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► To cite this version:

Aline Velho, Pamela Dall'asta, Marlon de Borba, Maryline Magnin-Robert, Philippe Reignault, et al.. Defense responses induced by ulvan in wheat against powdery mildew caused by *Blumeria graminis* f. sp. *tritici*. *Plant Physiology and Biochemistry*, 2022, 184, pp.14-25. 10.1016/j.plaphy.2022.05.012 . hal-03673184

HAL Id: hal-03673184

<https://hal.science/hal-03673184>

Submitted on 22 Jul 2024

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1 **Defense Responses Induced by Ulvan in Wheat Against Powdery Mildew Caused**
2 **by *Blumeria graminis* f. sp. *tritici***

3

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18

19 **ABSTRACT**

20 Ulvan is a water-soluble sulfated heteropolysaccharide extracted from the cell walls of
21 the green seaweeds *Ulva* spp. This polysaccharide is known to induce resistance and
22 protect plants against a broad range of plant pathogenic fungi, such as *Blumeria*
23 *graminis* f. sp. *tritici* (*Bgt*), the causal agent of powdery mildew in wheat. We aimed to
24 study the defense mechanisms induced by ulvan against *Bgt* in susceptible wheat by
25 investigating the defense-related gene expression, enzymes activity, accumulation of
26 phenolic compounds and hydrogen peroxide (H₂O₂), as well as the development of *Bgt*
27 infection structures *in vitro* and *in planta*. Symptoms were reduced by 42% in ulvan-
28 treated plants. *In vitro*, ulvan did not inhibit conidial germination of *Bgt* but *in planta*,
29 increased the appressorial germ tubes without haustorium. Ulvan increased the presence
30 of fluorescent papillae and accumulation of H₂O₂ at the penetration sites of *Bgt*, as well
31 as the content of phenolic compounds. POX, PAL and LOX activities were stimulated

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

41

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

44

45 **1. INTRODUCTION**

46

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

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

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).

66 Due to the rapid evolution of the pathogen and the emergence of new virulent
67 isolates, many race-specific (R) resistance genes introduced in wheat cultivars have
68 become ineffective for controlling powdery mildew development (Kang et al., 2020). In
69 this case, synthetic fungicides are commonly used to control this disease in wheat,
70 which are not healthy and environmentally friendly. For this reason, using alternative
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
73 obtained from marine macroalgae are interesting sources of active molecules to control
74 powdery mildew on various crops (Paulert et al., 2010; Jaulneau et al., 2011; Stadnik
75 and de Freitas, 2014). Algae extracts are a source of a wide range of chemically active
76 metabolites. These molecules possess various biological properties including
77 antimicrobial, antioxidant, antifungal effects, and resistance inducer capacity *via* the
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.,
81 2004; Jaulneau et al., 2011; Stadnik and de Freitas, 2014; de Borba et al., 2021) can be
82 used in plant protection.

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

96 The extent of plant defense induction can lead to an increased resistance against
97 subsequent pathogen attack. In this sense, various compounds acting as elicitors have
98 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.

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

120 So far, studies to investigate the ulvan effect on wheat were restricted to the
121 evaluation of the efficacy of this polysaccharide in controlling powdery mildew caused
122 by *Bgt*. However, few studies have been carried out in order to clarify the mode of
123 action and the defense pathways involved in wheat resistance against this biotrophic
124 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
127 expression, enzymes activity, and the evolution of phenolic compounds in leaves. A
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 (H₂O₂) at the site of *Bgt* penetration attempts in response to ulvan
131 treatment. Finally, *in vitro* assay allowed to assess the ability of ulvan to inhibit directly
132 *Bgt* conidial germination.

133

134 **2. MATERIAL AND METHODS**

135

136 **2.1 Biological material**

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

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

149

150 **2.2 Ulvan obtention**

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

160

161 **2.3 Plant treatment**

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

166

167 **2.4 Inoculation and disease evaluation**

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

181 In order to evaluate the ulvan effect on conidial germination, plastic Petri plates
182 (5 cm) containing agar medium (12 g L^{-1}) in presence of water (control) or amended
183 with ulvan solution (1 mg mL^{-1}) were prepared. Then, conidia of *Bgt* were gently
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
186 plate, being that divided into four quadrants, using a light microscope (Olympus BX
187 40). The conidia were classified into five categories: non-germinated conidia (NG),
188 conidia with aborted germ tubes (AGT), with a single short germ tube (SGT, length <
189 $10 \mu\text{m}$), a single long germ tube (LGT, length > $10 \mu\text{m}$) and multiple germ tubes (MGT)
190 (Fig. 2).

191

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

193 In order to monitor the development of infection structures of *Bgt*, segments (1.5
194 cm length) from the 1st expanded wheat leaves treated and non-treated with ulvan were
195 excised at 24, 48, and 72 h after inoculation (hai). Leaf tissues were cleared in an acetic
196 acid: ethanol solution (v:v, 1:3), stained with trypan blue (w:v 0.1%) for 30 min,
197 mounted on glass slides with lactoglycerol, and observed with a light microscope
198 (Olympus BX 40). Different developmental stages were evaluated in epidermal leaf
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
202 AGT with branched hyphae (BH) (Table 1).

203

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

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

211

212 **2.8 Cytochemical detection of hydrogen peroxide (H₂O₂) accumulation**

213 Detection of H₂O₂ was carried out using the DAB staining method according to
214 Thordal-Christensen et al. (1997). For H₂O₂ visualization, leaf segments treated or non-
215 treated with ulvan were collected at 24, 48, and 72 hai and immediately submerged in
216 3,3-diaminobenzidine (DAB) solution (1 mg mL⁻¹, pH 3.8) for 4 h. Thereafter, tissues
217 were completely bleached in acetic acid: ethanol (v:v, 1:3) solution and stored with
218 lactoglycerol in the darkness at room temperature. H₂O₂ accumulation was identified by
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
221 BX 40) and classified into three classes according to their frequencies: no DAB
222 staining, staining localized at the penetration sites, and whole-cell DAB staining (Fig.
223 5).

224

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

226 Total phenolic compounds were determined using the Folin-Ciocalteu method
227 (Singleton and Rossi 1965) downscaled to a 96-well plate with minor modifications.
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
231 180 rpm in dark condition before two centrifugations at 13,000 × g for 5 min. The
232 supernatants were stored at –20°C until analysis. An aliquot (30 µL) of methanolic
233 extract was mixed with 150 µL of Folin-Ciocalteu reagent (10-times diluted) and after 5
234 min of incubation at room temperature, 120 µL of sodium carbonate solution (w:v 10%)
235 was added. The mixture was incubated at room temperature for 2 h, and the absorbance
236 was read at 750 nm. Total phenolic compounds were expressed as mg of Gallic acid
237 equivalents (GAE) per g of fresh tissue.

238

239 **2.10 Enzyme activities**

240 Three plant enzyme activities involved in defense mechanisms were assessed 24,
241 48, and 72 h after inoculation with *Bgt* in wheat leaves treated and non-treated with
242 ulvan.

243 Peroxidase (POX, EC 1.11.1.7) activity was assayed according to Mustafa et al.,
244 (2017) and was determined by monitoring the increase in absorbance at 412 nm for five
245 min. The activity was defined as the amount of enzyme that reduced the electron donor
246 at 1 mol s⁻¹ using ABTS (2, 2-azino-bis 3-ethylbenzothiazoline-6-sulfonic acid) as
247 substrate ($\epsilon = 32400 \text{ M}^{-1} \text{ cm}^{-1}$).

