



## **Evaluation of $\lambda$ -carrageenan, CpG-ODN, glycine betaine, *Spirulina platensis* and ergosterol as elicitors for the control of *Zymoseptoria tritici* in wheat**

Geraldine Le Mire, Ali Siah, Brice Marolleau, Matthieu Gaucher, Claude Maumené, Yves Brostaux, Sébastien Massart, Marie-Noëlle Brisset, M. Haissam Jijakli

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## Evaluation of $\lambda$ -carrageenan, CpG-ODN, glycine betaine, *Spirulina platensis* and ergosterol as elicitors for control of *Zymoseptoria tritici* in wheat

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First, seventh and ninth authors : Université de Liège - Gembloux Agro Bio-Tech – Centre de recherche TERRA – Laboratoire de phytopathologie intégrée et urbaine. Passage des déportés 2, 5030 Gembloux, Belgique. Second author : Institut Supérieur d'Agriculture (ISA) Lille – Institut de recherche Charles Violette (EA 7394), 48 Boulevard Vauban, F-59046 Lille cedex, France. Third, fourth and eighth authors: Institut de Recherche en Horticulture et Semences (IRHS) – INRA Angers – Equipe ResPOM, 42 rue Georges Morel, F-49071 Beaucouzé cedex, France. Fifth author: Arvalis-Institut du Végétal – Station expérimentale, 91720 Boigneville, France. Sixth author : Université de Liège - Gembloux Agro Bio-Tech – Centre de recherche TERRA – Statistiques, Informatiques et Mathématiques appliqués à la bioingénierie. Passage des déportés 2, 5030 Gembloux, Belgique.

Corresponding author: Geraldine Le Mire.

Tel: +32 472103615; Email address: [geraldine.lemire@gmail.com](mailto:geraldine.lemire@gmail.com)

## 1 ABSTRACT

2 Wheat crops are constantly challenged by the pathogen *Zymoseptoria tritici*  
 3 responsible for Septoria tritici Blotch (STB) disease. The present study reports the  
 4 evaluation of five elicitor compounds ( $\lambda$ -carrageenan, CpG-ODN, glycine betaine,  
 5 *Spirulina platensis* and ergosterol) for the protection of wheat against STB in order to  
 6 offer new alternative tools to farmers for sustainable crop protection. Screening of  
 7 elicitors of wheat defenses was carried out through a succession of experiments:  
 8 biocidal *in vitro* tests enabled to check for any fungicidal activities; glasshouse  
 9 experiments allowed to determine the efficacy of a given compound in protecting  
 10 wheat against STB; qRT-PCR biomolecular tests investigated the relative expression  
 11 of 23 defense genes in treated *versus* untreated plants. We therefore demonstrated  
 12 that  $\lambda$ -carrageenan, CpG-ODN, glycine betaine, *Spirulina platensis* and ergosterol  
 13 are potential elicitors of wheat defenses. Foliar treatment with these compounds  
 14 conferred protection of wheat by up to approximately 70 % against *Z. tritici* under  
 15 semi-controlled conditions and induced both SA- and/or JA-dependent signaling  
 16 pathways in the plant. These findings contribute to extend the narrow list of potential  
 17 elicitors of wheat defenses against *Z. tritici*.

## 19 KEYWORDS

20 *Triticum aestivum*; elicitor; *Zymoseptoria tritici*; Integrated pest management;  
 21 alternative plant protection tools

Wheat is the most cultivated crop in Europe and in the world, with up to 734 million tons produced in 2015-2016 (Fones and Gurr 2015; Satger 2016). This monocotyledonous plant must however 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). So far, fungicide use is the most effective control method as no wheat cultivar is currently totally resistant to *Z. tritici* (Fraaije et al. 2005; Palmer and Skinner 2002). About 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 and alternative plant protection tools which have a prominent place in current research (Lyon et al. 2014). Contrary to 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; Walters et al. 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, France) and BION®50WG (synthetic Acibenzolar-S-methyl elicitor; Syngenta, Switzerland) (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:  $\lambda$ -carrageenan, cytosine-phosphate-guanine oligodesoxynucleotide motifs (CpG ODN), *Spirulina (Arthrospira) platensis*, glycine betaine and ergosterol. As it appears, none of these compounds have been tested before as elicitors on the pathosystem wheat-*Z. tritici*, although previous research has indeed demonstrated their elicitor properties on other plant species and/or animals.  $\lambda$ -carrageenan stimulates the resistance of tobacco, tomato, and thale-cress plants against pathogens such as *Botrytis cinerea* and Tobacco mosaic virus (TMV) (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* (*Spirulina*) 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 (GB) 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. 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 INRA (France) 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 indeed triggers a characteristic cascade of defense signals resulting in plant induced resistance (Muthamilarasan et al. 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 cv. Avatar. Plants were grown in the glasshouse under semi-controlled conditions (natural photoperiod supplemented with artificial light if needed, with 20 °C  $\pm$  5 according to the sunlight). Screening experiments and plant defense induction experiments were undertaken independently. For the screening of elicitors for their protective efficacy, seeds were sown in 25 x 15 cm plastic pots filled with loam (10 plants per pot). The study of induced plant defenses was carried out at another location (INRA facilities in Angers) where seeds were sown in 30 x 20 cm plastic boxes filled with loam (40 plants per 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 eight days at 18 °C with a 12 h : 12 h day : night cycle. Inocula were prepared by washing the cultures with 10 ml sterile distilled water and the resulting spore suspension was adjusted to desired concentrations using Malassez cell.

## 101 Elicitor preparation

102 The sources and characteristics of the five compounds examined in this study are  
 103 provided in Table 1. To be noted that the compound CpG-ODN consists of CpG-28  
 104 (sequence 5'-TAAACGTTATAACGTTATGACGTCAT- 3') synthesized with a wholly  
 105 phosphorothioate backbone (Carpentier et al. 2003). For each compound, the tested  
 106 concentrations were not the same as we selected the average effective doses which  
 107 were generally used in previous studies to demonstrate their respective elicitor  
 108 potential. All treatment solutions were freshly prepared before use in distilled water  
 109 supplemented with 0.1 % (v : v) of spreading agent Break-Thru®S240 (polyether  
 110 trisiloxane, Evonik Industries), and 0.05 % (v : v) of solubilizing agent Tween 20  
 111 (polyoxyethylene-sorbitan monolaurate, Sigma Aldrich). Control plants were treated  
 112 with distilled water alone. In addition, other control plants were treated respectively  
 113 with 0.75 % (v : v) of the epoxiconazole-based fungicide Opus® (BASF Agro, France)  
 114 or with the synthetic elicitor Bion®50WG (abbreviated to Bion) at 0.6 mg ml<sup>-1</sup>  
 115 (recommended doses according to <https://ephy.anses.fr>). It should be noted that  
 116 preliminary *in vitro* experiments according to Siah et al (2010) have been conducted  
 117 to rule out any compound showing a direct biocidal activity towards the fungal  
 118 pathogen *Z. tritici* (see supplementary Fig. S1).

