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Defense Responses of Wheat Seedling under Biotic Stress Mimicked by the Linear β-(1,3)-Glucan

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Abstract- Reactive oxygen species (ROS) production causes damage, and to better deal with the toxic effects of ROS, the seeds have developed detoxification mechanisms, among which the enzymes of the antioxidant system (catalase, superoxide dismutase, ascorbate peroxidase). Another result supports the link between ROS and redox regulation catalyzed by redoxin family in the seed. Among which, thioredoxins (Trxs) and peroxiredoxins (Prxs), particularly 1-Cys-Prx, expressed during maturation and germination steps.

This study aim to assess the effect of the laminarin (β-(1,3)-glucan) as an elicitor in the triggering of the natural defenses of the wheat seedling. Also, the fungus *Fusarium oxysporum*, known to attack wheat during maturation, exhibit a high chitin / glucan ratio. Thus, we have followed the expression of several markers known for their roles in the protection against oxidative stress, including 1-Cys-Prx, a protein-specific of cereals seed, as well as catalase and ascorbate peroxidase enzymes of the antioxidant system. The β-(1,3)-glucan induce the expression of pathogenesis related genes known for their antimicrobial activities. The triggering of defense mechanisms using exogenous elicitors, allowed to developed new strategies of phytosanitary protection more respectful of the environment.

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I. Introduction

Wheat is the most important cereal crop worldwide in terms of production and utilization. It is the most widely grown cereals and is a one source of energy, protein, and dietary fiber in human nutrition. The gluten proteins constitute up to 80% to 85% of total flour protein, and confer properties of elasticity and extensibility that are essential for the functionality of wheat flours. The gliadins and glutenins constitute each around 50% of the gluten proteins. Consequently, the most exciting and most researched areas in cereal chemistry and technology are the composition of wheat storage proteins and finding the relation between composition and functional properties [1,2,3] to improve the technological and rheological qualities [4,5]. However, in addition to the composition and the structural organization of the storage compounds, wheat seed quality depends on a several parameters including the activation of the metabolism of seed cells upon imbibition and the protection of tissues against oxidative stress during seed germination and desiccation [6].

Germination is a very complex process and is affected by many factors [7]. Minimum accumulation of reactive oxygen species (ROS) and enhanced activity of enzymes affect seed quality; thus, germination potential [8]. Thus, redox control is a critical determinant of these processes. Redox control of enzyme activities by thiol/disulfide exchange provides a mechanism for equilibrating the oxidation state of proteins of the surrounding environment. Mainly related to the activity of many seed enzymes and storage proteins [9, 10]. The redoxin family characterized by the typical motif -CXXC- in which two cysteines are separated by two other residues are among the proteins involved in redox control [11,12]. This motif is found in proteins of the thioredoxin (Trxs) belonging to the redoxin family. Plant Trxs are classified into different types (*m*, *f*, *x*, *y*, *o*, and *h*), depending on their characteristic: primary structure and cellular location. Trx *h* proteins are abundant in cereal seeds both during development and in germinating seeds, which reduces storage and α-amylase inhibitors [13, 14, 15, 16]. Trxs *h* are involved in the mobilization of storage proteins at the early stages of germination [17]. This role is proved by the identification of a large number of proteins as Trxs targets in cereals grain [16, 18]. Down-regulation of wheat Trx*h* induced easily forming glutenin macropolymers and the resistance of storage proteins to degradation [19]. Trxs *h* are involved in a complex interplay with other redox regulators such as glutaredoxin (Grx), glutathione (GSH) or peroxiredoxins (Prxs), also implicated against the oxidative stress. The Prxs are thiol-dependent antioxidants, containing one (1-cysteine [1-Cys] or two (2-Cys) conserved Cys residues. In barley (*Hordeum Vulgare*), the 2-Cys BAS1 exhibit an antioxidant activity [20, 21]. The 1-Cys Prxs are involved in the protection of the aleurone and embryo cells under oxidative stress by using the NADPH-dependent thioredoxin reductase (*NTR*)/*Trx* system, which localized in the nucleus [22]. The expression of the 1-Cys-Prxs increase during the late seed development, the protein is detected in embryo of mature seeds and the level increase with development age [23]. Under normal

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growth conditions the 1-Cys-Prxs have been proposed in maintaining dormancy [23, 21]. Under unfavorable conditions, dormancy have a beneficial effect for the seed because this property improve the possibility of survival during germination process. However, 1-Cys-Prxs are involved in sensing environmental conditions by the inhibition of germination in oxidative stress [26]. Other results suggest that 1-Cys-Prxs function not only to relieve mild oxidative stress but also as molecular chaperones under severe conditions during seed germination and plant development [27].

