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Lamia Jemmali

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Ingénierie des Fonctions Biologiques (ULCO)**

Sujet de thèse

**Stimulateurs des défenses naturelles du blé dur
en Tunisie et du blé tendre en France contre la
septoriose causée par *Zymoseptoria tritici***

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Dédicaces

Je dédie cette thèse

A mon cher mari Hassen

A mes Parents, Habib et Jamila

A mes frères, Slim et Zouhaier

A mes beaux parents

A mes beaux frères et sœurs

A toute ma famille

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dans ma vie....*

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Résumé

Le blé dur (*Triticum durum* Desf, BD), tout comme le blé tendre (*Triticum aestivum* L. em Thell, BT), est une céréale très touchée par la septoriose, une maladie foliaire causée par le champignon hémibiotrophe *Zymoseptoria tritici*. D'une part, ce présent travail a permis d'étudier l'interaction compatible du blé-*Z. tritici*. L'étude de l'interaction compatible chez les pathosystèmes BD/St-08-46 et BT/TO1193 a révélé l'induction des mêmes voies de défense chez les deux pathosystèmes étudiés mais avec différentes intensités. Ensuite, l'étude de l'interaction de *Z. tritici* avec un cultivar résistant de blé dur a mis en évidence l'association de résistance au champignon est liée à l'inhibition de la pénétration directe, la sporulation et l'activité des enzymes fongiques de dégradation des parois cellulaires de la plante (endo- β -1,4-xylanase, endo- β -1,3-glucanase et protéase). Ces derniers sembleraient être fortement liés à la sévérité de *Z. tritici* aussi bien chez le dur que le blé tendre. En plus, on a pu démontrer moyennant des analyses qRT-PCR l'intervention de plusieurs gènes dans la résistance du blé dur à la septoriose à savoir les gènes *PR2* (β -1,3-glucanase), *Chi 4 precursor* (précurseur de chitinase de la classe IV), *Pox* (peroxydase), *Msr* (méthionine sulfoxide réductase) et *Bsil* (inhibiteur de protéases). D'autre part, le potentiel des stimulateurs des défenses naturelles de plantes (SDPs) à protéger aussi bien le blé dur que le blé tendre contre les maladies fongiques a été évalué. Trois extraits naturels dont les matières actives sont l'acide ascorbique (AA), des oligosaccharides de parois cellulaires de plantes (Oligos) et algue brune (*Ascophyllum nodosum*, *A. nod*) ont été testés pour la première fois sur blé. Leur effet antifongique (direct) ainsi que l'effet inducteur des mécanismes de défense du blé (indirecte) ont été bien caractérisés moyennant des analyses moléculaires, biochimiques et cytologiques. En effet, seul l'AA a montré un effet direct sur la germination des spores et la croissance mycélienne du *Z. tritici* associé à l'induction des mécanismes de défense du blé. Par contre, les protections obtenues par l'Oligos et l'*A. nod* semblent être exclusivement liées à leurs propriétés inductrices de la défense qui ont permis de ralentir le développement du champignon ainsi que d'inhiber l'activités des CWDEs fongiques et la sporulation. D'ailleurs, Il s'est avéré que les SDPs testés sembleraient agir sur les mêmes mécanismes de défense chez les deux espèces de blé. Ils pourraient induire l'activation des (i) protéines PR, (ii) les enzymes antioxydants (peroxydase et catalase), (iii) les protéines PAL et LOX (enzymes clés de la voie des phénylpropanoïdes et la voie des octadécanoïdes, respectivement) et (iv) l'accumulation des H₂O₂ et le dépôt des polyphénols au niveau des sites de pénétration du champignon, ont été mis en évidence. Egalement, ils pourraient empreinter les mêmes voies utilisées par le cultivar résistant Salim en réponse à l'infection par le champignon et pourraient même induire une réponse plus importante des gènes de défense du blé dur tels que les gènes *PR2*, *Pox*, *Msr*, *ATPase* et *Bsil*. De même, deux applications (préventif et curatif) des SDPs testés a révélé une protection intéressante contre la maladie associée, dans le cas de l'*A. nod* et AA, à une augmentation de la teneur en chlorophylle et l'amélioration de la quantité et la qualité du rendement du cultivar sensible Karim, par contre pour le cultivar résistant Salim l'application des SDPs semble être inutile. En conclusion, l'application des SDPs au bon stade et avec les bonnes concentrations sur des cultivars sensibles pourraient aboutir à des résultats d'efficacité et rendement similaires à celles des cultivars résistants. Ainsi, elle pourrait remplacer l'utilisation des cultivars résistants surtout avec l'absence de cultivars complètement résistants disponibles pour l'agriculteur en Tunisie.

Mots clés : Blé dur, blé tendre, *Zymoseptoria tritici*, stimulateurs de défense des plantes, efficacité, résistance variétale.

Abstract

The durum wheat (*Triticum durum* Desf, DW) as well as the bread wheat (*Triticum aestivum* L. em Thell, BW) is strongly affected by septoria leaf blotch (STB) caused by the hemibiotrophic fungus *Zymoseptoria tritici*. First, the present work was used to study of the compatible interaction wheat-*Z. tritici*. The study of the compatible interaction among pathosystems BD/St-08-46 *Z. tritici* strain and BT/TO1193 *Z. tritici* strain revealed the induction of defense pathways in both studied pathosystèmes, but with slight differences. Then, the study of the interaction of *Z. tritici* with a resistant durum wheat cultivar showed the fungus resistance of association is related to the inhibition of the direct penetration, sporulation and the activity of the fungal enzymes degrading plant cell walls (endo- β -1,4-xylanase, endo- β -1,3-glucanase and protease). They seem to be strongly related to the severity of *Z. tritici* in both BW and DW. In addition, this study revealed the involvement of several genes in the resistance of DW against *Z. tritici* such as *PR2* genes (β -1,3-glucanase), *Chi 4 precursor* (precursor of Class IV chitinase), *Pox* (peroxidase), *Msr* (methionine sulfoxide reductase) and *Bsi1* (protease inhibitor). On the other hand, the potential of resistance inducers (RIs) to protect BW and DW against STB disease was evaluated. Three natural extracts based on ascorbic acid (AA), plant cell wall oligosaccharides (Oligos) and brown algae (*Ascophyllum nodosum*, *A. nod*) were tested for the first time on wheat. Their antifungal effect (direct) and the effect of inducing wheat defense mechanisms (indirect) have been well characterized through molecular, biochemical and cytological. We recorded that only AA exhibited a direct effect on spore germination and hyphal growth of *Z. tritici* associated to the induction of wheat defense mechanisms. However, conferred protection by Oligos and *A. nod* appears to be exclusively related to their plant defense inducing properties witch promoted the decrease of fungal CWDE activities and sporulation. Moreover, tested SDPs seem to enhance same defense pathways in both wheat species. They could induce the activation of (i) PR proteins, (ii) the antioxidant enzymes (catalase and peroxidase), (iii) the protein PAL and LOX (key enzymes of the phenylpropanoid and octadecanoid pathways, respectively) and (iv) the cytological accumulation of H₂O₂ and polyphenols, were highlighted. Also, they seem to use same pathways involved in durum wheat resistance mecanisms and may even induce a higher response of defense-related genes as *PR2*, *Pox*, *Msr*, *ATPase* and *Bsi1*. In general, protection conferred by tested RIs seems to be dependent on their composition, but it remains constant whatever of the wheat species. Similarly, in filed tested RIs conferred an interesting protection against STB associated, in the case of the *A. nod* and AA, with increased chlorophyll content and improving yield quantity and quality of the susceptible cultivar Karim, while in the resistant cultivar Salim the application of RIs seems to be useless. In conclusion, protection conferred by tested RIs seems to be dependent on their composition, but it remains constant whatever of the wheat species. The use of RIs may improve the resistance level and yield of susceptible cultivars in order to obtain similar results to the resistant cultivars. Thus, it could replace the use of resistant cultivars especially with the lack of completely resistant cultivars available to farmers in Tunisia.

Key words: Durum wheat, bread wheat, *Zymoseptoria tritici*, resistance inducers, efficiency, cultivar resistance.

Abréviations

AA	Produit à base d'acide ascorbique
ABA	Acide Abscissique
ABC	ATP-Binding Cassette
ACT	Actine du blé
AFLP	<i>Amplified Fragment Length Polymorphism</i>
<i>A. nod</i>	Produit à base d' <i>Aschophyllum nodosum</i>
ANOVA	Analyse de la Variance
AOS	Allène Oxide Synthase
BA2H	<i>Benzoïc Acid 2 Hydroxylase</i>
BABA	<i>β-aminobutyric acid</i>
BW	Blé tendre (<i>Bread Wheat</i>)
BTH	Acibenzolar-S-methyl-benzothiadiazole
CAT	Catalase
CE	<i>Cathechin Equivalent</i>
CWDE	Enzymes de dégradation des parois (<i>Cell Wall Degrading Enzyme</i>)
DAB	3-3'Diaminobenzidine
DAMP	<i>Dammage Associated Motif Pattern</i>
DP	Penetration directe (<i>Direct Penetration</i>)
DW	Blé dur (<i>Durum Wheat</i>)
ETD	<i>Effector-Triggered Defense</i>
ETI	<i>Effector-Triggered Immunity</i>
ETS	<i>Effector-Triggered Trigger Susceptibility</i>
H ₂ O ₂	Peroxyde d'hydrogène
HR	Réaction d'hypersensibilité
HSA	heptanoïl d'acide salicylique (<i>Heptanoic Salicylic Acid</i>)
JA	Acide Jasmonique (<i>Jasmonic Acid</i>)
LOX	Lipoxygénase
MAMP	Motifs moléculaires associés aux microbes (<i>Microbe--Associated Molecular Pattern</i>)
MFS	<i>Major facilator superfamily</i>
Oligos	Produit à base d'oligosaccharides de paroi
OGA	Oligogalacturonides
OXO	Oxalate oxydase
PAL	Phénylalanine Ammonia Lyase
PAMP	motifs moléculaires associés aux pathogènes (<i>Pathogen-Associated Molecular Pattern</i>)

PC	Composés phénoliques totaux (<i>Polyphenolic Compounds</i>)
PCD	Mort cellulaire programmée (<i>Programmed Cell Death</i>)
PCR	<i>Polymerase Chain Reaction</i>
PDA	<i>Potato Dextrose Agar</i>
POX	Peroxydase
PR	<i>Pathogen Related</i>
PTD	<i>PAMP-Triggered Defense</i>
PTI	<i>PAMP-Triggered Immunity</i>
QTL	Locus de caractères quantitatifs (<i>Quantitative Trait Loci</i>)
REST	<i>Relative Expression Software Tool</i>
RFLP	<i>Restriction Fragment Length Polymorphism</i>
RI	Inducteur de résistance (<i>Resistance Inducer</i>)
ROS	Formes actives de l'oxygène (<i>Reactive Oxygen Species</i>)
RT	<i>Reverse Transcription</i>
SA	Acide Salicylique (<i>Salicylic Acid</i>)
SAR	réponse systémique acquise (<i>Systemic Acquired Resistance</i>)
SDP	Stimulateur de Défense des Plantes
STB	Septoriose du blé (<i>Septoria Tritici Blotch</i>)
TUB	Tubuline du blé

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Chapitre 5

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Fig. 1 Effect of tested treatments on spore germination of St-08-46 *Z. tritici* strain on Karim (S), Nasr (MR) and Salim (R). Three RIs were tested including Oligos, AA and *A. nod* were tested at greenhouse condition using respective concentrations, 50, 50 and 500 mg L⁻¹. Treated leaves were compared to the reference H₂O-treated (i) controls. The infection of three tested cultivars by St-08-46 *Z. tritici* strain was monitored at 2 days post treatment. The *Z. tritici* spore germination was investigated at 1 day post infection (dpi) using microscopic observation with Calcofluor fluorescence. Data represent means of two independent experiments with three independent replicates (\pm s.d). Means tagged with the same letter are not significantly different using the Tukey test at $P \leq 0.05$ 199

Fig. 2 Effect of tested treatments on leaf penetration of St-08-46 *Z. tritici* strain on Karim (S), Nasr (MR) and Salim (R). Three treatments were tested including Oligos, AA and *A. nod* were tested at greenhouse condition using respective concentrations, 50, 50 and 500 mg L⁻¹. Treated leaves were compared to the reference H₂O-treated (i) control. The infection of three tested cultivars by St-08-46 *Z. tritici* strain was monitored at 2 days post treatment. The *Z. tritici* leaf penetration was investigated at 4 day post infection (dpi) using microscopic observation with Trypan blue staining. NGS: not germinated spores; DP: spores leading to a direct penetration; SP: stomatal penetration; GSNP: germinated spores that did not penetrate. Data represent means of two independent experiments with three independent replicates (\pm s.d). Means tagged with the same letter are not significantly different using the Tukey test at $P \leq 0.05$ 200

Fig. 3 Effect of tested treatments on colonization and pycnidia formation of St-08-46 *Z. tritici* strain on Karim (S), Nasr (MR) and Salim (R). Three treatments as Oligos, AA and *A. nod* were tested at greenhouse condition using respective concentrations, 50, 50 and 500 mg L⁻¹. Treated leaves were compared to the reference H₂O-treated control. The infection of three tested cultivars by St-08-46 *Z. tritici* strain was monitored at 2 days post treatment. The *Z. tritici* colonization and fructification was investigated at 22 day post infection (dpi) using microscopic observation with Calcofluor fluorescence. NCS: non-colonized stomata; CS: colonized stomata but not yet transformed into pycnidia; P: colonized stomata transformed into pycnidia. Data represent means of two independent experiments with three independent replicates (\pm s.d). Means tagged with the same letter are not significantly different using the Tukey test at $P \leq 0.05$ 201

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Fig. 5 Effect of tested treatments on CWDE activities of St-08-46 strain on Karim (S), Nasr (MR) and Salim (R) at greenhouse condition. CWDE activities were measured at 4, 10, 16 and 22 days post inoculation (DPI). three *Z. tritici* CWDEs were investigated such as endo- β -1,4-xylanase (A), endo- β -1,3-glucanase (B) and protease (C) activities. Three treatments were tested including Oligos, AA and *A. nod* were used at respective concentrations, 50, 50 and 500 mg L⁻¹. The infection of three tested cv. by St-08-46 *Z. tritici* strain was monitored at 2 days post treatment. Data represent means of two independent experiments (\pm s.d). Means tagged with the same letter are not significantly different using the Tukey test at $P \leq 0.05$ 205

Fig. 6 Quantitative expression of PR genes analyzed by real time RT-PCR analysis at 1, 2 and 3 days post treatment by Oligos, AA and *A. nod* treatments on non infected (ni) Karim (susceptible) and Salim (resistant) compared to infected (i) Karim and Salim by St-08-46 *Z. tritici* strain. Oligos, AA and *A. nod* were used at respective concentrations, 50, 50 and 500 mg L⁻¹. Three genes were investigated such as *PR2* (A, B, C and D), *Chi 4 precursor* (E, F, G and H) and *Pox* (I, J, K and L)-encoding genes. Means of relative expression at each time point are indicated above the bars in each graph. The experiment was conducted in triplicate. Similar results were obtained at least twice. Results correspond to means (\pm s.d) of duplicate reactions..... 206

Fig. 7 Wheat defense genes expression analyzed by semi-quantitative RT-PCR analysis at 1, 2, 3, 4 and 7 days post treatment (DPT) by AA, Oligos and *A. nod* treatments on Karim (S) and Salim (R). Oligos, AA and *A. nod* were used at respective concentrations, 50, 50 and 500 mg L⁻¹. Three genes were investigated such as *Msr* (A), *ATPase* (B) and *Bsil* (C) -encoding genes. For each condition, treated leaves were compared to their reference H₂O-treated leaves. RT-PCR products were generated using primers specific for β -tubulin (*TUB*), *Msr*, *ATPase* and *Bsil* first-strand cDNAs obtained from total wheat RNA, and visualized on agarose gel (1.5%). *TUB*-encoding gene was used as a house-keeping gene. Experiment was repeated three times and only results which were similar at least twice were considered..... 208

Avant propos

Les travaux présentés dans ce manuscrit ont été réalisés au sein de trois laboratoires : le laboratoire d'amélioration génétique des céréales à l'Institut National Agronomique de Tunisie (Tunis, Tunisie), l'Unité de Chimie Environnementale et Interactions sur le Vivant (UCEIV) à l'Université du Littoral Côte d'Opale (Calais, France) et le laboratoire de Biotechnologie et Gestion des Agents Pathogènes en Agriculture (BioGAP) à l'Institut Supérieur d'Agriculture à Lille (France).

Dans cette étude, nous proposons de tester l'efficacité de quatre extraits biologiques testés pour la première fois dans le cadre du pathosystème blé-*Z. tritici* à savoir un produit à base de l'acide ascorbique (AA), un extrait de l'algue brune *Aschophyllum nodosum* (*A.nod*), un produit à base de d'oligosaccharides de paroi de plantes concentrés (Oligos) et le chitosan. À cet effet, des analyses biologiques, microscopiques, biochimiques et moléculaires ont été réalisées aussi bien en condition contrôlées qu'en plein champs. Les expérimentations ont été achevées avec les quatre produits simultanément.

Toutefois, dans la mesure où la stratégie de valorisation des travaux de thèse reste à définir produit par produit, il a été choisi de présenter les résultats obtenus pour chaque extrait de façon séparée, sous forme d'articles indépendants.

Introduction générale et objectifs

Le blé est un terme générique qui désigne plusieurs céréales appartenant au genre *Triticum*. Ce sont des plantes annuelles de la famille des graminées, cultivées dans de très nombreux pays. Le blé fait partie des trois grandes céréales avec le maïs et le riz. Deux espèces de blé ont une importance économique réelle à l'heure actuelle. Le blé dur (*Triticum turgidum* ssp *durum*) est à la base de la fabrication des semoules ainsi que des pâtes alimentaires. Alors que le blé tendre (*Triticum aestivum*) est cultivé pour faire la farine panifiable utilisée pour le pain. Selon un rapport diffusé par la FAO en 2012, l'agriculture mondiale doit relever plusieurs défis : augmenter la production alimentaire de 70% pour nourrir 2,3 milliards de personnes de plus d'ici 2050, intensifier la lutte contre la pauvreté et la faim, utiliser plus efficacement les ressources naturelles qui s'amenuisent et s'adapter au changement climatique. La production céréalière annuelle devra s'accroître de près d'un milliard de tonnes pour répondre à la demande pour l'alimentation humaine et animale (FAO, <http://fao.org/news/story/fr>). Néanmoins, la production du blé peut être limitée par des contraintes biotiques et abiotiques.

Parmi les facteurs biotiques les plus redoutables au blé, la septoriose causée par *Z. tritici* constitue la maladie foliaire la plus importante sur cette culture en Tunisie (Harrabi et Cherif, 1990). Les pertes de rendement peuvent atteindre 40% durant les années pluvieuses en particulier lorsque les pluies de printemps persistent après l'émergence de la feuille drapeau (Eyal, 1999). L'incidence élevée de la septoriose observée récemment, résulte en particulier de l'intensification de la culture des céréales. L'utilisation croissante de fertilisants, l'augmentation de la densité du semis, l'interligne étroite, le semis précoce et l'utilisation de variétés semi-naines ont abouti à la variation du microclimat de la culture qui est devenu favorable au développement de la septoriose (Camacho-Casas *et al.*, 1995).

Pour pouvoir maîtriser la maladie, l'utilisation des fongicides chimiques est le moyen le plus connu. En fait, nul ne peut nier que les pesticides, au sens large du terme, ont largement contribué à optimiser les ressources agricoles et ont résolu le problème de maintes attaques de pathogènes. Mais, il n'en reste pas moins vrai que de nombreux problèmes sensibilisent actuellement le secteur agricole. Ainsi, l'accumulation de résidus toxiques dans les différents organismes de la chaîne alimentaire et la résistance accrue de nombreux agents pathogènes aux doses couramment employées sont autant de difficultés qui ont favorisé l'émergence de nouvelles stratégies ayant pour objectifs principaux la fiabilité, l'efficacité et le respect de l'environnement. Parmi les approches offrant de grandes promesses d'avenir, il paraît clair que de citer la lutte biologique. Les percées

récentes en biologie moléculaire et en transformation des végétaux ont démontré que sensibiliser une plante à répondre plus rapidement à l'infection pouvait lui conférer une protection accrue contre des microorganismes pathogènes. Dans ce contexte, une avenue potentiellement attrayante au cœur des préoccupations et des réflexions des chercheurs est la résistance induite basée sur l'utilisation des stimulateurs des défenses de la plante (SDPs). L'application des SDPs constitue donc une alternative potentielle pour protéger les cultures contre les agents pathogènes (Benhamou et Picard, 2000). Il s'agit de produits contenant des matières actives d'origine naturelle ou de synthèse destinés à induire les mécanismes de défense naturelles de la plante. Ce type de produits, à activité indirecte vis à vis du pathogène, ne semble pas présenter de risque ni de toxicité pour l'environnement ni d'apparition des souches résistantes du champignon.

Les travaux effectués durant cette thèse ont pour objectif principal d'évaluer l'efficacité de nouveaux SDP à protéger le blé contre la septoriose, d'analyser leur mode d'action sur deux espèces de blé, à savoir le blé tendre (*Triticum aestivum*) et le blé dur (*Triticum turgidum* ssp *durum*) ainsi que leur interaction avec des cultivars présentant des niveaux de résistance différents à la maladie. Tout ceci vise à déterminer les couples SDP-cultivar les plus adaptés pour lutter efficacement au champ contre la septoriose. L'ensemble des travaux sont synthétisés dans ce manuscrit qui est organisé en 5 chapitres :

- **Chapitre 1** : il constitue une synthèse bibliographique comportant une présentation du pathosystème blé-septoriose, l'étude de la réponse du blé à la septoriose ainsi qu'une brève partie présentant les SDPs et leur utilisation contre les maladies du blé.

- **Chapitre 2** : dans cette partie, on expose un article concernant l'étude comparée de l'interaction compatible de *Z. tritici* vis-à-vis du blé dur et du blé tendre.

- **Chapitre 3** : il est consacré à l'étude du mode d'action et l'efficacité de quatre produits à savoir un produit à base de l'acide ascorbique (AA), un extrait pur d'algue brune *A. nodosum* (*A.nod*), un produit à base d'oligosaccharides de paroi de plantes concentrés (Oligos) et le chitosan, traité chacun dans un article indépendant. Les effets directs (antifongique) et indirects (stimulateur de défense de la plante) de chaque produit vont être discutés séparément dans chaque article. En plus, la réponse du blé tendre et du blé dur vis-à-vis des produits testés sera présentée.

- **Chapitre 4** : les différents mécanismes de résistance du blé dur vis-à-vis de la septoriose sont présentés sous forme d'un article séparé. Ici, le processus infectieux de *Z. tritici* ainsi que l'expression des gènes impliqués dans la défense de la plante en réponse à l'infection sont étudiés chez trois cultivars de blé dur à différents niveaux de résistance à la septoriose.

- **Chapitre 5:** il s'agit d'un article qui se focalise sur l'étude de l'interaction des SDP testés et trois cultivars tunisiens de blé dur en vue de contrôler la septoriose, en vue de mettre en évidence l'effet du génotype de blé sur l'efficacité des SDPs à contrôler la maladie, l'induction des gènes de défense du blé dur et l'amélioration de la teneur en chlorophylle et du rendement de blé dur.

Ensuite, on terminera par une conclusion générale où les résultats trouvés seraient combinés en vue de mieux comprendre l'interaction de la septoriose avec (i) différentes espèces de la même espèce de blé et (ii) différents cultivars de blé dur ayant différents niveaux de résistance à la maladie. Dans un deuxième temps, l'étude de l'efficacité et le mode d'action des produits testés et leur interaction avec les différents génotypes de blé sera abordée.

Chap I | *Bibliographie générale*

I. Pathosystème blé – *Z. tritici*

I. 1. Le blé

I. 1.1. Classification, historique et généalogie

Le blé est une plante céréalière monocotylédone, appartenant au règne des *Plantae*, sous règne des *Tracheobionta*, à la classe des *Liliopsida*, à la famille des *Paecae*, à la tribu des *Triticeae*, au genre des *Triticum*. Ce genre est divisé par Mackey (1966) en cinq espèces dont les plus importantes sont *T. aestivum* et *T. turgidum* L. subsp. *durum* (Desf.) Husn (Doussinaut et al., 2004). Ces différentes espèces de blé ont été générées par des événements successifs de polypléidisation intervenant après des croisements interspécifiques entre trois espèces ancestrales diploïdes (Chantret et al., 2005). Le premier événement, impliquant *T. monococcum* et *Aegilops speltoides*, a eu lieu il y a environ 500 000 ans et a conduit à l'apparition du blé dur tétraploïde : *T. dicoccocum* ($2n=28$). Le deuxième événement de polypléidisation a eu lieu au cours de la domestication, il y a environ 9000-12000 ans, entre le blé dur cultivé (tétraploïde) et un autre blé diploïde (*A. tauschii*) et a donné le blé tendre hexaploïde ($2n=42$) (Chantret et al., 2005). Le blé dur, *T. durum*, appelé ainsi en raison de la dureté de son grain, est issu du croisement entre deux espèces ancestrales ($2n=14$) avec deux génomes A et B provenant d'espèces diploïdes différentes (*T. turgidum* et *T. dicoccocum*)(Fig. 1).

I.1.2. Développement du blé

L'échelle de croissance du blé a été décrite par Zadoks (1974). Il est composée de 10 principaux stades de croissance qui correspondent au premier chiffre, divisés en stades de croissance secondaires qui constituent le deuxième chiffre (de 1 à 99) (Zadoks et al., 1974) (Fig. 2). Selon Gate (1995) le cycle de développement du blé peut être divisé en six phases principales (Fig. 2): (i) *Levée*: elle se déroule à l'automne, à partir du semis jusqu'au stade 2-3 feuilles. (ii) *Tallage*: il se déroule principalement de la fin de l'automne à la fin de l'hiver. La première talle (= ramification) apparaît à l'aisselle du maître-brin quand celui-ci possède 3 à 4 feuilles. (iii) *Montaison*: où la tige principale commence à s'allonger ce qui correspond au stade épi 1 cm (fin Mars). (iv) *Epiaison* : elle correspond à la sortie de l'épi hors de la gaine de la dernière feuille (feuille drapeau). (v) *Floraison* : elle indique le moment où les étamines sont visibles en dehors des glumelles. (vi) *Remplissage* : il correspond à l'étape ultime du cycle de développement où la graine se forme. A ce stade, les grains accumuleront des réserves en amidon jusqu'à maturité de l'épi. La couleur jaune doré de l'épi indique sa maturité et la période propice à la récolte (Fig. 2).

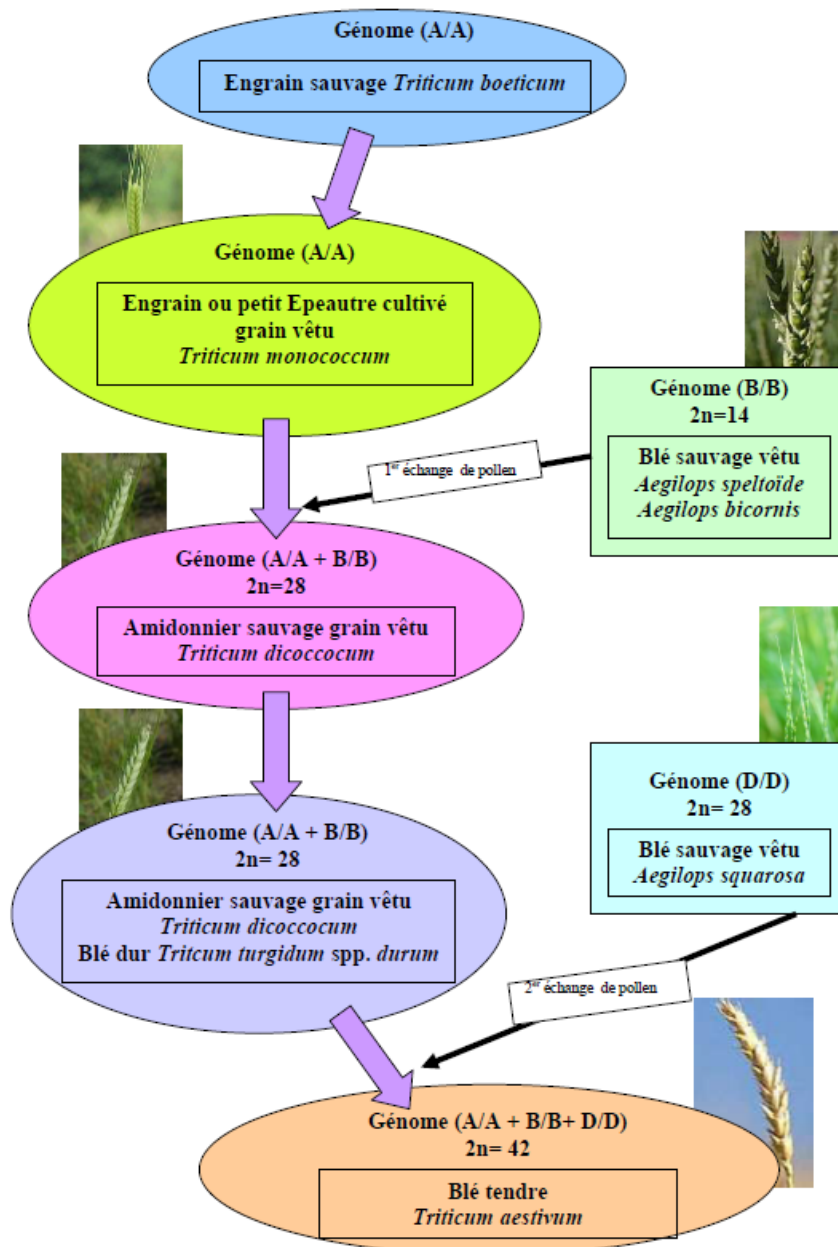


Fig. 1. Représentation schématique de l'évolution des espèces de blé (Chantret *et al.*, 2005).

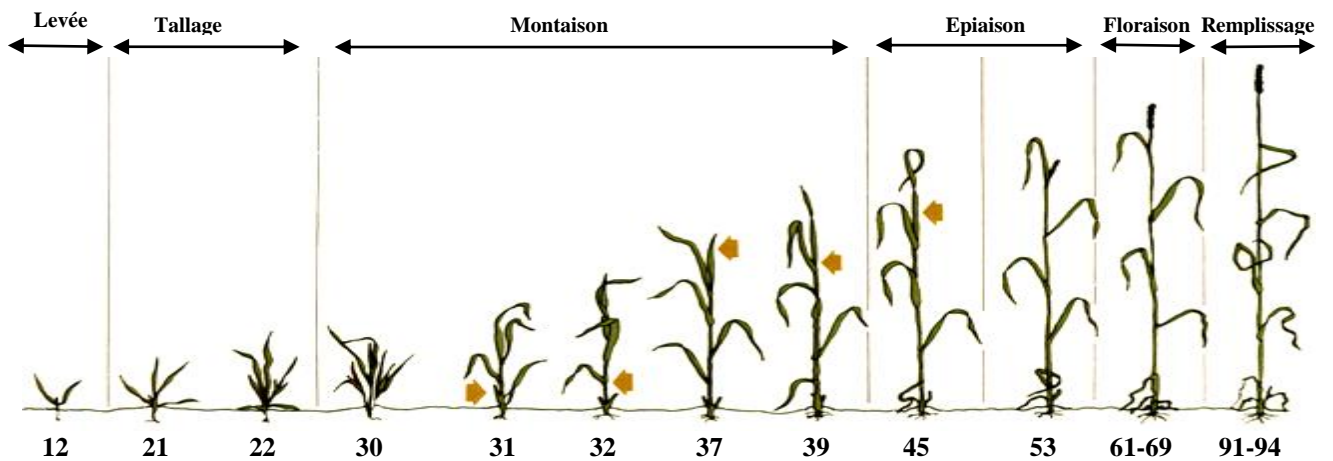


Fig. 2. Les principaux stades de développement des céréales selon l'échelle de Zadoks (Zadoks, 1974, Gate, 1995).

1.1.3. Importance économique du blé

La production céréalière mondiale et particulièrement celle du blé a régulièrement augmenté depuis un demi-siècle, pour atteindre 701.5 millions de tonnes (Mt) en 2011/2012, soit plus de 15 % de la surface agricole mondiale et trois fois plus que son niveau de 1960. Actuellement, la consommation mondiale de blé par habitant est d'environ 67 kg/an, et représente environ 70% de l'utilisation mondiale. Cette consommation est plus élevée pour les pays en voie de développement telle que la Tunisie (258 kg/habitant/an) (FAO; <http://www.fao.org>; ITES, 2014).

Pour la saison 2012/13, les superficies totales utilisées pour la production de blé et d'orge en Tunisie sont estimées à 1.13 million d'hectares (Mha), dont la majorité (47%) est consacré à la culture du blé dur. Or, la production céréalière totale en Tunisie est estimée en 2013 à 1.5 Mt. Les productions nationales n'ont pas permis la satisfaction des besoins domestiques. En effet, le blé dur, qui représente 70 % de la production des céréales, satisfait, en moyenne, 72 % de la demande nationale. Les importations des céréales ont donc constitué la solution pour combler les déficits enregistrés. Pour la saison 2012/13, les importations de blé sont estimées à 1.71 Mt (ITES, 2014).

Par contre, la situation en France est largement différente. En 2012/2013, environ 40 Mt de la production mondiale sont issues de l'agriculture française, ce qui fait de la France le 4^{ème} producteur mondial. En effet, le blé constitue la première ressource exportée de France, présentant ainsi une ressource agricole essentielle pour l'économie du pays. La superficie consacrée pour la culture des céréales en France est environ 9,4 Mha en 2012/2013, soit 15% du territoire français et 50% de la totalité des exploitations agricoles françaises. Environ 4.86 Mha (52%) sont consacrés à la production du blé tendre qui assure la majeure partie (52%) de la production des céréales (35.5 Mt), dont 45% est destinée à l'exportation (FAO ; <http://www.fao.org>).

Le potentiel de rendement est affecté par différents stress abiotiques et biotiques. Ces derniers peuvent être liés à des épidémies de maladies d'origines fongiques, bactériennes ou virales entre autres. La septoriose constitue la maladie foliaire la plus importante du blé. Elle peut être provoquée principalement par deux champignons : *Zymoseptoria tritici* (téléomorphe *Mycosphaerella graminicola*) et *Stagonospora nodorum* (téléomorphe *Phaeosphaeria nodorum*) (Eyal, 1999). *P. nodorum* infecte toutes les parties aériennes de la plante alors que *Z. tritici* se limite à s'attaquer aux feuilles. En France, *S. nodorum* est plutôt fréquent dans les zones du Sud, tandis que *Z. tritici* est présent sur l'ensemble du territoire. Par contre, en Tunisie *Z. tritici* est dominant. Les pertes de rendement, en conditions favorables de climat tempéré et humide, peuvent atteindre 50% en absence de traitement (Shaw *et al.*, 2008). Par conséquent, cette étude s'intéresse à la septoriose causée par *Z. tritici* sur blé tendre français et blé dur tunisien.

I. 2. *Zymoseptoria tritici* (téléomorphe *Mycosphaerella graminicola*)

I. 2.1. Taxonomie et biologie

Il s'agit d'un champignon ascomycètes caractérisé par un cycle de vie incluant une forme sexuée (Téломorphe) et une forme asexuée (Anamorphe). La première est appelée *Mycosphaerella graminicola*, mise en évidence par Seterson en 1976 en Nouvelle Zélande. Il appartient à la classe des Loculoascomycètes, ordre des Dothidéales (Goodwin *et al.*, 2004) a été récemment revu. L'analyse phylogénétique de plusieurs gènes montre que le genre *Mycosphaerella* appartient désormais à l'ordre des Capnodiales, (Schoch, *et al.*, 2006, Torriani *et al.*, 2008). Il présente le genre le plus large chez les Dothideales. Il s'agit d'un ordre extrêmement large et divers de champignons avec plus que 1000 espèces (Farr *et al.*, 1995). Hoorme *et al.* (2002) rapportent que les pseudothèces sont bruns sombres, la plupart du temps circulaires, légèrement immergés dans la gaine de la feuille, ayant une taille de 70 à 100 μm (Fig. 3A). Les asques mesurent 42 μm de long et chaque asque contient 8 ascospores. Ces dernières ont deux cellules inégales irrégulières et de taille variable (Fig. 3B). La deuxième a été désignée par *Septoria tritici*. Cette forme est décrite pour la première fois en 1842 par Desmazière (Halama, 1996). Récemment, les espèces du genre *Septoria* attaquant les céréales ont été regroupées sous le genre *Zymoseptoria* (Quaedvlieg *et al.*, 2011). L'espèce *Zymoseptoria tritici* appartient à la classe des Deutéromycètes, ordre des Sphaeropsidales, famille des Sphaerioidacées (Nasraoui, 2006). Elle est caractérisée par des pycnides de forme globuleuses, de couleur qui devient de plus en plus foncée suivant leur stade de maturité, et encastrés dans les tissus de l'épiderme et du mésophile (Fig. 3C). Ils libèrent les pycnidiospores. Ces dernières sont filiformes, enfermées dans des pycnides ayant une taille de 60 à 200 μm (Fig. 3D) (Eyal, 1999 ; Hoorme *et al.*, 2002). *Z. tritici* est un champignon hétérothalique, présentée sous deux Mating types, ou types sexués, contrôlés par deux différents allèles (idiomorphes) au niveau d'un seul locus (Kema *et al.*, 1996). La population française de *Z. tritici* présente un potentiel élevé de reproduction sexuée (Siah *et al.*, 2010a). En Tunisie, la coexistence des deux types sexués a été récemment détectée sur blé dur et blé tendre (Aouini, 2010 ; Ben Hassin et Hamada, 2014). De même, l'étude réalisée par Boukef *et al.* (2008), et qui a porté sur la distribution des deux types sexués de *Z. tritici* dans 5 régions de la Tunisie a montré qu'ils sont en équilibre panmictique.

I.2.2. Le cycle de développement et épidémiologie

L'origine d'une épidémie de *Z. tritici* se situe principalement en automne, lors de la levée du blé (Fig. 3E). Les contaminations primaires des jeunes plantules s'effectuent par les pycnidiospores qui sont issues de la multiplication asexuée au sein des pycnides, et qui sont excrétés par les ostioles des stomates dans un cirrhe. La dissémination ascendante de ces spores aux étages foliaires supérieurs est rendue possible par les éclaboussures de pluie.

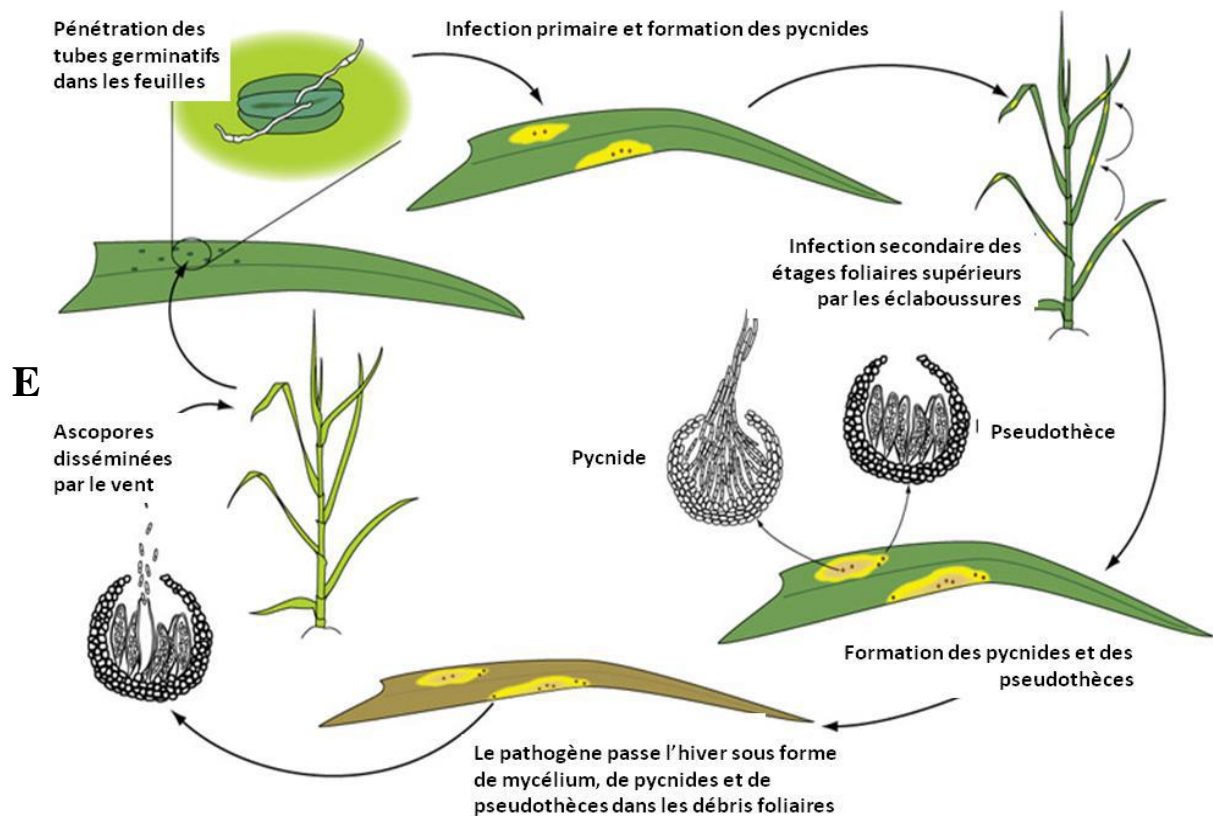
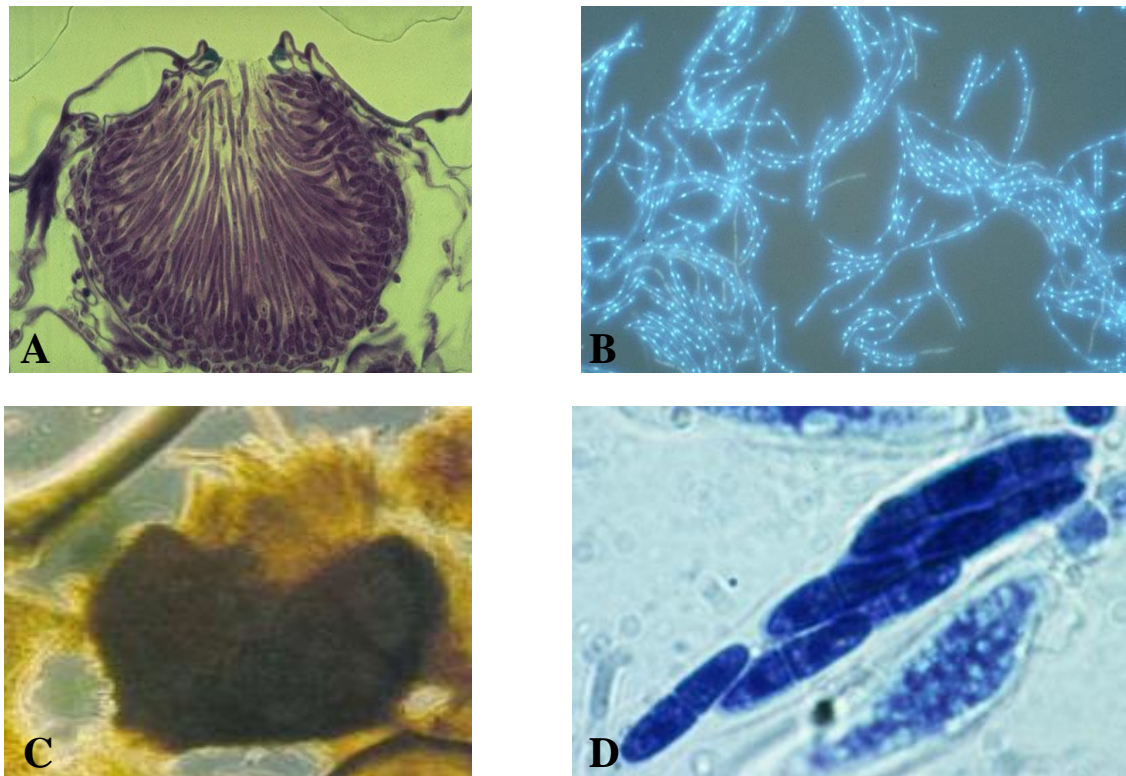


Fig. 3. Biologie et cycle de développement de *Z. tritici*. A : Structure d'un pycnide de *Zymoseptoria tritici* (Kema *et al.*, 1996a). B : : pycnidiospores de *S. tritici* (Kema *et al.*, 1996). C : Pseudothèque de *Z. tritici* (Eyal *et al.*, 1987). Détail d'un asque contenant les ascospores (Halama 1996). E : cycle de développement de *Z. tritici* présentant les deux phases : sexuée et asexuée (adapté de Ponomarenko *et al.*, 2011).

La contamination primaire peut aussi être assurée par les ascospores issues de la reproduction sexuée qui a lieu dans les pseudothèces, et disséminées par le vent sur de plus longues distances (Suffert et Sache, 2011; Suffert *et al.*, 2011). Cet inoculum primaire pourrait parvenir soit des résidus de cultures, soit de repousses ou d'adventices ou encore des parcelles de blé voisines (Eyal *et al.*, 1987 ; Suffert et Sache, 2011; Suffert *et al.*, 2011) (Fig. 3E). Par la suite, une contamination secondaire de la maladie prend place au printemps où les conditions d'hygrométrie et de température sont favorables au développement du parasite. La progression de la maladie s'effectue suivant deux types de gradients : un gradient vertical et un gradient horizontal. Le gradient vertical correspond à la dispersion des pycnidiospores à partir des étages foliaires inférieurs vers les étages foliaires supérieurs au cours des stades de tallage et de montaison du blé. Cette dispersion est assurée par les éclaboussures de pluie, entraînant avec elles des fractions du cirrhe contenant les pycnidiospores (Halama, 1996 ; Bannon et Cooke, 1998; Eyal, 1999). Le gradient horizontal, quant à lui, repose sur la dispersion des pycnidiospores de plante à plante. Cette dissémination est accomplie grâce aux phénomènes de ruissellement et de frottement des feuilles des étages supérieurs les unes contre les autres (Eyal, 1999) (Fig. 3E).

1.2.3. Processus d'infection et symptômes

L'espèce *Z. tritici* est un pathogène dimorphique. La transition de la forme sporale à la forme filamenteuse est alors importante pour l'initiation de l'infection. Après l'adhésion, sur une feuille de blé, les spores sécrètent une matrice extracellulaire afin de maintenir leur attachement à la surface foliaire jusqu'à la germination et la pénétration de l'hôte (Duncan et Howard, 2000). Des conditions d'hygrométrie suffisantes ainsi que des températures modérées, la germination des spores peut être initiée deux heures seulement après la contamination, par bourgeonnement d'un tube germinatif à partir des cellules apicales ou intermédiaires. La croissance des tubes germinatifs sur la surface foliaire peut donner naissance à des ramifications (Kema *et al.*, 1996; Shetty *et al.*, 2003). L'infection des tissus est initiée par la pénétration des tubes germinatifs dans les tissus de la plante. Cet événement a lieu rapidement habituellement dans les 24 - 48 h post inoculation (Fig. 4A). Le mode de pénétration de l'hôte par *Z. tritici* a été très discuté dans la littérature et a fait l'objet de controverses. Tout d'abord, seules des pénétrations directes à travers la cuticule de la feuille ont été observées. Par la suite, Kema *et al.* (1996) et Duncan et Howard (2000) ont rapporté que la pénétration foliaire se fait presque exclusivement à travers les stomates (Fig. 4A). Cependant, Shetty *et al.* (2003) ont observé des tentatives de pénétration directe. Désormais, les deux modes de pénétration, directe et stomatique, sont recensés dans la littérature, et les pénétrations directes peuvent s'effectuer au niveau des parois périclinales ou anticlinales des cellules de l'épiderme (Fig. 4B) (Siah *et al.*, 2010b ; El Chartouni *et al.*, 2012). Contrairement aux autres modèles de

champignon, tels que *Magnaporthe grisea* (Dean, 2005), *Z. tritici* ne présente pas de vrais appressoria mais pénètre plutôt par les stomates sans différentier des structures d'infection spécifiques (Goodwin *et al.*, 2011). Cependant, Rohel *et al.* (2001) ont observé des structures ressemblant à des appressoria, formant un renflement aux extrémités des tubes germinatifs, appelées « appressorium-like », au niveau des points de pénétration foliaire stomatique et directe.

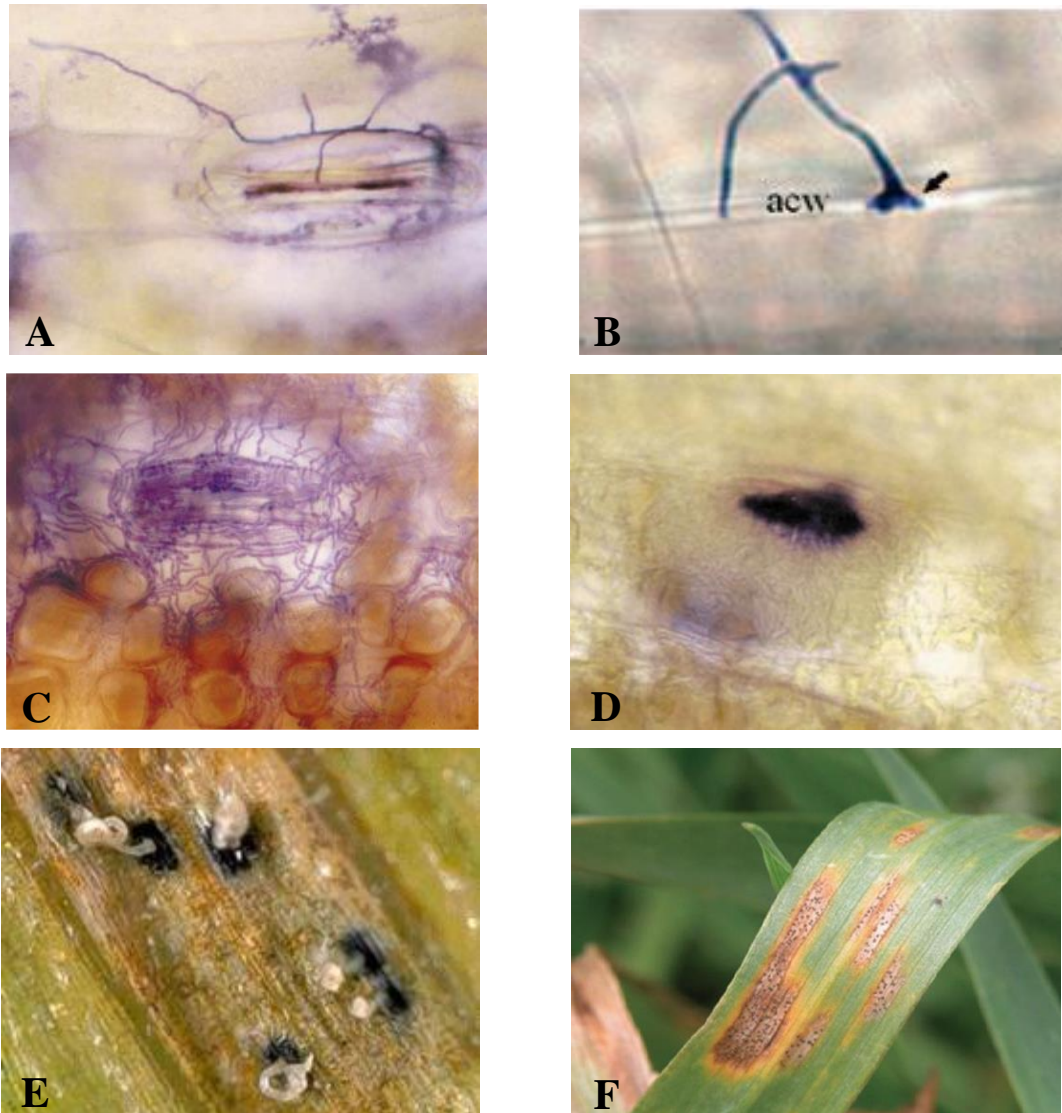


Fig. 4. Différentes étapes du processus infectieux et symptômes de *Z. tritici*. A : pénétration stomatique (Palmer and Skinner, 2002). B : pénétration directe (Siah *et al.*, 2010b). C : colonisation du mésophile (Palmer and Skinner, 2002). D : ébauche d'une pycnide formée dans la cavité sous-stomatique (Palmer et Skinner, 2002). E : cirrhes de *Z. tritici* (Vrancken *et al.*, 2008). F : tache produite par *Z. tritici* sur une feuille de blé et présentant des pycnides (Vrancken *et al.*, 2008).

En tant que champignon hémibiotrophe, ce pathogène présente une phase biotrophique d'environ 12 jours, où des hyphes infectieux se développent exclusivement dans l'espace intercellulaire et colonisent les chambres sous-stomatiques (Fig. 4C), sans causer de mort cellulaire ou de symptômes visibles à l'œil nu. Contrairement à certains champignons biotrophes, *Z. tritici* ne forme pas de structure spécialisée dans la nutrition, tel que l'haustorium. Le champignon survit

pendant cette phase probablement par l'assimilation des nutriments en solution dans l'apoplasme (Shetty *et al.*, 2003 ; Keon *et al.*, 2007 ; O'Driscoll *et al.*, 2015). À partir du 14^{ème} jour après inoculation, le champignon passe rapidement à la nécrotrophie. Cette phase nécrotrophique résulte des symptômes typiques de la maladie qui commence par des lésions chlorotiques qui se développent rapidement en taches nécrotiques contenant des pycnides (Fig. 4D). Ce qui suggère une intervention des composés toxiques dans l'infection (Eyal et Levy, 1987 ; Cohen et Eyal, 1993 ; Kema *et al.*, 1996d ; Duncan et Howard, 2000 ; Mehrabi *et al.*, 2006). À la faveur de l'humidité ambiante ou des pluies, les pycnides se gorgent d'eau, gonflent et les spores sont expulsées sous forme d'une gelée sporifère appelée «cirrhe» (Fig. 4E). L'enrobage mucilagineux de cette gelée alourdit considérablement les spores. Celles-ci sont donc disséminées vers les feuilles supérieures *via* les éclaboussures de pluie. La septoriose du blé causée par *Z. tritici* attaque exclusivement les feuilles et se manifeste par des lésions souvent ovales et parallèles aux tissus vasculaires portant des pycnides noirâtre (Fig. 4F) (Grieger, 2001). Cette maladie provoque des dégâts qui peuvent atteindre les 50 q/ha en situation de forte pression parasitaire et en absence de traitement (Shaw *et al.*, 2008).

1.2.4. Les facteurs impliqués dans le pouvoir pathogène de Z. tritici

Le contrôle de la maladie nécessite la compréhension des bases génétiques et biochimiques de la pathogénicité de *Z. tritici*. Le pouvoir pathogène de *Z. tritici* peut être défini comme étant sa capacité à induire les nécroses.

1.2.4.1. Les signaux de transduction

Les signaux de transduction jouent un rôle global dans la transmission du signal externe qui est à l'origine d'altération cellulaire. Parmi les signaux de transduction, on peut citer les *mitogen-activated protéines kinases (MAPKs)* et les *AMPC-dependent protein kinase A (PKA)* (Mehrabi, 2006). Les MAPKs nommés aussi les régulateurs de signaux extracellulaires, font partie d'une cascade de protéines récepteurs de signaux. Ces protéines activent séquentiellement et envoient des réponses cellulaires appropriées aux stimuli spécifiques (Blumer et Johnson, 1994). Mehrabi *et al.* (2006) ont identifié un gène codant pour une MAP Kinase et intervenant dans le pouvoir pathogène nommé *MgSlt2*. ils ont montré que ce dernier est essentiel pour la croissance et l'invasion de la plante hôte. On a signalé également, chez le mutant de ce gène, une hypersensibilité à la glucanase. Il s'agit d'une enzyme de digestion de paroi cellulaire produite par la plante. Ces résultats ont été confirmés par Zhao *et al.* (2007). De la même, Cousin *et al.* (2006), ont étudié le rôle du gène codant pour la protéine MAPK nommé *MgFus3*. Ils ont révélé que ce gène régule la pénétration du champignon et pourrait intervenir au niveau de la régulation de la fructification asexuel. Il est

considéré comme un facteur de pathogénicité multifonctionnel pour *Z. tritici*. De plus, les études réalisées par Mehrabi *et al.* (2006) et Zhao *et al.* (2007) ont décrit la caractérisation du gène codant pour une autre protéine MAPK nommé *MgHog1*. Ce gène a montré un rôle impératif dans la transition diomorphique et la pathogénicité du champignon. Il pourrait intervenir aussi au niveau de l'osmorégulation, la réponse au stress et la croissance du tube germinatif. Mehrabi et Kema (2006) ont identifié deux gènes *d'AMPc signaling* qui déterminent la pathogénicité de *Z. tritici* : *MgBcy1* et *MgTpk2*. Il a été démontré que ces gènes sont essentiels pour la fructification asexuée et pour la formation des pycnides. En outre, Mehrabi *et al.* (2009) ont décrit la caractérisation des gènes *MgGpa1*, *MgGpa2* et *MgGpa3* codant pour des protéines Ga et du gène *MgGpb1* codant pour une protéine Gβ chez *Z. tritici*. D'après cette étude, il s'est avéré que les gènes *MgGpa1*, *MgGpa3* et *MgGpb1* sont requis pour la pathogénicité de *Z. tritici* alors que *MgGpa2* n'est pas indispensable.

1.2.4.2. Les protéines de transport membranaire

Plusieurs recherches ont montré que les flux transporteurs sont devenus des facteurs de pathogénicité importants. On distingue la superfamille des ABC (*ATP-binding cassette*) et les MFS (*Major Facilitator Superfamily*) transporteurs. Ces protéines peuvent fonctionner comme un facteur de pathogénicité. En effet, Stergiopoulos *et al.* (2003) ont analysé le rôle des gènes ABC transporteurs *MgAtr1* à *MgAtr5* dans la pathogénicité de *Z. tritici*. Ils ont décrit le gène *MgAtr4* comme le premier facteur de pathogénicité attribué à *Z. tritici* alors que le rôle des gènes *MgAtr1*, *MgAtr2*, *MgAtr3* et *MgAtr5* est négligeable. En effet, il a été démontré que le gène *MgAtr4* intervient dans la colonisation et la formation des pycnides. En revanche, Zwiers *et al.* (2007) ont identifié et cloné un nouveau gène ABC transporteur (*MgAtr7*) qui n'est pas un facteur de pathogénicité de *Z. tritici*. Il est plutôt impliqué dans la régulation de l'homéostasie du fer.

1.2.4.3. Les enzymes de dégradation des parois cellulaires végétales (Cell Wall Degrading Enzymes : CWDEs)

Lors de l'infection de la plante, les champignons phytopathogènes produisent des enzymes de type CWDE qui dégradent les composants de la paroi cellulaire végétale. Parmi ces enzymes, on distingue deux classes: les enzymes de dégradation des carbohydrates (CAZymes) et celles impliquées dans la dégradation des protéines : les protéases. Les parois végétales sont constituées de plusieurs polysaccharides: la cellulose, les hémicelluloses et les pectines. Les CAZymes produites par les champignons phytopathogènes sont regroupées en trois grands groupes en fonction de leur substrat : les cellulases qui dégradent la cellulose, les hémicellulases qui dégradent les hémicelluloses et les pectinases qui dégradent les pectines. Ces groupes renferment différentes enzymes qui présentent des activités caractérisées par les types de liaisons qu'elles hydrolysent.

Parmi les cellulases, les endo- β -1,3-glucanases hydrolysent les liaisons β -1,4 entre les résidus de D-glucose. Les endo- β -1,4-xylanases sont considérées comme des éléments indispensables pour la dégradation des xylanes des hémicelluloses (Walton, 1994). De plus, parmi les hémicellulases, les endo- β -1,4-xylanases sont connues pour leur implication dans la pathogénicité de plusieurs micro-organismes, et plus particulièrement chez les champignons pathogènes des céréales. Les parois des Monocotylédones contiennent moins de pectines que celles des Dicotylédones. Ainsi, les agents pathogènes infectant les Monocotylédones produisent moins de pectinases (Zhao *et al.*, 2014). Ceci peut être expliqué par la composition plus riche en xylanes des parois cellulaires des Monocotylédones comparativement aux Dicotylédones (Zhao *et al.*, 2014). Lalaoui *et al.* (2000) ont montré l'importance de l'activité endo- β -1,4-xylanase dans la pathogénicité de *S. nodorum*. Kema *et al.* (2008) ont révélé l'importance de l'expression des gènes de *Z. tritici* codant pour les endo- β -1,4-xylanases *in planta*, durant la phase nécrotrophe du champignon. En plus, une corrélation entre l'activité endo- β -1,4-xylanase et la fréquence de l'apparition des nécroses provoquées par ces souches sur feuilles détachées a été mise en évidence par Douaiher *et al.* (2007). De même, l'étude réalisée par Siah *et al.* (2010b) a révélé une corrélation entre l'apparition des lésions et l'activité de cette enzyme mesurée *in planta* confirmant son implication dans le pouvoir pathogène de *Z. tritici*. Aussi, Tian *et al.* (2009) ont mesurées trois activités CWDE, xylanase, cellulase et protéase, dans des feuilles de blé provenant de cultivars présentant différents niveaux de résistance à *Z. tritici*, et ont observé que les trois activités CWDE (xylanase, cellulase et protéase) sont étroitement corrélées avec la biomasse fongique et les taux de la maladie. Les protéases, ou peptidases, sont des enzymes impliquées dans la dégradation des chaînes polypeptidiques en petits peptides et acides aminés. Chez les agents phytopathogènes, ces enzymes interviennent dans la dégradation de la paroi lors de la pénétration, dans l'adaptation aux mécanismes de défense de son hôte et dans la digestion des nutriments (North, 1982). Alors que la plupart des espèces fongiques contiennent plus de gènes codant pour des CAZymes que pour des protéases tel est le cas de *Fusarium graminearum*, l'inverse a été constaté chez *Z. tritici* (Goodwin *et al.*, 2011). Cette prédominance de gènes codant pour les protéases comparativement aux CWDEs chez *Z. tritici*, fréquemment observée chez les champignons endophytes comme *Trichoderma reesei*, suggère que *Z. tritici* dériverait d'un ancêtre endophyte, ou qu'il évoluerait vers un mode de vie endophyte (Goodwin *et al.*, 2011).

I. 2.5. Gènes de Z. tritici impliqués dans la tolérance aux toxines et fongicides

I. 2.5.1. Rôle des MAP Kinases

Plusieurs études récentes ont mis en évidence l'intervention de certaines protéines telles que les MAP Kinases et les transporteurs membranaires dans la résistance aux composés toxiques et

fongicides (Coleman et Mylonakis, 2009). Parmi les gènes codant pour les MAPKs on peut citer *MgHog1* et *MgSl2*. Mehrabi *et al.* (2006) ont montré que les mutants du gène *MgHog1* sont résistants aux phénylpyrroles et aux dicarboximides. Cette résistance pourrait être expliquée par l'osmosensibilité observée chez les mutants de ce gène. De même, Mehrabi *et al.* (2006) ont montré que le gène *MgSl2* permet de protéger le champignon contre les composés toxiques en fortifiant les murs cellulaires. Zhao *et al.* (2007) ont confirmé ce résultat et ont montré que le gène *MgSl2* diminue la sensibilité de *Z. tritici* aux miconazole et cyproconazole.

1.2.5.2. Rôle des protéines de transport membranaire

Les flux transporteurs permettent au champignon de contourner les toxines en inhibant leur accumulation dans les cellules fongiques. En effet, ces pompes de flux transmembranaires peuvent intervenir dans l'acquisition de la résistance aux fongicides en les empêchant d'atteindre leurs sites d'action et en les conduisant à l'extérieur de la cellule (Cannon *et al.*, 2009). On distingue deux superfamilles à savoir les ABC (*ATP-binding cassette*) et les MFS (*Major Facilitator Superfamily*) transporteurs. on peut citer le gène MFS transporteur (*MgMfs1*) qui permet l'induction de la résistance de *Z. tritici* aux strobilurines (Fernetez-Ortuno *et al.*, 2008). Roohparvar *et al.* (2006) ont pu montrer que ce gène est un vrai antiporteur de multiples de composés toxiques qui peut fonctionner comme un déterminant de la sensibilité ou la résistance du pathogène aux toxines fongiques tels que la cercosporine et aux fongicides de la famille des strobilurines à savoir le trifloystrobine et le krésoxim méthyle. De même, Cools *et al.* (2007) ont révélé l'intervention d'un gène ABC transporteur (*MgAtr3*), dans la résistance de *Z. tritici* aux triazoles comme l'époxiconazole. De plus, Zwiers *et al.* (2002) ont démontré que le gène *MgAtr1* est un facteur déterminant de la sensibilité de *Z. tritici* aux triazoles tel que le cyproconazole.

II. Interaction blé - *Z. tritici*

II.1. Résistance du blé à la septoriose

La résistance peut être définie comme la capacité d'une plante à prévenir ou empêcher l'infection ou la croissance de l'agent pathogène dans ses tissus. L'interaction blé – *Z. tritici* met en jeu deux types de résistance, qualitative (spécifique) et quantitative (non-spécifique) (Orton *et al.*, 2011). Ces deux formes de résistances présentent des caractéristiques génétiques différentes.

II.1. 1. La résistance spécifique

Il s'agit d'une résistance « race-spécifique » contrôlée par des gènes de résistance spécifique *Stb*, impliqués dans les interactions incompatibles de blé-*Z. tritici*. Ces gènes sont mis en évidence

dans les trois génomes A, B et D du blé (Simón *et al.*, 2012) et peuvent répondre au modèle de Flor (Flor, 1971) de la relation gène-pour-gène entre un gène de résistance spécifique et un gène d'avirulence. Dix huit gènes, notés de *Stb1* à *Stb18*, ont été identifiés et cartographiés à l'aide de marqueurs moléculaires de types microsatellites, AFLP (Amplified Fragment Length Polymorphism) ou RFLP (Restriction Fragment Length Polymorphism) (Goodwin, 2007 ; Goodwin et Thompson, 2011; Simón *et al.*, 2012).

II.1. 2. La résistance non-spécifique

Tandis que la résistance non-spécifique est par nature quantitative et partielle et non race-spécifique (Chartrain *et al.*, 2004a). Ce type de résistance est polygénique, ce qui la rend moins facilement contournable par le champignon, plus durable au champ et de ce fait également recherchée par les sélectionneurs (Orton *et al.*, 2011). La résistance non-spécifique est due à un ensemble de caractères de différentes natures contrôlés par des QTL, responsable de la variabilité de l'importance des symptômes observée durant les interactions compatibles.

II.2. Expression de la résistance lors de l'interaction plante-agent pathogène

II. 2. 1. La résistance physique

Lors de l'infection, le pathogène est confronté à des barrières physico-chimiques préexistantes. Elles sont constituées par les structures périphériques telles que les trichomes, les cires, la cuticule et les parois pecto-cellulosiques des cellules épidermiques. Dans l'interaction blé-*Z. tritici*, ces barrières sont surmontées par le champignon, qui adhère à la surface grâce au mucilage qui entoure les spores, germe rapidement et pénètre dans les tissus foliaires de l'hôte par voie stomatique ou directement au travers des parois des cellules de l'épiderme, quel que soit le niveau de résistance de la variété de blé (Shetty *et al.*, 2003).

II.2.2. Perception du signal et induction de la PTI

De façon générale, la résistance peut être induite par la reconnaissance du parasite, et plus spécifiquement par la reconnaissance par la plante des motifs microbiens qualifiés de « Microbe-Associated Molecular Patterns » (MAMP). Ces motifs sont de nature peptidique, lipidique ou polysaccharidique. Les MAMP sont présents aussi bien chez les microorganismes pathogènes - par conséquent appelés alors PAMP pour « Pathogen-Associated Molecular Patterns » que non-pathogènes. D'autre part, on distingue également les « Damage-Associated Molecular Patterns » (DAMP), qui sont des molécules, protéiques ou glucidiques, issues de la dégradation des parois végétales par les enzymes de l'agent pathogène, les « Cell Wall Degrading Enzymes » (CWDE).

Egalement appelés éliciteurs généraux, les MAMP/PAMP et les DAMP sont reconnus par des récepteurs transmembranaires, les Pathogenesis-Related Receptors (PRR), comme des marqueurs d'une attaque au moins potentielle, et induisent des réactions immunitaires innées dans le cadre de l'immunité déclenchée par les PAMP ou « PAMP-Triggered Immunity » (PTI) (Nürnberger and Scheel, 2001 ; Mengiste, 2012).

II.2.3. Production des effecteurs (*Effector-Triggered Susceptibility* : ETS)

Afin de surmonter la PTI, le bioagresseur produit et libère des effecteurs lui permettant de poursuivre sa colonisation, rendant à nouveau la plante sensible. On parle alors de sensibilité déclenchée par des effecteurs ou « Effector-Triggered Susceptibility » (ETS) (Li *et al.*, 2013). Le séquençage du génome de *Z. tritici* n'a révélé aucun gène codant pour ToxA qui est considérée comme un effecteur impliqué dans une relation gène-pour-gène inversée (Deller *et al.*, 2011; Goodwin *et al.*, 2011; Mengiste, 2012). Dans leur étude, Marshall *et al.* (2011) ont mis en évidence deux gènes homologues à *Epc6* (*extracellular protein 6*) notés *Mg1LysM* et *Mg3LysM* chez *Z. tritici*. Ces derniers possèdent une fonction protectrice des protéines à domaine LysM observée à ce jour que chez *Z. tritici* (Kombrink et Thomma, 2013).

II.2.4. Reconnaissance des effecteurs par la plante et mise en place de l'ETI

Lors de l'attaque par un agent pathogène biotrophe, la plante peut répondre à l'ETS par une deuxième vague de défense décrite comme l'immunité déclenchée par les effecteurs ou « Effector-Triggered Immunity » (ETI). Alors que la PTI permet de stopper la pénétration du parasite et la colonisation de la plante, l'ETI confine la colonisation des tissus au site initial d'infection. Un modèle dit « en zigzag » a été construit pour illustrer les quatre phases successives de la coévolution de l'interaction plante-microorganisme, tels que la PTI, l'ETS, l'ETI et l'alternance entre ETI et ETS qui résulte du contournement des gènes de résistance spécifique de la plante par le bioagresseur (Fig. 5). L'ETI caractérise les interactions de type « incompatible », il s'agit d'une réponse plus forte, plus rapide et plus localisée que la PTI et aboutit le plus souvent à une mort cellulaire programmée (Programmed Cell Death : PCD) localisée au niveau du site d'infection. Dans le cas de l'interactions blé-*Z. tritici*, l'induction de réponse précoce (PTI) par la reconnaissance de la chitine et des glucanes de *Z. tritici* a été mise en évidence (Lee *et al.*, 2014; Shetty *et al.*, 2009). Ce phénomène est surmonté par la mise en place d'effecteurs codés par les gènes *Mg1LysM* et *Mg3LysM* chez *Z. tritici* lui permettant d'échapper aux mécanismes de défense de la plante durant la phase biotrophe, et de provoquer l'entrée en nécrotrophie (ETS) (Marshall *et al.*, 2011).

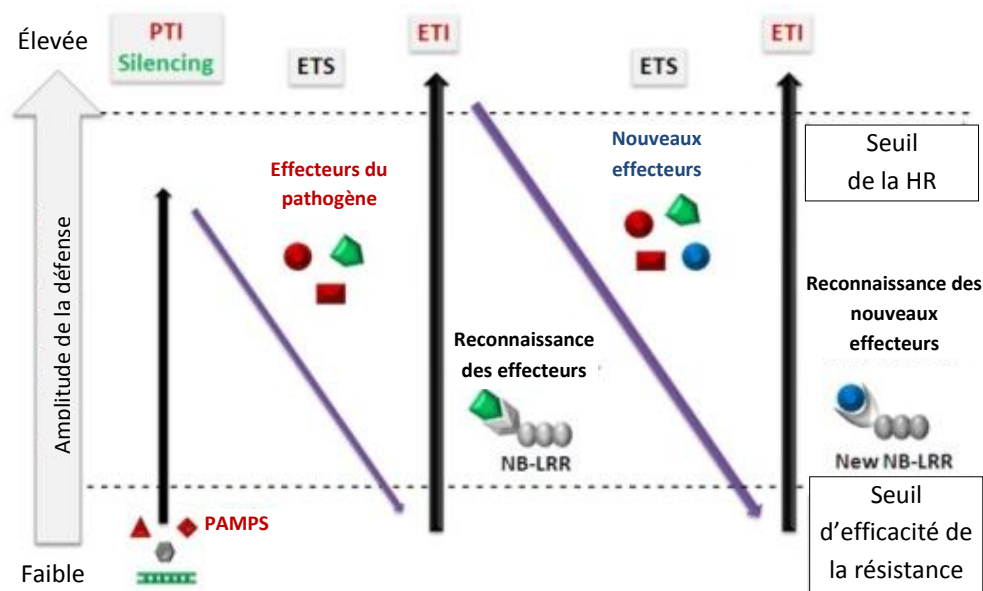


Fig. 5. Schéma représentant le modèle en « zigzag » selon lequel se réalise la coévolution de l'interaction plante-pathogène (adapté de Jones et Dangl, 2006).

II. 3. Les voies de défense du le blé contre *Z. tritici*

II.3.1 Les réponses cellulaires précoces

II.3.1.1. La cascade de phosphorylation : activation des MAPKs

Des cascades de signalisation faisant intervenir des protéines kinases comme les MAPKs sont activées rapidement lors des interactions plante-pathogène (Zhang et Klessig, 2001). C'est une composante importante qui assure la transduction des stimuli extracellulaires en réponses intracellulaires. Chez le blé, Rudd *et al.* (2008) sont les premiers à montrer l'implication des MAPK dans le pathosystème blé – *Z. tritici*. Ils ont identifié une MAPK nommée *TaMPK3*. Ils ont démontré que ce gène est induit durant l'interaction blé-*Z. tritici* soit lors des réactions de défense précoces issues de la reconnaissance des PAMPs par la plante (PTI), soit lors de l'entrée en nécrotrophie du champignon en favorisant la mort cellulaire chez la plante. Ensuite, l'étude de la phosphorylation des protéines menée par Yang *et al.* (2013b), a montré l'importance de la phosphorylation dans la résistance du blé à *Z. tritici*.

II.3.1.2. La vague oxydative et les formes activées de l'oxygène (ROS)

Le flux ionique transmembranaire déclenché par la reconnaissance du pathogène par la plante aboutit à une accumulation cytosolique des ions calcium (Ca^{2+}) capables de produire des réactions dans de multiples processus en aval. Une des conséquences est une vague oxydative qui génère rapidement une accumulation des ROS (*Reactive Oxygen Species*). Il s'agit du premier événement issu de la reconnaissance de l'agent pathogène. Ces composés sont impliqués dans la signalisation

aboutissant à l'expression des gènes de défense, à la HR, au renforcement pariétal, à la production de phytoalexines et à la réponse systémique acquise (SAR) (Parent *et al.*, 2008 ; O'Brien *et al.*, 2012). Les ROS sont aussi capables d'induire l'oxydation non-enzymatique des lipides, ce qui aboutit à la production d'un groupe d'oxylipines cyclopenténoniques. Au cours de cette étude on va s'intéresser à une ROS, le peroxyde d'hydrogène (H_2O_2), ainsi que deux enzymes impliquées dans le métabolisme des ROS à savoir la peroxydase (POX) et la catalase (CAT) :

Concernant le peroxyde d'hydrogène (H_2O_2), Hückelhoven *et al.* (2000) ont démontré que l'accumulation du H_2O_2 renforce les parois cellulaires et empêche le développement du champignon. A des concentrations importantes, le H_2O_2 provoque la mort cellulaire et limite la dissémination du champignon à l'intérieur du tissu si celui-ci est biotrophe (Levine *et al.*, 1994 ; Tenhaken *et al.*, 1995). Dans le cas d'un champignon hemibiotrophe, Shetty *et al.* (2003, 2007) ont révélé une accumulation précoce de cette molécule au cours de la phase biotrophe principalement chez les cultivars résistants permettant ainsi de limiter la pénétration foliaire et la croissance de *Z. tritici*. Par contre chez le cultivar sensible, le H_2O_2 s'accumule plus fortement et plus tardivement, au cours de la phase nécrotrophe qui correspond à l'augmentation de la biomasse du champignon dans les tissus foliaires, au moment de la mise en place des nécroses (Shetty *et al.*, 2003 ; Keon *et al.*, 2007 ; Yang *et al.*, 2013a). Cependant, le champignon peut résister à l'élévation de la teneur en H_2O_2 et dans certains cas son développement peut en être favorisé (Keon *et al.*, 2005 ; Mengiste, 2012).

Concernant la peroxydase (POX), il s'agit d'une protéine hémique qui produit du H_2O_2 à partir du fer superoxydé, selon la réaction de Fenton (O'Brien *et al.*, 2012). Cette enzyme est impliquée dans de multiples processus physiologiques dont font partie la lignification, l'oxydation des phénols et la subérisation. Ces processus sont notamment associés aux mécanismes de défense contre l'attaque par des agents pathogènes lors d'interactions incompatibles (Passardi *et al.*, 2005 ; O'Brien *et al.*, 2012). L'activité POX a été étudiée chez des cultivars de blé résistants ou sensibles aux septorioses du blé. Dans leur étude, Shetty *et al.* (2003) ont constaté, chez les cultivars résistants, une accumulation des transcrits et une activité POX plus importante que chez les cultivars sensibles après inoculation avec *Z. tritici*. Ceci coïncide avec l'apparition des premiers événements de pénétration. De plus, cette augmentation de l'activité POX apparaît précocement chez les cultivars résistants infectés par la septoriose, contrairement aux variétés sensibles, qui ne présentent pas de variation significative de l'activité POX tout au long du processus infectieux.

Concernant la catalase (CAT), elle est située principalement dans les peroxysomes. Son activité a été signalée comme étant étroitement liées à la disparition de H_2O_2 (Sandalio *et al.*, 2013). Chen *et al.* (1993,1995) suggèrent que l'acide salicylique (AS) bloquerait l'activité de la catalase,

entraînant une augmentation des niveaux d'H₂O₂ dans la plante. L'AS bloque l'activité CAT, entraînant une augmentation des niveaux d'H₂O₂ dans la plante. En outre, un traitement à base d'HSA (*Heptanoic Saclicylic Acid*) induit une réduction de l'activité catalase (CAT) hors contexte infectieux (Renard-Merlier *et al.*, 2009). Diani *et al.* (2009) a rapporté une augmentation de l'activité enzymatique des CAT suite à l'infection par *Z. tritici* chez les cultivars sensibles de blé tendre. Par contre, une réduction de cette activité est observée chez les cultivars résistants.

II.3.2. La transmission du signal

L'infection localisée peut induire un état de résistance général dans les parties non infectées de la plante. Cette réponse systémique est possible grâce à la production de messagers comme les phytohormones. En effet, les voies de signalisation liées aux réactions de défense chez les plantes sont régulées par 4 principales phytohormones : l'acide salicylique (AS), l'acide jasmonique (AJ), l'éthylène (ET) et l'acide abscissique (ABA). Dans le cadre de l'interaction blé - *Z. tritici*, seules les voies du SA et JA ont été explorées. Ainsi, nous limiterons notre étude au SA et JA.

II.3.2.1. L'acide salicylique (AS)

L'AS est un composé phénolique qui joue un rôle crucial dans la régulation des processus physiologiques et biochimiques de germination des graines, de floraison et de sénescence (Rivas-San Vicente et Plasencia, 2011). L'AS est reconnu pour son implication dans les événements biochimiques et moléculaires associés à l'induction des résistances à certaines maladies, qui suivent la PTI ou l'ETI. Durant l'interaction incompatible entre plante et agent pathogène, l'accumulation locale de l'AS est souvent associée à l'apparition d'une HR. Dans les minutes suivant la reconnaissance du parasite, l'AS est synthétisé à partir de l'acide benzoïque initialement présent dans la cellule, faisant intervenir la BA2H (Benzoic Acid 2 hydroxylase). La biosynthèse de l'AS peut avoir lieu selon deux voies : la voie de l'isochorismate, grâce à l'activité enzymatique de l'isochorismate-synthase (ICS) dans les chloroplastes, et la voie des phénylpropanoïdes. Dans cette seconde voie, la biosynthèse de l'AS débute par la conversion de la phénylalanine en acide trans-cinnamique *via* l'activité de la phénylalanine ammonia lyase (PAL) (Fig. 6) (Hammond-Kosack et Jones, 1996 ; Vlot *et al.*, 2009). L'acide cinnamique est ensuite hydroxylé en acide paracoumarique par l'action de la cinnamate. La voie de signalisation de l'AS interagit avec d'autres voies hormonales tels que le AJ et l'ET lors des réactions de défense de la plante aux pathogènes (Vlot *et al.*, 2009; Hammond-Kosack et Jones, 1996).