## 120 Glasshouse elicitor screening: plant treatment and inoculation

121 At the three-four leaf stage (third leaf fully expanded), the plants of each pot were  
 122 sprayed to run-off with 30 ml of the treatment solutions using a hand sprayer. Each  
 123 elicitor candidate was tested at three different concentrations in order to assess any  
 124 dose-dependent protection effects. Plant inoculation was performed five days after  
 125 treatment, by spraying the plants of each pot to run-off with 30 ml of a spore



suspension ( $10^6$  spores  $\text{ml}^{-1}$  in distilled water) amended with 0.05 % (v : v) Tween 20. Immediately after inoculation, each pot was covered with a transparent polyethylene bag for three 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 post-inoculation according to Siah et al. (2010). 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 (Gałeczki and Burzykowski 2013), and 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 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 the half of each



box were sprayed one day later with a solution containing 40 nm of hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) which mimics a pathogenic attack, as described by Dugé de Bernonville et al. (2014). Elicitor priming is characterized by the non-triggering 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). Such 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<sub>2</sub>O<sub>2</sub> shortly after would strongly induce the expression of defense genes. As a reactive oxygen species (ROS), H<sub>2</sub>O<sub>2</sub> is indeed involved in plant oxidative stress and acts as a key mediator in defense gene activation (Halliwell 2006). Besides, 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<sub>2</sub>O<sub>2</sub> in incompatible interactions (Shetty et al. 2007). We sampled the third leaf of five distinct seedlings at day 1 after treatment (*i.e.*, 24 hours after treatment), right before H<sub>2</sub>O<sub>2</sub> 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 treated with H<sub>2</sub>O<sub>2</sub> or not. All samples were immediately pooled, frozen and stored at - 80 °C until use. To be noted 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 RT-PCR

Total RNA was extracted from around 100 mg of plant tissue using the Nucleospin®RNA Plant Kit (Macherey-Nagel). Reverse-transcription of total RNA

176 was carried out using the M-MLV Reverse Transcriptase (ref M1701, Promega,  
 177 Madison USA), according to the manufacturer protocol. Real-time qPCR was  
 178 performed with MESA BLUE qPCR MasterMix (ref RT-SY2X-03+WOUFLB,  
 179 Eurogentec, Liège, Belgique) according to manufacturer instructions and using the  
 180 INRA biomolecular tool developed by Brisset and Duge De Bernonville (2011) on a  
 181 Biorad MyiC detection system. Cycling conditions consisted of a first step of DNA  
 182 polymerase activation (95 °C for 5 min), followed by 35 repeated cycles of  
 183 denaturing, annealing and extending (95 °C for 15 min, 60 °C for 1min). The  
 184 biomolecular study focused on twenty three different genes involved in several plant  
 185 defense mechanisms, including pathogenesis-related (PR) proteins, secondary  
 186 metabolism, oxidative stress, and defense signaling pathways (Van Loon and Van  
 187 Strien 1999; Vogt 2009; Weber 2002). Relative gene expression was obtained using  
 188 the  $2^{-\Delta\Delta Ct}$  method (Schmittgen and Livak 2008). Values correspond to the average  
 189 difference of expression of a given gene between treated and water control plants at  
 190 each time point (data obtained from the combination of two independent  
 191 experiments). Three internal reference genes were used for normalization (e.g.,  
 192 TubA, GAPDH, and Actin). The effect of plant treatment on the wheat defense  
 193 responses was evaluated by Multivariate ANOVA. In order to visualize and analyze  
 194 relative gene expression, a heatmap representation was performed using  
 195 dissimilarity distance (1-cor(X, Y)). Moreover, the identification of sets of genes that  
 196 may be similarly expressed across all conditions within the dataset was realized by  
 197 clustering gene expression. Finally, clustering results were confirmed by Principal  
 198 Component Analysis (PCA) of the gene expression data. PCA is a multivariate  
 199 statistical technique for simplifying complex data sets in which observations are  
 200 described by several dependent variables (in this study, the twenty three defense-

related genes) (Abdi and Williams 2010). The dimensionality of the data is 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 principal components 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 to the induction of defense responses in the treated wheat plant. Such correlation increases the chances of a compound of being short-listed for further experiments under practical conditions. We therefore examined for each compound 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) since they were only tested at this concentration during biomolecular experiments.

## RESULTS

### Protection efficacy against *Zymoseptoria 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 to 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 (B) and glycine betaine (D) was not statistically different to that of control plants at the following concentrations: medium concentration C2 for CpG-ODN ( $9.5 \times 10^{-4} \text{ g l}^{-1}$ ) and highest concentration C3 for CpG-ODN and glycine betaine ( $0.0095 \text{ g l}^{-1}$  and  $12 \text{ g l}^{-1}$  respectively).

## 251 Induction of plant immune responses

252 Independently from screening trials, we monitored the expression level of 23  
 253 defense-related genes of wheat at 1, 2 and 3 days after treatment with the five  
 254 compounds (labelled A to E; Table 1). MANOVA tests showed that plant treatment  
 255 had a significant effect on the expression of defense genes (Hotelling-Lawley test, *p*-  
 256 *value* < 10<sup>-3</sup>). We then measured the difference of average expression level of each  
 257 gene between treated plants and the water control, and represented it on a heatmap  
 258 profile (Fig. 2). Overall, elicitor treatments generally triggered plant defense  
 259 mechanisms at day 2 after treatment. Besides, no priming activities were observed  
 260 as the application of H<sub>2</sub>O<sub>2</sub> had no supplementary effect to the application of one of  
 261 the elicitor treatments on the expression of wheat defense genes. Three major  
 262 patterns were highlighted by the hierarchical clustering of genes according to their  
 263 expression levels. The first pattern (1) includes genes involved in cell wall  
 264 reinforcement, in the mevalonate pathway for the biosynthesis of isoprenoids and in  
 265 the phenylpropanoid pathway for the biosynthesis of antimicrobial compounds. In  
 266 particular, we observed that plants treated with the elicitor reference Bion showed a  
 267 3-fold upregulation of HMGR gene expression (hydroxymethyl glutarate-CoA  
 268 reductase) across all experimental conditions and a 2-fold downregulation of CHS  
 269 gene expression (chalcone synthase) at day 2 and 3 after treatment, with or without  
 270 subsequent application of H<sub>2</sub>O<sub>2</sub>. HMGR and CHS are key regulators of isoprenoid  
 271 and flavonoid biosynthesis respectively (Antolín-Llovera et al. 2011; Dao et al. 2011).  
 272 Conversely, plants treated with λ-carrageenan (A) showed a downregulation of these  
 273 two genes, at day 1 and 2 after treatment, with or without H<sub>2</sub>O<sub>2</sub>.  
 274 The second pattern (2) includes genes coding for PR proteins. A strong upregulation  
 275 of PR4 and PR5 gene expression across all experimental conditions was observed

276 for plants treated with  $\lambda$ -carrageenan (A), CpG-ODN (B), glycine betaine (D) and  
 277 ergosterol (E). These genes code respectively for the synthesis of hevein-like and  
 278 thaumatin-like proteins displaying antimicrobial activities. PR1 and PR8 gene  
 279 expression were also significantly upregulated by  $\lambda$ -carrageenan and ergosterol with  
 280 6-fold and 4-fold increase, respectively. PR1 is a well-known marker of salicylic acid-  
 281 dependent defense responses, while PR8 consists of class III chitinases (Van Loon  
 282 and Van Strien 1999). Conversely, spirulina (C) induced a 6-fold downregulation of  
 283 PR1 gene expression at day 2 after treatment, with or without H<sub>2</sub>O<sub>2</sub> applied  
 284 afterwards. Interestingly, plants treated with Bion and water-treated plants  
 285 subsequently sprayed with H<sub>2</sub>O<sub>2</sub> showed no difference in PR gene expression  
 286 compared to the control.

287 A last pattern (3) includes genes involved in anti-oxidative processes and plant  
 288 defense-signaling. The expression of LOX2 (13-lipoxygenase 2) was strongly  
 289 induced by Bion (10-fold upregulation) and by CpG-ODN, spirulina, GB and  
 290 ergosterol (between 7 and 9-fold upregulation). Similarly, a 6-fold upregulation of  
 291 LOX2 expression was induced by  $\lambda$ -carrageenan but only at day 1 after treatment. It  
 292 is noteworthy that the lipoxygenase enzyme coded by the LOX2 gene is involved in  
 293 jasmonic acid-dependent defense signaling (Wasternack and Hause 2013). On the  
 294 other hand, expression of genes PAL (phenylalanine ammonia-lyase), POX  
 295 (peroxidase) and PR15 (oxalate oxidase) were upregulated by Bion and by the other  
 296 tested compounds, with the exception of CpG-ODN. Such upregulation occurred  
 297 generally at day 1 and/or day 2 after treatment, with or without H<sub>2</sub>O<sub>2</sub> application. The  
 298 enzyme PAL is involved in the biosynthesis of phenolic compounds, including  
 299 salicylic acid, 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 principal components described the largest variability (26.8 % and 17.2 % of initial variation, respectively). They enabled to separate the treatments and cluster the genes into three similar groups with strong correlation coefficients ( $|r| > 0.5$ ,  $p\text{-value} < 10^{-3}$ ). Genes coding for PR proteins were once again clearly separated into a distinct group. Similarly, genes involved in anti-oxidative processes or in plant defense-signaling were gathered close to one another. Variables with significant correlation coefficients ( $p\text{-value} < 10^{-3}$ ) were those which showed strong gene up- and/or down-regulation in the heatmap profile.