248 Lipoxygenase (LOX, EC 1.13.11.12) was measured according to Randoux et al.
249 (2010) using linoleic acid as substrate. LOX activity was determined by monitoring
250 changes in absorbance at 234 nm for five min, and one unit was defined as the amount
251 of enzyme that catalyzes the conversion of the substrate (linoleic acid) at 1 mol s⁻¹ ($\epsilon =$
252 $23000 \text{ M}^{-1} \text{ cm}^{-1}$).

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⁻¹ ($\epsilon = 10000 \text{ M}^{-1} \text{ cm}^{-1}$).

257 Protein content was measured using the method of Lowry et al. (1951). The
258 standard curve was established with bovine serum albumin (BSA). POX, LOX, and
259 PAL activities were expressed as nanokatal mg⁻¹ protein, where 1 kat represents the
260 amount of enzyme that converts 1 mol of substrate s⁻¹ or forms 1 mol of product s⁻¹.

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

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

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

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

294

295 **2.12 Experimental design and statistical analyses**

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

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

308

309 **3. RESULTS**

310

311 **3.1 Disease protection**

312 At ten days after inoculation, the disease symptoms on wheat leaves were
313 visually evaluated and characterized by the presence of white to grayish powdery
314 colonies on the total surface of both faces of the first leaf of each plantlet. Control plants
315 exhibited on average 77 colonies per leaf, while ulvan-treated plants showed about 45
316 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***

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

326

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

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

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

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

351

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
358 cells (Fig. 4e).

359

360 **3.5 Cytochemical detection of H₂O₂ accumulation at *Bgt* penetration sites**

361 Upon *Bgt* infection of wheat leaves, H₂O₂ accumulation in the epidermal cell
362 wall beneath AGT was monitored by reddish-brown staining *via* DAB at 24, 48, and 72
363 hai. DAB staining was classified into three categories, no DAB staining (Fig. 5a),
364 staining localized at the penetration site of the fungus (Fig. 5c), and whole-cell DAB
365 staining (Fig. 5e). In control plants (water-sprayed), most penetration events led to no
366 visible DAB staining in the epidermal leaf cells. At 24 hai, the percentage of events
367 with no DAB staining was significantly lower by 30% in ulvan-treated plants than in
368 control ones (Fig. 5b). In contrast, ulvan-treatment increased the events with localized
369 DAB staining by 90% in comparison to control at 24 hai (Fig. 5d). Ulvan treatment also
370 increased the percentage of whole-cell DAB staining by 400, 83, and 48% at 24, 48, and
371 72 hai, respectively (Fig. 5f).

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
377 when compared to the control. The levels of phenolics in ulvan-treated plants were

378 about 13, 26, and 23% higher than control at 24, 48, and 72 h after inoculation (*i.e.* 72,
379 96, and 120 h after treatment). Under inoculated conditions, significant differences were
380 also observed, but only at 24 and 48 hai, when ulvan treatment increased total phenolic
381 compounds by 9 and 11%, respectively (Fig. 6)

382

383 **3.7 Enzyme activities**

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

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

394

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

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

401 Under non-inoculated conditions, ulvan induced the expression of 5 of the 6
402 genes involved in phenylpropanoid metabolism, *i.e.* *PAL*, *CHS*, *COMT*, *ANS*, and *FLS*
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
406 observed at 24 hat (3.8-fold) when a second up-regulation of this gene was observed at
407 96 hat (8.5-fold) (Table 2). The expression of genes encoding PR-proteins appeared also
408 to be modulated by the ulvan treatment. For *PR9*, an up-regulation was observed at 24
409 and 72 hat (5- and 7.5-fold, respectively), whereas the induction of *PR15* expression
410 was observed from 60 to 72 hat (5.6- and 3.6-fold, respectively) (Table 2). Finally, the
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 hai (29-, 127-
413 and 8-fold at 60, 72 and 96 hai, respectively) (Table 2).

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

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

435

436 **4. DISCUSSION**

437

438 In this study, ulvan treatment showed to be effective in reducing powdery
439 mildew infection in wheat. The pre-treatment of whole plants significantly reduced the
440 disease severity, but also induced numerous defense responses, such as an up-regulation
441 of defense-related genes, an increase in some enzyme activities involved in defense, and
442 the amount of total phenolic compounds. Moreover, at the microscopic scale, we
443 observed more autofluorescence in papillae, suggesting phenolic compounds
444 accumulation, and an accumulation of H₂O₂ in whole-cells at the site of *Bgt* penetration
445 attempts.

446 Ulvan has been known to show systemic effect in various plants and its
447 effectiveness is dependent on the time interval between applications, the dose, the
448 number of treatments, environmental conditions, and fungal species (Paulert et al.,
449 2010; Jaulneau et al., 2011; Stadnik and de Freitas, 2014). In cucumber and grapevine, a
450 reduction of powdery mildew severity was also observed when a crude extract of *Ulva*
451 was applied once at 3 days or twice at 3-6 days before fungal inoculation (Jaulneau et
452 al., 2011). In alfalfa, two ulvan treatments were more effective against *C. trifolii* than
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
457 protection in bean was found when ulvan was applied onto leaves but not *via* seed
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
461 associated with an atypical source of nutrition or under special conditions that impair
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
464 *C. fruticola*, where ulvan also stimulated the elongation of germ tubes (Gonçalves and
465 Stadnik, 2012). In contrast, *in planta* ulvan treatment did not affect conidial germination
466 and primary germ tube formation. Nevertheless, the percentage of appressorial germ
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
469 the formation of haustorium in epidermal leaf tissues. The treatment therefore indirectly
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
472 using ulvan in wheat against the hemibiotrophic fungus *Zymoseptoria tritici* (*Zt*), where
473 the treatment significantly reduced substomatal colonization and pycnidium formation
474 (de Borba et al., 2021). On the other hand, a commercial product based on *Ascophyllum*
475 *nodosum* (Dalgin Active[®]) did not affect fungal colonization but inhibited *Zt* sporulation
476 in bread and durum wheat (Somai-Jemmali et al., 2020).

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

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

484 In the present study, enzyme activity assays, defense-related gene expression
485 analyses, and microscopic observations provided additional information about the
486 defense mechanisms associated with ulvan treatment and probably contributing to the
487 reduction of *Bgt* symptoms development on susceptible wheat. Concerning the defense-
488 related enzymes, the activities of POX and LOX increased in ulvan-treated plants in
489 comparison to control at 24 and 48 h after inoculation (hai), whereas PAL activity
490 markedly increased only at 48 hai. A previous study on resistance induction by
491 acibenzolar-S-methyl (benzothiadiazole, Bion[®]) in wheat against powdery mildew
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.,
498 2001; Abouraïcha et al., 2015). In our study, the increase of POX and PAL activities
499 may be associated with the increase of phenolic compounds observed in ulvan-treated
500 and inoculated plants at 24 and 48 hai, which could explain the increase of fluorescent
501 papillae at the penetration sites of *Bgt* at 72 hai and contribute to impair fungal
502 penetration. Papillae are typically formed to overcome pathogen penetration or delay
503 the infection process of powdery mildew fungus, and their autofluorescence in
504 epidermal cells indicates accumulation of phenolic compounds (Hückelhoven et al.,
505 1999; Stadnik and Buchenauer, 1999, 2000; Reignault et al., 2001). Similar results were
506 observed on *Bgt*-infected wheat pre-treated with natural inducers like Iodus40[®] (a
507 product based on laminarin), trehalose (Renard-Merlier et al., 2007), and acetylated
508 oligogalacturonides -OGAs (Randoux et al., 2010). Thus, PAL and POX activities were
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 H₂O₂ accumulation, ulvan-treated plants showed more
512 DAB-stained cells during the 72 h after infection. Some elicitor treatments lead to ROS
513 accumulation, as a result of their recognition by the plant as a pathogen attack (Randoux
514 et al., 2010). The increase in whole-cell DAB staining from 24 hai may suggest that
515 ulvan pre-treatment leads to H₂O₂ accumulation in response to the attachment of PGTs