Antioxidant systems which act as ROS scavenger in seed biology play a major role in the growth processes during seed development. Some protective mechanisms involving ROS scavenging enzymes, such as catalase (CAT) [28] and, Ascorbate peroxidase (APX) [29]. Antioxidant enzyme CAT, which allows the tolerance of plant under stress conditions, is also recognized to be involved in the physiology of germination [30,31,32,33]. CAT activity in seeds and seedlings is also involved in the preservation of viability during storage, and necessary for seed germination and early seedling growth [34,35]. This enzyme has been used for determining the viability of seeds and germination capacity [36]. There are several studies about changes in enzyme activity that are related to germination, development, and tolerance of plants [37,38, 39, 40].

The importance of the CAT enzyme was signaled during the germination, the activity of CAT increases most drastically under unfavorable conditions such as saline stress during the early steps of germination [41,42], indicate that CAT is one of enzymes detoxifying hydrogen peroxide.

In addition to abiotic stress, biotic stress poses threats to wheat growth and production by reducing the germination of the seedling. Since the pathogenic fungi represent a significant constraint to wheat production. Pathogenesis related (PR) proteins involved in the general defense response of plants include glucanases and chitinases [43]. Generally, these enzymes increase in early plant-pathogens interactions. There are two distinct signal pathways in plant disease resistance; salicylic-dependent and jasmonic-acid-dependent pathways [44]. These two pathways induce different sets of defense-related genes. PR proteins, such as glucanase and chitinase, are generally induced by the salicylic-dependent pathway and degrade cell walls of fungal pathogens that contain glucan and chitin [45, 46].

As yet, most studies showed the effect of abiotic conditions in the expression of enzymes, and their responses to oxidative stress during different phases. In the present study, we attempt to gain more understanding on seed physiology. For this, wheat seeds (cv Soissons) were germinated under biotic stress mimicked by the linear β-(1,3)-glucan elicitor [47]. The objective of this study was to measure the response of wheat seedling by monitoring the expressions of 1-Cys-Prx, Trx *h*1, CAT, APX, and molecular markers of biotic stress such as the PR proteins (PR.1.1) and β-(1,3)-glucanase (Glu3). The final goal is to identify the markers able to enhance the germination of the wheat seeds under biotic stress.

II. Materials and Methods

a) Biological material and growth conditions

Seeds of Wheat (*Triticum aestivum*, cv Soisson) were sterilized by soaking in a 10% sodium hypochlorite for 20 min. Seeds were washed several times in sterile water and incubated in HCl 10 mM buffer for 5 min, followed by several rinses with sterile distilled water. The wheat seeds were germinated in the dark under controlled conditions. Biotic stress was simulated with a 200 μg. mL^{-1} of laminarin (linear β-(1,3)-glucan) from *laminaria digitata* algae (Sigma), and samples were collected at 0h, 12h, 24h, 48h and 72h after imbibition. Control was allowed germinating in the same conditions of biotic stress.

b) RNA extraction and Real-time quantitative PCR

Total RNA for qRT-PCR was isolated from wheat seedling after treatment with 200 μg.mL^{-1} of laminarin solution at 0h, 12h, 24h, 48h, and 72h. Total RNA was extracted using the RNeasy plant mini kit (Qiagen) following the manufacturer's instructions. Before qRT-PCR, RNA was treated with RNase-free DNase I (Qiagen) to eliminate any contaminating genomic DNA. cDNA was synthesized from 1 μg of total RNA using high capacity cDNA reverse transcription kit (Applied Biosystems) following the manufacturer's instructions. The PCR mix included 1X SYBR Green Master Mix (Applied Biosystems), cDNA template, and each of the forward and reverse primers (Table 1). The PCR cycling parameters were 20 s at 95 °C, followed by 40 cycles at 95 °C for 30 s, 60 °C for 30 s and finally 15 s at 95 °C. After completion of the cycling parameters, dissociation melt curve analyses (60 °C for 1 min, followed by 15 s at 95 °C and 15 s at 60 °C) were conducted to eliminate the effects of primers dimer formation and contamination. The qRT-PCR was performed in triplicate, and the negative controls included water and mRNA before reverse transcription. The genes investigated were 1-Cys-Prxs, CAT, PR.1.1 and Glu3. For the CAT gene, a TaqMan Universal PCR Master Mix (Applied Biosystems), gene primers and probes were used. 18S rRNA and β-tubulin genes were used as internal controls for the levels of cDNA. The relative fold changes were calculated according to the $2^{-\Delta\Delta CT}$ method [48].

