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Surfactin Protects Wheat against *Zymoseptoria tritici* and Activates Both Salicylic Acid- and Jasmonic Acid-Dependent Defense Responses

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Abstract: Natural elicitors induce plant resistance against a broad spectrum of diseases, and are currently among the most promising biocontrol tools. The present study focuses on the elicitor properties of the cyclic lipopeptide surfactin on wheat, in order to stimulate the defenses of this major crop against the challenging fungal pathogen *Zymoseptoria tritici*. The protection efficacy of surfactin extracted from the strain *Bacillus amyloliquefaciens* S499 was investigated through greenhouse trials. Surfactin protected wheat by 70% against *Z. tritici*, similarly to the chemical reference elicitor Bion[®] 50WG. In vitro biocidal assays revealed no antifungal activities of surfactin towards the pathogen. A biomolecular RT-qPCR based low-density microarray tool was used to study the relative expression of 23 wheat defense genes. Surfactin significantly induced wheat natural defenses by stimulating both salicylic acid- and jasmonic acid-dependent signaling pathways. Surfactin was successfully tested as an elicitor on the pathosystem wheat–*Z. tritici*. These results promote further sustainable agricultural practices and the reduction of chemical inputs.

Keywords: biocontrol; surfactin; induced resistance; winter wheat; *Zymoseptoria tritici*

1. Introduction

Biocontrol is an alternative plant protection method which promotes sustainable agricultural practices and contributes to reducing chemical inputs. Elicitors, in particular, are promising biocontrol tools which are currently the subject of intensive research within the framework of integrated pest management (IPM) strategies [1–3]. They correspond to natural molecules, generally emitted by pathogens or beneficial microorganisms, which induce a non-specific resistance of the plant against a broad spectrum of diseases [4,5]. Using elicitors as complements to fungicide applications thus offers the dual advantage of reducing the amount and application frequency of chemical inputs in the field, and of implementing sustainable plant protection methods in agricultural practices [6]. Among the numerous natural elicitors which have been identified up to now, major attention is today focused on surfactin. This cyclic lipopeptide consists of heptapeptides interlinked with a β -amino fatty acid chain of varying length to form a cyclic lactone ring structure [7,8]. In contrast to elicitor compounds secreted by pathogens (pathogenic-associated molecular patterns or PAMPs), surfactin is a microbe-associated

molecular pattern (MAMP) which is generally produced by plant growth-promoting rhizobacteria (PGPR) belonging to specific *Bacillus* strains [8–11]. Surfactin is a powerful biosurfactant which has mostly been studied for its antagonistic and cytotoxic activity against multiple pathogens [8,12]. However, research carried out in the past decade has also demonstrated that surfactin can act as an elicitor, by triggering an induced systemic resistance (ISR) of plants such as tomato, tobacco, bean, and beet against various diseases [7,13–15]. Jourdan et al. (2009) reported, for instance, that the application of surfactin at micromolar concentrations on tobacco cell suspensions led to the induction of early defense responses (ion fluxes across the plasma membrane and the production of reactive oxygen species) coupled with the activation of defense-related enzymes phenylalanine ammonia lyase (PAL) and lipoxygenase (LOX), and the production of the plant defense hormone salicylic acid [14]. It must, however, be noted that little research has been carried out so far concerning the potential of elicitors, such as surfactin, to induce resistance of major monocotyledonous plants [16]. In the present study, we investigated the elicitor potential of surfactin to protect winter wheat against the *Septoria tritici* blotch (STB) disease. Wheat is indeed one of the most cultivated crops in the world, with up to 734 million tons produced in 2015–2016 [17,18]. The STB disease caused by the fungal pathogen *Zymoseptoria tritici* (teleomorph: *Mycosphaerella graminicola*) represents a persistent threat each year to wheat crops all over Europe [17,19]. For instance, particularly strong STB pressures inflicted drastic yield losses during the 2016 season in Northern France: losses reached around 2.5 tons per hectare, which amounts to 36% of the total yield [20]. Furthermore, there are, so far, no wheat cultivars which are totally resistant to *Z. tritici*, and only the use of conventional fungicides can prevent massive yield losses [21,22]. The development of new and efficient biocontrol tools for wheat protection is thus critical [3].

