développement de

l'organisme et sont présents chez toutes les souches de cette espèce. Les 8 autres décrits comme facultatifs ou accessoires, pouvant être présents, absents ou réarrangés selon les isolats, et font de *Z. tritici* l'espèce de champignon filamenteux phytopathogène présentant le plus grand nombre de chromosomes facultatifs (Goodwin *et al.*, 2011 ; Yang *et al.*, 2020). Chez *Z. tritici*, la taille des chromosomes accessoires varie de 0,39 à 0,77 Mb et ils représentent environ 12 % du génome total du champignon, tout en étant composés en majorité de séquences répétées (Wittenberg *et al.*, 2009). Le rôle exact de ces chromosomes facultatifs reste encore à déterminer mais, selon Croll et McDonald (2012), ils pourraient, du fait de leur taux d'évolution rapide, servir de « berceau de l'évolution adaptative » pour le champignon et seraient un composant de sa redoutable plasticité sur le terrain. Ils seraient le siège de mutations rapides, fournissant ainsi au champignon des avantages phénotypiques lui permettant de répondre efficacement aux contraintes environnementales et aux réactions de défenses activées par l'hôte, tout en ayant, en conditions non-stressantes, un impact faible sur le métabolisme général du pathogène (Croll and McDonald, 2012 ; Testa *et al.*, 2015). Par ailleurs, des expériences d'infections de différents cultivars de blé avec des souches de *Z. tritici* ayant perdu un ou plusieurs de ces chromosomes accessoires ont démontré que certains d'entre eux ont un rôle dans l'interaction spécifique hôte-pathogène et/ou dans le déclenchement du passage de la phase biotrophe à la phase nécrotrophe (Habig *et al.*, 2017). Stewart *et al.* (2018), ont aussi récemment démontré l'association, significative mais faible, de quatre chromosomes accessoires avec des traits de virulence de *Z. tritici* grâce à une étude de cartographie de *quantitative trait loci* (QTL). Les deux études suggèrent ainsi le rôle potentiel des chromosomes accessoires dans la virulence du champignon et les interactions hôte-pathogène mais il reste encore beaucoup à comprendre sur le fonctionnement et le rôle de ces chromosomes facultatifs chez *Z. tritici*.

2.6. *Zymoseptoria tritici*, champignon dimorphe ou pléomorphe ?

Les champignons dimorphes sont des organismes ayant la capacité de changer entre deux morphologies, levure-like unicellulaire et hyphe pluricellulaire, lors de leur cycle de vie, en fonction des conditions environnementales (**Figure 13**) (Souza and Taborda, 2020). De nombreux champignons pathogènes majeurs, des animaux comme des végétaux, font partie de cette catégorie. C'est le cas par exemple de l'agent phytopathogène *Ustilago maydis*, responsable du charbon du maïs, ou des espèces appartenant au genre *Verticillium* (Ruiz-Herrera *et al.*, 1995 ; Nadal *et al.*, 2008). *Zymoseptoria tritici* est lui aussi considéré comme dimorphe, son nom de genre est basé notamment sur l'observation de sa morphologie de type levure, d'où le préfixe « *Zymo* » signifiant « levain, ferment » en grec (Quaedvlieg *et al.*, 2011). Certains gènes impliqués dans la morphogénèse de *Z. tritici* ont été identifiés et caractérisés, comme par exemple des gènes codant pour des *mitogen-activated protein kinases* (MAPK) ou pour des protéines impliquées dans la voie de signalisation de l'adénosine monophosphate cyclique (AMPC) (Mehrabi *et al.*, 2006a ; Mehrabi *et al.*, 2009). Les différentes morphologies peuvent jouer différents rôles durant le processus infectieux. La morphologie de type levure permettrait une rapide augmentation de la taille de la population et favoriserait sa dispersion, facilitant ainsi la colonisation de nouveaux milieux (Lin *et al.*, 2015). Chez *Z. tritici*, cette morphologie a surtout été observée *in vitro* et très rarement *in planta*. Récemment, Francisco *et al.* (2019), ont rapporté avoir observé ce mode de développement sur feuille de blé, mais sa contribution dans les processus infectieux et épidémique est encore inconnue. Les auteurs émettent l'hypothèse qu'il pourrait être

impliqué dans la dissémination de la maladie. Les hyphes sont, quant à eux, essentiels à la colonisation et à la pénétration du champignon dans les tissus foliaires ; les souches de *Z. tritici* incapables de produire des hyphes sont en effet incapables d'infecter la plante (Mehrabi *et al.*, 2006b ; Steinberg, 2015). Les hyphes sont aussi associés à des mécanismes d'évitement des conditions stressantes comme le stress oxydatif, les températures élevées, ainsi que le manque de ressources disponibles (Francisco *et al.*, 2019). Par exemple, Il a été démontré que la souche IPO323 de *Z. tritici* cultivée sur un milieu riche en nutriments avec des températures comprises entre 15°C et 18°C présente une morphologie levure-like, alors qu'elle se développe sous forme d'hyphe sur un milieu pauvre en ressources et avec des températures élevées, entre 25 et 28 °C (Motteram *et al.*, 2011 ; King *et al.*, 2017). La quantité de nutriments et leur biodisponibilité étant très limitées sur la surface des feuilles (Derridj, 1996), Francisco *et al.* (2019) suggèrent qu'après germination des spores sur la feuille de blé, celles-ci percevraient ce manque de ressources qui agirait comme un stimulus induisant la formation d'hyphes qui seraient plus à même d'explorer efficacement l'environnement à la recherche de sources d'énergie.

Récemment, Francisco *et al.* (2019) ont décrit deux autres morphologies exprimées par *Z. tritici*, les pseudo-hyphes, se différenciant des vrais hyphes par une absence de continuité cytoplasmique entre cellules-mères et filles, ainsi que des structures de type chlamydospores, cellules sphériques à parois épaisses riches en lipides (**Figure 13**) (Veses and Gow, 2009). Si le rôle des pseudo-hyphes reste encore inconnu, les chlamydospores, permettent au champignon, selon Francisco *et al.* (2019), de survivre dans des conditions stressantes (dessiccation, températures élevées ou choc de froid) beaucoup plus efficacement qu'en exprimant d'autres morphologies (levure-like et pseudo-hyphe). Selon ces auteurs, *Z. tritici* serait donc un champignon pléomorphe, capable de produire au moins quatre morphologies distinctes, chacune avec ses rôles spécialisés, en fonction des conditions environnementales. Cette caractéristique lui confère une plasticité phénotypique remarquable, qui associée à sa plasticité génétique et à son *fitness* élevé, le rendent difficile à contrôler au champ.

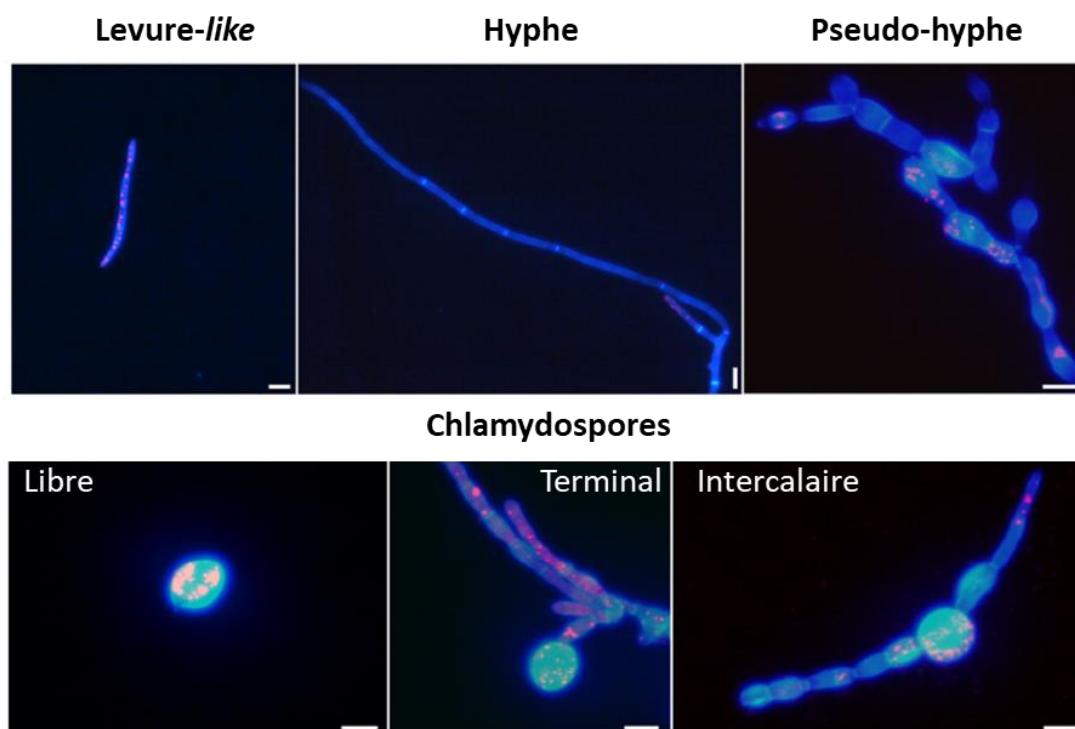


Figure 13 : Morphologies produites par *Zymoseptoria tritici* in vitro observées au microscope optique. La coloration bleue est due à l'utilisation de calcofluor white se liant à la chitine au niveau des parois cellulaires du champignon. Les gouttelettes lipidiques ont été colorées en rouge avec du rouge de Nil. Les structures de type chlamydospores ont été observées libres, situées aux extrémités terminales ou intercalées dans des hyphes ou des pseudo-hyphes (d'après **Francisco et al. 2019**).

3. INTERACTION BLÉ-ZYMOSEPTORIA TRITICI

Tout au long de leur cycle de vie, les plantes doivent faire face à un grand nombre d'agents pathogènes. Malgré l'absence d'un système immunitaire semblable à celui des animaux, les végétaux ont développé des mécanismes de défense interconnectés et complexes pour faire face aux diverses agressions extérieures. Ceux-ci reposent sur deux stratégies distinctes : les défenses constitutives et les défenses induites.

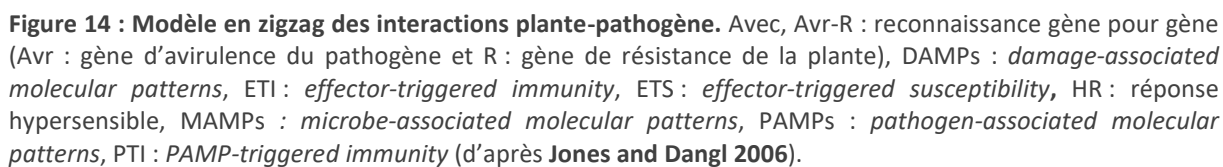
Les défenses constitutives, appelées aussi passives, correspondent aux barrières physiques (paroi, cuticule, trichome) et chimiques (métabolites secondaires) déployées par la plante, avant toute infection, pour se protéger des bio-agresseurs (Freeman and Beattie, 2008). Dans le cas du pathosystème blé-*Z. tritici*, ces défenses constitutives ne sont pas suffisantes pour protéger la plante de l'infection, les spores du champignon arrivant dans la majorité des cas à les contourner, germer et pénétrer dans les tissus foliaires (Shetty et al., 2003). Les défenses induites, appelées aussi actives, correspondent aux réponses (production de composés toxiques, enzymes, mort cellulaire programmée, etc.) mises en place par les cellules végétales après perception de l'invasion par les agents pathogènes. Ces mécanismes de défense active sont souvent coûteux d'un point de vue énergétique (Freeman and Beattie, 2008). Les réactions de défense induites chez le blé contre la septoriose seront développées et détaillées par la suite.

Les interactions plante-pathogène peuvent se dérouler de trois manières différentes : (i) relation non-hôte, (ii) relation incompatible et (iii) relation compatible. Concernant la relation non-hôte, le bio-agresseur (quel que soit son biotype) est incapable de pénétrer et de se développer au sein du végétal (quel que soit le cultivar). Les relations « hôte » sont dépendantes de l'association entre une souche de pathogène et une variété de plante. Si l'agent pathogène parvient à contourner les défenses constitutives de la plante et pénétrer dans les tissus végétaux mais que sa croissance et son développement sont inhibés par les mécanismes de défense induits mis en place par l'hôte, il s'agit d'une relation incompatible. Aucun symptôme n'est engendré lors de cette infection avortée ; le pathogène est qualifié d'avirulent (Reignault and Sancholle, 2005). Dans le cas d'une relation compatible, le pathogène est capable d'infecter son hôte sans que les mécanismes de défense de la plante ne puissent l'empêcher. Les symptômes sont visibles, le pathogène est alors qualifié de virulent (Reignault and Sancholle, 2005).

3.1. Réactions de défense induites du blé vis-à-vis de *Zymoseptoria tritici*

3.1.1. Perception de la présence du pathogène

La mise en place de réponses de défense induites nécessite tout d'abord que l'hôte détecte la présence de l'envahisseur. Pour ce faire, les végétaux sont capables de reconnaître des signaux moléculaires, de compositions chimiques diverses, qui peuvent être classés en trois catégories selon l'origine des composés. Si les molécules sont originaires de l'organisme pathogène lui-même, elles sont appelées *pathogen-associated molecular patterns* (PAMP). Si elles proviennent d'autres micro-organismes non pathogènes, il s'agit alors de *microbe-associated molecular patterns* (MAMP). La troisième et dernière catégorie correspond aux composés et débris cellulaires libérés lors de l'attaque par l'agent pathogène et issus de la plante elle-même. Ceux-ci sont nommés *damage-associated molecular patterns* (DAMP) et proviennent des cellules mortes ou endommagées par les toxines et enzymes du bio-agresseur (Henry *et al.*, 2012). Ces signaux sont reconnus par des récepteurs transmembranaires, les *pattern recognition receptors* (PRR), ce qui engendre par la suite des cascades de signalisation aboutissant à l'activation de voies de défense chez la plante. La mise en place de ces mécanismes suivant la reconnaissance de PAMPs par la cellule végétale est appelée *PAMP-triggered immunity* (PTI). Ce mécanisme de défense est non-spécifique mais peut parfois suffire pour empêcher l'invasion de l'agent phytopathogène (**Figure 14**). Celui-ci est toutefois capable de contourner la PTI en sécrétant des protéines effectrices. Celles-ci vont engendrer un phénomène appelé *effector-triggered susceptibility* (ETS) (**Figure 14**). Les rôles de ces effecteurs sont multiples, ils peuvent, par exemple, dissimuler la présence du champignon, inhiber la PTI et dégrader les cellules végétales (Duba *et al.*, 2018). Le rôle de certaines de ces protéines sera présenté et développé par la suite (**voir partie 3.3 Les effecteurs de *Zymoseptoria tritici***). La plante peut, dans certains cas, reconnaître spécifiquement ces effecteurs, ce qui déclenchera une nouvelle vague de défense appelée *effector-triggered immunity* (ETI) (**Figure 14**). La reconnaissance de ces protéines nécessite des récepteurs appelés *nucleotide binding leucine-rich repeat* (NB-LRR), localisés à l'intérieur des cellules et codés par des gènes de résistance (*R*). L'ETI se révèle généralement plus rapide, plus efficace et plus spécifique que la PTI pour lutter contre l'envahisseur (Jones and Dangl, 2006). L'ETI s'accompagne souvent de mort cellulaire chez l'hôte au niveau des zones les plus infectées, mécanisme aussi appelé réponse hypersensible ou *hypersensitive response* (HR). Le modèle en zigzag de Jones and Dangl (2006) intègre l'ensemble de ces interactions (PTI, ETS et ETI) (**Figure 14**).



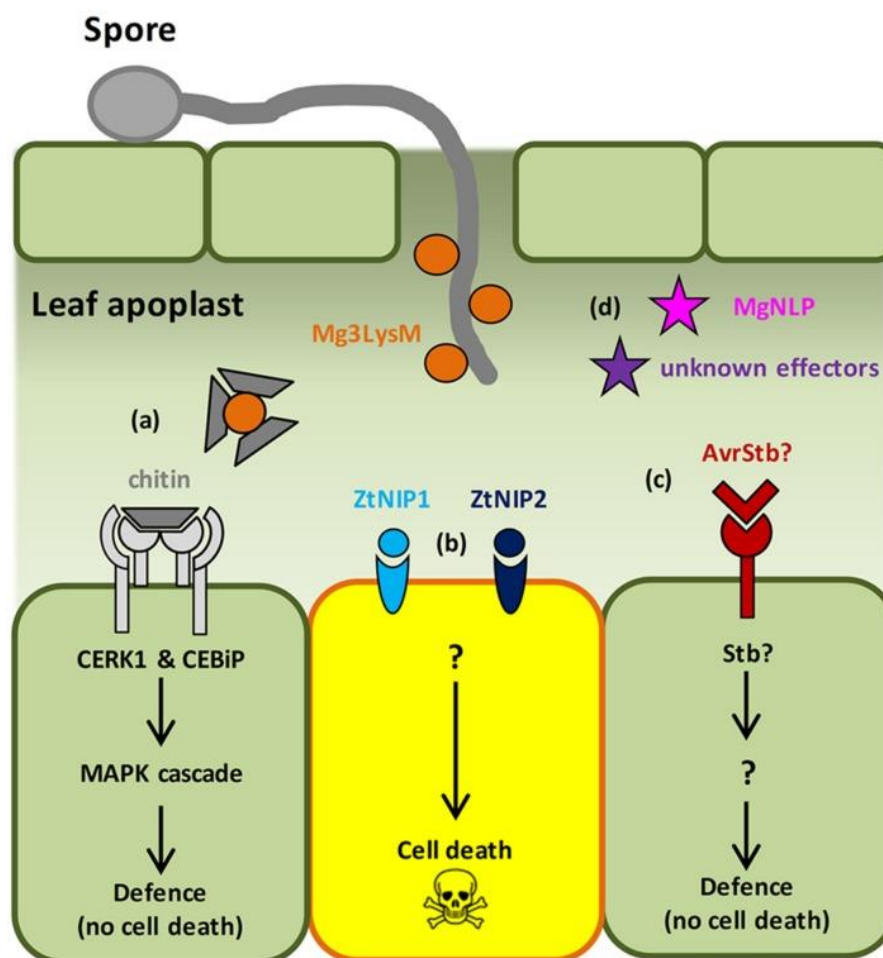


Figure 15 : Schéma des interactions moléculaires blé-*Z. tritici*. (a) La chitine est reconnue par les récepteurs membranaires *chitin elicitor binding protein* (CEBiP) et *chitin elicitor receptor kinase 1* (CERK1), déclenchant la PTI. L'effecteur Mg3LysM est capable de fixer la chitine inhibant l'immunité du blé et protégeant le champignon des chitinases de l'hôte. (b) Les effecteurs ZtNIP1 et ZtNIP, des facteurs nécrotiques, induisent la mort des cellules du blé *via* un mécanisme encore inconnu. (c) Gènes de résistance *Stb* possiblement induits après reconnaissance d'effecteurs (AvrSTB) sécrété dans l'apoplasme. Dans certains cas, ce mécanisme permet d'inhiber la croissance du pathogène. (d) Autres effecteurs impliqués dans l'infection de *Z. tritici*, comme la protéine *necrosis and ethylene-inducing peptide1* (NEP1), un autre potentiel facteur nécrotique (d'après Kettles and Kanyuka, 2016).

3.1.2. Réponses précoces du blé

A la suite de la reconnaissance des signaux moléculaires trahissant la présence de l'agent pathogène, des réponses induites précoces se mettent en place rapidement et localement chez la plante. Celles-ci nécessitent l'activation de cascades de phosphorylation qui impliquent notamment les MAPK (**Figure 15**). En effet, du fait de la phosphorylation ciblée de certaines protéines-clés, notamment des enzymes et des facteurs de transcription, les MAPK peuvent réguler, entre autres, la synthèse d'hormones et de métabolites de défense, l'activation de gènes de résistance, la fermeture des stomates ou encore la HR (Meng and Zhang, 2013). Dans le cadre de l'interaction blé-*Z. tritici*, plusieurs équipes ont montré qu'une MAPK, codée par le gène *TaMPK3*, s'accumulait dans les feuilles juste avant l'apparition des symptômes causés par *Z. tritici* (Rudd *et al.*, 2008 ; Yang *et al.*, 2013). Selon Rudd *et al.* (2008), cette protéine serait impliquée dans le déclenchement local de la HR-like au niveau

de l'infection, une réaction qui serait liée au développement des lésions nécrotiques sur les feuilles de blé lors de la phase nécrotrophe du champignon.

Une autre des réactions précoces ayant lieu dans les tissus végétaux en réponse à une infection est la libération de messagers secondaires, tels que les ions calcium (Ca^{2+}) qui régulent de nombreux processus cellulaires. Ces derniers peuvent notamment entraîner très rapidement la production et l'accumulation d'espèces réactives de l'oxygène (*reactive oxygen species*, ROS) aux niveaux intra- et extracellulaires. Les ROS regroupent plusieurs formes de molécules, extrêmement oxydantes, comme le radical superoxyde (O_2^-), le radical hydroxyle ($\text{OH}^\bullet$) et le peroxyde d'hydrogène (H_2O_2) (Görlach *et al.*, 2015). Le phénomène d'accumulation de ces composés au niveau cellulaire est souvent appelé *burst* oxydatif. Les ROS jouent différents rôles dans la réponse des plantes aux stress, qu'ils soient d'origine abiotique ou biotique. Dans le cadre des interactions plante-pathogène, les ROS peuvent présenter un effet direct antimicrobien, être impliqués dans l'induction de gènes de défense, la production de phytoalexines, le renforcement pariétal et la HR (Thordal-Christensen *et al.*, 1997 ; Gechev and Hille, 2005 ; Shetty *et al.*, 2007 ; Linley *et al.*, 2012 ; Lehmann *et al.*, 2015).

Dans le cas du pathosystème blé-*Z. tritici*, l'effet du H_2O_2 au niveau foliaire a été largement étudié. Shetty *et al.* (2003) ont ainsi démontré que chez les plantes résistantes, ce composé était accumulé rapidement et en grande quantité dès le début de l'infection. A l'inverse, chez les variétés sensibles, la concentration en H_2O_2 n'augmentait que lors du passage à la phase nécrotrophe du champignon. Les travaux de Shetty *et al.* (2007) ont aussi démontré que *Z. tritici* était sensible au H_2O_2 tout au long de son cycle de développement et qu'une accumulation importante de ce composé dans les tissus foliaires réduisait la colonisation, la production de biomasse fongique et allongeait la période de latence du champignon. Les auteurs ont toutefois noté que le champignon était plus tolérant au H_2O_2 lors du passage à la phase nécrotrophe, moment concomitant à la mort des cellules végétales et à la libération d'une grande quantité de ce composé.

Les ROS sont donc des composés majeurs de la défense des plantes. Toutefois, à fortes concentrations, ils sont hautement toxiques pour la plante elle-même. Une régulation fine de leur concentration cellulaire est donc nécessaire, rôle joué par de nombreuses enzymes comme la superoxyde dismutase (SOD), l'oxalate oxydase (OXO), la catalase (CAT) ou encore les peroxydases (POX). La SOD et l'OXO sont impliquées dans la formation du peroxyde d'hydrogène alors que la CAT et la POX interviennent dans sa détoxification et son utilisation en tant que substrat. Plus précisément, la SOD est une enzyme qui catalyse la dismutation du O_2^- en O_2 et H_2O_2 , et constitue la première ligne de détoxification des ROS (Gill *et al.*, 2015). L'oxalate oxydase est impliquée dans la réponse des plantes à différents stress et catalyse la transformation de l'acide oxalique en H_2O_2 et CO_2 (Delisle *et al.*, 2001). La catalase, située dans les peroxyssomes, permet la détoxification du H_2O_2 en eau et dioxygène. Les peroxydases, quant à elles, utilisent le H_2O_2 pour oxyder de nombreux substrats et sont impliquées dans de nombreuses réactions de défense majeures impliquant la lignification, l'oxydation de composés phénoliques et la subérisation (Pandey *et al.*, 2017).

Les rôles de la CAT et de la POX dans la résistance du blé vis-à-vis de la septoriose ont été étudiés et ont des effets bien différents (Shetty *et al.*, 2003 ; Shetty *et al.*, 2007). En ce qui concerne la CAT, on observe, chez les cultivars les plus résistants à la septoriose, une réduction de son activité après infection, corrélée avec un *burst* oxydatif précoce, suggérant un rôle négatif de cette enzyme dans la résistance du blé, lié probablement à la répression de l'accumulation de ROS en début d'infection.

(Shetty *et al.*, 2007). L'augmentation de l'activité POX dans les plantes, après infection, a plusieurs fois été associée avec une résistance accrue contre les agents pathogènes, comme, par exemple, lors de l'interaction riz-*Magnaporthe oryzae* (Hiraga *et al.*, 2001 ; Reimers *et al.*, 1992). De même, Shetty *et al.* (2003) ont rapporté une implication de la POX dans la résistance du blé vis-à-vis de la septoriose. Les auteurs ont en effet observé, après inoculation avec *Z. tritici*, une augmentation significative de l'activité de cette enzyme chez une variété résistante (Cv. Stakado), ce qui n'était pas le cas chez la variété sensible (Cv. Flame).

3.1.3. Transduction du signal et phytohormones

Après l'établissement des réponses précoces, le signal de stress perçu par les cellules végétales est transmis aux cellules et tissus adjacents, induisant ainsi la mise en place de la résistance locale acquise (*local acquired resistance*, LAR) au niveau du site de l'infection et/ou la résistance systémique acquise (*systemic acquired resistance*, SAR) transmettant la résistance au reste de la plante. Les messagers chimiques impliqués dans la transduction du signal sont très souvent des phytohormones. Les principales familles d'hormones observées chez les plantes sont l'auxine ou l'acide indole-acétique (*indole-3-acetic acid*, IAA), l'acide abscissique (ABA), l'acide salicylique (*salicylic acid*, SA), l'acide jasmonique (*jasmonic acid*, JA), les brassinostéroïdes (BR), les cytokinines (CK), l'éthylène (ET), les gibbérellines (GA) et les strigolactones (SL). Celles-ci jouent des rôles fondamentaux, allant de la division cellulaire et la régulation du métabolisme primaire jusqu'à l'adaptation des plantes aux différents stress environnementaux (Robert-Seilanianantz *et al.*, 2011).

Dans le cas des interactions blé-*Z. tritici*, les études visant à caractériser le rôle des phytohormones ont principalement été focalisées sur le JA et le SA. La biosynthèse et la signalisation du JA ont souvent été rapportées comme centrales dans la défense des plantes contre les pathogènes nécrotrophes, à l'inverse du SA qui est, lui, bénéfique pour lutter contre l'invasion d'agents biotrophes ou héli-biotrophes. Ce *crosstalk* hormonal entre ces deux molécules antagonistes a largement été discuté et parfois nuancé dans la littérature. De récentes études ont mis en lumière l'activation simultanée de ces deux voies hormonales dans certains cas, notamment durant l'ETI (Thaler *et al.*, 2012 ; Wasternack and Hause, 2013 ; Betsuyaku *et al.*, 2018).

La synthèse du JA dépend de la voie des octadécanoïdes et commence dans le chloroplaste avec un acide gras à 18 carbones, l'acide linoléique. Celui-ci est oxydé successivement par plusieurs enzymes clés de cette voie de biosynthèse, la lipoxygénase (LOX), l'allène oxyde synthase (AOS) et l'allène oxyde cyclase (AOC). Le produit de ces réactions est ensuite transféré dans le peroxyosome où il est finalement transformé en JA. Dans le cytosol, la phytohormone peut ensuite être convertie en plus de 30 dérivés. Les composés considérés les plus actifs biologiquement sont le JA libre, le méthyl-jasmonate (MeJA), le *cis*-jasmonate et le jasmonoyl-isoleucine (JA-Ile) (Wasternack and Hause, 2013 ; Ali and Baek, 2020).

La LOX joue un rôle prépondérant dans le cadre des interactions blé-*Z. tritici*. Ray *et al.* (2003) ont ainsi montré chez le blé une expression différentielle d'un gène codant pour une LOX en lien avec le niveau de résistance du cultivar infecté par *Z. tritici*. En effet, chez les cultivars résistants, les auteurs ont observé une diminution significative du nombre de transcrits plus rapidement que chez le cultivar sensible. Selon cette équipe, la LOX pourrait favoriser le développement et la colonisation du champignon chez l'hôte. L'inhibition de cette enzyme apporterait alors un avantage majeur à la plante

vis-à-vis du pathogène, faisant de ce mécanisme une caractéristique des variétés résistantes à la maladie.

Le SA, ou *2-hydroxybenzoic acid*, est un acide phénolique hydroxylé surtout connu pour son rôle en tant qu'hormone-clé des voies de défense chez les plantes. Ce composé est notamment impliqué dans la régulation des phénomènes HR et la mort cellulaire en réponse aux stress biotiques, ainsi que dans l'induction de la LAR et de la SAR (Malamy *et al.*, 1990 ; Gaffney *et al.*, 1993 ; Radojičić *et al.*, 2018). Le SA est généré à partir du chorismate, produit final de la voie du shikimate, et *via* deux voies métaboliques distinctes : celle de l'isochorismate (IC) et celle des phénylpropanoïdes (Ding and Ding, 2020) (**Figure 16**).

- La voie de l'IC comprend trois enzymes principales ; la première est l'isochorismate synthase (ICS1) qui catalyse la transformation du chorismate en isochorismate dans le chloroplaste, son homologue proche ICS2 joue un rôle similaire (Strawn *et al.*, 2007 ; Garcion *et al.*, 2008). La deuxième est l'EDS5 qui est un transporteur localisé dans la membrane du chloroplaste et permettant le transport de l'IC du chloroplaste vers le cytosol (Serrano *et al.*, 2013). La troisième est la PBS3 présentant une fonction isochorismoyl-glutamate synthase (IGS), permettant la transformation de l'IC en isochorismoyl-9-glutamate, qui se fragmente ensuite spontanément pour former le SA (Ding and Ding, 2020).
- La voie des phénylpropanoïdes se déroule majoritairement dans le cytosol et débute avec la transformation de la phénylalanine, dérivant du chorismate, en acide trans-cinnamique grâce à l'action de la phénylalanine ammonia lyase (PAL), enzyme-clé de la voie. Le produit est ensuite transformé, *via* différentes enzymes, d'abord en acide benzoïque puis en SA (Huang *et al.*, 2010) (**Figure 16**). Tout comme le JA, le SA peut ensuite être converti en plusieurs dérivés qui ont alors des rôles différents, comme le méthyl-salicylate (MeSA), une forme inactivée du SA qui pourrait servir de signal mobile déclencheur de la SAR, mais cette hypothèse reste toutefois à confirmer (Park *et al.*, 2007).

Les ROS peuvent influencer sur l'accumulation du SA. L' H_2O_2 entraîne une accumulation de l'acide benzoïque, un des intermédiaires réactionnels de la biosynthèse du SA dans la voie des phénylpropanoïdes. En retour, l'augmentation de la concentration cellulaire en SA conduit à une inhibition de l'activité de la CAT, induisant une augmentation de la concentration en H_2O_2 (Vidhyasekaran, 2015). En parallèle, le SA intervient notamment sur l'expression de gènes de défense comme la *pathogenesis-related protein 1* (PR1) (Ali *et al.*, 2018).

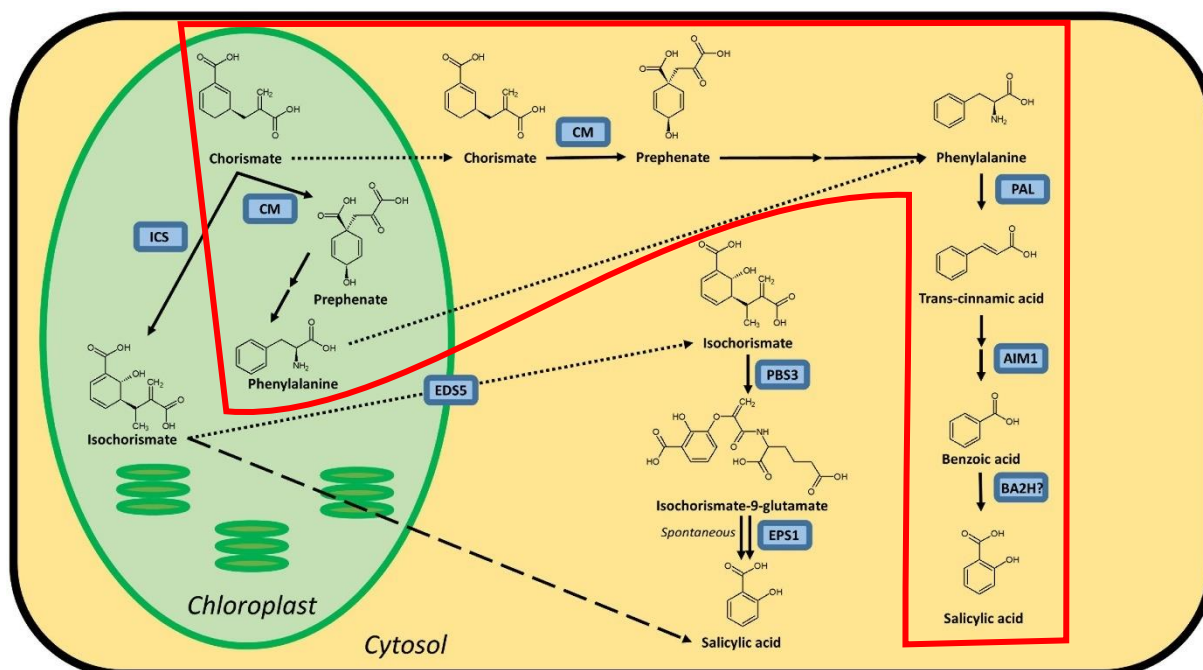


Figure 16 : Biosynthèse de l'acide salicylique. L'encart en rouge correspond à la voie des phénylpropanoïdes, les lignes en pointillées au transport de molécules du chloroplaste au cytosol et la ligne en tiret à une voie alternative encore inconnue, indépendante des voies connues (isochorismate et phénylpropanoïdes). AIM1 : *abnormal inflorescence meristem*, BA2H : *benzoic acid 2-hydroxylase*, CM : *chorismate mutase*, EDS5 : *enhanced disease susceptibility 5*, EPS1 : *enhanced pseudomonas susceptibility 1*, ICS : *isochorismate synthase*, PAL : *phenylalanine ammonia lyase*, PBS3 : *AvrPphB susceptible 3* (d'après Lefevre *et al.*, 2020).

3.1.4. Réponses tardives du blé

Après reconnaissance de l'agent pathogène, l'établissement de réponses précoces et la transduction du signal aux niveaux local et systémique, *via* notamment les phytohormones, la plante infectée peut développer des réponses de défense dites tardives. Les principaux mécanismes impliqués sont (i) le renforcement des barrières physiques, (ii) la production de métabolites secondaires antimicrobiens et (iii) l'accumulation de protéines PR (*pathogenesis-related*).

Les renforcements pariétaux

Après l'attaque, la paroi des cellules végétales est modifiée, voire remodelée, en profondeur, dans le but, notamment, de résister aux toxines et aux CWDEs produites par l'agent pathogène. De nombreux composés de défense, de natures chimiques variées, y sont incorporés, allant des polysaccharides comme la callose, en passant par des phénylpropanoïdes comme la lignine et la subérine, jusqu'aux glycoprotéines structurales comme les *hydroxyproline-rich glycoproteins* (HRGP) (Deepak *et al.*, 2010). La callose est un polymère glucidique, composé majoritairement de β -1,3-glucane, dont la déposition est induite au niveau des sites d'infection et régulée en partie par le SA (Luna *et al.*, 2011). La lignine est un polymère de monolignols dont les trois principaux représentants sont l'alcool coniférylique, l'alcool sinapylique et l'alcool coumarylique (**Figure 17**). Ces monolignols sont issus de la voie des phénylpropanoïdes, et leur synthèse nécessite l'intervention de plusieurs enzyme-clés, incluant la PAL, la *cinnamyl alcohol dehydrogenase* (CAD), la *caffeic acid O*-

Production de métabolites secondaires

Une autre des réponses tardives mises en place par la plante lors d'une agression est la synthèse et la libération de composés à faible poids moléculaire issus du métabolisme secondaire tels que les phénylpropanoïdes. Ces composés phénoliques sont impliqués dans la réponse aux stress qu'ils soient d'origine abiotique ou biotique. Cette famille regroupe, outre les monolignols qui interviennent dans la synthèse de la lignine, le SA et ses dérivés, les flavonoïdes et les phytoalexines.

Toutes ces molécules appartiennent à la voie des phénylpropanoïdes et leur synthèse nécessite souvent l'action de la PAL, une enzyme-centrale de cette voie. Adhikari *et al.* (2007) ont observé une augmentation significative du nombre de transcrits du gène codant pour la PAL très tôt, entre 3 et 6 heures après l'infection par *Z. tritici*, uniquement chez les variétés de blé résistantes (Tadinia and W7984), soulignant l'importance de ce gène dans la résistance de la plante à la maladie. Toutefois, Shetty *et al.* (2009) n'ont pas observé ce phénomène chez le cultivar résistant Stakado indiquant qu'il serait possiblement dépendant du cultivar. En ce qui concerne les flavonoïdes, et bien qu'ils soient connus pour leurs activités antifongiques, très peu de travaux se sont intéressés à leur rôle dans les interactions blé-pathogène (Mierziak *et al.*, 2014 ; Al Aboody and Mickymaray, 2020). Pour ce qui est des phytoalexines, seul un petit nombre a été observé et décrit chez le blé (Du Fall and Solomon, 2013).

Les protéines PR

Les protéines PR sont des molécules induites chez la plante en réponse à l'attaque d'agents pathogènes et sont des acteurs essentiels de la défense chez les plantes. Il s'agit de molécules à faible poids moléculaire, compris entre 6 et 43 KDa, s'accumulant principalement dans l'apoplasme et présentant des caractéristiques communes, notamment le fait d'être particulièrement stables. Elles sont ainsi thermostables, résistantes aux protéases et restent solubles en conditions acides (pH inférieur à 3) (Van Loon *et al.*, 1994). Ces protéines sont largement distribuées chez les plantes et ont été détectées dans tous les organes et particulièrement dans les feuilles où elles sont présentes en très grande quantité, représentant entre 5 et 10 % des protéines totales (Van Loon *et al.*, 1994). Les protéines PR ont des fonctions diverses et sont classées, selon celles-ci, en 17 catégories (**Tableau 1**) (Ali *et al.*, 2018). Il est à noter que le mode d'action exact de certaines de ces protéines, comme PR1, reste encore obscur à l'heure actuelle.

Tableau 1: Catégories, fonction et origine des protéines PR (d'après Ali *et al.*, 2018).

Protéine PR	Fonction	Origine	Référence
PR1	<i>Antifungal</i>	<i>Nicotiana tabacum</i>	<u>Antoniw <i>et al.</i> (1980)</u>
PR2	<i>β-1,3-glucanases</i>	<i>Nicotiana tabacum</i>	<u>Antoniw <i>et al.</i> (1980)</u>
PR3	<i>Class I, II, IV, V, VI, VII Chitinases</i>	<i>Nicotiana tabacum</i>	<u>Van Loon (1982)</u>
PR4	<i>Class I, II Chitinases</i>	<i>Nicotiana tabacum</i>	<u>Van Loon (1982)</u>
PR5	<i>Thaumatin-like proteins</i>	<i>Nicotiana tabacum</i>	<u>Van Loon (1982)</u>
PR6	<i>Proteinase inhibitor</i>	<i>Solanum lycopersicum</i>	<u>Green and Ryan (1972)</u>
PR7	<i>Endoproteinase</i>	<i>Solanum lycopersicum</i>	<u>Vera and Conejero (1988)</u>
PR8	<i>Class III Chitinase</i>	<i>Cucumis sativus</i>	<u>Metraux <i>et al.</i> (1988)</u>
PR9	<i>Peroxidase</i>	<i>Nicotiana tabacum</i>	<u>Lagrimini <i>et al.</i> (1987)</u>
PR10	<i>Ribonuclease-like proteins</i>	<i>Petroselinum crispum</i>	<u>Somssich <i>et al.</i> (1986)</u>
PR11	<i>Class I Chitinase</i>	<i>Nicotiana tabacum</i>	<u>Melchers <i>et al.</i> (1994)</u>
PR12	<i>Defensin</i>	<i>Raphanus raphanistrum</i>	<u>Terras <i>et al.</i> (1995)</u>
PR13	<i>Thionin</i>	<i>Arabidopsis thaliana</i>	<u>Epple <i>et al.</i> (1995)</u>
PR14	<i>Lipid-transfer protein</i>	<i>Hordeum vulgare</i>	<u>Garcia-Olmedo <i>et al.</i> (1995)</u>
PR15	<i>Oxalate oxidase</i>	<i>Hordeum vulgare</i>	<u>Zhang <i>et al.</i> (1995)</u>
PR16	<i>Oxidase-like</i>	<i>Hordeum vulgare</i>	<u>Wei <i>et al.</i> (1998)</u>
PR17	<i>Antifungal and antiviral</i>	<i>Nicotiana tabacum</i>	<u>Okushima <i>et al.</i> (2000)</u>

Le rôle de certaines de ces protéines PR dans les interactions blé-Z. *tritici* a été étudié. Plusieurs travaux ont montré que le niveau d'expression des gènes *PR1*, *PR2*, *PR3*, *PR5* et *PR9* après infection différait selon le niveau de résistance du cultivar (Ray *et al.*, 2003 ; Adhikari *et al.*, 2007 ; Shetty *et al.*, 2009). Selon Adhikari *et al.* (2007), le gène codant pour la *PR1* pourrait servir de biomarqueur pour distinguer un cultivar résistant d'un cultivar sensible selon son niveau d'expression à 12h après infection. Les *PR2* et *PR3* correspondent respectivement aux glucanases et chitinases, enzymes capables de dégrader les polymères de la paroi fongique, freinant ainsi son développement et libérant par la même occasion des fragments de chitine et de β -1,3-glucane qui pourront ensuite servir de PAMPs (**voir 3.1.1. Perception de la présence du pathogène**). Les *PR5* sont de petites molécules présentant une activité antimicrobienne (Wang *et al.*, 2010). Les *PR9* correspondent aux peroxydases dont le rôle a déjà été présenté (**voir 3.1.2. Réponses précoces du blé**). Ray *et al.* (2003) ont observé une augmentation significative du nombre de transcrits des *PR1*, *PR2* et *PR5*, 12h après infection, chez les cultivars les plus résistants comme les plus sensibles. Les auteurs indiquent, toutefois, une expression significativement plus marquée chez les cultivars résistants. Le niveau d'expression de ces protéines PR peut aussi varier durant le processus infectieux. En effet, Rudd *et al.* (2015) ont rapporté, un jour après infection une réduction de l'expression de gènes codant pour les *PR1*, *PR2*, *PR3*, *PR4* et *PR5* chez un cultivar sensible (Cv. Riband). Les auteurs émettent l'hypothèse que cette régulation négative serait causée par le champignon afin de manipuler et contourner les réactions de défense de la plante.

3.2. Résistance génétique structurale du blé vis-à-vis de *Zymoseptoria tritici*

Chez les plantes, la résistance peut être définie comme la capacité de la plante à empêcher ou réduire la colonisation ou la croissance d'un bio-agresseur dans ses tissus. D'un point de vue génétique, deux types de résistance existent : la résistance qualitative (spécifique) et la résistance quantitative (non-spécifique). Celles-ci ont, toutes deux, été observées dans le cas du pathosystème blé-*Z. tritici*.

3.2.1. La résistance qualitative

La résistance qualitative (spécifique) est contrôlée par un ou plusieurs gènes majeurs, appelés aussi gènes *R*, réagissant supposément selon la relation gène pour gène décrite par Flor (1971) (**Figure 14**). Les effets de protection de ces gènes sont majeurs mais souvent limités dans le temps, à cause du contournement des résistances par les agents pathogènes. Chez le blé, 21 gènes impliqués dans la résistance qualitative du blé vis-à-vis de la septoriose ont été rapportés et cartographiés. Ils sont appelés *Stb* (pour *Septoria tritici blotch*) (Brown *et al.*, 2015) (**Tableau 2**). Ces gènes sont fortement spécifiques et ne sont efficaces que contre un faible nombre de génotypes du champignon, généralement les avirulents. Parmi les 21 gènes identifiés, l'importance du *Stb6*, codant pour une *wall-associated receptor kinase*, a été largement étudié (Saintenac *et al.*, 2018). Arraiano *et al.* (2009) ont ainsi montré que la présence de ce gène était associée à une réduction de la sévérité de la maladie en moyenne de 19 % chez de nombreuses lignées de blé. La résistance apportée par ces gènes *Stb* est toutefois fréquemment contournée par le pathogène. Ainsi, la résistance quantitative, plus durable, a gagné de plus en plus en intérêt pour les scientifiques et les sélectionneurs (Pilet-Nayel *et al.*, 2017).

Tableau 2: Principaux gènes *R* impliqués dans la résistance qualitative du blé vis-à-vis de *Z. tritici* (d'après Brown *et al.*, 2015).

Gène	Chromosome	Marqueur	Souche de <i>Z. tritici</i>	Cultivar de blé	Référence
<i>Stb1</i>	5BL	Xbarc74 (2.8cM), Xgwm335 (7.4cM)	IN95-Lafayette- 1196-WW 1-4 et Purdue local	Bulgaria 88	Adhikari <i>et al.</i> (2004a)
<i>Stb2</i>	1BS	Xwmc406 (6cM), Xwmc230 (5cM)	Paskeville local et IPO92034	Veranopolis	Liu <i>et al.</i> (2013)
<i>Stb3</i>	7AS	Xwmc83	Paskeville local isolate	Israel 493	Goodwin and Thompson (2011)
<i>Stb4</i>	7DS	Xgwm111 (0.7cM)	IN95-Lafayette- 1196-WW-1-4, I-89, IPBr1	Tadinia	Adhikari <i>et al.</i> (2004c)
<i>Stb5</i>	7DS	Xgwm44 (7.2cM)	IPO94269	Synthetic 6x	Arraiano <i>et al.</i> (2001b)
<i>Stb6</i>	3AS	Xgwm369 (2cM)	IPO323	Flame, Hereward	Brading <i>et al.</i> (2002)
<i>Stb7</i>	4AL	Xwmc313 (0.3 à 0.5cM), Xwmc219 (1cM)	MG2 et IPO87019	ST6	McCartney <i>et al.</i> (2003)
<i>Stb8</i>	7BL	Xgwm146 (3.5cM), Xgwm577 (5.3cM)	IN95-Lafayette- 1196-WW 1-4	Synthetic W7984	Adhikari <i>et al.</i> (2003)
<i>Stb9</i>	2BL	Xfbb226 (3.6cM), Xwmc317, Xbarc0129	IPO89011	Courtot, Tonic	Chartrain <i>et al.</i> (2009)
<i>Stb10</i>	1Dc	Xgwm848	IPO94269 et ISR8036	Kavkaz-K4500	Chartrain <i>et al.</i> (2005c)
<i>Stb11</i>	1BS	Xbarc008 (1cM)	IPO90012	TE9111	Chartrain <i>et al.</i> (2005a)
<i>Stb12</i>	4AL	Xwmc219	ISR398 et ISR8036	Kavkaz-K4500	Chartrain <i>et al.</i> (2005c)
<i>Stb13</i>	7BL	Xwmc396 (7-9cM)	MG96-36, MG2	Salamouni	Cowling (2006)
<i>Stb14</i>	3BS	Xwmc500 (2cM), wmc632 (5cM)	MG2	Salamouni	Cowling (2006)
<i>Stb15</i>	6AS	Xpsr904 (14cM)	IPO88004	Arina, Riband	Arraiano <i>et al.</i> (2007b)
<i>StbSm3</i>	3AS	barc321 (1.9cM)	MG96-36, MG2	Salamouni	Cuthbert (2011)
<i>Stb16q</i>	3DL	Xgwm494 (4.3cM), Xbarc128 (9.9cM)	IPO88018 et IPO94218	SH M3	Tabib Ghaffary <i>et al.</i> (2012)
<i>Stb17</i>	5AL	Xhbg247 (3.1cM), Xgwm617 (38.3cM)	IPO88018	SH M3	Tabib Ghaffary <i>et al.</i> (2012)
<i>Stb18</i>	6DS	Xgpw5176, Xgpw3087	IPO323, IPO98022, IPO89011, IPO98046	Balance	Tabib Ghaffary <i>et al.</i> (2011)
<i>StbWW</i>	1BS	Xbarc119b (0.9– 4.1cM)	79, 2, 1A	WW1842, WW2449, WW2451	Raman <i>et al.</i> (2009)
<i>TmStb1</i>	7A ^m S	Xbarc174 (23.5cM)	IPO323	MDR043 (T. monococcum)	Jing <i>et al.</i> (2008)

3.2.2. La résistance quantitative

La résistance quantitative est gouvernée par l'action de différents ensembles de gènes et est généralement moins spécifique que la résistance qualitative, ce qui lui permet d'être souvent efficace contre un large panel de souches de *Z. tritici* et d'être plus difficilement contournée par le pathogène. Plusieurs travaux ont montré que la résistance qualitative chez le blé vis-à-vis de *Z. tritici* dépend de *quantitative trait loci* (QTL) qui ont pu être localisés et associés à certains marqueurs moléculaires (Eriksen *et al.*, 2003 ; Chartrain *et al.*, 2004 ; Brown *et al.*, 2015). Brown *et al.* (2015) rapportent que 89 régions génomiques, portant des QTL ou des méta-QTL, ont été identifiés comme étant impliqués dans la résistance du blé vis-à-vis de la maladie. Les auteurs indiquent que seul le chromosome 5D ne porte pas de QTL ou méta-QTL. Il est aussi à noter que plusieurs QTL et méta-QTL ont été cartographiés dans des régions du génome où des gènes de résistance spécifique (*Stb*) étaient présents. L'amélioration des connaissances sur la génétique des résistances qualitative et quantitative est d'une grande utilité pour les sélectionneurs pour développer des variétés plus résistantes à la maladie.

3.3. Les effecteurs de *Z. tritici*

Les effecteurs sont des protéines essentielles au bon déroulement de la colonisation et du développement de l'agent pathogène dans son hôte. Chez *Z. tritici*, des études de transcriptomique ont permis d'identifier une centaine de gènes candidats impliqués dans la virulence ou la pathogénicité (Rudd *et al.*, 2015 ; Kettles and Kanyuka, 2016). Aujourd'hui, plus d'une vingtaine de gènes codant pour des effecteurs ont été identifiés (**Tableau 3**) (Mirzadi Gohari *et al.*, 2014 ; Kettles and Kanyuka, 2016; Tiley *et al.*, 2019). Si beaucoup d'efforts ont été menés ces dernières décennies pour mieux comprendre le rôle de ces effecteurs, il reste encore beaucoup à découvrir pour mieux élucider leurs implications. Parmi les effecteurs majeurs identifiés, on peut citer MgAtr4, qui a été le premier effecteur découvert chez *Z. tritici* (Stergiopoulos *et al.*, 2003). Il s'agit d'un transporteur membranaire, membre de la famille des *ATP-binding cassette* (ABC) et dont le rôle serait de protéger l'envahisseur contre les molécules de défense produites par la plante hôte, comme les phytoalexines (Stergiopoulos *et al.*, 2003). La protéine MgHog1 est indispensable au champignon durant les débuts de l'infection, les mutants ne l'exprimant pas se révélant incapable de coloniser leur hôte. Mehrabi *et al.* (2006b) ont démontré qu'elle était impliquée dans la transition entre le tube germinatif et le développement de l'hyphe. MgSlt2 joue aussi un rôle fondamental, durant la phase asymptomatique, étant impliquée dans la ramification du mycélium, nécessaire à la colonisation des cavités sous-stomatiques (Mehrabi *et al.*, 2006a). D'autres travaux ont permis de mettre en évidence des effecteurs-clés de la phase nécrotrophe du champignon. Ainsi, M'Barek *et al.* (2015) ont montré que des filtrats de culture de *Z. tritici* contenaient des composés capables d'entraîner la mort des cellules foliaires quand elles étaient injectées dans les feuilles de blé. Les auteurs ont identifié deux effecteurs, potentiellement des *necrosis-inducing proteins*, nommés ZtNIP1 et ZtNIP2, qui viennent rejoindre le gène *NEP1* en tant que facteurs nécrotiques (Motteram *et al.*, 2009) (**Figure 15**). Le gène *ZtWor1* est quant à lui impliqué dans plusieurs mécanismes tels que la colonisation du mésophylle et la fructification asexuée du champignon (Mirzadi Gohari *et al.*, 2014). Plusieurs autres gènes de virulence sont impliqués dans la formation des pycnides, notamment *MgTpk2* et *MgBcy1* (Mehrabi and Kema, 2006). Très récemment, un nouvel effecteur a été identifié chez *Z. tritici*, il s'agit du gène de virulence *ZtvelB* qui est aussi

nécessaire pour la mise en place de pycnides. Il est à noter que celui-ci est aussi impliqué dans le développement du champignon sous forme levure-like (Tiley *et al.*, 2019).

Tableau 3: Liste de gènes codant pour des protéines effectrices chez *Z. tritici* (d'après Gohari *et al.*, 2015, modifié).

Gène	Catégorie	Référence
MgAtr4	ABC Transporter	Stergiopoulos <i>et al.</i> , 2003
MgFus3	Mitogen-activated protein kinase (MAPK)	Cousin <i>et al.</i> , 2006
MgSlt2	MAPK	Mehrabi <i>et al.</i> , 2006a
MgHog1	MAPK	Mehrabi <i>et al.</i> , 2006b
MgTpk2	Protein kinase A catalytic subunit	Mehrabi et Kema, 2006
MgBcy1	Protein kinase A regulatory subunit	Mehrabi et Kema, 2006
MgSTE11	MAPK kinase kinase	Kramer <i>et al.</i> , 2009
MgSTE50	Scaffold protein for MAPK signaling	Kramer <i>et al.</i> , 2009
MgSTE12	Transcription factor target of MAPK signaling	Kramer <i>et al.</i> , 2009
MgSTE7	MAPK kinase	Kramer <i>et al.</i> , 2009
MgNLP	Unknown	Motteram <i>et al.</i> , 2009
MgGpa1	G-protein alpha subunit	Mehrabi <i>et al.</i> , 2009
MgGpa3	G-protein alpha subunit	Mehrabi <i>et al.</i> , 2009
MgGpb1	G-protein alpha subunit	Mehrabi <i>et al.</i> , 2009
MCC1	c-type cyclin	Choi and Goodwin, 2010
Mg3LysM	Chitin binding effector protein	Marshall <i>et al.</i> , 2011
MgAlg2	Protein N-Glycosylation	Motteram <i>et al.</i> , 2011
ZtWor1	Transcription factor	Gohari <i>et al.</i> , 2014
ZtNIP1	Necrotic factor	M'Barek <i>et al.</i> , 2015
ZtNIP2	Necrotic factor	M'Barek <i>et al.</i> , 2015
Zt80707	Unknown/Secreted	Poppe <i>et al.</i> , 2015
Zt89160	Unknown	Poppe <i>et al.</i> , 2015
Zt103264	Unknown	Poppe <i>et al.</i> , 2015
ZtvelB	Transcription factor	Tiley <i>et al.</i> , 2019

4. LES MÉTHODES DE LUTTE CONTRE LA SEPTORIOSE

Actuellement, la lutte contre *Z. tritici* repose essentiellement sur l'utilisation des produits phytopharmaceutiques de synthèse mais aussi, dans une moindre mesure, sur le déploiement des résistances variétales (Torriani *et al.*, 2015). La protection octroyée par la lutte génétique est toutefois limitée et, à ce jour, aucune variété totalement résistante à la septoriose n'a pu être obtenue. D'autres méthodes par ailleurs existent pour protéger la culture et seront discutées ci-dessous.

4.1. La lutte chimique

La lutte chimique reste encore, et de loin, la principale méthode employée au champ pour lutter contre la septoriose. Dans l'Union européenne, jusqu'à 70 % des fongicides épandus dans les parcelles sont utilisés pour protéger le blé contre cette maladie (Fones *et al.*, 2017). Plusieurs familles de fongicides ont été utilisées pour contrôler *Z. tritici* au cours de ces dernières décennies.

Les premiers à avoir vu le jour sont les inhibiteurs multi-sites de contact, tels que le chlorothalonil ou le mancozèbe. Ces produits sont capables d'agir sur différentes cibles cellulaires du champignon et peuvent agir à la fois sur la germination des spores et la croissance mycélienne. Aucun phénomène de résistance marqué n'a été observé pour ces molécules, très probablement grâce à leur mode d'action non spécifique, ce qui leur confère un avantage certain (Leroux *et al.*, 2007). Néanmoins, ces produits présentent de nombreux inconvénients qui limitent fortement leur utilisation. En effet, pour être efficace, ils doivent être appliqués à fortes doses et sont facilement lessivables lors des épisodes de précipitation, ce qui peut entraîner une pollution environnementale conséquente. Leurs risques aux niveaux toxicologique et éco-toxicologique ont récemment conduit à leur interdiction d'utilisation par l'UE, respectivement en 2020 pour le chlorothalonil et 2021 pour le mancozèbe (Anses, 2019).

A partir des années 1960, de nouvelles molécules fongicides ont été élaborées. Il s'agit cette fois-ci de produits systémiques, donc pénétrants dans la plante, efficaces à faible dose et faiblement écotoxiques. Ces produits présentent aussi une double activité, préventive et curative. Leur mode d'action est cependant uni-site (mode d'action spécifique), ce qui, couplé à une absence de stratégie globale sur l'utilisation des fongicides et sur la gestion des résistances, a conduit très vite à une émergence et à une sélection de souches résistantes de *Z. tritici*, en Europe comme dans le reste du monde. Les premiers produits systémiques uni-sites à avoir été employés, dès fin 1960, sont les antiméiotiques. Ceux-ci sont capables d'empêcher la polymérisation des microtubules, inhibant *de facto* la division cellulaire. Fin 1970, une nouvelle catégorie de fongicides a été développée, les inhibiteurs de la 14 α -déméthylase CYP51 (*demethylation inhibitors*, DMI), enzyme-clé de la biosynthèse de l'ergostérol, un lipide membranaire essentiel chez les champignons (**Figure 18**). La famille des strobilurines, ou *quinone outside inhibitors* (QoI), qui sont capables d'inhiber la respiration du champignon en interagissant avec le complexe III de la chaîne de transporteurs d'électrons mitochondriale, sont utilisés, quant à eux, depuis la fin des années 1990. Enfin, la dernière grande famille de fongicides anti-*Z. tritici* à avoir vu le jour, dans les années 2000, est celle des inhibiteurs de la succinate déshydrogénase (*succinate dehydrogenase inhibitors*, SDHI), une protéine du complexe II de la membrane mitochondriale. Les SDHI sont donc aussi des inhibiteurs de la respiration cellulaire.

Aujourd'hui, la lutte chimique contre la septoriose repose majoritairement sur l'utilisation des DMI et des SDHI (Torriani *et al.*, 2015). L'application de traitements foliaires de DMI et de SDHI en mélange, a aussi été recommandé par certains auteurs, dans le but notamment de limiter le développement des populations résistantes à ces produits (Drummond *et al.*, 2015). Si aucun phénomène majeur de résistance n'a été observé chez *Z. tritici* contre les SDHI, ce n'est pas le cas pour les autres catégories de fongicides. Il n'aura fallu attendre que quelques années après le début de la commercialisation des QoI pour que des résistances à ce fongicide apparaissent dans des populations du Royaume-Uni et d'Irlande (McCartney *et al.*, 2007). Cheval *et al.* (2017) ont récemment montré que dans le Nord de la France, tous les isolats actuels du pathogène récoltés au champ sont totalement résistants à ces molécules.

En ce qui concerne les DMI, plusieurs équipes ont rapportés des diminutions inquiétantes de leur activité, d'abord en Europe, puis très vite dans le reste du monde (Cools *et al.*, 2013 ; McDonald *et al.*, 2019). Trois mécanismes associés à la résistance de *Z. tritici* envers les DMI ont été décrits dans la littérature, (i) des mutations du gène codant pour la protéine CYP51 engendrant une altération de

sa structure 3D, diminuant l'affinité entre le DMI et cette protéine et donc l'activité du fongicide, (ii) une surexpression du gène *CYP51* et (iii) une surexpression de gènes codant pour des transporteurs membranaires jouant le rôle de pompe à efflux permettant à la cellule d'évacuer les composés fongicides dans un objectif de détoxification (**Figure 18**) (Cools *et al.*, 2013). Les populations exprimant ce dernier phénotype sont appelées *multidrug resistant* (MDR). Certaines populations sont mêmes capables de cumuler ces différents mécanismes, expliquant en partie l'érosion de l'activité des DMI (Kildea *et al.*, 2019).

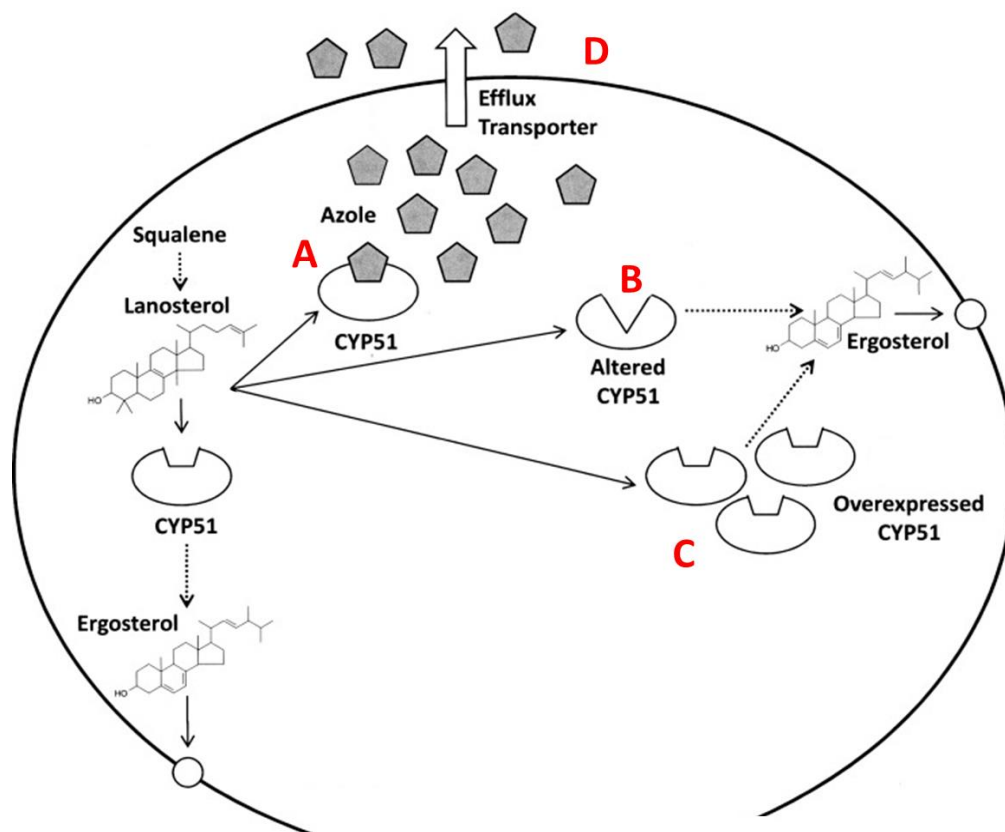


Figure 18 : Mécanismes de résistance aux DMI chez *Z. tritici*. La biosynthèse de l'ergostérol, composé lipidique membranaire majeur, nécessite l'activité de l'enzyme CYP51. (A) Inhibition de la protéine CYP51 par le fongicide DMI lorsque la souche n'est pas résistante. (B) CYP51 altérée et non-affectée par les DMI. (C) Surexpression du gène *CYP51* permettant la synthèse de l'ergostérol. (D) Surexpression de gènes codant pour des pompes à efflux (d'après Parker *et al.*, 2014).

4.2. La lutte variétale

Les cultivars de blé commercialisés jusqu'à très récemment étaient au mieux modérément résistants à la maladie et au pire sensibles. La création de variétés résistantes ou tolérantes à la septoriose n'étant pas la priorité des sélectionneurs, les fongicides de synthèse employés ces dernières décennies protégeant efficacement de la maladie (Fraaije *et al.*, 2012). Toutefois, avec le contexte sociétal et agroécologique actuel, le recours aux fongicides conventionnels se fait plus contraignant et les programmes d'amélioration du blé doivent désormais sérieusement prendre en compte cette maladie (Fraaije *et al.*, 2012). La tolérance à une maladie peut se définir comme la capacité d'un

cultivar à maintenir un rendement relativement élevé comparativement à un autre cultivar à niveau de sévérité égal (Bingham *et al.*, 2009). Des variétés hautement tolérantes à la septoriose ont ainsi déjà vu le jour, mais aucune totalement résistante n'a pu être obtenue (**Tableau 4**) (Castro and Simón, 2016). Nous avons vu qu'il existe chez le blé deux types de résistance à la septoriose : la résistance quantitative et la résistance qualitative (**voir 3.2. Résistance génétique structurale du blé vis-à-vis de *Zymoseptoria tritici***). Les avancées récentes dans l'identification des QTL du blé associés à la résistance vis-à-vis de la maladie ont permis de faire des progrès majeurs dans l'amélioration des variétés commerciales de cette céréale (Brown *et al.*, 2015). A l'inverse, les mécanismes de résistance qualitative, basés sur des gènes spécifiques, contournés de manière récurrente, n'ont pas permis d'aboutir à des cultivars résistants contre les populations de *Z. tritici* en Europe (Arraiano *et al.*, 2009). Dans le but d'exploiter la diversité génétique des populations ancestrales de blé afin de découvrir et de valoriser de nouvelles sources de résistance, les sélectionneurs s'intéressent à la production d'hybrides synthétiques (Tabib Ghaffary *et al.*, 2012).

Tableau 4 : Classement des variétés de blé tendre en France selon leur sensibilité à la septoriose, notée de 1 (variété sensible) à 9 (variété tolérante) (d'après Arvalis-Institut du végétal).

Très sensibles		Moyennement sensibles				Peu sensibles			
ADVISOR	5	ARKEOS	5,5	AUCKLAND	6	CAMPESINO	6,5	LG ABSALON ²	7,5
BERGAMO	5	BOREGAR⁶	5,5	COMPLICE	6	COLLECTOR	6,5	AMBOISE	7
CELLULE⁹	5	CHEVRON	5,5	CONCRET	6	FRUCTIDOR³	6,5	CHEVIGNON¹	7
OREGRAIN⁵	5	COSTELLO	5,5	CREEK	6	KWS DAKOTANA	6,5	CUBITUS	7
RUBISKO⁴	5	DIAMENTO	5,5	FLUOR	6	KWS TONNERRE	6,5	KWS EXTASE	7
APACHE	4,5	EXPERT	5,5	HYKING	6	LYRIK	6,5	RGT CESARIO	7
RGT VOLUPTO	4,5	FILON	5,5	MACARON	6	OXEBO	6,5	SY ADORATION	7
ALIXAN*	4	NEMO¹⁰	5,5	MONITOR	6	PASTORAL	6,5		
		OBIWAN	5,5	MUTIC	6	RGT PULKO	6,5		
		RGT KILIMANJARO	5,5	PROVIDENCE	6	SANREMO	6,5		
		RGT LEXIO	5,5	SORBET CS	6	SOPHIE CS	6,5		
		RGT LIBRAVO	5,5	TENOR	6				
		RGT SACRAMENTO⁸	5,5						
		UNIK	5,5						

(*) Le cultivar Alixan est celui qui sera utilisé dans le cadre des études décrites dans ce manuscrit.

(¹⁻¹⁰) Les numéros en exposant sur les variétés en gras, présentent le classement des variétés les plus cultivées en France, (d'après **FranceAgriMer, 2019**). La 7^{ème} variété, Syllon, n'est pas inscrite dans ce tableau.

Les trois variétés de blé les plus cultivées au niveau de l'assolement français sur la période 2019-2020 sont, dans l'ordre décroissant, Chevignon, LG Absalon et Fructidor (FranceAgriMer, 2019). Ces cultivars sont tous les trois peu sensibles à la maladie, en particulier le cultivar LG Absalon qui est l'une des variétés les plus tolérantes à la septoriose actuellement disponible sur le marché, montrant l'intérêt croissant donné à *Z. tritici* dans les programmes de sélection ces dernières années (**Tableau 4**) (Arvalis-Institut du végétal, 2019). Malgré ces améliorations dans la résistance et la tolérance du blé vis-à-vis du pathogène, celles-ci ne sont pas suffisantes pour protéger totalement la culture et il convient de mettre en place d'autres méthodes de lutte, dont la prophylaxie.

4.3. Mesures agronomiques prophylactiques

Plusieurs stratégies peuvent être mises en place afin, tout d'abord, de limiter la présence du champignon pathogène en début de saison. Nous avons vu que *Z. tritici* pouvait persister d'une année à l'autre sous forme de pycnidiospores et, surtout, d'ascospores dans de multiples réservoirs, notamment, les résidus de culture, les repousses et les hôtes alternatifs. Afin de réduire la pression de cet inoculum primaire, il est recommandé d'enfouir, grâce à un travail adapté du sol, ou de détruire les résidus de culture après récolte contenant ces spores, afin que ces dernières ne puissent plus contaminer la parcelle. La rotation des cultures et l'élimination rapide des repousses du blé et des adventices qui pourraient servir d'hôtes alternatifs à l'agent infectieux permettent également de casser le cycle de vie du champignon et ainsi de réduire l'inoculum primaire présent à la saison suivante (Suffert *et al.*, 2011). Décaler la date de semis est aussi recommandé, tout comme recourir à des variétés de blé plus tardives qui émergent en fin d'automne, quand les températures sont plus basses, limitant grandement la germination des spores et le niveau de sévérité final de la maladie (Gladders *et al.*, 2001). La densité des semis est aussi un facteur à prendre en compte ; un nombre important de feuilles sur une surface réduite favorise les contaminations secondaires *via* le *rain-splashing* ou simplement par contact (Eyal and Center, 1987). De plus, une densité foliaire forte entretient un taux d'humidité élevé au niveau foliaire, propice au développement de *Z. tritici*. La taille du cultivar utilisé semble aussi jouer un rôle. Les variétés naines étant particulièrement sensibles à la maladie, il est recommandé de recourir à l'emploi de variétés non-naines (Brown *et al.*, 2015). L'association de variétés en champ est, selon certains auteurs, une stratégie viable pour réduire l'impact de *Z. tritici*. Gigot *et al.* (2013) ont ainsi montré qu'un mélange de cultivars avec un ratio de 1:3 (cultivar sensible : cultivar résistant) diminuait la sévérité de la maladie d'un peu plus de 40 % pour le cultivar sensible. Cette association permettrait aussi de limiter la pression et la sélection de souches de *Z. tritici* virulentes sur les cultivars résistants.

4.4. Outils basés sur l'agriculture de précision

L'agriculture de précision vise à optimiser la gestion des intrants par les agriculteurs grâce à une meilleure compréhension de la variabilité intra-parcellaire associée à des outils d'aide à la décision (OAD). Dans la pratique, les parcelles sont scrutées à l'aide d'images satellites, de drones et/ou de différents senseurs. Les données obtenues sont ensuite analysées, et permettent d'avoir une vision globale de l'état des cultures, que ce soit au niveau du statut hydrique, des besoins azotés ou de la présence de pathogènes. Grâce à des modèles statistiques, les OAD permettent *in fine* aux agricultures d'apporter la dose optimale d'intrants, au moment opportun et uniquement sur les zones de la parcelle qui le nécessitent. Le but est ainsi de réduire la quantité d'intrants utilisés sans toutefois entraîner une baisse de rendements (Godwin *et al.*, 2003 ; Tackenberg *et al.*, 2016). Tackenberg *et al.* (2016) ont ainsi mis en place une méthode d'application de fongicides ciblée leur permettant d'économiser au total 8 % de produit comparé à une application conventionnelle uniforme. En ce qui concerne la septoriose, plusieurs OAD ont été développés pour lutter contre cette maladie (Ecophyto, 2018). Par exemple, le modèle d'Arvalis-Institut du végétal, Septo-LIS®, prend en compte la variété de blé cultivé, la date de semis, la localisation de la parcelle, les conditions météorologiques (*i.e* température et pluviométrie) ainsi que le stade de la culture et permet de déterminer des dates de traitements fongicides

recommandées (Arvalis-Institut du végétal, 2015). Les OAD peuvent aussi optimiser la fertilisation azotée, cette dernière étant connue pour accélérer le développement de la septoriose ; la réduction de ces intrants azotés aurait donc également un impact bénéfique sur la sévérité de la maladie (Simón *et al.*, 2003).