Table1: Primers used in qRT-PCR

Name	Primer sequence 5'>3'
rRNA18S	AGTAAGCGCGAGTCATCAGCT
	CATTCAATCGGTAGGAAGCGAC
TUBβ	TCCCAACAACATCCAGACCG
	TCCATACCCTCGCCAGTGTA
1-Cys-Prx	CGACCAGCTAGCTTTGATTG
	AAGCGCGGAGCTAGC
PR1.1	ACTACGACTACGGGTCCAACA
	TCGTAGTTGCAGGTGATGAAG
TRXh1	AAGAAGCTGGTGGTCATTGACTT
	AAGTCAATGACCACCAGCTTCTT
GLU3	CCTTGCCTCTTTGTATGCCTGA
	TCATCTTTTGTGGGTTCTTGC
CAT	CTCGGCCAGAAGCTCGC
	GATTTGCGCACTCCATGGA
	FAM AGC-CGT-CTC-AGC-TCC-AAG-CCG-A TAMRA

c) *Immunoblot analysis*

Proteins were extracted from wheat seedlings treated with 200 μg.mL^{-1} of the linear β-(1,3)-glucan solution and harvested at 0h, 12h, 24h, 48h, and 72h. Extraction was performed from each sample using 50 mM Tris-HCL (pH 8), one mM EDTA, and 0.5 mM PMSF. Proteins were separated in 4-12 % Nu-PAGES Gel using X-Cell SureLock™ Electrophoresis Cell (Invitrogen), transferred onto polyvinylidene fluoride membranes (Bio-Rad). Immuno-detection of protein was performed using antibodies against 1-Cys Prx and Trx*h*1 coupled with rabbit anti-chicken Ig Y (Ig G) (Sigma) and detected with sigma Fast ™ BCIP/NBT Tablets (Sigma). Finally, the quantification of the 1-Cys Prx and Trx*h*1 proteins were performed with the QuantiScan Software.

d) *Catalase and ascorbate peroxidase activities*

Proteins were extracted from wheat seedlings treated with 200 μg.mL^{-1} of the linear β-1,3-glucan solution and harvested at 0h, 12h, 24h, 48h, and 72h. Sample (0.5g) was ground in liquid nitrogen and then homogenized at 4°C in 1 ml of 0.2 M phosphate buffer pH 7.3 containing 1 mM EDTA, 1 mM ASA (ascorbate) and 2 mM 2-mercaptoethanol. The homogenate was rapidly centrifuged at 14000g for 15 min. The supernatant was used for enzyme assays and protein determination. Catalase activity was assayed measuring the rate of decrease of the absorbance of hydrogen peroxide at 240nm (ε= 39.4 M^{-1} cm^{-1}) in 3 ml of 0.2 M phosphate buffer pH 7 containing an aliquot of supernatant and 4 mM hydrogen peroxide [49]. For APX, activity was determined by measuring the decrease in absorbance at 290 nm, according to Chen et Asada (1989). The 1 ml reaction mixture contained 100 mM KPO4 (pH 7.5), 0.5 mM ascorbate, 0.2 mM H_2O_2, and 20 μl of extraction solution. APX activity was expressed as μmol ascorbate oxidized min^{-1}g^{-1}FW.

III. Results

Laminarin-treated seeds showed a development of stressed seeds compared to control (Fig. 1). This development of wheat seeds could be related to the fact that laminarin acts as an elicitor, inducing a mechanism at the molecular level which accelerates metabolic processes.

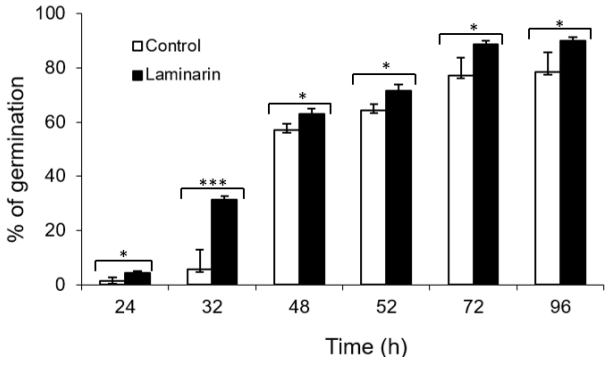

Figure 1: Monitoring germination of control and laminarin-treated wheat seeds.

a) *Study of Peroxyredoxine-1 (1-Cys-Prx)*

The expression and accumulation of 1-Cys-Prx transcripts and proteins during the maturation of wheat seed by qRT-PCR and western blot analysis, increases to reach its maximum at the end of this stage (35 DAA) (Fig. 2).