### Correlation between protection efficacy and gene induction

The PCA first component score described the largest variability (26.8%) and was thus 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. As it appears, 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,  $r^2 = 0.73$ ,  $p\text{-value} = 0.03$ ). Treatments with Bion and spirulina 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 ( $p\text{-value} > 0.05$ ).



## DISCUSSION

### Efficacious protection of wheat against STB under glasshouse conditions

Each of the five elicitor treatments containing respectively  $\lambda$ -carrageenan, CpG-ODN, spirulina, glycine betaine and ergosterol, significantly protected wheat against *Z. tritici* under semi-controlled conditions. The STB disease severity was broadly reduced by up to 70%, thus providing a consistent protection of the plant. Besides, these elicitor treatments were as efficient as the commercialized elicitor product Bion. We highlighted that high concentrations of applied CpG-ODN and GB did not effectively protect wheat against the pathogen. Such finding could be related to the fact that elicitor compounds are generally effective at low concentrations and within a given window (Trotel-Aziz et al. 2006; Thakur and Sohal 2013). Moreover, we demonstrated in preliminary assays that none of the compounds behaved as biofungicides at the concentrations used for glasshouse screening. It is therefore likely that all five tested compounds indeed acted as elicitors of wheat defenses. Such result is in line with previous researches which underlined their elicitor potential on other pathosystems.

For instance, among the three main types of marine carrageenans extracted from red algae,  $\lambda$ -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 (PAMP) in mammalian cells and is recognized by

pattern recognition receptor (PRR) Toll-Like Receptor 9 (TLR9) (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 spirulina are likely due its high content in secondary metabolites such as carotenoids, superoxide dismutase, glycolipids and sulfolipids, as suggested in other studies (Priyadarshani and Rath 2012). Most interestingly, and to our knowledge, the transkingdom potential of spirulina 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 spirulina as a plant resistance inducer (Kulik 1995). Concerning GB, 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 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 and/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; the expression of genes involved in the synthesis of signal hormones such as salicylic acid (SA) and jasmonic acid (JA) were induced concomitantly and/or at different time scales in treated wheat plants. Signaling pathways mediated by the hormones SA, JA and ethylene (ET) play indeed a major role in plant defense responses (Muthamilarasan et al. 2013).

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

399 In the present study, JA-dependent signaling was induced in plants treated with  $\lambda$ -  
 400 carrageenan during the first 24 hours before giving way to SA-dependent defense  
 401 responses. Previous studies have demonstrated that  $\lambda$ -carrageenan could protect  
 402 tomato plants and thale-cress by triggering the expression of JA-related genes  
 403 (Sangha et al. 2010, 2015). In addition, Vera et al (2011) showed that carrageenans  
 404 were able to suppress a disease at a systemic scale by triggering SA-related defense  
 405 responses such as an increased PAL enzymatic activity and the accumulation of  
 406 phenolic compounds (Vera et al. 2011). Besides, Ray et al (2003) reported that  
 407 infection of susceptible and resistant wheat cultivars by *Z. tritici* induced a strong  
 408 upregulation of the LOX gene expression up to 3 hours after plant infection before  
 409 quickly decreasing (Ray et al. 2003). On the other hand, SA and JA defense  
 410 signaling pathways were induced simultaneously in wheat plants treated with GB,  
 411 CpG-ODN or ergosterol. Our results therefore confirm the existence of intricate  
 412 hormone crosstalk in plant innate immunity (Balmer et al. 2013; Ding et al. 2016;  
 413 Lochman and Mikes 2006; Thaler et al. 2012). On the other hand, only JA-dependent  
 414 signaling seems to have been strongly induced in plants treated with spirulina and  
 415 Bion. The effect of spirulina on plant induced resistance had never been investigated  
 416 previously. However, numerous studies have already been dedicated to the elicitor  
 417 potential of Bion and our results are surprisingly in contradiction with these previous  
 418 findings. As a chemical elicitor consisting of Acibenzolar-S-methyl, Bion shows  
 419 functional analogy to the plant hormone SA and was shown to induce a long lasting  
 420 SAR with a characteristic accumulation of PR1 and PR5 proteins and an increase in  
 421 PAL and CHS activity (Görlach et al. 1996; Hofgaard et al. 2005). The fact that our  
 422 results rather suggest the involvement of JA mediated signaling in wheat treated with

423 Bion could be linked to the genotype of the cultivar used in this study (Ors et al.  
 424 2017) and/or to the fine tuning of signal hormones in the plant (Ding et al. 2016).  
 425 Further investigations, including biochemical experiments, would probably help to  
 426 better understand how these five elicitor treatments and Bion contributed to induce  
 427 such defense responses in this major European crop. Research on induced  
 428 resistance of monocots is slowly emerging (Balmer et al. 2013). Yet, the primary  
 429 objective of the present study was mainly to rapidly identify interesting elicitor  
 430 compounds which are effective to protect the wheat plant against STB. The qPFD  
 431 represented an interesting biomolecular tool as a useful complement to glasshouse  
 432 trials for thorough elicitor screening. The efficacy of the five elicitor treatments to  
 433 protect wheat under semi-controlled conditions and their ability to trigger SA and/or  
 434 JA signaling pathways in the plant make them attractive candidates for the  
 435 development of additional elicitor tools against STB. Our results are even more  
 436 interesting as these compounds are already available on the market. Their common  
 437 use has already required various toxicological tests (Bode et al. 2011; EFSA  
 438 FEEDAP Panel 2013; Marles et al. 2011; Weiner 2014). For instance,  $\lambda$ -carrageenan  
 439 is widely used in the food industry as an additive due to its jellifying and emulsifying  
 440 properties (Weiner 2014), whereas spirulina is used as a concentrated and nutritious  
 441 food supplement (Small 2011). CpG-ODN is generally used at low doses for medical  
 442 purposes as an adjuvant for vaccines targeting infectious diseases and cancer (  
 443 (Bode et al. 2011; Carpentier et al. 2003; Maubant et al. 2011). As an additional  
 444 product of the sugar beet processing industry, GB is a commercially important  
 445 compound with multiple applications in agriculture, medicine and animal husbandry  
 446 (Eklund et al. 2005; Mäkelä 2004). Ergosterol is generally extracted from yeast and is  
 447 of industrial and commercial importance as a precursor of therapeutically useful

substances such as vitamin D<sub>2</sub> (Ethiraj 2013). On a pragmatic basis, the main interest of 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 to this study in order to check the potential of these compounds under real conditions. Numerous environmental parameters such as plant developmental stage, plant genotype and disease pressure are indeed known to influence the protection efficacy of elicitors in the field (Walters et al. 2005).

