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Evaluation of λ-Carrageenan, CpG-ODN, Glycine Betaine, *Spirulina platensis*, and Ergosterol as Elicitors for Control of *Zymoseptoria tritici* in Wheat

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ABSTRACT

Wheat crops are constantly challenged by the pathogen *Zymoseptoria tritici*, responsible for Septoria tritici Blotch (STB) disease. The present study reports the evaluation of five elicitor compounds (λ-carrageenan, cytosine-phosphate-guanine oligodesoxynucleotide motifs [CpG ODN], glycine betaine, *Spirulina platensis*, and ergosterol) for the protection of wheat against STB in order to offer new alternative tools to farmers for sustainable crop protection. Screening of elicitors of wheat defenses was carried out through a succession of experiments: biocidal in vitro tests enabled checking for any fungicidal activities, glasshouse experiments allowed determination of the efficacy of a given compound in protecting wheat against STB, and quantitative reverse-transcription polymerase chain reaction biomolecular tests investigated the relative expression of 23 defense genes in treated versus untreated plants. Therefore, we demonstrated that λ-carrageenan, CpG-ODN, glycine betaine, *S. platensis*, and ergosterol are potential elicitors of wheat defenses. Foliar treatment with these compounds conferred protection of wheat by up to approximately 70% against *Z. tritici* under semicontrolled conditions and induced both salicylic acid- and jasmonic acid-dependent signaling pathways in the plant. These findings contribute to extending the narrow list of potential elicitors of wheat defenses against *Z. tritici*.

Wheat is the most cultivated crop in Europe and in the world, with up to 734 million tons produced in 2015 to 2016 (Fones and Gurr 2015; Satger 2016). However, this monocotyledonous plant must face a threatening disease in the field: Septoria tritici blotch (STB), caused by the fungal pathogen *Zymoseptoria tritici* (teleomorph: *Mycosphaerella graminicola*) (Torriani et al. 2015). STB is considered as the main disease affecting wheat crops in the European Union (EU) by inflicting up to 40% of yield losses each year (Eyal 1999; Rudd 2015). Thus far, fungicide use is the most effective control method because no wheat cultivar is currently totally resistant to *Z. tritici* (Fraaije et al. 2005; Palmer and Skinner 2002). Approximately 70% of EU fungicides are used to that end (Fones and Gurr 2015). However, reducing pesticide use in agricultural practices has become a key priority for multiple countries (including the EU) in the prospect of environmental sustainability and health protection (Dayan et al. 2009; European Commission 2012). In this respect, elicitors are promising alternative plant protection tools that have a prominent place in current research (Lyon et al. 2014). Unlike fungicides, elicitors are natural immune-stimulating compounds which indirectly target a broad spectrum of pathogens by enhancing the defensive state of the plant (Thakur and Sohal 2013). As such, elicitors are intended as preventive treatments complementary to the use of reduced fungicide rates (Walters et al. 2013, 2014). For now, few elicitor products are registered on the EU market for the sustainable management of wheat diseases, aside from Vacciplant (laminarin-based product; Goëmar) and BION50WG (synthetic acibenzolar-S-methyl elicitor; Syngenta) (Le Mire et al. 2016). The present study thus focuses on the screening of elicitors of wheat defenses against *Z. tritici* as a response to the urgent need for a larger panel of alternative tools for sustainable wheat protection (Walters et al. 2013). Five different compounds were selected for this work: λ-carrageenan, cytosine-phosphate-guanine oligodesoxynucleotide motifs (CpG ODN), *Spirulina* (*Arthrospira*) *platensis*, glycine betaine, and ergosterol. It appears that none of these compounds have been tested before as elicitors on the wheat–*Z. tritici* pathosystem, although previous research has indeed demonstrated their elicitor properties on other plant species or animals. λ-Carrageenan stimulates the resistance of tobacco, tomato, and thale-cress plants against pathogens such as *Botrytis cinerea* and *Tobacco mosaic virus* (Mercier et al. 2001; Sangha et al. 2010, 2015; Vera et al. 2011). A recent study reported that bacterial CpG-ODN induced defense responses in thale-cress (Yakushiji et al. 2009), while unmethylated CpG-ODN was also proven to induce animal innate immunity against pathogenic bacteria, viruses, and protozoans (Carrington and Secombes 2006; Covello et al. 2012). The cyanobacterium *S. platensis* showed beneficial health effects for humans and animals (poultry, mammals, and fish) thanks to its immune-stimulating properties, notably via the production of antibodies and cytokines (Farag et al. 2016; Wan et al. 2016). The recent work of Věchet and Šerá (2015) revealed that glycine betaine stimulates wheat defenses against powdery mildew (Věchet and Šerá 2015). Lastly, ergosterol is a well-known elicitor of dicotyledonous plants (e.g., tomato, tobacco, and sugar beet) (Amborabé et al. 2003; Lochman and Mikes 2006; Rossard et al.

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2010). This study was conducted in two steps. (i) We first investigated the efficacy of the five compounds in protecting wheat against STB under glasshouse conditions. Each compound was tested at three different concentrations in order to assess any dose-dependent effects. (ii) In order to confirm their elicitor potential, we carried out an independent experiment to explore the defense signaling pathways triggered in treated versus untreated plants. To that end, an innovative biomolecular tool developed by the Institut national de la recherche agronomique (INRA) was used to study the expression of 23 wheat defense genes (Brisset and Duge De Bernonville 2011; Dugé de Bernonville et al. 2014). The recognition of an elicitor by the plant does, indeed, trigger a characteristic cascade of defense signals, resulting in plant-induced resistance (Muthamilarasan and Prasad 2013). Conducting such biomolecular test for screening purposes provides further evidence as to the elicitor properties of the tested compounds. Moreover, we examined the correlation between the effectiveness of each compound to protect wheat and the expression of defense genes in the treated plants.

