

# Defense responses induced by ulvan in wheat against powdery mildew caused by Blumeria graminis f. sp. tritici

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Defense Responses Induced by Ulvan in Wheat Against Powdery Mildew Caused 1 2 by Blumeria graminis f. sp. tritici 3 Aline C. Velho<sup>1,3\*</sup>; Pamela Dall'Asta<sup>1,3</sup>; Marlon C. de Borba<sup>2,3</sup>; Maryline Magnin-4 Robert<sup>1</sup>; Philippe Reignault<sup>1</sup>; Ali Siah<sup>2</sup>, Marciel J. Stadnik<sup>3</sup>, Béatrice Randoux<sup>1\*</sup> 5 6 <sup>1</sup> Université du Littoral Côte d'Opale, Unité de Chimie Environnementale et 7 Interactions sur le Vivant (EA 4492), SFR Condorcet FR CNRS 3417, CS 80699, F-8 62228, Calais CEDEX, France. 9 10 <sup>2</sup> Joint Research Unit 1158 BioEcoAgro, Junia, Univ. Lille, Univ. Liège, UPJV, Univ. Artois, ULCO, INRAE, F-59000 Lille, France. 11 12 <sup>3</sup> Federal University of Santa Catarina, Agricultural Science Center (UFSC-CCA), Laboratory of Plant Pathology, Rod. Admar Gonzaga, 1346, 88034-001, Florianópolis-13 SC, Brazil. 14 15 \*Corresponding authors: Béatrice Randoux (beatrice.randoux@univ-littoral.fr), Aline 16 17 C. Velho (alinecristinav@gmail.com) 18 19 ABSTRACT Ulvan is a water-soluble sulfated heteropolysaccharide extracted from the cell walls of 20 21 the green seaweeds Ulva spp. This polysaccharide is known to induce resistance and protect plants against a broad range of plant pathogenic fungi, such as Blumeria 22 graminis f. sp. tritici (Bgt), the causal agent of powdery mildew in wheat. We aimed to 23 study the defense mechanisms induced by ulvan against Bgt in susceptible wheat by 24 investigating the defense-related gene expression, enzymes activity, accumulation of 25 phenolic compounds and hydrogen peroxide  $(H_2O_2)$ , as well as the development of Bgt 26 27 infection structures in vitro and in planta. Symptoms were reduced by 42% in ulvantreated plants. In vitro, ulvan did not inhibit conidial germination of Bgt but in planta, 28 increased the appressorial germ tubes without haustorium. Ulvan increased the presence 29 of fluorescent papillae and accumulation of  $H_2O_2$  at the penetration sites of Bgt, as well 30 as the content of phenolic compounds. POX, PAL and LOX activities were stimulated 31 © 2022 published by Elsevier. This manuscript is made available under the CC BY NC user license https://creativecommons.org/licenses/by-nc/4.0/

in ulvan-treated plants during the first 48 h after inoculation. However, few of defense-32 33 related genes studied were differentially expressed in infected plants after ulvan treatment. By contrast, in non-infected conditions, ulvan up-regulated the expression of 34 genes involved in phenylpropanoid metabolism, *i.e. PAL*, CHS, COMT, ANS and FLS, 35 genes encoding pathogenesis-related proteins, *i.e. PR1, PR9, PR15*, and LOX during the 36 first 96 h after treatment. This study provides new insights about the multiple ulvan 37 effects on wheat defense responses, and especially the elicitation of the 38 phenylpropanoid pathway leading to phenolic compounds accumulation, which could 39 be involved in cell wall reinforcement. 40

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42 Keywords: algal polysaccharides, *Blumeria graminis*, induced resistance, *Triticum*43 *aestivum* L., *Ulva fasciata*.

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- 45 1. INTRODUCTION
- 46

Bread wheat (*Triticum aestivum* L.) is the most widely cultivated cereal in the world (Kang et al., 2020). This crop is affected by a wide range of fungal diseases, and among them, powdery mildew has been considered one of the most important (Jankovics et al., 2015; Kang et al., 2020).

Powdery mildew causes severe yield losses in many wheat-growing regions and 51 the infection is favored by mild temperatures (15-22°C) and high humidity (above 52 70%). This disease is caused by the ascomycete *Blumeria graminis* f. sp. tritici (Bgt), a 53 54 biotrophic fungus widely distributed throughout the world. Bgt produces asexual spores known as conidia, and sexual ascospores in cleistothecia, which enable the fungus to 55 56 survive under adverse weather conditions (Jankovics et al., 2015). Disease symptoms on wheat appear between 7-10 days after infection and are characterized by the presence of 57 58 white or gravish powdery colonies on leaves and stems. These colonies have abundant 59 conidia that are easily spread by wind to long distances and promote secondary 60 infections throughout the wheat cropping (Jankovics et al., 2015). After infection, the fungus exclusively colonizes the cell walls of the epidermal cell layer of leaf tissues and 61 62 forms haustoria, the only fungal structures developing within the host tissues, but without breaking the cell membrane. Haustoria are at the center of the biotrophic 63

64 interaction of *Bgt* by taking up nutrients from the host and by delivering effectors in the
65 invaded cells to impair plant immunity (Jankovics et al., 2015).

Due to the rapid evolution of the pathogen and the emergence of new virulent 66 isolates, many race-specific (R) resistance genes introduced in wheat cultivars have 67 become ineffective for controlling powdery mildew development (Kang et al., 2020). In 68 this case, synthetic fungicides are commonly used to control this disease in wheat, 69 which are not healthy and environmentally friendly. For this reason, using alternative 70 71 control strategies that are compatible with a more sustainable agriculture have been 72 required (Renard-Merlier et al., 2007). In this scenario, oligo- and polysaccharides obtained from marine macroalgae are interesting sources of active molecules to control 73 74 powdery mildew on various crops (Paulert et al., 2010; Jaulneau et al., 2011; Stadnik and de Freitas, 2014). Algae extracts are a source of a wide range of chemically active 75 76 metabolites. These molecules possess various biological properties including antimicrobial, antioxidant, antifungal effects, and resistance inducer capacity via the 77 78 elicitation or priming of plant defense responses (Siah et al., 2018). In addition to 79 metabolites, polysaccharides from brown, red, and green seaweeds have been the most 80 studied ones. Among them, carrageenans, fucans, laminarins, and ulvans (Cluzet et al., 2004; Jaulneau et al., 2011; Stadnik and de Freitas, 2014; de Borba et al., 2021) can be 81 used in plant protection. 82

Plants are able to defend themselves during pathogen attacks, and the activation 83 of an effective plant immunity depends on pathogen recognition via the perception of 84 exogenous elicitors highly conserved and considered as essential structures for 85 pathogens survival (Newman et al., 2013), which are referred as pathogen-/microbe-86 associated molecular patterns (PAMPs or MAMPs). In addition to these molecules, 87 fragments of plant cell walls released by the action of hydrolytic enzymes secreted by 88 the pathogens, named damage-associated molecular patterns (DAMPs), represent a class 89 of endogenous elicitors (Trouvelot et al., 2014). As a consequence of PAMPs, MAMPs, 90 91 or DAMPs recognition by pattern recognition receptors (PRRs) occurring in the plant cell membrane, plants induce a broad range of defense reactions: transient oxidative 92 93 burst, alkalinization of the extracellular medium, influx of calcium, reinforcement of the 94 plant cell wall, production of antimicrobial compounds such as phytoalexins, and 95 induction of pathogenesis-related (PR) proteins (Trouvelot et al., 2014).