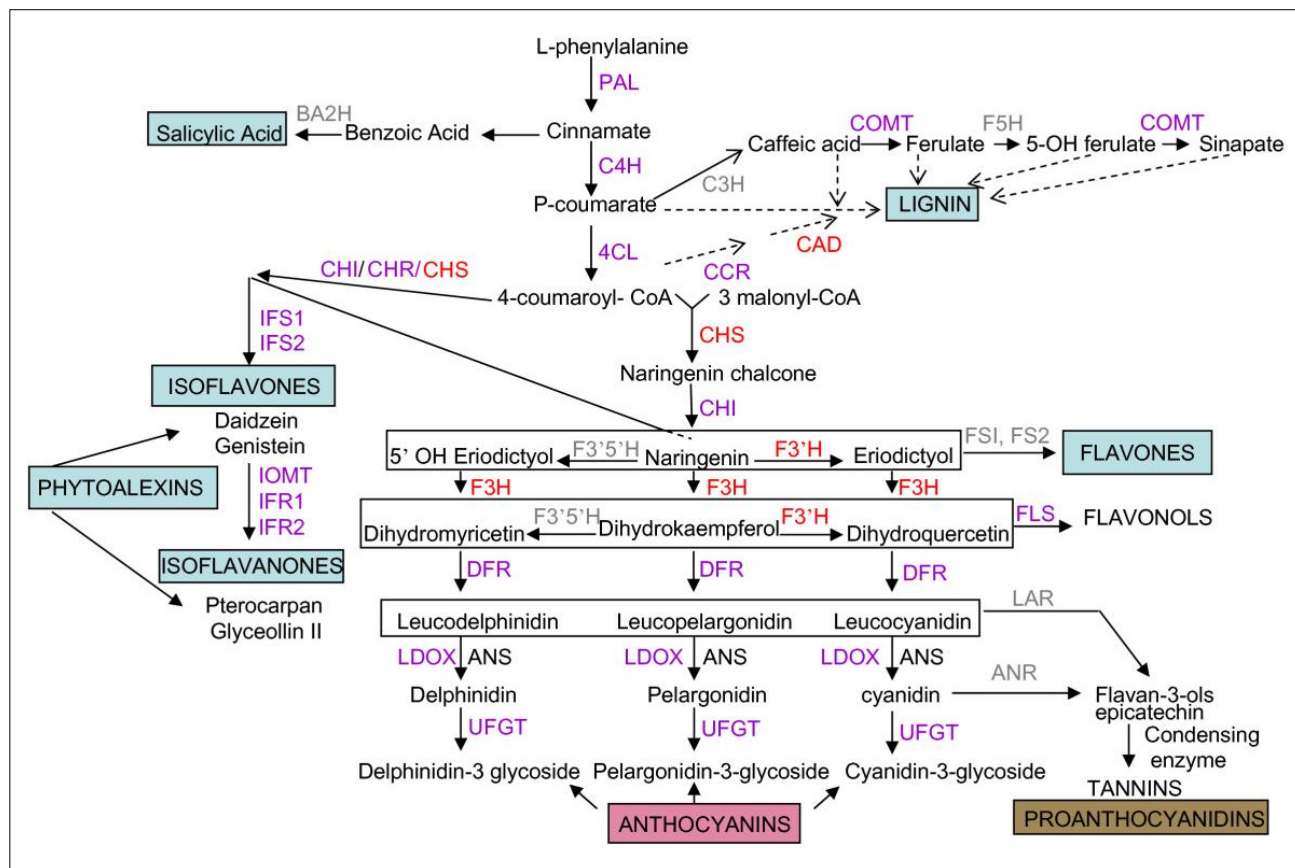


Fig. 6. Voie de synthèse des phénylpropanoïdes (Zabala *et al.*, 2006).

II.3.2.2. L'acide jasmonique (JA)

L'JA, le méthyle jasmonate et d'autres dérivés lipidiques sont impliqués dans la transduction du signal au niveau inter- et intracellulaire et peuvent être véhiculés par le phloème (Creelman and Mullet, 1997 ; Pieterse and van Loon, 1999). La biosynthèse de l'AJ à partir de l'acide linoléique dépend de la voie des octadécanoïdes présentée par la Fig. 7. La synthèse de l'AJ commence par l'insertion d'un atome d'oxygène sur l'acide α -linoléique par une lipoxigénase (LOX). Le résultat de cette insertion est la formation d'un hydroperoxyde qui sera rapidement converti en un allène oxydé par un allène oxide synthase (AOS) (Fig. 7). Par conséquent, cette étude s'intéresse à la LOX qui est l'enzyme clé de la voie des octadécanoïdes. La lipoxigénase (LOX), est impliquée lors des interactions plante – agent pathogène a été démontrée à plusieurs reprises. Dans le cadre du pathosystème blé-*Z. tritici*, Ray *et al.* (2003) ont montré que la transcription du gène LOX est induite chez les cultivars sensibles et résistants suite à l'inoculation. Le niveau de transcription est plus important chez le cultivar sensible comparativement au cultivar résistant. Par conséquent, ils ont suggéré que la LOX pourrait faciliter la colonisation et l'installation du champignon. L'inhibition rapide de l'activité LOX, chez les cultivars résistants, peut jouer un rôle dans le retard de la colonisation et paraît être une caractéristique des cultivars résistants à *Z. tritici* (Ray *et al.*, 2003). En plus, la LOX répond à plusieurs SDP appliqués sur blé tendre sensible à l'oïdium, tels

que le HSA et le Milsana. Ces derniers induisent une augmentation de l'activité LOX associée avec le niveau de protection induit contre *Blumeria graminis* (Randoux *et al.*, 2006; Renard-Merlier *et al.*, 2007).

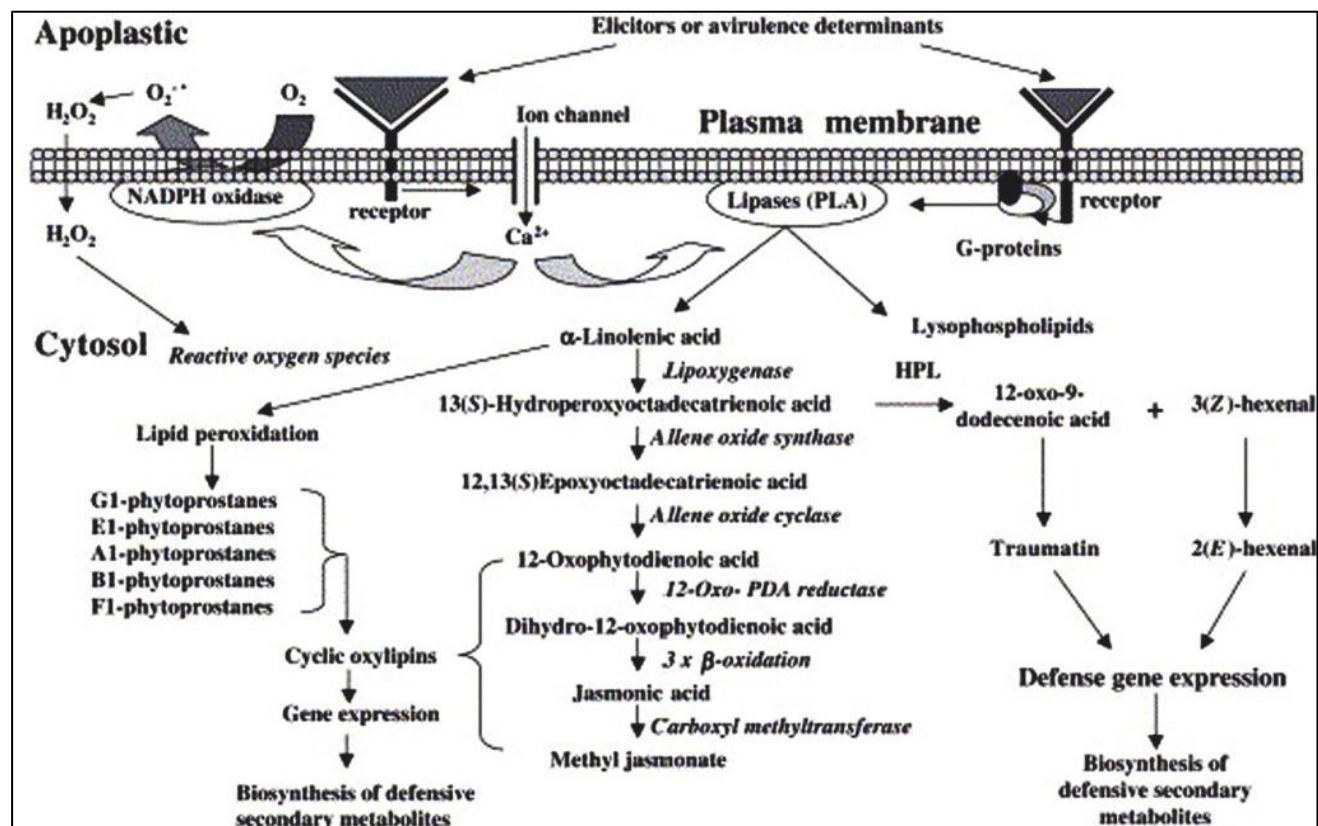


Fig. 7. Voie de synthèse des oxylipines, et plus particulièrement de l'acide jasmonique (Zhao *et al.*, 2005).

II.3.3. Les évènements tardifs de défense

II.3.3.1. Les phénylpropanoïdes

Les composés phénoliques sont des phénylpropanoïdes issus du métabolisme secondaire. Parmi eux, certains sont impliqués dans le renforcement des structures végétales (la lignine), dans la pigmentation (les anthocyanes), alors que d'autres régulent les réponses des plantes aux stress biotiques et abiotiques (phytoalexines, flavonoïdes, AS). La voie de biosynthèse des phénylpropanoïdes est initiée par la PAL et aboutit à l'accumulation de différents composés, dont la plupart sont synthétisées spécifiquement par certaines espèces végétales.

La *L*-phénylalanine ammonia lyase (PAL), est l'une des enzymes clés de la biosynthèse des composés phénoliques. Elle catalyse la réaction de désamination de la *L*-phénylalanine en acide trans-cinnamique, un précurseur de la majorité des composés phénoliques produits par les plantes, avec libération de NH₃. Cette première étape constitue une voie commune de biosynthèse des phénylpropanoïdes (Fig. 6). 4-hydroxylase (C4H), à l'origine de plusieurs produits comme les

flavonoïdes, l'AS (présenté dans la partie II.2.3.1), la lignine et les phytoalexines. L'expression d'un gène codant pour la PAL a été analysée chez différentes variétés de blé tendre infectées par *Z. tritici*. En contexte infectieux, l'induction de la transcription du gène codant pour la PAL a été enregistrée uniquement chez les variétés résistantes entre 3 et 6 heures après infection (Adhikari *et al.*, 2007). Par contre, dans l'étude menée par Shetty *et al.* (2009), le niveau de transcription du gène codant pour la PAL ne semble pas être induit par *Z. tritici*, ni chez le cultivar sensible, ni chez le cultivar résistant.

II.3.3.2. Les protéines PR

Les protéines liées à la pathogenèse (PR) sont induites chez la plante lors d'une interaction avec un agent pathogène ou suite à une blessure mécanique. Dans l'interaction blé - *Z. tritici*, l'induction de l'expression des gènes *PR1*, *PR2*, *PR3*, *PR5* et *PR9* diffère entre les interactions compatibles et incompatibles (Ray *et al.*, 2003; Adhikari *et al.*, 2007; Shetty *et al.*, 2009; Yang *et al.*, 2013b). Lors de ce projet de thèse, nous nous intéresserons principalement à l'étude de 3 protéines PR à savoir les PR2 (β -1,3-glucanases), les PR3 qui incluent les chitinases de la classe 4 (*Chi 4 precursor*) et les PR9 qui incluent les peroxydases (*Per*). Les glucanases et les chitinases sont des enzymes employées par la plante pour dégrader les parois fongiques libérant ainsi des monomères de chitine et de glucane pouvant à leur tour agir comme des PAMP et déclencher de nouvelles réactions de défense (PTI), tandis que les peroxydases sont associées au stress oxydatif (présenté dans la partie (II.3.1.2)).

Concernant les protéines *PR2*, ils incluent les β -1,3-glucanases de classe II qui sont des protéines acides sécrétées dans l'espace extracellulaire (Ferrari *et al.*, 2007). Ces dernières sont impliqués dans la dégradation des parois fongiques en catalysant le clivage hydrolytique des liaisons 1,3- β -D-glucosidiques et libérant les β -1,3-glucanes (Ryals *et al.*, 1996) qui vont agir comme des PAMP et déclencher les réactions de défense chez la plante (Shetty *et al.*, 2009). Les β -1,3-glucanases pourraient être impliquées dans la défense du blé contre les agents pathogènes (Mohammadi *et al.*, 2012; Cawood *et al.*, 2013). Outre leur rôle dans divers processus physiologique chez la plante (Sticher *et al.*, 1997), les β -1,3-glucanases végétales sont subdivisées en trois classes (I, II et III). Dans le cadre de l'interaction blé - *Z. tritici*, il a été mis en évidence une augmentation rapide de l'expression d'un gène codant pour la β -1,3-glucanase (*PR2*) chez le cultivar résistant de blé tendre, suivie par une accumulation de callose contribuant à l'arrêt de l'infection (Shetty *et al.*, 2009). Bien que, l'accumulation du transcrit et des protéines *PR2* est induite précocement et fortement lors des interactions incompatibles au cours de la phase biotrophe, une augmentation très importante est observée lors de la phase necroptrophe chez les cultivars sensibles par rapport aux cultivars résistants, qui est associée à l'augmentation rapide de la biomasse fongique (Shetty *et al.*, 2009; Yang *et al.*, 2013b).

Concernant les protéines *PR3*, ils hydrolysent la chitine des parois cellulaires fongiques et inhibent ainsi *in vitro* la croissance du champignon. De plus, les oligomères de chitine dégradée peuvent agir comme molécule signal pour stimuler des réactions de défense (Li *et al.*, 2001). Sept classes de chitinases de plantes ont été distinguées sur la base d'alignements de séquences et de leur distribution cellulaire (Singh *et al.*, 2007). Les protéines *PR3* incluent les chitinases de classe I, II, IV, V, VI et VII (van Loon et van Strien, 1999). Au cours de cette étude nous nous intéresserons aux chitinases de la classe IV qui sont apoplastiques et s'accumulent dans le milieu extracellulaire (Collinge *et al.*, 1993). Dans le cas de l'interaction blé-septoriose, Shetty *et al.* (2009) et Yang *et al.* (2013b), ont montré que la surexpression et l'activité enzymatique de la chitinase I est nettement supérieures chez des cultivars résistants que les cultivars sensibles de blé tendre au cours de la phase biotrophe de *Z. tritici*, alors que l'inverse est observé lors de la phase nécrotrophe. Ceci suggère que la chitinase pourrait être considérée comme un marqueur de susceptibilité variétale (Shetty *et al.*, 2009; Yang *et al.*, 2013b). Cependant, Adhikari *et al.* (2007) ont signalé une transcription plus élevée des chitinases II entre 3 heures et deux jours après l'inoculation chez les cultivars résistants comparés aux cultivars sensibles de blé tendre. Cependant, lors de la phase nécrotrophe du champignon aucune induction n'a été observée chez les cultivars résistants et sensibles.

III. Les stimulateurs de défenses des plantes (SDP)

Face à la pression de la maladie et au manque d'efficacité des résistances variétales au champ, la lutte contre la septoriose repose essentiellement sur l'utilisation de produits fongicides tels que les strobilurines et les triazoles. Néanmoins, le traitement parfois abusif des cultures avec les fongicides a entraîné l'émergence de souches résistantes de *Z. tritici* suite aux mutations ayant eu lieu au niveau des cibles de ces matières actives (Torriani *et al.*, 2009 ; Siah *et al.*, 2010b ; Omrane *et al.*, 2013). De plus, l'impact négatif des pesticides chimiques sur l'environnement et la santé humaine, en raison de leur toxicité, est de plus en plus en d'actualité. Par conséquent, plusieurs axes de recherche visent à réduire l'utilisation des produits phytosanitaires. C'est pour cela qu'un certain nombre de substances permettant de mimer l'attaque par un agent phytopathogène et d'activer les défenses chez la plante, ont vu le jour. Ces substances regroupées sous le nom de stimulateurs de défense des plantes (SDP) peuvent être utilisées dans la lutte contre les attaques des agents pathogènes. Le premier SDP homologué en Europe fut le Bion®, commercialisé par la société Syngenta, dont la matière active est l'acibenzolar-S-méthyl (ASM) ou BTH (Görlach *et al.*, 1996). Cette molécule, qui a été la plus étudiée, est capable d'induire la résistance systémique acquise (SAR) chez un grand nombre de plantes et se montre efficace contre un large spectre de bioagresseurs (virus, bactéries, champignons, nématodes etc...). Depuis, d'autres produits ont été

homologués comme Elexa® (Chitosan ; Safescience), Messenger® (Harpine ; Plant health care) et Vacciplant® (Laminarine; Goëmar). On distingue deux types de SDP, biotique et abiotique, qui ont été testés au laboratoire et au champ. Les inducteurs d'origine biotique regroupent des oligosaccharides de paroi (ex. les glucanes, les chitines, la laminarine), des glycoprotéines, des protéines ou peptides sécrétés (ex. les xylanases) ou encore des lipides membranaires (ex. l'ergostérol) d'origine végétale ou microbienne, alors que les inducteurs d'origine abiotiques regroupent les peptides synthétiques comme le β -aminobutyric acid (BABA) et les analogues de l'AS comme le benzothiazole (BTH) (Reignault et Walters, 2007; Benhamou et Rey, 2012; Thakur et Sohal, 2013; Tayeh *et al.*, 2013).

III.1. Principe

Au cours de leur évolution, les plantes ont développé de multiples mécanismes de défense pour se protéger des organismes phytopathogènes (décrits dans la partie II.2). L'induction de ces défenses passe par la détection des PAMPs et des DAMPs. En effet, les plantes sont capables de reconnaître des bioagresseurs potentiels par la perception de tels signaux moléculaires. Les molécules reconnues et permettant de mettre en place des défenses sont appelées « éliciteurs ». Les SDPs sont des produits capables d'induire les réactions de défense de la plante. C'est le phénomène d'élicitation. La potentialisation ou *priming* est un état physiologique acquis par la plante suite à un premier traitement, lui permettant de répondre plus rapidement et/ou plus fortement à l'agent pathogène par une réponse adaptée lors de l'infection. Cet état induit n'est pas très perceptible d'un point de vue moléculaire tant que l'agression par le parasite n'est pas ressentie (Jourdan *et al.*, 2008). Il permet cependant à l'hôte végétal de mettre en place de manière plus rapide et plus violente les différents mécanismes de défense et ainsi de répondre de manière plus efficace à une éventuelle agression par des pathogènes ou des insectes mais aussi à divers stress abiotiques (Conrath *et al.*, 2006). Tout SDP serait un potentialisateur selon la dose appliquée et l'hôte cible (Thulke et Conrath, 1998 ; Kohler *et al.*, 2002). Les inducteurs de réactions de défense ou de résistance ont une importance croissante sur le marché agricole car ils permettent d'améliorer les défenses des plantes face aux agresseurs des cultures et constituent un domaine de recherche important (Narayanasamy, 2002).

III.2 Inducteurs de défense déjà connus chez le blé

L'utilisation des SDP s'est avéré une stratégie de lutte complémentaire potentiellement intéressante pour protéger le blé contre les maladies tout en respectant l'environnement. Plusieurs chercheurs ont testé des SDP de différentes origines naturelles (biotiques) ou de synthèse (abiotiques), en vue de protéger le blé contre plusieurs pathogènes.

III.2.1. SDP abiotiques

Plusieurs SDPs abiotiques ont été testés sur blé et ont révélé une bonne efficacité pour contrôler différentes maladies telles que l'oïdium, la rouille et la septoriose. Rémus-Borel *et al.* (2005), ont montré qu'une application de silice induit la production de composés antifongiques chez le blé infecté par l'oïdium. Aussi, l'application foliaire de chlorure de potassium induirait un mécanisme osmotique impliqué dans le contrôle de l'oïdium du blé (Kettlewell *et al.*, 2000). De même, Deliopoulos *et al.* (2010) ont révélé que les sels bicarboniques (KHCO₃ et NaHCO₃) sont capables de protéger le blé contre la rouille et la silice le protège contre *S. nodorum*. Le BTH peut aussi induire une résistance contre l'oïdium, la rouille et la septoriose (Görlach *et al.*, 1996), mais pas contre la fusariose de l'épi (Yu et Muehlbauer, 2001). Bertini *et al.* (2003) ont mis en évidence une induction de l'expression du gène *PR4*, codant pour une chitinase, dans les coléoptiles de blé 10 jours après traitement avec le BTH. De même, une induction de l'expression de gènes de défense, codant pour une glucanase, une protéine PR1 et une lipoxygénase, est observée chez les feuilles de blé 24 heures après le traitement avec le BTH (Pasquer *et al.*, 2005). De plus, Dos Santos *et al.* (2011) ont confirmé la protection conférée par l'acibenzolar-Sméthyle ou Bion au blé vis-à-vis de l'oïdium et la rouille. Vechet *et al.* (2005) ont démontré que le BTH s'est révélé efficace dans la réduction de l'oïdium du blé. Renard-Merlier *et al.* (2007) et Tayeh *et al.* (2013) ont montré une efficacité de l'AS et HAS (heptanoate d'acide salicylique) pour contrôler l'oïdium du blé. Ces travaux ont révélé que le HAS augmente l'accumulation des H₂O₂ et la peroxydation des lipides. En outre, une étude récente a montré que l'application exogène de l'acide oxalique permet d'augmenter la tolérance du blé vis-à-vis de la septoriose au champ (Zhuk *et al.*, 2014).

III.2.2. SDP biotiques

Des études récentes ont utilisé des SDP biotiques à base d'extraits naturels de plantes. Renard-Merlier *et al.* (2007) ont testé l'Iodus40® (contenant de la laminarine, un polysaccharide issue d'une algue brune, *Laminaria digitata*) pour contrôler l'oïdium du blé et ont montré que les plantes traitées présentent une diminution du niveau de peroxydation lipidique. Reignault *et al.* (2001) ont montré une efficacité partielle du tréhalose (un disaccharide) contre l'oïdium du blé. Ce dernier induit l'augmentation des activités associées PAL et PO. De même, Tayeh *et al.* (2012), ont confirmé l'efficacité du tréhalose (un disaccharide) contre l'oïdium du blé ainsi que son effet stimulateur de défense. Ils ont montré l'induction des gènes codant pour les chitinases, l'oxalate oxydase (OXO) et LOX, ainsi que l'activation du métabolisme des lipides. Toujours dans le cadre du pathosystème blé-oïdium, Randoux *et al.* (2006) ont étudié l'effet de Milsana® qui est un extrait éthanolique foliaire d'une plante ligneuse, *Reynoutria sachalinensis*, comprenant des flavonoïdes et des anthraquinones antifongiques ainsi que des protéines, des glycoprotéines, des peptides et des

lipides. Une pulvérisation préventive avec ce produit confère une très bonne protection du blé contre cette maladie. Cependant, aucune activation des enzymes du métabolisme des ROS, comme la CAT, OXO, POX et SOD ne fut détectée. Toutefois, une légère augmentation de l'activité LOX est observée, 48h après le traitement. L'efficacité de ce produit est donc due essentiellement à son activité antifongique prononcée contre le champignon en inhibant la germination des conidies *in vitro* et *in planta*. En plus, des travaux ont testé l'efficacité de plusieurs inducteurs de résistance comme l'extrait du chêne pédonculé (*Quercus robur* L.), et des extraits de renouée de Sakhaline (*Reynoutria sachaliensis* L), du curcuma (*Curcuma longa* L.) et du gingembre (*Zingiber officinale* Roscoe). Tous ces produits se sont révélés efficaces dans la réduction de l'oidium chez un cultivar sensible de blé au laboratoire (Vechet *et al.*, 2005) et en plein champ (Vechet *et al.*, 2009). Également, Randoux *et al.* (2010) ont testé l'efficacité de 2 fractions d'oligogalacturonides (OGAs), avec ou sans groupement acétyle, dans la protection du blé contre l'oïdium. Hors contexte infectieux, les activités enzymatiques de l'OXO, POX et LOX sont augmentées suite à l'infiltration des 2 classes d'OGAs, suggérant l'implication du métabolisme des ROS et de la voie des oxylipines dans les réactions de défense portées par les OGAs. En contexte infectieux, les 2 fractions entraînent l'accumulation d'H₂O₂ au site de pénétration fongique. Cependant, seuls les OGAs acétylés induisent une accumulation importante de polyphénols au niveau des sites de pénétration.

Concernant le pathosystème blé-septoriose, les recherches qui visent l'utilisation des SDP biotiques sont très limitées. Tout d'abord, Joubert *et al.* (1998) ont montré l'efficacité des oligomères de β -1,3-glucan extrait d'une algue *L. digitata*, contre la septoriose du blé. Ensuite, Esnault *et al.* (2005) ont testé l'Iodus 40 contre la septoriose du blé et ont montré l'utilité de l'intégration de ce produit dans les stratégies de lutte contre cette maladie en France et en Angleterre. Par la suite, Shetty *et al.* (2009) ont montré dans leur étude, que l'application des glucanes extraits de parois de *Z. tritici* induit des mécanismes de défense du blé aussi bien chez le cultivar sensible de blé que le cultivar résistant, permettant le contrôle de la septoriose. Cette étude a mis en évidence l'induction de la surexpression des glucanases (PR2) associés au dépôt de callose. Une étude très récente, (Ors, 2015), a mis en évidence certains SDPs, à savoir un phosphite (Nutriphite®), un mélange d'extrait protéique-manganèse (Nectar®) et un polysaccharide extrait de parois (en cours de développement), qui ont montré des bonnes efficacités contre la septoriose du blé tendre en France. Cette étude a révélé que les mécanismes de défense sont induits différemment en fonction du cultivar et en fonction du SDP appliqué.

Alors que la septoriose représente à l'heure actuelle l'une des maladies occasionnant les plus fortes diminutions de rendement de blé, le nombre de SDP commercialisés et homologués pour lutter contre cette maladie est très limité. Dans le cadre du pathosystème blé-septoriose, l'utilisation de tels produits semble être une alternative prometteuse à celle des fongicides, mais assez peu

étudiée. L'induction des mécanismes de défense par un SDP pourrait contribuer à l'augmentation du niveau de résistance du blé et prévenir l'infection par *Z. tritici*. Le génotype peut donc influencer cette induction et par conséquent affecter l'efficacité de protection du SDP. Par conséquent, dans cette étude, nous proposons de tester quatre produits biostimulants naturels en cours de développement, testés pour la première fois dans le cadre du pathosystème blé-*Z. tritici*. Les effets directs (antifongique) et indirects (stimulateur de défense de la plante) de ces produits vont être discutés sur les deux espèces de blé à savoir le blé dur et le blé tendre.

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Chap II | Etude comparative de l'interaction compatible entre le blé tendre et le blé dur en réponse à la septoriose causée par *Zymoseptoria tritici*

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Problématique

Le blé dur, tout comme le blé tendre, est une céréale très touchée par la septoriose, une maladie foliaire causée par le champignon hémibiotrophe *Z. tritici*. Au vu de la littérature précédemment citée, on peut identifier un déficit majeur de connaissance concernant l'interaction blé dur-*Z. tritici* auquel nous allons tenter de répondre dans ce chapitre. Par conséquent, ce travail vise à étudier l'interaction d'un cultivar de blé dur (Karim) et une souche de *Z. tritici* (St-08-46), et la comparer à celle du pathosystème blé tendre (Premio)/ *Z. tritici* (T01193). A ce jour, les études ont le plus souvent été menées sur les réponses de défense de la plante lors d'interactions incompatibles blé - *M. graminicola* aboutissant à un arrêt précoce de la croissance de l'agent pathogène et de la colonisation de la plante-hôte. En revanche, l'étude présentée ici concerne exclusivement des interactions compatibles. D'une part, on va examiner les différents stades du processus infectieux ainsi que l'activité des CWDEs de la souche pathogène St-08-46 inoculée sur un cultivar sensible de blé dur, et les comparer à ceux de la souche pathogène T01193 inoculée sur un cultivar sensible de blé tendre. D'autre part, on va essayer de caractériser les réactions de défense mises en place par le cultivar étudié de blé dur et les comparer à celles détectées chez le cultivar étudié de blé tendre. Ce travail propose une étude inclusive des mécanismes de défense du blé au niveau moléculaire, biochimique et microscopique. En effet, trois voies de défense vont être explorés à savoir (i) les mécanismes de défense primaire par l'étude des enzymes de dégradation de la paroi fongique tels que les glucanases et les chitinases, (ii) le métabolisme des formes réactives d'oxygène (ROS) par l'investigation des enzymes antioxydants tels que la peroxydase et la catalase, ainsi que la visualisation microscopique de l'accumulation des H₂O₂, (iii) la voie des phénylpropanoïdes par l'examen de l'enzyme clé de cette voie (phénylalanine ammonia lyase, PAL), ainsi que la quantification des composés phénoliques solubles totaux, et la visualisation microscopique du dépôt des polyphénols, (iv) la voie des lipides ou octadécanoïdes par l'examen de l'enzyme clé de cette voie (la lipoxygénase, LOX).

Bien que cet article prend comme modèle d'étude un seul cultivar de chaque espèce de blé en interaction avec une seule souche de champignon adaptée à cette espèce, il pourra permettre de plus nous informer à propos de l'interaction blé-*Z. tritici*, surtout dans le cas du blé dur qui est très peu étudié et le comparer au blé tendre, le modèle d'étude le plus courant. Il pourra aussi nous orienter à propos du choix et la stabilité des SDPs qu'on peut intégrer dans les alternatives de lutte contre la septoriose chez les deux espèces de blé. Néanmoins, elle reste insuffisante et doit être confirmée par d'autres études similaires réalisées sur différents cultivars de chaque espèce en interaction avec plusieurs souches pathogènes de *Z. tritici*.

Publication 1: Similar infection process and induced defense patterns during compatible interactions between *Zymoseptoria tritici* and both bread and durum wheat species

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ABSTRACT

Bread wheat (BW) and durum wheat (DW) are both strongly affected by *Septoria tritici* blotch caused by the hemibiotrophic fungus *Zymoseptoria tritici*. However, only the BW-*Z. tritici* pathosystem has been well studied so far. Here, we compared compatible interactions between *Z. tritici* and both BW and DW species at the cytological, biochemical and molecular levels. Fungal infection process investigations showed close spore germination and leaf penetration features in both interactions, although differences in the patterns of these events were observed. During the necrotrophic phase, disease severity and sporulation levels were associated in both interactions with increases of the two cell-wall degrading enzyme activities endo- β -1,4-xylanase and endo- β -1,3-glucanase as well as protease. An analysis of plant defense responses during the first five days post inoculation revealed inductions of *PR2*, *Chi4 precursor*, *Pox* and *PAL* and a repression of *LOX* gene expressions in both wheat species, although differences in kinetics and levels of induction or repression were observed. In addition, peroxidase, catalase, glucanase, phenylalanine ammonia-lyase and lipoxygenase activities were induced in both wheat species, while only weak accumulations of hydrogen peroxide and polyphenol were detected at the fungal penetration sites. Our study revealed overall a similarity in *Z. tritici* infection process and triggered wheat defense pathways on both pathosystems.

Keywords *Zymoseptoria tritici*, *Mycosphaerella graminicola*, *Triticum aestivum*, *Triticum durum*, infection process, defense responses.

INTRODUCTION

With more than 215 million hectares sowed annually, wheat is the second most widely cultivated cereal in the world and provides around 20% of global calories for human consumption. Two distinct wheat species are mainly cultivated, *i.e.* bread wheat (*Triticum aestivum*, BW) and durum wheat (*Triticum durum*, DW). Both BW and DW are susceptible to a great number of fungal pathogens, including *Zymoseptoria tritici* (teleomorph *Mycosphaerella graminicola*) (Quaedvlieg et

al., 2011), previously named *Septoria tritici*, responsible for Septoria tritici blotch (STB). STB is the most important disease on DW in Tunisia (Berraies et al., 2014) and on BW in France (Gouache et al., 2013), with annual yield losses ranging from 30 to 50% (Eyal et al., 1987; Palmer and Skinner, 2002). *Z. tritici* is a hemibiotrophic pathogen possessing a long symptomless phase considered as biotrophic, which is followed by a necrotrophic phase. This latest phase leads to a tissue collapse, resulting in chlorosis and necrosis development and pycnidium formation on the leaves (Shetty et al., 2003, 2007).

The infection process involves germination of both ascospores and pycnidospores on the leaf surface (Kema et al., 1996). Spore germination occurs either by elongation or budding (Eyal et al., 1987), and leads to the formation of branched germ tubes or clusters of hyphae (Kema et al., 1996; Siah et al., 2010). Sometimes, appressorium-like structures are also differentiated within the penetration sites (Kema et al., 1996; Shetty et al., 2003; Siah et al., 2010). *Z. tritici* has been shown to penetrate the host through the stomata (Kema et al., 1996, Duncan and Howard, 2000; Shetty et al., 2003; Siah et al., 2010) or anticlinal cell wall grooves (Rohel et al., 2001; Siah et al., 2010). Growing exclusively intercellularly, infecting hyphae advance into the mesophyll during an asymptomatic phase without the formation of haustoria or other specialised feeding structures. After the biotrophic phase, *Z. tritici* switches to an aggressive necrotrophic lifestyle that leads to the characteristic lesions bearing spore-producing pycnidia along the leaf surface. This switch may be facilitated by a localized form of wheat programmed cell death, involving a loss of cell integrity and degradation of host macromolecules (Keon et al., 2007). During the necrotrophic phase, the fungus releases cell-wall degrading enzymes (CWDEs) such as endo- β -1,3-glucanases, cutinases and endo- β -1,4-xylanases, as well as proteases, leading to an increase of nutrient content for the fungus in the apoplastic space (Palmer and Skinner, 2002; Keon et al., 2007 ; Siah et al., 2010). It has been shown that the production of endo- β -1,4-xylanase and protease by *Z. tritici* can be correlated with symptom and sporulation levels (Douaiher et al., 2007; Tian et al., 2009; Siah et al., 2010).

During a plant-pathogen interaction, pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs) recognition leads to PAMPs-triggered immunity (PTI), resulting in the induction of basal defense that can halt further colonization. During host-pathogen interactions, the pathogens can counteract plant defenses *via* effector production, resulting in a compatible interaction, except for resistant cultivars that express an Effector-triggered immunity (ETI). This ETI can be considered as an accelerated and amplified PTI response, which is effective against biotrophic and hemibiotrophic pathogens (Jones and Dangl, 2006). However, ETI can differ mechanistically in the case of hemibiotrophic pathogens such as *Z. tritici*, since any programmed cell death was observed during the symptomless phase (Deller et al., 2011). In BW, susceptibility to *Z. tritici* is often described as the result of a lower or delayed defense induction

during the biotrophic phase, recalling an ineffective PTI. Marshall et al. (2011) and Lee et al. (2014) described the inactivation of PTI through the expression of Mg3LysM effector gene, which leads to the suppression of wheat defense chitin signaling. Concerning reactive oxygen species (ROS) metabolism, it has been reported that, in contrast to a susceptible cultivar, an early accumulation of H₂O₂ in a resistant cultivar inhibits infection through the arrest of hyphal growth within wheat leaf tissues, probably through cell wall cross-linking for reinforcement (Shetty et al., 2003). Many defense-related genes are early induced in resistant cultivars such as those encoding for phenylalanine ammonia-lyase, peroxidase, chitinase, and various other pathogenesis-related (PR) proteins (Ray et al., 2003; Adhikari et al., 2007; Shetty et al., 2009; Farsad et al., 2013), whereas only little or no induction of these defense-related genes is observed in susceptible cultivars.

All studies mentioned above investigated mechanisms involved in BW-*Z. tritici* interaction, whereas knowledge about the interaction between DW and *Z. tritici* is sparse. The objective of the present study was to characterize for the first time the fungal infection process and associated plant defense mechanisms during a compatible DW-*Z. tritici* interaction in comparison with a compatible BW-*Z. tritici* interaction. Our investigations were performed using a French BW cultivar (cv. Premio) inoculated with a French BW-adapted strain (T01193) and a Tunisian DW cultivar (cv. Karim) inoculated with a Tunisian DW-adapted strain (St-08-46). For this purpose, different aspects of these interactions were investigated at the molecular, biochemical and cytological levels. First, *Z. tritici* infection process was monitored through several parameters, such as spore germination, leaf penetration, mesophyll colonization, pycnidium formation and production of CWDE activities. Then, the induction of various wheat defense responses was studied during the early stages of infection through quantification of gene expression and enzyme activities involved in fungal cell wall degradation (endo- β -1,3-glucanase and chitinase), ROS metabolism (peroxidase and catalase), phenylpropanoid (phenylalanine ammonia lyase) and octadecanoid (lipoxygenase) pathways. H₂O₂ accumulation and polyphenol deposition were also monitored. Relationships between gene expression, enzyme activities and cytological response were discussed in both pathosystems.

MATERIALS AND METHODS

Plant material

Cultivars of the two wheat species known to be susceptible to STB were used in this study: the French BW cv. Premio and the Tunisian DW cv. Karim. Grains were pre-germinated in Petri dishes (12 x 12 cm) on moist filter paper in darkness at 20°C for 48 h. Germinated grains were then transplanted into compost filled in pots of 18 cm-diameter (12 grains/pot). The pots were placed in the greenhouse at 18°C and under day-night cycles of 16h/8h.

Fungal material and inoculation

Two *Z. tritici* strains were used in this study: the French strain T01193, isolated in 2009 from BW, which was used for the inoculation of the BW cultivar Premio and the Tunisian strain St-08-46, isolated in 2008 from DW, which was used for the inoculation of the DW cultivar Karim. Fungal cultures were grown on potatoes dextrose agar (PDA) medium for 10 days at 18°C and were stored at -80°C for further use. Plants were inoculated with 30 mL of a 10⁶ spores mL⁻¹ suspension in a 0.05% of Tween 20 (Sigma Aldrich, USA) in tap water using a hand sprayer. Control plants were sprayed with tap water containing 0.05% of Tween 20. Immediately after inoculation, each pot was covered with a clear polyethylene bag for 3 days in order to ensure a water-saturated atmosphere. Disease level was scored at 22 days post inoculation (dpi) by measuring percentage of leaf area with symptoms (necrosis) and the index of pycnidium coverage (which describes the importance of pycnidia in necrosis following a scale noted from 1 to 5) on the 3rd leaf of 25 plantlets (Siah et al., 2010). Data represent means of two independent experiments.

RNA extraction and analysis of gene expression by semi-quantitative RT-PCR and real-time RT-PCR

For plant gene expression study, three 3rd leaves were used as independent replicates. Samples were collected at 0, 1, 2 and 5 dpi. Total RNA was extracted from 100 mg of plant tissue using RNeasy Plant Mini Kit (Quiagen, The Netherlands). Genomic DNA contaminating the samples was removed by treatment with DNase using RNase-Free DNase Set (Quiagen, The Netherlands). Reverse transcription of total RNA was carried out using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA) according to the manufacturer's protocol. PCR reactions were conducted on subsequent obtained cDNA to amplify five wheat genes encoding for *PR2* (β -1,3-glucanase), *Chi4 precursor* (chitinase class IV precursor), *Pox* (peroxidase), *PAL* (phenylalanine ammonia-lyase) and *LOX* (lipoxygenase). *TUB* (β -tubulin) and *ACT* (actin) encoding genes were used as housekeeping genes. These analyses were performed using the gene-specific primers presented in Table 1. Primers pairs of *PR2*, *Chi4 precursor* and *Pox* were newly designed using the Primer Express® program tested for secondary structure using Net Primer® program. Gene expression was investigated by semi-quantitative Reverse-Transcription Polymerase Chain Reactions (RT-PCR) and was compared to *TUB* gene as reference. PCR was carried out in a 20 μ L reaction mixture containing 1x PCR buffer, 2 mM dNTPs, 10 μ M of each primer, 0.5 unit of Taq polymerase and 20 ng of cDNA using the following protocol: 94°C for 5 min, followed by 30 cycles each with 94°C for 1 min, 55°C for 1 min and 72°C for 1 min. The PCR reaction was terminated at 72°C for 7 min. PCR products were analyzed by electrophoresis on ethidium-bromide stained 1.5% agarose gels run in 0.5 x Tris-acetic acid-EDTA buffer at 100 V for 45 min. Gels were

visualized under UV light. The experiment was repeated three times and only results which were repeated at least twice were considered. Expression of *PAL* and *LOX* was performed by real-time RT-PCR analysis with pair of primers selected from previous published studies (Tayeh et al., 2013; 2015). Real-time RT-PCR (SYBR Green technique) was performed using ABI Prism 7300 detection system (Applied Biosystems, USA), using the following thermal profile : after incubation for 10 min at 95°C, 15s at 95°C (denaturation), 1 min at 60°C (annealing/extension) and 30s at 72°C (data acquisition) for 40 cycles. After real-time PCR, a melting curve was generated to verify the specificity of each amplification reaction. The experiment was conducted on the third leaves, in triplicate and only results which were repeated at least twice were considered. The results were normalized with the *TUB* and *ACT* genes and expressed relatively to the respective control, for each time point, corresponding to a fixed value of 1.

Table 1 Primers used in the semi-quantitative and quantitative reverse-transcription polymerase chain reactions studies

Gene	Primer sequences (5'-3')	Accession numbers
<i>TUB</i> (<i>beta tubulin</i> , housekeeping gene)	GGAGTACCCTGACCGAATGATG AACGACGGTGTCTGAGACCTTT	U76895
<i>ACT</i> (<i>actin</i> , housekeeping gene)	AACCTTCAGTTGCCAGCAA TGTTTCGACCGCTGGCATAAC	AB181991
<i>PR2</i> (β -1,3 glucanase)	CTCGACATCGGTAACGACCAG GCGGCGATGTACTTGATGTTT	Y18212
<i>Chi4 precursor</i> (<i>chitinase class IV precursor</i>)	TTCTGGTTCTGGATGACCAAC ACTGCTTGACAGTACTCCGTG	AF112966
<i>Pox</i> (<i>peroxidase</i>)	GAGATTCCACAGATGCAAACGAG GGAGGCCCTTGTTTCTGAATG	X56011
<i>PAL</i> (<i>phenylalanine ammonia lyase</i>)	CCCCCATTTGGTGTCTCCAT ACTGCGGAACATCAGCTT	AY005474
<i>LOX</i> (<i>lipoxygenase</i>)	GGGCACCAAGGAGTACAAGGA GCTCGTGATGGTGTGGATGA	U32428

Protein extraction and enzymatic assays

Three cell-wall degrading enzyme (CWDE) activities (endo- β -1,4-xylanase, endo- β -1,3-glucanase and protease) were measured at 4, 10, 16 and 22 dpi. Briefly, 2.5 g of third leaves were sampled and immediately frozen in liquid nitrogen for grinding in mortar. Endo- β -1,4-xylanase activity was measured at pH 4.8 and 45°C using xylan (1%, w/v) (Sigma Aldrich, USA) as substrate and xylose (10 mM) (Sigma Aldrich, USA) as reducing control sugar (Siah et al., 2010). Endo- β -1,3-glucanase activity was measured at pH 4.8 and 50 °C using Laminarin (Sigma Aldrich, USA) at pH 5 (0.5 %, w/v) as substrate and glucose (10 mM) as reducing control sugar (Douaiher et al., 2007). Absorbance was measured at 540 nm. One unit of endo- β -1,4-xylanase and endo- β -1,3-glucanase activities is defined as the amount of enzyme necessary to release 1 μ mol reducing sugar/p-nitrophenol $\text{min}^{-1} \text{mL}^{-1}$ of enzyme solution. Protease activity assays were assessed according to

Hellweg (2003). Absorbance was measured at 400 nm. One unit of protease activity is defined as a change in enzyme activity of 1 per hour. These activities were expressed in mU mg⁻¹ protein.

Five plant enzyme activities involved in plant defense mechanisms were measured at 0, 1, 2 and 5 dpi: β -1,3-glucanase (GLUC), catalase (CAT), peroxidase (POX), lipoxygenase (LOX) and phenylalanine ammonia-lyase (PAL). Each sample consisted of three medial segments excised from three third leaves. GLUC activity was analyzed according to Douaiher et al. (2007). CAT activity was assessed according to Randoux et al. (2010); one unit of CAT activity is defined as the amount of the enzyme which degrades H₂O₂ at 1 μ mol min⁻¹, ($\epsilon = 36 \text{ mM}^{-1} \text{ cm}^{-1}$). POX was estimated according to El Hadrami and Baaziz (1995) and expressed as nkat mg⁻¹ protein. One unit of POX activity is defined as the amount of enzyme that catalyses the conversion of guaiacol as substrate after 2 min of H₂O₂ addition. LOX activity was measured according to Randoux et al. (2010). One unit of LOX activity is defined as the amount of enzyme that catalyses the conversion of substrate (linoleic acid) at 1 mol s⁻¹ ($\epsilon = 23 \text{ mM}^{-1} \text{ cm}^{-1}$). PAL activity was calculated as described by Randoux et al. (2010). One unit of PAL activity is defined as the amount of enzyme that catalyses the formation of cinnamic acid at 1 mol s⁻¹ ($\epsilon = 10 \text{ mM}^{-1} \text{ cm}^{-1}$). CAT, POX, LOX and PAL activities were expressed in U mg⁻¹ protein.

Total protein concentration was measured at absorbance of 595 nm using bovine serum albumin as a standard (Bradford, 1976). For all tested activities, absorbance was measured using Thermo Scientific™ GENESYS 10S UV-Vis spectrophotometer. Data represent means of two independent experiments. Three independent replicates, which consisted on three 3rd leaves, were carried out for each experiment.

Cytological analysis

Two-cm long wheat leaf segments were harvested 1, 4 dpi and 22 dpi from inoculated and control H₂O-treated plants. Spore germination, penetration attempts, mesophyll colonization and pycnidial formation were assessed at 1, 4 or 22 dpi, using Trypan blue staining and/or Fluorescence Brightener 28 Calcofluor (Sigma Aldrich, USA) according to Siah et al. (2010). The accumulation of H₂O₂ was revealed by 3,3-diaminobenzidine (DAB) staining (red-brown staining) according to Randoux et al. (2010). Cytological deposition of the auto-fluorescent polyphenol deposition was visualized using a Nikon microscope (Champigny-sur-Marne, France) fitted for fluorescence (blue exciter filter, maximum transmittance 400 nm and barrier filter with a transmittance range of 500 to 800 nm). As described by Randoux et al. (2010), a yellow fluorescence visualized under UV-light microscopy indicates the presence of polymeric phenolic compounds. The visualization of H₂O₂ accumulation and polyphenol deposition were performed in 2-cm-long segment of third leaves, which was collected from the same plantlets used for enzymatic and molecular analyses and at the

same time points (0, 1, 2 and 5 dpi). Data represent means of two independent experiments. Three independent biological replicates were carried out for each experiment.

Phenolic compounds quantification

The quantification of the soluble phenolic compounds was determined according to the Folin-Ciocalteu method modified by El Hadrami et al. (1997). Samples were collected in the same conditions used for the enzymatic analysis. Foliar tissues (200 mg from 3rd leaf), were homogenized in an ice bath with 1.5 ml methanol. The homogenate was centrifuged three times at 10 000×g for 5 min; supernatants were recuperated each time. One hundred microliters of the supernatant was added to Folin-Ciocalteu reagent and sodium carbonate. The mixture was incubated at 40°C for 30 min and the blue color was read at 760 nm using catechin (Sigma Aldrich, USA) as a standard. The content of soluble phenolic compounds was expressed in mg-equivalents of catechin per gram of fresh weight (FW). Data represent means of two independent experiments. Three independent replicates were carried out for each experiment.

Data analyses

Statistical analyses of molecular and enzymatic assays were performed separately for each pathosystem (BW cv. Premio vs T01193 strain and DW cv. Karim vs St-08-46 strain), while microscopy and disease severity parameters were compared among the two pathosystems. For quantitative gene expression, the analyses were performed using the relative expression software tool REST® as described by Pfaffl et al. (2002) and the significant differences were tested at 95% ($P < 0.05$). For severity, enzymatic activities and microscopy parameters, an analysis of variance (ANOVA) was performed using the Tukey test at $P \leq 0.05$ using the XLSTAT software (Addinsoft, France).

RESULTS

Fungal infection process and CWDE activities

In order to investigate the infection process of T01193 and St-08-46 *Z. tritici* strains inoculated respectively on the BW cv. Premio and DW cv. Karim, microscopic observations were performed at 1, 4 and 22 dpi (Fig. 1). At 1 dpi, spores of T01193 formed germ tubes mainly at their tips (Fig. 1a), whereas the majority of spores of St-08-46 initiated three or four germ tubes simultaneously from the middle of the spore (Fig. 1b). However, no significant difference was observed regarding the spore germination rate of both strains (Table 2).

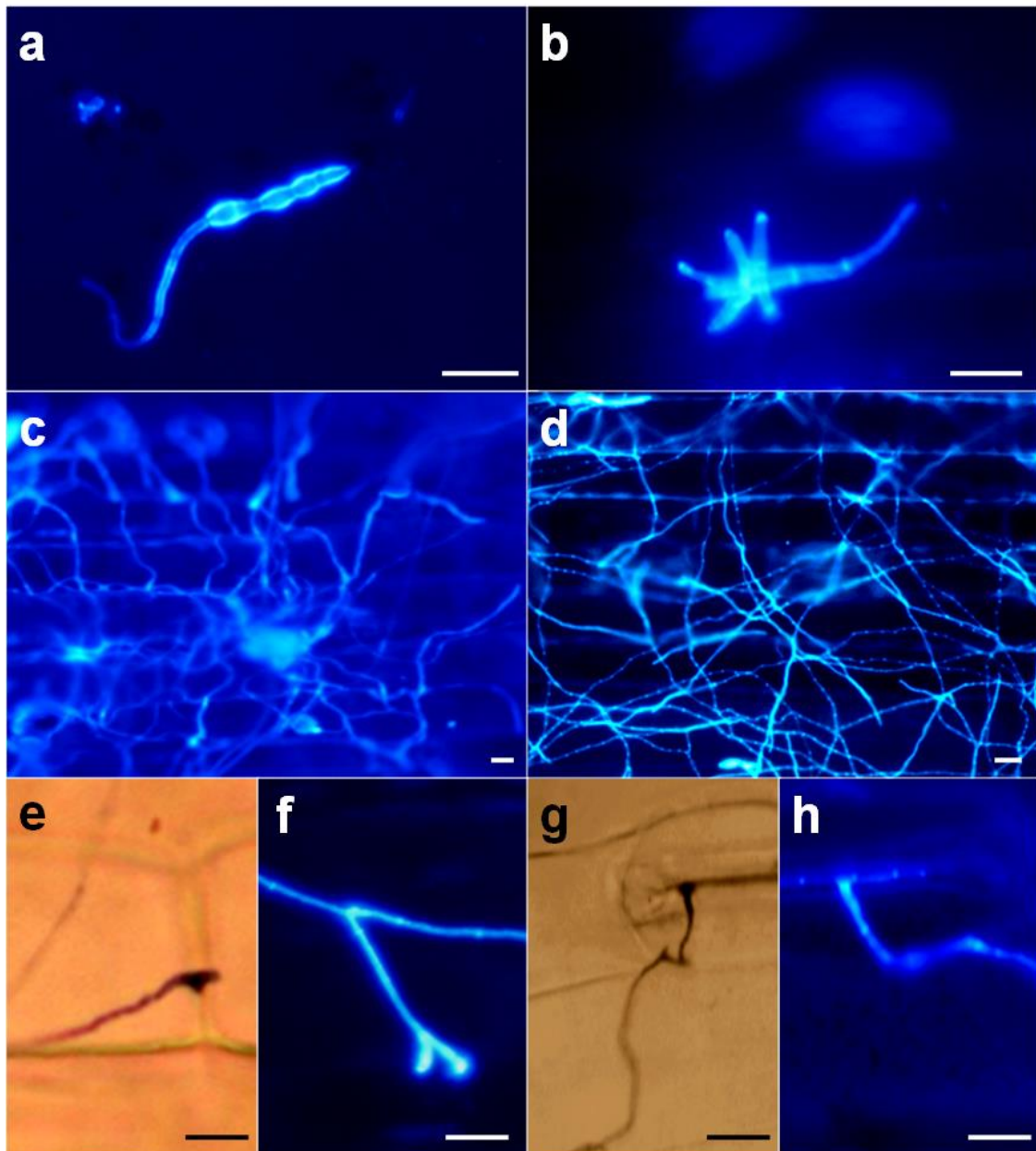


Fig. 1 Microscopic observation of spore germination, growth and penetration attempt of T01193 *Z. tritici* strain on the susceptible BW cv. Premio and St-08-46 *Z. tritici* strain on the susceptible DW cv. Karim. **a, b** spore germination of T01193 and St-08-46 strains observed at 1 dpi using Calcofluor fluorescence. **c, d** hyphal growth of T01193 and St-08-46 strains observed at 4 dpi using Calcofluor fluorescence. **e, g** direct penetration and stomatal penetration attempt of T01193 germ tube observed at 4 dpi using Trypan blue staining. **f, h** direct penetration and stomatal penetration attempt of St-08-46 germ tube observed at 4 dpi using Calcofluor fluorescence. Scale bar = 10 μ m

At 4 dpi, we observed a more extensive hyphal growth of St-08-46 (Fig. 1d), compared to T01193 (Fig. 1c). Both strains formed appressorium-like structures by swelling of the hyphae at the tip of germ tubes (Fig. 1e, f). These structures occurred at (i) the ridges of guard cell lips of stomata (Fig. 1g, h) which could lead to a stomatal penetration, and (ii) on both anticlinal (Fig. 1e) and periclinal (Fig. 1f) cell walls of adjacent epidermal cells which could lead to a direct penetration. The rate of spore germ tubes leading to stomatal penetration was 1.8-fold higher in BW than in DW

leaves, while spore germ tubes leading to a direct penetration was 2.8-fold higher in DW than in BW leaves at 4 dpi (Table 2).

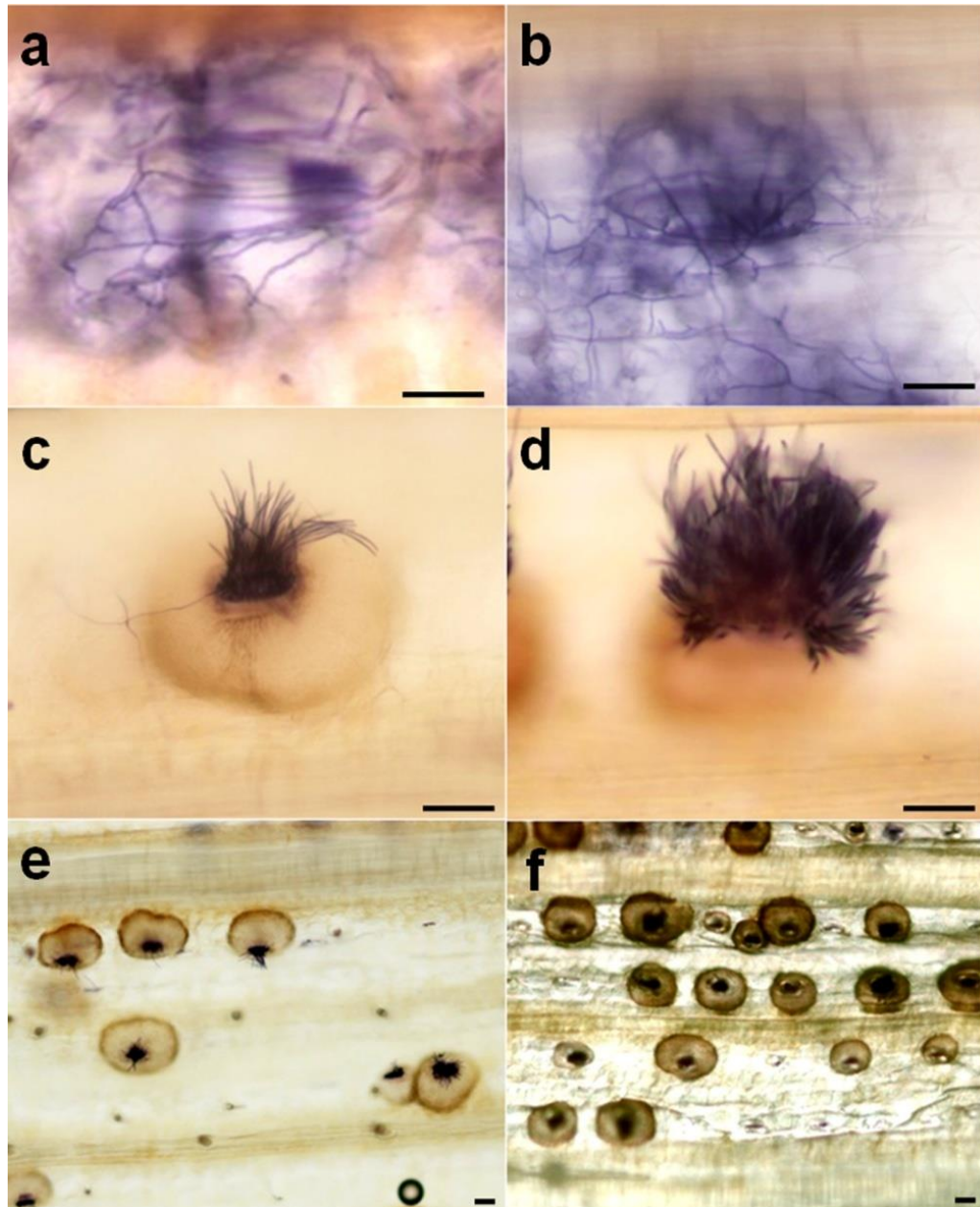


Fig. 2 Microscopic observation of mesophyll colonization, pycnidium structure and coverage of T01193 *Z. tritici* strain on the susceptible BW cv. Premio and St-08-46 *Z. tritici* strain on the susceptible DW cv. Karim at 22 dpi using Trypan blue staining. **a, b** mesophyll colonization by T01193 and St-08-46 strains. **c, d** pycnidia structure of T01193 and St-08-46 strains. **e, f** necrotic lesions bearing pycnidia on BW infected by T01193 strain and DW infected by St-08-46 strain. Scale bar = 10 μ m

At 22 dpi, both tested strains exhibited extensive intercellular hyphae with longitudinal and transversal growth between mesophyll cells (Fig. 2a,b). Hyphae usually aggregated within the substomatal cavities and eventually led to mature pycnidia bearing pycnidiospores (Fig. 2c,d). In DW leaves, pycnidia were darker (Fig. 2f) than BW leaves (Fig. 2e). The rates of uncolonized

stomata and colonized stomata that were not transformed into pycnidia were 1.5- and 1.4-fold higher in BW than in DW, respectively. Consequently, colonized stomata that were transformed into pycnidia were 1.3-fold less abundant in BW in comparison to DW leaves (Table 2).

Table 2 Percentages of different cytological parameters measured at 1, 4 and 22 dpi with T01193 and St-08-46 *Z. tritici* strains, respectively

Parameters (%)	dpi	T01193 / BW	St-08-46 / DW
GS	1	54.0±3.7 a	72.0±11.5 a
NGS	4	25.3±2.1 a	20.4±3.5 a
SP		17.9±1.7 a	9.8±0.2 b
DP		10.3±4.5 b	28.9±5.9 a
GSNP		46.4±3.3 a	41.0±7.2 a
NCS	22	20.6±3.5 a	13.8±0.2 b
CS		29.9±2.7 a	21.6±2.9 b
P		49.5±3.9 b	65.1±3.0 a

Data are mean values of two independent experiments (\pm s.d). Means tagged with the same letter are not statistically different between both wheat species (Tukey test, $P < 0.05$). GS: germinated spores, NGS: non germinated spore, DP: spore leading to a direct penetration, SP: spore leading to a stomatal penetration, GSNP: germinated spore without penetration, NCS: non-colonized stomata, CS: colonized stomata but not yet transformed into pycnidia, P: colonized stomata transformed into pycnidia

Disease severity assessment showed that the percentage of leaf area with symptoms and the index of pycnidium coverage were measurable from 16 dpi and showed maxima at 22 dpi (Table 3). Before 16 dpi, we didn't detect any visual symptoms of *Z. tritici* infection on both wheat species. Between 16 dpi and 22 dpi, the percentage of leaf area with symptoms and the index of pycnidium coverage increased by 2.1- and 1.4-folds on BW leaves and by 2- and 1.6-folds on DW leaves, respectively. At 22 dpi, both parameters were 1.2-fold more frequent on DW than on BW. Overall, disease establishment with St-08-46 strain appeared as more extensive and faster on DW leaves when compared to T01193 strain on BW leaves. Both CWDE activities, endo- β -1,4-endoxylanase and endo- β -1,3-glucanase, as well as protease activity, were measured at 4, 10, 16 and 22 dpi (Table 3). These three activities increased in both infected wheat species, but earlier in DW than in BW. Then, a significant production of both endo- β -1,3-glucanase and protease activities was observed at 16 dpi in infected leaves of DW only. At 22 dpi, endo- β -1,4-xylanase was 38-fold and 35-fold higher in inoculated BW and DW leaves compared to control leaves, respectively. Similar evolution was observed for both endo- β -1,3-glucanase and protease activities. Endo- β -1,3-glucanase activity was 12-fold and 8.5-fold higher at 22 dpi in inoculated BW and DW leaves compared to control leaves, respectively and protease activity was 176-fold and 130-fold higher in inoculated BW and DW leaves compared to control leaves, respectively.

Table 3 Evolution of disease severity parameters and CWDE activities in BW and DW at 4, 10, 16 and 22 dpi with T01193 and St-08-46 *Z. tritici* strains, respectively

Parameters	dpi	T01193 / BW		St-08-46 / DW	
		Control	T01193	Control	St-08-46
Severity of disease					
% of leaf with symptoms	4	-	nd	-	nd
	10	-	nd	-	nd
	16	-	23.8±14.6 c	-	29.4±14.7 c
	22	-	48.5±18.2 b	-	60.6±18.2 a
Index of pycnidium coverage	4	-	nd	-	nd
	10	-	nd	-	nd
	16	-	2.5±1.4 c	-	2.8±1.2 c
	22	-	3.5±1.1 b	-	4.4±0.7 a
CWDEs (mU mg ⁻¹ protein)					
Endo-β-1,4-xylanase	4	27.7±1.2 b	32.4±4.1 b	44.3±12.4 c	48.2±3.2 c
	10	14.5±3.8 b	30.5±3.1 b	33.1±5 c	60.1±2.5 bc
	16	15.3±12.1 b	110.8±70.9 b	30.6±19.9 c	246±36.6 b
	22	23.0±12.8 b	879.6±263.4 a	26.7±27.9 c	941.8±294.8 a
Endo-β-1,3-glucanase	4	34.2±4 b	38.6±2.8 b	152.8±26.7 c	170.3±23.7 c
	10	130±9.2 b	175.5±19 b	146.6±8.7 c	173.6±13.6 c
	16	101.9±11 b	221.3±111.8 b	127.8±35.9 c	345.4±43.1 b
	22	93.5±23.3 b	1136.3±472.2 a	120.3±26.9 c	1026.4±128 a
Protease	4	460.6±115.2 b	211.8±58.2 b	140.8±28.9 bc	166.5±31.6 bc
	10	88.3±53.4 b	425±62.5 b	98.8±87.1 bc	156.7±49.9 bc
	16	80.3±112.3 b	1108.3±338.1 b	59.4±12.7 bc	1740±1241.4 b
	22	64.2±42.5 b	11317.3±7854.2 a	50.6±35.9 c	6587.3±2689.6 a

(nd): not detected. Data are mean values of two independent experiments (±s.d). For severity parameters, statistics were realized for both wheat species and means tagged with the same letter are not statistically different between both wheat species (Tukey test, $P < 0.05$). For CWDE activities, statistics were realized separately for each wheat species and means tagged with the same letter are not statistically different in the same wheat species (Tukey test, $P < 0.05$).

Expression pattern of wheat defense-related genes

The expression of defense-related genes was monitored in BW and DW leaf tissues at 0, 1, 2 and 5 dpi. Semi-quantitative RT-PCR assay showed that *PR2*, *Chi4 precursor* and *Pox* were up-regulated in both wheat species in response to fungal infection, but with different expression patterns (Fig. 3). In BW leaves, *PR2* and *Pox* were up-regulated in response to infection at all tested time points, whereas the *Chi4 precursor* expression was highly induced at 1 and to a less extent at 2 dpi (Fig. 3a). On infected DW plants, *PR2*, *Chi4 precursor* and *Pox* transcript analysis revealed a transient up-regulation at 1 dpi (Fig. 3b). Quantitative RT-PCR analysis revealed that *LOX* expression was down-regulated the first two days post infection (1-2 dpi) in both wheat species; the repression reached at least 11- and 30-folds in BW and DW leaves, respectively (Fig. 4a). By contrast, *PAL* expression exhibited a slight transient induction with a relative expression close to 5.3 in BW leaves only, at 2 dpi (Fig. 4b).

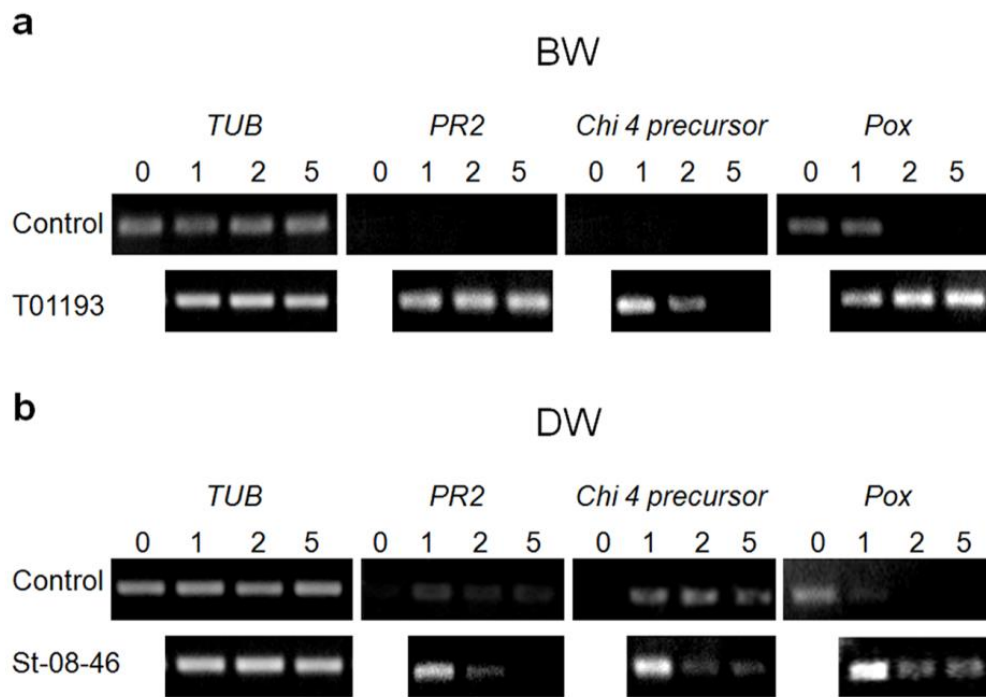


Fig. 3 Time course of *PR2*, *Chi4 precursor* and *Pox* expressions analyzed by semi-quantitative RT-PCR analysis on BW **a** and DW **b** leaves at 0, 1, 2 and 5 dpi with T01193 and St-08-46 strains. Leaf tissues for the 0 dpi were collected just prior inoculation. RT-PCR products were generated using primers specific for β -tubulin (*TUB*), *PR2*, *Chi 4 precursor* and *Pox* first-strand cDNAs obtained from total wheat RNA, and visualized on agarose gel (1.5%). *TUB*-encoding gene was used as a house-keeping gene. Experiment was repeated three times and only results which were similar from two independent experiments were considered

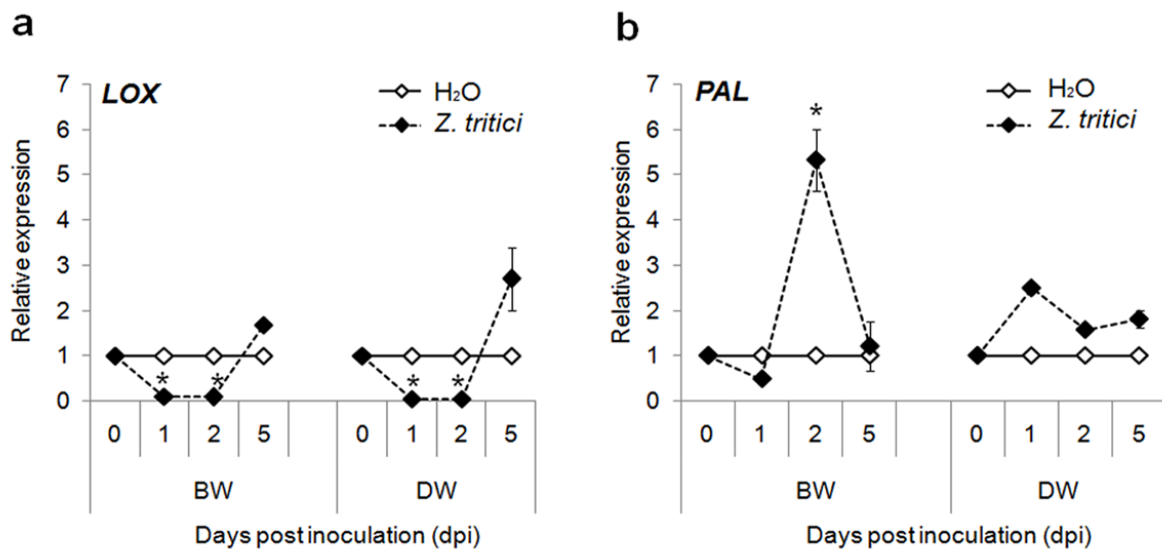


Fig. 4 Time course of *LOX* **a** and *PAL* **b** gene expressions analyzed by qRT-PCR analysis on BW and DW leaves at 0, 1, 2 and 5 dpi with T01193 and St-08-46 *Z. tritici* strains, respectively. Leaf tissues for the 0 dpi were collected just prior inoculation. Gene expression at each time point is represented relatively to the corresponding control and normalized *TUB* and *ACT*-encoding gene expression. The experiment was conducted in triplicate. Similar results were obtained at least twice. Results correspond to means (\pm s.d) of two technical replications from one biological replicate. Means of infected samples designed by asterisks are statistically differentially regulated (up- or down-) in comparison to their corresponding non-infected controls using Tukey test at $P \leq 0.05$

Plant enzymatic activities

In non-infectious conditions, GLUC, POX, CAT and PAL basal activities were almost similar between both wheat species, while basal LOX activity in BW was slightly higher than in DW leaves (Fig. 5).

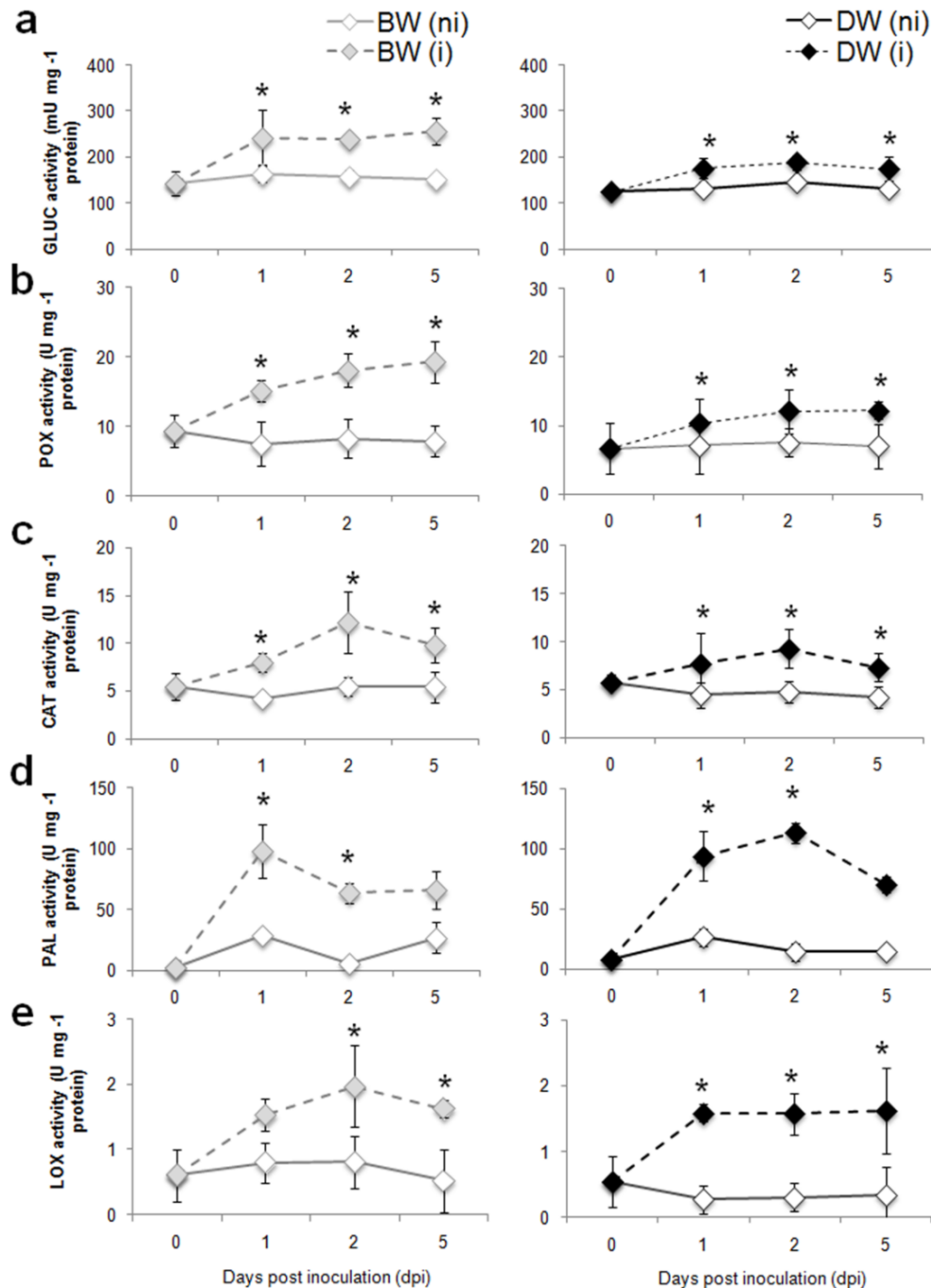


Fig. 5 Time course of five enzymatic activities on BW and DW leaves at 0, 1, 2 and 5 dpi with T01193 and St-08-46 *Z. tritici* strains, respectively. **a** β -1,3-glucanase (GLUC). **b** Catalase (CAT). **c** Peroxidase (POX). **d** Phenylalanine ammonia-lyase (PAL). **e** Lipoxygenase (LOX). Leaf tissues for the 0 dpi was collected just prior inoculation. Results correspond to means (\pm s.d) of two independent experiments using three independent biological replicates for each experiment. Means of infected samples designed by asterisks are significantly different from their corresponding non-infected controls using the Tukey test at $P \leq 0.05$

After infection, GLUC, POX, CAT and PAL activities significantly increased from 1 dpi in both species, and reached overall higher levels in BW leaves compared to DW leaves. GLUC, POX and CAT activities were induced at all tested time points with averages of 1.6-, 2.2- and 2-fold increases on BW leaves in response to infection by T01193 and with averages of 1.3-, 1.6- and 1.8-folds on DW leaves in response to infection by St-08-46. In addition, PAL activity was induced at 1 and 2 dpi with an average of 5.8- and 7.2-folds on BW and DW leaves, respectively. However, the induction of LOX activity was lower in infected BW leaves (with an increase average of 2.5-fold at 2 and 5 dpi) than in infected DW leaves (with an average of 5.3-fold at 1, 2 and 5 dpi) (Fig. 5).

H₂O₂ accumulation in the mesophyll

At the cytological level, H₂O₂ accumulation was revealed by DAB staining in BW and DW leaves (Fig. 1a, Annex 1). Four classes of events were distinguished: C1 (spore with non-penetrated germ tube), C2 (spore with penetrated germ tube without staining), C3 (penetrated germ tube with stained papilla), and C4 (penetrated germ tube with at least 1 whole stained mesophyll cell). Penetrated germ tubes with papilla or with one stained cell were observed from 2 dpi in very rare cases. The frequencies of all studied classes were not significantly different between both studied wheat species at all studied time points (Annex 1, Fig. 1b).

Polyphenol accumulation

Both local polyphenol-associated auto-fluorescence and total polyphenol content were evaluated. Local polyphenol deposition may be revealed as clear yellow fluorescence at the penetration sites by the fungus. Three types of events were observed and grouped by class, such as spore with non-penetrated germ tube (C1), spore with penetrated germ tube without fluorescence (C2), and penetrated germ tube with weak fluorescence (C3) (Fig. 2a, Annex 1). The frequencies of all studied classes were not significantly different at all monitored time points for both wheat species (Fig. 2b, Annex 1). On the other hand, the effect of the fungus on the total phenolic compound content was measured in non-infectious (ni) and in infectious (i) contexts (Table 4). No significant difference was observed between infected and control leaves on both wheat species. However, the basal content of phenolic compounds was always higher in BW leaves than in DW leaves (Table 1, Annex 3).

DISCUSSION

The infectious process of T01193 and St-08-46 *Z. tritici* strains was investigated by microscopy during a compatible interaction with the BW susceptible cv. Premio and the DW susceptible cv. Karim, respectively, in greenhouse conditions. The specificity of each *Z. tritici* strain to

corresponding wheat species was previously checked in same experimental conditions (data not shown).

Both fungal strains showed similar percentages of spore germination, while we detected some differences in the mode of germination. Regarding host penetration, both stomatal and direct penetrations were observed on both wheat species. Weber (1922) first reported that only direct penetrations took place, and more recently, Cohen and Eyal (1993), Kema et al. (1996) and Duncan and Howard (2000) observed that host penetration was strictly stomatal in susceptible BW plants. However, direct penetration attempts have also been reported (Rohel et al., 2001; Shetty et al., 2003). In our experiments, BW leaves infected by the T01193 strain exhibited more frequent leaf penetration attempts through the stomata, than those occurring through the cuticle and epidermal cell walls, unlike DW leaves infected by St-08-46 strain. However, appressorium-like swellings were differentiated by both strains at the penetration sites, whatever the penetration features. Such swellings, which could function as appressoria, have been previously reported (Kema et al., 1996; Duncan and Howard, 2000; Shetty et al., 2003; Siah et al., 2010). Just after penetration, the fungus behaves as a symptomless endophyte. In general, hyphae colonize intercellular spaces without causing cell death before necrosis and sporulation occurrence until 10 dpi, depending on growth conditions (Kema et al., 1996). For both wheat species, hyphae were strictly ectotrophic, and no haustoria were formed. Hyphae were observed between the epidermal cells, below the epidermal layer of cells and in the intercellular spaces of the mesophyll with longitudinal and transversal growth, as previously shown in BW by Shetty et al. (2003) and Siah et al. (2010). From 16 dpi, the investigation of the time-course of disease severity in our study showed that the percentage of leaf area with symptoms and the index of pycnidium coverage progressed in a similar manner, even though DW leaves exhibited faster infection and more extensive fructification than BW leaves. In sum, we presented here an example of compatible interactions of *Z. tritici* on BW and DW, which present similar infectious process with some differences in the mode of spore germination and leaf penetration attempts. These last features have to be checked in future studies, using various isolates of *Z. tritici* on each tested wheat species.

During the necrotrophic phase, the fungus invests more effort in producing its putative plant cell wall-attacking capability (Rudd et al., 2015). Several works suggested that CWDEs are important in *Z. tritici* pathogenicity and mediate disease symptom development through the breakdown of wheat leaf cell walls (Kema et al., 1996; Douaiher et al., 2007; Siah et al., 2010). Numerous CWDEs, such as endo- β -1,4-xylanases, endo- β -1,3-glucanases, and proteases have been identified in *Z. tritici* (Palmer and Skinner, 2002; Goodwin et al., 2011). Moreover, Tian et al. (2009) showed that endo- β -1,4-endoxylanase and protease activities are highly associated with susceptibility level of BW wheat cultivar. In our study, three activities (endo- β -1,4-xylanase, endo-

β -1,3-glucanase and protease), and mainly protease drastically increased during the necrotrophic phase of both tested strains, corresponding to the significant increase of necrosis development and pycnidium formation. Likewise, Rudd et al. (2015) observed the maximal secreted protease response of the fungus at the transition to disease symptoms, through an important accumulation of transcripts during compatible interaction between BW and *Z. tritici*. Thus, both tested pathosystems revealed a similar kinetic for studied CWDE activities. Nevertheless, on DW plants, the stimulation of fungal endo- β -1,4-xylanase and endo- β -1,3-glucanase activities occurred faster than on BW plants with an increase of these activities from 16 dpi. This faster induction of CWDEs on DW plants during the necrotrophic phase may explain the more important disease expression observed on DW compared to BW plants. The correlation between endo- β -1,4-xylanase activity and disease severity in *Z. tritici* was previously demonstrated *in vitro*, on detached BW leaves (Douaiher et al., 2007). *In planta*, Siah et al. (2010) described on BW a correlation between *Z. tritici* pathogenicity and the ability of the fungus to induce endo- β -1,4-xylanase activity.

Wheat gene expression assay highlighted an induction of two PR protein genes (*PR2* and *chi 4 precursor*) on both wheat species. The *chi 4 precursor* gene encodes a chitinase precursor class IV, which was known to hydrolyze β -1,4-glycoside bonds in chitin to produce N-acetylglucosamine and N, N+ diacetylchitobiose (Cohen-Kupiec and Chet, 1998). In infectious conditions, the expression of *chi 4 precursor* was weakly and transiently induced at 1 dpi in the leaves of both species. Several works reported weak transcript accumulation of chitinase class II (Adhikari et al., 2007) and chitinase class I (Yang et al., 2013) in response to *Z. tritici* inoculation in BW susceptible cultivars. Moreover, Ray et al. (2003), Adhikari et al. (2007) and Shetty et al. (2009) reported the involvement of chitinase in wheat defense against *Z. tritici*. The second targeted gene, *PR2*, encodes for a class II β -1,3-glucanase, which are acidic proteins secreted into the extracellular space. These proteins catalyze hydrolytic cleavage of the 1,3- β -D-glucosidic linkages in β -1,3 glucan (Ryals et al., 1996). In our conditions, this gene was differentially regulated in both wheat species. *PR2* gene expression was induced in BW leaves from 1 to 5 dpi, while it was only transiently induced at 1dpi in DW leaves. It has been shown that another gene encoding PR2 was induced on BW to higher levels during an incompatible interaction with *Z. tritici* than during a compatible one (Ray et al., 2003). Associated to the *PR2* gene up-regulation, a significant increase of total β -1,3-glucanase activity was observed in the infected leaves of both wheat species. By comparison between the period of *PR2* up-regulation (1 dpi) and the production of β -1,3-glucanase activity (1 to 5 dpi) in DW leaves, these results suggested that another gene encoding for β -1,3-glucanase may be strongly induced in leaf tissues during infection. Chitinases and β -1,3-glucanases have received particular attention because they are thought to contribute to the state of resistance (Shetty et al., 2009) and are known to exert synergistic antifungal effects when both enzymes are present (Mauch et al.,

1988). It is important to note that we measured gene expression and the total β -1,3-glucanase activity in infected leaves at an early biotrophic phase of *Z. tritici* development. Once fungal penetration has occurred, plant chitinases and β -1,3-glucanases secreted in intracellular space can release fungal chitin and β -1,3-glucan fragments from the pathogen and/or the host cell walls, regarded as PAMPs or DAMPs. These fragments may play an important role in the amplification of defense reactions and can therefore lead to better plant resistance. In this sense, purified β -1,3-glucan fragments isolated from cell walls of *Z. tritici* gave complete protection against this pathogen (Shetty et al., 2009).