In this study, we evaluated, in three steps, the biocontrol potential of surfactin to induce wheat resistance against *Z. tritici*: (i) we first investigated the efficacy of surfactin in protecting wheat against STB under glasshouse conditions. Three different concentrations of surfactin were tested in order to identify possible dose-dependent effects; (ii) the potential biocidal activity of surfactin directly against the pathogen was assessed through in vitro sensitivity bioassays. Such assays enabled us to check whether surfactin behaved as a fungicide and/or as an elicitor at the concentrations tested during greenhouse trials; (iii) biomolecular tests were finally carried out to provide further evidence as to the elicitor potential of surfactin to induce wheat defenses. The recognition of an elicitor by the plant triggers a cascade of defense mechanisms leading to induced resistance. We thus investigated the expression of 23 defense genes of wheat in treated versus untreated plants by using an innovative biomolecular tool developed by INRA [23,24]. Such tests provided useful information regarding the defense signaling pathways preferentially triggered in the plant by surfactin.

2. Materials and Methods

2.1. Plant and Fungal Materials

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

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

2.2. Screening Trials

2.2.1. Treatment Preparation

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

2.2.2. Plant Treatment, Inoculation, and Infection Level Assessment

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

2.3. In Vitro Sensitivity Bioassay

The potential direct effect of surfactin on *Z. tritici* fungal growth was assessed through in vitro bioassays. Such experiments enabled us to confirm that surfactin did not exert a fungicidal effect against the fungal pathogen, rather than an elicitor activity on wheat, at the concentrations tested during greenhouse screening trials. PDA plates were amended with different concentrations of surfactin, according to the method of Siah et al. [25]. Surfactin was first added at the highest concentration (0.1 mg mL^{-1}) to PDA medium at 30°C after autoclaving. It corresponds as well to the highest concentration tested in greenhouse trials. Successive dilutions were then carried out in order to test five decreasing concentrations (0.02 mg mL^{-1} , 0.01 mg mL^{-1} , 0.004 mg mL^{-1} , and 0.001 mg mL^{-1}). The control consisted of plates containing PDA only. The plates were subsequently spotted with $5 \mu\text{L}$ of 5×10^5 spores mL^{-1} suspension. Fungal growth was scored by measuring the colony perpendicular diameters of each spot at 10 days after incubation in the dark at 18°C . Values correspond to the average diameter of *Z. tritici* colonies scored on amended PDA media. The comparison of mean fungal growth was performed with the Tukey (ANOVA) test at $p = 0.05$. Three plates with five spots per plate were used as replicates for each condition, and two independent experiments were carried out.

2.4. Determination of Defense Gene Induction in Wheat

2.4.1. Plant Treatment

We investigated the defense signaling pathways that were potentially triggered in wheat following treatment with surfactin. For this experiment, treatments were prepared similarly to screening trials, although in this case, surfactin was tested only at the average concentration of 0.01 mg mL^{-1} , due to space limitations. Plants at the 3–4 leaf stage were either sprayed to runoff with surfactin (0.01 mg mL^{-1}), Bion (0.6 mg mL^{-1}), or water using an electric sprayer. Each treatment was applied on one box of 40 wheat plants (40 repetitions). One day after plant treatment, potential priming activities were also tested by applying a water solution containing 40 mM of hydrogen peroxide (H_2O_2) on the plants on each half of the box. Elicitor priming is a phenomenon whereby plant natural defenses are only activated when a subsequent challenge occurs, and not directly after elicitor recognition [26]. The exact molecular mechanisms involved in priming are still poorly understood. However, fitness benefits have been observed on primed plants in the field under high disease pressures as the energy of the plant remains devoted to its development until a biotic stress actually occurs [27,28]. In the present case, we used H_2O_2 to mimic a biotic stress comparable to a *Z. tritici* infection, as described by Dugé de Bernonville et al. [24]. Hydrogen peroxide is indeed a reactive oxygen species (ROS) which acts as a central player in the transduction of stress signals in the plant [29,30]. In the event that surfactin and/or the elicitor control Bion exert a priming activity, the expression of defense genes in the plant would be strongly induced in wheat after the application of H_2O_2 .