The extent of plant defense induction can lead to an increased resistance against subsequent pathogen attack. In this sense, various compounds acting as elicitors have been identified, consisting of lipids, glycopeptides, glycoproteins, and notably 99 carbohydrates. Depending on their characteristics, oligo- and polysaccharides tend to act
100 as non-specific plant defense elicitors, mimicking the pathogen attack (Paulert et al.,
101 2010). For example, laminarins, the β 1,3 glucans from *Laminaria digitata*, are thought
102 to mimic structurally fungal cell wall polysaccharides.

Over the past few decades, ulvan, a water-soluble sulfated heteropolysaccharide 103 104 extracted from the cell walls of green macroalgae Ulva spp., has shown promising effects on plant resistance induction as well as priming agent (Paulert et al., 2010). In 105 this way, treatments with ulvan are able to protect various crops against a wide range of 106 107 fungal diseases (Paulert et al., 2010; Stadnik and de Freitas, 2014). Previous studies 108 have shown that ulvan are effective in controlling powdery mildew in monocots like 109 wheat and barley (Paulert et al., 2010; Jaulneau et al., 2011), and dicots crops, such as 110 common bean (de Freitas and Stadnik, 2012; de Borba et al. 2019), cucumber and 111 grapevine (Jaulneau et al., 2011). Treatments with extracts of Ulva spp. provided protection to alfalfa against subsequent infection by Colletotrichum trifolii, which was 112 113 related to the biosynthesis of phytoalexins and the expression of defense-related genes encoding PR-proteins, such as *PR1*, *PR3*, and *PR10* in plant tissues (Cluzet et al., 2004). 114 115 Ulvan appeared also to stimulate various enzymes of defense responses, like peroxidase (POX) or glucanase (GLU) in bean and Arabidopsis, which may lead to the resistance 116 of these plants against various fungal pathogens, such as Colletotrichum 117 lindemuthianum (de Freitas and Stadnik, 2012), C. higginsianum and Alternaria 118 119 brassicicola (de Freitas and Stadnik, 2015), respectively.

So far, studies to investigate the ulvan effect on wheat were restricted to the evaluation of the efficacy of this polysaccharide in controlling powdery mildew caused by *Bgt*. However, few studies have been carried out in order to clarify the mode of action and the defense pathways involved in wheat resistance against this biotrophic pathogen in response to foliar application of ulvan.

125 Thus, the present work aimed to study the defense mechanisms induced by ulvan 126 against Bgt in bread wheat by investigating the activation of plant defense-related gene expression, enzymes activity, and the evolution of phenolic compounds in leaves. A 127 128 microscopic study was also performed to monitor the development of infection 129 structures in planta and the accumulation of defense compounds such as phenolics and 130 hydrogen peroxide  $(H_2O_2)$  at the site of Bgt penetration attempts in response to ulvan treatment. Finally, in vitro assay allowed to assess the ability of ulvan to inhibit directly 131 132 Bgt conidial germination.

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134 135

### 2. MATERIAL AND METHODS

#### 136 **2.1 Biological material**

Bread wheat (Triticum aestivum L.) cv. Pakito, provided by RAGT (Rodez, 137 France), was used in this study. This cultivar was considered highly susceptible to 138 Blumeria graminis f. sp. tritici (Bgt), exhibiting an estimating field resistance level at 4 139 on a 1-9 scale. Seeds were water soaked overnight in order to facilitate their 140 germination and then 24 seeds were sown in  $30 \times 12$  cm plastic trays with a commercial 141 substrate (Floradur® - Floragard, Germany). Plantlets were grown for 10 days in a 142 growth chamber with a 12 h photoperiod (light intensity 250  $\mu$ mol m<sup>-2</sup> s<sup>-1</sup>), 18°C dav 143 temperature, 12°C night temperature, and 70% relative humidity. 144

Blumeria graminis f. sp. tritici isolate MPEbgt1 was provided by M.P. Latorse
(Bayer CropScience, France). The fungus was inoculated and maintained on wheat
plants cv. Alixan (Limagrain, Saint-Beauzire, France) in a growth chamber under
constant light at 20°C.

149

### 150 **2.2 Ulvan obtention**

Ulvan was obtained as described by Paulert et al. (2010) and de Borba et al. 151 (2021). Briefly, dried algae (Ulva fasciata Delile) was suspended in distilled water (w:v, 152 1:10) and autoclaved at 110°C for 2 h. The resulting aqueous solution was filtered 153 through a cotton cloth and the polysaccharide precipitated with ethanol absolute (v:v, 154 1:3) for 48 h at -20°C. The polysaccharide obtained from the first precipitation was 155 collected, dried at 40-45°C, and ground to a fine powder. Thereafter, the polysaccharide 156 was dissolved in distilled water at 10 mg ml<sup>-1</sup>, dialyzed in a 3600 Da Mw cutoff dialysis 157 membrane for 72 h, and then lyophilized. The product was stored at -20°C in a 158 159 desiccator or silica until use.

160

### 161 **2.3 Plant treatment**

Ulvan treatment was previously dissolved in ultrapure water at 1 mg mL<sup>-1</sup>. For each tray, 10 mL of ulvan was applied on ten-day-old wheat plantlets using a sprayer connected to an air compressor (ITW Surfaces et Finitions, Valence, France) until all leaf area was covered. Plants sprayed with ultrapure water were used as control.

166

### 167 **2.4 Inoculation and disease evaluation**

Wheat plants were inoculated with a suspension of  $5 \times 10^5$  conidia mL<sup>-1</sup> at 48 h 168 after ulvan treatment. Conidia of Bgt were harvested from fifteen-day-old infected 169 170 wheat plantlets and immediately suspended in Fluorinert FC43 (heptacosafluorotributhylamine 3M, Cergy-Pontoise, France) (Randoux et al., 2006). 171 For each tray, 5 mL of inoculum was sprayed using a sprayer (ITW Surfaces et 172 Finitions, Valence, France) connected to an air compressor. After inoculation, plants 173 were kept under the same growth conditions described above. Disease symptoms were 174 evaluated at 10 days after inoculation by counting the number of Bgt colonies on the 175 176 fully expanded first leaf of plants, on both adaxial and abaxial surfaces. The ulvan effect on protection against powdery mildew was determined by assessing the number of Bgt 177 178 colonies per leaf in comparison with the control group (water sprayed).