MATERIALS AND METHODS

Plant and fungal materials. The experiments were conducted on wheat (*Triticum aestivum* L.) plants of the susceptible cultivar Avatar. Plants were grown in the glasshouse under semicontrolled conditions (natural photoperiod supplemented with artificial light if needed, at 20 ± 5°C according to the sunlight). Screening experiments and plant defense induction experiments were undertaken independently. For the screening of elicitors for their protective efficacy, seed were sown in 25-by-15-cm plastic pots filled with loam (10 plants/pot). The study of induced plant defenses was carried out at another location (INRA facilities in Angers) where seed were sown in 30-by-20-cm plastic boxes filled with loam (40 plants/box).

The *Z. tritici* strain T01187 (isolated in 2009 from northern France) was used for plant inoculation. Fungal culture was performed on potato dextrose agar medium for 8 days at 18°C with a cycle of 12 h/day and 12 h/night. Inocula were prepared by washing the cultures with 10 ml of sterile distilled water, and the resulting spore suspension was adjusted to desired concentrations using a Malassez cell.

Elicitor preparation. The sources and characteristics of the five compounds examined in this study are provided in Table 1. Note that the compound CpG-ODN consists of CpG-28 (sequence 5′-TAAACGTTATAACGTTATGACGTCAT-3′) synthesized with a wholly phosphorothioate backbone (Carpentier et al. 2003). For each compound, the tested concentrations were not the same because we selected the average effective doses which were generally used in previous studies to demonstrate their respective elicitor potential. All treatment solutions were freshly prepared before use in distilled water supplemented with 0.1% (vol/vol) spreading agent Break-ThruS240 (polyether trisiloxane; Evonik Industries), and 0.05% (vol/vol) solubilizing agent Tween 20 (polyoxyethylene-sorbitan monolaurate; Sigma-Aldrich). Control plants were treated with distilled water alone. In addition, other control plants were treated with 0.75% (vol/vol) of the epoxiconazole-based fungicide Opus (BASF Agro) or with the synthetic elicitor Bion50WG (abbreviated to Bion) at 0.6 mg ml^{-1} (recommended doses according to the E-Phy website [https://ephy.anses.fr). It should be noted that preliminary in vitro experiments according to Siah et al. (2010a) have been conducted to rule out any compound showing a direct biocidal activity toward the fungal pathogen *Z. tritici* (Supplementary Fig. S1).

Glasshouse elicitor screening: Plant treatment and inoculation. At the three- to four-leaf stage (third leaf fully expanded), the plants of each pot were sprayed to runoff with 30 ml of the treatment solutions using a hand sprayer. Each elicitor candidate was tested at three different concentrations in order to assess any dose-dependent protection effects. Plant inoculation was performed 5 days after treatment by spraying the plants of each pot to runoff with 30 ml of a spore suspension (10^6 spores ml^{-1} in distilled water) amended with 0.05% (vol/vol) Tween 20. Immediately after inoculation, each pot was covered with a transparent polyethylene bag for 3 days in order to ensure water-saturated conditions compatible with spore germination. A visual estimation of the disease severity was carried out at 28 days postinoculation according to Siah et al. (2010b). The disease severity was measured as the percentage of the third leaf area covered with symptomatic lesions (necrosis and chlorosis) bearing pycnidia. For each treatment, at least two independent biological experiments were performed in the glasshouse, with 40 technical repetitions (plants). An incomplete block design was carried out due to the large number of compounds to be tested (Lawal 2014). Results of combined experiments were analyzed with linear mixed-effect models (Gałecki and Burzykowski 2013), and analysis of variance (ANOVA) and the Tukey multiple comparison procedure at $P = 0.05$ were used to compare the mean disease severity of the plants treated with the different products. Reported values correspond to the average infection levels of the treated plants.

Induction of defense gene expression: Plant treatment and leaf sampling. The investigation of the plant signaling pathways triggered by the various elicitor treatments was performed in the glasshouse located in INRA facilities. The elicitor compounds were prepared and applied in the same way as in

TABLE 1. Characteristics of the compounds used in the study

Active ingredient	Supplier	Characteristics	General use	Concentration tested (g $liter^{-1}$)		
				C1	C2	C3
λ-Carrageenan	Sigma	Linear polysaccharide extracted from red algae	Gelling and emulsifying agent in the food industry	0.1	1	5
Cytosine-phosphate-guanine oligodesoxynucleotide motifs (CpG ODN)	Pr. A. Carpentier, Paris, France	Short single-stranded synthetic DNA molecules (CpG-28, sequence 5′-TAAACGTTATAACGTTATGACGTCAT-3′)	Human vaccine adjuvant	9.5×10^{-5}	9.5×10^{-4}	9.5×10^{-4}
Spirulina platensis	Djerbalgue, Tunisia	Dried cyanobacterium	Human dietary supplement	0.3	3	30
Glycine betaine	Ithec	Organic osmolyte extracted from beetroot	Protectant of plants against abiotic stress	0.12	1.2	12
Ergosterol	SIGMA	Fungal sterol	Therapeutic vitamin D precursor	0.002	0.08	0.03
BION50WG (acibenzolar-*S*-methyl; 50% [wt/wt])	Syngenta, Europe	Synthetic elicitor	Plant resistance inducer	0.6		
Fungicide Opus (epoxiconazole)	BASF Agro, France	Triazole fungicide	Broad-spectrum systemic fungicide	0.75 (vol/vol)		