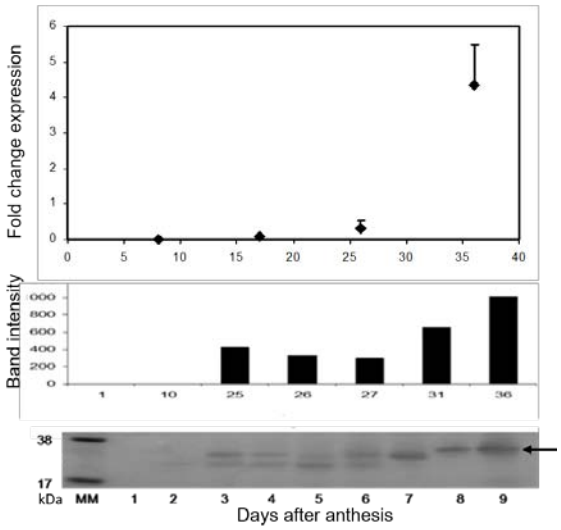

Figure 2: Expression pattern of soft wheat 1-Cys Prx transcript and protein in seed at the ripening stage.

b) 1-Cys-Prx transcript and protein expression

The *1-Cys Prx* gene expression is measured by qRT-PCR as described in experimental procedures, during seedling germination. Under laminarin treatment, the *1-Cys-Prxs* are highly expressed during the early steps of germination mainly at 24 and 48h (Fig. 3A), in comparison with the control whose expression decreases along with the germination (Fig.3A). To follow the accumulation of 1-Cys-Prxs proteins, a western blot analysis on extracts proteins from stressed and control seedling and quantification were performed on the band corresponding to 1-Cys-Prxs. The results show that 1-Cys-Prxs proteins increase gradually during the germination to reach a maximum at 48h and 72h (Fig. 3B, 3C), corresponding to the transduction phenomena. However; in the control, the proteins expression decreases throughout the germinating process. It is important to note that the gene expression of 1-Cys-Prxs decreases under normal conditions during germination.

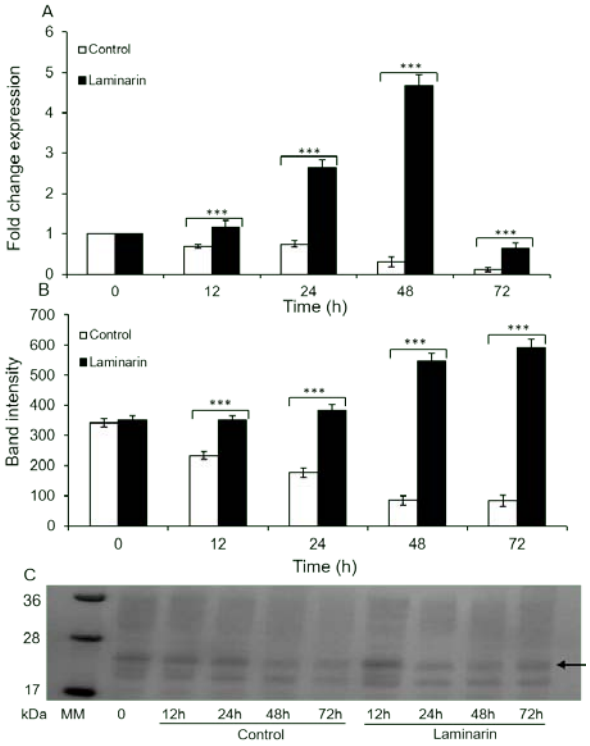

Figure 3: Wheat seeds were germinated in the presence of 200 $\mu g.mL^{-1}$ of laminarin solution, and samples were harvested at 0, 12h, 24h, 48h, and 72h after imbibition. A) Time course of 1-Cys Prxs gene expression using qRT-PCR in wheat seedling after treatment. B) Quantification of 1-Cys Prxs protein by QuantiScan software after western blot analysis. The values represent the mean from 3 biological replicates

c) Trx h1 gene and protein expressions

The result obtained by qRT-PCR showed that laminarin treatment has a strong effect on Trx *h*1 gene expression during the earliest stages of germination (Fig. 4A). However, the western blot analysis using an antibody against Trx*h*1 showed the detection of two bands (a) and (b) corresponding to the Trx*h*1 proteins isoforms. The expressions were identical between control and biotic stress until 48h (Fig. 4B, 4C), however; the accumulation of Trx*h*1 proteins increase considerably at 72h under laminarin treatment compared with the control.