179

### 180 2.5 Conidial germination of *B. graminis* f. sp. *tritici* (*Bgt*) *in vitro*

In order to evaluate the ulvan effect on conidial germination, plastic Petri plates 181 (5 cm) containing agar medium (12 g L<sup>-1</sup>) in presence of water (control) or amended 182 with ulvan solution (1 mg mL<sup>-1</sup>) were prepared. Then, conidia of Bgt were gently 183 184 tapped above the plates to allow a uniform conidia deposition and evaluated after 24 h 185 of incubation at 18°C. The germination was randomly determined on 400 conidia per plate, being that divided into four quadrants, using a light microscope (Olympus BX 186 40). The conidia were classified into five categories: non-germinated conidia (NG), 187 conidia with aborted germ tubes (AGT), with a single short germ tube (SGT, length <188  $10\mu m$ ), a single long germ tube (LGT, length > 10  $\mu m$ ) and multiple germ tubes (MGT) 189 190 (Fig. 2).

191

### 192 **2.6** Cytological observations of *Bgt* infection process

In order to monitor the development of infection structures of Bgt, segments (1.5 193 cm length) from the 1<sup>st</sup> expanded wheat leaves treated and non-treated with ulvan were 194 195 excised at 24, 48, and 72 h after inoculation (hai). Leaf tissues were cleared in an acetic acid: ethanol solution (v:v, 1:3), stained with trypan blue (w:v 0.1%) for 30 min, 196 mounted on glass slides with lactoglycerol, and observed with a light microscope 197 (Olympus BX 40). Different developmental stages were evaluated in epidermal leaf 198 199 cells and classified into six categories: non-germinated conidia, germinated conidia with 200 a primary germ tube (PGT), with an appressorial germ tube (AGT) without haustorium 201 (HS), AGT with HS in an early stage of development, AGT with fully formed HS and AGT with branched hyphae (BH) (Table 1). 202

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### 204 **2.7 Papilla autofluorescence at** *Bgt* **penetration sites**

Autofluorescent papillae were assessed using the same leaf segments collected to evaluate the infection structures development. Leaf tissues were observed using an Eclipse E600 microscope (Nikon) using a blue exciter filter, excitation 450–490nm, dichroic mirror 505, and barrier filter 20 nm with transmittance range 500 to 800 nm. The fluorescence beneath AGTs on epidermal cells was classified according to Randoux et al. (2006) as no fluorescence, fluorescent papilla, and whole fluorescent cell (Fig. 4).

211

### 212 **2.8** Cytochemical detection of hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) accumulation

213 Detection of H<sub>2</sub>O<sub>2</sub> was carried out using the DAB staining method according to Thordal-Christensen et al. (1997). For H<sub>2</sub>O<sub>2</sub> visualization, leaf segments treated or non-214 215 treated with ulvan were collected at 24, 48, and 72 hai and immediately submerged in 3.3-diaminobenzidine (DAB) solution (1 mg mL<sup>-1</sup>, pH 3.8) for 4 h. Thereafter, tissues 216 217 were completely bleached in acetic acid: ethanol (v:v, 1:3) solution and stored with lactoglycerol in the darkness at room temperature. H<sub>2</sub>O<sub>2</sub> accumulation was identified by 218 219 visualization of reddish-brown staining in all epidermal cells caused by oxidative 220 polymerization of DAB. Reactions were evaluated using a light microscope (Olympus BX 40) and classified into three classes according to their frequencies: no DAB 221 staining, staining localized at the penetration sites, and whole-cell DAB staining (Fig. 222 223 5).

224

#### 225 **2.9 Extraction and quantification of total phenolic compounds**

226 Total phenolic compounds were determined using the Folin-Ciocalteu method (Singleton and Rossi 1965) downscaled to a 96-well plate with minor modifications. 227 228 For this, leaves (treated or non-treated × inoculated and non-inoculated) were collected 229 at 24, 48, and 72 h after inoculation (hai). Powdered fresh leaf tissues (200 mg) were 230 mixed with 1 ml of methanol (MeOH) and the mixture was shaken for 1 h at 40°C under 180 rpm in dark condition before two centrifugations at  $13,000 \times g$  for 5 min. The 231 supernatants were stored at -20°C until analysis. An aliquot (30 µL) of methanolic 232 extract was mixed with 150 µL of Folin-Ciocalteu reagent (10-times diluted) and after 5 233 234 min of incubation at room temperature, 120 µL of sodium carbonate solution (w:v 10%) was added. The mixture was incubated at room temperature for 2 h, and the absorbance 235 236 was read at 750 nm. Total phenolic compounds were expressed as mg of Gallic acid equivalents (GAE) per g of fresh tissue. 237

238

### 239 2.10 Enzyme activities

Three plant enzyme activities involved in defense mechanisms were assessed 24, 48, and 72 h after inoculation with *Bgt* in wheat leaves treated and non-treated with ulvan.

Peroxidase (POX, EC 1.11.1.7) activity was assayed according to Mustafa et al., (2017) and was determined by monitoring the increase in absorbance at 412 nm for five min. The activity was defined as the amount of enzyme that reduced the electron donor at 1 mol s<sup>-1</sup> using ABTS (2, 2-azino-bis 3-ethylbenzothiazoline-6-sulfonic acid) as substrate ( $\mathcal{E}$  =32400 M<sup>-1</sup> cm<sup>-1</sup>).

Lipoxygenase (LOX, EC 1.13.11.12) was measured according to Randoux et al. (2010) using linoleic acid as substrate. LOX activity was determined by monitoring changes in absorbance at 234 nm for five min, and one unit was defined as the amount of enzyme that catalyzes the conversion of the substrate (linoleic acid) at 1 mol s<sup>-1</sup> ( $\epsilon$  = 23000 M<sup>-1</sup> cm<sup>-1</sup>).

253 Phenylalanine ammonia-lyase (PAL, EC 4.3.1.24) was assayed according to 254 Reignault et al. (2001). Enzyme activity was monitored by an increase in absorbance at 255 290 nm and one unit was defined as the amount of enzyme that catalyzes the formation 256 of cinnamic acid at 1 mol s<sup>-1</sup> ( $\epsilon = 10000 \text{ M}^{-1} \text{ cm}^{-1}$ ).