screening tests but were tested only at their medium concentration (C2) (Table 1) due to space limitations. Control plants were treated with distilled water alone. According to the INRA experimental design devoted to the use of the qPFD tool, each treatment was applied on one box of 40 plants (40 repetitions), and two independent biological experiments were performed. In order to test for priming activities, the plants on half of each box were sprayed 1 day later with a solution containing 40 nm of hydrogen peroxide (H_2O_2), which mimics a pathogenic attack, as described by Dugé de Bernonville et al. (2014). Elicitor priming is characterized by the nontriggering of plant defense mechanisms directly after elicitor recognition. Instead, strong and rapid host defense reactions are activated only upon a subsequent challenge (van Loon et al. 2006). This phenomenon prevents fitness costs to the plant in the absence of an infection. In the present case, if a tested compound exerts a priming activity, the application of H_2O_2 shortly after would strongly induce the expression of defense genes. As a reactive oxygen species, H_2O_2 is indeed involved in plant oxidative stress and acts as a key mediator in defense gene activation (Halliwell 2006). In addition, its use to mimic an STB infection is justified by the work of Shetty et al. (2007), who found that wheat infected by *Z. tritici* exhibited an important and early accumulation of H_2O_2 in incompatible interactions (Shetty et al. 2007). We sampled the third leaf of five distinct seedlings at day 1 after treatment (i.e., 24 h after treatment), right before H_2O_2 application on 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 treated with H_2O_2 or not. All samples were immediately pooled, frozen, and stored at –80°C until use. Note that supplementary experiments were carried out and confirmed that the application of water supplemented with the adjuvants (0.1% Break-Thru S240 and 0.05% Tween 20) on wheat plants did not induce the expression of defense genes (data not shown).

RNA extraction and quantification of gene expression by real-time reverse-transcription polymerase chain reaction. Total RNA was extracted from approximately 100 mg of plant tissue using the NucleospinRNA Plant Kit (Macherey-Nagel). Reverse-transcription (RT) of total RNA was carried out using the M-MLV RT (ref M1701; Promega Corp.), according to the manufacturer's protocol. Real-time quantitative polymerase chain reaction (qPCR) was performed with MESA BLUE qPCR MasterMix (ref RT-SY2X-03+WOUFLB; Eurogentec) according to manufacturer's instructions and using the INRA biomolecular tool developed by Brisset and Duge De Bernonville (2011) on a Biorad MyiC detection system. Cycling conditions consisted of a first step of DNA polymerase activation (95°C for 5 min), followed by 35 repeated cycles of denaturing, annealing, and extending (95°C for 15 min and 60°C for 1 min). The biomolecular study focused on 23 different genes involved in several plant defense mechanisms, including pathogenesis-related (PR) proteins, secondary metabolism, oxidative stress, and defense signaling pathways (van Loon and Van Strien 1999; Vogt 2010; Weber 2002). Relative gene expression was obtained using the $2^{-\Delta\Delta Ct}$ method (Schmittgen and Livak 2008). Values correspond to the average difference of expression of a given gene between treated and water control plants at each time point (data obtained from the combination of two independent experiments). Three internal reference genes were used for normalization (e.g., TubA, GAPDH, and Actin). The effect of plant treatment on the wheat defense responses was evaluated by multivariate ANOVA (MANOVA). In order to visualize and analyze relative gene expression, a heatmap representation was performed using dissimilarity distance [1 – cor(X,Y)]. Moreover, the identification of sets of genes that may be similarly expressed across all conditions within the dataset was realized by clustering gene expression. Finally, clustering results were confirmed by principal component analysis (PCA) of the gene expression data. PCA is a multivariate statistical technique for simplifying complex data sets in which observations are described by several dependent variables (in this study, the 23 defense-related genes) (Abdi and Williams 2010). The dimensionality of the data are reduced by identifying new variables, also called directions or principal components (PCs), which are uncorrelated and ordered. The first few PCs explain most of the data variation and are expected to show the cluster structure of the original data set. The first PCs are the directions along which samples show the greatest variation. Consequently, plotting samples along the first two PCs makes it possible to visually assess similarities and differences between samples. The statistical programming environment R was used to analyze the data for all experiments. The lme4 and multcomp packages were used to analyze greenhouse screening results and the FactoMineR package was used for the PCA.

Correlation between glasshouse and biomolecular results. Correlation analysis is a method of statistical evaluation used to study the strength of a relationship between at least two variables. The reliability of the elicitor screening experiments carried out in this study can be strengthened by checking that the protective effectiveness of a given compound is correlated with the induction of defense responses in the treated wheat plant. Such correlation increases the chances of a compound being short-listed for further experiments under practical conditions. Therefore, for each compound, we examined to what extent its protective efficacy was correlated with defense induction levels in treated wheat plants. The correlation was assessed at each qRT-PCR analysis date. The level of gene expression was estimated with the help of the PCA dimension which described the largest variability. The average level of gene expression used for the correlation test thus consisted of values of projected individuals (treatments) on the corresponding dimension. The protection efficacy values corresponded only to compounds tested at their medium concentration, C2 (Table 1), because they were only tested at this concentration during biomolecular experiments.

RESULTS

Protection efficacy against *Z. tritici* under glasshouse conditions. The mean disease severity assessed during these screening experiments corresponds to the percentage of symptomatic lesions scored on the third leaf surface of wheat plants. As a result, a mean disease severity of 23% was scored on the water control (Fig. 1). Plants treated with the fungicide epoxiconazole or the elicitor reference Bion showed an average disease severity of 0.1 and 6%, respectively. The mean disease severity of wheat plants treated with the five elicitor candidates ranged from 5 to 15%. Overall, plants sprayed with the various treatments showed significantly less disease symptoms of *Z. tritici* compared with the control (Tukey test, $P = 0.05$). The protection efficacy of a treatment corresponds to the difference of mean disease severity between the control and the treated plants. Consequently, the fungicide treatment showed the greatest protection efficacy (99.6%), followed by Bion (74%). All five elicitor treatments were as efficient as the commercial elicitor Bion in protecting wheat against STB: their protective effectiveness ranged between 69 and 72%. However, the disease severity scored on plants treated with CpG-ODN and glycine betaine was not statistically different from that of control plants at the following concentrations: medium concentration C2 for CpG-ODN (9.5×10^{-4} g liter^{-1}) and highest concentration C3 for CpG-ODN and glycine betaine (0.0095 g liter^{-1} and 12 g liter^{-1}, respectively).