ROS metabolism is a key element of plant defense responses, including PTI and ETI. ROS act as direct antimicrobial molecules or secondary messengers, and could be involved in metabolic pathways leading to resistance. Among them, H_2O_2 is described as a major determinant of defense reactions of wheat against *Z. tritici* during the biotrophic phase of the interaction (Shetty et al., 2003; 2007). During the necrotrophic phase, H_2O_2 accumulates, and even if the pathogen does not benefit from the released H_2O_2 , it can tolerate it (Shetty et al., 2007; Keon et al., 2007). Shetty et al. (2003) and Yang et al. (2013) detected, in a susceptible BW cultivar, a weak early H_2O_2 accumulation following *Z. tritici* infection. In our study, we detected weak H_2O_2 accumulation in papillae at penetration sites by the fungus from 2 to 5 dpi mainly on both wheat species. However, we investigated H_2O_2 metabolism in response to *Z. tritici* infection through POX and CAT activity assays. First, CAT is an H_2O_2 -scavenging enzyme. We detected a significant increase of CAT activity in both BW and DW leaves. Similarly, Diani et al. (2009) reported an increase in CAT activity in susceptible cultivars of BW from the fourth day post inoculation. These results suggest that H_2O_2 production in wheat leaves in response to *Z. tritici* infection quickly stimulates the antioxidant enzymes activities such as CAT, in order to degrade H_2O_2 and prevent its accumulation. Besides, POXs form a complex family of proteins that catalyze the oxido-reduction of various substrates using H_2O_2 . Diani et al. (2009) reported an early and localized necrosis and a fast and intense increase of the POX activity after inoculation in two BW cultivars resistant to *Z. tritici*. Here, total POX activity was enhanced in response to *Z. tritici* infection in both wheat species, which could participate to the lack of strong H_2O_2 accumulation in wheat leaves. Nevertheless in DW, only a weak induction of *Pox* expression was observed, suggesting that *Z. tritici* infection induces other POX-encoding genes in this wheat species. However, POX activity seems to be not involved in cell wall reinforcement by lignin deposition in both studied pathosystems since no polyphenol deposition in leaves of both wheat species was observed at the penetration sites by the fungus.

In order to investigate the induction of the phenylpropanoid pathway by *Z. tritici* infection in BW and DW, PAL activity, *PAL* gene expression, the content of total phenolic compounds and

polyphenol deposition at penetration sites of the fungus were monitored. PAL is one of the key enzymes acting upstream in the phenylpropanoid pathway, leading to the biosynthesis of a large array of phenolic compounds. In our study, while *PAL* gene is more responsive to *Z. tritici* in BW than in DW leaves, PAL activity is induced in both species. Nevertheless, no significant change in phenolic compound content and polyphenol deposition at fungal penetration sites were detected on both wheat species. Adhikari et al. (2007) and Shetty et al. (2009) showed that a PAL-encoding gene up-regulation was only observed in resistant BW cultivars following the infection by *Z. tritici*. This result suggests that the phenylpropanoid pathway plays a role in resistance observed during incompatible interactions. Nevertheless, our results revealed that PAL activation is not a marker of efficient protection against *Z. tritici* in both wheat species.

Moreover, we investigated the involvement of octadecanoid pathway in wheat-*Z. tritici* interaction by measuring total LOX activity and *LOX* gene expression. LOX acts upstream of the octadecanoid pathway and catalyses the dioxygenation of polyunsaturated fatty acid into either 9- or 13- hydroperoxyoctadecadi (tri) enoic acids. These highly reactive aliphatic molecules are then rapidly metabolized into oxylipins. Among them, jasmonic acid (JA) and its methyl ester, methyl jasmonate (MeJA), are involved in defense signaling. In our study, early down-regulation of *LOX* gene was observed in infected leaves of both wheat species (at 1 and 2 dpi). Similar transcriptional down-regulation of genes encoding for various LOXs and other enzymes involved in octadecanoid pathway (such as allene oxide synthase or 12-oxophytodienoate reductase) was detected in *Z. tritici* inoculated leaves of a susceptible BW cultivar at 1 dpi (Rudd et al., 2015). Interestingly, Ray et al. (2003) noticed a similar early down-regulation of this LOX-encoding gene in resistant cultivars at 6-12 h after infection with *Z. tritici*. These results indicated that the transcript analysis of this gene does not allow to differentiate between resistant and susceptible cultivars to *Z. tritici*. Moreover, total LOX activity was significantly induced in infected leaves, with higher level of this activity in DW leaves in comparison to BW ones. Various LOX isoforms may exhibit different pattern of gene induction in response to *Z. tritici* infection.

In conclusion, our study characterized for the first time the interaction between DW and *Z. tritici* and revealed close patterns of fungal infection process and host-defense reactions on both BW and DW wheat species. However, some differences between the two pathosystems were observed, but these variations could be attributed to a strain and/or a cultivar effect. Further investigations using larger sample sizes of fungal isolates and host cultivars of both pathosystems should be performed to confirm these findings. On the other hand, the similarities found in defense responses suggest the use of shared alternative control strategies on both wheat species, such as plant resistance inducers (called also elicitors) targeting the plant rather than the pathogen.

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Chap III | Induction de la résistance du blé tendre et du blé dur contre *Zymoseptoria tritici* par les SDPs

Les publications de ce chapitre ont eu l'objet des publications suivantes:

Somai-Jemmali L., Magnin-Robert M., Randoux B., Siah A., Tisserant B., Halama P., Reignault P., Hamada W., 2015. Biological control mechanisms of ascorbic acid against *Mycosphaerellagraminicola* in wheat. *Communications in Agricultural And Applied Biological Sciences* (In press).

Somai-Jemmali L., Magnin-Robert M., Randoux B., Siah A., Tisserant B., Halama P., Reignault P., Hamada W., 2015. Plant oligosaccharides enhancer of wheat defense response to Septoria leaf blotch. *Communications in Agricultural And Applied Biological Sciences*, Vol 80(2) (In press).

L. Somai-Jemmali, B. Randoux, A. Siah· M. Ors, P. Halama, Ph. Reignault and W. Hamada, 2014. Efficacy and modes of action of resistance inducers on two wheat species against *Zymoseptoria tritici*. *Communications in Agricultural And Applied Biological Sciences (indexed)*, Vol 79(3): 397-402.

Somai-Jemmali L., Salim S., Siah A. and Hamada W., 2014. Fungicide sensitivity of *Mycosphaerella graminicola* Tunisian isolates: the importance of drug transporter genes in the process of fungicide tolerance. *Phytopathologia Mediterranea (Impacted)* 53, 1: 83–93.

Les publications de ce chapitre ont été présentées dans des congrès internationaux:

Somai-Jemmali L., Magnin-Robert M., Randoux B., Siah A., Tisserant B., Halama P., Reignault P., Hamada W., 2015. Plant oligosaccharides enhancer of wheat defense response to Septoria leaf blotch. *Oral presentation in 67th International Symposium on Crop Protection 2015 Gent, Belgium (May 19)*.

Somai-Jemmali L., Magnin-Robert M., Randoux B., Siah A., Tisserant B., Halama P., Reignault P., Hamada W., 2015. Biological control mechanisms of ascorbic acid against *Mycosphaerellagraminicola* in wheat. *Oral presentation in 67th International Symposium on Crop Protection 2015 Gent, Belgium (May 19)*.

L. Somai, B. Randoux, A. Siah· M. Ors, P. Halama, Ph. Reignault and W. Hamada, 2014. Efficacy and modes of action of resistance inducers on two wheat species against *Zymoseptoria tritici*. *Oral presentation in 66th International Symposium on Crop Protection 2014 Gent, Belgium (May 20)*.

Somai L and Hamada W., 2013. Efficacy of ascorbic acid in controlling wheat against Septoria leaf blotch by direct and indirect effect. *Oral presentation in Crop protection management in Mediterranean organic agriculture international symposium, Sousse, Tunisia (May 14-16)*.

Problématique

Aujourd'hui, un nombre croissant de formulations contenant des SDPs qui permettent la réduction de l'utilisation des produits chimiques et qui apportent des solutions souvent innovantes dans le domaine de la protection des cultures tout en respectant l'environnement. L'exploitation de la résistance induite en agriculture biologique ou en agriculture raisonnée (alternance entre la lutte biologique et la lutte chimique) est une stratégie qui offre de grandes promesses d'avenir, car elle est essentiellement fondée sur la stimulation des mécanismes naturels de défense des plantes. Alors que la septoriose représente l'une des maladies occasionnant les plus fortes diminutions de rendement de blé, le nombre de SDPs actuellement commercialisés est très faible. Par conséquent, au cours de ce chapitre, on va présenter 4 nouvelles formulations de produits SDPs dont les matières actives sont testées pour la première fois contre la septoriose du blé, à savoir un produit à base de (i) l'acide ascorbique (AA), (ii) un extrait d'algue brune *A. nodosum* (*A.nod*), (iii) oligosaccharides de paroi de plantes (Oligos) et (iv) de chitosan. Les effets direct (antifongique) et indirect (stimulation des mécanismes de défense de la plante) de chaque produit seront discutés à part dans un article indépendant. En plus, les réponses du blé tendre et du blé dur vis-à-vis des produits testés seront discutées. Ainsi, cette étude pourra nous présenter de nouvelles matières actives biologiques qui pourraient être intéressantes comme alternatives de lutte contre la septoriose surtout avec le nombre limité de SDP commercialisés sur cereales.

Publication 2: Ascorbic acid-based efficiency to control *Zymoseptoria tritici* causal agent of STB disease and induce bread and durum wheat defense pathways

I: Ascorbic acid-based presents antifungal properties against *Zymoseptoria tritici* *in vitro* and in greenhouse condition

ABSTRACT

This study aims at characterizing the antifungal effect of an ascorbic acid (AA)-based against *Zymoseptoria tritici*, causal agent of the STB wheat disease. In greenhouse condition, a preventive foliar spray by 50 mg L⁻¹ of AA reduced the infection level by more than 75% on both bread and durum wheat susceptible cultivars. Microscopy revealed that AA inhibits *in planta* development and fructification. It also strongly decreased fungal cell wall degrading enzyme activities of *Z. tritici*. Inhibitor effect of AA was also confirmed *in vitro* experiments on seventeen *Z. tritici* strains, but it was irrespective of their pathogenicity and fungicide resistance levels.

Key words: ascorbic acid, *Zymoseptoria tritici*, biocontrol, antifungal activity, cell wall degrading enzyme

INTRODUCTION

Septoria tritici blotch (STB) is one of the most devastating foliar diseases of wheat caused by the hemibiotrophic fungus *Zymoseptoria tritici* [Quaedvlieg *et al.*, 2011], previously known as *Septoria tritici* (teleomorph *Mycosphaerella graminicola*). First, *Z. tritici* process a long symptomless phase considered as biotrophic, during which the fungal germ tube grows on the leaf surface, penetrates either through the stomata [Kema *et al.*, 1996, Duncan & Howard, 2000; Shetty *et al.*, 2003; Siah *et al.*, 2010a] or directly *via* the epidermal cell wall, and colonizes the intercellular space between mesophyll cells [Rohel *et al.*, 2001; Siah *et al.*, 2010a]. Then, it rapidly switches to a symptomatic necrotrophic phase characterized by the occurrence of chlorotic and/or necrotic lesions on the leaf surface, and the formation of pycnidia in colonized substomatal cavities [Kema *et al.*, 1996; Shetty *et al.*, 2003; Siah *et al.*, 2010a]. During the necrotrophic phase, the fungus releases cell-wall degrading enzymes (CWDEs) such as endo- β -1,3-glucanases and endo- β -1,4-xylanases, as well as proteases, leading to an increase of nutrient content for the fungus in the apoplastic space [Palmer & Skinner, 2002; Keon *et al.*, 2007; Siah *et al.*, 2010a]. CWDEs, as endo- β -1,4-xylanase and protease, produced by *Z. tritici*, were reported to be correlated with symptoms and sporulation levels [Douaiher *et al.*, 2007; Tian *et al.*, 2009; Siah *et al.*, 2010a].

Conventional fungicides (azoles and strobilurines) are extensively used to control STB but they are increasingly questioned because of their deleterious impacts on the environment and the decrease of their efficacy due to the rapid emergence of *Z. tritici* resistant strains in different

geographical regions over Europe [Gisi *et al.*, 2005; Torriani *et al.*, 2009]. The use of natural products to control pathogenic fungi, by acting *directly* upon fungal growth or *indirectly* by stimulation of plant defense, could be an interesting alternative to reduce the use of pesticides. In this sense, many studies revealed that exogenous application of AA conferred protection against plant pathogens, like insects and fungi [Nehal *et al.*, 2014; Elad, 1992; El-Korany *et al.*, 2008; Abdel-Kader *et al.*, 2012; Hassan *et al.*, 2014]. AA has now gained significant place in science, mainly due to its wide range of antimicrobial effects [Gupta *et al.*, 1941; Myrvik *et al.*, 1954, Eller *et al.*, 1968, Juven *et al.*, 1986; Obaleye *et al.*, 1994; Sánchez-Najera *et al.*, 2013]. Structurally, L-AA is one of the simplest vitamins. It is related to the C₆ sugars, being the aldono-1, 4-lactone of a hexonic acid (L-gluonic acid) and contains an enediol group on C₂ and C₃ (Fig. 1) [Khan *et al.*, 2011]. Eller and colleagues [1968] hypothesized that sporidical action observed on *Clodistrium* sp. in response to AA treatment is based on the formation of unknown oxidation products of AA. In this sense, the dehydroascorbic acid (DHA), a product formed during the oxidation of AA, was bactericidal to *Campylobacter jejuni* [Juven *et al.*, 1986]. These authors suggested that the cytotoxic effect of DHA towards *C. jejuni* is due to the interaction of these compounds with amino acids in vital proteins. AA was also able to inhibit the hyphal growth of *Candida albicans* through the interruption of hyphal formation signal [Ojha *et al.*, 2009]. Moreover, higher level of AA decrease the pathogenic attributes of opportunistic commensal *C. albicans*, through the reduction of proteinase secretion [Ojha *et al.*, 2009].

Drug transporters represent quantitative factors that can provide protection of organisms against natural toxic compounds. Two important families of drug transporters that mediate membrane transport are the ATP-Binding Cassette (ABC) [Bauer *et al.*, 1999; Theodoulou, 2000; Glavinas *et al.*, 2004] and the Major Facilitator Superfamily (MFS) [Peng *et al.*, 2011; Dos Santos, 2014] of transporters. Members of both classes can have broad and overlapping substrate specificities for natural toxic compounds and can be regarded as a “first-line defense barrier” in survival mechanisms. By directly influencing the effective concentration at target sites, membrane efflux systems act as crucial quantitative factors involved in sensitivity to natural toxic compounds. Many works revealed the induction of ABC transporter genes by organic acids such as *Pdr12* gene from *zygosaccharomyces witch* was induced by sorbic, benzoic and acetic acids [Piper, 2011] and *Ospdr9* gene from rice, which induce a resistance to AA [Moons, 2003]. For the case of *Z. tritici*, it was reported that *MgAtr1*, *MgAtr2* and *MgAtr4* genes were induced by organic acids as the fatty acids oleic, linoleic acid, the palmitic acid... [Zwiers & De Waard, 2000; Stergiopoulos *et al.*, 2002]. Moreover, several MFS transporters genes such as *MgMfs1* from *Z. tritici* and *BcMfs1* from *Botrytis cinerea*, have also been shown to be involved in resistance to natural plant toxins as the

mycotoxin cercosporin [Roohparvar *et al.*, 2007] and an alkaloid camptothecin [Hayashi *et al.*, 2002], respectively.

However, few studies investigated the antifungal effect of AA against plant pathogenic fungi and tested its further possible integration in biological control strategies against plant diseases and mainly cereal diseases. Therefore, the aim of our work was to evaluate the effect of AA-based on *Z. tritici* during a compatible interaction on two different wheat species, BW and DW. In this context, we investigated the effect of AA treatment *in vitro* conditions on (i) spore germination, hyphal growth and transcripts accumulation of fungal transporters genes implicated in detoxification of organic acids and natural toxins and *in planta* conditions (ii) on the fungal infectious disease processes in order to more understanding the fungal behavior in response to AA. Finally, we compared and discussed the direct effect of AA on both fungal species and its use to protect the both wheat species against *Z. tritici*.

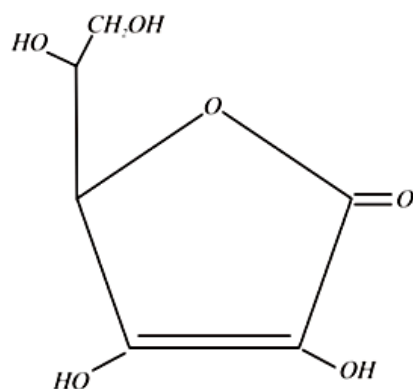


Fig.1. L-AA [Khan *et al.*, 2011].

MATERIAL AND METHODS

In vitro assays

Fungal isolation and inoculum production

Seventeen *M. graminicola* isolates collected from different regions in Tunisia [Somai-jemmali *et al.*, 2014]. To obtain the isolates, wheat leaves infected with STB were attached to moist filter paper and incubated in a damp environment at 18°C for 24 h to induce extrusion of cirri from pycnidia. Individual cirri were picked off and transferred to fresh potato dextrose agar PDA medium. After incubation for 7 days at 20°C, cultures obtained were transferred onto new PDA medium. Inoculum was produced by cultivating the isolates in liquid yeast-sucrose medium (YES: yeast extract 10 g L⁻¹, sucrose 10 g L⁻¹) for 7 d at 20°C on a rotary shaker at 100 rpm. Spores were collected by centrifugation at 5000 g for 5 min and washed twice with sterile distilled water. Spore suspensions were adjusted to 10⁵ spores mL⁻¹ for *in vitro* spore germination assays, and to 10⁷ spores mL⁻¹ for the *in planta* assays.

Fungicide sensitivity bioassays

The effects of azoxystrobin and tebuconazole on spore germination of each isolate was carried out on solid medium (10 g L⁻¹ glucose; 12.5 g L⁻¹ agar; K₂HPO₄ 2 g L⁻¹; KH₂PO₄ 2 g L⁻¹) amended with different concentrations of either of the two fungicides. Concentrations used for azoxystrobin were 0.0032, 0.016, 0.08, 0.4, 2, 5 or 10 mg L⁻¹, while those for tebuconazole were 0.0025, 0.025, 0.25, 1, 2.5, 3.5 or 5 mg L⁻¹. Both fungicides were added to the medium after autoclaving. Aliquots of 10⁵ spores mL⁻¹ of each isolate were sprayed on fungicide amended media in Petri dishes. Inoculated Petri dishes were incubated at 20°C in the dark for 2 days. Three dishes were used as replicates for each treatment. The percentage of germinated spores calculated from 100 spores was determined for each dish using a light microscope.

AA sensitivity bioassays

Tested AA product was provided by Sustainable Agro Solutions (Lerida, Spain) and consisted on a new formulation; based on natural AA (25%) associated with biological and inert formulates necessary to stabilize the product. AA was tested *in vitro* by measuring spore germination and fungal hyphal growth according to Siah *et al.* [2010b]. *Spore germination*: aliquots of 0.6 mL of each strain, adjusted at 5 x 10⁵ spores mL⁻¹, were added to PDA plates containing 10 final concentrations ranged between 0 and 150 mg L⁻¹ of AA. The plates were placed at 18°C in the dark for 24h. The percentages of germinated spores were then calculated for 100 spores, for each plate, using light microscope (Nikon, Eclipse 80i), in order to determinate the half maximal inhibitory concentration (IC₅₀) values. *Mycelium growth*: 5 µL of 5 x 10⁵ spores mL⁻¹ of each strain were spotted on the PDA plates containing the same concentrations of tested compounds. After incubation for 10 days at 18°C in the dark, hyphal growth were compared. Assays were repeated twice and for each condition, three plates with three spots per plate were used as triplicates.

RNA extraction and Reverse Transcription (RT) and analysis of gene expression by semi-quantitative RT-PCR

Fungal gene expression was investigated by semi-quantitative Reverse-Transcription Polymerase Chain Reactions (RT-PCR) for both *Z. tritici* strains, T01193 and St-08-46, according to Mehrabi & Kema [2006] and Roohparvar *et al.* [2007] with minor modifications. For each strain, 200 µL of a spore suspension, at a concentration of 10⁵ spores mL⁻¹, were added to 200 mL of liquid yeast-sucrose medium (YES: yeast extract 10g L⁻¹, sucrose 10 g L⁻¹) for 5 days at 18°C on a rotary shaker at 100 rpm. AA was then added to treated samples at a final concentration of 1 µg mL⁻¹. Total RNA was extracted from fungal material collected at 24, 48 and 72 hours by centrifugation at 5000g for 5

min and washed twice with sterilized water and were then ground to a fine powder in liquid nitrogen. RNA extraction was performed using RNA Easy Mini Kit (Invitrogen, UK) according to the manufacturer's protocol. Genomic DNA contaminating the samples was removed by treatment with DNase using RNase-Free DNase Set (Promega). RNA concentration was assessed with a spectrophotometer by measuring UV absorption at 260 nm. Reverse transcription of total RNA was carried out using cDNA Reverse Transcription Kit (Promega) according to the manufacturer's protocol. PCR reactions were conducted on subsequent obtained cDNA to amplify four *Z. tritici* target genes encoding for a MFS transporter protein (*MgMfs1*) and three ABC transporter proteins (*MgAtr1*, *MgAtr3* and *MgAtr4*) compared to the reference gene (*cytochrome b*, *CYT b*). Table 1 demonstrates used primers pairs of studied genes, which were newly designed using Bacon Design software and synthesized by Invitrogen™ Biotechnology. PCR was carried out in a 20 µL reaction mixture containing 1x PCR buffer, 2 mM dNTPs, 10 µM of each primer, 0.5 unit of Taq polymerase and 20 ng of cDNA using the following protocol: 94°C for 5 min, followed by 30 cycles each with 94°C for 1 min, 55°C for 1 min and 72°C for 1 min. The PCR reaction was terminated at 72°C for 7 min. PCR products were analyzed by electrophoresis on ethidium-bromide stained 1.5% agarose gels run in 0.5 x Tris-acetic acid-EDTA buffer at 100 V for 45 min. Gels were visualized under UV light. Experiment was repeated 3 times and only results which were repeated at least twice were considered.

Table 1 Primers used in the semi-quantitative reverse-transcription polymerase chain reaction studies

Gene	Primer sequences (5-3')	Accession number
<i>CYT b</i> (<i>cytochrome b</i> , housekeeping gene)	CTGATGATGGCAACCGCATTCTTA GCTGTATCGTGTAAGCTATTAGA	AY247413
<i>MgAtr1</i> (ABC transporter gene)	AGAGCGGACGAAAGTCCTTG GTGTTTCGTAGCCCTTTCGGA	AJ243112
<i>MgAtr3</i> (ABC transporter gene)	GTTTCGTCTTCGTCTTCTAC GATAGTTCTCACCTTGTCG	XM_003850890
<i>MgAtr4</i> (ABC transporter gene)	AGTGCTCCTCTGCGAATAG CGTTGTAGTGAATGGTGGTC	AF329852
<i>MgMfs1</i> (MFS transporter gene)	GTGTTGTGTATGGCGTTGG TGAGGTAGATGGTCTTGATGG	DQ661911

In planta assays

Plant material and AA foliar spray

Cultivars of the two wheat species known to be susceptible to STB were used for this study: *T. aestivum* cultivar Premio (BW) and *T. durum* cultivar Karim (DW). Grains were pre-germinated in Petri dishes (12 x 12 cm) on moist filter paper in darkness at 20°C for 24 h, at 4°C for 48 h and then at 20°C for 24 h. Germinated grains were then transplanted into soil in pots of 15 cm-diameter (12 grains/pot). The pots were placed in the greenhouse at 18°C and under day-night cycles of 16h /8h.

After 3 weeks, a foliar preventive AA spray at 50 mg L⁻¹ (0.283 mM) was applied before 2 days of the inoculation. The used concentration was that recommended by the manufacturer. Control plants were sprayed with tap water.

Fungal inoculation and pathogenicity level determination

Plantlets were inoculated when the third leaf was fully expanded (after 3 weeks) using a hand sprayer with 30 mL of spore suspension at 10⁶ spores mL⁻¹ amended with Tween 20 (0.05%, Sigma Aldrich, USA). Control plants (H₂O-treated) were sprayed with tap water amended with 0.05% of Tween 20. Immediately after inoculation, each pot was covered with a clear polyethylene bag for 3 days in order to ensure a water-saturated atmosphere to ensure better spore germination. Disease level was scored at 22 days post inoculation (dpi) by measuring percentage of leaf area with necrosis (PLN) and the index of pycnidium coverage (IPC) (which describes the importance of pycnidia in necrosis following a scale noted from 1 to 5) on the 3rd leaf of 25 plantlets (Siah et al., 2010). Data represent means of two independent experiments. For pathogenicity investigation, the Tunisian *Z. tritici* strains adapted to DW were inoculated on the susceptible DW cv. Karim. Also, *Z. tritici* strains adapted to BW were inoculated on the susceptible BW cv. Premio. The pathogenicity level of each strain was determined using the following scale: H: high pathogenicity (PLN>40); M: moderate pathogenicity (20<PLN<40); L: low pathogenicity (PLN<20).

Cytological analysis

In planta spore germination and leaf penetration by *Z. tritici* were monitored using Fluorescence Brightener 28 Calcofluor (Sigma Aldrich, USA) according to Siah *et al.* [2010a]. Two-centimeter-long wheat leaf segments were harvested 1 and 4 dpi from inoculated and control H₂O-treated plants. Mesophyll colonization and pycnidia formation were assessed at 22 dpi using Trypan blue staining according to Siah *et al.* [2010a]. Data represent means of two independent experiments. Three independent biological replicates were carried out for each experiment.

Protein extraction and enzyme assay

Three cell-wall degrading enzyme (CWDE) activities (endo-β-1,4-xylanase, endo-β-1,3-glucanase and protease) were measured at 4, 10, 16 and 22 dpi. Briefly, 2.5 g of third leaves were sampled and immediately frozen in liquid nitrogen for grinding in mortar. Endo-β-1,4-xylanase activity was measured at pH 4.8 and 45°C using xylan (1%, w/v) (Sigma Aldrich, USA) as substrate and xylose (10 mM) (Sigma Aldrich, USA) as reducing control sugar [Siah *et al.*, 2010a]. Endo-β-1,3-glucanase activity was measured at pH 4.8 and 50 °C using Laminarin (Sigma Aldrich, USA) at pH 5 (0.5 %, w/v) as substrate and glucose (10 mM) as reducing control sugar [Douaiher *et al.*, 2007].

Absorbance was measured at 540 nm. One unit of endo- β -1,4-xylanase and endo- β -1,3-glucanase activities is defined as the amount of enzyme necessary to release 1 μmol reducing sugar/p-nitrophenol $\text{min}^{-1} \text{mL}^{-1}$ of enzyme solution. Protease activity assays were assessed according to Hellweg [2003]. Absorbance was measured at 400 nm. One unit of protease activity is defined as a change in enzyme activity of 1 per hour. These activities were expressed in mU mg^{-1} protein. Total protein concentration was measured at A_{595} using bovine serum albumin as a standard [Bradford, 1976]. Data represent means of two independent experiments. Three independent replicates, which consisted on three 3rd leaves, were carried out for each experiment.

Statistical data analysis

For each strain, the AA and fungicides sensitivity levels were determined as half maximal inhibitory concentration (IC_{50}) values calculated from the corresponding dose-response curves using the GraphPad Prism 5 software (Hearne Scientific Software). For all data, an analysis of variance (ANOVA) was performed using the Tukey test at $P \leq 0.05$ using the XLSTAT software (Addinsoft, France).

RESULTS

In vitro effect of AA against *Z. tritici*

For *in vitro* assays, we used a collection of seventeen *Z. tritici* strains (14 strains were isolated from DW and 3 strains were isolated from BW). These strains were previously characterized for their pathogenicity and fungicides resistance levels [Siah *et al.*, 2010b; Somai-Jemmali *et al.*, 2014]. After 24 hours of incubation with AA, the percentage of spore germination was calculated in order to determinate the IC_{50} values of AA (Table 2). Three strains presented $\text{IC}_{50} \leq 1 \text{ mg L}^{-1}$, nine strains presented $1 \leq \text{IC}_{50} \leq 2 \text{ mg L}^{-1}$ and three strains presented $2 \leq \text{IC}_{50} \leq 3 \text{ mg L}^{-1}$. Only the T01193 and St-08-46 strains presented IC_{50} values $\geq 3 \text{ mg L}^{-1}$. In spite of their low levels of fungicides resistance and pathogenicity, St-08-6, St-08-12, St-08-21, St-08-34 and St-08-63 strains presented IC_{50} value higher than St-Q7-2 strain which had high level of fungicide resistance and pathogenicity. Therefore, no correlation was found between fungicides resistance, pathogenicity of the strains and IC_{50} values of AA.

For the following studies, only two *Z. tritici* strains were used, such as St-08-46 and T01193 strains. These last were chosen for their high level of pathogenicity, fungicide sensitivity and high IC_{50} value of AA. The Fig. 2 (b, d) illustrate the microscopic observation of T01193 and St-08-46 spores after 24 hours of incubation with 50 mg L^{-1} of AA. In the presence of AA, the majority of T01193 and St-08-46 spores were dehydrated (Fig. 2 b, d), stressed and not germinated compared

to normal spores observed in the unamended control, which are more swollen and longer (Fig. 2 a, c). Excepted T01193 strain, the hyphal growth of all studied strains was completely inhibited after 10 days of culture in plates with 5.5 mg L⁻¹ of AA. The hyphal growth of this last was completely inhibited from 16 mg L⁻¹ of AA (Fig. 3).

Table 2 IC₅₀ values of AA, pathogenicity and fungicide resistance level of seventeen *Z. tritici* strains

Strain	Origin	Host specie	Strobilurin resistance	Azole resistance	Pathogenicity	IC ₅₀ of AA
St-08-4			S	S	L	0.71 ± 0.32 i
St-08-2			S	S	H	0.76 ± 0.38 hi
St-08-5			S	S	L	0.90 ± 0.19 gh
St-08-31			S	S	M	1.15 ± 0.23 fgh
St-08-34			S	S	L	1.64 ± 0.58 efg
St-08-3			MR	MR	H	1.71 ± 0.38 efg
St-08-53			S	MR	H	1.71 ± 0.31 efg
St-08-6	Tunisia	DW	S	S	L	1.74 ± 0.48 def
St-08-21			S	S	L	1.87 ± 0.39 def
St-08-1			S	S	H	1.94 ± 0.21 def
St-08-12			S	S	L	1.96 ± 0.38 def
St-08-63			S	S	L	2.21 ± 0.54 cde
St-08-73			MR	S	H	2.54 ± 0.20 bcd
St-08-46			S	S	H	3.21 ± 0.41 ab
St-Q7-2	Germany		R	R	H	1.15 ± 0.68 fgh
IPO323	Netherland	BW	S	S	H	2.90 ± 0.12 bc
T01193	France		S	S	H	3.84 ± 0.58 a

Data are mean values of two independent experiments ± standard error. IC₅₀ is expressed in mg L⁻¹. Treatments with at least one similar letter didn't have significant difference at $P > 0.05$. S: sensitive, MR: moderate fungicide resistance, R: resistant; H: high pathogenicity; M: moderate pathogenicity; L: low pathogenicity. IC₅₀ is defined as the concentration of the inhibitor (AA) required to inhibit the spore germination rate to 50% of the maximal values (100 spores).

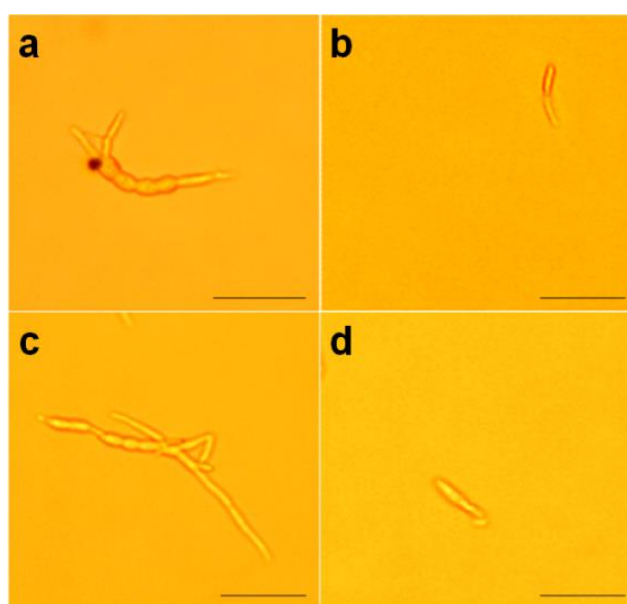


Fig. 2. Effect of AA on the spore germination of T01193 and St-08-46 *Z. tritici* strains *in vitro* condition after 24 hours of culture on PDA medium. a-c, T01193 and St-08-46 spores on the unamended control (PDA medium); b-d, T01193 and St-08-46 spores on PDA medium amended with 50 mg L⁻¹ of AA. Scale bar = 10 μm.

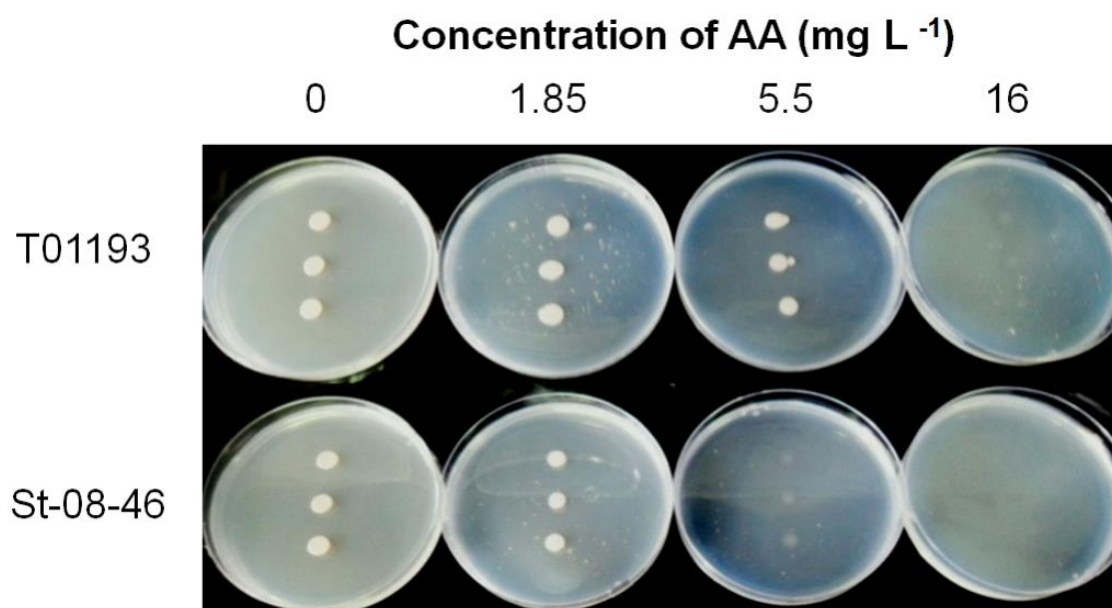


Fig. 3. Effect of AA on the hyphal growth of T01193 and St-08-46 *Z. tritici* strains *in vitro* condition after 24 hours of culture on PDA medium amended, from the left to the right, with 0, 1.85, 5.5 and 16 mg L⁻¹ of AA. For each tested concentration and strain, three spots were used as triplicates.

The expression patterns of four genes encoding *Z. tritici* drug transporter were studied for both strains T01193 and St-08-46 at 24, 48 and 72 hours (h) after *in vitro* incubation with 1 mg L⁻¹ of AA (Fig. 4). Excepted *MgAtr3*, the expression of all tested genes was induced in response to AA. *MgMfs1* seems to be the most up-regulated genes tested. *MgMfs1* gene up-regulation was observed from 24, 48 and 72 h, with a high induction observed at 48 h for T01193. The expression of *MgAtr1* gene was also up-regulated with a moderate up-regulation at 24 h for T01193 and at 48 h for St-08-46. In the case of *MgAtr4*, a slight induction of the gene expression was observed at 24 and 48 h for T01193 and at 24 h for St-08-46 (Fig. 4). No significant difference of the followed gene expression was observed between both tested strains (T01193 and St-08-46) (Fig 4).

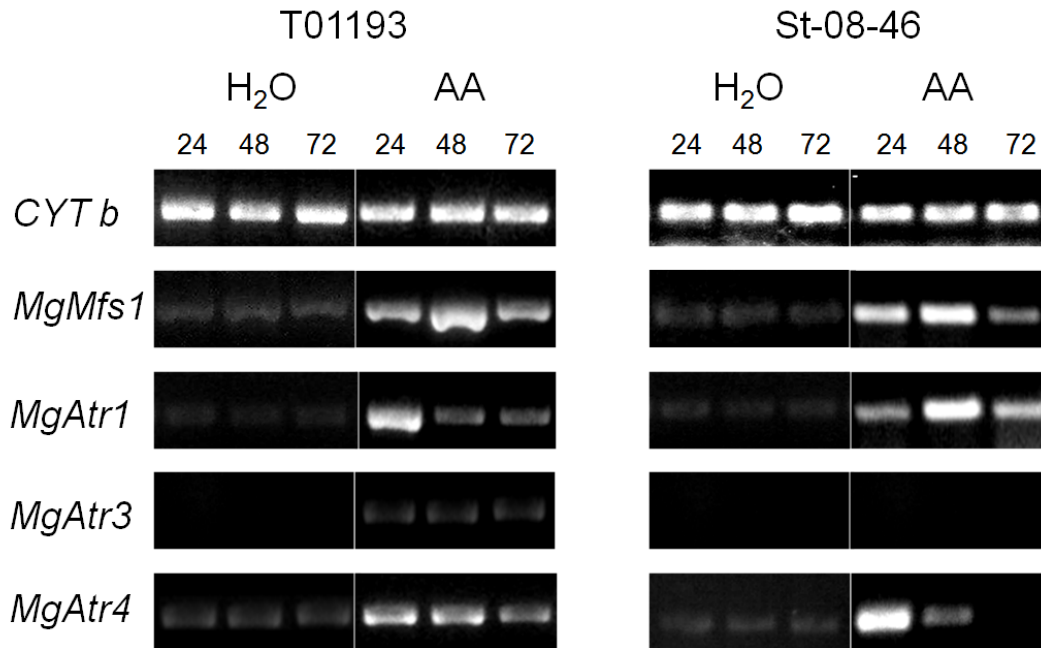


Fig. 4. Semi-quantitative analysis by RT-PCR of the *MgMfs1*-, *MgAtr1*-, *MgAtr3*-, *MgAtr4*- and cytochrome *b* (*CYT b*)-encoding genes expression of *Z. tritici* strains T01193 and St-08-46 grown on liquid artificial medium (YEG). Samples were collected at 24, 48 and 72 hours following the addition of 1 mg L⁻¹ of AA for treated samples or H₂O for control samples. *CYT b*-encoding gene was used as a house-keeping gene. Experiment was repeated three times and only results which were similar from two independent experiments were considered.

In planta effect of AA against *Z. tritici*

The effect of 50 mg L⁻¹ of AA was investigated on T01193 and St-08-46 *Z. tritici* strains inoculated respectively on BW and DW leaves. First, microscopic observation of (i) spore germination and hyphal growth at 4 dpi, (ii) mesophyll colonization and pycnidia formation at 22 dpi, were performed (Fig. 5 A, B). Microscopic observation assessed by Calcofluor staining at 4 dpi on AA-treated leaves described that the majority of spores were not germinated compared to H₂O-treated leaves where spores were germinated and hyphae are very developed. Moreover, cytological observation of mesophyll colonization and pycnidia observed at 22 dpi, by using Trypan blue staining, revealed that AA treatment significant reduced the fungal colonization of substomatal cavities and pycnidia formation compared to the control for BW (Fig. 5 A) and DW (Fig. 5 B) species. Secondly, Table 3 represents the rate of cytological parameters assessed for both tested pathosystems at : (i) 1 dpi: germinated spores (GS), (ii) 4 dpi: non germinated spores (NGS), spores leading to stomatal penetration (SP), spores leading to a direct penetration (DP), and germinated spores that did not penetrate (GSNP), (iii) 22 dpi: no colonized stomata (NCS), colonized stomata but not transformed into pycnidia yet (CS) and colonized stomata transformed into pycnidia (P). These results confirmed the inhibition of spore germination in response to AA treatment described both by a 61% decrease of GS on DW and BW at 1 dpi and by an increase of NGS on DW (64%)

and on BW (59%) at 4 dpi (Table 3). A perturbation of fungal penetration process on leaf tissues of both wheat species was observed in response to AA treatment, through the DP reduction on both wheat species (45% and 52 % on DW and BW, respectively). Moreover, significant reductions of GSNP and SP were reduced only on BW leaves at 4 dpi (by 79% and 54%, respectively) (Table 3), which may indicated a slightly different impact of AA treatment on the fitness of fungal strains adapted to DW or to BW. At 22 dpi, a reduction of pycnidia formation was observed in leaf tissues of both wheat strains in response to AA treatment, *via* a significant reduction of P, by 29% and 74% on DW and BW, respectively (Table 3). This reduction was associated with an increase of the NCS frequencies observed on the both wheat, 220% for DW and 330% for BW. Moreover, we noted that the percentage of CS was significantly decreased by 35% in BW leaves and increased by 73% in DW. These contrasting results could also involve an effect of AA application on the wheat itself.

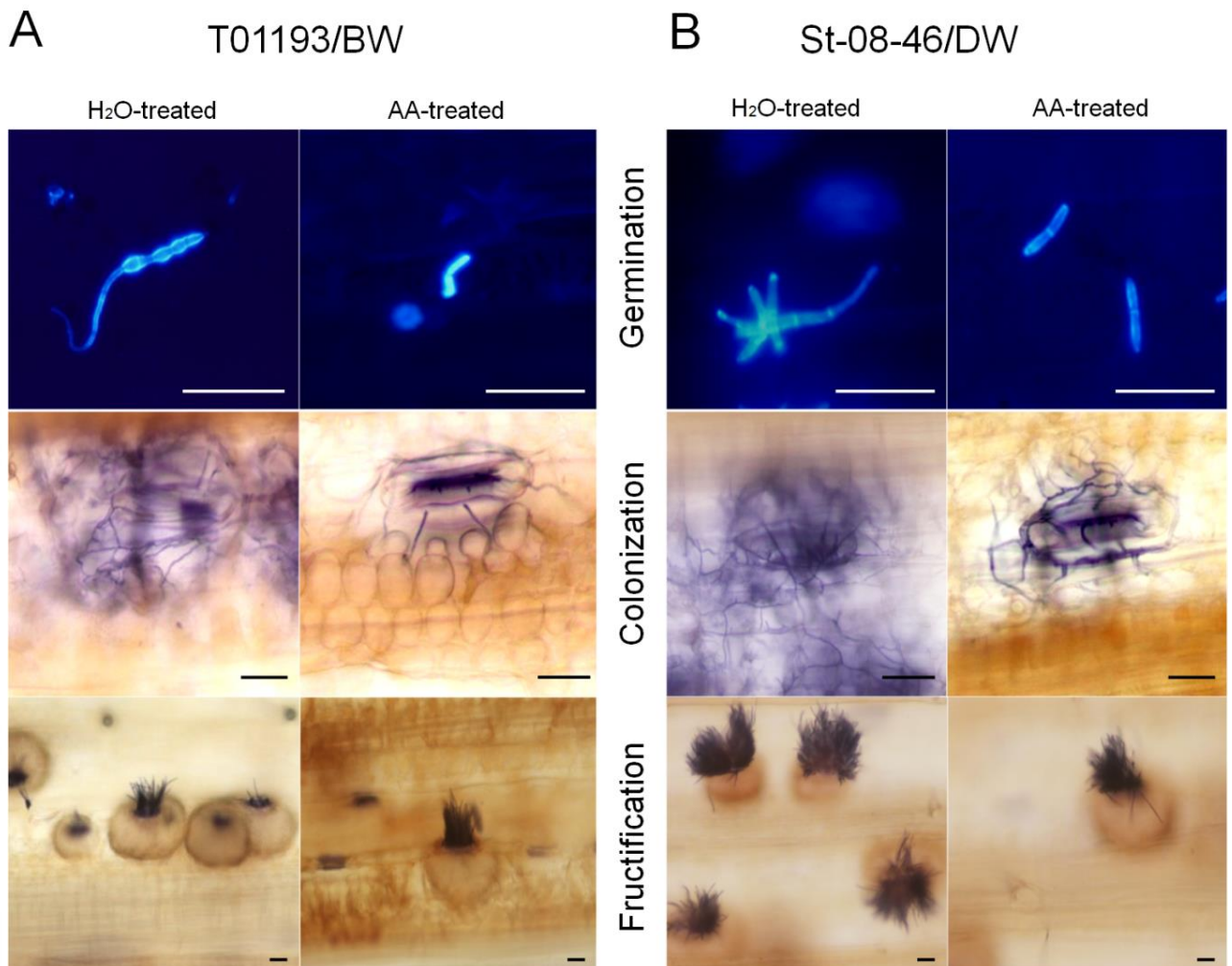


Fig. 5. Effect of 50 mg L⁻¹ of AA on the spore germination, mesophyll colonization and fructification at 22 dpi of *Z. tritici* on BW and DW leaves *in planta* condition. A, Leaves of susceptible BW cv. Premio infected by *Z. tritici* strain T01193. B, Leaves of susceptible DW cv. Karim infected by *Z. tritici* strain St-08-46. Microscopic observation of the spore germination of *Z. tritici* strains was monitored at 4 dpi using Calcofluor fluorescence. Microscopic observation of the mesophyll colonization and fructification was monitored at 22 dpi using Trypan blue staining. Scale bar = 10 µm.

Table 3 Percentages of different cytological parameters measured at 1, 4 and 22 days post infection (DPI) of BW and DW leaves by of T01193 and St-08-46 *Z. tritici* strains, respectively. At each time point, treated samples with 50 mg L⁻¹ of AA were compared to their corresponding control (H₂O-treated) samples

Parameters (%)	DPI	T01193 / BW		St-08-46 / DW	
		H ₂ O-treated	AA-treated	H ₂ O-treated	AA-treated
GS	1	54.1±3.7 a	21.3±4.0 b	72.1±11.5 a	28.1±16.1 b
NGS	4	25.3±2.1 b	69.5±2.7a	20.4±3.5 b	49.9±7.8 a
SP		17.9±1.7 a	3.8±1.8 b	9.8±0.2 a	10.1±2.2 a
DP		10.3±4.5 a	5.7±3.6 a	28.9±5.9 a	11.1±2.5 b
GSNP		46.5±3.3 a	21.1±2.4 b	41.0±7.2 a	29.1±7.0 a
NCS	22	20.6±3.5 b	45.4±1.6 a	13.8±0.2 b	46.6±3.3 a
CS		29.9±2.7 a	19.4±0.5 b	21.1±2.9 b	36.6±1.5 a
P		49.5±3.9 a	35.2±2.1 b	65.1±3.0 a	16.7±4.6 b

Data are mean values of two independent experiments (±sd). Statistics were made separately for each wheat specie. Means of AA-treated samples tagged with the same letter are not statistically different to their corresponding control in the same wheat specie (Tukey test, $P < 0.05$). GS: germinated spores, NGS: non germinated spore, DP: spore leading to a direct penetration, SP: spore leading to a stomatal penetration, GSNP: germinated spore without penetration, NCS: non-colonized stomata, CS: colonized stomata but not yet transformed into pycnidia, P: colonized stomata transformed into pycnidia.

Disease symptoms were significantly reduced in leaves of AA-treated plants compared to the control, through a high decrease of the percentage of leaf necrosis (76 and 79% on BW and DW, respectively) and the index of pycnidium coverage (74 and 75% on BW and DW, respectively) (Table 4, Fig. 6 A, B). These results indicate that AA treatment was effective to protect the both wheats against *Z. tritici*, the protection observed is greater than 70% in our conditions. We secondly studied CWDE activities on infected leaf tissues, especially at 10 and 22 dpi (Table 4). At 22 dpi, an important and significant decrease of CWDE activities was observed in infected tissues of wheat treated with AA in comparison to the control. Endo-β-1,4-xylanase activity was reduced by 87% on BW and 90% on DW in comparison to their respective control. Similarly, endo-β-1,3-glucanase and protease activities showed respective decreases (83% and 80%) and (84% and 90%) on DW and BW leaves. In our study, the weak CWDE activities were associated with a weak severity of symptoms (leaf necrosis and pycnidium coverage) in the both wheat species treated with AA.

Table 4 Evolution of disease severity parameters and CWDE activities at 4, 10, 16 and 22 days post inoculation (DPI) of BW and DW leaves by of T01193 and St-08-46 *Z. tritici* strains, respectively. At each time point, treated samples with 50 mg L⁻¹ of AA were compared to their corresponding control (H₂O-treated) samples

	DPI	T01193 /BW		St-08-46/DW	
		H ₂ O	AA	H ₂ O	AA
Severity of disease					
% of leaf with necrosis	4	nd	nd	nd	nd
	10	nd	nd	nd	nd
	16	24.1±14.6 b	2.9±3.4 d	28±14.7 b	3.8±4.4 d
	22	48.5±18.2 a	11.5±1.1 c	60.6±18.2 a	12.6±3.1 c
Index of pycnidium coverage	4	nd	nd	nd	nd
	10	nd	nd	nd	nd
	16	2.5±1.4 b	0.3±0.6 d	2.4±1.2 b	0.7±0.5 c
	22	3.5±1.1 a	0.9±0.3 c	4.4±0.7 a	1.1±0.3 c
CWDEs (mU mg⁻¹ protein)					
Endo-β-1.4-xylanase	4	32.4±4.1 b	31.1±2 b	48.2±3.2 c	42.6±6.4 c
	10	30.5±3.1 b	22.1±3.9 b	60.1±2.5 c	35.8±9.1 c
	16	110.8±40.3 b	36.1±17.5 b	246±36.6 b	57.8±25.5 c
	22	879.6±263.4 a	118.3±70.9 b	941.8±294.8 a	91.1±25.4 bc
Endo-β-1.3-glucanase	4	38.6±2.8 b	44.2±5.8 b	170.3±23.7 c	204.4±26.2 c
	10	175.5±19.0 b	130.5±5.3 b	173.6±13.6 c	197.3±5.4 c
	16	221.3±111.8 b	129.4±27.1 b	345.4±43.1 b	300.6±44.1 c
	22	1136.3±472.2 a	190.7±103.3 b	1026.4±128 a	208.3±86.4 c
Protease	4	211.8±58.2 b	70±57.4 b	166.5±31.6 bc	185.6±33.1 bc
	10	425±62.5 b	67.9±37.6 b	156.7±49.9 bc	148.1±24.2 c
	16	1108.3±338.1 b	207.5±157.0 b	1740±532.0 b	447.7±134.6 bc
	22	11317.3±867.0a	1765.2±548.3 b	6587.3±509.5 a	666.6±163.1 bc

(nd): not detected *Z. tritici* symptoms. Data are mean values of two independent experiments ± standard error. Means of AA-treated samples tagged with the same letter are not statistically different to their corresponding control in the same wheat specie (Tukey test, $P < 0.05$).

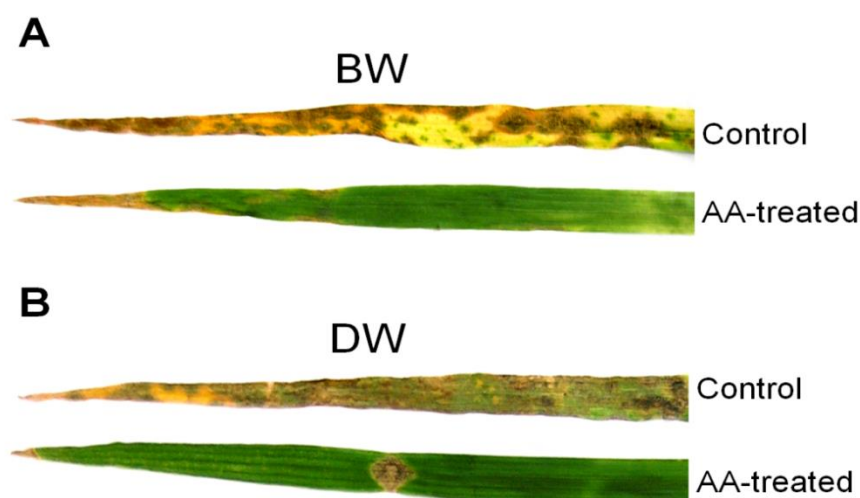


Fig. 6. Effect of 50 mg L⁻¹ AA on development of *Z. tritici* symptoms (chlorosis and necrosis) at 22 dpi on leaves of wheat seedlings assay in greenhouse condition. A, Leaves of susceptible BW cv. Premio infected by T01193 *Z. tritici* strain treated with AA compared to H₂O-treated leaves. B, Leaves of susceptible DW cv. Karim infected by St-08-46 *Z. tritici* strain treated with AA compared to H₂O-treated leaves.

DISCUSSION

First, the antifungal properties of AA were investigated against a collection of seventeen *Z. tritici* strains. Determined IC₅₀ values are including between 0.71 to 3.84 mg L⁻¹, which indicated different AA sensitivity depending on the *Z. tritici* strains tested. Among studied *Z. tritici* strains, T01193 was the most tolerant to AA followed by St-08-46. These two strains were selected for the continuation of this study. No relationship between the tolerance of *Z. tritici* strains to AA, the fungal resistance to fungicide and the level of strains pathogenicity was observed. These results clearly highlighted that the detoxification system induced to limit stress action of these various antifungal compounds (AA or fungicides) could be different and probably indicated another perturbations of these ones on fungal physiology.

In vitro conditions, we also showed an up-regulation of genes encoding drug transporter involved in resistance to fungicides. The gene encoding the MFS transporter (*MgMfs1*) was highly induced for the both tested strains (T01193 and St-08-46) in response to AA (1 mg L⁻¹), this one is a determinant of pathogen resistance to fungicides but also for the fungal resistance against organic toxins as the mycotoxin cercosporin produced by tomato [Roohparvar *et al.*, 2007]. Indeed, Hayashi *et al.* [2002] reported that *BcMfs1* from *B. cinerea*, the closest *MgMfs1* homologue [Roohparvar *et al.*, 2007], is involved in protection against natural toxic compound camptothecin, an alkaloid produced by the plant *Camptotheca acuminata*. In addition, our results indicate that ABC transporter genes *MgAtr1* and *MgAtr4* up-regulation could be enhanced by AA treatment. several research corroborate with our findings and reported the induction of *MgAtr1* by the fatty acids oleic and linoleic acid in yeast-like cells of *Z. tritici* [Zwiers and De Waard, 2000] and *MgAtr4* gene by the linoleic acid in *Z. tritici* mycelium [Stergiopoulos *et al.*, 2002]. Similarly, for other microorganisms as zygosaccharomyces, the ABC transporter gene (*Pdr12*) could promoted a resistance to many organic acids (sorbic, benzoic, acetic...) [Piper, 2011]. Overall, these results may indicate that AA could be a substrate for *MgMfs1*, *MgAtr1* and *MgAtr4* proteins and could be transporting by them. Another impact of AA may be hypothesized to explain the up-regulation of these genes, via the antioxidant activity of AA. In this sense, the addition of 5 mM of AA was found to induce the expression of an ABC transporter gene in rice roots (*Opsdr9*), which suggested a response to redox perturbations that favour the reduced state [Moons, 2003]. Cellular redox changes can affect the nuclear localization or the activity of transcription factors or activators and thus regulate gene expression.

Moreover, we demonstrated here that AA was able to inhibit hyphal development of all tested *Z. tritici* strains; at 16 mg L⁻¹ (90µM of AA) could completely inhibit hyphal growth of the most AA-tolerant *Z. tritici* strains, T01193. In this sense, *in vitro* assay realized with various fungi described that AA significantly inhibited fungal growth of necrotrophic fungi, *B. cinerea*,

Alternaria solani, *Fusarium oxysporum* and *F. solani* [Elad *et al.*, 1992; El-Korany *et al.*, 2008, Awadalla, 2008; Hassan *et al.*, 2014; Saad *et al.*, 2014], or hemibiotrophic ones, *Sclerotinia sclerotiorum* and *Colletotrichum musae* [Elad *et al.*, 1992, Khan *et al.*, 2001]. Similarly to AA effect described on *B. cinerea* [El-Korany *et al.*, 2008], sporulation and spore germination of *Z. tritici* T01193 and St-08-46 strains may be also impacted by AA treatment.

Our work also studied the efficacy of a 50 mg L⁻¹ (284 µM) of AA to control *Z. tritici* infectious process *in planta* under controlled conditions, BW was infected by T01193 strain and DW by St-08-46 strain. We showed that AA treatment dramatically altered the severity of symptoms caused by both *Z. tritici* strains (more than 70%). Our study is the first one to use AA for controlling a cereal disease. Many studies investigated the control of fungal dicotyledonic plant diseases by AA and confirmed the decrease of symptoms severity on cowpea, roselle, grapevine, tomato, cucumber, bean, eggplant, pepper, cantaloupe, strawberry... [Elad,1992; Tomader, 2005; Awadalla, 2008; Abdel-Kader *et al.*, 2012; Hassan *et al.*, 2014; Juber *et al.*, 2014]. Elad [1992] showed that a weak concentration of 1 mM AA solution could significantly reduce the severity of *Rhizopus stolonifer* infection on lettuce and of *B. cinerea* infection on tomato and cucumber. Moreover, Awadalla observed a significant reduction of the early blight disease incidence on tomato spraying with AA at 2.5 mM. Abdel-Kader *et al.* [2012] recorded that a foliar spray with 50 mM of a formulation based on AA could reduce by at least 30% the severity of powdery and downy mildew in pepper, cantaloupe and cucumber and early and late blight in tomato. Similarly, Juber *et al.* [2014] exhibited that 20 mg L⁻¹ of formulated AA decreased the severity of *F. oxysporum* f. sp. *Fragariae* on strawberry.

The AA efficacy to reduce symptoms expression could be explained by its direct inhibition of fungal spore germination and hyphal growth. The effect of AA on different infection stages was followed *in planta*, AA treatment overall decreases leaf penetration events on both wheat species, through the inhibition of direct penetration on DW leaves particularly, and inhibition of stomatal penetration on BW leaves. This could be explained by a different structure and morphology of BW and DW leaf surface or maybe a reaction of the plant tissues itself. Thus, AA treatment could influence the frequency and the mode of leaf penetration. According to previous studies, the mode of plant penetration by *Z. tritici* has been controversial. It has been reported that leaf penetration occurs through either stomata or direct ways across the epidermis [Kema *et al.*, 1996; Duncan & Howard, 2000; Mehrabi *et al.*, 2006] or *via* both stomata and epidermis [Cohen & Eyal, 1993; Dancer *et al.*, 1999; Rohel *et al.*, 2001; Siah *et al.*, 2010a]. Results found here confirm that this fungus could penetrate its host through both stomatal and direct ways. In addition, AA treatment slowed down mesophyll colonization and the differentiation of pycnidia. Mesophyll colonization is a crucial step for disease establishment: it allows further expansion of the fungus *via* the apoplast

and colonization of neighboring substomatal cavities [Cohen & Eyal, 1993; Duncan & Howard, 2000] and may represent a key step in the control of this fungus. The reduction of wheat mesophyll colonization by *Z. tritici* could be also explained by the induction of plant defense mechanisms in leaves such as the reinforcement of cell walls by accumulations of H₂O₂, callose and lignin in the mesophyll and epidermis cell walls which could stop the development and progression of the fungus [Vanacker *et al.*, 2000; Shetty *et al.*, 2003, 2007; Dogbo *et al.*, 2012]. Therefore, AA efficacy to control STB may be linked to a combination of direct antifungal effect and plant defense stimulation, like several other resistance inducer such as Milsana [Randoux *et al.*, 2006]. Thus, the study of defense mechanisms induction by tested AA formulation is required.

The transition from biotrophy to necrotrophy for *Z. tritici* French and Tunisian strains has been associated with a production of CWDEs such as endo- β -1,4-xylanase s, endo- β -1,3-glucanases and proteases. Cell wall degradation may be important to fungi not only for penetration and hyphal branching inside the plant tissue, but also for releasing nutrients from the wall polysaccharides that are necessary for growth [De Lorenzo *et al.*, 1997]. Douaiher *et al.* [2010] highlighted a range of CWDEs produced *in vitro* by *Z. tritici*, among which endo- β -1,4-xylanase activity was the most correlated enzyme with necrosis development in a detached-leaf pathogenicity assay. So far, few studies have been conducted *in planta* to observe the production of CWDEs and revealed that these enzymatic activities were closely correlated to the *Z. tritici* severity. In our study, AA treatment allowed to drastically decrease CWDE activities produced by T01193 and St-08-46 on BW and DW respectively at 22 dpi. Thus, AA efficacy to limit the fungal endo- β -1,4-xylanase, endo- β -1,3-glucanase and protease CWDE activities is clearly associated with the decrease of pycnidia formation and symptoms associated (necrosis lesions).

CONCLUSION

We established here that AA alters, in our conditions, the symptoms expression of STB, one of the major wheat diseases in Europe and Northern Africa. *In vitro* assay, AA shows strong inhibition of the spore germination and hyphal growth of seventeen *Z. tritici* strains. Indeed, AA could induce the activation of multidrug transporter-encoding genes that could allow its transport out of the fungal cells in the case of tolerant *Z. tritici* strains. *In planta* assay, AA slows down the infection and decreases disease severity through direct antifungal effects and consequently inhibits fungal colonization, fructification and CWDE activities (endo- β -1,4-xylanase, endo- β -1,3-glucanase and protease). Nevertheless some elements could indicate that the protective effect obtained by AA foliar spray may be also related to a plant defense stimulation, although there are still gaps to fill in order to elucidate the precise role of AA in enhancing the tolerance of plant to pathogenic stress.

Future research is now required to evaluate the induction of plant defense reaction in response to AA foliar spray and to determine the mechanisms by which AA induce plant resistance.

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Publication 2: Ascorbic acid-based efficiency to control *Zymoseptoria tritici* causal agent of STB disease and induce bread and durum wheat defense pathways

Part II: Ascorbic acid enhances wheat defense mechanisms by inducing ROS, phenylpropanoid and octadecanoid pathways

ABSTRACT

This study aims at characterizing the efficiency and the mode of action of an ascorbic acid (AA)-based against *Septoria tritici* blotch (STB), caused by *Zymoseptoria tritici*, during a compatible interaction. In non-inoculated context, *PR2* and *Chi 4 precursor* genes expression and peroxidase, catalase and lipoxygenase activities were induced in response to AA treatment. After inoculation, it potentiated all activities and transcript accumulation, except for lipoxygenase activity and *PAL* gene expression. It also induced cell wall H₂O₂ and polyphenol deposition in mesophyll cells at penetration sites. Moreover, BW and DW exhibited distinct response profiles in response to the AA treatment. Peroxidase, catalase, phenylalanine ammonia lyase and lipoxygenase activities were more responsive to AA in BW leaves, whereas the induction of β -1,3-glucanase activity, *LOX* gene expression and H₂O₂ and polyphenol deposition in mesophyll cells were stronger in DW leaves. Thus, AA could be used as an alternative to chemical control of STB on both BW and DW, because of its good protection level associated with a set of induced wheat defense mechanisms.

Key words: acid ascorbic, *Zymoseptoria tritici*, biocontrol, resistance inducer.

INTRODUCTION

Septoria tritici blotch (STB), caused by *Zymoseptoria tritici* (teleomorph *Mycosphaerella graminicola*), is one of the most devastating fungal foliar diseases on bread wheat (*Triticum aestivum* L. em Thell, BW) and durum wheat (*Triticum durum* Desf, DW). It causes annual yield losses ranged from 30 to 50% [Eyal *et al.*, 1987; Palmer and Skinner, 2002]. *Z. tritici* is a hemibiotrophic pathogen possessing a long symptomless phase considered as biotrophic, which is followed by a necrotrophic phase. This latest phase leads to a tissue collapse, resulting in chlorosis and necrosis development and pycnidium formation on the leaves [Shetty *et al.*, 2003, 2007].

Chemical fungicides are extensively used to control STB but they are increasingly questioned because of their deleterious impacts on the environment and the decrease of their efficacy due to the rapid emergence of *Z. tritici* resistant strains in different geographical regions over Europe [Gisi *et al.*, 2005; Torriani *et al.*, 2009]. The use of natural elicitors which could stimulate plant defense could be an interesting alternative to reduce the use of pesticides. Inducible defenses can be activated upon recognition of elicitors. The activation of plant defense by elicitors has been

correlated with a stimulation of (i) signaling processes including ion fluxes, mitogen-activated protein kinase (MAPK) cascade, the reactive oxygen species (ROS) [Garcia-Brugger *et al.*, 2006], and the reinforcement of cell walls by deposition of callose and lignin, phenolics and H₂O₂ [Moldenhauer *et al.*, 2006; Bhuiyan *et al.*, 2009; Zhao *et al.*, 2009; Shetty *et al.*, 2009; Yu *et al.*, 2010; Bi *et al.*, 2011; Wang *et al.*, 2013]. In wheat - *Z. tritici* interaction several elicitors were identified as chitin and glucans [Shetty *et al.*, 2009; Lee *et al.*, 2014]. Few studies investigated the stimulation of wheat resistance against *Z. tritici* [Joubert *et al.*, 1998; Jayaraj *et al.*, 2004; Shetty *et al.*, 2009] or other foliar diseases such as powdery mildew by elicitors, such as laminarin or β -1,3-glucan, Milsana®, trehalose, oligogalacturonides, exogenous salicylic acid or jasmonic acid [Reignault *et al.*, 2001; Randoux *et al.*, 2006; Randoux *et al.*, 2010; Tayeh *et al.*, 2013; Renard-Merlier *et al.*, 2007].

L-ascorbic acid (AA) has been reported to induce plant resistance against biotic stress due to its essential function as a co-factor for many enzymes in the biosynthesis of many phytohormones, its redox status and antioxidant activity. The endogenous level of AA has been suggested to be important in the regulation of plant defense against pathogens and was related to the overexpression of pathogenesis-related (PR) proteins in *Arabidopsis* challenged with *Pseudomonas syringae* [Pastori *et al.*, 2003; Barth *et al.*, 2004; Pavet *et al.*, 2005]. Similarly, Dias *et al.* [2011] suggested that AA is the main precursor of oxalic acid enhanced oxalic acid, an antioxidant enzyme involved in ROS biosynthesis as H₂O₂, in susceptible and resistant cacao infected by the hemibiotrophic fungus *Moniliophthora perniciosa*. Many works recorded induced plant resistance against abiotic stress by exogenous AA [Beltagi, 2008; Dolatabadian and Jouneghani, 2009; El Harriri *et al.*, 2010; Munir and Aftab, 2011; Ejaz *et al.*, 2012]. However little information is available about the stimulation of plant defense by exogenous AA against biotic stress. Katay *et al.* [2011] reported that the effect of chemical ascorbigen and 1-methyl ascorbigen pretreatment induced the double immune response leading to the biochemical immunization of bean plants against fungal pathogen *Uromyces phaseoli*. Besides, Tokunaga *et al.* [2007] demonstrated that the incubation of rice seedlings with AA could induce defense genes and mainly a novel XIP-type xylanase inhibitor protein (OsXIP) in rice [Dornez *et al.*, 2010].

Therefore, in this study we investigated the induction of wheat defense response in response to a preventive foliar spray by AA-based. The antifungal effect of AA against *Z. tritici* on wheat was already demonstrated by Somai-Jemmali *et al.* (Publication 2 Part 1) which recorded that foliar pretreatment by 50 mg L⁻¹ of AA. The aim of our work is to explain this good efficacy against *Z. tritici* and to understand its mode of action on wheat. Our investigations were performed using a French BW cultivar (cv. Premio) inoculated with a French BW-adapted strain (T01193) and a Tunisian DW cultivar (cv. Karim) inoculated with a Tunisian DW-adapted strain (St-08-46). The

AA eliciting – effect in non infected (ni) context - and potentiating -effect in infected (i) context were evaluated on molecular, enzymatic and cytological scale. We first studied the induction of defense related genes expression, *PR2* (β -1,3-glucanase), *Chi4 precursor* (chitinase class IV precursor), *Pox* (peroxidase); *PAL* (phenylalanine ammonia lyase) and *LOX* (lipoxygenase) encoding genes. Then, we also measured the enzymatic activity of β -1,3-glucanase (GLUC), for its involvement in degrading fungal cell walls. Furthermore, in order to investigate ROS metabolism, peroxidase (POX) and catalase (CAT) activities were also measured. Phenylpropanoid pathway study was performed by measuring (phenylalanine ammonia lyase) PAL activity and total polyphenol content. We also measured (lipoxygenase) LOX activity, which presents the key enzyme of the octadecanoid pathway. By microscopy, we observed the accumulation of H₂O₂ and polyphenol deposition after fungal penetration. Relationships between genes expression, enzyme activities and cytological response will be discussed.

MATERIALS AND METHODS

Plant material and tested product

Cultivars of the two wheat species known to be susceptible to STB were used for this study: *T. aestivum* cultivar Premio (BW) and *T. durum* cultivar Karim (DW). Grains were pre-germinated in Petri dishes (12 x 12 cm) on moist filter paper in darkness at 20°C for 24 h, at 4°C for 48 h and then at 20°C for 24 h. Germinated grains were then transplanted into soil in pots of 15 cm-diameter (12 grains/pot). The pots were placed in the greenhouse at 18°C and under day-night cycles of 16h /8h. Tested AA was provided by Sustainable Agro Solutions (Lerida, Spain) and consisted on a new formulation; based on natural AA (25%) associated with biological and inert formulates necessary to stabilize the product. After 3 weeks, when the third leaf was fully expanded, plants were sprayed 50 mg L⁻¹ (0.283 mM) of AA. The concentration used was that recommended by the manufacturer. Control plants were sprayed with tap water.

Fungal material and inoculation

Two *Z. tritici* strains, T01193 and St-08-46, were chosen for their high pathogenicity level and were inoculated respectively on BW and DW. Cultures were grown for 3 weeks at 18°C and were stored at -80°C for further use. Two days post treatment (dpt), plants were inoculated with 30 mL of a 10⁶ spores mL⁻¹ suspension in a 0.05% of Tween 20 (Sigma Aldrich, USA) solution using a hand sprayer. Control plants were sprayed with tap water containing 0.05% of Tween 20. Immediately after inoculation, each pot was covered with a clear polyethylene bag for 3 days in order to ensure a water-saturated atmosphere.

RNA extraction and Reverse Transcription (RT)

For plant genes expression study, samples were collected from non inoculated (ni) and inoculated plants (i). At (ni) conditions, samples were collected at 1, 2, 3, 4 and 7 dpt. For (i) conditions, samples were collected at 0, 1, 2 and 5 dpi (corresponding to 2, 3, 4 and 7 dpt). Total RNA was extracted from 100 mg of plant tissue using RNeasy Plant Mini Kit (Quiagen, The Netherlands). Genomic DNA contaminating the samples was removed by treatment with DNase using RNase-Free DNase Set (Quiagen, The Netherlands). Reverse transcription of total RNA was carried out using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA) according to the manufacturer's protocol.

Analysis of gene expression by semi-quantitative RT-PCR and real-time RT-PCR

PCR reactions were conducted on subsequent obtained cDNA to amplify five wheat genes encoding for *PR proteins 2* (β -1,3glucanase), *Chi 4 precursor* (chitinase IV precursor), *Pox* (peroxidase); *PAL* (phenylalanine ammonia lyase) and *LOX* (lipoxygenase). These analyses were performed using the following gene-specific primers presented in Table 1. The primers pairs were designed using the Primer Express® program and were tested for secondary structure using Net Primer® program. The gene expression of *PR2*, *Chi 4 precursor* and *Pox* was investigated by semi-quantitative RT-PCR. PCR was carried out in a 20 μ L reaction mixture containing 1x PCR buffer, 2 mM dNTPs, 10 μ M of each primer, 0.5 unit of Taq polymerase and 20 ng of cDNA using the following protocol: 94°C for 5 min, followed by 30 cycles each with 94°C for 1 min, 55°C for 1 min and 72°C for 1 min. The PCR reaction was terminated at 72°C for 7 min. PCR products were analyzed by electrophoresis on ethidium-bromide stained 1.5% agarose gels run in 0.5 x Tris-acetic acid-EDTA buffer at 100 V for 45 min. Gels were visualized under UV light. *TUB* was used as an internal control in semi-quantitative Reverse-Transcription Polymerase Chain Reactions (RT-PCR) analysis. Experiment was repeated 3 times and only results which were repeated at least twice were considered. The investigation of *PAL* and *LOX* expression was performed by real time-RT-PCR analysis. The amplification specificity of each qRT-PCR was checked by a single peak in melt curve analysis, and no primer dimers were detected using agarose gel electrophoresis. Real-time RT-PCR was performed using ABI Prism 7300 detection system (Applied Biosystems, USA), using the following thermal profile : after incubation for 10 min at 95°C, 15s at 95°C (denaturation), 1 min at 60°C (annealing/extension) for 40 cycles and 30s at 72°C (data acquisition). Relative expression for each cDNA was calculated for each time point relative to control at the same time. It corresponds to $2^{-\Delta\Delta Ct}$ described by Pfaffl *et al.* (2002) and was calculated using the relative expression software tool REST®. $\Delta\Delta Ct = [Ct \text{ Target (Sample)} - Ct \text{ Reference (Sample)}] - [Ct \text{ Target (Control)} - Ct \text{ Reference (Control)}]$. *TUB* is used as reference gene. The results were normalized with the *TUB* and

ACT genes and expressed relatively to the control, for each time point, corresponding to a fixed value of 1. After qPCR, a melt curve was generated to verify the specificity of each amplification reaction. The experiment was conducted in triplicate and only results which were repeated at least twice were considered.

Table 1 List of primers encoding for wheat genes analyzed by RT-PCR.

Gene	Primer sequences (5'-3')	Accession numbers
<i>TUB</i> (<i>beta tubulin</i> , housekeeping gene)	GGAGTACCCTGACCGAATGATG AACGACGGTGTCTGAGACCTTT	U76895
<i>ACT</i> (<i>actin</i> , housekeeping gene)	AACCTTCAGTTGCCAGCAA TGTTTCGACCGCTGGCATAAC	AB181991
<i>PR2</i> (β -1,3 glucanase)	CTCGACATCGGTAACGACCAG GCGGCGATGTACTTGATGTTC	Y18212
<i>Chi4 precursor</i> (<i>chitinase class IV precursor</i>)	TTCTGGTTCTGGATGACCAAC ACTGCTTGCAGTACTCCGTG	AF112966
<i>Pox</i> (<i>peroxidase</i>)	GAGATTCCACAGATGCAAACGAG GGAGGCCCTTGTTTCTGAATG	X56011
<i>PAL</i> (<i>phenylalanine ammonia lyase</i>)	CCCCATTGGTGTCTCCAT ACTGCGCGAACATCAGCTT	AY005474
<i>LOX</i> (<i>lipoxygenase</i>)	GGGCACCAAGGAGTACAAGGA GCTCGTGATGGTGTGGATGA	U32428

Enzyme activities

Total activity of 5 enzymes involved in plant defense mechanisms were measured: β 1,3-glucanase (GLUC), peroxidase (POX), catalase (CAT), phenylalanine ammonia lyase (PAL) as well as lipoxygenase (LOX). Samples were collected at the same timing used for gene expression analysis. Each sample consisted of three medial segments excised from three third leaves. β -1,3-glucanase activity was analyzed according to Douaiher *et al.* [2007a, b]. CAT activity was assessed according to Randoux *et al.* [2010] by recording decrease in A_{240} ($\epsilon = 36 \text{ mM}^{-1}\text{cm}^{-1}$) produced by H_2O_2 breakdown. One unit of the CAT activity is defined as the amount of the enzyme which formed H_2O_2 at $1 \mu\text{mol min}^{-1}$. POX was estimated according to El Hadrami and Baaziz [1995] and expressed as $\text{nkcat mg of protein}^{-1}$. One unit of POX activity is defined as the amount of enzyme that catalyses the conversion of guaiacol as substrate at A_{470} after 2 minutes of H_2O_2 addition. LOX activity was measured according to Randoux *et al.* [2010]. One unit of LOX activity is defined as the amount of enzyme that catalyses the conversion of substrate (linoleic acid) at 1 mol s^{-1} ($\epsilon = 23 \text{ mM}^{-1}\text{cm}^{-1}$). PAL activity was calculated as described by Randoux *et al.* [2010]. One unit of PAL activity is defined as the amount of enzyme that catalyses the formation of cinnamic acid at 1 mol s^{-1} ($\epsilon = 10 \text{ mM}^{-1}\text{cm}^{-1}$). All enzymatic activities were expressed in mU mg^{-1} of proteins.

Total protein concentration was measured at A₅₉₅ using bovine serum albumin as a standard (Bradford, 1976). Data represent means of two independent experiments. Three independent replicates were carried out for each experiment.

Cytological analysis

The accumulation of H₂O₂ was revealed by 3,3-diaminobenzidine (DAB) staining (red-brown staining) according to Randoux *et al.* [2010]. For polyphenol deposition visualization, leaves stained with Trypan Blue were used for fluorescence microscopy using a Nikon microscope (Champigny-sur-Marne, France) fitted for fluorescence (blue exciter filter, maximum transmittance 400 nm and barrier filter with a transmittance range of 500 to 800 nm). As described by Randoux *et al.* [2010], a yellow fluorescence visualized under UV-light microscopy indicates the presence of polymeric phenolic compounds. H₂O₂ and polyphenol visualization were detected at fungal penetration sites in 2-cm-long segment of 3rd leaves. Leaf samples were collected from the same plantlets used for enzymatic and molecular analysis and at the same timing. Data represent means of two independent experiments. Three independent replicates were carried out for each experiment.

Total polyphenolic compounds quantification

The quantification of the content of total soluble phenol compounds was determined according to the Folin-Ciocalteu method modified by El Hadrami *et al.* (1997). Leaf samples were collected from the same plantlets used for enzymatic analysis. Foliar tissues (200 mg from 3rd leaf), were homogenized in an ice bath with 1.5 ml methanol. The homogenate was centrifuged three times at 10000g for 5 min; supernatants were recuperated each time. One hundred microlitres of the supernatant was added to Folin-ciocalteu reagent and sodium carbonate. The mixture was incubated at 40°C for 30 min and the blue color was read at 760 nm using Catechin (Sigma Aldrich, USA) as a standard. The polyphenol content was determined for the experiments described above and was expressed in mg-equivalents of Catechin (Sigma Aldrich, USA) (CE) per gram of fresh weight (FW). Data represent means of two independent experiments. Three independent replicates were carried out for each experiment.

Statistical data analysis

Statistical data analyses of molecular and enzymatic assays were made separately for each wheat species (BW cv. Premio/T01193 strain and DW cv. Karim/St-08-46 strain). For quantitative gene expression, the analyses were performed using the relative expression software tool REST® as described by Pfaffl *et al.* (2002) and the significant differences were tested at 95% ($P < 0.05$). For

severity parameters, enzymatic activities and microscopy, an analysis of variance (ANOVA) was performed using Tukey test at $P \leq 0.05$ using the XLSTAT software (Addinsoft, France).

RESULTS

Wheat defense-related genes expression

Three PR-protein-encoding genes expression, *PR2*, *Chi 4 precursor* and *Pox*, was studied by semi-quantitative RT-PCR analysis (Fig. 1). At (ni) condition, *PR2* expression was strongly induced in response to AA treatment from 1 to 4 dpt in BW leaves and from 1 to 3 dpi in DW leaves. Similarly, AA-treated BW leaves recorded strong up-regulation of *Chi 4 precursor* gene expression at 1-2 dpt, while in AA-treated DW leaves a peak of *Chi 4 precursor* up-regulation was observed at 3 dpt. Again, the *Pox* expression was strongly up-regulated at 2 and 3 dpt in BW and DW leaves, respectively. Following *Z. tritici* infection, AA treated recorded strong induction of *PR2* and *Chi 4 precursor* genes at all studied time points for both wheat species, while *Pox* gene was up-regulated from 3 to 7 dpt (1 to 5 dpi) on BW leaves and strongly enhanced at 4 dpt (2 dpi) which reduced at 7 dpi (5 dpi) on DW leaves (Fig. 1).

The time course of *LOX*- and *PAL*-encoding genes' expression was studied by quantitative RT-PCR analysis (Fig. 2). In (ni) condition, *LOX* expression was induced by 29.6-, 10.3- and 19-fold (at 1, 2 and 4 dpt) on BW leaves and by 9.3, 62.4-fold increases (at 1 and 2 dpt) in DW leaves. After *Z. tritici* infection, AA-treated leaves recorded higher 11.4- and 90.6-fold increases on BW and DW leaves, respectively. A strong significant increase of *LOX* expression was observed in DW and BW leaves, respectively. In (ni) condition, *PAL* expression was transiently repressed by 6.7-fold at 1 dpt in DW leaves, while no modification of *PAL* expression was observed on BW leaves treated with AA in comparison to control. At infectious context, *PAL* expression was down-regulated on both BW (4.29- and 6.71-folds at 4-7 dpt) and DW (7.94-folds at 1 dpi) leaves.