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## LITERATURE CITED

- Abdi, H., and Williams, L. J. 2010. Principal component analysis. Wiley Interdiscip. Rev. Comput. Stat. 2:433–459. doi: 10.1002/wics.101
- Adie, B., Chico, J., Rubio-Somoza, I., and Solano, R. 2007. Modulation of plant defenses by ethylene. J. Plant Growth Regul. 26:160–177. doi: 10.1007/s00344-007-0012-6
- Amborabé, B.-E., Rossard, S., Pérault, J.-M., and Roblin, G. 2003. Specific perception of ergosterol by plant cells. C. R. Biol. 326:363–370.
- Antolín-Llovera, M., Leivar, P., Arró, M., Ferrer, A., Boronat, A., and Campos, N. 2011. Modulation of plant HMG-CoA reductase by protein phosphatase 2A. Plant Signal. Behav. 6:1127–1131. doi: 10.4161/psb.6.8.16363

- 472 Balmer, D., Planchamp, C., and Mauch-Mani, B. 2013. On the move: Induced  
473 resistance in monocots. *J. Exp. Bot.* 64:1249–1261. doi: 10.1093/jxb/ers248
- 474 Bode, C., Zhao, G., Steinhagen, F., Kinjo, T., and Klinman, D. M. 2011. CpG DNA as  
475 a vaccine adjuvant. *Expert Rev. Vaccines.* 10:499–511. doi: 10.1586/erv.10.174
- 476 Carpentier, A. F., Auf, G., and Delattre, J.-Y. 2003. CpG-oligonucleotides for cancer  
477 immunotherapy: Review of the literature and potential applications in malignant  
478 glioma. *Front. Biosci.* 8:e116–e128.
- 479 Carrington, A. C., and Secombes, C. J. 2006. A review of CpGs and their relevance  
480 to aquaculture. *Vet. Immunol. Immunopathol.* 112:87–101. doi:  
481 10.1016/j.vetimm.2006.03.015
- 482 Covello, J. M., Friend, S. E., Purcell, S. L., Burka, J. F., Markham, R. J. F., Donkin, A.  
483 W., Groman, D. B., and Fast, M. D. 2012. Effects of orally administered  
484 immunostimulants on inflammatory gene expression and sea lice (*Lepeophtheirus*  
485 *salmonis*) burdens on Atlantic salmon (*Salmo salar*). *Aquaculture.* 366:9–16. doi:  
486 10.1016/j.aquaculture.2012.08.051
- 487 Dao, T., Linthorst, H., and Verpoorte, R. 2011. Chalcone synthase and its functions  
488 in plant resistance. *Phytochem. Rev.* 10:397–412. doi: 10.1007/s11101-011-9211-7
- 489 Dayan, F. E., Cantrell, C. L., and Duke, S. O. 2009. Natural products in crop  
490 protection. *Bioorg. Med. Chem.* 17:4022–4034. doi: 10.1016/j.bmc.2009.01.046
- 491 Ding, L.-N., Yang, G.-X., Yang, R.-Y., Cao, J., and Zhou, Y. 2016. Investigating  
492 interactions of salicylic acid and jasmonic acid signaling pathways in monocots  
493 wheat. *Physiol. Mol. Plant Pathol.* 93:67–74. doi: 10.1016/j.pmpp.2016.01.002
- 494 Dugé de Bernonville, T., Marolleau, B., Staub, J., Gaucher, M., Brisset, M.-N. 2014.  
495 Using molecular tools to decipher the complex world of plant resistance inducers: an  
496 apple case study. *J. Agric. Food Chem.* 62:11403–11411.



- 497 EFSA FEEDAP Panel (EFSA Panel on Additives and Products or Substances used  
498 in Animal Feed). 2013. Scientific Opinion on the safety and efficacy of betaine  
499 (betaine anhydrous and betaine hydrochloride) as a feed additive for all animal  
500 species based on a dossier submitted by VITAC EEIG. EFSA J. 11:3210. doi:  
501 10.2903/j.efsa.2013.3210
- 502 Eklund, M., Bauer, E., Wamatu, J., and Mosenthin, R. 2005. Potential nutritional and  
503 physiological functions of betaine in livestock. Nutr. Res. Rev. 18:31–48. doi:  
504 10.1079/NRR200493
- 505 Ethiraj, S. 2013. Fermentative production of ergosterol using *Saccharomyces*  
506 *cerevisiae*. J. Appl. Sci. Res. 9:1214–1221.
- 507 Eyal, Z. 1999. The *Septoria tritici* and *Stagonospora nodorum* Blotch diseases of  
508 wheat. Eur. J. Plant Pathol. 105:629–641. doi: 10.1023/A:1008716812259
- 509 Farag, M. R., Alagawany, M., El-Hack, M. E. A., and Dhama, K. 2016. Nutritional and  
510 healthical aspects of *Spirulina (Arthrospira)* for poultry, animals and human. Int. J.  
511 Pharmacol. 12:36–51. doi: 10.3923/ijp.2016.36.51
- 512 Fones, H., and Gurr, S. 2015. The impact of *Septoria tritici* Blotch disease on wheat:  
513 An EU perspective. Fungal Genet. Biol. 79:3–7. doi: 10.1016/j.fgb.2015.04.004
- 514 Fraaije, B. A., Cools, H. J., Fountaine, J., Lovell, D. J., Motteram, J., West, J. S., and  
515 Lucas, J. A. 2005. Role of ascospores in further spread of Qol-resistant cytochrome  
516 b alleles (G143A) in field populations of *Mycosphaerella graminicola*.  
517 Phytopathology. 95:933–941. doi: 10.1094/PHYTO-95-0933
- 518 Gałecki, A., and Burzykowski, T. 2013. Linear mixed-effects model. In Linear Mixed-  
519 Effects Models Using R, Springer Texts in Statistics, Springer New York, p. 245–273.  
520 doi: 10.1007/978-1-4614-3900-4\_13

- 521 Glazebrook, J. 2005. Contrasting mechanisms of defense against biotrophic and  
 522 necrotrophic pathogens. *Annu. Rev. Phytopathol.* 43:205–227. doi:  
 523 10.1146/annurev.phyto.43.040204.135923
- 524 Görlach, J., Volrath, S., Knauf-Beiter, G., Hengy, G., Beckhove, U., Kogel, K. H.,  
 525 Oostendorp, M., Staub, T., Ward, E., Kessmann, H., and Ryals, J. 1996.  
 526 Benzothiadiazole, a novel class of inducers of systemic acquired resistance,  
 527 activates gene expression and disease resistance in wheat. *Plant Cell.* 8:629–643.  
 528 doi: 10.1105/tpc.8.4.629
- 529 Halliwell, B. 2006. Reactive species and antioxidants. Redox biology is a  
 530 fundamental theme of aerobic life. *Plant Physiol.* 141:312–322. doi:  
 531 10.1104/pp.106.077073
- 532 Hofgaard, I. S., Ergon, Å., Wanner, L. A., and Tronsmo, A. M. 2005. The effect of  
 533 chitosan and Bion on resistance to pink snow mould in perennial ryegrass and winter  
 534 wheat. *J. Phytopathol.* 153:108–119. doi: 10.1111/j.1439-0434.2005.00937.x
- 535 Kogel, K-H., Langen, G. 2005. Induced disease resistance and gene expression in  
 536 cereals. *Cell Microbio.* 7:1555–1564. doi:10.1111/j.1462-5822.2005.00592.x
- 537 Kulik, M. M. 1995. The potential for using cyanobacteria (blue-green algae) and  
 538 algae in the biological control of plant pathogenic bacteria and fungi. *Eur. J. Plant*  
 539 *Pathol.* 101:585–599. doi: 10.1007/BF01874863
- 540 La Camera, S., Gouzerh, G., Dhondt, S., Hoffmann, L., Fritig, B., Legrand, M., et al.  
 541 2004. Metabolic reprogramming in plant innate immunity: the contributions of  
 542 phenylpropanoid and oxylipin pathways. *Immunol. Rev.* 198:267–284. doi:  
 543 10.1111/j.0105-2896.2004.0129.x