Induction of plant immune responses. Independently from screening trials, we monitored the expression level of 23 defense-related genes of wheat at 1, 2, and 3 days after treatment with the five compounds (Table 1, labeled A to E). MANOVA tests showed that plant treatment had a significant effect on the expression of defense genes (Hotelling-Lawley test, P value $< 10^{-3}$). We then measured the difference of average expression level of each gene between treated plants and the water control, and represented it on a heatmap

profile (Fig. 2). Overall, elicitor treatments generally triggered plant defense mechanisms at day 2 after treatment. In addition, no priming activities were observed because the application of H_2O_2 had no supplementary effect to the application of one of the elicitor treatments on the expression of wheat defense genes. Three major patterns were highlighted by the hierarchical clustering of genes according to their expression levels. The first pattern (i) includes genes involved in cell wall reinforcement, in the mevalonate pathway for the biosynthesis of isoprenoids, and in the phenylpropanoid pathway for the biosynthesis of antimicrobial compounds. In particular, we observed that plants treated with the elicitor reference Bion showed a threefold upregulation of hydroxymethyl glutarate-CoA reductase (HMGR) gene expression across all experimental conditions and a twofold downregulation of chalcone synthase (CHS) gene expression at day 2 and 3 after treatment, with or without subsequent application of H_2O_2. HMGR and CHS are key regulators of isoprenoid and flavonoid biosynthesis, respectively (Antolín-Llovera et al. 2011; Dao et al. 2011). Conversely, plants treated with λ-carrageenan showed a downregulation of these two genes at day 1 and 2 after treatment, with or without H_2O_2.

The second pattern (ii) includes genes coding for PR proteins. A strong upregulation of PR4 and PR5 gene expression across all experimental conditions was observed for plants treated with λ-carrageenan, CpG-ODN, glycine betaine, and ergosterol. These genes code for the synthesis of hevein-like and thaumatin-like proteins displaying antimicrobial activities. PR1 and PR8 gene expression were also significantly upregulated by λ-carrageenan and ergosterol, with sixfold and fourfold increase, respectively. PR1 is a well-known marker of salicylic acid (SA)-dependent defense responses, while PR8 consists of class III chitinases (van Loon and Van Strien 1999). Conversely, *S. platensis* induced a sixfold downregulation of PR1 gene expression at day 2 after treatment, with or without H_2O_2 applied afterward. Interestingly, plants treated with Bion and water-treated plants subsequently sprayed with H_2O_2 showed no difference in PR gene expression compared with the control.

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

Confirmation of clustering results was provided by PCA (Fig. 3). Genes were used as variables and treatments as individuals. The first two PCs described the largest variability (26.8 and 17.2% initial variation, respectively). They enabled separation of the treatments and clustering of the genes into three similar groups with strong correlation coefficients ($|r| > 0.5$, P value $< 10^{-3}$). Genes coding for PR proteins were once again clearly separated into a distinct group. Similarly, genes involved in antioxidative processes or in plant defense signaling were gathered close to one another. Variables with significant correlation coefficients (P value $< 10^{-3}$) were those