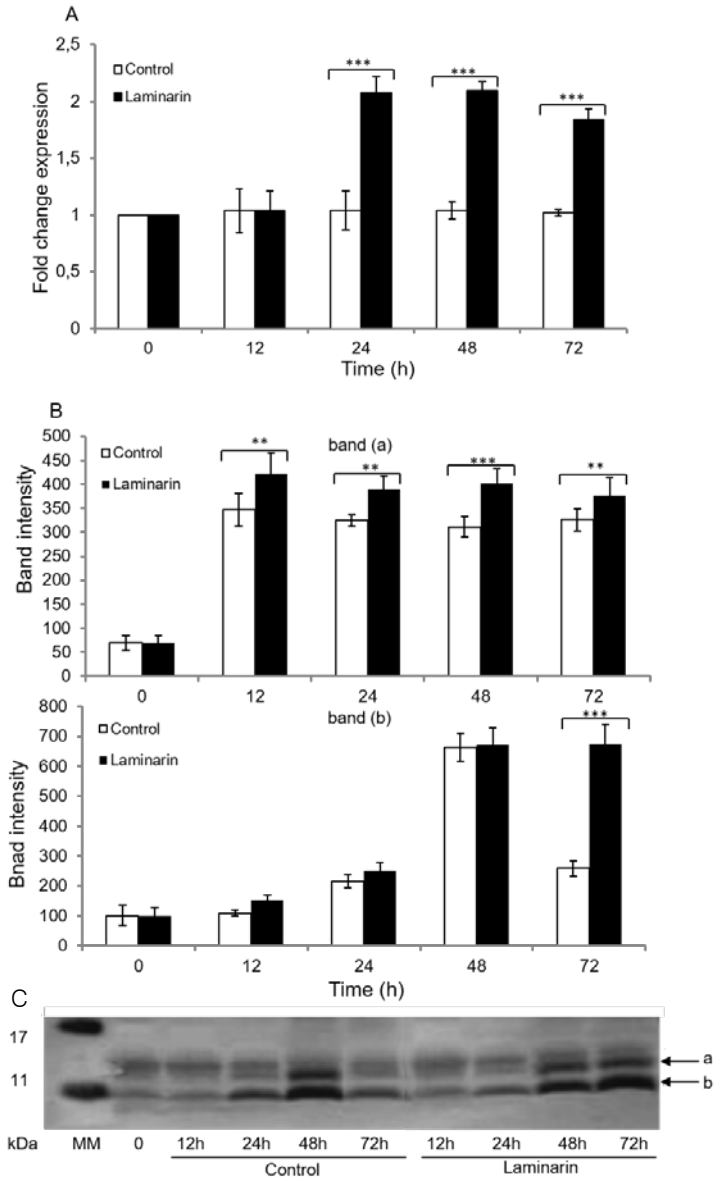

Figure 4: Wheat seeds (*Triticum aestivum*, cv Soisson) were allowed to germinate in the presence of 200 µg.mL-1 of laminarin solution, and samples were harvested at 0h, 12h, 24h, 48h, and 72h after imbibition. A) Time course of Trxh1 gene expression using qRT-PCR in wheat seedling after treatment and B) Western blot analysis of Trxh1 expression using the anti-Trxh1 antibody. a and b) Quantification of Trx *h*1 protein isoforms (a and b) by QuantiScan software after western blot analysis. The values represent the mean from 3 biological replicates

d) Catalase expression

The mRNA transcripts catalase increases during germination under laminarin treatment (Fig. 5A). The higher levels were observed at 24h and 48h (fig. 5A) before decrease afterward. However, in control, the expression remains almost unchanged along with the germination. The measure of CAT activity in the presence of hydrogen peroxide showed that under biotic stress, the activity increase throughout the germination to reach a maximum at 72h (fig. 5B).