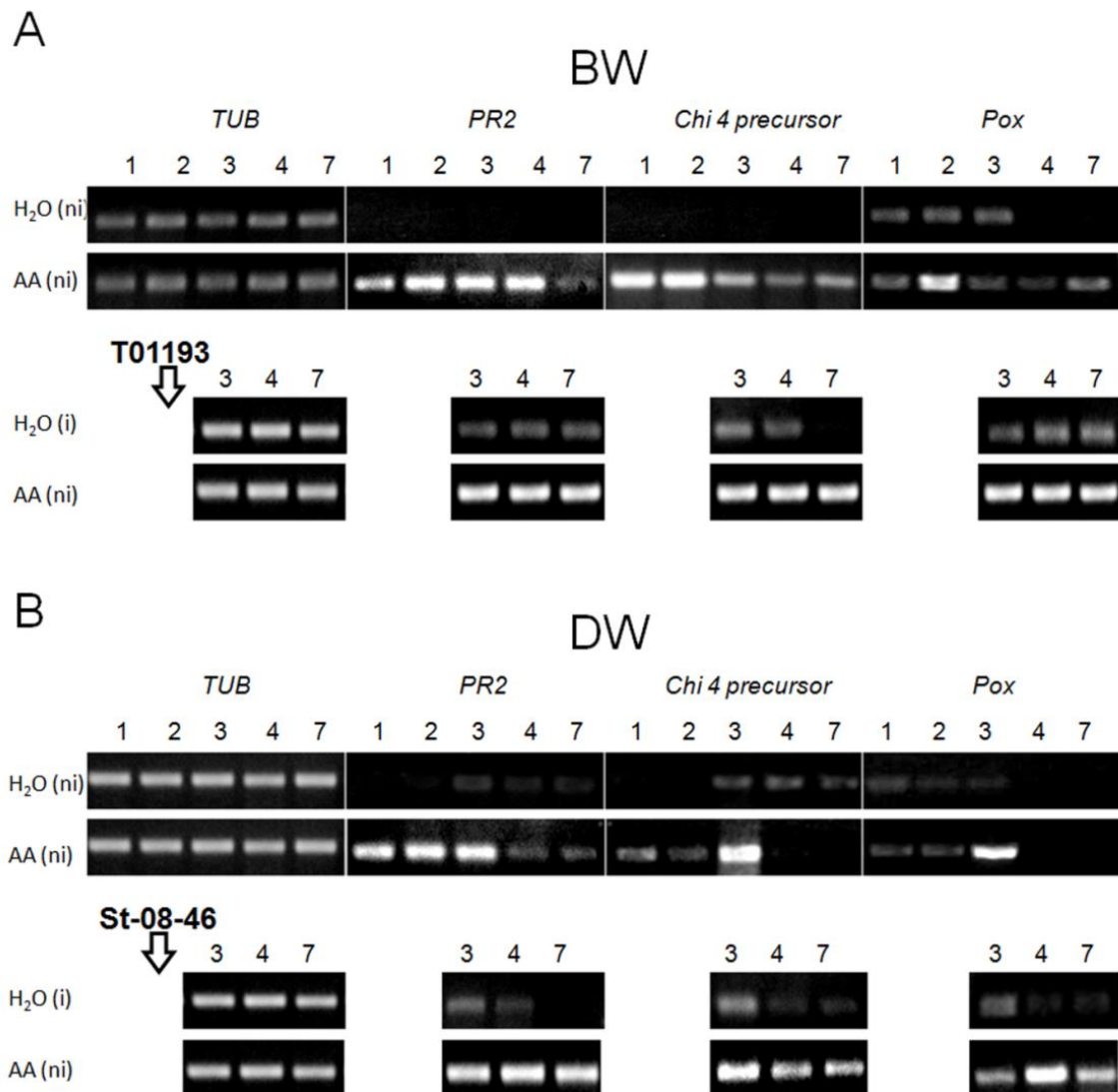


Fig. 1. Effect of foliar AA treatment (50 mg L^{-1}) on *pathogenesis-related 2 (PR2)*-, *Chi4 precursor*- and *Pox*-encoding genes expression in BW (A) and DW (B) leaves revealed by semi-quantitative RT-PCR. Four plant treatments were designed in this assay for each cultivar: non-treated and non-infected [H_2O (ni)], AA-treated and non-infected [AA (ni)], non-treated and *Z. tritici*-infected [H_2O (i)], AA-treated and *Z. tritici*-infected [AA (i)]. For infected samples, the inoculation was carried out 48 hours post AA treatment. Wheat leaves were collected at 1, 2, 3, 4 and 7 days post AA treatment in (ni) and (i) conditions. For each condition, AA-treated leaves were compared to their reference H_2O -treated leaves. RT-PCR products were generated using primers specific for β -tubulin (*TUB*), *PR2*, *Chi 4 precursor* and *Pox* first-strand cDNAs obtained from total wheat RNA, and visualized on agarose gel (1.5%). *TUB*-encoding gene was used as a house-keeping gene. Experiment was repeated three times and only results which were similar from two independent experiments were considered.

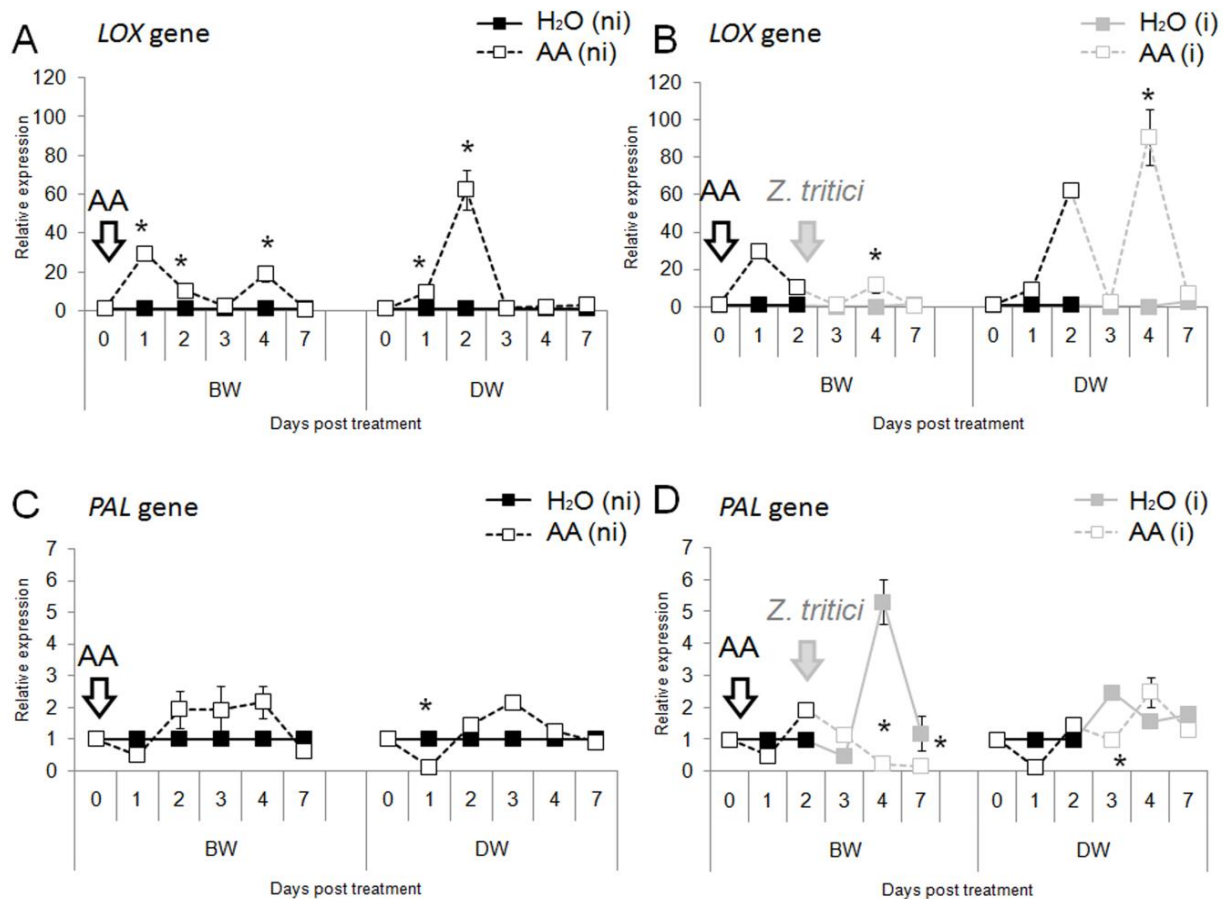


Fig. 2. Effect of foliar AA treatment (50 mg L^{-1}) on *LOX* and *PAL* expressions in BW and DW leaves revealed by quantitative RT-PCR. (A) *LOX* gene expression in (ni) condition. (B) *LOX* gene expression in (i) condition. (C) *PAL* gene expression in (ni) condition. *PAL* gene expression in (i) condition. Four plant treatments were designed in this assay for each cultivar: non-treated and non-infected [H_2O (ni)], AA-treated and non-infected [AA (ni)], non-treated and *Z. tritici*-infected [H_2O (i)], AA-treated and *Z. tritici*-infected [AA (i)]. For infected samples, the inoculation was carried out 48 hours post AA treatment. Wheat leaves were collected after 1, 2, 3, 4 and 7 days post AA treatment. The experiment was conducted in triplicate. Similar results were obtained at least twice. Results correspond to means (\pm s.d) of duplicate reactions. Means of infected samples designed by asterisks are statistically differentially regulated (up- or down-) in comparison to their corresponding H_2O -treated controls using Tukey test at $P \leq 0.05$.

Enzymatic activities

At the enzymatic level, the effect of AA treatment on GLUC, POX, CAT, PAL and LOX enzymatic activities was studied in (ni) and (i) conditions (Fig. 3). In (ni) context, all enzymatic activities, excepted PAL, were transiently stimulated at 1 day post AA treatment on both wheat species compared to reference H_2O -treated (ni) leaves. GLUC, POX, CAT and LOX activities were stimulated in BW leaves (1.6-, 4.9-, 3.4- and 4-fold increases, respectively) and DW (1.4-, 2.2-, 2.8- and 2.7-fold increases, respectively) (Fig. 3A, B, C, E). In detail, the peak of POX activity was observed from 1 to 2 dpt in DW leaves of AA-treated wheat (Fig. 3B). Moreover, a second peak of CAT activity was observed at 3 dpt (2.4-fold) in BW leaves of AA-treated wheat (Fig. 3C).

At (i) context, AA-treated BW leaves recorded a stimulation of POX (2.3-, 2.1- and 2.3-folds) and CAT (1.7-, 1.7- and 2.6-folds) activities from 1 to 5 dpi (3 to 7 dpt), respectively (Fig. 3B, C), followed by a stronger enhancement of PAL activity (2.98- and 3.48-fold increases from 2 to 5 dpi, respectively) (Fig. 3D). Similarly, AA-treated DW leaves recorded an increased POX and CAT activities from 1 dpi (3 dpt) which marked maxima at 5 dpi (7 dpt) (2.2- and 2.5-fold increases, respectively) (Fig. 3B, C), while GLUC and PAL activities were later stimulated at 5 dpi (7 dpt) (1.3- and 1.9-folds, respectively) (Fig. 3A, D).

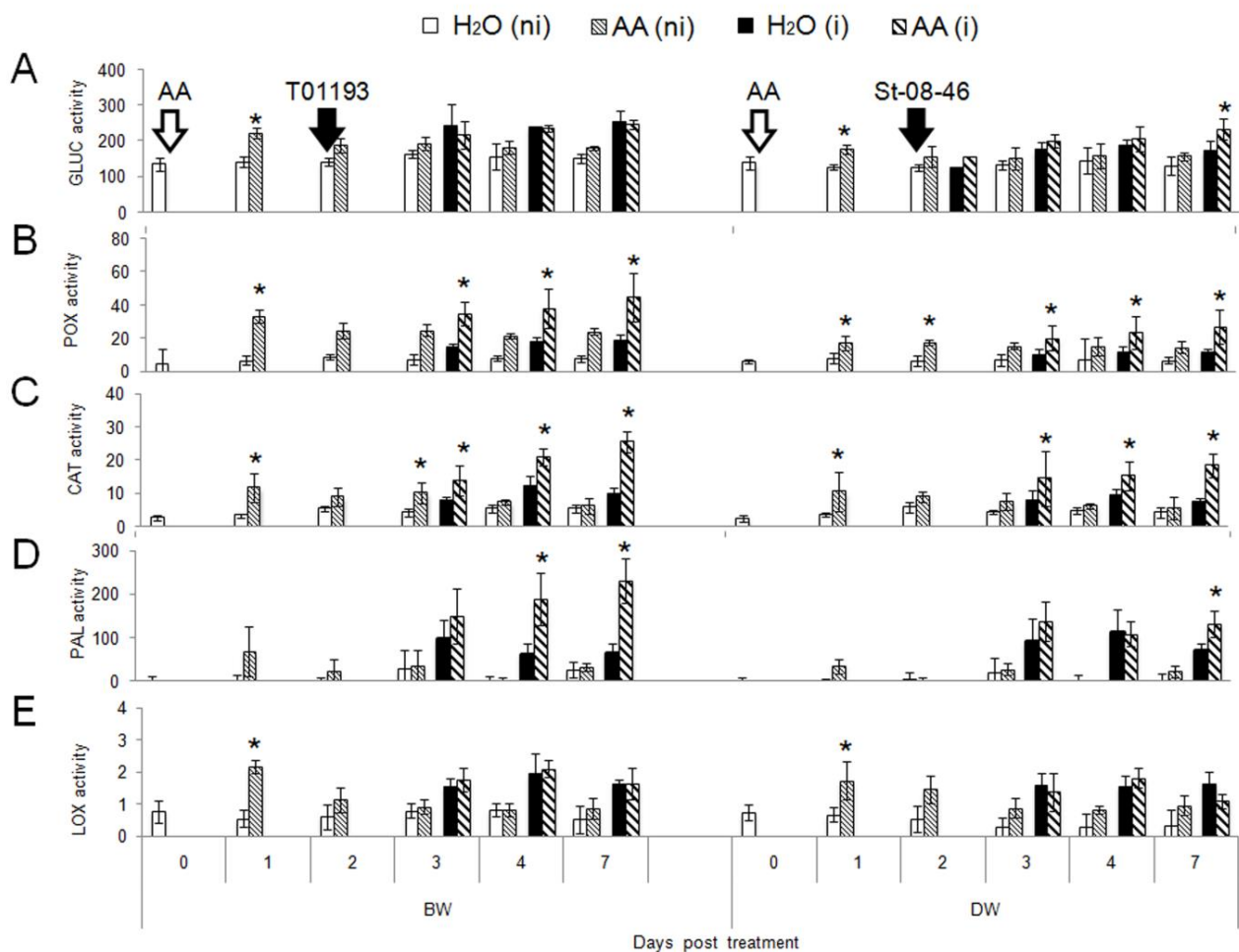


Fig. 3. Effect of foliar AA treatment (50 mg L⁻¹) on five defense-related enzyme activities (expressed in U mg⁻¹ of proteins) on BW and DW leaves. (A) GLUC (β -1,3-glucanase) activity. (B) POX (peroxidase) activity. (C) CAT (catalase) activity. (D) PAL (phenylalanine-ammonia-lyase) activity. (E) LOX (lipoxygenase) activity. Four plant treatments were designed in this assay for each cultivar: non-treated and non-infected [H₂O (ni)], AA-treated and non-inoculated [AA (ni)], non-treated and *Z. tritici*-infected [H₂O (i)], AA-treated and *Z. tritici*-infected [AA (i)]. For infected samples, the inoculation was carried out 48 hours post AA treatment. Wheat leaves were collected after 1, 2, 3, 4 and 7 days post AA treatment. Results correspond to means (\pm s.d) of two independent experiments using three independent replicates for each experiment. AA-treated samples with an asterisk are different from their corresponding H₂O-treated controls (Tukey's test, $P \leq 0.05$).

Mesophyll H_2O_2 accumulation

At the cytological level, H_2O_2 accumulation was revealed by 3, 3-diaminobenzidine (DAB) staining (red-brown staining) in BW and DW leaves. Four classes of *Z. tritici* spores penetration events were divided into four classes (Fig. 4A): C1 (spores with non-penetrated germ tube), C2 (spore with penetrated germ tube without staining), C3 (penetrated germ tube with papilla), C4 (penetrated germ tube with at least 1 whole stained mesophyll cell).

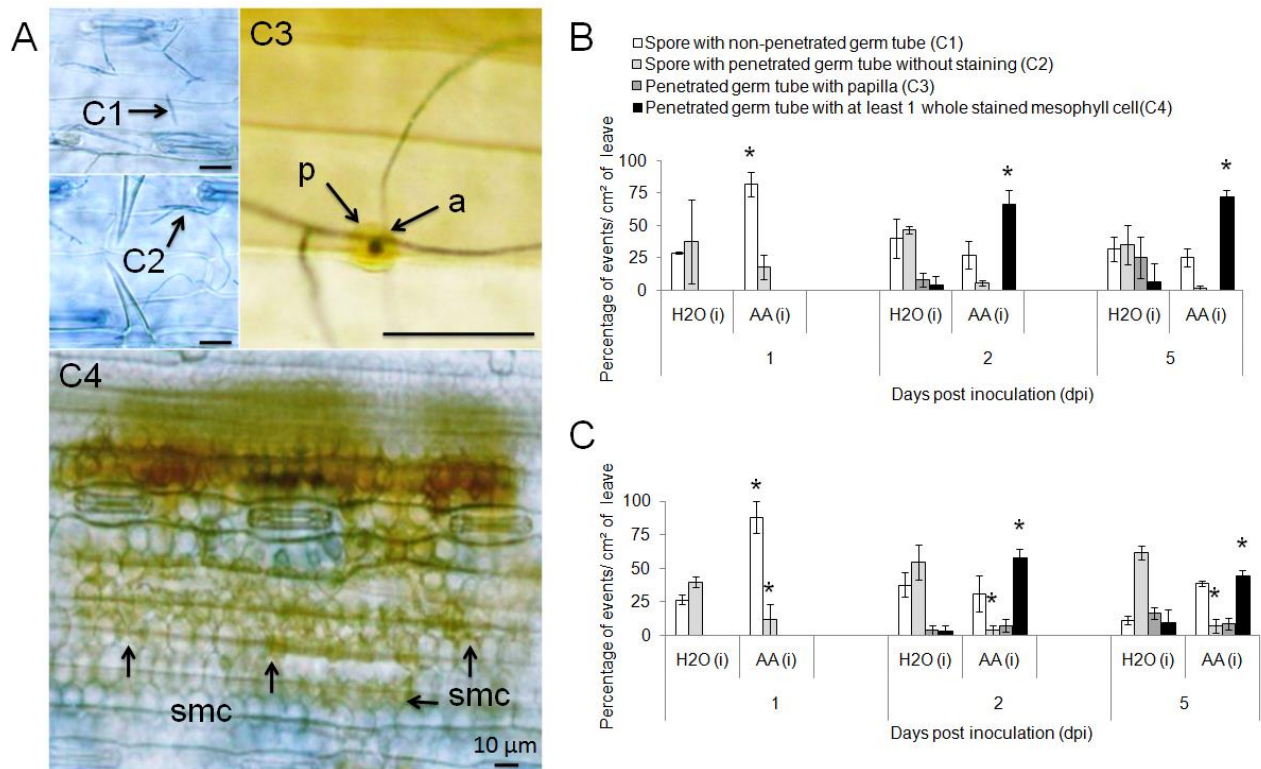


Fig. 4. Effect of AA treatment (50 mg L^{-1}) on H_2O_2 accumulation visualized by DAB staining at the T01193 and St-08-46 *Z. tritici* strains penetration sites on epidermal cells of BW and DW leaves, respectively. The inoculation was carried out 48 hours post AA treatment. Wheat leaves were collected after at 0, 1, 2 and 5 days post inoculation. (A) Microscopic observations of measured classes of H_2O_2 accumulation according to DAB staining intensity: (C1) spore with non-penetrated germ tube, (C2) spore with penetrated germ tube without staining, (C3) penetrated germ tube with papilla and (C4) penetrated germ tube with at least 1 whole stained mesophyll cells. (a) Appressorium-like structure. (p) Papilla. (smc) Stained mesophyll cells. Bar scale = $10 \mu\text{m}$. (B) Percentages of measured classes in BW leaves. (C) Percentages of measured classes in DW leaves. Results correspond to means (\pm s.d) of two independent experiments using three independent replicates for each experiment. AA-treated (i) samples with an asterisk are different from their corresponding H_2O -treated (i) controls (Tukey's test, $P \leq 0.05$).

Results exhibited that AA treatment induced 2.8- and 3.3-fold increases in BW and DW leaves, respectively, associated with 3.4-, 15.1- and 8.3-fold decreases of C2 at 1-2-5dpi, respectively, only in DW leaves. From 2 dpi, AA treatment enhanced significant increase of H_2O_2 accumulation in both wheat species. AA-treated leaves exhibited stronger induction of C4 in BW

leaves (16.1- and 10.8-folds) than DW leaves (14.9- and 4.4-folds), at 2-5 dpi, respectively (Fig. 4B, C).

Polyphenol accumulation

Both local polyphenol-associated auto-fluorescence and total polyphenol content were evaluated. Local polyphenol deposition may be revealed as clear yellow fluorescence at the penetration sites by the fungus. Four types of events were observed, such as C1 (spore with non-penetrated germ tube), C2 (spore with penetrated germ tube without fluorescence), C3 (penetrated germ tube with weak fluorescence) and C4 (penetrated germ tube with strong fluorescence) (Fig. 5A).

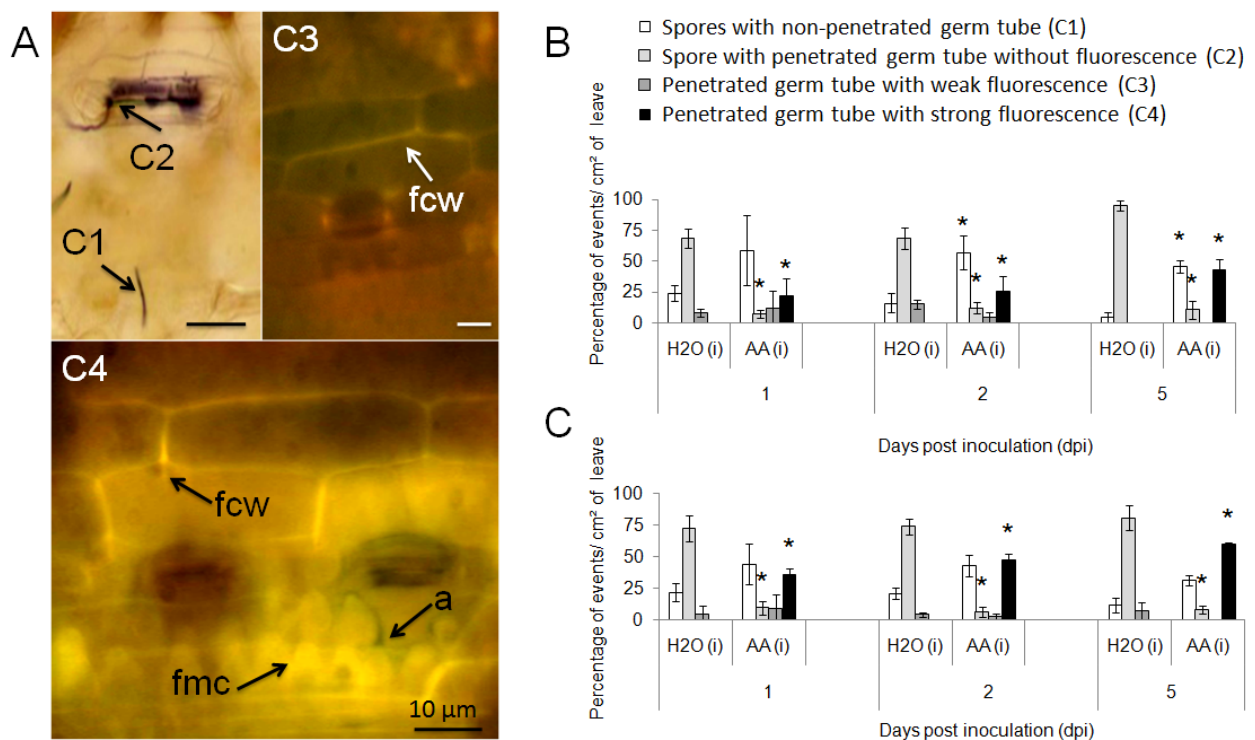


Fig. 5. Effect of AA treatment (50 mg L⁻¹) on the deposition of polyphenol at the T01193 and St-08-46 *Z. tritici* penetration sites on epidermal cells of BW and DW leaves, respectively. The inoculation was carried out 48 hours post AA treatment. Wheat leaves were collected after at 0, 1, 2 and 5 days post inoculation. (A) Microscopic observations of polyphenol deposition classes according to polyphenol-associated autofluorescence intensity: (C1) Spore with non-penetrated germ tube, (C2) Spore with penetrated germ tube without fluorescence, (C3) Penetrated germ tube with weak fluorescence, (C4) Penetrated germ tube with strong fluorescence. (fmc) Fluorescent cell walls. (fcw) Fluorescent mesophyll cells. (a) Appressorium-like structure. Bar scale = 10 μm. (B) Percentages of measured classes in BW leaves. (C) Percentages of measured classes in DW leaves. Results correspond to means (±s.d) of two independent experiments using three independent replicates for each experiment. AA-treated (i) samples with an asterisk are different from their corresponding H₂O-treated (i) controls (Tukey’s test, $P \leq 0.05$).

Results exhibited that AA treatment induced decrease of C2 in both BW (9.8-, 5.7- and 9-folds) and DW (7.6-, 11.7- and 9.8-folds). Moreover, AA treated leaves revealed increased polyphenol deposition (C4) which was higher in DW (36.4, 48 and 60.2% at 1-2-5 dpi) than BW (22.5, 26.2 and 43.7% at 1-2-5 dpi). Second, the effect of AA treatment on polyphenol content was

also measured in whole 3rd leaf of both wheat species at (ni) and (i) context (Fig. 6). In (ni) context, AA-treated exhibited earlier increase of polyphenol content in DW leaves (1.7- and 1.5-folds at 4-7 dpt) than BW (1.8-folds at 7 dpt) (Fig. 6). Similarly, in infected leaves, AA treatment generated the increase of polyphenol content at 4-7 dpt which was stronger in BW (1.51- and 1.64-folds) than DW (1.49- and 1.51-folds). However, the level of polyphenol content was always higher in BW than DW at all time points (Fig. 6).

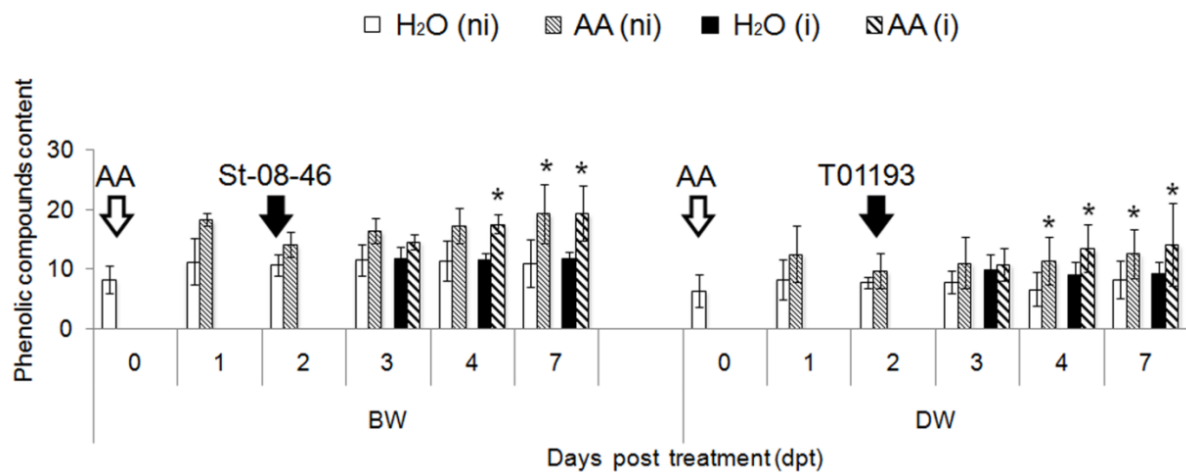


Fig. 6. Effect of AA treatment (50 mg L⁻¹) on the content of phenolic compounds (expressed in mg of CE g⁻¹ of FW) on BW and DW leaves. For infected samples, the inoculation was carried out 48 hours post AA treatment. Four plant treatments were designed in this assay for each cultivar: non-treated and non-infected [H₂O (ni)], AA-treated and non-inoculated [AA (ni)], non-treated and *Z. tritici*-infected [H₂O (i)], AA-treated and *Z. tritici*-infected [AA (i)]. Plants were infected by *Z. tritici* 48 hours after AA treatments. Wheat leaves were collected after 1, 2, 3, 4 and 7 days post AA treatment in (ni) and (i) conditions. Results correspond to means (±s.d) of two independent experiments using three independent replicates for each experiment. AA-treated samples with an asterisk are different from their corresponding H₂O-treated controls (Tukey's test, $P \leq 0.05$).

DISCUSSION

Numerous works has been reported that ascorbic acid was able to protect many dicotylidone crops against fungal diseases [Abdel-Kader *et al.*, 2012; Juber *et al.*, 2014]. Somai-Jemmali *et al.* (submitted publication) described the antifungal effect of ascorbic acid against *Z. tritici* on wheat. Tokunaga *et al.* [2007] recorded that ascorbic acid could also stimulate plant defenses mechanisms. They demonstrated that the incubation of rice seedlings with 20 mM of ascorbic acid could induce defense genes and mainly a novel XIP-type xylanase inhibitor protein (OsXIP) in rice, which inhibits endogenous xylanases [Dornez *et al.*, 2010]. Therefore, the aim of our work was to study the response of several wheat defense pathways to a preventive foliar spray by 50 mgL⁻¹ of ascorbic acid based formulation (AA). Two wheat species were studied including one French BW cultivar (Premio) and one Tunisian DW cultivar (Karim). Each studied wheat specie was inoculated by a

specific *Z. tritici* strain (T01193 and St-08-46, respectively). Since the stimulation of natural defenses is potentially the most useful during a compatible interaction [Tayeh *et al.*, 2013], we proposed to study the elicitor and the potentiating effect of AA in the case of a compatible host-pathogen interaction. We refer here as the elicitor effect, the physiological changes caused by the treatment, in the absence of a pathogen, whereas priming or potentiating allows the treated plants to respond faster and/or stronger after a pathogen infection [Jourdan *et al.*, 2008].

Gene expression assay highlighted an induction of two PR protein genes (*PR2* and *chi 4 precursor*) on both wheat species. We showed here that AA treatment induces *PR2* gene expression and corresponding enzymatic activity in both (ni) and (i) contexts. This last encodes for a class II β -1,3-glucanase, which are acidic proteins secreted into the extracellular space. These proteins catalyze hydrolytic cleavage of the 1,3- β -D-glucosidic linkages in β -1,3 glucan (Ryals *et al.*, 1996). It also induced up-regulation of the *Chi 4 precursor* gene, which encodes a class IV chitinase precursor, in (ni) and (i) contexts. The *Chi 4 precursor* gene encodes a chitinase precursor class IV, which was known to hydrolyze β -1,4-glycoside bonds in chitin to produce N-acetylglucosamine and N, N+ diacetylchitobiose (Cohen-Kupiec and Chet, 1998). Therefore, AA treatment triggers fungal cell wall degrading enzymes which could contribute to the primary defense against the fungi and explain the inhibited leaf penetration and intercellular growth.

Then, we investigated the effect of AA treatment on ROS metabolism through the elicitation of peroxidase, catalase and H_2O_2 accumulation. ROS act as direct antimicrobial molecules or signal molecules involved in metabolic pathways leading to resistance [Hückelhoven, 2007]. First, CAT is an H_2O_2 -scavenging enzyme, while peroxidase catalyzes the oxido-reduction of various substrates as ascorbate using H_2O_2 [Thordal-Christensen *et al.*, 1997]. Many studies investigated the role of ROS metabolism in wheat resistance against *Z. tritici*. They exhibited increased POX and CAT activities and H_2O_2 accumulation in resistant cultivars [Shetty *et al.*, 2003, 2007; Diani *et al.*, 2009]. In our experiment, ROS metabolism was enhanced following foliar spray with AA treatment at (ni) and (i) context. The foliar spray with 50 mgL^{-1} of AA generated an early elicitation of a lignin-forming peroxidase-encoding (*Pox*) gene up-regulation, which could participate in cell wall reinforcement by catalyzing phenolics deposition such as lignin [Van Loon *et al.*, 2006], associated with enhanced peroxidase and catalase enzymes. At cytological scale, AA-treated leaves revealed an important and fast increase of H_2O_2 accumulation in response to fungal penetration. Shetty *et al.* [2007] concluded that H_2O_2 is very important in wheat defense mechanisms against *Z. tritici* during the biotrophic phase of the fungus. Therefore we could suggest here, that the mechanism of oxidative stress occurring after AA treatment was probably caused by the generation of reactive oxygen species with ascorbic acid oxidation or recycling. Our results corroborate several recent studies which confirmed the induction of oxidative stress by exogenous ascorbic acid in other crops

such as corn, sugarcane and maize [Dolatabadian and Jouneghani, 2009; Munir and Aftab, 2011; Ejaz *et al.*, 2012; Darvishan *et al.*, 2013; Ahmad *et al.*, 2013]. Although, Tokunaga *et al.* [2007] didn't detect any induction of oxidative stress in rice seedlings following incubation with ascorbic acid.

Moreover, we investigated the involvement of octadecanoid pathway in wheat-*Z. tritici* interaction by measuring total LOX activity and *LOX* gene expression. LOX acts upstream of the octadecanoid pathway, this enzyme catalysis the dioxygenation of polyunsaturated fatty acid into either 9- or 13- hydroperoxyoctadecadi (tri) enoic acids. These highly reactive aliphatic molecules are then rapidly metabolized into critical defense signals as jasmonic acid (JA) and its methyl ester, methyl jasmonate [Holtman *et al.*, 2000]. In addition to these signals molecules, the actions of LOX may also contribute to defense responses by synthesizing cutin monomers (cutin is the framework of cuticle) or antimicrobial compounds. Two gene families known as *LOX-1* and *LOX-2* encode isoforms of this enzyme [Rouster *et al.*, 1997]. We showed here that AA treatment presented a strong and transient elicitor effect on the expression of *LOX* and total LOX activity. After inoculation, only *LOX* gene expression was induced. However, Tokunaga *et al.* [2007] didn't detect any induction of lipid peroxidation in rice roots following incubation with ascorbic acid.

In order to investigate the induction of the phenylpropanoid pathway by *Z. tritici* infection in BW and DW, (i) phenylalanine-ammonia-lyase (PAL) activity, (ii) *PAL* gene expression and (iii) polyphenol content, were monitored. PAL is one of the key enzymes acting upstream in the phenylpropanoid pathway, leading to the biosynthesis of a large array of phenolic compounds [Zabala *et al.*, 2006]. Many studies exhibited that PAL is associated with in cereals resistance against pathogens such as *Fusarium graminearum*, *F. culmorum*, *Puccinia hordei*... [Prats *et al.*, 2007; Beccari *et al.*, 2011; Orzali *et al.*, 2014]. Our results show that, in (ni) condition, AA treatment did not generate any significant change of PAL activity, *PAL* gene expression and total polyphenol content and deposition in mesophyll cells. However, after inoculation, AA-treated leaves revealed enhanced PAL enzyme activity as well as phenolic compounds accumulation, whereas *PAL* expression was not affected. AA therefore only acts as a priming molecule on total PAL activity and polyphenol content and deposition, whilst *PAL* gene expression did not correlate with phenolic compounds biosynthesis pathway. Several studies confirmed the correlation of PAL activity and polyphenol content in incompatible interaction with *Z. tritici* [Hassan *et al.*, 2007; Adhikari *et al.*, 2007].

In conclusion, AA treatment resulted in higher elicitor and potentiating effects on *Pox* gene expression, total POX, CAT, PAL, LOX activities and total polyphenol content in BW leaves. Ascorbic acid is known to be a good inducer of ROS metabolism witch could interferes with lipid peroxidation or penylpropanoid pathways through H₂O₂ or peroxidase [Marrs, 1996; Thordal-

Christensen *et al.*, 1997; Bucciarelli *et al.*, 1998; Dolatabadian *et al.* 2008; Dolatabadian and Saleh Jouneghani, 2009]. Thus, we suggest here that octadecanoids and phenylpropanoids stimulation could not directly caused by AA treatment but it is probably a consequence of ROS metabolism activation. This study allowed explaining the good protection of AA treatment previously recorded by Somai-Jemmali *et al.* (Publication 2 Part 1) on both wheat species against *Z. tritici*, which resulting in decreased fungal leaf penetration and mesophyll colonization. Indeed, enhanced fungal cell wall degrading enzymes, H₂O₂ and polyphenol deposition at fungal penetration sites could contribute to the antifungal effect of AA already demonstrated by Somai-Jemmali *et al.* (Publication 2 Part 1) and stop the development of the fungus within the plant cells [Vanacker *et al.*, 2000]. In addition, our work promotes a comparative study of defense mechanism response to exogenous resistance inducers between BW (*T. aestivum*) and DW (*T. durum*). Despite their similar level of susceptibility to *Z. tritici*, BW and DW tested cultivars exhibited distinct responses to the treatment. Therefore, ROS metabolism, phenylpropanoids and total LOX activity were more responsive to AA in BW leaves, whereas the stimulation of *PR2* activity *LOX* gene expression and cell wall straitening by H₂O₂ and polyphenol deposition was higher in DW leaves. Few works investigated the comparison of physiological defenses response to biotic stresses, between different wheat species and our results are supported by Ibrahim *et al.* [2013] who showed that the antioxidant activities were stimulated in response to UV and/or heat stresses. We confirm that ROS metabolism is more responsive to stress in DW than in BW plants. Moreover, Gogoi *et al.* [2001] compared phenol compounds accumulation and POX activity in response to *Neovossia indica* in resistant BW and DW cultivars and in susceptible ones. Likewise, resistant cultivars exhibited a stronger induction of phenolic compounds and POX activity in BW than in DW plants.

Hence, AA can act efficiently in wheat as a resistance inducer when applied at the appropriate concentration and the current stage of development, although further investigations using more susceptible cultivars of of BW and DW in interaction with more pathogenic *Z. tritici* strains are required to confirm our results.

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Publication 3: *Aschophyllum nodosum* extract induces defense response in durum and bread wheat and confers protection against *Zymoseptoria tritici* causal agent of STB disease under greenhouse condition

ABSTRACT

This study aims at characterizing the antifungal effect and resistance inducer effect of *Aschophyllum nodosum* extract-based formulation (*A. nod*), against *Zymoseptoria tritici* major pathogen of bread wheat (BW) and durum wheat (DW). Results exhibited that tested product affected leaf penetration, mesophyll colonization and pycnidium formation and could at least 2-fold reduce severity associated with a strong decrease of *Z. tritici* cell wall degrading enzymes (CWDE) activities, such as endo- β -1,4-xylanase, endo- β -1,3-glucanase and protease, during necrotrophic phase on both wheat species. However, *A. nod* treatment induced the up-regulation of β -1,3-glucanase (*PR2*)-, peroxidase (*Pox*)-, lipoxygenase (*LOX*)- and phenylalanine ammonia lyase (*PAL*)-encoding genes and enhanced glucanase, peroxidase, catalase, PAL and LOX proteins on both wheat species. At cytological level, H₂O₂ and polyphenol deposition were enhanced following fungal penetration. Therefore, *A. nod* induced wheat defense response probably by enhanced oxidative burst associated with activated phenylpropanoid and octadecanoid pathways. These defense pathways were triggered in both BW and DW cultivars with slight difference. We recorded more responsive ROS and lipoxygenase on tested BW cultivar, while the tested DW cultivar revealed more responsive phenylpropanoids and β -1,3-glucanase. Hence, *A. nod* could present a good protection level exclusively due to its ability to induce a set of induced wheat defense pathways. Therefore, it could present an interesting alternative to control *Z. tritici* whatever the wheat specie. Further investigations of more susceptible BW and DW cultivars response to *A. nod* treatment must be performed in order to confirm our results.

Key words: *Zymoseptoria tritici*, *Aschophyllum nodosum*, antifungal, resistance inducer, wheat species.

INTRODUCTION

Septoria tritici blotch (STB) caused by *Zymoseptoria tritici* (Quaedvlieg *et al.*, 2011) previously known as *Septoria tritici* (teleomorph *Mycosphaerella graminicola*) is one of the most devastating fungal foliar disease on bread wheat (*Triticum aestivum* L. em Thell, BW) and Durum wheat (*Triticum durum* Desf, DW). It causes annual yield losses which could reach 50% during outbreaks (Babadoost and Herbert, 1984; Eyal *et al.*, 1987; Palmer and Skinner, 2002). *Z. tritici* is a hemibiotrophic fungus which process a long symptomless phase considered as biotrophic, followed by a symptomatic necrotrophic phase characterized by the occurrence of chlorotic and/or necrotic

lesions on the leaf surface, and the formation of pycnidia (Kema *et al.*, 1996; Shetty *et al.*, 2003; Siah *et al.*, 2010b). During the necrotrophic phase, the fungus releases cell-wall degrading enzymes (CWDEs) such as endo- β -1,3-glucanases and endo- β -1,4-xylanases, as well as proteases, leading to an increase of nutrient content for the fungus in the apoplastic space (Palmer and Skinner, 2002; Keon *et al.*, 2007; Siah *et al.*, 2010b).

In literature, elicitors containing carbohydrate, such as seaweeds extract, has been reported to be inducers of plant resistance to various pathogens (Vallad and Goodman, 2004). These compounds are an environmentally friendly and allowed reducing the use of chemicals. *Aschphyllum nodosum* is the most widely used brown seaweed in agriculture and grows along the Atlantic coastlines of North America and Europe (McLachlan, 1985; Ugarte *et al.*, 2006; Rayorath *et al.*, 2008). Many recent works reported that *A. nodosum* treatment could stimulate growth and leaf chlorophyll levels in spinach and mango (Fan *et al.*, 2013; Morales-Payan, 2013), growth of spinach, strawberry, Arabidopsis and *Brassica napus* (Rayorath *et al.*, 2008; Jannin *et al.*, 2013; Alam *et al.*, 2013; Xu and Leskovar, 2015), yield quality improvement in strawberry, onion, Arabidopsis, wheat and spinach (Rayorath *et al.*, 2008; Dogra and Mandradia, 2012; Fan *et al.*, 2013; Alam *et al.*, 2013; Sen *et al.*, 2015) and resistance to abiotic stresses (drought, salinity, temperature extremes) in eggplant, orange, *Festuca arundinacea* (Ayad, 1998; Spann and Little, 2010; Hegazi *et al.*, 2014).

Moreover, *A. nodosum* extract has been reported to reduce the severity of many plant diseases caused by nematodes such as *Meloidogyne javanica* in tomato (Whapam *et al.*, 1994), viruses such as Tobacco Mosaic Virus in tobacco (Klarzynski *et al.*, 2003), bacteria such as *Pseudomonas syringae* in *Arabidopsis thaliana* and *P. aeruginosa* in *Caenorhabditis elegans* (Subramanian *et al.*, 2011; Kandasamy *et al.*, 2012) and fungi such as *Alternaria radicina* and *Botrytis cinerea* in carrots (Jayaraj *et al.*, 2008), *A. cucumerinum*, *Didymella applanata*, *Fusarium oxysporum* and *Botrytis cinerea* in cucumber (Jayaraj *et al.*, 2011), *Sclerotinia sclerotiorum* in tomato (Subramanian *et al.*, 2011) and *A. solani* in tomato (Hernández-Herrera *et al.*, 2014). These properties could be attributed to the presence of fucans (4–10%) and laminarins (1–7%) in *A. nodosum* extract which have been characterized by an interesting antimicrobial and defense inducer effect (Baardseth *et al.*, 1975; Patier *et al.*, 1993; Mercier *et al.*, 2001; Klarzynski *et al.*, 2000, 2003; Khan *et al.*, 2009; Vera *et al.*, 2011). Moreover, laminarin could control *Z. tritici* in wheat (Esnault *et al.*, 2005). At our knowledge, there is no work which reported the use of *A. nodosum* extract to control wheat diseases. However, other types of algae such as the green algae *Ulva fasciata* and brown algae *Laminaria digitata* were efficient against wheat diseases like *Z. tritici* and *Blumeria graminis f. sp. tritici* (Joubert *et al.*, 1998; Esnault *et al.*, 2005; Renard-Merlier *et al.*, 2007; Paulert *et al.*, 2010). Few studies investigated the elicitor effect of plant diseases. It was reported that seaweed

polysaccharides trigger an initial oxidative burst at local level and the activation of salicylic (SA), jasmonic acid (JA) and/or ethylene signaling pathways at systemic level. The activation of these signaling pathways leads to an increased expression of genes encoding: (i) Pathogenesis-Related (PR) proteins with antifungal and antibacterial activities; (ii) defense enzymes such as phenylalanine ammonia lyase (PAL) and lipoxygenase (LOX) which determine accumulation of phenylpropanoid compounds (Jayaraj *et al.*, 2008, 2011; Vera *et al.*, 2011). Considering all these observations, the aims of this current study is to focus on the potential antifungal and plant resistance inducer properties of a based-*A. nodosum* extract (*A. nod*) against *Z. tritici* on susceptible bread wheat (BW) and durum wheat (DW) cultivars. The difference in response of both wheat species to *A. nod* will be discussed.

MATERIALS AND METHODS

Fungal material and inoculation

For *in vivo* assays, the two *Z. tritici* T01193 and St-08-46 strains were chosen for their high level of pathogenicity, respectively on BW and DW. Cultures were grown for 3 weeks at 18°C and were stored at -80°C for further use. Plants were inoculated at 2 days post treatment (dpt) with 30 mL of a 10^6 spores mL⁻¹ suspension in a 0.05% of Tween 20 (Sigma Aldrich, USA) solution using a hand sprayer. Control plants were sprayed with tap water containing 0.05% of Tween 20. Immediately after inoculation, each pot was covered with a clear polyethylene bag for 3 days in order to ensure a water-saturated atmosphere. Disease level was scored at 22 days post inoculation (dpi) by measuring percentage of leaf area with symptoms (necrosis and pycnidium) and the index of pycnidium coverage (which describes the importance of pycnidia in necrosis following a scale noted from 1 to 5) within necrotic lesions on the 3rd leaf of 25 plantlets. Data represent means of two independent experiments.

Direct effect assessment on PDA medium

For this work we used a new formulation based on *A. nodosum* (*A.nod*) extract (25%) provided by CODA (Lerida, Spain). It was tested *in vitro* by measuring spore germination and fungal hyphal growth according to Siah *et al.* (2010a). *Spore germination*: aliquots of 0.6 mL of each strain, adjusted at 5×10^5 spores mL⁻¹, were sprayed on PDA plates containing various concentrations of tested product, which corresponded to 0.07; 0.22; 0.6; 2; 6.1; 18.5; 55.5; 160; 500 and 1500 mg L⁻¹ of *A. nodosum* extract. The plates were placed at 18°C in the dark for 24h. The percentages of germinated spores were then calculated for 100 spores, for each plate, using light microscope (Nikon, Eclipse 80i). *Mycelium growth*: 5 µL of 5×10^5 spores mL⁻¹ of each strain were spotted on

the PDA plates containing the same concentrations of tested compounds. After incubation for 10 days at 18°C in the dark, hyphal growth were compared. Assays were repeated twice and for each condition, three plates with three spots per plate were used as triplicates.

Plant material and tested product

Cultivars of the two wheat species known to be susceptible to STB were used for this study: bread wheat (BW) cultivar Premio and durum wheat (DW) cultivar Karim. Grains were pre-germinated in Petri dishes (12 x 12 cm) on moist filter paper in darkness at 20°C for 24 h, at 4°C for 48 h and then at 20°C for 24 h. Germinated grains were then transplanted into soil in pots of 15 cm-diameter (12 grains/pot). The pots were placed in the greenhouse at 18°C and under day-night cycles of 16h /8h. After 3 weeks, when the third leaf was fully expanded, plants were sprayed with 500 mg L⁻¹ of *A. nod.*

RNA extraction and Reverse Transcription (RT)

For plant genes expression study, samples were collected from non inoculated (ni) and inoculated plants (i). At (ni) conditions, samples were collected at 1, 2, 3, 4 and 7 dpt. For (i) conditions, samples were collected at 0, 1, 2 and 5 dpi (corresponding to 2, 3, 4 and 7 dpt). Total RNA was extracted from 100 mg of plant tissue using RNeasy Plant Mini Kit (Quiagen, The Netherlands). Genomic DNA contaminating the samples was removed by treatment with DNase using RNase-Free DNase Set (Quiagen, The Netherlands). Reverse transcription of total RNA was carried out using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA) according to the manufacturer's protocol.

Analysis of gene expression by semi-quantitative RT-PCR and real-time RT-PCR

PCR reactions were conducted on subsequent obtained cDNA to amplify five wheat genes encoding for *PR proteins 2* (β -1,3glucanase), *Chi 4 precursor* (chitinase IV precursor), *Pox* (peroxidase); *PAL* (phenylalanine ammonia lyase) and *LOX* (lipoxygenase). These analyses were performed using the following gene-specific primers presented in Table 1. The primers pairs were designed using the Primer Express® program and were tested for secondary structure using Net Primer® program. The gene expression of *PR2*, *Chi 4 precursor* and *Pox* was investigated by semi-quantitative RT-PCR. PCR was carried out in a 20 μ L reaction mixture containing 1x PCR buffer, 2 mM dNTPs, 10 μ M of each primer, 0.5 unit of Taq polymerase and 20 ng of cDNA using the following protocol: 94°C for 5 min, followed by 30 cycles each with 94°C for 1 min, 55°C for 1 min and 72°C for 1 min. The PCR reaction was terminated at 72°C for 7 min. PCR products were analyzed by electrophoresis on ethidium-bromide stained 1.5% agarose gels run in 0.5 x Tris-acetic acid-EDTA buffer at 100 V for

45 min. Gels were visualized under UV light. *TUB* was used as an internal control in semi-quantitative Reverse-Transcription Polymerase Chain Reactions (RT-PCR) analysis. Experiment was repeated 3 times and only results which were repeated at least twice were considered. The investigation of *PAL* and *LOX* expression was performed by real time-RT-PCR analysis. The amplification specificity of each qRT-PCR was checked by a single peak in melt curve analysis, and no primer dimmers were detected using agarose gel electrophoresis. Real-time RT-PCR was performed using ABI Prism 7300 detection system (Applied Biosystems, USA), using the following thermal profile : after incubation for 10 min at 95°C, 15s at 95°C (denaturation), 1 min at 60°C (annealing/extension) for 40 cycles and 30s at 72°C (data acquisition). Relative expression for each cDNA was calculated for each time point relative to control at the same time. It corresponds to $2^{-\Delta\Delta Ct}$ described by Pfaffl *et al.* (2002) and was calculated using the relative expression software tool REST®. $\Delta\Delta Ct = [Ct \text{ Target (Sample)} - Ct \text{ Reference (Sample)}] - [Ct \text{ Target (Control)} - Ct \text{ Reference (Control)}]$. *TUB* is used as reference gene. The results were normalized with the *TUB* and *ACT* genes and expressed relatively to the control, for each time point, corresponding to a fixed value of 1. After qPCR, a melt curve was generated to verify the specificity of each amplification reaction. The experiment was conducted in triplicate and only results which were repeated at least twice were considered.

Table 1 Primers used in the semi-quantitative and quantitative reverse-transcription polymerase chain reactions studies

Gene	Primer sequences (5'-3')	Accession numbers
<i>TUB</i> (<i>beta tubulin</i> , housekeeping gene)	GGAGTACCCTGACCGAATGATG AACGACGGTGTCTGAGACCTTT	U76895
<i>ACT</i> (<i>actin</i> , housekeeping gene)	AACCTTCAGTTGCCAGCAA TGTTTCGACCGCTGGCATAAC	AB181991
<i>PR2</i> (β -1,3 glucanase)	CTCGACATCGGTAACGACCAG GCGGCGATGTAAGTATGATGTTT	Y18212
<i>Chi4 precursor</i> (<i>chitinase class IV precursor</i>)	TTCTGGTTCTGGATGACCAAC ACTGCTTGCAGTACTCCGTG	AF112966
<i>Pox</i> (<i>peroxidase</i>)	GAGATTCCACAGATGCAAACGAG GGAGGCCCTTGTTTCTGAATG	X56011
<i>PAL</i> (<i>phenylalanine ammonia lyase</i>)	CCCCCATTTGGTGTCTCCAT ACTGCGCGAACATCAGCTT	AY005474
<i>LOX</i> (<i>lipoxygenase</i>)	GGGCACCAAGGAGTACAAGGA GCTCGTGATGGTGTGGATGA	U32428

Protein extraction and enzymatic assays

Fungal enzyme assays: Three fungal cell-wall degrading enzyme (CWDE) activities (endo- β -1,4-xylanase, endo- β -1,3-glucanase and protease) were measured at 4, 10, 16 and 22 dpi. Briefly, 2.5 g of third leaves were sampled and immediately frozen in liquid nitrogen for grinding in mortar. Endo- β -1,4-xylanase activity was measured at pH 4.8 and 45°C using xylan (1%, w/v) (Sigma) as

substrate and xylose (10 mM) (Sigma Aldrich, USA) as reducing control sugar (Siah *et al.*, 2010). Endo- β -1,3-glucanase activity was measured at pH 4.8 and 50 °C using Laminarin (Sigma Aldrich, USA) at pH 5 (0.5 %, w/v) as substrate and glucose (10 mM) as reducing control sugar (Douaiher *et al.*, 2007). Absorbance was measured at 540 nm. One unit of endo- β -1,4-xylanase and endo- β -1,3-glucanase activities is defined as the amount of enzyme necessary to release 1 μ mol of reducing sugar/p-nitrophenol $\text{min}^{-1} \text{mL}^{-1}$ of enzyme solution. Protease activity assays were assessed according to Hellweg (2003). Absorbance was measured at 400 nm. One unit of protease activity is defined as a change in enzyme activity of 1 Pox hour. These activities were expressed in mU mg^{-1} of proteins.

Plant enzyme assays: Five plant enzyme activities involved in defense mechanisms were measured: β -1,3-glucanase (GLUC), catalase (CAT), peroxidase (POX), lipoxygenase (LOX) and phenylalanine ammonia-lyase (PAL). Samples were collected at the same timing used for gene expression analysis. Each sample consisted of three medial segments excised from three third leaves. GLUC activity was analyzed according to Douaiher *et al.* (2007). CAT activity was assessed according to Randoux *et al.* (2010); one unit of CAT activity is defined as the amount of the enzyme which degrades H_2O_2 at 1 $\mu\text{mol min}^{-1}$, ($\epsilon = 36 \text{ mM}^{-1}\text{cm}^{-1}$). POX was estimated according to El Hadrami and Baaziz (1995) and expressed as $\text{nkcat mg of protein}^{-1}$. One unit of POX activity is defined as the amount of enzyme that catalyses the conversion of guaiacol as substrate at A_{470} after 2 min of H_2O_2 addition. LOX activity was measured according to Randoux *et al.* (2010). One unit of LOX activity is defined as the amount of enzyme that catalyses the conversion of substrate (linoleic acid) at 1 mols^{-1} ($\epsilon = 23 \text{ mM}^{-1}\text{cm}^{-1}$). PAL activity was calculated as described by Randoux *et al.* (2010). One unit of PAL activity is defined as the amount of enzyme that catalyses the formation of cinnamic acid at 1 mols^{-1} ($\epsilon = 10 \text{ mM}^{-1}\text{cm}^{-1}$). POX, CAT, LOX and PAL activities were expressed in U mg^{-1} of proteins.

Total protein concentration was measured at absorbance of 595 nm using bovine serum albumin as a standard (Bradford, 1976). For all tested activities, absorbance was measured using Thermo Scientific™ GENESYS 10S UV-Vis spectrophotometer. Data represent means of two independent experiments. Three independent replicates were carried out for each experiment.

Cytological analysis

The accumulation of H_2O_2 was revealed by 3,3-diaminobenzidine (DAB) staining (red-brown staining) according to Randoux *et al.* [2010]. For polyphenol deposition visualization, leaves stained with Trypan Blue were used for fluorescence microscopy using a Nikon microscope (Champigny-sur-Marne, France) fitted for fluorescence (blue exciter filter, maximum transmittance 400 nm and barrier filter with a transmittance range of 500 to 800 nm). As described by Randoux *et*

al. [2010], a yellow fluorescence visualized under UV-light microscopy indicates the presence of polymeric phenolic compounds. H₂O₂ and polyphenol visualization were detected at fungal penetration sites in 2-cm-long segment of 3rd leaves. Leaf samples were collected from the same plantlets used for enzymatic and molecular analysis and at the same timing. Data represent means of two independent experiments. Three independent replicates were carried out for each experiment.

Total polyphenolic compounds quantification

The quantification of the content of total soluble phenol compounds was determined according to the Folin-Ciocalteu method modified by El Hadrami *et al.* (1997). Leaf samples were collected from the same plantlets used for enzymatic analysis. Foliar tissues (200 mg from 3rd leaf), were homogenized in an ice bath with 1.5 ml methanol. The homogenate was centrifuged three times at 10000g for 5 min; supernatants were recuperated each time. One hundred microlitres of the supernatant was added to Folin-ciocalteu reagent and sodium carbonate. The mixture was incubated at 40°C for 30 min and the blue color was read at 760 nm using Catechin (Sigma Aldrich, USA) as a standard. The polyphenol content was determined for the experiments described above and was expressed in mg-equivalents of Catechin (Sigma Aldrich, USA) (CE) per gram of fresh weight (FW). Data represent means of two independent experiments. Three independent replicates were carried out for each experiment.

Statistical data analysis

Statistical data analyses of molecular and enzymatic assays were made separately for each wheat species (BW cv. Premio/T01193 strain and DW cv. Karim/St-08-46 strain). For quantitative gene expression, the analyses were performed using the relative expression software tool REST® as described by Pfaffl *et al.* (2002) and the significant differences were tested at 95% ($P < 0.05$). For severity parameters, enzymatic activities and microscopy, an analysis of variance (ANOVA) was performed using Tukey test at $P \leq 0.05$ using the XLSTAT software (Addinsoft, France).

RESULTS

Direct effect of A. nod on T01193 and St-08-46 Z. tritici strains

First, the verification of the *in vitro* effect of *A. nod* on the spore germination and hyphal growth of T01193 and St-08-46 strains in PDA medium plates didn't show any significant difference compared to reference plates without treatment (Annex 2). Second, the direct effect of 500 mg L⁻¹ of *A. nod* was investigated on T01193 and St-08-46 *Z. tritici* strains inoculated respectively on BW and DW leaves. Microscopic observation of (i) spore germination and hyphal

growth at 4 dpi, (ii) mesophyll colonization and pycnidium formation at 22 dpi, were performed on BW (Fig. 1A) and DW (Fig. 1B). Microscopic observation assessed by Calcofluor staining at 4 dpi showed similar hyphal growth in treated and untreated leaves, while at 22 dpi *A. nod*-treated leaves revealed reduced colonization and sporulation compared to reference H₂O-treated leaves on both wheats. Secondly, Table 2 represents the rate of cytological parameters assessed for both tested pathosystems at : (i) 1 dpi: germinated spores (GS), (ii) 4 dpi: non germinated spores (NGS), spores leading to stomatal penetration (SP), spores leading to a direct penetration (DP), and germinated spores that did not penetrate (GSNP), (iii) 22 dpi: no colonized stomata (NCS), colonized stomata but not transformed into pycnidia yet (CS) and colonized stomata transformed into pycnidia (P).

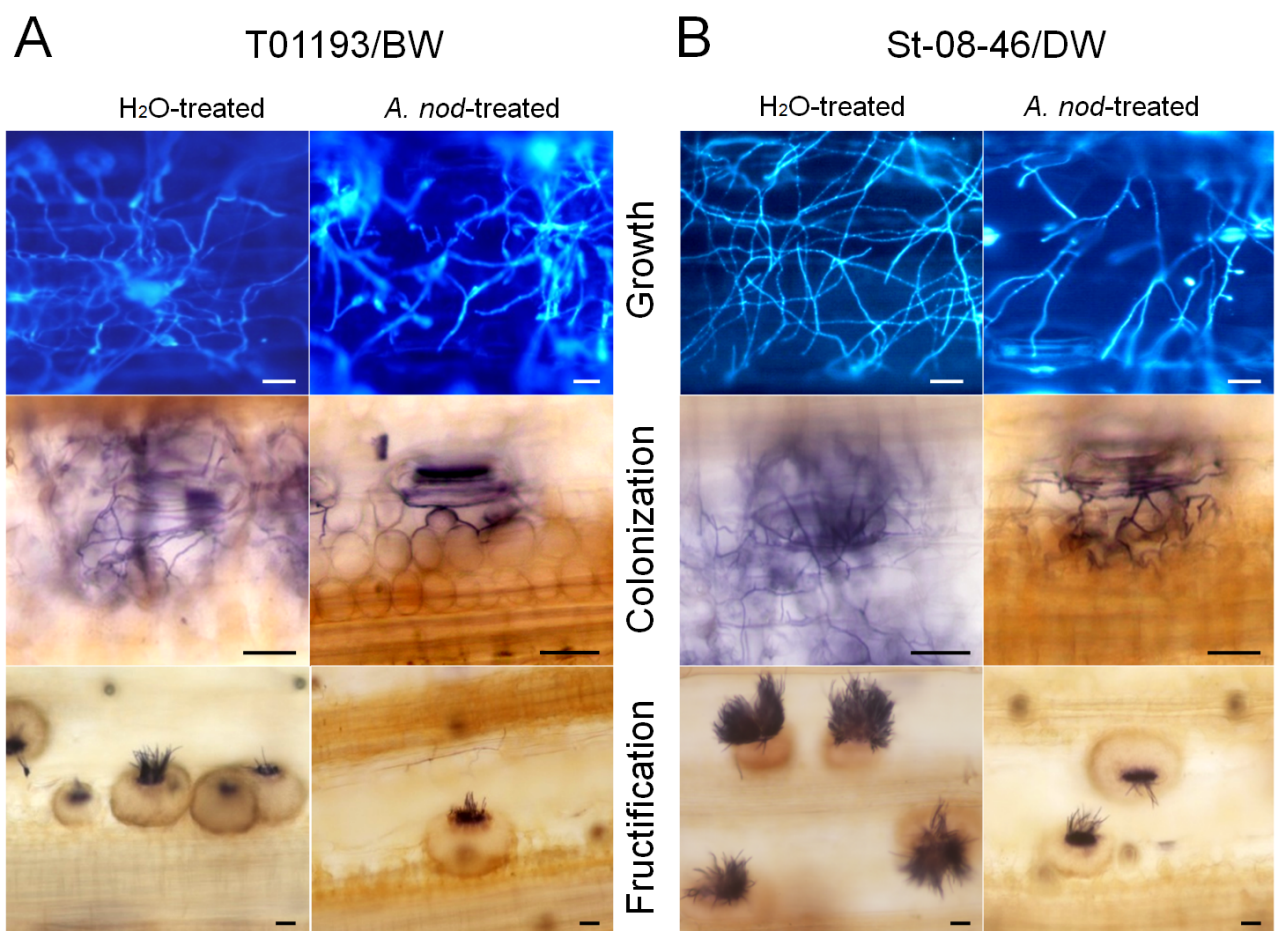


Fig. 1. Effect of 500 mg L⁻¹ of *A. nod* on the spore germination, mesophyll colonization and fructification at 22 dpi of *Z. tritici* on DW and BW. A, Susceptible BW cv. Premio infected by T01193 *Z. tritici* strain. B, Susceptible DW cv. Karim infected by St-08-46 *Z. tritici* strain. Microscopic observation of the spore germination of both *Z. tritici* strains was monitored at 4 dpi using Calcofluor fluorescence. Microscopic observation of the mesophyll colonization and fructification was monitored at 22 dpi using Trypan blue staining. Scale bar = 10 µm.

At 1 dpi, *A. nod* treatment didn't generate any significant difference of spore germination compared to reference H₂O-treated leaves on both wheat species (Table 2). At 4 dpi, *A. nod*

treatment caused 1.9- and 1.1-fold decreases of SP and DP, respectively, only in DW leaves. No significant difference of NGS and GSNP was observed. At 22 dpi, *A. nod*-treated leaves exhibited 2.99-fold decreased NCS on BW leaves, while it was 1.8-fold increased on DW leaves. Also, CS was 2.19- and 2.10-fold increased on BW and DW leaves, respectively. However, P was 1.81- and 2.17-fold decreased on BW and DW leaves, respectively.

Table 2 Percentages of different cytological parameters measured at 1, 4 and 22 days post infection (DPI) of BW and DW leaves by of T01193 and St-08-46 *Z. tritici* strains, respectively. At each time point, treated samples with 500 mg L⁻¹ of *A. nod* were compared to their corresponding reference H₂O-treated samples.

Parameters (%)	DPI	T01193 / BW		St-08-46 / DW	
		H ₂ O-treated	<i>A. nod</i> -treated	H ₂ O-treated	<i>A. nod</i> -treated
GS	1	54±3.7 a	57.7±2.7 a	72±11.5 a	61.1±0.9 a
NGS	4	25.3±2.1 a	21.1±10.9 a	20.4±3.5 a	24.3±4.3 a
SP		17.9±1.7 a	17.1±1.1 a	9.8±0.2 a	5.1±1.2 b
DP		10.3±4.5 a	9.8±2.1 a	28.9±5.9 a	26.5±6.1 b
GSNP		46.4±3.3 a	52±9.8 a	41±7.2 a	44.1±1.2 a
NCS	22	20.6±3.5 a	6.9±1.7 b	13.8±0.2 b	24.6±6.3 a
CS		29.9±2.7 b	65.6±7.2 a	21.6±2.9 b	45.4±6.4 a
P		49.5±3.9 a	27.4±5.6 b	65.1±3 a	30±3.2 b

GS: germinated spores. NGS: non germinated spore. DP: spore leading to a direct penetration. SP: spore leading to a stomatal penetration. GSNP: germinated spore without penetration. NCS: non-colonized stomata. CS: colonized stomata but not yet transformed into pycnidia. P: colonized stomata transformed into pycnidia. Data are mean values of two independent experiments (±s.d). Statistics were made separately for each wheat specie. Means of *A. nod*-treated samples tagged with the same letter are not statistically different to their corresponding H₂O-treated samples in the same wheat specie (Tukey test, $P < 0.05$).

Moreover, Disease severity screening showed that the Percentage of Leaf Necrosis (PLN) and the Index of Pycnidium Coverage (IPC) were measurable from 16 dpi and showed maxima at 22 dpi (Table 3). At 16 dpi, *A. nod*-treated leaves showed 4.6- and 5.8-fold decreases of PLN and 4- and 8.3-fold decreases of IPC, on BW and DW leaves, respectively. Similarly, at 22 dpi, *A. nod*-treated leaves exhibited 2.7- and 3-fold decreases of PLN and 2.3- and 2.4-fold decreases of IPC, on BW and DW leaves, respectively. Moreover, *Z. tritici* CWDE activities such as endo- β -1,4-xylanase and endo- β -1,3-glucanase and protease were measured at 4, 10, 16 and 22 dpi (Table 3). The CWDE activities levels were not significantly different from H₂O-treated (ni) leaves until 16 dpi, which corresponded to the beginning of pycnidia formation. The highest activity levels were found in the controls at 22 dpi associated with the maximal IPC and PLN. *A. nod*-treated leaves exhibited 8.8- and 5.9-fold decreases of endo- β -1,4-xylanase activity, 6.1- and 3.4-fold decrease of endo- β -1,3-glucanase activity and 5.7- and 8.3-fold decreases of protease activity, on BW and DW leaves, respectively.

Table 3 Evolution of disease severity parameters and CWDE activities at 4, 10, 16 and 22 days post inoculation (DPI) of BW and DW leaves by of T01193 and St-08-46 *Z. tritici* strains, respectively. At each time point, treated samples with 500 mg L⁻¹ of *A. nod* were compared to their corresponding reference H₂O-treated samples.

Parameters	DPI	T01193/BW		St-08-46/DW	
		H ₂ O-treated	<i>A. nod</i> -treated	H ₂ O-treated	<i>A. nod</i> -treated
Severity of disease (%)					
% of leaf with necrosis	4	nd	nd	nd	nd
	10	nd	nd	nd	nd
	16	24.1±14.6 b	5.2±3.1 c	28±14.7 b	4.8±5.7 c
	22	48.5±18.2 a	17.8±3.8 c	60.6±18.2 a	20.4±16.3 b
Index of pycnidium coverage	4	nd	nd	nd	nd
	10	nd	nd	nd	nd
	16	2.5±1.4 b	0.3±0.9 d	2.4±1.2 b	0.6±0.6 d
	22	3.5±1.1 a	1.5±1 c	4.4±0.7 a	1.8±1.1 c
CWDEs (mU mg ⁻¹ protein)					
Endo-β-1,4-xylanase	4	32.4±4.1 b	25.8±1.1 b	48.2±3.2 c	42.9±3 c
	10	30.5±3.1 b	23.2±8 b	60.1±2.5 c	51.9±7.3 c
	16	110.8±40.3 b	39.1±14.4 b	246±1.36.6 b	81.1±29.6 bc
	22	879.6±263.4 a	99.8±7.5 b	941.8±294.8 a	158.1±33.5 bc
Endo-β-1,3-glucanase	4	38.6±2.8 b	40.5±3.3 b	170.3±23.7 c	124.7±6.7 c
	10	175.5±19 b	86.1±7.2 b	173.6±13.6 c	124.7±11.3 c
	16	221.3±111.8 b	113.9±15.6 b	345.4±43.1 b	197.3±52.2 c
	22	1136.3±472.2 a	187.9±63.6 b	1026.4±128 a	300.6±103.7 b
Protease	4	211.8±58.2 b	48.7±12.6 b	166.5±31.6 bc	84.8±15.7 c
	10	425±62.5 b	83±39.4 b	156.7±49.9 bc	55±7 c
	16	1108.3±338.1 b	207.7±59.8 b	1740±532 b	254.9±115.2 bc
	22	11317.3±867 a	1357.1±181.1 b	6587.3±509.5 a	1157.3±97.4 bc

(nd): not detected *Z. tritici* symptoms. Data are mean values of two independent experiments (± s.d). Means of *A. nod*-treated samples tagged with the same letter are not statistically different to their corresponding H₂O-treated samples in the same wheat specie (Tukey test, $P < 0.05$).

Enhanced defense response on BW and DW

Wheat defense-related genes expression

Three PR-protein-encoding genes expression, *PR2*, *Chi 4 precursor* and *Pox*, was studied by semi-quantitative RT-PCR analysis, in (ni) and (i) conditions. Expression of *TUB*-encoding gene is used as housekeeping gene. The expression pattern of *A. nod*-treated leaves was compared to corresponding reference H₂O-treated leaves on both BW and DW leaves (Fig. 2). In (ni) condition, *A. nod*-treated leaves exhibited significant up-regulation of *PR2*-encoding gene expression at 1-4 dpt and 4 dpt on BW and DW leaves, respectively. Likewise, *Pox*-encoding gene was significantly up-regulated at 1-3 dpt and 4 dpt on BW and DW leaves, respectively. However, little up regulation of *Chi 4 precursor*-encoding gene expression was detected only in BW leaves at 1-3 dpt. In (i) context, for *A. nod*-treated DW leaves, *PR2*-encoding gene's expression was strongly up-regulated at 3-7 dpt (1-5 dpi). Besides, *Pox*-encoding gene's expression was up-regulated at 3-4 and 4 dpt on

BW and DW leaves, respectively. However, *Chi 4 precursor*-encoding gene's expression was little up-regulated at 7 and 4 dpt on BW and DW leaves, respectively.

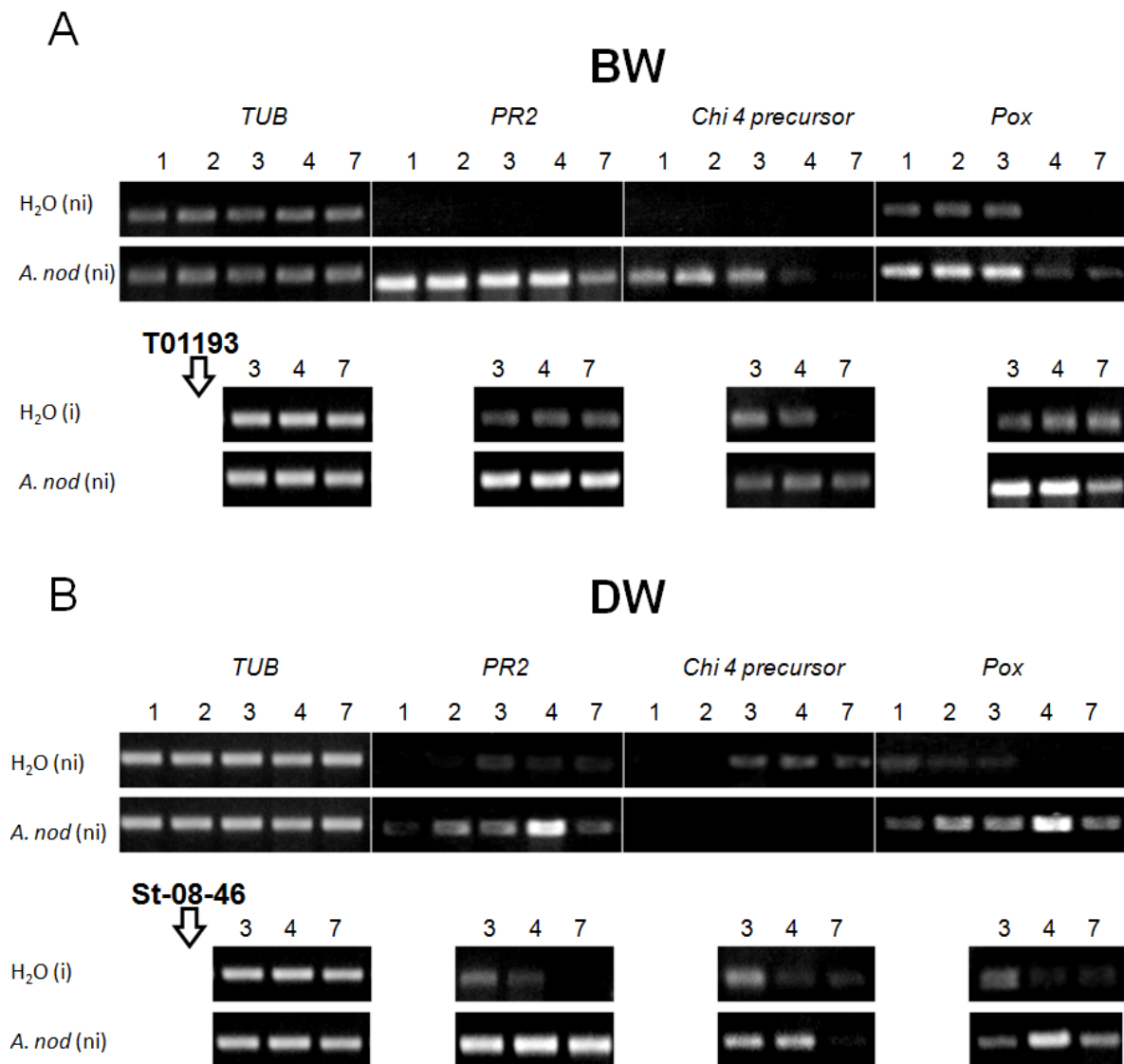


Fig. 2. Effect of foliar *A. nod* treatment (500 mg L⁻¹) on *pathogenesis-related 2* (*PR2*)-, *Chi4 precursor*- and *Pox*-encoding genes expression in BW (A) and DW (B) leaves revealed by semi-quantitative RT-PCR. Four plant treatments were designed in this assay for each cultivar: non-treated and non-infected [H₂O (ni)], *A. nod*-treated and non-infected [*A. nod* (ni)], non-treated and *Z. tritici*-infected [H₂O (i)], *A. nod*-treated and *Z. tritici*-infected [*A. nod* (i)]. For infected samples, the inoculation was carried out 48 hours post *A. nod* treatment. Wheat leaves were collected at 1, 2, 3, 4 and 7 days post *A. nod* treatment in (ni) and (i) conditions. For each condition, *A. nod*-treated leaves were compared to their reference H₂O-treated leaves. RT-PCR products were generated using primers specific for β -tubulin (*TUB*), *PR2*, *Chi 4 precursor* and *Pox* first-strand cDNAs obtained from total wheat RNA, and visualized on agarose gel (1.5%). *TUB*-encoding gene was used as a house-keeping gene. Experiment was repeated three times and only results which were similar from two independent experiments were considered.

The time course of *LOX*- and *PAL* encoding genes' expression was studied by quantitative RT-PCR analysis for their sensibility. Their expression was investigated in (ni) and (i) conditions

and was normalized to the *TUB* and *ACT* reference gene expression (Fig. 2). The study of *LOX* expression pattern showed that, in (ni) condition, *A. nod* treatment generated 59.5- and 8.8-fold significant up-regulations only on BW leaves at 1 and 3 dpt, respectively (Fig. 3 A), while *PAL*-encoding gene's expression was 4-fold significant up-regulated at 2 dpt, only in DW leaves (Fig. 3 C). After inoculation, *LOX* expression pattern showed strong 50.9-fold up-regulation at 7 dpt, only in DW leaves (Fig. 3 B), while *PAL*-encoding gene's expression was significantly 5.3- and 5.2-fold induced at 4 dpt (2 dpi), on BW and DW leaves (Fig. 3 D).

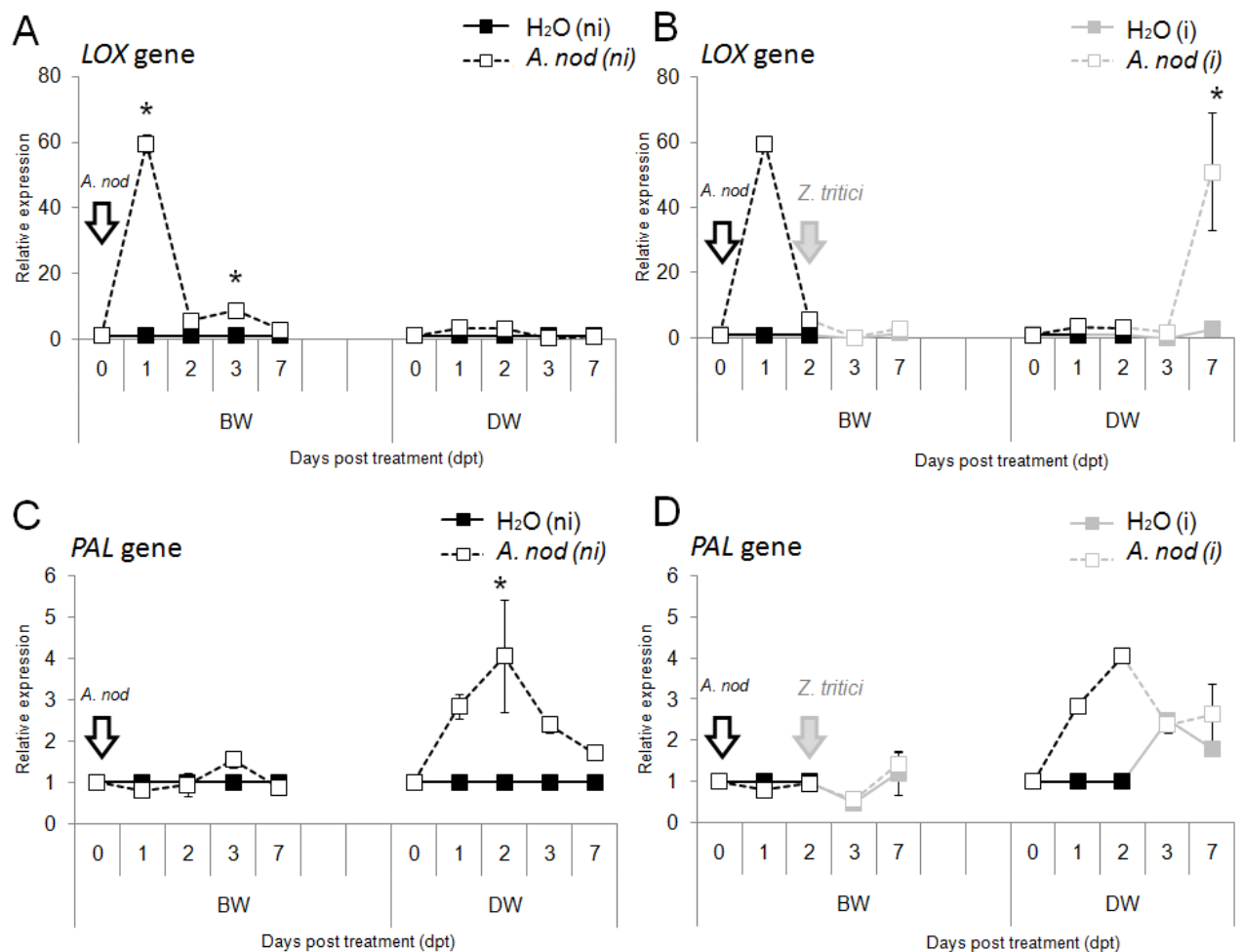


Fig. 3. Effect of foliar *A. nod* treatment (500 mg L^{-1}) on *LOX*- and *PAL*-encoding genes expression in BW and DW leaves revealed by quantitative RT-PCR. (A) *LOX* gene expression in (ni) condition. (B) *LOX* gene expression in (i) condition. (C) *PAL* gene expression in (ni) condition. *PAL* gene expression in (i) condition. Four plant treatments were designed in this assay for each cultivar: non-treated and non-infected [H_2O (ni)], *A. nod*-treated and non-infected [*A. nod* (ni)], non-treated and *Z. tritici*-infected [H_2O (i)], *A. nod*-treated and *Z. tritici*-infected [*A. nod* (i)]. For infected samples, the inoculation was carried out 48 hours post *A. nod* treatment. Wheat leaves were collected after 1, 2, 3 and 7 days post *A. nod* treatment. The experiment was conducted in triplicate. Similar results were obtained at least twice. Results correspond to means (\pm s.d) of duplicate reactions. Means of infected samples designed by asterisks are statistically differentially regulated (up- or down-) in comparison to their corresponding H_2O -treated controls using Tukey test at $P \leq 0.05$.

Enzymatic activities

At the enzymatic level, the effect of *A. nod* treatment on β -1,3-glucanase (GLUC), peroxidase (POX), catalase (CAT), phenylalanine ammonia-lyase (PAL) and lipoxygenase (LOX) enzymatic activities was studied in (ni) and (i) conditions (Fig. 3). *A. nod*-treated leaves were compared to reference H₂O-treated leaves for both wheat species (Fig. 4).