4.5. Le biocontrôle : une méthode de lutte alternative

4.5.1. Définitions et catégories

D'après le ministère de l'agriculture et de l'alimentation français, le biocontrôle peut se définir comme « un ensemble de méthodes de protection des végétaux basé sur l'utilisation de mécanismes naturels ». Toujours selon eux, les produits de biocontrôle sont définis à l'article L. 253-6 du code rural et de la pêche maritime comme « des agents et des produits utilisant des mécanismes naturels dans le cadre de la lutte intégrée contre les ennemis des cultures. Ils comprennent en particulier : les macro-organismes ; et les produits phytopharmaceutiques qui sont composés de micro-organismes, de médiateurs chimiques tels que les phéromones et les kairomones, ou de substances naturelles d'origine végétale, animale ou minérale » (Ministère de l'agriculture et de l'alimentation, 2021). Actuellement, la place du biocontrôle dans le marché de la protection des cultures au niveau mondial ne représente que 5 %, soit environ 3 milliards de dollars, mais est en croissance continue chaque année (IBMA, 2017). De plus, considérant les limites de la lutte chimique et la mise en place de stratégies globales visant à réduire l'utilisation de produits phytopharmaceutiques de synthèse chimique en agriculture (plan Ecophyto 2025), il est fort probable que la croissance du marché du biocontrôle se poursuive dans les années à venir. Il existe quatre grandes catégories de produits de biocontrôle : les macroorganismes, les microorganismes, les médiateurs chimiques et les substances d'origine naturelle. En France, la législation définit cette dernière catégorie, de la manière suivante : « on entend par substance naturelle toute substance naturellement présente et qui a été identifiée en l'état dans la nature. Cette substance est soit extraite d'un matériau source naturel ; soit obtenue par synthèse chimique et strictement identique à une substance naturelle telle que décrite ci-dessus » (articles L.253-5 et L.253-7 du code rural et de la pêche maritime). Les produits de biocontrôle issus de cette dernière catégorie peuvent présenter différents modes d'actions, parfois complémentaires, pour protéger les cultures contre les agents pathogènes, notamment fongiques. Ils peuvent être, soit bio-fongicides, soit stimulateurs de défense des plantes (SDP), soit être les deux (**Figure 19**).

Les bio-fongicides sont des substances d'origine naturelle agissant directement sur le champignon, en inhibant sa croissance et/ou son développement grâce à leur activité fongicide ou fongistatique. Les SDP sont, quant à eux, des composés, d'origine naturelle ou synthétique, qui agissent indirectement pour protéger la plante, en induisant ses réactions de défense. Seules les SDP d'origine naturelles sont classés parmi les produits de biocontrôle. Deux grandes catégories de SDP peuvent être différenciés selon leur mode d'action, les éliciteurs stricts et les potentialisateurs.

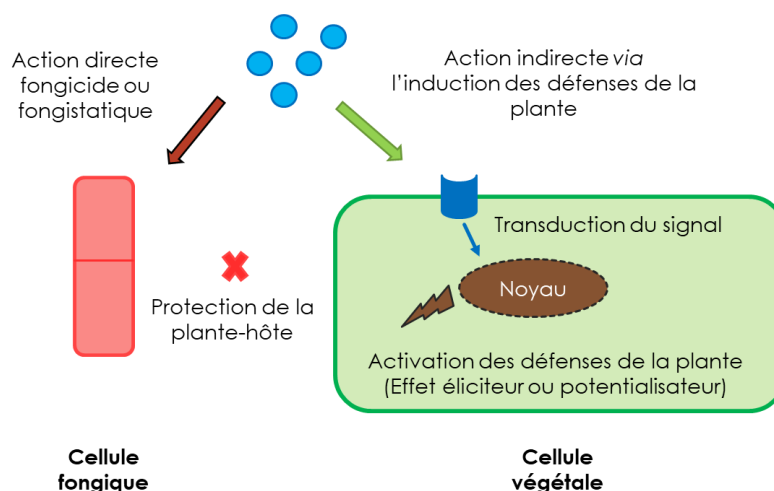


Figure 19 : Les modes d'action des produits de biocontrôle ciblant les agents pathogènes. Le produit (en bleu) peut agir directement sur le champignon, et/ou indirectement en stimulant les réactions de défense du végétal, conduisant à la protection de la plante contre l'agression.

4.5.2. Les éliciteurs stricts *versus* potentialisateurs

Après traitement avec un éliciteur strict et reconnaissance de ce composé par la plante, les voies de défense de cette dernière sont directement et fortement activées, qu'il y ait infection par la suite ou non. L'élicitation des défenses de la plante peut certes conduire à une protection de l'organisme végétal mais au prix d'un coût énergétique potentiellement conséquent et d'une réorientation métabolique pouvant nuire au rendement final de la culture. L'importance de ce coût dépend grandement du contexte environnemental et agronomique (Steppuhn and Baldwin, 2008 ; Cipollini *et al.*, 2017).

Les composés potentialisateurs, quant à eux, protègent la plante par effet *priming*. Celui-ci est établi chez les plantes préalablement exposées à un stimulus, dit primaire, de niveau faible, qui seront capables de le mémoriser et de rentrer alors dans un « état d'alerte » ou *primed state*. Dans cet état, la plante est capable de réagir à une infection ou une agression, beaucoup plus rapidement et plus fortement qu'une plante non stimulée au préalable, appelée naïve, mais aussi de reconnaître la présence de l'agresseur à des niveaux de signaux beaucoup plus faibles (**Figure 20**). Ce *primed state* dure souvent quelques jours à quelques semaines mais peut parfois durer plus longtemps et même être transmis à la descendance (Conrath *et al.*, 2015 ; Hilker *et al.*, 2016). Après le stimulus initial et durant l'« état d'alerte », et contrairement à l'effet que peuvent entraîner les éliciteurs stricts, seule une activation faible et transitoire, voire inexistante, est observée (**Figure 21**). L'effet *priming* n'entraîne pas de modifications au niveau de l'ADN de la plante et est réversible.

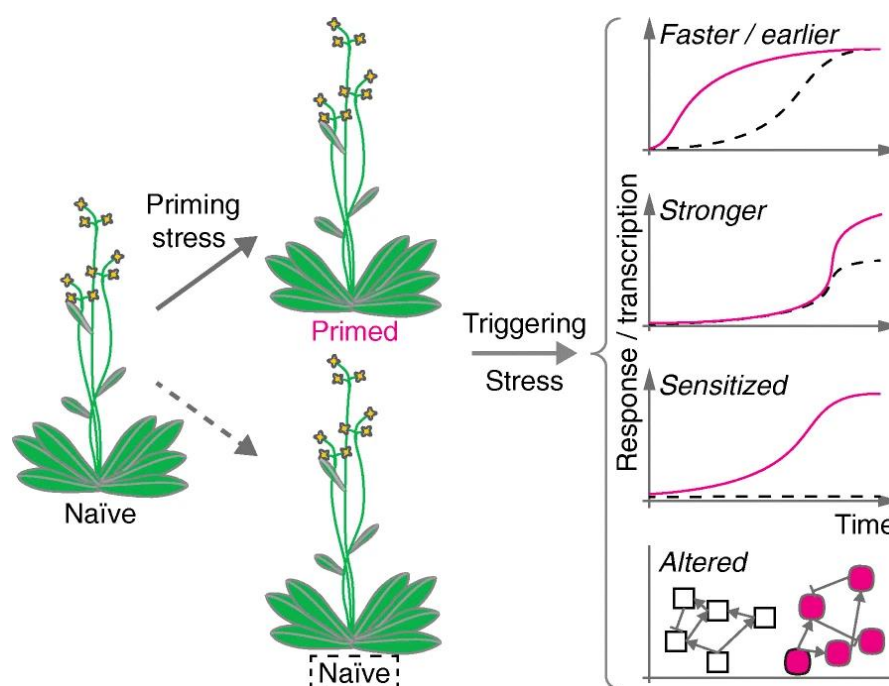


Figure 20 : Illustration de l'effet *priming* chez les plantes. Les plantes stimulées, *primed*, voient leurs réponses à une agression modifiées par rapport à des plantes naïves. Ces premières peuvent ainsi réagir plus vite ou plus fort ou à des niveaux plus faibles que les secondes. Les voies métaboliques engagées durant la réponse au bio-agresseur peuvent aussi différer entre une plante *primed* et une naïve. Des combinaisons de ces mécanismes peuvent avoir lieu lors de l'attaque par le pathogène. Les courbes en pointillées indiquent les réponses d'une plante naïve à l'infection et celles en rose correspondent aux réponses des plantes *primed* (d'après Lämke and Bäurle, 2017).

4.5.3. Les particularités des potentialisateurs

Dans le cas du *priming* par les potentialisateurs, les mécanismes liés à la mémorisation du signal et à cet état physiologique unique sont encore peu connus, mais des hypothèses ont pu être avancées par certains auteurs. Parmi celles-ci, Conrath *et al.* (2006) proposent comme mécanisme biologique l'accumulation de protéines signal, telles que les *calcium dependant protein kinases* (CDPK) ou les *heat-shock proteins* (HSP), qui seraient produites sous forme inactivée lors du stimulus initial et activées uniquement lors de l'attaque par le bio-agresseur. Les auteurs suggèrent aussi que l'accumulation de facteurs de transcription serait impliquée lors du phénomène de *priming* (Ptashne, 2008). Le stimulus initial modifie l'expression des gènes de la plante, dont les facteurs de transcription qui, à leur tour, pourront modifier l'expression d'autres gènes, notamment au moment de l'infection post-stimulus. Des mécanismes de boucles rétroactives impliquant ces facteurs de transcription pourraient aussi contribuer à la rémanence dans le temps de cet « état d'alerte » (Ptashne, 2008).

Selon Bruce *et al.* (2007), des régulations épigénétiques pourraient aussi jouer un rôle dans la mémorisation de cet état *primed*. La définition moderne d'épigénétique se réfère à l'étude des modifications agissant directement sur l'expression des gènes mais qui ne sont pas dues à des modifications dans la séquence de l'ADN (Gayon, 2016). Dans le cas du *priming*, le stimulus initial entraînerait notamment des changements dans la conformation 3D de l'ADN par méthylation, modifications des histones ou au niveau de la condensation de la chromatine, modifiant ainsi

l'accessibilité de certaines portions de l'ADN par les ARN polymérases et l'expression des gènes, sans toutefois altérer la séquence nucléotidique (Madlung and Comai, 2004).

Il est à noter que lors d'un effet *priming*, les tissus et organes n'ayant pas reçu le stimulus initial sont souvent aussi dans cet « état d'alerte » et ainsi plus résistants aux futures agressions, évoquant un phénomène de type SAR (Conrath *et al.*, 2006). Les plantes stimulées (*primed*) expriment souvent des réponses modifiées de celles mises en places par les naïves lors de l'infection (**Figure 20**) (Lämke and Bäurle, 2017).

Les potentialisateurs présentent donc de nombreux avantages théoriques par rapport aux éliciteurs stricts, un coût énergétique *a priori* moindre et donc potentiellement moins impactant au niveau du rendement, ainsi qu'une mise en place plus rapide et/ou plus forte des mécanismes de défense permettant de contrer plus efficacement l'agent pathogène (**Figure 21**). Ces molécules SDP capables de déclencher le *priming* chez les plantes sont donc particulièrement recherchées dans le cadre de la protection des cultures.

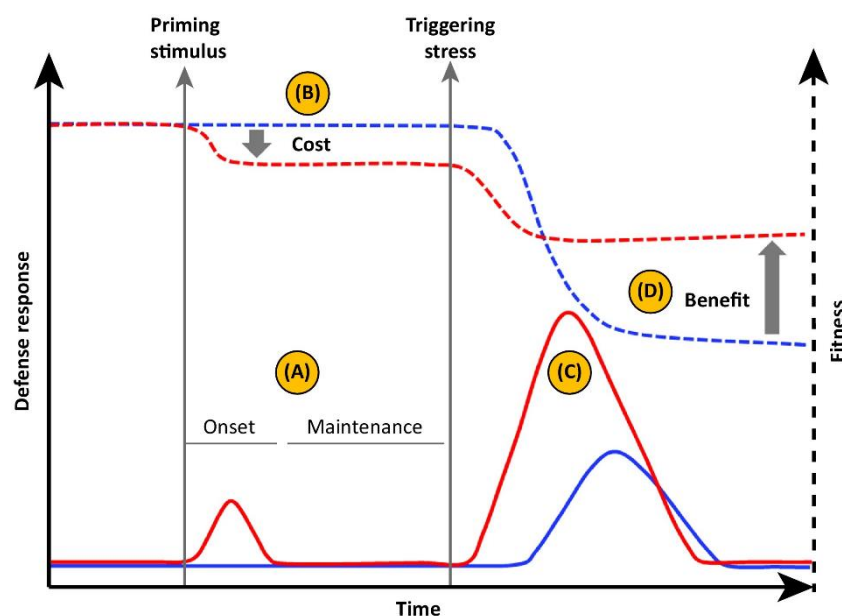


Figure 21 : Schéma de la relation entre *priming* (courbes pleines) et *fitness* (tirets) chez les plantes *primed* (en rouge) versus naïves (en bleu). (A) le stimulus initial déclenche de faibles, voire inexistantes, réactions de défense chez la plante qui est alors en « état d'alerte ». (B) Ces réactions de défense peuvent présenter un coût énergétique et métabolique significatif même si bien inférieur à celui causé par l'élicitation stricte des défenses. (C) Lors de l'infection, les plantes *primed* peuvent mobiliser leurs réactions de défense plus rapidement et plus fortement que les plantes naïves. (D) Les plantes *primed* sont plus à même de se défendre face à l'infection avec un ratio bénéfice/coûts > 1 (d'après Martinez-Medina *et al.*, 2016).

Les SDP présentent des origines et des structures moléculaires très variées. Ils peuvent être d'origine synthétique (*i.e.* sulfamethoxazole), abiotique, comme les minéraux et leurs ions associés (*i.e.* cuivre ou phosphites) ou encore biotique (Bektas and Eulgem, 2015 ; Siah *et al.*, 2018). Les composés provenant d'êtres vivants sont les plus variés et regroupent notamment des extraits de plantes (algues ou spermatophytes), des co-produits issus des industries agroalimentaires, des métabolites bactériens (rhamnolipides, lipopeptides, harpines, *etc.*), des extraits de parois et membranes microbiennes (chitosan, lipopolysaccharides, peptidoglycanes, flagellines,

exopolysaccharides, ergostérol, *etc.*) et encore d'autres catégories de molécules organiques tels que les vitamines, les acides aminés et les sucres (Siah *et al.*, 2018b). Les SDP d'origine biotique peuvent aussi être classés selon leur origine au niveau des interactions plante-pathogène comme PAMP, MAMP ou DAMP.

4.5.4. Le biocontrôle de *Z. tritici*

A l'heure actuelle, très peu de produits de biocontrôle sont homologués et disponibles pour protéger les grandes cultures. Dans le cadre de la lutte contre la septoriose, à part le soufre, bio-fongicide bien connu, seul le Vacciplant® (précédemment Iodus 40®, Goëmar, Arista LifeScience, France), est homologué et est disponible sur le marché européen. Il s'agit d'un SDP d'origine biotique à base de laminarine, un polymère glucidique de β -1,3-glucane, extrait de l'algue brune *Laminaria digitata* (Renard-Merlier *et al.*, 2007). Pour rappel, Shetty *et al.* (2009) ont montré que des extraits de β -1,3-glucane provenant de la paroi de *Z. tritici* permettait la mise en place de réactions de défense chez le blé conduisant à sa protection vis-à-vis de la septoriose. Plusieurs équipes ont par ailleurs rapporté une induction des défenses par cet SDP chez d'autres plantes, telles que la vigne et le tabac (Klarzynski *et al.*, 2000 ; Aziz *et al.*, 2003). Ainsi, Aziz *et al.* (2003) ont montré que la laminarine était, chez les feuilles de vigne, capable d'augmenter l'expression de gènes de défense comme ceux codant pour la β -1,3-glucanase, la chitinase ou encore d'inhibiteurs de protéases à sérine, et d'induire une protection partielle contre *Botrytis cinerea* et *Plasmopara viticola*.

Jusqu'en 2017, le Bion® (Syngenta, Suisse) contenant de l'acibenzolar-S-méthyl, un analogue fonctionnel du SA synthétisé chimiquement, était aussi homologué comme éliciteur du blé et permettait de lutter contre la septoriose et d'autres maladies affectant cette culture, telles que l'oïdium et la rouille (Görlach *et al.*, 1996a). Cet éliciteur n'a, toutefois, jamais été homologué comme produit de biocontrôle au niveau de la réglementation.

Les microorganismes bénéfiques

Les plantes peuvent dans certains cas coopérer avec des microorganismes épiphytes ou endophytes, vivants respectivement à l'extérieur ou à l'intérieur des tissus de l'hôte sans lui causer de dommages. Ces microorganismes symbiotiques peuvent fournir différents avantages à la plante, comme l'amélioration de sa croissance, son apport en eau et en composés minéraux et sa résilience face aux stress biotiques et abiotiques (Pérez-Montaña *et al.*, 2014). Lors d'une attaque par un agent pathogène, les microorganismes bénéfiques peuvent intervenir dans la protection de la plante de différentes manières, notamment *via* la production de composés toxiques vis-à-vis du bio-agresseur, la compétition directe vis-à-vis du pathogène ou encore en activant les réactions de défense de la plante. L'induction de phénomènes de résistance par les microorganismes bénéfiques peut se produire au niveau de la rhizosphère et entraîner une résistance globale et durable chez la plante, phénomène alors appelé résistance systémique induite (*induced systemic resistance*, ISR). L'ISR a, pendant un temps, été considéré comme dépendante de la voie de biosynthèse et de la perception du JA/ET et n'induisant pas l'expression de protéines PR (Pieterse *et al.*, 1998). Toutefois, des travaux ont pu mettre en évidence l'inverse ; lors de certains phénomènes d'ISR, la voie du SA était impliquée, ainsi que la production de protéines PR, mais pas celle du JA/ET (Barriuso *et al.*, 2008). D'autres études ont

montré que les deux voies antagonistes, JA/ET et SA, pouvaient être induites ensemble durant l'activation de l'ISR (Walters and Bennett, 2014).

Dans le cadre de la lutte contre *Z. tritici*, plusieurs agents de biocontrôle potentiels ont été découverts ces dernières décennies. En ce qui concerne l'utilisation de bactéries vivantes, plusieurs espèces de *Pseudomonas* se sont révélées efficaces contre le champignon, que ce soit *in vitro* ou *in planta* (Levy *et al.*, 1988 ; Levy *et al.*, 1992 ; Kildea *et al.*, 2008 ; Bricout, 2020). Par exemple, selon Flaishman *et al.* (1996), l'application de la souche de *P. putida*, BK8661, connue pour sécréter des sidérophores et du cyanide d'hydrogène (HCN), directement sur des feuilles de plantules de blé, est capable de réduire, en chambre de culture, la densité des pycnides de 20 % et la sévérité de la maladie de 71 %. Par ailleurs, le traitement de feuilles de blé avec des suspensions de *Paenibacillus polymyxa* et de *Bacillus cereus* ont montré des efficacités de protection allant jusqu'à 55 % en serre (Alippi *et al.*, 2000). Plus récemment, Kildea *et al.* (2008) ont identifié la souche MKB135 de *Bacillus megaterium*, qui lorsque appliquée sur les feuilles de blé, réduit la sévérité de la maladie très fortement, jusqu'à 80 % au champ. Des bactéries lactiques ont aussi montré une efficacité de protection du blé vis-à-vis de la septoriose (Lynch *et al.*, 2016). D'autres équipes se sont intéressées à l'utilisation de champignons et principalement aux effets potentiels des espèces de *Trichoderma*. En effet, Cordo *et al.* (2007) ont identifié plusieurs souches de *Trichoderma* sp., agissant comme éliciteurs des réactions de défense chez le blé vis-à-vis de *Z. tritici*, en stimulant notamment l'activité protéolytique antifongique des protéines situées dans l'apoplasme. Parmi les souches testées, *T. harzanium* Th5 était le plus efficace lorsqu'appliqué sous forme d'enrobage de semence avec une activité de protection d'environ 26 %. D'autres souches de *T. harzanium*, 123 et 170, se sont montrées encore plus prometteuses dans la lutte contre la septoriose, en réduisant la sévérité de la maladie respectivement de 80 % et 90 % (Stocco *et al.*, 2016). Pour finir, des enrobages de semence avec des souches de *T. konigii* se sont aussi révélés efficaces pour réduire le développement de la maladie (Perelló *et al.*, 2009). Plus récemment, le rôle potentiel de l'ISR induite par *Paenibacillus* sp. strain B2 dans la réduction de la biomasse fongique de *Z. tritici* a été rapporté (Samain *et al.*, 2019).

Les substances d'origine naturelle

Les recherches menées par différentes équipes sur le pathosystème blé-*Z. tritici* ont permis d'identifier de nouvelles substances d'origine naturelle prometteuses, qu'elles soient bio-fongicides, SDP ou les deux. Ces composés sont d'origines végétale ou microbienne et peuvent même provenir de *Z. tritici* lui-même, comme le β -1,3-glucane provenant de la paroi du champignon (Shetty *et al.*, 2009). Récemment, Le Mire *et al.*, (2019) ont identifié de nouveaux SDP contre la septoriose, le λ -carraghénine (polysaccharide extrait d'algues rouges), le CpG-Oligo désoxynucléotide, la glycine bêtaïne, l'ergostérol (composé essentiel des membranes fongiques) et la spiruline (*Spirulina platensis*). Leurs résultats ont montré que ces éliciteurs induisaient à la fois la voie du SA et du JA et permettait de protéger le blé de la septoriose jusqu'à 70 % en conditions semi-contrôlées, sans présenter d'activité antifongique. Les activités d'extraits de rhizomes de *Juncus maritimus* ont été testés pour leur activité directe *in vitro*. Les auteurs ont pu mettre en évidence un dérivé phénanthrène particulièrement efficace, l'effusol, qui présentait une concentration minimal inhibitrice (CMI) égale à 19 mg.L⁻¹ (Sahli *et al.*, 2018). Bocquet *et al.* (2018) ont aussi rapporté une activité antifongique *in vitro* de la part d'extraits de cônes et d'huile essentielle de houblon, même si les concentrations requises

étaient relativement élevées. Le rôle des lipopeptides produit par *Bacillus subtilis* dans le biocontrôle de *Z. tritici* a récemment été souligné. En effet, Mejri *et al.*, (2018) ont rapporté un effet bio-fongicide marqué de la mycosubtiline à la fois *in vitro*, $CI_{50} = 1,4 \text{ mg.L}^{-1}$ (concentration inhibitrice à 50 % de la croissance fongique), et *in planta*. A l'inverse aucun effet direct n'a été observé avec la surfactine et la fengycine. L'application foliaire de ces composés à 100 mg.L^{-1} a permis de montrer que les trois lipopeptides protégeaient le blé vis-à-vis de la septoriose, avec une efficacité de protection plus élevée conférée par la mycosubtiline. Au vu de leur absence d'activité *in vitro*, les auteurs suggèrent que le mode d'action de la surfactine et la fengycine est basé sur une activité SDP. Les travaux de Le Mire *et al.* (2018) confirment cette hypothèse et montrent que la surfactine extraite de la souche S499 de *Bacillus amyloliquefaciens* protège le blé en induisant les voies de défense dépendantes du SA et du JA de manière conjointe. D'autres extraits de bactéries, notamment de *Bacillus* spp. ont aussi pu montrer des activités directes antifongiques *in vitro*, soulignant l'intérêt potentiel de ces bactéries dans le biocontrôle de la septoriose du blé (Alippi *et al.*, 2000 ; Kildea *et al.*, 2008).

Les biosurfactants microbiens : des biomolécules prometteuses pour le biocontrôle

Les biosurfactants sont des molécules tensio-actives qui sont produites par un large panel de microorganismes. Les principales classes de biosurfactants sont représentées par les lipides mannosylérythritol, les tréhaloses dimycolates, les tréhalolipides, les sophorolipides, les rhamnolipides et les lipopeptides (Crouzet *et al.*, 2020). Ces molécules peuvent présenter des structures très diverses mais sont toutes amphiphiles, composés d'une partie hydrophobe et d'une partie hydrophile qui leur donnent leurs caractéristiques physico-chimiques particulières. Ces composés sont utilisés dans une large gamme de champs d'applications tels que l'industrie alimentaire, la santé humaine, l'industrie cosmétique, l'industrie pétrolière, la remédiation des eaux et des sols contaminés, les nanotechnologies et, en ce qui nous concerne, l'agriculture (Sachdev and Cameotra, 2013 ; Lourith and Kanlayavattanukul, 2009 ; Singh *et al.*, 2019). Les biosurfactants sont des composés prometteurs en biocontrôle car ils sont considérés comme facilement biodégradables, présentent une faible toxicité sur la santé humaine et l'environnement et plusieurs études ont déjà montré des effets significatifs de ces biomolécules en protection des plantes (Crouzet *et al.*, 2020a). Les principales familles étudiées dans ce contexte sont les lipopeptides et les rhamnolipides, qui présentent de nombreux avantages, notamment leur production à une échelle industrielle, leur efficacité et leur faible rémanence dans l'environnement, faisant d'eux des candidats particulièrement intéressants en biocontrôle (Crouzet *et al.*, 2020a). Certaines études se sont toutefois intéressées aux autres familles de biosurfactants en agriculture (Yoshida *et al.*, 2015 ; Sen *et al.*, 2017 ; Chen *et al.*, 2020).

Les rhamnolipides

Les rhamnolipides sont des glycolipides, produits, notamment, par des espèces de *Pseudomonas* sp. et de *Burkholderia* sp., constitués d'une ou plusieurs têtes rhamnose hydrophiles et d'une ou plusieurs chaînes d'acide gras hydrophobes (**Figure 22**). En protection de cultures, plusieurs études ont rapporté leurs activités antimicrobiennes et d'induction des voies de défense chez les plantes (Vatsa *et al.*, 2010). Les rhamnolipides sont efficaces en tant que bio-fongicides et ont montré une activité

directe contre un large spectre d'agents phytopathogènes : *Botrytis* sp., *Rhizoctonia* sp., *Fusarium* sp., *Alternaria* sp., *Pythium* sp., *Phytophthora* sp., et *Plasmopara* sp. (Stanghellini and Miller, 1997 ; Abalos *et al.*, 2001 ; Crouzet *et al.*, 2020). Les rhamnolipides sont aussi capables d'induire des phénomènes de résistance chez les plantes comme chez la vigne, *Brassica napus* ou *Arabidopsis thaliana*, notamment en déclenchant des influx de Ca^{2+} , l'accumulation de ROS, la déposition de callose ou la production d'hormones de défense (**Figure 23**) (Varnier *et al.*, 2009 ; Sanchez *et al.*, 2012 ; Monnier *et al.*, 2018). Cette double activité des rhamnolipides, bio-fongicide et SDP, pourrait s'expliquer par la nature amphiphile de ces composés. Plusieurs auteurs émettent l'hypothèse que ces molécules seraient capables d'interagir avec les membranes des plantes comme celles des agresseurs fongiques (Monnier *et al.*, 2019). Chez les champignons pathogènes, ces glycolipides s'intercaleraient dans leurs membranes, au niveau des bicouches de phosphatidylcholine et de phosphatidyléthanolamine, causant *in fine* leur déstabilisation et leur lyse cellulaire (**Figure 23**) (Stanghellini and Miller, 1997 ; Sánchez *et al.*, 2010 ; Monnier *et al.*, 2019). Chez les plantes, le mécanisme de reconnaissance des rhamnolipides est encore inconnu. Il est, cependant, suggéré que les molécules interagiraient aussi avec la membrane des plantes mais avec des conséquences moins radicales, entraînant seulement l'induction des voies de défense (**Figure 23**) Monnier *et al.*, 2019).

Les lipopeptides

Les lipopeptides, produits par de nombreuses espèces de champignons et de bactéries, sont composés d'une chaîne lipidique hydrophobe liée à un oligopeptide linéaire (LLP) ou cyclique (CLP) (**Figure 22**). L'activité antimicrobienne de ces composés est très bien documentée et leur potentiel dans le domaine du biocontrôle n'est plus à démontrer (Ongena and Jacques, 2008 ; Geudens and Martins, 2018 ; Caulier *et al.*, 2019). Les CLP bactériens sont de puissants biosurfactants qui sont supposément capables d'engendrer d'importantes déstabilisations membranaires, comme les rhamnolipides, conduisant à la perte d'intégrité cellulaire et ainsi à la mort des champignons pathogènes (**Figure 22**; Crouzet *et al.*, 2020). Toutefois, d'après plusieurs équipes, l'activité antimicrobienne des CLP ne serait pas seulement due aux interactions avec les membranes, mais aussi à des modifications et des altérations des fonctions intracellulaires des microorganismes ciblés (Latoud *et al.*, 1987 ; Qi *et al.*, 2010). Plusieurs CLP sont aussi capables de stimuler les voies de défense chez les plantes. Il a ainsi été montré que la fengycine et/ou la surfactine protégeaient le haricot, la tomate, la laitue, le melon, le citron, le ray-grass, la vigne, la betterave, la fraise, le coton et, bien sûr, le blé contre différentes maladies (Ongena *et al.*, 2007 ; Desoignies *et al.*, 2013 ; García-Gutiérrez *et al.*, 2013 ; Chowdhury *et al.*, 2015 ; Waewthongrak *et al.*, 2014 ; Han *et al.*, 2015 ; Rahman *et al.*, 2015 ; Yamamoto *et al.*, 2015 ; Le Mire *et al.*, 2018 ; Li *et al.*, 2019). Chez le riz, des CLP comme le WLIP, la lokisin et l'entolysin ont réussi à induire des phénomènes de résistance contre *M. oryzae* (Omoboye *et al.*, 2019). Ces CLP protégeraient les plantes vis-à-vis des infections principalement par l'intermédiaire de l'ISR. Les lipopeptides induiraient les réactions de défense chez les plantes d'une manière similaire aux rhamnolipides, en interagissant avec les membranes végétales (**Figure 23**) (Henry *et al.*, 2011 ; Schellenberger *et al.*, 2019). Il est à noter que dans le cas des rhamnolipides comme des lipopeptides, la structure de la molécule influe grandement sur les activités de ces molécules (Henry *et al.*, 2011 ; Kawagoe *et al.*, 2015 ; Luzuriaga-Loaiza *et al.*, 2018 ; Robineau *et al.*, 2020). Plusieurs espèces de *Bacillus* ont été identifiées comme prometteuses pour la production de lipopeptides pouvant

présenter des structures différentes, en particulier des CLP appartenant à 3 principales familles, les iturines (mycosubtiline, bacillomycine D), les surfactines et les fengycines (**Figure 22**).

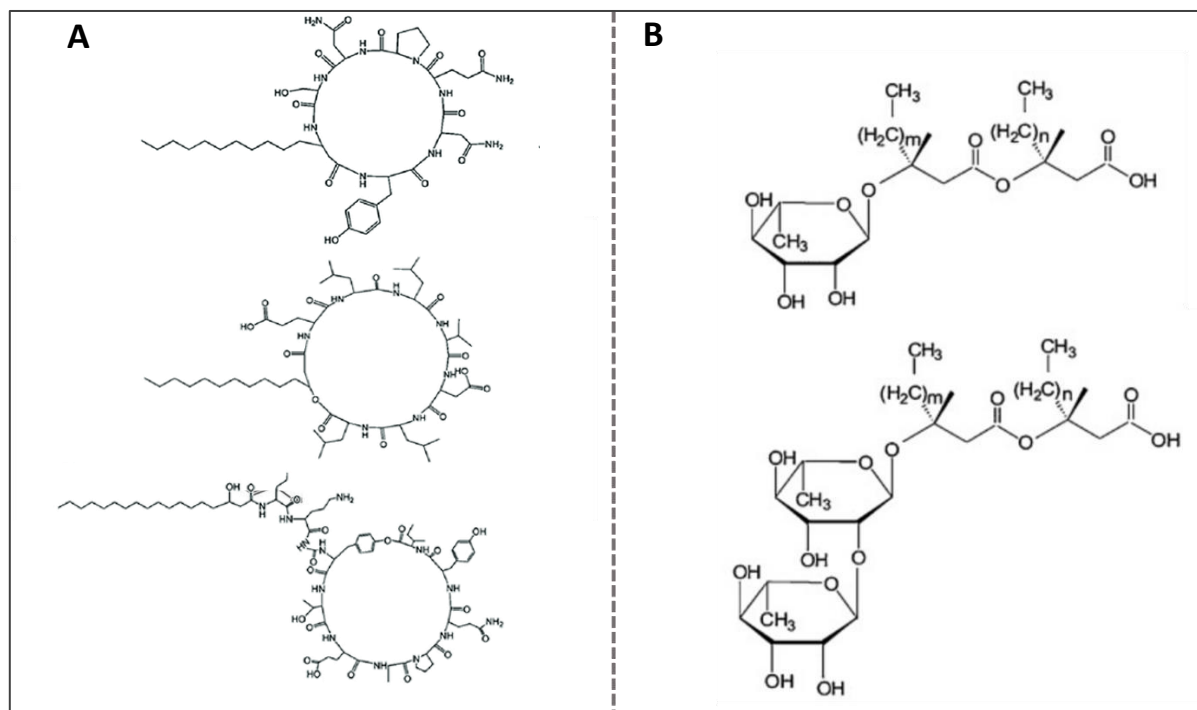


Figure 22: Structure générale des (A) principaux lipopeptides produits par *Bacillus* sp. et des (B) rhamnolipides. Avec, en (A) de haut en bas : la structure d'une iturine, d'une surfactine et d'une fengycine et en (B) en haut à droite, la structure générale des mono-rhamnolipides et en bas des di-rhamnolipides (d'après Hamley et al., 2013 et Dobler et al., 2016).

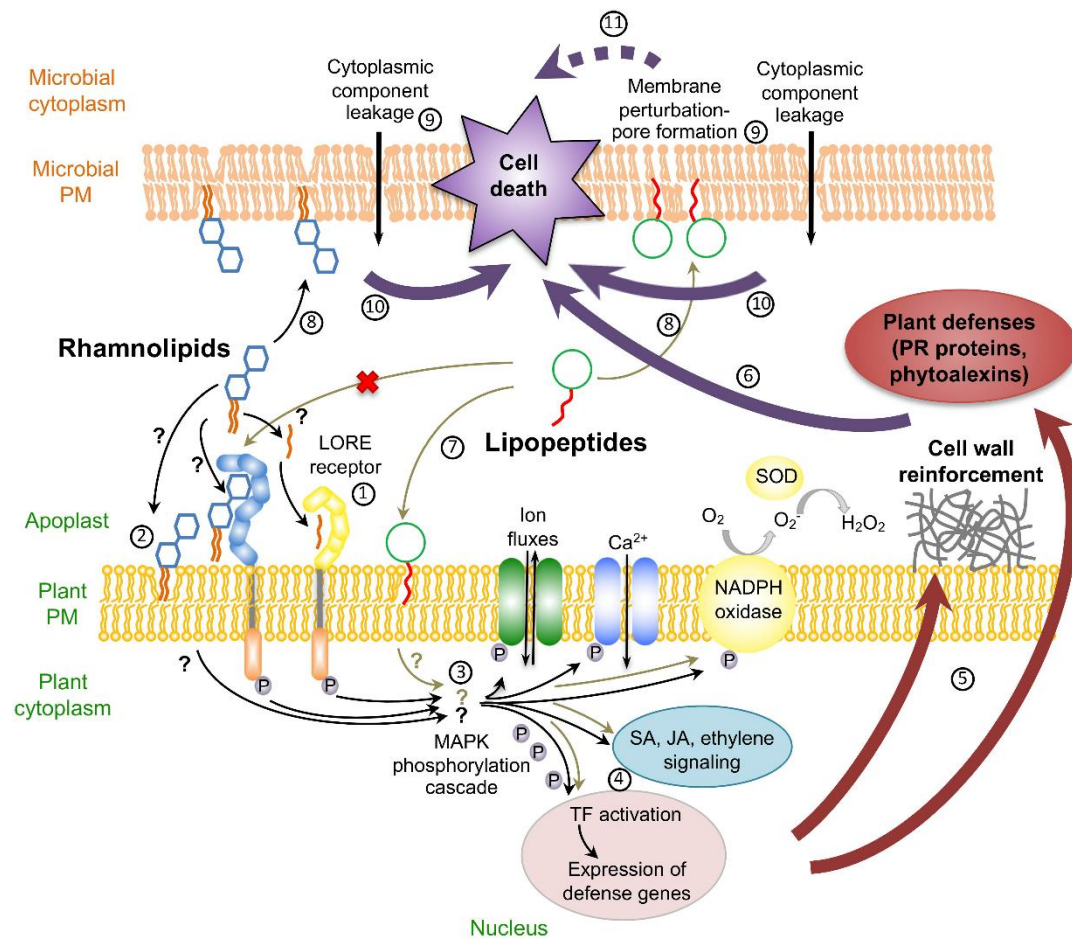


Figure 23 : Effets des rhamnolipides et des lipopeptides, à la fois direct sur l'agent pathogène et indirect *via* la stimulation des défenses de la plante. (1) La partie mc-3-OH-acyl du rhamnolipide est perçue par le récepteur LORE de la plante. (2) Le rhamnolipide pourrait être reconnu directement par la cellule de la plante au niveau de la membrane végétale. (3) La reconnaissance du rhamnolipide conduit à l'établissement de réponses précoces. (4) Mise en place d'une transduction du signal à l'issue de ces réponses précoces. (5) Etablissement des réponses tardives, comme l'expression de protéines PR et la production de phytoalexines permettant (6) l'inhibition de développement du pathogène. (7) Les lipopeptides peuvent aussi interagir avec la membrane plasmique de la cellule végétale pour induire les réactions de défense. (8) Les rhamnolipides et les lipopeptides peuvent désorganiser directement la membrane du pathogène, ce qui (9) induit la formation de pores au niveau de la membrane plasmique et une perte d'intégrité cellulaire chez le microorganisme et finit par (10) conduire à la mort cellulaire de l'agent pathogène. (11) La mort cellulaire causée par les lipopeptides peut aussi être due à l'action de ces biomolécules sur d'autres mécanismes intra-cellulaires (d'après **Crouzet et al., 2020**).

CONTEXTE ET OBJECTIFS GÉNÉRAUX DU PROJET DE THÈSE

Actuellement, la lutte contre *Z. tritici* sur les cultures de blé repose essentiellement sur l'utilisation des fongicides conventionnels. Toutefois, ces produits sont de plus en plus controversés à cause de leurs effets négatifs potentiels sur l'environnement et la santé humaine, mais aussi en raison de l'émergence fréquente de résistances à ces produits chez les populations de l'agent pathogène. La réduction de leur utilisation est ainsi fortement encouragée dans l'Union Européenne (UE). En France, le plan Ecophyto a été mis en place et s'inscrit dans cette démarche. Celui-ci, qui a débuté en 2008, visait tout d'abord à réduire de 50% l'utilisation des produits phytosanitaires de synthèse pour 2018. Le plan fut révisé en 2015, l'objectif est maintenu mais désormais à l'horizon 2025 (Ecophyto II, 2016). A l'heure actuelle, 70% des fongicides épandus dans l'UE le sont pour lutter uniquement contre *Z. tritici* (Fones and Gurr, 2015a). Au vu de la quantité de fongicides utilisés contre cette maladie, il est urgent de développer d'autres méthodes de lutte pour y faire face. Une alternative prometteuse à l'utilisation des pesticides conventionnels dans la gestion des agents phytopathogènes est le recours aux agents ou produits de biocontrôle. Actuellement, seuls le soufre et le Vacciplant® sont homologués en tant que produits de biocontrôle contre la septoriose du blé. L'identification de nouveaux composés ou principes actifs de biocontrôle ciblant la septoriose du blé est ainsi fortement encouragée pour offrir aux agriculteurs de nouvelles bio-solutions leur permettant de lutter durablement contre cette maladie. Par ailleurs, si certains travaux menés par la communauté scientifique ont permis de mettre en évidence des microorganismes et des substances naturelles prometteurs pour contrôler la septoriose du blé, leur nombre reste toutefois limité (e.g. Lynch *et al.*, 2016 ; Stocco *et al.*, 2016 ; Bocquet *et al.* 2018 ; Le Mire *et al.*, 2018 ; Mejri *et al.* 2018 ; Le Mire *et al.*, 2019 ; Ors *et al.*, 2019 ; Samain *et al.*, 2019). Par ailleurs, les connaissances sur les mécanismes de résistance induite déclenchés chez le blé, contre cette maladie, par les microorganismes bénéfiques et les composés éliciteurs ou potentialisateurs, restent, aujourd'hui encore, largement lacunaires (Le Mire *et al.*, 2018 ; Le Mire *et al.*, 2019; Ors *et al.*, 2019; Samain *et al.*, 2019). En outre, aucune étude à l'aide d'outils Omics n'a été jusqu'ici conduite pour caractériser plus finement ces mécanismes chez le blé vis-à-vis de la septoriose. Seuls quelques travaux ont utilisé ces approches pour décrypter les mécanismes de résistance induite chez le blé vis-à-vis des agents pathogènes, comme *Blumeria graminis* (Chain *et al.*, 2009) et *Xanthomonas translucens* (Fiorilli *et al.*, 2018). Ce manque d'information concernant les modes d'action des produits de biocontrôle dans leur ensemble constitue actuellement un frein au développement de leur utilisation.

Les objectifs principaux de ce projet de thèse étaient donc d'identifier de nouveaux produits de biocontrôle efficaces sur le blé vis-à-vis de la septoriose et de caractériser leurs modes d'action, en particulier au niveau de l'induction des mécanismes de défense de l'hôte.

Cette thèse s'inscrit dans le cadre du portefeuille de projets Smartbiocontrol, financé par Interreg V, qui vise à promouvoir l'utilisation et le développement de produits de biocontrôle sur les cultures d'intérêt présentes au niveau de la région transfrontalière franco-belge (Smartbiocontrol, 2017). Plusieurs pathosystèmes y ont été ciblés, dont, celui qui nous intéresse ici, le pathosystème blé-*Z. tritici*. Ce portefeuille de projets est mené en collaboration entre 26 partenaires français et belges,

dont l'UMR Transfrontalière BioEcoAgro site ISA-Junia (**Figure 24**). Smartbiocontrol est composé de cinq projets :

- Bioscreen : Criblage et identification de nouveaux composés de biocontrôle
- Bioprod : Optimisation de la production et de la formulation des composés
- Bioprotect : Optimisation de l'application des composés en serre et au champ
- Biosens : Détection des agents pathogènes au champ pour optimiser le positionnement
- Biocomgest : Coordination

Plus précisément, mon projet de thèse s'inscrit dans le projet Bioscreen, qui vise principalement à cribler et identifier de nouveaux composés de biocontrôle (bio-fongicides et/ou inducteurs de résistance). Le coût complet du projet Bioscreen est de 3 097 785 €, avec une aide de 1 703 781 € par le FEDER (le budget octroyé à l'ISA-Junia est de 143 991 € pour un coût complet de 261 801 €). Dans le cadre de mes travaux, j'ai eu accès, grâce notamment aux différents partenaires de Bioscreen, à une bibliothèque de 181 composés d'origines variées (extraits microbiens purifiés ou non, molécules synthétiques bio-inspirées, etc.). Dans un premier temps, un criblage de leurs activités biologiques sur le pathosystème d'intérêt blé-*Z. tritici* a été réalisé, principalement sur la base de leur activité antifongique directe *in vitro*. L'efficacité de protection des composés les plus prometteurs a ensuite été vérifiée *in planta* en serre. Enfin, parmi les 181 composés initialement mis à disposition, deux molécules (*i.e.* un lipopeptide et un rhamnolipide) se sont révélées particulièrement actives pour protéger le blé contre *Z. tritici*, supposément grâce à leur double activité (antifongique et activation des défenses de l'hôte). Les mécanismes liés à leurs modes d'action ont finalement été caractérisés, en particulier concernant leur activité possible de stimulation des défenses du blé. Les principaux résultats obtenus dans le cadre de ces travaux sont ou seront valorisés sous forme d'articles scientifiques présentés ci-après.



Figure 24: Carte présentant les 26 partenaires franco-belges du portefeuille de projets Smartbiocontrol, financé par Interreg V (www.smartbiocontrol.eu/fr).

RÉSULTATS

RESUMÉ DU CRIBLAGE DES COMPOSÉS D'ORIGINE MICROBIENNE ÉTUDIÉS

Un total de 181 composés (bactéries, molécules ou extraits) a été fourni par les différents partenaires du projet Bioscreen, afin de réaliser un criblage de leurs activités biologiques sur le pathosystème blé-*Z. tritici*. L'objectif était de déterminer quels composés pouvaient être les plus prometteurs pour lutter contre la septoriose du blé. Si ces composés ont tous des origines, des niveaux de pureté et des propriétés physico-chimiques bien différents, ceux-ci présentent toutefois une même caractéristique commune, celle de provenir de micro-organismes bactériens ou fongiques ou alors de mimer des métabolites provenant de ceux-ci. Nous avons ainsi à disposition :

- 146 extraits d'espèces fongiques différentes provenant de la mycothèque de l'Université Catholique de Louvain, Belgique.
- 2 souches de *Bacillus velezensis*, 1 souche de *B. amyloliquefaciens* et 2 souches de *Paenibacillus polymyxa*, fournies par l'Institut National de la Recherche Agronomique (INRA) de Kenitra, Maroc.
- 8 lipodepsipeptides cycliques extraits et purifiés de *Pseudomonas* spp., fournis par l'Université de Gand, Belgique.
- 1 lipopeptide, la mycosubtiline, extrait et purifié à partir d'une souche de *Bacillus subtilis*, fournis par Lipofabrik, France.
- 21 rhamnolipides et molécules associées, dont 18 bio-inspirés et synthétisés par chimie verte, ainsi qu'un mix de rhamnolipides naturels produits par *P. aeruginosa* et deux molécules associées, fournis par l'Université d'Artois, France.

L'activité *in vitro* antifongique directe des différents composés a été déterminée en mesurant la croissance des colonies de *Z. tritici* sur milieu solide (*potato dextrose agar*, PDA), en plaques 12 puits, contenant les molécules ou extraits d'intérêt. Les tests d'activité antifongique ont tous été réalisés vis-à-vis de la souche T02596 de *Z. tritici*, virulente sur le cultivar de blé Alixan (Mejri *et al.*, 2018). Les concentrations initiales des composés étaient de 30% pour les surnageants bactériens et fongiques, de 50 µg.mL⁻¹ pour les métabolites microbiens purifiés (lipodepsipeptides cycliques et mycosubtiline) et de 1500 µM pour les rhamnolipides bio-inspirés. Les observations ont été effectuées 10 jours après inoculation des plaques avec la suspension de spores du champignon (5.10⁵ spores/mL) et les résultats obtenus sont présentés en **Annexe 1**. Ce criblage a permis de mettre en évidence un nombre conséquent de composés efficaces *in vitro* contre l'agent pathogène. Sur les 146 extraits fongiques testés, 11 ont montré une inhibition totale de la croissance fongique et 12 un taux d'inhibition allant de 80 à 99% (concentration de 30% (v/v)). Six rhamnolipides et molécules associées ont aussi induit son inhibition totale à la concentration de 1500 µM. Aucun effet significatif n'a été observé avec les six lipodepsipeptides cycliques extraits de *Pseudomonas* spp., contrairement à la mycosubtiline, qui a montré une activité inhibitrice totale de la croissance du champignon à la concentration de 50 µg.mL⁻¹. Les surnageants de cinq souches de *Bacillus* spp. ont, quant à eux, tous inhibé à 100% la croissance de l'agent phytopathogène (**Annexe 1**).

Afin de caractériser plus finement l'activité antifongique des composés les plus efficaces *in vitro* vis-à-vis de *Z. tritici*, leur concentration minimale inhibitrice (CMI) et d'inhibition à 50% (CI₅₀) ont été déterminées dans les mêmes conditions que précédemment, avec une concentration maximale de 1500 µM pour les rhamnolipides, 100 µg/mL pour la mycosubtiline et 30% (v/v) pour les extraits

(fongiques et bactériens) (**Tableau 5**). La CMI représente la concentration minimale d'un composé empêchant totalement la croissance de *Z. tritici*, alors que la CI_{50} correspond à la concentration théorique inhibant 50% de la croissance. Les CI_{50} des extraits fongiques présentés ci-dessous ont été calculées à l'aide du logiciel XLSTAT (Addinsoft, France) alors que les autres CI_{50} (pour les filtrats de culture bactériens, rhamnolipides, mycosubtiline) l'ont été avec le logiciel GraphPad Prism (USA).

Tableau 5 : Résumé des CI_{50} et CMI des composés les plus efficaces in vitro contre *Z. tritici*

Composés	CI_{50}	CMI
Extraits fongiques		
<i>Penicillium griseofulvum</i>	2.1%	7.5%
<i>Trichoderma Virens</i>	2.9%	15%
<i>Aspergillus niger</i>	3.6%	15%
<i>Myrothecium verrucaria</i>	4.5%	15%
<i>Lecanicillium longisporum</i>	5.9%	30%
<i>Penicillium cyclopium</i>	6.0%	15%
<i>Trichothecium roseum</i>	7.3%	15%
<i>Emericella rugulosa</i>	8.0%	>30%
<i>Paecilomyces lilacinus</i>	15.3%	>30%
<i>Penicillium aurantiogriseum</i>	16.3%	>30%
<i>Penicillium purpurogenum</i>	19.5%	>30%
<i>Sporothrix insectorum</i>	19.9%	>30%
<i>Talaromyces flavus</i>	22.6%	>30%
<i>Laetiporus sulphureus</i>	29.6%	>30%
<i>Penicillium brevicompactum</i>	33.7%	>30%
<i>Chaetomium globosum</i>	43.4%	>30%
Surnageants bactériens		
<i>Bacillus velezensis</i> S1	1.4%	3.7%
<i>Bacillus velezensis</i> S6	7.4%	15%
<i>Paenibacillus polymyxa</i> M2	Nd*	30%
<i>Paenibacillus polymyxa</i> AMESA	Nd*	15%
<i>Bacillus amyloliquefaciens</i> I3	26.7%	30%
Rhamnolipides et molécules associées		
Rhamnose-Ester-C12	75 μ M	133 μ M
Rhamnose-Ether-C12	81 μ M	450 μ M
Acide laurique	109 μ M	200 μ M
Rhamnose-Ether-C10	158 μ M	200 μ M
Dodécanol	164 μ M	200 μ M
Rhamnose-Succinate-C8	1399 μ M	1500 μ M
Lipopeptide cyclique purifié		
Mycosubtiline	0.57 μ g.mL ⁻¹	0.78 μ g.mL ⁻¹

*Les CI_{50} des surnageants bactériens de *Paenibacillus polymyxa* n'ont pas pu être déterminées en raison des faibles rendements en filtrats obtenus avec ces souches.

Plusieurs composés ont ainsi montré une activité antifongique significative dans ces conditions. Parmi ceux-ci, huit extraits fongiques (*Penicillium griseofulvum*, *Trichoderma virens*, *Aspergillus niger*, *Myrothecium verrucaria*, *Lecanicillium longisporum*, *Penicillium cyclopium*, *Trichothecium roseum*, *Emericella rugulosa*) ont présenté des CI_{50} inférieures à 10% (v/v). Après élimination des candidats fongiques pouvant potentiellement produire des métabolites toxiques, notamment pour l'homme, trois espèces semblaient prometteuses pour lutter contre la septoriose du blé, *Trichoderma virens*, *Lecanicillium longisporum* et *Emericella rugulosa*. Les surnageants bactériens provenant des souches S1 et S6 de *Bacillus velezensis* se sont révélés particulièrement efficaces *in vitro*, avec des CI_{50} de 1,4% et de 7,4%, respectivement. Les CI_{50} des surnageants bactériens provenant des deux souches de *Paenibacillus polymyxa* n'ont pas pu être calculées en raison d'une quantité trop limitée de filtrats utilisables. En effet, la viscosité de leurs milieux de culture après quelques heures était beaucoup trop élevée. Pour produire des surnageants provenant de ces souches en quantité et qualité convenable, il serait donc recommandé d'optimiser les conditions de culture (milieux, température, pH, etc.). En ce qui concerne les rhamnolipides et molécules associées, seuls six ont présenté une activité antifongique significative *in vitro*. Nous avons aussi retrouvé une CI_{50} et une CMI particulièrement basse pour la mycosubtiline, confirmant les résultats rapportés par Mejri *et al.* (2018) (**Tableau 5**).

Les composés les plus prometteurs au cours de cette étape ont ainsi été sélectionnés pour de plus amples analyses visant à (i) vérifier leur activité de protection *in planta*, (ii) déterminer si certains d'entre eux étaient capables d'induire les défenses du blé vis-à-vis de la septoriose, (iii) comprendre leurs modes d'action et (iv) caractériser leurs composants principaux dans le cas des surnageants bruts. Les méthodologies utilisées, les caractéristiques des biomolécules et extraits ainsi que les principaux résultats obtenus seront présentés et discutés dans les chapitres suivants :

- Dans le **chapitre 1**, nous nous intéresserons aux activités biologiques (antifongique, élicitation des défenses du blé et activité de protection) des rhamnolipides naturels et bio-inspirés et verrons, grâce à l'étude de leur relation structure-activité, l'importance de la longueur de la chaîne carbonée dans leur efficacité.
- Dans le **chapitre 2**, le potentiel du rhamnolipide le plus efficace pour protéger le blé contre *Z. tritici*, le Rhamnose-Ester-C12 (Rh-Est-C12), de stimulation des défenses de l'hôte, sera exploré à l'aide d'une approche combinée de transcriptomique et de métabolomique.
- Dans le **chapitre 3**, l'activité élicitrice et potentialisatrice de, cette fois-ci, la mycosubtiline, sera étudiée à l'aide d'approches transcriptomiques, métabolomiques, mais aussi cytologiques.
- Dans le **chapitre 4**, la caractérisation métabolique des souches S1 et S6 de *Bacillus velezensis*, présentant les plus fortes efficacités directes *in vitro* et dont les surnageants sont aisés à produire, sera abordée en lien avec leurs activités antifongiques.

CHAPITRE 1

Importance de la chaîne C12 dans les activités biologiques des rhamnolipides sur le pathosystème blé-*Zymoseptoria tritici*

Les rhamnolipides sont des métabolites secondaires amphiphiles, produits par plusieurs espèces bactériennes appartenant, notamment, aux genres *Pseudomonas* et *Burkholderia*. Cette famille de glycolipides, généralement considérée comme faiblement toxique et écotoxique, a déjà montré des activités antimicrobiennes significatives vis-à-vis d'autres agents pathogènes ainsi que des activités inductrices de défense chez certains organismes végétaux (Vatsa *et al.*, 2010 ; Crouzet *et al.*, 2020). Leur potentiel en tant que produit de biocontrôle n'avait toutefois jamais été exploré sur le pathosystème blé-*Z. tritici*. Lors du criblage initial, nous avons mis en évidence que seule une partie des rhamnolipides à notre disposition était capable d'inhiber significativement la croissance du champignon pathogène *in vitro*, ceux présentant une chaîne carbonée longue de 10 ou 12 carbones. Afin d'aller plus loin, nous avons décidé de déterminer les activités biologiques (antifongique, élévation des défenses du blé et activité de protection) de ces composés et d'étudier ainsi leur relation structure-activité. Les résultats obtenus sont présentés et discutés dans l'article suivant. Brièvement, nous avons pu montrer que les rhamnolipides présentant des liaisons éthers ou esters et avec des chaînes carbonées à 12 carbones étaient globalement les plus efficaces pour toutes les activités biologiques étudiées. Aussi, nous avons mis en évidence que le groupement rhamnose était essentiel pour l'activité de ces composés, notamment de protection. Le Rhamnose-Ester-C12 (Rh-Est-C12) s'est révélé être le composé le plus prometteur parmi de tous ceux dont nous disposions et des analyses supplémentaires ont été réalisées afin d'explorer plus en profondeur son potentiel pour protéger le blé vis-à-vis de *Z. tritici*, notamment sa capacité à inhiber la croissance de souches de l'agent pathogène hautement résistantes (*multi-drug resistant*, MDR) aux fongicides de synthèse de la famille des inhibiteurs de la déméthylation (DMI).

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Article

Importance of the C12 Carbon Chain in the Biological Activity of Rhamnolipids Conferring Protection in Wheat against *Zymoseptoria tritici*

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Abstract: The hemibiotrophic fungus *Zymoseptoria tritici*, responsible for Septoria tritici blotch, is currently the most devastating foliar disease on wheat crops worldwide. Here, we explored, for the first time, the ability of rhamnolipids (RLs) to control this pathogen, using a total of 19 RLs, including a natural RL mixture produced by *Pseudomonas aeruginosa* and 18 bioinspired RLs synthesized using green chemistry, as well as two related compounds (lauric acid and dodecanol). These compounds were assessed for *in vitro* antifungal effect, *in planta* defence elicitation (peroxidase and catalase enzyme activities), and protection efficacy on the wheat-*Z. tritici* pathosystem. Interestingly, a structure-activity relationship analysis revealed that synthetic RLs with a 12 carbon fatty acid tail were the most effective for all examined biological activities. This highlights the importance of the C12 chain in the bioactivity of RLs, likely by acting on the plasma membranes of both wheat and *Z. tritici* cells. The efficacy of the most active compound Rh-Est-C12 was 20-fold lower in *planta* than *in vitro*; an optimization of the formulation is thus required to increase its effectiveness. No *Z. tritici* strain-dependent activity was scored for Rh-Est-C12 that exhibited similar antifungal activity levels towards strains differing in their resistance patterns to demethylation inhibitor fungicides, including multi-drug resistance strains. This study reports new insights into the use of bio-inspired RLs to control *Z. tritici*.

Keywords: Wheat, *Zymoseptoria tritici*, rhamnolipids, elicitors, structure-activity relationship

1. Introduction

Wheat is one of the most produced and consumed crops worldwide along with maize and rice. For the 2018-2019 period, the Food and Agriculture Organisation estimated that global wheat production

reached 732.1 million tons [1]. This crop is cultivated in many different geoclimatic areas and ecosystems, and it is vastly used for human food as well as livestock feed. Hence, ensuring a safe and sufficient production of this cereal is a vital matter. Although wheat is susceptible to various pathogenic agents, Septoria tritici blotch (STB), caused by the hemibiotrophic fungus *Zymoseptoria tritici*, is considered as the most impacting foliar disease on wheat and is of major concern for wheat production worldwide. Currently, strategies to control this phytopathogen rely mainly on the application of conventional fungicides and, to a lower extent, on the use of partially resistant cultivars. In Western Europe, where the climate is particularly conducive for fungal development, STB is of critical importance, both agronomically and economically, leading to yield losses up to 50%. Thus, 70% of fungicides applied in the European Union are used for the control of this disease, for a cost of approximately 1.2 billion dollars per year [2,3].

The main conventional fungicides used currently for STB control are (i) succinate dehydrogenase inhibitors (SDHIs), carboxamide molecules inhibiting succinate dehydrogenase of the fungal mitochondrial respiratory chain, and (ii) demethylation inhibitor (DMI) fungicides, which target the 14 α -demethylase CYP51 protein involved in the biosynthesis of ergosterol, a major lipid compound of fungal plasma membrane. Although the decreasing sensitivity in *Z. tritici* populations to SDHIs in the field is still being contained, reports regarding the resistance of this fungus towards DMIs have increased in the recent years. Indeed, serious breakdowns in azole fungicide efficacy appeared, firstly in Europe and then all over the world [4-6]. Concerning DMI resistance, three mechanisms of resistance have been described: (i) alteration of the CYP51 protein due to mutations in the corresponding gene, causing a decrease of affinity between the fungicide and its target, the protein CYP51, (ii) overexpression of the CYP51 gene, and (iii) enhanced efflux activity in fungal cells due to the overexpression of genes coding for membrane transporters, leading to populations exhibiting multi-drug resistance (MDR) phenotypes [4]. Because of this fungicide resistance issue and the increasing societal demand to implement a more sustainable agriculture, there is a strong need to develop alternatives to control STB in the field.

In the last decades, many studies have highlighted the potential of green surfactants in agriculture. Biosurfactants are surface-active compounds produced by a wide range of microorganisms. Even though they may be chemically very diverse, they are all amphiphilic molecules, consisting of hydrophobic and hydrophilic moieties. They may be used in agriculture for different applications like bioremediation, stimulating interactions between plants and beneficial microorganisms, as well as in crop protection by acting as biofungicides or/and as plant resistance inducers [7-9]. Rhamnolipids (RLs) are secondary metabolites with biosurfactant properties, naturally produced by bacteria belonging to the genera *Pseudomonas* sp. and *Burkholderia* sp. They are naturally biosynthesized as a mixture of compounds with one or two rhamnose residues (mono- or di-RLs), forming a polar hydrophilic head and linked through a beta-glycosylic bond to one or two 3-hydroxy fatty acids, as hydrophobic tails. Rhamnolipids are one of the most intensively studied classes of surfactant glycolipids. Like other biosurfactants, they may potentially be used in a broad range of areas, including medicine, cosmetics, food processing, petroleum industries, and agriculture [10-12]. In crop protection, previous studies reported the antimicrobial properties of natural RLs, as well as their capacity to induce plant resistance mechanisms, as reviewed by Vatsa et al. [13]. For instance, they were demonstrated to inhibit the growth of various phytopathogenic fungi, such as *Botrytis cinerea* and *Fusarium solani*, and to control oomycete phytopathogens, such as *Phytophthora* sp., by lysing zoospores [14-17]. Natural RLs are also able to protect plants, such as grapevine and *Brassica napus*, against *B. cinerea*, by triggering defense responses, such as ROS production [17,18]. Natural RLs were also reported to induce defense mechanisms in *Arabidopsis thaliana* against three phytopathogens, including *Pseudomonas syringae* pv. *tomato*, *Hyaloperonospora arabidopsidis*, and *B. cinerea* [19]. RLs are considered as easily biodegradable and exhibit low toxicity and ecotoxicity, making them promising compounds for plant protection [20,21].

Nevertheless, even though RLs are known to be synthesized by various bacterial species, they are still mostly produced using *Pseudomonas aeruginosa*, a species that cannot be used as biocontrol agent

because it is an opportunistic human pathogen. In addition, large-scale industrial production is difficult because of low yields obtained with *P. aeruginosa*. To overcome these issues, three methods may be proposed, such as (i) the use of non-pathogenic natural RLs-producing bacteria such as *Burkholderia* sp. [22], (ii) their heterologous synthesis with genetically modified microorganisms generally recognized as safe, such as *Saccharomyces cerevisiae* [23], or (iii) their synthesis using green chemistry. Recently, synthetic environmentally-friendly RLs produced by green chemistry were found to be able to trigger ROS production in *A. thaliana*, showing a promising potential as plant resistance inducers [24]. Moreover, Robineau et al. [25] demonstrated that synthetic mono-RLs confer protection to tomato against *B. cinerea* by both direct antifungal activity and host defense mechanism activation.

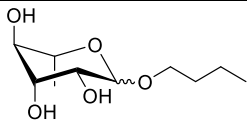
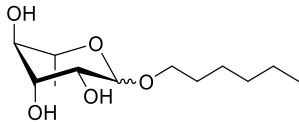
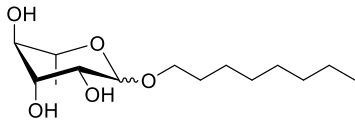
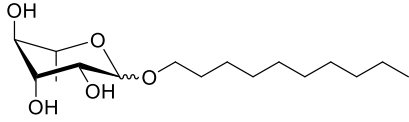
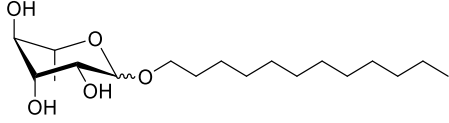
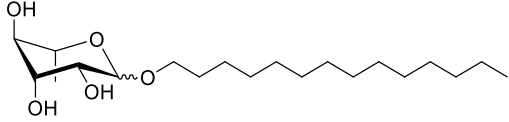
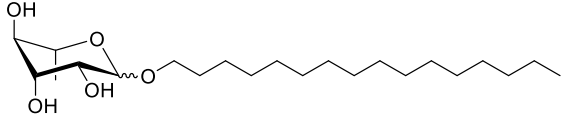
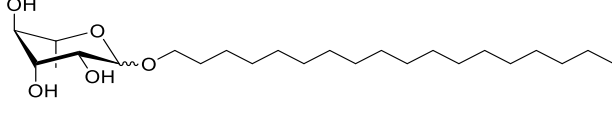
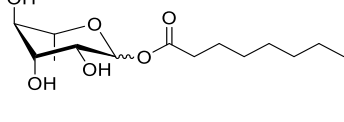
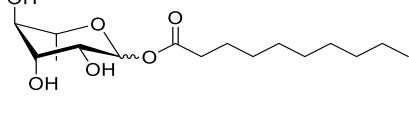
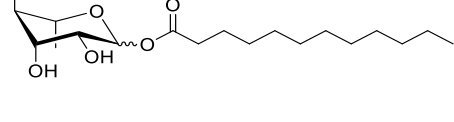
So far, biological activity of RLs has never been explored in the wheat-*Z. tritici* pathosystem. The objective of the present study was thus to assess the direct antifungal activity, the plant eliciting effect, as well as the protection efficacy of 19 RLs (either naturals, produced by *P. aeruginosa*, or synthetic (bio-inspired) obtained by green chemistry), as well as lauric acid and dodecanol (C12-carbon chain molecules) on the wheat-*Z. tritici* model. A structure-activity relationship analysis of these compounds was performed in order to highlight the chemical radicals responsible for the RL activities. Moreover, the efficacy of the most active RL was compared between *in vitro* (direct antifungal activity) and *in planta* (disease symptom reduction) conditions. Finally, a possible strain-dependant effect of the RL activity was investigated by examining the *in vitro* antifungal activity of the most active RL toward 21 *Z. tritici* strains that differ in their resistance level to DMI fungicides.

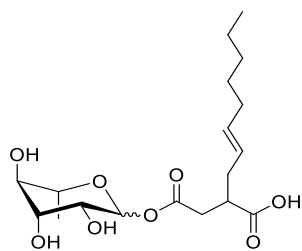
2. Results

2.1. Chemical description of the synthesized RLs

A total of 18 RLs were obtained using green chemistry to investigate their biological activities on the wheat-*Z. tritici* pathosystem (Table 1). Three series of rhamnose derivatives, differing by the function linking the hydrophobic chain (ether, ester, or succinates), were successfully synthesized. A first series of ether rhamnose derivatives (Rh-Eth), synthesized without using solvent from the L-rhamnose and a fatty alcohol with various chain lengths (4 to 18 carbon atoms), was produced (Table 1). Then, a second series of RLs, with an ester link (Rh-Est), was obtained (8 to 12 carbons). Finally, succinate rhamnose derivatives (Rh-Suc), including mono-rhamnosyl (alkenyl)succinate (Rh-Suc-C8, Rh-Suc-C12, and Rh-Suc-C12b) and mono-rhamnosyl (alkenyl)alkylsuccinate (Rh-Suc-C8-C8, Rh-Suc-C8-C12, Rh-Suc-C12-C8, and Rh-Suc-C12-C12) derivatives, were generated (Table 1). Among the synthesized RLs, nine of them (Rh-Eth-C4, Rh-Eth-C6, Rh-Eth-C8, Rh-Eth-C10, Rh-Eth-C12, Rh-Eth-C14, Rh-Eth-C16, Rh-Est-C12, and Rh-Suc-C12) have already been reported [25], while the nine other RLs, belonging for a large part to the new RL family mono-rhamnosyl (alkenyl)alkylsuccinate, were obtained for the first time (Rh-Eth-C18, Rh-Est-C8, Rh-Est-C10, Rh-Suc-C8, Rh-Suc-C12b, Rh-Suc-C8-C8, Rh-Suc-C8-C12, Rh-Suc-C12-C8, Rh-Suc-C12-C12). For mono-rhamnosyl (alkenyl)alkylsuccinate RLs, they were obtained after esterification of L-rhamnose with octenylsuccinic or dodecenylsuccinic anhydride in *N,N*-dimethylformamide (DMF) in presence of *N,N*-dimethylpyridin-4-amine (DMAP), followed by a second esterification by using alkyl bromide. Then, by combining the use of an alkylated succinic anhydride with 8 or 12 carbon atoms and an octanoic or lauric acid chloride during the synthesis from rhamnose, four new derivatives of rhamnose (Rh-Suc-C8-C8, Rh-Suc-C8-C12, Rh-Suc-C12-C8 and Rh-Suc-C12-C12), with a yield comprised between of 34 and 39%, were successfully developed.

Table 1. List, structure and characteristics of the eighteen synthesized RLs as well as lauric acid, dodecanol and natural RLs.

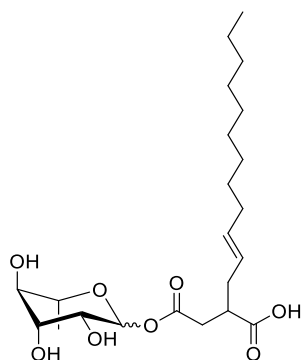
Scheme	Compound	Name	Molecular weight (g.mol ⁻¹)
	Rh-Eth-C4	Butyl α/β -L-rhamnopyranoside	220
	Rh-Eth-C6	Hexyl α/β -L-rhamnopyranoside	248
	Rh-Eth-C8	Octyl α/β -L-rhamnopyranoside	276
	Rh-Eth-C10	Decyl α/β -L-rhamnopyranoside	304
	Rh-Eth-C12	Dodecyl α/β -L-rhamnopyranoside	332
	Rh-Eth-C14	Tetradecyl α/β -L-rhamnopyranoside	360
	Rh-Eth-C16	Hexadecyl α/β -L-rhamnopyranoside	388
	Rh-Eth-C18*	Octadecyl α/β -L-rhamnopyranoside	416
	Rh-Est-C8*	Octanoyl α/β -L-rhamnopyranoside	290
	Rh-Est-C10*	Decanoyl α/β -L-rhamnopyranoside	318
	Rh-Est-C12	Dodecanoyl α/β -L-rhamnopyranoside	346

α/β -L-rhamnopyranoside


Rh-Suc-C8*

 Octenylsuccinate α/β -L-rhamnopyranoside

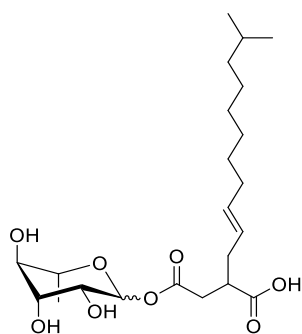
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Rh-Suc-C12

 Dodecenylsuccinate α/β -L-rhamnopyranoside

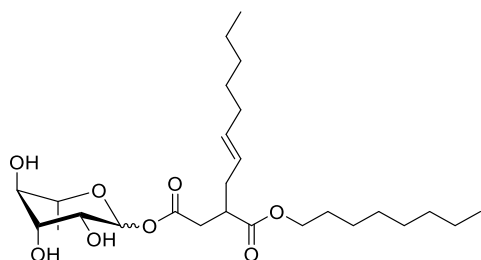
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Rh-Suc-C12b*

 Iso-Dodecenylsuccinate α/β -L-rhamnopyranoside

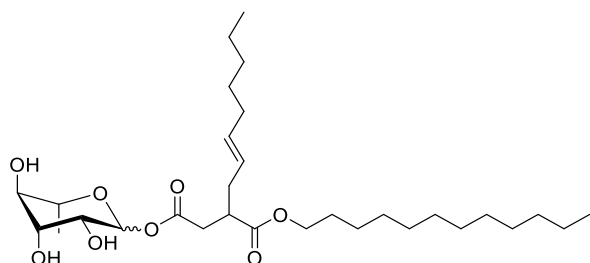
430



Rh-Suc-C8-C8*

 3-Octenyl octylsuccinate α/β -L-rhamnopyranoside

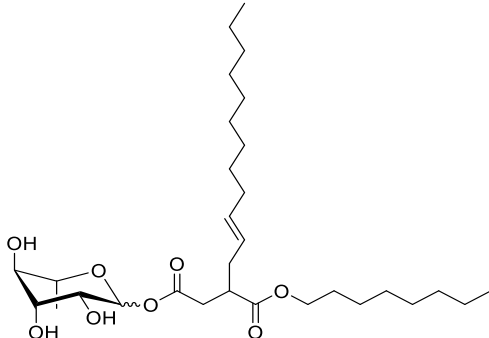
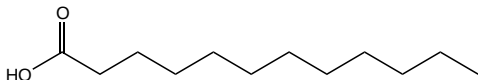
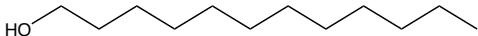
486



Rh-Suc-C8-C12*

 3-Octenyl dodecylsuccinate α/β -L-rhamnopyranoside

542

	Rh-Suc-C12-C8*	3-Dodecenyloctylsuccinate α/β -L-rhamnopyranoside	542
	Rh-Suc-C12-C12*	3-Dodecenyldodecylsuccinate α/β -L-rhamnopyranoside	598
	Lauric acid	Lauric acid	200
	Dodecanol	Dodecanol	186
Mixture of rhamnolipids Agae Technologies LLC	(data from Nat-Rh	Bacterial rhamnolipids (see “Materials and methods”)	578

* Molecules newly synthesized in this study, while the other molecules were already reported in Robineau et al. [25].