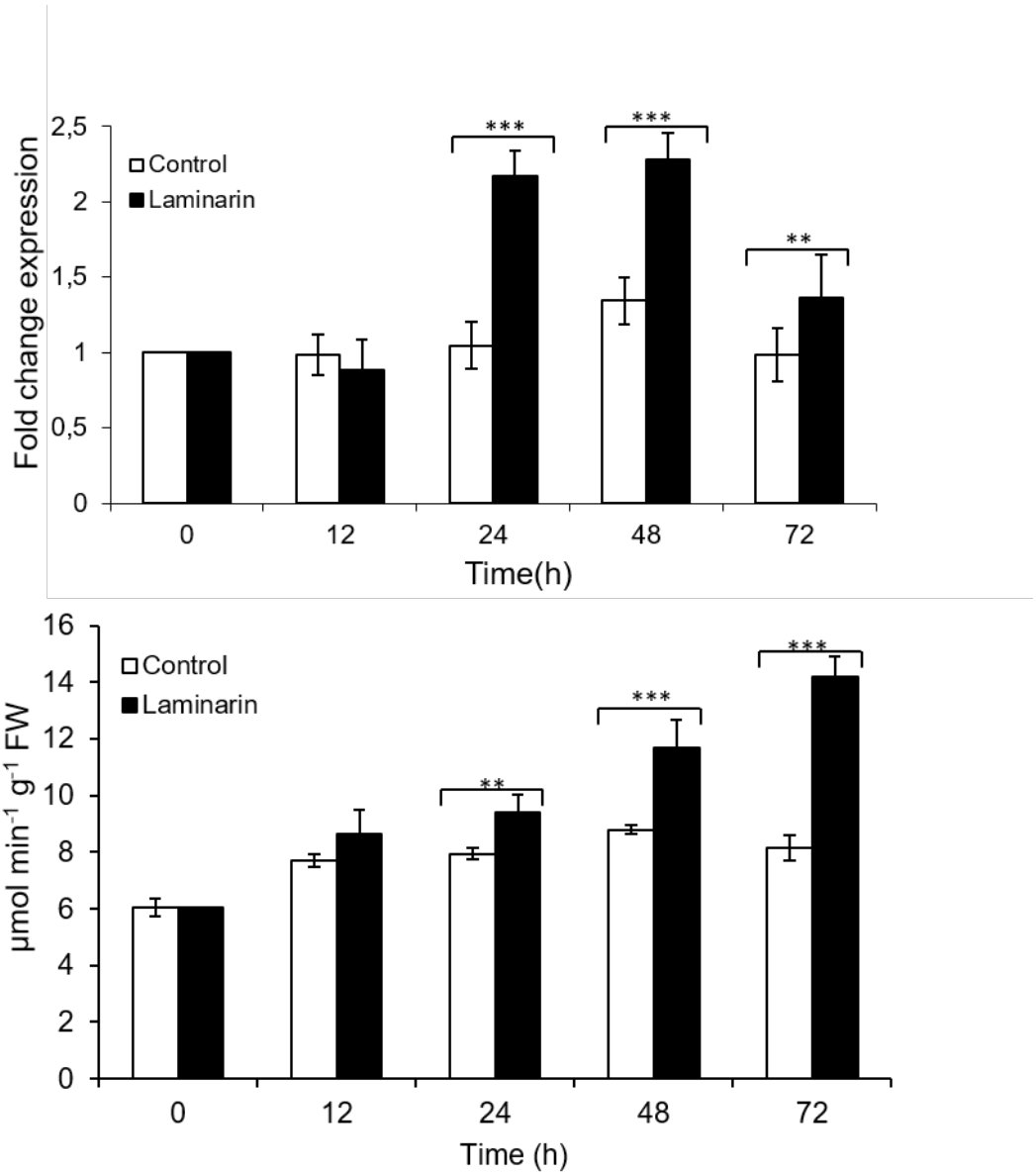

Figure 5: Wheat seed were germinated in the presence of 200 μg.mL^{-1} of laminarin solution, and samples were harvested at 0h, 12h, 24h, 48h, and 72h after imbibition. A) Time course of catalase genes expression using qRT-PCR in wheat seedling after treatment. B) Catalase activity measured the rate of decrease of the absorbance of hydrogen peroxide. The values represent the mean from 3 biological replicates

e) Ascorbate peroxidase

The enzymatic activity does not show any differences between the control and stressed samples up to 48 hours, after which the activity increase to reach a maximum at 72 hours in the presence of laminarin (Fig. 6).

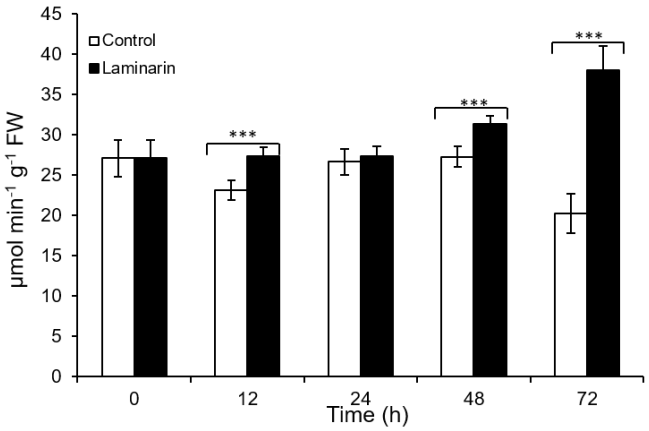

Figure 6: Wheat seeds were germinated in the presence of 200 μg.mL^{-1} of laminarin solution, and samples were harvested at 0h, 12h, 24h, 48h, and 72h after imbibition. APX activity measured the rate of decrease of the absorbance of hydrogen peroxide. The values represent the mean from 3 biological replicates

f) The molecular marker of biotic stress

To give proof that the germination process takes place in conditions of biotic stress simulated by the linear β-(1,3)-glucan. The measure of gene expression showed an increase of *PR.1.1* and *Glu3* expression at 72h, in comparison with the control characterized by an unchanged profile (Fig. 7A, 7B).