2.4.2. RNA Extraction and Quantification of Gene Expression by Quantitative RT-PCR

For each condition (e.g., water only, Bion or surfactin), the third leaf of five distinct seedlings was sampled at day 1 after plant treatment, right before H_2O_2 application on the half of each box. Similarly, the third leaf of five distinct seedlings was sampled at day 2 and day 3 after treatment on the whole boxes, for plants which received H_2O_2 or were untreated. All samples were immediately pooled, frozen, and stored at -80°C until use. Total RNA was extracted from 100 mg of plant tissue using the Nucleospin[®] RNA Plant Kit (Macherey-Nagel, Düren, Germany). Reverse-transcription of total RNA was carried out using the M-MLV Reverse Transcriptase (ref M1701, Promega, Madison, WI, USA), according to the manufacturer's protocol. Real-time qPCR was performed with MESA BLUE qPCR MasterMix (ref RT-SY2X-03 + WOUFLB, Eurogentec, Liège, Belgium) according the manufacturer's instructions, using the biomolecular tool described by Brisset and Dugé de Bernonville (2011), on a Biorad MyiC detection system [23]. The qRT-PCR bioassay focused on twenty-three different genes involved in various wheat defense mechanisms. These include pathogenesis-related (PR) proteins, oxidative stress, and defense signaling pathways (e.g., salicylic acid, jasmonic acid, and ethylene) [31–34]. Relative changes in defense gene expression of treated plants were compared to the relative expression of the same genes in water control plants by using the $2^{-\Delta\Delta\text{Ct}}$ method described by Schmittgen & Livak [35]. Three internal reference genes were used for normalization (e.g., TubA, GAPDH, and actin). Relative defense gene expression was calculated for each time point. The gene expression levels were obtained from two independent biological experiments, with three technical replicates.

The effect of plant treatment on wheat defense responses was evaluated by multivariate ANOVA. In order to visualize and analyze gene expression, a heatmap representation was performed using dissimilarity distance ($1 - \text{cor}(X, Y)$). Moreover, the identification of sets of genes that may be similarly expressed across all conditions within the dataset (relationship discovery) was realized by hierarchical clustering of gene expression. Hierarchical clustering analysis is a stepwise algorithm which merges two gene variables at each step, the two of which have the least dissimilarity distance. Such distance between clusters of genes was defined using the complete linkage method (using the "hclust" function in the R statistical software). In addition, the "pvclust" package in R was used to calculate the

probability values (p -values) for each cluster using bootstrap resampling techniques. Gene clusters which were not significantly supported by the data were rejected with a significance level of 0.05.

3. Results

3.1. Screening and Biocidal Results

The efficacy of surfactin to protect winter wheat against *Z. tritici* was assessed through greenhouse trials (Figure 1). Control plants were infected on up to 20% of their third leaf surface by the pathogen. On the other hand, symptomatic lesions occurred only on 6% to 8% of the leaf surface of plants treated with surfactin, regardless of its concentration. Finally, plants treated with Bion had barely 6% of their leaf surface covered with lesions. The disease severity was significantly lower ($p = 0.05$) on plants treated with Bion or with surfactin when compared to control plants. Hence, wheat was similarly protected by surfactin and the elicitor control Bion, with a protection efficacy of up to 70% and 69%, respectively.

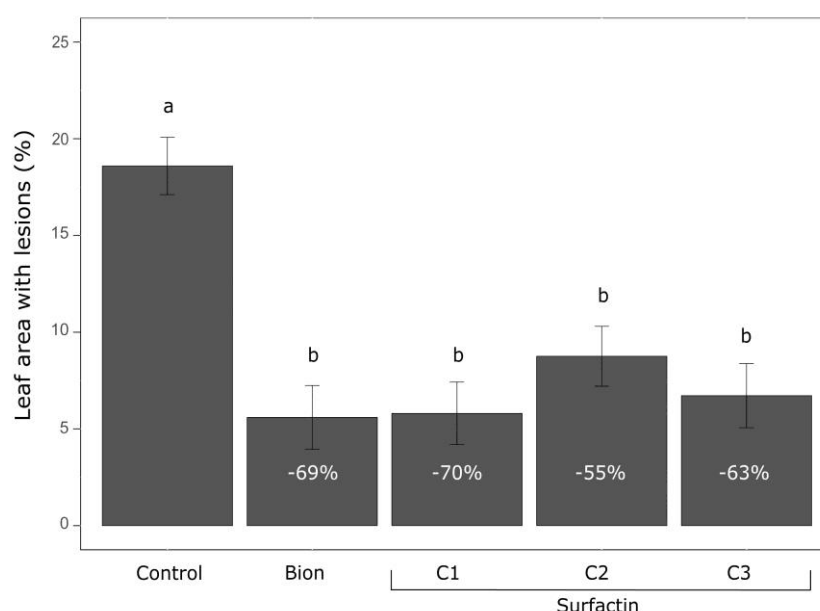


Figure 1. Mean disease severity of *Zymoseptoria tritici* on treated wheat plants. Data corresponds to the average percentage of the third leaf surface of wheat plants exhibiting symptomatic lesions (necrosis and/or chlorosis) bearing pycnidia. Plants were treated at the 3–4 leaf stage and five days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

The potential direct effect of surfactin on *Z. tritici* was studied through in vitro sensitivity bioassays (Figure 2). Such experiments are a first indication to understand if the protective efficacy of surfactin assessed during greenhouse trials was potentially due to a direct fungicidal effect against the pathogen.