Protein content was measured using the method of Lowry et al. (1951). The standard curve was established with bovine serum albumin (BSA). POX, LOX, and PAL activities were expressed as nanokatal  $mg^{-1}$  protein, where 1 kat represents the amount of enzyme that converts 1 mol of substrate s<sup>-1</sup> or forms 1 mol of product s<sup>-1</sup>.

261

### 262 2.11 RNA extraction and RT-qPCR gene expression analysis

263 First wheat leaves (treated and non-treated  $\times$  inoculated and non-inoculated) were collected at 0, 24, 48, 60, 72, and 96 h after treatment, snap-frozen in liquid 264 nitrogen, and kept at -80 °C until processing. Total RNA was extracted from 100 mg of 265 plant tissue using RNeasy Plant Mini Kit (Qiagen, Germany). Genomic DNA 266 contaminating the samples was removed by treatment with DNase using RNase-Free 267 DNase Set (Qiagen, The Netherlands). In total, 2 µg of RNA was reverse-transcribed 268 using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA) 269 270 according to the manufacturer's protocol.

The expression of 10 selected target genes was tracked by quantitative RT-PCR using the primers reported in Supplementary Table 1 (phenylalanine ammonia-lyase -

PAL, chalcone synthase - CHS, flavonol synthase - FLS1, dihydroflavonol-4-reductase -273 DFR, anthocyanidin synthase - ANS, caffeic acid 3-O-methyltransferase - COMT, 274 275 lipoxygenase - LOX, pathogenesis-related protein 1 - PR1, oxalate oxidase - PR8, and peroxidase - PR9). Among them, eight genes (PAL, CHS, FLS, ANS, LOX, PR1, PR8, 276 PR9) and the housekeeping genes (ATPAAA, ATPase AAA Family. PetA, class A 277 Apetala 2) were selected from previous studies performed on wheat-pathogenic fungus 278 interactions (Khong et al., 2013; Tayeh et al., 2013, 2014 and 2015; Ma et al., 2014; 279 Sharma et al., 2016; Giménez et al., 2011; Ors et al., 2018; de Borba et al., 2021). 280 281 Primers pairs of two targeted genes (DFR and COMT) were newly designed using the Oligo Explorer 1.1.0 software and Primer3Plus website (Supplementary Table 1). 282

283 Reactions were carried out in a real-time PCR detector CFX Connect (Bio-Rad) using the following thermal profile: 15 s at 95°C (denaturation) and 1 min at 60°C 284 285 (annealing/extension) for 40 cycles. PCR reactions were performed in duplicate with the SsoAdvanced Universal SYBR Green Supermix (Bio-rad, USA) according to the 286 287 manufacturer's protocol. Melting curve assay was performed from 65° to 95°C at 0.5°C/s. Melting peaks were visualized for checking the specificity of each 288 289 amplification. The data were analyzed using the CFX Manager software, and the 290 relative levels of gene expression were determined following the method of Hellemans et al. (2007) with ATPAAA and PetA serving as two internal reference genes. The results 291 were expressed relative to the respective control, for each sampling time, corresponding 292 293 to a fixed value of 1.

294

### 295 2.12 Experimental design and statistical analyses

The experiments were arranged in a completely randomized design using three replicates for each treatment and repeated three times. Each replication corresponded to one plastic tray with 24 wheat plants. All data were subjected to analysis of variance (ANOVA) in order to determine the significance of differences. Means were compared by Student's t-test ( $p \le 0.05$ ) using the SAS program (v.9.1 SAS Institute, Inc., Cary, NC). Total phenolic compounds were analyzed using the Tukey test ( $p \le 0.05$ ).

For the relative expression of targeted genes, three replicates corresponded to three independent leaves for each condition and each sampling time. The experiments were conducted three times and only results that were repeated at least twice were considered. Bonferonni's multiple comparison test ( $p \le 0.05$ ) was used for gene expression analysis. The expression was considered as up- or down-regulated to the 1x controls, when changes in relative expression were >2x or >0.5x, respectively. 308

#### **309 3. RESULTS**

310

### 311 **3.1 Disease protection**

At ten days after inoculation, the disease symptoms on wheat leaves were visually evaluated and characterized by the presence of white to grayish powdery colonies on the total surface of both faces of the first leaf of each plantlet. Control plants exhibited on average 77 colonies per leaf, while ulvan-treated plants showed about 45 colonies, corresponding to a significant 42% reduction of infection (Fig. 1a and 1b).

317

### 318 **3.2** Ulvan effect on conidial germination of *Bgt in vitro*

Conidial germination of *Bgt* was slightly affected by ulvan treatment *in vitro*. In the control condition, 52.8% of conidia were non-germinated (NG), 20% presented a single short germ tube (SGT), 19% multiple germ tubes (MGT), 6.5% a single long germ tube (LGT), and 1.8% of aborted germ tubes (AGT). Ulvan treatment significantly decreased the percentage of NG conidia by 34% and stimulated the development of MGT by 81% in comparison to control. No significant effect of ulvan was observed for conidia with AGT, SGT, or LGT (Fig. 2).

326

### 327 **3.3** Ulvan effect on the development of *Bgt* infection structures

In our conditions, during the first 24 h after inoculation, the conidia deposited on 328 329 the leaf surface germinated and formed one short germ tube, named primary germ tube (PGT) (Fig. 3a). This step was rapidly followed by the emergence of a secondary germ 330 331 tube, which swells at the tip and this was then named appressorial germ tube (AGT) (Fig. 3b). Subsequently, AGT produced a penetration peg, which breaks through the 332 333 subjacent cell wall. Between 24 h and 48 h, the first haustorium was formed without disrupting the plasma membrane of the invaded epidermal cell (Fig. 3c). Once the 334 335 haustorium was successfully developed (Fig. 3d), a secondary epiphytic hypha emerged from AGT, and thereafter branched hyphae (BH) were formed (Fig. 3e). 336

No impact of ulvan treatment on *Bgt* conidial germination was observed on wheat leaves at 24, 48, and 72 h after inoculation (Table 1). Concerning the infection process of the fungus on leaf tissues, a similar percentage of conidia with only a PGT was observed in ulvan-treated plantlets and non-treated ones. However, the percentage of conidia with an AGT but without HS was 42, 36, and 75% higher at 24, 48, and 72 hai on leaves of ulvan-treated wheat plants when compared to non-treated ones,

respectively. In contrast, the percentage of conidia with an AGT and with HS at an early 343 344 stage of development decreased by 34, 25, and 13% at 24, 48, and 72 hai, respectively, on ulvan-treated wheat leaves in comparison to the control ones. No conidium with an 345 AGT and a fully formed HS was detected before 48 hai, in treated and non-treated 346 leaves, and no significant difference was found in the percentages observed in both 347 cases at 48 and 72 hai. Conidia with AGT and BH were observed only at 72 hai. The 348 percentage of these structures decreased by 76% on epidermal cells of ulvan-treated 349 350 plants in comparison to the control ones (Table 1).