2.2. Biological activities of RLs differ according to the length of their carbon chain

A total of 21 molecules, including 18 synthesized RLs and three related molecules (a mixture of natural RLs, lauric acid, and dodecanol) were examined for both their direct (antifungal) and indirect (plant defence elicitation) activities, as well as for their protection efficacy in the greenhouse, on the wheat-*Z. tritici* pathosystem. Regarding the *in vitro* antifungal activity, among all tested RLs, from C4 to C18-derived molecules, only Rh-Eth-C10, Rh-Eth-C12, and Rh-Est-C12 drastically inhibited fungal growth (Table 2, Figure 1). Succinate-RLs did not significantly reduce fungal growth, except for Rh-Suc-C8, which showed an inhibitory effect only at the highest tested concentration of 1500 μ M (Table 2). Concerning the ester-RL family, Rh-Est-C10 had no antifungal activity, while Rh-Est-C12 was found to be the most active molecule among all tested RLs (Figure 1). On the other hand, lauric acid and dodecanol displayed a marked *in vitro* antifungal effect against *Z. tritici*, with IC_{50} and MIC values close to those of the active RLs. Natural RLs did not show any direct antimicrobial effect.

Concerning the ability of the molecules to elicit plant defenses, activity of two plant enzymes involved in ROS scavenging, catalase (CAT) and peroxidase (POX), was investigated two days after treatment. Only three molecules significantly modified at least one enzymatic activity (Table 2). Rh-Eth-

C12 increased CAT activity by 1.86 fold compared to the control, whereas Rh-Suc-C12b enhanced POX activity by 2.26 fold compared to the control. Rh-Suc-C12, whose chemical structure is close to Rh-Suc-C12b, did not exhibit any induction of the targeted enzymes in our conditions. Finally, Rh-Est-C12 induced both CAT activity and POX activities by 1.71 and 2.27 folds, respectively, when compared to the control. Lauric acid and dodecanol did not cause any significant change in the investigated enzyme activities.

None of the tested molecule displayed *in planta* phytotoxicity at the tested concentration 1.5 mM. Five tested RLs caused a significant reduction in disease symptoms (Table 2). Among them, the four molecules Rh-Eth-C12, Rh-Suc-C12b, Rh-Eth-C10, and Natural-Rh conferred only a slight protection effect, with a decrease in disease severity ranging from 15.5 to 17.4%. On the other hand, disease severity reduction obtained with Rh-Est-C12 treatment was significantly different from all other treatments and exhibited the highest activity, with 35.6% of disease symptom reduction (approximately 2 fold higher than the protection efficacies conferred by the other active RLs). On the contrary to RLs with C12-carbon chain, lauric acid and dodecanol did not display any significant protection activity.

Table 2. Biological activities, including *in vitro* direct antifungal activity, *in planta* defense activation and *in planta* protection efficacy of the eighteen synthesized RLs, as well as lauric acid, dodecanol and natural RLs, at 1.5 mM, on the wheat-*Zymoseptoria tritici* pathosystem.

Molecule	<i>In vitro</i> antifungal activity		<i>In planta</i> defense elicitation activity		<i>In planta</i> protection efficacy
	IC ₅₀	MIC	Catalase activity	Peroxidase activity	Leaf diseased area (%)•
	(μM)	(μM)	(U.min ⁻¹ .g ⁻¹ proteins)	(U.min ⁻¹ .g ⁻¹ proteins)	
Control	-	-	83.4 ^{ab}	51.7 ^{bc}	55.7 ^a (0%)
Natural-Rh	>1500	>1500	71.9 ^a	53.4 ^c	46.3^b (-17.0%)
Rh-Eth-C4	>1500	>1500	110.4 ^{abcd}	64.8 ^{bc}	51.4 ^{ab} (-7.8%)
Rh-Eth-C6	>1500	>1500	111.7 ^{abcd}	55.9 ^{bc}	54.4 ^{ab} (-2.3%)
Rh-Eth-C8	>1500	>1500	121.1 ^{abcd}	74.2 ^{abc}	54.3 ^{ab} (-2.6%)
Rh-Eth-C10	158	200	131.6 ^{bcd}	54.2 ^{bc}	46.0^b (-17.4%)
Rh-Eth-C12	81	450	155.2^d	71.0 ^{abc}	46.2^b (-15.5%)
Rh-Eth-C14	>1500	>1500	99.2 ^{abcd}	71.2 ^{abc}	49.4 ^{ab} (-11.3%)
Rh-Eth-C16	>1500	>1500	130.9 ^{bcd}	67.7 ^{bc}	50.3 ^{ab} (-9.7%)
Rh-Eth-C18	>1500	>1500	Nd*	Nd*	Nd*
Rh-Est-C8	>1500	>1500	112.9 ^{abcd}	44.9 ^c	50.6 ^{ab} (-9.1%)
Rh-Est-C10	>1500	>1500	98.4 ^{abcd}	58.2 ^{bc}	50.6 ^{ab} (-9.3%)
Rh-Est-C12	75	133	142.5^{cd}	117.3^a	35.9^c (-35.6%)
Rh-Suc-C8	1399	1500	123.3 ^{abcd}	60.3 ^{bc}	54.1 ^{ab} (-3.0%)
Rh-Suc-C12	>1500	>1500	124.9 ^{abcd}	76.6 ^{abc}	50.3 ^{ab} (-9.7%)
Rh-Suc-C12b	>1500	>1500	96.8 ^{abc}	116.9^a	46.2^b (-16.2%)
Rh-Suc-C8-C8	>1500	>1500	102.4 ^{abcd}	62.1 ^{bc}	52.4 ^{ab} (-6.0%)
Rh-Suc-C8-C12	>1500	>1500	113.7 ^{abcd}	96.9 ^{ab}	51.2 ^{ab} (-8.1%)
Rh-Suc-C12-C8	>1500	>1500	120.1 ^{abcd}	79.9 ^{abc}	55.8 ^a (+0.1%)
Rh-Suc-C12-C12	>1500	>1500	Nd*	Nd*	Nd*
Lauric acid	109	200	94.0 ^{abc}	76.8 ^{abc}	50.9 ^{ab} (-8.7%)
Dodecanol	164	200	107.4 ^{abcd}	51.1 ^{bc}	54.4 ^{ab} (-2.4%)

(*) No data were obtained for two RLs because of solubilization issues. Different letters indicate significant differences between treatments according to ANOVA followed by Tukey's test at $P \leq 0.05$. Values in bold indicate significant effects. (•) Values between brackets stand for the percentage of disease symptom reduction compared to the control condition.

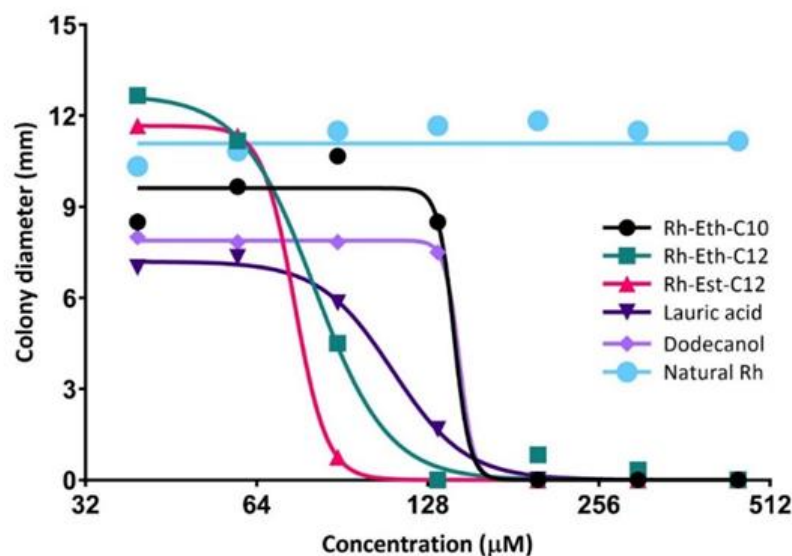


Figure 1. *In vitro* dose-response curves displaying the antifungal effect of the three most efficient RLs, as well as dodecanol and lauric acid, against *Zymoseptoria tritici* (strain T02596) compared to the natural RLs.

2.3. Correlations between *in vitro* antifungal activity, in planta defence elicitation and protection efficacy

A correlative analysis was performed to determine whether *in vitro* antifungal and elicitation activities were relevant to explain disease symptom reduction caused by the treatments. The statistical analyses revealed significant positive correlations between both IC_{50} and MIC and the disease severity reduction ($r=0.46$) (Table 3). However, no significant correlation was found between CAT activity and protection efficacy, while POX activity and disease symptoms were significantly and negatively correlated ($r=-0.51$).

Table 3. Correlations between protection efficacy and both direct (IC_{50} and MIC) and indirect (plant catalase and peroxidase activities) effects of the tested RLs, as well as dodecanol and lauric acid, obtained on the wheat-*Zymoseptoria tritici* pathosystem.

	IC_{50}	MIC	Catalase activity	Peroxidase activity
IC_{50}	-	-	-	-
MIC	0.99	-	-	-
Catalase activity	-0.40	-0.35	-	-
Peroxidase activity	-0.10	-0.10	0.19	-
Leaf diseased area	0.46	0.46	-0.25	-0.51

Significant values according to the Pearson test at $P \leq 0.05$ are indicated in bold

2.4. Comparison of *in vitro* versus *in planta* antifungal effects of Rh-Est-C12

According to the structure-activity relationship study above, Rh-Est-C12 appeared to be the most active RL for protection efficacy, *in vitro* antifungal effect, and elicitation activity. Hence, we focused on this molecule in order to compare its direct activity *in vitro* and *in planta*. *In planta*, the protective effect of foliar treatment with Rh-Est-C12 on wheat against *Z. tritici* was assessed at different concentrations. There was no significant protection with applications of Rh-Est-C12 at 50, 100, 200, and 400 μ M of the RL. However, Rh-Est-C12 treatments with 800, 1600, and 3200 μ M significantly reduced disease

symptoms on wheat leaves, with protection rates of 19.9%, 53.3%, and 78.9% respectively (Figure 2). No phytotoxicity was observed at any of the tested concentrations. The *in planta* IC₅₀ value of this molecule (the concentration of Rh-Est-C12 reducing by half the disease symptom level compared to the non-treated control) was calculated and was determined as 1510 μ M. Rh-Est-C12 exhibited an *in planta* IC₅₀ value approximatively 20-fold higher than the IC₅₀ value obtained *in vitro* for the molecule, revealing a major difference in the activity of the molecule between the two conditions.

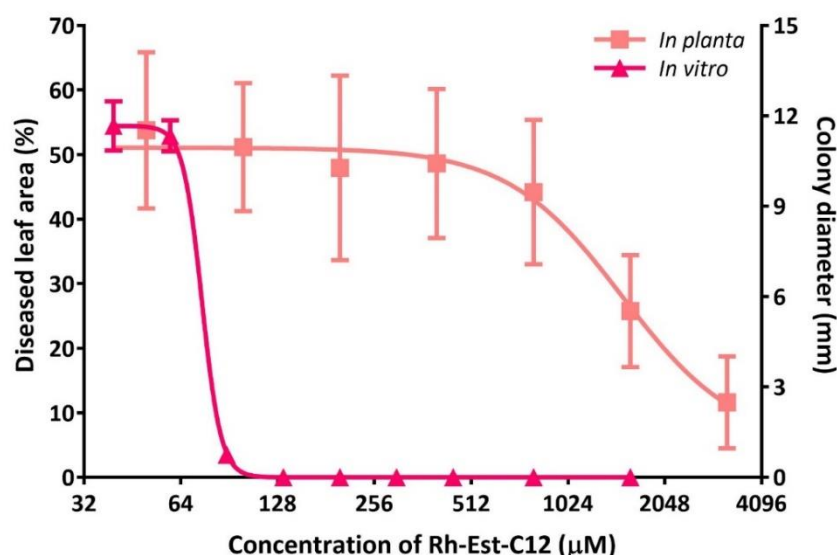


Figure 2. Comparison of *in planta* and *in vitro* dose-response curves of Rh-Est-C12 on the wheat-*Zymoseptoria tritici* pathosystem. Wheat cultivar Alixan and *Z. tritici* strain T02596 were used. The *in planta* activity of the compound was assessed by measuring the percentage of chlorosis and necrosis on the third leaves of each plant at 21 days after inoculation. Bars stand for standard deviations.

Additionally, the effect of Rh-Est-C12 on *in planta* spore germination and fungal hyphal growth was assessed at three different concentrations (50, 400, and 3200 μ M) (Figure 3). Whereas no significant activity was observed after application of Rh-Est-C12 at 50 and 400 μ M, a strong antifungal effect was highlighted for this compound at 3200 μ M. The proportion of non-germinated spores in the treated plants was 2-fold higher than in the control. Moreover, a reduction by 4-fold of the class 4 spore proportion (germinated spores with a strongly developed germ tube) was also scored in the plants treated with 3200 μ M Rh-Est-C12 compared to the control (Figure 3). Again, the *in planta* direct activity of Rh-Est-C12 on hyphal growth appeared weaker than its direct antifungal effect obtained *in vitro* on fungal colony development.

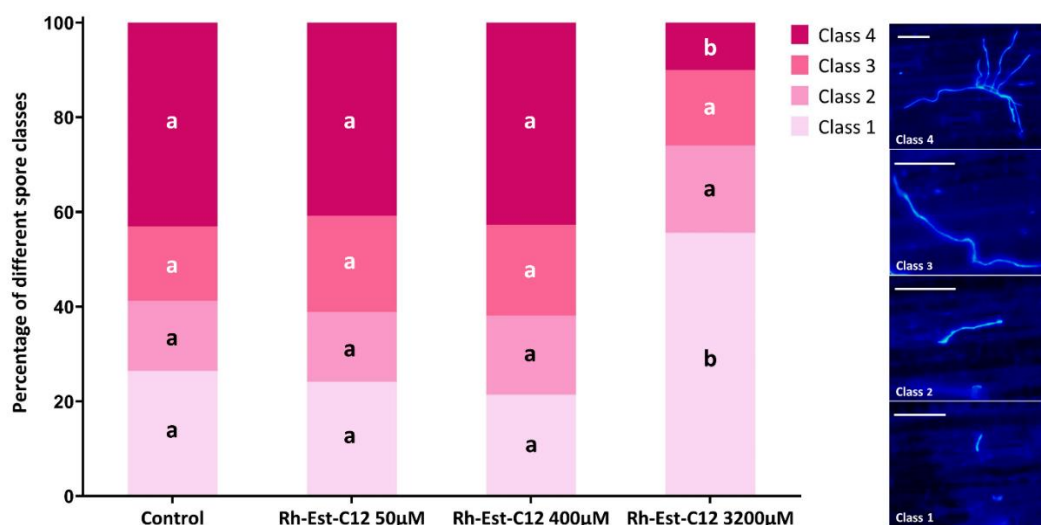


Figure 3. *In planta* spore germination and hyphal growth of *Zymoseptoria tritici* on wheat leaves treated or not with Rh-Est-C12. Wheat cultivar Alixan and *Z. tritici* strain T02596 were used. Percentage of four different classes of Calcofluor-stained spores was assessed three days after inoculation on wheat third leaves. The right panel shows a representative picture of each spore class (scale bar = 25  m). Class 1: non germinated spore; Class 2: germinated spore with small germ tube; Class 3: germinated spore with developed germ tube; Class 4: germinated spore with a strongly developed germ tube. For each condition, 100 different spores from six different leaves were assessed. Within each class, different letters indicate significant differences between treatments according to ANOVA followed by the Tukey’s test at $P \leq 0.05$.

2.5. Direct *in vitro* antifungal activity of Rh-Est-C12 does not vary depending on *Z. tritici* strains

To examine any possible strain effect of Rh-Est-C12 direct *in vitro* antifungal activity, 21 different *Z. tritici* strains showing different resistance levels towards DMI fungicides, were investigated for their resistance level to this RL. Overall, no major difference were observed between all tested strains regarding the IC_{50} values of Rh-Est-C12 (Figure 4A, Supplementary Tables 1 and 2). Resistance factors (ratio between the IC_{50} value of a given strain and the IC_{50} value of the sensitive reference strain IPO323) ranged from 0.7 to 1.2 for Rh-Est-C12, indicating no strain effect on the direct activity of the molecule. By contrast, a strong strain effect was highlighted for the five tested DMI fungicides, with resistance factors ranging from 3.8 to 1071.4 for tebuconazole, from 1.0 to 150.8 for metconazole, from 28.6 to 501.8 for epoxiconazole, 37.4 to 74753.2 for prothioconazole, and from 5.1 to 266.2 for prochloraz (Figure 4A, Supplementary Tables 1 and 2). Rh-Est-C12 displayed similar antifungal activity levels on both (i) non-MDR and non *Cyp51*-overexpressing strains, and (ii) the highly resistant strains with MDR character or overexpressing the *Cyp51* gene (Figure 4B, Supplementary Tables 1 and 2).

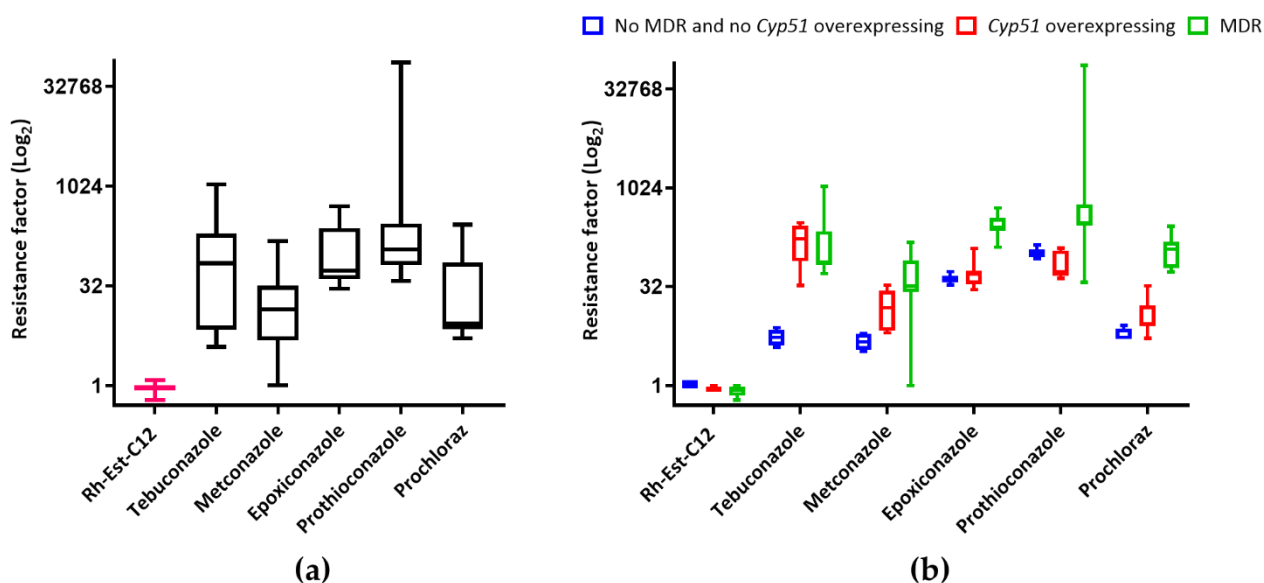


Figure 4. Boxplots showing the resistance factors obtained for 21 different *Zymoseptoria tritici* strains analyzed together (a) or distinguished according to their DMI resistance profiles (seven MDR strains, seven strains overexpressing the *Cyp51* gene, and seven strains without these resistance mechanisms) (b), against five DMI fungicides as well as the RL molecule Rh-Est-C12. Resistance factors correspond to the ratio between the IC₅₀ value of a given strain and the IC₅₀ value of the sensitive reference strain IPO323.

3. Discussion

We explored here, for the first time, the potential of RLs to protect wheat against *Z. tritici*, a major pathogen on wheat crops, using a panel of 21 molecules, including 18 bioinspired RLs synthesized using green chemistry and three related molecules (a mixture of natural RLs from *P. aeruginosa*, lauric acid, and dodecanol). Natural bacterial RLs have already been reported to show antimicrobial activity against a wide range of plant pathogenic fungi [13]. For instance, Varnier et al. [17] reported a significant antigerminative effect of 0.1 mg.mL⁻¹ of bacterial RL against the ascomycete polyphagous fungus *B. cinerea*. Moreover, natural RLs were found particularly effective against certain oomycetes, such as *Phytophthora* sp., by lysing zoospores, hypothetically by intercalating into oomycete cell membranes, leading to their disorganization [14]. However, in our conditions, and even with concentrations up to 0.87 mg.mL⁻¹ (*i.e.* 1500 μ M) no activity was observed for the tested mixture of natural RLs. Among all tested compounds, only a minority exhibited significant *in vitro* antifungal activity. The structure-activity relationship analysis revealed that bioinspired RLs with 4, 6, 8, 14, 16, and 18 carbon fatty acid tails did not display any significant direct effect. Similarly, succinate-derived molecules were also unable to inhibit fungal *in vitro* growth, except Rh-Suc-C8 which exhibited a very slight effect. The most effective RLs were ether and ester-derived compounds, namely Rh-Eth-C10, Rh-Eth-C12, and Rh-Est-C12. Notably, Robineau et al. [25] also described the antifungal activity of these three compounds against *B. cinerea*. However, they observed a more pronounced effect with Rh-Eth-C10. In the present work, Rh-Eth-C10 was not as effective as Rh-Est-C12 that displayed the lowest IC₅₀ among all the tested active compounds (158 and 75 μ M, corresponding to 0.048 and 0.026 mg.mL⁻¹, for Rh-Eth-C10 and Rh-Est-C12, respectively) (Table 1). Moreover, Rh-Est-C10 did not exhibit any inhibition of fungal growth. Hence, we conclude that 12 carbon is the optimum fatty acid tail length for both ether and ester-RLs regarding their direct activity against *Z. tritici*. Thereafter, we assessed the antifungal potential of chemically simple molecules with 12 carbon chain, lauric acid and dodecanol. Interestingly, these two molecules exhibited a significant direct effect, but their IC₅₀ are higher than those of Rh-Est-C12 and Rh-Eth-C12 (Table 2). These results confirm the importance of the 12-carbon chain in the direct activity of RLs towards *Z. tritici*.

We also investigated the elicitor potential of RLs on non-infected wheat using two key enzyme biomarkers of plant defence. Only three molecules significantly enhanced the activity of at least one out of the two targeted plant defense-related enzymes CAT and POX. Rh-Eth-C12 was able to stimulate CAT activity, Rh-Suc-C12b induced POX activity, and Rh-Est-C12 increased both (Table 2). These enzymes are involved in many plant defense responses including ROS scavenging, plant signaling, and hypersensitive response [26,27]. These results suggest an increase of ROS production in wheat plants treated with these three compounds. Since large amounts of ROS in plant tissues can be harmful to the plant, wheat plants likely responded by increasing the activity of ROS scavenging enzymes. Interestingly, natural and synthetic RLs were reported to confer resistance and trigger oxidative burst in other plants such as *A. thaliana* and grapevine [17,24]. Robineau et al. [25] also described a significant increase of ROS production in *A. thaliana* leaf disks treated with Rh-Eth-C12 and Rh-Est-C12. Accumulation of H₂O₂ in wheat leaves during infection with *Z. tritici* was reported to increase plant resistance against the pathogen [28]. While the role of CAT in wheat defense against *Z. tritici* is still unclear, Shetty and colleagues [29] reported an involvement of POX in wheat innate immunity. The authors observed a significant induction of this enzyme in resistant cultivars when infected with *Z. tritici*, compared to susceptible cultivars. This may be explained by the specific roles of POX, such as the reinforcement of cell walls and deposition of polyphenolics [30]. This link between POX and innate resistance of wheat to *Z. tritici* is supported by the positive correlation scored between the induction of POX enzymatic activity and plant protection, *i.e.* negative correlation between POX activity and disease symptoms (Table 3).

As for the *in vitro* direct antifungal activity, interestingly, our results revealed that RLs with C12 fatty acid chain were the most effective regarding the induction of the targeted wheat defense biomarkers (CAT and POX). This dual activity on both the host (wheat) and the pathogen (*Z. tritici*) is more likely due to RL interaction with biological plasma membranes of both organisms. Indeed, Monnier and colleagues [31] demonstrated, using *in silico* models, that RLs can act on both plant and fungal membranes by insertion within the lipid bilayers, near the lipid phosphate group of the phospholipid bilayers, nearby phospholipid glycerol backbones. Even though RLs fit into both membranes, RLs seem to differently affect their fluidity. Indeed, the authors suggested that, in the plant membrane, RLs did not significantly impact lipid dynamics inside the lipid bilayer, whereas, in the fungal membrane, a clear fluidity increase was observed, due probably to the presence of ergosterol. These subtle changes in lipid dynamics could explain why the effect of RLs is less drastic on the plant (only triggering defense reactions) than on the fungus that leads to a strong membrane destabilization causing cell death [31]. The importance of the RL carbon chain in plant defense activation has already been reported in previous studies. For instance, Luzuriaga-Loaiza et al. [24] highlighted different results with synthetic RL bolaforms (SRBs) in *A. thaliana*, where SRBs with 14-carbon fatty acid tails were found to be the most effective to induce ROS production in the plant petioles. Regarding other surfactant agents, it has been shown that for surfactin, a lipopeptide produced by *Bacillus subtilis*, the molecules with chain length of 14 or 15 carbons were found more active than surfactin with 12 carbons to enhance production of H₂O₂ in tobacco cells [32]. On the other hand, we found that lauric acid and dodecanol were not able to elicit POX- and CAT-based defense reactions, suggesting a major role of the sugar head in plant defense induction. Although further investigations are needed to decipher the modes of action of RLs on wheat defense induction, such a result suggests the major importance of both rhamnose head and fatty acid tail in the biological activities of RLs on this plant model. Moreover, the precise structure of the RL carbon chain is also crucial, since we observed a significant enhancement of POX activity when using Rh-Suc-C12b compared to its isoform Rh-Suc-C12, which showed no elicitation activity in the treated wheat plants.

Our results revealed that Rh-Eth-C10, Rh-Eth-C12, Rh-Est-C12, Rh-Suc-C12b, and natural RLs are able to reduce *Z. tritici* disease severity on wheat plants. Overall, four categories of protective RLs were observed: (i) protective RLs exhibiting only antifungal activity (Rh-Eth-C10); (ii) protective RLs displaying only elicitation effect (Rh-Suc-C12b); (iii) protective RLs with both activities (Rh-Eth-C12 and Rh-Est-C12), and (iv) protective RLs displaying neither direct nor indirect effects (Natural-Rh), implying

that this latter compound could induce plant defenses through mechanisms not involving our two targeted enzymatic biomarkers. Statistical analyses revealed a positive correlation between POX activity and protection efficacy, as well as between *in vitro* direct activity and protection efficacy. Surprisingly, even though lauric acid and dodecanol were able to inhibit fungal growth *in vitro* with IC₅₀ values closes to those of protective RLs, they were unable to protect wheat against the disease, highlighting again the role of rhamnose head in RL protective activity. As discussed above, our structure-activity relationship study reveals the predominant importance of 12 carbon chain in RL biological activities in the wheat-*Z. tritici* model. Likewise, Robineau et al. [25] also described the important role of 12 carbon fatty acid tails in the bioactivity of RLs on the *A. thaliana*-*B. cinerea* pathosystem.

The mono-RL Rh-Est-C12 was found to be the most effective RL to protect wheat against *Z. tritici*, displaying a reduction of disease symptoms up to 78.9% when applied at 1.1 mg.mL⁻¹ (*i.e.* 3200 µM). This product is able to act dually, by inhibiting fungal growth and eliciting plant defenses by enhancing ROS scavenging enzyme activities. Nevertheless, a significant gap between the *in vitro* and *in planta* efficacies of this molecule was highlighted. A ratio of approximately 20-fold separates the *in vitro* IC₅₀ value of Rh-Est-C12 and the needed concentration to reduce disease symptoms by 50% *in planta*. This difference of activity may be explained by several factors, including the adherence level of Rh-Est-C12 on wheat leaves during spraying, biodegradability as well as photodecomposition, and penetration properties of the molecule in wheat leaves. Further studies on formulation may increase and optimize the *in planta* efficacy of Rh-Est-C12. Another explanation of the gap between *in vitro* and *in planta* activities of Rh-Est-C12 is the difference between the physiological state of the fungus *in vitro* and *in planta*. Indeed, the fungus develops *in planta* hyphae which can be considered as more robust (since it is in its wild environment, *i.e.* within its host) than the yeast-like morphotype formed by *Z. tritici* *in vitro*. Another major difference between *in vitro* and *in planta* conditions is that *Z. tritici* develops *in planta* several specialized structures such as pseudo-appressoria and pycnidia that it cannot form *in vitro*, because of the absence of the host in this latter condition.

Considering the high diversity of *Z. tritici* populations that can be found in a single geographic area [33], we assessed the variability of the direct antifungal activity of Rh-Est-C12 towards 21 different strains of the fungus differing in their resistance level to DMI fungicides, the most used chemical class in the field to control this pathogen. Although the *in vitro* IC₅₀ value of Rh-Est-C12 was, as expected, much higher than those of conventional fungicides on the reference strain IPO323 (54.3 µg.mL⁻¹, *i.e.* 157 µM, for the RL *vs* 0.001 to 0.07 µg.mL⁻¹ depending on the considered fungicide, Supplementary Table 1), it is noteworthy that no strain effect was observed for Rh-Est-C12. By contrast, strong resistance factors were scored for all tested DMI molecules, thus confirming previous studies reporting high rates of DMI resistance in *Z. tritici* populations [34]. Hence, we could assume that Rh-Est-C12 display a mode of action on *Z. tritici* different from that of DMI fungicides, which inhibit the sterol 14 α-demethylase, CYP51, preventing the fungus to produce ergosterol [35]. Here, we suggest that Rh-Est-C12 interacts directly with *Z. tritici* cell membranes, resulting in an inhibition of the fungal growth, as previously suggested for other fungi [14,18].

The environmentally-friendly properties of RLs, the duality of Rh-Est-C12 biological activities (direct and indirect activities), an absence of strain specific resistance within the examined *Z. tritici* collection, together with the low-cost production and industrial feasibility of bioinspired synthetic RLs compared to bacterial production and purification, makes Rh-Est-C12 a promising candidate to control *Z. tritici* in the field. Nevertheless, additional investigations on the formulation aiming at reducing the concentration of the compound *in planta* are required.

4. Materials and Methods

4.1. Chemical compounds used

A total of 18 RLs varying in their structures (chain length and/or linker) synthesized using green chemistry, as well as lauric acid and dodecanol (Sigma-Aldrich, France), were used in order to investigate the structure-activity relationship of RLs on the wheat-*Z. tritici* pathosystem (Table 1). Moreover, commercial RLs purchased from Agae Technologies LLC (Corvallis, USA) were used as a natural RL reference. This natural RL is a mixture of RLs of different structures, composed by decanoic acid, 3-((6-deoxy-2-O-(6-deoxy- α -L-mannopyranosyl)- α -L-mannopyranosyl)oxy)-, 1-(carboxymethyl)octyl ester (Rh-Rh-C10-C10), and 1-(carboxymethyl)octyl 3-((6-deoxy- α -L-mannopyranosyl)oxy)decanoate (Rh-C10-C10), and produced by *P. aeruginosa* with a purification rate of 90% (data from Agae Technologies LLC). According to the provided information, its average molar mass is estimated to 577.72 g.mol⁻¹.

The conversions and the purities of the synthesized products were determined by NMR spectroscopy. NMR spectra were recorded on a Bruker DRX300 spectrometer operating at 300 MHz for ¹H nuclei and 75 MHz for ¹³C nuclei. CDCl₃ (99.50% isotopic purity) were purchased from Euriso-Top. The progress of the reactions was checked by thin layer chromatography (TLC) on Merck silica gel 60 glass plates. Detection was carried out by spraying the chromatograms with 10% ethanolic sulfuric acid and heating them to 100 °C. Flash column chromatography was performed with silica gel (40–100 μ m, Merck): all chemicals were of reagent grade and used without further purification.

4.2. General procedures for RL synthesis

Among the 18 synthesized RLs, nine belonging to ether (butyl α / β -L-rhamnopyranoside (Rh-Eth-C4), hexyl α / β -L-rhamnopyranoside (Rh-Eth-C6), octyl α / β -L-rhamnopyranoside (Rh-Eth-C8), decyl α / β -L-rhamnopyranoside (Rh-Eth-C10), dodecyl α / β -L-rhamnopyranoside (Rh-Eth-C12), tetradecyl α / β -L-rhamnopyranoside (Rh-Eth-C14), hexadecyl α / β -L-rhamnopyranoside (Rh-Eth-C16)), ester (dodecanoyl α / β -L-rhamnopyranoside (Rh-Est-C12)), and mono-rhamnosyl (alkenyl)succinate (dodecenylsuccinate α / β -L-rhamnopyranoside (Rh-Suc-C12)) families, have already been described in Robineau et al. [25]. The nine new synthesized RLs in the current study were octadecyl α / β -L-rhamnopyranoside (Rh-Eth-C18) (ether family), octanoyl α / β -L-rhamnopyranoside (Rh-Est-C8), decanoyl α / β -L-rhamnopyranoside (Rh-Est-C10) (ester family), octenylsuccinate α / β -L-rhamnopyranoside (Rh-Suc-C8), isododecenylsuccinate α / β -L-rhamnopyranoside (Rh-Suc-C12b) (mono-rhamnosyl (alkenyl)succinate family), 3-octenyloctylsuccinate α / β -L-rhamnopyranoside (Rh-Suc-C8-C8), 3-octenyldodecylsuccinate α / β -L-rhamnopyranoside (Rh-Suc-C8-C12), 3-dodecenylsuccinate α / β -L-rhamnopyranoside (Rh-Suc-C12-C8), and 3-dodecenylsuccinate α / β -L-rhamnopyranoside (Rh-Suc-C12-C12) (mono-rhamnosyl (alkenyl)alkylsuccinate family).

The general procedures for rhamnose ether, ester and mono-rhamnosyl (alkenyl)succinate molecule syntheses have been reported in Robineau et al. [25], while the general procedure for mono-rhamnosyl (alkenyl)alkylsuccinate derivative synthesis will be described as follow. First, alkenylsuccinic anhydride (12.2 mmol, 1 eq.) was added dropwise to a stirred solution of L-rhamnose (2.0 g, 12.2 mmol, 1 eq.) and DMAP (1.8 g, 14.6 mmol, 1.2 eq.) in DMF (20 mL). After 16 h at 60°C, DMAP (1.5 g, 12.2 mmol, 1 eq.) and alkyl bromide (14.6 mmol, 1.2 eq.) was added slowly to the reaction mixture and stirred overnight. The reaction was cooled and H₂SO₄ 10% was added to reached pH 5. Ethyl acetate was added and the organic phase was washed with a saturated NaCl solution and HCl 1 M. The organic layer was dried with MgSO₄ and concentrated under reduced pressure. The rhamnose alkylsuccinate was isolated after purification by column chromatography (EtOAc/Petroleum spirit 5:5).

4.3. RL chemical analysis

The chemical analyses of the previously reported RLs Rh-Eth-C4, Rh-Eth-C6, Rh-Eth-C8, Rh-Eth-C10, Rh-Eth-C12, Rh-Eth-C14, Rh-Eth-C16, Rh-Est-C12, Rh-Suc-C12 were already performed [25]. For the nine new synthesized RLs in the present study, their chemical analyses are described below.

4.3.1. Octadecyl α/β -L-rhamnopyranoside (Rh-Eth-C18)

White solid (β/α ratio 2:8, 30%); Rf 0.70 (AcOEt/MeOH 9/1); ^1H NMR (CDCl_3): 4.73 (m, 2H), 3.91 (m, 2H), 3.77-3.73 (m, 2H), 3.66-3.59 (m, 4H), 3.46-3.34 (m, 4H), 1.57-1.53 (m, 4H), 1.31-1.25 (m, 66H), 0.87 (m, 6H, CH_3) ^{13}C NMR (CDCl_3): δ 100.3, 99.6, 73.4, 72.4, 71.4, 70.9, 69.7, 68.2, 68.1, 31.0-21.9 (CH_2 alkyl chain), 17.9, 17.3, 14.4.

4.3.2. Octanoyl α/β -L-rhamnopyranoside (Rh-Est-C8)

Yellowish oil (β/α ratio 3:7, 61%); Rf 0.41 (AcOEt/MeOH 9/1); ^1H NMR (CDCl_3): 5.06-4.79 (m, 2H), 4.06-3.87 (m, 4H), 3.63-3.41 (m, 4H), 2.41-2.35 (m, 4H), 1.63-1.59 (m, 4H), 1.31-1.23 (m, 22H), 0.92 (m, 6H, CH_3) ^{13}C NMR (CDCl_3): δ 174.2, 172.0, 93.2, 92.9, 75.8, 74.2, 73.3, 72.8, 71.1, 69.8, 68.6, 68.1, 34.5, 34.1, 32.0-21.6 (CH_2 alkyl chain), 17.5, 17.1, 13.3.

4.3.3. Decanoyl α/β -L-rhamnopyranoside (Rh-Est-C10)

Yellowish oil (β/α ratio 3:7, 58%); Rf 0.44 (AcOEt/MeOH 9/1); ^1H NMR (CDCl_3): 5.07-4.78 (m, 2H), 4.03-3.89 (m, 4H), 3.65-3.60 (m, 2H), 3.47-3.39 (m, 2H), 2.39-2.34 (m, 4H), 1.63-1.58 (m, 4H), 1.31-1.23 (m, 30H), 0.94 (m, 6H, CH_3) ^{13}C NMR (CDCl_3): δ 174.5, 172.1, 93.7, 93.0, 75.9, 74.1, 73.6, 72.6, 71.2, 69.8, 68.6, 68.0, 34.4, 34.3, 32.0-21.6 (CH_2 alkyl chain), 17.7, 17.5, 13.4.

4.3.4. Octenylsuccinate α/β -L-rhamnopyranoside (Rh-Suc-C8)

Colorless oil (β/α ratio 2:8, 59%); Rf 0.19 (AcOEt/MeOH 9/1); ^1H NMR (CDCl_3): 5.47 (m, 2H), 5.29 (m, 2H), 5.20-5.14 (m, 2H), 4.07-4.03 (m, 2H), 3.69-3.64 (m, 4H), 3.44-3.41 (m, 4H), 2.89-2.84 (m, 2H), 2.71-2.62 (m, 4H), 2.23-1.98 (m, 8H), 1.32-1.08 (m, 18H), 0.91 (m, 6H, CH_3) ^{13}C NMR (CDCl_3): δ 178.2, 175.6, 174.8, 134.8, 125.0, 94.4, 91.0, 74.2, 73.8, 73.5, 71.0, 70.7, 44.3, 31.7-21.9 (CH_2 alkyl chain), 16.8, 16.2, 13.8.

4.3.5. Iso-Dodecenylsuccinate α/β -L-rhamnopyranoside (Rh-Suc-C12b)

Yellowish solid (β/α ratio 2:8, 55%); Rf 0.23 (AcOEt/MeOH 9/1); ^1H NMR (CDCl_3): 5.49 (m, 2H), 5.33 (m, 2H), 5.18-5.11 (m, 2H), 4.09-4.02 (m, 2H), 3.71-3.65 (m, 4H), 3.45-3.41 (m, 4H), 2.90-2.84 (m, 2H), 2.71-2.62 (m, 4H), 2.23-1.98 (m, 8H), 1.65-1.61 (m, 2H), 1.32-1.08 (m, 26H), 0.91 (m, 6H, CH_3) ^{13}C NMR (CDCl_3): δ 178.2, 175.6, 174.9, 134.6, 125.2, 94.4, 91.1, 74.1, 73.8, 73.4, 71.0, 70.7, 44.4, 31.8-21.8 (alkyl chain), 17.0, 16.9, 16.7, 16.4, 13.7.

4.3.6. 3-Octenylsuccinate α/β -L-rhamnopyranoside (Rh-Suc-C8-C8)

Yellowish oil (β/α ratio 2:8, 39%); Rf 0.46 (AcOEt/MeOH 9/1); ^1H NMR (CDCl_3): 5.46 (m, 2H), 5.28 (m, 2H), 5.21-5.12 (m, 2H), 4.09-4.02 (m, 2H), 3.71-3.62 (m, 4H), 3.45-3.40 (m, 4H), 2.90-2.83 (m, 2H), 2.73-2.63 (m, 4H), 2.40-1.98 (m, 12H), 1.61-1.58 (m, 4H), 1.32-1.08 (m, 40H), 0.92 (m, 6H, CH_3), 0.89 (m, 6H, CH_3) ^{13}C NMR (CDCl_3): δ 178.2, 175.6, 174.8, 134.8, 125.0, 94.4, 91.0, 74.2, 73.8, 73.5, 71.0, 70.7, 44.3, 34.4, 34.2, 31.7-21.4 (CH_2 alkyl chain), 17.4, 17.2, 16.8, 16.2, 13.8, 13.3.

4.3.7. 3-Octenylsuccinate α/β -L-rhamnopyranoside (Rh-Suc-C8-C12)

Yellowish oil (β/α ratio 2:8, 38%); Rf 0.45 (AcOEt/MeOH 9/1); ^1H NMR (CDCl_3): 5.46 (m, 2H), 5.28 (m, 2H), 5.21-5.12 (m, 2H), 4.09-4.02 (m, 2H), 3.71-3.62 (m, 4H), 3.45-3.40 (m, 4H), 2.90-2.83 (m, 2H), 2.72-2.63 (m, 4H), 2.41-1.97 (m, 12H), 1.65-1.57 (m, 4H), 1.32-1.08 (m, 56H), 0.92 (m, 12H, CH_3), ^{13}C NMR (CDCl_3): δ 178.2, 175.6, 174.8, 134.8, 125.0, 94.4, 91.0, 74.2, 73.8, 73.5, 71.0, 70.7, 44.3, 34.4, 34.3, 32.0-21.6 (CH_2 alkyl chain), 17.6, 17.3, 16.8, 16.2, 13.8, 13.3.

4.3.8. 3-Dodecenyloctylsuccinate α/β -L-rhamnopyranoside (Rh-Suc-C12-C8)

Yellowish oil (β/α ratio 2:8, 37%); Rf 0.47 (AcOEt/MeOH 9/1); ^1H NMR (CDCl_3): 5.50 (m, 2H), 5.33 (m, 2H), 5.17-5.10 (m, 2H), 4.09-4.02 (m, 2H), 3.71-3.64 (m, 4H), 3.45-3.41 (m, 4H), 2.92-2.85 (m, 2H), 2.69-2.60 (m, 4H), 2.41-2.34 (m, 4H), 2.23-1.99 (m, 8H), 1.63-1.59 (m, 4H), 1.32-1.06 (m, 56H), 0.92 (m, 12H, CH_3) ^{13}C NMR (CDCl_3): δ 178.0, 175.4, 174.6, 134.6, 125.0, 94.4, 91.1, 74.2, 73.9, 73.4, 71.3, 70.9, 44.3, 32.1-21.6 (CH_2 alkyl chain), 17.7, 17.3, 17.0, 16.6, 13.8, 13.4.

4.3.9. 3-Dodecenyldodecylsuccinate α/β -L-rhamnopyranoside (Rh-Suc-C12-C12)

Yellowish oil (β/α ratio 2:8, 34%); Rf 0.43 (AcOEt/MeOH 9/1); ^1H NMR (CDCl_3): 5.51 (m, 2H), 5.34 (m, 2H), 5.18-5.09 (m, 2H), 4.09-4.03 (m, 2H), 3.70-3.58 (m, 6H), 3.49-3.41 (m, 6H), 2.91-2.84 (m, 2H), 2.71-2.63 (m, 4H), 2.40-2.32 (m, 4H), 2.25-2.00 (m, 8H), 1.66-1.59 (m, 4H), 1.33-1.08 (m, 72H), 0.92 (m, 12H, CH_3) ^{13}C NMR (CDCl_3): δ 178.1, 175.5, 174.8, 134.7, 125.1, 94.5, 91.0, 74.2, 73.9, 73.4, 71.1, 70.8, 44.3, 34.5, 34.2, 31.9-21.5 (CH_2 alkyl chain), 17.6, 17.3, 16.9, 16.4, 13.9, 13.7.

4.4. Plant treatment and inoculation

All in *planta* assays were carried out using the pathogenic *Z. tritici* strain T02596 isolated in 2014 from Northern France, and the susceptible wheat cultivar Alixan (Limagrain, France) showing compatible interaction with the strain T02596 [36]. The experiments were performed in the greenhouse at $21 \pm 2^\circ\text{C}$ with a 16/8 h day/night cycle. Wheat seeds were pre-germinated in square Petri dishes on moist filter paper, as described by Siah et al. [37]. Germinated grains were placed into 3-L pots filled with universal loam (Gamm Vert, France) and put into the greenhouse. For each condition, three pots containing 12 plants each, meaning 36 plants in total, were used as replicates. Three weeks after sowing, when third-leaves are fully expanded, 30 mL of a solution of 1.5 mM of each RL, as well as lauric acid and dodecanol, were hand-sprayed on the plants of each pot. These molecules were first dissolved in dimethyl sulfoxide (DMSO), with final concentration of DMSO at 0.2%, before being supplemented with 0.05% polyoxyethylene-sorbitan monolaurate (Tween 20, Sigma-Aldrich, USA) as a wetting agent. Control plants were treated with a solution of 0.2% DMSO, supplemented with 0.05% Tween 20. Due to solubilization difficulties at 1.5 mM, treatments with Rh-Eth-C18 and Rh-Suc-C12-C12 were not performed. Two days after treatment, 30 mL of fungal spore suspension (10^6 spores.mL $^{-1}$), also supplemented with 0.05% of Tween 20, were hand-sprayed on the plants of each pot. *Zymoseptoria tritici* spores were harvested after being cultivated on potato dextrose agar medium (PDA) plates for one week in dark conditions, according to Siah et al. [37]. Immediately after inoculation, each pot was covered with a clear polyethylene bag for 3 days in order to ensure a high humidity level allowing a good plant infection. Three weeks after inoculation, the disease severity was measured on the third leaf by assessing the area covered by lesions (chlorosis or necrosis) bearing or not pycnidia. This experiment was repeated twice. In the case of Rh-Est-C12, the most efficient RL in our conditions, the protection efficacy of this compound was assessed at different concentrations, ranging from 50 μM to 3200 μM (50, 100, 200, 400, 800, 1600, and 3200 μM). This experiment underwent the same conditions as described above, except for DMSO that was used at 0.1% final concentration in the treatment solutions.

4.5. Plant defence-related enzyme assays

Evaluation of the indirect effect of RLs, as well as lauric acid and dodecanol, was performed by assessing the activity of two antioxidant key-enzymes involved in plant defense mechanisms: catalase (CAT; EC: 1.11.1.6) and peroxidase (POX; EC: 1.11.1.7). Wheat plants grown under greenhouse conditions treated or not with 1.5 mM of RLs, as described above, were used to examine the elicitation activity of the compounds. Plant third-leaves were harvested two days after treatment and 100 mg were immediately frozen in liquid nitrogen and stored at -80 °C for further analyses. Three leaves, sampled randomly from three different pots (one leaf per pot), were used as repetitions for each condition. Leaf segments were later ground with a mortar and pestle in liquid nitrogen. The powder obtained was resuspended in 1 mL of 50 mM ice-cold potassium phosphate buffer, pH 7.0, and centrifuged at 12000 g for 10 min at 4°C. The supernatant was used to evaluate the activity of CAT or POX. POX activity was assessed based on the procedure of Mitchell et al. [38] and Shindler et al. [39]. The reactive medium consisted of 0.5 mM 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS) and 0.25 mM H₂O₂ in 25 mM sodium acetate buffer, pH 4.4. This assay was performed in flat-bottomed polystyrene 96-well microplates (Corning Costar®, Corning, USA). To initiate the reaction, 10 µL of supernatant was added to 190 µL of the reactive mix in plate wells. The increased absorbance at 412 nm, produced by the formation of the radical cation, was monitored kinetically for 5 minutes at 25 °C. POX activity was expressed as change in absorbance at 412 nm.min⁻¹.g⁻¹ protein. CAT activity was determined according to Beers and Sizer [40] and García-Limones et al. [41] with slight modifications. To initiate the reaction, 10 µL of wheat leaf supernatant diluted in sterile water 1:4 (v/v) were added to the wells containing 190 µL of reactive medium consisting of 50 mM H₂O₂ in 50 mM potassium phosphate buffer, pH 7.0. 96-well microplates (Greiner UV-Star®, Greiner Bio-One GmbH, Austria), allowing measurements up to 200 nm, were used. The decreased absorbance at 240 nm resulting from the degradation of H₂O₂ was measured kinetically during 5 minutes at 25 °C. CAT activity was expressed as change in absorbance at 240 nm.min⁻¹.g⁻¹ protein. Protein content of wheat leaf supernatants was assessed using the method of Bradford [42] with bovin serum albumin (BSA) as a standard.

4.6. In planta antifungal effect of Rh-Est-C12

Wheat plants grown under greenhouse conditions, sprayed or not with different concentrations of Rh-Est-C12 (0, 50, 400, and 3200 µM) and inoculated with spore suspension of the T02596 *Z. tritici* strain, were used to assess *in planta* the activity of this molecule on spore germination and hyphal growth of the phytopathogen. The three concentrations 50, 400, and 3200 µM were chosen because they correspond to the lowest, median, and highest concentrations tested *in planta* for this RL, respectively. The chitin staining dye Fluorescent Brightener 28 (Calcofluor, Sigma-Aldrich, USA) was used to stain the fungus on the leaf surfaces, according to Siah et al. [37]. Three days after inoculation, wheat third-leaf segments (2 cm) were harvested and immersed for five minutes in a solution of 0.1% (w/v) Calcofluor and 0.1 M Tris-HCl buffer, pH 8.5. Leaf segments were then washed twice in sterile osmotic water for two minutes. After being dried in dark conditions at room temperature, they were placed on a glass slide, covered with a cover slip and observed microscopically (Nikon, Eclipse 80i) under UV-light. For each leaf segment, the percentage of different spore classes was calculated on 100 observed spores (class 1: non germinated spore; class 2: germinated spore with a small germ tube; class 3: germinated spore with a developed germ tube; class 4: germinated spore with a strongly developed germ tube). Six third-leaf segments from three different pots (two segments per pot) were used as replicates for each condition. Pictures were taken with a digital camera (DXM1200C) using image capture software (NIS-Elements BR).

4.7. In vitro direct antifungal effect of rhamnolipids

In vitro antifungal activity of all tested RLs, as well as dodecanol and lauric acid, was assessed by measuring the growth of *Z. tritici* colonies on solid medium in sterile 12-well plates (Cellstar standard®, Greiner Bio-One GmbH, Austria). Plate wells were filled with 3 mL of PDA, autoclaved

beforehand, mixed with our molecules of interest at approximately 40°C. The compounds were first dissolved in DMSO (0.1% final concentration in the wells). After PDA cooling down, 5 µL of the *Z. tritici* strain T02596 spore suspension (5.10^5 spores.mL⁻¹) were added to each well, according to Siah et al. [37]. Ten different concentrations (39.5, 59.3, 88.9, 133.3, 200, 300, 450, 666.7, 1000, and 1500 mM) were used for each molecule. Moreover, control mediums supplemented with 0.1% of DMSO, with or without fungal inoculation, were used. After inoculation, plates were incubated for 10 days in growth chamber in dark conditions at $20 \pm 1^\circ\text{C}$, after which two perpendicular diameters of fungal colonies were measured visually. This experiment was repeated three times and three wells were used as replicates for each condition.

4.8. Fungal strain effect of Rh-Est-C12 antifungal activity

To assess the fungal strain effect of the most active RL Rh-Est-C12 regarding its antifungal direct activity, a total of 21 single-conidial *Z. tritici* strains (Zt.1 to Zt.21) isolated in 2016 from Arras location in northern France, and the reference strain IPO323 isolated in the Netherland in 1981 [41], were assessed for their variability in sensitivity to Rh-Est-C12. These *Z. tritici* strains were chosen because they displayed, in preliminary assays, different resistance levels towards five fungicide molecules belonging to DMI class (tebuconazole, metconazole, epoxiconazole, prothioconazole, prochloraz). The sensitivity of the strains towards these fungicides was evaluated in 96-well microplates, as described in Siah et al. [37], at the concentrations 75, 25, 8.33, 2.78, 0.93, 0.31, 0.10, and 0.03 mg.L⁻¹. The antifungal bioassay for Rh-Est-C12 against the 22 *Z. tritici* strains was performed at the concentrations 450, 300, 200, 133.3, 88.9, 59.3, and 39.5 µM.

4.9. Statistical analyses

Data obtained for *in planta* protection efficacy, enzyme activity, and *in planta* spore germination assays were subjected to analysis of variance (ANOVA) followed by Tukey's test at $P \leq 0.05$, using the XLSTAT software (Addinsoft, Paris, France). For *in vitro* antifungal activity assays, the minimal inhibitory concentration (MIC), defined as the minimal tested concentration totally inhibiting fungal growth, was scored for each molecule. In addition, half-maximal inhibitory concentration (IC₅₀) was calculated using GraphPad Prism software version 8 (GraphPad Software Inc., USA) for each molecule and tested fungal strain. In the strain effect assay, resistance factors were calculated by dividing the IC₅₀ of each strain by the IC₅₀ of the reference strain IPO323 for each tested compound. Correlations between protection efficacies, *in vitro* direct activity (IC₅₀ and MIC), and *in planta* indirect effect (CAT and POX activities) within all the tested molecules, was carried out with principal component analysis (PCA) based on the Pearson correlation test, using XLSTAT software (Addinsoft, Paris, France).

Supplementary Materials: The Supplementary Materials are available online. Supplementary Table 1: Half-maximal inhibitory concentration (IC₅₀) values obtained for 22 *Zymoseptoria tritici* strains differing in their resistance level to DMI fungicides, towards five DMI molecules and the rhamnolipid molecule Rh-Est-C12. Supplementary Table 2: Resistance factors obtained for 22 *Zymoseptoria tritici* strains differing in their resistance level to DMI fungicides, towards five DMI molecules and the rhamnolipid molecule Rh-Est-C12, calculated from the IC₅₀ values presented in the Supplementary Table 1. Resistance factors correspond to the ratio between the IC₅₀ value of a given strain and the IC₅₀ value of the sensitive reference strain IPO323.

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Sample Availability: Samples of the compounds are not available from the authors.



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Supplementary Table 1. Half-maximal inhibitory concentration (IC₅₀) values obtained for 22 *Zymoseptoria tritici* strains differing in their resistance level to DMI fungicides, towards five DMI molecules and the rhamnolipid molecule Rh-Est-C12.

<i>Zymoseptoria tritici</i> isolates		IC ₅₀ (mg.L ⁻¹)					
		Tebuconazole	Metconazole	Epoxiconazole	Prothioconazole	Prochloraz	Rh-Est-C12
Control strain	IPO323	0.07	0.04	0.03	0.001	0.02	54.3
No MDR or <i>Cyp51</i> gene overexpressing strains	Zt.1	0.26	0.14	1.12	0.10	0.12	49.9
	Zt.2	0.54	0.13	1.39	0.10	0.17	65.4
	Zt.3	0.49	0.20	1.22	0.09	0.16	64.1
	Zt.4	0.48	0.25	1.18	0.14	0.17	50.3
	Zt.5	0.38	0.25	1.63	0.12	0.12	59.0
	Zt.6	0.36	0.18	1.35	0.08	0.17	47.4
	Zt.7	0.29	0.14	1.03	0.12	0.20	60.3
<i>Cyp51</i> gene overexpressing strains	Zt.8	5.42	0.25	1.05	0.12	0.19	49.7
	Zt.9	9.42	0.61	1.45	0.11	0.12	49.3
	Zt.10	15.54	0.56	1.69	0.05	0.39	44.2
	Zt.11	12.02	0.72	1.62	0.05	0.20	51.7
	Zt.12	18.85	1.10	1.48	0.08	0.25	48.7
	Zt.13	21.07	1.35	3.63	0.05	0.79	49.2
	Zt.14	2.33	0.27	0.86	0.04	0.20	45.3
MDR strains	Zt.15	5.91	1.30	3.82	0.28	3.66	49.2
	Zt.16	4.86	1.27	7.47	75.00	3.13	44.2
	Zt.17	5.26	0.04	6.72	0.28	1.94	40.0
	Zt.18	3.53	1.05	8.03	0.30	1.28	32.0
	Zt.19	75.00	6.02	15.05	0.57	1.48	56.6
	Zt.20	15.51	3.17	10.56	0.04	6.38	47.5
	Zt.21	4.82	1.54	7.61	0.33	2.86	46.2

Supplementary Table 2. Resistance factors obtained for 22 *Zymoseptoria tritici* strains differing in their resistance level to DMI fungicides, towards five DMI molecules and the rhamnolipid molecule Rh-Est-C12, calculated from the IC₅₀ values presented in the Supplementary Table 1.

<i>Zymoseptoria tritici</i> isolates		Resistance factors*					
		Tebuconazole	Metconazole	Epoxiconazole	Prothioconazole	Prochloraz	Rh-Est-C12
Control strain	IPO323	1 (0.07)	1 (0.04)	1 (0.03)	1 (0.001)	1 (0.02)	1 (54.29)
No MDR or <i>Cyp51</i> gene overexpressing strains	Zt.1	3.8	3.6	37.2	102.4	5.1	0.9
	Zt.2	7.6	3.3	46.2	104.4	7.2	1.2
	Zt.3	7.0	4.9	40.7	89.3	6.7	1.2
	Zt.4	6.8	6.2	39.5	137.5	7.2	0.9
	Zt.5	5.4	6.1	54.3	117.0	5.1	1.1
	Zt.6	5.2	4.6	45.2	84.5	6.9	0.9
	Zt.7	4.1	3.5	34.2	117.8	8.2	1.1
<i>Cyp51</i> gene overexpressing strains	Zt.8	77.6	6.3	35.0	123.3	8.1	0.9
	Zt.9	134.6	15.3	48.2	112.8	5.2	0.9
	Zt.10	222.0	14.0	56.3	54.3	16.5	0.8
	Zt.11	171.7	18.0	53.9	46.9	8.3	1.0
	Zt.12	269.3	27.6	49.3	75.9	10.6	0.9
	Zt.13	301.0	33.8	121.1	53.8	33.0	0.9
	Zt.14	33.3	6.8	28.6	42.5	8.5	0.8
MDR strains	Zt.15	84.4	32.5	127.3	276.5	152.6	0.9
	Zt.16	69.5	31.7	249.1	74753.2	130.6	0.8
	Zt.17	75.1	1.0	224.1	274.3	80.8	0.7
	Zt.18	50.4	26.3	267.5	298.6	53.5	0.6
	Zt.19	1071.4	150.8	501.8	564.9	61.8	1.0
	Zt.20	221.5	79.3	351.9	37.4	266.2	0.9
	Zt.21	68.9	38.6	253.5	328.6	119.2	0.9

*Resistance factors correspond to the ratio between the IC₅₀ value of a given strain and the IC₅₀ value of the sensitive reference strain IPO323.

CHAPITRE 2

Le rhamnolipide Rh-Est-C12 protège le blé vis-à-vis de *Zymoseptoria tritici* via principalement une activité antifongique directe sans perturbation majeure de la physiologie foliaire

Lors du précédent chapitre, nous avons montré que le Rh-Est-C12 était le rhamnolipide le plus efficace à notre disposition, présentant une activité antifongique *in vitro* et *in planta* significative, mais aussi d'éllicitation des activités CAT et POX chez les feuilles de blé, suggérant que ce composé serait capable de déclencher un *burst* oxydatif chez la plante traitée. Ce rhamnolipide présentait aussi l'efficacité de protection la plus élevée de tous les composés testés, même si des travaux restent encore nécessaires afin de l'améliorer au vu de la différence d'activité conséquente observée entre les conditions *in vitro* et *in planta* (facteur 20). Enfin, nous avons montré que le Rh-Est-C12 présente le même niveau d'activité antifongique vis-à-vis de souches de *Z. tritici* plus ou moins résistantes aux DMI, même vis-à-vis de celles avec un phénotype MDR. En raison de ces résultats encourageants, nous avons mis en place d'autres analyses visant à caractériser plus finement le mode d'action indirect du Rh-Est-C12 sur notre pathosystème. Nous avons cherché à déterminer si, comme le suggère l'induction des activités CAT et POX, ce rhamnolipide bio-inspiré est capable de stimuler les défenses du blé. Dans le but de répondre à cette question, ainsi que pour observer l'effet global de l'application de ce composé sur le métabolisme des feuilles de blé, une approche combinée de transcriptomique et de métabolomique a été menée. Aucune étude ne s'est intéressée, jusque-ici, à l'effet d'un potentiel stimulateur de défenses sur le transcriptome et le métabolome du blé vis-à-vis de *Z. tritici*. Malgré la protection observée avec le traitement, le Rh-Est-C12, dans nos conditions, n'a entraîné que de faibles modifications sur le niveau d'expression des gènes et l'accumulation des métabolites dans les feuilles de blé. Ces résultats indiquent que ce composé ne semble pas entraîner d'altérations coûteuses au niveau énergétique pour l'hôte, ce qui est une propriété bénéfique dans le cadre de son utilisation en tant que composé protecteur du blé. Par ailleurs, ces résultats suggèrent que l'activité de protection du Rh-Est-C12 serait principalement due à son activité antifongique directe, et, dans une moindre mesure, à l'induction des réactions de défense de l'hôte. L'effet de l'infection du blé par *Z. tritici*, durant sa phase biotrophe, sur le transcriptome et le métabolome de l'hôte, sera aussi discuté.

Les résultats obtenus dans le cadre de ce chapitre sont en préparation en vue d'une soumission pour publication dans la revue Frontiers in Plant Science (IF = 4,30).

Bioinspired rhamnolipid protects wheat against *Zymoseptoria tritici* through mainly a direct antifungal activity and without major disorganization of the leaf physiology

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ABSTRACT

Rhamnolipids (RLs) are glycolipids naturally biosynthesized by bacteria from *Pseudomonas* sp. and *Burkholderia* sp. genera and have been shown to display activities against a wide range of pathogens. Most of previous studies on RLs focused on their direct antimicrobial activity, while only few reports describe the mechanisms by which the RLs induce resistance in plants against pathogens and the related fitness cost on plant physiology. We combined here transcriptomic and metabolomic approaches to unravel the mechanisms underlying a RL-associated induced resistance in wheat against the hemibiotrophic fungus *Zymoseptoria tritici*, a major pathogen on this crop. The investigations were performed by treating wheat plants with a bioinspired synthetic mono-RL with a 12-carbon fatty acid tail (Rh-Est-C12) in both infectious and non-infectious conditions, to assess its potential wheat defence eliciting as well as priming bioactivities. Whereas Rh-Est-C12 conferred to wheat a significant protection against *Z. tritici* (41% disease severity reduction), only a slight effect of the RL on wheat leaf gene expression and metabolite accumulation was obtained. A subset of 24 differentially expressed genes (DEGs) and 11 differentially accumulated metabolites (DAMs) were scored in elicitation modalities examined at two, five and fifteen days post-treatment (dpt), and 25 DEGs and 17 DAMs were recorded in priming modalities tested at five and fifteen dpt. Most of the changes are down-regulations and only few DEGs and DAMs associated with resistance to pathogens were identified, while DEGs and DAMs related to other physiological functions were recorded. Nevertheless, a transient early regulation in gene expression was noticed at two dpt (*e.g.* genes involved in signaling, transcription, translation, cell-wall structure and function), suggesting a perception of the RL by the plant upon treatment. Further *in vitro* and *in planta* bioassays showed that Rh-Est-C12 displays direct antimicrobial activity towards *Z. tritici*, thus confirming previous reports. Taken together, our results suggest that Rh-Est-C12 protects wheat against *Z. tritici* though mainly a direct antifungal activity and to a lesser extent *via* the induction of plant defenses, without causing major alterations on the plant metabolism. This study provides new insights into the modes of action of RLs on the wheat-*Z. tritici* pathosystem and reveals the interest to use this low- fitness cost protective molecule to control the pathogen.

Key words: Wheat, *Zymoseptoria tritici*, plant bioprotection, rhamnolipids, plant defense mechanisms, omics, transcriptomic, metabolomic.

INTRODUCTION

Biosurfactants, also referred to as green surfactants, are amphiphilic surface-active molecules that include a hydrophilic head and a hydrophobic tail (Desai and Banat, 1997). They are produced by a wide range of microorganisms, including bacteria, yeasts, and fungi, and they are classified according to their molecular structure into, mainly, mannosylerythritol lipids, trehalose dimycolate, trehalolipids, sophorolipids, lipopeptides, and rhamnolipids (Crouzet *et al.*, 2020). Biosurfactants have been extensively studied for decades because of their high potential of application in diverse areas, including human health, cosmetic, food industry, petroleum industry, soil and water remediation, nanotechnology, and agriculture (Lourith and Kanlayavattanakul, 2009 ; Sachdev and Cameotra, 2013 ; Shekhar *et al.*, 2015 ; Singh *et al.*, 2019). Besides, these biomolecules are gaining more and more attention due to their high biodegradability as well as their low toxicity and ecotoxicity, making them an ecofriendly alternative to synthetic molecules in several areas, such as for the development of sustainable agriculture (Abdel-Mawgoud *et al.*, 2010 ; Shekhar *et al.*, 2015).

Rhamnolipids (RLs) are glycolipids with biosurfactant properties, naturally biosynthesized as secondary metabolites by bacteria from *Pseudomonas* sp. and *Burkholderia* sp. genera. First report of RLs goes back to 1946, from an *in vitro* culture of *Pseudomonas pyocyanea* (now *P. aeruginosa*) (Bergström *et al.*, 1946). Since then, RLs are one of the most intensively studied classes of surfactant glycolipids. Bacteria often produce RLs as a mixture of molecules with one or two rhamnose residues (mono- or di-RLs), forming a polar hydrophilic head and linked through a beta-glycosylic bond to one or two 3-hydroxy fatty acids, as hydrophobic tails. RL analyses and natural variations in the chemical structures of bacterially produced RLs have led to the identification of a pool of almost 60 different RLs (Abdel-Mawgoud *et al.*, 2010). The differences among these homologs come from modifications in the glycon and/or the aglycon parts; however, the biodiversity of RLs is mostly due to variations in the aglycon parts (Abdel-Mawgoud *et al.*, 2010). Like other biosurfactants, RLs were explored for their use in several areas, including agriculture and sustainable crop protection. Hence, their potential efficacy for the biocontrol of plant pathogens has been reported in several studies, as recently reviewed by Crouzet *et al.* (2020). Most of the studies described the activities of RLs against a large panel of phytopathogenic fungi and oomycetes, while no direct evidence has been reported about their bioactivities towards bacteria and virus infecting plants (Crouzet *et al.*, 2020b). The mode of action of RLs may be direct towards the pathogen, through an antifungal activity, and/or indirect *via* an induction of plant immunity reactions, as it was, for instance, recently reported for the *Brassica napus*-*Leptosphaeria maculans* pathosystem using semi-purified RL mixtures from *P. aeruginosa* (Monnier *et al.*, 2020).

The compounds activating plant defense reactions against biotic stress could be distinguished into two categories, elicitors or priming agents, and both can be referred to as resistance inducers. Elicitors induce plant defense responses immediately after their perception by the plant, whereas the priming agents usually activate marked defense responses, not directly after their application, but only once the plant is attacked by a pathogen, making priming agents more suitable than elicitors because of their expected lower fitness cost on the plant physiology (Paré *et al.*, 2005). Primed plants set up stronger and/or faster response patterns and may detect the pathogen attack at a lower threshold, hence reacting in a more sensitized way when compared to naïve (unprimed) plants (Paré *et al.*, 2005; Lämke and Bäurle, 2017). The activation of plant defense responses could rely on either the specific

recognition, by transmembrane plant protein recognition receptors, of conserved molecular motifs from a pathogen, the plant itself or a beneficial microbe (Jones and Dangl, 2006), or be triggered by the exogenous application of natural or synthetic molecules, possibly not perceived by specific plant transmembrane receptors, such as phytohormones and biosurfactants (Siah et al., 2018b). Perception of these initial stimuli by the plant often leads to the onset of numerous defense mechanisms, including earlier (influx of calcium, rapid extracellular alkalinization, mitogen-activated protein kinase cascade involved in phosphorylation, oxidative burst, *etc.*) and subsequent (transcriptional changes in attack-responsive genes, biosynthesis of pathogenesis-related (PR) proteins, production of low-molecular mass secondary metabolites such as phytoalexins or antimicrobial peptides, *etc.*) cellular events (Bolwell *et al.*, 1995 ; Felle, 2001 ; Pedley and Martin, 2005 ; van Loon *et al.*, 2006 ; Galon *et al.*, 2010).

Wheat, along with maize and rice, is one of the most cultivated crops worldwide. It is used as a primary ingredient for both human and livestock foods as well as in several agro-industrial areas. Nevertheless, this crop is challenged in the field by several bio-aggressors, which significantly impact wheat grain quality and quantity (Savary *et al.*, 2019). Septoria tritici blotch, caused by the fungal ascomycete *Zymoseptoria tritici*, is since few decades one of the most frequently occurring and damaging diseases on wheat crop, causing yield losses of up to 50% especially in areas where environmental conditions are suitable for disease establishment (Fones and Gurr, 2015). *Zymoseptoria tritici* is a hemibiotrophic fungus that is characterized by an initial asymptomatic biotrophic phase lasting approximately two weeks, followed by a visually symptomatic necrotrophic phase of around one week (Siah et al., 2010a). However, the duration of these two phases may vary depending on the wheat cultivar, fungal strain, and environmental conditions. The control of *Z. tritici* relies mainly on the use of chemical fungicides, such as in Europe, where 70% of the total applied fungicides target this pathogen (Fones and Gurr, 2015), and to a lower extent on resistant cultivars, as no completely resistant cultivar to *Z. tritici* is available. Meanwhile, the durability of these management strategies is often compromised in the field since *Z. tritici* frequently develops resistances to fungicides and regularly overcomes host resistances (*e.g.* Cowger *et al.*, 2000; Cheval *et al.*, 2017). Hence, looking for new eco-friendly and sustainable protection alternative tools for wheat protection against *Z. tritici*, such as bio-sourced resistance inducers, is strongly encouraged. However, the literature regarding the induced resistance in wheat against this major fungal pathogen is still poor, while defense mechanisms of wheat triggered in response to *Z. tritici* infection are extensively studied at the plant-pathogen interaction level. The objective of the present study was, thus, to unravel defense mechanisms induced, in wheat towards *Z. tritici*, by a bioinspired synthetic mono-RL with a C12-carbon chain (Rh-Est-C12), which was recently shown to be the most active and protective RL among a series of 19 RLs tested on this pathosystem by performing a structure-activity relationship study (Platel *et al.*, 2021). The investigations were performed in greenhouse conditions using a combined transcriptomic and metabolomic approach, in both non-infectious and infectious conditions, to assess the potential eliciting and priming effects of the molecule.