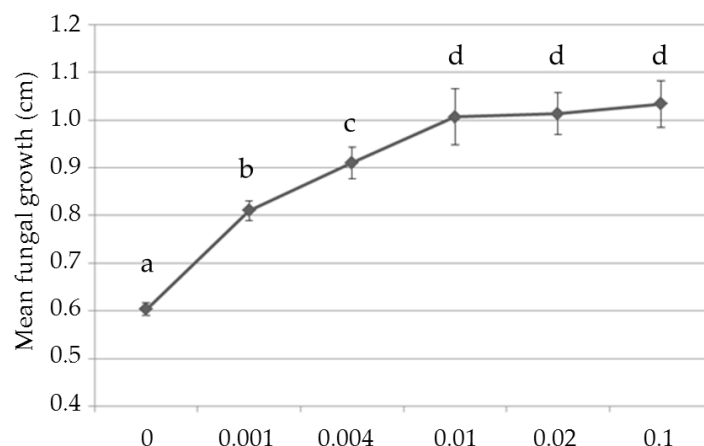


Figure 2. Biocidal effect of surfactin on the in vitro fungal growth of *Zymoseptoria tritici*. Potato dextrose agar (PDA) medium was amended with five decreasing concentrations of surfactin: 0.1 mg mL⁻¹, 0.02 mg mL⁻¹, 0.01 mg mL⁻¹, 0.004 mg mL⁻¹, and 0.001 mg mL⁻¹. The control corresponds to PDA medium without surfactin (0 mg mL⁻¹). Means tagged with the same letters are not significantly different using the Tukey test at $p = 0.05$.

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

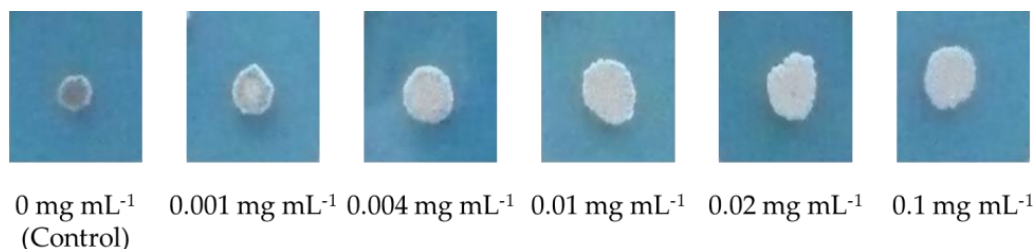


Figure 3. Illustration of *Zymoseptoria tritici* fungal growth on PDA medium amended with surfactin at six concentrations: 0.1 mg mL⁻¹, 0.02 mg mL⁻¹, 0.01 mg mL⁻¹, 0.004 mg mL⁻¹, 0.001 mg mL⁻¹, and 0 mg mL⁻¹ (Control).

3.2. Induction of Defense Responses

The expression level of 23 defense-related genes of wheat was monitored 1, 2, and 3 days after treatment with either Bion or surfactin. The treatments applied on wheat plants had a significant effect on the expression of defense genes (MANOVA, p -value < 0.05). For each gene, the average expression level measured in treated plants was compared to the water control (which received no H₂O₂) and represented on a heatmap profile (Figure 4). The average expression level of genes for water-treated plants which received hydrogen peroxide (labelled “+H₂O₂”) after 1 day was similarly compared to the water control. Hierarchical clustering of genes according to their expression levels revealed five gene clusters which were significantly supported by the data (p -value ≤ 0.05).