- 544 Lawal, B. 2014. Incomplete block design. In *Applied Statistical Methods in*  
 545 *Agriculture, Health and Life Sciences*, Springer, Cham, p. 639–659. doi:  
 546 10.1007/978-3-319-05555-8\_16
- 547 Le Mire, G., Nguyen, M., Fassotte, B., Du Jardin, P., Verheggen, F., Delaplace, P.,  
 548 and Jijakli, M. H. 2016. Implementing plant biostimulants and biocontrol strategies in  
 549 the agroecological management of cultivated ecosystems. A review. *Biotechnol.*  
 550 *Agron. Société Environ.* 20(S1):299–313.
- 551 Lochman, J., and Mikes, V. 2006. Ergosterol treatment leads to the expression of a  
 552 specific set of defence-related genes in tobacco. *Plant Mol. Biol.* 62:43–51. doi:  
 553 10.1007/s11103-006-9002-5
- 554 Lyon, G. D., Newton, A. C., and Walters, D. R. 2014. Induced resistance in crop  
 555 protection: The future, drivers and barriers. In *Induced Resistance for Plant Defense*,  
 556 eds. Dale R. Walters, Adrian C. Newton, and Gary D. Lyon. John Wiley & Sons, Ltd,  
 557 p. 316–325. doi: 10.1002/9781118371848.ch14
- 558 Mäkelä, P. 2004. Agro-industrial uses of glycine betaine. *Sugar Tech.* 6:207–212.  
 559 doi: 10.1007/BF02942500
- 560 Marles, R. J., Barrett, M. L., Barnes, J., Chavez, M. L., Gardiner, P., Ko, R., Mahady,  
 561 G. B., Low Dog, T., Sarma, N. D., Giancaspro, G. I., Sharaf, M., and Griffiths, J.  
 562 2011. United States pharmacopeia safety evaluation of *Spirulina*. *Crit. Rev. Food Sci.*  
 563 *Nutr.* 51:593–604. doi: 10.1080/10408391003721719
- 564 Maubant, S., Banissi, C., Beck, S., Chauvat, A., and Carpentier, A. F. 2011. Adjuvant  
 565 properties of cytosine-phosphate-guanosine oligodeoxynucleotide in combination  
 566 with various polycations in an ovalbumin-vaccine model. *Nucleic Acid Ther.* 21:231–  
 567 240. doi: 10.1089/nat.2011.0291

- Mercier, L., Lafitte, C., Borderies, G., Briand, X., Esquerré-Tugayé, M. T., and Fournier, J. 2001. The algal polysaccharide carrageenans can act as an elicitor of plant defence. *New Phytol.* 149:43–51. doi: 10.1046/j.1469-8137.2001.00011.x
- Muthamilarasan, M., and Prasad, M. 2013. Plant innate immunity: An updated insight into defense mechanism. *J. Biosci.* 38:433–449.
- Niu, D. D., Liu, H. X., Jiang, C. H., Wang, Y. P., Wang, Q. Y., Jin, H. L., and Guo, J. H. 2011. The plant growth-promoting rhizobacterium *Bacillus cereus* AR156 induces systemic resistance in *Arabidopsis thaliana* by simultaneously activating salicylate- and jasmonate/ethylene-dependent signaling pathways. *Mol. Plant. Microbe Interact.* 24:533–542. doi: 10.1094/MPMI-09-10-0213
- Ors, M. E., Randoux, B., Selim, S., Couleaud, G., Maumené, C., and Reignault, P. 2017. Cultivar-dependent partial resistance and associated defence mechanisms in wheat against *Zymoseptoria tritici*. *Plant Pathol.* doi: 10.1111/ppa.12760
- Palmer, C. L., and Skinner, W. 2002. *Mycosphaerella graminicola*: Latent infection, crop devastation and genomics. *Mol. Plant Pathol.* 3:63–70. doi: 10.1046/j.1464-6722.2002.00100.x
- Priyadarshani, I., and Rath, B. 2012. Bioactive compounds from microalgae and cyanobacteria: Utility and applications. *Int. J. Pharm. Sci. Res.* 3:4123–4130. doi: 10.13040/IJPSR.0975-8232.3(11).4123-30
- Ray, S., Anderson, J., Urmeev, F., and Goodwin, S. 2003. Rapid induction of a protein disulfide isomerase and defense-related genes in wheat in response to the hemibiotrophic fungal pathogen *Mycosphaerella graminicola*. *Plant Mol. Biol.* 53:741–754. doi: 10.1023/B:PLAN.0000019120.74610.52

- 591 Rossard, S., Roblin, G., and Atanassova, R. 2010. Ergosterol triggers characteristic  
592 elicitation steps in *Beta vulgaris* leaf tissues. J. Exp. Bot. 61:1807–1816. doi:  
593 10.1093/jxb/erq047
- 594 Rudd, J. J. 2015. Previous bottlenecks and future solutions to dissecting the  
595 *Zymoseptoria tritici*–wheat host-pathogen interaction. Fungal Genet. Biol. 79:24–28.  
596 doi: 10.1016/j.fgb.2015.04.005
- 597 Sangha, J. S., Ravichandran, S., Prithiviraj, K., Critchley, A. T., and Prithiviraj, B.  
598 2010. Sulfated macroalgal polysaccharides  $\lambda$ -carrageenan and  $\iota$ -carrageenan  
599 differentially alter *Arabidopsis thaliana* resistance to *Sclerotinia sclerotiorum*. Physiol.  
600 Mol. Plant Pathol. 75:38–45. doi: 10.1016/j.pmpp.2010.08.003
- 601 Sangha, J. S., Kandasamy, S., Khan, W., Bahia, N. S., Singh, R. P., Critchley, A. T.,  
602 et al. 2015.  $\lambda$ -Carrageenan suppresses tomato chlorotic dwarf viroid (TCDVd)  
603 replication and symptom expression in tomatoes. Mar. Drugs. 13:2875–2889. doi:  
604 10.3390/md13052875
- 605 Schenk, P. M., Kazan, K., Wilson, I., Anderson, J. P., Richmond, T., Somerville, S.  
606 C., and Manners, J. M. 2000. Coordinated plant defense responses in *Arabidopsis*  
607 revealed by microarray analysis. Proc. Natl. Acad. Sci. 97:11655–11660. doi:  
608 10.1073/pnas.97.21.11655
- 609 Schmittgen, T., and Livak, K. 2008. Analyzing real-time PCR data by the comparative  
610 CT method. Nat. Protoc. 3:1101–8. doi: 10.1038/nprot.2008.73
- 611 Shetty, N. P., Mehrabi, R., Lütken, H., Haldrup, A., Kema, G. H. J., Collinge, D. B.,  
612 and Jørgensen H. J. 2007. Role of hydrogen peroxide during the interaction between  
613 the hemibiotrophic fungal pathogen *Septoria tritici* and wheat. New Phytol. 174:637–  
614 647. doi: 10.1111/j.1469-8137.2007.02026.x

- 615 Shukla, P. S., Borza, T., Critchley, A. T., and Prithiviraj, B. 2016. Carrageenans from  
 616 red seaweeds as promoters of growth and elicitors of defense response in plants.  
 617 Front. Mar. Sci. 3:81. doi: 10.3389/fmars.2016.00081
- 618 Siah, A., Deweer, C., Morand, E., Reignault, P., and Halama, P. 2010. Azoxystrobin  
 619 resistance of French *Mycosphaerella graminicola* strains assessed by four *in vitro*  
 620 bioassays and by screening of G143A substitution. Crop Prot. 29:737–743. doi:  
 621 10.1016/j.cropro.2010.02.012
- 622 Siah, A., Deweer, C., Duyme, F., Sanssené, J., Durant, R., Halama, P., and  
 623 Reignault, P. 2010. Correlation of in planta endo-beta-1,4-xylanase activity with the  
 624 necrotrophic phase of the hemibiotrophic fungus *Mycosphaerella graminicola*. Plant  
 625 Pathol. 59:661-670.
- 626 Small, E. 2011. 37. Spirulina – food for the universe. Biodiversity. 12:255–265. doi:  
 627 10.1080/14888386.2011.642735
- 628 Thakur, M., and Sohal, B. S. 2013. Role of elicitors in inducing resistance in plants  
 629 against pathogen infection: A review. ISRN Biochem. 2013:762412. doi:  
 630 10.1155/2013/762412
- 631 Thaler, J. S., Humphrey, P. T., and Whiteman, N. K. 2012. Evolution of jasmonate  
 632 and salicylate signal crosstalk. Trends Plant Sci. 17:260–270. doi:  
 633 10.1016/j.tplants.2012.02.010
- 634 Torriani, S. F. F., Melichar, J. P. E., Mills, C., Pain, N., Sierotzki, H., and Courbot, M.  
 635 2015. *Zymoseptoria tritici*: A major threat to wheat production, integrated approaches  
 636 to control. Fungal Genet. Biol. 79:8–12. doi: 10.1016/j.fgb.2015.04.010
- 637 Trotel-Aziz, P., Couderchet, M., Vernet, G., and Aziz, A. 2006. Chitosan stimulates  
 638 defense reactions in grapevine leaves and inhibits development of *Botrytis cinerea*.  
 639 Eur. J. Plant Pathol. 114:405–413. doi: 10.1007/s10658-006-0005-5