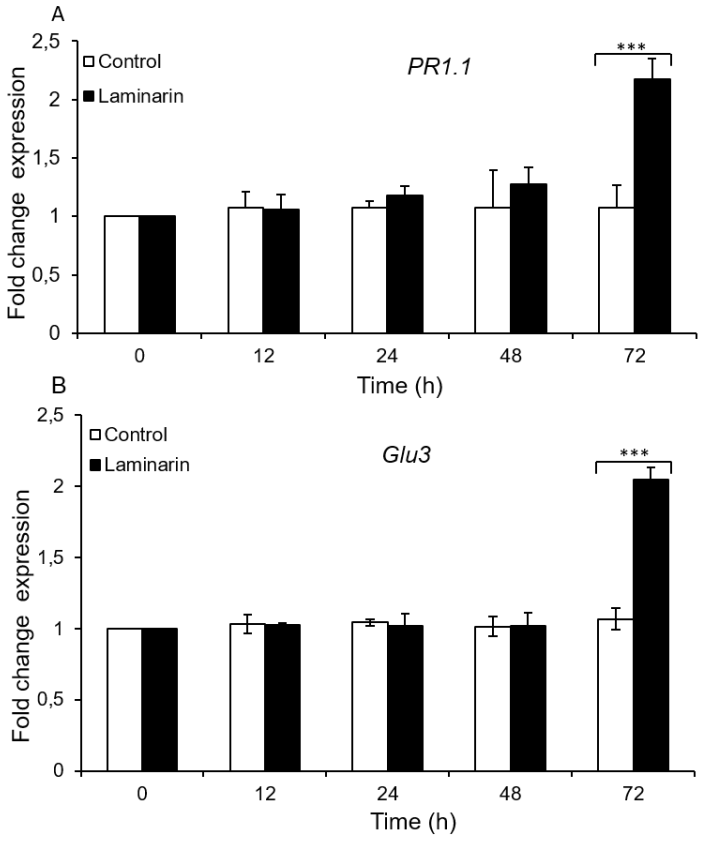

Figure 7: Wheat seeds were germinated in the presence of 200 μg.mL^{-1} of laminarin solution, and samples were harvested at 0h, 12h, 24h, 48h, and 72h after imbibition. Time course of A) *PR1.1* and B) *Glu3* genes expression using qRT-PCR in wheat seedling after treatment. The values represent the mean fold-changes from 3 biological replicates.

IV. Discussion

Germination is a very complex process and is affected by many factors [7]. Minimum accumulation of ROS and enhanced activity of enzymes affect seed quality, thus germination potential [8].

In the present study, stress conditions induced by laminarin treatment caused an increase of enzyme activities during seedling germination however, each enzyme showed a various expression profile. The 1-Cys-Prxs known to be specific of wheat seeds, constituted the main enzyme against stress oxidative [22], due to those expressions during the earliest stage of germination [26]. Despite the expression of 1-Cys-Prx decrease during the germination, the utilization of laminarin allows maintaining 1-Cys-Prx at a higher level. Results already confirmed [50], the rice mRNA of 1-Cys-Prxs level became significantly diminished immediately after seeds germination under normal conditions. [23] showed that 1-Cys-Prxs are highly expressed during late seed development and desiccation stage, suggesting the implication of the 1-Cys-Prxs against oxidative stress in this stage caused by dehydration. The function of oxidative stress was confirmed in rice by the production of transgenic R1-Cys Prxs plants, exhibiting enhanced resistance against stress [50]. These results suggest that 1-Cys-Prxs used in the germination process were accumulated during the desiccation stage, and after imbibition, the transcript level becomes dramatically reduced and completely disappears once the germination process ends. Since the use of molecules as laminarin maintains a high expression of 1-Cys-Prxs involved against oxidative stress during unfavorable conditions.