516 or AGTs, which helps to avoid pathogen infection. This is corroborated by the up-
517 regulation of *PR15*, encoding an oxalate oxidase activity producing H₂O₂, just after
518 inoculation in ulvan-treated plants. Indeed, ulvan is highly associated with enhanced
519 resistance against powdery mildew in wheat and barley plants by priming an oxidative
520 burst in suspension-cultured cells (Paulert et al., 2010). Moreover, the accumulation of
521 ROS, such as H₂O₂, may also play a role in the induction of defense-signaling pathway.
522 Previous studies have clearly shown that the accumulation of H₂O₂ is one of the earliest
523 observable plant defenses strategy. H₂O₂ is also involved in cell wall crosslinking and
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, H₂O₂ accumulation induced by ulvan treatment
527 can provoke cell death, as an attempt to restrict pathogen growth, since, with the host
528 cell death, the nutrient supply becomes unavailable for biotrophic fungi (Thordal-
529 Christensen et al., 1997).

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

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,

551 ulvan-treatment up-regulated *PR9* and *LOX* expression 24 h after treatment, and later,
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
554 h after treatment. The first transient induction of *PR9* and *LOX* could be related to
555 signalization, leading later to the establishment of defense responses. Subsequently,
556 *PRI* was notably induced, which confirms its role in the activation of plant defense.
557 *PRI* is known as a marker for SA signaling pathway and is frequently induced in
558 response to fungal infection by activating SAR, especially against biotrophic pathogens
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
564 involved in the phenylpropanoid pathway, we studied genes encoding enzymes involved
565 in different branches. PAL is the enzyme acting upstream of the phenylpropanoid
566 pathway, whereas acting downstream, we can find CHS (chalcone synthase), followed
567 by ANS (anthocyanidin synthase) or FLS (flavonol synthase), which are enzymes
568 involved in the flavonoids and isoflavonoid-type phytoalexins biosynthesis pathway.
569 COMT (caffeic acid 3-O-methyltransferase) is a key enzyme for another branch of the
570 phenylpropanoid pathway, leading to monolignol synthesis (Nguyen et al., 2016).
571 Several studies have shown the role of these enzymes in plants under abiotic and biotic
572 stress conditions. According to our results, *CHS*, *ANS*, and *FLS* expressions were up-
573 regulated without pathogen challenge and after ulvan treatment in order to help wheat
574 plants to accumulate more flavonoids and probably enhance the resistance to *Bgt*. For
575 example, *CHS* and *CHI* (chalcone isomerase) were also associated with Milsana-
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
579 the main monolignols involved in lignin synthesis in wheat (Nguyen et al., 2016). The
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 *PRI5*, encoding an oxalate
585 oxidase producing H₂O₂, and *PR9* encoding a peroxidase using H₂O₂ for lignification

586 seems to confirm this hypothesis. Moreover, we observed that global phenolic
587 compounds already accumulated in wheat in non-infected conditions, which confirms
588 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

596 **FUNDING**

597

598 This work was supported by the International Cooperation Program
599 CAPES/COFECUB - Bioestimalg Project (Grant: 88887.130209/2017-01).

600

601 **AUTHOR'S CONTRIBUTION**

602

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

609

610 **ACKNOWLEDGEMENTS**

611

612 The authors thank the Brazilian agency CAPES (Coordination for the
613 Improvement of Higher Education Personnel) for financial support and scholarships.
614 M.J.S is a research member of the CNPq (National Council for Scientific and
615 Technological Development).