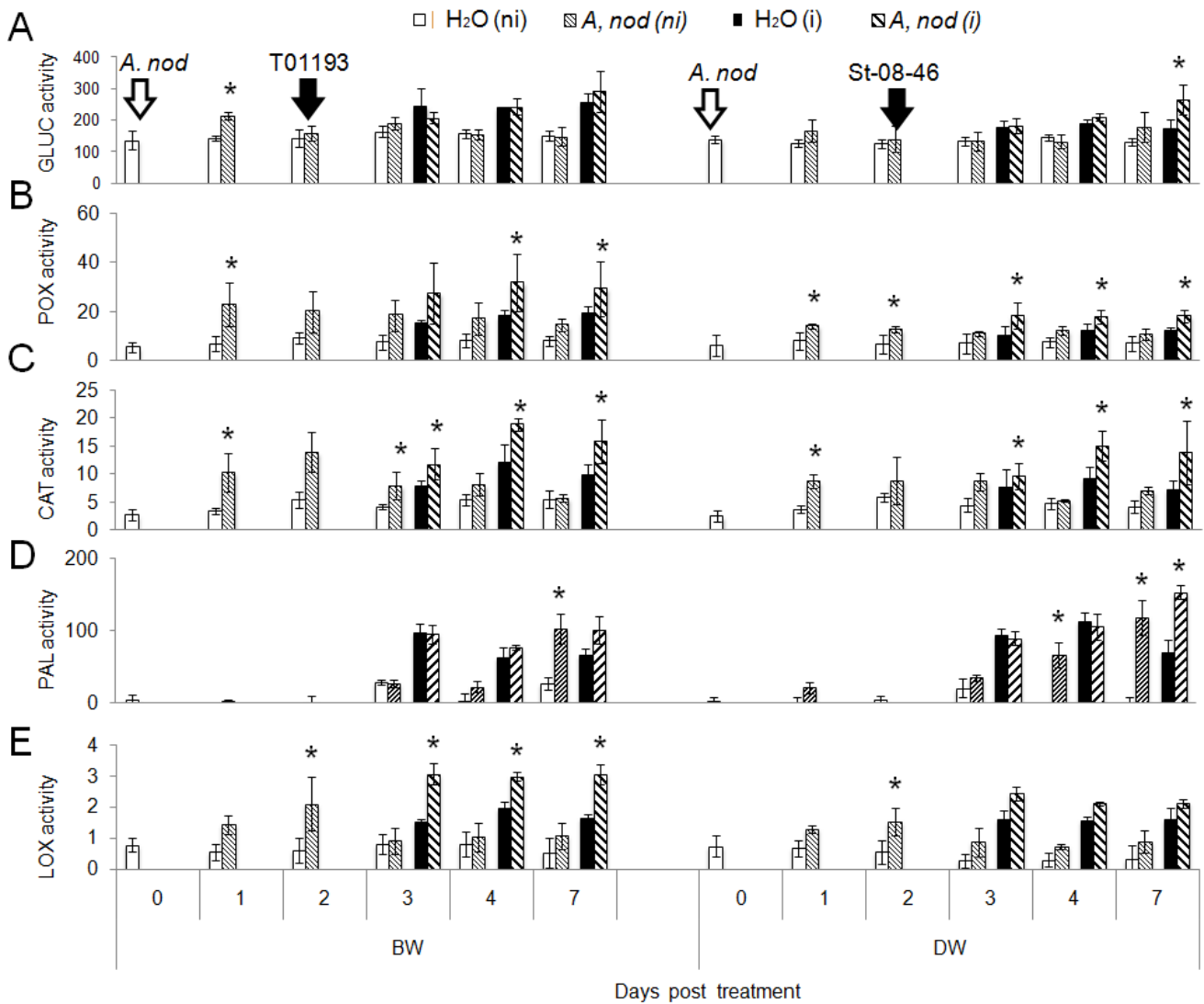


Fig. 4. Effect of foliar *A. nod* treatment (500 mg L⁻¹) on five defense-related enzyme activities (expressed in U mg⁻¹ of proteins) on BW and DW leaves. (A) GLUC (β -1,3-glucanase) activity. (B) POX (peroxidase) activity. (C) CAT (catalase) activity. (D) PAL (phenylalanine-ammonia-lyase) activity. (E) LOX (lipoxygenase) activity. Four plant treatments were designed in this assay for each cultivar: non-treated and non-infected [H₂O (ni)], *A. nod*-treated and non-inoculated [*A. nod* (ni)], non-treated and *Z. tritici*-infected [H₂O (i)], *A. nod*-treated and *Z. tritici*-infected [*A. nod* (i)]. For infected samples, the inoculation was carried out 48 hours post *A. nod* treatment. Wheat leaves were collected after 1, 2, 3, 4 and 7 days post *A. nod* treatment. Results correspond to means (\pm s.d) of two independent experiments using three independent replicates for each experiment. *A. nod*-treated samples with an asterisk are different from their corresponding H₂O-treated controls (Tukey's test, $P \leq 0.05$).

In (ni) context, treated leaves exhibited 1.5-fold increase of wheat GLUC activity at 1 dpt only in BW leaves, respectively (Fig. 4A). Similarly, POX activity showed significant, 3.3-fold increase at 1 dpt, in BW leaves and 1.8- and 1.9-fold increases at 1 and 2 dpt, in DW leaves (Fig. 4 B). Again, CAT activity, was significantly 2.4-fold induced at 1 dpt in DW leaves and 3- and 2.6-fold at 1 and 2 dpt in BW leaves. Besides, *A. nod* treatment enhanced 7.4-fold increase of PAL accumulation at 7 dpt in BW leaves and 67- and 118.2-fold increase in DW leaves at 4 and 7 dpt, respectively. Indeed, LOX activity was 3.5- and 2.8-fold increased at 2 dpt in BW and DW leaves, respectively. In (i) context, *A. nod* treatment induced 1.51-fold increase of wheat GLUC activity only in DW leaves at 7dpt (Fig. 4 A). For POX activity, *A. nod* -treated leaves exhibited significant 1.8-, 1.5- and 1.5-fold increases in DW leaves at 3, 4 and 7 dpt, respectively, and 1.8- and 1.6-fold increase in BW leaves at 4 and 7 dpt, respectively (Fig. 4 B). CAT activity was 1.6-fold increase in BW leaves at 4 and 7 dpt, respectively and 1.6- and 1.9-fold increase in DW leaves (Fig. 4 C). Besides, PAL activity was significantly 2.2-fold induced at 7 dpt only in DW leaves. However, significant 2-, 1.5- and 1.9-fold increase of LOX activity was observed only in BW leaves at 3, 4 and 7 dpt (Fig. 4 D).

Mesophyll H₂O₂ accumulation

At the cytological level, H₂O₂ accumulation was revealed by 3, 3-diaminobenzidine (DAB) staining (red-brown staining) in BW and DW leaves. Four classes of *Z. tritici* spores were distinguished: C1 (spores with non-penetrated germ tube) (Fig. 5a), C2 (spore with penetrated germ tube without staining) (Fig. 5b), C3 (penetrated germ tube with papilla) (Fig. 5c), C4 (penetrated germ tube with at least 1 whole stained mesophyll cell) (Fig. 5d). Results exhibited that *A. nod* treatment didn't present any significant effect on C1, C2 and C3 spores, while C4 were significantly 2.8-fold increased in DW leaves at 2 dpi and 1.9- and 2.5-fold increased in BW leaves at 2 and 5 dpi corresponding to 4 and 7 dpt (Table 4).

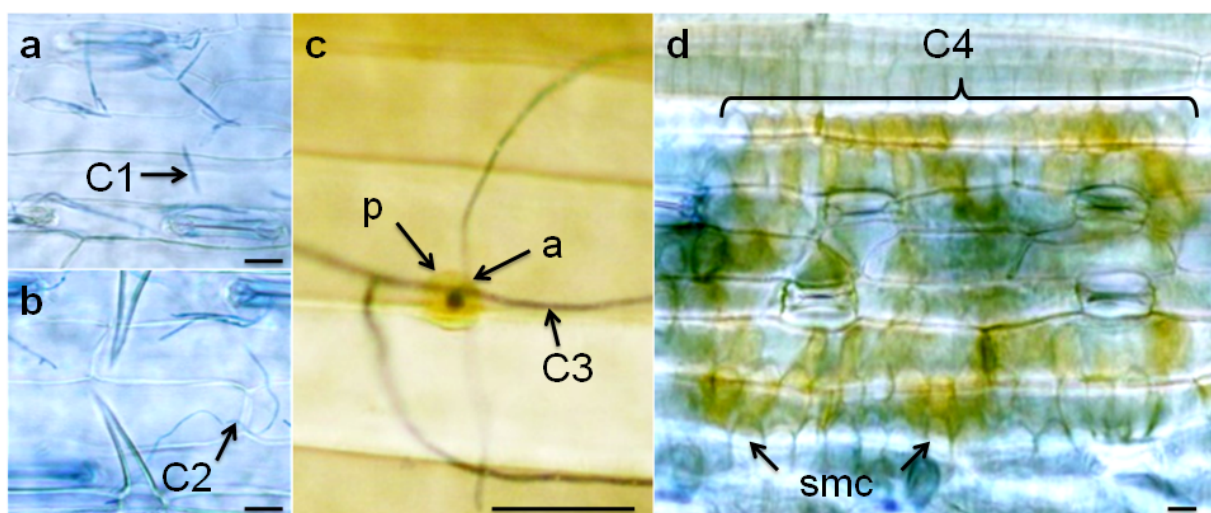


Fig. 5. Microscopic observations of H_2O_2 accumulation visualized by DAB staining displayed at BW and DW leaves following foliar spray by 500 mg L^{-1} of *A. nod*. The inoculation of BW and DW leaves by T01193 and St-08-46 *Z. tritici* strains, respectively, was carried out 48 hours post *A. nod* treatment. Wheat leaves were collected after at 0, 1, 2 and 5 days post inoculation. Four classes of *Z. tritici* spores were observed: (C1) spore with non-penetrated germ tube, (C2) spore with penetrated germ tube without staining, (C3) penetrated germ tube with papilla and (C4) penetrated germ tube with at least 1 whole stained mesophyll cells. (a) Appressorium-like structure. (p) Papilla. (smc) Stained mesophyll cells. Bar scale = $10 \mu\text{m}$.

Table 4 Percentages of measured classes of H_2O_2 accumulation according to DAB staining intensity displayed at BW and DW leaves following foliar spray with 500 mg L^{-1} of *A. nod*. The inoculation of BW and DW leaves by T01193 and St-08-46 *Z. tritici* strains, respectively, was carried out 48 hours post *A. nod* treatment.

Wheat species	Classes (%)	Days post inoculation					
		1		2		5	
		H ₂ O (i)	<i>A. nod</i> (i)	H ₂ O (i)	<i>A. nod</i> (i)	H ₂ O (i)	<i>A. nod</i> (i)
BW	C1	29.1±1.1 a	0±0 a	40.5±15.3 a	22.2±3.3 a	32.1±9.6 a	9.7±4.5 a
	C2	37.6±1.1 ab	0±0 b	46.8±2.9 a	18.5±3.5 ab	35.5±15 ab	18.5±6.5 ab
	C3	0±0 b	0±0 a	8.6±5.4 a	17.3±4.3 a	25.8±16.1 a	10.5±2.3 a
	C4	0±0 d	0±0 c	4.2±7.2 c	42±4.6 b	6.7±14.1 c	61.3±9.3 a
DW	C1	26.8±3.8 a	13.0±22.5 a	37.8±9.2 a	36.5±4 a	11.7±3 a	29.8±2.7 a
	C2	39.9±3.8 a	20.4±35.3 a	54.6±12.9 a	12.6±7.6 a	61.4±5.1 a	24.7±8.8 a
	C3	0±0 b	0±0 b	3.7±3.6 ab	19.3±9.8 ab	16.7±4.1 ab	23.3±9.2 a
	C4	0±0 c	0±0 c	3.9±3.8 c	31.6±9.8 a	10.1±9.4 bc	22.2±4.1 ab

C1: spore with non-penetrated germ tube. C2: spore with penetrated germ tube without staining. C3: penetrated germ tube with papilla. C4: penetrated germ tube with at least 1 whole stained mesophyll cells. Results correspond to means (\pm s.d) of two independent experiments using three independent replicates for each experiment. *A. nod*-treated (i) samples with an asterisk are different from their corresponding H₂O-treated (i) controls (Tukey's test, $P \leq 0.05$)

Phenolic compounds accumulation

Both polyphenol-associated auto-fluorescence and total polyphenol content were evaluated. First, polyphenol deposition may be revealed as clear yellow fluorescence at the penetration sites by the fungus. Four types of events were observed, such as C1 (spore with non-penetrated germ tube) (Fig. 6a), C2 (spore with penetrated germ tube without fluorescence) (Fig. 6a), C3 (penetrated germ tube with weak fluorescence) (Fig. 6b) and C4 (penetrated germ tube with strong fluorescence) (Fig. 6c).

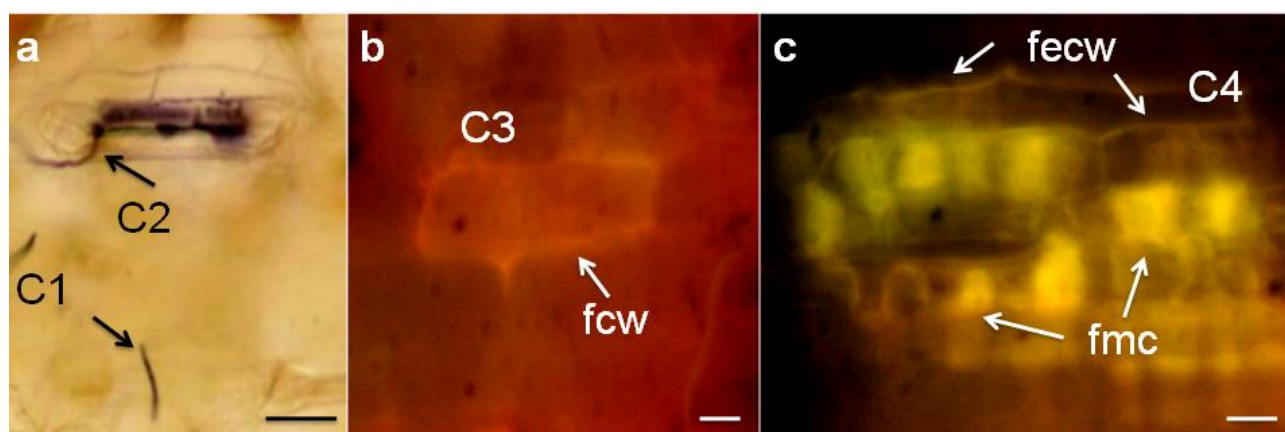


Fig. 6. Microscopic observations of polyphenol-associated autofluorescence displayed at BW and DW leaves following foliar spray with 500 mg L⁻¹ of *A. nod*. The inoculation of BW and DW leaves by T01193 and St-08-46 *Z. tritici* strains, respectively, was carried out 48 hours post *A. nod* treatment. Wheat leaves were collected after at 0, 1, 2 and 5 days post inoculation. Four classes of *Z. tritici* spores were observed: (C1) Spore with non-penetrated germ tube, (C2) Spore with penetrated germ tube without fluorescence, (C3) Penetrated germ tube with weak fluorescence, (C4) Penetrated germ tube with strong fluorescence. (fmc) Fluorescent cell walls. (fcw) Fluorescent mesophyll cells. Bar scale = 10 µm.

Results exhibited that only in *A. nod*-treated BW leaves, C1 were significantly 2.4- and 3.1-fold higher than H₂O-treated controls at 1 and 2 dpi, respectively. But, *A. nod* treatment generated significant decrease of C2 by 6.9- and 4.2-fold at 1 and 2 dpi in DW leaves and by 1.5-, 1.6- and 2.2-fold decrease at 1, 2 and 5 dpi in BW leaves. However, it induced significant 7.4-fold increase of C3 only in DW leaves at 2 dpi. After treatment, wheat leaves showed increasing C4. This last was 17.4- and 43-fold increased at 2 and 5 dpi, respectively, in DW leaves and 32-fold decreased at 5 dpi in BW leaves (Table 5).

Table 5 Percentages of measured classes of polyphenol deposition according to polyphenol-associated autofluorescence intensity displayed at BW and DW leaves following foliar spray with 500 mg L⁻¹ of *A. nod*. The inoculation of BW and DW leaves by T01193 and St-08-46 *Z. tritici* strains, respectively, was carried out 48 hours post *A. nod* treatment.

Wheat species	Classes (%)	Days post inoculation					
		1		2		5	
		H ₂ O (i)	<i>A. nod</i> (i)	H ₂ O (i)	<i>A. nod</i> (i)	H ₂ O (i)	<i>A. nod</i> (i)
BW	C1	23.7±6.4 b	55.6±8.8 a	16.3±8.1 b	50.6±9.2 ab	4.8±4.2 b	22.4±8.2 ab
	C2	68.5±8 b	44.4±8.8 b	68.4±8.7 b	41.9±7.3 c	95.2±4.2 a	43±12.5 c
	C3	7.8±3.3 ab	0±0 b	15.3±3.7 a	7.5±7.2 a	0±0 b	2.6±4.4 ab
	C4	0±0 b	0±0 c	0±0 b	0±0 b	0±0 b	32±11.1 a
DW	C1	22.3±7.1 ab	43.2±6.6 a	21.3±4.3 ab	38.4±19.6 a	11.7±5.6 b	24.9±7.6 b
	C2	72.7±10.4 ab	55±4.4 c	74.2±6.2 a	10.8±2.2 c	80.9±9.7 a	19.2±3 c
	C3	5±6.6 b	1.8±3 b	4.5±1.8 b	33.4±15.2 ab	7.4±6.7 b	12.9±2.7 b
	C4	0±0 c	0±0 b	0±0 c	17.4±5.3 b	0±0 c	43±6.6 a

C1: Spore with non-penetrated germ tube. C2: Spore with penetrated germ tube without fluorescence. C3: Penetrated germ tube with weak fluorescence. C4: Penetrated germ tube with strong fluorescence. Results correspond to means (±s.d) of two independent experiments using three independent replicates for each experiment. *A. nod*-treated (i) samples with an asterisk are different from their corresponding H₂O-treated (i) controls (Tukey's test, $P \leq 0.05$)

Second, the effect of *A. nod* treatment on polyphenol content was also measured in whole 3rd leaf of both wheat species at (ni) and (i) context. Results exhibited that *A. nod* treatment didn't

generate any significant changes compared to reference H₂O-treated leaves in both wheat species (Annex 3, table 2).

DISCUSSION

Antifungal activity of A. nod against T01193 and St-08-46 Z. tritici strains

First, this study demonstrated that *A. nod* didn't exhibit any *in vitro* antifungal effect on spore germination and hyphal growth of both tested *Z. tritici* strains. Similarly, Joubert *et al.* (1998) didn't find direct antifungal activity of β -1,3-glucan extracted from brown algae against *Z. tritici*. However, *A. nodosum* extracts exhibited *in vitro* suppression of several plant pathogenic bacteria such as *Pseudomonas aeruginosa* (Kandasamy *et al.*, 2012) and fungi such as *Sclerotinia minor* (Scott *et al.*, 2014).

The effect of *A. nod* on different infection stages was assessed here and we showed that *A. nod* didn't affect spore germination or hyphal growth, while it could decrease the intensity of colonization in penetration sites and the number of colonized stomata transformed into pycnidia. Indeed, we noted a decrease of mesophyll penetration and the number of colonized stomata only in DW leaves, while in *A. nod*-treated BW leaves exhibited increased number of colonized stomata. This could be explained by a different structure and morphology of BW and DW leaf surface. Moreover, the decrease of mesophyll penetration and colonization could be explained by the installation of defense mechanisms in wheat leaves (Vanacker *et al.*, 2000; Shetty *et al.*, 2003, 2007; Dogbo *et al.*, 2012).

Furthermore, CWDEs were reported as pathogenicity factor of *Z. tritici* and were associated with its fructification in necrotrophic phase (Kema *et al.*, 1996; Douaiher *et al.*, 2007b; Tian *et al.* (2009) and Siah *et al.* (2010b). Here, *A. nod* treatment allowed to drastically decrease CWDE activities produced by St-08-46 and T01193 on DW and BW respectively. Thus, *A. nod* efficacy is associated to fungal CWDE activities such as endo- β -1,4-xylanase, endo- β -1,3-glucanase and protease inhibition during the necrotrophic phase. Consequently, pycnidium coverage and necrotic leaf area were significantly increased. Therefore, our study highlighted a good efficacy of *A. nod* when applied at 500 mg L⁻¹ whatever the wheat specie (more than 50%). Our study is the first work to report the efficiency of *A. nodosum* to control cereal disease, although few works reported it as a good biocontrol agent against fungal diseases in dicotyledons plant such as *Verticillium* in pepPox (Garcia-Mina *et al.*, 2004), *A. radicina* and *B. cinerea* in carrots (Jayaraj *et al.*, 2008), *A. cucumerinum*, *D. applanata*, *F. oxysporum* and *B. cinerea* in cucumber (Jayaraj *et al.*, 2011), *S. sclerotiorum* in tomato (Subramanian *et al.*, 2011) and *A. solani* in tomato (Hernández-Herrera *et al.*, 2014). Few studies investigated the elicitor effect of seaweed in wheat such as the green algae

Ulva fasciata against *Blumeria graminis* (Paulert *et al.*, 2010). Natural molecules, β 1-3 glucan and laminarin, extracted from brown algae *L. digitata* were efficient against *Z. tritici* and *Blumeria graminis f. sp. tritici* (Joubert *et al.*, 1998; Esnault *et al.*, 2005; Renard-Merlier *et al.*, 2007)

Elicitation of BW an DW defense pathways by A. nod treatment

In the present study, we investigated the induction of two plant degrading fungal cell walls enzymes such as β -1,3-glucanase (GLUC) and chitinase. The involvement of glucanases and chitinases in wheat defense against pathogens was confirmed in many studies (Bertini *et al.*, 2003; Lu *et al.*, 2006; Singh *et al.*, 2007; Cawood *et al.*, 2013). We found that *A. nod* treatment present elicitor and potentiating effect β -1,3-glucanase at molecular and enzymatic level. It enhanced *PR2*-encoding gene up-regulation in both wheat species, while total glucanase activity was significantly induced only in BW leaves. β -1,3-glucanase catalyzes hydrolytic cleavage of the β -1,3-D-glucosidic linkages in β -1,3-glucan, which is a component of fungal cell walls (Ryals *et al.*, 1996). Besides their role in diverse physiological, developmental processes in uninfected plants (Sticher *et al.*, 1997), β -1,3-glucanase presents a possible response to plants to microbial pathogens. Our finding were supported by Patier *et al.* (1993), Jayaraj *et al.* (2008, 2011) studies which reported increased activity of β -1,3-glucanase by *A. nodosum* extract in *Rubus fructus*, carrots and cucumber, respectively.

In this study we demonstrated that *A. nod* weakly affect *chitinase (class IV) precursor* - encoding gene's expression in BW leaves. This result didn't corroborate with the findings of Jayaraj *et al.* (2008, 2011) studies which reported enhanced total chitinase enzymatic activity by *A. nodosum* extract carrots and cucumber, respectively. Therefore we suggested that *A. nod* couldn't affect studied chitinase-precursor gene's expression but could trigger other PR genes encoding for other types of chitinases. Indeed, Neuhaus *et al.* (1999) divided isozymes of chitinases into seven classes (class I–VII) on the basis of their structural properties. The above enzymes are involved with defense reactions and further have significant antimicrobial activities (Vidhyasekaran, 1997; Jayaraj *et al.*, 2004). In addition, hydrolytic activities of chitinases and glucanases on pathogen cell wall chitin and glucan would result in the release of oligosaccharides, which may in turn act as elicitors and thereby sustain the induction levels of defense reactions (Jayaraj *et al.*, 2004). In our experiments, *A. nod* treatment enhanced accumulation of *Pox*-encoding gene's transcript and total peroxidase (POX) and catalase (CAT) enzymatic activities in (ni) and (i) context. Our result were supported by Jayaraj *et al.* (2008, 2011) and Lizzi *et al.* (1998) which reported increased POX activities following *A. nodosum* extract treatment in carrots, cucumber and pepper, respectively. At cytological scale, *A. nod* induced an important and fast increase of H₂O₂ accumulation at the fungal penetration sites mainly in BW leaves. This induction was observed in DW leaves as well as BW

leaves. Our results corroborate with the findings of Jayaraj *et al.* (2008) in carrot. Many authors demonstrated that POX and CAT are antioxidant enzymes which could use H₂O₂ to oxidize a large variety of hydrogen donors such as phenolic substances, amines, ascorbic acid, indole, and certain inorganic ions (Van Huystee, 1987; Caruso *et al.* (2001). Moreover, many works reported the involvement of POX, CAT activities and H₂O₂ accumulation in the wheat defense mechanisms against *Z. tritici* (Diani *et al.*, 2009; Shetty *et al.*, 2003, 2007). Several other carbohydrates could induced ROS metabolism in wheat pathogenic fungi, such as β -1,3-glucan, OGAs, trehalose or, laminarin (Renard-Merlier *et al.*, 2007; Shetty *et al.*, 2003; Randoux *et al.*, 2010; Tayeh *et al.*, 2013).

Phenylalanine ammonia lyase (PAL) is one of the key enzymes acting upstream in the phenylpropanoid pathway and the biosynthesis of phenolic compounds. It catalyzes the deamination reaction from L-phenylalanine to trans-cinnamic acid, a precursor of the majority of the phenolic compounds produced by plants (Zabala *et al.*, 2006). PAL also plays a crucial role in the biosynthesis of lignin, which consists of phenolic polymers which reinforce pectocellulosic cell walls and limit the invasion of plant tissues by pathogens (Dogbo *et al.*, 2012). Many studies exhibited that PAL is associated with in cereals resistance against pathogens such as *Fusarium graminearum*, *F. culmorum*, *Puccinia hordei*... (Prats *et al.*, 2007; Beccari *et al.*, 2011; Orzali *et al.*, 2014). Several studies confirmed the correlation of PAL activity and phenolic compounds accumulation in incompatible interaction with *Z. tritici* (Hassan *et al.*, 2007; Adhikari *et al.*, 2007). Therefore, in this study we investigated *PAL* gene's expression, total PAL enzymatic activity and phenolic compounds accumulation (by the quantification of polyphenol content and the visualization of cellular polyphenol deposition). Our results revealed that *A. nod* treatment enhanced significant up-regulation of *PAL* gene only in DW leaves, followed by a later PAL activity mainly in DW leaves in (ni) and (i) context. This PAL induction was associated with polyphenol deposition at penetration sites of *Z. tritici*, earlier and stronger in DW than BW leaves. Although, *A. nod* treatment didn't affect polyphenol content. Similarly, Jayaraj *et al.* (2008, 2011) studies reported enhanced PAL activity and phenolic levels by *A. nodosum* extract in carrots and cucumber, respectively. However, Mercier *et al.* (2001) reported that *A. nodosum* extract-induced resistance did not involve the SA pathway.

We investigated the induction by the *A. nod*-treatment of octadecanoid pathway by measuring total lipoxygenase (LOX) activity and *LOX* gene expression. LOX enzyme catalyzes the first step in the oxygenation of polyunsaturated fatty acids (Holtman *et al.*, 2000). There are numerous reports describing the induction of LOX at molecular or/and enzymatic scale increases during pathogen infection (Ohata *et al.*, 1991; Kolomiets *et al.*, 2000; Ray *et al.*, 2003; Sandhu *et al.*, 2007). We showed here that *A. nod*-treatment enhanced LOX at molecular and enzymatic scale. In DW leaves,

only potentiating effect on *LOX* gene's expression was observed, while in BW leaves, only elicitor effect was detected. Besides, *A. nod* treatment generated elicitor and potential effect on LOX activity, whereas in DW leaves only elicitor effect was observed. Therefore, we could suggest that *A. nod*-treatment affects lipids metabolism. This hypothesis is supported by Jayaraj *et al.* (2008, 2011) studies which reported increased LOX activity by *A. nodosum* extract in carrots and cucumber, respectively. Besides, Subramanian *et al.* (2011) reported enhanced expression of jasmonic acid related gene transcripts in *A. thaliana* with *A. nodosum* extract. Other SDPs such as Heptanoyl salicylic acid (HSA), salicylic acid (SA), OGA, (Renard-Merlier *et al.*, 2007; Randoux *et al.*, 2010; Tayeh *et al.*, 2013) activate lipids metabolism by increasing LOX-encoding gene expression and corresponding LOX activity and confer good protection of wheat against powdery mildew. However, LOX was none responsive to other elicitors such as laminarin-based product (Renard-Merlier *et al.*, 2007). These plant defense elicitor properties could be attributed to the presence of fucans and laminarins. Patier *et al.* (1993), Klarzynski *et al.* (2000), Vera *et al.* (2011) and Mercier *et al.* (2001) reported that laminarin (β -D-1,3-glucan) were capable of eliciting β -D-(1,3) glucanases activities and accumulation of SA in blackberry and tobacco. Moreover, laminarin could control *Z. tritici* in wheat (Esnault *et al.*, 2005). Furthermore, fucans enhanced the accumulation of PR1, PR2, PR3 and PR5 proteins and induced PAL and LOX activity on tobacco against TMV (Klarzynski *et al.*, 2003).

As a conclusion, this work presents an interesting resistance inducer witch could control STB disease on BW and DW when applied at the appropriate concentration and the current stage of development, although further investigations using more susceptible cultivars of each wheat specie and more pathogenic *Z. tritici* strains are required.

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Publication 4: Oligosaccharides-based exhibits high protection against *Zymoseptoria tritici* and induces various related-defense pathways in bread and durum wheat under greenhouse condition

ABSTRACT

The aim of this work is to investigate the elicitation and protection effects of preventive treatments with oligosaccharides (20%)-based new formulation (Oligos) against *Zymoseptoria tritici* major pathogen of bread wheat (BW) and durum wheat (DW). Our findings suggested that Oligos treatment led to strongly reduced hyphal growth, penetration, mesophyll colonization and fructification. Consequently, it also drastically decreased the production of CWDE activities by *Z. tritici*, such as endo- β -1,4-xylanase and endo- β -1,3-glucanase in both wheat species, during necrotrophic phase suggesting their correlation with disease severity and their potential use as markers of resistance inducer efficiency. *PR2*-, *Chi 4 precursor*-, *Pox*- and *LOX*-encoding genes up-regulation, GLUC, CAT and LOX proteins and the content of total soluble phenolic compounds were enhanced in (ni) and (i) context. However, induced PAL enzymatic activity, H₂O₂ accumulation and polyphenol deposition in mesophyll cells, were observed only in (i) context. Overall, Oligos treatment seems to induce the same pathways in tested BW and DW cultivars, but not with the same importance. In this experiment, LOX, PAL and phenolic compounds accumulation were earlier and higher in tested BW cultivar, while GLUC, CAT and H₂O₂ accumulation could be more responsive in tested DW cultivar. Further investigations using more susceptible BW and DW cultivars are required. Thus, tested Oligos product presents an interesting resistance inducer characterized by a high and stable efficiency when it used at appropriate concentration and development stage. It could be integrated into common control strategies against STB disease.

Key words: *Mycosphaerella graminicola*, oligosaccharides, resistance, inducer, wheat species.

INTRODUCTION

Wheat is one of the most important cereal crops worldwide. Two wheat species are mainly cultivated, *i.e.* bread wheat (*Triticum aestivum* L. em Thell, BW) and Durum wheat (*Triticum durum* Desf, DW). *Zymoseptoria tritici* (Quaedvlieg *et al.*, 2011) previously known as *Septoria tritici* (teleomorph *Septoria tritici* blotch (STB) caused by *Zymoseptoria tritici* (Quaedvlieg *et al.*, 2011) previously known as *Septoria tritici* (teleomorph *Mycosphaerella graminicola*) is one of the most devastating foliar disease on wheat which and causes yield losses which could reach 50% in high rainfall seasons (Babadoost and Herbert, 1984; Eyal *et al.*, 1987; Palmer and Skinner, 2002).

To date, fungicides are used to control fungal development but their negative impacts on the environment and human health justify a decrease of used quantities. Thus, alternative or nonpollutant-associated strategies are being developed, including stimulation of plant defenses by a wide range of elicitors. Among them, oligosaccharides have been abundantly studied for their action on growth regulation, plant development, elicitation of defense systems against biotic, or abiotic stress in plants. Oligosaccharides frequently investigated were notably glucans, pectic, xylan, chitin polysaccharides, oligogalacturonic acids (OGAs), (Jayaraj *et al.*, 2004; Aziz *et al.*, 2007; Courtois *et al.*, 2009; Hadrami *et al.*, 2010; Caillot *et al.*, 2012; Ferrari *et al.*, 2013). They can be produced naturally or may derive from polysaccharides after chemical, physical, or biochemical degradations. Oligosaccharides can be found in plants as trehalose (Tayeh, 2014) or fungal cell walls like chitin oligosaccharides from *Puccinia graminis* (Bohland *et al.*, 1997), or crustaceans such as chitosan (Trotel-Aziz *et al.*, 2006). Furthermore, oligosaccharides could induce host defense responses in both monocotyledons and dicotyledons. When oligosaccharides elicit defense responses in plants, the same phenomenon as those induced in the plant when a pathogen is recognized, may occur. Within a few minutes, the early defense responses triggered by the pathogen may be a modification of ion fluxes across the plasma membrane, phosphorylation and dephosphorylation of signaling proteins, production of reactive oxygen species. Few hours later, a broad spectrum of defense reaction may be induced to confer local resistance against microorganisms. These defense reactions may be: cell wall reinforcement through lignin synthesis and crosslinking of macromolecular constituents, accumulation of metabolites with antimicrobial properties as phytoalexins, production of salicylic acid (SA) and jasmonate which are secondary metabolites resulting from the phenylpropanoid and octadecanoid pathways (Vasuikova *et al.*, 2001; Jayaraj *et al.*, 2004; Aziz *et al.*, 2006; Faoro *et al.*, 2008; Hadrami *et al.*, 2010; Li and Zhu, 2013).

The first elicitor-active oligosaccharide was degraded from the fungal cell walls of phytopathogen *Phytophthora sojae*, which is able to induce the synthesis of phytoalexins in plant cells to prevent fungal infection (Sharp *et al.*, 1984). Since that, numerous reports demonstrated that oligosaccharides were able to induce a variety of defense reactions conferring resistance to viral, bacterial and fungal pathogens in many crops including tobacco (Falcón-Rodríguez *et al.*, 2009), Arabidopsis (Ménard *et al.*, 2004), rice (Yamaguchi and Hirano, 1997), tomato (Benhamou *et al.*, 1994), potato (Li *et al.*, 2014), carrots (Jayaraj *et al.*, 2009), grapevine (Aziz *et al.*, 2006, 2007; Caillot *et al.*, 2012), strawberry (Eikemo *et al.*, 2003), cucumber (Ben-Shalom *et al.*, 2003), barley (Faoro *et al.*, 2008) and wheat (Bohland *et al.*, 1997; Renard-Merlier *et al.*, 2007; Randoux *et al.*, 2010). In case of wheat, oligosaccharides could induce resistance against abiotic stress (El-Bashiti *et al.*, 2005). Moreover, they could partially induced fungal diseases including β 1-3 glucan

oligomers against *Z. tritici* (Joubert *et al.*, 1998; Esnault *et al.*, 2005), chitosan, trehalose and OGAs against *Blumeria graminis* f. sp. *tritici* (Reignault *et al.*, 2001; Renard-Merlier *et al.*, 2007; Rondoux *et al.*, 2010; Tayeh, 2013). Nevertheless, little is known about disease resistance inducers and, more specifically, about the biological activities from oligosaccharides in wheat.

Considering all these observations, this work proposes to study the potential antifungal and elicitor activities of plant cell wall oligosaccharides extract-based against *Z. tritici* in susceptible wheat cultivars. Moreover, difference in bread wheat (BW) and durum wheat (DW) response to studied oligosaccharides will be discussed.

MATERIALS AND METHODS

Fungal material and inoculation

For *in vivo* assays, the two *Z. tritici* T01193 and St-08-46 strains were chosen for their high level of pathogenicity, respectively on BW and DW. Cultures were grown for 3 weeks at 18°C and were stored at -80°C for further use. Plants were inoculated at 2 days post treatment (dpt) with 30 mL of a 10^6 spores mL⁻¹ suspension in a 0.05% of Tween 20 (Sigma Aldrich, USA) solution using a hand sprayer. Control plants were sprayed with tap water containing 0.05% of Tween 20. Immediately after inoculation, each pot was covered with a clear polyethylene bag for 3 days in order to ensure a water-saturated atmosphere. Disease level was scored at 22 days post inoculation (dpi) by measuring percentage of leaf area with symptoms (necrosis and pycnidium) and the index of pycnidium coverage (which describes the importance of pycnidium in necrosis following a scale noted from 1 to 5) within necrotic lesions on the 3rd leaf of 25 plantlets. Data represent means of two independent experiments.

Direct effect assessment on PDA medium

For this study, we used new formulation based on a plant cell wall oligosaccharides(20%) (Oligos) provided by CODA (Lerida, Spain). It was tested *in vitro* by measuring spore germination and fungal hyphal growth according to Siah *et al.* (2010a). *Spore germination*: aliquots of 0.6 mL of each strain, adjusted at 5×10^5 spores mL⁻¹, were sprayed on PDA plates containing various concentrations of tested product. Eight concentrations of Oligos (0.06; 0.2; 0.61; 1.85; 5.55; 16; 50 and 150 mg L⁻¹) were used. The plates were placed at 18°C in the dark for 24h. The percentages of germinated spores were then calculated for 100 spores, for each plate, using light microscope (Nikon, Eclipse 80i), in order to determinate the half maximal inhibitory concentration (IC₅₀) values. *Mycelium growth*: 5 µL of 5×10^5 spore mL⁻¹ of each strain were spotted on the PDA plates containing the same concentrations of tested compounds. After incubation for 10 days at 18°C in

the dark, hyphal growth were compared. Assays were repeated twice and for each condition, three plates with three spots per plate were used as triplicates.

Plant material and tested product

Cultivars of the two wheat species known to be susceptible to STB were used for this study: bread wheat (BW) cultivar Premio and durum wheat (DW) cultivar Karim. Grains were pre-germinated in Petri dishes (12 x 12 cm) on moist filter paper in darkness at 20°C for 24 h, at 4°C for 48 h and then at 20°C for 24 h. Germinated grains were then transplanted into soil in pots of 15 cm-diameter (12 grains/pot). The pots were placed in the greenhouse at 18°C and under day-night cycles of 16h /8h. After 3 weeks, when the third leaf was fully expanded, plants were sprayed with 50 mg L⁻¹ of Oligos.

RNA extraction and Reverse Transcription (RT)

For plant genes expression study, samples were collected from non inoculated (ni) and inoculated plants (i). At (ni) conditions, samples were collected at 1, 2, 3, 4 and 7 dpt. For (i) conditions, samples were collected at 0, 1, 2 and 5 dpi (corresponding to 2, 3, 4 and 7 dpt). Total RNA was extracted from 100 mg of plant tissue using RNeasy Plant Mini Kit (Quiagen, The Netherlands). Genomic DNA contaminating the samples was removed by treatment with DNase using RNase-Free DNase Set (Quiagen, The Netherlands). Reverse transcription of total RNA was carried out using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA) according to the manufacturer's protocol.

Analysis of gene expression by semi-quantitative RT-PCR and real-time RT-PCR

PCR reactions were conducted on subsequent obtained cDNA to amplify five wheat genes encoding for *PR proteins 2* (β -1,3glucanase), *Chi 4 precursor* (chitinase IV precursor), *Pox* (peroxidase); *PAL* (phenylalanine ammonia lyase) and *LOX* (lipoxygenase). These analyses were performed using the following gene-specific primers presented in Table 1. The primers pairs were designed using the Primer Express® program and were tested for secondary structure using Net Primer® program. The gene expression of *PR2*, *Chi 4 precursor* and *Pox* was investigated by semi-quantitative RT-PCR. PCR was carried out in a 20 μ L reaction mixture containing 1x PCR buffer, 2 mM dNTPs, 10 μ M of each primer, 0.5 unit of Taq polymerase and 20 ng of cDNA using the following protocol: 94°C for 5 min, followed by 30 cycles each with 94°C for 1 min, 55°C for 1 min and 72°C for 1 min. The PCR reaction was terminated at 72°C for 7 min. PCR products were analyzed by electrophoresis on ethidium-bromide stained 1.5% agarose gels run in 0.5 x Tris-acetic acid-EDTA buffer at 100 V for 45 min. Gels were visualized under UV light. *TUB* was used as an internal control in semi-

quantitative Reverse-Transcription Polymerase Chain Reactions (RT-PCR) analysis. Experiment was repeated 3 times and only results which were repeated at least twice were considered. The investigation of *PAL* and *LOX* expression was performed by real time-RT-PCR analysis. The amplification specificity of each qRT-PCR was checked by a single peak in melt curve analysis, and no primer dimers were detected using agarose gel electrophoresis. Real-time RT-PCR was performed using ABI Prism 7300 detection system (Applied Biosystems, USA), using the following thermal profile : after incubation for 10 min at 95°C, 15s at 95°C (denaturation), 1 min at 60°C (annealing/extension) for 40 cycles and 30s at 72°C (data acquisition). Relative expression for each cDNA was calculated for each time point relative to control at the same time. It corresponds to $2^{-\Delta\Delta Ct}$ described by Pfaffl *et al.* (2002) and was calculated using the relative expression software tool REST®. $\Delta\Delta Ct = [Ct \text{ Target (Sample)} - Ct \text{ Reference (Sample)}] - [Ct \text{ Target (Control)} - Ct \text{ Reference (Control)}]$. *TUB* is used as reference gene. The results were normalized with the *TUB* and *ACT* genes and expressed relatively to the control, for each time point, corresponding to a fixed value of 1. After qPCR, a melt curve was generated to verify the specificity of each amplification reaction. The experiment was conducted in triplicate and only results which were repeated at least twice were considered.

Table 1 List of primers encoding for wheat genes analyzed by quantitative and semi-quantitative RT-PCR.

Gene	Primer sequences (5'-3')	Accession numbers
<i>TUB</i> (<i>beta tubulin</i> , housekeeping gene)	GGAGTACCCTGACCGAATGATG AACGACGGTGTCTGAGACCTTT	U76895
<i>ACT</i> (<i>actin</i> , housekeeping gene)	AACCTTCAGTTGCCAGCAA TGTTTCGACCGCTGGCATAAC	AB181991
<i>PR2</i> (β -1,3 <i>glucanase</i>)	CTCGACATCGGTAACGACCAG GCGGCGATGTACTTGATGTTT	Y18212
<i>Chi4 precursor</i> (<i>chitinase class IV precursor</i>)	TTCTGGTTCTGGATGACCAAC ACTGCTTGCACTACTCCGTG	AF112966
<i>Pox</i> (<i>peroxidase</i>)	GAGATTCCACAGATGCAAACGAG GGAGGCCCTTGTTTCTGAATG	X56011
<i>PAL</i> (<i>phenylalanine ammonia lyase</i>)	CCCCATTGGTGTCTCCAT ACTGCGGAACATCAGCTT	AY005474
<i>LOX</i> (<i>lipoxygenase</i>)	GGGCACCAAGGAGTACAAGGA GCTCGTGATGGTGTGGATGA	U32428

Protein extraction and enzymatic assays

Fungal enzyme assays: Three fungal cell-wall degrading enzyme (CWDE) activities (endo- β -1,4-xylanase, endo- β -1,3-glucanase and protease) were measured at 4, 10, 16 and 22 dpi. Briefly, 2.5 g of third leaves were sampled and immediately frozen in liquid nitrogen for grinding in mortar. Endo- β -1,4-xylanase activity was measured at pH 4.8 and 45°C using xylan (1%, w/v) (Sigma) as substrate and xylose (10 mM) (Sigma Aldrich, USA) as reducing control sugar (Siah *et al.*, 2010). Endo- β -1,3-glucanase activity was measured at pH 4.8 and 50 °C using Laminarin (Sigma Aldrich,

USA) at pH 5 (0.5 %, w/v) as substrate and glucose (10 mM) as reducing control sugar (Douaiher *et al.*, 2007). Absorbance was measured at 540 nm. One unit of endo- β -1,4-xylanase and endo- β -1,3-glucanase activities is defined as the amount of enzyme necessary to release 1 μ mol of reducing sugar/p-nitrophenol $\text{min}^{-1} \text{mL}^{-1}$ of enzyme solution. Protease activity assays were assessed according to Hellweg (2003). Absorbance was measured at 400 nm. One unit of protease activity is defined as a change in enzyme activity of 1 per hour. These activities were expressed in mU mg^{-1} of proteins.

Plant enzyme assays: Five plant enzyme activities involved in defense mechanisms were measured: β -1,3-glucanase (GLUC), catalase (CAT), peroxidase (POX), lipoxygenase (LOX) and phenylalanine ammonia-lyase (PAL). Samples were collected at the same timing used for gene expression analysis. Each sample consisted of three medial segments excised from three third leaves. GLUC activity was analyzed according to Douaiher *et al.* (2007). CAT activity was assessed according to Randoux *et al.* (2010); one unit of CAT activity is defined as the amount of the enzyme which degrades H_2O_2 at 1 $\mu\text{mol min}^{-1}$, ($\epsilon = 36 \text{ mM}^{-1}\text{cm}^{-1}$). POX was estimated according to El Hadrami and Baaziz (1995) and expressed as $\text{nkat mg of protein}^{-1}$. One unit of POX activity is defined as the amount of enzyme that catalyses the conversion of guaiacol as substrate at A_{470} after 2 min of H_2O_2 addition. LOX activity was measured according to Randoux *et al.* (2010). One unit of LOX activity is defined as the amount of enzyme that catalyses the conversion of substrate (linoleic acid) at 1 mols^{-1} ($\epsilon = 23 \text{ mM}^{-1}\text{cm}^{-1}$). PAL activity was calculated as described by Randoux *et al.* (2010). One unit of PAL activity is defined as the amount of enzyme that catalyses the formation of cinnamic acid at 1 mols^{-1} ($\epsilon = 10 \text{ mM}^{-1}\text{cm}^{-1}$). CAT, POX, LOX and PAL activities were expressed in U mg^{-1} of proteins.

Total protein concentration was measured at absorbance of 595 nm using bovine serum albumin as a standard (Bradford, 1976). For all tested activities, absorbance was measured using Thermo Scientific™ GENESYS 10S UV-Vis spectrophotometer. Data represent means of two independent experiments. Three independent replicates were carried out for each experiment.

Cytological analysis

The accumulation of H_2O_2 was revealed by 3,3-diaminobenzidine (DAB) staining (red-brown staining) according to Randoux *et al.* [2010]. For polyphenol deposition visualization, leaves stained with Trypan Blue were used for fluorescence microscopy using a Nikon microscope (Champigny-sur-Marne, France) fitted for fluorescence (blue exciter filter, maximum transmittance 400 nm and barrier filter with a transmittance range of 500 to 800 nm). As described by Randoux *et al.* [2010], a yellow fluorescence visualized under UV-light microscopy indicates the presence of polymeric phenolic compounds. H_2O_2 and polyphenol visualization were detected at fungal

penetration sites in 2-cm-long segment of 3rd leaves. Leaf samples were collected from the same plantlets used for enzymatic and molecular analysis and at the same timing. Data represent means of two independent experiments. Three independent replicates were carried out for each experiment.

Total polyphenolic compounds quantification

The quantification of the content of total soluble phenol compounds was determined according to the Folin-Ciocalteu method modified by El Hadrami et al. (1997). Leaf samples were collected from the same plantlets used for enzymatic analysis. Foliar tissues (200 mg from 3rd leaf), were homogenized in an ice bath with 1.5 ml methanol. The homogenate was centrifuged three times at 10000g for 5 min; supernatants were recuperated each time. One hundred microlitres of the supernatant was added to Folin-ciocalteu reagent and sodium carbonate. The mixture was incubated at 40°C for 30 min and the blue color was read at 760 nm using Cathechin (Sigma Aldrich, USA) as a standard. The polyphenol content was determined for the experiments described above and was expressed in mg-equivalents of Cathechin (Sigma Aldrich, USA) (CE) per gram of fresh weight (FW). Data represent means of two independent experiments. Three independent replicates were carried out for each experiment.

Statistical data analysis

Statistical data analyses of molecular and enzymatic assays were made separately for each wheat species (BW cv. Premio/T01193 strain and DW cv. Karim/St-08-46 strain). For quantitative gene expression, the analyses were performed using the relative expression software tool REST® as described by Pfaffl *et al.* (2002) and the significant differences were tested at 95% ($P < 0.05$). For severity parameters, enzymatic activities and microscopy, an analysis of variance (ANOVA) was performed using Tukey test at $P \leq 0.05$ using the XLSTAT software (Addinsoft, France).

RESULTS

Antifungal effect of Oligos against St-08-63 and T01193 Z. tritici strains

The verification of the *in vitro* effect of Oligos on the spore germination and hyphal growth of T01193 and St-08-46 strains in PDA medium plates didn't show any significant difference compared to reference plates without treatment (Annex 2). The direct effect of 50 mg L⁻¹ of Oligos was investigated on T01193 and St-08-46 *Z. tritici* strains inoculated respectively on BW and DW leaves. Microscopic observation of (i) spore germination and hyphal growth at 4 dpi, (ii) mesophyll colonization and pycnidium formation at 22 dpi, were performed on BW (Fig. 1A) and DW (Fig. 1B). First, microscopic observation of (i) spore germination and hyphal growth (Fig. 1a, b, c, d) at 4

dpi, (ii) mesophyll colonization (Fig. 1e, f, g, h) and pycnidium formation (Fig. 1i, j, k, l) at 22 dpi, were performed. At 4 dpi, we observed partially reduced hyphal growth in treated (Fig. 1 b, d) and untreated leaves (Fig. 1 a, c). At 22 dpi, Oligos-treated leaves showed considerably reduced mesophyll cells colonization (Fig. 1 f, h) and pycnidium formation (Fig. 1 j, l) compared to reference H₂O-treated leaves (Fig. 1 e, g, i and k).

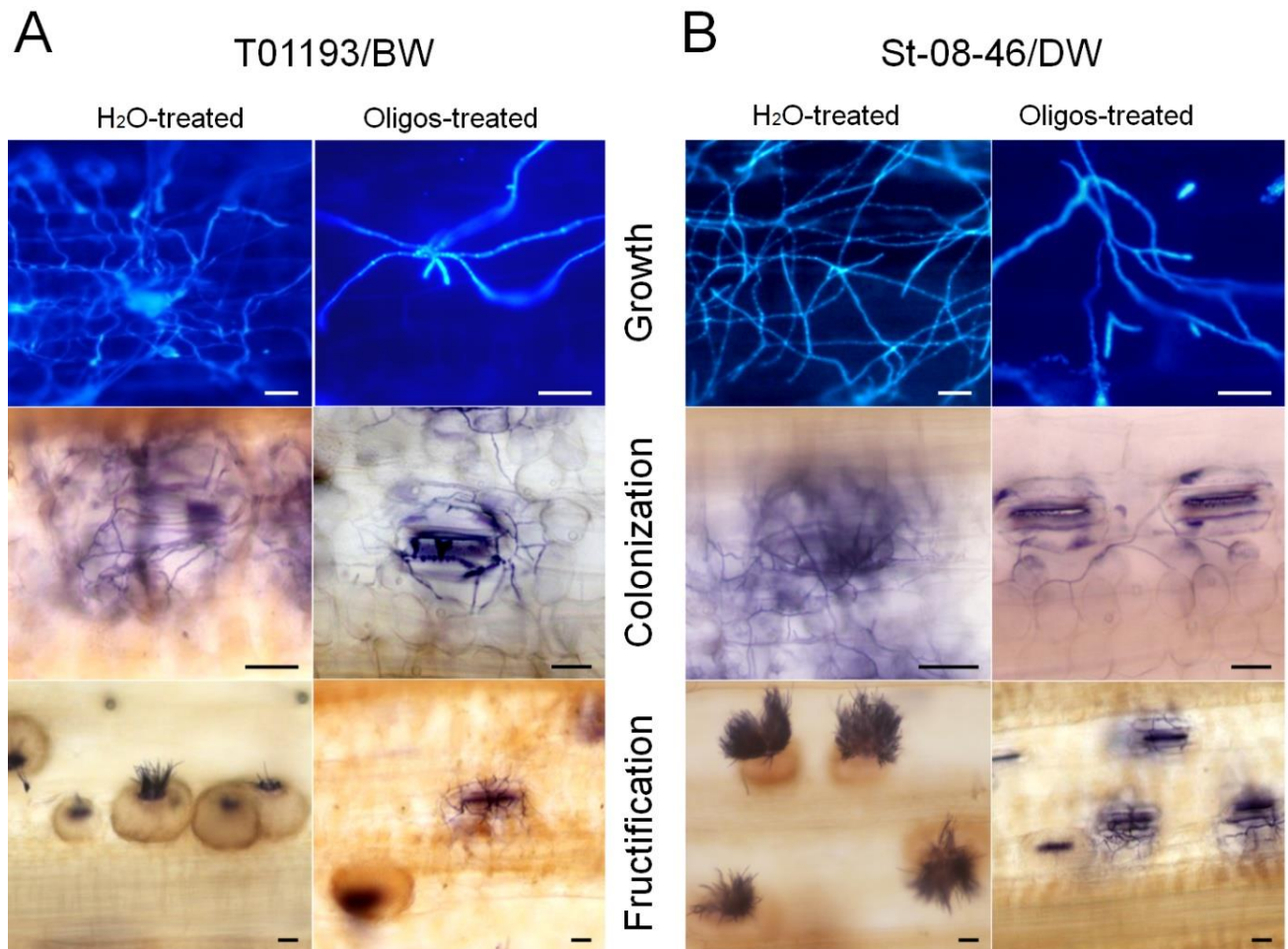


Fig. 1. Effect of 50 mg L⁻¹ of Oligos on the spore germination, mesophyll colonization and fructification at 22 dpi of *Z. tritici* on DW and BW. (A) Susceptible BW cv. Premio infected by T01193 *Z. tritici* strain. (B) Susceptible DW cv. Karim infected by St-08-46 *Z. tritici* strain. Microscopic observation of the spore germination of both *Z. tritici* strains was monitored at 4 dpi using Calcofluor fluorescence. Microscopic observation of the mesophyll colonization and fructification was monitored at 22 dpi using Trypan blue staining. Scale bar = 10 µm.

Besides, Table 2 represents the rate of cytological parameters assessed for both tested pathosystems at : (i) 1 dpi: germinated spores (GS), (ii) 4 dpi: non germinated spores (NGS), spores leading to stomatal penetration (SP), spores leading to a direct penetration (DP), and germinated spores that did not penetrate (GSNP), (iii) 22 dpi: no colonized stomata (NCS), colonized stomata but not transformed into pycnidia yet (CS) and colonized stomata transformed into pycnidia (P). Results exhibited that Oligos treatment didn't affect GS compared to reference H₂O-treated leaves in both tested pathosystems at 1 dpi (Table 2). Then, at 4 dpi, Oligos-treated DW leaves exhibited

1.75-fold increases of NGS and 3.56-fold decrease of DP, while Oligos-treated BW leaves revealed 1.37-fold increases of NGS associated with 2.29-fold decrease SP in BW leaves. At 22 dpi, Oligos-treated DW leaves presented 2.30-fold increase of NCS, 2.89-fold of CS and 11.22- and 3.81-fold decreased P. However, in BW leaves we detected 1.59-fold increase of NCS, 1.82-fold of CS and 3.81-fold decreased P.

Table 2 Percentages of different cytological parameters measured at 1, 4 and 22 days post inoculation (DPI) of BW and DW leaves by of T01193 and St-08-46 *Z. tritici* strains, respectively. At each time point, treated samples with 50 mg L⁻¹ of Oligos were compared to their corresponding reference H₂O-treated samples.

Parameters (%)	DPI	T01193 / BW		St-08-46 / DW	
		H ₂ O-treated	Oligos-treated	H ₂ O-treated	Oligos-treated
GS	1	54±3.7 a	55.8±2.4 a	72±11.5 a	54±6.1 a
NGS	4	25.3±2.1 b	34.8±9.1 a	20.4±3.5 b	35.8±6.6 a
SP		17.9±1.7 a	7.8±1.3 b	9.8±0.2 a	11.5±2.9 a
DP		10.3±4.5 a	4.1±1.6 a	28.9±5.9 a	8.1±4.2 b
GSNP		46.4±3.3 a	53.4±8 a	41±7.2 a	44.6±3.3 a
NCS	22	20.6±3.5 b	32.7±3.7 a	13.8±0.2 b	31.7±8.1 a
CS		29.9±2.7 b	54.3±2.7 a	21.6±2.9 b	62.5±1.8 a
P		49.5±3.9 a	13±1.6 b	65.1±3 a	5.8±9.4 b

GS: germinated spores. NGS: non germinated spore. DP: spore leading to a direct penetration. SP: spore leading to a stomatal penetration. GSNP: germinated spore without penetration. NCS: non-colonized stomata. CS: colonized stomata but not yet transformed into pycnidia. P: colonized stomata transformed into pycnidia. Data are mean values of two independent experiments (±s.d). Statistics were made separately for each wheat specie. Means of Oligos-treated samples tagged with the same letter are not statistically different to their corresponding H₂O-treated samples in the same wheat specie (Tukey test, $P < 0.05$).

Disease severity screening showed that the Percentage of Leaf Necrosis (PLN) and the Index of Pycnidium Coverage (IPC) were measurable from 16 dpi and showed maxima at 22 dpi (Table 3). At 22 dpi, Oligos-treated leaves exhibited 6.3- and 5.3-fold decreases of PLN and 4.4- and 3.4-fold decreases of IPC, on BW and DW leaves, respectively. Moreover, *Z. tritici* CWDE activities such as endo-β-1,4-xylanase, endo-β-1,3-glucanase and protease were measured at 4, 10, 16 and 22 dpi (Table 3). The CWDE activities levels were not significantly different from H₂O-treated (ni) leaves until 16 dpi, which corresponded to the beginning of pycnidia formation. The highest activity levels were found in the controls at 22 dpi associated with the maximal IPC and PLN. Oligos-treated leaves revealed 15.3- and 12.3-fold decrease of xylanase activity, 7.4- and 7.2-fold decrease of endo-β-1,3-glucanase activity and 11.1- and 17.7-fold decrease of protease activity, on BW and DW leaves, respectively.

Table 3 Evolution of disease severity parameters and CWDE activities at 4, 10, 16 and 22 days post inoculation of BW and DW leaves by of T01193 and St-08-46 *Z. tritici* strains, respectively. At each time point, treated samples with 50 mg L⁻¹ of Oligos were compared to their corresponding reference H₂O-treated samples.

Parameters	DPI	T01193/BW		St-08-46/DW	
		H ₂ O-treated	Oligos-treated	H ₂ O-treated	Oligos-treated
Severity of disease (%)					
% of leaf with necrosis (PLN)	4	nd	nd	nd	nd
	10	nd	nd	nd	nd
	16	24.1±14.6 b	1.6±2.9 d	28±14.7 b	7.2±3.2 c
	22	48.5±18.2 a	9.1±3.6 c	60.6±18.2 a	9.6±11.4 c
Index of pycnidium coverage (IPC)					
	4	nd	nd	nd	nd
	10	nd	nd	nd	nd
	16	2.5±1.4 b	0.3±0.1 d	2.4±1.2 b	1.1±1 c
	22	3.5±1.1 a	0.8±1.1 c	4.4±0.7 a	1.3±1 c
CWDEs activities (mU mg ⁻¹ of protein)					
Endo-β-1.4-xylanase	4	32.4±4.1 c	20.4±1 c	48.2±3.2 d	29.8±6.1 d
	10	30.5±3.1 c	17.2±2.8 c	60.1±2.5 d	44.5±3.8 d
	16	110.8±40.3 c	39.5±20.4 c	246±36.6 c	56.2±18.4 d
	22	879.6±263.4 a	71.3±21.5 c	941.8±294.8 a	61.6±34.3 d
Endo-β-1.3-glucanase	4	38.6±2.8 c	37.4±3.4 c	170.3±23.7 de	120.9±5.9 e
	10	175.5±19 c	125±18.4 c	173.6±13.6 de	146.4±11.8 de
	16	221.3±111.8 c	122.2±8.8 c	345.4±43.1 c	159.9±30.1 de
	22	1136.3±472.2 a	158±44 c	1026.4±128 a	137.9±7.3 de
Protease	4	211.8±58.2 b	117.3±38.1 b	166.5±31.6 cd	99.9±19.9 d
	10	425±62.5 b	102.3±58.1 b	156.7±49.9 cd	56.1±10.4 d
	16	1108.3±338.1 b	253.9±107.3 b	1740±532 bc	241±20 cd
	22	11317.3±867 a	1015.8±311.6 b	6587.3±509.5 a	373.2±159 cd

(nd): not detected *Z. tritici* symptoms. Data are mean values of two independent experiments (±s.d). Means of Oligos-treated samples tagged with the same letter are not statistically different to their corresponding H₂O-treated samples in the same wheat specie (Tukey test, $P < 0.05$).

Enhanced wheat defense response by Oligos treatment

Wheat defense-related genes up regulation

The time course of three PR genes expression *PR2*, *Chi4 precursor* and *Pox*, was studied by semi-quantitative RT-PCR analysis in (ni) and (i) conditions, compared to the *TUB* gene expression as reference. The expression pattern of Oligos-treated leaves was compared to reference H₂O-treated leaves for both BW and DW leaves (Fig. 2). Results exhibited the induction of up-regulation of all studied PR genes expression by Oligos treatment in (ni) and (i) condition, on both wheat species. In (ni) conditions, PR gene expression was assessed at 1, 2, 3, 4 and 7 dpt. *PR2*-encoding gene's expression was up-regulated at 1-7 dpt in both wheat species. Also, *Chi4 precursor*-encoding gene expression was induced at 3 and 7 dpt on BW and DW leaves, respectively. Besides, *Pox*-encoding gene was significantly up-regulated in 2 and 3-7 dpt on BW and DW leaves, respectively. In (i) context, PR gene expression was measured at 1, 2 and 5 dpt (3, 4 and 7 dpt). BW leaves revealed *PR2*, *Chi4 precursor* and *Pox* genes expression induction at 3-4 dpt, 3-7 dpt and 3-7 dpt,

respectively. Similarly, DW leaves revealed *PR2*, *Chi4 precursor* and *Pox* gene induction at 3-7 dpt, 3-7 dpt and 4 dpt, respectively.

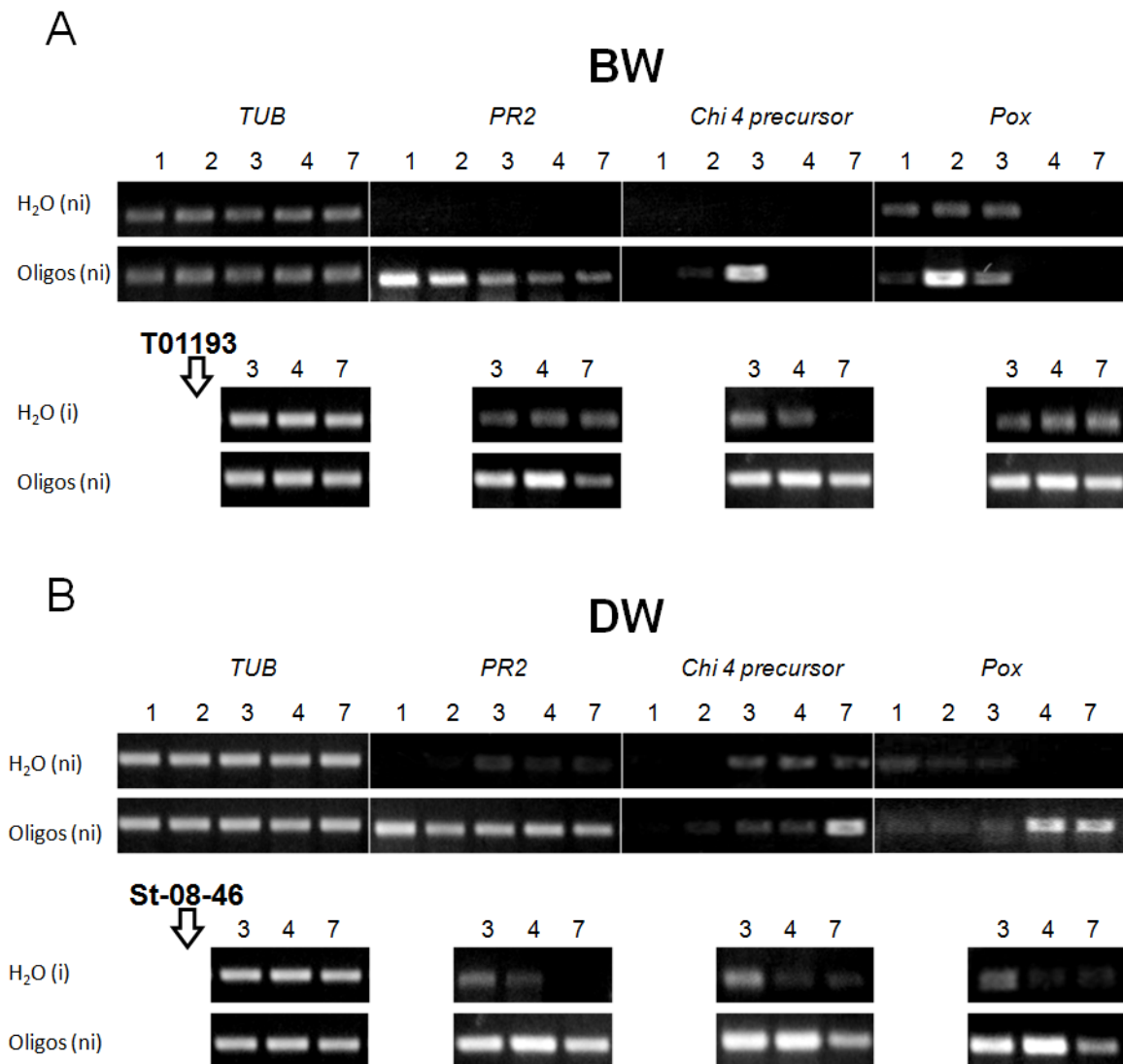


Fig. 2. Effect of foliar Oligos treatment (50 mg L^{-1}) on *pathogenesis-related 2 (PR2)*-, *Chi4 precursor*- and *Pox*-encoding genes expression in BW (A) and DW (B) leaves revealed by semi-quantitative RT-PCR. Four plant treatments were designed in this assay for each cultivar: non-treated and non-infected [H_2O (ni)], Oligos-treated and non-infected [Oligos (ni)], non-treated and *Z. tritici*-infected [H_2O (i)], Oligos-treated and *Z. tritici*-infected [Oligos (i)]. For infected samples, the inoculation was carried out 48 hours post Oligos treatment. Wheat leaves were collected at 1, 2, 3, 4 and 7 days post Oligos treatment in (ni) and (i) conditions. For each condition, Oligos-treated leaves were compared to their reference H_2O -treated leaves. RT-PCR products were generated using primers specific for β -tubulin (*TUB*), *PR2*, *Chi 4 precursor* and *Pox* first-strand cDNAs obtained from total wheat RNA, and visualized on agarose gel (1.5%). *TUB*-encoding gene was used as a house-keeping gene. Experiment was repeated three times and only results which were similar from two independent experiments were considered.

The time course of *LOX*- and *PAL*-encoding gene's expression was studied by quantitative RT-PCR analysis. Their expression was investigated in (ni) and (i) conditions and was normalized

to the *TUB* and *ACT* reference gene expression (fig. 3). In (ni) condition, after 1 day post Oligos treatment *LOX* expression pattern was 74.7- and 5.9-fold up-regulated on BW and DW leaves, respectively, while *PAL*-encoding gene's expression pattern didn't reveal any significant change compared to reference H₂O-treated (ni) leaves on both wheat species. After inoculation, was 4-fold significant up-regulated at 2 dpt, only in DW leaves. After inoculation, at 4 dpt corresponding to 2 dpi, on BW leaves weaker 3.7-up-regulation was noted, while *LOX* expression pattern showed strong 87.7-fold up-regulation on DW leaves. However, *PAL*-encoding gene's expression pattern didn't reveal any significant change compared to reference H₂O-treated (ni) and (i) leaves on both wheat species.

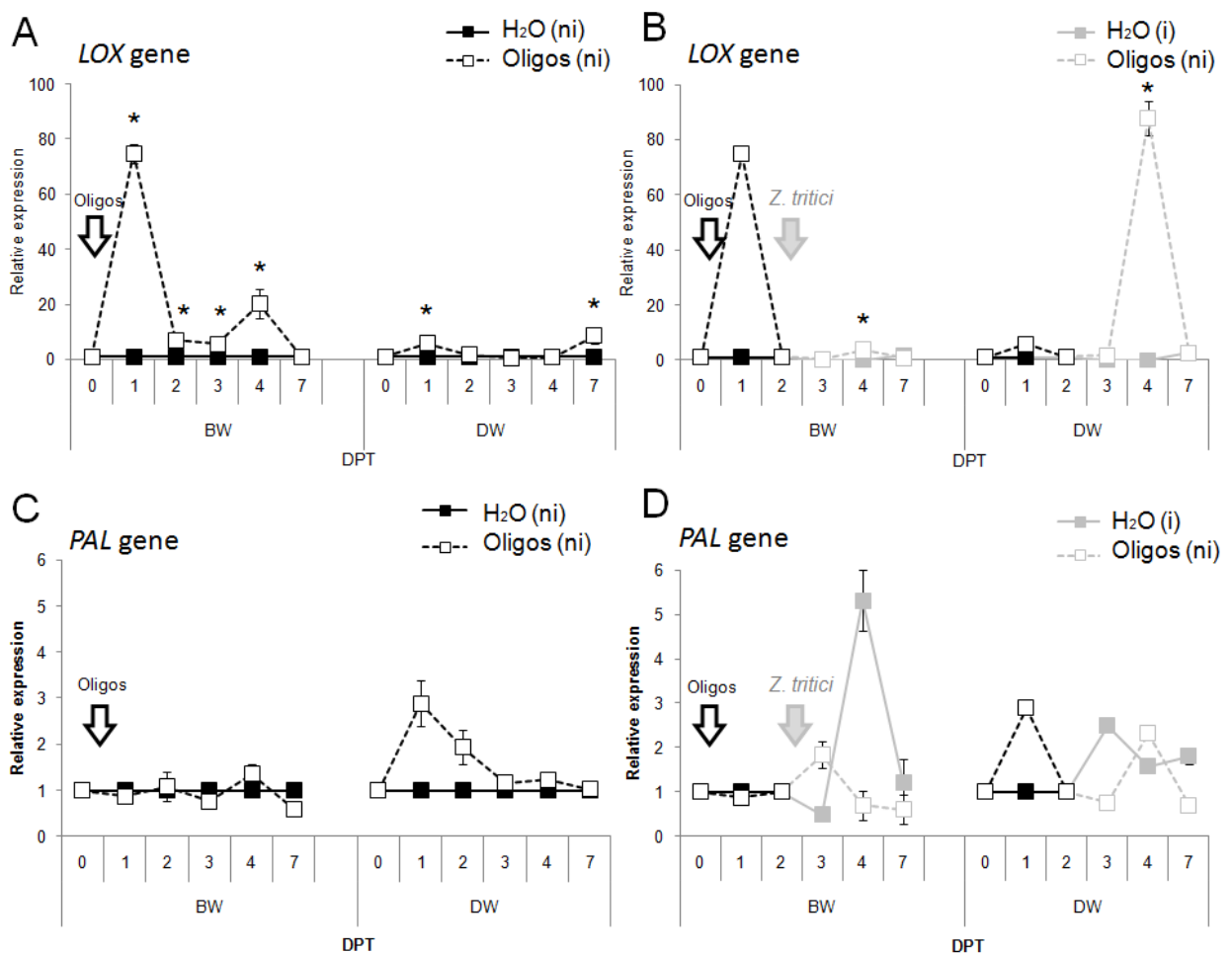


Fig. 3. Effect of foliar Oligos treatment (50 mg L⁻¹) on *LOX* and *PAL* genes expression in BW and DW leaves revealed by quantitative RT-PCR. (A) *LOX* gene expression in (ni) condition. (B) *LOX* gene expression in (i) condition. (C) *PAL* gene expression in (ni) condition. *PAL* gene expression in (i) condition. Four plant treatments were designed in this assay for each cultivar: non-treated and non-infected [H₂O (ni)], Oligos-treated and non-infected [Oligos (ni)], non-treated and *Z. tritici*-infected [H₂O (i)], Oligos-treated and *Z. tritici*-infected [Oligos (i)]. For infected samples, the inoculation was carried out 48 hours post Oligos treatment. Wheat leaves were collected after 1, 2, 3, 4 and 7 days post Oligos treatment. The experiment was conducted in triplicate. Similar results were obtained at least twice. Results correspond to means (\pm s.d) of duplicate reactions. Means of infected samples designed by asterisks are statistically differentially regulated (up- or down-) in comparison to their corresponding H₂O-treated controls using Tukey test at $P \leq 0.05$.

Enzymatic activities

At the enzymatic level, the effect of Oligos treatment on β -1,3-glucanase (GLUC), peroxidase (POX), catalase (CAT), phenylalanine ammonia-lyase (PAL) and lipoxygenase (LOX) enzymatic activities was studied in (ni) context (elicitor effect) (Table 4) and (i) context (potentiating effect) (Table 5).

Table 4 Effect of foliar Oligos treatment (50 mg L⁻¹) on five defense-related enzyme activities on BW and DW leaves at (ni) context. Wheat leaves were collected after 1, 2, 3, 4 and 7 days post Oligos treatment.

Activities (U mg ⁻¹ of proteins)	Days post treatment	T01193/BW		St-08-46/DW	
		H ₂ O (ni)	Oligos (ni)	H ₂ O (ni)	Oligos (ni)
GLUC	0	135.7±29.7	-	138.9±11.7	-
	1	141.9±7.1	225.3±10.6*	126.2±10.8	218.5±46.9*
	2	142±11.5	149.3±9.4	124.9±13	139±13.9
	3	163.6±11	180.7±26.6	132.6±13	103.4±19.2
	4	157.1±36	149.4±16.8	145.3±37.8	157.3±23.4
	7	150.8±12	173.5±16.1	130.1±26.4	144.3±10.7
POX	0	5.5±1.9	-	6.3±4.3	-
	1	6.9±3	12.4±3.6	8.1±3.7	9.9±1.7
	2	9.3±1.6	12.2±1.9	6.6±3.4	11.3±0.8
	3	7.5±3.2	13.3±2.7	7.1±3.8	10±1.4
	4	8.3±1.4	14.6±6	7.5±12.2	10.2±1.3
	7	7.9±2.2	12±3.7	7±2.3	11.5±2.8
CAT	0	2.7±1	-	2.5±1	-
	1	3.4±0.6	5.6±1.2	3.7±0.6	5.2±1.1
	2	5.5±1.4	5.2±1.1	5.8±0.8	5.7±0.8
	3	4.2±0.5	7.5±0.9	4.4±1.3	8.4±2.5*
	4	5.4±1	6.8±2.3	4.8±1.1	5.2±1
	7	5.5±1.6	7.1±1.5	4.2±1.1	5.6±0.7
PAL	0	4.1±8.4	-	3.1±4.7	-
	1	0±0	46.3±19.7	0±0	41.7±43.2
	2	0±0	10.2±8.9	5.1±14.9	0±0
	3	29±43.3	38.5±26.7	20.8±32.7	21.4±35.3
	4	3.0±9.7	5.9±13.2	0±0	0±0
	7	27.2±18.2	29.6±6.1	1.5±17	0±0
LOX	0	0.8±0.6	-	0.7±0.4	-
	1	0.5±0.7	1.9±0.7*	0.7±0.3	1.9±0.9
	2	0.6±0.4	1.6±0.4	0.5±0.4	3±0.8*
	3	0.8±0.3	1.3±0.2	0.3±0.2	1.3±0.8
	4	0.8±0.4	1.1±0.3	0.3±0.2	1.2±0.7
	7	0.5±0.5	1±0.5	0.3±0.4	1.1±0.4

Results correspond to means (\pm s.d) of two independent experiments using three independent replicates for each experiment. Statistics were made separately for each wheat specie. For each wheat specie, Oligos-treated samples with an asterisk (*) are different from their corresponding H₂O-treated controls (Tukey's test, $P \leq 0.05$).

The study of eliciting effect of tested oligosaccharides was investigated by comparing enzymatic activities in Oligos-treated (ni) leaves to corresponding reference H₂O-treated (ni) leaves at 1, 2, 3, 4 and 7 dpt (Table 4). Results exhibited that in BW (ni) leaves, Oligos treatment enhanced GLUC activity (1.6-fold at 1 dpt) and LOX activity (3.8-fold increase at 1 dpt). Although, we didn't detect significant elicitation of POX, CAT and PAL activities. Likewise, DW Oligos-treated (ni)

leaves exhibited remarkable increase of wheat GLUC activity (1.7-fold at 1 dpt), CAT activity (1.9-fold at 3 dpt), LOX activity (6-fold increase at 2 dpt). However, no significant elicitation was noted for POX and PAL activities.

Table 5 Time course of enzymatic activities at 1, 2 and 5 days post inoculation of BW and DW leaves by of T01193 and St-08-46 *Z. tritici* strains, respectively. The inoculation was carried out 48 hours post Oligos treatment. At each time point, treated samples with 50 mg L⁻¹ of Oligos were compared to their corresponding reference H₂O-treated samples.

Activities (U mg ⁻¹ of proteins)	Days post inoculation	T01193/BW		St-08-46/DW	
		H ₂ O (i)	Oligos (i)	H ₂ O (i)	Oligos (i)
GLUC	1	242.7±59.1	267.8±49.4	176±21.1	220±12.1*
	2	239.1±3.1	276.3±9	188.6±15.1	198.1±18.2
	5	256±28.5	305.1±12.1*	174.6±25.8	216.2±14.9*
POX	1	15.1±1.5	23.7±11.8	10.3±3.7	14.9±2.3
	2	18.1±2.4	20.9±11.2	12.1±3.2	15.1±4
	5	19.3±3	29.3±18.7	12.2±1.2	15±3
CAT	1	8±0.9	11.1±1.9	7.7±3.2	8.7±3.9
	2	12.1±3.2	10.4±1.8	9.3±2.1	11.3±3.6
	5	9.8±1.8	13.3±3.1*	7.3±1.4	11.9±2.8*
PAL	1	98.4±41.8	171.7±48.9*	94±48.8	121.9±47.6
	2	63.6±23.2	93.9±45.9	113.3±52.2	77±34.5
	5	66.6±19.5	177±54.1*	70.5±17.8	92±45.5
LOX	1	1.5±0.3	3.3±0.1*	1.6±0.4	2.7±0.5
	2	2±0.6	4.2±0.9*	1.6±0.3	3.2±1.3*
	5	1.6±0.1	3.9±1*	1.6±0.4	3.6±1.1*

Results correspond to means (±s.d) of two independent experiments using three independent replicates for each experiment. Statistics were made separately for each wheat specie. For each wheat specie, Oligos-treated samples with an asterisk (*) are different from their corresponding H₂O-treated controls (Tukey's test, $P \leq 0.05$).

The investigation of the potentiating effect of tested oligosaccharides was investigated by comparing enzymatic activities in Oligos-treated (i) leaves to corresponding reference H₂O-treated (i) leaves at 1, 2 and 5 dpi (3, 4 and 7 dpt) (Table 5). Data showed that on BW leaves, we detected significant increase of GLUC activity (1.19-fold at 5 dpi), CAT activity (1.36-fold at 5 dpi), PAL activity (1.74- and 2.66-fold at 1-5 dpi) and LOX activity (2.20-, 2.10- and 2.44-fold at 1-2-5 dpi). No significant potentiating effect on POX activity was observed. Similarly, on DW leaves, Oligos treatment induced strongly enhanced GLUC activity (1.25- and 1.24-fold at 1-2 dpi), CAT activity (1.63-fold induced at 5 dpi) and LOX activity (2- and 2.25-fold at 1-2 dpi). No significant potentiating effect on POX and PAL activity was observed.

Mesophyll H₂O₂ accumulation

At the cytological level, H₂O₂ accumulation was revealed by 3, 3-diaminobenzidine (DAB) staining (red-brown staining) in BW and DW leaves. Four classes of *Z. tritici* spores were distinguished: C1 (spores with non-penetrated germ tube) (Fig. 4a), C2 (spore with penetrated germ tube without staining) (Fig. 4b), C3 (penetrated germ tube with papilla) (Fig. 4c), C4 (penetrated germ tube with at least 1 whole stained mesophyll cell) (Fig. 4d). Results exhibited that Oligos treatment induced significant 15.6-fold increase of H₂O₂ accumulation, only in infected DW leaves at 2 dpi (4 dpt) (Table 6).