MATERIALS AND METHODS

Bioinspired rhamnolipid synthesis

The RL used in the present study (dodecanoyl α/β -L-rhamnopyranoside, hereafter referred to as Rh-Est-C12) is bioinspired from the RLs naturally produced by *P. aeruginosa* (**Figure 1A**) and was synthesized as described previously by Robineau *et al.* (2020) and Platel *et al.* (2021). The conversion and the purity of the product was assessed using nuclear magnetic resonance (NMR) spectroscopy and confirmatory analyses were performed using elemental analysis (vario MICRO cube CHNS/O, Elementar, Lyon, France) and NMR spectroscopy (NMR, Bruker 400 MHz spectrometer). Briefly, chemical analysis of the obtained molecule Rh-Est-C12 consists of : yellowish oil (β/α ratio 3:7, 52%); Rf 0.48 (AcOEt/MeOH 9/1); ^1H NMR (CDCl_3): 5.09–4.77 (m, 2H), 4.06–3.87 (m, 4H), 3.66–3.59 (m, 2H), 3.48–3.41 (m, 2H), 2.40–2.33 (m, 4H), 1.65–1.58 (m, 4H), 1.30–1.25 (m, 38H), 0.92 (m, 6H, CH_3) ^{13}C NMR (CDCl_3): δ 174.7, 172.0, 93.5, 92.8, 75.9, 74.2, 73.4, 72.6, 71.3, 69.9, 68.7, 68.1, 34.4, 34.3, 32.0–21.6 (CH_2 alkyl chain), 17.6, 17.4, 13.6. The molecular weight of the obtained molecule is 346 g.mol^{-1} .

Plant and fungal materials

The experiments were conducted using seeds of the wheat cultivar (cv.) Alixan, susceptible to *Z. tritici*, provided by the breeding company Limagrain Europe (Verneuil l'Étang, France). All *in vitro* and *in planta* bioassays were carried out using the pathogenic *Z. tritici* single-spore strain T02596, isolated in 2014 in Northern France from a wheat field non-treated with fungicides. Since its isolation, the strain was stored in cryotube aliquots at -80°C in order to avoid genetic drift of the strain due to *in vitro* subcultures. The fungal inoculum was produced by growing the strain on potato dextrose agar (PDA) medium during one week in dark conditions.

In vitro antifungal bioactivity assay

The direct activity of Rh-Est-C12 RL towards *Z. tritici* growth was assessed on PDA medium, as described by Platel *et al.* (2021), at different concentrations (1.9, 3.9, 7.8, 15.6, 31.2, 62.5, 125, 250, 500, and 1000 mg.L^{-1}) of the RL. Briefly, the RL was first dissolved in DMSO (0.1 % final DMSO concentration within the medium) before being mixed at approximately 40°C with an autoclaved PDA medium. The experiment was carried out in sterile 12-well plates (Cellstar standard®, Greiner Bio-One GmbH, Kremsmünster, Austria); each well was filled with 3 mL of the mixture, each treatment was replicated three times. Plate inoculation was performed by spotting a drop of $5 \mu\text{L}$ of *Z. tritici* spore suspension at $5.10^5 \text{ spores.mL}^{-1}$ at the center of each well. Control wells filled or not with 0.1 % DMSO and inoculated or not with fungal spores were used. After 10-day incubation at $20 \pm 1^\circ\text{C}$ in dark conditions, fungal growth was evaluated by measuring the two perpendicular diameters of each fungal colony.

Plant treatment and infection

Wheat seeds were first pregerminated in square Petri dishes ($12 \times 12 \text{ cm}$) on moist filter paper, as described by Siah *et al.* (2010), before being transferred into three-liter pots filled with universal loam (Gamm Vert, France) and placed in the greenhouse at $21 \pm 2^\circ\text{C}$ with a 16/8 h day-night cycle. Three pots of 12 wheat plants each ($n=36$) were used as replicates for each modality. After three weeks of plant growth, *i.e.* when the third leaves were fully expended (corresponding to day 0, D0), the plants

were pretreated with Rh-Est-C12 at 500 mg.L⁻¹ or with a mock treatment as control, using a hand sprayer. A volume of 30 mL was used for the treatment of each pot. Rh-Est-C12 was firstly dissolved in dimethyl sulfoxide (DMSO, Sigma-Aldrich, Saint-Louis, USA), with a final concentration of DMSO of 0.1 % in the treatment solution, supplemented with 0.05% of polyoxyethylene-sorbitan monolaurate (Tween 20, Sigma-Aldrich, Saint-Louis, USA) as a wetting agent. Mock treatment consisted of a solution of 0.1% DMSO supplemented with 0.05% of Tween 20. At two days post-treatment (D2), both RL-treated and mock-treated plants were inoculated by spraying 30 mL of 10⁶ spores.mL⁻¹ *Z. tritici* suspension. Non-inoculated plants were mock-inoculated by spraying 30 mL of a 0.05 % Tween 20 solution on the plants of each pot. After inoculation, the plants were covered with a clear polyethylene bag for three days, to ensure a high humidity level on the surface of the leaves, a required condition for the disease establishment. Disease severity level was scored on the plant third leaf at 23 days after treatment (D23), corresponding to 21 days post-inoculation, by assessing the leaf areas covered by lesions (chlorosis and necrosis) bearing or not pycnidia.

***In planta* fungal staining assay**

The effect of Rh-Est-C12 on *in planta* spore germination and epiphytic fungal growth on the leaf surface was examined at D5 (*i.e.* three days after inoculation) using the chitin dye Fluorescent Brightener 28 (Calcofluor, Sigma-Aldrich, Saint-Louis, USA). Three-centimeter segments, harvested from third leaves of wheat plants, grown in the greenhouse, inoculated with *Z. tritici* and treated or not with Rh-Est-C12 at 500 mg.L⁻¹ as described above, were sampled. The segments were immersed for 5 min in a solution of 0.1% (w/v) Calcofluor with 0.1 M Tris-HCl buffer at pH 8.5, before being rinsed twice in sterile osmosed water for 2 min and dried at room temperature. Leaf segments were then deposited on a glass slide, covered with cover slip, and observed with an optic microscope (Eclipse 80i, Nikon, Champigny-sur-Marne, France) under UV-light. Four germinated spore classes were assessed during notations under the microscope: class 1, non-germinated spore; class 2, germinated spore with a small germ tube; class 3, germinated spore with a developed germ tube; and class 4, germinated spore with a strongly developed germ tube. Nine leaf segments, randomly selected from three different pots (three segments per pot), were used as replicates for each condition. The frequencies of spore classes were determined from the analyses of 100 distinct spores on each leaf segment ($n_{\text{spores}} = 900$). Pictures were obtained using digital camera (DXM1200C, Nikon, Champigny-sur-Marne, France) coupled with the image capture software NIS-Elements BR (Nikon, Champigny-sur-Marne, France).

RNA extraction and transcriptomic analysis

Total RNA from wheat leaves was extracted using RNeasy Plant Mini Kit (Qiagen, Courtaboeuf, France). The quality of RNAs was measured with Nanodrop by analyzing their absorbance ratios A260/280 and A260/230, which were found to range between 2.0 and 2.2. Besides, RNA quality was also examined with Bioanalyzer 2100 (Agilent, France) and a minimal RNA integrity number (RIN) of 0.8 was required for all samples. Non-targeted gene expression analysis was performed using Microarrays GE 4x44 chip purchased from Agilent (Santa Clara, CA, USA). The hybridization for all conditions were carried out in triplicate with three samples of total RNA extracted from bulked wheat leaves (with three different leaves from different pots per bulk). All the nine wheat leaves used for RNA extraction are originating from different pots. All steps of RNA amplification, staining, hybridization, and washing were performed according to the manufacturer's indications. Slides were

scanned at five μm /pixel resolution using the GenePix 4000B scanner (Molecular Devices Corporation, Sunnyvale, CA, USA) and the images were used for grid alignment and expression data digitization by using the software GenePix Pro 6.0 (Molecular Devices Corporation, Sunnyvale, CA, USA).

Metabolite extraction and analysis

Metabolite extraction was performed from powdered freeze-dried wheat leaves (30-50 mg per sample) using 25 μL of methanol per mg dry weight. The extract was then incubated in an ultrasound bath for 10 minutes, before centrifugation at 13000 g at 10 °C for 10 minutes. The supernatant was analyzed using a Dionex Ultimate 3000 UHPLC system (Thermo Fisher Scientific, USA). The chromatographic separations were performed on a Nucleodur C18 HTec column (150 $\times$ 2 mm, 1.8 μm particle size; Macherey-Nagel, Germany) maintained at 30 °C. The mobile phase consisted of acetonitrile/formic acid (0.1%, v/v, eluant A) and water/formic acid (0.1%, v/v, eluant B) at a flow rate of 0.3 mL min⁻¹. The gradient elution was programmed as follows: 0 to 1 min, 95% B; 1 to 2 min, 95% to 85% B; 2 to 7 min, 85% to 0% B; 7 to 9 min, 100% A. The sample volume injected was 1 μL . The UHPLC system was coupled to an Exactive Orbitrap mass spectrometer (Thermo Fischer Scientific, USA), equipped with an electrospray ionization (ESI) source operating in positive mode. Parameters were set at 300 °C for ion transfer capillary temperature and 2500 V for needle voltages. Nebulization with nitrogen sheath gas and auxiliary gas were maintained at 60 and 15 arbitrary units, respectively. The spectra were acquired within the m/z (mass-to-charge ratio) mass ranging from 100 to 1000 atomic mass units (a.m.u.), using a resolution of 50,000 at m/z 200 a.m.u. The system was calibrated internally using dibutyl-phthalate as lock mass at m/z 279.1591, giving a mass accuracy lower than 1 ppm. The instruments were controlled using the Xcalibur software (Thermo Fischer Scientific, USA). LC-MS grade methanol and acetonitrile were purchased from Roth Sochiel (France); water was provided by a Millipore water purification system. Apigenin and chloramphenicol (Sigma-Aldrich, France) was used as internal standard.

Statistical analyses

Protection efficacy conferred by Rh-Est-C12 on wheat plants in the greenhouse was analyzed using a One-Way analysis of variance (ANOVA) at $P \leq 0.05$, using GraphPad Prism software version 9 (GraphPad Software Inc., San Diego, USA). *In planta* spore germination and hyphal growth were analyzed using the Tukey test at $P \leq 0.05$, using GraphPad Prism software version 9. Half-maximal inhibitory concentration (IC_{50}) was calculated from the dose-response curve of Rh-Est-C12 using the GraphPad Prism software version 9.

Gene expression data in the transcriptomic assay were normalized by Quantile algorithm. The three control samples were filtered for $P < 0.05$ and the average was calculated for each gene. A fold change value was calculated for each gene between individual treated samples and the mean of corresponding controls. Differentially expressed genes (DEGs) were selected for a significant threshold > 2.0 or < 0.5 ($P < 0.05$). Functional annotations of DEGs were based on NCBI GenBank and related-genes physiological processes were assigned with NCBI, AmiGO 2 Gene Ontology and UniProt. Kyoto encyclopedia of genes and genomes (KEGG) pathway analysis was also used to identify relevant biological pathways for the selected genes. However, DEGs may be involved in more than one biological process, especially when it comes to stress responses, hence the determined functional annotation could be subjected to modifications in the future.

In the metabolomic assay, metabolite identification and their classification into different chemical families were based on previously published works on benzoxazinoids (de Bruijn *et al.*, 2016), flavonoids (Wojakowska *et al.*, 2013) and hydroxycinnamic acid amides (Li *et al.*, 2018) from wheat. Besides, the proposed putative metabolite identifications was based on expertized analysis of the corresponding mass spectra and comparison with published reports in literature. Additional information was retrieved from the KEGG and PubChem databases. The relative quantification of the selected metabolites was performed using the software Xcalibur. The identity of some metabolites was confirmed using the corresponding standards provided by Sigma-Aldrich (France). Differential metabolomic analyses among the different conditions were performed using the Tukey's Honest Significant Difference method followed by a false discovery rate (FDR) correction using the Benjamini-Hochberg procedure (Benjamini and Hochberg, 1995). Metabolites of interest were considered differentially accumulated when the false discovery rate was below 5 % (FDR $\leq$ 0.05).

RESULTS

Rh-Est-C12 displays direct antifungal activity and reduces disease severity caused by *Z. tritici*

Rh-Est-C12 induced-protection in wheat against *Z. tritici* was assessed in the greenhouse. At 23 days after treatment (D23), *i.e.* 21 days after inoculation, disease symptom level scored on the third leaves of the non-treated inoculated control plants consisted of 55.7% of diseased leaf area. Preventive treatment two days prior to inoculation with *Z. tritici* with a Rh-Est-C12 solution at 500 mg.L⁻¹ resulted in a significant reduction of the disease symptoms caused by the pathogen, with only 32.8% of diseased leaf area on the treated third leaves, corresponding to a reduction of 41.1% (**Figure 1B**). No phytotoxic effect was observed on wheat leaves at the tested concentration. On the other hand, *in vitro* bioassays were performed in order to assess the direct antifungal effect of Rh-Est-C12 on the fungus. Dose-response curve revealed a total inhibition of the *in vitro* fungal growth by the concentration of 31.2 mg.L⁻¹ and an IC₅₀ value of 26.8 mg.L⁻¹ (**Figure 1C and D**). Finally, *in planta* cytological staining was carried out to examine the RL effect on *Z. tritici* early infection process. The results revealed that Rh-Est-C12 impacts at D5 (five days after treatment) both spore germination and the epiphytic hyphal growth of the fungus. The proportion of each class of spores was significantly different between Rh-Est-C12-treated and control plants (**Figure 1E**). On control plants at D5, *Z. tritici* spores were mainly germinated with a developed or strongly developed germ tube (class 1 and 2), while on Rh-Est-C12 treated plants, *Z. tritici* spores were less developed and were mostly present as non-germinated spores or germinated spores but only with a small germ tube (class 3 and 4) (**Figure 1E**).

Rh-Est-C12 did not induce any major modifications in wheat leaf gene expression

RNA microarray chip assay was performed to investigate the effect of Rh-Est-C12 on wheat transcriptome under absence or presence of *Z. tritici*, at two days after treatment (D2) in non-infectious conditions, and at five days after treatment (D5), *i.e.* three days after inoculation, in both non-infectious and infectious conditions. These two time points were chosen because they correspond to early stages of both *Z. tritici* infection (D5) and plant defense mechanism induction (D2). The eliciting potential of Rh-Est-C12 was assessed at D2 and D5 by comparing treated and non-treated plants under non-infectious conditions. The priming of plant defenses was evaluated at D5 by comparing treated and non-treated plants under infectious conditions (treated and inoculated plants vs non-treated

inoculated plants), by also taking into account the potential elicitation effect of Rh-Est-C12 at D2 and D5. Moreover, additional comparisons were performed (treated and inoculated plants vs treated non-inoculated plants, and treated and inoculated plants vs non-treated non-inoculated plants) to get a deeper understanding of the potential priming effect of Rh-Est-C12 on wheat plants (**Supplementary Table S2, Supplementary Figures S1 and S2**). Thereafter, the different comparisons will be referred to as eliciting, fungal, and priming effects, corresponding to the elicitation, fungal infection alone, and priming modalities, respectively.

Overall, out of the 43,803 wheat probes available on the RNA microarray chip, 78 differentially expressed genes (DEGs) were highlighted when taking into account all studied modalities and performed comparisons, and at all time points (**Supplementary Table S1**). Overall, only a weak regulation of gene expression was observed in both elicitation (24 DEGs, at D2 and D5) and priming (25 DEGs, at D5) modalities, when compared to the transcriptional effect of the fungal infection alone (38 DEGs, D5) (**Figure 2B**). Only two common DEGs were identified at D5 between priming and fungal effect modalities (**Figure 2A**). Interestingly, most of the changes observed in gene expression profiles correspond to downregulations, except at D2 in eliciting modality, where a higher number of upregulated genes was found when compared to downregulated ones (**Figure 4**).

When considering the eliciting modality, at D5, almost no transcriptional effect was observed in the treated wheat plants. We could notice a downregulation of the expression of two DEGs, one encodes for class 1 chitinase, and the other encodes for “rDNA tandem repeat with genes for 18S, 5.8S and 26S rRNA” (**Figure 4**). However, at an earlier post-treatment stage (D2), 22 DEGs were found. These genes encode for proteins involved in responses to abiotic and oxidative stresses, growth and development, cell-wall structure and function, pigment biosynthesis and chloroplast structure, light-harvesting, photosynthesis and carbohydrate metabolism, protein metabolism, as well as signaling transcription, and translation (**Figure 3**). Regarding the priming modality at D5, among the 25 DEGs highlighted, only seven were up-regulated, while 18 were down-regulated. All of these DEGs were different from the ones found in eliciting and fungal effect modalities, except two DEGs encoding for heat shock protein 80 and heat shock protein 70-like, which were also deregulated in fungal effect modality (**Figures 2 and 4**). Genes regulated in the priming modality encode for proteins involved in diverse functions and belong to all targeted functional groups of genes. Finally, fungal infection alone displayed the most important effect on the wheat leaf transcriptome when compared to eliciting and priming modalities. Again, most of the changes observed were due to gene downregulations (six genes upregulated vs 32 genes downregulated). All functional groups of genes were represented in this modality, except the gene functional group light-harvesting, photosynthesis and carbohydrate metabolism (**Figure 3 and 4**).

Rh-Est-C12 also did not cause marked change in wheat leaf metabolite accumulation

An UHPLC-MS analysis was carried out to examine the effect of Rh-Est-C12 on the wheat leaf metabolome, at the same time points as those selected for transcriptomic analysis (D2 and D5) and an additional time point at 15 days after treatment (D15), *i.e* thirteen days after inoculation. This time should coincide with the moment preceding the switch of the fungus from the biotrophic to the necrotrophic phase. All comparisons aiming at investigating the eliciting, priming, and fungal effects were undertaken according to the same pairwise comparison performed above in transcriptomic

assay. A total of 54 metabolites was identified when considering all modalities and performed comparisons, and all time points (**Supplementary Table S3**). PCA analysis revealed a significant clustering of the treated and/or inoculated plants, indicating that Rh-Est-C12 and/or *Z. tritici* affect wheat metabolite patterns (**Figure 5**).

When focusing on eliciting modality, only few differentially accumulated metabolites (DAMs) within the treated non-inoculated leaves were highlighted at the three targeted time points (**Figure 6**). At D2, one amino acid (asparagine) was found under-accumulated, whereas at D5, the concentrations of two phytohormones and precursors (OPDA and MeJA) were significantly decreased. Moreover, two amino acids (valine and proline) were over-accumulated at this later time-point. At D15, six metabolites were significantly under-accumulated, one amino acid (valine), one phytohormone, abscisic acid glucose ester (ABA-Glc), and four flavonoids, luteolin-6-C-hexoside (luteolin-6-C-Glc), luteolin-C-hexosyl-deoxyhexoside (luteolin-C-hexo-O-deoxyhexo), luteolin-C-hexosyl-C-pentoside (luteolin-C-hexo-C-pento) and triclin. In priming modality, more DAMs were observed than in eliciting modality, but at D5 only. At D5, 13 DAMs were under-accumulated, seven of them are amino acids, leucine-isoleucine, phenylalanine, tyrosine, arginine, histidine, lysine, and asparagine, five are benzoxazinoids, HBOA, HBOA-glucoside (HBOAGlc), HMBOA-hexoside (HMBOAGlc), HM2BOA, HM2BOA-hexoside (HM2BOAGlc) and one is an hydroxycinnamic acid amide, feruloylagmatine. It is to notice that most of these under-accumulated DAMs were the same than those found over-accumulated in fungal effect modality. The overaccumulation of only one metabolite, the amino acid aspartic acid, can be associated to the priming modality. No DAMs belonging to the flavonoid and phytohormone families were highlighted at this time point. At D15, however, an increase in the concentration of three flavonoids, luteolin-6-C-Glc, luteolin-C-hexo-C-pento, and chrysoeriol-6-C-hexoside (chrysoeriol-6-C-Glc), was observed. Fungal infection alone caused more changes in leaf metabolite concentrations than eliciting and priming modalities. At D5, among the 54 identified metabolites, 24 were differently accumulated (17 over-accumulated, seven under-accumulated). At D15, considerably less modifications were detected, with seven DAMs only, among them six under-accumulated and one over-accumulated (**Figure6**).

DISCUSSION

We explored here, using a combined transcriptomic and metabolomic approach, the ability of a bioinspired mono-RL (Rh-Est-C12) to trigger defense reactions in wheat towards *Z. tritici*, a significantly damaging pathogen on this major agro-economic crop. The results confirmed that Rh-Est-C12 significantly protects wheat against the pathogen by reducing disease severity by almost half, and displays a direct antimicrobial activity towards the fungus both *in vitro* and *in planta*, thus agreeing with a previous report testing the potential activity of this RL, as well as other RLs with different structures, on the wheat-*Z. tritici* pathosystem (Platel *et al.* 2021). The protective and antimicrobial activity of RLs against phytopathogens, including fungi and oomycetes, have been reported in many studies, as reviewed recently by Crouzet *et al.* (2020). However, studies investigating the RL-induced resistance using omics approaches are lacking. Our results reveal that the examined mono-RL, Rh-Est-C12, does not induce major modifications in either the patterns of gene expression or metabolite accumulation, when considering both eliciting and priming modalities.

Surprisingly, only a slight number of DEGs associated with response to pathogens was observed, while a more important set of genes involved in other functions was recorded in both eliciting and priming modalities. Among the DEGs detected in the eliciting modality, a much higher number (22) was scored at D2 than at D5 (2), suggesting that the slight eliciting effect potentially conferred by Rh-Est-C12 at the earlier time point D2 is not persistent and seems to disappear three days later, at D5. Out of the 22 detected DEGs, two encode for heat shock proteins (HSPs), 22.3kDa heat-shock protein and heat shock protein 80. These chaperone proteins are crucial molecules involved in plant defense against abiotic and biotic stresses. As reviewed by ul Haq *et al.* (2019), HSPs may increase ROS scavenging by enhancing plant antioxidant enzymes. Interestingly, we have previously found that Rh-Est-C12 can increase catalase and peroxidase activities in wheat (Platel *et al.*, 2021). Besides, HSPs are also considered to play a role in membrane stability (ul Haq *et al.*, 2019). Remarkably, RLs are supposed to inhibit microbial pathogens and trigger plant immunity defenses by interacting directly with their plasma membranes (Crouzet *et al.*, 2020). Hence, the accumulation of HSP transcripts could suggest that Rh-Est-C12 can affect wheat-cell membrane stability, by mimicking an abiotic stress. This potential “abiotic stress-like” mode of action of Rh-Est-C12 could explain the deregulation of abiotic stress response-associated DEGs observed in this modality, such as cold acclimation protein WCOR518, fasciclin-like protein FLA15, β -carotene hydroxylase, peroxidase 6, β -amylase, and myb-related protein MYBAS1 (Davison *et al.*, 2002 ; Kaplan and Guy, 2004 ; Svensson *et al.*, 2006 ; You and Chan, 2015 ; Zang *et al.*, 2015 ; Peixoto-Junior *et al.*, 2018). At the metabolome level, we detected, at D5, the accumulation of two amino acids, valine and proline, and the under-accumulation of two phytohormones and precursors, OPDA and MeJA. Proline is an osmolyte metabolite involved in adaptation and tolerance to a large variety of abiotic stresses, such as osmotic, freezing, and cold stress (Szabados and Savouré, 2010 ; Lv *et al.*, 2011). The accumulation of this metabolite could likely be a consequence of the Rh-Est-C12 “abiotic stress-like” effect on wheat. At D15 also, the concentration of only a few DAMs was altered. Taken as a whole, these results suggest that Rh-Est-C12 treatment, in the elicitation modality, triggers some modifications in the wheat plant transcriptome and metabolome but its effect is very limited, especially when compared to the deregulation levels induced by other elicitors in other plants. For instance, Landi *et al.* (2017) recently reported, using RNA-seq, that preharvest treatment of strawberry fruit with chitosan and BTH, induced modifications in the expression of 5,062 and 5,210 transcripts, respectively, at 6, 12 and 24 hours post-treatment, compared to control conditions. In grapevine, plant treatment with the beta-glucan laminarin and its sulfated derivative (PS3) induced the regulation of 94 and 132 genes at 12 hours post-treatment, respectively, highlighted Gauthier *et al.* (2014) using a microarray chip. The fact that Rh-Est-C12 induces only slight alterations on the plant transcriptome and metabolome, even with the high used concentration (500 mg.L⁻¹), indicates that this RL can provide protection to wheat against *Z. tritici* without a high fitness cost on the plant. Such a positive property is sought after for crop protection compounds, since yield should not be negatively impacted by compounds having insignificant impact on the plant physiology. (Cipollini *et al.*, 2017). At the opposite, elicitors displaying a large extent of alterations on the host physiology, as those presented above, may lead to protection but with, possibly, higher allocation costs within the plant (Tripathi *et al.*, 2019). The suggested neutral effect of Rh-Est-C12 on wheat fitness cost should furthermore be ascertained on wheat grain yield.

In the modality assessing the potential priming effect of Rh-Est-C12, we detected significant downregulation of 18 genes at D5, among them six are involved in response to oxidative stress

(encoding mainly for peroxidases) and five related to light harvesting, photosynthesis, and carbohydrate metabolism (encoding for chlorophyll a/b-binding protein WCAB precursor, chloroplast pigment-binding protein CP24, light-harvesting complex I, light-induced protein 1-like and light-regulated protein precursor). The other downregulated DEGs were associated with other functions. Two of these DEGs are particularly interesting in plant defense against pathogens, (i) the xylanase inhibitor TAXI-III that was reported as being potentially involved in durum wheat resistance against *Fusarium graminearum*, and (ii) the papain-like cysteine peptidase that was often described as a central key-enzyme in plant immunity (Moscetti *et al.*, 2013 ; Misas-Villamil *et al.*, 2016). Additionally, six DEGs were over-expressed (genes encoding for aquaporin PIP1, ATP-dependent RNA helicase, probable pyridoxal biosynthesis protein PDX1.1, sterol desaturase family protein, S-adenosylmethionine synthetase and heat-shock protein 80). Among these genes, some may be involved in plant defense against biotic stress, like those encoding for aquaporin PIP1 and ATP-dependent RNA helicase (Zhang *et al.*, 2014 ; Li *et al.*, 2020). Remarkably, out of all the 24 detected DEGs in the priming modality, only the one encoding for heat-shock protein 80 was found in another condition (*Z. tritici* infection alone) at D5. In addition, the RL effect in priming modality seems weak, with only a few of the detected DEGs thought to be involved in plant resistance to pathogens. On the wheat metabolome, also, only a few changes were observed. At D5, aspartic acid was the only metabolite significantly over-accumulated, whereas 13 DAMs were under-accumulated. Most of them, however, were found over-accumulated in fungal effect modality and totally absent in the comparison treated-inoculated vs non-treated non-inoculated (**Supplementary Figure S3**), suggesting that the observed metabolite under-accumulations in the priming modality at D5 are not conferred by the RL treatment but rather by the reduced fungal infection only. Finally, at D15, three flavonoid metabolites were over-accumulated. Flavonoids are secondary metabolites biosynthesized from phenylpropanoid pathway, which have been described as antimicrobial and antioxidant compounds that could be involved in wheat defense against biotic stress (Pietta, 2000).

Insights regarding the effect of *Z. tritici* alone on the wheat transcriptome and metabolome in the case of a compatible interaction have also been acquired. At D5, we observed alterations in the accumulation of transcripts of 43 genes (with all functional groups represented, except light harvesting, photosynthesis, carbohydrate metabolism) and 26 metabolites (all functional or chemical families represented), much more than in the other modalities (*i.e* elicitation and priming ones). The regulation of these DEGs and DAMs may be caused by (i) plant recognition of the fungus and (ii) the subsequent defense response deployment, *i.e* upregulation of genes encoding for pathogenesis-related (PR) proteins, chitinases and thaumatin-like proteins, as well as over-accumulation of specific amino acids, benzoxazinoids, and hydroxycinnamic acid amides. Noteworthy, PR-proteins are key-components of the plant immune system (Ali *et al.*, 2018). Benzoxazinoids are plant defense metabolites found in many *Poaceae* species and are known to display antimicrobial and allelopathic activities, as well as to regulate callose deposition, like in maize (Hashimoto and Shudo, 1996 ; De Bruijn *et al.*, 2018). Hydroxycinnamic acid amides are another family of plant secondary metabolites reported to be positively correlated with plant resistance in several pathosystems (Muroi *et al.*, 2009 ; Gunnaiah *et al.*, 2012 ; Yogendra *et al.*, 2014). Seybold *et al.* (2020) described significant modifications in the accumulation of benzoxazinoids and hydroxycinnamic acid amides during *Z. tritici* infection, between susceptible and resistant cultivars, underlining the potential importance of these metabolites in wheat resistance towards the pathogen. Interestingly, we did not observe any significant

accumulation of benzoxazinoids at D15. Seybold *et al.* (2020) also observed an inhibition of benzoxazinoid accumulation in a susceptible wheat cultivar (Obelisk) at an early stage of *Z. tritici* infection only, hypothetically explained by fungal effector secretion and host-defense manipulation by the pathogen during the studied interaction.

Another possible explanation of the observed modification of DEGs and DAMs in wheat leaves during the infection alone is the “hijacking” of plant metabolism by the fungus to facilitate its invasion. Indeed, still at D5, 37 out of the 43 detected DEGs were downregulated. These genes were particularly associated with response to stress (abiotic, oxidative, pathogens) and secondary metabolism, as well as cell-wall structure and function and amino acid and protein metabolism, suggesting a deleterious effect of the fungus on these functions. Our results suggest that *Z. tritici* could impair cell-wall strengthening in Alixan cultivar. Indeed, the fungal infection induced downregulation of six DEGs associated with cell wall structure and function, including genes encoding for cinnamyl alcohol dehydrogenase and non-specific lipid transfer proteins and precursors. Cinnamyl alcohol dehydrogenase is a key enzyme of lignin biosynthesis pathway, for which encoding genes were also found downregulated by Rudd *et al.* (2015) on wheat at one day after infection with *Z. tritici*. Seybold *et al.* (2020) showed lignin under-accumulation in a susceptible cultivar (Obelisk) compared to a resistant one (Chinese spring), suggesting the role of this crucial monolignol polymer in wheat resistance to *Z. tritici*. Moreover, non-specific lipid transfer proteins (nsLTPs) also play critical roles in protective mechanisms, such as cell-wall organization, against pathogens and have even been classified as part of the PR-14 family. The other functions of this protein family, such as antimicrobial activity or systemic acquired resistance (SAR) signaling, in plant response to biotic stress, have been reviewed by Liu *et al.* (2015). Moreover, we observed, at D15, an under-accumulation of five flavonoids. Flavonoids are known to accumulate in cell walls during infection (Mierziak *et al.*, 2014). Decrease in their concentration may be caused by an overcoming of wheat defense reactions by the fungus. In addition, we observed significant under-accumulation of MeJA and MeSA at D5, suggesting a repression of jasmonic acid (JA) and salicylic acid (SA) pathways at this time point. These phytohormones are particularly crucial in defenses against pathogen attacks. Usually, SA is often considered to be the key hormone regulating defenses against biotrophic pathogens, while JA would trigger defense mechanisms towards necrotrophic invaders. MeSA, a methyl form of SA, would be responsible for SAR (Thaler *et al.*, 2012 ; Chen *et al.*, 2019). In the wheat-*Z. tritici* pathosystem, Rudd *et al.* (2015) also detected a downregulation of SA and JA pathways at the beginning of *Z. tritici* biotrophic phase on the susceptible cultivar Riband. The authors also reported an SA accumulation during the fungal necrotrophic phase, whereas we observed a significant increase in SA concentration in wheat leaves at D15, *i.e* at the end of the biotrophic phase, suggesting that SA regulation during the *Z. tritici* infection is cultivar-dependent.

In conclusion, although Rh-Est-C12 treatment triggers significant regulations, especially on the wheat leaf gene expression at D2, possibly caused by “abiotic stress-like” mimicking due to its interaction with plant plasma membranes, it would appear that the RL protects wheat against *Z. tritici* without causing major alterations on the plant metabolism. Our results suggest that (i) Rh-Est-C12 likely displays a neutral fitness cost on the plant at the tested concentration and (ii) that the protection efficacy conferred by this molecule is mainly due to its direct antifungal activity against the pathogen

and to a lesser extent to an induction of plant defenses. This study also provides new insights into the molecular bases of the wheat-*Z. tritici* interaction.

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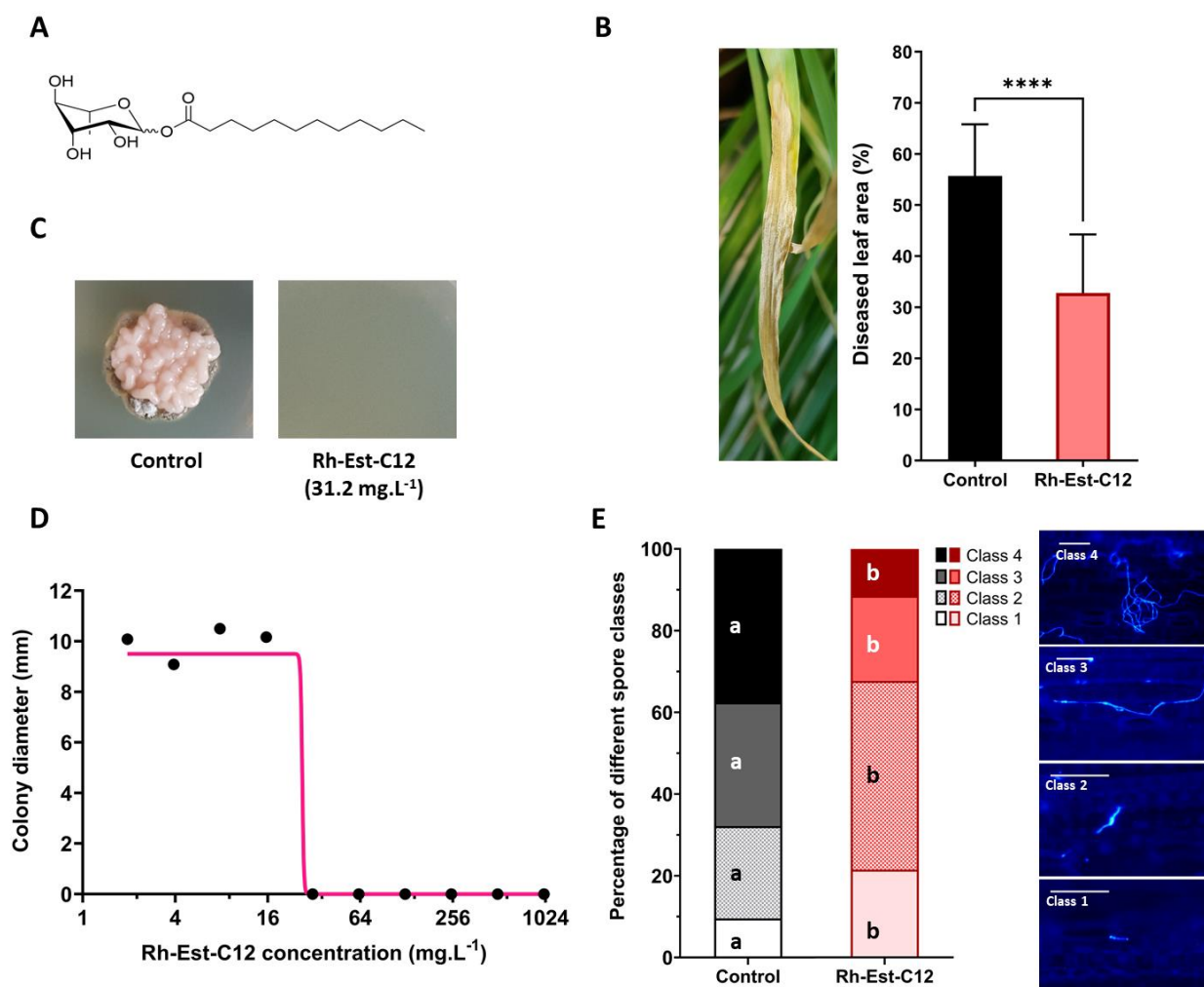
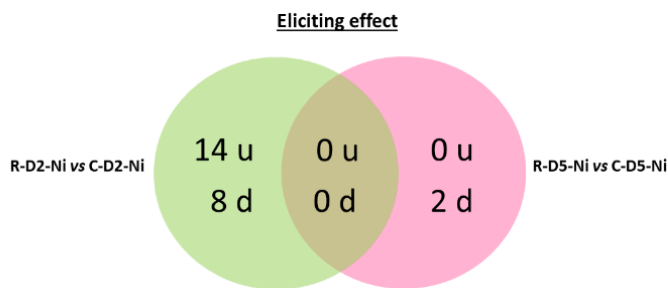


Figure 1. Protective and antifungal effect of the Rh-Est-C12 on wheat (Cv. Alixan) towards *Zymoseptoria tritici* (T02596 strain). **(A)** Molecular structure of the bioinspired synthetic rhamnolipid (RL) Rh-Eth-C12 used in this study. The RL is composed by a rhamnose linked to a C12-carbon chain with an ester bond. **(B)** The disease severity level of septoria tritici blotch was assessed at 23 days after treatment (D23) with Rh-Est-C12 at 500 mg.L⁻¹ or mock solution, *i.e* at 21 days after inoculation with the pathogen, by scoring the area chlorosis and necrosis on wheat third-leaves. Results obtained with treated plants were significantly different from control plants according to unpaired t-test ($P \leq 0.05$). Bars stand for standard-deviation ($n=36$). Representative picture of STB symptoms at D23 on non-treated wheat is shown at the left of the figure. Pictures illustrating colonies in the below *in vitro* assay are presented in **(C)**. **(D)** *In vitro* antifungal dose-response curve of Rh-Est-C12 against *Zymoseptoria tritici* (T02596 strain). The direct antifungal activity of the rhamnolipid against the fungal pathogen was assessed by measuring perpendicular diameters of *Z. tritici* colonies after 10 days on PDA medium plates supplemented with different concentrations of the surfactant ($n=6$). Non-linear regression was designed using GraphPad Prism software version 9. **(E)** *In planta* epiphytic spore development state (class 1: non-germinated spore; class 2: germinated spore with small germ tube; class 3: germinated spore with developed germ tube; class 4: germinated spore with a strongly developed germ tube) was assessed using Calcofluor dye at 5 days after treatment with the Rh-Est-C12 at 500 mg.L⁻¹, *i.e* 3 days after inoculation. The class of 100 distinct spores, selected randomly, was determined, for each condition, on each of the nine third-leaf segments sampled. Within each spore class, the presence of different letters indicates a significant difference according to the Tukey test at $P \leq 0.05$. Scale bar = 25 μ m.

A



B

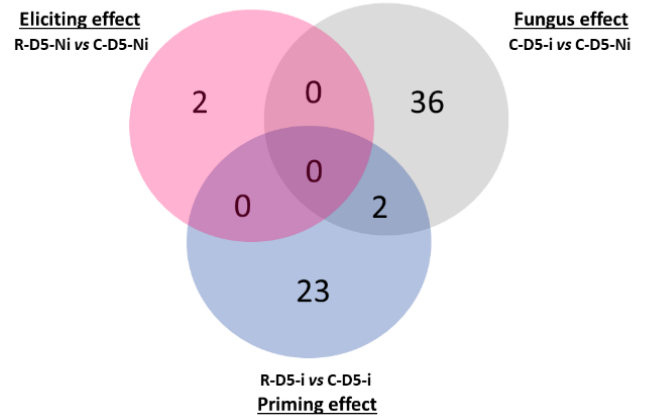


Figure 2: Venn diagrams of the differentially expressed gene number observed during Rh-Est-C12 eliciting, priming as well as *Z. tritici* effects. (A) The effect of the rhamnolipid alone, applied at 500 mg.L⁻¹, on the number of up-regulated (u) and down-regulated (d) was explored at 2 and 5 days after treatment (R-D2-Ni vs C-D2-Ni and R-D5-Ni vs C-D5-Ni respectively). (B) The eliciting (R-D5-Ni vs C-D5-Ni), fungal (C-D5-i vs C-D5-Ni) and priming effects (R-D5-i vs C-D5-i) on significantly deregulated gene number in wheat third-leaves (Cv. Alixan) were compared at five days after treatment with Rh-Est-C12, *i.e* at three days after infection with *Z. tritici* (T02596 strain). About the codification, C stands for treatment with mock solution while R is for Rh-Est-C12 application. Ni concerns mock inoculated wheat while i means inoculated plants with *Z. tritici*. D2 or D5 indicate that third-leaves were respectively collected at 2 or 5 days after treatment.

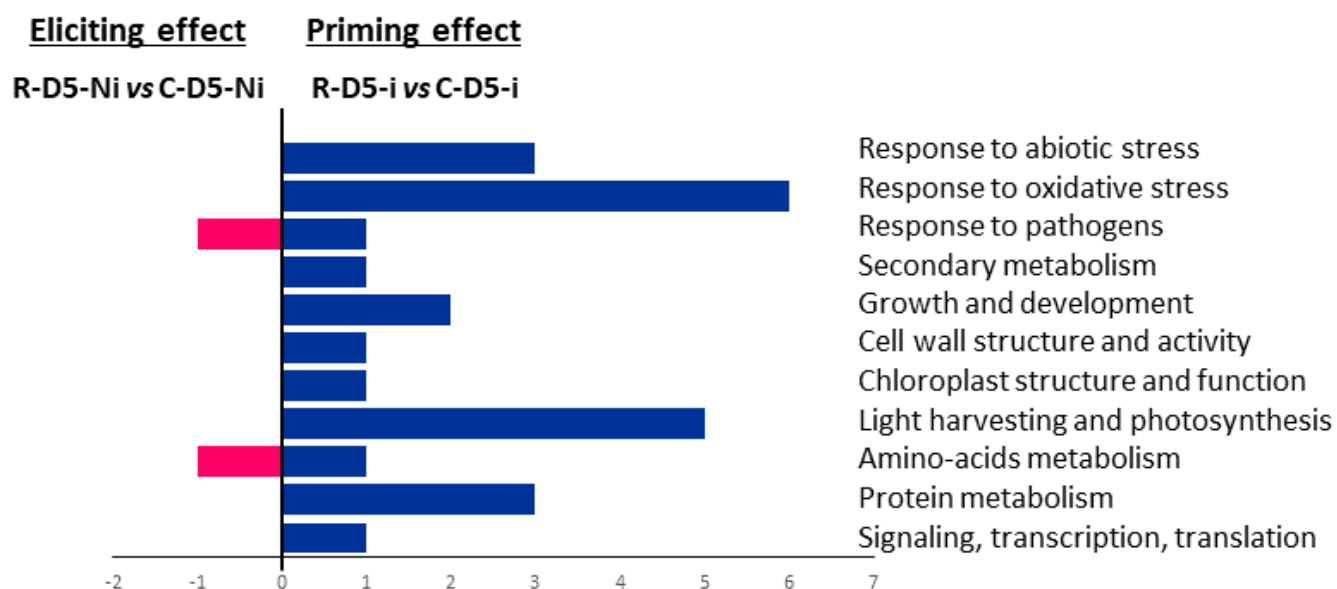


Figure 3: Functional groups of differentially expressed gene number observed in eliciting conditions (R-D5-Ni vs C-D5-Ni) compared to priming (R-D5-i vs C-D5-i). DEGs were classified in the different functional groups according to NCBI GenBank. About the codification, C stands for treatment with mock solution while R is for Rh-Est-C12 application. Ni concerns mock inoculated wheat while i means inoculated plants with *Z. tritici*. D2 and D5 indicate that third-leaves were respectively collected at 2 and 5 days after treatment.

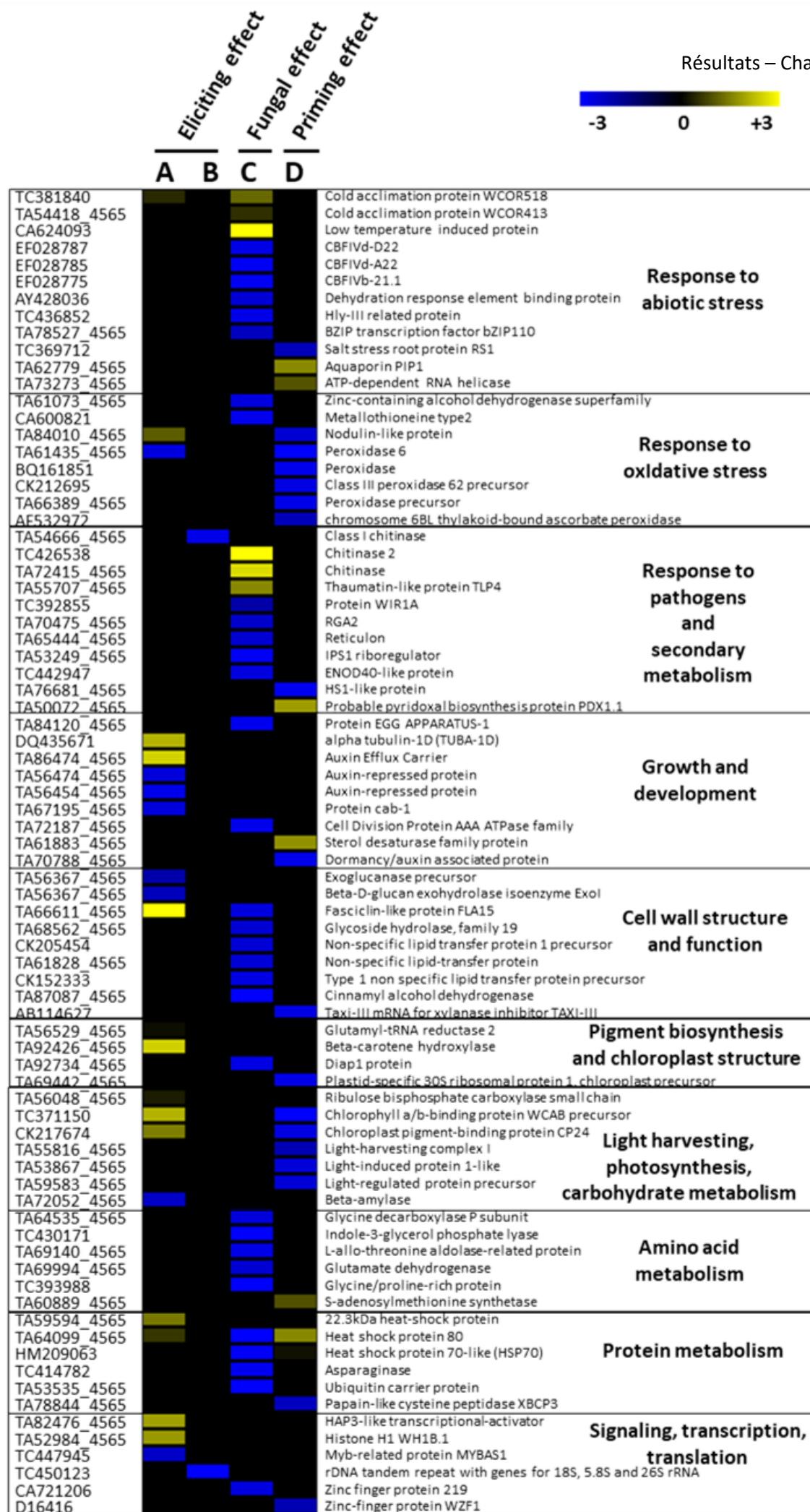


Figure 4: Heatmap representation of gene expression deregulation in wheat leaves (Cv. Alixan) caused by the foliar application of 500 mg.L⁻¹ Rh-Est-C12 and/or inoculation with *Z. tritici* (T02596 strain) at two (A) and five days after treatment (B, C, D). Columns (A) and (B) illustrate the effect of the rhamnolipid without further inoculation with the fungus, *i.e* the eliciting effect, respectively at two (R-D2-Ni vs C-D2-Ni) and five days (R-D5-Ni vs C-D5-Ni) after treatment. In (C) is represented the fungal action alone (C-D5-i vs C-D5-Ni) while (D) shows the potential priming activity of Rh-Est-C12 (R-D5-i vs C-D5-i). Gene-related physiological processes are represented on the right part of the heatmap and were determined using NCBI, AmiGO 2 Gene Ontology, KEGG and UniProt. Significant relative change in gene transcription is expressed in Log₂ ratio, according to the yellow-blue color scale, using the WebMev software. C stands for treatment with mock solution while R is for Rh-Est-C12 application. Ni concerns mock inoculated wheat while i means inoculated plants with *Z. tritici*. D2 and D5 indicate that third-leaves were respectively collected at 2 and 5 days after treatment.

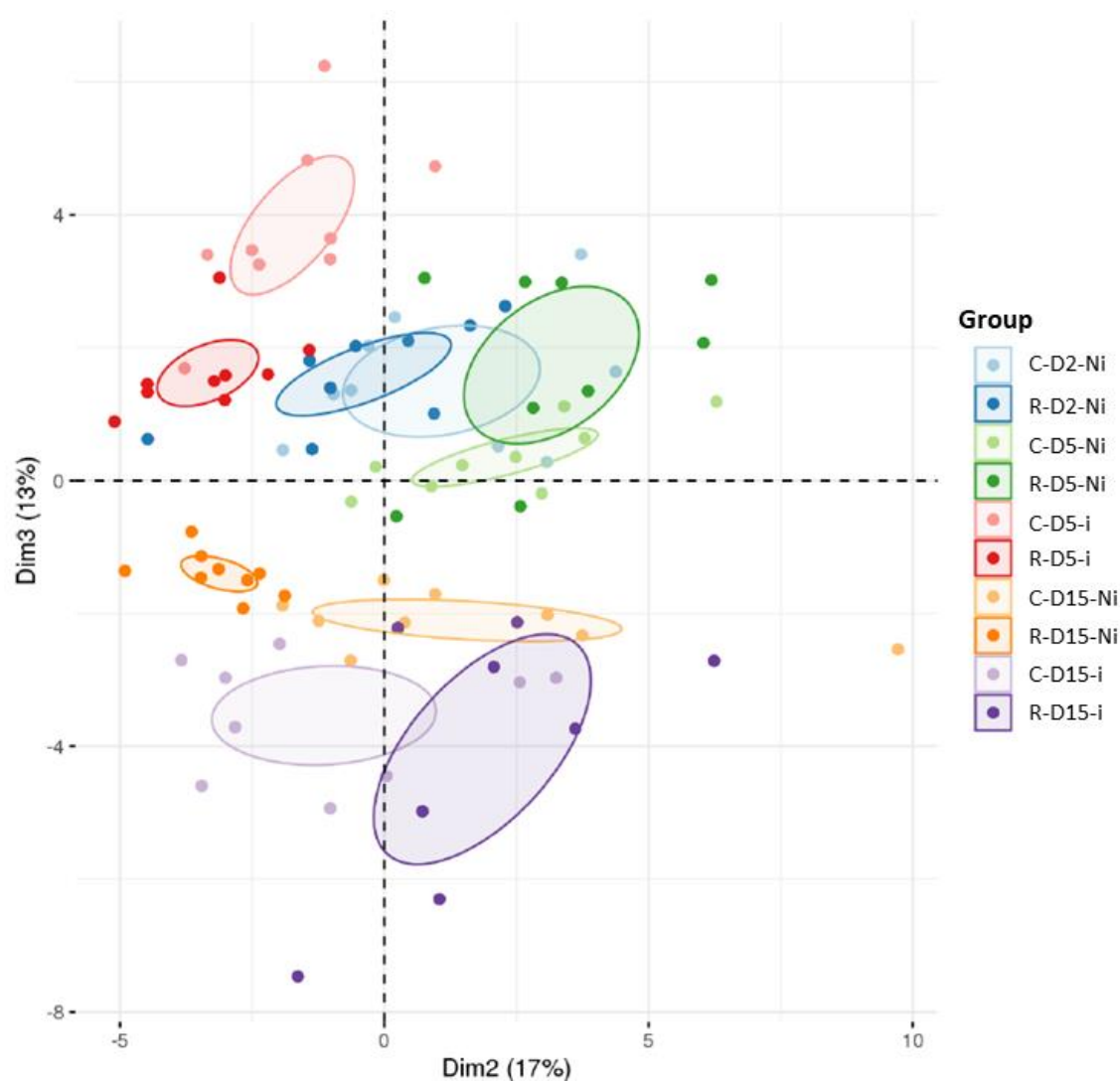
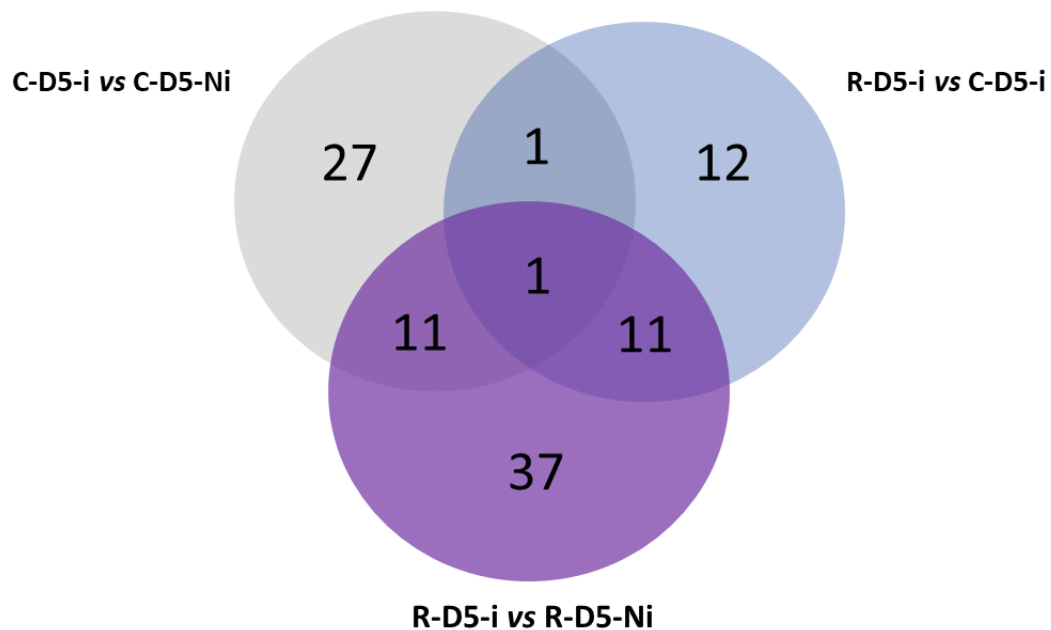


Figure 5: Principal component analysis (PCA) of targeted metabolomic data sets from wheat leaves treated or not with Rh-Est-C12 and/or inoculated with *Z. tritici*. For each modality, nine biological replicates were used. C stands for treatment with mock solution while R is for Rh-Est-C12 application. Ni concerns mock inoculated wheat while i means inoculated plants with *Z. tritici*. D2, D5 and D15 indicate that third-leaves were respectively collected at 2, 5 and 15 days after treatment.



Figure 6: Heatmap representation of differentially accumulated metabolites within wheat third-leaves (Cv. Alixan) treated or not with Rh-Est-C12 and infected with *Z. tritici* (T02596 strain) or not, at different time-points. With metabolite relative accumulation changes in the (A) eliciting modalities, from the left to the right, R-D2-Ni vs C-D2-Ni, R-D5-Ni vs C-D5-Ni and R-D15-Ni vs C-D15-Ni; (B) fungal modalities, C-D5-i vs C-D5-Ni and C-D15-i vs C-D15-Ni; (C) priming modalities, R-D5-i vs C-D5-i and R-D15-i vs C-D15-i. C stands for treatment with mock solution while R is for Rh-Est-C12 application. Ni concerns mock inoculated wheat while i means inoculated plants with *Z. tritici*. D2, D5 and D15 indicate that third-leaves were respectively collected at 2, 5 and 15 days after treatment. Log2 of significant metabolite fold changes for indicated pairwise comparisons are given by shades of red or blue colors according to the corresponding scale bar. Metabolites were grouped according to their functional or chemical family as amino acids, benzoxazinoids, flavonoids, hormones and hydroxycinnamic acid amides. Data represent mean values of nine biological replicates for each condition and time point. Statistical analyses were performed using the Tukey's Honest Significant Difference method followed by a false discovery rate (FDR) correction, with $FDR < 0.05$. For $FDR \geq 0.05$, Log2 fold changes were set to 0.



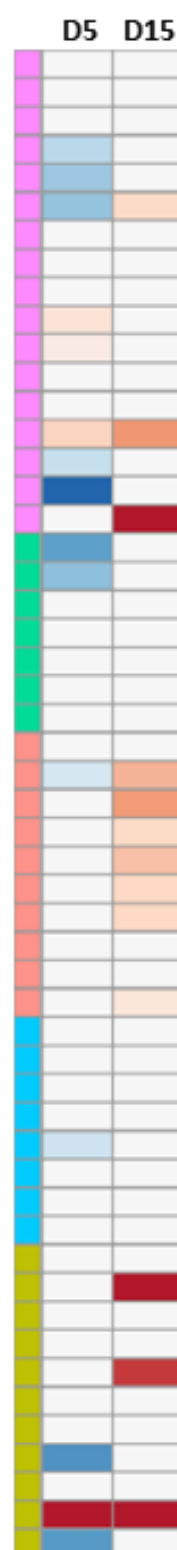
Supplementary Figure S1: Venn diagram of the number of DEGs observed during the fungus effect (C-D5-i vs C-D5-Ni), the priming effect (R-D5-i vs C-D5-i) and the whole deregulation on gene expression caused by the rhamnolipid treatment followed by *Z. tritici* infection (R-D5-i vs C-D5-Ni) at five days after treatment. Wheat cultivar Alixan, *Z. tritici* T02596 strain and a rhamnolipid concentration of 500 mg.L⁻¹ were used. C stands for treatment with mock solution while R is for Rh-Est-C12 application. Ni concerns mock inoculated wheat while i means inoculated plants with *Z. tritici*. D5 indicate that third-leaves were respectively collected at 5 days after treatment.



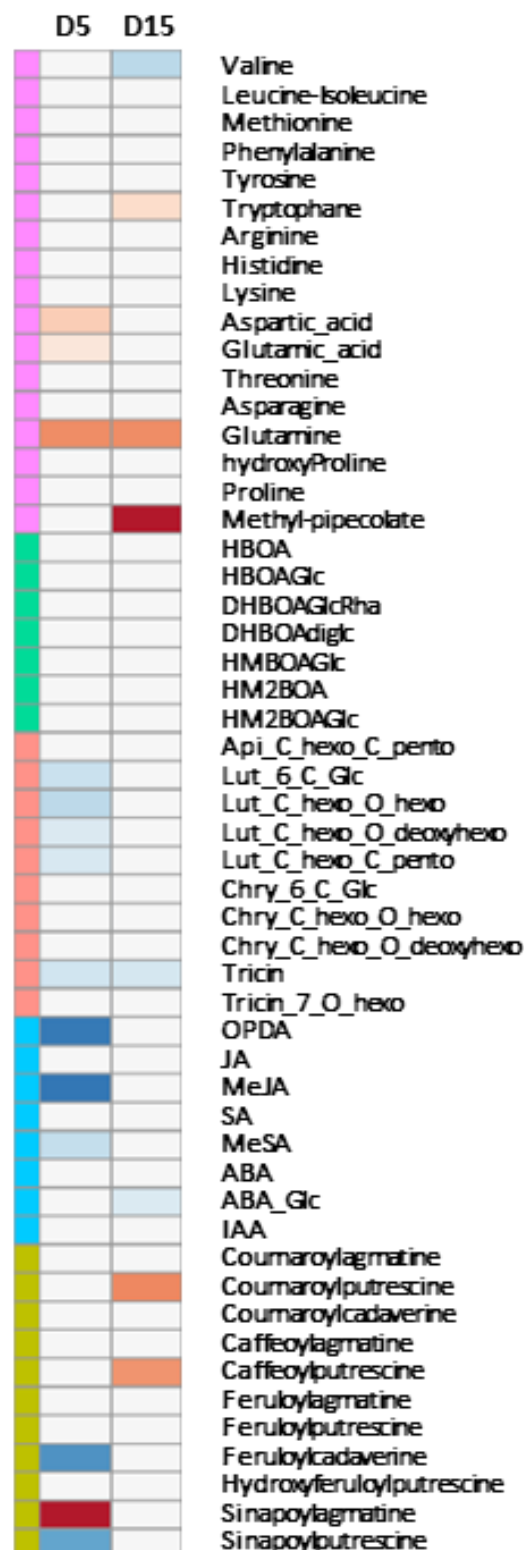
A	B		
TA54418_4565		Cold acclimation protein WCOR413	Response to abiotic stress
U73211		Cold acclimation protein WCOR410c (Wcor410c)	
CA624093		Low temperature induced protein	
AF058794		COR39 (cor39)	
TA73273_4565		ATP-dependent RNA helicase	
TA84187_4565		Protein phosphatase type 2-C	
TA92949_4565		Efflux transporter, RND family, MFP subunit	
TA62230_4565		NAC transcription factor	
CF133748		Myb-related protein	
DQ286566		WRKY transcription factor (WRKY)	
TC421665		Transcription factor Myb2	
AY781354		DREB transcription factor 4A (DREB4)	
EF028787		CBFIVd-D22	
EF028785		CBFIVd-A22	
TC369712		Salt stress root protein RS1	
TC436852		Hly-III related proteins	
TA59927_4565		Monodehydroascorbate reductase	
TA59060_4565		22 kDa drought-inducible protein	
TA102260_4565		Nitrite reductase apoprotein	Response to oxidative stress
TC401354		Metallothionein-like protein type 3	
BQ161851		Peroxidase	
CK212695		Class III peroxidase 62 precursor	
TA61435_4565		Peroxidase 6	
TC384087		Glutathione S-transferase	
TA61129_4565		Glutathione transferase	
CA600821		Metallothioneine type2	
TC429241		Peptide methionine sulfoxide reductase	
TA51226_4565		Glutaredoxin	
TC426538		Chitinase 2	Response to pathogens
TA72415_4565		Chitinase	
TC442947		ENOD40-like protein	
EF570122		Pathogen-inducible transcription factor ERF3	
TA76681_4565		HS1-like protein	
TA57993_4565		MutT/nudix-like	
TA84120_4565		Protein EGG APPARATUS-1	Flowering and seed maturation
TC404192		AAA ATPase, central region (50.1 kD)-like protein	
TA70093_4565		U2AF small subunit	
TA86268_4565		CONSTANS interacting protein 5	
TA70788_4565		Dormancy/auxin associated protein	Growth and development
TA61980_4565		Abscisic stress ripening-like protein	
DQ435671		Alpha tubulin-1D (TUBA-1D)	
TC405303		Mitogen-activated protein kinase 7	
L27516		Wcs66	
TA61828_4565		Non-specific lipid-transfer protein	Cell wall structure and function
TA66611_4565		Fasciclin-like protein FLA15	
CK152333		Type 1 non specific lipid transfer protein precursor	
TA87087_4565		Cinnamyl alcohol dehydrogenase	
CK207368		Cellulose synthase BoCesA7	
TA77295_4565		3-ketoacyl-CoA synthase	
TA77906_4565		Hydroxyproline-rich glycoprotein-like	
TA60114_4565		UDP-D-xylose epimerase 2	
TA61116_4565		Non-specific lipid-transfer protein 4.3 precursor	
TA92734_4565		Diap1 protein	Chloroplast structure, light harvesting, carbohydrate metabolism
TC371150		Chlorophyll a/b-binding protein WCAB precursor	
CK217674		Chloroplast pigment-binding protein CP24	
TA55816_4565		Light-harvesting complex I	
TA61423_4565		Chloroplast pigment-binding protein CP24	
TA59583_4565		Light-regulated protein precursor	
EU492898		Ribulose-1,5-bisphosphate carboxylase	
TA70102_4565		Phosphoenolpyruvate carboxykinase	
TA91251_4565		Isoform ERG1b of A2WVV5	
TA52838_4565		Glycine rich protein	Amino acid metabolism
TC393988		Glycine/proline-rich protein	
TA69140_4565		L-allo-threonine aldolase-related protein	
TA69994_4565		Glutamate dehydrogenase	
TA82049_4565		Bifunctional aspartokinase/homoserine dehydrogenase 2	Protein metabolism
TA67255_4565		Heat-shock protein precursor	
HM209063		Heat shock protein 70-like (HSP70)	
TC391450		ATP-dependent Clp protease ATP-binding subunit	
JX104551		SNAP34	Lipid metabolism
TA53535_4565		Ubiquitin carrier protein	
TA58123_4565		Fatty acid desaturase	
TA87662_4565		Acyl-ACP thioesterase	
TA52495_4565		Lysophospholipase-like protein	Signaling, transcription, translation, etc.
TA81385_4565		Phosphoenolpyruvate carboxylase	
TA68843_4565		Exonuclease family protein	
TA54635_4565		EF-hand Ca2+-binding protein CCD1	
TA73684_4565		Serine/threonine protein kinase	
D16416		Zinc-finger protein WZF1	
AY881102		Putative MAPK protein kinase (MAPK1a)	
TA74203_4565		Receptor-like protein kinase	
TA85618_4565		DEAD-box ATP-dependent RNA helicase 26	
CA721206		Zinc finger protein 219	
EF040602		ZIM motif-containing protein	
TA51878_4565		Susceptibility homeodomain transcription factor	
TC389894		Tetratricopeptide repeat (TPR)-containing protein-like	

Supplementary Figure S2: Heatmap representation of differentially expressed genes in (A) wheat leaves treated with Rh-Est-C12 consecutively infected with *Z. tritici*, at five days after treatment, compared to non-infected-leaves that underwent only rhamnolipid application (R-D5-i vs R-D5-Ni). In (B), the whole response specter of differential gene expression in wheat leaves caused by foliar application of Rh-Est-C12 and infection with *Z. tritici* compared to control plants is shown (R-D5-i vs C-D5-Ni). Wheat cultivar Alixan, *Z. tritici* T02596 strain and a rhamnolipid concentration of 500 mg.L⁻¹ were used. Gene-related physiological processes are represented on the right part of the heatmap and were determined using NCBI, AmiGO 2 Gene Ontology, KEGG and UniProt. Significant relative change in gene transcription is expressed in Log2 ratio, according to the yellow-blue color scale, using the WebMev software. C stands for treatment with mock solution while R is for Rh-Est-C12 application. Ni concerns mock inoculated wheat while i means inoculated plants with *Z. tritici*. D5 indicate that third-leaves were respectively collected at 5 days after treatment.

A) R-D-i vs R-D-Ni

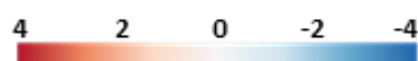


B) R-D-i vs C-D-Ni



Group

- Amino acids
- Benzoxazinoids
- Flavonoids
- Hormones
- Hydroxycinnamic acid amides



Supplementary Figure S3: Heatmap of significant relative changes in metabolite accumulation pattern within wheat third-leaves (Cv. Alixan) following Rh-Est-C12 application at different time-points. Pairwise comparisons performed, from the left to the right, correspond to (A) R-D5-i vs R-D5-Ni, R-D15-i vs R-D15-Ni and (B) R-D5-i vs C-D5-Ni, R-D15-i vs C-D15-Ni. C stands for treatment with mock solution while R is for Rh-Est-C12 application. Ni concerns mock inoculated wheat while i means inoculated plants with *Z. tritici*. D2, D5 and D15 indicate that third-leaves were respectively collected at 2, 5 and 15 days after treatment. Log2 of significant metabolite fold changes for indicated pairwise comparisons are given by shades of red or blue colors according to the corresponding scale bar. Metabolites were grouped according to their functional or chemical family as amino acids, benzoxazinoids, flavonoids, hormones and hydroxycinnamic acid amides. Data represent mean values of nine biological replicates for each condition and time point. Statistical analyses were performed using the Tukey's Honest Significant Difference method followed by a false discovery rate (FDR) correction, with $FDR < 0.05$. For $FDR \geq 0.05$, Log2 fold changes were set to 0.

Supplementary Table S1: Alterations in gene expression pattern responses of wheat leaves caused by the eliciting effect of Rh-Est-C12 at two (A) and five (B) days after treatment, respectively (R-D2-Ni vs C-D2-Ni) and (R-D5-Ni vs C-D5-Ni), the *Zymoseptoria tritici* infection (C-D5-i vs C-D5-Ni) and observed during (D) rhamnolipid priming conditions (R-D5-i vs C-D5-i). This table is related to Figure 4. Wheat cultivar Alixan, *Z. tritici* T02596 strain and a rhamnolipid concentration of 500 mg.L⁻¹ were used. Gene-related physiological processes are represented on the right part of the table and were determined using NCBI, AmiGO 2 Gene Ontology, KEGG and UniProt. C stands for treatment with mock solution while R is for Rh-Est-C12 application. Ni concerns mock inoculated wheat while i means inoculated plants with *Z. tritici*. D2 and D5 indicate that third-leaves were respectively collected at 2 and 5 days after treatment. Top Hit, ID and reference numbers are also presented.