At the early steps of germination, Trx *h* is involved in the mobilization of storage proteins and oxidative stress by the reduction of the 1-Cys-Prxs [22]. Our work exhibit the effect of laminarin on Trx*h*1 protein expression, suggesting an improvement of the mobilization of storage and the reduction of 1-Cys-Prxs. The acceleration of germination in transgenic barley lines overexpressing Trx *h* [51], as well as the retardation of germination in transgenic wheat lines with suppressed Trx *h* expression [52], confirms this function of the Trx system in cereals seeds. The Trx *h* controls the oxidative stress in the living tissues, specifically in the scutellum and the aleurone layer [22]. However, under abiotic stress, the analysis of the expression of Trx *h* in seedling shows a difference in the expression profile in the aleurone compared with other tissues. The aleurone of cells suffering oxidative stress is characterized by the presence of the NTR/Trx *h* system [53; 22], suggests that it is associated with antioxidant response. Moreover, in dry seeds, the NTR/Trx *h* and 1-Cys-Prxs are localized in the nucleus of the seed and the NTR-dependent activity of the 1-Cys-Prxs [22].

One of the factors that determine seed quality is the presence of CAT enzyme [37]. In addition to allow the tolerance of the plants under stress conditions, the antioxidant enzymes CAT is reported to be involved in the physiology of germination [30, 32, 33]. Our study showed the effect of laminarin in inducing CAT gene expression and activity during the early steps of germination. In this subject, many studies have demonstrated that enzymes CAT, are related to various stresses during the germination [54,55], the activities were increased significantly in roots and shoots. Among the antioxidant enzymes, CAT activity increase most drastically under abiotic and biotic stress due to its specificity to detoxifying the hydrogen peroxide (H_20_2), suggesting that the CAT enzyme is a major enzyme involved in oxidative stress. After anthesis and during maturation steps, the dehydration steps caused a stress for seeds, and the detoxification potential of seeds might be strongly altered if these enzymes were to undergo to some damage during seeds storage, leading to a reduction of seeds vigor. Under biotic or abiotic stresses, the activatation of antioxidant defense mechanisms, contribute in maintaining the structural integrity of the cell and decrease oxidative damage [56]. In rice plants, an important cereal model, increased expression levels of antioxidant enzymes and genes have been related to the response to stress factors [57, 58].

The genes *PR1.1* and *Glu-3* are generally considered as molecular markers of the JA and SA-dependent pathway. Our data show that transcriptional responses to the β-(1.3)-glucan induce the expression of *PR1.1* and *Glu3* related to biotic stress. The sulfated β-(1,3)-glucan induces the production of acidic isoforms of PR proteins *via* the salicylic acid (SA) and ET dependent signaling pathways in tobacco, Arabidopsis, and grapevine [59,60]. The biotic markers genes are expressed rather than the detoxifying enzymes, suggesting that laminarin causes stress conditions and the generation of reactive oxygens species. The induction of antioxidant enzymes in plants may protect themselves from the active oxygen damage due to pathogen invasion. However, PR proteins, such as β-1-3-glucanase and chitinase, degrade cell walls of fungal pathogens that contain glucan and chitin. Since the use of β-(1-3)-glucan elicit the full cascade of specific defense responses.

All these results suggest that the accumulation of 1-Cys-Prxs, Trx *h*1, and the antioxidant enzymes as CAT and APX during seed maturation allowed to protect seedlings against oxidative stress at the early stage of germination under biotic stress. Since laminarin use as an elicitor could improve seed germination quality and vigor and protect wheat seedling against pathogens attacks.

V. Conclusion

The use of compounds like laminarin seems to promote the natural defenses mechanisms in wheat seeds. Thus, we have highlighted the importance of the proteins of 1-Cys-Prx and the PR proteins in the protection against possible stress linked to the presence of pathogens. In cereals, because 1-Cys-Prx is seed-specific, the use of external substances such as laminarin is a means for stimulating this protein and thus improving protection against the possible attacks by fungi, such as Fusarium wilt which attacks common wheat.

This study mainly allowed to discriminate the implication of markers of redoxins and those of the antioxidant system in the study of seeds physiology. 1-Cys-Prx seems to be well placed to address this physiology and oxidative stress in particular during germination in the presence of biotic stress in relation to the antioxidant system. The importance of Trxs *h* seems to be secondary, due to their involvement in the mobilization of storage proteins. Thus, 1-Cys-Prx can be used as a marker indicating the seeds redox state during the first stages of germination. The use of external treatments with oligosaccharide substances capable of stimulating the natural defenses constitutes a plant protection strategy.

This approach, which reconciles agriculture and environment constitute an alternative in plant protection. The strategy of stimulating plant defense mechanisms using natural compounds differs from the use of pesticides, by stimulating the plant defense and weakening against pathogens. The use of substances such as laminarin has the considerable advantage of not unnecessarily mobilizing the metabolism of the plant before defense.

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