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- 352

### 3.4 Autofluorescence at Bgt penetration sites

353 The occurrence of autofluorescence at the penetration sites of the fungus was 354 evaluated and the frequency of fluorescent papillae and whole fluorescent cells were 355 recorded (Fig. 4a-4c). The ulvan treatment significantly increased the presence of 356 fluorescent papillae by 13% in comparison to control plants (water-sprayed) at 72 hai 357 (Fig. 4d). In contrast, no significant effect of ulvan was observed for whole fluorescent cells (Fig. 4e). 358

359

#### 360 3.5 Cytochemical detection of H<sub>2</sub>O<sub>2</sub> accumulation at Bgt penetration sites

Upon Bgt infection of wheat leaves,  $H_2O_2$  accumulation in the epidermal cell 361 wall beneath AGT was monitored by reddish-brown staining via DAB at 24, 48, and 72 362 hai. DAB staining was classified into three categories, no DAB staining (Fig. 5a), 363 staining localized at the penetration site of the fungus (Fig. 5c), and whole-cell DAB 364 staining (Fig. 5e). In control plants (water-sprayed), most penetration events led to no 365 visible DAB staining in the epidermal leaf cells. At 24 hai, the percentage of events 366 with no DAB staining was significantly lower by 30% in ulvan-treated plants than in 367 368 control ones (Fig. 5b). In contrast, ulvan-treatment increased the events with localized DAB staining by 90% in comparison to control at 24 hai (Fig. 5d). Ulvan treatment also 369 370 increased the percentage of whole-cell DAB staining by 400, 83, and 48% at 24, 48, and 72 hai, respectively (Fig. 5f). 371

372

373 **3.6 Total phenolic compounds** 

374 The total phenolic compounds were analyzed in wheat leaves of ulvan-treated or 375 water-sprayed plants and inoculated or not with *Bgt*. Under non-inoculated conditions, 376 the content of phenolic compounds was slightly higher in leaves of ulvan-treated plants when compared to the control. The levels of phenolics in ulvan-treated plants were 377

about 13, 26, and 23% higher than control at 24, 48, and 72 h after inoculation (*i.e.* 72,
96, and 120 h after treatment). Under inoculated conditions, significant differences were
also observed, but only at 24 and 48 hai, when ulvan treatment increased total phenolic
compounds by 9 and 11%, respectively (Fig. 6)

382

### **383 3.7 Enzyme activities**

Two days after ulvan-treatment or water-spraying (control) and before the *Bgt* inoculation in wheat plants (48 hat or 0 hai), the activity levels of POX, LOX and PAL were measured. At this sampling time, no significant stimulation of the three enzymatic activities was observed in ulvan-treated plants compared to control ones (Fig. 7).

Under inoculated conditions, ulvan treatment led to an increase of POX activity by about 60% and 55%, at 24 and 48 hai, respectively when compared to the control (Fig. 7a). Similarly, LOX activity in ulvan-treated plants was 45% and 63% higher than controls at 24 and 48 hai, respectively (Fig. 7b). Concerning PAL activity, a significant increase was observed at 48 hai in leaves of ulvan-treated plants by about 2.5-fold in comparison to control (Fig. 7c).

394

#### 395 **3.8** Induction of molecular defense markers in wheat leaves pre-treated with ulvan

The impact of ulvan-treatment on the expression of genes involved in defense was evaluated under non-inoculated and inoculated conditions. These genes were involved in the phenylpropanoid pathway (*PAL*, *CHS*, *COMT*, *ANS*, *DFR*, and *FLS*), the octadecanoid pathway (*LOX*) or encoded for pathogenesis-related proteins (*PR1*, *PR9*, and *PR15*).

Under non-inoculated conditions, ulvan induced the expression of 5 of the 6 401 genes involved in phenylpropanoid metabolism, *i.e. PAL*, CHS, COMT, ANS, and FLS 402 403 at 72 h and/or 96 h after treatment (hat) in comparison to the non-treated and non-404 inoculated wheat plants (Table 2). Concerning CHS, its expression was transiently 405 down-regulated at 60 hat. In the case of LOX, a first and transient induction was observed at 24 hat (3.8-fold) when a second up-regulation of this gene was observed at 406 407 96 hat (8.5-fold) (Table 2). The expression of genes encoding PR-proteins appeared also to be modulated by the ulvan treatment. For PR9, an up-regulation was observed at 24 408 409 and 72 hat (5- and 7.5-fold, respectively), whereas the induction of PR15 expression was observed from 60 to 72 hat (5.6- and 3.6-fold, respectively) (Table 2). Finally, the 410 411 expression of *PR1* was clearly impacted by ulvan, with a down-regulation of the gene

412 from 24 to 48 h (0.2-fold), followed by an up-regulation from 60 to 96 hat (29-, 127413 and 8-fold at 60, 72 and 96 hat, respectively) (Table 2).

Bgt infection of the control plants (water-sprayed) clearly modulated the 414 expression of the targeted genes (Table 3). Concerning the genes encoding proteins 415 involved in the phenylpropanoid pathway, the Bgt infection transiently induced an up-416 regulation of ANS (2.6-fold) at 24 hai and COMT (2.9-fold) at 48 hai in comparison to 417 control (non-inoculated). In the case of PAL and CHS, the gene appeared to present a 418 different pattern with an induction at 24 and 48 hai (11.6- and 4.2-fold, 14- and 12-fold, 419 420 respectively). No significant modulation of DFR and FLS was observed on the kinetic points studied (Table 3). The expression of LOX was induced from 24 hai, reaching a 421 422 higher accumulation at 48 hai (4.7-fold) (Table 3). Concerning the PR-proteins, various 423 patterns were observed according to the type of *PR* genes analyzed. Two peaks of high 424 transcripts accumulation of PR1 were observed, with a first transient peak at 12 hai (7,341-fold) and a second one at 48 hai (22,566-fold). The expression of PR9 was 425 426 induced on all points of the kinetic (from 12 to 48 hai, by about 1,600-, 2,300- and 3,300-fold, respectively). Similar to PR9, PR15 was induced on all points of the kinetic, 427 428 with the higher transcript accumulation at 12 hai (60-fold) (Table 3).

In general, under inoculated conditions, similar patterns were observed in leaves of ulvan-treated and control plants, except for *CHS*, *COMT*, and *PR1* wherein the expression was significantly lower in infected leaves of ulvan-treated than in control ones at 48 hai (Table 3). For *ANS* and *PR15*, the expression appeared to be significantly higher in leaves of infected plants pretreated with ulvan than in infected control at 12 hai (Table 3).