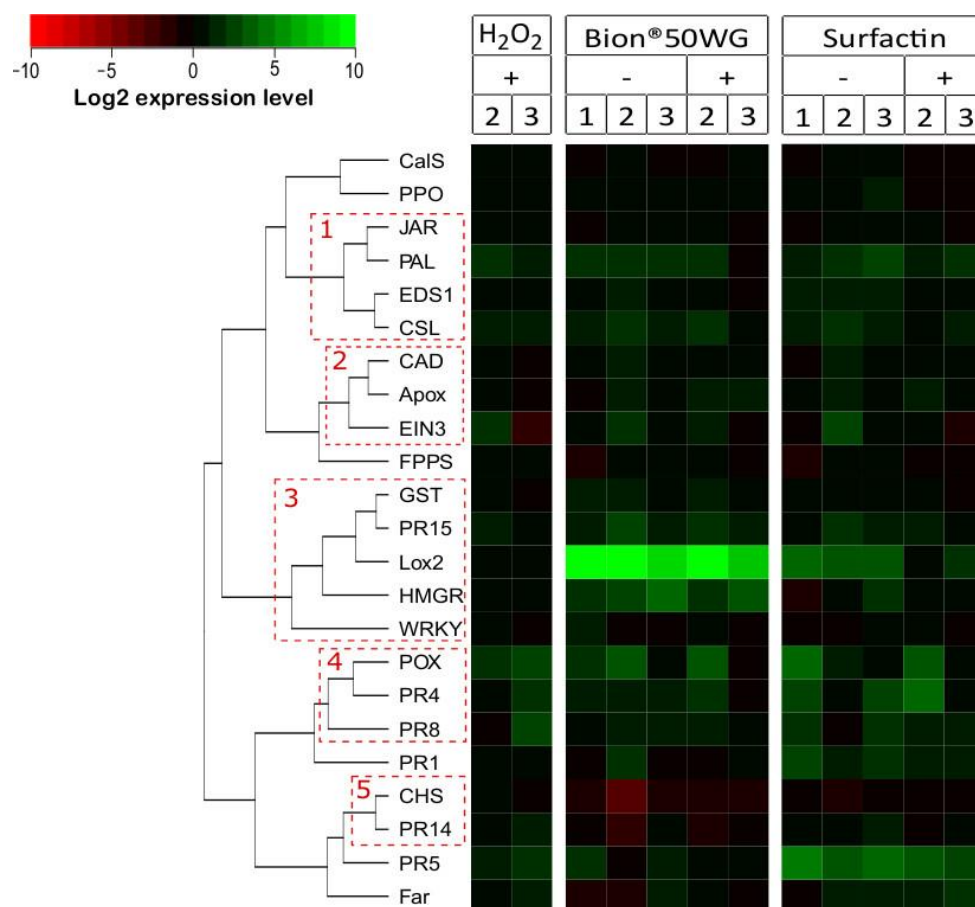


Figure 4. Heatmap profiling across all experimental conditions (product, $\pm$ H₂O₂, day post-treatment) with hierarchical clustering of 23 defense-related genes of wheat [23]: *Apox*, ascorbate peroxidase; *CalS*, callose synthase; *CHS*, chalcone synthase; *CAD*, cinnamyl-alcohol dehydrogenase; *CSL*, cysteine sulfoxide; *EIN3*, EIN3-binding F box protein; *EDS1*, enhanced disease susceptibility 1; *Far*, (E,E)- α -farnesene synthase; *FPPS*, farnesyl pyrophosphate synthase; *GST*, glutathione S-transferase; *HMGR*, hydroxymethyl glutarate-CoA reductase; *JAR*, jasmonate resistant 1; *Lox2*, 13-lipoxygenase 2; *PAL*, phenylalanine ammonia-lyase; *PR*, pathogenesis-related protein; *PPO*, polyphenol oxidase; *POX*, peroxidase; *WRKY*, WRKY transcription factor 30. The averaged defense gene expression profiles are compared to a water control, and were obtained after plant treatment at the 3–4 leaf stage. Treatments consisted of water control plants subsequently treated with hydrogen peroxide after 1 day (H₂O₂), the elicitor reference BION® applied at 0.6 mg mL⁻¹, or surfactin applied at 0.01 mg mL⁻¹.