- 640 Van der Ent, S., Van Wees, S. C. M., and Pieterse, C. M. J. 2009. Jasmonate  
641 signaling in plant interactions with resistance-inducing beneficial microbes.  
642 *Phytochemistry*. 70:1581–1588. doi: 10.1016/j.phytochem.2009.06.009
- 643 Van Loon, L. C., and Van Strien, E. A. 1999. The families of pathogenesis-related  
644 proteins, their activities, and comparative analysis of PR-1 type proteins. *Physiol.*  
645 *Mol. Plant Pathol.* 55:85–97. doi: 10.1006/pmpp.1999.0213
- 646 Van Loon, L. C., Rep, M., and Pieterse, C. M. J. 2006. Significance of inducible  
647 defense-related proteins in infected plants. 44:135–162. doi:  
648 10.1146/annurev.phyto.44.070505.143425
- 649 Van Wees, S. C. M., De Swart, E. A. M., Van Pelt, J., Van Loon, L. C., and Pieterse,  
650 C. M. J. 2000. Enhancement of induced disease resistance by simultaneous  
651 activation of salicylate- and jasmonate-dependent defense pathways in *Arabidopsis*  
652 *thaliana*. *Proc. Natl. Acad. Sci. U. S. A.* 97:8711–8716. doi: 10.1073/pnas.130425197
- 653 Věchet, L., and Šerá, B. 2015. Effectiveness of both synthetic compounds and  
654 biological extracts against powdery mildew (*Blumeria graminis* f. sp. *tritici*) on winter  
655 wheat. *Agrociencia*. 49:77–85.
- 656 Vera, J., Castro, J., Gonzalez, A., and Moenne, A. 2011. Seaweed polysaccharides  
657 and derived oligosaccharides stimulate defense responses and protection against  
658 pathogens in plants. *Mar. Drugs*. 9:2514–2525. doi: 10.3390/md9122514
- 659 Vogt, T. 2009. Phenylpropanoid biosynthesis. *Mol. Plant*. 3:2–20. doi:  
660 10.1093/mp/ssp106
- 661 Walters, D., Walsh, D., Newton, A., and Lyon, G. 2005. Induced resistance for plant  
662 disease control: Maximizing the efficacy of resistance elicitors. *Phytopathology*.  
663 95:1368–1373. doi: 10.1094/PHYTO-95-1368



- 664 Walters, D. R., Ratsep, J., and Havis, N. D. 2013. Controlling crop diseases using  
665 induced resistance: challenges for the future. *J. Exp. Bot.* 64:1263–1280. doi:  
666 10.1093/jxb/ert026
- 667 Walters, D. R., Havis, N. D., Paterson, L., Taylor, J., Walsh, D. J., and Sablou, C.  
668 2014. Control of foliar pathogens of spring barley using a combination of resistance  
669 elicitors. *Front. Plant Sci.* 5:241. doi: 10.3389/fpls.2014.00241
- 670 Wan, D., Wu, Q., and Kuča, K. 2016. Chapter 42 - Spirulina. In *Nutraceuticals*, ed.  
671 Ramesh C. Gupta. Boston: Academic Press, p. 569–583.
- 672 Wasternack, C., and Hause, B. 2013. Jasmonates: biosynthesis, perception, signal  
673 transduction and action in plant stress response, growth and development. An  
674 update to the 2007 review in *Annals of Botany*. *Ann. Bot.* 111:1021–1058. doi:  
675 10.1093/aob/mct067
- 676 Weber, H. 2002. Fatty acid-derived signals in plants. *Trends Plant Sci.* 7:217–224.
- 677 Weiner, M. L. 2014. Food additive carrageenan: Part II: A critical review of  
678 carrageenan *in vivo* safety studies. *Crit. Rev. Toxicol.* 44:244–269. doi:  
679 10.3109/10408444.2013.861798
- 680 Yakushiji, S., Ishiga, Y., Inagaki, Y., Toyoda, K., Shiraishi, T., and Ichinose, Y. 2009.  
681 Bacterial DNA activates immunity in *Arabidopsis thaliana*. *J. Gen. Plant Pathol.*  
682 75:227–234. doi: 10.1007/s10327-009-0162-4
- 683
- 684 **Author-Recommended Internet Resources**
- 685 Brisset, M. N., and Duge De Bernonville, T. 2011. Patent WO/2011/161388. Device  
686 for determining or studying the state of stimulation of the natural defences of plants  
687 or portions of plants.  
688 <https://patentscope.wipo.int/search/en/detail.jsf?docId=WO2011161388&recNum=21>

0&docAn=FR2011051470&queryString=(CL/antibod\*)%2520OR%2520(DE/antibod\*)  
 %2520OR%2520(ET/antibod\*)%2520&maxRec=145490.

European Commission. 2012. Sustainable agriculture for the future we want. In Rio:  
 European Commission. [http://ec.europa.eu/agriculture/events/2012/rio-side-  
 event/brochure\\_en.pdf](http://ec.europa.eu/agriculture/events/2012/rio-side-event/brochure_en.pdf)

Satger, O. 2016. L'offre mondiale excédentaire en 2015/2016 et la perspective de  
 bonnes récoltes en 2016 continuent de faire pression sur les prix des grains.

Agreste. 287:6. <http://agreste.agriculture.gouv.fr/IMG/pdf/conjsynt287201605cult.pdf>

715 **Table 1.** Characteristics of the compounds used in the study

Active ingredient	Supplier	Characteristics	General use	Concentration tested (g l <sup>-1</sup> )		
				C1	C2	C3
λ-carrageenan	SIGMA	Linear polysaccharide extracted from red algae	Gelling and emulsifying agent in the food industry	0.1	1	5
CpG-ODN	Pr. A. Carpentier, Paris, France	Short single-stranded synthetic DNA molecules (CpG-28, sequence 5'-TAAACGTTATAAC GTTATGACGTCAT-3')	Human vaccine adjuvant	9.5 x 10 <sup>-5</sup>	9.5 x 10 <sup>-4</sup>	9.5 x 10 <sup>-4</sup>
<i>Spirulina platensis</i>	Djeralbague, Tunisia	Dried cyanobacterium	Human dietary supplement	0.3	3	30
Glycine betaine	lthec	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
BION®50WG (Acibenzolar-S-methyl; 50 % w : w)	Syngenta, Europe	Synthetic elicitor	Plant resistance inducer		0.6	
Fungicide Opus® (Epoxiconazole)	BASF Agro, France	Triazole fungicide	Broad-spectrum systemic fungicide		0.75 (v :v)	

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**Fig. 1.** Efficacy of five candidate elicitors ( $\lambda$ -carrageenan, CpG-ODN, Spirulina, Glycine betaine, 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 *Z. tritici* disease severity compared to 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 and/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 two independent experiments at least per treatment). Boxes tagged with the same letters correspond to means that are not significantly different using the Tukey test at  $P = 0.05$ .