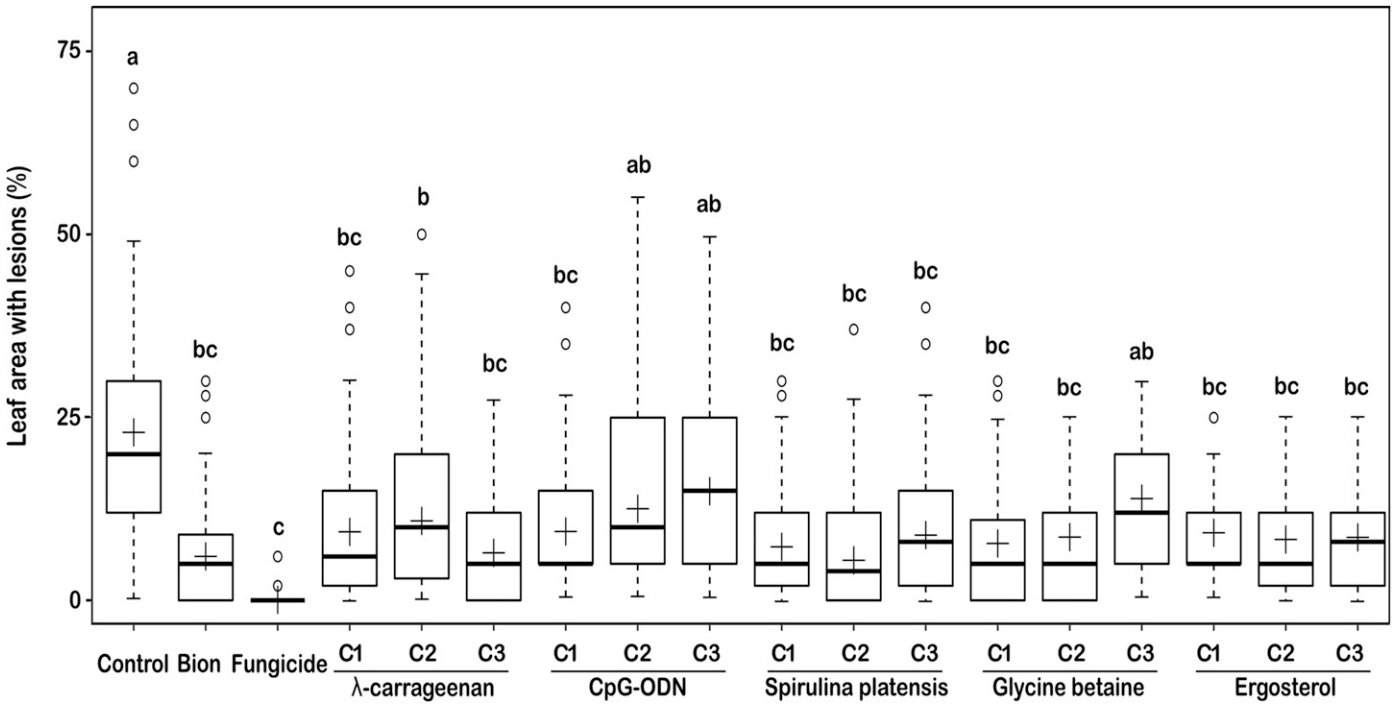

Fig. 1. Efficacy of five candidate elicitors (λ-carrageenan, cytosine-phosphate-guanine oligodesoxynucleotide motifs [CpG ODN], *Spirulina platensis*, glycine betaine, and ergosterol) tested at three different concentrations to protect wheat leaves against the disease Septoria tritici blotch (STB) under glasshouse conditions. Preventive treatment of wheat with various compounds significantly reduced *Zymoseptoria tritici* disease severity compared with the control. Compounds CpG-ODN and glycine betaine showed no difference with the control at high concentrations. Data are percentages of the third leaf surface of plants exhibiting symptomatic STB lesions (necrosis or chlorosis). Medians are represented by black horizontal lines in each box, and means are represented by "+" symbol ($n \geq 80$) (e.g., incomplete block design with five pots of eight plants and at least two independent experiments per treatment). Boxes tagged with the same letters correspond to means that are not significantly different using the Tukey test at $P = 0.05$.

which showed strong gene up- or downregulation in the heatmap profile.

Correlation between protection efficacy and gene induction. The PCA first component score described the largest variability (26.8%) and, thus, was used for the correlation test (Fig. 4). A correlation found between two variables is characterized by the fact that a change in one is accompanied by a change in another. It appears that the higher the coordinates were on dimension 1, the higher the protective efficacy. Overall, protection efficacy and defense induction were positively correlated at each qRT-PCR sampling date for all compounds. The strongest and most significant correlation was obtained for day 1 (linear regression analysis, $r^2 = 0.73$, P value = 0.03). Treatments with Bion and *S. platensis* were the most effective to protect wheat against STB while at the same time inducing significant defense responses in the plant. For the two other sampling dates, the correlations were positive but less significant (P value > 0.05).

DISCUSSION

Efficacious protection of wheat against STB under glasshouse conditions. Each of the five elicitor treatments (containing λ-carrageenan, CpG-ODN, *S. platensis*, glycine betaine, and ergosterol) significantly protected wheat against *Z. tritici* under semicontrolled conditions. The STB disease severity was broadly reduced by up to 70%, thus providing a consistent protection of the plant. In addition, these elicitor treatments were as efficient as the commercialized elicitor product Bion. We noted that high concentrations of applied CpG-ODN and glycine betaine did not effectively protect wheat against the pathogen. This finding could be related to the fact that elicitor compounds are generally effective at low concentrations and within a given window (Thakur and Sohal 2013; Trotel-Aziz et al. 2006). Moreover, in preliminary assays, we demonstrated that none of the compounds behaved as a biofungicide at the concentrations used for glasshouse screening. Therefore, it is likely that all five tested compounds did, indeed, act as elicitors of wheat defenses. This result is in line with previous research, which underlined their elicitor potential on other pathosystems.

For instance, among the three main types of marine carrageenans extracted from red algae, λ-carrageenan was proven to be the most efficient in stimulating plant defense responses due to its high degree of sulfation (41% of total weight) (Shukla et al. 2016; Vera et al. 2011). We could assume that its efficacy to protect wheat against *Z. tritici* could be linked to such high sulfate content, and further studies would allow a better understanding of its mode of action. Concerning CpG-ODN, no research has yet been reported for its ability to protect a crop plant against a fungal pathogen. However, previous studies have demonstrated that CpG-ODN acts as a pathogen-associated molecular pattern in mammalian cells and is recognized by pattern recognition receptor Toll-like receptor 9 (Carrington and Secombes 2006). A similar recognition process of CpG-ODN by specific wheat receptors can be assumed. On the other hand, the elicitor properties of *S. platensis* are likely due its