The first cluster (1) includes gene *EDS1* (enhanced disease susceptibility 1) involved in the production of the defense hormone salicylic acid (SA), *JAR* (jasmonate resistant 1) involved in jasmonic acid (JA)-related defense signaling, *PAL* (phenylalanine ammonia lyase) involved in the phenylpropanoid pathway, and *CSL* (cysteine sulfoxide) involved in antioxidative stress. The expression level of genes *EDS1* and *JAR* was similar between the control and the treated plants. On the other hand, Bion and surfactin induced about a 3-fold upregulation of *PAL* and *CSL* gene expression compared to the control, whether the plants were later sprayed with H₂O₂ or not. A similar 2- to 3-fold upregulation of *PAL* and *CSL* occurred for “+H₂O₂” plants which were treated only with water before being sprayed with H₂O₂ one day later. The second gene cluster (2) includes *EIN3* (EIN3-binding F box protein) involved in ethylene (ET)-related defense signaling, *CAD* (cinnamyl-alcohol dehydrogenase) involved in cell wall reinforcement, and *Apox* (ascorbate peroxidase) involved in antioxidative stress. Wheat plants treated with Bion or surfactin showed a significant upregulation (3- to 4-fold increase) of the expression level of these three genes at day 2 after

treatment. On the other hand, “+H₂O₂” plants showed no difference with the control, except for a 4-fold upregulation of *EIN3* expression level at day 2.

A third cluster (3) includes the *WRKY* transcription factor 30 gene involved in defense signaling, *HMGR* (hydroxymethyl glutarate-CoA reductase) involved in the mevalonate pathway leading to biosynthesis of terpenoid defense compounds, *LOX2* (Lipoxygenase 2) involved in the octadecanoid pathway leading to the biosynthesis of the defense hormone JA, and genes *PR15* (pathogenesis-related protein 15) and *GST* (glutathione S-transferase) which are involved in antioxidative stress. Both Bion and surfactin induced a significant upregulation of *HMGR*, *LOX2*, and *PR15* gene expression in wheat from day 1 to day 3 after treatment. Such upregulation occurred whether the corresponding plants later received H₂O₂ or not. Notably, the upregulation of *LOX2* gene expression was particularly strong: 10-fold increase for wheat treated with Bion and about 8-fold increase for plants treated with surfactin. On the other hand, “+H₂O₂” plants showed no difference with the control. Another cluster (4) includes genes *PR4* and *PR8* which both code for antimicrobial chitinases, and gene *POX* (peroxidase) involved in antioxidative stress. The expression level of these three genes was significantly upregulated by 5- to 6-fold in plants treated with Bion or with surfactin compared to the control. Finally, a last cluster (5) includes gene *PR14* coding for a lipid-transfer protein and gene *CHS* (chalcone synthase) involved in the flavonoid/isoflavonoid pathway and SA-related defense signaling. In particular, Bion induced a strong 7-fold downregulation of *CHS* (chalcone synthase) gene expression up to three days after treatment, while surfactin induced a 4-fold downregulation of *CHS* only at day 2. On the other hand, “+H₂O₂” water-treated plants showed no difference with the control.

Overall, treatment of wheat with either Bion or surfactin induced a significant upregulation of the expression level of several genes involved in key defense mechanisms, notably genes involved in SA- and JA-related signaling pathways, oxidative stress, and cell wall reinforcement. Moreover, the application of H₂O₂ on water-treated plants was successful in being recognized as an attack by the plant by inducing the expression of defense genes, such as *PAL* and *POX*. On the other hand, the application of H₂O₂ on plants treated with Bion or surfactin showed no difference in terms of defense gene expression compared to treated plants which received no hydrogen peroxide, thus suggesting that nor Bion nor surfactin exerted a priming activity.

4. Discussion

Previous studies have demonstrated that pure surfactin extracted from strains of non-pathogenic *Bacillus* could significantly protect thale-cress, bean, tomato and tobacco plants against the fungal pathogen *Botrytis cinerea* [10,13,14]. Surfactin was also proven to efficaciously protect sugar beet against the virus *Polymyxa betae* [15] and strawberry plants against *Colletotrichum gloeosporioides* [36]. More recently, Mejri et al. (2017) reported that surfactin extracted from the *Bacillus subtilis* strain BBG131 and applied at 0.1 mg mL⁻¹ on the susceptible wheat cultivar “Alixan” could efficaciously protect the plant by up to 35% against *Z. tritici* [37]. Our results are thus in accordance with previous research, as we demonstrated that surfactin applied at low doses (e.g., 0.001, 0.01, and 0.1 mg mL⁻¹) efficaciously protected wheat by up to 70% against *Z. tritici*. Moreover, surfactin was as efficacious as the synthetic elicitor control Bion. In the present study, such high protection efficacy of surfactin could be linked to the mixture of homologues extracted from the *B. amyloliquefaciens* strain S499 and/or to the wheat cultivar “Avatar” that was used for greenhouse trials. The efficacy of a given elicitor can indeed be cultivar-dependent [38,39], and the elicitor activity of surfactin was proven to rely on specific structural traits such as the length of the fatty acid [7].