Résultats – Chapitre 2

	Eliciting effect		Fungal effect	Priming effect				
	A	B	C	D				
Name	R-D2-Ni vs C-D2-Ni	R-D5-Ni vs C-D5-Ni	C-D5-i vs C-D5-Ni	R-D5-i vs C-D5-i	Description	TopHit	ID	RefNumber
Response to abiotic stress								
TC381840	2,4760	1,0000	3,0605	1,0000	Cold acclimation protein WCOR518	tc TC381840	A_99_P421457	19082
TA54418_4565	1,0000	1,0000	2,5297	1,0000	Cold acclimation protein WCOR413	ta TA54418_4565	A_99_P210931	43524
CA624093	1,0000	1,0000	8,8824	1,0000	Low temperature induced protein	ta CA624093 ta C	A_99_P459967	11840
EF028787	1,0000	1,0000	0,3043	1,0000	CBFIVd-D22	gb EF028787 ta T	A_99_P405352	948
EF028785	1,0000	1,0000	0,2543	1,0000	CBFIVd-A22	gb EF028785 gb E	A_99_P140518	15660
EF028775	1,0000	1,0000	0,2535	1,0000	CBFIVb-21.1	gb EF028775 tc T	A_99_P012439	20101
AY428036	1,0000	1,0000	0,3768	1,0000	Dehydration response element binding protein	gb AY428036 gb E	A_99_P074325	23922
TC436852	1,0000	1,0000	0,2955	1,0000	Hly-III related protein	tc TC436852	A_99_P511722	6696
TA78527_4565	1,0000	1,0000	0,4594	1,0000	BZIP transcription factor bZIP110	ta TA78527_4565	A_99_P289066	42528
TC369712	1,0000	1,0000	1,0000	0,4755	Salt stress root protein RS1	tc TC369712	A_99_P407642	6772
TA62779_4565	1,0000	1,0000	1,0000	3,4587	Aquaporin PIP1	ta TA62779_4565	A_99_P234106	9903
TA73273_4565	1,0000	1,0000	1,0000	2,8643	ATP-dependent RNA helicase	ta TA73273_4565	A_99_P430447	24209
Response to oxidative stress								
TA61073_4565	1,0000	1,0000	0,3344	1,0000	Zinc-containing alcohol dehydrogenase superfamily	ta TA61073_4565	A_99_P408522	21811
CA600821	1,0000	1,0000	0,2760	1,0000	Metallothioneine type2	ta CA600821 tc T	A_99_P460107	16140
TA84010_4565	2,9456	1,0000	1,0000	0,3796	Nodulin-like protein	ta TA84010_4565	A_99_P307876	15025
TA61435_4565	0,3348	1,0000	1,0000	0,1487	Peroxidase 6	ta TA61435_4565	A_99_P415477	32597
BQ161851	1,0000	1,0000	1,0000	0,2850	Peroxidase	ta BQ161851 tc T	A_99_P431937	25512
CK212695	1,0000	1,0000	1,0000	0,3180	Class III peroxidase 62 precursor	ta CK212695 tc T	A_99_P407997	44165
TA66389_4565	1,0000	1,0000	1,0000	0,2922	Peroxidase precursor	ta TA66389_4565	A_99_P247391	14997
AF532972	1,0000	1,0000	1,0000	0,4802	chromosome 6BL thylakoid-bound ascorbate peroxidase	gb AF532972 gb A	A_99_P259786	38976
Response to pathogens								
TA54666_4565	1,0000	0,2924	1,0000	1,0000	Class I chitinase	ta TA54666_4565	A_99_P211671	41435
TC426538	1,0000	1,0000	13,5307	1,0000	Chitinase 2	tc TC426538	A_99_P488752	20923
TA72415_4565	1,0000	1,0000	4,8597	1,0000	Chitinase	ta TA72415_4565	A_99_P268406	11345
TA55707_4565	1,0000	1,0000	3,4425	1,0000	Thaumatococcus-like protein TLP4	ta TA55707_4565	A_99_P509202	23176
TC392855	1,0000	1,0000	0,5507	1,0000	Protein WIR1A	tc TC392855	A_99_P060636	28568
TA70475_4565	1,0000	1,0000	0,4104	1,0000	RGA2	ta TA70475_4565	A_99_P433007	20640
TA65444_4565	1,0000	1,0000	0,3907	1,0000	Reticulon	ta TA65444_4565	A_99_P412857	7188
TA53249_4565	1,0000	1,0000	0,2556	1,0000	IPS1 riboregulator	ta TA53249_4565	A_99_P207336	20707
TC442947	1,0000	1,0000	0,3281	1,0000	ENOD40-like protein	tc TC442947	A_99_P525387	1183
TA76681_4565	1,0000	1,0000	1,0000	0,2666	HS1-like protein	ta TA76681_4565	A_99_P194333	16022
Secondary metabolism								
TA50072_4565	1,0000	1,0000	1,0000	3,6839	Probable pyridoxal biosynthesis protein PDX1.1	ta TA50072_4565	A_99_P197801	18956
Flowering and seed maturation								
TA84120_4565	1,0000	1,0000	0,2751	1,0000	Protein EGG APPARATUS-1	ta TA84120_4565	A_99_P308216	36050
Growth and development								
DQ435671	4,0224	1,0000	1,0000	1,0000	alpha tubulin-1D (TUBA-1D)	gb DQ435671 ta T	A_99_P012999	6685
TA86474_4565	4,6137	1,0000	1,0000	1,0000	Auxin Efflux Carrier	ta TA86474_4565	A_99_P192117	12326
TA56474_4565	0,3507	1,0000	1,0000	1,0000	Auxin-repressed protein	ta TA56474_4565	A_99_P215841	35480
TA56454_4565	0,2896	1,0000	1,0000	1,0000	Auxin-repressed protein	ta TA56454_4565	A_99_P215761	27870
TA67195_4565	0,3445	1,0000	1,0000	1,0000	Protein cab-1	ta TA67195_4565	A_99_P250166	16476
TA72187_4565	1,0000	1,0000	0,2876	1,0000	Cell Division Protein AAA ATPase family	ta TA72187_4565	A_99_P267636	41732
TA61883_4565	1,0000	1,0000	1,0000	3,5486	Sterol desaturase family protein	ta TA61883_4565	A_99_P231241	32885
TA70788_4565	1,0000	1,0000	1,0000	0,2813	Dormancy/auxin associated protein	ta TA70788_4565	A_99_P262826	45151

Résultats – Chapitre 2

	Eliciting effect		Fungal effect	Priming effect			
	A	B	C	D			
Name	R-D2-Ni vs C-D2-Ni	R-D5-Ni vs C-D5-Ni	C-D5-i vs C-D5-Ni	R-D5-i vs C-D5-i	Description	TopHit	ID
							RefNumber
Cell wall structure and function							
TA56367_4565	0,5407	1,0000	1,0000	1,0000	Exoglucanase precursor	ta TA56367_456 A_99_P215601	6504
TA56367_4565	0,4738	1,0000	1,0000	1,0000	Beta-D-glucan exohydrolase isoenzyme Exol	ta TA56367_456 A_99_P433432	15270
TA66611_4565	8,6860	1,0000	0,3372	1,0000	Fascidin-like protein FLA15	ta TA66611_456 A_99_P248201	22500
TA68562_4565	1,0000	1,0000	0,3766	1,0000	Glycoside hydrolase, family 19	ta TA68562_456 A_99_P255031	1863
CK205454	1,0000	1,0000	0,3699	1,0000	Non-specific lipid transfer protein 1 precursor	ta CK205454 tc A_99_P427502	36085
TA61828_4565	1,0000	1,0000	0,3668	1,0000	Non-specific lipid-transfer protein	ta TA61828_456 A_99_P231006	27677
CK152333	1,0000	1,0000	0,3202	1,0000	Type 1 non specific lipid transfer protein precursor	ta CK152333 tc A_99_P446157	36915
TA87087_4565	1,0000	1,0000	0,0592	1,0000	Cinnamyl alcohol dehydrogenase	ta TA87087_456 A_99_P318481	8569
AB114627	1,0000	1,0000	1,0000	0,3116	Taxi-III mRNA for xylanase inhibitor TAXI-III	gb AB114627 gb A_99_P195998	22657
Pigment biosynthesis							
TA56529_4565	2,1599	1,0000	1,0000	1,0000	Glutamyl-tRNA reductase 2	ta TA56529_456 A_99_P216016	43656
TA92426_4565	4,5534	1,0000	1,0000	1,0000	Beta-carotene hydroxylase	ta TA92426_456 A_99_P336181	23802
Chloroplast structure and function							
TA92734_4565	1,0000	1,0000	0,3150	1,0000	Diap1 protein	ta TA92734_456 A_99_P337136	42984
TA69442_4565	1,0000	1,0000	1,0000	0,2943	Plastid-specific 30S ribosomal protein 1, chloroplast precursor	ta TA69442_456 A_99_P258121	37451
Light harvesting and photosynthesis							
TA56048_4565	2,3370	1,0000	1,0000	1,0000	Ribulose bisphosphate carboxylase small chain	ta TA56048_456 A_99_P444042	23343
TC371150	4,0099	1,0000	1,0000	0,1805	Chlorophyll a/b-binding protein WCAB precursor	tc TC371150 A_99_P409112	19146
CK217674	3,3076	1,0000	1,0000	0,3216	Chloroplast pigment-binding protein CP24	tc CK217674 tc A_99_P437157	39278
TA55816_4565	1,0000	1,0000	1,0000	0,5050	Light-harvesting complex I	ta TA55816_456 A_99_P214401	27084
TA53867_4565	1,0000	1,0000	1,0000	0,3512	Light-induced protein 1-like	ta TA53867_456 A_99_P209081	23883
TA59583_4565	1,0000	1,0000	1,0000	0,3590	Light-regulated protein precursor	ta TA59583_456 A_99_P223636	16866
Carbohydrate metabolism							
TA72052_4565	0,4187	1,0000	1,0000	1,0000	Beta-amylase	ta TA72052_456 A_99_P009886	12260
Amino-acids metabolism							
TA64535_4565	1,0000	1,0000	0,3402	1,0000	Glycine decarboxylase P subunit	ta TA64535_456 A_99_P240211	15342
TC430171	1,0000	1,0000	0,0893	1,0000	Indole-3-glycerol phosphate lyase	tc TC430171 A_99_P496502	8009
TA69140_4565	1,0000	1,0000	0,3061	1,0000	L-allo-threonine aldolase-related protein	ta TA69140_456 A_99_P449637	41850
TA69994_4565	1,0000	1,0000	0,3764	1,0000	Glutamate dehydrogenase	ta TA69994_456 A_99_P259951	10977
TC393988	1,0000	1,0000	0,1308	1,0000	Glycine/proline-rich protein	tc TC393988 A_99_P543657	7174
TA60889_4565	1,0000	1,0000	1,0000	2,8554	S-adenosylmethionine synthetase	ta TA60889_456 A_99_P227511	9678
Protein metabolism							
TA59594_4565	3,2559	1,0000	1,0000	1,0000	22.3kDa heat-shock protein	ta TA59594_456 A_99_P223676	44575
TA64099_4565	2,6016	1,0000	0,1519	3,4096	Heat shock protein 80	ta TA64099_456 A_99_P238761	26
HM209063	1,0000	1,0000	0,2354	2,1997	Heat shock protein 70-like (HSP70)	gb HM209063 g A_99_P234541	16785
TC414782	1,0000	1,0000	0,2639	1,0000	Asparaginase	tc TC414782 A_99_P466942	23822
TA53535_4565	1,0000	1,0000	0,1923	1,0000	Ubiquitin carrier protein	ta TA53535_456 A_99_P208256	24763
TA78844_4565	1,0000	1,0000	1,0000	0,4606	Papain-like cysteine peptidase XBCP3	ta TA78844_456 A_99_P290171	29285
Signaling, transcription, translation							
TA82476_4565	3,7353	1,0000	1,0000	1,0000	HAP3-like transcriptional-activator	ta TA82476_456 A_99_P302726	42546
TA52984_4565	3,6271	1,0000	1,0000	1,0000	Histone H1 WH1B.1	ta TA52984_456 A_99_P497187	27663
TC447945	0,4220	1,0000	1,0000	1,0000	Myb-related protein MYBAS1	tc TC447945 A_99_P538902	19305
TC450123	1,0000	0,0664	1,0000	1,0000	rDNA tandem repeat with genes for 18S, 5.8S and 26S rRNA	tc TC450123 A_99_P544312	866
CA721206	1,0000	1,0000	0,3296	1,0000	Zinc finger protein 219	ta CA721206 tc A_99_P533942	44850
D16416	1,0000	1,0000	1,0000	0,5072	Zinc-finger protein WZF1	gb D16416 ta T.A_99_P489622	10858

Supplementary Table S2: Alterations in gene expression pattern responses illustrating (A) R-D5-i versus R-D5-Ni as well as (B), the whole response of wheat to the rhamnolipid and the pathogen, R-D5-i versus C-D5-Ni. This table is related to Supplementary Figure S4 . Wheat cultivar Alixan, *Z. tritici* T02596 strain and a rhamnolipid concentration of 500 mg.L⁻¹ were used. Gene-related physiological processes are represented on the right part of the table and were determined using NCBI, AmiGO 2 Gene Ontology, KEGG and UniProt. C stands for treatment with mock solution while R is for Rh-Est-C12 application. Ni concerns mock inoculated wheat while i means inoculated plants with *Z. tritici*. D5 indicate that third-leaves were collected at 5 days after treatment. Top Hit, ID and reference numbers are also presented.

	A	B				
Name	R-D5-i vs R-D5-Ni	R-D5-i vs C-D5-Ni	Description	TopHit	ID	RefNumber
Response to abiotic stress						
TA54418_4565	7,4785	1,0000	Cold acclimation protein WCOR413	ta TA54418_456	A_99_P210931	43524
U73211	2,6271	1,0000	Cold acclimation protein WCOR410c (Wcor410c)	gb U73211 gb	LA_99_P509672	30914
CA624093	3,1237	6,3201	Low temperature induced protein	ta CA624093 ta	A_99_P459967	11840
AF058794	3,2216	2,8069	COR39 (cor39)	gb AF058794 gb	A_99_P000281	39752
TA73273_4565	4,6451	4,6272	ATP-dependent RNA helicase	ta TA73273_456	A_99_P430447	24209
TA84187_4565	0,3340	1,0000	Protein phosphatase type 2-C	ta TA84187_456	A_99_P308421	21815
TA92949_4565	0,2647	1,0000	Efflux transporter, RND family, MFP subunit	ta TA92949_456	A_99_P144213	15689
TA62230_4565	0,3470	1,0000	NAC transcription factor	ta TA62230_456	A_99_P232346	6986
CF133748	0,5054	1,0000	Myb-related protein	ta CF133748 tc	A_99_P453952	5264
DQ286566	0,3439	0,4157	WRKY transcription factor (WRKY)	gb DQ286566 ta	A_99_P455767	42312
TC421665	0,4667	0,5449	Transcription factor Myb2	tc TC421665 tc	A_99_P479147	37654
AY781354	0,4658	0,4330	DREB transcription factor 4A (DREB4)	gb AY781354 gb	A_99_P197071	14918
EF028787	0,3331	0,2706	CBFIVd-D22	gb EF028787 ta	A_99_P405352	948
EF028785	0,3488	0,2629	CBFIVd-A22	gb EF028785 gb	A_99_P140518	15660
TC369712	0,2693	0,2597	Salt stress root protein RS1	tc TC369712	A_99_P407642	6772
TC436852	1,0000	0,3734	Hly-III related proteins	tc TC436852	A_99_P511722	6696
TA59927_4565	1,0000	0,4435	Monodehydroascorbate reductase	ta TA59927_456	A_99_P224741	7355
TA59060_4565	1,0000	0,3945	22 kDa drought-inducible protein	ta TA59060_456	A_99_P222071	24327
Response to oxidative stress						
TA102260_4565	7,7725	7,2280	Nitrite reductase apoprotein	ta TA102260_45	A_99_P366657	8326
TC401354	0,4472	1,0000	Metallothionein-like protein type 3	tc TC401354	A_99_P446677	44649
BQ161851	0,4760	0,4200	Peroxidase	ta BQ161851 tc	A_99_P431937	25512
CK212695	0,4174	0,3184	Class III peroxidase 62 precursor	ta CK212695 tc	A_99_P407997	44165
TA61435_4565	0,1741	0,2027	Peroxidase 6	ta TA61435_456	A_99_P415477	32597
TC384087	0,4739	0,4275	Glutathione S-transferase	tc TC384087	A_99_P411017	6865
TA61129_4565	0,4062	0,4092	Glutathione transferase	ta TA61129_456	A_99_P228341	15064
CA600821	0,4414	0,3408	Metallothioneine type2	ta CA600821 tc	A_99_P460107	16140
TC429241	1,0000	0,4463	Peptide methionine sulfoxide reductase	tc TC429241	A_99_P494392	29872
TA51226_4565	1,0000	0,4196	Glutaredoxin	ta TA51226_456	A_99_P200897	45008
Response to pathogens						
TC426538	3,2690	6,6861	Chitinase 2	tc TC426538	A_99_P488752	20923
TA72415_4565	1,0000	3,1080	Chitinase	ta TA72415_456	A_99_P268406	11345
TC442947	0,3054	0,3054	ENOD40-like protein	tc TC442947	A_99_P525387	1183
EF570122	0,3191	0,3950	Pathogen-inducible transcription factor ERF3	gb EF570122 gb	A_99_P177974	11088
TA76681_4565	0,3038	1,0000	HS1-like protein	ta TA76681_456	A_99_P194333	16022
TA57993_4565	0,3685	1,0000	MutT/nudix-like	ta TA57993_456	A_99_P219466	4113
Flowering and seed maturation						
TA84120_4565	0,3312	0,3269	Protein EGG APPARATUS-1	ta TA84120_456	A_99_P308216	36050
TC404192	1,0000	0,4137	AAA ATPase, central region (50.1 kD)-like protein	tc TC404192	A_99_P450607	15744
TA70093_4565	1,0000	0,4476	U2AF small subunit	ta TA70093_456	A_99_P260356	14065
TA86268_4565	1,0000	6,3553	CONSTANS interacting protein 5	ta TA86268_456	A_99_P315626	21018

	A	B				
Name	R-D5-i vs R-D5-Ni	R-D5-i vs C-D5-Ni	Description	TopHit	ID	RefNumber
Growth and development						
TA70788_4565	0,3873	0,4173	Dormancy/auxin associated protein	ta TA70788_456	A_99_P262826	45151
TA61980_4565	1,0000	0,3849	Abscisic stress ripening-like protein	ta TA61980_456	A_99_P231656	43647
DQ435671	1,0000	0,3752	Alpha tubulin-1D (TUBA-1D)	gb DQ435671 ta	A_99_P012999	6685
TC405303	0,3142	1,0000	Mitogen-activated protein kinase 7	tc TC405303	A_99_P452302	22398
L27516	3,3956	1,0000	Wcs66	gb L27516 gb V	A_99_P167734	4367
Cell wall structure and activity						
TA61828_4565	1,0000	0,3999	Non-specific lipid-transfer protein	ta TA61828_456	A_99_P231006	27677
TA66611_4565	1,0000	0,2492	Fasciclin-like protein FLA15	ta TA66611_456	A_99_P248201	22500
CK152333	1,0000	0,2855	Type 1 non specific lipid transfer protein precursor	ta CK152333 tc	A_99_P446157	36915
TA87087_4565	1,0000	0,0944	Cinnamyl alcohol dehydrogenase	ta TA87087_456	A_99_P318481	8569
CK207368	1,0000	0,5232	Cellulose synthase BoCesA7	ta CK207368 tc	A_99_P424332	22342
TA77295_4565	1,0000	0,4688	3-ketoacyl-CoA synthase	ta TA77295_456	A_99_P284791	23697
TA77906_4565	0,4527	0,4223	Hydroxyproline-rich glycoprotein-like	ta TA77906_456	A_99_P286926	32415
TA60114_4565	0,4000	0,4045	UDP-D-xylose epimerase 2	ta TA60114_456	A_99_P412027	14821
TA61116_4565	0,4933	0,3082	Non-specific lipid-transfer protein 4.3 precursor	ta TA61116_456	A_99_P228291	22943
Chloroplast structure and function						
TA92734_4565	1,0000	0,1752	Diap1 protein	ta TA92734_456	A_99_P337136	42984
Light harvesting and photosynthesis						
TC371150	0,2371	0,2858	Chlorophyll a/b-binding protein WCAB precursor	tc TC371150	A_99_P409112	19146
CK217674	0,3347	0,3749	Chloroplast pigment-binding protein CP24	ta CK217674 tc	A_99_P437157	39278
TA55816_4565	1,0000	0,4759	Light-harvesting complex I	ta TA55816_456	A_99_P214401	27084
TA61423_4565	1,0000	0,4533	Chloroplast pigment-binding protein CP24	ta TA61423_456	A_99_P229541	44166
TA59583_4565	0,4288	1,0000	Light-regulated protein precursor	ta TA59583_456	A_99_P223636	16866
EU492898	0,4845	1,0000	Ribulose-1,5-bisphosphate carboxylase	gb EU492898 ta	A_99_P625436	18029
Carbohydrate metabolism						
TA70102_4565	0,2493	0,3105	Phosphoenolpyruvate carboxykinase	ta TA70102_456	A_99_P260386	18755
TA91251_4565	0,2911	0,3341	Isoform ERG1b of A2WWV5	ta TA91251_456	A_99_P332361	17064
Amino-acids metabolism						
TA52838_4565	4,0093	4,5867	Glycine rich protein	ta TA52838_456	A_99_P163692	5193
TC393988	0,2677	0,2183	Glycine/proline-rich protein	tc TC393988	A_99_P543657	7174
TA69140_4565	0,2624	0,2234	L-allo-threonine aldolase-related protein	ta TA69140_456	A_99_P449637	41850
TA69994_4565	0,2422	0,2621	Glutamate dehydrogenase	ta TA69994_456	A_99_P259951	10977
TA82049_4565	1,0000	2,7976	Bifunctional aspartokinase/homoserine dehydrogenase 2, chloroplast prec	ta TA82049_456	A_99_P163227	42192

Name	A R-D5-i vs R-D5-Ni	B R-D5-i vs C-D5-Ni	Description	TopHit	ID	RefNumber
Protein metabolism						
TA67255_4565	3,3495	3,5074	Heat-shock protein precursor	ta TA67255_456	A_99_P250376	10187
HM209063	0,5698	0,5177	Heat shock protein 70-like (HSP70)	gb HM209063 g	A_99_P234541	16785
TC391450	0,2458	1,0000	ATP-dependent Clp protease ATP-binding subunit	tc TC391450	A_99_P433687	20222
JX104551	0,3942	1,0000	SNAP34	gb JX104551 ta	A_99_P292561	27821
TA53535_4565	1,0000	0,3973	Ubiquitin carrier protein	ta TA53535_456	A_99_P208256	24763
Lipid metabolism						
TA58123_4565	0,4510	1,0000	Fatty acid desaturase	ta TA58123_456	A_99_P219611	11349
TA87662_4565	1,0000	0,4479	Acyl-ACP thioesterase	ta TA87662_456	A_99_P437477	32120
TA52495_4565	1,0000	0,4527	Lysophospholipase-like protein	ta TA52495_456	A_99_P204796	24058
Signaling, transcription, translation						
TA81385_4565	5,0694	4,3236	Phosphoenolpyruvate carboxylase	ta TA81385_456	A_99_P299011	44004
TA68843_4565	0,4674	0,3908	Exonuclease family protein	ta TA68843_456	A_99_P256071	41928
TA54635_4565	0,2322	0,3794	EF-hand Ca ²⁺ -binding protein CCD1	ta TA54635_456	A_99_P165947	28159
TA73684_4565	0,3243	0,3771	Serine/threonine protein kinase	ta TA73684_456	A_99_P423972	31763
D16416	0,2334	0,2611	Zinc-finger protein WZF1	gb D16416 ta T.	A_99_P489622	10858
AY881102	0,4348	1,0000	Putative MAPK protein kinase (MAPK1a)	gb AY881102 ta	A_99_P014394	16503
TA74203_4565	0,3949	1,0000	Receptor-like protein kinase	ta TA74203_456	A_99_P274251	4201
TA85618_4565	1,0000	3,9979	DEAD-box ATP-dependent RNA helicase 26	ta TA85618_456	A_99_P313296	14881
CA721206	1,0000	0,4445	Zinc finger protein 219	ta CA721206 tc	A_99_P533942	44850
EF040602	1,0000	0,4563	ZIM motif-containing protein	gb EF040602 ta	A_99_P614787	18006
TA51878_4565	1,0000	0,4278	Susceptibility homeodomain transcription factor	ta TA51878_456	A_99_P202881	23811
Other functions						
TC389894	3,9449	4,0264	Tetratricopeptide repeat (TPR)-containing protein-like	tc TC389894	A_99_P431697	38421

Supplementary Table S3: List of the 54 metabolites detected in wheat leaves (cv. Alixan) with ultra-high-performance liquid chromatography-mass spectrometry (UHPLC-MS), using a targeted metabolomic approach. Metabolites were classified according to KEGG (Kyoto Encyclopedia of Genes and Genomes; <http://www.genome.ad.jp/kegg>) and PubChem (<http://pubchem.ncbi.nlm.nih.gov>) databases. Id: identifier; m/z: mass-to-charge ratio; m/z error (difference between the measured m/z and the calculated m/z of an ion, in ppm); RT: retention time. (*) correspond to metabolites confirmed with authentic standards.

Id	Metabolite	Formula	Class ^a	Detected <i>m/z</i>	<i>m/z</i> error (ppm)	RT (min)	KEGG ID	PubChem CID
1	Arginine	C ₆ H ₁₄ N ₄ O ₂	Amino acids	175.1190	0.20	0,85	C00062	6322
2	Asparagine	C ₄ H ₈ N ₂ O ₃	Amino acids	133.0609	0.86	0,98	C00152	6267
3	Aspartic acid	C ₄ H ₇ NO ₄	Amino acids	134.0449	0.69	1,02	C00049	5960
4	Glutamic acid	C ₅ H ₉ NO ₄	Amino acids	148.0604	0,77	1,01	C00025	4525487
5	Glutamine	C ₅ H ₁₀ N ₂ O ₃	Amino acids	147.0764	0,09	0,99	C00064	5961
6	Histidine	C ₆ H ₉ N ₃ O ₂	Amino acids	156.0769	0.68	1,01	C00135	6274
7	Hydroxyproline	C ₅ H ₉ NO ₃	Amino acids	132.0656	0.70	1,01	C01157	5810
8	Isoleucine	C ₆ H ₁₃ NO ₂	Amino acids	132.1020	0.76	1,56	C00407	6306
9	Leucine	C ₆ H ₁₃ NO ₂	Amino acids	132.1020	0.76	1,62	C00123	6106
10	Lysine	C ₆ H ₁₄ N ₂ O ₂	Amino acids	147.1129	0.44	0.84	C00047	5962
11	Methionine	C ₅ H ₁₁ NO ₂ S	Amino acids	150.0584	0.36	2.04	C00073	6137
12	Phenylalanine	C ₉ H ₁₁ NO ₂	Amino acids	166.0862	-0.53	4.30	C00079	6140
13	Proline	C ₅ H ₉ NO ₂	Amino acids	116.0708	1.69	1.23	C00148	145742
14	Threonine	C ₄ H ₉ NO ₃	Amino acids	120.0657	1.22	1.08	C00188	6288
15	Tryptophan	C ₁₁ H ₁₂ N ₂ O ₂	Amino acids	205.0973	0.51	4.75	C00078	6305
16	Tyrosine	C ₉ H ₁₁ NO ₃	Amino acids	182.0813	0.54	3.20	C00082	6057
17	Valine	C ₅ H ₁₁ NO ₂	Amino acids	118.0863	0.48	1.14	C00183	6287
18	Methylpipercolic acid	C ₇ H ₁₃ NO ₂	Amino acid derivatives	144.1019	0.27	1.08	C10172	115244
19	DHBOA-hexosyl-hexoside	C ₁₄ H ₁₇ NO ₉	Benzoxazinoids	523.1773	0.59	4.48		
20	DHBOA-hexosyl-deoxyhexoside	C ₈ H ₇ NO ₄	Benzoxazinoids	490.1557	0.59	4.71		
21	HBOA	C ₈ H ₇ NO ₃	Benzoxazinoids	166.0499	0.34	5.92	C15769	322636
22	HBOA-glucoside	C ₁₄ H ₁₇ NO ₈	Benzoxazinoids	328.1027	0.09	4.71		14605136
23	HM2BOA	C ₁₀ H ₁₁ NO ₅	Benzoxazinoids	226.0709	-0.61	5.51		11064107
24	HMBOA-hexoside	C ₁₀ H ₁₁ NO ₅	Benzoxazinoids	358.1241	-0.61	5.06		
25	HM2BOA-hexoside	C ₁₆ H ₂₁ NO ₁₀	Benzoxazinoids	388.1245	1.86	5.36		
26	Apigenin-6-C-hexoside-C-pentoside	C ₂₆ H ₂₈ O ₁₄	Flavonoids	565,1553	0,09	4,66		
27	Chrysoeriol-6-C-hexoside	C ₂₂ H ₂₂ O ₁₁	Flavonoids	463.1237	0.43	5.35	C05990	442611
28	Chrysoeriol-C-hexosyl-O-deoxyhexoside	C ₂₈ H ₃₂ O ₁₅	Flavonoids	609.1817	0.50	5.24		
29	Chrysoeriol-C-hexosyl-O-hexoside	C ₂₈ H ₃₂ O ₁₆	Flavonoids	625.1766	0.42	5.17		72193674
30	Luteolin-C-hexosyl-deoxyhexoside	C ₂₇ H ₃₀ O ₁₅	Flavonoids	595.1660	0.45	5.02		
31	Luteolin-C-hexosyl-C-pentoside	C ₂₆ H ₂₈ O ₁₅	Flavonoids	581,1504	0.51	4.97		44258081
32	Luteolin-C-hexosyl-O-hexoside	C ₂₇ H ₃₀ O ₁₆	Flavonoids	611,1608	0,23	4,98		
33	Luteolin-6-C-hexoside	C ₂₁ H ₂₀ O ₁₁	Flavonoids	449,108	0,36	5,00	C01750	5280459
34	Tricin	C ₁₇ H ₁₄ O ₇	Flavonoids	331.0812	0,09	6,45	C10193	5281702
35	Tricin-7-O-hexoside	C ₂₃ H ₂₄ O ₁₂	Flavonoids	493,1343	0,5	5,56		5322022
36	Abscisic acid	C ₁₅ H ₂₀ O ₄	Phytohormones	265,14343	0,53	6,06		
37	Abscisic acid glucose ester	C ₂₁ H ₃₀ O ₉	Phytohormones	427,19625	0,35	4,33		
38	Indole acetic acid	C ₁₀ H ₉ NO ₂	Phytohormones	176,0706	1,16	1,71		
39	OPDA	C ₁₈ H ₂₈ O ₃	Phytohormones	293,211121	0,89	7,42		
40	Jasmonic acid	C ₁₂ H ₁₈ O ₃	Phytohormones	211,1329	0,14	6,27	C08491	5281166
41	Methyljasmonate	C ₁₃ H ₂₀ O ₃	Phytohormones	225,148521	0,31	6,88		
42	Salicylic acid	C ₇ H ₆ O ₃	Phytohormones	139,039	0,21	4,5	C00805	338
43	Methylsalicylate	C ₈ H ₈ O ₃	Phytohormones	153,05462	0,56	5,39		
44	Coumaroylagmatine	C ₁₄ H ₂₀ N ₄ O ₂	Hydroxycinnamic acid amides	277,1659	0,01	4,66	C04498	5280691
45	Coumaroylputrescine	C ₁₃ H ₁₈ N ₂ O ₂	Hydroxycinnamic acid amides	235,1441	0,02	4,50		
46	Coumaroylcadaverine	C ₁₄ H ₂₀ N ₂ O ₂	Hydroxycinnamic acid amides	249,15975	0,56	3,97		
47	Caffeoylagmatine	C ₁₄ H ₂₀ N ₄ O ₃	Hydroxycinnamic acid amides	293,1608	0,06	4,52		
48	Caffeoylputrescine	C ₁₃ H ₁₈ N ₂ O ₃	Hydroxycinnamic acid amides	251,139	0,08	4,47	C03002	
49	Feruloylagmatine	C ₁₅ H ₂₂ N ₄ O ₃	Hydroxycinnamic acid amides	307,1765	0,11	4,57	C18325	46173376
50	Feruloylputrescine	C ₁₄ H ₂₀ N ₂ O ₃	Hydroxycinnamic acid amides	265,1548	0,49	4,70	C10497	5281796
51	Feruloylcadaverine	C ₁₅ H ₂₂ N ₂ O ₃	Hydroxycinnamic acid amides	279,1702	0,43	4,85		
52	Hydroxyferuloylputrescine	C ₁₄ H ₂₀ N ₂ O ₄	Hydroxycinnamic acid amides	281,14955	0,12	3,81		
53	Sinapoylagmatine	C ₁₆ H ₂₄ N ₄ O ₄	Hydroxycinnamic acid amides	337,187	0,09	4,65		
54	Sinapoylputrescine	C ₁₅ H ₂₂ N ₂ O ₄	Hydroxycinnamic acid amides	295,1653	0,22	4,56		

^a Metabolites was classified according to KEGG (Kyoto Encyclopedia of Genes and Genomes; <http://www.genome.ad.jp/kegg/>) and PubChem (<http://pubchem.ncbi.nlm.nih.gov>) databases. Id: identifier; *m/z*: mass-to-charge ratio; *m/z* error (difference between the measured *m/z* and the calculated *m/z* of an ion, represented in ppm); RT: retention time. Compounds indicated in bold were confirmed with authentic standards.

CHAPITRE 3

Le lipopeptide mycosubtiline potentialise les réponses immunitaires du blé vis-à-vis de *Zymoseptoria tritici*

Dans le chapitre précédent, nous avons montré que le Rh-Est-C12 protégeait le blé vis-à-vis de *Z. tritici* sans entraîner d'altérations majeures chez l'hôte, que ce soit au niveau de l'expression de gènes, ou de l'accumulation de métabolites dans les feuilles de blé. Les résultats obtenus suggèrent que la protection conférée par le rhamnolipide provenait majoritairement de son activité antifongique et, plus minoritairement, de son activité d'induction des défenses du blé. Dans ce chapitre, nous avons cherché à déterminer si la mycosubtiline, un autre composé surfactant, était capable d'induire des réactions de défense chez le blé, toujours dans le cadre d'une interaction compatible avec *Z. tritici*. La mycosubtiline est un lipopeptide cyclique (CLP), produit notamment par *Bacillus subtilis* et présentant une activité antifongique directe vis-à-vis de *Z. tritici* (Mejri *et al.*, 2018). Par ailleurs, Farace *et al.* (2015) ont montré que ce CLP induisait des réactions de défense *in vitro* chez des cellules de vigne. Ici, nous avons voulu déterminer si la mycosubtiline présentait, ou non, un double mode d'action pour protéger le blé vis-à-vis de l'agent phytopathogène (antifongique et stimulation des défenses de la plante). Pour ce faire, nous avons mené une approche combinée de transcriptomique et de métabolomique, comme dans le chapitre précédent, mais aussi associée à des analyses cytologiques. Contrairement au Rh-Est-C12, le traitement foliaire du blé avec la mycosubtiline a entraîné des modifications conséquentes dans l'accumulation de transcrits et de métabolites dans les conditions traitées. En particulier, nos résultats suggèrent que la mycosubtiline serait capable de *primer* le blé, ce qui est généralement reconnu comme plus avantageux en termes de coûts énergétiques que l'élicitation des défenses de la plante (Martinez-Medina *et al.*, 2016). Le mécanisme par lequel la mycosubtiline déclenche la potentialisation du blé sera discuté. Celle-ci pourrait être causée par l'interaction de la mycosubtiline avec les membranes cytoplasmiques de l'hôte modifiant sa fluidité, comme un stress abiotique-*like*. Ce stimulus permettrait de déclencher des réactions de défense chez le blé, notamment les voies de réponses associées à la phytohormone acide abscissique (ABA).

Les résultats obtenus dans le cadre de ce chapitre sont en préparation en vue de leur publication dans la revue Plant Physiology (IF = 6,90).

Deciphering defense mechanisms primed by the *Bacillus subtilis* lipopeptide mycosubtilin in wheat towards *Zymoseptoria tritici*

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ABSTRACT

Induced immunity in plants is a valuable eco-friendly tool that fits with sustainable agriculture and healthy food. However, despite the agro-economic significance of a major crop as wheat, the mechanisms underlying the induction of its defense responses are still unclear. Here, we show that mycosubtilin, a lipopeptide from *Bacillus subtilis*, protects wheat against the hemibiotrophic fungal pathogen *Zymoseptoria tritici* through dual (direct and indirect) modes of action and that the indirect one relies mainly on priming rather than the elicitation of plant defense-related mechanisms. Indeed, foliar application of the biomolecule primed, during the early stages of infection, the expression of 80 genes associated with sixteen functional groups, including responses to pathogens, abiotic and oxidative stresses, secondary metabolism, cell-wall structure and function, and primary metabolic pathways (carbohydrate, amino acid, protein, lipid, and energy metabolisms). More specifically, genes involved in abscisic acid (ABA) biosynthesis and ABA-associated signaling pathways are regulated, suggesting a role of this phytohormone in the indirect activity of mycosubtilin. Interestingly, mycosubtilin also primed the accumulation of several flavonoids during the period preceding the fungal switch to the necrotrophic phase. This priming-based bioactivity of mycosubtilin against a biotic stress could result from an interaction of the biomolecule with leaf cell plasma membranes that may mimic an abiotic stress-like stimulus in wheat plants, as supported by cytological bioassays, as well as the regulation of genes associated with abiotic stress-responses and early signal transduction in the treated plants. Further bioassays showed that mycosubtilin displays a direct antifungal activity against *Z. tritici* *in vitro* and *in planta* and reduces disease severity caused by the pathogen. This study provides new insights into induced immunity in wheat and opens new perspectives for the use of mycosubtilin lipopeptide as a biocontrol compound with two distinct modes of action against *Z. tritici*.

INTRODUCTION

Wheat is one of the most cultivated cereal crops worldwide, serving as primary ingredient in human diet, food industry, and livestock feed. Wheat cultivation has to cope with a wide range of phytopathogenic microorganisms impacting its growth, productivity and quality of production (Savary *et al.*, 2019). The most frequently occurring and damaging pathogen on wheat crop is the hemibiotrophic fungus *Zymoseptoria tritici*, responsible for Septoria tritici blotch (STB) disease, causing severe yield losses of up to 50 % during high-level pressure years (Fones and Gurr, 2015). The infection process of hemibiotrophic pathogens consists of an initial asymptomatic biotrophic phase followed by a necrotrophic phase, the latter being characterized by visual symptom apparition (Koeck *et al.*, 2011). Even if progresses have been accomplished in wheat resistance breeding during the last decades, with 21 resistance genes described against *Z. tritici*, disease control of STB relies mainly on synthetic fungicides (Brown *et al.*, 2015 ; Torriani *et al.*, 2015). In Europe, 70 % of the total currently applied fungicides are used to protect wheat against *Z. tritici* (Fones and Gurr, 2015). Nevertheless, fungicide resistance developed by *Z. tritici* populations is an increasing concern for wheat producers (Cools *et al.*, 2013 ; Cools and Fraaije, 2013 ; McDonald *et al.*, 2019 ; Steinhauer *et al.*, 2019). Therefore, a strong need for substitutes and alternatives have emerged in the recent years, reinforced by all the concerns around the potential impacts of chemical inputs on the environment and human health.

Biosurfactants, or green surfactants, have been reported as ecofriendly promised candidates for biocontrol of crop diseases (Ongena and Jacques, 2008 ; D'aes *et al.*, 2010 ; Vatsa *et al.*, 2010 ; Sachdev and Cameotra, 2013 ; Shekhar *et al.*, 2015 ; Chen *et al.*, 2017 ; Crouzet *et al.*, 2020). They are surface-active biomolecules produced by a broad range of microorganisms, including bacteria and fungi. These molecules are often classified according to their chemical structure, such as mannosylerythritol lipids, trehalose dimycolate, trehalolipids, sophorolipids, rhamnolipids, and lipopeptides (Crouzet *et al.*, 2020). Even though they may be very diverse both in their structure and origin, they all are amphiphilic molecules, composed by hydrophilic and hydrophobic moieties. They also exhibit a high potential of application in numerous fields, including human health, cosmetic, food industry, petroleum industry, soil and water remediation, nanotechnology, and agriculture (Lourith and Kanlayavattanukul, 2009 ; Sachdev and Cameotra, 2013 ; Shekhar *et al.*, 2015 ; Singh *et al.*, 2019). Regarding their use in crop protection, many studies have reported their significant efficacy in plant protection against pathogens (Ongena and Jacques, 2008 ; D'aes *et al.*, 2010 ; Vatsa *et al.*, 2010 ; Sachdev and Cameotra, 2013 ; Chen *et al.*, 2017 ; Crouzet *et al.*, 2020). Moreover, green surfactants have been described as displaying high biodegradability, as well as low toxicity and ecotoxicity, two major advantages required for the development of sustainable agriculture (Shekhar *et al.*, 2015).

The possible modes of action of biosurfactants in plant protection against pathogens are diverse. For instance, they can display direct antimicrobial activity towards pathogens, modify the bio-availability of nutrients used by the pathogens, and/or induce plant immune defenses (D'aes *et al.*, 2010). The compounds inducing the plant immune defenses may be distinguished into two categories, including elicitors, that directly activate host defense responses after their application, and priming agents, that require additional signals, such as pathogen recognition, to trigger the full defense responses (Paré *et al.*, 2005). Primed plants may develop a stronger and/or faster response pattern than so-called naïve (unprimed) plants. They also may detect the pathogen invasion at a lower

threshold and hence react in a more sensitized way. Finally, primed plants may exhibit other response networks, involving specific defense pathways (Paré *et al.*, 2005; Lämke and Bäurle, 2017).

Bacillus spp. are considered as microbial factories for the production of metabolites of interest, especially biosurfactant molecules such as lipopeptides. Lipopeptides consist of hydrophobic fatty acid tail linked to a short linear or cyclic oligopeptide (CLPs), and were, at first, mostly studied for their direct antimicrobial activities against phytopathogenic agents. However, many investigations were also performed on their stimulating effect of host defense mechanisms (Ongena and Jacques, 2008 ; Raaijmakers *et al.*, 2010; Rabbee *et al.*, 2019 ; Crouzet *et al.*, 2020). Three main groups of CLPs were reported to exhibit significant biological activities, including surfactins, iturins and fengycins. These CLPs possess a significant antimicrobial activity against a wide range of fungi and oomycetes, due probably to their ability to interact directly with pathogen plasma membranes, hence resulting in their destabilization, pore formation and cytoplasmic leakage, leading *in fine* to cell death or to an inhibition of spore germination (Chitarra *et al.*, 2003 ; Romero *et al.*, 2007 ; Etchegaray *et al.*, 2008; Pérez-García *et al.*, 2011 ; Qian *et al.*, 2016 ; Desmyttere *et al.*, 2019). However, some authors suggest that CLPs could also display antifungal activity by interacting with fungal intracellular targets (Qi *et al.*, 2010). Concerning their ability to induce plant immunity system, many studies have highlighted the potential of mainly two families of CLPs, fengycin and surfactin, to trigger defense reactions in various plants, such as bean, citrus, grapevine, lettuce, tomato, melon, rye grass, sugar beet, and wheat, as reviewed by Crouzet *et al.* (2020). Mycosubtilin, another CLP was also shown to induce defense responses in grapevine cells (Farace *et al.*, 2015). Supposedly, CLPs are able to fit into the lipid bilayer of host plant plasma membranes, slightly altering the lipid dynamics, hence leading to the activation of defense reactions, as eliciting or priming agents (Deleu *et al.*, 2008 ; Nasir *et al.*, 2010 ; Schellenberger *et al.*, 2019).

Although the innate immunity of wheat towards *Z. tritici* is extensively studied (*e.g.* Rudd *et al.*, 2015; Seybold *et al.*, 2020), the literature regarding the induced resistance in wheat against this major fungal pathogen, with exogenous treatments, is relatively poor. Regarding CLPs, only one study showing the potential of surfactin to activate defense mechanisms in wheat against *Z. tritici* has been recently reported (Le Mire *et al.*, 2018). Nevertheless, the ability of mycosubtilin, a CLP from the iturin family, to induce defense reactions in wheat against this disease has never been investigated, while its direct antimicrobial activity against *Z. tritici* has already been examined (Mejri *et al.*, 2018). Omics tools, including transcriptomics and metabolomics, have significantly improved these later years the understanding of plant-pathogen cross-talks. Although such approaches have already been used to investigate the interactions between wheat and *Z. tritici*, they have not been deployed so far to examine the induced resistance in this pathosystem. The aim of the present study was thus to determine whether mycosubtilin is able to trigger immunity reactions in wheat towards *Z. tritici* as an elicitation (in absence of infection) or a priming (in presence of infection) molecule, by using transcriptomic and metabolomic tools. The analyses allowed to decipher the mechanisms as well as the plant-defense related pathways induced by the biomolecule. Cytological bioassays were also performed to better understand the mode of action of mycosubtilin on both the fungus and the host plant.

MATERIALS AND METHODS

Plant growth, treatment and inoculation

Seeds of the cultivar (cv.) Alixan (Limagrain, France), susceptible to STB, were pregerminated in square Petri dishes (12 × 12 cm) on moist filter paper as described by Siah *et al.* (2010), and germinated grains were delicately transferred into three-liter pots filled with universal loam (Gamm Vert, France). The prepared pots were then placed in the greenhouse at $21 \pm 2^\circ\text{C}$ with a 16/8 h day-night cycle. For each condition and each sampling modality, three pots harboring 12 wheat plants each ($n=36$), were used. At day 0 (DO), corresponding to three weeks after sowing, plants of each pot were hand-sprayed with 30 mL of either a solution of 100 mg.L^{-1} mycosubtilin from *Bacillus subtilis* (Lipofabrik, Lesquin, France) or mock (control) treatment. Mycosubtilin was firstly dissolved in dimethyl sulfoxide (DMSO, Sigma-Aldrich, Saint-Louis, USA), leading to a final DMSO concentration of 0.1 % in the treatment solution, also supplemented with 0.05 % of polyoxyethylene-sorbitan monolaurate (Tween 20, Sigma-Aldrich, Saint-Louis, USA) serving as wetting agent. Mock treatment consisted of a solution of 0.1 % DMSO supplemented with 0.05 % of Tween 20. At two days after treatment (D2), half of the plants treated with mycosubtilin, as well as control plants, were inoculated by hand-spraying 30 mL of *Z. tritici* spore suspension ($10^6 \text{ spores.mL}^{-1}$), mixed with 0.05 % of Tween 20, on the plants of each pot. Fungal spores were obtained by growing the *Z. tritici* single-spore strain T02596 (isolated in 2014 in Northern France) on potato dextrose agar (PDA) medium during one week in dark conditions, according to Siah *et al.* (2010). The other half of the plants were mock inoculated by hand spraying 30 mL of a 0.05 % Tween 20 solution on the plants of each pot. All pots, even the mock inoculated ones, were then immediately covered with a clear polyethylene bag for three days in order to ensure a high humidity level on the surface of the leaves, a required condition for the success of the infection. At 23 days after treatment (D23), the disease severity level was assessed by scoring, on the plant third leaves, the area of lesions (chlorosis and necrosis) bearing or not pycnidia. Leaf samples were harvested at two (D2), five (D5) and fifteen (D15) days after treatment for further cytological, biochemical, or molecular analyses. In addition to the protection efficacy assay, a phytotoxicity biotest was performed in order to evaluate the impact of mycosubtilin at different concentrations (0, 0.8, 4, 20, 100, and 500 mg.L^{-1}) on leaf appearance and plant fresh biomass. The assay was carried out in the greenhouse using the same conditions of plant growth and treatment described above. Leaf appearance was assessed on the third leaves at two, seven, and 15 days after treatment, while the plant fresh biomass was determined at 15 days after treatment. Three pots of nine plants each (27 plants in total) were used as replicates for each molecule concentration condition. The global design of the whole study is presented in **Supplementary Figure S1**.

In vitro antifungal activity assay in solid medium

In vitro antifungal effect of mycosubtilin was determined by measuring the growth of the *Z. tritici* strain T02596 on PDA medium, as described by Platel *et al.* (2021). Briefly, mycosubtilin was dissolved in DMSO (0.1 % final DMSO concentration) and different concentrations of this biomolecule (0.19, 0.39, 0.78, 1.56, 3.125, 6.25, 12.5, 25, 50, and 100 mg.L^{-1}) were mixed at approximately 40°C with an autoclaved PDA medium. Each well of sterile 12-well plates (Cellstar standard®, Greiner Bio-One GmbH, Kremsmünster, Austria) were filled with three mL of the mixture. After PDA polymerization, a drop of 5 μL of *Z. tritici* spore suspension at $5.10^5 \text{ spores.mL}^{-1}$ was spotted on the center of each plate

well. After drop drying in sterile conditions, the plates were incubated in a growth chamber at $20 \pm 1^\circ\text{C}$ in dark conditions. Ten days later, the growth of fungal colonies was assessed by measuring their two perpendicular diameters. Control wells supplemented or not with 0.1 % DMSO and inoculated or not with fungal spores were also used. This experiment was repeated twice and three wells were used as replicates for each condition.

***In vitro* direct activity bioassays in liquid medium**

In vitro direct antifungal activity of mycosubtilin in liquid medium was assessed by measuring cell viability of the *Z. tritici* strain T02596 using resazurin staining, as proposed by Siah *et al.* (2010b). Sterile 12-well plates (Cellstar standard®, Greiner Bio-One GmbH, Austria) were used for the bioassay. Plate wells were filled with 2 mL of autoclaved glucose peptone medium supplemented with mycosubtilin at different concentrations (0.8, 4, 20, 100, and 500 mg.L⁻¹), before inoculation with fungal spore suspension already prepared in sterile glucose peptone medium, for a final concentration of 5.10^4 spores.mL⁻¹. Mycosubtilin was first dissolved in DMSO at a final concentration of 0.5 % in the wells. Controls with and without 0.5 % DMSO and with or without the fungus, were also used. After incubation for ten days at $20 \pm 1^\circ\text{C}$ under agitation at 150 rpm in dark conditions, 140 µL of the content of each well were sampled and deposited in flat-bottomed polystyrene 96-well microplates (Corning Costar®, Corning, USA). Then, 10 µL Alamar blue (AbD Serotec, UK) were added to each well before incubating the microplates for 4h in the same conditions described above. Cell viability was evaluated by measuring the absorbance at 540 nm using a microplate reader (Multiskan GO, Thermo Fischer Scientific, France). Six biological replicates were used as repetitions for each condition.

The inhibitory effect of mycosubtilin towards unique wheat cells (cv. Alixan) was assessed in sterile 12-well plates (Cellstar standard®, Greiner Bio-One GmbH, Austria). Wheat unique cells were obtained using the protocol of Biesaga-Kościelniak *et al.* (2008), with few modifications. Briefly, wheat caryopses were plunged for five minutes in 70% ethanol, rinsed twice with sterile osmotic water, and disinfected for 20 min in a 15% calcium hypochlorite solution, before being cleansed again at least twice. Mature embryos were delicately separated from the rest of the grain and were gently ground in a mortar. Fifty embryos were required to initiate wheat-cell suspension in 200 mL Erlenmeyer flask. These flasks contained 20 mL of autoclaved Gamborg B5 medium including vitamins (Duchefa Biochemie B.V, Netherlands), supplemented with 20 g.L⁻¹ sucrose and 3.5 mg.L⁻¹ 2,4-dichlorophenoxyacetic acid (Thermo Fischer Scientific, France). Wheat cell suspensions were incubated at $20 \pm 1^\circ\text{C}$ in dark conditions under agitation at 80 rpm. At three and six days after inoculation, 20 mL of sterile medium were added to each flask. At 10 days, the mixture was filtered through a one mm sieve, to get rid of ungrounded agglomerate tissue. Fresh medium was added to the filtrates to obtain 80 mL suspension in each flask. Seven days later, 20 mL of Gamborg B5 fresh medium were supplemented. Finally, every week, 25 % of the suspension was discarded and replaced by fresh medium. After two months of subculture, 12-well plate wells were filled with two mL of wheat cell suspension as well as two mL of fresh Gamborg B5 medium supplemented with mycosubtilin at different concentrations (0.8, 4, 20, 100, and 500 mg.L⁻¹). The plates were, then, placed into growth chamber at $20 \pm 1^\circ\text{C}$ in the dark with an agitation of 80 rpm. Three weeks after incubation, the number of wheat cells were counted for each condition using a Malassez hemocytometer under a light microscope (Nikon, Champigny-sur-Marne, France). Representative pictures were obtained using

digital camera (DXM1200C, Nikon, Champigny-sur-Marne, France) coupled with the image capture software NIS-Elements BR (Nikon, Champigny-sur-Marne, France).

***In planta* cytological assays**

The direct antifungal activity of mycosubtilin at 100 mg.L⁻¹ on *Z. tritici* spore germination as well as on the epiphytic hyphal growth of the fungus on the leaf surface was assessed *in planta* at D5 using Fluorescent Brightener 28 (Calcofluor, Sigma-Aldrich, Saint-Louis, USA), a chitin staining dye. Third-leaf segments (2 cm) harvested from wheat plants grown in the greenhouse, inoculated with *Z. tritici* and treated or not with mycosubtilin in the same conditions described above, were sampled and immersed for five minutes in a solution of 0.1 % (w/v) Calcofluor buffered with 0.1 M Tris-HCl, pH 8.5. Leaf segments were then rinsed twice in sterile osmosed water for two minutes before being dried in dark conditions at room temperature. Finally, they were deposited on a glass slide, covered with cover slip, and observed with an optic microscope (Eclipse 80i, Nikon, Champigny-sur-Marne, France) under UV-light. The proportion of different spore classes was determined from 100 distinct spores on each third-leaf segment. Spore classes were defined as followed; class 1, non-germinated spore; class 2, germinated spore with a small germ tube; class 3, germinated spore with developed germ tube; and class 4, germinated spore with a strongly developed germ tube. Nine leaf segments, randomly selected from three different pots (three segments per pot), were used as replicates for each condition. Pictures were obtained using digital camera (DXM1200C, Nikon, Champigny-sur-Marne, France) coupled with the image capture software NIS-Elements BR (Nikon, Champigny-sur-Marne, France).

Sampling design for transcriptomic and metabolomic analyses

Samples were collected at two days (D2) and five days (D5) after treatment for transcriptomic analyses. For each condition, *i.e.* plants treated with mycosubtilin (M) or not (control, C), and inoculated with *Z. tritici* (i) or not (Ni), nine third leaves sampled from three different pots (three leaves per pot), were randomly harvested, bulked per pot, immediately frozen in liquid nitrogen and stored at -80°C for further analyses. Thereafter, 100 mg of frozen pooled leaves were ground in a mortar with liquid nitrogen for RNA extraction. Regarding metabolomic analyses, samples were collected at D2, D5, as well as fifteen days after treatment (D15). For each modality, nine third leaves were randomly harvested from three different pots (three leaves per pot), immediately frozen in liquid nitrogen, and stored at -80°C for further analyses. Leaves were later freeze-dried using Alpha 2-4 LSCplus lyophilizer (Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany) and ground with iron beads using a MM2 Retsch mixer-mill (Retsch GmbH, Haan, Germany) in order to obtain a fine powder, suitable for analyses. Sampling design for both transcriptomic and metabolomic analyses is illustrated in **Supplementary Figure S1**.

RNA extraction and microarray analyses

Total RNA was extracted from wheat leaves using RNeasy Plant Mini Kit (Qiagen, Courtaboeuf, France). RNA quality was determined with Nanodrop by analyzing their absorbance ratios A260/280 and A260/230, which were found to range between 2.0 and 2.2. Moreover, RNA quality was also examined with Bioanalyzer 2100 (Agilent, France) and a minimal RNA integrity number (RIN) of 0.8 was required for all samples. For microarray analyses, hybridization for all conditions were performed in

triplicate with three sets of total RNA extracted from bulked wheat leaves (see above Sampling design section). Wheat Gene Expression Microarrays GE 4x44 (Agilent, Santa Clara, CA, USA) were used to study the gene expression profile between the different conditions. RNA amplification, staining, hybridization, and washing steps were conducted according to the manufacturer's specifications. Slides were scanned at five $\mu\text{m}/\text{pixel}$ resolution using the GenePix 4000B scanner (Molecular Devices Corporation, Sunnyvale, CA, USA). Images were used for grid alignment and expression data digitization with GenePix Pro 6.0 software (Molecular Devices Corporation, Sunnyvale, CA, USA). Gene expression data were normalized by Quantile algorithm. The three control samples were filtered for $P < 0.05$ and the average was calculated for each gene. A fold change (FC) value was calculated for each gene between individual treated samples and the mean of corresponding controls. Differentially expressed genes (DEGs) were selected for a significant threshold > 2.0 or < 0.5 ($P < 0.05$). Functional annotation of DEGs was based on NCBI GenBank and related-genes physiological processes were assigned with NCBI, AmiGO 2 Gene Ontology and UniProt. KEGG pathway analysis was also used to identify relevant biological pathways for the selected genes. All microarray data have been submitted to the NCBI GEO: archive for functional genomics data with the accession number.

Metabolite extraction and UHPLC-MS analyses

Metabolites were extracted from powdered freeze-dried wheat leaves (30-50 mg per sample) using 25 μL of methanol per mg dry weight. The extract was then incubated in an ultrasound bath for 10 minutes, before centrifugation at 13000 g at 10 °C for 10 minutes. The supernatant was analyzed using a Dionex Ultimate 3000 UHPLC system (Thermo Fisher Scientific, USA). The chromatographic separations were performed on a Nucleodur C18 HTec column (150 $\times$ 2 mm, 1.8 μm particle size; Macherey-Nagel, Germany) maintained at 30 °C. The mobile phase consisted of acetonitrile/formic acid (0.1%, v/v, eluant A) and water/formic acid (0.1%, v/v, eluant B) at a flow rate of 0.3 mL min⁻¹. The gradient elution was programmed as follows: 0 to 1 min, 95% B; 1 to 2 min, 95% to 85% B; 2 to 7 min, 85% to 0% B; 7 to 9 min, 100% A. The sample volume injected was 1 μL . The UHPLC system was coupled to an Exactive Orbitrap mass spectrometer (Thermo Fischer Scientific, USA), equipped with an electrospray ionization (ESI) source operating in positive mode. Parameters were set at 300 °C for ion transfer capillary temperature and 2500 V for needle voltages. Nebulization with nitrogen sheath gas and auxiliary gas were maintained at 60 and 15 arbitrary units, respectively. The spectra were acquired within the m/z (mass-to-charge ratio) mass ranging from 100 to 1000 atomic mass units (a.m.u.), using a resolution of 50,000 at m/z 200 a.m.u. The system was calibrated internally using dibutyl-phthalate as lock mass at m/z 279.1591, giving a mass accuracy lower than 1 ppm. The instruments were controlled using the Xcalibur software (Thermo Fischer Scientific, USA). LC-MS grade methanol and acetonitrile were purchased from Roth Sochiel (France); water was provided by a Millipore water purification system. Apigenin and chloramphenicol (Sigma-Aldrich, France) was used as internal standard.

Metabolites belonging to different chemical families were identified based on published works about benzoxazinoids (de Bruijn *et al.*, 2016), flavonoids (Wojakowska *et al.*, 2013) and hydroxycinnamic acid amides (Li *et al.*, 2018) from wheat. Putative metabolite identifications were proposed based on expertized analysis of the corresponding mass spectra and comparison with published literature. Further information was retrieved from the KEGG (Kyoto Encyclopedia of Genes and Genomes, <http://www.genome.ad.jp/kegg/>) and PubChem (<http://pubchem.ncbi.nlm.nih.gov>)

databases. Relative quantification of the selected metabolites was performed using the Xcalibur software. For some metabolites, identity was confirmed with the corresponding standard provided by Sigma-Aldrich (France).

Statistical analyses

Protection efficacy data set was analyzed using One-Way analysis of variance (ANOVA) at $P \leq 0.05$, while data obtained for *in planta* spore germination and hyphal growth were analyzed using ANOVA followed by the Tukey's test at $P \leq 0.05$, using GraphPad Prism software version 9 (GraphPad Software Inc., San Diego, USA). Regarding the *in vitro* antifungal activity assay, the half-maximal inhibitory concentration (IC_{50}) was also determined with the GraphPad Prism software version 9. For transcriptomic assay, DEGs were selected for a significant threshold > 2.0 or < 0.5 ($P < 0.05$). Functional annotations of DEGs were determined based on NCBI GenBank, AmiGO 2 Gene Ontology, UniProt and KEGG pathways. However, DEGs may be involved in more than one biological process, especially when it comes to stress responses, hence the determined functional annotation could be subjected to modifications in the future. Differential metabolomic analyses were performed with the W4M platform (Guitton *et al.*, 2017), using the Tukey's Honest Significant Difference method followed by a false discovery rate (FDR) correction using the Benjamini-Hochberg procedure (Benjamini and Hochberg, 1995). Metabolites of interest were considered differentially accumulated when the false discovery rate was below 5 % ($FDR \leq 0.05$).

RESULTS

Foliar application of mycosubtilin protects wheat against *Z. tritici* and reduces fungal spore germination and leaf epithetic growth

The ability of mycosubtilin to confer protection to wheat against *Z. tritici* was assessed in the greenhouse using the wheat cv. Alixan and the pathogenic *Z. tritici* strain T02596. At 21 days after inoculation, the level of disease severity consisted of 55.7% of diseased leaf area in the non-treated inoculated plants. Preventive foliar application of mycosubtilin at 100 mg.L^{-1} two days before inoculation resulted in a significant disease reduction (23.3% of diseased leaf area, corresponding to a 58.1% decrease) at 21 days after inoculation, *i.e.* 23 days after treatment (D23) in treated plants when compared to non-treated inoculated plants (**Figure 1A**). *In planta* fungal staining assays using Calcofluor revealed that the treatment with mycosubtilin significantly reduces the rates of both spore germination and epithetic growth of *Z. tritici* at three days after inoculation, *i.e.* five days after treatment (D5) (**Figure 1B**). In treated wheat plants, non-germinated spores (class 1) were significantly more abundant (approximatively five-fold) than in the control plants. Moreover, the numbers of germinated spores with either developed germ tube (class 3) or strongly developed germ tube (class 4) were significantly reduced (by three- and seven-folds, respectively) on treated plants when compared to the control ones.

Mycosubtilin impacts the *in vitro* growth of both *Z. tritici* spores and wheat single cells, but at different concentration thresholds

The effect of mycosubtilin on the *Z. tritici* *in vitro* growth was evaluated on solid PDA medium in 12-well plates. The biomolecule exhibited a strong antifungal activity against the pathogen, with IC₅₀ and MIC values of 0.57 and 0.78 mg.L⁻¹, respectively (**Supplementary Figure S2**). In order to gain more insights into the mode of action of mycosubtilin on the host plant as well as on the pathogen, further bioassays were performed in liquid medium to examine the effect of the lipopeptide on the development (cell multiplication) of either wheat single cells or *Z. tritici* spores. Microscopic observation of the fungal spores grown in glucose peptone liquid medium showed that mycosubtilin totally inhibits the fungal growth from the concentration of 4 mg.L⁻¹. Besides, the lipopeptide exhibits also an effect on the growth of wheat single cells cultivated in suspension in MS liquid medium, but with a total inhibiting concentration of 500 mg.L⁻¹ (**Figure 2**). Hence, the activity threshold of mycosubtilin seems to be 125-fold higher towards wheat single cells than against *Z. tritici* spores in liquid medium. However, no phytotoxic effect of mycosubtilin was observed at this concentration when the biomolecule is applied on wheat leaves. Indeed, no visible leaf necrosis and no significant effect on the total fresh biomass were noticed on the treated plants at all tested concentrations (**Figure 2**).

Mycosubtilin regulates several defense-associated genes in wheat against *Z. tritici*

Transcriptomic analyses using RNA microarray assay were performed in order to examine both eliciting and priming effects of mycosubtilin in wheat towards *Z. tritici* during the early stages of the fungal infection. The bioassay was performed in non-infectious conditions at two days after treatment (D2), and in both non-infectious and infectious conditions at five days after treatment (D5), *i.e.* three days after inoculation. The eliciting effect was examined at D2 and D5 by comparing the treated non-inoculated plants to non-treated and non-inoculated plants. The priming effect was investigated at D5 by comparing treated and inoculated plants to non-treated inoculated plants, by taking into account the elicitation modality at both D2 and D5. Moreover, in order to have a reliable conclusion and comprehensive view on the data linked to the priming effect, additional comparisons (treated and inoculated plants *versus* treated non-inoculated plants, and treated and inoculated plants *versus* non-treated non-inoculated plants), were also performed (**Supplementary Table S2, Supplementary Figure S4**). The fungal effect was investigated at D5 by comparing non-treated and inoculated plants to non-treated and non-inoculated ones. The different comparisons will thereafter be referred to as eliciting, fungal, and priming effects, corresponding to eliciting, infection alone, and priming modalities, respectively.

Among the 43,803 wheat probes represented in the RNA microarray chip, a total of 130 genes were regulated (*i.e.* when taking into account both up and down regulations) when examining eliciting, fungal, and priming effects and considering all time points (**Supplementary Table S1**). Overall, treatment with mycosubtilin led to a broader gene regulation response in priming modality at D5 when compared to both elicitation modalities at D2 or D5 (**Figures 3, 4 and 5**). When considering the eliciting effect at both D2 and D5, only 40 differentially expressed genes (DEGs) were highlighted, with 18, 6, and 16 DEGs specifically scored at D2, both D2 and D5, and D5, respectively. Among them, 28 were upregulated and 12 were downregulated (**Figure 3A**). At D5, priming modality displayed considerably

more DEGs (80) when compared to the eliciting effect at D5 (22), with 13 DEGs found in common among the two modalities. Globally, when considering all investigated modalities at D5 (eliciting, fungal, and priming effects), a total of 116 DEGs were recorded (**Figure 3B**). Out of the detected 116 DEGs, 8, 27 and 59, were specifically noticed during either elicitation, fungal or priming effect modalities, respectively. (**Figure 3B**).

Functional groups of DEGs recorded during wheat eliciting or priming by mycosubtilin at D5 were compared (**Figure 4**). Consistently with the findings highlighted above in Venn diagrams, priming conditions clearly exhibited more DEGs annotated in the highlighted functional groups when compared to the eliciting conditions at D5. Among the sixteen identified functional groups of DEGs, those linked to responses to stress were the most regulated upon treatment with mycosubtilin, especially in priming conditions, followed by those involved in growth and development, and chloroplast and light harvesting. Few functional groups of DEGs were found only in priming modality, including those related to pigment biosynthesis, photosynthesis, flowering and seed maturation, cell wall structure and function, and primary metabolic pathways (carbohydrate, amino acid, protein, and lipid metabolisms). Moreover, a relative strong increase in the number of DEGs involved in transcription regulation, RNA-processing and translation, as well as secondary metabolism, was observed in priming when compared to elicitation modalities (**Figures 4**).

When examining the gene regulation in detail, mycosubtilin-induced eliciting effect observed at D2 mainly involved DEGs associated to responses to pathogens, oxidative stress, and abiotic stress; most of them were significantly upregulated, except *peroxidase* and *peroxidase 2*, which were significantly downregulated (**Figures 5, Supplementary Table S1**). Noticeably, three DEGs involved in signaling, transcription, and translation (*Ca²⁺/H⁺ exchanging protein*, *zinc finger protein WZF1*, and *histone H1 WH1A.3*) were found overexpressed at D2 (**Figure 5**). Additionally, at D5, a downregulation of eight others DEGs, three associated with responses to pathogens (*thaumatin-like protein TL4*, *chitinase*, and *chitinase2*), as well as an upregulation of eight DEGs, with, among them, two linked with responses to pathogens, were recorded. In the priming modality at D5, mycosubtilin induced the regulation of a substantial number of genes related to several physiological pathways. Among them, a subset of 26 genes are linked to responses to stresses, including thirteen to oxidative stress, nine to pathogens, and four to abiotic stress. Interestingly, several genes involved in abscisic acid (ABA) phytohormone biosynthesis (*beta-carotene hydroxylase* and *lycopene β -cyclase*) and ABA-associated signaling pathways (*ARK protein*, *aquaporins*, *aldose reductase*, *ABC1 family protein-like*, *glutathione peroxidase-like protein GPX15Hv*, *nicotianamine synthase 3*, *chlorophyll a/b binding protein of LHCII type III chloroplast precursor*, *light-harvesting complex 1* and *S-adenosylmethionine synthetase*) were also regulated in priming modality at D5 (**Figure 5**). Regarding the effect of the fungal infection alone on the wheat leaf transcriptome at D5, only an upregulation of six DEGs involved in response to abiotic stress and pathogens was obtained, whereas a set of 32 DEGs was downregulated by the fungus alone at this time point, among them some are also related to responses to abiotic stress and pathogens. However, the most remarkable effect of *Z. tritici* is the downregulation of an important number of genes involved in cell-wall structure, amino acid metabolism, and protein metabolism (**Figure 5**).

Mycosubtilin primes flavonoid accumulation in wheat against *Z. tritici*

Metabolomic analyses using UHPLC-MS was undertaken to assess the effect of mycosubtilin on the wheat leaf metabolome in both non-infectious and infectious conditions, and at the same time points than those targeted in the above transcriptomic assay (D2 and D5), with an additional time point corresponding to 15 days after treatment (D15), *i.e* 13 days after inoculation, corresponding to the late stage of *Z. tritici* biotrophic phase. The eliciting effect was investigated at D2, D5, and D15 by comparing the treated non-inoculated plants to non-treated and non-inoculated plants. The priming effect was examined at D5 as well as D15, on the one hand, by comparing treated and inoculated plants to non-treated inoculated plants, and on the other hand, by considering the elicitation modalities at D2, D5, and D15. Besides, further comparisons (treated and inoculated plants *versus* treated non-inoculated plants, and treated and inoculated plants *versus* non-treated non-inoculated plants), were also carried out in order to have a comprehensive picture on the data linked to the priming effect on leaf metabolome (**Supplementary Figure 5**). As described in the transcriptomic assay, the different comparisons in the metabolomic assay will also be referred to as eliciting, fungal, and priming effects, corresponding to eliciting, infection alone, and priming modalities, respectively.

The analyses were targeted on 54 metabolites belonging to major chemical families that were detected and identified using UHPLC-MS (**Figure 6, Supplementary Table S3**). Plant treatment with mycosubtilin and/or inoculation with *Z. tritici* resulted in marked differentiations among wheat leaf metabolite patterns, as highlighted by the PCA analysis (**Supplementary Figure S6**). The largest changes in the accumulation of the selected metabolites were observed during fungal infection alone or during priming modalities, whereas only minor modifications were scored in eliciting modalities (**Figure 6**). In all eliciting modalities, only eight differentially accumulated metabolites (DAMs) were recorded, among them four were under-accumulated at D2 (asparagine and chry-C-hexo-O-hexo), D5 (MeJA), and D15 (tricin), and four were over-accumulated at D15, including three amino acids (glutamic acid, threonine and proline) and one hormone (ABA_Glc). However, in the priming modalities, a higher number of DAMs was scored, including 16 DAMs at D5 and 16 DAMs at D15 (**Figure 6**). At D5, only metabolite down-accumulation was detected (corresponding mainly to those over-accumulated in the fungal modalities), whereas at D15, both increases and decreases in metabolite concentration were observed. At D15, a decrease in the concentration of three hydroxycinnamic acid amides (coumaroylputrescine, coumaroylcadaverine, and caffeoylputrescine) and five amino acids and derivatives (leucine-isoleucine, arginine, threonine, glutamine, and methyl-pipecolate) were found. Interestingly, seven metabolites belonging to flavonoids and one benzoxazinoid were over-accumulated specifically in the priming modality at D15 (**Figure 6**).

Fungal infection alone induced substantial modifications in metabolite accumulation patterns at D5 and D15, with marked changes at D5, corresponding to the early stages of infection (**Figure 6**). Indeed, at D15, only significant changes in the concentration of glutamine, methyl-pipecolate, salicylic acid, and four hydroxycinnamic acid amides were noticed. However, at D5, a total of 26 DAMs, about half of the quantified molecules, were significantly regulated, with 18 molecules over-accumulated and eight under-accumulated. Seven amino acids, six benzoxazinoids, and five hydroxycinnamic acid amides were more concentrated, while three phytohormone precursors or derivatives (OPDA, MeJA and MeSA), five flavonoids, four luteolin derivatives, and one chrysoeriol derivative were down-accumulated (**Figure 6**). The proposed model on the effects of mycosubtilin-induced priming and *Z.*

tritici infection at D5 and D15 on both hydroxycinnamic acid amide and flavonoid biosynthesis pathways within wheat leaves is illustrated (Figure 7).

DISCUSSION

We combined here transcriptomic and metabolomic approaches to unravel the resistance mechanisms induced in wheat, by using the *B. subtilis* lipopeptide mycosubtilin as a treatment and *Z. tritici* as a phytopathogen model. Only few previous works have used Omics when characterizing induced resistance in plants, whilst such tools could be helpful and informative on the molecular and physiological changes occurring during plant resistance activation using exogenous treatments (e.g. Gauthier *et al.*, 2014 ; Fiorilli *et al.*, 2018). Our results revealed that mycosubtilin likely acts on the wheat-*Z. tritici* pathosystem through a double (direct and indirect) activity and that the indirect activity relies mainly on the priming rather than the elicitation of wheat defense mechanisms.