616

617 **CONFLICTS OF INTEREST**

618

619 The authors declare no conflicts of interest.

620

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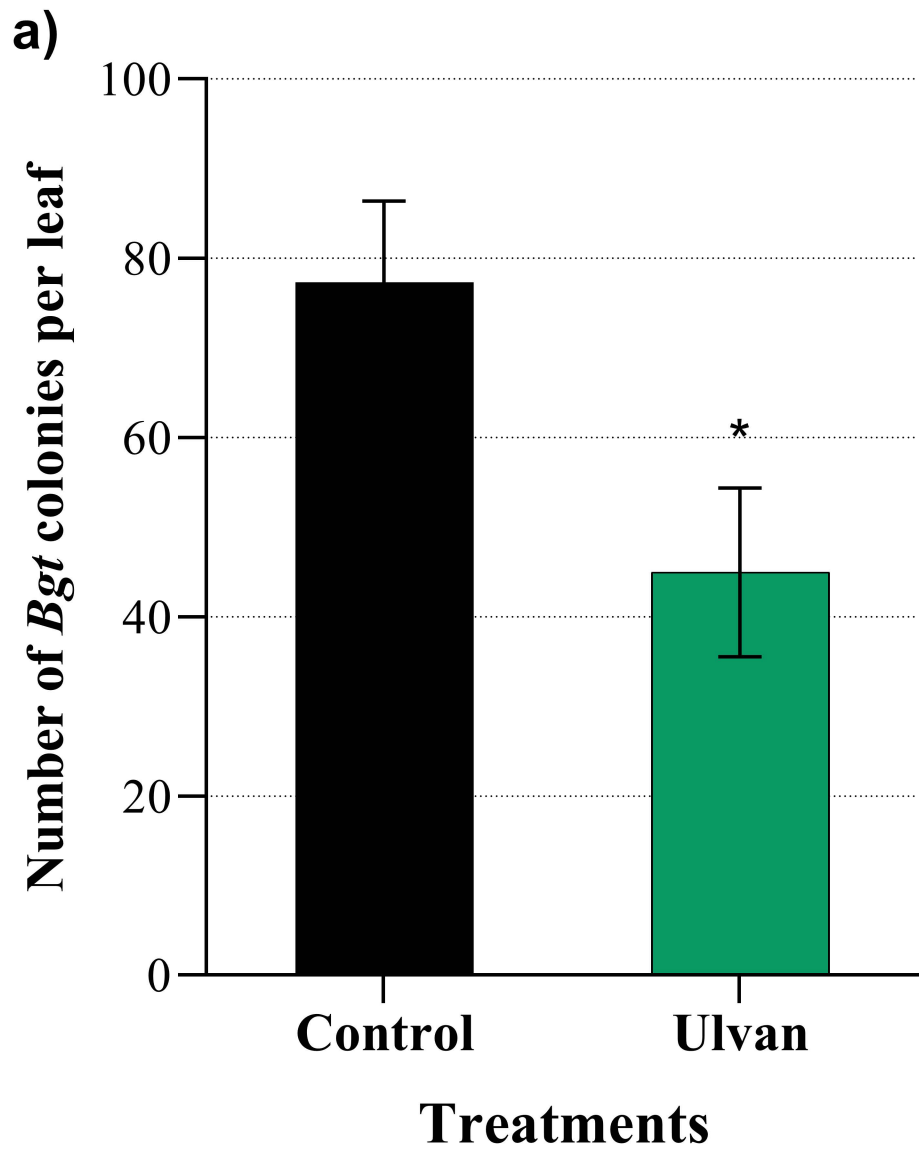
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