In addition to greenhouse trials, we showed that surfactin had no direct in vitro biocidal effect against the pathogen at the concentrations tested in the greenhouse. Rather, it appears that high concentrations of surfactin promoted the in vitro growth of *Z. tritici*. This lipopeptide is indeed a powerful amphiphilic biosurfactant involved in bacterial mobility and in the formation of biofilms, pellicles, and fruiting bodies of *Bacillus* [8,9]. It is therefore likely that the surface tension of PDA media

containing surfactin was lowered, thereby allowing a better spreading of the inoculum droplets on the plates during inoculation.

The reduction of the surface tension was likely enhanced by increasing concentrations of surfactin in the PDA media. Then, such increased fungal growth might probably be due to the physicochemical properties of surfactin, rather than to a growth-promoting effect. These results are once again in accordance with previous studies. Indeed, Mejri et al. (2017) demonstrated the lack of direct antifungal activity of surfactin against the pathogen *Z. tritici* in both in vitro and in planta bioassays [37]. Actually, the fungitoxic effects of surfactin have never been reported [12,13] except in the work of Tendulkar et al. (2007) on rice. They showed that surfactin extracted from *Bacillus licheniformis* BC98 exhibited an in vitro direct fungicidal activity against the rice blast disease *Magnaporthe grisea* [40]. Taken together, the findings of greenhouse trials and in vitro biocidal assays of the present study confirm that surfactin likely protects wheat against *Z. tritici* by inducing plant resistance.

Going one step further, our biomolecular tests on wheat immune responses confirmed that surfactin was indeed perceived by the plant as an elicitor. Surfactin stimulated wheat defense mechanisms by inducing the expression of various defense genes coding for antimicrobial compounds, regulators of oxidative stress, and enzymes involved in defense signaling [41]. The induction of plant resistance by an elicitor is indeed characterized by a complex spatio-temporal network of metabolic modifications. Early events, such as protein phosphorylation, ion fluxes across the plasma membrane, and a burst of ROS, occur in a matter of seconds after elicitor recognition by plant receptors [1,42]. Proteins such as POX and PR15 are set to work, in order to control the oxidative burst [43]. After a few hours, defense genes involved in the biosynthesis of phytohormones and antimicrobial compounds are activated [1]. The hormones SA, JA, and ET are considered as the three crucial primary signals which regulate plant defenses against biotic stress [33,44]. Finally, physical and biochemical changes, such as cell wall reinforcement through callose apposition and PR protein biosynthesis, occur several hours to several days after elicitor recognition [45]. Previous research has shown that plant resistance against biotrophic and hemi-biotrophic pathogens is generally regulated by SA, while resistance against necrotrophic pathogens and chewing insects is regulated by JA and ET [33,46]. Depending on the triggered signaling pathway, a different set of genes encoding PR proteins are expressed [45,47]. Induced resistance depending on SA, also called systemic acquired resistance (SAR), involves the marker protein PR1 and the enzymes PAL and CHS [32,48]. Conversely, JA-dependent defense responses induced by MAMPS generally lead to rhizobacteria-mediated induced systemic resistance (ISR), and go hand in hand with the expression of the genes *LOX2* and *PR4* [33,47]. The LOX enzyme catalyzes the deoxygenation of polyunsaturated fatty acids, leading to the downstream biosynthesis of JA [31]. However, most studies on SA/JA crosstalk and the corresponding responsive genes have been carried out on dicotyledonous plants, and less is known concerning monocotyledonous plants. Still, it appears that similar hormone interactions may be involved in cereals. Indeed, a recent work carried out by Ding et al. (2016) showed that SA and JA were able to act antagonistically or synergistically on the expression of wheat defense genes [49]. They also reported that gene *PR5* was specifically induced by SA in the plant, while *LOX2* was specifically induced by JA, and that gene *PR1* could actually be induced simultaneously by both hormones.