Mycosubtilin displays direct antifungal towards *Z. tritici* and likely interacts with wheat leaf cells without causing *in planta* phytotoxicity

Foliar application of mycosubtilin at 100 mg.L⁻¹ decreased by more than half (58.1%) *Z. tritici* symptoms on wheat plants, thus agreeing with previous results obtained on this pathosystem (Mejri *et al.*, 2018). Mycosubtilin has also been reported to biocontrol other pathogens, such as *Bremia lactucae* on lettuce, *Botrytis cinerea* on grapevine, and *Fusarium oxysporum* f. sp. *iridacearum* on *Iris* sp. (Deravel *et al.*, 2014; Farace *et al.*, 2015; Mihalache *et al.*, 2018). Our *in vitro* and *in planta* bioassays showed that mycosubtilin exhibits a direct antifungal effect on both spore germination and epiphytic growth of *Z. tritici* on wheat leaves, hence corroborating previous findings on this fungus (Mejri *et al.*, 2018). Interestingly, our results revealed that mycosubtilin exhibits growth inhibiting activity on fungal as well as wheat single cells grown in suspension, suggesting that the lipopeptide interacts with the cells of both organisms with a common process. Indeed, it has been reported that the mode of action of mycosubtilin may rely on the destabilization of cytoplasmic membranes (Nasir *et al.*, 2010 ; Nasir and Besson, 2012), that could lead to the loss of cell integrity, cytoplasm leakage, and ultimately cell death. We can, thus, hypothesize that the biological activities of mycosubtilin on both *Z. tritici* and wheat cells may be associated with its ability to interact with the plasma membranes of both organisms. Nevertheless, the *in vitro* threshold concentration of the molecule activity on both species varies strongly, with a difference of 125-fold between *Z. tritici* and wheat cells, despite that fungal cells growing in contact with the biomolecule during the bioassay were protected by cell-walls, while wheat cells grown in suspension didn't likely develop cell-walls. However, no visible phytotoxicity caused by mycosubtilin on the whole plants was observed even at the highest tested concentration (500 mg.L⁻¹), presumably because leaves are overall more robust (presence of cuticle, etc.) than single wheat cells growing *in vitro*. An effect of mycosubtilin at 50 mg.L⁻¹ on the growth of grapevine single cells has already been observed, with a significant increase in the amounts of cell death (23% of the total cells compared with 11% in the control) observed at 24h post-treatment, thus agreeing with our results (Farace *et al.*, 2015). Taken together, these results suggest that, when applied on wheat plants at 100 mg.L⁻¹, mycosubtilin is able, on the one hand, to display a direct antimicrobial activity towards the pathogen and, on the other hand, to interact with the plasma membranes of plant leaf cells without causing damages that could lead to their death. More likely, this interaction of the molecule with the

plant cell plasma membranes could be the initial stimulus triggering the downstream defense reactions highlighted in the wheat plants in the transcriptomic and metabolomic assays. This defense-initiating stimulus could be attributed, as suggested in other plant models, to the potential biophysical and biochemical interactions of mycosubtilin with the lipid bilayer of the leaf cell plasma membranes, hence modifying their fluidity as an “abiotic stress-like” agent, like thermal or drought stress (Uemura and Yoshida, 1986 ; Niu and Xiang, 2018 ; Couchoud *et al.*, 2019 ; Crouzet *et al.*, 2020). This “abiotic stress-like” mechanism could, hence, explain the accumulation of gene transcripts involved in plant defenses against abiotic and oxidative stresses found in eliciting conditions.

Perception of mycosubtilin by wheat leaves leads to the elicitation of few genes associated with plant defense

Transcriptomic analyses revealed that in wheat plant treated with mycosubtilin (100 mg.L⁻¹), the expression of genes involved in plant responses to stresses and other biological functions was significantly regulated. At D2, in the elicitation modality, 24 DEGs were recorded, among them three, involved in signaling, transcription and translation were upregulated, including gene encoding for Ca²⁺/H⁺ exchanging protein, a crucial regulator protein of cytoplasm calcium homeostasis. Moreover, we found in the same modality and time point an overexpression of a *CBL-interacting protein kinase* (*CIPK*) gene, encoding for a protein functioning as a calcium sensor displaying a large range of activity in plant responses against stresses (Wang *et al.*, 2016; Cui *et al.*, 2018). Calcium influx into the cytoplasm is considered as one of the first cellular reactions after stress signal perception, that can trigger subsequent downstream defense reactions upon detection of the elevation of calcium concentration, by calcium-dependent protein kinases (CDPKs) (Harmon *et al.*, 2000). The accumulation of *Ca²⁺/H⁺ exchanging protein* and *CIPK* transcripts detected in our conditions could likely be linked to the mycosubtilin-induced initial stimulus suggested above on the wheat cell plasma membranes, leading to a modification in Ca²⁺ concentration inside wheat cells and hence to the subsequent triggered defense reactions observed in wheat leaves. In addition, among the DEGs still highlighted at D2 in the elicitation modality, 14 are associated to responses to either oxidative, pathogen, or abiotic stresses, suggesting that mycosubtilin confers to wheat an increased resistance level to these stresses. Notably, the upregulation of three genes encoding for a *Pathogenesis-related (PR) protein* or precursors (*pathogen-related protein*, *chitinase II precursor*, and *PR17d precursor*), known to play a major role in plant resistance to pathogens, was observed. Chitinases are enzymes known to be involved in wheat defenses against *Z. tritici* by degrading the fungal cell-wall chitin (Kettles and Kanyuka, 2016). PR-17 is a class of PR proteins with an unknown mode of action, discovered by Christensen *et al.* (2002) and which could be involved in wheat defense against powdery mildew (Görlach *et al.*, 1996b). WIR1 is a membrane protein able to fortify plant cell wall and that was reported to contribute to wheat defense responses against fungal infections, including powdery mildew and stripe rust (Bull *et al.*, 1992 ; Coram *et al.*, 2008). The accumulation of these transcripts at D2, *i.e* just before the moment of plant inoculation, may contribute to wheat protection against *Z. tritici*. Nevertheless, further investigations are needed to determine the role of these proteins during the studied compatible interaction.

At D5, still in the elicitation context, 22 DEGs were found, among them six were also reported at D2, including three involved in responses to oxidative stress (*peroxidase*, *peroxidase 6* and

metallothionein-like protein type 3) and two in growth and development (*ZIM motif family protein* and *auxin-repressed protein*). Genes encoding for ZIM motif family protein were described as key repressors of the jasmonic acid (JA) signaling pathway (Chini *et al.*, 2009), being, hence, involved in the regulation of many physiological processes, especially in the mediation of plant responses to biotic and abiotic stresses (Sun *et al.*, 2017; Ruan *et al.*, 2019). A large number of genes belonging to this family were found to be regulated by other hormones such as gibberellins and ABA (Zheng *et al.*, 2020). Considering DEGs involved in the growth and development functional group, the upregulation of 1-aminocyclopropane-1-carboxylate (ACC) oxidase is to be noticed. This enzyme has been shown to be of prior importance for ethylene (ETH) production in plants (Houben and Van de Poel, 2019). As ETH is a gaseous plant hormone playing a crucial role in several biological processes, including tolerance to stresses, wheat responses to mycosubtilin could hence include ETH-responses (B. Adie *et al.*, 2007). Remarkably, Chandler *et al.* (2015) showed that mycosubtilin induces gene expression modifications *in vitro* in rice cells, with, especially, an increase in the quantity of ACC synthase (ACS1) transcripts, another key enzyme in ETH synthesis. Regarding DEGs involved in response to pathogens, genes encoding for PR proteins (*thaumatin-like protein TLP4* and *chitinases*) were found downregulated, whereas *MLA7*, an homolog of a resistance gene against powdery mildew in barley (Caldo *et al.*, 2004), and *hydrolase alpha/beta fold family-like*, encoding for a protein family displaying large varieties of functions in plants (Mindrebo *et al.*, 2016), were upregulated. In addition to this ability of mycosubtilin to regulate wheat defenses against biotic stress, we also observed significant modifications in the expression of genes involved in responses to abiotic stress, suggesting that mycosubtilin triggers multifaceted cross-talks in wheat defense pathways. In fact, three DEGs involved in response to abiotic stress were upregulated in the elicitation modality at D5, including genes encoding for transcription factor (TF) MYB2 (myeloblastosis), MYB-related protein, and ATP-dependent RNA helicase. MYB TFs were particularly studied for their critical importance in plant growth and stress tolerance, as reviewed by Ambawat *et al.*, (2013). More precisely, a MYB2 was reported to participate in rice tolerance to salt, cold, and dehydration stress (Yang *et al.*, 2012). The authors also showed that this TF was regulated by ABA. ATP-dependent helicases are major actors involved in RNA whole life, such as RNA splicing and decay as well as translation initiation (Nakamura *et al.*, 2004). Zhang *et al.* (2014) reported that wheat RNA helicase 1 (*TaRH1*) may play a regulatory role during plant responses to both biotic and abiotic stress.

Mycosubtilin primes genes associated with several metabolic pathways in wheat

In the priming modality at D5, we observed different DEG patterns than those detected in the elicitation ones at D2 and D5, with a number of DEGs drastically higher than in the other tested modalities, suggesting that mycosubtilin would act on wheat as a priming compound. This hypothesis is supported by the upregulation of the gene encoding for histone H1 WH1A.3 recorded at D2 in elicitation modality. Indeed, histone modifications are supposed to play a central role in priming memorization and transcription reprogramming after the stressing cue, corresponding here to mycosubtilin application (Lämke and Bäurle, 2017). Out of the 80 DEGs noticed in the priming modality, 26 are involved in responses to stresses (oxidative, pathogen and abiotic stresses). Among them, we observed significant downregulation of the expression of genes encoding for chitinases, glucan endo-1,3- beta glucosidase isoenzyme 1 and a germin-like protein. In addition, as observed in elicitation modalities, transcripts of *peroxidase* were also downregulated, suggesting that mycosubtilin may

negatively regulate peroxidase-related defense mechanisms. On the other hand, mycosubtilin priming led to the overexpression of DEGs involved in responses to pathogens such as *harpin binding protein* (*HrBP1-1*), a gene encoding for a protein located in plant cell walls known to promote anti-pathogen responses and SAR in plants (Chen *et al.*, 2012). We also found DEGs potentially involved in carotenoids and ABA biosynthesis, such as *beta-carotene hydroxylase* and *lycopene β -cyclase* (Du *et al.*, 2010 ; Kang *et al.*, 2018 ; Finkelstein, 2013). Remarkably, a significant number of DEGs described to play a part in ABA signaling and response pathways were regulated, such as genes encoding for ARK protein, aquaporins, aldose reductase, ABC1 family protein-like, glutathione peroxidase-like protein GPX15Hv, nicotianamine synthase 3, chlorophyll a/b binding protein of LHCII type III chloroplast precursor, light-harvesting complex 1 and S-adenosylmethionine synthetase (Karuna Sree *et al.*, 2000 ; Inoue *et al.*, 2003 ; Liu *et al.*, 2013 ; Zhai *et al.*, 2013 ; Kim *et al.*, 2015 ; Saruhashi *et al.*, 2015 ; Hwang *et al.*, 2016 ; Fang *et al.*, 2019). The expression of some of these genes could potentially lead to significant protective activity in wheat. For instance, aquaporins (AQPs) are membrane proteins, existing in a large variety of organisms, mainly known for their role in water transport (Verkman, 2013). As reviewed recently by Li *et al.* (2020), a member of AQP subfamily PIP1, AtPIP1;4, found in *Arabidopsis thaliana*, was shown to play a central part in pathogen associated-molecular patterns (PAMPs)-triggered immunity (PTI) and SAR in the plant, by regulating H₂O₂ transport across plasma membrane, from apoplast to cytoplasm, hence playing an active role in triggering H₂O₂-dependent host defenses. Another AQP, AtPIP1;2, has been reported to be involved in PTI and ABA-dependent stomatal closure in *A. thaliana* (Rodrigues *et al.*, 2017). Another identified relevant DEG in the priming modality is *ABC1-family like*. Indeed, Wang *et al.* (2013) have recently reported that *TaAbc1* upregulation is associated with hypersensitive response against stripe rust in wheat. Additionally, genes involved in carbohydrate metabolism were over-transcribed in the priming modality. ABA has been described as primordial in the regulation of carbohydrate metabolism during plant stresses (Kempa *et al.*, 2008). We also found an overexpression of DEGs involved in amino acid, protein, lipid and energy metabolisms, that could be regulated by mycosubtilin to compensate the potential deleterious effect of *Z. tritici* infection on wheat metabolism. Finally, four DEGs associated with signaling, transcription and translation were upregulated in the priming modality, highlighting the significant broader amplitude of responses displayed in mycosubtilin-primed and infected wheat plants than in naïve infected plants.

Mycosubtilin did not elicit marked metabolite regulation but primes flavonoid accumulation in wheat leaves

Metabolomic analyses showed a substantially higher number of DAMs in the priming modality than in the elicitation ones, thus agreeing with transcriptomic findings. Indeed, only few metabolites were regulated upon treatment with mycosubtilin in the elicitation modality, such as methyl jasmonate (MeJA) at D5 belonging to jasmonate family, hence corroborating with transcriptomic findings suggesting the involvement of JA pathway in mycosubtilin-induced defenses. At D15, a flavonoid (tricin) was found under-accumulated, whereas four metabolites were detected in higher concentrations, *i.e* glutamic acid, threonine as well as, remarkably, ABA-glucoside (ABA-Glc), and proline. Proline is an osmolyte amino acid involved in adaptation to abiotic stress, especially salt, osmotic, and freezing stresses (Szabados and Savaouré, 2010 ; Lv *et al.*, 2011). Proline accumulation is supposed to be biosynthesized from glutamic acid and regulated by ABA (Stewart, 1980 ; Cao *et al.*, 2020). Although the concentration of ABA and derivatives was not significantly regulated in treated

wheat leaves, except at D15 for ABA-Glc, many DEGs and DAMs associated to ABA-dependent pathways were scored, suggesting that wheat responses to mycosubtilin may involve this hormone (both in elicitation and priming modalities). Whereas the role of ABA in plant tolerance to abiotic stress, such as drought, salinity, cold, and heavy metals has been extensively investigated, its effect on plant pathogen resistance remains obscure (Asselbergh *et al.*, 2008 ; Vishwakarma *et al.*, 2017). As reviewed by Lievens *et al.* (2017), a negative effect of this phytohormone on plant resistance was reported for many pathosystems, such as tomato-*B. cinerea* and rice-*Magnaporthe oryzae*. In the rice-*M. grisea* pathosystem, ABA was shown to compromise rice resistance against the pathogen by acting antagonistically towards SA-dependent pathway (Jiang *et al.*, 2010). Some phytopathogens were even able to biosynthesize their own ABA, such as *B. cinerea* and *M. oryzae*, most likely to modulate host defenses and enhance their pathogenicity (Ding *et al.*, 2015 ; Spence *et al.*, 2015). However, in the later years, this phytohormone was described to also display positive effect on plant resistance in other pathosystems, such as *A. thaliana*-*Pythium irregulare* and *Brassica napus*-*Leptosphaeria maculans* (Adie *et al.*, 2007b ; Kaliff *et al.*, 2007). Two ABA-dependent modes of action on plant resistance against pathogens were highlighted, including regulation of stomatal closure in order to prevent pathogens from invading the plant, and callose deposition (Ton *et al.*, 2009). In addition, ABA was also recently proposed as a potential key actor in molecular cross-talks for plant-microbe symbiosis at the rhizosphere level (Stec *et al.*, 2016). In the wheat-*Z. tritici* pathosystem, the role of ABA, as well as ethylene, in the host resistance mechanisms has, up to now, been clearly overlooked. Hence, further investigations focusing on this phytohormone may provide new insights into its role in wheat resistance against *Z. tritici*.

In the priming modality at D5, only an under-accumulation of metabolites was observed, with patterns of amino acids and benzoxazinoids in opposition to those scored in the fungal effect modality at the same time point. This result suggests that mycosubtilin treatment (weakening the fungus pathogenicity) may reduce the expected fungal effect on wheat benzoxazinoid and amino acid pathways, likely due (i) to its direct antifungal activity and/or (ii) to a countering by the plant of *Z. tritici* effect on these two pathways thanks to a compensatory mechanism. However, at D15, we observed remarkable significant changes in metabolite accumulation. The most remarkable result was clearly the increased accumulation of almost all targeted flavonoids, especially luteolin-derivatives (**Figure 6 and 7**). Indeed, we detected an over-accumulation of lut-6-C-Glc, lut-C-hexo-O-hexo, lut-C-hexo-O-deoxyhexo, lut-C-hexo-C-pento, chry-6-C-Glc, chry-C-hexo-O-hexo, and tricin-7-O-hexo (**Figure 6 and 7**). Remarkably, at D5 in the priming modality, genes encoding for chalcone synthase 1 and 2, enzymes involved in flavonoid biosynthesis, were significantly upregulated (**Figure 7**). Flavonoids are known to exhibit a range of biological activities in plant protection, not only antimicrobial activity but also strong antioxidant activity and cell-wall reinforcing (Lam *et al.*, 2015 ; Lan *et al.*, 2015 ; Al Aboody and Mickymaray, 2020). Accumulation of compounds displaying these three activities may be greatly beneficial for the host, especially just at the moment preceding the *Z. tritici* switch to necrotrophic phase. Indeed, at this time, *Z. tritici* growth increases, and the fungus produces toxins to induce wheat cell apoptosis, in order to feed on the released nutrients (Steinberg, 2015b). Hence, cell-wall strengthening and the increase in antioxidant capacity may very likely help wheat mesophyll cells to resist to the pathogen attack as well as apoptotic-like cell death initiating symptom development. On the other hand, hydroxycinnamic acid amides (coumaroylputrescine, coumaroylcadaverine, and caffeoylputrescine) were significantly under-accumulated. The decrease in the concentration of these

metabolites in wheat leaves may be linked with the hypotheses regarding mycosubtilin effect on plant metabolome during the priming modality emitted above (due to direct mycosubtilin antifungal effect and/or plant compensatory mechanism). Nevertheless, another reason would be the reorganization of metabolic pathways due to the mycosubtilin priming effect, as illustrated in **Figure 7**. Hence, during infection, flavonoid biosynthesis pathway would be enhanced at the detriment of the hydroxycinnamic acid amide pathway, leading to this differential accumulation in wheat leaves in the priming modalities. Interestingly, flavonoid biosynthesis may be induced by ABA, highlighting once more that ABA-dependent responses may be crucial to understand wheat responses to mycosubtilin (Gai *et al.*, 2020). Finally, other metabolomic responses associated with the mycosubtilin-primed effect is the under-accumulation of leucine-isoleucine, arginine, threonine, glutamine, methyl-pipecolate and DHBOAdiglc, likely explained by the antifungal and/or compensatory hypotheses launched above (**Figure 6**).

In conclusion, this study provides new insights into the mechanisms underlying the bioactivity of the *B. subtilis* lipopeptide mycosubtilin on the wheat-*Z. tritici* pathosystem. This promising biomolecule likely confers protection through a double activity, *i.e.* directly by displaying an antimicrobial activity against the fungus, and indirectly by priming defense responses in wheat that can help the plant to deal with the pathogen. Taken together, these findings reveal that mycosubtilin acts on wheat as a priming agent rather than an eliciting agent, likely by involving ABA, JA, ETH biosynthesis and signaling pathways. The stimulation of the plant immune system by mycosubtilin probably results from the interaction of the biomolecule with plasma membranes of leaf cells leading to the activation of abiotic stress-like responses in the plant. However, further investigations are required to unravel the precise mechanisms by which wheat leaf cells perceive mycosubtilin within the membranes.

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CONFLICT OF INTEREST

Philippe Jacques is a co-founder of Lipofabrik and Lipofabrik Belgium and a member of the scientific advisory board of both companies.

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Figures

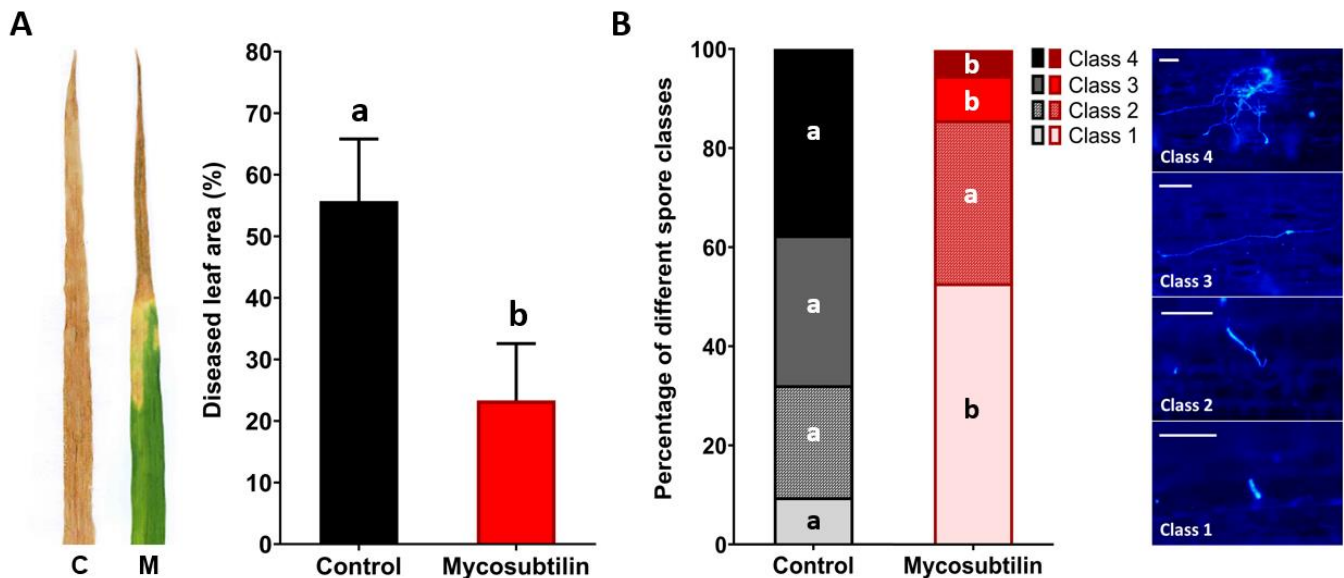


Figure 1. Disease severity level (**A**) and rates of *in planta* spore germination and epiphytic growth (**B**) of *Zymoseptoria tritici* (T02596 strain) on wheat leaves (cv. Alixan) pre-treated with mycosubtilin (M) at 100 mg.L⁻¹ or not (C, control). (**A**) Disease severity level was scored at 21 day safter inoculation (*i.e* 23 days after mycosubtilin treatment) by assessing the area of lesions (chlorosis and necrosis) on wheat third leaf of each plant (n=36). Means tagged with the same letter are not significantly different according to One-way ANOVA test ($P \leq 0.05$). (**B**) Proportions of four different development stages of fungal spores was assessed by using Calcofluor staining recorded at three days after inoculation (*i.e* five days after mycosubtilin treatment). The different spore development classes are defined as followed: Class 1, non-germinated spore; Class 2, germinated spore with small germ tube; Class 3, germinated spore with developed germ tube; Class 4, germinated spore with a strongly developed germ tube. For each condition, the different classes were determined from 100 distinct spores chosen randomly on each third-leaf segment. Within each spore class, the presence of different letters indicates a significant difference according to the Tukey test at $P \leq 0.05$. Scale bar = 25 µm.

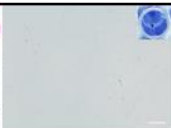
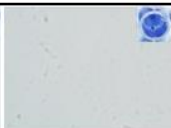
Mycosubtilin (mg. L ⁻¹)	Control	DMSO	0.8	4	20	100	500
Fungal cell suspension							
ΔDO (540 nm)	1.39 (± 0.10)	1.47 (± 0.20)	1.36 (± 0.23)	1.36 (± 0.23)	~0	~0	~0
Wheat cell suspension							
Wheat cell (cells mL ⁻¹)	12x10 ⁴ (± 11,4x10 ⁴)	10x10 ⁴ (± 8,2x10 ⁴)	11x10 ⁴ (± 11,0x10 ⁴)	12x10 ⁴ (± 10,3x10 ⁴)	7x10 ⁴ (± 10,6x10 ⁴)	1x10 ⁴ (± 3,2x10 ⁴)	< 1x10 ⁴
Wheat leaf surface							
Visible tissue damage	No necrosis	No necrosis	No necrosis	No necrosis	No necrosis	No necrosis	No necrosis
Wheat plant biomass							
Fresh weight (g per pot)	102.3 (± 11.5)	102.0 (± 15.6)	103.7 (± 6.7)	102.0 (± 7.2)	98.9 (± 8.5)	101.4 (± 8.8)	102.5 (± 10.2)

Figure 2. Effect of mycosubtilin at different concentrations on the growth of either *Z. tritici* or wheat cells grown *in vitro* in liquid medium in twelve well microplates, and on leaf appearance and plant fresh biomass of whole plants grown in the greenhouse. Fungal growth was assessed using resazurin staining and optical density measurement at 10 days after inoculation (dai) of glucose peptone medium with *Z. tritici* spores (n=6), while wheat cell growth was evaluated using Malassez hemocytometer at 21 days after inoculation of Gamborg B5 medium with wheat cell suspension (n=10). Leaf appearance was assessed visually at two, seven and fifteen days after plant treatment (n=27), while the fresh biomass was determined by weighting nine whole plants from each pot (n=3). Representative pictures of either *Z. tritici* or wheat cell cultures observed under light microscope at magnification 10x and 20x respectively, as well as the microplate wells used for *Z. tritici* culture medium staining with resazurin, are shown. Likewise, representative pictures of leaf appearance and aerial plant fresh biomass, at fifteen days after treatment, are also presented.

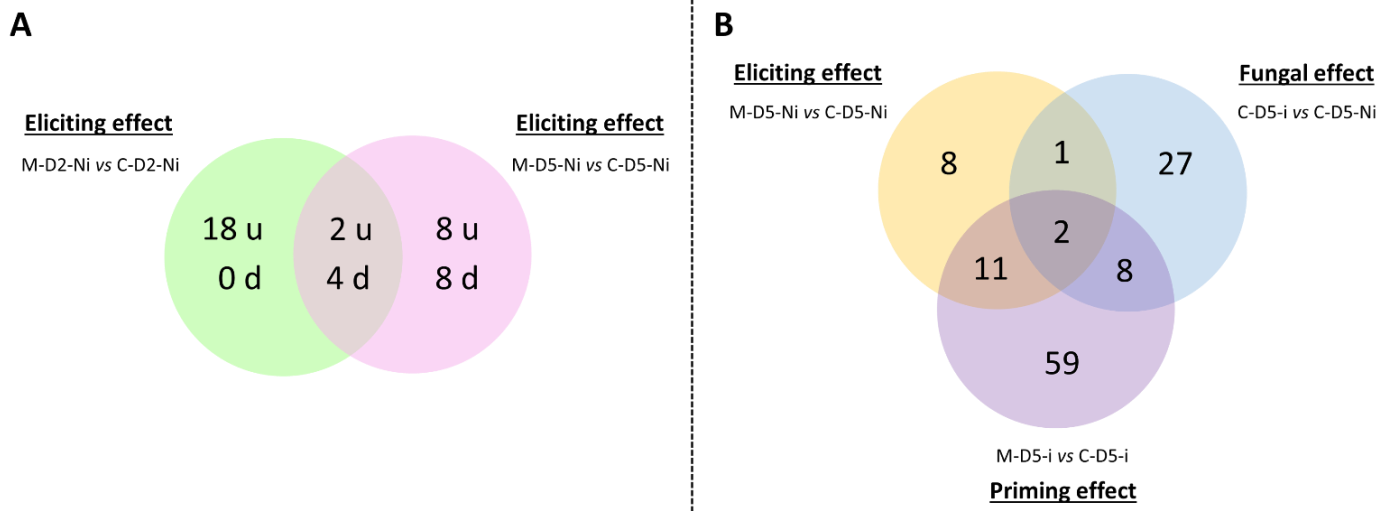


Figure 3. Venn diagrams underlying (A) the number of up-regulated (u) or down-regulated (d) genes observed after mycosubtilin treatment in non-infectious conditions by *Zymoseptoria tritici* as well as (B) the number of differentially expressed genes in the different tested conditions at five days after treatment with mycosubtilin (*i.e.* three days after wheat inoculation with *Z. tritici*). In (A), the potential early elicitation effect of mycosubtilin at two days after treatment (M-D2-Ni vs C-D2-Ni) on the levels of wheat leaf gene expression is compared to the later one at five days after treatment (M-D5-Ni vs C-D5-Ni). In (B), The eliciting effect of mycosubtilin (M-D5-Ni vs C-D5-Ni) is compared to the fungus effect (C-D5-i vs C-D5-Ni) and to the priming effect of mycosubtilin (M-D5-i vs C-D5-i). M means treated with mycosubtilin towards mock treated C. Ni stands for mock inoculated whereas i indicates that plants were infected with *Z. tritici*. D2 and D5 means that leaves were sampled respectively at two and five days after mycosubtilin treatment.

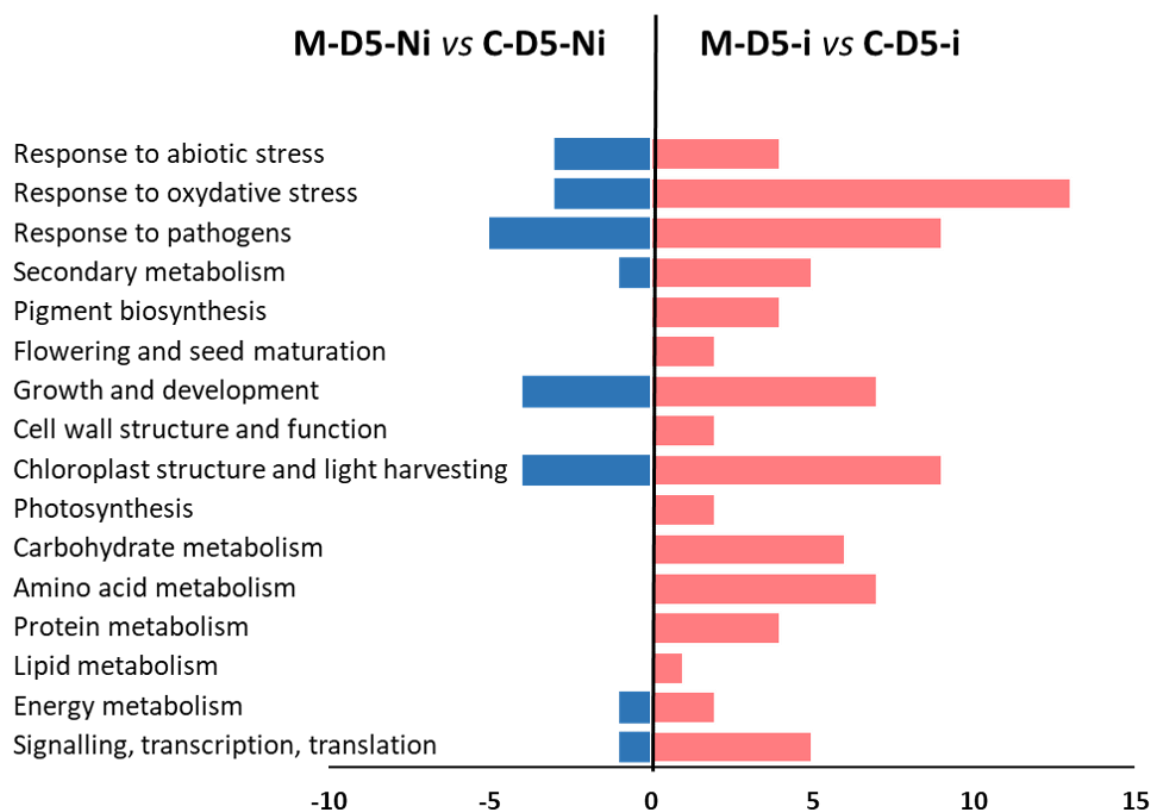


Figure 4. Functional groups of differentially expressed genes highlighting eliciting (M-D5-Ni vs C-D5-Ni) or priming (M-D-5i vs C-D5-i) effect of wheat defenses after treatment with mycosubtilin. Functional annotation of DEGs was performed based on NCBI GenBank and related-gene physiological processes were assigned with NCBI, AmiGO 2 Gene Ontology and UniProt. M means treated with mycosubtilin towards mock treated C. Ni stands for mock inoculated whereas i indicates that plants were infected with *Z. tritici*. D5 means that leaves were sampled at five days after mycosubtilin treatment.

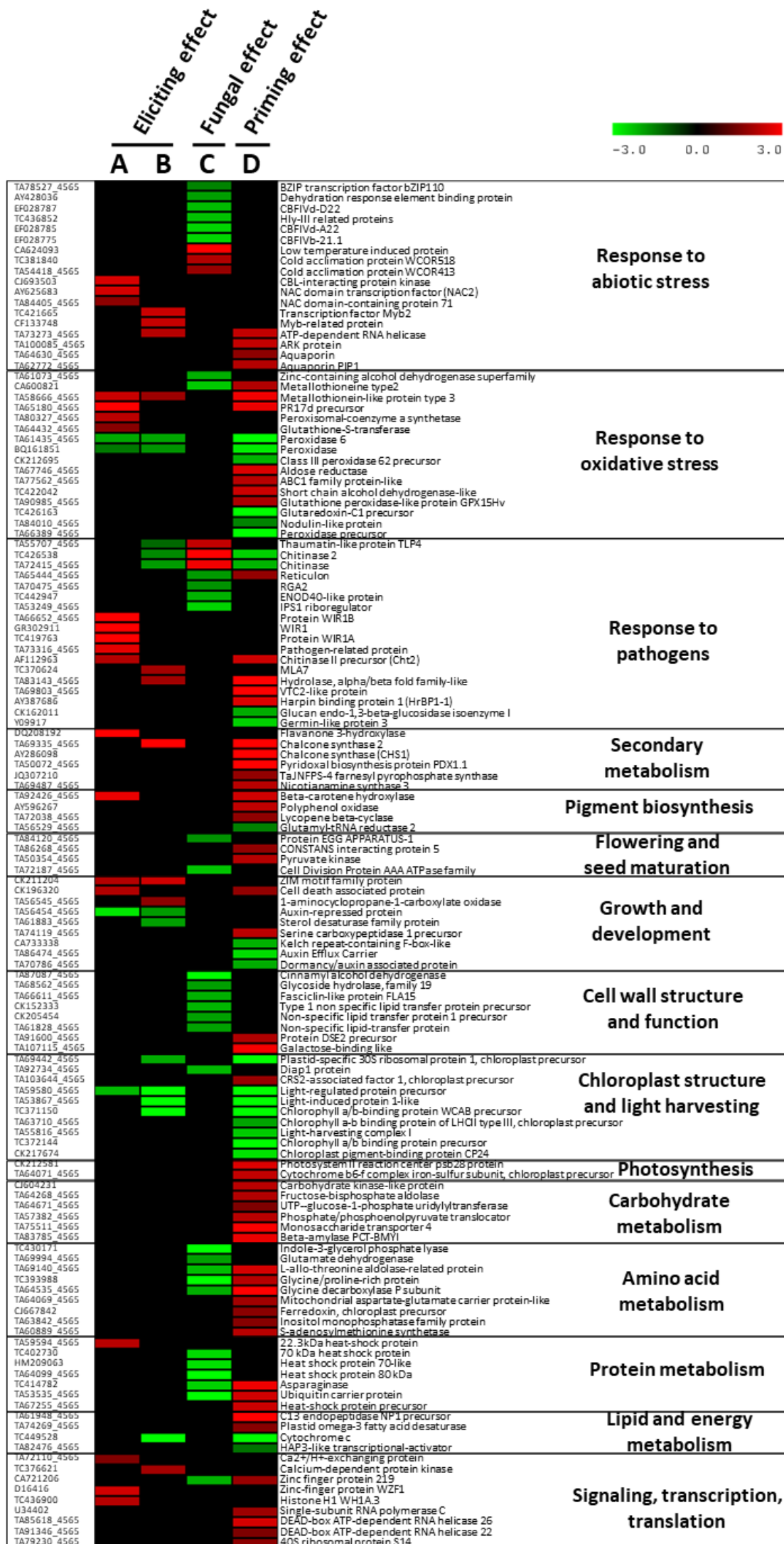
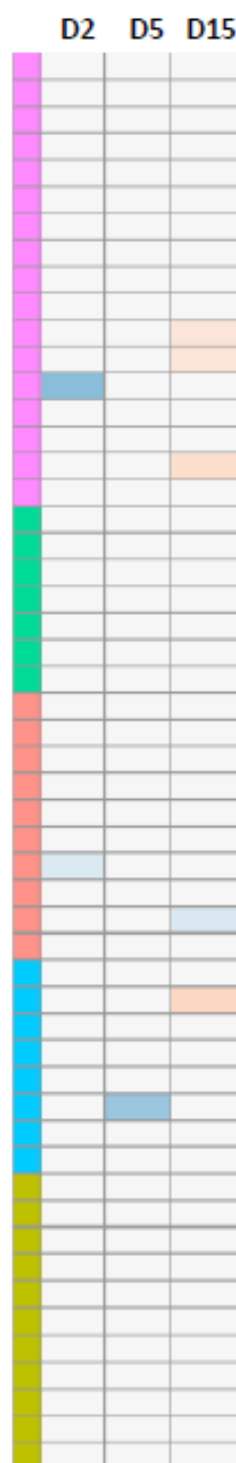
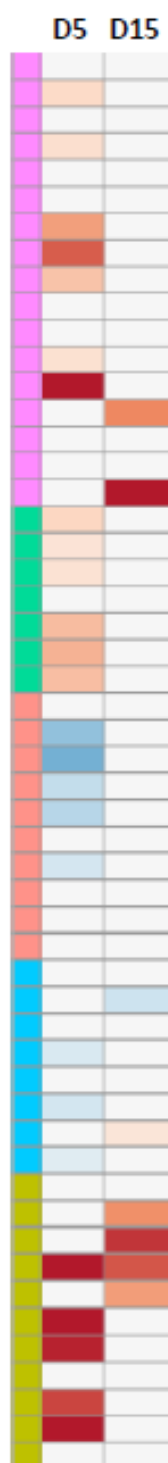


Figure 5. Heatmap showing significantly up- or down-regulated genes in wheat third-leaves (cv. Alixan) treated or not with 100 mg.L⁻¹ mycosubtilin and infected or not with *Zymoseptoria tritici* (strain T02596), recorded at two days after treatment in noninfectious conditions (**A** and **B**) and at five days after treatment (*i.e.* three days after inoculation) in infectious conditions (**C** and **D**). **A** and **B** highlight the eliciting effect, **C** shows the fungal effect, while **D** reveals the priming effect induced by mycosubtilin. Gene-related physiological processes are represented on the right part of the graph and were determined using NCBI, AmiGO 2 Gene Ontology, KEGG and UniProt. **A**, M-D2-Ni vs C-D2-Ni; **B**, M-D5-Ni vs C-D5-Ni; **C**, C-D5-i vs C-D5-Ni; **D**, M-D5-i vs C-D5-i. Significant relative change in gene expression is presented in Log2 ratio, according to the color scale, using the WebMev software. M means treated with mycosubtilin towards mock treated C. Ni stands for mock inoculated whereas i indicated that plants were infected with *Z. tritici*. D2 and D5 means that leaves were sampled respectively at two and five days after mycosubtilin treatment.

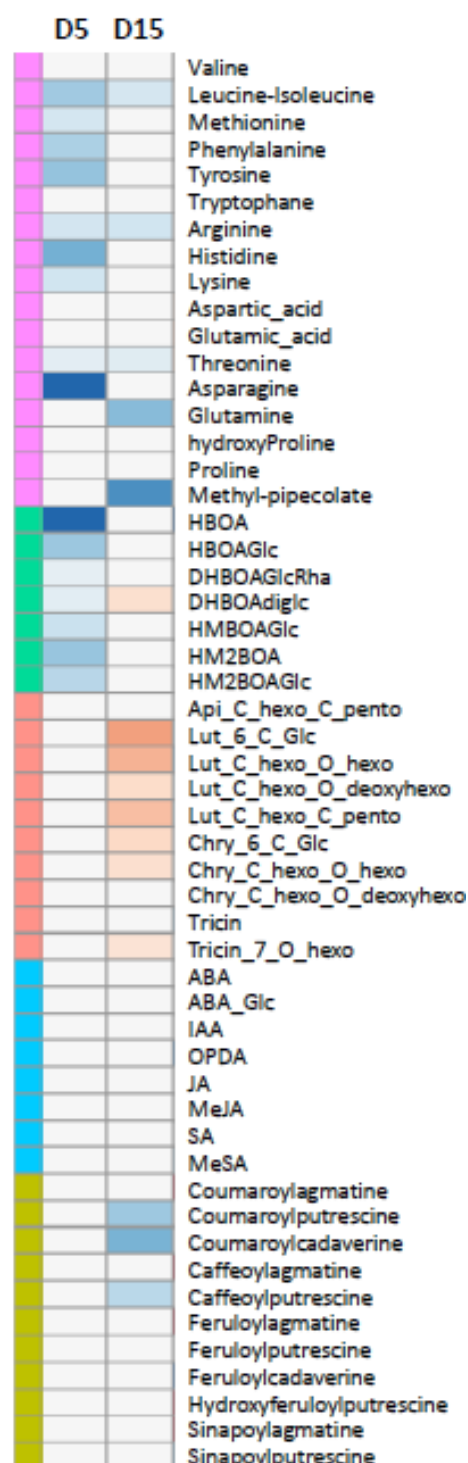
A) Eliciting effect



B) Fungal effect



C) Priming effect



Group

- Amino acids
- Benzoxazinoids
- Flavonoids
- Hormones
- Hydroxycinnamic acid amides

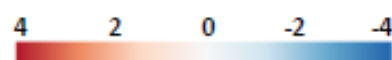
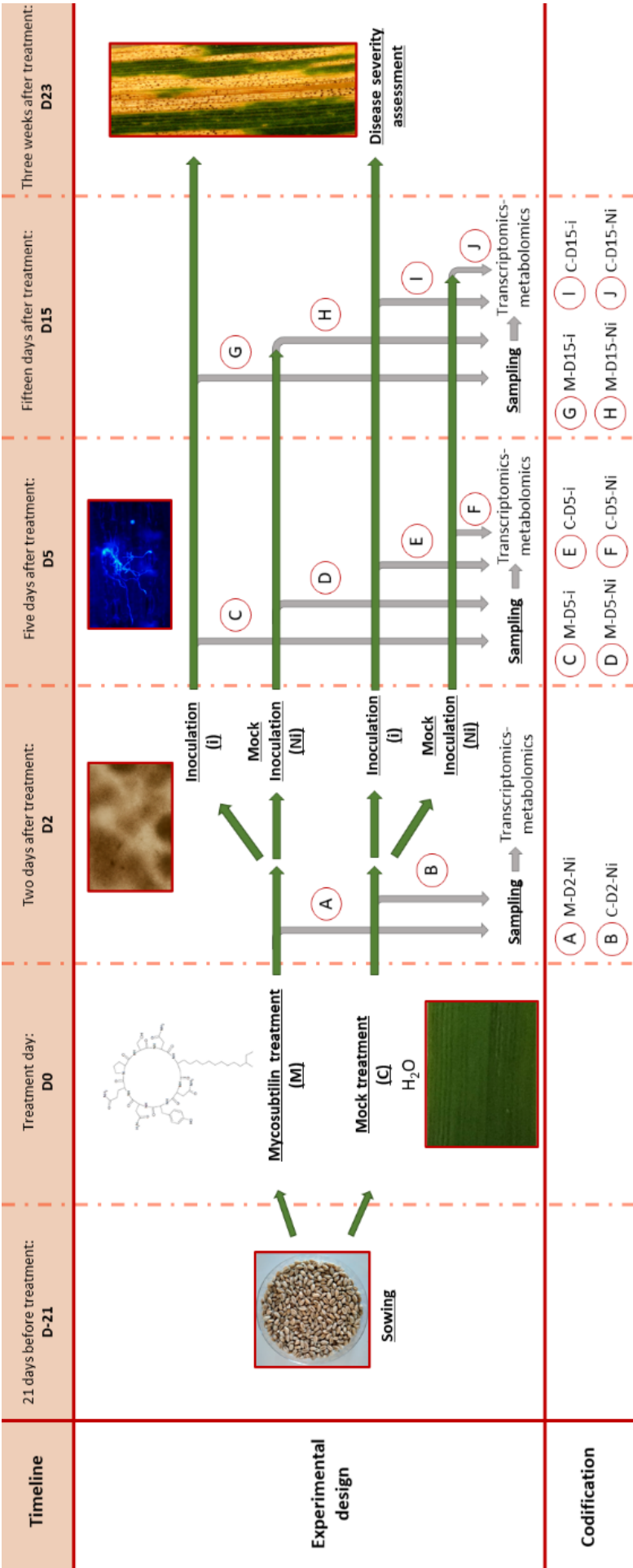


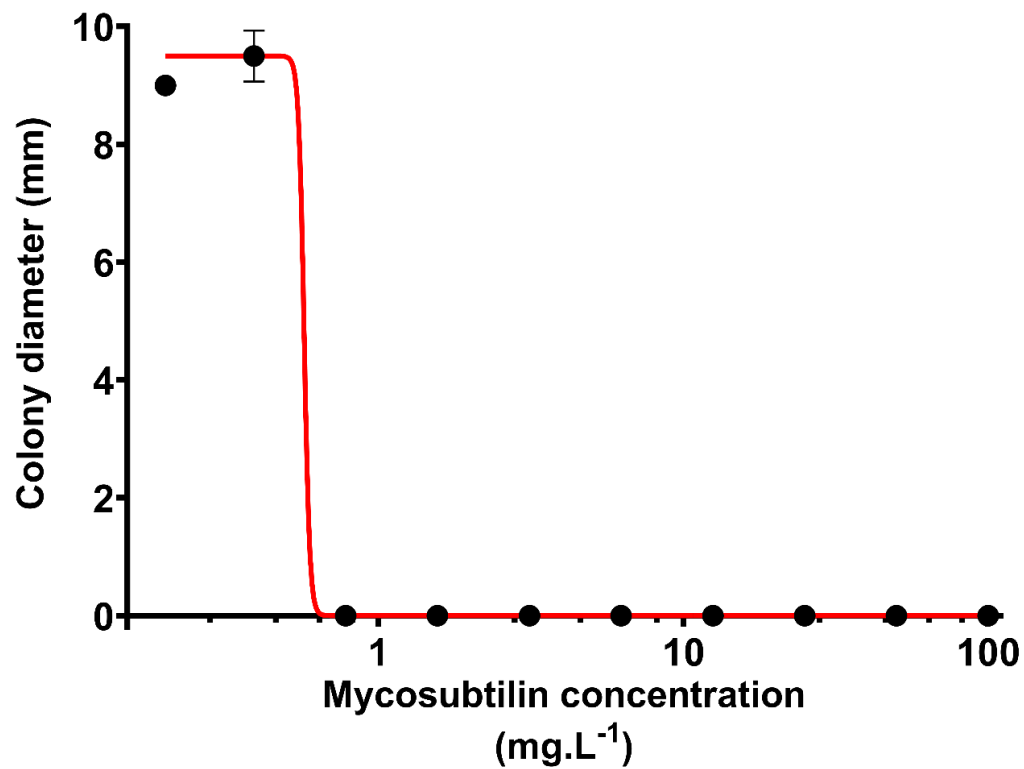
Figure 6. Heatmap of significant relative changes in metabolite patterns within wheat third-leaves (cv. Alixan) treated or not with 100 mg.L⁻¹ mycosubtilin and infected or not with *Zymoseptoria tritici* (strain T02596) at different time points (n = 9 plants). (A), potential elicitation effect of mycosubtilin in noninfectious conditions at 2 (M-D2-Ni vs C-D2-Ni), 5 (M-D5-Ni vs C-D5-Ni) and 15 (M-D15-Ni vs C-D15-Ni) days after treatment (dat). B, Fungal effect on wheat leaf metabolome at 5 (C-D5-i vs C-D5-Ni) and 15 (C-D15-i vs C-D15-Ni) dat, *i.e.* at 3 and 13 days after wheat inoculation (dai) with *Z. tritici*, respectively. C, Priming effect of mycosubtilin in infectious conditions at 5 (MD5i vs CD5i) and 15 (MD15i vs CD15i) dat (*i.e.* at 3 and 13 dai, respectively). M means treated with mycosubtilin towards mock treated C. Ni stands for mock inoculated whereas i indicates that plants were infected with *Z. tritici*. D2, D5 and D15 mean that leaves were sampled respectively at two, five and fifteen days after mycosubtilin treatment. Log2 of significant metabolite fold changes for indicated pairwise comparisons are given by shades of red or blue colors according to the scale bar. Metabolites were grouped according to their functional or chemical family as amino acids, benzoxazinoids, flavonoids, hormones and hydroxycinnamic acid amides. Data represent mean values of nine biological replicates for each condition and time point. Statistical analyses were performed using the Tukey's Honest Significant Difference method followed by a false discovery rate (FDR) correction, with FDR < 0.05. For FDR ≥ 0.05, Log2 fold changes were set to 0.



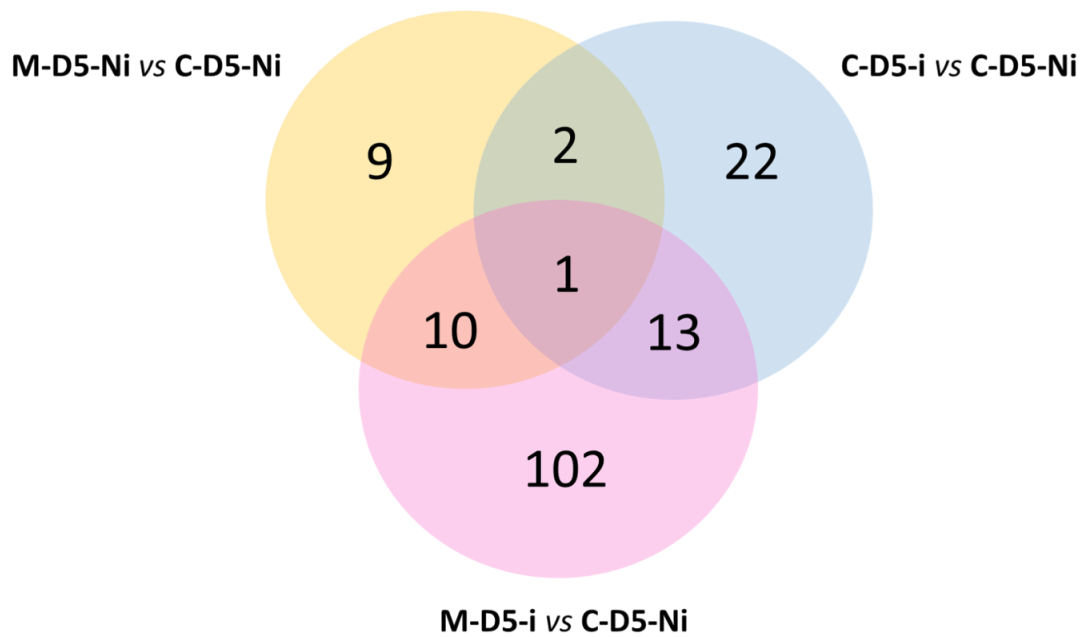
Figure 7. Metabolic map of the biosynthesis pathways and patterns of accumulation of major hydroxycinnamic acid amides and flavonoids within wheat third leaves (cv. Alixan) inoculated with *Zymoseptoria tritici* (strain T02596) pre-treated or not with mycosubtilin. Metabolic pathways are based on KEGG pathways (map00310, map00330, map00940, map00941, map00944). Amino acids, diamines, hydroxycinnamic acid amides and flavonoids metabolite families are separated and indicated in block of different colors, respectively purple, black, green and orange. Heatmaps show significant Log₂ fold changes in metabolite accumulation, according to the corresponding scale bar. For each analyzed metabolite, the two upper squares represent the fungus effect on the relative accumulation of the compound, from the left to the right, at five (C-D5-i vs C-D5-Ni) and fifteen (C-D15-i vs C-D15-Ni) days after treatment (dat), *i.e* 13 days after inoculation (dai). The two lower squares represent the effect of application of mycosubtilin (priming) prior to infection with *Z. tritici* on the wheat metabolome at five (M-D5-i vs C-D5-i) and fifteen (M-D15-i vs C-D15-i) dat. Metabolites in grey were not analyzed in this study. Solid arrows stand for enzymatic reactions completely described in the abovementioned KEGG pathways whereas dashed arrows are for suggested ones. Double arrows represent two or more metabolic steps. With ACT: agmatine N⁴-coumaroyltransferase, ADC: arginine decarboxylase, CCOAOMT: caffeoyl-CoA O-methyltransferase, CHS: chalcone synthase, F3'5'H: flavonoid 3',5'-hydroxylase, F3'H: flavonoid 3'-monooxygenase, FOMT: flavone 3'-O-methyltransferase, LDC: lysine decarboxylase. Genes coding for chalcone synthase were significantly upregulated when plants were primed with mycosubtilin at five days after treatment (M-D5-i vs C-D5-i), hence CHS is presented in red. M means treated with mycosubtilin towards mock treated C. Ni stands for mock inoculated whereas i indicates that plants were infected with *Z. tritici*. D5 and D15 mean that leaves were sampled respectively at five and fifteen days after mycosubtilin treatment.



Supplementary figure S1. Experimental design illustrating the main steps of the whole study. After pre-germination in square petri dishes, wheat seeds (cv. Alixan) were grown in greenhouse for 21 days. At days 0 (DO), plants were either sprayed with a solution of mycosubtilin at 100 mg.L^{-1} or with a mock treatment. Photography of wheat third-leaves epidermis observed with an optic microscope is shown. At two days after treatment (D2), third leaves were harvested for each condition and were used for further analyses (transcriptomics and metabolomics). After this, remaining plants were inoculated, or not, with a spore suspension of the *Zymoseptoria tritici* strain T02596 (concentration: 10^6 spores mL^{-1}). Photography of *Z. tritici* growing on PDA medium and observed microscopically is presented. At five days (D5) and fifteen days (D15) after treatment, wheat third leaves were sampled for further analyses; assessment of the *in planta* infection process of the fungus at D5 was investigated as well as transcriptomic analyses whereas metabolomic assays were performed at D5 and D15. Finally, at 23 days after treatment (D23), disease severity level was scored by measuring the third-leaf area with disease lesions. Codification used for each modality in the present study is shown at the bottom of the figure.



Supplementary figure S2. Dose-response curve obtained *in vitro* for mycosubtilin towards the *Zymoseptoria tritici* strain T02596. The fungal growth was assessed on PDA medium amended or not with different concentrations of mycosubtilin and scored 10 days of incubation by measuring fungal colony perpendicular diameters (n=6 colonies). Non-linear regression was performed and IC₅₀ was determined at 0.57 mg.L⁻¹ using GraphPad Prism software version 9.



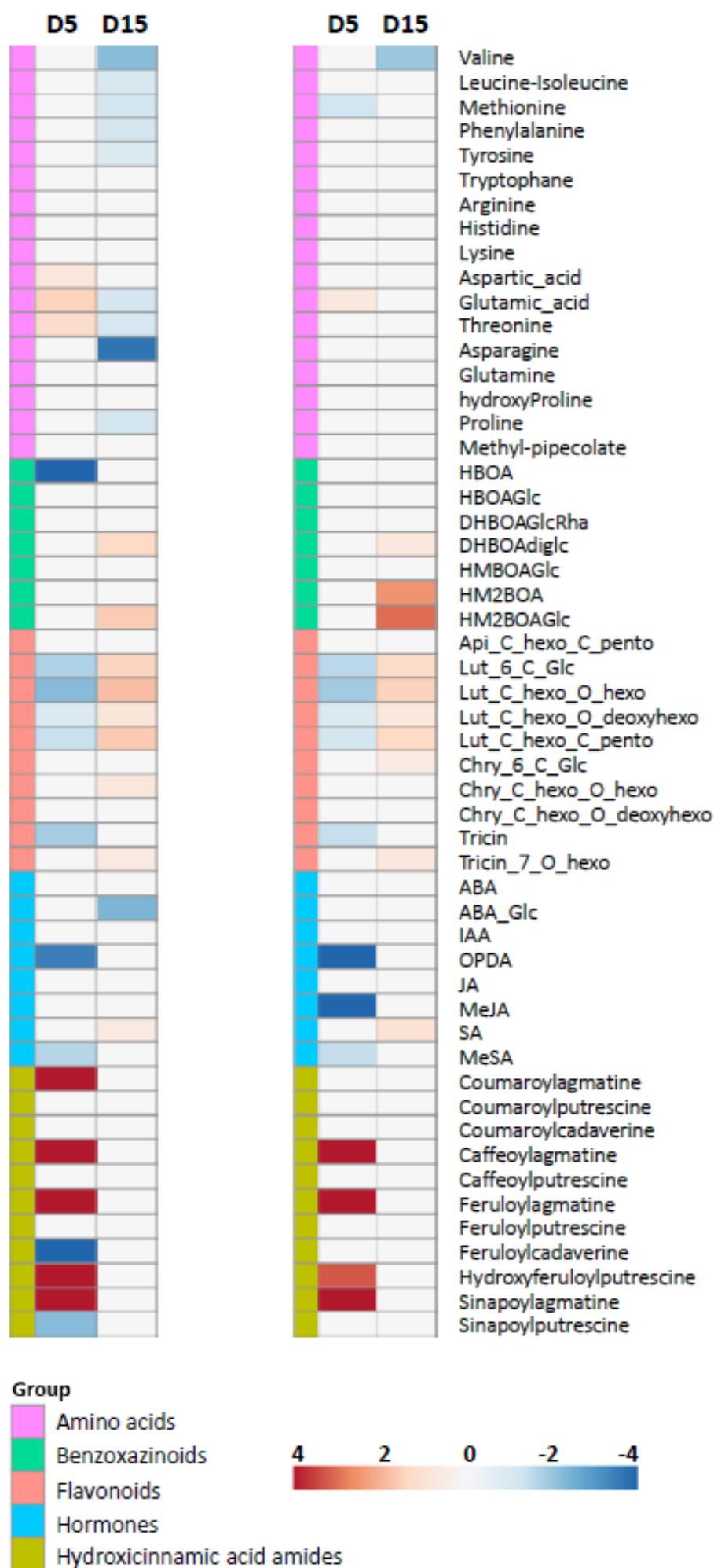
Supplementary figure S3. Venn diagram of the number of differentially expressed genes observed when investigating the eliciting effect of mycosubtilin (M-D5-Ni vs C-D5-Ni), *Zymoseptoria tritici* effect (C-D5-i vs C-D5-Ni) and the combined effect of both mycosubtilin and *Z. tritici* (M-D5-i vs M-D5-Ni) at five days after treatment with mycosubtilin (*i.e.* three days after wheat inoculation with *Z. tritici*). M means treated with mycosubtilin towards mock treated C. Ni stands for mock inoculated whereas i indicates that plants were infected with *Z. tritici*. D5 mean that leaves were sampled at five days after mycosubtilin treatment.



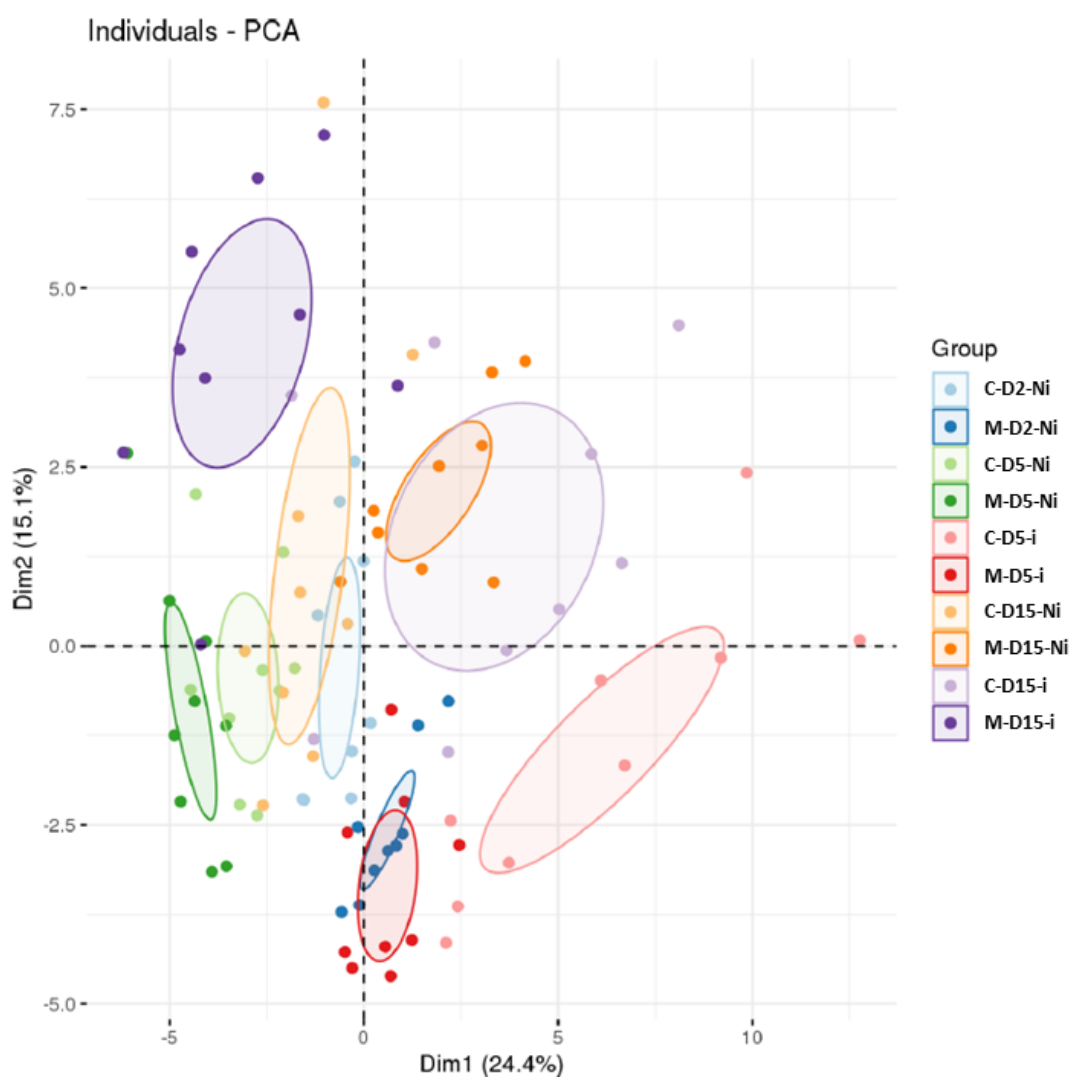
A B

CA624093 TA73273_4565 TA54418_4565 TA84187_4565 TA92949_4565 AY785903 TA67331_4565 TC381840 TA100085_4565 TA79737_4565 TA57180_4565 TA74058_4565 TA53066_4565			Low temperature induced protein ATP-dependent RNA helicase Cold acclimation protein WCOR413 Protein phosphatase type 2-C Efflux transporter, RND family, MFP subunit TaCBF6 Proline-rich protein precursor Cold acclimation protein WCOR518 ARK protein Importin beta-like protein Actin Serine hydroxymethyltransferase Metathionin-like protein type 3	Response to abiotic stress
TA102260_4565 TA77625_4565 TA65180_4565 TA67746_4565 TA64544_4565 TA100790_4565 TA65542_4565 TA61435_4565 BO161851 CK212695			Nitrite reductase apoprotein Chloroplast glutathione reductase PR17d precursor Aldose reductase Mitochondrial lipamide dehydrogenase Cytochrome P450 family protein GMC oxidoreductase Peroxidase 6 Peroxidase Class III peroxidase 62 precursor	Response to oxidative stress
TA55707_4565 TA69803_4565 TC426538 TA72415_4565 GR302911 TC419763 TA76681_4565 EF570122 AF112963 TC370624 TA83143_4565 AY387686 TC404042 TA53249_4565 DO208192			Thaumatin-like protein TLP4 VTC2-like protein Chitinase 2 Chitinase WIR1 Protein WIR1A HS1-like protein Pathogen-inducible transcription factor ERF3 Chitinase II precursor (Cht2) MLA7 Hydrolase, alpha/beta fold family-like Harpin binding protein 1 (HrBP1-1) Phytosulfolinkine IPSL riboregulator Havanone 3-hydroxylase Chalcone synthase 2 Chalcone synthase (CHS1) Pyridoxal biosynthesis protein PDX1.1 TaNFPS-4 farnesyl pyrophosphate synthase GGDP synthase Isochorismate synthetase CONSTANS interacting protein 5 Protein EGG APPARATUS-1 Pyruvate kinase	Response to pathogens
TA99155_4565 TC405303 CK211204 CK196320 TA84990_4565 TC380019 CA612835			DNA replication and repair protein RecF Mitogen-activated protein kinase 7 ZIM motif family protein Cell death associated protein CDK5RAP1-like protein SAP-like protein BP-73 Absciscic stress ripening protein-like protein	Secondary metabolism
TA87087_4565 TA66611_4565 CK152333 CK205454 TA61828_4565 TC383836 TA102372_4565 TA91609_4565 TA66617_4565 TA95179_4565 CD879285 TA103644_4565 TA92734_4565 TA92426_4565 AY596267 TA70845_4565 TA58881_4565 TA89084_4565 TA72038_4565			Cinnamyl alcohol dehydrogenase Fasciclin-like protein FLA15 Type 1 non specific lipid transfer protein precursor Non-specific lipid transfer protein 1 precursor Non-specific lipid transfer protein Nucleosome assembly protein 1-like protein 2 Hydroxyproline-rich glycoprotein DZ-HRGP precursor Protein DSE2 precursor Plastid-specific 30S ribosomal protein 3, chloroplast precursor Amidophosphoribosyl-transferase Triosephosphate isomerase, chloroplast precursor CRS2-associated factor 1, chloroplast precursor Diap1 protein Beta-carotene hydroxylase Polyphenol oxidase Porphobilinogen deaminase Glutamate-1-semialdehyde 2,1-aminomutase, chloroplast precursor S-adenosyl-L-methionine Mg-protoporphyrin IX methyltransferase Lycopene beta-cyclase	Flowering and seed maturation
TC371150 TA63710_4565 TA55816_4565 TC372144 CK217674			Chlorophyll a/b-binding protein WCAB precursor Chlorophyll a-b binding protein of LHCl type III, chloroplast precursor Light-harvesting complex I Chlorophyll a/b binding protein precursor Chloroplast pigment-binding protein CP24 Photosystem reaction center P680 protein	Growth and development
CA612581 TA64071_4565 AE251264 CJ604231 TA83009_4565 TA64268_4565 TA64671_4565 TA57382_4565 TA75511_4565 TA83785_4565			Cytochrome b6-f complex iron-sulfur subunit, chloroplast precursor Ribulose biphosphate carboxylase activase B (RcaB) Carbohydrate kinase-like protein FKBP-type peptidyl-prolyl cis-trans isomerase 3, chloroplast precursor Fructose-bisphosphate aldolase UTP--glucose-1-phosphate uridylyltransferase Phosphate/phosphoenolpyruvate translocator Monosaccharide transporter 4 Beta-amylase PCT-BMYI	Cell wall structure and function
DO139267 TA82049_4565 TC430171 TA69994_4565 TA64535_4565 EU236151 TA64069_4565 CJ667842 TA63842_4565 TC369894 TC448142 TC413834 TA79034_4565			4-hydroxyphenylpyruvate dioxygenase Bifunctional aspartokinase/homoserine dehydrogenase 2, chloroplast precursor Indole-3-glycerol phosphate lyase Glutamate dehydrogenase Glycine decarboxylase P subunit Arginine decarboxylase Mitochondrial aspartate-glutamate carrier protein-like Ferredoxin, chloroplast precursor Inositol monophosphatase family protein Glycine/D-amino acid oxidases-like Glycine cleavage system H protein, mitochondrial precursor S-adenosylmethionine:2-demethylmenaquinone methyltransferase-like 3-dehydroquinate dehydratase/shikimate 5-dehydrogenase	Chloroplast structure and pigment biosynthesis
TA67255_4565 TA70500_4565 TA61948_4565 TC402730 HM209063 TA64099_4565 TA74269_4565 TC449528			Heat shock protein precursor ATP-dependent Clp protease proteolytic subunit C13 endopeptidase NP1 precursor 70 kDa heat shock protein Heat shock protein 70-like Heat shock protein 80 Plastid omega-3 fatty acid desaturase Cytochrome c	Light harvesting
TA82827_4565 TA51150_4565 U34402 TA85618_4565 TA91346_4565 D38485 TA71493_4565 TA88703_4565 TA85526_4565 CA502352 TA79230_4565 TA53517_4565 TA70216_4565 TA68774_4565 TA71488_4565 TA61485_4565 TA66609_4565 TA50993_4565 TA56520_4565 TA61855_4565 TA58672_4565 TA58281_4565 CV767873 TA61981_4565 CK214923 TA64720_4565 TC413718 TA61076_4565 TA68490_4565 TA58879_4565 TA54070_4565			Heat shock protein precursor ATP-dependent Clp protease proteolytic subunit C13 endopeptidase NP1 precursor 70 kDa heat shock protein Heat shock protein 70-like Heat shock protein 80 Plastid omega-3 fatty acid desaturase Cytochrome c GTP-binding protein-related Serine/threonine kinase Single-subunit RNA polymerase C DEAD-box ATP-dependent RNA helicase 26 DEAD-box ATP-dependent RNA helicase 22 Ps16 protein Elongation factor Ts Elongation factor G, mitochondrial precursor Translation Initiation factor IF-2 Elongation factor 40S ribosomal protein S14 Ribosomal protein L34 30S ribosomal protein S17, chloroplast precursor 50S ribosomal protein L1, chloroplast precursor 50S ribosomal protein L35 Ribosomal protein s6 RPS6-2 Ribosome-like protein Large subunit 26S ribosomal RNA gene Ribosomal protein L35A Ribosomal protein L13a Ribosomal protein 60S ribosomal protein L44 60S ribosomal protein L27 Large subunit 26S ribosomal RNA gene Ribosomal protein L11 50S ribosomal protein L21, chloroplast precursor 60S acidic ribosomal protein P3 60S ribosomal protein L10-2 50S ribosomal protein L28, chloroplast precursor Ribosomal protein L37 Ribosomal protein L10A C3meo4	Photosynthesis
TA91877_4565 TC389894 AF110180			Tetratricopeptide repeat (TPR)-containing protein-like High-affinity phosphate transporter PT1	Carbohydrate metabolism
				Amino acid metabolism
				Protein metabolism
				Lipid and energy metabolism
				Signaling, transcription, translation
				Other functions

Supplementary figure S4. Heatmap of differentially expressed genes in wheat leaves (Cv.Alixan) after *Zymoseptoria tritici* (T02596 strain) infection in presence of mycosubtilin, at five days after treatment (*i.e.* three days after inoculation). (A) Comparison in gene expression between wheat leaves treated with mycosubtilin and inoculated with the fungus *versus* leaves treated with mycosubtilin and non-inoculated (M-D5-i vs M-D5-Ni). (B) whole specter of gene regulation induced by the combined effect of both mycosubtilin and *Z. tritici* (MD5i vs CD5Ni). Gene-related physiological processes are represented on the right part of the graph and were determined using NCBI, AmiGO 2 Gene Ontology, KEGG and UniProt. Significant relative changes in gene expression are presented in Log2 ratio, according to the corresponding color scale, using the WebMev software. M means treated with mycosubtilin towards mock treated C. Ni stands for mock inoculated whereas i indicates that plants were infected with *Z. tritici*. D5 means that leaves were sampled at five days after mycosubtilin treatment.



Supplementary figure S5. Heatmap of significant relative changes in metabolite pattern accumulation within wheat third-leaves (Cv. Alixan) following mycosubtilin application and/or *Zymoseptoria tritici* (T02596 strain) infection, at different time points (n=9 plants). (A) From the left to the right, the following conditions are presented, M-D5-i vs M-D5-Ni and M-D15-i vs M-D15-Ni. (B) Whole specter of metabolite accumulation modifications induced by the combined effect of mycosubtilin treatment and *Z. tritici* infection (from the left to the right M-D5-i vs C-D5-Ni and M-D15-i vs C-D15-Ni). M means treated with mycosubtilin towards mock treated C. Ni stands for mock inoculated whereas i indicates that plants were infected with *Z. tritici*. D5 and D15 mean that leaves were sampled respectively at five and fifteen days after mycosubtilin treatment. Log2 of significant metabolite fold changes for indicated pairwise comparisons are given by shades of red or blue colors according to the scale bar. Metabolites were grouped according to their functional or chemical family as amino acids, benzoxazinoids, flavonoids, hormones and hydroxycinnamic acid amides. Data represent mean values of nine biological replicates for each condition and time point. Statistical analyses were performed using the Tukey's Honest Significant Difference method followed by a false discovery rate (FDR) correction, with $FDR < 0.05$. For $FDR \geq 0.05$, Log2 fold changes were set to 0.



Supplementary Figure S6. Principal component analysis (PCA) displaying the global impact of mycosubtilin treatment and infection with *Zymoseptoria tritici* on wheat leaf metabolome. PCA was performed on relative amounts of all analyzed compounds in the different groups of samples. For each group, nine biological replicates were used. The first two principal components explain 24.4% and 15.1% of the variance separating the groups of samples, respectively.