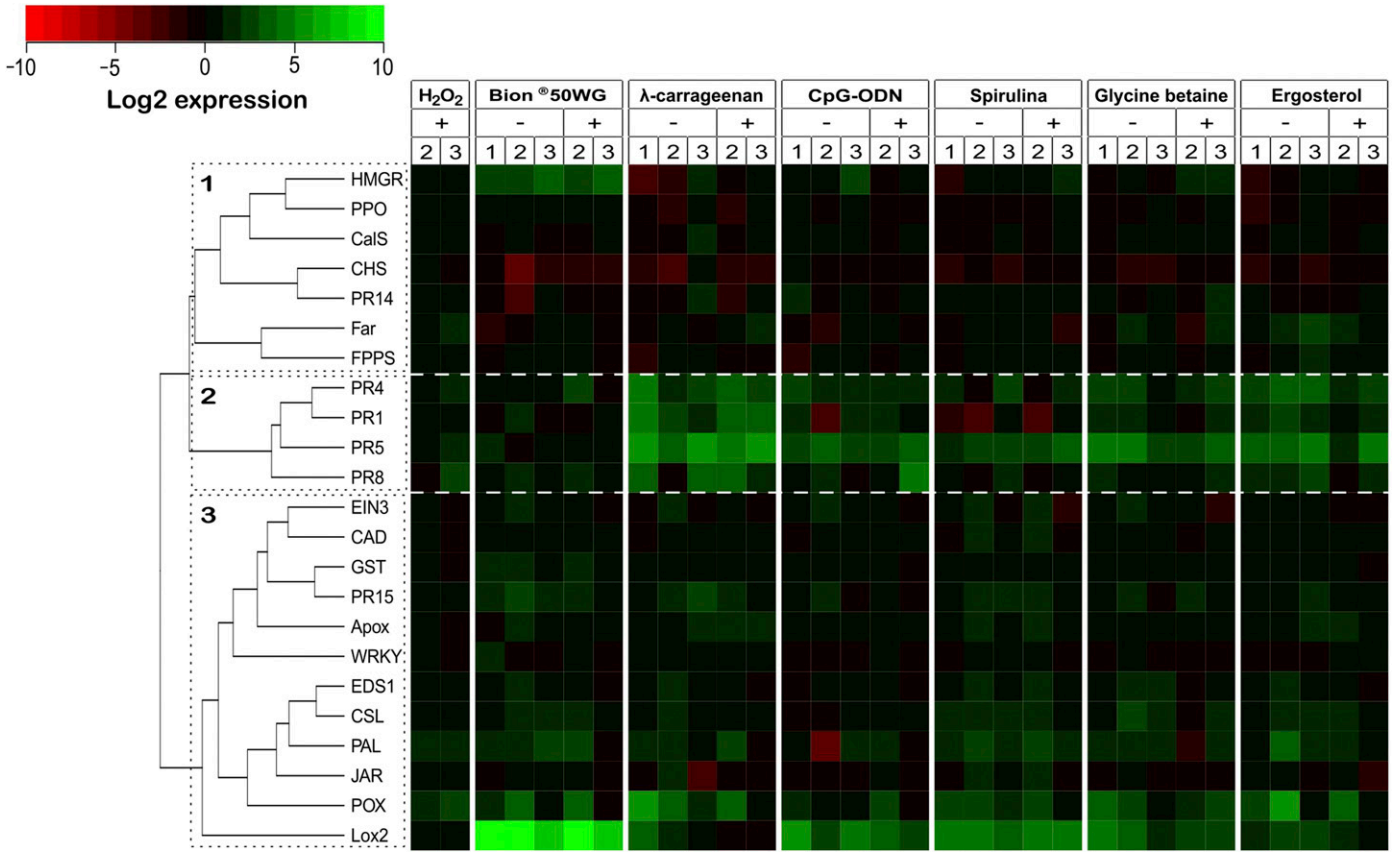

Fig. 2. Heatmap profiling of the average expression level of 23 defense-related genes of wheat across all experimental conditions (product, ± hydrogen peroxide [H_2O_2], and day posttreatment). Data obtained by the combination of two independent experiments ($n = 32$). Hierarchical clustering of gene expression highlighted three main patterns. The log2 expression levels above or below 0 represent an induction (in green) or a repression (in red), respectively, of gene expression in treated plants compared with the water control. H_2O_2 was applied (+) or not (–) on plants at 1 day after treatment to mimic a pathogen attack. Genes examined in this study: 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, and WRKY = WRKY transcription factor 30.

high content in secondary metabolites such as carotenoids, superoxide dismutase, glycolipids, and sulfolipids, as suggested in other studies (Priyadarshani and Rath 2012). Most interestingly, to our knowledge, the transkingdom potential of *S. platensis* in inducing the defense mechanisms of both animals and plants had never been established. In 1995, Kulik already assumed that cyanobacteria could represent valuable biocontrol agents in agriculture but, since then, no studies had yet demonstrated the potential of *S. platensis* as a plant resistance inducer (Kulik 1995). Concerning glycine betaine, most studies carried out up to now have reported its potential to induce plant resistance to abiotic stresses. The fact that this plant osmolyte may also play a role in enhancing plant defenses against diseases opens up a whole new range of possibilities in integrated pest management (IPM) strategies. Conversely, ergosterol is a well-known elicitor and our results confirm that this compound presents a clear benefit for sustainable plant protection extended to crop plants.

SA or JA defense signaling pathways are triggered in wheat. Biomolecular tests on wheat defense responses confirm that each elicitor treatment was indeed perceived by the plant: the expression of genes coding for antimicrobial compounds was upregulated, and the expression of genes involved in the synthesis of signal hormones such as SA and JA were induced concomitantly or at different time scales in treated wheat plants. Signaling pathways mediated by the hormones SA, JA, and ethylene (ET) play a major role in plant defense responses (Muthamilarasan and Prasad 2013).