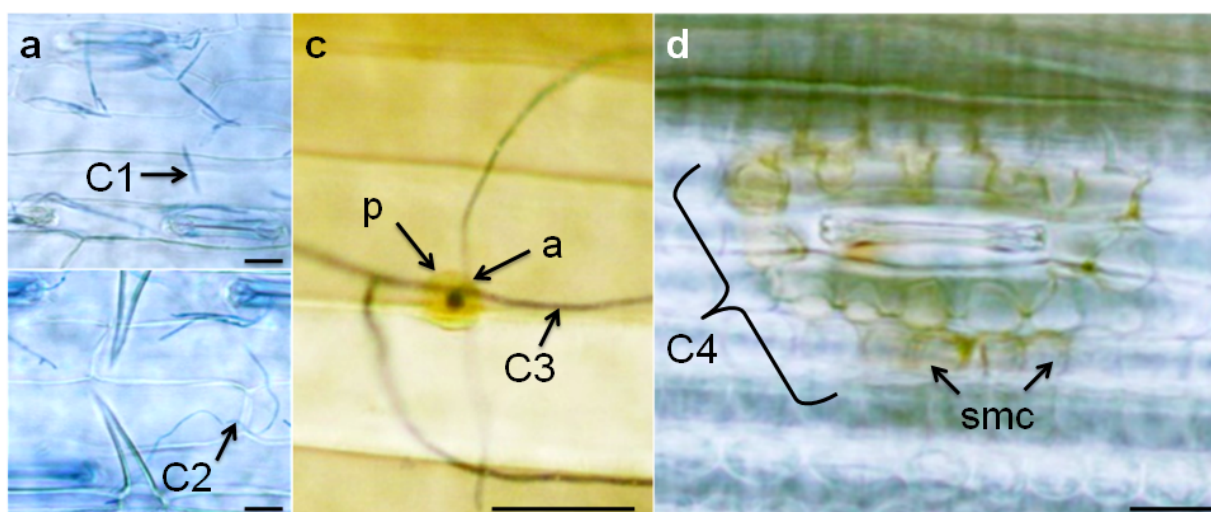


Fig. 4. Microscopic observations of H₂O₂ accumulation visualized by DAB staining displayed at BW and DW leaves following foliar spray by 50 mg L⁻¹ of Oligos. The inoculation of BW and DW leaves by T01193 and St-08-46 *Z. tritici* strains, respectively, was carried out 48 hours post Oligos treatment. Wheat leaves were collected after at 0, 1, 2 and 5 days post inoculation. a: (C1) spore with non-penetrated germ tube. b: (C2) spore with penetrated germ tube without staining. c: (C3) penetrated germ tube with papilla. d: (C4) penetrated germ tube with at least 1 whole stained mesophyll cells. (a) Appressorium-like structure, (p) Papilla. (smc) Stained mesophyll cells. Bar scale = 10 µm.

Table 6 Percentages of measured classes of H₂O₂ accumulation according to DAB staining intensity displayed at BW and DW leaves following foliar spray with 50 mg L⁻¹ of Oligos. The inoculation was carried out 48 hours post Oligos treatment.

Wheat species	Classes (%)	Days post inoculation		2		5	
		1		H ₂ O (i)	Oligos (i)	H ₂ O (i)	Oligos (i)
BW	C1	29.1±1.1 ab	71.1±11.7 a	40.5±15.3 ab	26.7±3.2 b	32.1±9.6 b	43.4±7.5 ab
	C2	37.6±1.1 a	28.9±11.7 ab	46.8±2.9 ab	34.1±7.7 ab	35.5±15 ab	26.3±7.5 b
	C3	0±0 a	0±0 a	8.6±5.4 a	24.5±9 a	25.8±16.1 a	14.9±7 a
	C4	0±0 a	0±0 a	4.2±7.2 a	14.7±0.6 a	6.7±14.1 a	15.3±7.6 a
DW	C1	26.8±3.8 ab	26.1±6.7 ab	37.8±9.2 ab	8.2±2.2 b	11.7±3 ab	43.4±7.2 a
	C2	39.9±3.8 abc	73.9±6.7 a	54.6±12.9 abc	8.2±6.7 c	61.4±5.1 ab	26.3±15.9 bc
	C3	0±0 b	0±0 b	3.7±3.6 ab	22.7±8.9 a	16.7±4.1 ab	14.9±7 ab
	C4	0±0 c	0±0 c	3.9±3.8 bc	60.8±0.8 a	10.1±9.4 bc	15.3±7.6 b

C1: spore with non-penetrated germ tube. C2: spore with penetrated germ tube without staining. C3: penetrated germ tube with papilla. C4: penetrated germ tube with at least 1 whole stained mesophyll cells. Results correspond to means (±s.d) of two independent experiments using three independent replicates for each experiment. Oligos-treated (i) samples with an asterisk are different from their corresponding H₂O-treated (i) controls (Tukey's test, $P \leq 0.05$).

Phenolic compounds accumulation

Both local polyphenol-associated auto-fluorescence and total polyphenol content were evaluated. Local polyphenol deposition may be revealed as clear yellow fluorescence at the penetration sites by the fungus. Four types of events were observed, such as C1 (spores with non-penetrated germ tube) (Fig. 5a), C2 (spore with penetrated germ tube without fluorescence) (Fig. 5a), C3 (penetrated germ tube with weak fluorescence) (Fig. 5b) and C4 (penetrated germ tube with strong fluorescence) (Fig. 5c).

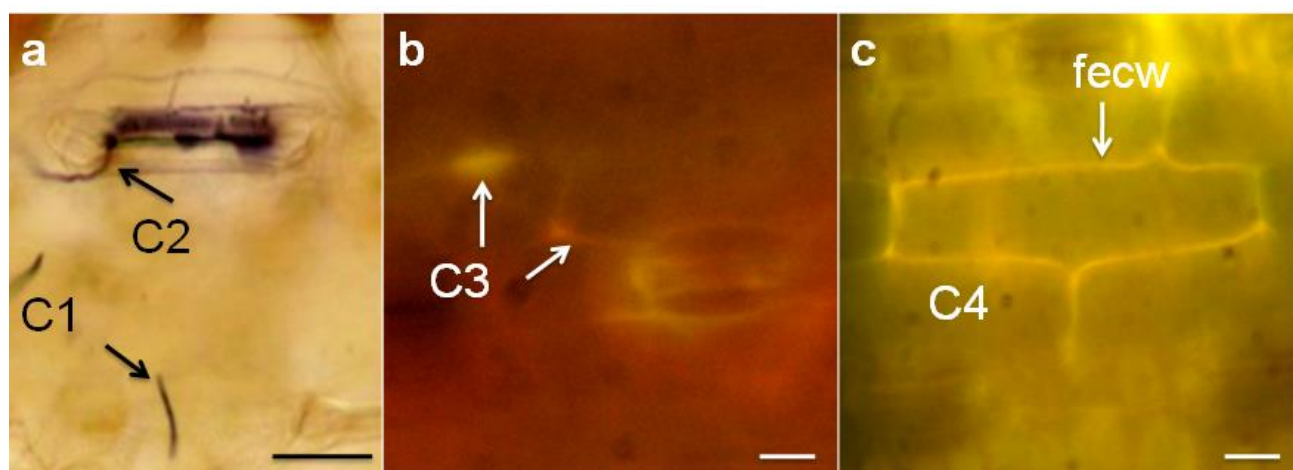


Fig. 5. Microscopic observations of polyphenol-associated autofluorescence displayed at BW and DW leaves following foliar spray with 50 mg L⁻¹ of Oligos. The inoculation of BW and DW leaves by T01193 and St-08-46 *Z. tritici* strains, respectively, was carried out 48 hours post Oligos treatment. Wheat leaves were collected after at 0, 1, 2 and 5 days post inoculation. Four classes of *Z. tritici* spores were observed: (C1) spore with non-penetrated germ tube, (C2) spore with penetrated germ tube without fluorescence, (C3) penetrated germ tube with weak fluorescence, (C4) penetrated germ tube with strong fluorescence. (p) Papilla. (fchw) Fluorescent mesophyll cells. Bar scale = 10 µm.

Table 7 Percentages of measured classes of polyphenol deposition according to polyphenol-associated autofluorescence intensity displayed at BW and DW leaves following foliar spray with 50 mg L⁻¹ of Oligos. The inoculation of BW and DW leaves by T01193 and St-08-46 *Z. tritici* strains, respectively, was carried out 48 hours post Oligos treatment.

Wheat species	Classes (%)	Days post inoculation					
		1		2		5	
		H ₂ O (i)	Oligos(i)	H ₂ O (i)	Oligos(i)	H ₂ O (i)	Oligos(i)
BW	C1	23.7±5.3 b	47.1±5.1 a	16.3±7.2 bc	22.9±1.9 b	4.8±15.3 c	15.8±3.6 bc
	C2	68.5±10.3 b	20.3±11.9 c	68.4±11.6 b	29±4.2 c	95.2±5.8 a	25.9±4.6 c
	C3	7.8±5.1 a	16.1±4.1 a	15.3±2.9 a	12.5±21.7 a	0±0 a	14.3±4.1 a
	C4	0±0 b	16.5±15 ab	0±0 b	35.6±20.2 a	0±0 b	44.0±8.5 a
DW	C1	22.3±7.1 a	21.1±12.6 a	21.3±4.3 a	32.6±9.0 a	11.7±5.6 a	17.3±4.6 a
	C2	72.7±10.4 a	42.2±16.8 b	74.2±6.2 a	28.3±13.9 b	80.9±9.7 a	28.1±4.5 b
	C3	5±6.6 a	26.7±24 a	4.5±1.8 a	32.1±23.5a	7.4±6.7 a	7±2.6 a
	C4	0±0 b	10±10 b	0±0 b	7±12.2 b	0±0 b	47.7±9 a

C1: Spore with non-penetrated germ tube. C2: Spore with penetrated germ tube without fluorescence. C3: Penetrated germ tube with weak fluorescence. C4: Penetrated germ tube with strong fluorescence. Results correspond to means (±s.d) of two independent experiments using three independent replicates for each experiment. Oligos-treated (i) samples with an asterisk are different from their corresponding H₂O-treated (i) controls (Tukey's test, $P \leq 0.05$).

In Oligos-treated leaves, we noted significant increase of C1 at 1 dpi only in BW leaves and decrease of C2 at 1-2-5 dpi (3-4-7 dpt) on both wheat species. Polyphenol deposition was detected from 1 dpi (3 dpt). In BW leaves, C4 was 35.6- and 44-fold increased at 2-5 dpi (4-7) dpt, while in DW leaves, it was 47.7-fold increased at 5 dpi (7 dpt). However, no significant difference of C3 was detected on both wheat species. Second, the effect of Oligos treatment on polyphenol content was also measured in whole 3rd leaf of both wheat species at (ni) and (i) context. In, BW Oligos-treated (ni) leaves results exhibited that the content of total soluble polyphenol was 1.7-fold increased at 5 dpi (7 dpt), while in DW Oligos-treated (ni), it was 1.9- and 1.8-fold induced at 4-7 dpt. After inoculation, Oligos treatment induced, 1.6-fold increase of polyphenol content at 5 dpi (7 dpt), in BW leaves, whereas it generated 1.4- and 1.5-fold increase at 2-5 dpi (4-7 dpt) in DW leaves (Table 6).

Table 8 Polyphenol content (expressed in mg of CE g⁻¹ of FW) on BW and DW leaves, at 0, 1, 2, 3, 4 and 7 days post treatment (DPT) by Oligos in (ni) and (i) condition. The inoculation of BW and DW leaves by T01193 and St-08-46 *Z. tritici* strains, respectively, was carried out 48 hours post Oligos treatment.

Pathosystem	DPT	Non infectious context		Infectious context	
		H ₂ O (ni)	Oligos (ni)	H ₂ O (i)	Oligos (i)
T01193/BW	0	8.2±2.3	8.2±2.3	-	-
	1	11.3±3.9	13.6±4.2	-	-
	2	10.7±1.8	14.4±3.8	-	-
	3	11.6±2.6	15.2±3.6	11.8±1.9	12.7±3.9
	4	11.4±3.4	16.3±5.3	11.6±1.1	16.5±7.9
	7	11.0±4.1	18.7±6.5*	11.8±1.1	18.4±8.5*
St-08-46/DW	0	6.4±2.7	6.4±2.7	-	-
	1	8.3±3.4	11.2±2.3	-	-
	2	7.7±0.9	8.7±1.5	-	-
	3	7.8±1.9	10.3±3.6	9.9±2.6	12.1±2.2
	4	6.7±2.9	12.8±3.4*	9.1±2.2	13.1±2.1*
	7	8.3±3.1	14.5±4.7*	9.3±2	13.5±3.5*

Results correspond to means (±s.d) of two independent experiments using three independent replicates for each experiment. Statistics were made separately for each wheat specie. For each wheat specie, Oligos-treated samples with an asterisk (*) are different from their corresponding H₂O-treated controls (Tukey's test, $P \leq 0.05$).

DISCUSSION

Here, we examined antifungal and resistance inducer effect of based – oligosaccharides (20%) (Oligos) against *Z. tritici*. Tested oligosaccharide consists on plant cell wall extracted from plants as glucan, chitin... Besides, differences in response of BW/T01193 and DW/St-08-46 pathosystems to Oligos treatment were discussed.

Antifungal activity of Oligos against T01193 and St-08-46 Z. tritici strains

At tested concentrations, Oligos didn't show any significant *in vitro* antifungal effect neither on spore germination nor on hyphal growth of both tested *Z. tritici* strains. Similarly, Joubert *et*

al. (1998) which didn't find direct antifungal activity of GLUC an extracted from brown algae against *Z. tritici*. Although, *in vitro* inhibitory effect of chitin-oligosaccharides has been documented against various species of bacteria, fungi and oomycetes (Hadrami *et al.*, 2010). Direct toxicity of oligosaccharides remains dependent on properties such as the concentration applied, the molecular weight (No *et al.*, 2002), degree of acetylation or polymerisation (Rahman *et al.*, 2014), solvent, pH and viscosity (Chung *et al.*, 2003), which could explain absence of *in vitro* inhibitory effect of tested oligosaccharides against *Z. tritici*. *In vivo* condition, Oligos treatment allowed to reduce hyphal growth, leaf penetration, mesophyll colonisation intensity and pycnidium coverage, in both wheat species. This observed fungal inhibition could be attributed to chito-oligosaccharides presence which were reported to exert an inhibitory action on the hyphal growth and/or spore germination of numerous pathogenic fungi, including root and necrotrophic pathogens, such as *Fusarium oxysporum*, *Botrytis cinerea*, *Monilina laxa*, *Alternaria alternate*, *A. radicina*, *Pythium aphanidermatum*, *B. graminis* f. sp. *tritici* *Phytophthora cactorum* and *P. fragariae* var. *fragariae* and could protect many crops including carrots, strawberry, cucumber, tobacco... (Eikemo *et al.*, 2003; Ben-Shalom *et al.*, 2003; El Hassni *et al.*, 2004; Jayaraj *et al.*, 2004; El Hassni *et al.*, 2005; Liu *et al.*, 2007; Jayaraj *et al.*, 2009).

In our experiment, Oligos treatment, applied at 50 mg L⁻¹ in greenhouse condition, was able, to efficiently slowing fungal progression and the transformation of stomata in pycnidia. Therefore, Oligos treatment allowed to markedly decreasing the fructification and fungal necrosis, coorelated to the strong inhibition of CWDE activities (endo-β-1,4-xylanase, endo-β-1,3-glucanase and protease) at necrotrophic stage, in both wheat species. This suggests the correlation of these CWDEs with disease severity and their potential use as markers of resistance inducer efficiency.

Numerous reports demonstrated that oligosaccharides confer resistance to fungal pathogens in many crops including cereals. Chitin and chitosan oligosaccharides were reported to control powdery mildew in barley (Faoro *et al.*, 2008), *Fusarium gramineum* (Orzali *et al.*, 2014), *Alternaria alternata* (Wachowska *et al.*, 2013) in wheat. Moreover, glucans could control *Z. tritici* in wheat (Esnault *et al.*, 2005; Shetty *et al.*, 2009). Other oligosaccharides as OGAs and trehalose , has been reported as a good alternative to control of *B. graminis* f. sp. *tritici* in wheat (Reignault *et al.*, 2001; Renard-Merlier *et al.*, 2007; Randoux *et al.*, 2010). The absence of *in vitro* inhibition of spore germination and hyphal growth of both *Z. tritici* strains by Oligos addition, suggests that it could operate indirectly *via* other means such as the enhancement of host resistance. This hypothesis will be investigated here, by assessing defense related proteins and metabolites at different scales.

Elicitation of BW and DW defense pathways by oligosaccharides

In the current study, Oligos treatment, applied at 50 mg L⁻¹ in greenhouse condition, allowed enhancement of defense mechanisms in both BW/T01193 and DW/St-08-46 pathosystems, including the induction of plant degrading fungal cell walls enzymes, modifications in ROS metabolism, phenylpropanoid and octadecanoid pathways. This stimulation of wheat defense pathways was detected in (ni) condition (elicitor effect) and (i) condition (potentiating effect).

In one hand, chitinases hydrolyze β -1,4-glycoside bonds in chitin to produce N-acetylglucosamine and N, N+ diacetylchitibiose (Cohen-Kupiec *et al.*, 1998). The involvement of chitinases in BW defense against pathogens was confirmed in many studies (Bertini *et al.*, 2003; Singh *et al.*, 2007; Lu *et al.*, 2006; Cawood *et al.*, 2010, 2013). Chitinase isozymes have been divided into seven classes (class I–VII) on the basis of their structural properties (Neuhaus *et al.*, 1999). In this study, we were interested to *Chi 4 precursor*-encoding gene which encode an apoplatic chitinase (Class IV) precursor (Benhamou *et al.*, 1990; Dore *et al.*, 1991). Several works reported weak enhancement of chitinase of class I (Shetty *et al.*, 2009; Yang *et al.*, 2013), and II (Adhikari *et al.*, 2007) gene expression and enzymatic activity in response to *Z. tritici* inoculation in a bread wheat susceptible cultivar. In the other hand, β -1,3-glucanase (PR-2) catalyzes hydrolytic cleavage of the 1,3- β -D-glucosidic linkages in β -1,3 glucan, which is a component of fungal cell walls (Ryals *et al.*, 1996). A correlation between constitutive β -1,3-glucanase levels in leaves and resistance to *Alternaria solani* has been established in tomato (Lawrence *et al.* 1996). Numerous reports demonstrated the involvement of β -1,3-glucanase in wheat defense against pathogens including *Z. tritici* (Shetty *et al.*, 2009), *Puccinia triticina* (Cawood *et al.*, 2010, 2013) and *Puccinia recondita* f. sp. *tritici* (Anguelova *et al.* 1999). In our experiment, we revealed that Oligos treatment enhanced the production of cell walls degrading enzymes as primary defense against *Z. tritici*, including β -1,3-glucanase and chitinase IV precursor (*Chi 4 precursor*). It exhibited an early elicitation of *PR2* transcripts and β -1,3-glucanase enzymatic activity, in both wheat species, associated with later *Chi4 precursor*-encoding gene's up-regulation. Besides, Oligos treatment induced a good potentiating effect of both cell wall degrading enzymes. Similarly, it has been demonstrated that treatment of a susceptible wheat cultivar with purified β -1,3-glucanase a treatment was accompanied by increased gene expression of β -1,3-glucanase and chitinase I transcripts and proteins accumulation associated with the deposition of callose (Shetty *et al.*, 2009). Similarly, it was reported that trehalose (TR: a nonreducing disaccharide) elicited the expression of genes encoding chitinase (*chi*, *chi1*, and *chi4 precursor*) (Tayeh *et al.*, 2014). Besides, Falcón-Rodríguez *et al.* (2009) and Jayaraj *et al.* (2009) demonstrated the induction of β -1,3-glucanase proteins accumulation by chitin oligosaccharides in tobacco and carrots. Moreover, early and late β -1,3-glucanase and chitinase accumulation were enhanced by oligosaccharides as Chitin and glucan

an treatment of grapevine against gray mold and downy mildew (Aziz *et al.*, 2006) and Potato against *Phytophthora infestans* (Zhu *et al.*, 2014). Moreover, it has been reported that other elicitor such as Jasmonic acid and salicylic acid induced accumulation of β -1,3-glucanase in wheat and enhance resistance against *Stagonospora nodorum* (Jayaraj *et al.*, 2004).

Reactive oxygen species (ROS) metabolism is a key element of nonspecific plant defense responses. ROS act as direct antimicrobial molecules or secondary messengers, or are involved in metabolic pathways leading to resistance (Hückelhoven, 2007). The production of ROS, as H_2O_2 , via consumption of oxygen in a so-called oxidative burst, is one of the earliest cellular responses following successful pathogen recognition (Apel and Hirt, 2004). Here, we investigate the response of ROS metabolism to Oligos treatment, in both wheat species, through the elicitation of POX and CAT activity and H_2O_2 accumulation at penetration sites of the fungus. First, CAT is an H_2O_2 -scavenging enzyme, mainly located in peroxisomes. Its activity was reported as closely related to H_2O_2 disappearance (Sandalio *et al.*, 2013). As CAT degrades H_2O_2 without any reducing power, this enzyme provides plants with an energy-efficient way to remove H_2O_2 . Besides, POXs form a complex family of proteins that catalyze the oxidoreduction of various substrates using H_2O_2 (Passardi *et al.* 2004). Diani *et al.* (2009) reported induction of POX activity in BW resistance against *Z. tritici*. Also, Farsad *et al.* (2013) and Adhikari *et al.* (2007) works revealed the involvement of *Pox* gene expression in BW resistance against *Z. tritici*. In addition, H_2O_2 is very important in wheat defense during the biotrophic phase and contribute to resistance induction against *Z. tritici* infection (Shetty *et al.*, 2003; Yang *et al.*, 2013). Here, we detected early and short elicitation of CAT activity in response to Oligos treatment, only in DW leaves. In (i) context, Oligos treatment enhanced H_2O_2 accumulation at penetration sites of *Z. tritici*, followed by potentiation of CAT activity, mainly in DW leaves. Therefore, we suggested that the increase of H_2O_2 accumulation following treatment, enhanced antioxidant enzyme as CAT, in order to degrade H_2O_2 . However, POX enzymes activities were not significantly affected by Oligos treatment, while *Pox*-encoding gene up-regulation was enhanced mainly in BW. The *Pox* gene encodes for a specific type of peroxidase (lignin forming peroxidase) that could act in cell wall reinforcement by catalyzing phenolics deposition as lignin (Van Loon *et al.*, 2006; Taheri and Tarighi, 2011). Therefore, we suggest that this treatment triggers peroxidase involved in lignification or phenolics deposition, but didn't induce all peroxidases proteins accumulation. Several other oligosaccharides induced ROS metabolism in wheat pathogenic fungi, such as trehalose and oligogalacturonides (OGAs) in BW leaves in response to *Blumeria graminis f. sp. tritici*. TR triggered ROS metabolism in wheat through inducing oxalate oxidase (*oxo*) gene up-regulation (Tayeh *et al.*, 2014) and POX activity (Reignault *et al.*, 2001). Randoux *et al.* (2010) reported that

OGAs treatment enhanced OXO and POX activities associated with H₂O₂ accumulation at fungal penetration sites.

Phenylpropanoid pathway enhancement by Oligos treatment was investigated through measuring (i) *PAL*-encoding gene expression, (ii) total PAL activity, (iii) polyphenol content and (iv) polyphenol deposition in mesophyll cells. PAL is the key enzyme acting upstream in the phenylpropanoid pathway and the biosynthesis of phenolic compounds. It catalyzes the deamination reaction from L-phenylalanine to trans-cinnamic acid, a precursor of the majority of the phenolic compounds produced by plants (Zabala *et al.*, 2006). Several studies confirmed the correlation of PAL activity and total phenolic compounds accumulation in incompatible interaction with *Z. tritici* (Hassan *et al.*, 2007; Adhikari *et al.*, 2007). In our experiment, Oligos treatment didn't affect *PAL*-encoding gene, whereas it revealed potentiation of PAL activity, only in BW leaves. Other oligosaccharides as TR induced increase of PAL activity in BW in response to *Blumeria graminis f. sp. tritici* (Reignault *et al.*, 2001). Moreover, we observed that Oligos treatment enhanced polyphenol deposition in mesophyll cells at penetration sites of the fungus associated with elicitation and potentiation of total polyphenol content.

Octadecanoid pathway response to Oligos treatment was assessed by measuring enzymatic lipoxygenase (LOX) activity and *LOX* gene expression. LOX is involved in octadecanoid pathway, which mediates defense responses. Corresponding enzymes catalyze the first step in the oxygenation of polyunsaturated fatty acids (Holtman *et al.*, 2000). Treatment with methyl jasmonate stimulates barley *LOX* (Rouster *et al.*, 1997) (Holtman *et al.*, 1997). Ray *et al.* (2003) found out a down regulation of a *LOX*-encoding gene expression in susceptible wheat cultivar after infection by *Z. tritici*. However, the increase in LOX activity in the cereals-disease interaction has been demonstrated on several occasions (Ohata *et al.*, 1991; Avdiushko *et al.*, 1993; Sulsarenko, 1996; Ray *et al.*, 2003; Sandhu *et al.*, 2007). In the current study, tested Oligos strongly induced *LOX* gene up-regulation and total LOX activity in (ni) an (i) context, mainly in BW leaves. This suggests that lipid peroxidation and JA biosynthesis could be triggered by this treatment. Likewise, lipid metabolism was shown to be altered by TR spraying *via* the up-regulation of lipoxygenase (*lox*) and lipid-transfer protein (*ltp*)-encoding gene expression. Indeed, after *Blumeria graminis f. sp. tritici* infection, TR spraying enhanced up-regulations of *Chi4 precursor* and *lox* gene expression as well as an induction of the LOX activity (Tayeh *et al.*, 2014). Lipoxygenase activity was also triggered by Milsana (an ethanolic extract from leaves of *Reynoutria sachalinensis*) (Randoux *et al.*, 2006) and OGAs (Randoux *et al.*, 2010) against powdery mildew on wheat.

Overall, our investigation promotes the characterization of a based-oligosaccharids and highlighted enhanced related defense pathways in both BW and DW. However, our work must be

completed by further investigations using more susceptible cultivars of BW and DW in interaction with more pathogenic *Z. tritici* strains in order to prove our results.

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Publication 5: Chitosan enhances defense response in *Triticum durum* and *Triticum aestivum* and induces protection against *Zymoseptoria tritici* causal agent of STB disease under greenhouse condition

ABSTRACT

The objective of the current study is to explore the antifungal activity of chitosan against *Zymoseptoria tritici* and the possibility of defense stimulation ability on durum and bread wheat. In one hand, chitosan could control both wheat species against *Z. tritici* and allows to drastically decrease the production of fungal CWDE activities, such as endo- β -1,4-xylanase, endo- β -1,3-glucanase activities in both wheat species and protease activity only in DW leaves, during necrotrophic phase. On the other hand, chitosan treatment enhanced β -1,3-glucanase (GLUC) activity, phenolic compounds deposition and H₂O₂ accumulation at penetration sites of the fungi in both wheat species coincided with the first contact with the fungus in order to decrease its penetration and limit its progress, followed by peroxidase (POX), catalase (CAT), phenylalanine ammonia lyase (PAL) and lipoxygenase (LOX) activities in both wheat species which will activate ROS, phenylpropanoid and octadecanoid pathways. Besides, BW and DW exhibited distinct response profiles to tested treatments. Moreover, our experiment showed that both studied wheat species response exhibited some similarities as showed for CAT and LOX activities. However, they responded differentially for others as POX and PAL activities and H₂O₂ accumulation which were more responsive in BW, while GLUC activity and polyphenol deposition were more responsive in DW. Therefore, chitosan formulation could be considered as a good resistance inducer (RI) and could help in protecting susceptible BW and DW cultivars against *Z. tritici*, when applied at the appropriate concentration and timing as preventive manner. Further analysis are needed in order to elucidate with different wheat species and genotypes effect on defense mechanisms induction by RI, are required.

Key words: *Zymoseptoria tritici*, chitosan, defense, durum wheat, bread wheat.

INTRODUCTION

Septoria tritici blotch (STB) caused by *Zymoseptoria tritici* (Quaedvlieg *et al.*, 2011) previously known as *Septoria tritici* (teleomorph *Mycosphaerella graminicola*) is one of the most devastating fungal foliar diseases on bread wheat (*Triticum aestivum* L. em Thell, BW) and Durum wheat (*Triticum durum* Desf, DW). It causes annual yield losses ranging from 30 to 50% (Eyal *et al.*, 1987; Palmer and Skinner, 2002). Chemical control is the common way to control STB disease. However, it is limited by the negative impact of chemical fungicides on the environment and the

decrease of their efficiency caused by the emergence of resistant strains. Therefore, alternative of nonpollutant-associated strategies are being developed, including stimulation of plant defenses by a wide range of elicitors.

Chitosan oligosaccharides which are a partially deacetylated polymer of *N*-acetyl glucosamine, which is obtained after alkaline deacetylation of the chitin derived from the exoskeletons of crustaceans and arthropods. This last have been abundantly studied for its action on growth regulation, plant development, and elicitation of defense systems against biotic or abiotic stress in plants (Hadrami *et al.*, 2010). Furthermore, chitosan could induce host defense responses in both monocotyledons and dicotyledons. When oligosaccharides elicit defense responses in plants, the same phenomenon as those induced in the plant when a pathogen is recognized, may occur. Within a few minutes, the early defense responses triggered by the pathogen may be a modification of ion fluxes across the plasma membrane, phosphorylation and dephosphorylation of signaling proteins, production of reactive oxygen species. Few hours later, a broad spectrum of defense reaction may be induced to confer resistance against microorganisms. These defense reactions may be: cell wall strengthening through lignin synthesis and cross linking of macromolecular constituents, accumulation of metabolites with antimicrobial properties as phytoalexins, production of salicylic acid and jasmonate which are secondary metabolites resulting from the phenylpropanoid and octadecanoid pathways (Vasukova *et al.*, 2001; Jayaraj *et al.*, 2004; Aziz *et al.*, 2006; Faoro *et al.*, 2008; Hadrami *et al.*, 2010; Li and Zhu, 2013).

Numerous reports demonstrated that chitosan was able to induce a variety of defense reactions conferring resistance to viral, bacterial and fungal pathogens in many crops including tobacco (Falcón-Rodríguez *et al.*, 2009), rice (Inui *et al.*, 1997), tomato (Benhamou *et al.*, 1994), carrots (Jayaraj *et al.*, 2009), grapevine (Aziz *et al.*, 2006), strawberry (Eikemo *et al.*, 2003), cucumber (Ben-Shalom *et al.*, 2003), barley (Faoro *et al.*, 2008) and wheat (Bohland *et al.*, 1997; Renard-Merlier *et al.*, 2007; Randoux *et al.*, 2010). Moreover, other oligosaccharides could partially induce fungal diseases including β -1,3-glucan oligomers against *Z. tritici* (Joubert *et al.*, 1998; Esnault *et al.*, 2005), chitosan, trehalose and OGAs against *Blumeria graminis* f. sp. *tritici* (Reignault *et al.*, 2001; Renard-Merlier *et al.*, 2007; Rondoux *et al.*, 2010; Tayeh, 2013). Nevertheless, little is known about disease resistance inducers and, more specifically, about the biological activities from oligosaccharides in wheat.

Considering all these observations, in the present study we investigated the potential antifungal and wheat defense stimulator activities of based-chitosan against *Z. tritici*. Moreover, difference in BW and DW response to studied chitin oligosaccharides will be discussed.

MATERIALS AND METHODS

Fungal material and inoculation

For *in vivo* assays, the two *Z. tritici* T01193 and St-08-46 strains were chosen for their high level of pathogenicity, respectively on BW and DW. Cultures were grown for 3 weeks at 18°C and were stored at -80°C for further use. Plants were inoculated at 2 days post treatment (dpt) with 30 mL of a 10^6 spores mL⁻¹ suspension in a 0.05% of Tween 20 (Sigma Aldrich, USA) solution using a hand sprayer. Control plants were sprayed with tap water containing 0.05% of Tween 20. Immediately after inoculation, each pot was covered with a clear polyethylene bag for 3 days in order to ensure a water-saturated atmosphere. Disease level was scored at 22 days post inoculation (dpi) by measuring percentage of leaf area with symptoms (necrosis and pycnidia) and the index of pycnidium coverage (which describes the importance of pycnidia in necrosis following a scale noted from 1 to 5) within necrotic lesions on the 3rd leaf of 25 plantlets. Data represent means of two independent experiments.

Direct effect assessment on PDA medium

Here we investigated a chitosan (10%)-based formulation provided by CODA (Lerida, Spain). It was tested *in vitro* by measuring spore germination and fungal hyphal growth according to Siah *et al.* (2010a). *Spore germination*: aliquots of 0.6 mL of each strain, adjusted at 5×10^5 spores mL⁻¹, were sprayed on PDA plates containing various concentrations of tested product. Eight concentrations of chitosan (0.1; 0.4; 1.2; 3.6; 11; 33; 100 and 300 mg L⁻¹) were used. The plates were placed at 18°C in the dark for 24h. The percentages of germinated spores were then calculated for 100 spores, for each plate, using light microscope (Nikon, Eclipse 80i), in order to determinate the half maximal inhibitory concentration (IC₅₀) values. *Mycelium growth*: 5 µL of 5×10^5 spore mL⁻¹ of each strain were spotted on the PDA plates containing the same concentrations of tested compounds. After incubation for 10 days at 18°C in the dark, hyphal growth were compared. Assays were repeated twice and for each condition, three plates with three spots per plate were used as triplicates.

Plant material and tested product

Cultivars of the two wheat species known to be susceptible to STB were used for this study: bread wheat (BW) cultivar Premio and durum wheat (DW) cultivar Karim. Grains were pre-germinated in Petri dishes (12 x 12 cm) on moist filter paper in darkness at 20°C for 24 h, at 4°C for 48 h and then at 20°C for 24 h. Germinated grains were then transplanted into soil in pots of 15 cm-diameter (12 grains/pot). The pots were placed in the greenhouse at 18°C and under day-night cycles of 16h /8h.

After 3 weeks, when the third leaf was fully expanded, plants were sprayed with 100 mg L⁻¹ of chitosan.

Protein extraction and enzymatic assays

Fungal enzyme assays: Three fungal cell-wall degrading enzyme (CWDE) activities (endo- β -1,4-xylanase, endo- β -1,3-glucanase and protease) were measured at 4, 10, 16 and 22 dpi. Briefly, 2.5 g of third leaves were sampled and immediately frozen in liquid nitrogen for grinding in mortar. Endo- β -1,4-xylanase activity was measured at pH 4.8 and 45°C using xylan (1%, w/v) (Sigma) as substrate and xylose (10 mM) (Sigma Aldrich, USA) as reducing control sugar (Siah *et al.*, 2010). Endo- β -1,3-glucanase activity was measured at pH 4.8 and 50 °C using Laminarin (Sigma Aldrich, USA) at pH 5 (0.5 %, w/v) as substrate and glucose (10 mM) as reducing control sugar (Douaiher *et al.*, 2007). Absorbance was measured at 540 nm. One unit of endo- β -1,4-xylanase and endo- β -1,3-glucanase activities is defined as the amount of enzyme necessary to release 1 μ mol of reducing sugar/p-nitrophenol min⁻¹ mL⁻¹ of enzyme solution. Protease activity assays were assessed according to Hellweg (2003). Absorbance was measured at 400 nm. One unit of protease activity is defined as a change in enzyme activity of 1 per hour.

Plant enzyme assays: Five plant enzyme activities involved in defense mechanisms were measured: β -1,3-glucanase (GLUC), catalase (CAT), peroxidase (POX), lipoxygenase (LOX) and phenylalanine ammonia-lyase (PAL). Samples were collected from non inoculated (ni) and inoculated plants (i). At (ni) conditions, samples were collected at 1, 2, 3, 4 and 7 dpt. For (i) conditions, samples were collected at 0, 1, 2 and 5 dpi (corresponding to 2, 3, 4 and 7 dpt). Each sample consisted of three medial segments excised from three third leaves. GLUC activity was analyzed according to Douaiher *et al.* (2007). CAT activity was assessed according to Randoux *et al.* (2010); one unit of CAT activity is defined as the amount of the enzyme which degrades H₂O₂ at 1 μ mol min⁻¹, ($\epsilon = 36 \text{ mM}^{-1}\text{cm}^{-1}$). POX was estimated according to El Hadrami and Baaziz (1995) and expressed as nkat mg of protein⁻¹. One unit of POX activity is defined as the amount of enzyme that catalyses the conversion of guaiacol as substrate at A₄₇₀ after 2 min of H₂O₂ addition. LOX activity was measured according to Randoux *et al.* (2010). One unit of LOX activity is defined as the amount of enzyme that catalyses the conversion of substrate (linoleic acid) at 1 mols⁻¹ ($\epsilon = 23 \text{ mM}^{-1}\text{cm}^{-1}$). PAL activity was calculated as described by Randoux *et al.* (2010). One unit of PAL activity is defined as the amount of enzyme that catalyses the formation of cinnamic acid at 1 mols⁻¹ ($\epsilon = 10 \text{ mM}^{-1}\text{cm}^{-1}$).

All enzymatic activities were expressed in mU mg⁻¹ of proteins. Total protein concentration was measured at A₅₉₅ nm using bovine serum albumin as a standard (Bradford, 1976). For all tested activities, absorbance was measured using Thermo Scientific™ GENESYS 10S UV-

Vis spectrophotometer. Data represent means of two independent experiments. Three independent replicates were carried out for each experiment.

Cytological analysis

The accumulation of H₂O₂ was revealed by 3,3-diaminobenzidine (DAB) staining (red-brown staining) according to Randoux *et al.* [2010]. For polyphenol deposition visualization, leaves stained with Trypan Blue were used for fluorescence microscopy using a Nikon microscope (Champigny-sur-Marne, France) fitted for fluorescence (blue exciter filter, maximum transmittance 400 nm and barrier filter with a transmittance range of 500 to 800 nm). As described by Randoux *et al.* [2010], a yellow fluorescence visualized under UV-light microscopy indicates the presence of polymeric phenolic compounds. H₂O₂ and polyphenol visualization were detected at fungal penetration sites in 2-cm-long segment of 3rd leaves. Leaf samples were collected from the same plantlets used for enzymatic and molecular analysis and at the same timing. Data represent means of two independent experiments. Three independent replicates were carried out for each experiment.

Total polyphenolic compounds quantification

The quantification of the content of total soluble phenol compounds was determined according to the Folin-Ciocalteu method modified by El Hadrami *et al.* (1997). Leaf samples were collected from the same plantlets used for enzymatic analysis. Foliar tissues (200 mg from 3rd leaf), were homogenized in an ice bath with 1.5 ml methanol. The homogenate was centrifuged three times at 10000g for 5 min; supernatants were recuperated each time. One hundred microlitres of the supernatant was added to Folin-ciocalteu reagent and sodium carbonate. The mixture was incubated at 40°C for 30 min and the blue color was read at 760 nm using Catechin (Sigma Aldrich, USA) as a standard. The polyphenol content was determined for the experiments described above and was expressed in mg-equivalents of Catechin (Sigma Aldrich, USA) (CE) per gram of fresh weight (FW). Data represent means of two independent experiments. Three independent replicates were carried out for each experiment.

RESULTS

Direct effect of chitosan on T01193 and St-08-46 Z. tritici strains

The verification of the *in vitro* effect of Chitosan on the spore germination and hyphal growth of T01193 and St-08-46 strains in PDA medium plates didn't show any significant difference compared to reference plates without treatment (Annex 2). The direct effect of 50 mg L⁻¹ of Chitosan was investigated on T01193 and St-08-46 *Z. tritici* strains inoculated respectively on BW

and DW leaves. Microscopic observation of (i) spore germination and hyphal growth at 4 dpi, (ii) mesophyll colonization and pycnidium formation at 22 dpi, were performed on BW (Fig. 1A) and DW (Fig. 1B). The microscopic observation of (i) spore germination and hyphal growth at 4 dpi, (ii) mesophyll colonization and pycnidia formation at 22 dpi, were performed. Both wheat species exhibited lower hyphal growth propagation in treated wheat leaves compared to untreated leaves, at 4 dpi, and decreased mesophyll colonization and the frequency of pycnidia in treated leaves species comparing to untreated leaves, at 22 dpi.

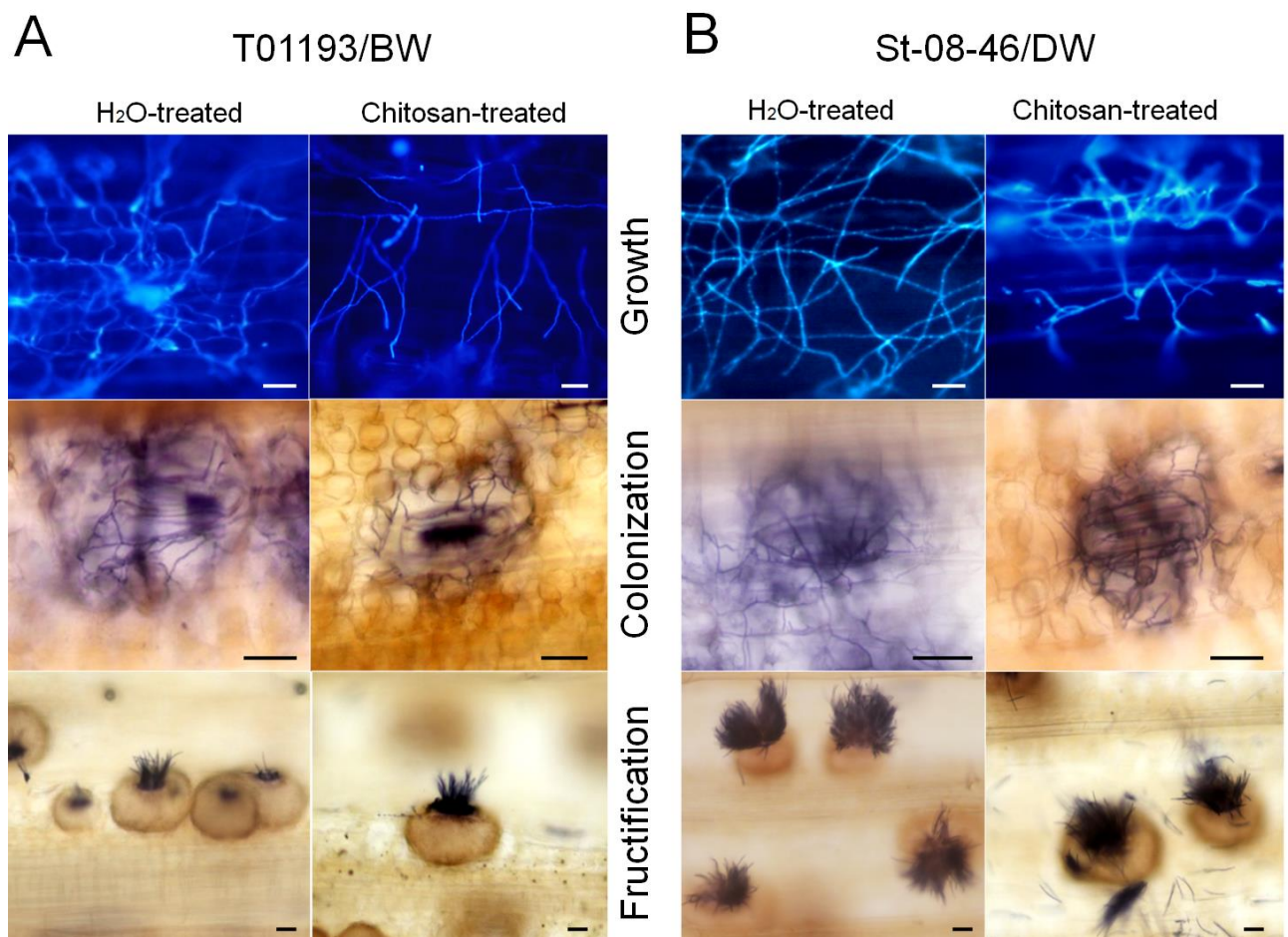


Fig. 1. Effect of 100 mg L⁻¹ of Chitosan on the spore germination, mesophyll colonization and fructification at 22 dpi of *Z. tritici* on BW and DW. (A) Susceptible BW cv. Premio infected by T01193 *Z. tritici* strain. (B) Susceptible DW cv. Karim infected by St-08-46 *Z. tritici* strain. Microscopic observation of the spore germination of both *Z. tritici* strains was monitored at 4 dpi using Calcofluor fluorescence. Microscopic observation of the mesophyll colonization and fructification was monitored at 22 dpi using Trypan blue staining. Scale bar = 10 µm.

Secondly, Table 1 represents the rate of cytological parameters assessed for both tested pathosystems at : (i) 1 dpi: germinated spores (GS), (ii) 4 dpi: non germinated spores (NGS), spores leading to stomatal penetration (SP), spores leading to a direct penetration (DP), and germinated spores that did not penetrate (GSNP), (iii) 22 dpi: no colonized stomata (NCS), colonized stomata

but not transformed into pycnidia yet (CS) and colonized stomata transformed into pycnidia (P). Results exhibited that chitosan treatment didn't affect GS compared to reference H₂O-treated leaves in both tested pathosystems at 1 dpi (Table 1). Results exhibited a similar rate of GS for between treated and untreated leaves for both tested pathosystems, at 1 dpi (Table 1). At 4 dpi, chitosan treatments induced 1.67-fold increases of NGS and 2.09-fold decrease of SP in DW leaves, while BW chitosan-treated leaves exhibited 2.36-fold decrease of SP. At 22 dpi, DW leaves showed 1.49-fold increase of CS and 1.26-fold decrease of P at 22 dpi, but didn't generate significant change of DP, GSNP and NCS. However, BW leaves exhibited 2.51-fold decrease of NCS, 1.68-fold increase of CS and 1.19-fold decrease of P.

Table 1 Percentages of different cytological parameters measured at 1, 4 and 22 days post inoculation (DPI) of BW and DW leaves by of T01193 and St-08-46 *Z. tritici* strains, respectively. At each time point, treated samples with 100 mg L⁻¹ of Chitosan were compared to their corresponding reference H₂O-treated samples.

Parameters (%)	DPI	T01193 / BW		St-08-46 / DW	
		H ₂ O	Chitosan	H ₂ O	Chitosan
GS	1	54±3.7a	57.1±5a	72±11.5a	58.1±4.3a
NGS	4	25.3±2.1a	33.9±1.4a	20.4±3.5b	34.1±5.3a
SP		17.9±1.7a	7.6±3.4b	9.8±0.2a	4.7±5.4b
DP		10.3±4.5a	14.7±0.8a	28.9±5.9a	21.9±5.5a
GSNP		46.4±3.3a	43.9±2.9a	41±7.2a	39.3±3.7a
NCS	22	20.6±3.5a	8.2±6.2b	13.8±0.2a	16.1±1.9a
CS		29.9±2.7b	50.3±8.5a	21.6±2.9b	32.2±1.2a
P		49.5±3.9a	41.5±11.8b	65.1±3a	51.7±1.1b

GS: germinated spores. NGS: non germinated spore. DP: spore leading to a direct penetration. SP: spore leading to a stomatal penetration. GSNP: germinated spore without penetration. NCS: non-colonized stomata. CS: colonized stomata but not yet transformed into pycnidia. P: colonized stomata transformed into pycnidia. Data are mean values of two independent experiments (±s.d). Statistics were made separately for each wheat specie. Means of Chitosan-treated samples tagged with the same letter are not statistically different to their corresponding H₂O-treated samples in the same wheat specie (Tukey test, $P < 0.05$).

Disease severity screening showed that the Percentage of Leaf Necrosis (PLN) and the Index of Pycnidia Coverage (IPC) were measurable from 16 dpi and showed maxima at 22 dpi (Table 2). At 22 dpi, chitosan-treated leaves exhibited 1.6- and 1.8-fold decreases of PLN and 1.7- and 1.6-fold decreases of IPC, on BW and DW leaves, respectively. Moreover, *Z. tritici* CWDE activities such as endo- β -1,4-endoxylanase, endo- β -1,3-glucanase and protease were measured at 4, 10, 16 and 22 dpi (Table 2). The CWDE activities levels were not significantly different from H₂O-treated (ni) leaves until 16 dpi, which corresponded to the beginning of pycnidia formation. The highest activity levels were found in the controls at 22 dpi associated with the maximal IPC and PLN. Chitosan-treated leaves revealed 2.2- and 1.9-fold decrease of endo- β -1,4-xylanase activity and 1.8- and 1.6-fold for endo- β -1,3-glucanase activity, on BW and DW leaves, respectively. It also induced 2.4-fold decrease of protease activity only in DW leaves.

Table 2 Evolution of disease severity parameters and CWDE activities at 4, 10, 16 and 22 days post inoculation of BW and DW leaves by of T01193 and St-08-46 *Z. tritici* strains, respectively. At each time point, treated samples with 100 mg L⁻¹ of Chitosan were compared to their corresponding reference H₂O-treated samples.

Parameters	DPI	T01193/BW		St-08-46/DW	
		H ₂ O	Chitosan	H ₂ O	Chitosan
Severity of disease (%)					
% of leaf with necrosis	4	nd	nd	nd	nd
	10	nd	nd	nd	nd
	16	24.1±14.6 b	13.5±11.1 c	28±14.7 b	15.4±8.5 c
	22	48.5±18.2 a	30.4±11.1 b	60.6±18.2 a	33.8±19.9 b
Index of pycnidium coverage	4	nd	nd	nd	nd
	10	nd	nd	nd	nd
	16	2.5±1.4 b	1.3±0.91 c	2.4±1.2 b	2.3±0.9 b
	22	3.5±1.1 a	2.1±1.1 b	4.4±0.7 a	2.8±1.1 b
CWDEs (mU mg ⁻¹ of protein)					
Endo-β-1.4-xylanase	4	32.4±4.1 c	22.9±0.8 c	48.2±3.2 d	35.1±1.4 d
	10	30.5±3.1 c	21.8±14.2 c	60.1±2.5 cd	72±9.9 cd
	16	110.8±40.3 c	51.2±15.2 c	246±36.6 c	114.6±51.8 cd
	22	879.6±263.4 a	404.2±35.7b	941.8±294.8 a	494.8±75.3 b
Endo-β-1.3-glucanase	4	38.6±2.8 c	42.4±3.3 c	170.3±23.7 d	160.9±25.3 d
	10	175.5±19 c	140.3±7.2 c	173.6±13.6 d	131.7±8 d
	16	221.3±111.8 c	161.2±15.6 c	345.4±43.1 c	241.1±32.7 cd
	22	1136.3±472.2 a	625.2±34.5 b	1026.4±128 a	657.9±163.5 b
Protease	4	211.8±58.2 b	165.6±17.8 b	166.5±31.6 c	318.9±210 c
	10	425±62.5 b	215.6±60.7 b	156.7±49.9 c	48±23.5 c
	16	1108.3±338.1 b	409.8±178.4 b	1740±532 bc	935.9±636 c
	22	11317.3±867 a	6803.7±506.2 a	6587.3±509.5 a	2706.4±235.3 b

(nd): not detected *Z. tritici* symptoms. Data are mean values of two independent experiments (±s.d). Means of Chitosan-treated samples tagged with the same letter are not statistically different to their corresponding H₂O-treated samples in the same wheat specie (Tukey test, $P < 0.05$).

Enhanced wheat defense response by chitosan treatment

Enzymatic activities

At the enzymatic level, the effect of chitosan treatment on β-1,3-glucanase (GLUC), peroxidase (POX), catalase (CAT), phenylalanine ammonia-lyase (PAL) and lipoxygenase (LOX) enzymatic activities was studied in (ni) context (elicitor effect) (Table 3) and (i) context (potentiating effect) (Table 4). The study of chitosan eliciting effect was investigated by comparing enzymatic activities in chitosan-treated (ni) leaves to corresponding reference H₂O-treated (ni) leaves at 1, 2, 3, 4 and 7 dpt. Results exhibited that chitosan treatment induced 1.5-fold increase of GLUC activity at 1dpt on DW leaves and 1.63- and 1.40-fold increase at 1 and 3dpt, respectively, on BW leaves. Likewise, POX activity was 2.5-, 2.6- and 2.7-fold induced at 2-3-7 dpt only on BW leaves. Moreover, CAT activity was 3.6- and 3.2-fold induced at 1 dpt on BW and DW leaves, respectively. Also, LOX

activity was 5.2- and 3.8-fold increase at 1-2 dpt, on BW leaves, and 4.3, 4.6 and 7-fold enhanced at 1-2-3 dpt, in DW leaves. However, chitosan treatment didn't generate significant change of PAL activity in (ni) context for both wheat species.

Table 3 Effect of foliar Chitosan treatment (100 mg L⁻¹) on five defense-related enzyme activities on BW and DW leaves at (ni) context. Wheat leaves were collected after 1, 2, 3, 4 and 7 days post Chitosan treatment.

Activities (U mg ⁻¹ of proteins)	Days post treatment	T01193/BW		St-08-46/DW	
		H ₂ O (ni)	Chitosan (ni)	H ₂ O (ni)	Chitosan (ni)
GLUC	0	135.7±29.7	-	138.9±11.7	-
	1	141.9±7.1	231.2±59.1*	126.2±10.8	190.1±38*
	2	142±11.5	146.4±18.3	124.9±13	158.3±14.8
	3	163.6±11	229.6±19.1*	132.6±13	157.4±37.8
	4	157.1±36	187.6±9.2	145.3±37.8	164.6±32.5
	7	150.8±12	178.1±11.4	130.1±26.4	170.3±10
POX	0	5.5±1.9	-	6.3±4.3	-
	1	6.9±3	16.2±1	8.1±3.7	14.7±1
	2	9.3±1.6	23±9.3*	6.6±3.4	13.5±0.5
	3	7.5±3.2	19.1±7.6*	7.1±3.8	12.6±1.5
	4	8.3±1.4	18±7.1	7.5±12.2	12.4±1.5
	7	7.9±2.2	21.4±10.4*	7±2.3	12.7±1.2
CAT	0	2.7±1	-	2.5±1	-
	1	3.4±0.6	12.1±1.3*	3.7±0.6	12±4.9*
	2	5.5±1.4	7.3±1.8	5.8±0.8	8.9±1.8
	3	4.2±0.5	6.8±1	4.4±1.3	8.1±3.9
	4	5.4±1	7.4±0.9	4.8±1.1	5.2±0.7
	7	5.5±1.6	5.6±0.5	4.2±1.1	5.8±0.4
PAL	0	4.1±8.4	-	3.1±4.7	-
	1	0±0	30.2±13.2	0±0	15±9.7
	2	0±0	11.6±16.9	5.1±14.9	22.1±35.1
	3	29±43.3	22.9±33.1	20.8±32.7	28.2±15.2
	4	3.0±9.7	0±0	0±0	0±0
	7	27.2±18.2	45.2±17.1	1.5±1.7	18±17.6
LOX	0	0.8±0.6	-	0.7±0.4	-
	1	0.5±0.7	2.6±0.9*	0.7±0.3	3±1.1*
	2	0.6±0.4	2.3±1.1*	0.5±0.4	2.3±1*
	3	0.8±0.3	1.1±0.2	0.3±0.2	2.1±0.8*
	4	0.8±0.4	1.6±0.8	0.3±0.2	1.3±0.3
	7	0.5±0.5	1.1±0.3	0.3±0.4	1.1±0.2

Results correspond to means (±s.d) of two independent experiments using three independent replicates for each experiment. Statistics were made separately for each wheat specie. For each wheat specie, Chitosan-treated samples with an asterisk (*) are different from their corresponding H₂O-treated controls (Tukey's test, $P \leq 0.05$).

The investigation of the chitosan potentiating effect was investigated by comparing enzymatic activities in Chitosan-treated (i) leaves to corresponding reference H₂O-treated (i) leaves at 1, 2 and 5 dpi corresponding to 3, 4 and 7 dpt. Data showed that chitosan-treated leaves exhibited 1.3- and 1.4-fold increase of GLUC activity at 1-2 dpi only on DW leaves. For CAT activity, chitosan treatment generated significant 3.5-fold increase at 5 dpi, on BW leaves and 1.9- and 2.4-fold increase at 2-5 dpi, on DW leaves. Besides, for PAL activity, we noted significant 1.8- and 2.1-fold increase at 2-5 dpi on BW leaves and 2.6-fold increase at 5 dpi on DW leaves. Indeed, at 1-2-5 dpi,

LOX activity was significantly 2.5-, 2.1 and 2.1-fold increased on BW leaves and 2.6-, 2- and 2-fold induced on DW leaves. However, chitosan treatment didn't affect POX activity on both wheat species.

Table 4 Time course of enzymatic activities at 1, 2 and 5 days post inoculation of BW and DW leaves by of T01193 and St-08-46 *Z. tritici* strains, respectively. The inoculation was carried out 48 hours post Chitosan treatment. At each time point, treated samples with 100 mg L⁻¹ of Chitosan were compared to their corresponding reference H₂O-treated samples.

Activities (U mg ⁻¹ of proteins)	Days post inoculation	T01193/BW		St-08-46/DW	
		H ₂ O (i)	Chitosan (i)	H ₂ O (i)	Chitosan (i)
GLUC	1	242.7±59.1	280.1±108.7	176±21.1	232.4±21*
	2	239.1±3.1	286.3±82.4	188.6±15.1	204.1±53.9
	5	256±28.5	313.3±95.8	174.6±25.8	249.9±35.4*
POX	1	15.1±1.5	23.4±9.6	10.3±3.7	13.8±5.8
	2	18.1±2.4	27.9±11.1	12.1±3.2	18.6±5.8
	5	19.3±3	29.2±1.2	12.2±1.2	18±8
CAT	1	8±0.9	10.9±2.8	7.7±3.2	11.8±2.9
	2	12.1±3.2	15.4±5.2	9.3±2.1	17.4±5.8*
	5	9.8±1.8	33.8±9.1*	7.3±1.4	17.2±2.1*
PAL	1	98.4±41.8	175.3±59.4*	94±48.8	137±36.5
	2	63.6±23.2	120.8±33.1	113.3±52.2	136.2±55.3
	5	66.6±19.5	139.6±30.8*	70.5±17.8	181±60.9*
LOX	1	1.5±0.3	3.8±0.8*	1.6±0.4	4.2±1.2*
	2	2±0.6	4.1±1.2*	1.6±0.3	3.2±1.2*
	5	1.6±0.1	3.3±0.6*	1.6±0.4	3.2±0.6*

Results correspond to means (±s.d) of two independent experiments using three independent replicates for each experiment. Statistics were made separately for each wheat specie. For each wheat specie, Chitosan-treated samples with an asterisk (*) are different from their corresponding H₂O-treated controls (Tukey's test, $P \leq 0.05$).

Mesophyll H₂O₂ accumulation

At the cytological level, H₂O₂ accumulation was revealed by 3, 3-diaminobenzidine (DAB) staining (red-brown staining) on BW and DW leaves. Four classes of *Z. tritici* spores were distinguished: C1 (spore with non-penetrated germ tube) (Fig. 2a), C2 (spore with penetrated germ tube without staining) (Fig. 2b), C3 (penetrated germ tube with papilla) (Fig. 2c), C4 (penetrated germ tube with at least 1 whole stained mesophyll cells) (Fig. 2d). Our findings exhibited that in BW leaves, chitosan treatment induced significant increase of C4 at 2-5 dpi, respectively on both BW (12.7- and 8.8-folds) and DW (12.2- and 3-folds).

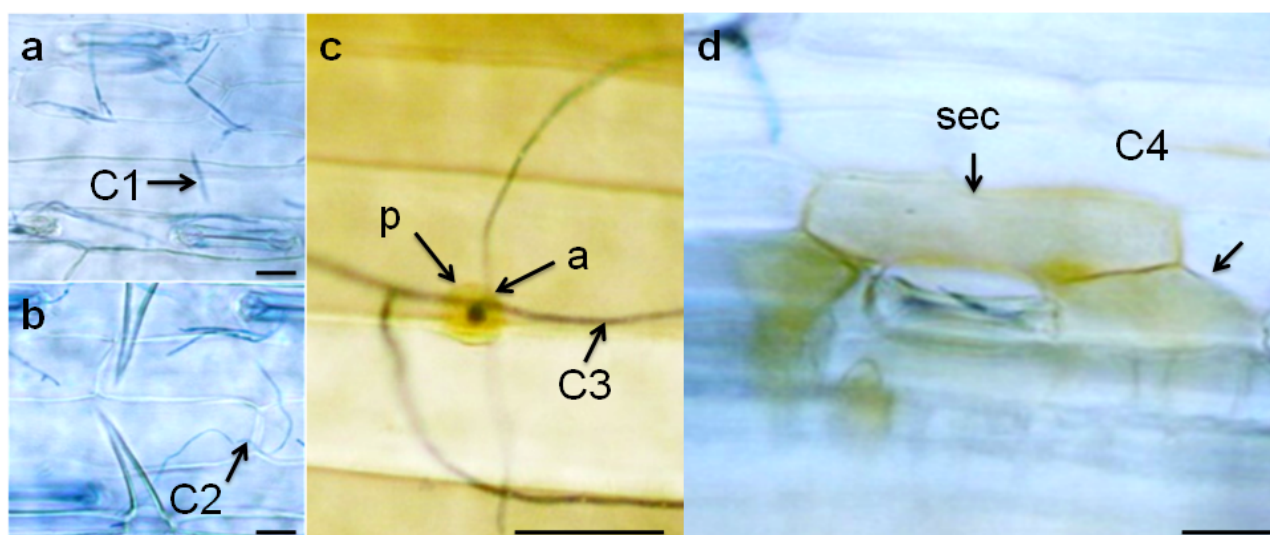


Fig. 2. Microscopic observations of H_2O_2 accumulation visualized by DAB staining displayed at BW and DW leaves following foliar spray by 100 mg L^{-1} of Chitosan. The inoculation of BW and DW leaves by T01193 and St-08-46 *Z. tritici* strains, respectively, was carried out 48 hours post Chitosan treatment. Wheat leaves were collected after at 0, 1, 2 and 5 days post inoculation. Four classes of *Z. tritici* spores were observed: (C1) spore with non-penetrated germ tube, (C2) spore with penetrated germ tube without staining, (C3) penetrated germ tube with papilla and (C4) penetrated germ tube with at least 1 whole stained mesophyll cells. (a) Appressorium-like structure, (p) Papilla. (sec) Stained epidermal cell. Bar scale = $10 \mu\text{m}$.

Table 5 Percentages of measured classes of H_2O_2 accumulation according to DAB staining intensity displayed at BW and DW leaves following foliar spray with 100 mg L^{-1} of Chitosan. The inoculation of BW and DW leaves by T01193 and St-08-46 *Z. tritici* strains, respectively, was carried out 48 hours post Chitosan treatment.

Wheat species	Classes (%)	Days post inoculation		2		5	
		1		H ₂ O (i)	Chitosan (i)	H ₂ O (i)	Chitosan (i)
BW	C1	29.1±1.1 a	80.1±6.7 a	40.5±15.3 a	8.1±5.0 a	32.1±9.6 a	17.9±7.9 a
	C2	37.6±1.1 ab	19.9±6.7 a	46.8±2.9 ab	13.7±5 b	35.5±15 ab	9.5±2.5 ab
	C3	0±0 a	0±0 a	8.6±5.4 a	25.1±12.4 a	25.8±16.1 a	14±4.8 a
	C4	0±0 c	0±0 c	4.2±7.2 c	53±5.9 a	6.7±14.1 c	58.6±6.4 b
DW	C1	26.8±3.8 b	31.9±6.4 a	37.8±9.2 ab	23.4±5.7 b	11.7±3 b	21.1±10.8 b
	C2	39.9±3.8 a	68.1±6.4 a	54.6±12.9 a	20.1±2.4 a	61.4±5.1 a	48.6±4.5 a
	C3	0±0 a	0±0 a	3.7±3.6 a	8.9±7.8 a	16.7±4.1 a	0±0 a
	C4	0±0 b	0±0 b	3.9±3.8 b	47.5±4.9 a	10.1±9.4 b	30.3±8.9 a

C1: spore with non-penetrated germ tube. C2: spore with penetrated germ tube without staining. C3: penetrated germ tube with papilla. C4: penetrated germ tube with at least 1 whole stained mesophyll cells. Results correspond to means (\pm s.d) of two independent experiments using three independent replicates for each experiment. Chitosan-treated (i) samples with an asterisk are different from their corresponding H₂O-treated (i) controls (Tukey's test, $P \leq 0.05$).

Phenolic compounds accumulation

Both local polyphenol-associated auto-fluorescence and total polyphenol content were evaluated. Local polyphenol deposition may be revealed as clear yellow fluorescence at the penetration sites by the fungus. Four types of events were observed, such as C1 (spore with non-penetrated germ tube) (Fig. 5a), C2 (spore with penetrated germ tube without fluorescence) (Fig. 5a), C3 (penetrated germ tube with weak fluorescence) (Fig. 5b) and C4 (penetrated germ tube with strong fluorescence) (Fig. 5c). Only C2 and C4 were affected by chitosan treatment. Treated BW leaves

exhibited 6.28-, 2.38- and 5.57-fold decrease of C2 and 18.8-, 47- and 53.4-fold increase of C4, at 1-2-5 dpi, respectively. Similarly, treated DW leaves exhibited 2.21-, 3.27- and 3.04-fold decrease of C2 and 48.3-, 39.3- and 61.2-fold increase of C4, at 1-2-5 dpi, respectively (Table 6). Second, the effect of Chitosan treatment on polyphenol content was also measured in whole 3rd leaf of both wheat species at (ni) and (i) context. Results showed that chitosan treatment didn't generate any significant changes compared to reference H₂O-treated leaves in both wheat species (Annex 3, table 3).

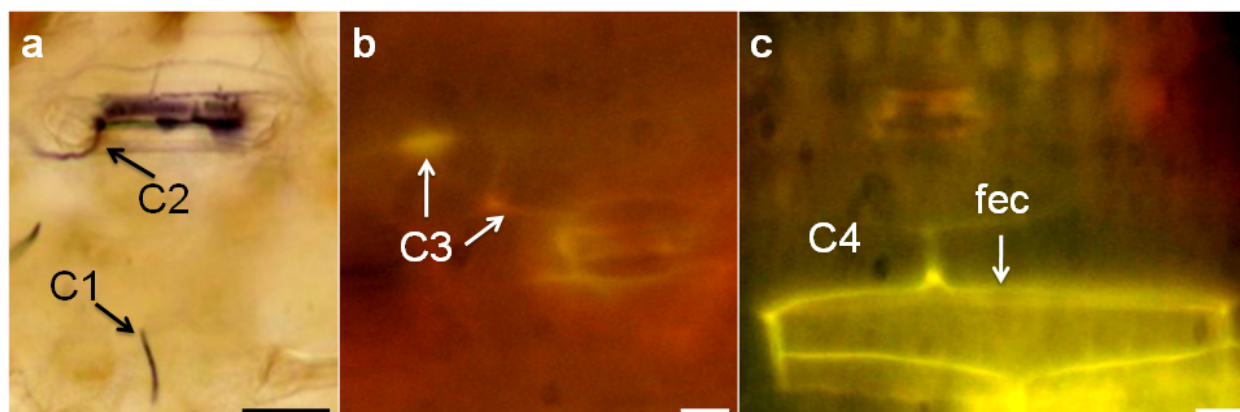


Fig. 3. Microscopic observations of polyphenol-associated autofluorescence displayed at BW and DW leaves following foliar spray with 100 mg L⁻¹ of Chitosan. The inoculation of BW and DW leaves by T01193 and St-08-46 *Z. tritici* strains, respectively, was carried out 48 hours post Chitosan treatment. Wheat leaves were collected after at 0, 1, 2 and 5 days post inoculation. Four classes of *Z. tritici* spores were observed: (C1) spore with non-penetrated germ tube, (C2) spore with penetrated germ tube without fluorescence, (C3) penetrated germ tube with weak fluorescence, (C4) penetrated germ tube with strong fluorescence. (fec) Fluorescent epidermal cell. Bar scale = 10 µm.

Table 6 Percentages of measured classes of polyphenol deposition according to polyphenol-associated autofluorescence intensity displayed at BW and DW leaves following foliar spray with 100 mg L⁻¹ of Chitosan. The inoculation of BW and DW leaves by T01193 and St-08-46 *Z. tritici* strains, respectively, was carried out 48 hours post Chitosan treatment.

Wheat species	Classes (%)	Days post inoculation					
		1		2		5	
		H ₂ O (i)	Chitosan (i)	H ₂ O (i)	Chitosan (i)	H ₂ O (i)	Chitosan (i)
BW	C1	23.7±5.3 abc	43.7±8.1 a	16.3±7.2 bc	16.8±8.6 bc	4.8±15.3 c	29.5±8.3 ab
	C2	68.5±10.3 b	10.9±3.9 c	68.4±11.6 b	28.8±7.9 c	95.2±5.8 a	17.1±7.3 c
	C3	7.8±5.1 b	26.6±2.8 a	15.3±2.9 ab	7.4±12.8 b	0±0 b	0±0 b
	C4	0±0 c	18.8±11.3 b	0±0 c	47±2.8 a	0±0 c	53.4±5.5 a
DW	C1	22.3±7.1 ab	16.0±5.8 ab	21.3±4.3 ab	24.3±1.8 a	11.7±5.6 ab	11.1±5.1 b
	C2	72.7±10.4 a	32.9±3.8 b	74.2±6.2 a	22.7±10.6 b	80.9±9.7 a	26.6±5.4 b
	C3	5±6.6 a	2.7±2.5 a	4.5±1.8 a	13.8±4.6 a	7.4±6.7 a	1±1.8 a
	C4	0±0 c	48.3±8 ab	0±0 c	39.3±7.8 b	0±0 c	61.2±1.9 a

C1: Spore with non-penetrated germ tube. C2: Spore with penetrated germ tube without fluorescence. C3: Penetrated germ tube with weak fluorescence. C4: Penetrated germ tube with strong fluorescence. Results correspond to means (±s.d) of two independent experiments using three independent replicates for each experiment. Chitosan-treated (i) samples with an asterisk are different from their corresponding H₂O-treated (i) controls (Tukey's test, $P \leq 0.05$).

DISCUSSION.*Antifungal activity of chitosan against T01193 and St-08-46 Z. tritici strains*

At tested concentrations, chitosan didn't exhibit any *in vitro* antifungal effect neither on spore germination nor on hyphal growth of both tested *Z. tritici* strains. Although, *in vitro* inhibitory effect of chitosan has been documented against various species of bacteria, fungi and oomycetes (Hadrami *et al.*, 2010). For instance, chitosan was reported to exert an inhibitory action on the hyphal growth and/or spore germination of numerous pathogenic fungi, including root and necrotrophic pathogens, such as *Fusarium oxysporum*, *Botrytis cinerea*, *Monilina laxa*, *Alternaria alternate*, *A. radicina*, *Pythium aphanidermatum*, *B. graminis* f. sp. *tritici* *Phytophthora cactorum* and *P. fragariae* var. *fragariae* and could protect many crops including carrots, strawberry, cucumber and tobacco (Eikemo *et al.*, 2003; Ben-Shalom *et al.*, 2003; El Hassni *et al.*, 2004; Jayaraj *et al.*, 2004; El Hassni *et al.*, 2005; Liu *et al.*, 2007; Jayaraj *et al.*, 2009). The absence of *in vitro* inhibitory effect of tested oligosaccharids against *Z. tritici* could be explained by the fact that the direct toxicity of chitosan oligosaccharides remains dependent on properties such as the concentration applied, the molecular weight, degree of acetylation, solvent, pH and viscosity (No *et al.*, 2002; Chung *et al.*, 2003). Rahman *et al.* (2014) demonstrated that the degree of polymerization of chitosan could affect the *in vitro* antifungal effect on germination of *Botrytis cinerea*.

In greenhouse condition, chitosan treatment allowed reducing germination of spores only in DW leaves, and reducing stomatal penetration in both wheat species. This could be explained by difference in morphology of cuticle surface as the importance of wax which provides an important barrier to the passage of water from the surface through the cuticular membrane; the transit of nutrients and other substances in the reverse direction may be similarly obstructed. Consequently, cuticular wax could enhance drought tolerance and reduce fungal spore germination and leaf penetration through the stomata and therefore, it could control the success and failure of the infection process (Martin, 1964; Araus *et al.*, 1991; Clarke *et al.*, 1993; Zhang *et al.*, 2013). Das *et al.* (1999) supported our hypothesis by demonstrating that leaf surface waxes enhancing resistance against spot blotch (*Drechslera sorokiniana*) in wheat.

Moreover, microscopic examination of treated leaves revealed partially reduced mesophyll colonisation intensity and pycnidia coverage. Besides, we demonstrated that chitosan treatment increased colonized stomata associated with decreased frequency of pycnidia suggests potential slowing of fungal progression and the transformation of stomata in pycnidia. Consequently, fructification and fungal necrosis were partially reduced mainly in DW leaves, accompanied by inhibition of tested CWDE activities in both wheat species except protease which was significantly reduced only in DW leaves. Thus, chitosan treatment was more efficient against St-08-46 strain on

DW leaves than T01193 strain on BW leaves. Chitosan efficacy is associated to fungal CWDE activities such as endo- β -1,4-endoxylanase, endo- β -1,3-glucanase and protease inhibition during the necrotrophic phase, suggesting their correlation with disease severity and their potential use as markers of RI efficiency.

Our work is the first experiment that demonstrated the efficiency of *Z. tritici* in both wheat species by 100 mgL⁻¹ of chitosan. Numerous reports demonstrated that chitosan confers resistance to fungal pathogens in many other crops including tobacco (Falcón-Rodríguez *et al.*, 2009), rice (Inui *et al.*, 1997), tomato (Benhamou *et al.*, 1994), carrots (Jayaraj *et al.*, 2009), grapevine (Aziz *et al.*, 2006), strawberry (Romanazzi *et al.*, 2012) and barley (Faoro *et al.*, 2008). Few works reported chitosan as good alternative to control other wheat diseases including *Fusarium gramineum*. Chitosan seed treatment induced a decrease in disease severity and enhanced quantitative yield parameters, suggesting the possibility of the use of chitosan as a seed treatment in crop protection in order to improve the plant defense response (Reddy *et al.*, 1999; Orzali *et al.*, 2014). However, other reports demonstrated that chitosan did not suppress the growth of *Fusarium* species and *Alternaria alternata* on wheat spikes (Wachowska *et al.*, 2013). Other elicitors such as β -1,3-glucans from laminarin could control *Z. tritici* in wheat (Esnault *et al.*, 2005; Shetty *et al.*, 2009). Few works reported oligosaccharides as good alternative to control other wheat diseases including chitosan, OGAs and trehalose against *B. graminis* f. sp. *tritici* (Reignault *et al.*, 2001; Renard-Merlier *et al.*, 2007; Randoux *et al.*, 2010; Tayeh, 2012). The absence of *in vitro* direct antifungal effect of chitosan against both *Z. tritici* strains, suggests that these products could operate indirectly *via* other means such as the enhancement of host resistance. This hypothesis was confirmed here, by assessing defense related proteins and metabolites at different scales.

Elicitation of BW an DW defense mechanisms by chitosan

Our study indicates enhancing wheat defense-related proteins and metabolites accumulation by chitosan treatment, at different scales, in (ni) context (elicitor effect) and (i) context potentiating effect).

Our work demonstrated that chitosan treatment present elicitor effect on β -1,3-glucanase in both wheat species but only with potentiating effect, on DW leaves. Enzymes able to degrade fungal cell walls directly may represent an important defense mechanism of plants. β -1,3-glucanases (GLUC) catalyzes hydrolytic cleavage of the 1,3- β -D-glucosidic linkages in β -1,3 glucan, which is a component of fungal cell walls (Ryals *et al.*, 1996). Besides, their role in diverse physiological, developmental processes in uninfected plants (Sticher *et al.*, 1997). Velazhahan *et al.* (2003) demonstrated that β -1,3-glucanase purified from sorghum leaves inhibited efficiently growth of *Trichoderma viride* *in vitro*. Furthermore, β -1,3-glucanases are known to release

oligosaccharides from the cell walls of fungi, which in turn, act as signals in the elicitation of host defense responses (Takeuchi *et al.*, 1990, Yoshikawa *et al.*, 1990; Ham *et al.*, 1991). A correlation between constitutive β -1,3-glucanase levels in leaves and resistance to *Alternaria solani* has been established in tomato (Lawrence *et al.* 1996). The involvement of β -1,3-glucanase in wheat defense against pathogens was confirmed in many studies (Shetty *et al.*, 2009; Cawood *et al.*, 2010, 2013). At least four β -1,3-glucanases have been reported in wheat (Muthukrishnan *et al.* 2001). Also, it has been shown that β -1,3-glucanase is involved in the defense response in wheat to leaf rust caused by *Puccinia recondita* f. sp. *tritici* (Anguelova *et al.*, 1999). Our findings corroborates with those of Falc3n-Rodr3guez *et al.* (2009) and Jayaraj *et al.* (2009) witch detected the induction of β -1,3-glucanase activity in other crops including tobacco and carrots. Moreover, it has been reported that Jasmonic acid and salicylic acid used as elicitors enhanced β -1,3-glucanase activity in wheat and enhance resistance against *Stagonospora nodorum* (Jayaraj *et al.*, 2004). Besides, it has been demonstrated that treatment of a susceptible wheat cultivar with purified β -1,3-glucan fragments from cell walls of *Z. tritici* gave complete protection against disease and this was followed by increased gene expression of β -1,3-glucanase and the deposition of callose (Shetty *et al.*, 2009).

ROS has been suggested to be the first defense line against pathogen invasion by directly killing the pathogen or slowing down its progress. ROS also participates in other resistance mechanisms including cell wall lignification, phytoalexin production, hypersensitive response and defense signaling (Vidhyasekaran, 1997; Iriti and Faoro, 2003). In our experiments, chitosan treatment generated elicitation and potentiating of total catalase (CAT) activity, in both wheat species but only elicitation of peroxidase (POX) activity in BW leaves. Moreover, chitosan treatment induced strong H₂O₂ accumulation at penetration sites of the fungus from 48 hours post inoculation, mainly in BW leaves. Many authors demonstrated that POX and CAT are antioxidant enzymes which could use H₂O₂ to oxidize a large variety of hydrogen donors such as phenolic substances, amines, ascorbic acid, indole, and some inorganic ions (Van Huystee, 1987; Caruso *et al.*, 2001). Similarly, Ben-Shalom *et al.* (2003) and Jayaraj *et al.* (2009) reported the induction of POX activity by chitosan treatment in other crops including carrots (against *Alternaria radicina* and *B. cinerea*) and cucumber (against *B. cinerea*). Moreover, many works reported the involvement of POX, CAT activities and H₂O₂ accumulation in the wheat defense mechanisms against *Z. tritici* (Diani *et al.*, 2009; Shetty *et al.*, 2003, 2007). Several other oligosaccharides could induce ROS metabolism in wheat against pathogenic fungi, such as β -1,3-glucan, Heptanoyl salicylic acid (HSA), salicylic acid (SA), oligogalacturonides, trehalose or, laminarin (Shetty *et al.*, 2003; Renard-Merlier *et al.*, 2007; Randoux *et al.*, 2010; Tayeh *et al.*, 2013).

Phenylalanine ammonia lyase (PAL) is one of the key enzymes acting upstream of the phenylpropanoid pathway and the biosynthesis of phenolic compounds. It catalyzes the deamination

reaction from L-phenylalanine to trans-cinnamic acid, a precursor of the majority of the phenolic compounds produced by plants (Zabala *et al.*, 2006). Many studies exhibited that PAL is associated with cereals resistance against pathogens such as *F. graminearum*, *F. culmorum* and *Puccinia hordei* (Prats *et al.*, 2007; Beccari *et al.*, 2011; Orzali *et al.*, 2014). Several studies confirmed the correlation of PAL activity and total phenolic compounds accumulation in incompatible interaction with *Z. tritici* (Hassan *et al.*, 2007; Adhikari *et al.*, 2007a,b). Therefore, in this study we investigated PAL enzymatic activity, polyphenol content and polyphenol deposition at *Z. tritici* penetration sites. Results exhibited that chitosan treatment induced only potentiating of PAL enzymatic activity, in both wheat species, associated with polyphenol deposition at penetration sites of *Z. tritici* from 24 hours post inoculation, but it has not affected polyphenol content in wheat leaves. Falcón-Rodríguez *et al.* (2009) and Jayaraj *et al.* (2009) which detected the induction of PAL activity in other crops including tobacco and carrots. Besides, the application of chitin and chitosan to soybean leaf tissues caused increased activity of PAL enzymes associated with increased total phenolic content of soybean leaves (Khan *et al.*, 2002). Also, Benhamou *et al.* (1994) reported that chitosan oligosaccharides treatment was able to induce several defense responses, including an accumulation of phenolics, in tomato which led to resistance to fungal infection.

Lipoxygenase (LOX) is a key enzyme of octadecanoid pathway by catalyzing the first step in the oxygenation of polyunsaturated fatty acids (Holtman *et al.*, 2000). The octadecanoic pathway represents the series of metabolic steps through which jasmonates are synthesized following oxidation of linolenic acid. Doares *et al.* (1995) reported the importance of this pathway in signaling induced by oligosaccharides. Application of chitosan to plants through cut stems, led to a rapid increase in jasmonic acid content, confirming the activation of the octadecanoic pathway. Chitosan is also reported to increase the endogenous levels of jasmonic acids in many species including rice Rakwal *et al.* (2002), leading to the activation of the octadecanoic acid pathway. The defense responses relying on this pathway include LOX activity that have been shown to be induced by chitosan in many crops including *Citrus* and *Fragaria* species and carrots (Jayaraj *et al.*, 2009; Hadrami *et al.*, 2010; Wolski *et al.*, 2010). Moreover, the behaviour of LOX activity was reported in tobacco cell suspensions elicited with chitosan (Pospieszny *et al.*, 1991). Similarly, here, we investigated the induction by the chitosan -treatment of octadecanoid pathway by measuring total LOX activity. We showed that chitosan-treated wheat leaves exhibited strong elicitation and potentiation of LOX activity, for both wheat species. This result was previously demonstrated in wheat by Bohland *et al.* (1997) which showed that three isoforms of LOX were differentially induced in wheat leaves, in response to various eliciting treatments (rust fungus elicitor, chitin oligosaccharides, chitosan, and methyl jasmonate) applied by infiltration. Therefore, we could suggest that chitosan-treatment affects lipid metabolism. Other SDPs such as Heptanoyl salicylic

acid, salicylic acid (SA) (Tayeh *et al.*, 2013) activate lipids metabolism by increasing LOX-encoding gene expression and corresponding LOX activity in wheat interaction with powdery mildew. However, LOX was none responsive to other elicitors such as laminarin-based product (Renard-Merlier *et al.*, 2007).

In conclusion, the same pathways were induced in both studied wheat species but not with the same importance. We noted similar response of CAT and LOX in both wheat species. POX and PAL activities and H₂O₂ accumulation were more responsive in BW, while β -1,3-glucanase activity and phenolic compounds deposition in penetration sites of *Z. tritici* were more responsive in DW. Our work must be completed by further investigations using more susceptible cultivars of each wheat specie and more pathogenic *Z. tritici* strains in order to prove our results.