Supplementary Table S1. Relative gene expression profiles in wheat third-leaves for mycosubtilin early (A) or later elicitation (B), fungus inoculation (C) and priming (D). This table is related to figure 6. Gene-related physiological processes are represented on the right part of the table and were determined using NCBI, AmiGO 2 Gene Ontology, KEGG and UniProt. With A: M-D2-Ni vs C-D2-Ni; B: M-D5-Ni vs C-D5-Ni; C: C-D5-i vs C-D5-Ni and D: M-D5-i vs C-D5-i. Log2 ratios >2 correspond to up-regulated genes (in red) and log2 ratios <0.5 to down-regulated genes (in green). Log2 ratios <2 and >0.5 were conventionally referred to 1. M means treated with mycosubtilin towards mock treated C. Ni stands for mock inoculated whereas i indicates that plants were infected with *Z. tritici*. D2 and D5 mean that leaves were sampled respectively at two and five days after mycosubtilin treatment.

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Name	A MNiD0 vs CNiD0	B MNiD3 vs CNiD3	C CiD3 vs CNiD3	D MiD3 vs CiD3	Description	TopHit	ID	RefNumber
Response to abiotic stress								
TA78527_4565	1,0000	1,0000	0,4594	1,0000	BZIP transcription factor bZIP110	ta TA78527_4565	A_99_P289066	42528
AY428036	1,0000	1,0000	0,3768	1,0000	Dehydration response element binding protein	gb AY428036 gb E A_99_P074325		23922
EF028787	1,0000	1,0000	0,3043	1,0000	CBFIVd-D22	gb EF028787 ta T A_99_P405352		948
TC436852	1,0000	1,0000	0,2955	1,0000	Hly-III related proteins	tc TC436852 A_99_P511722		6696
EF028785	1,0000	1,0000	0,2543	1,0000	CBFIVd-A22	gb EF028785 gb E A_99_P140518		15660
EF028775	1,0000	1,0000	0,2535	1,0000	CBFIVb-21.1	gb EF028775 tc T A_99_P012439		20101
CA624093	1,0000	1,0000	8,8824	1,0000	Low temperature induced protein	ta CA624093 ta C A_99_P459967		11840
TC381840	1,0000	1,0000	3,0605	1,0000	Cold acclimation protein WCOR518	tc TC381840 A_99_P421457		19082
TA54418_4565	1,0000	1,0000	2,5297	1,0000	Cold acclimation protein WCOR413	ta TA54418_4565 A_99_P210931		43524
CJ693503	3,9743	1,0000	1,0000	1,0000	CBL-interacting protein kinase	ta CJ693503 tc T A_99_P431532		1799
AY625683	3,9232	1,0000	1,0000	1,0000	NAC domain transcription factor (NAC2)	gb AY625683 ta T A_99_P425752		32825
TA84405_4565	2,3672	1,0000	1,0000	1,0000	NAC domain-containing protein 71	ta TA84405_4565 A_99_P423302		13839
TC421665	1,0000	3,6317	1,0000	1,0000	Transcription factor Myb2	tc TC421665 tc T A_99_P479147		37654
CF133748	1,0000	3,5665	1,0000	1,0000	Myb-related protein	ta CF133748 tc T A_99_P453952		5264
TA73273_4565	1,0000	3,1435	1,0000	3,4706	ATP-dependent RNA helicase	ta TA73273_4565 A_99_P271141		28142
TA100085_4565	1,0000	1,0000	1,0000	3,4983	ARK protein	ta TA100085_4565 A_99_P360266		21911
TA64630_4565	1,0000	1,0000	1,0000	2,3547	Aquaporin	ta TA64630_4565 A_99_P240586		20137
TA62772_4565	1,0000	1,0000	1,0000	3,1293	Aquaporin PIP1	ta TA62772_4565 A_99_P234076		10538
Response to oxydative stress								
TA61073_4565	1,0000	1,0000	0,3344	1,0000	Zinc-containing alcohol dehydrogenase superfamily	ta TA61073_4565 A_99_P408522		21811
CA600821	1,0000	1,0000	0,2760	3,0496	Metallothioneine type2	ta CA600821 tc T A_99_P460107		16140
TA58666_4565	3,5920	2,6363	1,0000	4,7939	Metallothionein-like protein type 3	ta TA58666_4565 A_99_P446552		19674
TA65180_4565	11,1557	1,0000	1,0000	4,6107	PR17d precursor	ta TA65180_4565 A_99_P242791		20412
TA80327_4565	3,3133	1,0000	1,0000	1,0000	Peroxisomal-coenzyme A synthetase	ta TA80327_4565 A_99_P295351		41985
TA64432_4565	2,2668	1,0000	1,0000	1,0000	Glutathione-S-transferase	ta TA64432_4565 A_99_P239886		42661
TA61435_4565	0,3513	0,3554	1,0000	0,0971	Peroxidase 6	ta TA61435_4565 A_99_P552147		16980
BQ161851	0,4950	0,4049	1,0000	0,2188	Peroxidase	ta BQ161851 tc T A_99_P431937		25512
CK212695	1,0000	1,0000	1,0000	0,3268	Class III peroxidase 62 precursor	ta CK212695 tc T A_99_P407997		44165
TA67746_4565	1,0000	1,0000	1,0000	4,1175	Aldose reductase	ta TA67746_4565 A_99_P252121		11372
TA77562_4565	1,0000	1,0000	1,0000	3,2047	ABC1 family protein-like	ta TA77562_4565 A_99_P285771		37317
TC422042	1,0000	1,0000	1,0000	3,6762	Short chain alcohol dehydrogenase-like	tc TC422042 A_99_P152844		16834
TA90985_4565	1,0000	1,0000	1,0000	2,8981	Glutathione peroxidase-like protein GPX15Hv	ta TA90985_4565 A_99_P491687		28365
TC426163	1,0000	1,0000	1,0000	0,1801	Glutaredoxin-C1 precursor	tc TC426163 A_99_P487912		11412
TA84010_4565	1,0000	1,0000	1,0000	0,4558	Nodulin-like protein	ta TA84010_4565 A_99_P307876		15025
TA66389_4565	1,0000	1,0000	1,0000	0,1906	Peroxidase precursor	ta TA66389_4565 A_99_P247391		14997
Response to pathogens								
TA55707_4565	1,0000	0,5486	3,4425	1,0000	Thaumatin-like protein TLP4	ta TA55707_4565 A_99_P509202		23176
TC426538	1,0000	0,4366	13,5307	0,2663	Chitinase 2	tc TC426538 A_99_P488752		20923
TA72415_4565	1,0000	0,3820	4,8597	0,3235	Chitinase	ta TA72415_4565 A_99_P268406		11345
TA65444_4565	1,0000	1,0000	0,3907	2,4595	Reticulon	ta TA65444_4565 A_99_P412857		7188
TA70475_4565	1,0000	1,0000	0,4104	1,0000	RGA2	ta TA70475_4565 A_99_P433007		20640
TC442947	1,0000	1,0000	0,3281	1,0000	END40-like protein	tc TC442947 A_99_P525387		1183
TA53249_4565	1,0000	1,0000	0,2556	1,0000	IPS1 riboregulator	ta TA53249_4565 A_99_P207336		20707
TA66652_4565	17,1386	1,0000	1,0000	1,0000	Protein WIR1B	ta TA66652_4565 A_99_P248341		33427
GR302911	13,8584	1,0000	1,0000	1,0000	WIR1	gb GR302911 tc T A_99_P488332		14017
TC419763	10,4972	1,0000	1,0000	1,0000	Protein WIR1A	tc TC419763 A_99_P475682		36715
TA73316_4565	4,4605	1,0000	1,0000	1,0000	Pathogen-related protein	ta TA73316_4565 A_99_P271256		41870
AF112963	3,1035	1,0000	1,0000	3,6904	Chitinase II precursor (Cht2)	gb AF112963 ta T A_99_P000381		24453
TC370624	1,0000	2,7363	1,0000	1,0000	MLA7	tc TC370624 A_99_P408547		22360
TA83143_4565	1,0000	2,7211	1,0000	5,7061	Hydrolase, alpha/beta fold family-like	ta TA83143_4565 A_99_P304906		15369
TA69803_4565	1,0000	1,0000	1,0000	5,8659	VT2-like protein	ta TA69803_4565 A_99_P259271		42520
AY387686	1,0000	1,0000	1,0000	4,1747	Harpin binding protein 1 (HrBP1-1)	gb AY387686 ta T A_99_P645996		44828
CK162011	1,0000	1,0000	1,0000	0,3420	Glucan endo-1,3-beta-glucosidase isoenzyme I	ta CK162011 tc T A_99_P492787		8899
Y09917	1,0000	1,0000	1,0000	0,2637	Germin-like protein 3	gb Y09917 gb FJ8 A_99_P546587		21507
Secondary metabolism								
DQ208192	9,6489	1,0000	1,0000	1,0000	Flavanone 3-hydroxylase	gb DQ208192 ta T A_99_P163772		30321
TA69335_4565	1,0000	11,9472	1,0000	10,5210	Chalcone synthase 2	ta TA69335_4565 A_99_P174849		25042
AY286098	1,0000	1,0000	1,0000	6,3104	Chalcone synthase (CHS1)	gb AY286098 gb A A_99_P163037		29628
TA50072_4565	1,0000	1,0000	1,0000	5,7942	Pyridoxal biosynthesis protein PDX1.1	ta TA50072_4565 A_99_P197801		18956
JQ307210	1,0000	1,0000	1,0000	2,4647	TaJNFP5-4 farnesyl pyrophosphate synthase	gb JQ307210 ta T A_99_P255071		6487
TA69487_4565	1,0000	1,0000	1,0000	3,2033	Nicotianamine synthase 3	ta TA69487_4565 A_99_P258261		20065
Pigment biosynthesis								
TA92426_4565	4,6192	1,0000	1,0000	3,7490	Beta-carotene hydroxylase	ta TA92426_4565 A_99_P336181		23802
AY596267	1,0000	1,0000	1,0000	3,3863	Polyphenol oxidase	gb AY596267 ta T A_99_P081185		22056
TA72038_4565	1,0000	1,0000	1,0000	2,4004	Lycopene beta-cyclase	ta TA72038_4565 A_99_P567096		33927
TA56529_4565	1,0000	1,0000	1,0000	0,4659	Glutamyl-tRNA reductase 2	ta TA56529_4565 A_99_P216016		43656
Flowering and seed maturation								
TA84120_4565	1,0000	1,0000	0,4140	1,0000	Protein EGG APPARATUS-1	ta TA84120_4565 A_99_P308216		36050
TA86268_4565	1,0000	1,0000	1,0000	2,4193	CONSTANS interacting protein 5	ta TA86268_4565 A_99_P315626		21018
TA50354_4565	1,0000	1,0000	1,0000	3,1442	Pyruvate kinase	ta TA50354_4565 A_99_P558877		37866
Growth and development								
TA72187_4565	1,0000	1,0000	0,2876	1,0000	Cell Division Protein AAA ATPase family	ta TA72187_4565 A_99_P267636		41732
CK211204	3,1279	3,8023	1,0000	1,0000	ZIM motif family protein	ta CK211204 ta B A_99_P469812		31070
CK196320	3,0527	1,0000	1,0000	2,4193	Cell death associated protein	ta CK196320 tc T A_99_P536337		8482
TA56545_4565	1,0000	2,3451	1,0000	1,0000	1-aminocyclopropane-1-carboxylate oxidase	ta TA56545_4565 A_99_P216071		11632
TA56454_4565	0,2222	0,3825	1,0000	1,0000	Auxin-repressed protein	ta TA56454_4565 A_99_P215761		27870
TA61883_4565	1,0000	0,3739	1,0000	1,0000	Sterol desaturase family protein	ta TA61883_4565 A_99_P231241		32885
TA74119_4565	1,0000	1,0000	1,0000	3,2686	Serine carboxypeptidase 1 precursor	ta TA74119_4565 A_99_P273981		5019
CA733338	1,0000	1,0000	1,0000	0,3309	Kelch repeat-containing F-box-like	ta CA733338 tc T A_99_P567897		2248
TA86474_4565	1,0000	1,0000	1,0000	0,2405	Auxin Efflux Carrier	ta TA86474_4565 A_99_P192117		12326
TA70786_4565	1,0000	1,0000	1,0000	0,3417	Dormancy/auxin associated protein	ta TA70786_4565 A_99_P262816		1345

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Name	A MNiD0 vs CNiD0	B MNiD3 vs CNiD3	C CiD3 vs CNiD3	D MiD3 vs CiD3	Description	TopHit	ID	RefNumber
Cell wall structure and function								
TA87087_4565	1,0000	1,0000	0,0592	1,0000	Cinnamyl alcohol dehydrogenase	ta TA87087_4565	A_99_P318481	8569
TA68562_4565	1,0000	1,0000	0,3766	1,0000	Glycoside hydrolase, family 19	ta TA68562_4565	A_99_P255031	1863
TA66611_4565	1,0000	1,0000	0,3372	1,0000	Fasciclin-like protein FLA15	ta TA66611_4565	A_99_P248201	22500
CK152333	1,0000	1,0000	0,3202	1,0000	Type 1 non specific lipid transfer protein precursor	ta CK152333 tc TCA_99_P446157		36915
CK205454	1,0000	1,0000	0,3699	1,0000	Non-specific lipid transfer protein 1 precursor	ta CK205454 tc TCA_99_P427502		36085
TA61828_4565	1,0000	1,0000	0,3668	1,0000	Non-specific lipid-transfer protein	ta TA61828_4565	A_99_P231006	27677
TA91600_4565	1,0000	1,0000	1,0000	2,9867	Protein DSE2 precursor	ta TA91600_4565	A_99_P333491	35745
TA107115_4565	1,0000	1,0000	1,0000	5,0040	Galactose-binding like	ta TA107115_4565	A_99_P386442	35996
Chloroplast structure and function								
TA69442_4565	1,0000	0,3415	1,0000	0,1868	Plastid-specific 30S ribosomal protein 1, chloroplast protein	ta TA69442_4565	A_99_P258121	37451
TA92734_4565	1,0000	1,0000	0,3150	1,0000	Diap1 protein	ta TA92734_4565	A_99_P337136	42984
TA103644_4565	1,0000	1,0000	1,0000	2,5178	CRS2-associated factor 1, chloroplast precursor	ta TA103644_4565	A_99_P010186	14454
Light harvesting								
TA59580_4565	0,3099	0,1806	1,0000	0,1687	Light-regulated protein precursor	ta TA59580_4565	A_99_P223621	14346
TA53867_4565	1,0000	0,1549	1,0000	0,1783	Light-induced protein 1-like	ta TA53867_4565	A_99_P209081	23883
TC371150	1,0000	0,1464	1,0000	0,0368	Chlorophyll a/b-binding protein WCAB precursor	tc TC371150	A_99_P409112	19146
TA63710_4565	1,0000	1,0000	1,0000	0,3636	Chlorophyll a-b binding protein of LHCl type III, chloroplast	ta TA63710_4565	A_99_P237481	36780
TA55816_4565	1,0000	1,0000	1,0000	0,2774	Light-harvesting complex I	ta TA55816_4565	A_99_P214401	27084
TC372144	1,0000	1,0000	1,0000	0,1915	Chlorophyll a/b binding protein precursor	tc TC372144	A_99_P410077	391
CK217674	1,0000	1,0000	1,0000	0,2346	Chloroplast pigment-binding protein CP24	ta CK217674 tc TCA_99_P437157		39278
Photosynthesis								
CK212581	1,0000	1,0000	1,0000	4,1967	Photosystem II reaction center psb28 protein	ta CK212581 tc TCA_99_P424262		24845
TA64071_4565	1,0000	1,0000	1,0000	3,4588	Cytochrome b6-f complex iron-sulfur subunit, chloroplast	ta TA64071_4565	A_99_P238726	35747
Carbohydrate metabolism								
CJ604231	1,0000	1,0000	1,0000	3,2506	Carbohydrate kinase-like protein	ta CJ604231 tc TCA_99_P441937		19794
TA64268_4565	1,0000	1,0000	1,0000	3,0694	Fructose-bisphosphate aldolase	ta TA64268_4565	A_99_P239376	27725
TA64671_4565	1,0000	1,0000	1,0000	2,1494	UTP--glucose-1-phosphate uridylyltransferase	ta TA64671_4565	A_99_P004441	24422
TA57382_4565	1,0000	1,0000	1,0000	3,2052	Phosphate/phosphoenolpyruvate translocator	ta TA57382_4565	A_99_P217791	33087
TA75511_4565	1,0000	1,0000	1,0000	5,1904	Monosaccharide transporter 4	ta TA75511_4565	A_99_P278701	5738
TA83785_4565	1,0000	1,0000	1,0000	4,6539	Beta-amylase PCT-BMYI	ta TA83785_4565	A_99_P307146	32293
Amino acid metabolism								
TC430171	1,0000	1,0000	0,0893	1,0000	Indole-3-glycerol phosphate lyase	tc TC430171	A_99_P496502	8009
TA69994_4565	1,0000	1,0000	0,3764	1,0000	Glutamate dehydrogenase	ta TA69994_4565	A_99_P259951	10977
TA69140_4565	1,0000	1,0000	0,3061	3,9418	L-allo-threonine aldolase-related protein	ta TA69140_4565	A_99_P449637	41850
TC393988	1,0000	1,0000	0,1308	3,1335	Glycine/proline-rich protein	tc TC393988	A_99_P543657	7174
TA64535_4565	1,0000	1,0000	0,3402	6,8149	Glycine decarboxylase P subunit	ta TA64535_4565	A_99_P240211	15342
TA64069_4565	1,0000	1,0000	1,0000	2,7421	Mitochondrial aspartate-glutamate carrier protein-like	ta TA64069_4565	A_99_P238716	33459
CJ667842	1,0000	1,0000	1,0000	2,2975	Ferredoxin, chloroplast precursor	ta CJ667842 tc TCA_99_P436607		43120
TA63842_4565	1,0000	1,0000	1,0000	2,2437	Inositol monophosphatase family protein	ta TA63842_4565	A_99_P237761	10378
TA60889_4565	1,0000	1,0000	1,0000	3,1980	S-adenosylmethionine synthetase	ta TA60889_4565	A_99_P227511	9678
Protein metabolism								
TA59594_4565	3,2151	1,0000	1,0000	1,0000	22.3 kDa heat-shock protein	ta TA59594_4565	A_99_P223676	44575
TC402730	1,0000	1,0000	0,2499	1,0000	70 kDa heat shock protein	tc TC402730	A_99_P448647	36262
HM209063	1,0000	1,0000	0,2354	1,0000	Heat shock protein 70-like	gb HM209063 gb	A_99_P234541	16785
TA64099_4565	1,0000	1,0000	0,1519	1,0000	Heat shock protein 80 kDa	ta TA64099_4565	A_99_P238761	26
TC414782	1,0000	1,0000	0,2639	4,7982	Asparaginase	tc TC414782	A_99_P466942	23822
TA53535_4565	1,0000	1,0000	0,1923	3,8264	Ubiquitin carrier protein	ta TA53535_4565	A_99_P208256	24763
TA67255_4565	1,0000	1,0000	1,0000	3,7019	Heat-shock protein precursor	ta TA67255_4565	A_99_P250376	10187
TA61948_4565	1,0000	1,0000	1,0000	5,9779	C13 endopeptidase NP1 precursor	ta TA61948_4565	A_99_P231506	7777
Lipid metabolism								
TA74269_4565	1,0000	1,0000	1,0000	2,2655	Plastid omega-3 fatty acid desaturase	ta TA74269_4565	A_99_P274486	33811
Energy metabolism								
TC449528	1,0000	0,1814	1,0000	0,0777	Cytochrome c	tc TC449528	A_99_P542662	24896
TA82476_4565	1,0000	1,0000	1,0000	0,5086	HAP3-like transcriptional-activator	ta TA82476_4565	A_99_P302726	42546
Signaling								
TA72110_4565	2,1573	1,0000	1,0000	1,0000	Ca2+/H+-exchanging protein	ta TA72110_4565	A_99_P267366	40677
TC376621	1,0000	2,7894	1,0000	1,0000	Calcium-dependent protein kinase	tc TC376621	A_99_P415112	24028
Transcription regulation								
CA721206	1,0000	1,0000	0,3296	2,5283	Zinc finger protein 219	ta CA721206 tc TCA_99_P533942		44850
D16416	3,8660	1,0000	1,0000	1,0000	Zinc-finger protein WZF1	gb D16416 ta TA7_	A_99_P489622	10858
TC436900	3,1349	1,0000	1,0000	1,0000	Histone H1 WH1A.3	tc TC436900	A_99_P511852	5211
Transcription, RNA-processing and translation								
U34402	1,0000	1,0000	1,0000	2,6546	Single-subunit RNA polymerase C	gb U34402 ta TA8_	A_99_P000396	29420
TA85618_4565	1,0000	1,0000	1,0000	3,9276	DEAD-box ATP-dependent RNA helicase 26	ta TA85618_4565	A_99_P313296	14881
TA91346_4565	1,0000	1,0000	1,0000	2,1117	DEAD-box ATP-dependent RNA helicase 22	ta TA91346_4565	A_99_P332671	20451
TA79230_4565	1,0000	1,0000	1,0000	2,3422	40S ribosomal protein S14	ta TA79230_4565	A_99_P165432	22492

Supplementary Table S2. Relative gene expression profiles illustrating fungus effect in presence of mycosubtilin (M-D5-i vs M-D5-Ni) and the whole specter of plant responses to mycosubtilin and fungus (M-D5-i vs C-D5-Ni). This table is related to supplementary figure S5. Gene-related physiological processes are represented on the right part of the graph and were determined using NCBI, AmiGO 2 Gene Ontology, KEGG and UniProt. Log2 ratios >2 correspond to up-regulated genes (in red) and log2 ratios <0.5 to down-regulated genes (in green). Log2 ratios <2 and >0.5 were conventionally referred to 1. M means treated with mycosubtilin towards mock treated C. Ni stands for mock inoculated whereas i indicates that plants were infected with *Z. tritici*. D5 means that leaves were sampled at five days after mycosubtilin treatment.

Name	A Mid3 vs MniD3	B Mid3 vs CNiD3	Description	TopHit	ID	RefNumber
Response to abiotic stress						
CA624093	10,8866	7,9255	Low temperature induced protein	ta CA624093 ta C A_99_P459967		11840
TA73273_4565	3,1771	10,1871	ATP-dependent RNA helicase	ta TA73273_4565 A_99_P271141		28142
TA54418_4565	5,3330	1,0000	Cold acclimation protein WCOR413	ta TA54418_4565 A_99_P210931		43524
TA84187_4565	0,4464	1,0000	Protein phosphatase type 2-C	ta TA84187_4565 A_99_P308421		21815
TA92949_4565	0,2966	1,0000	Efflux transporter, RND family, MFP subunit	ta TA92949_4565 A_99_P144213		15689
AY785903	0,3354	1,0000	TaCBF6	gb AY785903 gb C A_99_P000681		22350
TA67331_4565	0,3060	1,0000	Proline-rich protein precursor	ta TA67331_4565 A_99_P498102		7990
TC381840	1,0000	2,6078	Cold acclimation protein WCOR518	tc TC381840_A_99_P421457		19082
TA100085_4565	1,0000	3,6298	ARK protein	ta TA100085_4565_A_99_P360266		21911
TA79737_4565	1,0000	3,9456	Importin beta-like protein	ta TA79737_4565 A_99_P293321		35865
TA57180_4565	1,0000	2,7545	Actin	ta TA57180_4565 A_99_P217276		4091
TA74058_4565	1,0000	2,5253	Serine hydroxymethyltransferase	ta TA74058_4565 A_99_P008296		33374
Response to oxydative stress						
TA58666_4565	4,1559	8,3937	Metallothionein-like protein type 3	ta TA58666_4565 A_99_P446552		19674
TA102260_4565	7,2071	5,8849	Nitrite reductase apoprotein	ta TA102260_4565_A_99_P366657		8326
TA77625_4565	1,0000	3,3397	Chloroplast glutathione reductase	ta TA77625_4565 A_99_P285981		31266
TA65180_4565	1,0000	6,9092	PR17d precursor	ta TA65180_4565 A_99_P242791		20412
TA67746_4565	1,0000	3,0000	Aldose reductase	ta TA67746_4565 A_99_P252121		11372
TA64544_4565	1,0000	2,9082	Mitochondrial lipamide dehydrogenase	ta TA64544_4565 A_99_P240241		32379
TA100790_4565	1,0000	2,8398	Cytochrome P450 family protein	ta TA100790_4565_A_99_P362271		32984
TA65542_4565	1,0000	2,7234	GMC oxidoreductase	ta TA65542_4565 A_99_P244206		18477
TA61435_4565	1,0000	0,1966	Peroxidase 6	ta TA61435_4565 A_99_P552147		16980
BQ161851	1,0000	0,4719	Peroxidase	ta BQ161851 tc T A_99_P431937		25512
CK212695	1,0000	0,4214	Class III peroxidase 62 precursor	ta CK212695 tc T A_99_P407997		44165
Response to pathogens						
TA55707_4565	7,4080	3,7743	Thaumatin-like protein TLP4	ta TA55707_4565 A_99_P509202		23176
TA69803_4565	6,4953	5,3893	VTC2-like protein	ta TA69803_4565 A_99_P259271		42520
TC426538	4,2133	1,0000	Chitinase 2	tc TC426538_A_99_P488752		20923
TA72415_4565	5,5826	1,0000	Chitinase	ta TA72415_4565 A_99_P268406		11345
GR302911	5,8764	1,0000	WIR1	gb GR302911 tc T A_99_P488332		14017
TC419763	9,3819	1,0000	Protein WIR1A	tc TC419763_A_99_P475682		36715
TA76681_4565	0,4121	1,0000	HS1-like protein	ta TA76681_4565 A_99_P194333		16022
EF570122	0,3030	1,0000	Pathogen-inducible transcription factor ERF3	gb EF570122 gb H A_99_P177974		11088
AF112963	1,0000	4,3880	Chitinase II precursor (Cht2)	gb AF112963 ta T A_99_P000381		24453
TC370624	1,0000	3,4177	MLA7	tc TC370624_A_99_P408547		22360
TA83143_4565	1,0000	3,0760	Hydrolase, alpha/beta fold family-like	ta TA83143_4565 A_99_P304906		15369
AY387686	1,0000	4,0090	Harpin binding protein 1 (HrBP1-1)	gb AY387686 ta T A_99_P645996		44828
TC404042	1,0000	3,8154	Phytosulfokine	tc TC404042_A_99_P450387		20130
TA53249_4565	1,0000	0,2250	IPS1 riboregulator	ta TA53249_4565 A_99_P207336		20707
Secondary metabolism						
DQ208192	12,9069	7,4518	Flavanone 3-hydroxylase	gb DQ208192 ta T A_99_P163772		30321
TA69335_4565	1,0000	10,9130	Chalcone synthase 2	ta TA69335_4565 A_99_P174849		25042
AY286098	1,0000	5,4080	Chalcone synthase (CHS1)	gb AY286098 gb A_99_P163037		29628
TA50072_4565	1,0000	5,9485	Pyridoxal biosynthesis protein PDX1.1	ta TA50072_4565 A_99_P197801		18956
JQ307210	1,0000	2,2196	TaJNFPS-4 farnesyl pyrophosphate synthase	gb JQ307210 ta T A_99_P255071		6487
TA83418_4565	1,0000	2,8848	GDP synthase	ta TA83418_4565 A_99_P305831		14702
TA83418_4565	1,0000	2,3471	Isochorismate synthetase	ta TA83418_4565 A_99_P565517		1945
Flowering and seed maturation						
TA86268_4565	5,3738	11,9770	CONSTANS interacting protein 5	ta TA86268_4565 A_99_P315626		21018
TA84120_4565	0,4894	0,5134	Protein EGG APPARATUS-1	ta TA84120_4565 A_99_P308216		36050
TA50354_4565	1,0000	4,0211	Pyruvate kinase	ta TA50354_4565 A_99_P558877		37866
Growth and development						
TA99155_4565	6,4739	7,5675	DNA replication and repair protein RecF	ta TA99155_4565 A_99_P357521		34334
TC405303	0,2207	1,0000	Mitogen-activated protein kinase 7	tc TC405303_A_99_P452302		22398
CK211204	0,4144	1,0000	ZIM motif family protein	ta CK211204 ta B A_99_P469812		31070
CK196320	1,0000	2,3513	Cell death associated protein	ta CK196320 tc T A_99_P536337		8482
TA84990_4565	1,0000	2,8643	CDK5RAP1-like protein	ta TA84990_4565 A_99_P311161		19118
TC380019	1,0000	2,5327	SAP-like protein BP-73	tc TC380019_A_99_P419147		17470
CA612835	1,0000	0,5152	Abscisic stress ripening protein-like protein	ta CA612835 tc T A_99_P512357		23935
Cell wall structure and activity						
TA87087_4565	0,2972	1,0000	Cinnamyl alcohol dehydrogenase	ta TA87087_4565 A_99_P318481		8569
TA66611_4565	0,2902	0,2694	Fascidin-like protein FLA15	ta TA66611_4565 A_99_P248201		22500
CK152333	0,4777	0,3021	Type 1 non specific lipid transfer protein precursor	ta CK152333 tc T A_99_P446157		36915
CK205454	1,0000	0,4856	Non-specific lipid transfer protein 1 precursor	ta CK205454 tc T A_99_P427502		36085
TA61828_4565	1,0000	0,3846	Non-specific lipid-transfer protein	ta TA61828_4565 A_99_P231006		27677
TC383836	1,0000	2,4738	Nucleosome assembly protein 1-like protein 2	tc TC383836_A_99_P423887		18218
TA102372_4565	1,0000	3,1164	Hydroxyproline-rich glycoprotein DZ-HRGP precursor	ta TA102372_4565_A_99_P039309		28910
TA91600_4565	1,0000	3,1924	Protein DSE2 precursor	ta TA91600_4565 A_99_P333491		35745
Chloroplast structure and function						
TA66617_4565	1,0000	2,8274	Plastid-specific 30S ribosomal protein 3, chloroplast	ta TA66617_4565 A_99_P248226		33266
TA95179_4565	1,0000	2,9719	Amidophosphoribosyl-transferase	ta TA95179_4565 A_99_P345026		40498
CD879285	1,0000	2,6638	Triosephosphate isomerase, chloroplast precursor	ta CD879285 tc T A_99_P434992		19245
TA103644_4565	1,0000	3,0541	CRS2-associated factor 1, chloroplast precursor	ta TA103644_4565_A_99_P010186		14454
TA92734_4565	1,0000	0,4010	Diap1 protein	ta TA92734_4565 A_99_P337136		42984

	A	B				
Name	Mid3 vs MniD3	Mid3 vs CniD3	Description	TopHit	ID	RefNumber
Pigment biosynthesis						
TA92426_4565	1,0000	3,3226	Beta-carotene hydroxylase	ta TA92426_4565	A_99_P081185	22056
AY596267	1,0000	3,8471	Polyphenol oxidase	gb AY596267 ta TA92426_4565	A_99_P081185	22056
TA70845_4565	1,0000	3,2178	Porphobilinogen deaminase	ta TA70845_4565	A_99_P155942	10728
TA58881_4565	1,0000	2,8977	Glutamate-1-semialdehyde 2,1-aminomutase, chl	ta TA58881_4565	A_99_P419897	32620
TA89084_4565	1,0000	2,4213	S-adenosyl-L-methionine Mg-protoporphyrin IX m	ta TA89084_4565	A_99_P494142	24157
TA72038_4565	1,0000	2,7744	Lycopene beta-cyclase	ta TA72038_4565	A_99_P267096	33927
Light harvesting						
TC371150	1,0000	0,0737	Chlorophyll a/b-binding protein WCAB precursor	tc TC371150	A_99_P409112	19146
TA63710_4565	1,0000	0,4580	Chlorophyll a-b binding protein of LHClI type III, c	ta TA63710_4565	A_99_P237481	36780
TA55816_4565	1,0000	0,3900	Light-harvesting complex I	ta TA55816_4565	A_99_P214401	27084
TC372144	1,0000	0,3827	Chlorophyll a/b binding protein precursor	tc TC372144	A_99_P410077	391
CK217674	1,0000	0,3345	Chloroplast pigment-binding protein CP24	ta CK217674 tc TC	A_99_P437157	39278
Photosynthesis						
CK212581	1,0000	3,3828	Photosystem II reaction center psb28 protein	ta CK212581 tc TCA	A_99_P424262	24845
TA64071_4565	1,0000	3,5968	Cytochrome b6-f complex iron-sulfur subunit, chl	ta TA64071_4565	A_99_P238726	35747
AF251264	1,0000	3,8899	Ribulose bispophosphate carboxylase activase B (Rc	gb AF251264 ta T	A_99_P449812	42320
Carbohydrate metabolism						
CJ604231	1,0000	3,5308	Carbohydrate kinase-like protein	ta CJ604231 tc TC	A_99_P441937	19794
TA83009_4565	1,0000	2,6181	FKBP-type peptidyl-prolyl cis-trans isomerase 3, d	ta TA83009_4565	A_99_P430677	38537
TA64268_4565	1,0000	2,7123	Fructose-bisphosphate aldolase	ta TA64268_4565	A_99_P239376	27725
TA64671_4565	1,0000	2,0409	UTP--glucose-1-phosphate uridylyltransferase	ta TA64671_4565	A_99_P004441	24422
TA57382_4565	1,0000	4,1155	Phosphate/phosphoenolpyruvate translocator	ta TA57382_4565	A_99_P217791	33087
TA75511_4565	1,0000	4,8683	Monosaccharide transporter 4	ta TA75511_4565	A_99_P278701	5738
TA83785_4565	1,0000	4,4735	Beta-amylase PCT-BMYI	ta TA83785_4565	A_99_P307146	32293
Amino-acids metabolism						
DQ139267	2,7893	4,3191	4-hydroxyphenylpyruvate dioxygenase	gb DQ139267 ta TA	A_99_P637271	37769
TA82049_4565	4,4783	2,7594	Bifunctional aspartokinase/homoserine dehydrog	ta TA82049_4565	A_99_P163227	42192
TC430171	0,4417	1,0000	Indole-3-glycerol phosphate lyase	tc TC430171	A_99_P496502	8009
TA69994_4565	0,5428	1,0000	Glutamate dehydrogenase	ta TA69994_4565	A_99_P259951	10977
TA64535_4565	1,0000	3,1452	Glycine decarboxylase P subunit	ta TA64535_4565	A_99_P240211	15342
EU236151	1,0000	3,3617	Arginine decarboxylase	gb EU236151 ta T	A_99_P229841	38415
TA64069_4565	1,0000	5,2741	Mitochondrial aspartate-glutamate carrier protei	ta TA64069_4565	A_99_P238716	33459
CJ667842	1,0000	3,3303	Ferredoxin, chloroplast precursor	ta CJ667842 tc TC	A_99_P436607	43120
TA63842_4565	1,0000	2,3311	Inositol monophosphatase family protein	ta TA63842_4565	A_99_P237761	10378
TC369894	1,0000	3,3870	Glycine/D-amino acid oxidases-like	tc TC369894	A_99_P427247	29953
TC448142	1,0000	2,8702	Glycine cleavage system H protein, mitochondrial	tc TC448142	A_99_P538777	8089
TC413834	1,0000	2,5071	S-adenosylmethionine:2-demethylmenaquinone r	tc TC413834	A_99_P465342	10889
TA79034_4565	1,0000	2,2333	3-dehydroquinate dehydratase/shikimate 5-dehy	ta TA79034_4565	A_99_P290801	15040
Protein metabolism						
TA67255_4565	1,0000	6,2782	Heat-shock protein precursor	ta TA67255_4565	A_99_P250376	10187
TA70500_4565	1,0000	4,3298	ATP-dependent Clp protease proteolytic subunit	ta TA70500_4565	A_99_P261831	33261
TA61948_4565	1,0000	3,5141	C13 endopeptidase NP1 precursor	ta TA61948_4565	A_99_P231506	7777
TC402730	0,4696	0,3776	70 kDa heat shock protein	tc TC402730	A_99_P448647	36262
HM209063	1,0000	0,3461	Heat shock protein 70-like	gb HM209063 gb	A_99_P234541	16785
TA64099_4565	1,0000	0,3827	Heat shock protein 80 kDa	ta TA64099_4565	A_99_P238761	26
Lipid metabolism						
TA74269_4565	1,0000	2,9766	Plastid omega-3 fatty acid desaturase	ta TA74269_4565	A_99_P274486	33811
Energy metabolism						
TC449528	1,0000	0,2457	Cytochrome c	tc TC449528	A_99_P542662	24896
Signaling						
TA82827_4565	1,0000	3,5853	GTP-binding protein-related	ta TA82827_4565	A_99_P303856	12334
TA51150_4565	1,0000	3,0011	Serine/threonine kinase	ta TA51150_4565	A_99_P200651	28794
Transcription, RNA-processing, translation						
U34402	1,0000	5,1196	Single-subunit RNA polymerase C	gb U34402 ta TA8	A_99_P000396	29420
TA85618_4565	3,8503	8,0637	DEAD-box ATP-dependent RNA helicase 26	ta TA85618_4565	A_99_P313296	14881
TA91346_4565	1,0000	2,9475	DEAD-box ATP-dependent RNA helicase 22	ta TA91346_4565	A_99_P332671	20451
D38485	1,0000	3,8436	Ps16 protein	gb D38485 ta TA6	A_99_P243491	32092
TA71493_4565	1,0000	4,3047	Elongation factor Ts	ta TA71493_4565	A_99_P265276	14490
TA88703_4565	1,0000	2,1987	Elongation factor G, mitochondrial precursor	ta TA88703_4565	A_99_P323926	25452
TA85526_4565	1,0000	4,3363	Translation initiation factor IF-2	ta TA85526_4565	A_99_P426897	36495
CA502352	1,0000	3,3990	Elongation factor	ta CA502352 tc T	A_99_P433232	2769
TA79230_4565	1,0000	4,0916	40S ribosomal protein S14	ta TA79230_4565	A_99_P165432	22492
TA53517_4565	1,0000	3,7743	Ribosomal protein I34	ta TA53517_4565	A_99_P428417	18198
TA70216_4565	1,0000	3,6376	30S ribosomal protein S17, chloroplast precursor	ta TA70216_4565	A_99_P260786	41398
TA68774_4565	1,0000	3,5124	50S ribosomal protein L1, chloroplast precursor	ta TA68774_4565	A_99_P255791	32662
TA71488_4565	1,0000	3,4800	50S ribosomal protein L35	ta TA71488_4565	A_99_P265261	23498
TA61485_4565	1,0000	3,4580	Ribosomal protein s6 RP56-2	ta TA61485_4565	A_99_P229796	30672
TA66609_4565	1,0000	3,3720	Ribosome-like protein	ta TA66609_4565	A_99_P441707	27111
TA50993_4565	1,0000	3,2869	Large subunit 26S ribosomal RNA gene	ta TA50993_4565	A_99_P568177	38089
TA56520_4565	1,0000	3,2492	Ribosomal protein L35A	ta TA56520_4565	A_99_P215981	843
TA61855_4565	1,0000	3,1757	Ribosomal protein L13a	ta TA61855_4565	A_99_P231131	34917
TA58672_4565	1,0000	2,9515	Ribosomal protein	ta TA58672_4565	A_99_P221126	44143
TA58281_4565	1,0000	2,7390	60S ribosomal protein L44	ta TA58281_4565	A_99_P220136	28679
CV767873	1,0000	2,5830	60S ribosomal protein L27	ta CV767873 tc T	A_99_P424047	37514
TA61981_4565	1,0000	2,5522	Large subunit 26S ribosomal RNA gene	ta TA61981_4565	A_99_P410577	31986
CK214923	1,0000	2,5427	Ribosomal protein L11	ta CK214923 ta C	A_99_P479112	18063
TA64720_4565	1,0000	2,5323	50S ribosomal protein L21, chloroplast precursor	ta TA64720_4565	A_99_P240931	20989
TC413718	1,0000	2,4327	60S acidic ribosomal protein P3	tc TC413718 tc T	A_99_P421697	43054
TA61076_4565	1,0000	2,3721	60S ribosomal protein L10-2	ta TA61076_4565	A_99_P228111	36378
TA68490_4565	1,0000	2,3161	50S ribosomal protein L28, chloroplast precursor	ta TA68490_4565	A_99_P254736	20009
TA58879_4565	1,0000	2,2323	Ribosomal protein L37	ta TA58879_4565	A_99_P221536	19773
TA54070_4565	1,0000	2,1438	Ribosomal protein L10A	ta TA54070_4565	A_99_P209766	19576
Other functions						
TA91877_4565	1,0000	2,5423	C3meo4	ta TA91877_4565	A_99_P334381	24012
TC389894	5,7677	5,7787	Tetratricopeptide repeat (TPR)-containing protein	tc TC389894	A_99_P431697	38421
AF110180	4,6138	1,0000	High-affinity phosphate transporter PT1	gb AF110180	A_99_P026169	24145

Supplementary Table S3. List of the 56 metabolites detected in wheat leaves (cv. Alixan) with ultra-high-performance liquid chromatography-mass spectrometry (UHPLC-MS), using a targeted metabolomic approach. Metabolites were classified according to KEGG (Kyoto Encyclopedia of Genes and Genomes; <http://www.genome.ad.jp/kegg>) and PubChem (<http://pubchem.ncbi.nlm.nih.gov>) databases. Id: identifier; m/z: mass-to-charge ratio; m/z error (difference between the measured m/z and the calculated m/z of an ion, in ppm); RT: retention time. * indicates that compounds were confirmed with authentic standards.

Id	Metabolite	Formula	Class ^a	Detected <i>m/z</i>	<i>m/z</i> error (ppm)	RT (min)	KEGG ID	PubChem CID
1	Arginine	C ₆ H ₁₄ N ₄ O ₂	Amino acids	175.1190	0.20	0,85	C00062	6322
2	Asparagine	C ₄ H ₈ N ₂ O ₃	Amino acids	133.0609	0.86	0,98	C00152	6267
3	Aspartic acid	C ₄ H ₇ NO ₄	Amino acids	134.0449	0.69	1,02	C00049	5960
4	Glutamic acid	C ₅ H ₉ NO ₄	Amino acids	148.0604	0,77	1,01	C00025	4525487
5	Glutamine	C ₅ H ₁₀ N ₂ O ₃	Amino acids	147.0764	0,09	0,99	C00064	5961
6	Histidine	C ₆ H ₉ N ₃ O ₂	Amino acids	156.0769	0.68	1,01	C00135	6274
7	Hydroxyproline	C ₅ H ₉ NO ₃	Amino acids	132.0656	0.70	1,01	C01157	5810
8	Isoleucine	C ₆ H ₁₃ NO ₂	Amino acids	132.1020	0.76	1,56	C00407	6306
9	Leucine	C ₆ H ₁₃ NO ₂	Amino acids	132.1020	0.76	1,62	C00123	6106
10	Lysine	C ₆ H ₁₄ N ₂ O ₂	Amino acids	147.1129	0.44	0.84	C00047	5962
11	Methionine	C ₅ H ₁₁ NO ₂ S	Amino acids	150.0584	0.36	2.04	C00073	6137
12	Phenylalanine	C ₉ H ₁₁ NO ₂	Amino acids	166.0862	-0.53	4.30	C00079	6140
13	Proline	C ₅ H ₉ NO ₂	Amino acids	116.0708	1.69	1.23	C00148	145742
14	Threonine	C ₄ H ₉ NO ₃	Amino acids	120.0657	1.22	1.08	C00188	6288
15	Tryptophan	C ₁₁ H ₁₂ N ₂ O ₂	Amino acids	205.0973	0.51	4.75	C00078	6305
16	Tyrosine	C ₉ H ₁₁ NO ₃	Amino acids	182.0813	0.54	3.20	C00082	6057
17	Valine	C ₅ H ₁₁ NO ₂	Amino acids	118.0863	0.48	1.14	C00183	6287
18	Methylpipecolic acid	C ₇ H ₁₃ NO ₂	Amino acid derivatives	144.1019	0.27	1,08	C10172	115244
19	DHBOA-hexosyl-hexoside	C ₁₄ H ₁₇ NO ₉	Benzoxazinoids	523.1773	0.59	4.48		
20	DHBOA-hexosyl-deoxyhexoside	C ₈ H ₇ NO ₄	Benzoxazinoids	490.1557	0.59	4.71		
21	HBOA	C ₈ H ₇ NO ₃	Benzoxazinoids	166.0499	0.34	5.92	C15769	322636
22	HBOA-glucoside	C ₁₄ H ₁₇ NO ₈	Benzoxazinoids	328.1027	0.09	4.71		14605136
23	HM2BOA	C ₁₀ H ₁₁ NO ₅	Benzoxazinoids	226.0709	-0.61	5.51		11064107
24	HMBOA-hexoside	C ₁₀ H ₁₁ NO ₅	Benzoxazinoids	358.1241	-0.61	5.06		
25	HM2BOA-hexoside	C ₁₆ H ₂₁ NO ₁₀	Benzoxazinoids	388.1245	1.86	5.36		
26	Apigenin-6-C-hexoside-C-pentoside	C ₂₆ H ₂₈ O ₁₄	Flavonoids	565,1553	0,09	4,66		
27	Chrysoeriol-6-C-hexoside	C ₂₂ H ₂₂ O ₁₁	Flavonoids	463.1237	0.43	5.35	C05990	442611
28	Chrysoeriol-C-hexosyl-O-deoxyhexoside	C ₂₈ H ₃₂ O ₁₅	Flavonoids	609.1817	0.50	5.24		
29	Chrysoeriol-C-hexosyl-O-hexoside	C ₂₈ H ₃₂ O ₁₆	Flavonoids	625.1766	0.42	5.17		72193674
30	Luteolin-C-hexosyl-deoxyhexoside	C ₂₇ H ₃₀ O ₁₅	Flavonoids	595.1660	0.45	5.02		
31	Luteolin-C-hexosyl-C-pentoside	C ₂₆ H ₂₈ O ₁₅	Flavonoids	581,1504	0.51	4.97		44258081
32	Luteolin-C-hexosyl-O-hexoside	C ₂₇ H ₃₀ O ₁₆	Flavonoids	611,1608	0.23	4.98		
33	Luteolin-6-C-hexoside	C ₂₁ H ₂₀ O ₁₁	Flavonoids	449,108	0.36	5.00	C01750	5280459
34	Tricin	C ₁₇ H ₁₄ O ₇	Flavonoids	331.0812	0,09	6.45	C10193	5281702
35	Tricin-7-O-hexoside	C ₂₃ H ₂₄ O ₁₂	Flavonoids	493,1343	0,5	5.56		5322022
36	Abscisic acid	C ₁₅ H ₂₀ O ₄	Phytohormones	265,14343	0.53	6,06		
37	Abscisic acid glucose ester	C ₂₁ H ₃₀ O ₉	Phytohormones	427,19625	0.35	4,33		
38	Indole acetic acid	C ₁₀ H ₉ NO ₂	Phytohormones	176,0706	1,16	1,71		
39	OPDA	C ₁₈ H ₂₈ O ₃	Phytohormones	293,211121	0,89	7,42		
40	Jasmonic acid	C ₁₂ H ₁₈ O ₃	Phytohormones	211,1329	0,14	6.27	C08491	5281166
41	Methyljasmonate	C ₁₃ H ₂₀ O ₃	Phytohormones	225,148521	0,31	6,88		
42	Salicylic acid	C ₇ H ₆ O ₃	Phytohormones	139,039	0,21	4,5	C00805	338
43	Methylsalicylate	C ₈ H ₈ O ₃	Phytohormones	153,05462	0,56	5,39		
44	Coumaroylagmatine	C ₁₄ H ₂₀ N ₄ O ₂	Hydroxycinnamic acid amides	277,1659	0,01	4,66	C04498	5280691
45	Coumaroylputrescine	C ₁₃ H ₁₈ N ₂ O ₂	Hydroxycinnamic acid amides	235,1441	0,02	4.50		
46	Coumaroylcadaverine	C ₁₄ H ₂₀ N ₂ O ₂	Hydroxycinnamic acid amides	249,15975	0.56	3,97		
47	Caffeoylagmatine	C ₁₄ H ₂₀ N ₄ O ₃	Hydroxycinnamic acid amides	293,1608	0.06	4.52		
48	Caffeoylputrescine	C ₁₃ H ₁₈ N ₂ O ₃	Hydroxycinnamic acid amides	251,139	0.08	4.47	C03002	
49	Feruloylagmatine	C ₁₅ H ₂₂ N ₄ O ₃	Hydroxycinnamic acid amides	307,1765	0,11	4.57	C18325	46173376
50	Feruloylputrescine	C ₁₄ H ₂₀ N ₂ O ₃	Hydroxycinnamic acid amides	265,1548	0,49	4.70	C10497	5281796
51	Feruloylcadaverine	C ₁₅ H ₂₂ N ₂ O ₃	Hydroxycinnamic acid amides	279,1702	0.43	4.85		
52	Hydroxyferuloylputrescine	C ₁₄ H ₂₀ N ₂ O ₄	Hydroxycinnamic acid amides	281,14955	0.12	3,81		
53	Sinapoylagmatine	C ₁₆ H ₂₄ N ₄ O ₄	Hydroxycinnamic acid amides	337,187	0.09	4.65		
54	Sinapoylputrescine	C ₁₅ H ₂₂ N ₂ O ₄	Hydroxycinnamic acid amides	295,1653	0.22	4.56		

^a Metabolites was classified according to KEGG (Kyoto Encyclopedia of Genes and Genomes; <http://www.genome.ad.jp/kegg/>) and PubChem (<http://pubchem.ncbi.nlm.nih.gov>) databases. Id: identifier; *m/z* : mass-to-charge ratio; *m/z* error (difference between the measured *m/z* and the calculated *m/z* of an ion, represented in ppm); RT: retention time. Compounds indicated in bold were confirmed with authentic standards.

CHAPITRE 4

**Isolement et identification de souches de *Bacillus velezensis*
produisant des lipopeptides et présentant une activité antifongique
vis-à-vis de *Zymoseptoria tritici***

Dans le chapitre précédent, nous avons montré que la mycosubtiline, un lipopeptide cyclique, était capable de protéger le blé vis-à-vis de *Z. tritici* grâce à un double mode d'action (activité antifongique directe et stimulation des défenses de l'hôte *via* la potentialisation). Ici, deux souches de bactéries (S1 et S6) provenant de la phyllosphère de blé, ont été isolées au Maroc. Ces bactéries ont été identifiées comme appartenant à l'espèce de *Bacillus velezensis* et se sont révélées prometteuses pour le biocontrôle de *Z. tritici*. Les deux souches bactériennes sélectionnées, S1 et S6, ont montré une activité antagoniste significative vis-à-vis de *Z. tritici in vitro*. Nous avons ensuite produit les filtrats de culture dont l'activité antifongique a ensuite été déterminée. Les résultats obtenus sont très encourageants et l'activité antifongique *in vitro*, en particulier du surnageant produit avec la souche S1, vis-à-vis de l'agent phytopathogène, était forte. La caractérisation des métabolites produits par ces souches a été réalisée et a permis de montrer que la souche S1 biosynthétisait différents types de lipopeptides, notamment plusieurs bacillomycines D putatives. Le rôle potentiel de ces composés dans l'activité antifongique des surnageants sera discuté.

Cette étude a été menée en collaboration avec plusieurs partenaires ayant réalisé un certain nombre de travaux :

- INRA de Kenitra (Maroc) : Isolement des souches
- URCA Reims : Identification des souches
- Université de Lille : Caractérisation des lipopeptides produits par les souches
- ULCO Calais et Junia Lille : Activités biologiques, coordination et rédaction du manuscrit

Les résultats obtenus dans le cadre de ce chapitre sont en cours de consolidation et de préparation en vue de leur publication dans la revue Agronomy (IF = 2,60).

Isolation and Identification of lipopeptide-producing *Bacillus velezensis* strains from wheat phyllosphere with antifungal activity against the wheat pathogen *Zymoseptoria tritici*

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ABSTRACT

Septoria tritici blotch, caused by the fungal pathogen *Zymoseptoria tritici*, is a highly significant disease on wheat crops worldwide, causing yield losses of up to 50% during severe epidemics. Disease management relies mainly on the use of chemical fungicides and to a lower extent on the use of resistant cultivars. Because of the potential negative impacts of agrochemicals on both the environment and human health, a development of ecofriendly crop protection tools, such as the use of compounds with biocontrol properties, is hence strongly recommended. The objective of the present study was thus to search for new bacterial strains with promising antimicrobial activity against *Z. tritici*. Two phyllospheric bacteria (S1 and S6) were isolated from wheat ears in Morocco and were identified as *Bacillus velezensis* strains according to 16s rRNA sequencing. Antagonistic assays performed with either living strains or cell-free culture filtrates showed significant antifungal effects against *Z. tritici*. For the culture filtrate, the half-maximal inhibitory dilution and the minimal inhibitory dilution consisted of 1.4% and 3.7 % for the strain S1, and 7.4% and 15% for the strain S6, respectively. Matrix assisted laser desorption Ionization-time of flight (MALDI-ToF) analysis revealed that both strains synthesize cyclic lipopeptides from different families. The strain S1 produces surfactins, iturins as well as fengycins, while the strain S6 seems to synthesize only surfactins and iturins. Interestingly, iturins produced by the strain S1 are putative bacillomycin D, while those from the strain S6 are putative iturin A or mycosubtilin. Such patterns could explain the difference observed in the activity of the culture filtrates from the two strains. This study allows the identification of new lipopeptide-producing strains of *B. velezensis* with a high potential of application for the biocontrol of *Z. tritici*.

Keywords : *Zymoseptoria tritici*, *Bacillus velezensis*, antimicrobial agents, cyclic lipopeptides, Bacillomycin D.

INTRODUCTION

Bacillus sp. are ubiquitous Gram-positive bacteria occurring in diverse ecological niches and are known for their ability to produce a wide array of metabolites with applications in several areas, like crop bio-protection against pests and diseases. *Bacillus* genus harbors more than 300 described species (www.bacterio.net/bacillus.html) but constitutes a phylogenetically incoherent group with two main reported clades (*B. subtilis* and *B. cereus* clades) (Bhandari *et al.*, 2013). Most of the exploited *Bacillus* species for the production of biologically active lipopeptides are *B. subtilis*, *B. pumilus*, *B. licheniformis*, *B. velezensis*, and *B. amyloliquefaciens*, although the classification of these later species is confusing and still not unanimous within the scientific community (Fan *et al.*, 2017). *Bacillus* species dedicate approximatively 5 to 8% of their total genome to the biosynthesis of bioactive secondary metabolites, such as peptides and lipopeptides, polyketides, bacteriocins, and siderophores (Hamley *et al.* 2015). Lipopeptides from *Bacillus* sp. are synthesized by multi-enzymatic proteins named non-ribosomal peptide synthetases, which confer substantial structural diversity to the molecules and results in the production of linear, branched, or cyclic compounds usually considered as low toxic (Strieker *et al.*, 2010 ; Deravel *et al.*, 2014). Lipopeptides are classified into three main families according to their amino acid sequence, including iturins (mycosubtilin, iturin A, and bacillomycin D), surfactin, and fengycin (Xunchao *et al.*, 2013; Guo *et al.* 2014). The surfactin family consists of heptapeptides containing a β -hydroxy fatty acid, while iturin family includes heptapeptides with a β -amino fatty acid (Romero *et al.*, 2007 ; Jacques, 2011). Molecules belonging to the fengycin family, as well as the related plipastatin, are decapeptides linked to a β -hydroxy fatty acid. Lipopeptides have been demonstrated to show a broad spectrum of antimicrobial activity towards a wide range of phytopathogenic fungi, oomycetes, bacteria, and viruses through direct antagonistic effect and/or *via* the stimulation of the plant immunity (Crouzet *et al.*, 2020a). These modes of action seem to result mainly from the interaction of the lipopeptides with the cell plasma membranes of the targeted organisms, because of their amphiphilic properties conferring them an affinity with cell membranes (Fira *et al.*, 2018 ; Crouzet *et al.*, 2020) .

Wheat is one of the most cultivated and consumed cereals worldwide, used as basic human and livestock foods in several regions around the world. *Zymoseptoria tritici*, causing Septoria tritici blotch, is one of the most occurring and devastating pathogens on wheat crops, able to induce grain yield losses up to 50%, especially in regions where the environmental conditions are favorable for disease development (Ponomarenko *et al.*, 2011 ; Fones and Gurr, 2015). *Zymoseptoria tritici* is a hemibiotrophic fungus, with a biotrophic phase of about two weeks, followed by a necrotrophic phase of one week (Siah *et al.*, 2010). However, this time lapse varies depending on the host cultivar, the fungal strain, and the environmental conditions (Lovell *et al.*, 2004). Since host resistance to *Z. tritici* is not fully effective in most wheat cultivars, disease control relies mainly of the use of synthetic chemical fungicides. However, the use of such products is increasingly controversial because of their potential negative and harmful impacts on both the environment and human health. Besides, *Z. tritici* frequently develops resistance to fungicides and regularly overcomes host-resistance genes, making it one of the most difficult plant pathogens to control in the field (Cowger *et al.*, 2000; Cheval *et al.*, 2017). In this context and in the framework of sustainable agriculture, it is urgent to develop and promote alternative control strategies against this disease. One of the most promising alternatives to allow a reduction of chemical pesticides is the biocontrol of plant pathogens using microorganisms or natural

substances. Such an eco-friendly solution received increasing attention because of its potential improved safety when compared to conventional pesticides (Beneduzi *et al.*, 2012). The main objective of the present study was thus to isolate new biocontrol agents producing metabolites with antimicrobial bioactivity against *Z. tritici*, by focusing on the bacterial species *B. velezensis*, which has never been investigated for its antifungal activity towards the pathogen. Although previous reports on *B. velezensis* were performed with strains collected mainly from soil or rhizosphere, we isolated here bacterial strains from wheat phyllosphere (plant ear). The assays consisted to isolate, identify, and characterize the antimicrobial activity of the isolated strains against *Z. tritici*. Moreover, the lipopeptide metabolites produced by the bacteria were also identified using matrix assisted laser desorption ionization-time of flight (MALDI-ToF) analysis.

MATERIALS AND METHODS

Bacillus sp. strain isolation

Both S1 and S6 *B. velezensis* strains used in the present study were isolated from wheat ears in August 2016 in Morocco. The strain S1 was isolated in Ait Mellol location (GPS coordinates 033°14'44 006°22'52 251,33') while the strain S6 was isolated in Marrakech location (GPS coordinates 031°26'61 006°50'50 716,81). One wheat ear was sampled from unknown cultivar in each location and then immediately immersed in laboratory conditions in a solution of 250 mL of physiological water, before being submitted for agitation for 2h. Serial dilution was then performed from each sample until reaching 10^{-6} of the mother solution, then 100 μ L of the obtained diluted solution (10^{-6}) was spread on a Petri plate amended with potato dextrose agar (PDA) medium. After an incubation period of 24h at 25°C in dark conditions, characteristic colonies of *B. velezensis* were collected under sterile conditions and transferred into new PDA plates, before conservation at -80 C° in cryotubes for further analyses.

Culture filtrate production and preparation

Culture filtrates (free cell supernatants) from the S1 and S6 *B. velezensis* strains were produced in sterile conditions using a modified Landy's liquid medium (Landy *et al.*, 1948), containing 20 g.L⁻¹ glucose, 5 g.L⁻¹ glutamic acid, 1 g.L⁻¹ yeast extract, 1 g.L⁻¹ K₂HPO₄, 0.5 g.L⁻¹ MgSO₄.7H₂O, 0.5 g.L⁻¹ KCl, 1.6 mg.L⁻¹ CuSO₄.5H₂O, 0.4 mg.L⁻¹ FeSO₄.7H₂O, 1.2 mg.L⁻¹ MnSO₄.H₂O, and 3-(N-morpholino)propanesulfonic acid buffer at 100 mM pH 7.0. A preculture of each strain was prepared in 100 mL Erlenmeyer containing 10 mL of modified Landy's medium inoculated with frozen stocked cells of each bacterial strain and incubated at 30 C° under 160 rpm shaking for 16h. Then, 10 mL of each preculture were used to inoculate 250 mL Erlenmeyer amended with 100 mL of modified Landy's medium, which then were incubated at 30 C° and 160 rpm shaking for 48h. The first step of the filtration consisted to adjust the concentration of the cultures regarding the bacterial cells into 0.8 optical density at 600 nm using a Spectrophotometer, in order to allow a better and reliable further filtration process. The adjusted cultures were then centrifuged at 10,000 g for 10 min before being sterilized by filtration across a 0.22 μ m cellulose acetate membrane and stored at -20 C°.

In vitro bacterial antagonism activity and antifungal activity bioassays of culture filtrates

Antagonism activity of both *B. velezensis* strains S1 and S6 and the antifungal activity of their culture filtrates were assessed using the *Z. tritici* single-conidial strain T02596 isolated in 2014 from northern France (Platel *et al.*, 2021). The antagonism assays were performed by plate confrontation between the bacterial strains and the fungus. The fungus was grown on PDA medium in Petri dishes incubated at 20°C. After five days of incubation, fungal spores were collected by washing the cultures with 10 mL of sterile distilled water, and then, the concentration of spore suspensions was adjusted to 1×10^6 spores.mL⁻¹. An aliquot of 0.5 mL of spore suspension was spread on PDA Petri dish before being incubated at 20°C for 24h in the dark. Then, 10 µL of bacterial suspension (0.8 optical density, corresponding to 7.10^6 CFU/mL) were deposited on the center of the dish. This bacterial suspension was obtained with a pre-culture of each strain in 25 mL Erlenmeyer containing 5 mL of lysogeny broth (LB) medium inoculated with frozen stocked cells of each bacterial strain (300 µL) and incubated at 28°C for 24h under 180 rpm shaking. The antagonism effect was evaluated by observing an inhibition zone after 13 days of incubation at 28°C in the dark. Three Petri dishes were used as replicates for each condition.

The assays regarding the antifungal activity of the bacterial filtrates were performed in 12-well plates amended with PDA medium supplemented with different concentrations of each culture filtrate (final dilutions in PDA medium corresponding to 0.9, 1.9, 3.8, 7.5, 15, and 30% of culture filtrate). The plates were prepared, inoculated, and incubated according to Platel *et al.* (2021). Three plate wells were used as repetitions for each condition, including the control without culture filtrate. The effect of the culture filtrate on fungal growth was noticed by measuring visually the two perpendicular diameters of each developed *Z. tritici* colony. Dose-response curve and half-maximal inhibitory dilution (ID₅₀) value of each strain were performed using the GraphPad Prism software version 9 (GraphPad Software Inc., San Diego, USA).

MALDI-ToF assay

Bacterial colonies grown on PDA medium at 30 °C for 72h h were spread onto a MALDI ground steel target plate (Bruker Daltonik, Bremen, Germany). They were washed twice with a 99.9/0.1 (v/v) H₂O:Trifluoroacetic acid (TFA) solution and treated with a 70/30 (v/v) formic acid:H₂O solution. They were then coated with a solution of α-cyano-4-hydroxycinnamic acid matrix (10mg/mL in H₂O:acetonitrile:TFA, 50/47.5/2.5 v/v/v). Analyses were realized with the Bruker Daltonik Autoflex Speed MALDI-ToF/ToF mass spectrometer. Calibration of the device was performed with the Peptide Calibration Standard II kit from Bruker Daltonik composed of bradykinin 1-7, angiotensin I, angiotensin II, substance P, bombesin, rening substrate, ACTH clip 1-17, ACTH clip 18-39, and somatostatin. Analyses were carried out in the reflector and positive mode in the m/z 700-5000 mass range. Mass spectra were obtained by accumulating 2000 lasers shots at 50% of intensity.

Molecular identification of S1 and S6 strains

Genomic DNA from exponentially growing S1 and S6 cultures was extracted using the Wizard Genomic Purification DNA Kit (Promega Corp., Madison, WI, USA) following instructions from the supplier. The strains S1 and S6 were identified by sequencing the 16S rRNA gene using 16S forward 5'AGAGTTTGATCATGGCTCAG and 16S reverse 5'ACGTTACCTTGTTACGACTT primers (Esmaeel *et al.*, 2020), and the following PCR cycles: one cycle at 94°C for 5min, 30 cycles (94°C, 1min; 56°C, 30s; 72°C, 2min), and a final extension at 72°C for 10 min. The resulting PCR product sizes were purified with the

GeneJET Gel Extraction Kit (Thermo Scientific Fermentas, Waltham, USA) and then sequenced at GENWIZ Co., Ltd. (Leipzig, Germany) using Sanger sequencing technology. The sequencing reaction was performed in two directions using the forward and reverse primers (FD1, RP1) to cover the length of the 16S rRNA gene. After trimming, forward and reverse sequences were aligned and then assembled to obtain the full contiguous sequence. The sequences were then submitted to blast basic using local alignment search tool (BLAST) provided online by the national center for biotechnology information (NCBI, Bethesda, MD, USA). The nearest 16S rRNA gene sequences were downloaded and the phylogenetic tree was constructed using the neighbor-joining algorithms and kimura two-parameter model *via* MEGA version X (Kumar *et al.*, 2018). The dataset was boot-strapped 1000 times. The 16S rRNA gene sequences of strains S1 and S6 have been deposited in GenBank under accession numbers MW931521 and MW931522, respectively.

RESULTS

S1 and S6 strains are new unique *B. velezensis* genotypes

Based on the obtained 16S rRNA gene sequences, strains S1 and S6 shared high sequence similarity (>99%) with *B. velezensis*. The phylogenetic analysis constructed using MEGA version X with neighbor-joining method indicated that strains S1 and S6 belong to the genus *Bacillus* and form a monophyletic cluster with *B. velezensis* with a high bootstrap score (>95%) (**Figure 1**). Moreover, sequence alignment of 16S rRNA gene sequence between strains S1 and S6 showed that both strains shared 99.86% sequence identity. Therefore, the following names *B. velezensis* strain S1 and *B. velezensis* strain S6 are proposed. They also shared high sequence identity with other species of *Bacillus* sp., including *B. vallismortis*, *B. subtilis*, *B. mojavensis*, and *B. halotolerans* (**Figure 1**).

S1 strain shows stronger antifungal activity towards *Z. tritici* compared to S6 strain

The antagonistic activity of S1 and S6 strains towards *Z. tritici* was performed by depositing a bacterial suspension on the center of PDA-amended Petri plates already inoculated with *Z. tritici*. Results revealed that both strains exhibit antagonistic activity against the pathogen and form a visible halo around the formed bacterium colonies (**Figure 2A,B**), which revealing a release and diffusion of antimicrobial compounds into the surrounding medium. On the other hand, culture filtrates from the two strains were produced and tested for their antimicrobial activity against the fungus on PDA medium. Dose-response curves revealed that culture filtrates from both strains are able to inhibit fungal growth, but the S1 culture filtrate showed higher antifungal activity when compared to S6 culture filtrate (**Figure 2C,D**). The minimal-inhibitory dilution (MID) values for S1 and S6 culture filtrates correspond to 3.75% and 15%, while the half-maximal inhibitory dilution (ID₅₀) values for S1 and S6 culture filtrates correspond to 1.4% and 7.4%, respectively (**Figure 2C,D**). In order to examine the effect of culture media on the growth of the two bacterial strains, three growth media were tested (King's B medium, KB; plate count agar, PCA, and lysogeny broth, LB). Results highlighted that LB is the most suitable among the three tested media for *in vitro* growth of both *B. velezensis* strains (**Supplementary Figure S1**).

Both S1 and S6 strains produce cyclic lipopeptides but with distinct patterns

Lipopeptides produced by the two *B. velezensis* strains S1 and S6 grown on solid medium were assessed using MALDI-ToF analysis. Both strains produced cyclic lipopeptides from different families, with several putative chemical structures within each family (**Table 1, Figure 3**). Even if common metabolites were detected among the two bacterial strains, they overall displayed different putative metabolite patterns (**Table 1**). The strain S1 produced surfactins, iturins as well as fengycins, while the strain S6 biosynthesized only surfactins and iturins. Interestingly, iturins produced by the strain S1 are, putatively, bacillomycin D, while those produced by the strain S6 are iturin A or mycosubtilin. Surfactins produced by both strains are surfactin or pumilacidin (**Table 1**).

DISCUSSION

We isolated here two wheat phyllosphere-associated bacterial strains identified as two strains of *B. velezensis* (S1 and S6), and exhibiting potent antagonistic activity against *Z. tritici*, a major pathogen on wheat crops. Our results are in agreements with previous reports showing that this beneficial bacterial species can display antagonistic activity against crop phytopathogens. For instance, the *B. velezensis* strain FZB42 (previously named as *B. amyloliquefaciens* strain FZB42) has been shown to exhibit antagonistic activity against another wheat pathogen, *Fusarium graminearum*, as well as the phytopathogenic bacterium *Ralstonia solanacearum* (Gu *et al.*, 2017). We suggest that the antagonistic activity of the *B. velezensis* strains S1 and S6 observed here against *Z. tritici* is due to the capacity of the strains to produce antimicrobial compounds, such as lipopeptides. Indeed, lipopeptides are known to be the major contributor to *Bacillus* sp. biocontrol activity (Abderrahmani *et al.*, 2011). Various strains of *B. velezensis* have recently received considerable attention since this species can produce different types of biologically active secondary metabolites that can suppress plant pathogens (Rabbee *et al.*, 2019). It has been reported *B. velezensis* strains are able to synthesize various bioactive lipopeptides, like amylocyclicin, bacilysin, bacillomycin D, bacillibactin, bacillaene, difficidin, fengycin, macrolactin, plantazolicin, and surfactin (Chen *et al.*, 2017). Today, several products based on *Bacillus* spp., such as *B. amyloliquefaciens*, *B. pumilus* or *B. subtilis*, are marketed as a biopesticides (Pérez-García *et al.*, 2011; Rabbee *et al.*, 2019).

Our results showed that the culture filtrate from the *B. velezensis* strain S1 displayed a strong and a higher antifungal activity against *Z. tritici* when compared to the strain S6. This marked difference could be explained by the differential composition in the lipopeptides synthesized by the two strains. Indeed, MALDI-ToF analysis revealed a presence of fengycin and bacillomycin D (an antimicrobial lipopeptide belonging to the iturin family) only in the most active filtrate, from the strain S1. The importance of these two lipopeptides in the antifungal activity of *B. velezensis* has already been reported with the *B. velezensis* strain FZB42, in which a double mutant that was deficient in both bacillomycin D and fengycin synthesis ($\Delta bmyA \Delta fenA$) heavily impaired its ability to inhibit the growth of *F. oxysporum*, thereby indicating synergistic effects among such lipopeptides against this targeted fungal pathogen (Koumoutsi *et al.*, 2004). In the same way, Romero and colleagues (2007) reported that the lipopeptides from iturin and fengycin families have a major role in the antimicrobial activity of *Bacillus* sp. strains on *Podosphaera fusca*. However, other studies seem to point out that the presence of bacillomycin as being solely responsible for the antifungal activity. For instance, bacillomycin D deficient mutant strains of *B. velezensis* strain FZB42 or strain SQR9 exhibited severely impaired antifungal activities, which indicated that the bacillomycin D appear to be the major

contributor to the antifungal activity against *F. oxysporum* (Chen *et al.*, 2009; Xu *et al.*, 2013). Cao *et al.*, (2018) suggest that the relative contribution of each lipopeptide to the antimicrobial activity may also be dependent on the species of the targeted plant pathogen. These authors have reported that iturin and fengycin are functionally redundant in antagonism against the bacterium *Ralstonia solanacearum*, when only iturin family metabolites appear to have an antagonism activity against the fungus *Fusarium oxysporum*.