435

### 436 4. DISCUSSION

437

In this study, ulvan treatment showed to be effective in reducing powdery 438 439 mildew infection in wheat. The pre-treatment of whole plants significantly reduced the disease severity, but also induced numerous defense responses, such as an up-regulation 440 441 of defense-related genes, an increase in some enzyme activities involved in defense, and the amount of total phenolic compounds. Moreover, at the microscopic scale, we 442 443 observed more autofluorescence in papillae, suggesting phenolic compounds accumulation, and an accumulation of  $H_2O_2$  in whole-cells at the site of Bgt penetration 444 445 attempts.

Ulvan has been known to show systemic effect in various plants and its 446 447 effectiveness is dependent on the time interval between applications, the dose, the number of treatments, environmental conditions, and fungal species (Paulert et al., 448 2010; Jaulneau et al., 2011; Stadnik and de Freitas, 2014). In cucumber and grapevine, a 449 reduction of powdery mildew severity was also observed when a crude extract of Ulva 450 451 was applied once at 3 days or twice at 3-6 days before fungal inoculation (Jaulneau et al., 2011). In alfalfa, two ulvan treatments were more effective against C. trifolii than 452 453 one single treatment because some plant defense genes were particularly induced after 454 two consecutive ulvan applications (Cluzet et al., 2004). In common bean, one, two, or 455 three applications of ulvan were able to reduce the *Fusarium oxysporum* f. sp. *phaseoli* 456 colonization at earlier, but not at later phenological stages. Furthermore, higher protection in bean was found when ulvan was applied onto leaves but not via seed 457 458 soaking or soil irrigation (de Borba et al. 2019).

459 Ulvan did not inhibit conidial germination of Bgt in vitro, but increased the 460 formation of multiple germ tubes. Abnormal formation of germ tubes usually can be associated with an atypical source of nutrition or under special conditions that impair 461 462 conidial germination, *i.e.* when pre-infection structures did not recognize the surface 463 (Nielsen et al. 2000). Similar effects have been found out for another fungal pathogen as C. fructicola, where ulvan also stimulated the elongation of germ tubes (Gonçalves and 464 Stadnik, 2012). In contrast, in planta ulvan treatment did not affect conidial germination 465 and primary germ tube formation. Nevertheless, the percentage of appressorial germ 466 467 tubes (AGTs) without haustorium increased. These results suggest that ulvan does not 468 impair the initial germination in planta, but at later stages of infection when Bgt starts the formation of haustorium in epidermal leaf tissues. The treatment therefore indirectly 469 470 affects the fungal colonization, providing evidence that ulvan is able to induce plant 471 defense mechanisms and acts as a resistance inducer. Similar effects were observed using ulvan in wheat against the hemibiotrophic fungus Zymoseptoria tritici (Zt), where 472 473 the treatment significantly reduced substomatal colonization and pycnidium formation (de Borba et al., 2021). On the other hand, a commercial product based on Ascophyllum 474 *nodosum* (Dalgin Active<sup>®</sup>) did not affect fungal colonization but inhibited Zt sporulation 475 in bread and durum wheat (Somai-Jemmali et al., 2020). 476

Previous works have shown that algal polysaccharides, as ulvan, are considered
as an important source of plant resistance inducers (Paulert et al., 2010; de Freitas and
Stadnik, 2012; de Borba et al., 2021). Corresponding induced plant responses include
the biosynthesis of phytoalexins, pathogenesis-related (PR) proteins (Cluzet et al.,

2004), induction of enzyme activities as peroxidases, and accumulation of phenolic 481 482 compounds (Abouraïcha et al., 2015). However, molecular mechanisms underlying the ulvan-induced defense to powdery mildew pathogens are still unknown. 483

484 In the present study, enzyme activity assays, defense-related gene expression analyses, and microscopic observations provided additional information about the 485 defense mechanisms associated with ulvan treatment and probably contributing to the 486 reduction of Bgt symptoms development on susceptible wheat. Concerning the defense-487 related enzymes, the activities of POX and LOX increased in ulvan-treated plants in 488 489 comparison to control at 24 and 48 h after inoculation (hai), whereas PAL activity markedly increased only at 48 hai. A previous study on resistance induction by 490 acibenzolar-S-methyl (benzothiadiazole, Bion<sup>®</sup>) in wheat against powdery mildew 491 492 showed a PAL activity peak at 24 hai, which occurred during the formation of AGTs 493 (Stadnik and Buchenauer, 1999). PAL seems to be central for systemic acquired 494 resistance (SAR) because its inhibition suppresses induced resistance (Stadnik and 495 Buchenauer, 2000; Bhuiyan et al., 2009). POX and PAL are involved in the branch of 496 the phenylpropanoid pathway leading to the biosynthesis of lignin but also other 497 secondary metabolites, such as antimicrobial and phenolic compounds (Reignault et al., 2001; Abouraïcha et al., 2015). In our study, the increase of POX and PAL activities 498 may be associated with the increase of phenolic compounds observed in ulvan-treated 499 and inoculated plants at 24 and 48 hai, which could explain the increase of fluorescent 500 papillae at the penetration sites of Bgt at 72 hai and contribute to impair fungal 501 502 penetration. Papillae are typically formed to overcome pathogen penetration or delay the infection process of powdery mildew fungus, and their autofluorescence in 503 504 epidermal cells indicates accumulation of phenolic compounds (Hückelhoven et al., 1999; Stadnik and Buchenauer, 1999, 2000; Reignault et al., 2001). Similar results were 505 observed on Bgt-infected wheat pre-treated with natural inducers like Iodus40<sup> $^{(R)}$ </sup> (a 506 product based on laminarin), trehalose (Renard-Merlier et al., 2007), and acetylated 507 oligogalacturonides -OGAs (Randoux et al., 2010). Thus, PAL and POX activities were 508 509 stimulated in ulvan-treated plants during the first 48 h after infection probably leading 510 to the accumulation of phenolic compounds and to a cell-wall reinforcement.