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Chap IV | Mécanismes de résistance du blé dur à la septoriose causée par *Zymoseptoria tritici*

Des parties de ce chapitre ont été présentées dans les congrés suivants:

L. Somai, S. Farjaoui, A. Siah, P. Halama, Ph. Reignault and W. Hamada, 2015. Lutte intégrée contre la septoriose du blé dur en Tunisie moyennant l'utilisation des stimulateurs de défense de la plante associés à la résistance variétale. *Présentation orale dans le Premier Symposium National sur la Protection Intégrée des Plantes SYNPIP-2015, Sousse (20-21 Avril).*

L. Somai, A. Siah, P. Halama, Ph. Reignault and W. Hamada, 2014. Effet de la résistance variétale sur l'efficacité de certains SDP contre la septoriose du blé dur en Tunisie et sa corrélation avec l'activité des CWDE. *Oral presentation in 25^{ème} forum international des sciences biologiques et de biotechnologie de l'ATSB, Hammamet, Tunisie (24-27 Mars).*

Problématique

La résistance spécifique du blé à la septoriose conférée par les gènes de résistance spécifique *Stb*, impliqués dans les interactions incompatibles de blé-*Z. tritici* de type gène-pour-gène, ce qui la rend facilement contournable par le champignon. Par contre, la résistance non-spécifique, reposant sur un nombre de gènes plus important impliqués dans la résistance et s'exprimant chez le blé par différents niveaux de résistance et/ou sensibilité, paraît être moins facilement contournée par le champignon et plus durable. Ainsi, la caractérisation et l'introgression des gènes impliqués dans la résistance non-spécifique au sein d'une variété de blé permettraient d'augmenter la durabilité de sa résistance vis-à-vis de *Z. tritici*. Par conséquent, au cours de ce chapitre nous proposons d'étudier les réponses de défense de la plante et les paramètres liés à la pathogénicité de *Z. tritici* chez trois cultivars de blé dur présentant des niveaux de résistance différents, à savoir un cultivar très sensible (Karim), un cultivar moyennement résistant (Nasr) et un cultivar résistant (Salim). Ces derniers sont inoculés par une souche tunisienne de *Z. tritici* (St-08-46) spécifique du blé dur et connue par un niveau élevé de pathogénicité. Ce travail présente l'effet du génotype du blé dur sur le processus infectieux et l'expression des gènes intervenant dans la défense non-spécifique du blé. La compréhension des mécanismes d'incompatibilité devrait permettre de cibler les voies de défenses à stimuler afin d'obtenir une meilleure protection du blé contre cette maladie.

Publication 6: Quantitative expression analysis of candidate genes involved in resistance of Tunisian durum wheat (*Triticum turgidum* L. var. *durum*) against *Zymoseptoria tritici*

ABSTRACT

In Tunisia, *Septoria tritici* blotch (STB), causal agent *Zymoseptoria tritici* (Desm.) (Quaedvlieg *et al.*, 2011) previously known as *Septoria tritici* (teleomorph *Mycosphaerella graminicola* (Fuckel) J. Schröt.), has become a major disease of durum wheat (*Triticum turgidum* L. subsp. *durum* Desf.). Knowledge regarding mechanisms involved in general resistance against this disease is required to breed new and more resistant wheat cultivars. In the present study, three wheat cultivars including Karim (susceptible: S), Nasr (moderately resistant: MR) and Salim (resistant: R) were inoculated by a high pathogenic *Z. tritici* strain (St-08-46). A characterization of the fungal infection process using cytological approaches demonstrated that the high level of cultivar resistance could affect the mode of fungal penetration by inhibiting direct penetration and increasing stomatal penetration. In addition, significant distinct amounts of symptoms and sporulation were observed in accordance with the expected resistance levels of the cultivars. The assessment of fungal cell wall degrading enzymes (CWDEs) using biochemical approaches revealed that endo- β -1,4-xylanase, endo- β -1,3-glucanase and protease activities were closely correlated to the importance of sporulation and fungal biomass and inversely proportional to the level of cultivar resistance. Furthermore, in this research, an expression analysis of six wheat defense-related genes including *PR2* (endo- β -1,3-glucanase), *Chi 4 precursor* (chitinase class IV precursor), *Pox* (peroxidase), *Msr* (methionine sulfoxide reductase), *ATPase* (vacuolar ATP synthase) and *Bsi1* (Putative protease inhibitor) was conducted in the wheat cultivars Karim (susceptible) and Salim (resistant). An expression pattern was studied during the first steps of the infection process: at 24, 48 and 72 hours after inoculation (hai). Significant differential expression patterns of all tested genes, except ATPase, was observed between the resistant and susceptible wheat cultivars after inoculation with *Z. tritici* suggests that these genes may play a major role in the early defense and could be directly involved in resistance mechanisms of durum wheat.

Key words: *Triticum durum*, *Zymoseptoria tritici*, defense, genes, expression.

INTRODUCTION

Septoria tritici blotch (STB), caused by *Zymoseptoria tritici* (Desm.) (Quaedvlieg *et al.*, 2011) previously known as *Septoria tritici* (teleomorph *Mycosphaerella graminicola* (Fuckel) J. Schröt.), is an important wheat disease in the Mediterranean region. *Z. tritici* is a hemibiotrophic pathogen with a long symptomless phase considered as biotrophic during which the fungal germ tube grows on the leaf surface, penetrates either through the stomata or directly *via* the epidermal cell wall, and colonizes the intercellular space between mesophyll cells. The fungus then rapidly switches to

necrotrophic growth, associated to an increase of the fungal biomass, chlorotic and/or necrotic lesions on the leaf surface, and the formation of pycnidium in colonized substomatal cavities (Cohen and Eyal, 1993; Kema *et al.*, 1996; Duncan and Howard, 2000; Siah *et al.*, 2010). Although no haustoria are differentiated, *Z. tritici* obtains nutrients from the apoplastic space during the biotrophic period, whereas during the necrotrophic phase, the fungus releases cell wall degrading enzymes (CWDEs) leading to an increase of nutrient content in the apoplastic space (Keon *et al.*, 2005). It has been demonstrated that *Z. tritici* was able to produce a battery of CWDEs capable of degrading cell-wall polymers such as endo- β -1,4-xylanase, endo- β -1,3-glucanase and protease... (Palmer and Skinner, 2002). Douaiher *et al.* (2007) and Siah *et al.* (2010) revealed that endo- β -1,4-xylanase, by *Z. tritici* is correlated with symptom and sporulation levels. In their study, Tian *et al.* (2009) showed that endo- β -1,4-xylanase and protease activities are highly associated with susceptibility level of wheat cultivar.

In Tunisia, durum wheat (*Triticum turgidum* L. subsp. *durum* [Desf.] Husn.) is the primary cultivated crop and occupies more than half of the area dedicated to cereal crops (Bnejdi and El Gazzah, 2008). STB has become a major disease of durum wheat particularly during favourable growing seasons where it causes significant yield losses which can easily reach up to 50% (Gharbi *et al.*, 2008; Berraies *et al.*, 2014a). In our country, fungicide application is commonly used, as a control strategy. However, this strategy is costly, not reliable from year to year, and has become less effective over the years (Somai-Jemmali *et al.*, 2014). Hamada *et al.* (2008) reported the coexistence of both sexual mating types *Z. tritici* has been reported in Tunisian and suggested the contribution of the sexual cycle of *Z. tritici* in primary infections of the wheat crop and its subsequent dissemination in Tunisia. Ben hassin and Hamada (2014) collected for the first time *M. graminicola* ascospores in Tunisia and demonstrated therefore the occurrence of sexual reproduction thereby increasing genetic recombination causing the emergence of new resistant strains.

Host resistance is a major component of sustainable integrated disease management (IDM) strategies; hence breeding for resistance to STB has been a major component of the national breeding program of Tunisia. However, the availability of effective sources of resistance is a key component of the breeding program, but limited sources of resistance are known and often confer only partial resistance (Goodwin, 2007; Arraiano *et al.*, 2007). In the last decade, few novel durum wheat cultivars, characterized by a better resistance to STB were registered such as Nasr (in 2004), Maali (in 2007) and Salim (in 2010) (INRAT, 2014). These cultivars can be also used as parental material to eventually diversify resistance sources in breeding for resistance to STB (Berraies *et al.*, 2014b). Furthermore, a high level of resistance to a virulent Tunisian isolates has been detected in a local durum wheat cultivar Ajili. Fergaoui *et al.* (2011, 2015) crossed high yielding but susceptible

durum wheat cultivars Karim and Khiar with the resistant Agili. These studies showed that the old local durum wheat germplasm might harbor novel resistance genes that can be deployed in durum wheat breeding programs. They also demonstrated that this resistance is controlled by a dominant gene and confirmed the gene-for-gene interaction in wheat-*Z. tritici* pathosystem.

Overall, full resistance to STB has rarely been described in commercialized Tunisian durum wheat cultivars. Indeed, the specific resistance of wheat septoria conferred by the specific resistance genes *Stb* could be easily overcome by the fungus. However, the non-specific resistance, based on a larger number of genes involved in resistance and which is expressed in the wheat by different levels of strength and/or sensitivity, appears to be less easily bypassed by the fungus and more durable. Therefore, the characterization and introgression of non-specific genes involved could increase the durability of cultivar resistance against STB disease. Improving resistance would be a great advantage for farming areas where this disease causes significant financial losses. In this sense, the main objective of this research was to reveal genes involved in non-specific durum wheat resistance.

The development of reliable methods of quantitative RT-PCR has opened up many further possibilities and promoted the expression patterns of defense-related genes in order to reveal their involvement in bread wheat resistance against STB disease (Adhikari *et al.*, 2007; Farsad *et al.*, 2013). The early and strong accumulation of many defense-related genes-encoding transcripts was detected in compatible interactions between wheat and *Z. tritici* leading to full resistance, Pathogenesis-related (PR)-protein including *PR1* (unknown function), *PR2* (endo- β -1,3-glucanase), *Chi 4 precursor* (chitinase IV precursor), *PR4* (chitinase II), *PR5* (thaumatin-like proteins) and *Pox* (peroxidase) encoding genes (Ray *et al.*, 2003; Adhikari *et al.*, 2007; Shetty *et al.*, 2009; Mouhamadi *et al.*, 2012; Farsad *et al.*, 2013). Indeed, other defense-related genes were differentially expressed between compatible and incompatible interactions in bread wheat such as *Msr* (methionin sulfoxide reductase), *vacuolar ATPase* (vacuolar ATP synthase) and *Bsi1* (Putative protease inhibitor)-encoding genes suggesting their contribution in resistance to STB in bread wheat (Adhikari *et al.*, 2007; Farsad *et al.*, 2013). The main objective of this research was to compare the effect of cultivar resistance level on *Z. tritici* infection process and its CWDEs activities on durum wheat plants. The secondary goal was to examine the involvement of *PR2*, *Chi 4 precursor*, *Pox*, *Msr*, *ATPase* and *Bsi1* genes in the durum wheat resistance mechanisms against *Z. tritici*.

MATERIALS AND METHODS

Plant and fungal growths and pathogenicity tests

For this study we used three durum wheat cultivar selected for their different level of *Z. tritici* resistance, such as a susceptible (S) cv. Karim, a moderately resistant (MR) cv. Nasr and a resistant

(R) cv. Salim (IRESA, 2014). Wheat grains were germinated in Petri dishes on moist filter paper in darkness at 20 °C for 24 h, at 4 °C for 48 h and then at 20 °C for 24 h and 12 germinated grains were then placed into pots filled with 3 L of loam in the greenhouse at 18 °C ($\pm 2^{\circ}\text{C}$) with a 16/8 h photoperiod with supplementary illumination. After 3 weeks (when the third leaf from the base of plant was fully expanded), plants of each pot were inoculated using hand sprayer with 30 mL of spore suspension in distillate water (10^6 spores mL^{-1}) amended with 0.05% of Tween 20 (Sigma Aldrich, USA). Control plants were treated with sterile distillate water containing Tween 20. Immediately after inoculation, each pot was covered with a clear polyethylene bag for 3 days in order to ensure a water-saturated atmosphere compatible with a good fungal germination. The disease level was scored at 22 days post infection (dpi) by noting the percentage of the third leaf area with symptoms (chlorosis and necrosis) and by noting pycnidium density on a scale from 0 (absence of pycnidia) to 5 (all leaf areas with symptoms contain pycnidia) (Siah *et al.*, 2010).

Histopathological analysis

Monitoring of leaf penetration by *Z. tritici* was performed using Fluorescence Brightener 28 (Calcofluor, Sigma Aldrich) according to Siah *et al.* (2010). Segments (2 cm) were harvested from the third fully expanded leaf at 5 dpi from inoculated plants and then immersed for 5 min in a solution of 0.1 % (w/v) Calcofluor in 0.1 M Tris-HCl buffer pH 8.5. Leaf segments were washed for 2 min with sterile distilled water. After drying, they were placed on a glass slide, covered with a cover slip and observed microscopically (Nikon, Eclipse 80i) under UV illumination. Mesophyll colonization was investigated using Trypan blue (Shetty *et al.*, 2003; Siah *et al.*, 2010) with few modifications. Leaf segments (2 cm) were harvested from both control and inoculated leaves and cleared in a mixture of absolute ethanol: glacial acetic acid (3: 1, v/v) overnight. Cleared leaves were rehydrated for 4 h in distilled water and then fixed for 20 min in lactoglycerol (lactic acid: glycerol: water, 1: 1: 1, v/v/v). Staining of the fungus was carried out at 70 °C by immersing the leaf segments in 0.1% Trypan blue dissolved in lactophenol-ethanol (1:2, v/v) for 6 h. After washing, leaf segments were fixed in lactoglycerol, placed on a glass slide, covered with a cover slip and then observed microscopically under white light illumination. Spores were recorded for each cultivar on three leaf segments and subsequently 150 randomly chosen spores were studied. Data represent means of two independent experiments. Three independent replicates were carried out for each experiment.

Protein extraction and enzymatic assays

Three CWDE activities (endo-1,4-xylanase, endo- β -1,3-glucanase and protease) were assessed. Total protein extractions were carried out at 4, 10, 16 and 22 dpi as previously described by Siah *et*

al. (2010) with few modifications. Briefly, 2.5 g of inoculated and control third leaves were removed and immediately frozen in liquid nitrogen prior to grinding in a mortar. The resulting powder was then dispersed in 5 mL 0.05 M Tris–HCl buffer pH 7.8 containing 0.1 M KCl and 0.5 % cysteine. After cooling for 30 min at 4 °C, the mixture was squeezed through three layers of cheesecloth and centrifuged at 13 000 g for 20 min at 4 °C. From the supernatant, 100 µL were transferred to an Eppendorf tube and stored at 4°C for total protein quantification. Total protein extracts were then dialyzed against 0.5 L of distilled water for 24 h at 4 °C overnight (SpectrumLab Inc.). The dialyzed solution was then subjected immediately to endo-β-1,3-glucanase and endo-β-1,4-xylanase activity quantification using a modified 3,5-dinitrosalicylic acid (DNS) method (Douaiher *et al.*, 2007; Siah *et al.*, 2010). endo-β-1,4-xylanase activity was measured at pH 4.8 and 45 °C using xylan (1%, w/v) (Sigma Aldrich, USA) as substrate and xylose (10 mM) (Sigma Aldrich, USA) as reducing control sugar (Siah *et al.*, 2010). endo-β-1,3-glucanase activity was measured at pH 4.8 and 50 °C using Laminarin (Sigma Aldrich, USA) at pH 5 (0.5 %, w/v) as substrate and glucose (10 mM) as reducing control sugar (Miller *et al.*, 1959; Douaiher *et al.*, 2007a, b). One unit of endo-1,4-xylanase and endo-β-1,3-glucanase activity is defined as the amount of enzyme necessary to release 1 µmol of reducing sugar/p-nitrophenol min⁻¹ mL⁻¹ of enzyme solution. Absorbance was measured at 540 nm. Protease activity assays were assessed according to Hellweg (2003). Six aliquots of 100 µL of total protein were heated to 80 °C for 10 min to inactivate enzyme activity. After cooling, 50 µL of azocasein solution (20 mg/mL Azocasein, Sigma Aldrich) and 50 µL of incubation buffer (200 mM Tris, 20 mM CaCl₂ pH 7.8) were added. After incubation at 40 °C under gentle agitation for 30 to 60 min, 500 µL of 5 % aqueous trichloroacetic acid were added to precipitate non-digested protein and the mixture was incubated for 10 min at room temperature. The precipitate was pelleted by centrifuging for 5 min at 13000 g and 500 µL of test buffer were added at 400 µL of the supernatant. Absorbance was measured at 400 nm. Measures were performed in triplicates with denatured samples as comparative samples using an azo-dye labelled substrate. One unit of protease activity is defined as a change in enzyme activity of 1 Pox hour. Total protein concentrations were determined at 595 nm using bovine serum albumin as a standard (Bradford, 1976).

RNA extraction and analysis of gene expression by semi-quantitative RT-PCR and real-time RT-PCR

For genes expression study, three third leaves were used as independent replicates. Samples were collected at 1, 2 and 3 days post inoculation (dpi) from treated and control (H₂O-treated) plants. Total RNA was extracted from 100 mg of plant tissue using RNeasy Plant Mini Kit (Quiagen, The Netherlands). Genomic DNA contaminating the samples was removed by treatment with DNase

using RNase-Free DNase Set (Quiagen, The Netherlands). Reverse transcription of total RNA was carried out using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA) according to the manufacturer's protocol.

PCR reactions were conducted on subsequent obtained cDNA to amplify five wheat genes encoding for *PR2* (β -1,3-glucanase), *Chi 4 precursor* (chitinase class IV precursor), *Pox* (peroxidase), *Msr* (methionine sulfoxide reductase), *ATPase* (vacuolar ATP synthase) and *Bsi1* (Putative protease inhibitor). *TUB* encoding gene is used as housekeeping gene. Primers pairs used for amplifying *PR2* (U76895), *Chi 4 precursor* (AF112966), *Pox* (X56011) and *TUB* (U76895) were designed using the Primer Express® program and were tested for secondary structure using Net Primer® program. *Msr*, *ATPase* and *Bsi1* primers were published by Adhikari *et al.* (2007). The following gene-specific primers are presented in Table 1.

Table 1 List of primers encoding for wheat genes analyzed by quantitative and semi-quantitative RT-PCR.

Gene	Primer sequences (5'-3')
<i>TUB</i> (<i>beta-tubulin</i> , housekeeping gene)	GGAGTACCCTGACCGAATGATG AACGACGGTGTCTGAGACCTTT
<i>PR2</i> (β -1,3-glucanase)	CTCGACATCGGTAACGACCAG GCGGCGATGTACTIONTGTGTTTC
<i>Chi4 precursor</i> (<i>chitinase class IV precursor</i>)	TTCTGGTTCTGGATGACCAAC ACTGCTTGCAGTACTCCGTG
<i>Pox</i> (<i>peroxidase</i>)	GAGATTCCACAGATGCAAACGAG GGAGGCCCTTGTCTTCTGAATG
<i>Msr</i> (<i>putative methionine sulfoxide reductase</i>)	CATGCAGATGTTTCGGACAAA ACCATCGCGTCCCATGTAAA
<i>ATPase</i> (<i>vacuolar ATP synthase</i>)	GCTTTCGGATCTCTGGCAATT TGATGAGCACGGCCAGATT
<i>Bsi1</i> (<i>putative protease inhibitor</i>)	GGGCCCTGCAAGAAGTACTG ACACGCATAGGCACGATGAC

The expression of *Msr*, *ATPase* and *Bsi1* genes was investigated by semi-quantitative RT-PCR. PCR was carried out in a 20 μ L reaction mixture containing 1x PCR buffer, 2 mM dNTPs, 10 μ M of each primer, 0.5 unit of Taq polymerase and 20 ng of cDNA using the following protocol: 94°C for 5 min, followed by 30 cycles each with 94°C for 1 min, 55°C for 1 min and 72°C for 1 min. The PCR reaction was terminated at 72°C for 7 min. PCR products were analyzed by electrophoresis on ethidium-bromide stained 1.5% agarose gels run in 0.5 x Tris-acetic acid-EDTA buffer at 100 V for 45 min. Gels were visualized under UV light. Experiment was repeated 3 times and only results which were repeated at least twice were considered. The investigation of *PR2*, *Chi 4 precursor* and *Pox*-encoding gene expression was performed by real time-RT-PCR analysis. The amplification specificity of each qRT-PCR was checked by a single peak in melt curve analysis, and no primer dimmers were detected using agarose gel electrophoresis. Real-time RT-PCR was performed using ABI Prism 7300 detection system (Applied Biosystems, USA), using the following

thermal profile : after incubation for 10 min at 95°C, 15s at 95°C (denaturation), 1 min at 60°C (annealing/extension) for 40 cycles and 30s at 72°C (data acquisition). Relative expression for each cDNA were calculated for each time point relative to control at the same time It corresponds to $2^{-\Delta\Delta Ct}$ described by Pfaffl *et al.* (2002) and was calculated using the relative expression software tool REST®. $\Delta\Delta Ct = [Ct \text{ Target (Sample)} - Ct \text{ Reference (Sample)}] - [Ct \text{ Target (Control)} - Ct \text{ Reference (Control)}]$. *TUB* is used as reference gene. For each time point and for each wheat cultivar, the control (ni) corresponds to a fixed value of 1. After qPCR, a melt curve was generated to verify the specificity of each amplification reaction. The experiment was conducted in triplicate and only results which were repeated at least twice were considered.

Statistical data analysis

For quantitative gene expression, the analyses were performed using the relative expression software tool REST® as described by Pfaffl *et al.* (2002) and the significant differences were tested at 95% ($P < 0.05$). For severity parameters, enzymatic activities and microscopy, an analysis of variance (ANOVA) was performed using Tukey test at $P \leq 0.05$ using the XLSTAT software (Addinsoft, France).

RESULTS

Comparative study of Z. tritici infection in Karim (S), Nasr (MR) and Salim (R)

Infectious process

The present study allowed the investigation of the cultivar resistance level on *Z. tritici* infection process. Therefore, several cytological parameters related to the development of the Tunisian *Z. tritici* strain (St-08-46), were quantified in Karim, Nasr and Salim at different time points (Table 2). at : (i) 1 dpi: germinated spores (GS), (ii) 4 dpi: non germinated spores (NGS), spores leading to stomatal penetration (SP), spores leading to a direct penetration (DP), and germinated spores that did not penetrate (G SNP), (iii) 22 dpi: none colonized stomata (NCS), colonized stomata but not yet transformed into pycnidia (CS) and colonized stomata transformed into pycnidia (P). Our results exhibited that tested cultivars presented closed and non-significantly different percentages of GS at 1dpi. At 4 dpi, percentages of SP were significantly higher in Salim (13.8%) than in Karim and Nasr (9.8% and 5.8%), while DP were significantly privileged in Karim and Nasr (28.9% and 27.5%) than in Salim (11.4%). However, no significant differences were recorded for the NGS and G SNP. At 22 dpi, the percentage of NCS and CS were not significantly different between Karim, Nasr and Salim, while Karim revealed the highest percentage of P (65.1%) followed by Nasr (41.4%) and Salim (21.3%).

Table 2 Frequencies of cytological parameters measured at 1, 4 and 22 dpi in Karim (S), Nasr (MR) and Salim (R).

Parameters (%)	DPI	Durum wheat cultivars		
		Karim	Nasr	Salim
GS	1	72±11.5a	67.7±0.6a	70.7±1.9a
NGS	4	20.4±3.5a	24.2±11.3a	26.4±0.8a
SP		9.8±0.2b	5.8±1.7c	13.8±0.6a
DP		28.9±5.9a	27.5±7.3a	11.4±2.2b
GSNP		41±7.2a	42.4±4.2a	48.4±2.5a
NCS	22	13.8±0.2a	17.4±2.1a	33.0±13.2a
CS		21.6±2.9a	41.2±4.4a	45.6±17.2a
P		65.1±3a	41.4±5.3b	21.3±4.3c

Results with one similar letter did not have significant difference at $P \leq 0.05$. GS: germinated spores; NGS: not germinated spores; DP: spores leading to a direct penetration; SP: stomatal penetration; GSNP: germinated spores that did not penetrate; NCS: non-colonized stomata; CS: colonized stomata but not yet transformed into pycnidia; P: colonized stomata transformed into pycnidia.

Disease levels

Disease severity was estimated by measuring the percentage of leaf necrosis (PLN) (Fig. 1A) and Pycnidium coverage index (PCI) (Fig. 1B), at 4, 10, 16 and 22 dpi for three durum wheat cultivar Karim (S), Nasr (MR) and Salim (R) on the 3rd leaf (Fig. 1A). Symptoms were detected from 16 dpi. At 16 and 22 dpi, the PLN and PCI were significantly different between three tested cultivar. At 16 dpi, Karim revealed the highest percentage of PLN and PCI (63.5% and 4.5%), followed by Nasr (30.8% and 3.8%) and Salim (13.4% and 1.9%).

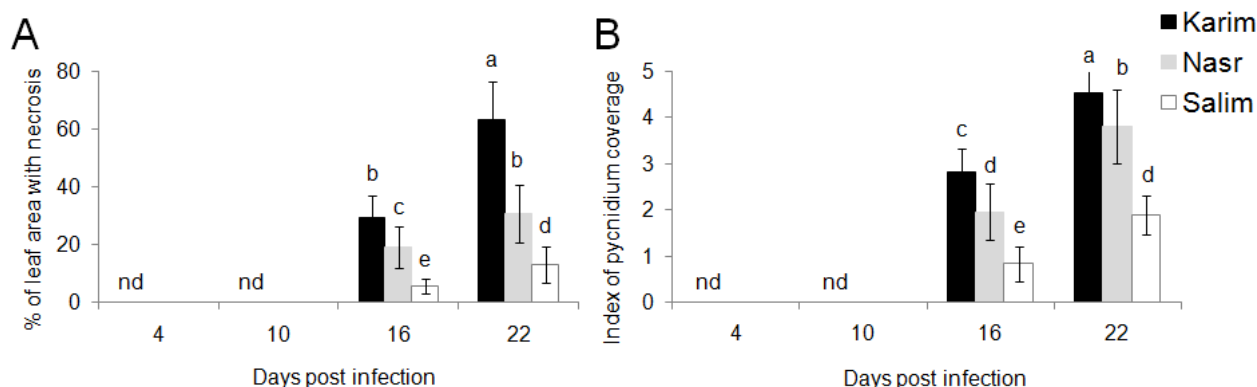


Fig. 1 Evolution of disease severity of St-08-46 strain on Karim (S), Nasr (MR) and Salim (R) durum wheat cultivar. The percentage of leaf necrosis (A) and Pycnidium coverage index (B) were evaluated at 4, 10, 16, 22 dpi. Data represent means of two independent experiments (\pm s.d.). Means tagged with the same letter are not significantly different using the Tukey test at $P \leq 0.05$.

Fungal Cell-Wall Degrading Enzyme (CWDE) activities

Endo- β -1,4-xylanase, endo- β -1,3-glucanase and protease activities produced by the fungus in plant tissues were monitored during the infection process at 4, 10, 16 and 22 dpi (Fig. 2). At 4 and 10 dpi,

infected (i) leaves exhibited weak CWDE activities levels which were not significantly different from those obtained in non-inoculated (ni) control plants. At 16 dpi, only endo- β -1,4-xylanase activity recorded significant difference between infected and non-infected Karim and Nasr leaves. At 22 dpi, we detected a strong increase of all CWDE activities in three tested cultivar except protease activity in Salim plants. At 22 dpi, endo- β -1,4-xylanase activity was 33.1-, 18.6- and 2.8-fold higher in (i) Karim, Nasr and Salim leaves, respectively (than corresponding (ni) leaves) (Fig. 2A). Similarly, endo- β -1,3-glucanase activity was 7.9-, 7.6- and 5.2-fold increased in infected Karim, Nasr and Salim leaves, respectively (Fig. 2B). Besides, protease activity level recorded 4172.2, 2976.5 and 530.7 mU mg⁻¹ of proteins, in infected Karim, Nasr and Salim leaves, respectively (Fig. 2C). Moreover, endo- β -1,4-xylanase and endo- β -1,3-glucanase activities were significantly different between three durum wheat cultivar and were higher in Karim, followed by Nasr and Salim plants, while for protease activity, Karim and Nasr leaves were significantly higher than Salim plants.

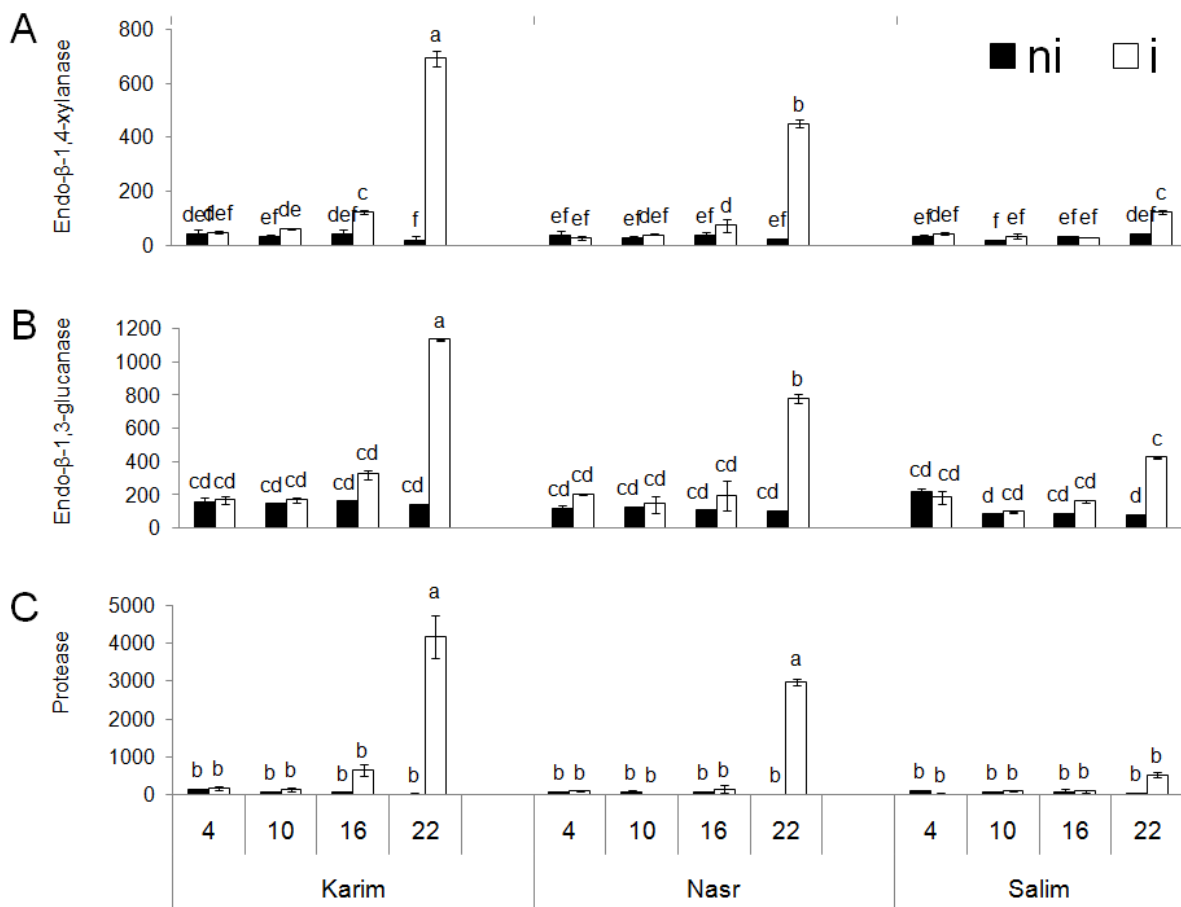


Fig. 2 Fungal endo- β -1,4-xylanase (A), endo- β -1,3-glucanase (B) and protease (C) activities measured at 4, 10, 16 and 22 days post inoculation (DPI) of Karim (S), Nasr (MR) and Salim (R) by *Z. tritici* St-08-46 strain. Data represent means of two independent experiments (\pm s.d). Means tagged with the same letter are not significantly different using the Tukey test at $P \leq 0.05$.

Induction of defense-related genes by *Z. tritici* infection in Salim (R) and Karim(S)

We established transcript profiles of a set of six distinct genes including *PR2* (endo- β -1,3-glucanase), *Chi 4 precursor* (chitinase class IV precursor), *Pox* (peroxidase), *Msr* (methionine sulfoxide reductase), *ATPase* (vacuolar ATP synthase) and *Bsi1* (Putative protease inhibitor) in susceptible wheat cultivar (Karim) (Fig. 3A) compared to resistant wheat cultivar(Salim) (Fig. 3B). Gene expression profiles in inoculated (I) plants of each cultivar were followed at 24, 48 and 72 hours post inoculation (hai) and were compared with their respective non-inoculated (ni) controls. The quantitative expression of *PR2*, *Chi 4 precursor* and *Pox*-encoding genes, using real time RT-PCR analysis, revealed their induction in infected (i) Salim (R) leaves, compared to (i) Karim (S) leaves. Indeed, we found that *PR2* gene was up-regulated at 48 and 72 hai in both wheat cultivars, while this induction was higher in Salim (9.0- and 44.9-fold), than Karim (3.4- and 13.2-fold) (Fig. 3A). However, *Chi 4 precursor* and *Pox* genes were significantly down-regulated in Karim leaves at 24-48-72 hai (3.5-, 4.7- and 3.9-folds, Fig. 3B) and 72 hai (3.37-folds, Fig. 3C), respectively. Although, in Salim leaves, *Chi 4 precursor* and *Pox* genes were highly up-regulated at 24, 48 and 72 hai and marked maximal relative expression at 48 hai (292.1-folds, Fig. 3B) and 72 hai (91.5-folds, Fig. 3C), respectively.

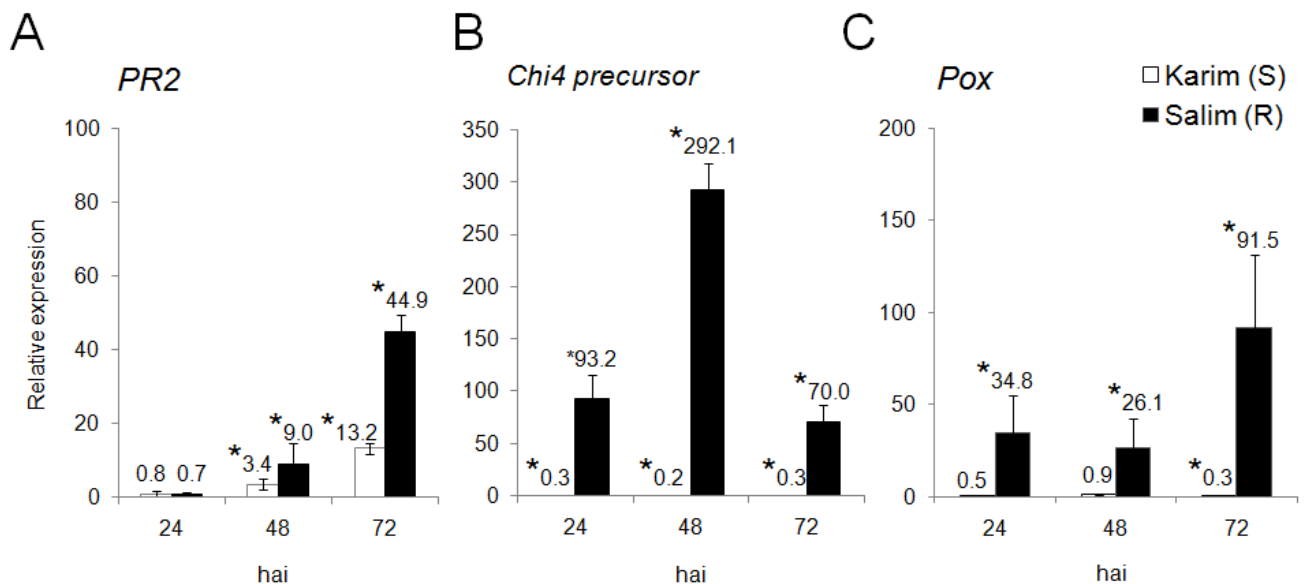


Fig. 3 Quantitative expression of PR genes analyzed by real time RT-PCR at 0, 24, 48 and 72 hours after infection (hai) of Karim (S) and Salim (R) by *Z. tritici* St-08-46 strain. Three genes were investigated such as *PR2* (A), *Chi 4 precursor* (B) and *Pox* (C)-encoding genes. Mean of relative expression at each time point are indicated above the bars in each graph. The experiment was conducted in triplicate. Similar results were obtained at least twice. Results correspond to means (\pm s.d) of duplicate reactions.

The semi-quantitative RT-PCR analysis exhibited that the expression of *Msr*, *ATPase* and *Bsil*-encoding genes were not induced in Karim leaves compared to (ni) control. However, in infected Salim leaves *Msr* gene was significantly enhanced at 72 hai, *Bsil* gene was highly up-regulated at 24 and 48 hai, whereas no significant change *ATPase* gene expression was observed at all studied time points (Fig. 4).

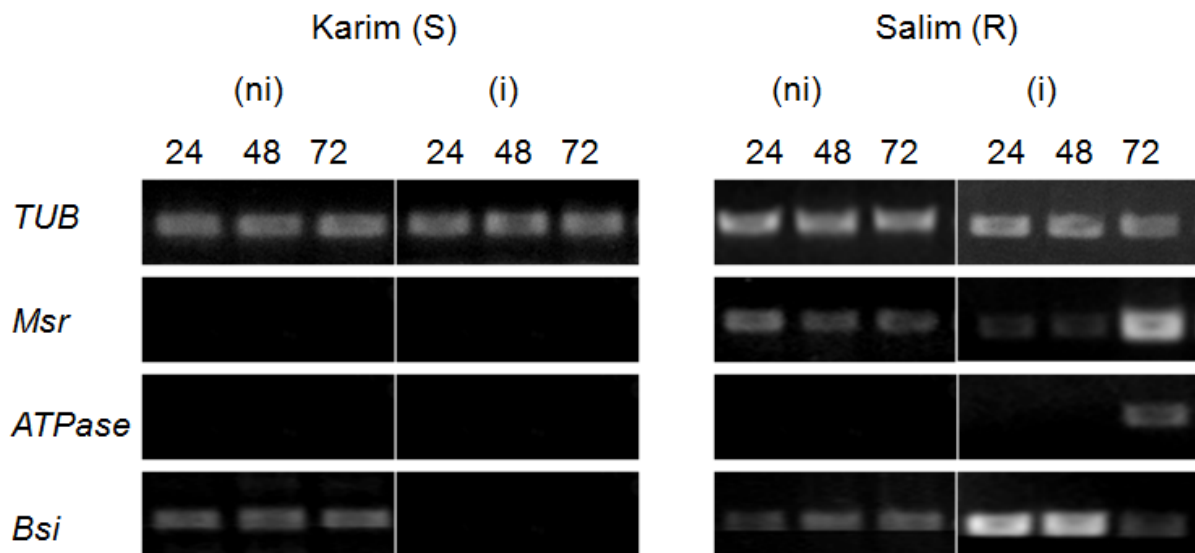


Fig. 4 Expression of *Msr*, *ATPase* and *Bsil*-encoding genes expression analyzed by semi-quantitative RT-PCR analysis at 0, 24, 48 and 72 hours after infection (hai) of Karim (S) and Salim (R) by *Z. tritici* St-08-46 strain. At each time point, infected leaves (i) were compared to their reference non infected (ni) leaves. RT-PCR products were generated using primers specific for β -tubulin (*TUB*), *Msr*, *ATPase* and *Bsil* first-strand cDNAs obtained from total wheat RNA, and visualized on agarose gel (1.5%). *TUB*-encoding gene was used as a house-keeping gene. Experiment was repeated three times and only results which were similar at least twice were considered.

DISCUSSION

In the present study, three durum wheat cultivars characterized by different level of *Z. tritici* resistance, have been compared, such as Karim (S), Nasr (MR) and Salim (R), infected by a high pathogenic Tunisian *Z. tritici* strain (St-08-46). The aim of this study is the first to investigate the effect of durum wheat cultivar resistance on fungal infection process and CWDE activities by the *Z. tritici* St-08-46 strain. We also explored the involvement of six defense-related genes in durum wheat resistance against STB disease.

Durum wheat resistance level affect mode of leaf penetration, colonization and sporulation

No significant difference of spore's germination capacity and total penetrating spores between the susceptible and resistant durum wheat cultivars agreeing the findings of Shetty *et al.* (2003) and Siah *et al.* (2010) in bread wheat. However, in susceptible Karim and moderately resistant Nasr

cultivars we observed important direct penetration, whereas Shetty *et al.* (2003) and Siah *et al.* (2010) studies which recorded rare penetration occurred through the epidermis. Besides, the penetration of *Z. tritici* via stomata was more frequent than direct penetration in resistant Salim than Karim and Nasr durum wheat cultivars, while Shetty *et al.* (2003) reported the inverse in bread wheat cultivars. Furthermore, the apparition and extension of the necrotic symptoms and the importance of the plant invasion by the parasite was faster in Karim (S) cultivar followed by Nasr (MR) and Salim (R) cultivars. At microscopic level, no significant difference of colonized and none colonized stomata were found between three wheat cultivar at 22 dpi, whereas the frequency of pycnidia were significantly different in tested cultivars and were correlated to the pycnidium coverage index at the macroscopic level. Indeed, at macroscopic scale, mycelial penetration was accompanied by rare and small necrotic lesions bearing few pycnidia we observed in resistant Salim cultivar, in Nasr (MR) necrosis were large and bearing more pycnidia but not generalized on the totality of leaf surface, while in susceptible Karim cultivar, large necrotic spots bearing a lot of pycnidia and generalized to all the leaf was frequently observed. This result suggests that in Salim (R), the activation of defense mechanisms are more efficient to reduce necrosis and pycnidium coverage, followed by Nasr (MR) and Karim (S). This observation could be explained, as reported by Adhikari *et al.* (2007) and Ray *et al.* (2003), by the fact that wheat plants could activate their defense mechanisms before penetration by the pathogen.

Durum wheat resistance level is associated with reductions of CWDE activities of St-08-46 strain

Defense mechanisms take likely place during the early stages of the infection, whose effective expression can lead to an inhibition of intercellular hyphal growth, fungal biomass and fungal pathogenicity compounds such as CWDEs, impacting disease symptoms and fungal sporulation. It has been previously shown that CWDE activities was associated with *Z. tritici* pathogenicity and impacts the disease level of wheat (Douaiher *et al.*, 2007; Siah *et al.*, 2010; Tian *et al.*, 2009).

In the present work, we highlighted significantly higher levels of β -1,4-xylanase, endo- β -1,3-glucanase and protease activities produced by the fungus in Karim, the most susceptible cultivar, from 16 dpi, at which the symptoms begin to be observed. These CWDEs reached their highest activity levels in Karim at 22 dpi and were associated with the highest pycnidium coverage index and percentage of leaf necrosis. endo- β -1,4-xylanase activity also increased in partially resistant cv. Nasr, from 16 dpi, while endo- β -1,3-glucanase and protease activities were significantly increased only at 22 dpi, but at lower levels than in Karim. However, in resistant cv. Salim, significant increase of endo- β -1,4-xylanase and endo- β -1,3-glucanase was detected only at 22 dpi, whereas no significant changes of protease activity was observed. Therefore, β -1,4-endoxylanase, endo- β -1,3-glucanase and protease activities seem to be correlated with the degree of fungal sporulation and

symptoms. These observations confirm the studies of Siah *et al.* (2010) and Tian *et al.* (2009), where CWDE activities by *Z. tritici* was associated with sporulation level, and negatively correlated with the resistance level.

This finding supports the hypothesis that defense mechanisms affect disease development and pycnidium formation. In response to fungal CWDE activities, plants have evolved a diverse battery of defense responses including protein inhibitors of these enzymes such as TAXI (*Triticum aestivum* Xylanase Inhibitor) and XIP (Xylanase Inhibitor Protein) (Debyser and Delcour, 1998; Debyser *et al.*, 1999; McLauchlan *et al.*, 1999). Here, we could suggest that the little and insignificant increase of protease activity in resistant cultivar Salim plants could be due early up-regulation of *Bsil* gene which could enhance the biosynthesis of proteases inhibitors proteins. The discovery of these plant inhibitors and the recent resolution of their three-dimensional structures, free or in complex with their target enzymes, provide new lines of evidence regarding their function and evolution in plant–pathogen interactions.

Effect of cultivar resistance on defense–related gene expression

To our knowledge, all works investigated the expression patterns of defense–related genes in bread wheat resistance against *Z. tritici*, but, no information is available concerning durum wheat. Therefore, this study allowed highlight several defense-related genes that could promote Tunisian durum wheat resistance against Tunisian *Z. tritici* strain (St-08-46). Therefore, we investigated expression profiles of six distinct genes including *PR2* (endo- β -1,3-glucanase), *Chi 4 precursor* (chitinase class IV precursor), *Pox* (peroxidase), *Msr* (methionine sulfoxide reductase), *ATPase* (vacuolar ATP synthase) and *Bsil* (Putative protease inhibitor) in a high resistant cultivar (Salim) to a high susceptible cultivar (Karim).

The endo- β -1,3-glucanase catalyzes hydrolytic cleavage of the 1,3- β -D-glucosidic linkages in β -1,3 glucan, which is a component of fungal cell walls (Ryals *et al.*, 1996). Here, the expression of *PR-2* gene was significantly induced in both susceptible and resistant cultivars. Higher level of expression was noted in the resistant wheat cultivar (Salim) than in a susceptible cultivar (Karim) at 48-72 hai and reached a maximum at 72 hai, coincided with spore generation on leaf surface, production of many branched hypha and leaf penetration by surface hyphae. *PR-2* gene which encode for class II endo- β -1,3-glucanase, which are acidic proteins secreted into the extracellular space (Ferrari *et al.*, 2007). Our results indicated that this gene might have an important role in the defense response and may directly inhibit the pathogen and protect the host against fungal enzymes. Our findings corroborate with that of Shetty *et al.* (2009) showed that endo- β -1,3-glucanase gene up-regulation correlates well with wheat resistance against *Z. tritici*. Similar result was reported by Ray *et al.* (2003) showed accumulation of endo- β -1,3-glucanase during the early stages of infection. Its level was significantly higher in resistant wheat cultivar suggesting its potential role in

resistance against *Z. tritici*. Besides, Mohammadi *et al.* (2012) found earlier *PR2* accumulation of transcripts in resistant genotype suggesting the role of glucanase enzyme in degradation of pathogen cell walls and preventing of its growth. Chitinases hydrolyze β -1,4-glycoside bounds in chitin to produce N-acetylglucosamine and N, N+ diacetylchitibiose (Cohen-Kupiec and Chet, 1998). In our experiment, *Chi 4 precursor* -encoding gene was significantly down regulated in susceptible cultivar at all studied time points, whereas it was strongly up-regulated in resistant cultivar following *Z. tritici* infection. *Chi 4 precursor* -encoding gene encodes a precursor of an anaplastic class IV chitinase. Once fungal penetration has occurred, apoplastic chitinases release eliciting molecules from the hyphae that penetrate into the intracellular space (Dore *et al.*, 1991). Shetty *et al.* (2009) corroborates with our findings and showed the induction of *Chi 4 precursor* gene up-regulation in resistant wheat cultivar at 24-72 hai. They also concluded that chitinase appears to be less important than glucanase, but, they suggested a synergistic effect between them. However, in this study, *Chi 4 precursor* gene showed the highest up-regulation, which could be explained by the importance of chitin in *Z. tritici* cell wall. However, a synergistic effect could be envisaged. Moreover, Adhikari *et al.* (2007) demonstrated the early and strong induction of *chitinase II (PR4)* gene expression following inoculation of the two resistant cultivars with *Z. tritici*. The peroxidase which use hydrogen peroxide (H_2O_2) as a substrate in reactions involved in biosynthesis of lignin and other phenolic compounds that act as additional antimicrobial barriers (O'Brien *et al.*, 2012; Passardi *et al.*, 2005). In this experiment, we observed significant down regulation in susceptible cultivar, while it was strongly enhanced following the infection at studied time points and reached a maximum at 72 hai. The observed early induction of *Pox* gene in the present study suggests that this gene might play a significant role in the generation of H_2O_2 and lignin deposition during resistant wheat defense response which could decrease stop fungal progress. Adhikari *et al.* (2007) also found early induction (3 hai) of *Pox* gene up-regulation in resistant cultivars with *Z. tritici*. Farsad *et al.* (2013) indicated the higher up-regulation of *Pox* gene in resistant cultivar suggesting its effectiveness in the conferral of resistance to STB in wheat. Similar to our findings, Shetty *et al.* (2009) found significant down-regulation of *Chi 4 precursor* gene following infection in susceptible wheat cultivars. However, Adhikari *et al.* (2007) and Farsad *et al.* (2013) didn't show down-regulation of *Chi 4 precursor* and *Pox* genes. They exhibited slight induction of these genes in susceptible cultivars, but much lower level compared to those of the resistant cultivars.

A number of plant proteinase inhibitors have been shown to possess antifungal activity (Lorito, 1994). Although the function of this gene remains unknown, it might be part of the plant's general response to stress and might not play a specific role in defense against *Z. tritici* (Adhikari *et al.*, 2007). In this study, we detected an early and strong up-regulation of *Bsi1* gene (at 24-48 hai) coincided with the spore germination and propagation on the leaf surface and the beginning of

penetration into the wheat leaves. Therefore, we could suggest that *Bsi1* might have an antifungal activity and could inhibit growth of the penetrating hyphae within the cell wall as previously proposed by Stevens *et al.* (1996). Our findings confirm those of Adhikari *et al.* (2007) and Farsad *et al.* (2013) which also showed early induction of *Bsi1* gene up-regulation in resistant bread wheat cultivars suggesting its association with the wheat resistance against *Z. tritici*. The Msr enzyme catalyzes the reduction of methionine sulfoxides to methionine. Consequently, it is indicated to act as a last-chance antioxidant that repairs proteins damaged by oxidative stress (Romero *et al.*, 2004). Up regulation of the *Msr* gene in the resistant wheat cultivar suggests that the product of this gene might repair proteins that have been damaged following *Z. tritici* inoculation. Here, we demonstrated that *Msr* gene was up-regulated at 72 hai only in resistant durum wheat cultivar, suggesting its involvement in decreasing leaf penetration and inhibiting mesophyll colonization. Our results were supported by Adhikari *et al.* (2007) and Farsad *et al.* (2013) which concluded that *Msr* gene could be correlated to bread wheat resistance against *Z. tritici*. ATP synthases are multi-subunit enzyme complexes that help to maintain the acidic environment of cellular components by transferring protons across membranes through ATP hydrolysis (Wassmer *et al.*, 2005). Wilkens *et al.* (2004) reported that the vacuolar ATPase protein complexes are several units that function as ATP hydrolysis pumps and transporters proton H⁺. ATPase is involved in several vital processes including active transport proteins and metabolites. In this experiment, we detected weak and insignificant expression of *ATPase* gene at 72 hai. The study of Adhikari *et al.* (2007) supports our results and shows a very low up-regulation of vacuolar *ATPase* gene at biotrophic phase, followed by a strong up-regulation at necrotrophic phase, in resistant genotypes. Therefore, we suggest that the vacuolar *ATPase* gene could not be involved in the early protection when the fungus enters the stomatal cavities of wheat leaves, but could have an important role at necrotrophic phase in order to prevent the fungus from growing or producing disease symptoms.

In summary, the obtained molecular data explained the infection process and the inhibition of the pathogen in resistant cultivar. During the first 24 h after inoculation, the fungus finds and enters the stomatal cavities of wheat leaves. This initial contact stimulates induction of PR proteins as *PR2*, *Chi 4 precursor*, *Pox* and other defense associated genes as *Msr* and *Bsi1* genes, in the resistant lines. Susceptible cultivar Karim responded weakly or not at all, either because the pathogen was not recognized or it was able to shut off the defense response. At this stage, we detected that, in resistant cultivar, the fungus prefers the penetration through the substomatal cavities, suggesting that enhanced cell wall degrading proteins and cell wall reinforcement makes stomatal penetration easier than direct penetration for the fungus. This early defense response explains the decrease of mesophyll invasion and sporulation as seen at microscopic and

macroscopic scale in resistant cultivar. However, vacuolar *ATPase* gene seems to be not involved in early durum wheat defense against *Z. tritici* infection.

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Chap V | Stratégie de lutte intégrée moyennant l'application des SDPs associée à l'utilisation des cultivars résistants de blé dur pour contrôler la septoriose en Tunisie

Des parties de ce chapitre ont été présentées dans les congrès suivants:

L. Somai, S. Farjaoui, A. Siah, P. Halama, Ph. Reignault and W. Hamada, 2015. Lutte intégrée contre la septoriose du blé dur en Tunisie moyennant l'utilisation des stimulateurs de défense de la plante associés à la résistance variétale. *Présentation orale dans le Premier Symposium National sur la Protection Intégrée des Plantes SYNPIP-2015, Sousse (20-21 Avril).*

L. Somai, A. Siah, P. Halama, Ph. Reignault and W. Hamada, 2014. Effet de la résistance variétale sur l'efficacité de certains SDP contre la septoriose du blé dur en Tunisie et sa corrélation avec l'activité des CWDEs. *Oral presentation in 25^{ème} forum international des sciences biologiques et de biotechnologie de l'ATSB, Hammamet, Tunisie (24-27 Mars).*

Problématique

La sélection de cultivars tolérants ou résistants et l'emploi de fongicides constituent les moyens de lutte les plus efficaces pour réduire les pertes de rendements dues à la septoriose. Cependant, le brassage allélique généré chez *Z. tritici* lors de la reproduction sexuée se traduit par le contournement des résistances de la plante hôte, remettant en cause la durabilité de la résistance spécifique du blé conférée par les gènes *stb*. D'autre part, l'activation des réactions de défense des plantes par l'application des inducteurs de résistance constitue l'une des stratégies potentielles de lutte alternative permettant de réduire l'emploi des fongicides. Dans le cadre du pathosystème blé-septoriose, l'utilisation de tels produits semble être une alternative prometteuse à celle des fongicides, mais assez peu étudiée. L'induction des mécanismes de défense par un SDP pourrait contribuer à l'augmentation du niveau de résistance du blé et prévenir l'infection par *Z. tritici*. Il s'agit ici de comparer l'induction des résistances par un SDP entre différents cultivars de blé dur tunisien à savoir un cultivar très sensible (Karim), un cultivar moyennement résistant (Nasr) et un cultivar résistant (Salim) inoculés par une souche tunisienne de *Z. tritici* (St-08-46). Les expérimentations ont été réalisées simultanément avec 3 SDPs (AA, Oligos et *A. nod*), correspondant à des formulations testées pour la première fois contre la septoriose du blé. Ces produits ont été testés en condition semi-contrôlée (serre vitrée) et en plein champs. Les essais réalisés en condition semi-contrôlée vont permettre de caractériser l'efficacité de ces produits, leur effet sur le processus infectieux de *Z. tritici* comme la germination des spores, la pénétration foliaire, la colonisation des cavités sous-stomatiques, la sporulation et la production de CWDEs (tels que la β -1,4-endoxylyanase, la β -1,3-glucanase et la protéase) ainsi que l'induction de 6 gènes impliqués dans la défense du blé en réponse à ces traitements, à savoir *PR2* (β -1,3-glucanase), *Chi 4 précurseur* (précurseur de chitinase de la classe IV), *Per* (peroxidase), *Msr* (méthionine sulfoxyde réductase), *ATPase* (ATP synthase vacuolaire) and *Bsi1* (inhibiteur de protéases). Tandis que les essais en plein champs vont nous permettre de confirmer l'efficacité des SDP testés en condition naturelles, ainsi que leur effet sur l'amélioration de la teneur en chlorophylle et le rendement du blé en quantité et qualité. Cette étude pourra nous permettre de révéler l'effet des SDP testés sur l'amélioration de la teneur en chlorophylle et le rendement en quantité et qualité par rapports au témoin non traité. Ainsi, ce chapitre pourra révéler des marqueurs moléculaires et biochimiques d'efficacités des SDP ainsi que des combinaisons « SDP-cultivar » intéressants qui pourront être intégrées dans le cadre d'une lutte intégrée contre la septoriose du blé.

Publication 7: Integrated control strategies using resistance inducers associated to cultivar resistance against STB on Tunisian durum wheat (*Triticum turgidum* L. var. *durum*)

ABSTRACT

Septoria tritici blotch (STB), causal agent *Zymoseptoria tritici* is one of the most devastating foliar diseases of wheat. Plant resistance inducers (RIs) are developed as an alternative to conventional fungicides. Their efficiency could be affected by many factors as the level of cultivar resistance. Therefore, we studied here the response of three Tunisian durum wheat cultivars differing in their resistance levels to *Z. tritici*, as Karim (susceptible), Nasr (moderately resistant) and Salim (resistant) to three RIs based on plant cell wall oligosaccharides (Oligos), ascorbic acid (AA) and *A. nodosum* algae extract (*A. nod*). In greenhouse condition, foliar preventive spray by Oligos, AA and *A. nod* at 50, 50 and 500 mgL⁻¹, respectively, could alter *Z. tritici* infectious process. Our results showed that only AA could significantly affect spore germination of St-08-46 *Z. tritici* spores. Besides, Oligos and AA treatments leaves could affect leaf penetration by reducing the direct penetration at 4 dpi in Karim (S) and Nasr (MR) leaves. The investigation of defense-related gene expression using RT-PCR analysis during biotrophic phase demonstrated an elicitor effect of *PR2*, *Chi4 precursor*, *Pox*, *Msr*, *ATPase* and *Bsil* genes by tested RIs which could explain their antifungal effect. Consequently, at 22 dpi, all tested RIs induced a significant decrease of fungal CWDE activities, disease symptoms and sporulation in the susceptible cultivar Karim. Similarly, in field tested RIs conferred an interesting protection against STB associated, in the case of the *A. nod* and AA, with increased chlorophyll content and improving yield quantity and quality of the susceptible cultivar Karim, while in the resistant cultivar Salim the application of RIs seems to be useless. In conclusion, protection conferred by tested RIs seems to be dependent on their composition, but it remains constant whatever of the wheat species. The use of RIs may improve the resistance level and yield of susceptible cultivars in order to obtain similar results to the resistant cultivars. Thus, it could replace the use of resistant cultivars especially with the lack of available completely resistant cultivars in Tunisia.

Key words: *Zymoseptoria tritici*, wheat, cultivar, resistance inducers, defense genes, pathogen-related genes, RT-PCR analysis.

INTRODUCTION

During induced resistance upon abiotic or biotic stimulation, plant increases their resistance level against a pathogen attack. Such an induction of plant resistance with either natural or synthetic

compounds offers an opportunity to reduce the use of conventional fungicides, since major crop diseases are currently mainly controlled by the use of chemicals. The extensive and systematic use of conventional fungicides is currently questioned because of its negative impacts on the environment and human health and because of the emergence and spreading of resistant strains of pathogens (Walters *et al.*, 2005). Septoria tritici blotch (STB) is a widespread and destructive disease of wheat caused by the hemibiotrophic ascomycete fungus *Zymoseptoria tritici* (Desm.) (Quaedvlieg *et al.*, 2011) previously known as *Septoria tritici* (teleomorph *Mycosphaerella graminicola* (Fuckel) J. Schröt.). Due to a lack of commercialized fully resistant wheat cultivars and the emergence of fungal strains resistant to fungicides, STB is one of the most significant threats to the potential yield of wheat crops. In Tunisia, durum wheat (*Triticum turgidum* L. subsp. *durum*) is the primary cultivated crop and occupies more than half of the area dedicated to cereal crops (Bnejdi and El Gazzah, 2008).

STB has become a major disease of durum wheat particularly during favorable growing seasons where it causes significant yield losses which can easily reach up to 50% (Gharbi *et al.*, 2008; Berraies *et al.*, 2014). In wheat, the induction of defense responses has been demonstrated after treatments with a set of different resistance inducers (Renard-Merlier *et al.*, 2007; Hofgaard *et al.*, 2010; Tayeh *et al.*, 2013, 2014). In their study, Shetty *et al.* (2009) showed that the application of a β -1,3-glucan extract from *Z. tritici* cell wall elicits wheat defense mechanisms and increases the resistance level of wheat against *Z. tritici* (Shetty *et al.*, 2009). Moreover, Zhuk *et al.* (2014) reported that wheat treated with oxalic acid at the field level was more tolerant to STB. These studies suggest that treatment with resistance inducers could be an alternative to the use of fungicides to protect wheat against STB. However, the use of resistance inducers at the field level revealed that the plant genotype may influence the expression of induced resistance (Bruce, 2014; Walters *et al.*, 2005). Likewise, Walters *et al.* (2011) found, in controlled conditions, a correlation between protection efficacy conferred by elicitors and resistance level of different spring barley cultivars to *Rhynchosporium secalis* and *Blumeria graminis*.

The present study aims at identifying the protection efficacy conferred resistance inducers (RIs) treatment on Tunisian durum wheat cultivars exhibiting different resistance levels against STB. The effect of cultivar resistance on the antifungal and wheat defense elicitor properties of tested RIs was discussed.

MATERIALS AND METHODS

Essay in greenhouse condition

Three durum wheat cultivars were used and selected for their different level of *Z. tritici* resistance, such as a susceptible (S) cv. Karim, a moderately resistant (MR) cv. Nasr and a resistant (R) cv.

Salim. Wheat grains were germinated in Petri dishes on moist filter paper in darkness at 20 °C for 24 h, at 4 °C for 48 h and then at 20 °C for 24 h and 12 germinated grains were then placed into pots filled with 3 L loam in the greenhouse at 18 °C ($\pm 2^{\circ}\text{C}$) with a 16 / 8 h photoperiod with supplementary illumination. After 3 weeks, when the third leaf was fully expanded, plants were sprayed with tested products including AA (50 mg L⁻¹ of ascorbic acid), Oligos (50 mg L⁻¹ of concentrated plant cell wall oligosaccharides) and *A. nod* (500 mg L⁻¹ of *Aschophyllum nodosum* extract). Used concentration was determined following the manufacture. A Tunisian *Z. tritici* strain (St-08-46), chosen for its high level of pathogenicity was inoculated at 48 hours post treatment using hand sprayer. It was grown on Potato dextrose Agar (PDA) medium (Sigma) for 10 days at 18°C with a 12 /12-h day / night cycle. Inoculation was prepared by washing cultures with 10 mL sterile distilled water. The obtained suspensions were then adjusted to 10⁶ spores mL⁻¹. Immediately after inoculation, each pot was covered with a clear polyethylene bag for 3 days in order to ensure a water-saturated atmosphere compatible with a good fungal germination. The disease level was scored at 22 days post infection (dpi) by noting the percentage of the third leaf of 25 plants area with symptoms (chlorosis and necrosis) and by noting pycnidia density on a scale from 0 (absence of pycnidia) to 5 (all leaf areas with symptoms bearing pycnidia).

Histopathological analysis

Monitoring of leaf penetration by *Z. tritici* was performed using Fluorescence Brightener 28 (Calcofluor, Sigma) according to Siah *et al.* (2010). Segments (2 cm) were harvested from the third fully expanded leaf at 5 dai from inoculated plants and then immersed for 5 min in a solution of 0.1 % (w/v) Calcofluor in 0.1 M Tris–HCl buffer pH 8.5. Leaf segments were washed for 2 min with sterile distilled water. After drying, they were placed on a glass slide, covered with a cover slip and observed microscopically (Nikon, Eclipse 80i) under UV illumination. Mesophyll colonization was investigated using Trypan blue (Shetty *et al.*, 2003; Siah *et al.*, 2010) with few modifications. Leaf segments (2 cm) were harvested from both control and inoculated leaves and cleared in a mixture of absolute ethanol: glacial acetic acid (3: 1, v/v) overnight. Cleared leaves were rehydrated for 4 h in distilled water and then fixed for 20 min in lactoglycerol (lactic acid: glycerol: water, 1: 1: 1, v/v/v). Staining of the fungus was carried out at 70 °C by immersing the leaf segments in 0.1% Trypan blue dissolved in lactophenol-ethanol (1:2, v/v) for 6 h. After washing, leaf segments were fixed in lactoglycerol, placed on a glass slide, covered with a cover slip and then observed microscopically under white light illumination. Spores were recorded for each cultivar on three leaf segments and subsequently 150 randomly chosen spores were studied. Data represent means of two independent experiments. Three independent replicates were carried out for each experiment.

Protein extraction and enzymatic assays

Three CWDE activities (endo- β -1,4-xylanase, endo- β -1,3-glucanase and protease) were assessed. Total protein extractions were carried out at 4, 10, 16 and 22 dpi as previously described by Siah *et al.* (2010) with few modifications. Briefly, 2.5 g of inoculated and control third leaves were removed and immediately frozen in liquid nitrogen prior to grinding in a mortar. The resulting powder was then dispersed in 5 mL 0.05 M Tris-HCl buffer pH 7.8 containing 0.1 M KCl and 0.5 % cysteine. After cooling for 30 min at 4 °C, the mixture was squeezed through three layers of cheesecloth and centrifuged at 13 000 g for 20 min at 4 °C. From the supernatant, 100 μ L were transferred to an Eppendorf tube and stored at 4°C for total protein quantification. Total protein extracts were then dialyzed against 0.5 L of distilled water for 24 h at 4 °C overnight (SpectrumLab Inc.). The dialyzed solution was then subjected immediately to endo- β -1,3-glucanase and endo- β -1,4-xylanase activity quantification using a modified 3,5-dinitrosalicylic acid (DNS) method (Douaiher *et al.*, 2007; Siah *et al.*, 2010). Endo- β -1,4-xylanase activity was measured at pH 4.8 and 45 °C using xylan (1%, w/v) (Sigma) as substrate and xylose (10 mM) (Sigma Aldrich, USA) as reducing control sugar (Siah *et al.*, 2010). Glucanase activity was measured at pH 4.8 and 50 °C using Laminarin (Sigma Aldrich, USA) at pH 5 (0.5 %, w/v) as substrate and glucose (10 mM) as reducing control sugar (Miller *et al.*, 1959; Douaiher *et al.*, 2007a, b). One unit of endo- β -1,4-xylanase and glucanase activity is defined as the amount of enzyme necessary to release 1 μ mol of reducing sugar/p-nitrophenol $\text{min}^{-1} \text{mL}^{-1}$ of enzyme solution. Absorbance was measured at 540 nm. Protease activity assays were assessed according to Hellweg (2003). Six aliquots of 100 μ L of total protein were heated to 80 °C for 10 min to inactivate enzyme activity. After cooling, 50 μ L of azocasein solution (20 mg/mL Azocasein, SIGMA) and 50 μ L of incubation buffer (200 mM Tris, 20 mM CaCl_2 pH 7.8) were added. After incubation at 40 °C under gentle agitation for 30 to 60 min, 500 μ L of 5 % aqueous trichloroacetic acid were added to precipitate non-digested protein and the mixture was incubated for 10 min at room temperature. The precipitate was pelleted by centrifuging for 5 min at 13000 g and 500 μ L of test buffer were added at 400 μ L of the supernatant. Absorbance was measured at 400 nm. Measures were performed in triplicates with denatured samples as comparative samples using an azo-dye labeled substrate. One unit of protease activity is defined as a change in enzyme activity of 1 per hour. All enzymatic activities were expressed in mU mg^{-1} of proteins. Total protein concentration was measured at A_{595} using bovine serum albumin as a standard (Bradford, 1976). For all tested activities, absorbance was measured using Thermo Scientific™ GENESYS 10S UV-Vis spectrophotometer. Data represent means of two independent experiments. Three independent replicates were carried out for each experiment.

RNA extraction and analysis of gene expression by semi-quantitative RT-PCR and real-time RT-PCR

Gene's expression study was monitored at third leaf with three replicates. Samples were collected at 1, 2, 3, 4 and 7 days post treatment (dpt) from treated and control (H₂O-treated) plants. Total RNA was extracted from 100 mg of plant tissue using RNeasy Plant Mini Kit (Quiagen, The Netherlands). Genomic DNA contaminating the samples was removed by treatment with DNase using RNase-Free DNase Set (Quiagen, The Netherlands). Reverse transcription of total RNA was carried out using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA) according to the manufacturer's protocol. PCR reactions were conducted on subsequent obtained cDNA to amplify five wheat genes encoding for *PR2* (β -1,3-glucanase), *Chi 4 precursor* (chitinase class IV precursor), *Pox* (peroxidase), *Msr* (methionine sulfoxide reductase), *ATPase* (vacuolar ATP synthase) and *Bsi1* (Putative protease inhibitor). *TUB* encoding gene is used as housekeeping gene. Primers pairs used for amplifying *PR2* (U76895), *Chi 4 precursor* (AF112966), *Pox* (X56011) and *TUB* (U76895) were designed using the Primer Express® program and were tested for secondary structure using Net Primer® program. *Msr*, *ATPase* and *Bsi1* primers were published by Adhikari *et al.* (2007). The following gene-specific primers are presented in Table 1.

Table 1 List of primers encoding for wheat genes analyzed by quantitative and semi-quantitative RT-PCR.

Gene	Primer sequences (5'-3')
<i>TUB</i> (<i>beta tubulin</i> , housekeeping gene)	GGAGTACCCTGACCGAATGATG AACGACGGTGTCTGAGACCTTT
<i>PR2</i> (<i>endo-β-1,3-glucanase</i>)	CTCGACATCGGTAACGACCAG GCGGCGATGTACTTGATGTTC
<i>Chi4 precursor</i> (<i>chitinase class IV precursor</i>)	TTCTGGTCTGGATGACCAAC ACTGCTTGCACTACTCCGTG
<i>Pox</i> (<i>peroxidase</i>)	GAGATTCCACAGATGCAAACGAG GGAGGCCCTTGTTTCTGAATG
<i>Msr</i> (<i>putative methionine sulfoxide reductase</i>)	CATGCAGATGTTTCGGACAAA ACCATCGCGTCCCATGTAAA
<i>ATPase</i> (<i>vacuolar ATP synthase</i>)	GCTTTCGGATCTCTGGCAATT TGATGAGCACGGCCAGATT
<i>Bsi1</i> (<i>putative protease inhibitor</i>)	GGGCCCTGCAAGAAGTACTG ACACGCATAGGCACGATGAC

The expression of *Msr*, *ATPase* and *Bsi* genes was investigated by semi-quantitative RT-PCR. PCR was carried out in a 20 μ L reaction mixture containing 1x PCR buffer, 2 mM dNTPs, 10 μ M of each primer, 0.5 unit of Taq polymerase and 20 ng of cDNA using the following protocol: 94°C for 5 min, followed by 30 cycles each with 94°C for 1 min, 55°C for 1 min and 72°C for 1 min. The PCR reaction was terminated at 72°C for 7 min. PCR products were analyzed by electrophoresis on ethidium-bromide stained 1.5% agarose gels run in 0.5 x Tris-acetic acid-EDTA buffer at 100 V for

45 min. Gels were visualized under UV light. Experiment was repeated 3 times and only results which were repeated at least twice were considered. The investigation of *PR2*, *Chi 4 precursor* and *Pox*-encoding gene expression was performed by real time-RT-PCR analysis. The amplification specificity of each qRT-PCR was checked by a single peak in melt curve analysis, and no primer dimmers were detected using agarose gel electrophoresis. Real-time RT-PCR was performed using ABI Prism 7300 detection system (Applied Biosystems, USA), using the following thermal profile : after incubation for 10 min at 95°C, 15s at 95°C (denaturation), 1 min at 60°C (annealing/extension) for 40 cycles and 30s at 72°C (data acquisition). Relative expression for each cDNA were calculated for each time point relative to control at the same time It corresponds to $2^{-\Delta\Delta Ct}$ described by Pfaffl *et al.* (2002) and was calculated using the relative expression software tool REST®. $\Delta\Delta Ct = [Ct \text{ Target (Sample)} - Ct \text{ Reference (Sample)}] - [Ct \text{ Target (Control)} - Ct \text{ Reference (Control)}]$. *TUB* is used as reference gene. For each time point and for each wheat cultivar, the control H₂O-treated (ni) corresponds to a fixed value of 1. After qPCR, a melt curve was generated to verify the specificity of each amplification reaction. The experiment was conducted in triplicate and only results which were repeated at least twice were considered. The experiment was conducted in triplicate and only results which were repeated at least twice were considered.

Field trials

The trial was conducted during the 2011-2012 and 2013-2014 growing seasons at Regional Research Centre Crops (CRRGC) in Beja experimental station, located at North West Tunisia (36°43'30" N, 9°10'55" E; 9°11'20"). This station is located in the sub-humid region characterized by high rainfall with mild winter which is favourable conditions for natural infection of septoria. Three durum wheat cultivars were cultivated as, including Karim, Nasr and Salim. The trial was conducted in randomized complete block design with three replications of 3 m² spaced by 1.5 m, with a planting density of 320 grains/m². Seeding was mechanically performed in mid-December. Fertilizer (ammonium nitrate) and weed control (glyphosate) were those recommended for Beja area. Two applications of three tested products (AA, Oligos and *A. nod*) were realized at tillering stage (GS22) and flag leaf stage (GS39) (Zadoks *et al.*, 1974). We used the same concentrations previously applied in greenhouse test. The severity of disease was scored visually after 1 moth of the second application of treatments (ear emergence stage, GS 61-69).

At this stage, we also estimated the chlorophyll content using the SPAD Chlorophyll Meter which is an effective tool for rapid and non destructive estimation of relative chlorophyll and nitrogen content in sugar maple leaves during the growing season. Van der Berg and Perkins (2004) demonstrated that the relationship between total extractable chlorophyll and chlorophyll content index given by SPAD Chlorophyll Meter was significantly linear. At maturity, each plot was

harvested with a combine. The grains were cleaned and the quantity harvested was weighed and reported to the harvested area. The weight of 1000 grains was determined from three samples of 1000 grains. The yield (g/ m²) was determined. Data represent means of two independent experiments. Three independent replicates were carried out for each experiment.

Statistical data analysis

For semi quantitative gene expression, experiment was repeated 3 times and only results which were repeated at least twice were considered. For quantitative gene expression studies, the analyses were performed using the relative expression software tool REST® as described by Pfaffl *et al.* (2002). For all data, an analysis of variance (ANOVA) was performed using a Tukey test at a significance level of $P \leq 0.05$. All data were analyzed by XLSTAT software (Sigma Plus, France).

RESULTS

In greenhouse condition

Alteration of the *Z. tritici* infectious process

In order to evaluate the impact of the preventive treatment by three RIs (Oligos, AA and *A. nod* used at respective concentrations, 50, 50 and 500 mg L⁻¹) on the development of St-08-46 *Z. tritici* strain in three durum wheat cultivars, Karim (S), Nasr (MR) and Salim (R), several cytological parameters related to the development of the Tunisian *Z. tritici* strain (St-08-46), were quantified tested cultivars at different time points (Fig. 1). At 1 dpi, H₂O (i) control leaves of the three cultivars exhibited percentages of germination close to 70 % (Fig. 1). Only AA treatment revealed significant decrease of spore germination compared to corresponding H₂O (i) controls (between 2.6-, 2.6- and 4.5-folds respectively in Karim, Nasr and Salim). At 4 dpi, only AA-treated leaves exhibited significant 2.4-, 2.5- and 2.6-fold increases of the percentage of non germinated spores (NGS), respectively in Karim, Nasr and Salim (Fig. 2A). The percentage of germinated spores leading to a stomatal penetration (SP) was not affected by tested RIs, while the percentage of germinated spores leading to a direct penetration (DP) were significantly reduced in AA- and Oligos-treated Karim (2.6- and 3.6-folds) and Nasr (2.7-folds) (Fig. 2B). In addition, the percentage of germinated spores that did not penetrate (GSNP) was 2.3- and 4.3-fold reduced following AA treatment in Nasr and Salim, respectively, while *A. nod* treatment caused 1.7-fold decrease of GSNP in Nasr (Fig. 2C).

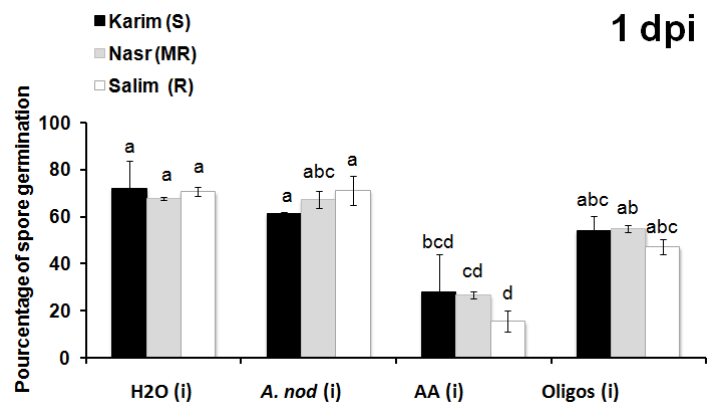


Fig. 1 Effect of tested treatments on spore germination of St-08-46 *Z. tritici* strain on Karim (S), Nasr (MR) and Salim (R). Three RIs were tested including Oligos, AA and *A. nod* were tested at greenhouse condition using respective concentrations, 50, 50 and 500 mg L⁻¹. Treated leaves were compared to the reference H₂O-treated (i) controls. The infection of three tested cultivars by St-08-46 *Z. tritici* strain was monitored at 2 days post treatment. The *Z. tritici* spore germination was investigated at 1 day post infection (dpi) using microscopic observation with Calcofluor fluorescence. Data represent means of two independent experiments with three independent replicates (±s.d). Means tagged with the same letter are not significantly different using the Tukey test at $P \leq 0.05$.

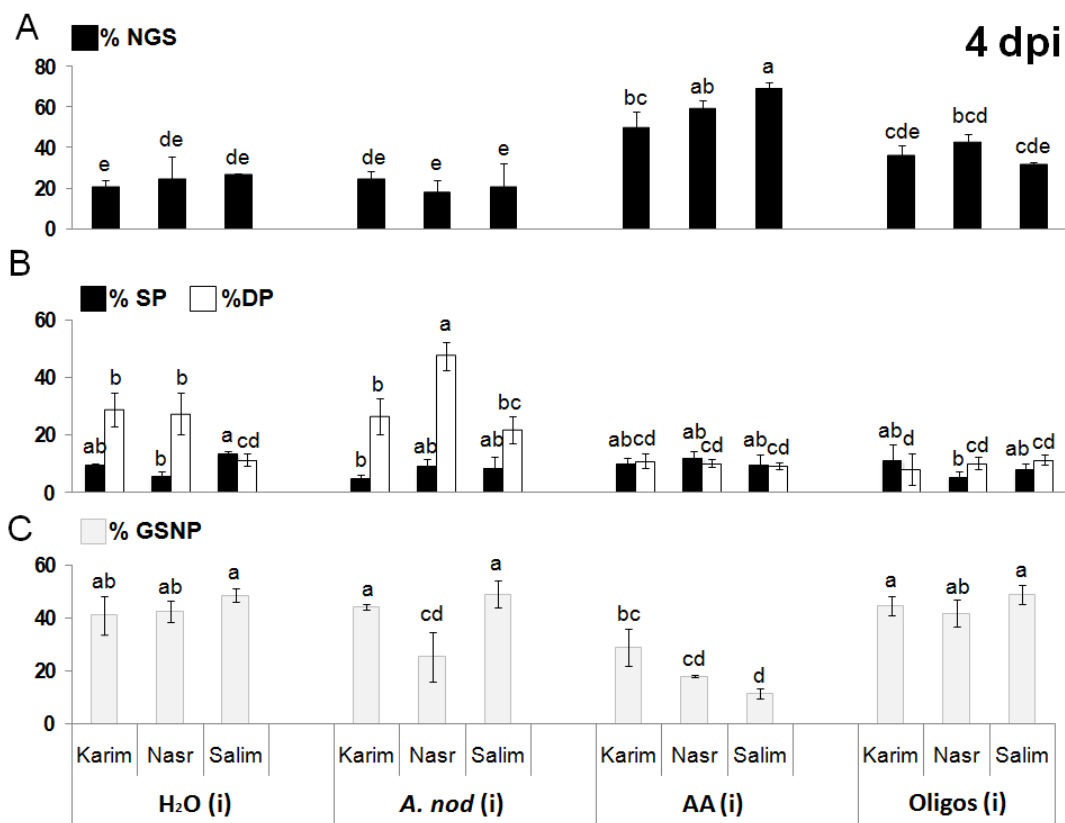


Fig. 2 Effect of tested treatments on leaf penetration of St-08-46 *Z. tritici* strain on Karim (S), Nasr (MR) and Salim (R). Three treatments were tested including Oligos, AA and *A. nod* were tested at greenhouse condition using respective concentrations, 50, 50 and 500 mg L⁻¹. Treated leaves were compared to the reference H₂O-treated (i) control. The infection of three tested cultivars by St-08-46 *Z. tritici* strain was monitored at 2 days post treatment. The *Z. tritici* leaf penetration was investigated at 4 day post infection (dpi) using microscopic observation with Trypan blue staining. NGS: not germinated spores; DP: spores leading to a direct penetration; SP: stomatal penetration; GSNP: germinated spores that did not penetrate. Data represent means of two independent experiments with three independent replicates (±s.d). Means tagged with the same letter are not significantly different using the Tukey test at $P \leq 0.05$.

At 22 dpi, the percentage of none colonized stomata (NCS) and colonized stomata but not yet transformed into pycnidia (CS) were affected only by AA treatment. NCS was 3.4-, 3.9- and 1.8-fold increased in Karim, Nasr and Salim respectively, while CS was 1.7-fold increased in Karim and 1.3- and 1.5-fold decreased in Nasr and Salim respectively (Fig. 3A). However, the percentage of colonized stomata transformed into pycnidia (P) was affected by all tested RIs in three tested cultivars. Oligos exhibited higher decreases (11.1-, 6.4- and 12.2-folds in Karim, Nasr and Salim, respectively), followed by AA (3.9-, 3.3- and 2.0-folds) and *A. nod* (1.6-, 1.4- and 1.9-folds) (Fig. 3B).