The effect of purified lipopeptides from *B. subtilis* have been previously examined against *Z. tritici*. It has been shown that mycosubtilin, a lipopeptide belonging to iturin family, displayed a strong antifungal activity against *Z. tritici* (half-maximal inhibitory concentration of 1.4 mg L^{-1}), while neither surfactin nor fengycin showed significant activity towards the pathogen (Mejri *et al.*, 2018). On the other hand, bacterium cell-free filtrates of a mutant strain of *B. subtilis* overproducing mycosubtilin showed an antagonistic activity against several phytopathogenic fungi, including *Pythium aphanidermatum*, *Botrytis cinerea*, and *F. oxysporum*, with growth inhibition zones significantly larger than those induced by the wild-type supernatant (Leclère *et al.*, 2005). Taken together, these results suggest that lipopeptides of the iturin family may display an interesting antifungal activity towards plant phytopathogens. Moyne *et al.* (2001) reported that analogues of bacillomycin and/or length of the lipid chain may have an influence on the antifungal activity of this lipopeptide on various phytopathogenic fungi, such as *Alternaria solani*, *Aspergillus flavus*, *Colletotrichum gloeosporioides*, *Phomopsis gossypii*, and *Sclerotium rolfsii*. The mechanism underlying the bacillomycin D antifungal activity was performed on *F. graminearum* and revealed an inhibition of the formation and germination of conidia (Gu *et al.*, 2017). In details, the mycelia structure swollen in the presence of Bacillomycin D. Moreover, observations with electronic microscopy revealed substantial destruction of hyphae and conidia, with loose of cell walls and irregular shapes, which indicate that the cytoplasm was leaking out of the cells (Gu *et al.*, 2017).

This study highlighted that the two strains isolated from wheat plant ears and identified as *B. velezensis* strains S1 and S6 exhibit antimicrobial activity against the wheat pathogen *Z. tritici*, and that their biological activity seems to be related to the production of lipopeptides. The higher antifungal activity observed for the culture filtrate S1 could, thus, be due to the different lipopeptides it may produce, in particular the various analogues of bacillomycin D. Hence, further investigations using purified lipopeptides are needed to confirm the antifungal activity of bacillomycin D, alone or in mixture with other lipopeptides, against *Z. tritici*. On the other hand, (Chowdhury *et al.*, 2015a) have suggested that *B. velezensis*, and notably the strain FBZ42, may trigger pathways of induced systemic resistance (ISR) which can also contribute to the biocontrol activity of FBZ42. Indeed, sub-lethal concentrations of lipopeptides and volatiles produced by plant-associated *Bacilli* trigger pathways of ISR, which protect plants against attacks of pathogenic microbes, viruses, and nematodes (Chowdhury *et al.*, 2015). In this sense, the ability of the S1 and S6 *B. velezensis* strains to induce ISR in wheat against *Z. tritici* should be investigated.

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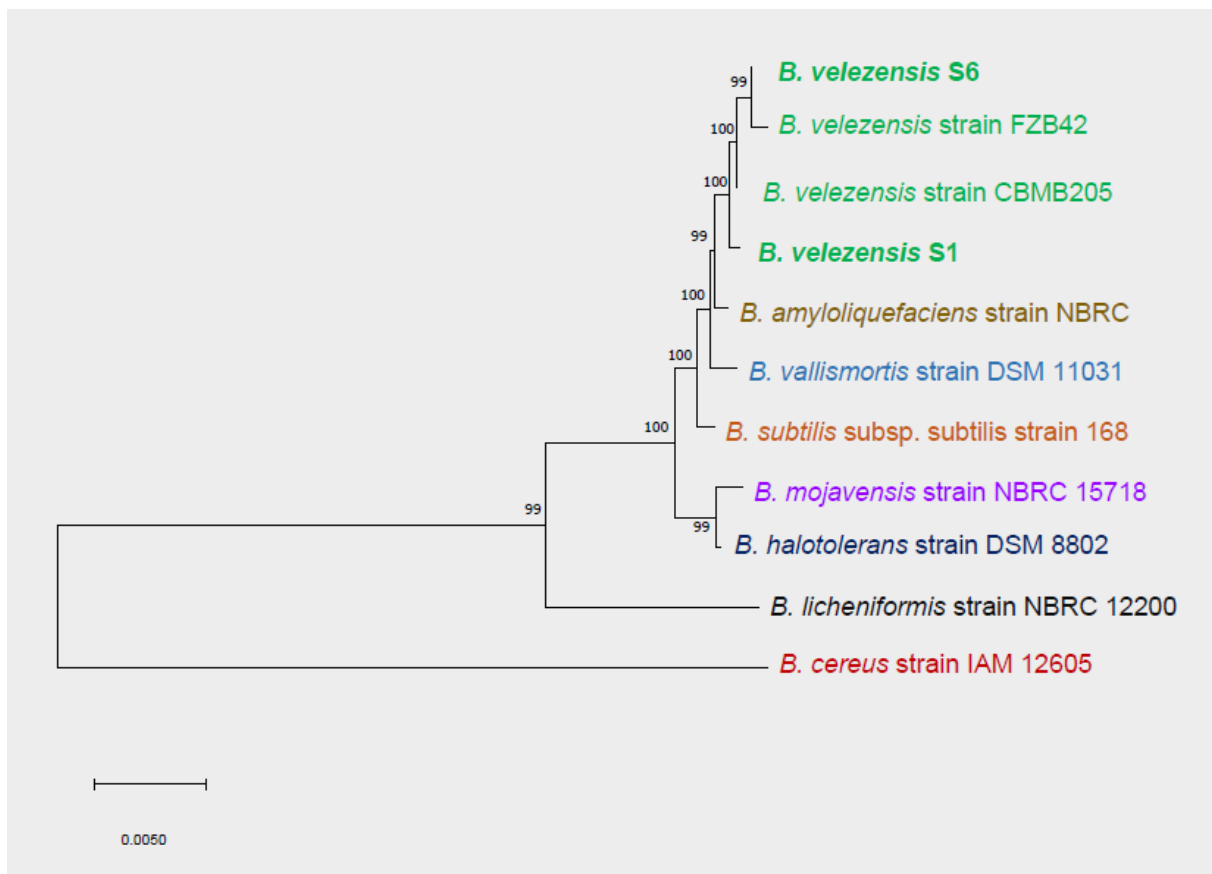


Figure 1. Phylogenetic tree of the S1 and S6 *Bacillus velezensis* strains. The tree was built based on 16S rRNA gene sequences using MEGA version X with neighbor-joining method. Values at nodes indicate bootstrap values out of 1000 resampling. *Bacillus cereus* IAM 12605 was used as an outgroup. S1 and S6 are shown in bold.

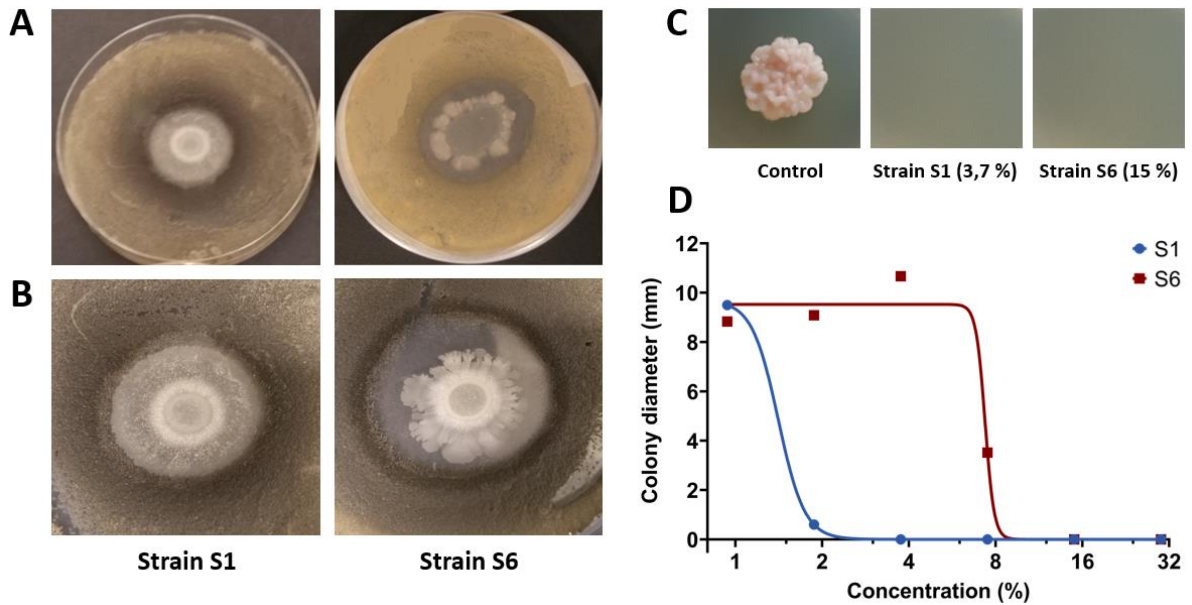


Figure 2. Antifungal activity of S1 and S6 *Bacillus velezensis* strains against the *Zymoseptoria tritici* strain T02596. **A and B**, Antagonistic activity of S1 and S6 strains on solid PDA medium at 13 days after Petri plate inoculation (B is a zoom of A). The inhibiting effect is visualized by the formation of a halo around the bacterial colonies. **C**, illustration of the antifungal effect of the S1 and S6 culture filtrates on *Z. tritici* observed on PDA medium. **D**, *in vitro* dose-response curves of the antifungal effect of the S1 and S6 culture filtrates on *Z. tritici* growth assessed by measuring the fungal colony diameters at 10 days after plate inoculation. Dose-response curves were performed using GraphPad Prism software v 9.0. Three repetitions were used as replicates for each concentration of tested culture filtrate.

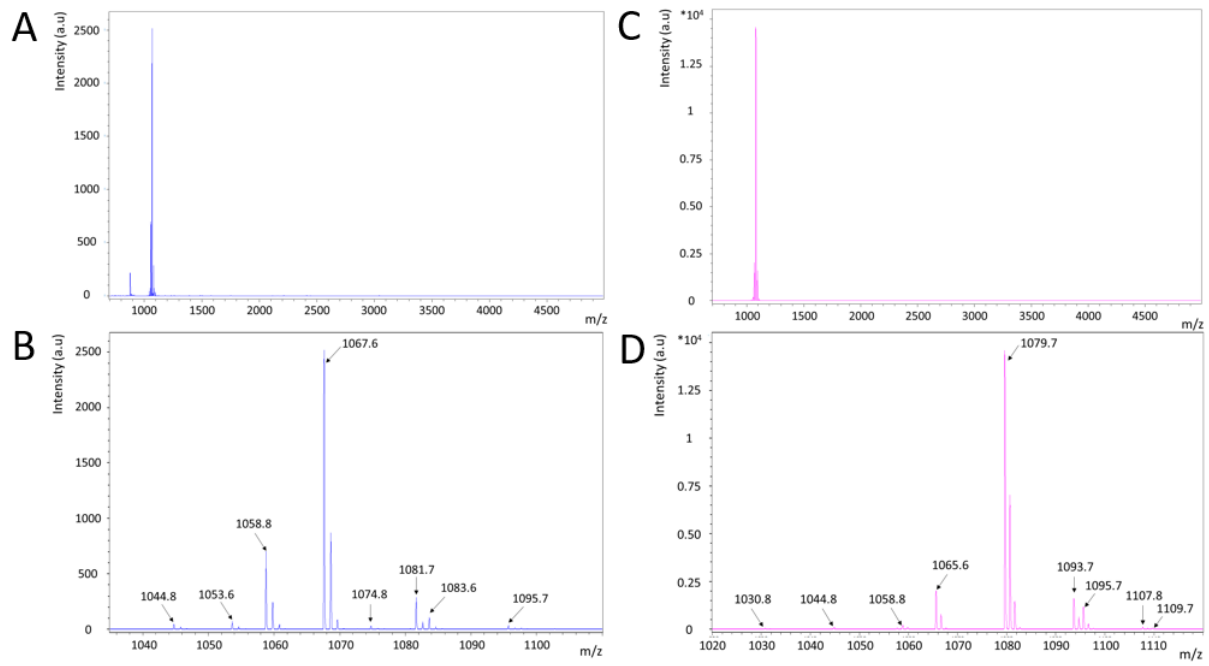
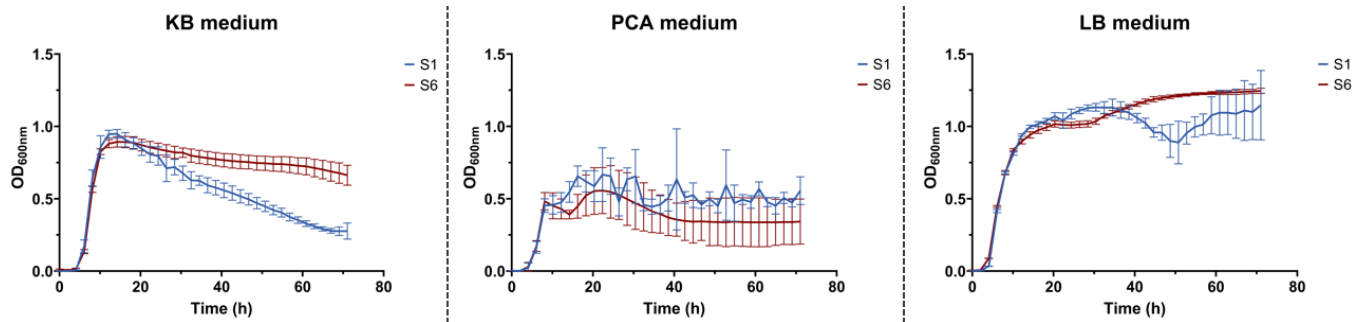


Figure 3. Whole-cell MALDI-ToF mass spectra of the two *Bacillus velezensis* strains S1 (A, B) and S6 (C, D). B and D are a zoom on A and D, respectively.

Table 1. Putative metabolites produced by the two *Bacillus velezensis* bacterial strains S1 and S6 grown on PDA medium, detected by MALDI-TOF.

<i>Bacillus velezensis</i> S1		
m/z	Putative assigned lipopeptide	Lipopeptide family
1044.8	Surfactin C14 or [Ile7] Surfactin C14 or [Val7] Surfactin C15 or [Val7] Pumilacidin C14 [M+Na] ⁺	Surfactin
1058.8	Surfactin C15 or [Ile7] Surfactin C15 or [Ile7] Pumilacidin C14 or [Val7] Pumilacidin C15 [M+Na] ⁺	Surfactin
1074.8	[Ile7] Surfactin C15 or [Ile7] Pumilacidin C14 or [Val7] Pumilacidin C15 [M+K] ⁺	Surfactin
1053.6	Bacillomycin D C14 [M+Na] ⁺	Iturin
1067.7	Bacillomycin D C15 [M+Na] ⁺	Iturin
1083.7	Bacillomycin D C15 [M+K] ⁺	Iturin
1081.7	Bacillomycin D C16 [M+Na] ⁺	Iturin
1095.7	Bacillomycin D C17 [M+Na] ⁺	Iturin
1486.0	Fengycin A C16 [M+Na] ⁺ or Fengycin A C15 (insat) [M+K] ⁺	Fengycin
1499.8	Fengycin A C17 [M+Na] ⁺ or Fengycin AC16 (insat) [M+K] ⁺ or Fengycin B C15 (insat) [M+Na] ⁺	Fengycin
<i>Bacillus velezensis</i> S6		
m/z	Putative assigned lipopeptide	Lipopeptide family
1030.8	Surfactin C13 or [Val7] Surfactin C14 or [Ile7] Surfactin C13 or [Ala4] Surfactin C15 [M+Na] ⁺	Surfactin
1044.8	Surfactin C14 or [Val7] Surfactin C15 or [Ile7] Surfactin C14 or [Val7] Pumilacidin C14 [M+Na] ⁺	Surfactin
1058.8	Surfactin C15 or [Ile7] Surfactin C15 or [Ile7] Pumilacidin C14 or [Val7] Pumilacidin C15 [M+Na] ⁺	Surfactin
1065.6	Iturin A C14 or Mycosubtilin C14 [M+Na] ⁺	Iturin
1079.6	Iturin A C15 or Mycosubtilin C15 [M+Na] ⁺	Iturin
1095.6	Iturin A C15 or Mycosubtilin C15 [M+K] ⁺	Iturin
1093.6	Iturin A C16 or Mycosubtilin C16 [M+Na] ⁺	Iturin
1109.6	Iturin A C16 or Mycosubtilin C16 [M+K] ⁺	Iturin
1107.6	Iturin A C17 or Mycosubtilin C17 [M+Na] ⁺	Iturin



Supplementary Figure S1. Kinetics of S1 and S6 *Bacillus velezensis* strains growth in different liquid media. These two strains were inoculated in Erlenmeyer containing one of the three different media, from the left to the right, King's B (KB), Plate Count Agar (PCA) and lysogeny broth (LB) for three days. Bacterium growth was assessed by measuring the optical density in each well at 600 nm. Three repetitions were used for each condition. Optical density was also measured for controls (i.e media without bacteria) and these data were used to correct optical density means presented in this figure. Bars stand for standard deviation (n=3).

DISCUSSION GÉNÉRALE ET PERSPECTIVES

Dans le cadre de ce projet de thèse, nous avons démarré les travaux avec une bibliothèque de 181 métabolites (d'origines naturelles ou synthétiques) ou extraits microbiens. Nous avons initié les travaux par le criblage de ces composés sur la base de leur activité antifongique directe *in vitro* vis-à-vis de *Z. tritici*. Cette étape a permis de mettre en évidence plusieurs biomolécules et extraits microbiens prometteurs. Parmi les 146 extraits fongiques testés, huit (provenant de *Penicillium griseofulvum*, *Trichoderma virens*, *Aspergillus niger*, *Myrothecium verrucaria*, *Lecanicillium longisporum*, *P. cyclopium*, *Trichothecium roseum*, *Emericella rugulosa*) se sont révélés significativement actifs *in vitro* vis-à-vis de l'agent pathogène, avec des CI_{50} inférieures à 10% (v/v). Néanmoins, certains surnageants présentaient le risque de contenir des mycotoxines dangereuses pour la santé humaine (Sorenson *et al.*, 1975 ; Northolt *et al.*, 1979 ; Nielsen *et al.*, 2009 ; Banani *et al.*, 2016 ; Siciliano *et al.*, 2017), et ont donc été écartés du criblage. Finalement, trois extraits provenant des espèces fongiques *T. virens*, *L. longisporum* et *E. rugulosa*, ne comprenant pas, à notre connaissance, de composés toxiques ou écotoxiques majeurs, et inhibant de manière significative la croissance de *Z. tritici*, se sont révélés être les plus prometteurs parmi les surnageants d'origine fongique obtenus à l'issue de ce criblage. *Trichoderma virens* est un champignon filamenteux tellurique bien connu pour produire des métabolites antibiotiques, parasiter des champignons pathogènes et déclencher des phénomènes d'ISR chez certains hôtes (Burns and Benson, 2000 ; Djonović *et al.*, 2006). Il est d'ores et déjà utilisé en tant qu'agent de biocontrôle pour lutter contre *Rhizoctonia solani*, *Sclerotium rolfsii*, et *Pythium* spp (Mukherjee and Kenerley, 2010). Si plusieurs travaux ont montré l'efficacité d'autres espèces de *Trichoderma* dans la lutte contre la septoriose du blé, en particulier *T. harzanium*, le potentiel de *T. virens* en tant que solution de biocontrôle n'avait jusqu'alors jamais été rapporté sur notre pathosystème d'étude. *L. longisporum* est un champignon entomopathogène commercialisé en tant qu'agent de biocontrôle pour lutter contre les pucerons. Plusieurs études ont aussi démontré sa capacité à agir contre des agents phytopathogènes responsables de l'oïdium chez plusieurs espèces cultivées dont le concombre (Kim *et al.*, 2008), mais aucune n'a visé des espèces fongiques pathogènes infectant le blé. Peu d'informations sont disponibles dans la littérature à propos d'*E. rugulosa*, mais il semblerait que celui-ci produise des composés fongicides efficaces contre l'agent responsable de la fusariose de la tomate *Fusarium oxysporum* f.sp. *lycopersici* (Sibounnavong *et al.*, 2011). De plus amples analyses ont été initiées afin de déterminer l'effet de ces extraits *in planta*, notamment dans le but de déterminer leur efficacité de protection ainsi que leur potentiel effet éliciteur *via* l'étude de l'activité d'enzymes-clés impliquées dans les voies de défense du blé (catalase, peroxydase, β -1,3-glucanase et chitinase). Les résultats de ces expériences n'étaient toutefois pas concluants et ne seront pas présentés dans le présent manuscrit. En effet, nous avons observé des différences significatives majeures entre les activités des différents lots d'extraits fournis par l'UCLouvain. Les analyses concernant les activités biologiques de ces extraits sont donc en hiatus le temps d'obtenir une répétabilité de production satisfaisante. Des études complémentaires visant à caractériser les composants majoritaires des différents extraits d'intérêt pourraient ici avoir un double rôle, permettre de comprendre les différences d'efficacité entre les lots et identifier les biomolécules responsables de leurs activités.

Aucun des lipopeptides cycliques testés (N1, N2, N8, WLIP, orfamide, putisolvin, lokisin et xantholysin) n'a montré d'activité significative directe *in vitro* vis-à-vis de *Z. tritici* à l'issue de ce criblage, alors que plusieurs de ceux-ci ont déjà été rapportés comme présentant des activités antimicrobiennes, notamment vis-à-vis des agents phytopathogènes *Botrytis cinerea* et *Magnaporthe*

oryzae (Geudens and Martins, 2018; Omoboye *et al.*, 2019). Omoboye *et al.* (2019) ont aussi récemment démontré que les métabolites suivants, WLIP et lokisin, étaient capables de déclencher des mécanismes d'ISR chez le riz contre *M. oryzae* lorsqu'appliqués au niveau racinaire. Il pourrait donc être intéressant de réaliser des expériences similaires sur le pathosystème blé-*Z. tritici* afin de faire avancer nos connaissances sur le potentiel de ces métabolites en tant que composés de biocontrôle dans le cadre de la lutte contre ce pathogène.

D'un point de vue global, sélectionner les composés d'intérêt uniquement selon ce criblage *in vitro* ne nous a permis de discerner que ceux présentant une activité antifongique, mais pas ceux capables d'induire les réactions de défense de l'hôte. Afin d'obtenir le plus large panel de composés prometteurs pour protéger le blé contre la septoriose, il aurait aussi été pertinent de réaliser un criblage de ceux-ci selon leur activité indirecte. Cette étape était initialement prévue dans le cadre de cette thèse et aurait dû permettre d'identifier les biomolécules ou extraits capables d'influer sur les voies de défenses de la plante. Pour ce faire, nous avons prévu de déterminer leur effet *in planta* sur l'activité de six enzymes présentes dans les feuilles de blé et jouant un rôle-clé dans les réactions de défense chez les plantes, avec deux impliquées dans la détoxification des ROS, la CAT et la SOD ; la LOX, qui intervient dans la biosynthèse du JA ; la PAL, enzyme centrale de la voie de biosynthèse des phénylpropanoïdes ; et deux protéines PR, chitinase et β -1,3-glucanase (Halliwell, 2006 ; Huang *et al.*, 2010 ; Wasternack and Hause, 2013 ; Gill *et al.*, 2015). En raison du nombre élevé de composés à tester, nous avons cherché à mettre en place une méthode de criblage à haut-débit utilisant la station de pipetage automatisée de la plateforme Realcat (Université de Lille). Des protocoles automatisés utilisant ces robots ont donc été établis pour toutes ces enzymes. Ceux-ci ont demandé un nombre extrêmement conséquent de sous-étapes d'optimisation et de validation. Finalement, à cause de contraintes techniques, l'optimisation des protocoles visant à déterminer les activités de la lipoxygénase et de la phénylalanine-ammonia lyase n'a pas été concluante. Ces enzymes ont été abandonnées en tant que biomarqueurs et ont été substituées par la POX, une enzyme impliquée dans la détoxification des ROS, mais aussi la lignification, ainsi que l'oxydation de composés phénoliques (Pandey *et al.*, 2017). Néanmoins, en raison de nombreuses contraintes techniques, de temps, et finalement sanitaires, le criblage des composés selon leur activité indirecte n'a pas pu être réalisé. Les travaux effectués pour la mise en place de cette méthode automatisée et innovante de criblage pourraient être utiles dans d'autres futurs projets. Les compétences acquises dans l'analyse des activités enzymatiques du blé m'ont aussi été utiles dans l'étude des activités biologiques des rhamnolipides sur le pathosystème blé-*Z. tritici*.

Lors de l'étape de criblage des rhamnolipides selon leur activité directe contre *Z. tritici*, nous avons pu remarquer une grande diversité dans leur efficacité d'inhibition alors même que ces molécules ne présentaient souvent entre elles que de faibles différences structurales. Nous avons alors décidé d'étudier la relation entre leurs structures et leurs activités biologiques (antifongique, élévation des défenses, protection) sur notre pathosystème, comme présenté dans le **chapitre 1**. Nous avons pu montrer que parmi les 19 rhamnolipides à notre disposition, ceux présentant des chaînes carbonées à 12 carbones ainsi que des liaisons éthers ou esters étaient les plus efficaces pour les différentes activités biologiques étudiées. Aucun rhamnolipide avec un nombre impair de carbones dans leur chaîne carbonée n'a été synthétisé par les partenaires de l'Université d'Artois, il aurait pourtant été judicieux de déterminer les activités de mono-rhamnolipides avec des chaînes carbonées

à 11 ou 13 carbones pour déterminer quelle longueur de chaîne était vraiment la plus optimale sur notre pathosystème. Par ailleurs, l'absence de protection et d'éllicitation des défenses du blé observée après traitement avec l'acide laurique et le dodécanol, respectivement un acide gras et un alcool à 12 carbones, ont permis de mettre en évidence le rôle capital du groupement rhamnose dans l'activité des rhamnolipides. Il pourrait être pertinent de déterminer si d'autres familles de molécules présentant des queues hydrophobes (comme les lipopeptides) seraient plus efficaces pour protéger le blé vis-à-vis de la septoriose si elles présentaient des chaînes carbonées de 12 carbones. Le mélange de rhamnolipides naturels produit par *P. aeruginosa* n'a, quant à lui, montré aucun effet antifongique direct *in vitro* vis-à-vis de *Z. tritici* et aucune induction des activités CAT et POX du blé, même à des concentrations allant jusqu'à 1,5 mM. Toutefois, à cette même concentration, nous avons observé une réduction significative de la sévérité de la maladie sur les feuilles de blé. Plusieurs hypothèses pourraient expliquer cela. Une des plus plausibles est que ce composé serait capable de stimuler les défenses de l'hôte sans modifier l'activité des deux enzymes susmentionnées, soulignant les faiblesses de n'utiliser qu'un faible nombre de biomarqueurs différents à un seul moment, deux jours après traitement. Des analyses plus poussées ciblant les modes d'action de ce composé pourraient nous fournir plus d'informations sur son activité protectrice. Enfin, nous avons pu mettre en évidence que le Rh-Est-C12 était le rhamnolipide le plus efficace, à notre disposition, pour protéger le blé de la septoriose. Ce rhamnolipide est d'autant plus intéressant dans la lutte contre cette maladie qu'il est aussi actif sur des souches hautement résistantes aux DMI, une famille de fongicides de synthèse largement utilisée sur la culture du blé (Cools *et al.*, 2013 ; McDonald *et al.*, 2019). En raison de l'induction des activités de la catalase et de la peroxydase observée chez le blé après traitement avec cette molécule, notre hypothèse était que le mode d'action du Rh-Est-C12 était double (activité directe antifongique et indirecte *via* éllicitation des défenses de l'hôte).

Nous avons donc cherché à caractériser de manière plus fine, dans le **chapitre 2**, le mode d'action indirect du rhamnolipide Rh-Est-C12, à l'aide d'une approche combinée de transcriptomique et de métabolomique ciblée. Nous avons pu montrer que le traitement foliaire du blé avec le Rh-Est-C12 déclenchait des altérations significatives au niveau du transcriptome des feuilles de blé deux jours après traitement, avec une modification de l'expression de 20 gènes, dont un certain nombre était lié à des réponses de la plante aux stress abiotiques. Ces altérations pourraient être causées par l'interaction du rhamnolipide avec les membranes plasmiques végétales, conduisant à leur déstabilisation et à ces réponses de la part du blé. En effet, plusieurs auteurs ont suggéré que l'induction des défenses des plantes par les rhamnolipides était déclenchée selon ce mécanisme (Crouzet *et al.*, 2020). Néanmoins, que ce soit en conditions éllicitation (feuilles traitées mais non-inoculées) ou *priming* (feuilles traitées puis inoculées), l'effet du traitement sur l'accumulation des métabolites détectés et sur le niveau de transcription des gènes était globalement faible. Les résultats ainsi obtenus suggèrent que l'activité protectrice de ce rhamnolipide, lorsqu'appliqué préventivement sur les feuilles de blé, serait principalement liée à son activité antifongique directe. Une autre possibilité, toutefois, est que ce rhamnolipide serait capable d'induire des phénomènes de résistance chez le blé sans entraîner de modifications physiologiques majeures.

Pour aller plus loin dans l'exploration du potentiel des rhamnolipides en protection des cultures, d'autres travaux ont été réalisés. Ainsi, nous avons, en collaboration avec l'Université de Gand, comparé l'effet de l'application des rhamnolipides produits par *P. aeruginosa* (rhamnolipides dits

« naturels ») ou du Rh-Est-12, entre les deux pathosystèmes blé-*Zymoseptoria tritici* et riz-*Magnaporthe oryzae*. Ces derniers présentent plusieurs caractéristiques communes. En effet, le riz (*Oryza sativa* L.) est, tout comme le blé, une espèce appartenant à la famille des *Poaceae*, massivement cultivée au niveau mondial. *M. Oryzae* (anamorphe : *Pyricularia oryzae*) est, comme *Z. tritici*, un champignon phytopathogène hémibiotrophe majeur de cette culture. Cet agent pathogène est responsable de pertes de rendement annuelles dans les pays producteurs estimées entre 10% et 30% en moyenne (Skamnioti and Gurr, 2009). Plusieurs analyses comparatives ont ainsi été menées, et les résultats obtenus sont résumés en **annexe 2 (Tableau supplémentaire 2)**. Globalement, les rhamnolipides naturels n'ont présenté aucune activité bénéfique pour la protection du blé (**Annexe 2**). A l'inverse, sur le pathosystème riz-*M. oryzae*, ces rhamnolipides étaient beaucoup plus intéressants. Au niveau de leurs propriétés antifongiques, ceux-ci étaient capables d'inhiber la germination des spores et la croissance mycélienne de *M. oryzae* *in vitro* (**Annexe 2 ; Tableau supplémentaire 2, Figure supplémentaire 2**). Aussi, la co-application de ces rhamnolipides naturels avec les spores lors de l'infection foliaire du riz diminuait le nombre des lésions causées par la maladie, même à la concentration la plus basse testée (50 μ M). Nous avons, de plus, observé une réduction significative des symptômes lorsque ce composé était appliqué au niveau racinaire. L'efficacité de ce composé varie donc grandement entre les deux pathosystèmes. Le Rh-Est-C12 s'est montré, quant à lui, actif sur les deux pathosystèmes (i) au niveau antifongique, (ii) lorsqu'appliqué au niveau racinaire et (iii) en co-application avec les spores du champignon alors que les concentrations utilisées pour la co-application étaient plus faibles que celles efficaces dans le cadre du **chapitre 1**, où le Rh-Est-C12 ne présentait une activité de protection significative sur le blé qu'à partir de 800 μ M. Notre hypothèse actuelle pour expliquer les raisons de la protection observée lors des expériences de co-application rhamnolipides/spores est que les rhamnolipides, en s'intercalant dans les membranes fongiques, induiraient des fuites cellulaires chez les spores de *Z. tritici* et *M. oryzae*, conduisant à la libération de signaux moléculaires de type PAMP, qui déclencheraient la résistance chez les plantes-hôtes étudiées. Afin de tester cette hypothèse, des travaux supplémentaires visant à étudier l'effet de cette co-application (rhamnolipides avec les spores de *Z. tritici* ou *M. Oryzae*) sur la production de marqueurs cytologiques de l'induction des défenses des hôtes, comme la production de ROS ou le dépôt de callose, vont bientôt être initiés. L'ensemble des travaux réalisés sur les deux pathosystèmes sera valorisé sous forme d'article scientifique une fois les résultats complémentaires obtenus.

Un autre surfactant d'intérêt dont le mode d'action a été particulièrement étudié dans le cadre de cette thèse est la mycosubtiline, un lipopeptide cyclique produit notamment par *Bacillus subtilis*. Nous avons pu, dans le **chapitre 3**, montrer que ce composé, lorsqu'appliqué au niveau foliaire, protégeait le blé vis-à-vis de *Z. tritici* grâce à un double mode d'action, antifongique direct et indirect *via* potentialisation. Ainsi, nous avons observé, après traitement et dans les premières phases de l'infection, la modification significative du niveau d'expression de 80 gènes, dont une partie est impliquée dans les réponses aux stress abiotiques et biotiques. Plusieurs gènes d'intérêt impliqués dans ces réactions de défense ont ainsi été mis en évidence, notamment des gènes impliqués dans les voies de réponse à l'ABA. Une accumulation de flavonoïdes, une famille de molécules connue pour présenter des activités antifongiques, antioxydantes, et de renforcement des parois des cellules végétales, a aussi été observée (Al Aboody and Mickymaray, 2020; Lam *et al.*, 2015; Lan *et al.*, 2015). Ces travaux ont permis d'identifier des gènes et métabolites potentiellement centraux dans la résistance induite du blé vis-à-vis de la septoriose et qui mériteraient sans aucun doute de voir leur

rôle étudié plus en détail dans des projets futurs (e.g l'ABA, la famille des flavonoïdes, les gènes codants pour la protéine WIR1, le précurseur de la protéine PR17d, MLA7, et bien d'autres). Si nous avons pu mettre en évidence l'effet potentialisateur de la mycosubtiline lorsqu'appliqué en traitement foliaire, il serait pertinent de déterminer ultérieurement si les mécanismes de résistance impliqués peuvent être catégorisés de type LAR ou SAR à l'aide d'un protocole expérimental adapté. Notre hypothèse est que la mycosubtiline potentialiserait le blé *via* son interaction avec les membranes plasmiques du blé, déclenchant un stimulus semblable à celui causé par un stress abiotique, ce qui expliquerait les nombreuses modifications métaboliques liés aux réponses à ces types de stress, dont

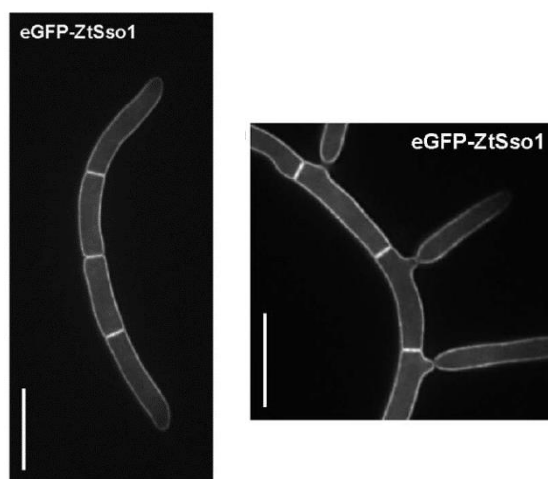


Figure 26 : Cellules de *Zymoseptoria tritici* exprimant le marqueur de membrane plasmique eGFP-ZtSso1. Barres d'échelle = 10 μ M

les réponses à l'ABA (Vishwakarma *et al.*, 2017). Les lipopeptides et les rhamnolipides supposément agissent vis-à-vis des champignons pathogènes en s'intercalant dans leur membrane, les déstabilisant et conduisant *in fine* à leur mort cellulaire. Ils stimuleraient aussi les défenses de l'hôte *via* des mécanismes similaires d'interaction avec les membranes végétales mais avec des impacts différents en raison des différences de compositions membranaires (Crouzet *et al.*, 2020). Dans notre cas, si le Rh-Est-C12 et la mycosubtiline présentent tous deux des activités antifongiques significatives, et que leur traitement semble avoir déclenché un stimulus semblable à un stress abiotique dans les deux cas, seul la mycosubtiline semble stimuler de façon marquée les défenses. Une des perspectives possibles de ces travaux serait de vérifier l'hypothèse concernant les interactions rhamnolipide et lipopeptide avec les membranes du blé ou de *Z. tritici*, par exemple en produisant des composés d'intérêt marqués pour déterminer, par autoradiographie, leurs cibles cellulaires. Il pourrait aussi être possible de travailler avec des cellules modifiées génétiquement pour produire des marqueurs fluorescents des membranes plasmiques, comme cela a récemment été effectué avec la souche de *Z. tritici* IPO323_eGFP-Sso1 transformée pour exprimer une protéine membranaire marquée (Kilaru *et al.*, 2017 ; Steinberg *et al.*, 2020).

Ainsi, la mycosubtiline et le Rh-Est-C12 partagent un certain nombre de points communs. Bien que les démarches aient pu différer concernant l'analyse des activités biologiques de ces composés, nous tenterons de comparer de manière non exhaustive le potentiel de ces composés pour lutter contre *Z. tritici* chez le blé. Pour commencer, il convient de rappeler que la mycosubtiline est produite à partir de *Bacillus subtilis* alors que le Rh-Est-C12 l'est par chimie verte. Comme mentionné ci-dessus,

ces deux composés présentent une activité antifongique significative vis-à-vis de *Z. tritici* due, supposément, à leur capacité à interagir avec les membranes plasmiques fongiques. La mycosubtiline s'est néanmoins révélée plus efficace pour inhiber la croissance de l'agent pathogène *in vitro* que le Rh-Est-C12, avec des CMI de 0.78 mg.L⁻¹ et 31.2 mg.L⁻¹ et des CI₅₀ de 0.57 mg.L⁻¹ et 26.8 mg.L⁻¹, respectivement. Aussi, l'activité de protection conférée par la mycosubtiline (58,1%) après application foliaire était supérieure à celle obtenue après traitement avec le Rh-Est-C12 (41,1%), alors même que la concentration testée pour le lipopeptide (100 mg.L⁻¹) était inférieure à celle pour le rhamnolipide (500 mg.L⁻¹). Cette différence entre activités de protection pourrait s'expliquer, en partie, par les modes d'action des deux composés. En effet, contrairement au Rh-Est-C12, la mycosubtiline présenterait un double mode d'action : antifongique et potentialisateur des défenses du blé, rendant ce composé particulièrement prometteur pour lutter contre *Z. tritici*. Le rhamnolipide présente toutefois d'autres activités biologiques d'intérêt. Nous avons ainsi mis en évidence que son application racinaire, à de faibles concentrations, était capable de réduire significativement la sévérité de la maladie sur les feuilles de blé. Par ailleurs, il était aussi efficace pour inhiber la croissance de souches de l'agent pathogène hautement résistantes aux DMI. Ce composé, pourrait ainsi, selon son utilisation, présenter d'autres avantages dans la lutte contre ce champignon. L'étude des dernières activités biologiques décrites n'a pas été réalisée avec la mycosubtiline dans le cadre de ce projet de thèse. Afin d'aller plus loin dans ce travail de comparaison, il pourrait être pertinent d'étudier, cette fois, l'effet d'un mélange Rh-Est-C12 et mycosubtiline pour protéger le blé, ce qui permettrait d'en apprendre plus sur leurs possibles cibles cellulaires communes. Une autre possibilité pourrait être de suivre une approche d'évolution expérimentale *in vitro* en mettant en contact des souches de *Z. tritici* avec le Rh-Est-C12 et/ou la mycosubtiline. Ces travaux pourraient permettre, d'une part, de comprendre et, potentiellement, prévenir l'apparition de phénomènes de résistance chez le champignon vis-à-vis de ces composés, mais aussi de déterminer si les résistances développées contre l'un ou l'autre de ces surfactants apporteraient des avantages évolutifs vis-à-vis de l'autre, ce qui supporterait l'hypothèse d'une cible cellulaire commune.

Enfin, dans le cadre du **chapitre 4**, deux souches de *Bacillus velezensis* provenant de la phyllosphère de blé, ont été isolées et identifiées. Celles-ci, nommées S1 et S6, présentaient une activité antagoniste significative vis-à-vis de *Z. tritici*. Leurs surnageants ont, tous les deux, significativement inhibé la croissance de l'agent pathogène *in vitro*, et celui provenant de la souche S1 était le plus efficace des deux, avec une CI₅₀ de 1,4%. Les métabolites produits par ces deux souches ont été caractérisés par MALDI-TOF et nous avons pu mettre en évidence que la S1 biosynthétise probablement des bacillomycines D, un lipopeptide membre de la famille des iturines, dont l'activité antimicrobienne a déjà été rapportée vis-à-vis d'autres agents phytopathogènes mais jamais vis-à-vis de *Z. tritici* (Moyné *et al.*, 2001 ; Gu *et al.*, 2017). Des travaux additionnels concernant cette souche sont prévus et viseront notamment à purifier les métabolites qu'elle produit afin de déterminer leurs activités biologiques, notamment de protection *in planta*.

Plusieurs composés d'intérêt sur le pathosystème blé-*Z. tritici* ont donc été mis en évidence dans le cadre de ce projet de thèse, et les modes d'action de deux d'entre eux, le Rh-Est-C12 et la mycosubtiline, deux molécules amphiphiles surfactantes, ont été particulièrement étudiées. Certains mécanismes liés à la résistance induite chez le blé ont aussi été mis en lumière. Les résultats obtenus ainsi que la philosophie suivie tout au long de cette thèse s'inscrivent dans une démarche globale

d'identification d'outils durables pour lutter contre *Z. tritici*, en particulier en raison des résistances déployés par celui-ci contre les fongicides de synthèse (Leroux *et al.*, 2007; Cheval *et al.*, 2017). L'étude des composés d'intérêt devra, par la suite, se poursuivre au champ afin de déterminer leur efficacité de protection en conditions non-contrôlées, celle-ci pouvant grandement différer de celle observée en serre. Dans ce cadre, et même si les lipopeptides et rhamnolipides sont généralement décrits comme respectueux de l'environnement (Crouzet *et al.*, 2020), il sera primordial de vérifier la toxicité et l'éco-toxicité de ces composés ainsi que leur rémanence dans l'environnement et leur accumulation dans les parties comestibles des plantes cultivées. En effet, il convient de rappeler que produits d'origine « naturelle » n'est pas antinomique de toxique pour la santé humaine et l'environnement. Une analyse de leur cycle de vie, allant de leur production jusqu'à leur purification et en passant par la gestion des déchets pourrait aussi être envisagée afin de déterminer leur impact sur l'environnement au sens large. Des travaux visant à améliorer la formulation des composés d'intérêts surfactants seront aussi nécessaires, au vu de la différence d'activité significative notamment observée avec le Rh-Est-C12 *in vitro* et *in planta*. Ceux-ci devraient être mis en place prochainement au laboratoire en collaboration avec l'Université de Gand. Des travaux complémentaires visant à déterminer la durée d'efficacité des traitements, ainsi qu'à optimiser les conditions et modes d'application des différents composés, pourraient aussi être imaginés. En particulier, des concentrations trop élevées de mycosubtiline et du Rh-Est-C12 pourraient se révéler néfastes sur le blé et sa production en raison de leur mode d'action qui, supposément, pourrait agir comme un stress abiotique. Une gestion parcimonieuse des traitements sera aussi à mettre en place afin de limiter l'apparition de phénomènes de résistance de la part du champignon pathogène. Aussi, il conviendra de chercher à limiter les pertes d'efficacité souvent observés lors du passage de la serre au champ avec les produits de biocontrôle. Malgré tout cela, il semble peu probable que ces composés de biocontrôle, pris indépendamment, se révèlent aussi efficaces que les fongicides de synthèse pour lutter contre la septoriose du blé dans les années à venir. Ceux-ci pourraient toutefois faire partie d'une combinaison de leviers agroécologiques, incluant par exemple, le recours à des variétés plus résistantes ou le recours à des mesures prophylactiques (*e.g.* décalage des dates de semis, association de variétés, enfouissement des résidus de culture, *etc.*) qui pourraient ainsi limiter la pression et la nuisibilité de la maladie.

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ANNEXES

ANNEXE 1 : RÉSULTATS DU CRIBLAGE DES COMPOSÉS POUR LEUR ACTIVITÉ ANTIFONGIQUE *IN VITRO*

L'activité antifongique des surnageants fongiques et bactériens ainsi que des métabolites purifiés ou synthétisés par chimie verte a été déterminée *in vitro*. Les tests concernant les extraits fongiques ont été réalisés en plaques 96-puits (**Figure 1**) alors que ceux pour les autres composés ont été effectués en boîtes de Petri. L'ensemble des résultats est présenté dans le **Tableau 1**.

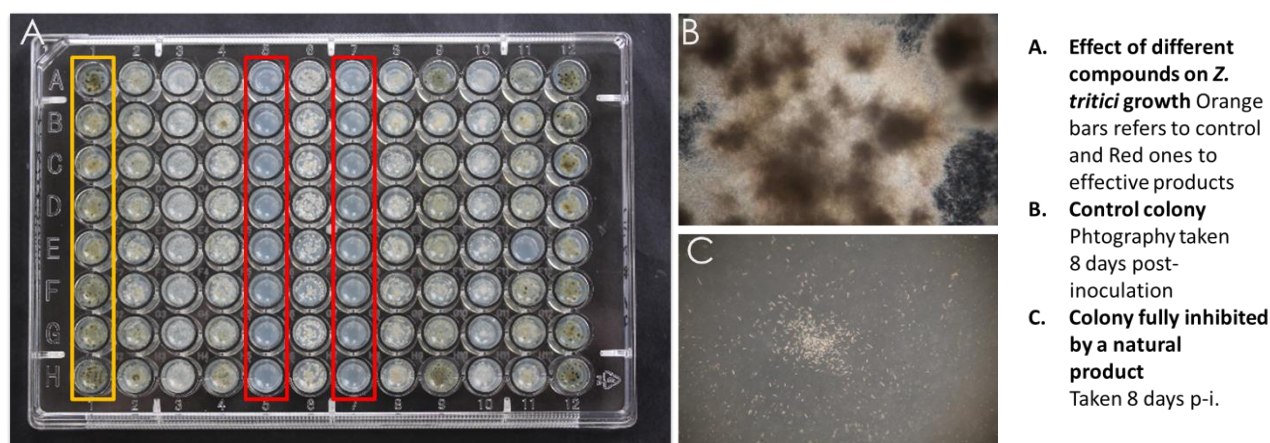


Figure supplémentaire 1 : Photographie illustrant l'évaluation *in vitro* de l'activité antifongique des extraits fongiques fournis par l'Université Catholique de Louvain en microplaque 96-puits.

Tableau supplémentaire 1 : Activité antifongique *in vitro* des 181 composés testés à une seule concentration.

Composés	Concentration	Inhibition de la croissance de <i>Z. tritici</i> <i>in vitro</i>
Extraits fongiques		
<i>Aspergillus niger</i>	30%	100%
<i>Lecanicillium longisporum</i>	30%	100%
<i>Paecilomyces lilacinus</i>	30%	100%
<i>Penicillium aurantiogriseum</i>	30%	100%
<i>Penicillium brevicompactum</i>	30%	100%
<i>Penicillium griseofulvum</i>	30%	100%
<i>Trichoderma Virens</i>	30%	100%
<i>Trichothecium roseum</i>	30%	100%
<i>Myrothecium verrucaria</i>	30%	100%
<i>Laetiporus sulphureus</i>	30%	100%
<i>Penicillium cyclopium</i>	30%	100%
<i>Chaetomium globosum</i>	30%	80%-99%
<i>Emericella rugulosa</i>	30%	80%-99%
<i>Penicillium purpurogenum</i>	30%	80%-99%
<i>Sporothrix insectorum</i>	30%	80%-99%
<i>Talaromyces flavus</i>	30%	80%-99%
<i>Aspergillus flavus</i>	30%	80%-99%
<i>Beauveria brongniartii</i>	30%	80%-99%
<i>Hypomyces chrysospermus</i>	30%	80%-99%

<i>Chaetomium globosum</i> ²	30%	80%-99%
<i>Hypomyces rosellus</i>	30%	80%-99%
<i>Penicillium funiculosum</i>	30%	80%-99%
<i>Penicillium viridicatum</i>	30%	80%-99%
<i>Dacrymyces stillatus</i>	30%	50%-79%
<i>Paecilomyces farinosus</i>	30%	50%-79%
<i>Chaetomium cochliodes</i>	30%	50%-79%
<i>Chondrostereum purpureum</i>	30%	50%-79%
<i>Fusarium proliferatum</i>	30%	50%-79%
<i>Fusarium oxysporum</i>	30%	50%-79%
<i>Metarhizium anisopliae</i>	30%	50%-79%
<i>Sordaria fimicola</i>	30%	50%-79%
<i>Trichoderma koningii</i>	30%	50%-79%
<i>Trichoderma polysporum</i>	30%	50%-79%
<i>Ulocladium atrum</i>	30%	50%-79%
<i>Candida ethanolica</i>	30%	50%-79%
<i>Colletotrichum coccodes</i>	30%	50%-79%
<i>Trichoderma pseudokoningii</i>	30%	50%-79%
<i>Trichoderma arundinaceum</i>	30%	50%-79%
<i>Acremonium alternatum</i>	30%	0%-49%
<i>Alternaria alternata</i>	30%	0%-49%
<i>Ascoidea hylecoeti</i>	30%	0%-49%
<i>Aspergillus ustus</i>	30%	0%-49%
<i>Aureobasidium pullulans</i>	30%	0%-49%
<i>Beauveria bassiana</i>	30%	0%-49%
<i>Beauveria felina</i>	30%	0%-49%
<i>Botrytis cinerea</i>	30%	0%-49%
<i>Bulleromyces albus</i>	30%	0%-49%
<i>Candida glabrata</i>	30%	0%-49%
<i>Candida melibiosica</i>	30%	0%-49%
<i>Candida parapsilosis</i>	30%	0%-49%
<i>Candida tenuis</i>	30%	0%-49%
<i>Candida butyri</i>	30%	0%-49%
<i>Candida oleophila</i>	30%	0%-49%
<i>Candida saitoana</i>	30%	0%-49%
<i>Candida sake</i>	30%	0%-49%
<i>Cephaloascus fragrans</i>	30%	0%-49%
<i>Chaetomium cupreum</i>	30%	0%-49%
<i>Chaetomium cupreum</i> ²	30%	0%-49%
<i>Cladosporium cladosporioides</i>	30%	0%-49%
<i>Cladosporium fulvum</i>	30%	0%-49%
<i>Clonostachys compactiuscula</i>	30%	0%-49%
<i>Coniothyrium concentricum</i>	30%	0%-49%
<i>Cryptococcus flavescens</i>	30%	0%-49%
<i>Dioszegia hungarica</i>	30%	0%-49%
<i>Drechslera poae</i>	30%	0%-49%
<i>Emericella nidulans</i>	30%	0%-49%
<i>Epicoccum nigrum</i>	30%	0%-49%
<i>Epicoccum purpurascens</i>	30%	0%-49%
<i>Fellomyces polyborus</i>	30%	0%-49%
<i>Filobasidium floriforme</i>	30%	0%-49%
<i>Fusarium chlamydosporum</i>	30%	0%-49%

<i>Fusarium culmorum</i>	30%	0%-49%
<i>Fusarium graminearum</i>	30%	0%-49%
<i>Galactomyces geotrichum</i>	30%	0%-49%
<i>Gliocladium catenulatum</i>	30%	0%-49%
<i>Hanseniaspora uvarum</i>	30%	0%-49%
<i>Hansfordia pulvinata</i>	30%	0%-49%
<i>Hyalodendron lignicola</i>	30%	0%-49%
<i>Hypholoma fasciculare</i>	30%	0%-49%
<i>Hypocrea pachybasioides</i>	30%	0%-49%
<i>Irpex lacteus</i>	30%	0%-49%
<i>Lecanicillium dimorphum</i>	30%	0%-49%
<i>Melanospora argentinensis</i>	30%	0%-49%
<i>Metarhizium anisopliae</i> var. <i>majus</i>	30%	0%-49%
<i>Metschnikowia fructicola</i>	30%	0%-49%
<i>Microdochium bolleyi</i>	30%	0%-49%
<i>Nectria ochroleuca</i>	30%	0%-49%
<i>Nigrospora sacchari</i>	30%	0%-49%
<i>Metschnikowia pulcherrima</i>	30%	0%-49%
<i>Paecilomyces fumorosoroseus</i>	30%	0%-49%
<i>Galactomyces geotrichum</i>	30%	0%-49%
<i>Penicillium striatisporum</i>	30%	0%-49%
<i>Pestalotiopsis disseminata</i>	30%	0%-49%
<i>Phlebiopsis spissa</i>	30%	0%-49%
<i>Phytophthora cryptogea</i>	30%	0%-49%
<i>Pleurotus ostreatus</i>	30%	0%-49%
<i>Pseudozyma fusiformata</i>	30%	0%-49%
<i>Pyricularia oryzae</i>	30%	0%-49%
<i>Pythium aphanidermatum</i>	30%	0%-49%
<i>Rhizoctonia solani</i>	30%	0%-49%
<i>Rhodospiridium paludigenum</i>	30%	0%-49%
<i>Rhodotorula glutinis</i>	30%	0%-49%
<i>Laetarsia fuciformis</i>	30%	0%-49%
<i>Rhodotorula mucilaginosa</i>	30%	0%-49%
<i>Saccharomyces cerevisiae</i>	30%	0%-49%
<i>Sclerotinia minor</i>	30%	0%-49%
<i>Trametes versicolor</i>	30%	0%-49%
<i>Trichoderma asperellum</i>	30%	0%-49%
<i>Trichoderma atroviride</i>	30%	0%-49%
<i>Trichoderma hamatum</i>	30%	0%-49%
<i>Trichoderma harzianum</i>	30%	0%-49%
<i>Trichoderma longibrachiatum</i>	30%	0%-49%
<i>Ulocladium oudemansii</i>	30%	0%-49%
<i>Verticillium chlamydosporium</i>	30%	0%-49%
<i>Verticillium lecanii</i>	30%	0%-49%
<i>Alternaria longipes</i>	30%	0%-49%
<i>Aphanocladium album</i>	30%	0%-49%
<i>Chaetomium piluliferum</i>	30%	0%-49%
<i>Cladosporium herbarum</i>	30%	0%-49%
<i>Clonostachys rosea</i>	30%	0%-49%
<i>Rhodospiridium diobovatum</i>	30%	0%-49%
<i>Cylindrocarpon destructans</i>	30%	0%-49%
<i>Heteroconium chaetospira</i>	30%	0%-49%

<i>Nomuraea rileyi</i>	30%	0%-49%
<i>Ochroconis constricta</i>	30%	0%-49%
<i>Penicillium frequentans</i>	30%	0%-49%
<i>Penicillium oxalicum</i>	30%	0%-49%
<i>Waitea circinata</i>	30%	0%-49%
<i>Tilletiopsis pallescens</i>	30%	0%-49%
<i>Trichoderma reesei</i>	30%	0%-49%
<i>Trichoderma viride</i>	30%	0%-49%
<i>Pythium ultimum</i>	30%	0%-49%
<i>Pleurotus eryngii</i>	30%	0%-49%
<i>Pleurotus pulmonarius</i>	30%	0%-49%
<i>Coprinus comatus</i>	30%	0%-49%
<i>Oudemansiella mucida</i>	30%	0%-49%
<i>Nigrospora sphaerica</i>	30%	0%-49%
<i>Acremonium sordidulum</i>	30%	0%-49%
<i>Ampelomyces quisqualis</i>	30%	0%-49%
<i>Coniothyrium minitans</i>	30%	0%-49%
<i>Moesziomyces rugulosus</i>	30%	0%-49%
<i>Pythium oligandrum</i>	30%	0%-49%
<i>Phlebiopsis gigantea</i>	30%	0%-49%
<i>Iriformospora indica</i>	30%	0%-49%
<i>Verticillium biguttatum</i>	30%	0%-49%
<i>Stachybotrys bisbyi</i>	30%	0%-49%
Extraits bactériens		
<i>Bacillus velezensis</i> S1	30%	100%
<i>Bacillus velezensis</i> S6	30%	100%
<i>Paenibacillus polymyxa</i> M2	30%	100%
<i>Paenibacillus polymyxa</i> AMESA	30%	100%
<i>Bacillus amyloliquefaciens</i> I3	30%	100%
Lipodepsipeptides cycliques purifiés		
N1	50 µg.ml ⁻¹	0%
N2	50 µg.ml ⁻¹	0%
N8	50 µg.ml ⁻¹	0%
WLIP	50 µg.ml ⁻¹	0%
Orfamide	50 µg.ml ⁻¹	0%
Putisolvin	50 µg.ml ⁻¹	0%
Lokisin	50 µg.ml ⁻¹	0%
Xantholysin	50 µg.ml ⁻¹	0%
Rhamnolipides et molécules associées		
Rhamnolipides naturels	1500 µM	0%
Rh-Eth-C4	1500 µM	0%
Rh-Eth-C6	1500 µM	0%
Rh-Eth-C8	1500 µM	0%
Rh-Eth-C10	1500 µM	100%
Rh-Eth-C12	1500 µM	100%
Rh-Eth-C14	1500 µM	0%
Rh-Eth-C16	1500 µM	0%
Rh-Eth-C18	1500 µM	0%
Rh-Est-C8	1500 µM	0%
Rh-Est-C10	1500 µM	0%
Rh-Est-C12	1500 µM	100%
Rh-Suc-C8	1500 µM	100%

Rh-Suc-C12	1500 μM	0%
Rh-Suc-C12b	1500 μM	0%
Rh-Suc-C8-C8	1500 μM	0%
Rh-Suc-C8-C12	1500 μM	0%
Rh-Suc-C12-C8	1500 μM	0%
Rh-Suc-C12-C12	1500 μM	0%
Acide laurique	1500 μM	100%
Dodécanol	1500 μM	100%
Liopeptide cyclique purifié		
Mycosubtiline	50 $\mu\text{g.ml}^{-1}$	100%

ANNEXE 2 : COMPARAISON DES ACTIVITÉS BIOLOGIQUES DES RHAMNOLIPIDES ENTRE LES PATHOSYSTÈMES BLÉ-ZYMOSEPTORIA TRITICI ET RIZ-MAGNAPORTE ORYZAE

Tableau supplémentaire 2 : Résumé des activités biologiques du mix de rhamnolipides produits par *Pseudomonas aeruginosa* et du Rh-Est-C12 observées sur les pathosystèmes riz-*Magnaporthe oryzae* et blé-*Zymoseptoria tritici*

Compound	Natural Rhamnolipids				Rh-Est-C12			
Pathosystem	Rice- <i>M. oryzae</i>		Wheat- <i>Z. tritici</i>		Rice- <i>M. oryzae</i>		Wheat- <i>Z. tritici</i>	
Concentration (μM)	50	200	50	200	50	200	50	200
<i>In vitro</i> antifungal assays								
Spore germination	+	+	0	0	0	+	0	+
Appressorium formation	+ / 0	+ / 0	Nd*	Nd*	+	+	Nd*	Nd*
Mycelium growth	+	+	Nd*	Nd*	0	+	Nd*	Nd*
Spore cell leakage	0	0	0	0	0	0	0	+
Mycelium cell leakage	0	0	Nd*	Nd*	0	+	Nd*	Nd*
<i>In planta</i> fungal spore and rhamnolipid co-application								
Spore germination	In progress	In progress	0	0	In progress	In progress	+	+
Disease severity	+	+	0	0	+	+	+	+
Callose deposition	In progress	In progress	In progress	In progress	In progress	In progress	In progress	In progress
ROS production	In progress	In progress	In progress	In progress	In progress	In progress	In progress	In progress
<i>In planta</i> rhamnolipid root application								
Disease severity	+	+	0	0	+	+	+	+

Avec (+) effet significatif observé, (0) sans effet significatif observé et (+/0) effet à vérifier. (Nd*) Les analyses indiquées n'ont pas pu être réalisées avec *Z. tritici* car ce champignon ne forme pas d'appressorium et se développe *in vitro* avec une morphologie de type levure, ne produisant pas de mycélium dans ces conditions.

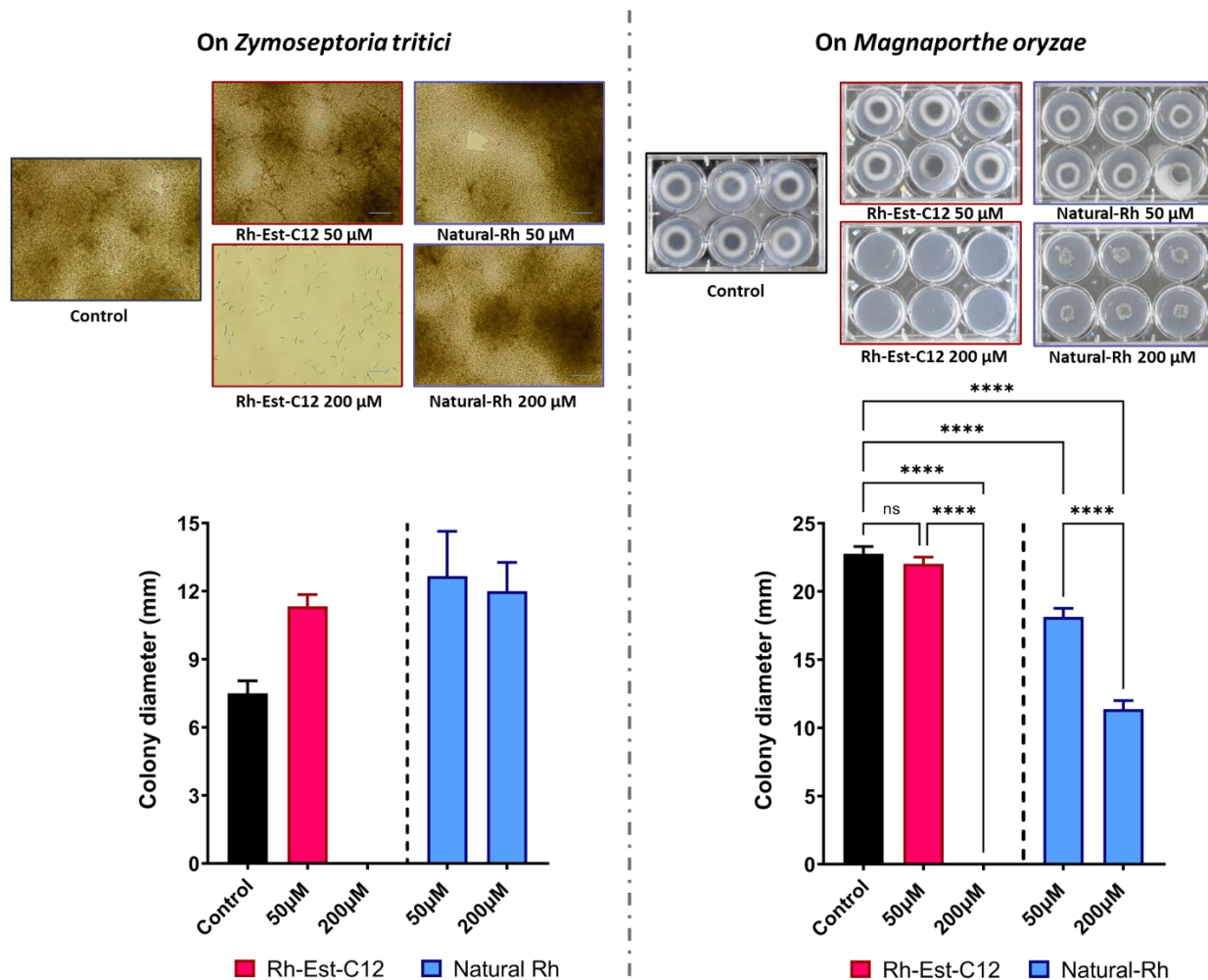


Figure supplémentaire 2 : Activité antifongique in vitro du Rh-Est-C12 et du mix de rhamnolipides produits par *Pseudomonas aeruginosa* vis-à-vis de *Z. tritici* (à gauche) et *M. oryzae* (à droite). Les photographies de *Z. tritici* ont été prises 3 jours après inoculation au microscope avec une magnification X10 et les mesures des diamètres des colonies de ce champignon ont été réalisées 10 jours après inoculation avec des spores de *Z. tritici* (5×10^5 spores.mL⁻¹). Les photographies et mesures des diamètres des colonies de *M. oryzae* ont été réalisées 5 jours après inoculation (5×10^4 spores.mL⁻¹). Les barres d'erreur des graphiques représentent les écarts-types. Les analyses statistiques ont consisté en une Anova One-way sur chaque jeu de données suivi par un test de Tukey. Les **** correspondent à des différences significatives ($P < 0.0001$).

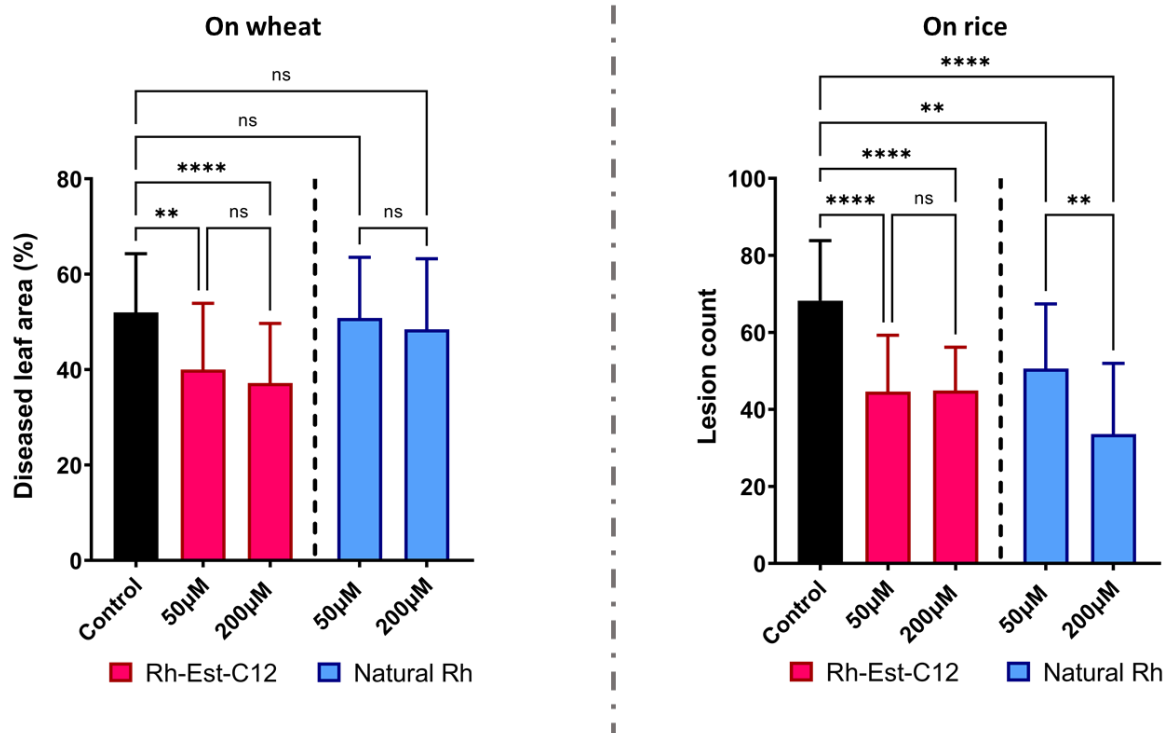


Figure supplémentaire 3 : Effet de la co-inoculation de spores de *Z. tritici* ou de *M. oryzae* avec différents rhamnolipides sur la sévérité de leur infection. L'activité in planta des composés sur blé a été déterminée 21 jours après leur co-application avec les spores fongiques (10×10^5 spores.mL⁻¹) en mesurant le pourcentage de lésions chlorotiques et nécrotiques sur les troisièmes feuilles de chaque plante. La quantité de lésions sur les feuilles de riz a été déterminée 6 jours après co-application des spores de *M. oryzae* (5×10^4 spores.mL⁻¹) avec les rhamnolipides sur les deuxièmes feuilles de chaque plante. Les barres d'erreur des graphiques représentent les écarts-types. Les analyses statistiques ont consisté en une Anova One-way sur chaque jeu de données suivi par un test de Tukey. Les ** correspondent aux différences significatives entre conditions ($P < 0.01$) et **** ($P < 0.0001$).

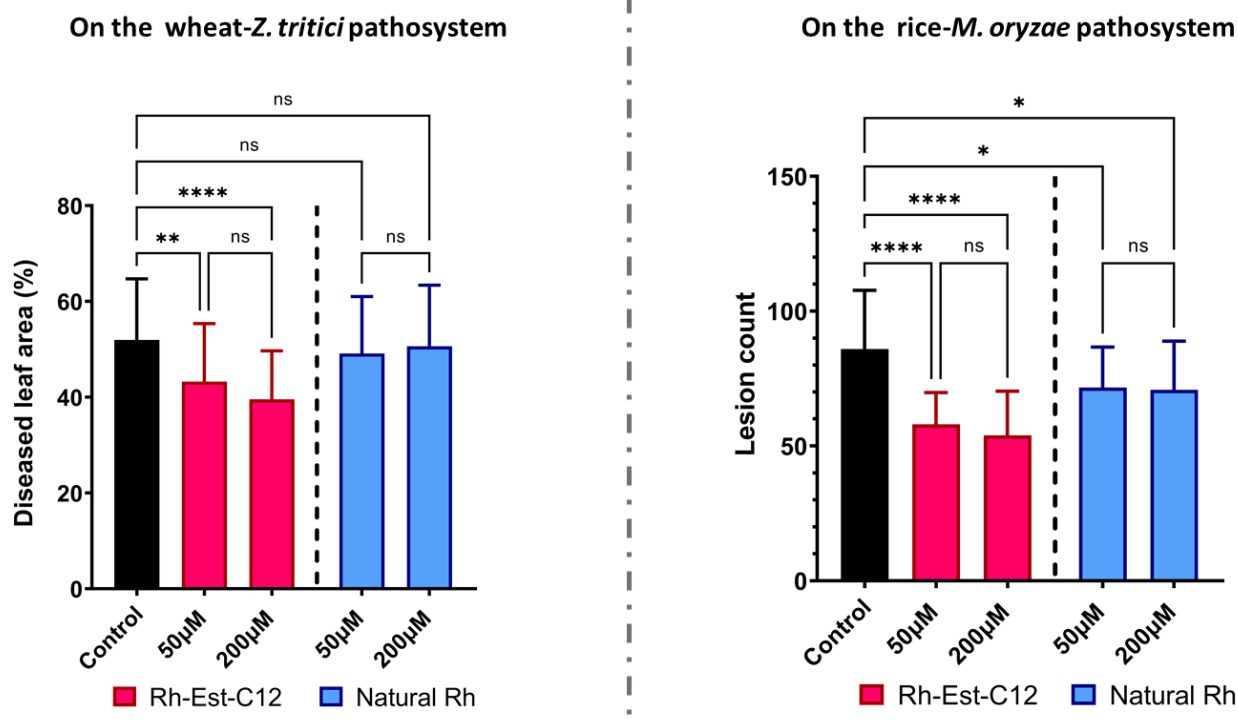


Figure supplémentaire 4 : Effet de l'application racinaire du Rh-Est-C12 et du mix de rhamnolipides produits par *Pseudomonas aeruginosa* sur la protection du blé et du riz vis-à-vis, respectivement, de *Z. tritici* (à gauche) et *M. oryzae* (à droite). Avant semis, le sol a été mélangé avec les rhamnolipides aux concentrations indiquées et les graines pré-germées de blé comme de riz ont été plongées dans une solution contenant le rhamnolipide à la concentration testée. Trois jours avant inoculation avec l'agent pathogène, les rhamnolipides ont été appliqués une nouvelle fois au niveau du sol. Le pourcentage de lésions chlorotiques et nécrotiques sur les troisièmes feuilles de chaque plant de blé a été déterminé 21 jours après infection avec les spores de *Z. tritici* (10×10^5 spores.mL⁻¹). La quantité de lésions sur les feuilles de riz a été déterminée 6 jours après infection avec les spores de *M. oryzae* (5×10^4 spores.mL⁻¹) sur les deuxièmes feuilles de chaque plante. Les barres d'erreur des graphiques représentent les écarts-types. Les analyses statistiques ont consisté en une Anova One-way sur chaque jeu de données suivi par un test de Tukey. Les * correspondent aux différences significatives entre conditions ($P < 0.05$), ** ($P < 0.05$) et **** ($P < 0.0001$).