511 Similarly, regarding H<sub>2</sub>O<sub>2</sub> accumulation, ulvan-treated plants showed more 512 DAB-stained cells during the 72 h after infection. Some elicitor treatments lead to ROS accumulation, as a result of their recognition by the plant as a pathogen attack (Randoux 513 514 et al., 2010). The increase in whole-cell DAB staining from 24 hai may suggest that ulvan pre-treatment leads to H<sub>2</sub>O<sub>2</sub> accumulation in response to the attachment of PGTs 515

or AGTs, which helps to avoid pathogen infection. This is corroborated by the up-516 517 regulation of *PR15*, encoding an oxalate oxidase activity producing H<sub>2</sub>O<sub>2</sub>, just after inoculation in ulvan-treated plants. Indeed, ulvan is highly associated with enhanced 518 519 resistance against powdery mildew in wheat and barley plants by priming an oxidative burst in suspension-cultured cells (Paulert et al., 2010). Moreover, the accumulation of 520 521 ROS, such as  $H_2O_2$  may also play a role in the induction of defense-signaling pathway. Previous studies have clearly shown that the accumulation of H<sub>2</sub>O<sub>2</sub> is one of the earliest 522 observable plant defenses strategy. H<sub>2</sub>O<sub>2</sub> is also involved in cell wall crosslinking and 523 524 lignification for strengthening at the infection sites, participating for instance in 525 monolignols oxidation by peroxidases before polymerization into lignin (Bhuiyan et al., 526 2009; Nguyen et al., 2016). However, H<sub>2</sub>O<sub>2</sub> accumulation induced by ulvan treatment can provoke cell death, as an attempt to restrict pathogen growth, since, with the host 527 528 cell death, the nutrient supply becomes unavailable for biotrophic fungi (Thordal-Christensen et al., 1997). 529

530 In contrast to PAL, LOX is involved in the octadecanoid pathway leading to the synthesis of oxylipins and jasmonic acid (JA) accumulation. This enzyme plays an 531 532 important role in plant response to elicitor treatments and resistance against powdery 533 mildew in wheat (Jaulneau et al., 2011). Similarly, the increase of LOX activity was also associated with a reduction in the number of Bgt colonies in wheat plants treated 534 with trehalose, a non-reducing disaccharide found in a wide range of organisms (Tayeh 535 et al., 2014), with Milsana®, an ethanolic extract from the plant Reynoutria 536 sachalinensis (Randoux et al., 2006) and with OGAs (Randoux et al., 2010). On the 537 other hand, no significant effect on the regulation of LOX was found in wheat treated 538 with the algal-based product Dalgin<sup>®</sup> and infected with the hemibiotrophic Z. tritici 539 (Somai-Jemmali et al., 2021). In alfalfa, ulvan also induced expression of the β-540 541 glucuronidase (GUS) gene placed under the control of lipoxygenase gene promoter, and as the expression profiling was similar to that observed upon methyl jasmonate 542 543 treatment (MeJA), the authors suggested for the first time the involvement of the JApathway in ulvan-induced immunity (Jaulneau et al., 2010). 544

545 Surprisingly, our findings mainly revealed that ulvan induced the expression of 546 several genes involved in plant defense responses independently of pathogen infection. 547 Most of the genes studied were up-regulated after ulvan treatment, without any 548 challenge by the pathogen, while further inoculation did not activate most of them 549 anymore. First, the expression profiles of PRs exhibited a certain degree of overlap 550 between the activation of both JA and SA pathways. In non-inoculated conditions,

ulvan-treatment up-regulated PR9 and LOX expression 24 h after treatment, and later, 551 552 the expression of five genes involved in phenylpropanoid metabolism, one in the 553 octadecanoid pathway and three genes encoding PR-proteins, mainly between 60 and 96 h after treatment. The first transient induction of PR9 and LOX could be related to 554 signalization, leading later to the establishment of defense responses. Subsequently, 555 556 *PR1* was notably induced, which confirms its role in the activation of plant defense. PR1 is known as a marker for SA signaling pathway and is frequently induced in 557 response to fungal infection by activating SAR, especially against biotrophic pathogens 558 559 (Cluzet et al., 2004).

560 As described above, the phenylpropanoid pathway is known to produce phenolic 561 compounds, which can be involved in many vital processes of plants like lignin 562 biosynthesis and cell wall reinforcement at sites of attempted penetration (Hückelhoven 563 et al., 1999; Stadnik and Buchenauer, 2000; Bhuiyan et al., 2009). Among genes involved in the phenylpropanoid pathway, we studied genes encoding enzymes involved 564 565 in different branches. PAL is the enzyme acting upstream of the phenylpropanoid pathway, whereas acting downstream, we can find CHS (chalcone synthase), followed 566 567 by ANS (anthocyanidin synthase) or FLS (flavonol synthase), which are enzymes 568 involved in the flavonoids and isoflavonoid-type phytoalexins biosynthesis pathway. COMT (caffeic acid 3-O-methyltransferase) is a key enzyme for another branch of the 569 570 phenylpropanoid pathway, leading to monolignol synthesis (Nguyen et al., 2016). Several studies have shown the role of these enzymes in plants under abiotic and biotic 571 stress conditions. According to our results, CHS, ANS, and FLS expressions were up-572 regulated without pathogen challenge and after ulvan treatment in order to help wheat 573 574 plants to accumulate more flavonoids and probably enhance the resistance to Bgt. For example, CHS and CHI (chalcone isomerase) were also associated with Milsana-575 576 induced resistance against powdery mildew in cucumber plants since flavonoids were 577 found in the haustorial complex of the Podosphaera fuliginea (Fofana et al., 2002). 578 COMT is also involved in sinapaldehyde synthesis, leading to sinapyl alcohol, one of the main monolignols involved in lignin synthesis in wheat (Nguyen et al., 2016). The 579 580 up-regulation of COMT in non-infected conditions suggests an accumulation of 581 monolignols, which could be rapidly oxidized and polymerized for lignin synthesis in 582 the case of pathogen attack. Interestingly, monolignol biosynthesis genes have been 583 known to be induced in both susceptible and resistant wheat cultivars after Bgt attack 584 (Bhuiyan et al., 2009). The concomitant up-regulation of PR15, encoding an oxalate oxidase producing  $H_2O_2$ , and *PR9* encoding a peroxidase using  $H_2O_2$  for lignification 585

seems to confirm this hypothesis. Moreover, we observed that global phenolic
compounds already accumulated in wheat in non-infected conditions, which confirms
the importance of the elicitation of the phenylpropanoid pathway in wheat for defense.

589 Our study clearly indicates that ulvan treatment first elicits plant defense 590 responses independently of fungal infection. The phenylpropanoid pathway seems to be 591 largely involved in the protection conferred by ulvan against *Bgt* in wheat. Nevertheless, 592 further investigations, including different genotypes, and non-targeted approaches like 593 metabolomics and global hormonal signaling pathways could be an exciting challenge 594 for future research.

595

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### 601 AUTHOR'S CONTRIBUTION

602

Aline C. Velho performed all experiments and wrote the manuscript. Pamela Dall'Asta and Maryline Magnin-Robert contributed to gene expression analysis, phenolic compounds assay and manuscript writing. Marlon C. de Borba contributed to enzyme activities and manuscript writing. Philippe Reignault, Ali Siah, Marciel J. Stadnik and Béatrice Randoux supervised this study and contributed to manuscript writing.