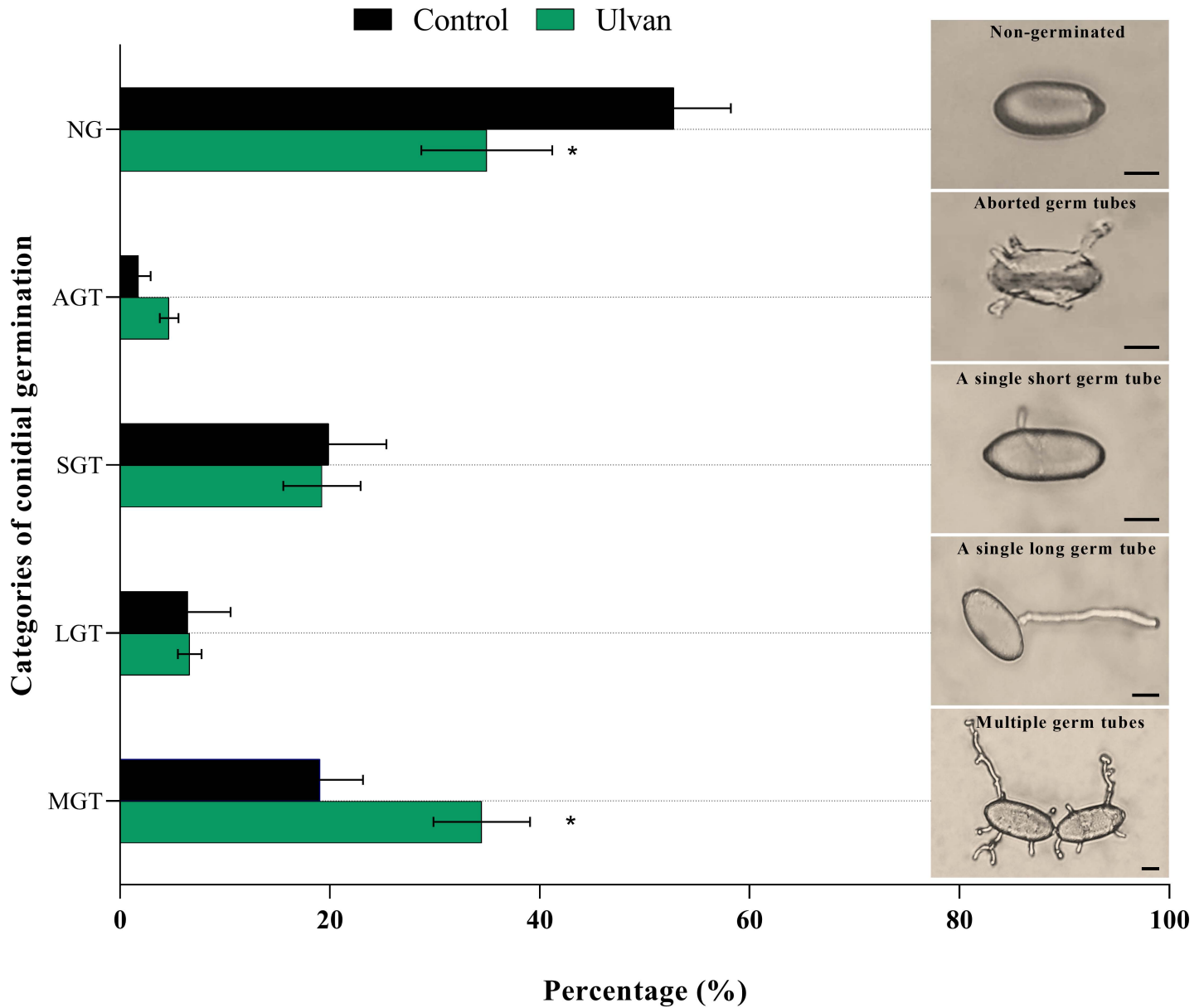
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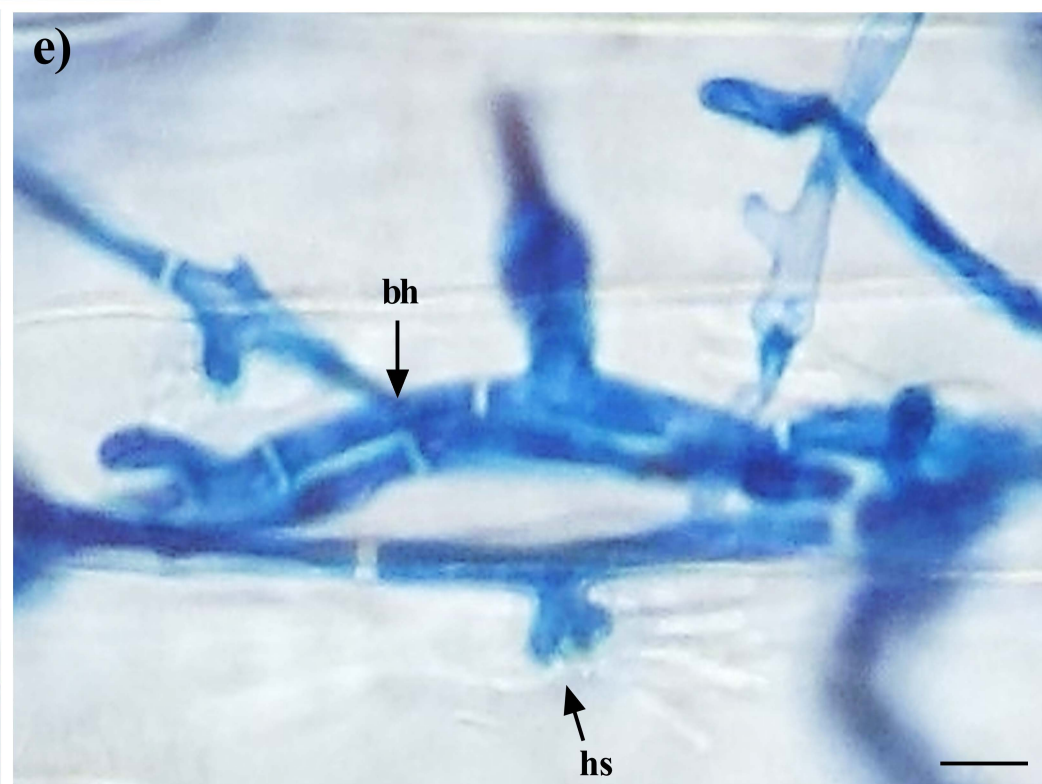
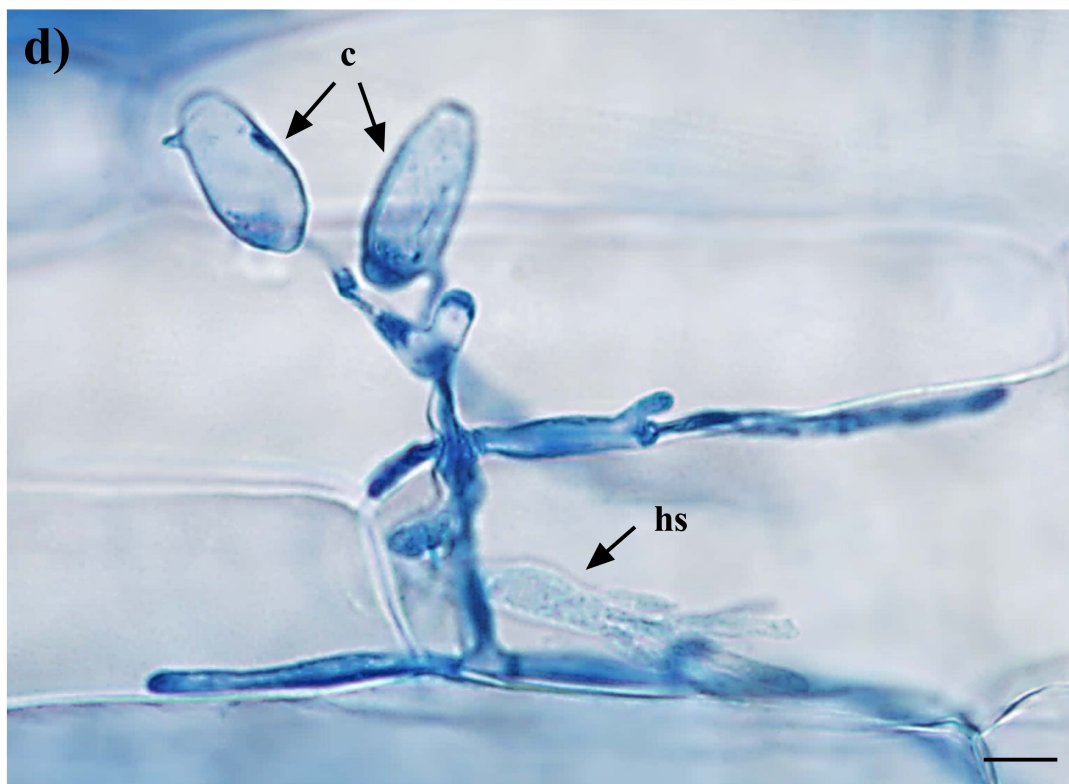
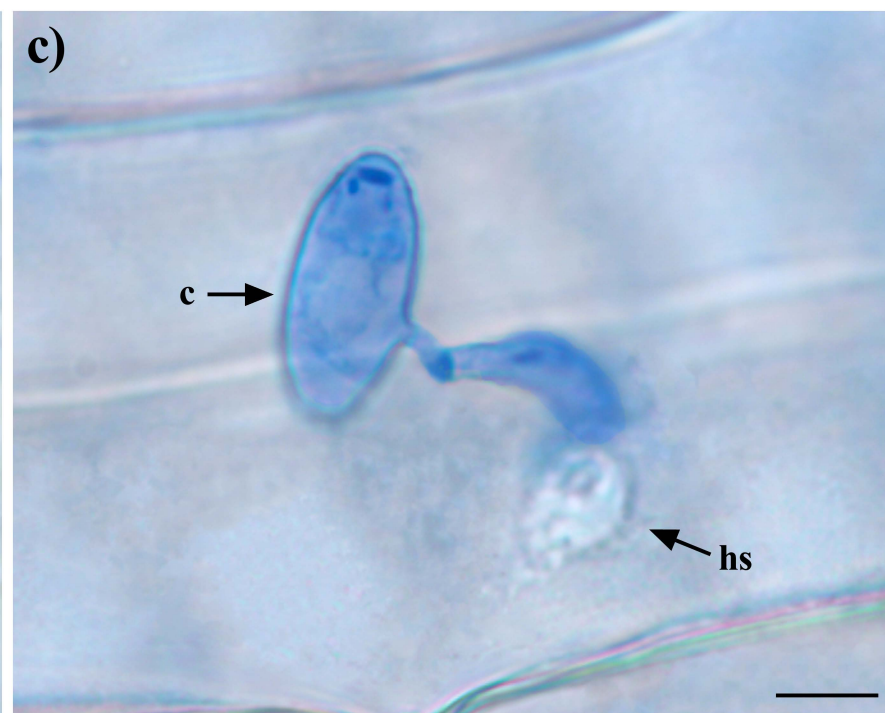