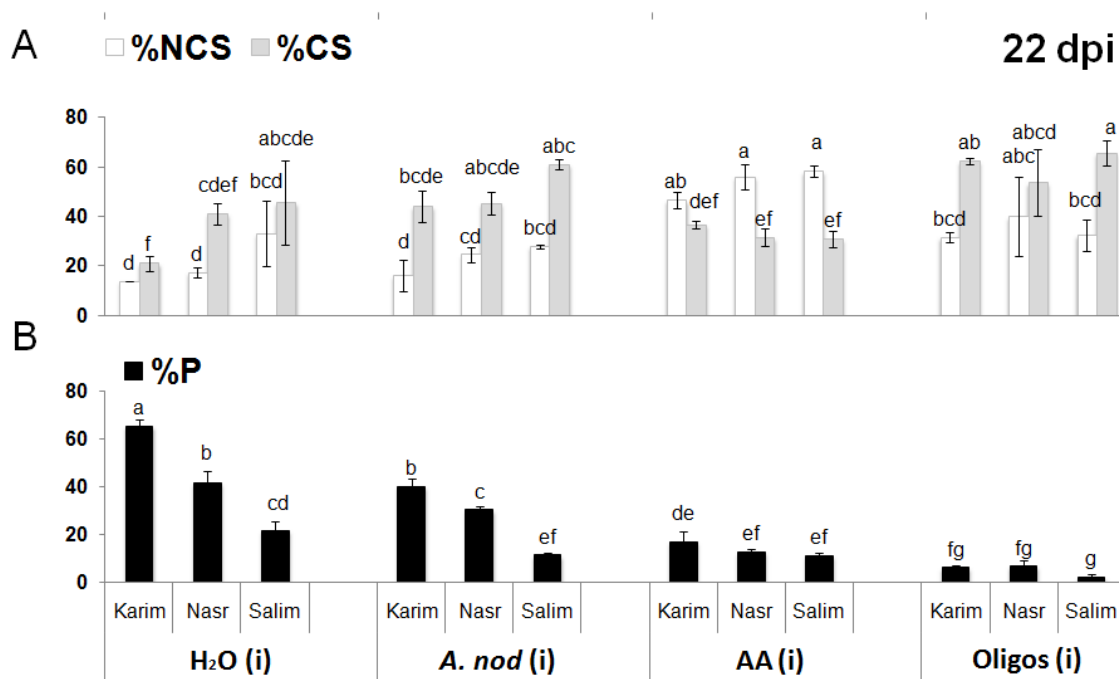


Fig. 3 Effect of tested treatments on colonization and pycnidia formation of St-08-46 *Z. tritici* strain on Karim (S), Nasr (MR) and Salim (R). Three treatments as Oligos, AA and *A. nod* were tested at greenhouse condition using respective concentrations, 50, 50 and 500 mg L⁻¹. Treated leaves were compared to the reference H₂O-treated control. The infection of three tested cultivars by St-08-46 *Z. tritici* strain was monitored at 2 days post treatment. The *Z. tritici* colonization and fructification was investigated at 22 day post infection (dpi) using microscopic observation with Calcofluor fluorescence. NCS: non-colonized stomata; CS: colonized stomata but not yet transformed into pycnidia; P: colonized stomata transformed into pycnidia. Data represent means of two independent experiments with three independent replicates (±s.d). Means tagged with the same letter are not significantly different using the Tukey test at $P \leq 0.05$.

Protection efficacy of tested RIs

In order to evaluate the protection efficacy of (Oligos, AA and *A. nod*) to control St-08-46 *Z. tritici* strain infection on Karim (S), Nasr (MR) and Salim (R), the percentage of leaf area with necrosis and the index of pycnidium coverage were assessed on the third wheat leaves at 22 dpi (Fig. 1). Our findings revealed that Oligos, AA and *A. nod* treatments promoted a significant reduction of leaf

area with necrosis mainly in Karim (S) (by 5-, 4.3- and 2.5-folds), followed by Nasr (MR) (by 4.0-, 3.6- and 1.7-folds) and Salim (R) (by 2.5-, 2.9 and 1.4)(Fig. 4A). Likewise, Oligos, AA and *A. nod* treatments induced a significant reduction of the index of pycnidium coverage mainly in Karim (S) (by 5-, 4.3- and 2.5-folds), followed by Nasr (MR) (by 4.0-, 3.6- and 1.7-folds) and Salim (R) (by 2.5-, 2.9 and 1.4)(Fig. 4A). Likewise, revealed that Oligos and AA treatment promoted a significant reduction of the index of pycnidium coverage in Karim (S) (by 3.5-, 3.1- and 1.9-folds), Nasr (MR) (2.3-, 2.8- and 1.6-folds) and Salim (R) (2.0-, 2.0- and 1.2-folds)(Fig. 4B). Therefore, Oligos and AA conferred a good protection while *A. nod* showed lower protection. Overall, higher protection of RI was shown in the susceptible cultivar Karim, while the lowest protection was observed in the resistant cultivar Salim.

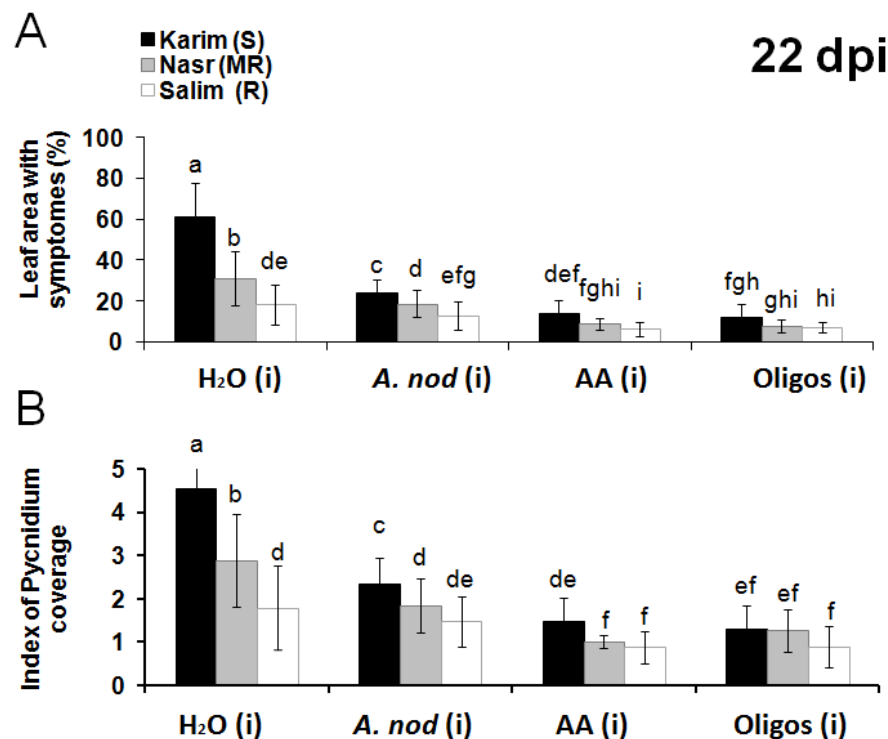


Fig. 4 Protection conferred by tested treatments on Karim (S), Nasr (MR) and Salim (R) at greenhouse condition. Disease level of *Z. tritici* St-08-46 strain was investigated at 22 dpi. Three treatments were tested including Oligos, AA and *A. nod* were used at respective concentrations, 50, 50 and 500 mg L⁻¹. Treated leaves were compared to the reference H₂O-treated control. The infection of three tested cultivars by St-08-46 *Z. tritici* strain was monitored at 2 days post treatment. Data represent means of two independent experiments (\pm s.d). Means tagged with the same letter are not significantly different using the Tukey test at $P \leq 0.05$.

Cell-wall degrading enzyme activities

Three CWDE activities, including endo- β -1,4-xylanase, endo- β -1,3-glucanase and protease, produced by the fungus in plant tissues were monitored during the infection process at 4, 10, 16 and 22 dpi (Fig. 5). At 4, 10 and 16 dpi, in Oligos-, AA- and *A. nod*-treated leaves, all tested CWDEs

presented weak activity levels, which were not significantly different from H₂O-treated (i) controls (Annex 4). At 22 dpi, all tested RIs caused significant decrease of these activities compared to H₂O-treated (i) in three tested cultivars (Fig. 5A, B and C).

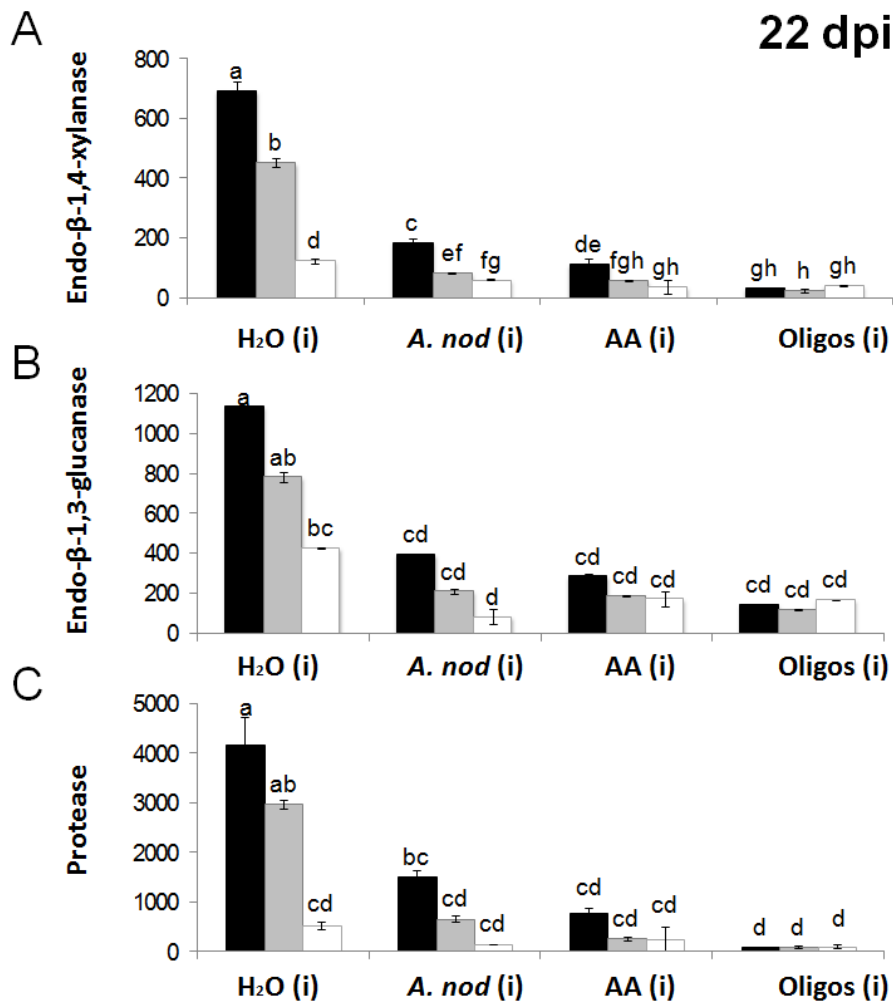


Fig. 5 Effect of tested treatments on CWDE activities of St-08-46 strain on Karim (S), Nasr (MR) and Salim (R) at greenhouse condition. CWDE activities were measured at 4, 10, 16 and 22 days post inoculation (DPI). three *Z. tritici* CWDEs were investigated such as endo-β-1,4-xylanase (A), endo-β-1,3-glucanase (B) and protease (C) activities. Three treatments were tested including Oligos, AA and *A. nod* were used at respective concentrations, 50, 50 and 500 mg L⁻¹. The infection of three tested cv. by St-08-46 *Z. tritici* strain was monitored at 2 days post treatment. Data represent means of two independent experiments (±s.d). Means tagged with the same letter are not significantly different using the Tukey test at $P \leq 0.05$.

Results indicated that treated Salim leaves recorded lower reduction of CWDEs than Nasr and Karim leaves. Oligos-, AA- and *A. nod*-treated Salim leaves exhibited 3.0-, 3.5- and 2.1-fold reduction of endo-β-1,4-xylanase activity (Fig. 5A), 2.5-, 2.5- and 5.1-fold decreases of endo-β-1,3-glucanase activity (Fig. 5B) and 5.0-, 2.2- and 3.6-fold reduction of protease activity (Fig. 5C). However, in Nasr treated leaves Oligos-, AA- and *A. nod* generated 19.1-, 8.0- and 5.5-fold reduction of endo-β-1,4-xylanase activity, 6.5-, 4.2- and 3.7-fold decreases of endo-β-1,3-glucanase

activity and 32.0-, 11.3- and 4.5-fold reduction of protease activity. Karim treated leaves revealed the highest reduction of endo- β -1,4-xylanase (21.2-, 6.3- and 3.7-folds in Oligos-, AA- and *A. nod*-treated leaves), endo- β -1,3-glucanase (8.0-, 4.0- and 2.9-folds in Oligos-, AA- and *A. nod*-treated leaves) and protease (49.8-, 5.3- and 2.8-folds in Oligos-, AA- and *A. nod*-treated leaves) activities. Furthermore, Oligos treatment caused the highest 14.5-, 5.7- and 28.9-fold reduction averages of endo- β -1,4-xylanase, endo- β -1,3-glucanase and protease, respectively. Then, AA caused 5.9-, 3.6- and 6.3-fold decrease averages of endo- β -1,4-xylanase, endo- β -1,3-glucanase and protease, respectively. *A. nod*-treated leaves showed the lowest reduction averages (3.8-, 3.9- and 3.6-folds of endo- β -1,4-xylanase, endo- β -1,3-glucanase and protease, respectively). Expression of defense-related genes by RIs in Karim (S) and Salim (R)

We established transcript profiles of a set of wheat genes involved in defense mechanisms against *Z. tritici* infection. The induction of these genes was monitored in the resistant cultivar Salim and the susceptible cultivar Karim leaves, at non infectious context. Treated leaves were compared to untreated leaves.

Pathogen-related (PR) genes

Three PR genes, including *PR2* (β -1,3-glucanase), *Chi 4 precursor* (chitinase class IV precursor) and *Pox* (peroxidase), were studied using real time PCR analysis at 1, 2 and 3 dpt.

Concerning *PR2*-encoding gene, our findings exhibited that in Salim (R) leaves, Oligos treatment induced *PR2* gene up regulation at all time points and marked maxima at 2 dpi (190 folds), while in Karim (S) leaves, it induced similar up regulation at 1 dpi (8 folds) which will disappear later (Fig. 6B). Following AA treatment, we noted, in Salim (R) leaves, a significant up-regulation at 1 dpi (26 folds) which will decrease after that, while in Karim (S) it induce later up-regulation at 3 dpi (20 folds) (Fig. 6C). Besides, *A. nod* treatment showed increased *PR2* gene up regulation in resistant cv. Salim at all time points which marked maxima at 3 dpi (314 folds), while, in Karim (S) revealed weak up-regulation at 1 dpi (9 folds) which will disappear later (Fig. 6D).

Concerning *Chi 4 precursor* -encoding gene, results revealed that in Salim (R) leaves, all RIs treatments induced significant early and strong up regulation of *Chi 4 precursor* gene expression at all time points, while in Karim (S) leaves; lower up-regulation was noted. Following Oligos treatment, *Chi 4 precursor* gene expression was 28-, 12- and 29-folds up-regulated at 1-2-3 dpt, whereas in Karim (S), it was 7- and 10-folds up-regulated at 1-2 dpt (Fig. 6F). Similarly, in AA-treated Salim (R) leaves *Chi 4 precursor* gene marked maximal up-regulation at 1 dpt (66-folds) which will decrease later (29- and 10-folds at 2-3 dpt, respectively) (Fig. 6G). However, in AA-treated Karim (S) leaves *Chi 4 precursor* gene expression was not affected. Moreover, in Salim (R) leaves recorded early 10- and 8-fold up-regulation of *Chi 4 precursor* gene at 1-2 days post *A. nod*

treatment, but *A. nod* –treated Karim (S) revealed lower up-regulation at 1 dpt (4-folds) which increased at 2-3 dpt (9- and 10-folds, respectively) (Fig. 6H).

Concerning *Pox*-encoding gene, in Salim (R) leaves, Oligos treatment induced increased up-regulation of *Pox* gene expression (29-, 12- and 96-folds at 1-2-3 dpt), whereas in Karim (S), *Pox* gene expression was lower induced (5-, 4-fold at 1-2 dpt, respectively) (Fig. 6J). Likewise, AA-treated Salim (R) leaves recorded early 30-fold up-regulation of *Pox* gene at 1 dpt, which decreased at 2-3 dpt (3- and 12-folds, respectively), but, in AA–treated Karim (S), this up-regulation was induced later and marked maxima at 3 dpt (27-folds) (Fig. 6K). The highest accumulation of *Pox* transcripts was noted following *A. nod* treatment in Salim (R) leaves, which marked maxima at 1 dpi (263-folds) and decreased later (47- and 197-folds at 2-3 dpt), while in *A. nod* –treated Karim (S), this gene was significantly lower up-regulated at 2 and 3 dpt (3- and 13-folds) (Fig. 6L).

Otherwise, the comparison of PR genes relative expression were compared between (ni) wheat leaves treated with RIs and infected wheat leaves by St-08-46 *Z. tritici* strain. Our results indicated that Oligos- and *A. nod*-treated Salim leaves exhibited higher and earlier up-regulation of *PR2* gene than that observed in resistant cultivar Salim following *Z. tritici* infection. Whereas, AA-treated Salim leaves revealed earlier but lower *PR2* gene up-regulation than that observed in Salim (i) leaves (Fig. 6A). However, *Chi 4 precursor* gene seems to be more responsive to *Z. tritici* infection than tested RIs (Fig. 6B). Furthermore, *Pox* gene was higher and earlier up-regulated in Salim treated by *A. nod* than infected Salim leaves. Oligos treated Salim leaves recorded closed *Pox* gene expression pattern than that observed in infected Salim leaves. However, in Salim (ni) AA treatment enhanced *Pox* gene up-regulation at 1 dpt, which declined at 2 and 3 dpt instead of Salim (i) leaves which recorded increasing *Pox* gene up-regulation (Fig. 6I).

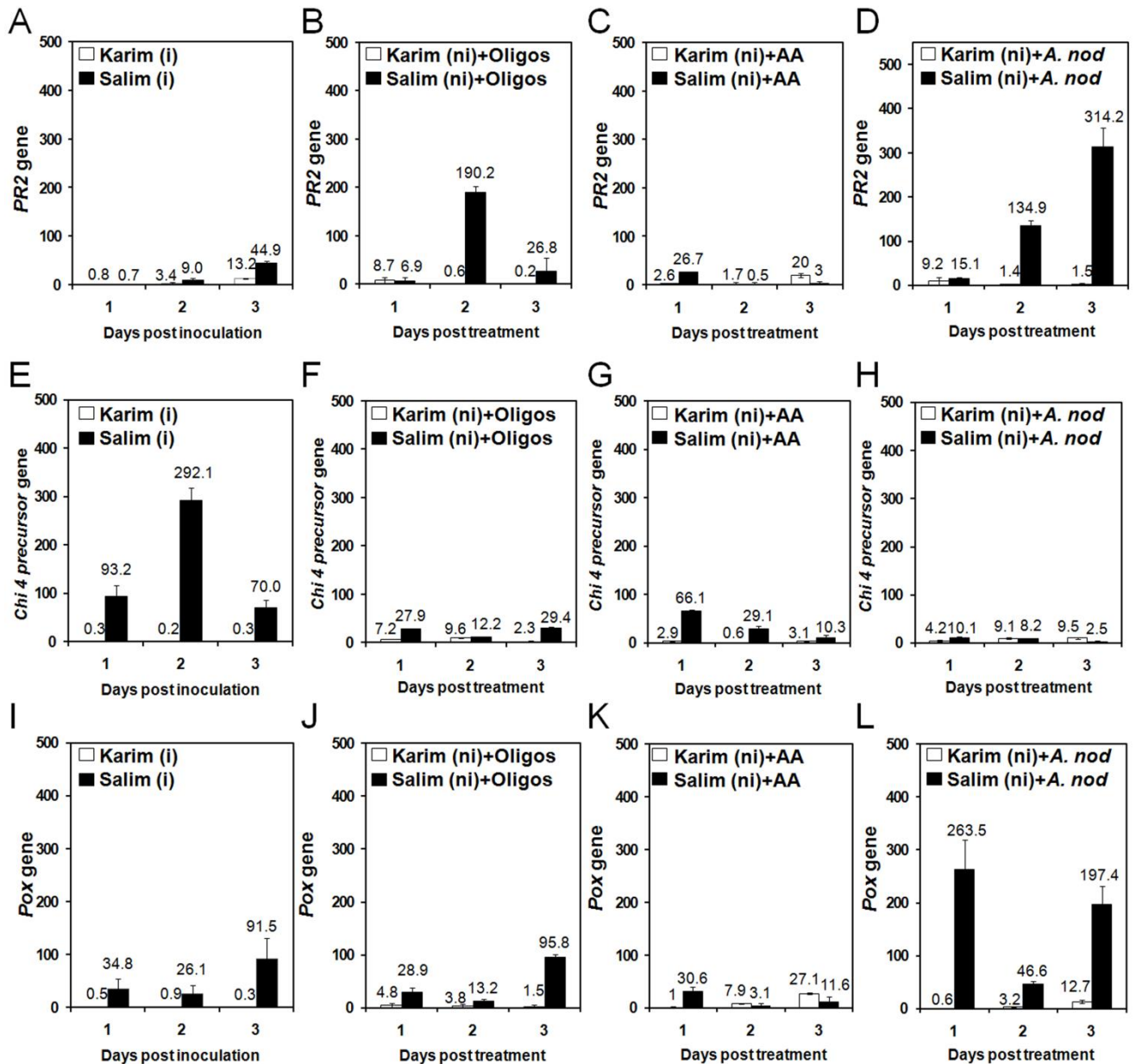


Fig. 6 Quantitative expression of PR genes analyzed by real time RT-PCR analysis at 1, 2 and 3 days post treatment by Oligos, AA and *A. nod* treatments on non infected (ni) Karim (susceptible) and Salim (resistant) compared to infected (i) Karim and Salim by St-08-46 *Z. tritici* strain. Oligos, AA and *A. nod* were used at respective concentrations, 50, 50 and 500 mg L⁻¹. Three genes were investigated such as *PR2* (A, B, C and D), *Chi 4 precursor* (E, F, G and H) and *Pox* (I, J, K and L)-encoding genes. Means of relative expression at each time point are indicated above the bars in each graph. The experiment was conducted in triplicate. Similar results were obtained at least twice. Results correspond to means (\pm s.d) of duplicate reactions.

Msr-, *ATPase*- and *Bsil*-encoding genes

In addition, three other genes involved in wheat defense mechanisms, as *Msr* (*methionine sulfoxide reductase*), *ATPase* (*vacuolar ATP synthase*) and *Bsil* (*Putative protease inhibitor*), were investigated using semi-quantitative RT-PCR analysis in susceptible cv. Karim compared to

resistant cv. Salim, at non infectious context. Gene expression profiles treated plants of each cultivar were followed at 1, 2, 3, 4 and 7 dpt and were compared with their respective H₂O-treated controls (Fig. 7).

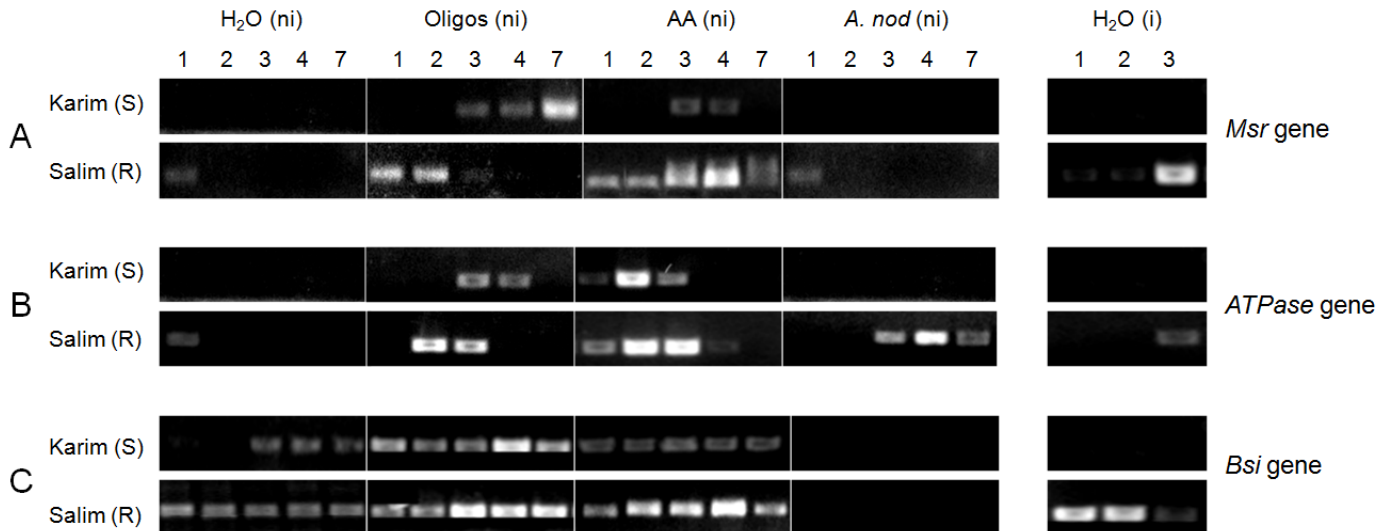


Fig. 7 Wheat defense genes expression analyzed by semi-quantitative RT-PCR analysis at 1, 2, 3, 4 and 7 days post treatment (DPT) by AA, Oligos and *A. nod* treatments on Karim (S) and Salim (R). Oligos, AA and *A. nod* were used at respective concentrations, 50, 50 and 500 mg L⁻¹. Three genes were investigated such as *Msr* (A), *ATPase* (B) and *Bsi1* (C) -encoding genes. For each condition, treated leaves were compared to their reference H₂O-treated leaves. RT-PCR products were generated using primers specific for β -tubulin (*TUB*), *Msr*, *ATPase* and *Bsi1* first-strand cDNAs obtained from total wheat RNA, and visualized on agarose gel (1.5%). *TUB*-encoding gene was used as a house-keeping gene. Experiment was repeated three times and only results which were similar at least twice were considered.

Concerning Msr-encoding gene, Oligos treatment enhanced strong up-regulation at 1-2 dpt in Salim (R), while in Karim (S), later and weaker up-regulation was noted at 3-4-7 dpt and marked maxima at 7 dpt. Similarly, in AA-treated Salim (R) leaves *Msr* gene expression was up-regulated at all tested time points and marked maxima at 4 dpt, whereas AA-treated Karim (S) leaves revealed later and lower up-regulation (at 3-4 dpt). However, no significant change was noted following *A. nod* treatment in both cultivars (Fig. 7A).

Concerning ATPase-encoding gene, Oligos-treated Salim (R) leaves recorded early and strong up-regulation at 2-3 dpt, while Oligos-treated Karim (S) revealed later and weaker up-regulation at 3-4 dpt. Likewise, in AA-treated Salim (R) leaves *ATPase* gene expression was highly up-regulated at 2-3 dpt, whereas AA-treated Karim (S) leaves revealed high up-regulation only at 2 dpt which will decrease later. Besides, *ATPase* gene up-regulation was enhanced following *A. nod* treatment in Salim (R) leaves from 3 dpt and marked maximum at 4 dpt, but we didn't detect any expression of this gene in *A. nod*-treated Karim (S) (Fig. 7B).

Concerning *BsiI*-encoding gene, it was highly up-regulated following Oligos treatment in Salim (R) leaves at 3-4-7 dpt, while in Oligos-treated Karim (S) it was induced only at 4 dpt. Moreover, in AA-treated Salim (R) leaves *BsiI* gene was strongly up-regulated at 2-3-4 dpt marked maximum at 4 dpt, but AA-treated Karim (S) leaves revealed weaker up-regulation at 1-2 dpt. Although, no expression of *BsiI* gene was observed following *A. nod* treatment in both tested cultivars, while in H₂O-treated controls it was weakly induced from 1 to 7 dpt in Salim (R) leaves and at 3-4-7 dpt in Karim (S) leaves. Therefore, this gene could be down-regulated by *A. nod* treatment (Fig. 7C).

Furthermore, the comparison of *Msr*, *ATPase* and *BsiI* genes relative expression in (ni) wheat leaves treated with RIs with wheat leaves infected by St-08-46 *Z. tritici* strain revealed that *Msr* and *ATPase* up-regulation was earlier and stronger in Oligos and AA-treated Karim and Salim leaves, while *BsiI* gene was higher up-regulated following *Z. tritici* infection in resistant wheat Salim than Oligos, AA and *A. nod* treatments.

In field

Protection efficacy and yield improvement of tested RIs

In field, our findings indicated that all tested RIs, applied at tillering (22) and stem elongation (37) stages (Zadoks *et al.*, 1974), allowed to significant decreases of *Z. tritici* severity respectively on the susceptible cv. Karim and the moderate resistant cv. Nasr compared to H₂O-treated (i) controls, while reduced infection showed on the resistant cv. Salim was not significantly different (Fig. 8A). Oligos-, AA- and *A. nod*-treated leaves recorded 2.2-, 2.0- and 1.7-fold decreases in Karim, 2.1-, 2.5- and 1.6-fold decreases in Nasr leaves and 2.0-, 1.9- and 1.6-fold decreases in Salim leaves (Fig. 8A).

Chlorophyll content

At flowering (61, Zadoks *et al.*, 1974), the estimation of chlorophyll content using SPAD values at flag leaf revealed that *A. nod* treatment induced increased SPAD values in Karim (S) and Nasr (MR) (by 55.8% and 28.6%, respectively) compared to H₂O-treated (i) controls, while AA-treated leaves recorded increased SPAD values in Karim (S) and Salim (R) (by 39.7% and 20.8%, respectively). However, Oligos-treated leaves didn't exhibit any significant increase of SPAD values (Fig. 8B). Therefore, *A. nod* treatment could improve chlorophyll content mainly in susceptible cultivars and allowed to achieve closed levels observed in resistant cultivar Salim, while AA treatment could get better chlorophyll content even in resistant cultivar Salim. In contrast, Oligos treatment seems not affect chlorophyll content.

Yield parameters

The effect of tested RIs on durum wheat yield was assessed yield quantity (g/m²) (Fig. 8C) and quality (the weight of 1000 seeds) (Fig. 8D) in *A. nod*, AA and Oligos treated (i) leaves compared to H₂O-treated (i) control leaves. Results exhibited significant increased yield quantity (g/m²) (by 37.1%, 38.7% and 31.3%) and the weight of 1000 seeds (by 13.3 %, 14.0% and 10.8%) of Karim (S) cultivar by *A. nod*, AA and Oligos treatments, respectively. But, only *A. nod* and AA treatments induced 1.2- and 1.3-fold increases of yield quantity (g/m²) of Nasr (MR) cultivar. However, all tested RIs didn't induce any significant change in Salim (R) cultivar yield quantity and quality (Fig. 8C and D).

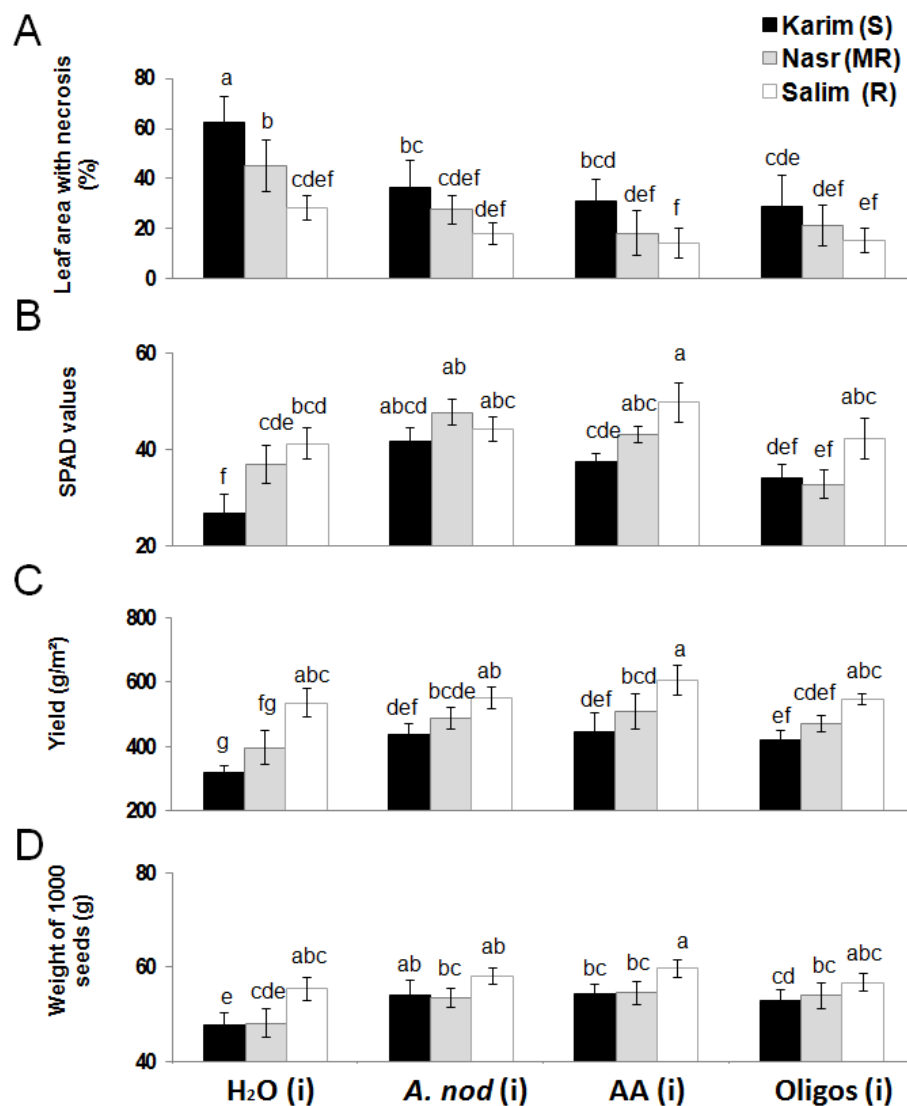


Fig. 8. Effect of tested treatments on disease severity and yield parameters on Karim (S), Nasr (MR) and Salim (R) at field condition. Three treatments were tested including Oligos, AA and *A. nod* were used at respective concentrations, 50, 50 and 500 mg L⁻¹. A: Leaf area with symptoms. B: Chlorophyll content estimation using SPAD values. C: Yield quantity (g). D: Weight of 1000 seeds (g). Each treatment was applied twice (at tillering (22) and stem elongation (37) stages according to Zdock's scale). Data represent means of two independent experiments (\pm s.d) conducted during the 2011-2012 and 2013-2014 growing seasons in Beja experimental station. For each time point, means tagged with the same letter are not significantly different using the Tukey test at $P \leq 0.05$.

DISCUSSION

Because of the impacts on the environment and human health caused by the extensive use of agrochemicals, new integrative alternatives are required to control plant diseases. The use of resistance inducers is one of these strategies that could lead to a more durable management of wheat diseases. Because of their plant resistance-inducing activities against pathogens, several protein extract-based products have been studied so far (Kamoun *et al.*, 1993; Takenaka *et al.*, 2003; Dutsadee and Nunta, 2008; Kulye *et al.*, 2012; Zhang *et al.*, 2010, 2014a, 2014b). Therefore, the present study aims to evaluate the potential of three natural resistance inducers (RIs) based on plant cell wall oligosaccharids (Oligos), ascorbic acid (AA) and *A. nodosum* algae extract (*A. nod*), on Tunisian durum wheat to control STB disease. For this study, three Tunisian durum cultivars, characterized by different resistance levels to *Z. Tritici*, were used such as Karim (susceptible), Nasr (moderate resistant) and Salim (resistant) cultivars level of STB resistance was previously demonstrated in tested cultivars (Publication 6).

Diverse events of the infectious process of *Z. tritici* were investigated on Karim (S), Nasr (MR) and Salim (R) cultivars treated with 50, 50 and 500 mg L⁻¹ of Oligos, AA and *A. nod* treatments, respectively compared to corresponding H₂O-treated controls (i). Our results showed that only AA could significantly affect spore germination at 1 dpi and 4 dpi which could be explained by its direct antifungal effect previously demonstrated against *Z. tritici* (Publication 2, Part 1). This inhibition was improved in Salim (R) cultivar suggesting that the cultivar resistance level could affect the germination of St-08-46 *Z. Tritici* spores. Besides, reduced leaf penetration through the adjacent epidermal cells (direct penetration) was noted in Oligos- and AA-treated leaves at 4 dpi. This phenomenon was already observed in resistant untreated cultivar Salim suggesting that it is could be closely related to wheat resistance. Further studies investigating multiple resistant cultivars in interaction with more *Z. tritici* strains are required in order to confirm these results.

During the necrotic phase of *Z. tritici*, our results recorded that all RIs were able to reduce fungal CWDE activities including endo- β -1,4-xylanase, endo- β -1,3-glucanase and protease. It has been shown that, in the wheat-*Z. tritici* pathosystem, CWDEs production is associated with both the virulence level of the fungal strain and with the susceptibility level of the wheat cultivar (Siah *et al.*, 2010; Tian *et al.*, 2009). In our experiments, we confirmed this correlation in susceptible and moderately resistant cultivars Karim and Nasr witch exhibited closed CWDE activities to disease symptoms and sporulation suggesting their use as a an marker of RIs efficacy. However, this correlation was not available in the resistant cultivar Salim, the decrease of CWDE activities was not correlated to the efficacy level of tested RIs.

Furthermore, the investigation of related-defense genes expression in treated susceptible cultivar Karim and resistant cultivar Salim was monitored at non-infectious (ni) context. The infected (i) and untreated control was used as reference. Six genes were studied such as *PR2*, *Chi 4 precursor*, *Pox*, *Msr*, *ATPase* and *Bsi1* genes. *PR2* gene encodes for class II β -1,3-glucanase, which are acidic proteins secreted into the extracellular space (Ferrari *et al.*, 2007). The β -1,3-glucanase catalyzes hydrolytic cleavage of the 1,3- β -D-glucosidic linkages in β -1,3 glucan, which is a component of fungal cell walls (Ryals *et al.*, 1996). *Chi 4 precursor* gene encodes a precursor of an anapoplatic class IV chitinase. Chitinases hydrolyze β -1,4-glycoside bounds in chitin to produce N-acetylglucosamine and N, N+ diacetylchitibiose (Cohen-Kupiec and Chet, 1998). *Pox* gene encodes a peroxidase proteins which use hydrogen peroxide (H_2O_2) as a substrate in reactions involved in biosynthesis of lignin and other phenolic compounds that act as additional antimicrobial barriers (O'Brien *et al.*, 2012; Passardi *et al.*, 2005). Moreover, the *Msr* gene encodes a methionine sulfoxide reductase enzyme catalyzes the reduction of methionine sulfoxides to methionine. It acts as a last-chance antioxidant that repairs proteins damaged by oxidative stress (Romero *et al.*, 2004). *ATPase* encodes ATP synthases witch are multi-subunit enzyme complexes that help to maintain the acidic environment of cellular components by transferring protons across membranes through ATP hydrolysis (Wassmer *et al.*, 2005). *Bsi1* gene encodes a proteinase inhibitor that has been shown to possess antifungal activity (Lorito, 1994).

In our experiment, studied defense genes were differentially responsive to tested RIs. Oligos and AA treatment induced all tested genes, while *A. nod* treatment triggered only PR genes and *ATPase* gene. The elicitation of gene encoding for fungal cell wall degrading enzymes (glucanase and chitinase) and protease inhibitors could explain the observed antifungal effect of AA and Oligos on stomatal penetration, colonization and sporulation. Overall, our results highlighted differential expression of induced resistance in susceptible and resistant durum wheat cultivars. The expression of all studied defense genes was always earlier and significantly stronger in Salim (R) than Karim (S). In this sense, Walters *et al.* (2005, 2011); Bruce (2014) reported that the plant genotype could influence the induced resistance and the protection efficacy conferred by resistance inducers. Previous reviews demonstrated the association of PR genes as *PR2* (Ray *et al.*, 2003; Shetty *et al.*, 2009; Mohammadi *et al.*, 2012), *PR4* (Adhikari *et al.*, 2007; Shetty *et al.*, 2009) and *Pox* (Adhikari *et al.*, 2007; Farsad *et al.*, 2013) genes with wheat resistance against *Z. tritici*. Adhikari *et al.* (2007) and Farsad *et al.* (2013) supported our findings and showed early and strong induction of *Msr*, *ATPase* and *Bsi1* genes up-regulation in resistant bread wheat cultivars suggesting their association with the wheat resistance against *Z. tritici*. Also, in this study we demonstrated that tested RIs enhanced the same related-defense gene involved in durum wheat-*Z. tritici* interaction. In resistant cultivar Salim, *PR2* and *Pox* were more responsive to *A. nod* and AA than the infection by St-08-46

Z. tritici strain, while *Chi 4 precursor* was more responsive to *Z. tritici* than tested RIs probably due to the high presence of chitin in fungal cell wall.

In addition, in this study Oligos, AA and *A. nod* treatments used at 50, 50 and 500 mgL⁻¹, respectively, were able to decrease sporulation and necrosis at greenhouse and field. Oligos and AA treatments were always more efficient than *A. nod* treatment. Significant decrease of *Z. tritici* severity was mainly observed in the susceptible cultivar Karim suggested that the application of RIs on resistant cultivars is useless. Besides, in field AA and *A. nod* treatments improved chlorophyll content of wheat leaves. Likewise, Beltagi, (2008), Dolatabadian *et al.* (2008) and El Harriri *et al.* (2010) reported increased chlorophyll content by exogenous AA in many crops such as chick pea, canola and flax. Similarly, Fan *et al.* (2013) and Morales-Payan (2013) recorded increased chlorophyll levels by *A. nodosum* treatment in spinach and mango. Moreover, tested RIs could be able to increase Karim yield quantity and quality while they could not significantly affect resistant cultivar Salim. *A. nod* and AA were more effective to improve durum wheat yield than Oligos. Many works corroborate with our findings and demonstrated that *A. nod* and AA treatment could improve yield of many crops (Rayorath *et al.*, 2008; El Harriri *et al.*, 2010; Dogra and Mandradia, 2012; Fan *et al.*, 2013; Alam *et al.*, 2013; Sen *et al.*, 2015).

Overall, this study revealed interesting RIs to control STB in durum wheat. Their effect was mainly observed in the susceptible cultivar Karim, while in resistant cultivar Salim their application seems to be useless. They could improve resistance level in susceptible cultivars and allowed to obtain yield quantity and quality closed to the resistant cultivar. Therefore, the use of RIs at the right stage and concentration could replace the use of the resistant cultivars especially with their high cost and their high cost and the continuous decreasing of their level of resistance.

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Conclusion générale et perspectives

Le déficit continu entre la plante et le pathogène nous pousse à explorer de nouvelles alternatives de la protection des plantes. Ainsi, au cours des dernières décennies, des avancées spectaculaires sur les mécanismes impliqués dans la résistance induite chez les plantes ont favorisé le développement d'initiatives visant à valoriser la stratégie de défense naturelle des végétaux dans un contexte conjuguant la préservation de l'environnement et la production intensive des produits agricoles. Aujourd'hui, un nombre croissant de formulations contenant des stimulateurs des défenses naturelles de plantes (SDP) qui permettent la réduction de l'utilisation produits chimiques «conventionnels», tout en apportant des solutions souvent innovantes dans le domaine de la protection des cultures ont vu le jour. Un SDP est un produit biologique capable de déclencher les événements moléculaires, biochimiques et cytologiques menant à l'expression de la résistance chez une plante. Il s'agit donc d'une sorte de « vaccin » capable d'activer le « système immunitaire » de la plante de telle sorte que si elle est initialement sensible à un agent pathogène elle devienne résistante. L'exploitation de la résistance induite en agriculture biologique ou en agriculture raisonnée (intégration entre la lutte biologique et la lutte chimique) est une stratégie qui pourrait offrir de grandes promesses d'avenir, comme elle est essentiellement fondée sur la stimulation des mécanismes naturels de défense des plantes.

Les travaux présentés ici pourraient contribuer à envisager l'usage des SDP dans le cadre des grandes cultures tel que le blé dans l'objectif de protéger contre l'une des principales maladies foliaires les plus dommageables, à savoir la septoriose du blé. Cette dernière est causée par le champignon hémibiotrophe *Z. tritici* (telomorphe *M. graminicola*). Notre objectif principal visait à l'identification de nouveaux SDPs efficaces pour protéger le blé contre la septoriose tout en analysant leur mode d'action aussi bien chez le blé dur (*Triticum durum*) que blé tendre (*Triticum aestivum*). Le deuxième objectif de ce travail vise à mieux comprendre l'interaction compatible du blé dur-*Z. tritici* tout en les comparant à celles du blé tendre ainsi que la caractérisation des mécanismes de défense impliqués dans ces interactions ce qui pourra nous orienter à propos du choix et la stabilité des SDPs qu'on peut intégrer dans les alternatives de lutte contre la septoriose chez les deux espèces de blé.

Après à un premier chapitre introductif, le deuxième chapitre, différents modes de pénétration foliaire adoptés par les deux souches de *Z. tritici* étudiées a pu être mis en évidence. Ce qui suggère que les génotypes de blé peuvent influencer le mode de pénétration foliaire. Aussi, nous avons pu confirmer la corrélation entre l'augmentation de l'activité de trois CWDEs fongiques (endo- β -1,4-xylanase, endo- β -1,3-glucanase et protéase) et la sporulation du champignon au cours de la phase

nécrotrophe. Cette corrélation a été déjà démontrée dans la littérature sur le blé tendre à plusieurs reprises et elle a pu être confirmée chez le blé dur pour la première fois. Ainsi, ces trois enzymes peuvent être considérés comme étant des marqueurs fiables de la sévérité et la sporulation du champignon. Dans la littérature, la majorité des travaux qui ont étudiés l'interaction blé- *Z. tritici* est réalisée sur des cultivars de blé tendre alors que très peu d'information est disponible concernant la réponse moléculaire, biochimique et cytologique du blé dur. Par conséquent, cette partie d'étude a permis de prendre connaissance et de caractériser les réponses de défense basales survenant chez cultivars sensibles à la septoriose à savoir un cultivar de blé dur (Karim) et un cultivar de blé tendre (Premio). Les cultivars Karim et Premio ont été inoculés respectivement par une souche pathogène adaptée au blé dur (St-08-46) et une souche pathogène adaptée au blé tendre (T01193). Au cours de ce chapitre, on a pu conclure que l'infection par les deux souches de *Z. tritici* semble activer les mêmes mécanismes de défense au niveau des cinq premiers jours suivant l'inoculation chez les deux espèces de blé. En effet, suite à l'infection la sur-expression des gènes *PR2*, *Chi4 precursor*, *Pox* et *PAL* associée à l'augmentation de l'activité de plusieurs enzymes tels que la peroxydase, catalase, glucanase, phénylalanine ammonia-lyase et lipoxygénase, ont été notés. Par contre, l'expression du gène *LOX* a été réprimée chez les deux espèces. En revanche, on n'a pas détecté d'accumulation significative de H_2O_2 ni de polyphénols au niveau des sites de pénétration du champignon chez les deux espèces de blé. En conclusion, on peut envisager les mêmes stratégies de lutte par l'utilisation des même SDP pour la protection du blé dur et du blé tendre contre la septoriose.

Au niveau du troisième chapitre, on a présenté 4 SDPs à savoir un produit à base de l'acide ascorbique (AA), d'algue brune *A. nodosum* (*A.nod*), d'oligosaccharides de paroi de plantes (Oligos) et de chitosan ont testés pour la première fois contre la septoriose du blé. L'AA et l'Oligos ont révélé une très bonne efficacité de protection, suivis de l'*A. nod* et ensuite du chitosan. La caractérisation de l'effet antifongique des produits testés a montré l'altération de certaines phases du processus infectieux de *Z. tritici* comme pénétration foliaire, la colonisation des cavités sous-stomatiques, la sporulation et l'activité des CWDEs. Seuls l'effet sur la fréquence et le mode pénétration foliaire sont espèce-dépendants, probablement à cause des différences morphologiques des deux espèces de blé. Seul l'AA a montré un effet sur la germination des spores. En effet, on a pu mettre en évidence que ce produit est capable de générer un stress comparable à une toxine par l'induction de l'expression des gènes de transporteurs membranaires (*MgMfs1*, *MgAtr1* et *MgAtr4*) intervenants dans la détoxification de la cellule fongique et sa protection contre les toxines. Ainsi, l'efficacité élevée de l'AA semblé être due à un effet antifongique directe associé à un effet stimulateur de défense. Par contre, l'efficacité des trois autres produits pourrait être due exclusivement à leur effet stimulateur des mécanismes de défense du blé. Ensuite, nous avons

étudié l'effet des 4 SDP testés sur la stimulation des mécanismes de défense du blé. Cette étude a été menée aussi bien en absence qu'en présence du champignon dans un souci de mesurer l'effet exclusivement éliciteur et/ou potentialisateur de ces produits. Il s'est avéré que la nature du produit peut influencer l'intensité et le type de mécanismes de défense induits chez le blé, ce qui pourrait expliquer la différence d'efficacité observée.

En effet, les 4 produits testés semblent agir par l'induction de la surexpression des gènes PR codant pour la β -1,3-glucanases (*PR2*), précurseur de chitinase de la classe IV (*Chi4 precursor*) et peroxydase (*Pox*) et le gène *LOX* (codant pour l'isoforme 1 de la lipoxygénase) en contexte non infectieux et infectieux. Par contre, le gène *PAL* (codant pour l'isoforme 1 de la phénylalanine ammonia lyase) n'a pas été élicité que suite au traitement à base de l'*A. nod* en contexte infectieux. Aussi, les 4 produits testés auraient une action stimulatrice des activités enzymatiques. En contexte non infectieux, les activités β -1,3-glucanase (GLUC), catalase (CAT) et lipoxygénase (LOX) sont induites par tous les 4 produits testés. La peroxydase (POX) a été induite par tous les produits sauf l'Oligos. Seul l'*A. nod* a présenté un effet éliciteur sur l'activité phénylalanine ammonia lyase (PAL). Tandis qu'en contexte infectieux, les feuilles de blé traitées préventivement et inoculées, ont montré une augmentation des activités testées comparées aux feuilles témoins excepté la GLUC, la POX et la LOX. L'AA semble ne pas avoir un effet potentialisateur sur la GLUC et la LOX. Par contre, l'Oligos et le chitosan n'ont pas montré un effet potentialisateur sur l'activité POX. Concernant l'accumulation des métabolites, seuls l'AA et l'Oligos ont été capables de stimuler l'accumulation des composés phénoliques totaux, aussi bien en absence qu'en présence du champignon. Au niveau cytologique, les feuilles de blé traitées par les 4 produits et principalement l'AA et le chitosan ont révélé une accumulation significative du H₂O₂ et polyphénols en réponse à la pénétration du tube germinatif du champignon dans le mésophile. Ainsi les produits testés semblent affecter le mécanisme de défense primaire, le métabolisme des ROS, la voie des octadécanoïdes ainsi que la voie des phénylpropanoïdes. La rapidité et l'intensité de cet effet est influencé par l'espèce de blé considéré et la présence du champignon. D'autre part, la réponse d'une espèce de blé peut être différente en fonction de la nature du produit appliqué. En revanche, chaque SDP semble induire les mêmes métabolismes de défense chez les deux espèces de blé étudiées.

Le quatrième chapitre de ce manuscrit a permis de caractériser les mécanismes de défense mis en jeu naturellement chez les trois génotypes de blé dur tunisien caractérisés par différents niveaux de résistance vis-à-vis de la septoriose à savoir un cultivar très sensible (Karim), un cultivar moyennement résistant (Nasr) et un cultivar résistant (Salim). En condition semi-contrôlée, ces cultivars ont été inoculés par une souche tunisienne de *Z. tritici* (St-08-46) connue par un niveau élevé de pathogénicité. Concernant les essais au champ, la contamination par la septoriose est naturelle. Au cours de ce chapitre on a pu révéler que le génotype de blé peut affecter le mode de

pénétration foliaire, la sporulation ainsi que l'activité des CWDEs (tels que la endo- β -1,4-xylanase, la β -1,3-glucanase et la protéase) du champignon. La réduction des activités CWDEs au niveau de la phase néotrophe du champignon est étroitement corrélée à la diminution de la sporulation et la sévérité de la maladie. Ce qui suggère qu'ils pourraient être considérés comme étant des marqueurs de résistance variétale. D'autre part, l'expression de 6 gènes impliqués dans la défense du blé, à savoir *PR2* (β -1,3-glucanase), *Chi 4 precursor* (précurseur de chitinase de la classe IV), *Pox* (peroxydase), *Msr* (methionine sulfoxide reductase), *ATPase* (ATP synthase vacuolaire) and *Bsi1* (inhibiteur de protéases), a été réalisée au niveau des 5 premiers jours suivant l'inoculation avec la souche St-08-46 de *Z. tritici* au cours de la phase biotrophe chez le cultivar le plus sensible (Karim) et le cultivar le plus résistant (Salim). Tous les gènes étudiés présentent différents niveaux d'expression basiques chez le cultivar sensible Karim et le cultivar résistant Salim. Dès 24 heures après l'inoculation, on a détecté une surexpression plus importante et plus précoce de tous les gènes étudiés, excepté l'ATPase, chez le cultivar résistant comparé au cultivar sensible. Ainsi, cette étude a permis de mettre en évidence des gènes de défense qui pourraient intervenir dans les mécanismes de défense précoce du blé au cours de la phase biotrophe du développement du champignon et qui expliqueraient l'effet observé sur la pénétration foliaire. Ces gènes pourraient être ainsi considérés comme étant des facteurs de résistance à la septoriose.

Au niveau du cinquième chapitre, une tentative de caractérisation des interactions entre génotype et SDP dans le cadre de la protection du blé dur contre la septoriose en Tunisie a été abordée. La comparaison des différentes combinaisons «SDP-cultivar» montre que la résistance induite par 3 SDPs (AA, Oligos et *A. nod*) vis-à-vis de *Z. tritici* est conditionnée par le génotype de la plante et par la nature du SDP appliqué. Cette étude a révélé une protection de la maladie par les SDPs testés associée à des altérations de certaines phases du processus infectieux ainsi que l'induction de la surexpression des gènes de défense du blé. En effet, seul l'AA a révélé un effet inhibiteur de la germination des spores qui été plus important chez le cultivar résistant. Aussi, les feuilles traitées par l'AA et l'Oligos ont montré une diminution de la pénétration foliaire directe. Ce phénomène a été observé aussi chez le cultivar résistant non traité (Salim) et pourrait être expliqué par l'activation des mécanismes de défense au niveau des cellules du mésophile. Au niveau de la phase nécrotrophe du développement du champignon, les 3 SDP testés ont permis l'inhibition de la colonisation et la sporulation et la sévérité de la maladie associé à une réduction très importante des CWDEs testés surtout au niveau du cultivar sensible Karim. Aussi, l'effet éliciteur des SDPs testés a été mis en évidence. Six gènes impliqués dans la défense du blé, à savoir les gènes *PR2*, *Chi 4 précurseur*, *Pox*, *Msr*, *ATPase* et *Bsi1*. Il s'est avéré que pour un même SDP, le cultivar résistant (Salim) a montré une induction de l'expression des gènes étudiés plus rapide et plus intense que celle observée chez le cultivar sensible (Karim). Par conséquent, la réponse d'un cultivar diffère en

fonction de la nature du produit appliqué. En effet, la nature du SDP et le cultivar de blé pourraient présenter deux facteurs primordiaux affectant chacun la résistance induite obtenue et dont les effets pourraient interagir. En plus, l'efficacité des SDPs testés a conservé le même ordre aussi bien en condition semi-contrôlée qu'en plein champs. Les traitements à base d'AA et Oligos ont permis une bonne efficacité pour contrôler la maladie, suivies de l'*A. nod* qui a montré une efficacité moyenne. Par contre, seuls les traitements à base de l'AA et l'*A. nod* ont permis une amélioration significative de la teneur en chlorophylle et le rendement en quantité et qualité principalement chez le cultivar sensible Karim et ont permis d'obtenir des résultats comparable à celles du cultivar résistant Salim. Par conséquent, l'application des SDPs testés pourraient permettre améliorer la tolérance des cultivars sensibles à la maladie et de remplacer l'utilisation des cultivars résistants surtout avec l'absence en Tunisie de cultivars commercialisés complètement résistant ainsi que la décroissance continue du niveau de résistance des cultivars de blé disponibles. Ainsi, les travaux de ce chapitre nous permettent de conclure que l'efficacité de la protection conférée par les SDPs et la stimulation des mécanismes de défense contre *Z. tritici* dépend du SDP et du cultivar de blé considérés. Ces résultats soulignent l'importance du choix variétal dans le cadre d'une lutte raisonnée associant des SDPs pour lutter contre la septoriose du blé. De façon globale, l'efficacité de protection contre la septoriose est influencée ici par un facteur intrinsèque - le génotype de blé - et par un facteur extrinsèque - la nature du SDP. Ainsi, notre étude révèle des interactions entre les SDPs et les génotypes de blé qui affectent la protection induite contre *Z. tritici*. Ce résultat pourrait être expliqué par le fait que les SDPs pourraient être reconnus par la plante comme des PAMP. Ceci suggère que la réponse du génotype de blé aux SDPs repose sur leur reconnaissance par des récepteurs dont l'abondance et la spécificité peuvent varier selon le génotype de blé. De même, la rapidité de la réponse pourrait être due aux signaux de transduction et/ou protéines de transport présents chez l'un et pas l'autre.

Enfin, l'utilisation des SDP dans le cadre du pathosystème blé-*Z. tritici* paraît être une solution envisageable pour réduire l'utilisation des fongicides au champ puisque nous avons montré ici que l'activation préventive des mécanismes de défense du blé surtout chez les cultivars les plus sensibles pourrait permettre d'augmenter le niveau de résistance vis-à-vis de la septoriose. Le choix de la variété et celui du SDP sont donc des paramètres influençant l'efficacité de la protection, dont il faudrait impérativement tenir compte lors du passage au champ. Par conséquent, les travaux engagés au cours de cette thèse ont permis de souligner la complexité des interactions en jeu dans le pathosystème *Z. tritici* et a révélé d'une part, un certain nombre de marqueurs de résistance du blé à la septoriose et d'autre part, des marqueurs de d'efficacité des SDP contre cette maladie.

Cependant, cette étude a pris un seul modèle d'interaction blé dur-St-08-46 et blé tendre-T01193. L'investigation de la réponse de plusieurs cultivars sensibles de chaque espèce de blé à une

même souche ainsi que l'étude de l'interaction de plusieurs souches du champignon sur un même cultivar, sont indispensables pour confirmer les conclusions tirées de cette étude. Egalement, plusieurs approches sont envisageables ultérieurement en vue de caractériser d'avantage chacune des voies de défenses étudiées, par la réalisation d'autres analyses complémentaires tels que le dosage de la teneur en SA et en JA qui pourrait confirmer l'implication de la voie des phénylpropanoïdes et des octadécanoïdes dans les mécanismes de défense mis en jeu chez le blé contre la septoriose. Il serait aussi judicieux d'explorer les voies de défense étudiées dans cette thèse durant la phase nécrotrophe de *Z. tritici* où ces voies - ainsi que d'autres - pourraient être sollicitées différemment en réponse au changement du mode de vie du champignon. En vue de mieux valoriser et confirmer le rôle des gènes de blé impliqués dans résistance à la septoriose, il serait possible d'envisager l'utilisation de mutants de blé déficients de ces gènes qui sont aujourd'hui disponibles par la technique de TILLING. Une étude complémentaire de l'expression de ces gènes à l'aide des techniques à haut débit telles que les puces à ARN ou Microarray pourrait être très utile. Ces outils permettraient d'identifier la largeur du spectre d'action des SDPs sur d'autres mécanismes de défense, et peut être d'identifier chez le blé des marqueurs de la résistance induite. Par ailleurs, il serait intéressant d'explorer d'autres mécanismes de défense, telle que l'évaluation d'une possible systémie des réactions de défense induites par les SDP testés en les examinant aussi au niveau de la feuille drapeau ou des racines. Une autre perspective intéressante serait d'optimiser l'usage de ces inducteurs de résistance en testant des combinaisons des différents SDPs étudiés (AA, Oligos, *A. nod* et chitosan) ce qui permettrait éventuellement l'établissement d'une synergie entre les différents mécanismes de défense induits par chacun des éliciteurs et ainsi procurer au blé une protection maximale et plus durable contre la septoriose. Dans le même esprit, l'intégration de ces SDPs dans les programmes classiques de protection des cultures avec les fongicides conventionnels serait aussi envisageable. Concernant l'effet sur la colonisation par le champignon, l'utilisation de la souche GFP de ce champignon permettrait de mieux caractériser la pénétration cellulaire. A ce titre aussi, des variétés de blé transgéniques avec des constructions portant à la fin le gène rapporteur GFP fusionné à chacun des gènes de défense étudiés serait très utile à caractériser le lieu et la progression de la transmission du signal de défense.

Annexes

Annexe 1

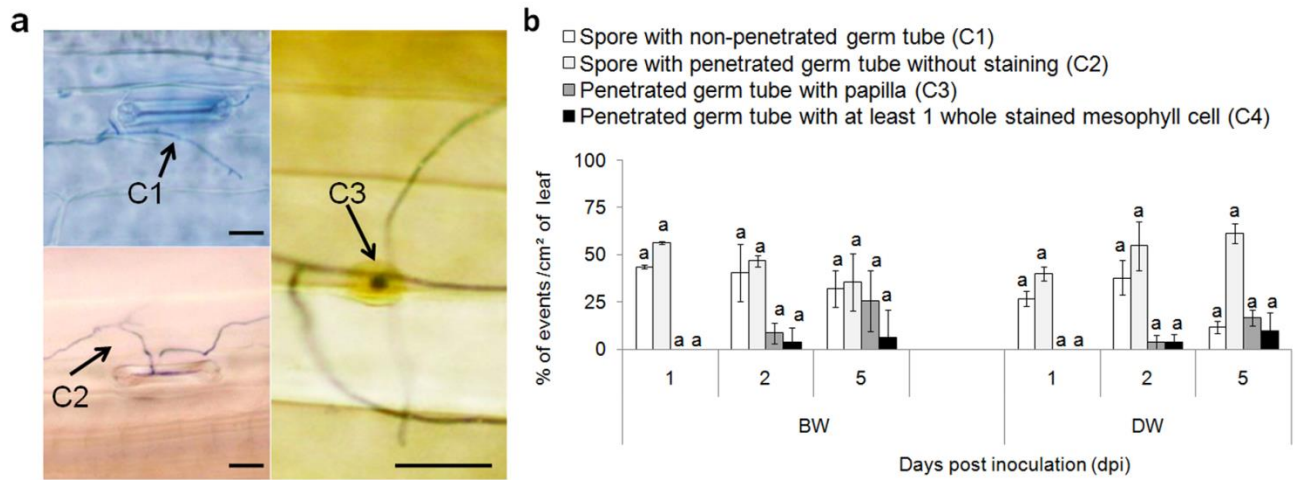


Fig. 6 H₂O₂ accumulation in BW and DW leaves infected by *Z. tritici* strains T01193 and St-08-46, respectively. **a** Microscopic observations of measured classes, spore with non-penetrated germ tube (C1), spore with penetrated germ tube without staining (C2), penetrated germ tube with stained papilla (C3) and penetrated germ tube with at least 1 whole stained mesophyll cell (C4). Bar scale = 10 μm. **b** Percentages of each class in BW and DW leaves. Results correspond to means (±s.d) of two independent experiments using three independent replicates for each experiment. For each class, bars with the same letter are significantly similar between both wheat species using the Tukey test at $P \leq 0.05$

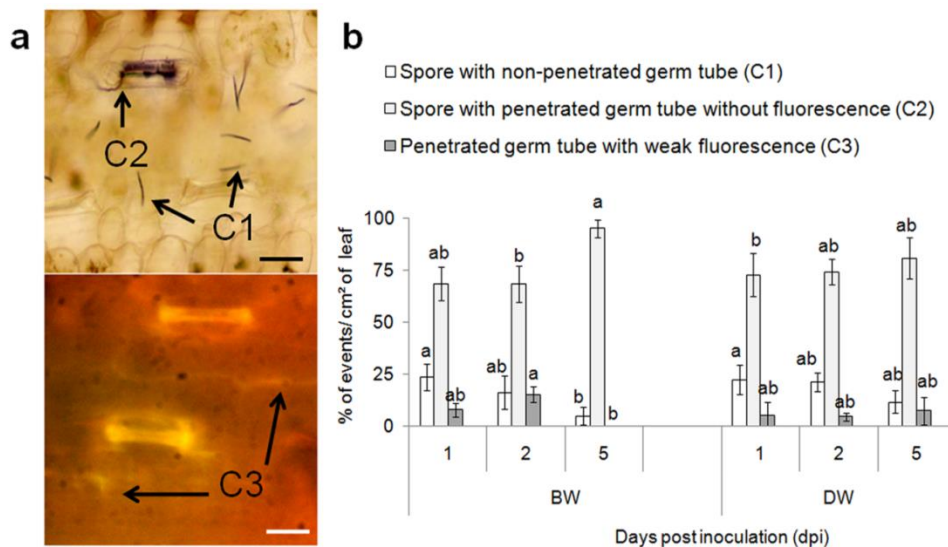


Fig. 7 Polyphenols-associated autofluorescence observed in BW and DW leaves infected by *Z. tritici* strains T01193 and St-08-46, respectively. **a** Microscopic observations of polyphenol accumulation according to autofluorescence visualization in BW and DW leaves: spore with non-penetrated germ tube (C1), spore with penetrated germ tube without fluorescence (C2), penetrated germ tube with weak fluorescence (C3). Bar scale = 10 μm. **b** Percentages of each classes in BW and DW leaves. Results correspond to means (±s.d) of two independent experiments using three independent replicates for each experiment. For each class, bars with the same letter are significantly similar between both wheat species using the Tukey test at $P \leq 0.05$

Annexe 2

A

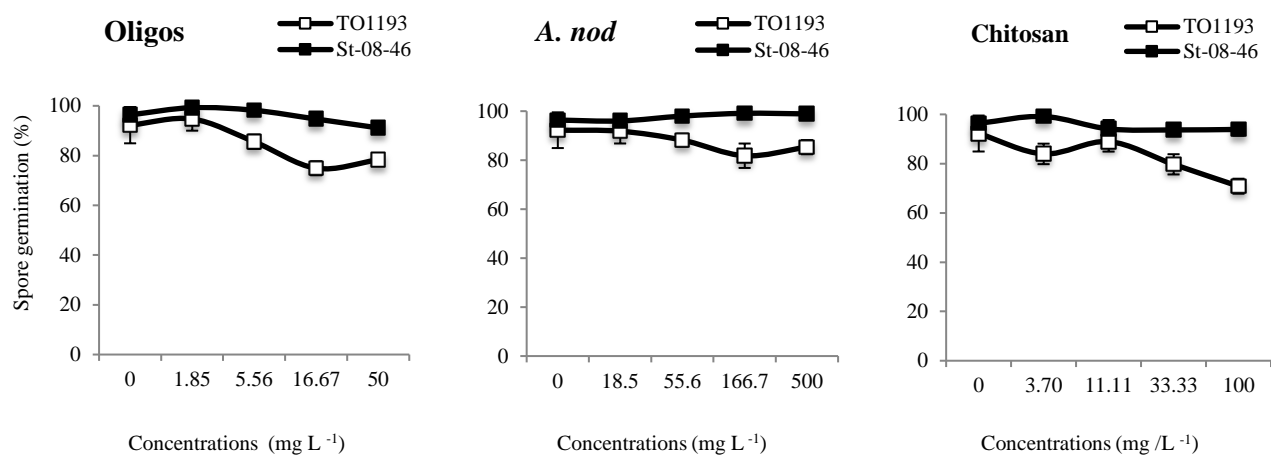


Fig. 1 Percentage of spore germination of T01193 (a, b) and St-08-46 *Z. tritici* strains *in vitro* condition after 24 hours of incubation on PDA medium amended with different concentrations of Oligos, *A. nod* et chitosan.

B

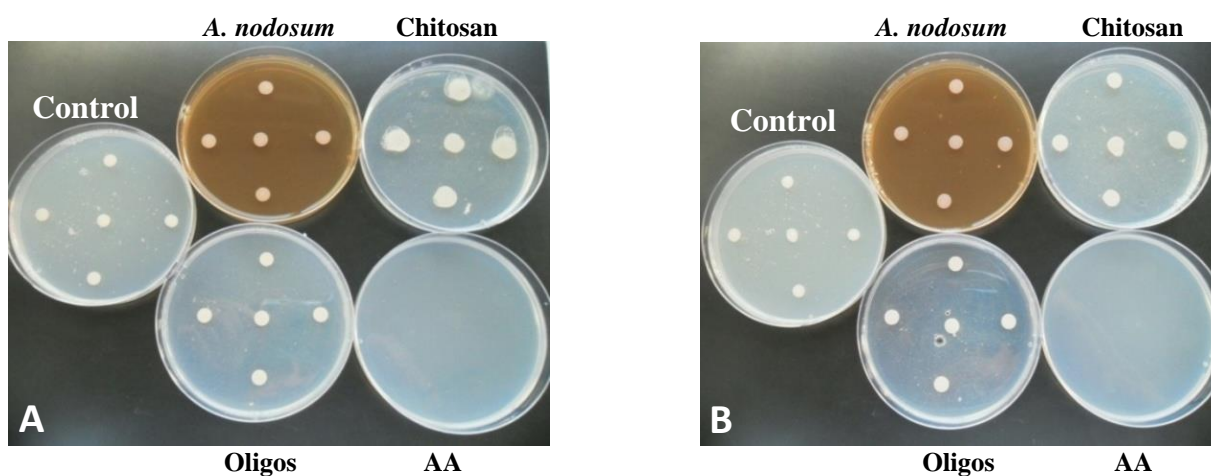


Fig. 2 Hyphal growth of T01193 (a, b) and St-08-46 *Z. tritici* strains *in vitro* condition after 10 days of culture on PDA medium amended with 50, 50, 500 and 100 mg L⁻¹ of AA, Oligos, *A. nod* et chitosan, respectively.

Annexe 3

Table 1 Phenols content in BW and DW leaves at 0, 1, 2 and 5 dpi with T01193 and St-08-46 *Z. tritici* strains, respectively. It is expressed in mg-equivalents of catechin (CE) per gram of fresh weight (FW)

	dpi	BW		DW	
		H ₂ O	T01193	H ₂ O	St-08-46
Polyphenol content (mg CE mg ⁻¹ FW)	0	10.7±1.8 b	10.7±1.8 b	7.7±0.9 ab	7.7±0.9 ab
	1	11.6±2.6 ab	11.8±1.9 a	7.8±1.9 ab	9.9±2.6 a
	2	11.4±3.4 ab	11.6±1.1 a	6.7±2.9 b	9.1±2.2 ab
	5	11.0±4.1 ab	11.8±1.1 a	8.3±3.1 ab	9.3±2 ab

Data are mean values of two independent experiments (±s.d). Statistics were realized separately for each wheat specie and means tagged with the same letter are not statistically different in the same wheat species (Tukey test, $P < 0.05$)

Table 2 Phenols content (expressed in mg of CE g⁻¹ of FW) on BW and DW leaves, at 0, 1, 2, 3, 4 and 7 days post treatment (DPT) by *A. nod* in (ni) and (i) condition. The inoculation of BW and DW leaves by T01193 and St-08-46 *Z. tritici* strains, respectively, was carried out 48 hours post *A. nod* treatment.

Path system	DPT	Non infectious context		Infectious context	
		H ₂ O (ni)	<i>A. nod</i> (ni)	H ₂ O (i)	<i>A. nod</i> (i)
T01193/BW	0	8.2±2.3 a	8.2±2.3 a	-	-
	1	11.3±3.9 a	12.1±2.8 a	-	-
	2	10.7±1.8 a	13.3±3 a	-	-
	3	11.6±2.6 a	14.3±2.8 a	11.8±1.9 a	12.1±2.7 a
	4	11.4±3.4 a	12.0±3.1 a	11.6±1.1 a	11.8±3.5 a
	7	11.0±4.1 a	11.6±3.1 a	11.8±1.1 a	12.3±2.4 a
St-08-46/DW	0	6.4±2.7 a	6.4±2.7 a	-	-
	1	8.3±3.4 a	9.3±1.7 a	-	-
	2	7.7±0.9 a	8.9±1.9 a	-	-
	3	7.8±1.9 a	7.8±1.2 a	9.9±2.6 a	9.9±1.4 a
	4	6.7±2.9 a	9.1±2.6 a	9.1±2.2 a	10±2.4 a
	7	8.3±3.1 a	7.8±3.1 a	9.3±2 a	11±2.2 a

Results correspond to means (±s.d) of two independent experiments using three independent replicates for each experiment. Statistics were made separately for each wheat specie. For each wheat specie, *A. nod*-treated samples with an asterisk (*) are different from their corresponding H₂O-treated controls (Tukey's test, $P \leq 0.05$).

Table 3 Phenols content (expressed in mg of CE g⁻¹ of FW) on BW and DW leaves, at 0, 1, 2, 3, 4 and 7 days post treatment (DPT) by Chitosan in (ni) and (i) condition. The inoculation of BW and DW leaves by T01193 and St-08-46 *Z. tritici* strains, respectively, was carried out 48 hours post Chitosan treatment.

Pathosystem	DPT	Non infectious context		Infectious context	
		H ₂ O (ni)	Chitosan (ni)	H ₂ O (i)	Chitosan (i)
T01193/BW	0	8.2±2.3 a	8.2±2.3 a	-	-
	1	11.3±3.9 a	12.8±6.3 a	-	-
	2	10.7±1.8 a	11.1±3.4 a	-	-
	3	11.6±2.6 a	13.3±4 a	11.8±1.9 a	14.6±1.2 a
	4	11.4±3.4 a	11.7±3.9 a	11.6±1.1 a	14±0.6 a
	7	11.0±4.1 a	12.2±3.4 a	11.8±1.1 a	16.4±4.7 a
St-08-46/DW	0	6.4±2.7 a	6.4±2.7 a	-	-
	1	8.3±3.4 a	8.5±1.5 a	-	-
	2	7.7±0.9 a	7.8±1.1 a	-	-
	3	7.8±1.9 a	11.1±2.8 a	9.9±2.6 a	9.5±1 a
	4	6.7±2.9 a	9.9±1.9 a	9.1±2.2 a	9.9±2.1 a
	7	8.3±3.1 a	10.2±3.7 a	9.3±2 a	10±0.8 a

Results correspond to means (±s.d) of two independent experiments using three independent replicates for each experiment. Statistics were made separately for each wheat specie. For each wheat specie, Chitosan-treated samples with an asterisk (*) are different from their corresponding H₂O-treated controls (Tukey's test, $P \leq 0.05$).

Annexe 4

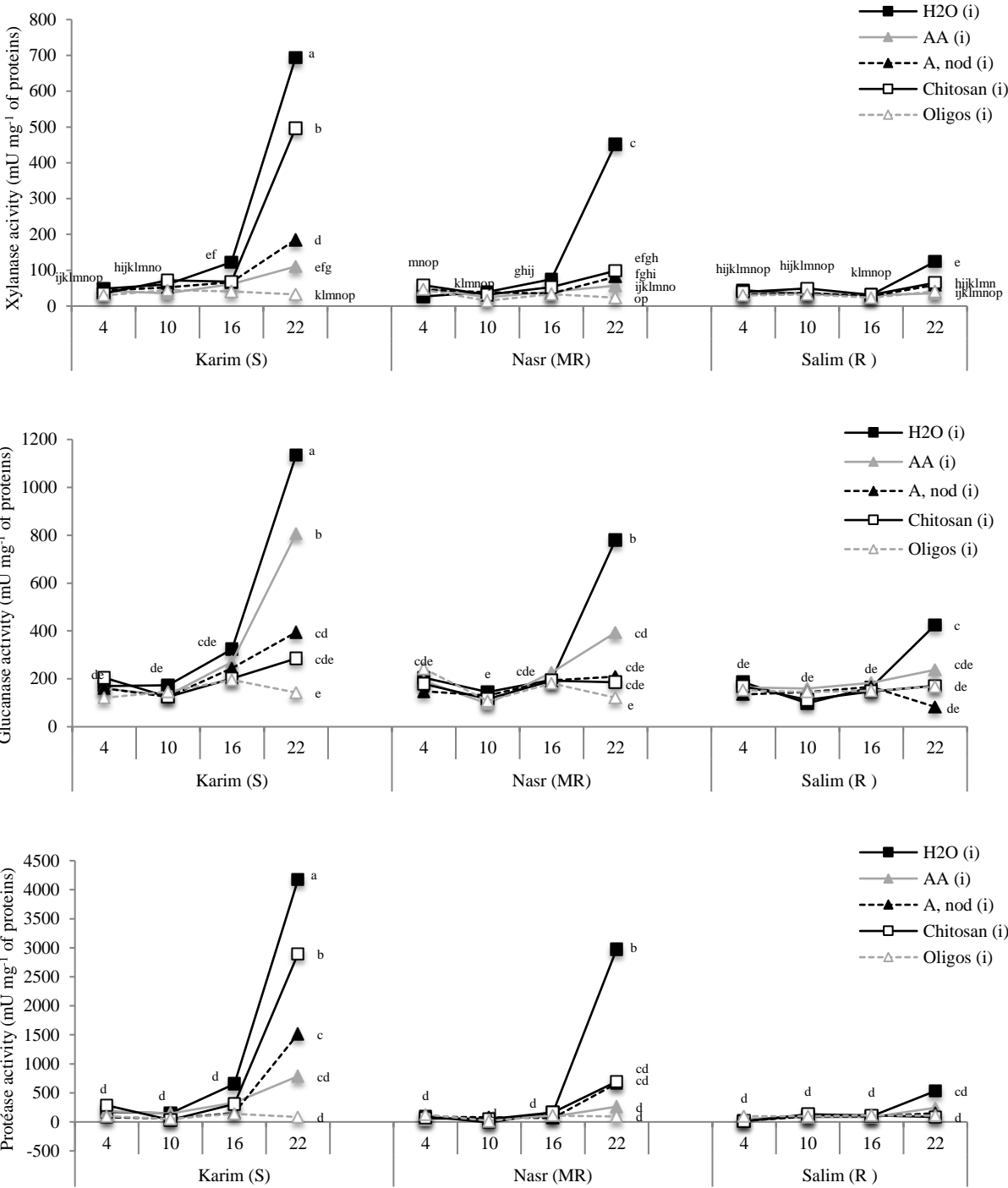


Fig. 1 CWDE activities produced by St-08-46 strain at 4, 10, 16 and 22 dpi on three durum wheat cultivars (Karim (S), Nasr (MR) and Salim (R)) treated with Oligos, AA and *A. nod* treatments compared to the reference H₂O-treated (i) control. For each time point, means tagged with the same letter are not significantly different using the Tukey test at $P \leq 0.05$

Stimulateurs des défenses naturelles du blé dur en Tunisie et du blé tendre en France contre la septoriose causée par *Zymoseptoria tritici*

Le blé dur (*Triticum durum* Desf, BD), tout comme le blé tendre (*Triticum aestivum* L. em Thell, BT), est une céréale très touchée par la septoriose, une maladie foliaire causée par le champignon hémibiotrophe *Zymoseptoria tritici*. D'une part, ce présent travail a permis d'étudier l'interaction compatible du blé-*Z. tritici*. L'étude de l'interaction compatible chez les pathosystèmes BD/St-08-46 et BT/TO1193 a révélé l'induction des mêmes voies de défense chez les deux pathosystèmes étudiés mais avec différentes intensités. Ensuite, l'étude de l'interaction de *Z. tritici* avec un cultivar résistant de blé dur a mis en évidence l'association de résistance au champignon est liée à l'inhibition de la pénétration directe, la sporulation et l'activité des enzymes fongiques de dégradation des parois cellulaires de la plante (endo- β -1,4-xylanase, endo- β -1,3-glucanase et protéase). Ces derniers sembleraient être fortement liés à la sévérité de *Z. tritici* aussi bien chez le dur que le blé tendre. En plus, on a pu démontrer moyennant des analyses qRT-PCR l'intervention de plusieurs gènes dans la résistance du blé dur à la septoriose à savoir les gènes *PR2* (β -1,3-glucanase), *Chi 4 precursor* (précurseur de chitinase de la classe IV), *Pox* (peroxydase), *Msr* (méthionine sulfoxide réductase) et *Bsi1* (inhibiteur de protéases). D'autre part, le potentiel des stimulateurs des défenses naturelles de plantes (SDPs) à protéger aussi bien le blé dur que le blé tendre contre les maladies fongiques a été évalué. Trois extraits naturels dont les matières actives sont l'acide ascorbique (AA), des oligosaccharides de parois cellulaires de plantes (Oligos) et algue brune (*Ascophyllum nodosum*, *A. nod*) ont été testés pour la première fois sur blé. Leur effet antifongique (direct) ainsi que l'effet inducteur des mécanismes de défense du blé (indirecte) ont été bien caractérisés moyennant des analyses moléculaires, biochimiques et cytologiques. En effet, seul l'AA a montré un effet direct sur la germination des spores et la croissance mycélienne du *Z. tritici* associé à l'induction des mécanismes de défense du blé. Par contre, les protections obtenues par l'Oligos et l'*A. nod* semblent être exclusivement liées à leurs propriétés inductrices de la défense qui ont permis de ralentir le développement du champignon ainsi que d'inhiber l'activités des CWDEs fongiques et la sporulation. D'ailleurs, Il est avéré que les SDPs testés sembleraient agir sur les mêmes mécanismes de défense chez les deux espèces de blé. Ils pourraient induire l'activation des (i) protéines PR, (ii) les enzymes antioxydants (peroxydase et catalase), (iii) les protéines PAL et LOX (enzymes clés de la voie des phénylpropanoïdes et la voie des octadécanoïdes, respectivement) et (iv) l'accumulation des H₂O₂ et le dépôt des polyphénols au niveau des sites de pénétration du champignon, ont été mis en évidence. Egalement, ils pourraient empreinter les mêmes voies utilisées par le cultivar résistant Salim en réponse à l'infection par le champignon et pourraient même induire une réponse plus importante des gènes de défense du blé dur tels que les gènes *PR2*, *Pox*, *Msr*, *ATPase* et *Bsi1*. De même, deux applications (préventif et curatif) des SDPs testés a révélé une protection intéressante contre la maladie associée, dans le cas de l'*A. nod* et AA, à une augmentation de la teneur en chlorophylle et l'amélioration de la quantité et la qualité du rendement du cultivar sensible Karim, par contre pour le cultivar résistant Salim l'application des SDPs semble être inutile. En conclusion, l'application des SDPs au bon stade et avec les bonnes concentrations sur des cultivars sensibles pourraient aboutir à des résultats d'efficacité et rendement similaires à celles des cultivars résistants. Ainsi, elle pourrait remplacer l'utilisation des cultivars résistants surtout avec l'absence de cultivars complètement résistants disponibles pour l'agriculteur en Tunisie.

Mots clés : Blé dur, blé tendre, *Zymoseptoria tritici*, stimulateurs de défense des plantes, efficacité, résistance variétale.