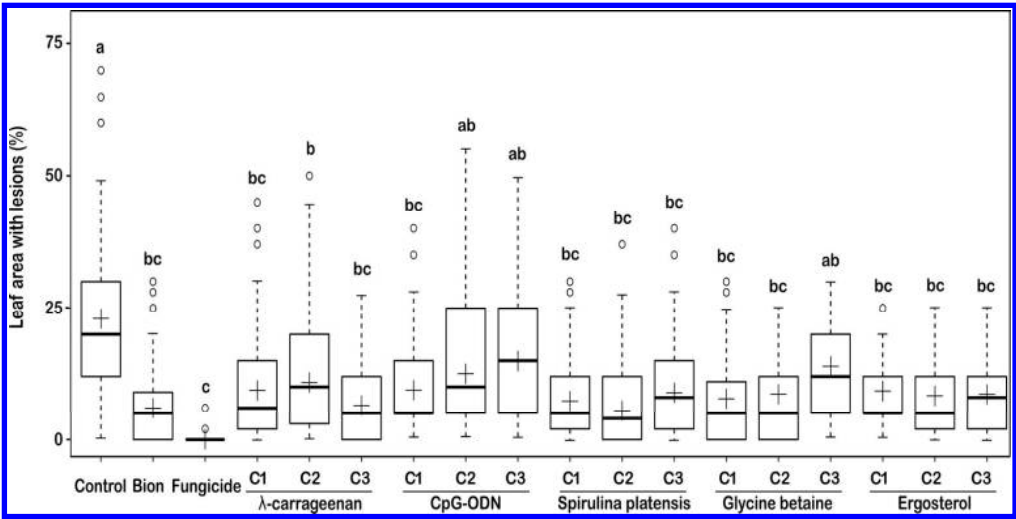
**Fig. 2.** Heatmap profiling of the average expression level of 23 defense-related genes of wheat across all experimental conditions (product,  $\pm$  H<sub>2</sub>O<sub>2</sub>, day post-treatment). Data obtained by the combination of two independent experiments ( $n = 32$ ). Hierarchical clustering of gene expression highlighted three main patterns. The Log<sub>2</sub> expression levels above or under 0 represent respectively an induction (in green) or a repression (in red) of gene expression in treated plants compared to the water control. Hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) was applied (+) or not (-) on plants at 1 day after treatment to mimic a pathogen attack. The list of genes examined in this study is the following: 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)- $\alpha$ -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.

**Fig. 3.** Principal component analysis (PCA) of gene expression across all  
experimental conditions compared to water control (product,  $\pm$  H<sub>2</sub>O<sub>2</sub>, day post-  
treatment). Three major groups are highlighted in different colors (red, blue and  
black). The 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 to one of the dimensions.

**Fig. 4.** Relationship between the expression of wheat defense genes and the efficacy  
of five compounds in protecting wheat against *Zymoseptoria tritici*:  $\lambda$ -carrageenan  
(A); CpG-ODN (B); Spirulina (C); Glycine betaine (D); Ergosterol (E). Positive  
correlations are highlighted and are significant at day 1 after plant treatment. Gene  
expression was obtained by qRT-PCR while protection efficacy data was a result of  
glasshouse elicitor screening. Hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) applications were realized  
to test for priming activity: grey, without; black, with. Coordinates on 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).

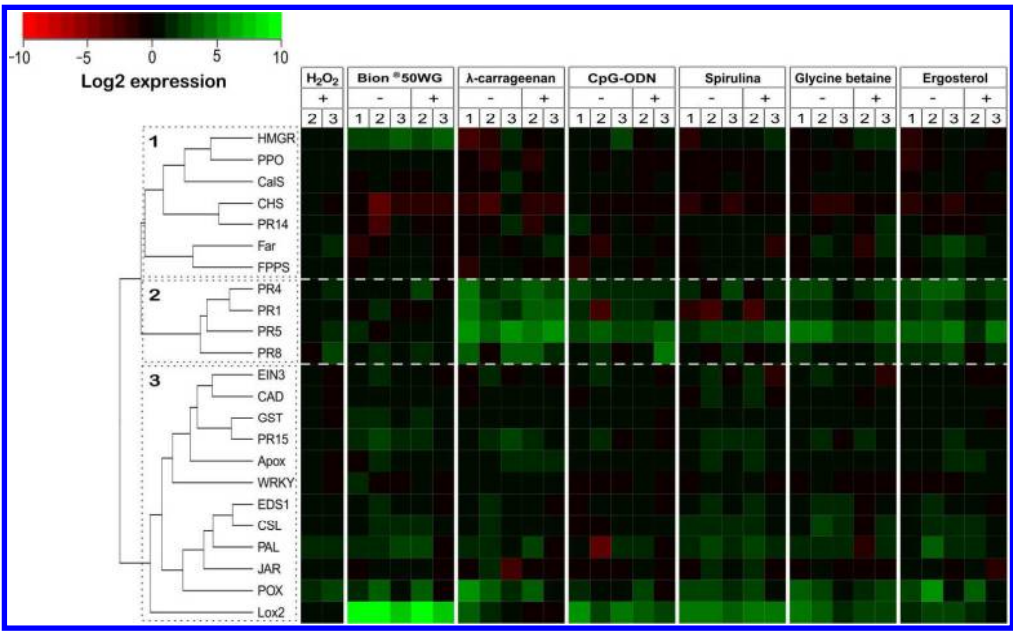
771 **Fig. S1.** *In vitro* assessment of the biocidal effect of  $\lambda$ -carrageenan, CpG-ODN,  
 772 *Spirulina platensis*, glycine betaine and ergosterol towards the pathogen  
 773 *Zymoseptoria tritici*. Values correspond, respectively, to the average percentage of  
 774 germinated spores and average fungal diameter (cm) of the pathogen scored on  
 775 potato dextrose agar (PDA) culture medium amended with the five different  
 776 compounds. For each compound, five decreasing concentrations were tested, with C  
 777 being the highest concentration:  $\lambda$ -carrageenan, 5 g l<sup>-1</sup>; CpG-ODN, 0.0095 g l<sup>-1</sup>;  
 778 *Spirulina platensis*, 30 g l<sup>-1</sup>; glycine betaine, 12 g l<sup>-1</sup>; ergosterol, 0.03 g l<sup>-1</sup>). The  
 779 control corresponds to PDA medium without tested compounds (0 g l<sup>-1</sup>). Means  
 780 tagged with the same letters are not significantly different using the Tukey test at  $P =$   
 781 0.05.



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279x140mm (191 x 191 DPI)