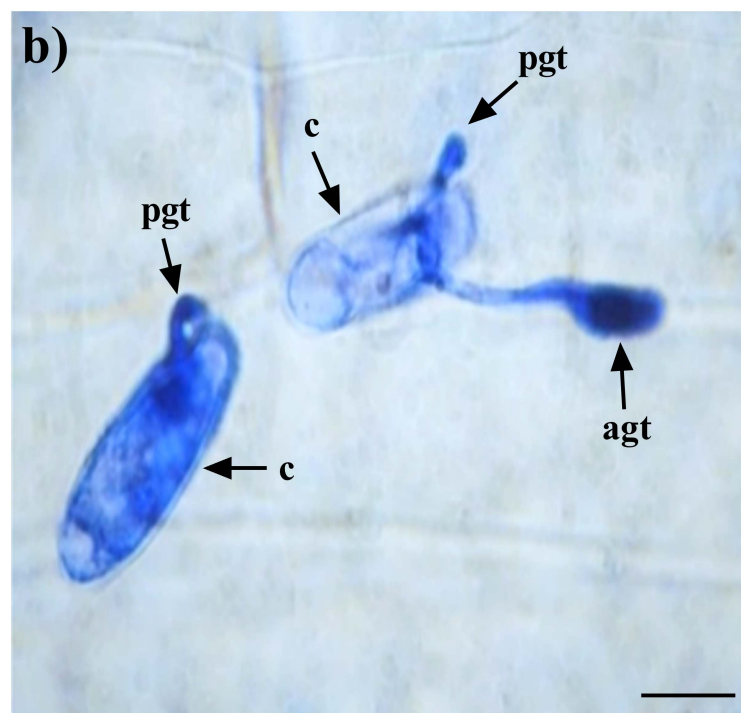
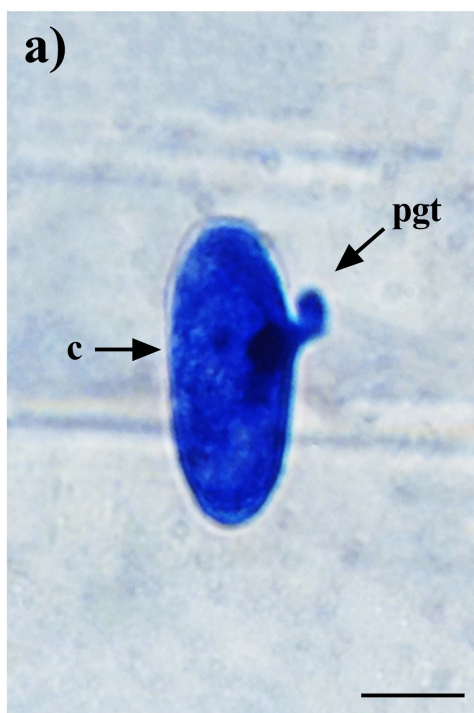
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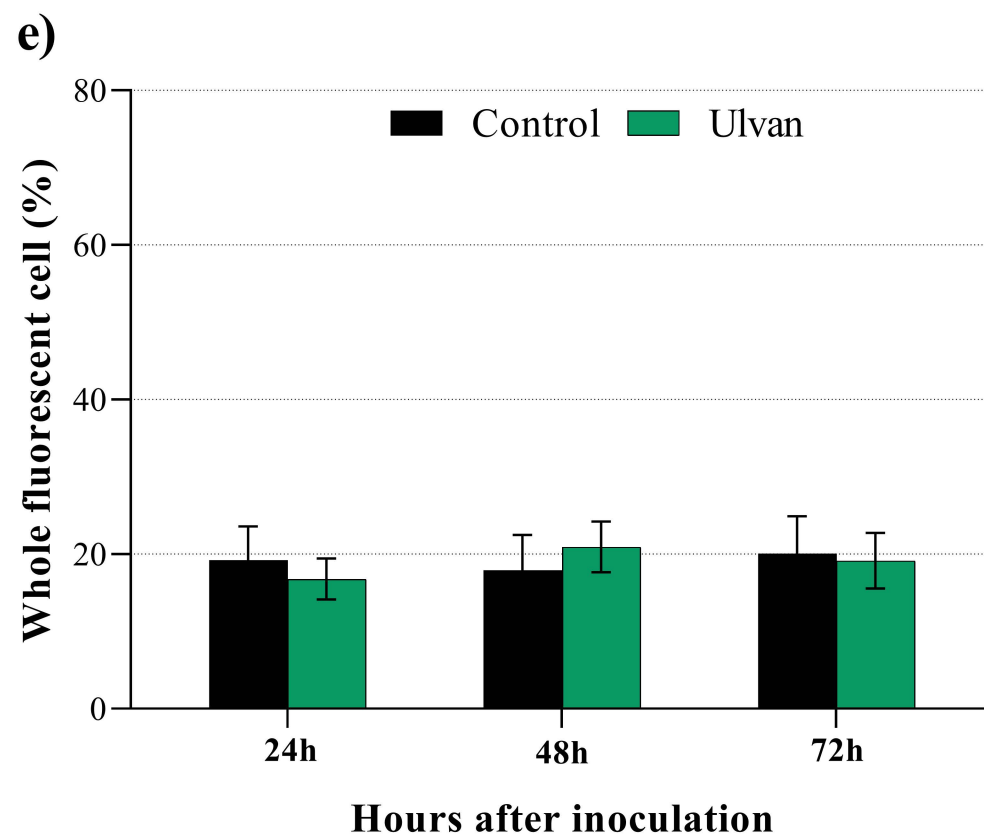
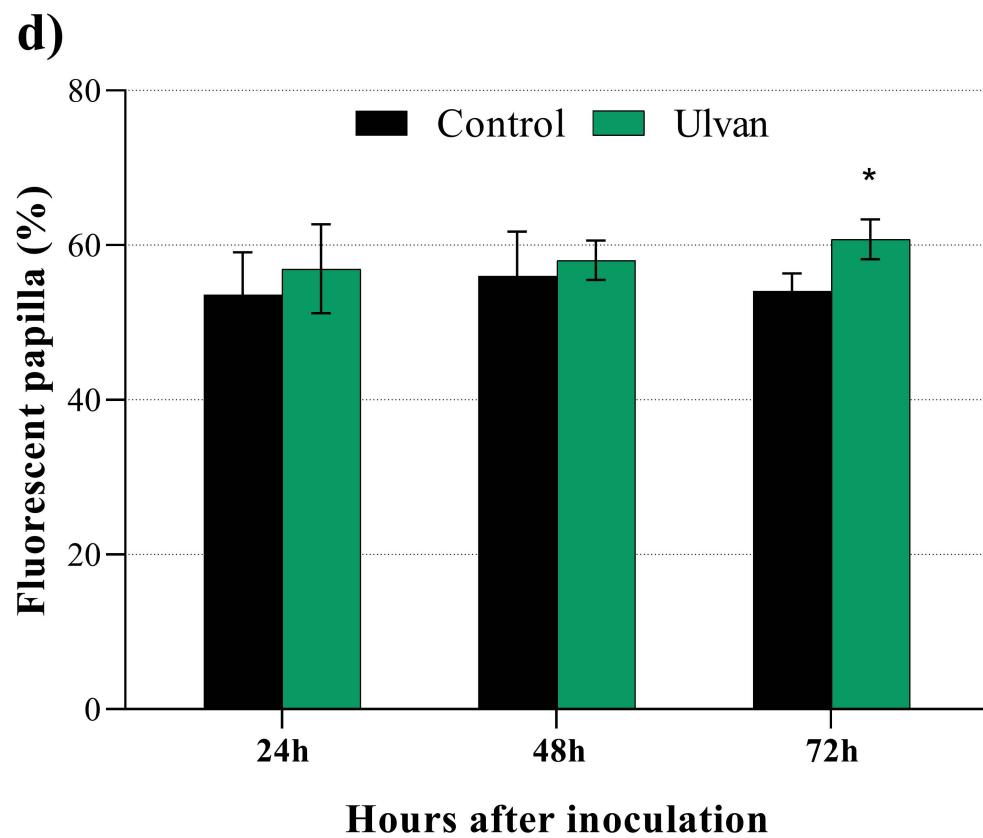