Based on that knowledge, our biomolecular findings suggest that surfactin induced both SA- and JA-dependent defense responses in wheat, as it triggered a significant upregulation of the expression level of genes *PR5* and *LOX2*. Interestingly, surfactin produced by the antagonistic strain *Bacillus subtilis* UMAF6639 was also shown to protect melon plants against powdery mildew, by similarly inducing both SA and JA defense signaling pathways, along with the production of ROS and the reinforcement of the plant cell wall [50]. The simultaneous induction of SA- and JA-dependent defense responses by some elicitors has also been reported in previous studies on dicotyledonous plants [51–53]. However, in model plants, the elicitor potential of surfactin has been associated with JA-dependent responses. For instance, it was proven to stimulate the activity of the LOX enzyme and

the synthesis of numerous active secondary metabolites in tomato plants [13], and the activity of both PAL and LOX enzymes in tobacco cells [14,54].

It is clear that plant-induced resistance involves intricate hormonal crosstalk, including in wheat, and there is no established boundary between SAR and ISR in plants [47,55]. Other hormones which were not investigated by qRT-PCR might also be involved (e.g., auxin, abscisic acid, cytokinin, gibberellin) [56,57]. A better insight into the mode of action of surfactin to induce wheat defense mechanisms would require some additional biochemical experiments on the activity of key defense enzymes. However, the primary objective of this study was to confirm the elicitor potential of surfactin for sustainably protecting wheat. Interestingly, Henry et al. (2011) suggested that, depending on the specific features of the plant plasma membrane (e.g., organization and composition of the lipid bilayer), surfactin could be perceived at the plant cell surface by interacting with the lipids at the plasma membrane level [7]. This mode of perception can be considered unusual, since most identified elicitors, such as flagellin or chitin, are known to be recognized by high affinity protein receptors [7,16].

Concerning Bion, our findings suggest that this synthetic elicitor induced both SA and JA defense signaling pathways in wheat, with JA signaling clearly outweighing SA signaling up to 3 days after plant treatment. Such results are in contrast with previous studies, as Bion has been reported, up to now, to induce solely SA-dependent defense responses in plants [58–60]. As a chemically synthesized elicitor consisting of acibenzolar-S-methyl, Bion shows indeed a functional analogy to the plant hormone SA, and is thus well known to trigger SA-responsive genes, notably robust SAR markers, such as *PR1*, *PR2*, and *PR5* [6,33,60]. Our results might be explained by the complex hormonal crosstalk involved in wheat defense signaling. Finally, concerning the investigation of potential priming activities, we demonstrated that the application of H₂O₂ following plant treatment with either surfactin or Bion exerted no additional effect on the expression of wheat defense genes. Plants which are primed following elicitor perception activate faster and stronger defense responses upon a second pathogen challenge, rather than directly inducing their defense mechanisms [55,61]. It thus appears that neither Bion nor surfactin had a priming effect on wheat defenses. However, it would be interesting to carry out a similar experiment by replacing H₂O₂ with a real pathogen attack (e.g., an actual inoculation of *Z. tritici*), and to investigate the induction of wheat defenses over more sampling times (e.g., at 6, 12, and 96 h after treatment, for example).

5. Conclusions

This study provides further insight into the remarkable elicitor properties of surfactin by demonstrating its ability to efficaciously protect wheat by up to 70% against the fungal pathogen *Z. tritici*. The stimulation of wheat defense mechanisms appears to involve both SA and JA defense signaling pathways. Research on induced resistance in monocots remains elusive, and is still an emerging field [16,62]. Both monocots and dicots have undergone evolutionary adaptations which may involve the triggering of distinct sets of defense gene expression after elicitor recognition [16]. It is noteworthy that previous studies on the elicitor potential of surfactin have mostly been dedicated to the protection of dicot plants [13–15]. Further research is thus still needed to understand the exact modes of action of surfactin to induce wheat resistance. Besides, field trials are now required to confirm the reliability of this lipopeptide elicitor in efficaciously protecting wheat crops. Several environmental parameters, such as the weather and disease pressure, are indeed known to influence the efficacy of an elicitor in the open field [6,63]. These results open the way towards the development of novel surfactin-based biocontrol tools for wheat protection, in order to enhance the sustainability of current agricultural practices. Moreover, the elicitor potential of surfactin for other cultivated monocots, such as barley and rice, and against others diseases, deserves to be explored.

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Brice Marolleau and Matthieu Gaucher conceived, designed and performed biomolecular experiments; Geraldine Le Mire performed the screening and in vitro biocidal experiments and analyzed the data; Magali Deleu provided surfactin; Geraldine Le Mire wrote the paper.

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