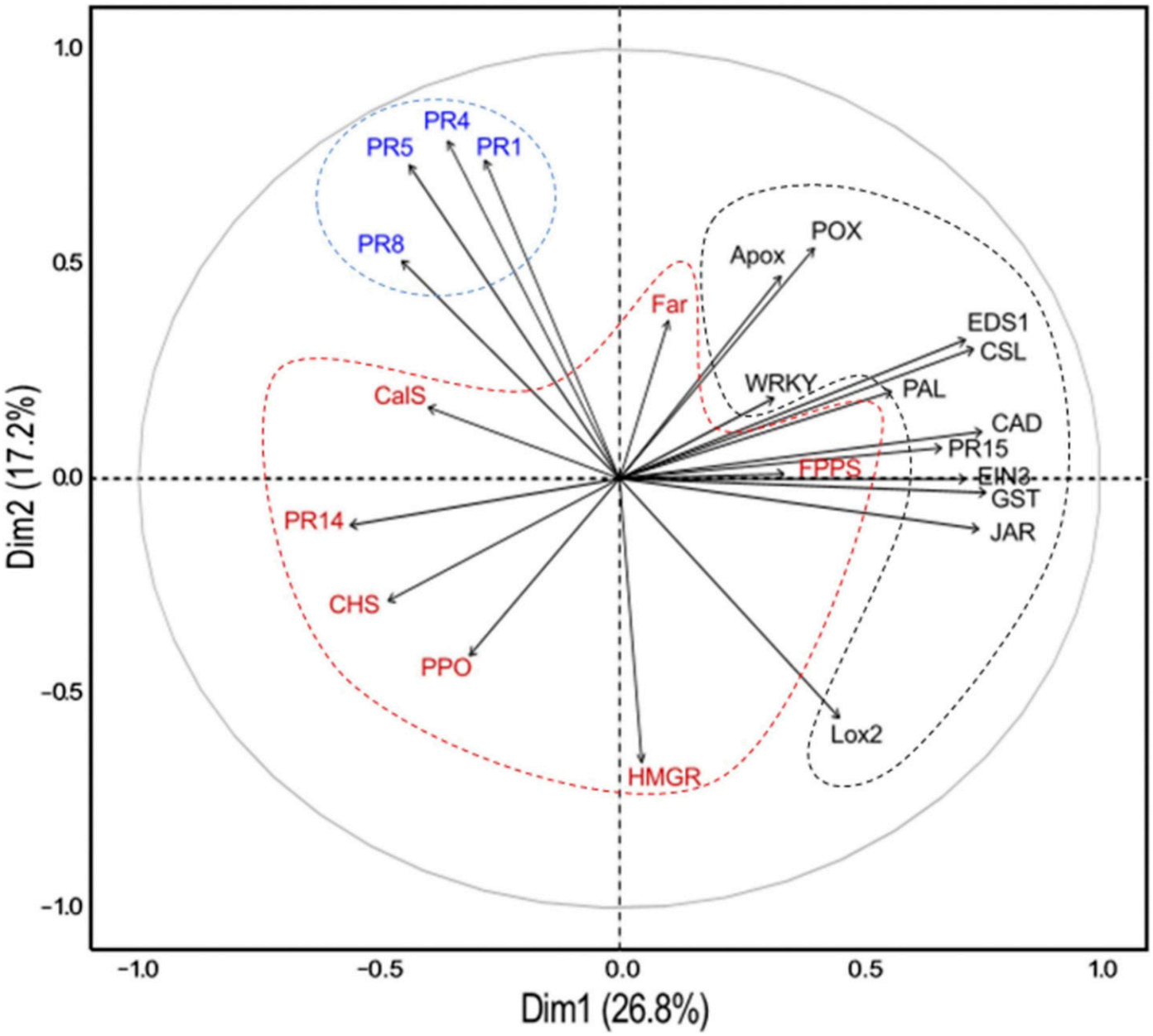

Fig. 3. Principal component analysis (PCA) of gene expression across all experimental conditions compared with water control (product, ± hydrogen peroxide [H_2O_2], and day posttreatment). Three major groups are highlighted in different colors (red, blue, and black). Variables correspond to the 23 defense-related genes of wheat examined by heatmap profiling ($n = 32$) and hierarchical clustering. Variables are projected on the first and second principal component scores (Dim1 and Dim2) and were significantly correlated with one of the dimensions. 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, and WRKY = WRKY transcription factor 30.

In dicotyledonous plants, SA-dependent defense signaling occurs upon infection by biotrophic or hemibiotrophic pathogens (Glazebrook 2005), whereas defense responses mediated by JA and ET are triggered upon infection by necrotrophic pathogens and phloem-feeding insects (Adie et al. 2007; Van der Ent et al. 2009). Defense genes can be differentially expressed through these defense signaling pathways. PR1 and PR5 are systemic acquired resistance (SAR) marker genes which are characteristically induced during SA-dependent defense responses, whereas LOX2 and PR4 induction is generally linked to JA-dependent signaling (Glazebrook 2005; van Loon and Van Strien 1999). Most studies have reported the existence of a mutual antagonism between SA- and JA-signaling pathways, although synergistic interactions have also been described in thale-cress (Niu et al. 2011; Schenk et al. 2000; van Wees et al. 2000). However, most investigations have been carried out on dicotyledonous plants and less is known about SA/JA crosstalk in monocotyledonous plants. Central elements of induced resistance pathways are conserved between dicotyledonous and monocotyledonous plants but their regulation and interaction with other plant pathways seem to have undergone particular evolutionary adaptations (Balmer et al. 2013; Kogel and Langen 2005). Knowledge of chemical and molecular mechanisms of induced resistance in monocotyledonous plants is still missing. For instance, the role of SA during SAR has yet to be elucidated, and the PR1 family has undergone important diversifications (Balmer et al. 2013). Recently, Ding et al. (2016) showed that wheat was able to finely tune its defense responses depending on its pathogenic invader (Ding et al. 2016). They reported that SA and JA were able to act synergistically or antagonistically in order to influence the expression of wheat defense genes.

In the present study, JA-dependent signaling was induced in plants treated with λ-carrageenan during the first 24 h, before giving way to SA-dependent defense responses. Previous studies have demonstrated that λ-carrageenan could protect tomato plants and thale-cress by triggering the expression of JA-related genes (Sangha et al. 2010, 2015). In addition, Vera et al. (2011) showed that carrageenans were able to suppress a disease at a systemic scale by triggering SA-related defense responses such as an increased PAL enzymatic activity and the accumulation of phenolic compounds (Vera et al. 2011). In addition, Ray et al. (2003) reported that infection of susceptible and resistant wheat cultivars by *Z. tritici* induced a strong upregulation of the LOX gene expression up to 3 h after plant infection before quickly decreasing (Ray et al. 2003). On the other hand, SA and JA defense signaling pathways were induced simultaneously in wheat plants treated with glycine betaine, CpG-ODN, or ergosterol. Therefore, our results confirm the existence of intricate hormone crosstalk in plant innate immunity (Balmer et al. 2013; Ding et al. 2016; Lochman and Mikes 2006; Thaler et al. 2012). On the other hand, only JA-dependent signaling seems to have been strongly induced in plants treated with *S. platensis* and Bion. The effect of *S. platensis* on plant induced resistance had never been investigated previously. However, numerous studies have already been dedicated to the elicitor potential of Bion and our results are surprisingly in contradiction to these previous findings. As a chemical elicitor consisting of acibenzolar-S-methyl, Bion shows functional analogy to the plant hormone SA and was shown to induce a long-lasting SAR with a characteristic accumulation of PR1 and PR5 proteins and an increase in PAL and CHS activity (Görlach et al. 1996; Hofgaard et al. 2005). The fact that our results, instead, suggest the involvement of JA-mediated signaling in wheat treated with Bion could be linked to the genotype of the cultivar used in this study (Ors et al. 2018) or to the fine tuning of signal hormones in the plant (Ding et al. 2016).