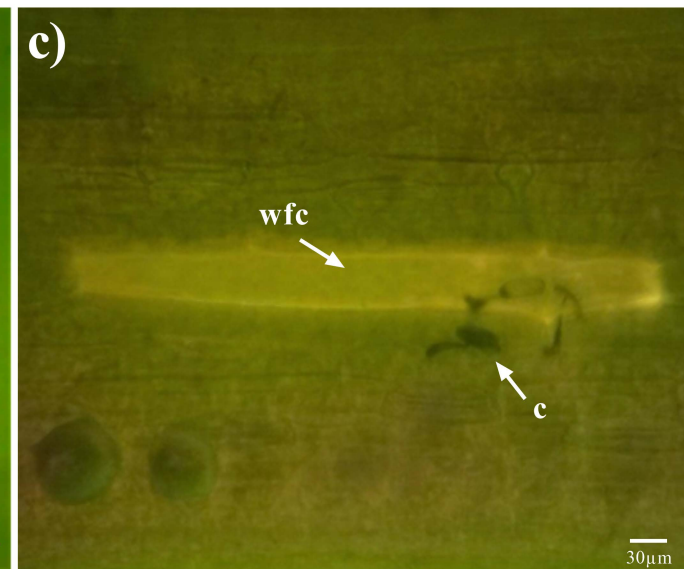
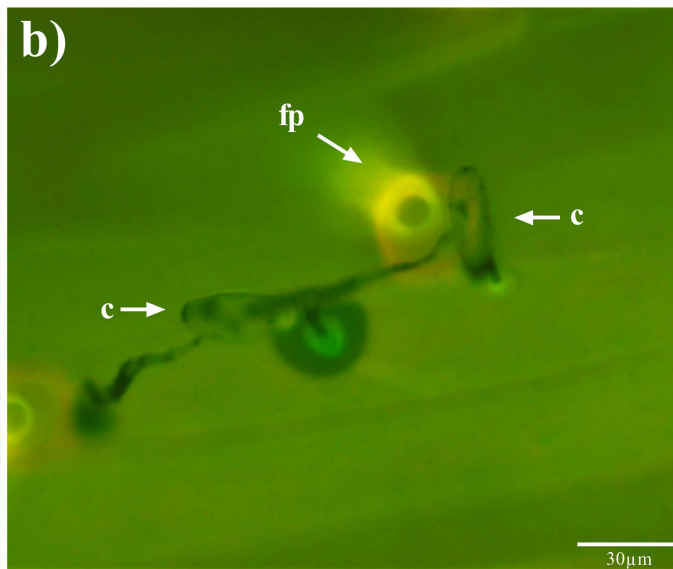
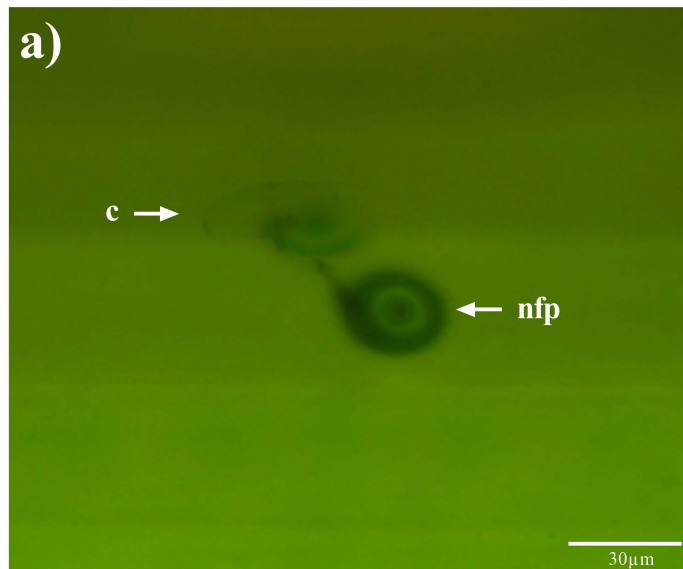
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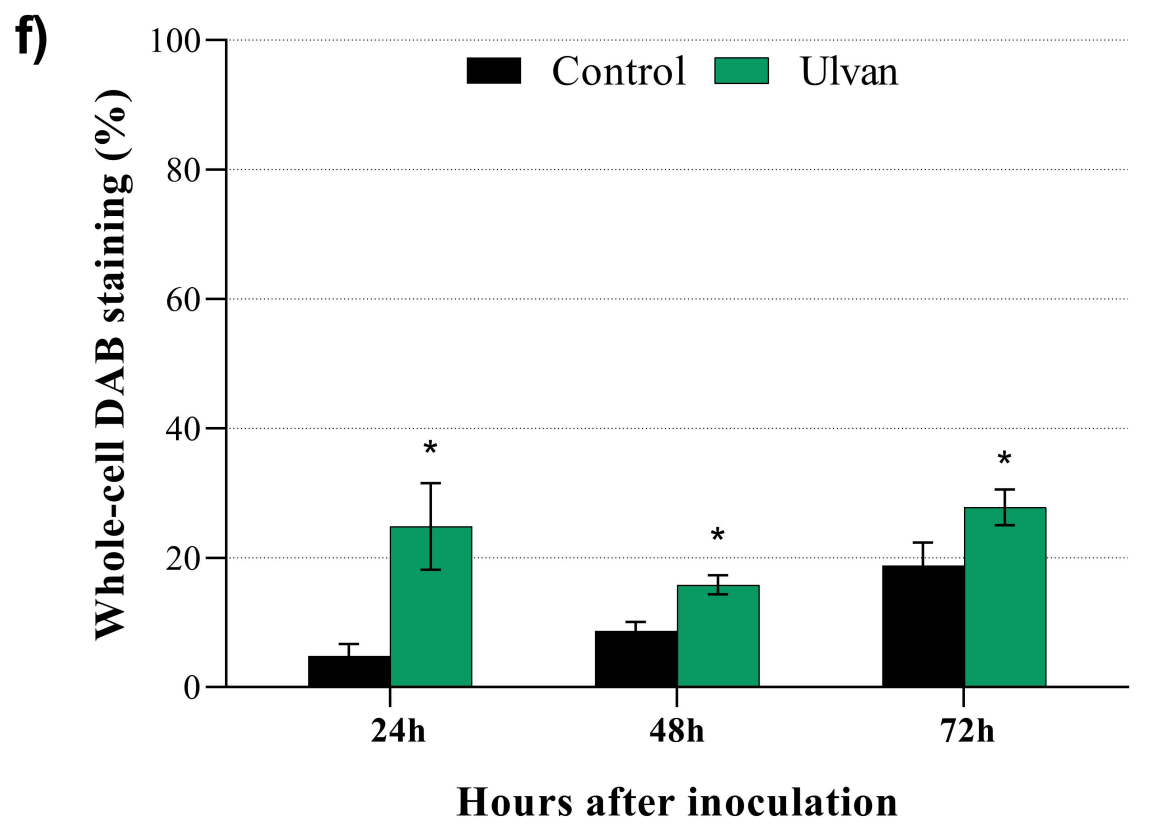
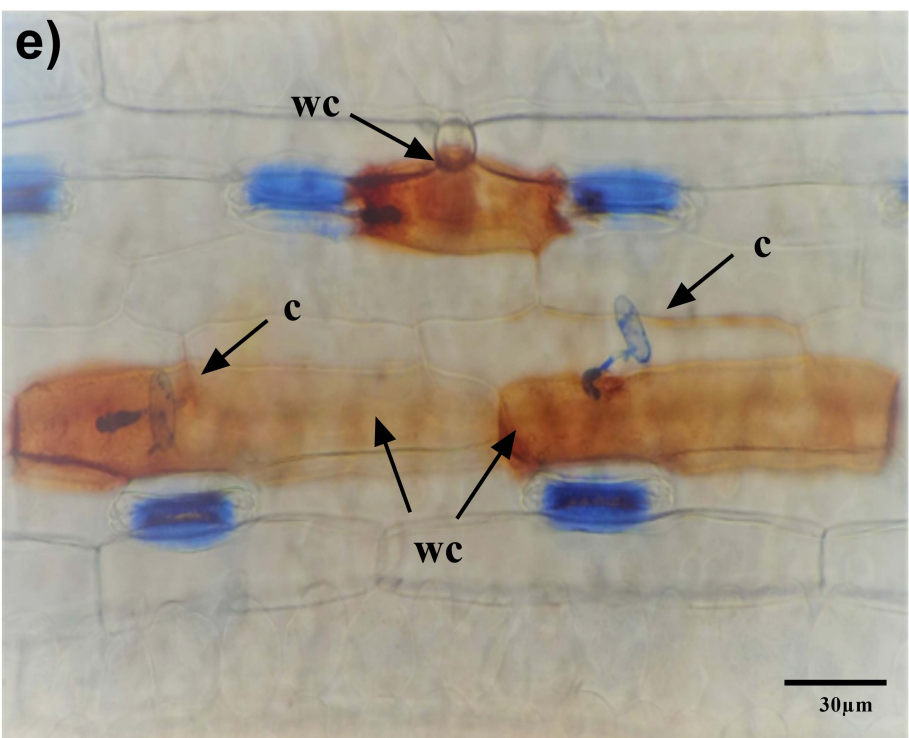