609

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### 617 CONFLICTS OF INTEREST

618

The authors declare no conflicts of interest.

620

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781







Percentage (%)







Hours after inoculation

Hours after inoculation



Hours after inoculation



Hours after inoculation



Hours after inoculation

**Table 1**. Percentages of the different developmental stages of *Blumeria graminis* f. sp. *tritici* (*Bgt*) on epidermal leaf cells of both ulvan-treated or water-sprayed (control) wheat plantlets at 24, 48, and 72 h after inoculation. Susceptible plants were sprayed with water or ulvan 48 h before inoculation ( $5 \times 10^5$  spores mL<sup>-1</sup>). Means followed by different letters in the column, within each category, indicate values significantly different compared to control according to Student's t-test ( $p \le 0.05$ ).

Development stages	Treatmonts	Hours after inoculation				
Development stages	Treatments	24h	<b>48h</b>	72h		
Non-germinated	Control	$2.1 \pm 1.5 \text{ ns}$	3.1 ±0.7 ns	$2.4 \pm 1.0 \text{ ns}$		
	Ulvan	3.1 ± 1.6	3.1 ±1.8	4.8 ±3.5		
PGT only (%)	Control	2.2 ±0.9 ns	3.9 ±1.8 ns	1.4 ±0.8 ns		
	Ulvan	4.3 ±3.3	5.3 ±3.1	2.4 ±0.8		
AGT without HS (%)	Control	38.4 ±3.3 b	30.9 ±6.7 b	14.9 ±6.0 b		
	Ulvan	54.6 ±6.3 a	42.1 ±0.9 a	26.1 ±5.9 a		
AGT with HS (early stage) (%)	Control	57.3 ±2.9 b	57.5 ±8.2 b	64.9 ±4.0 b		
	Ulvan	38.0 ±9.1 a	43.2 ±2.5 a	56.2 ±2.8 a		
AGT with fully formed HS (%)	Control	nd	4.7 ±2.2 ns	10.7 ±2.2 ns		
	Ulvan	nd	6.3 ±1.2	9.3 ±3.6		
AGT with BH	Control	nd	nd	5.6 ±2.2 b		
	Ulvan	nd	nd 1.3 ±1.2 a			

Abbreviations: PGT = primary germ tube, AGT = appressorial germ tube, HS = haustorium, BH = branched hyphae, ns=non-significant, nd = non-detected.

**Table 2.** Quantitative real-time reverse-transcription polymerase chain reaction (RT-qPCR) experiments of transcripts levels of a set of defense genes in susceptible wheat (cv. Pakito) after ulvan treatment in non-inoculated condition<sup>x</sup>.

hat <sup>y</sup>	PAL	CHS	COMT	ANS	DFR	FLS	LOX	PR1	PR9	<b>PR15</b>
24	1.50 NS	1.30 NS	0.80 NS	1.32 NS	0.96 NS	1.77 NS	3.76 A	0.17 A	5.08 A	0.54 NS
48	1.15 NS	1.99 NS	1.04 NS	1.82 NS	1.64 NS	0.99 NS	0.76 NS	0.23 NS	0.98 NS	2.24 NS
60	0.51 NS	0.11 A	0.50 NS	0.61 NS	0.58 NS	0.28 NS	0.33 NS	29.02 A	2.58 NS	5.55 A
72	1.38 NS	8.58 A	1.35 NS	4.93 A	0.97 NS	4.91 A	1.24 NS	127.07 A	7.45 A	3.61 A
96	2.57 A	4.64 A	3.83 A	1.36 NS	1.05 NS	4.70 A	8.52 A	8.21 A	1.21 NS	1.25 NS

<sup>x</sup>: Values shown represent fold up-regulation in ulvan-treated compared with water-sprayed plants after normalization to *PetA* and *ATPAAA* genes. A = significant change, NS = non-significant change. Gene expression was considered as up- or down-regulated to the 1x controls when changes in relative expression were >2x or >0.5x, respectively.

<sup>Y</sup>: hours after treatment.

**Table 3.** Quantitative real-time reverse-transcription polymerase chain reaction (RT-qPCR) experiments of transcripts levels of a set of defense genes in susceptible wheat (cv. Pakito) after ulvan treatment in inoculated condition<sup>x</sup>.

	hai/hat <sup>y</sup>	PAL	CHS	COMT	ANS	DFR	FLS	LOX	PR1	PR9	<b>PR15</b>
Bgt	12 /NT	0.73 NS	0.40 NS	0.35 NS	0.74 NS	0.57 NS	0.27 NS	1.30 NS	7,341 A	1,594 A	58.90 A
	24 /NT	11.60 A	13.73 A	0.94 NS	2.58 A	0.64 NS	0.71 NS	1.78 NS	340 NS	2,332 A	7.70 A
	48 /NT	4.16 A	11.91 A	2.86 A	0.55 NS	0.92 NS	1.77 NS	4.68 A	22,566 A	3,372 A	10.65 A
Ulvan + Bgt	12 /60	0.76 NS	0.11 NS	0.53 NS	2.24 NS*	0.90 NS	0.30 NS	0.37 NS	5,606 A	2,547 A	155.1 A*
	<b>24</b> /72	14.13 A	9.89 A	0.73 NS	3.84 A	0.59 NS	1.44 NS	3.24 A	398 NS	3,251 A	10.88 A
	<b>48</b> /96	3.76 A	3.54 A*	1.31 NS*	0.72 NS	0.93 NS	2.43 A	8.22 A	7,546 A*	2,557 A	15.92 A

<sup>x</sup>: Values shown represent fold up-regulation in leaves of water-sprayed *Bgt* infected and ulvan-treated *Bgt* infected plants compared with watersprayed non-infected plants after normalization to *PetA* and *ATPAAA* genes. Gene expression was considered as up- or down-regulated to the 1x controls when changes in relative expression were >2x or >0.5x, respectively. A = significant change, NS = non-significant change. Statistical analyses were performed if the relative expression difference between ulvan-treated and infected wheat and the infected control wheat were >2x or >0.5x, \* indicates a significant difference.

<sup>Y</sup>: hours after inoculation /equivalent to hours after treatment with ulvan.

NT: Non-treated with ulvan.



- Conidial germination of Blumeria graminis
  - f. sp. tritici (Bgt)
- Development of multiple germ tubes



- Severity of powdery mildew
- Haustorium development
- Accumulation of H<sub>2</sub>O<sub>2</sub>
- Frequency of fluorescent papillae
- Total phenolic compounds
- POX, PAL and LOX activities
- Ulvan elicit the expression of defense-related genes in non-infected conditions Phenylpropanoid pathway - PAL, CHS, COMT, ANS and FLS **Octadecanoid pathway - LOX** Defense-related proteins - PR1, PR9 and PR15