Further investigations, including biochemical experiments, would probably help to better understand how these five elicitor treatments and Bion contributed to induce such defense responses in this major European crop. Research on induced resistance of monocots is slowly emerging (Balmer et al. 2013). However, the primary objective of the present study was mainly to rapidly identify interesting elicitor compounds which are effective in protecting the wheat plant against STB. The qPFD represented an interesting biomolecular tool as a useful complement to glasshouse trials for thorough elicitor screening. The efficacy of the five elicitor treatments to protect wheat under semicontrolled conditions and their ability to trigger SA or JA signaling pathways in the plant make them attractive candidates for the development of additional elicitor

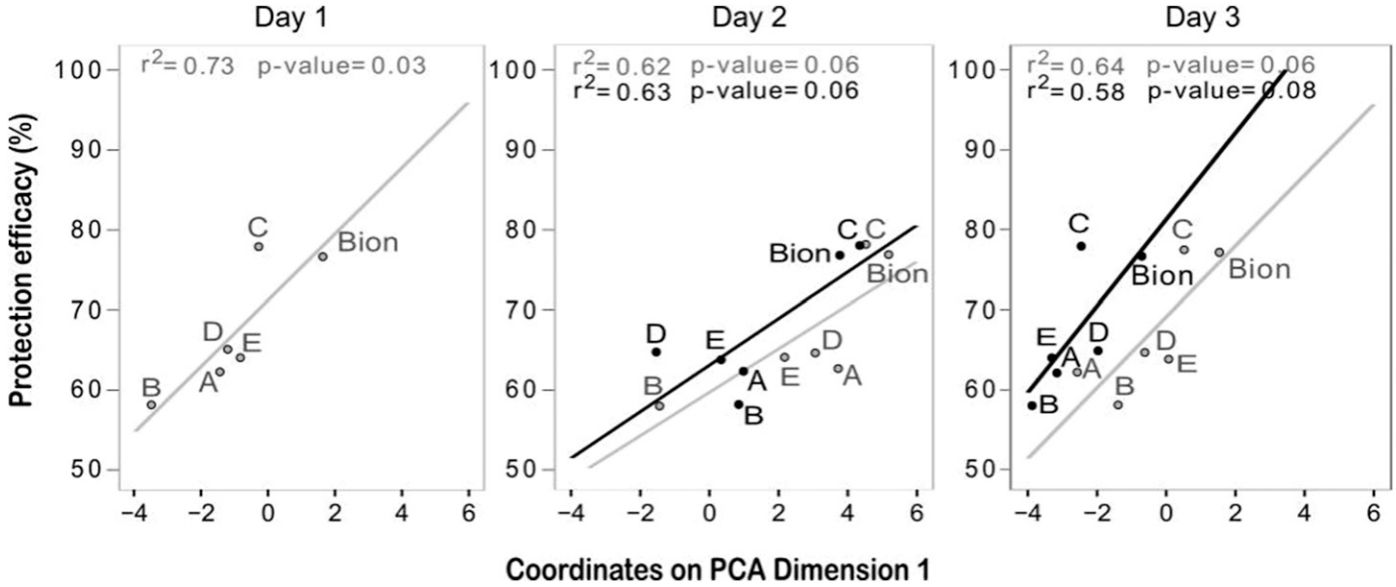

Fig. 4. Relationship between the expression of wheat defense genes and the efficacy of five compounds in protecting wheat against *Zymoseptoria tritici*: λ-carrageenan (A); cytosine-phosphate-guanine oligodesoxynucleotide motifs (CpG ODN) (B); *Spirulina platensis* (C); glycine betaine (D); and ergosterol (E). Positive correlations are highlighted and are significant at day 1 after plant treatment. Gene expression was obtained by quantitative reverse-transcription polymerase chain reaction while protection efficacy data were a result of glasshouse elicitor screening. Hydrogen peroxide (H_2O_2) applications were realized to test for priming activity: gray = without and black = with. Coordinates on the principal component analysis (PCA) first component score are averaged across biological replicates and were used as a measure of induced gene expression. Correlation coefficients and *P* values of linear models are indicated in each plot ($n \geq 80$ for screening tests and $n = 32$ for gene expression studies).

tools against STB. Our results are even more interesting because these compounds are already available on the market. Their common use has already required various toxicological tests (Bode et al. 2011; EFSA Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) 2013; Marles et al. 2011; Weiner 2014). For instance, λ-carrageenan is widely used in the food industry as an additive due to its jellifying and emulsifying properties (Weiner 2014), whereas *S. platensis* is used as a concentrated and nutritious food supplement (Small 2011). CpG-ODN is generally used at low doses for medical purposes as an adjuvant for vaccines targeting infectious diseases and cancer (Bode et al. 2011; Carpentier et al. 2003; Maubant et al. 2011). As an additional product of the sugar beet processing industry, glycine betaine is a commercially important compound with multiple applications in agriculture, medicine, and animal husbandry (Eklund et al. 2005; Mäkelä 2004). Ergosterol is generally extracted from yeast and is of industrial and commercial importance as a precursor of therapeutically useful substances such as vitamin D_2 (Ethiraj 2013). On a pragmatic basis, the main interest in implementing elicitors as alternative plant protection tools in agriculture is to reduce the use of agrochemicals in the field and promote environment-friendly IPM strategies. Consequently, field trials would represent the next logical step after this study in order to check the potential of these compounds under real conditions. Indeed, numerous environmental parameters such as plant developmental stage, plant genotype, and disease pressure are known to influence the protection efficacy of elicitors in the field (Walters et al. 2005).

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