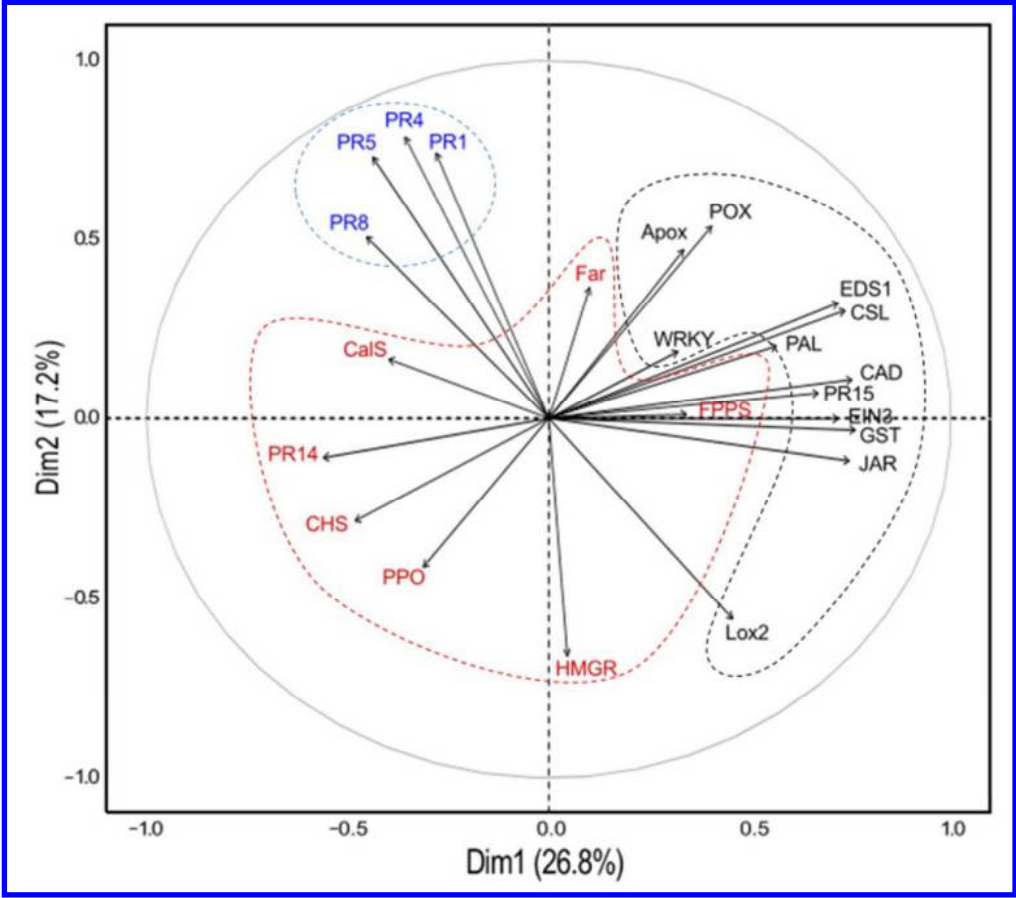




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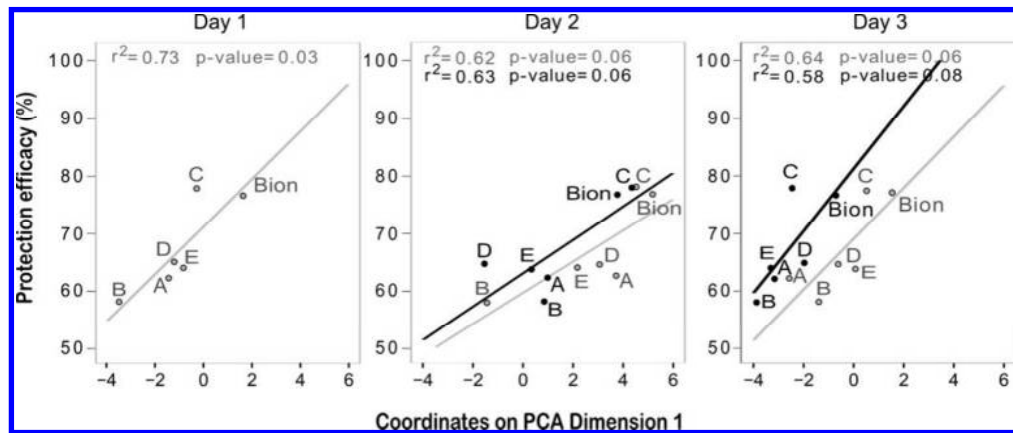
269x164mm (198 x 198 DPI)

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This paper has been peer reviewed and accepted for publication but has not yet been copyedited or proofread. The final published version may differ.



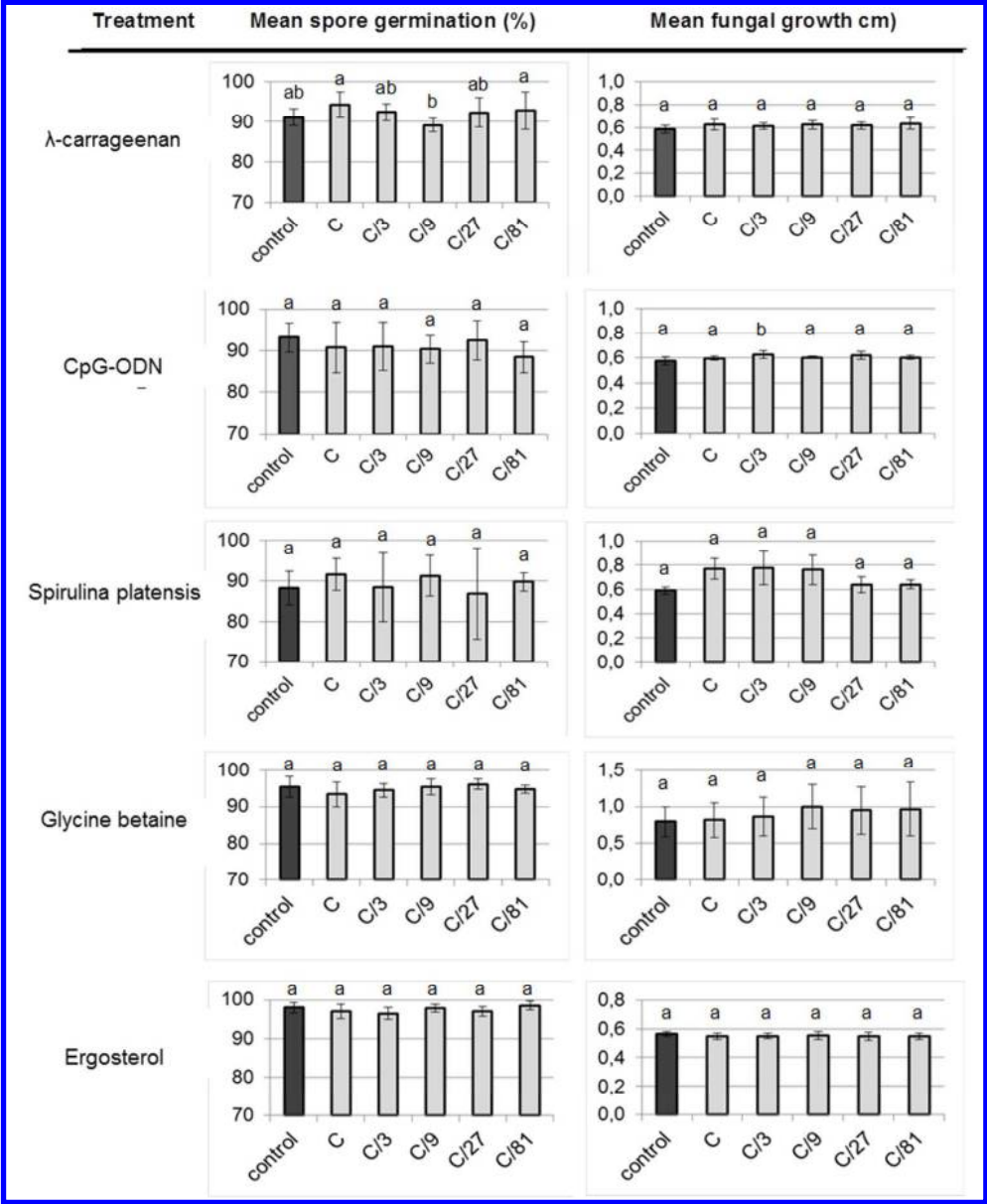
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88x78mm (300 x 300 DPI)



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236x98mm (113 x 113 DPI)



**Fig. S1.** *In vitro* assessment of the biocidal effect of λ-carrageenan, CpG-ODN, *Spirulina platensis*, glycine betaine and ergosterol towards the pathogen *Zymoseptoria tritici*. Values correspond, respectively, to the average percentage of germinated spores and average fungal diameter (cm) of the pathogen scored on potato dextrose agar (PDA) culture medium amended with the five different compounds. For each compound, five decreasing concentrations were tested, with C being the highest concentration: λ-carrageenan, 5 g l<sup>-1</sup>; CpG-ODN, 0.0095 g l<sup>-1</sup>; *Spirulina platensis*, 30 g l<sup>-1</sup>; glycine betaine, 12 g l<sup>-1</sup>; ergosterol, 0.03 g l<sup>-1</sup>. The control corresponds to PDA medium without tested compounds (0 g l<sup>-1</sup>). Means tagged with the same letters are not significantly different using the Tukey test at *P* = 0.05.

355x434mm (150 x 150 DPI)