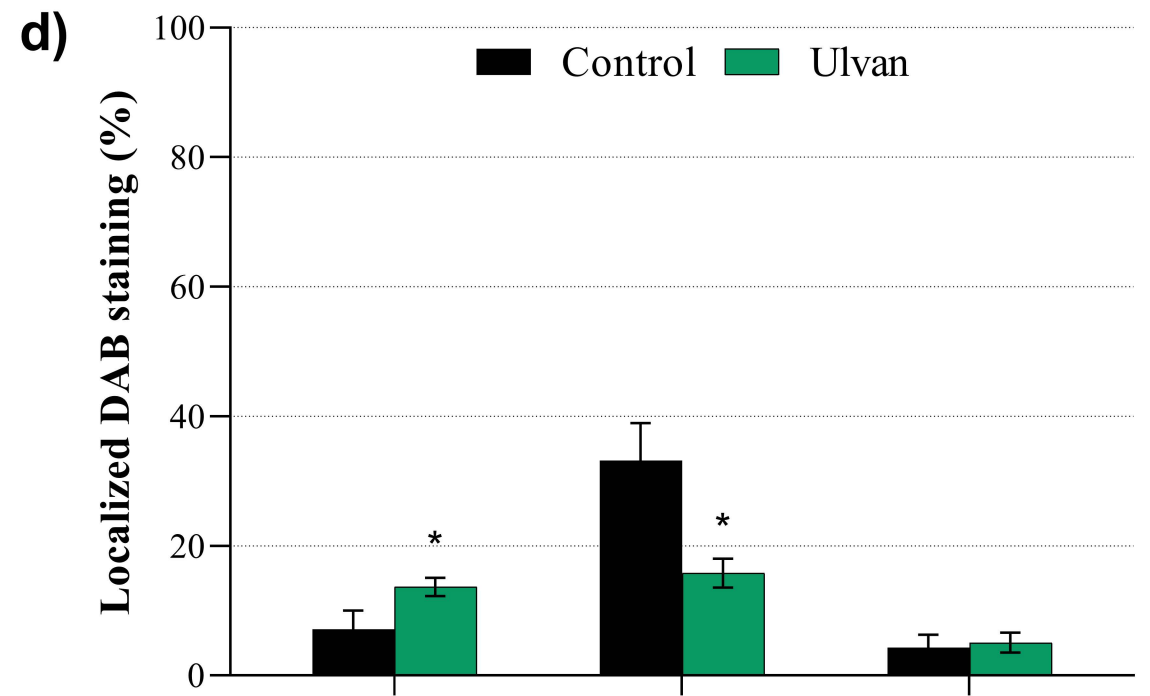
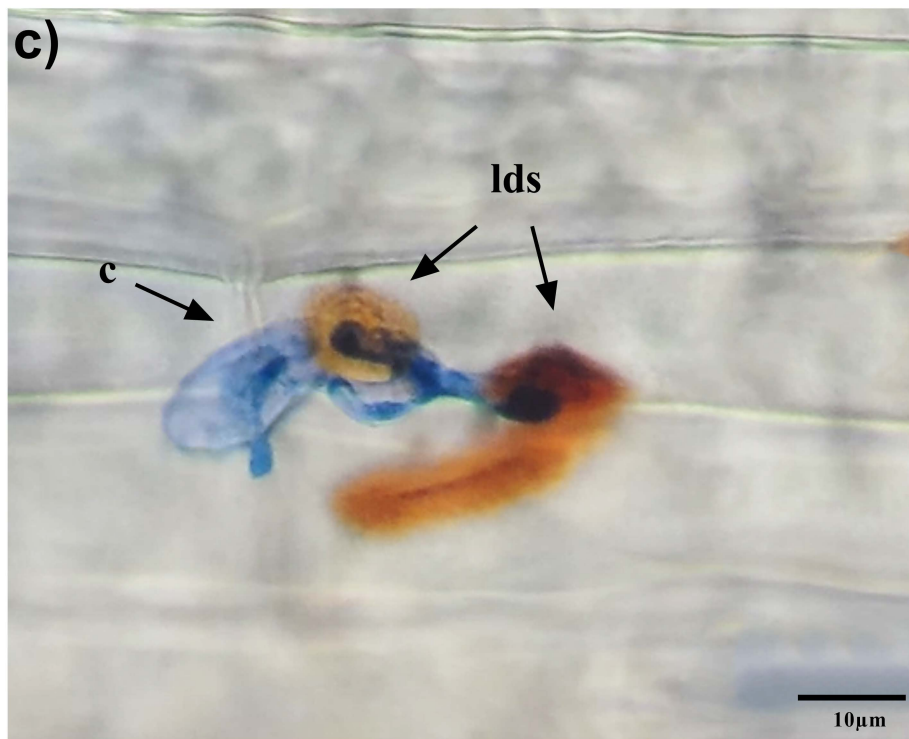
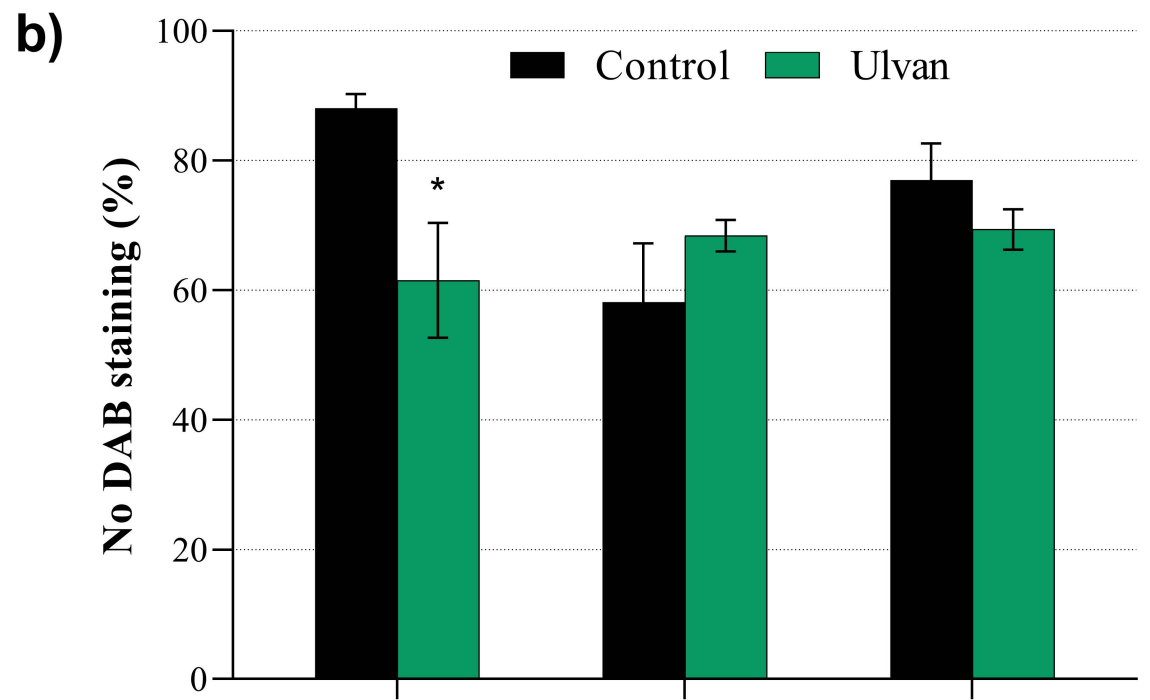
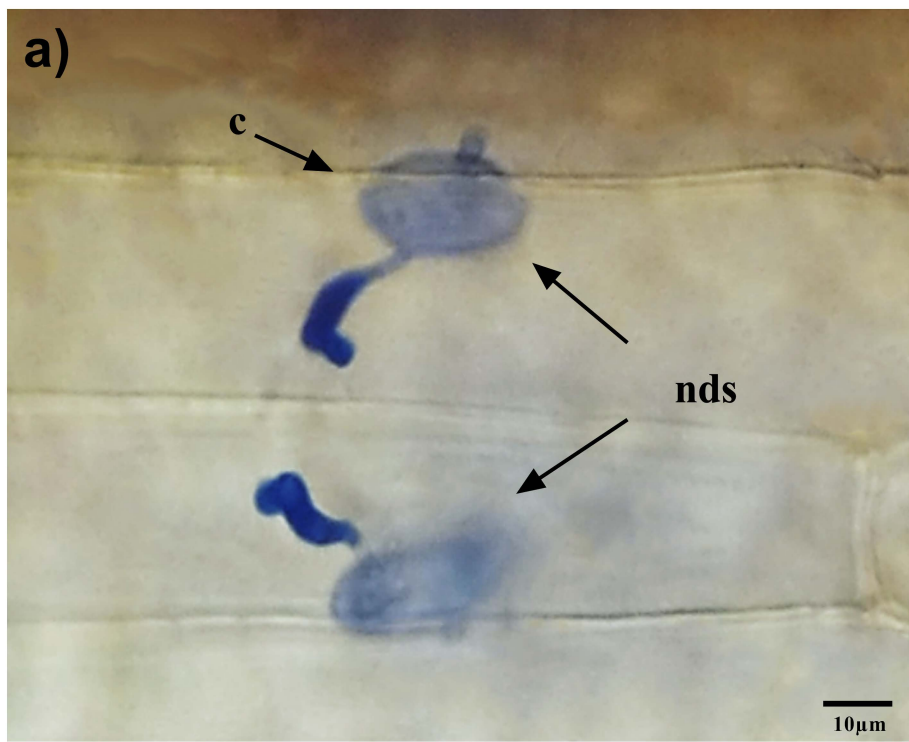
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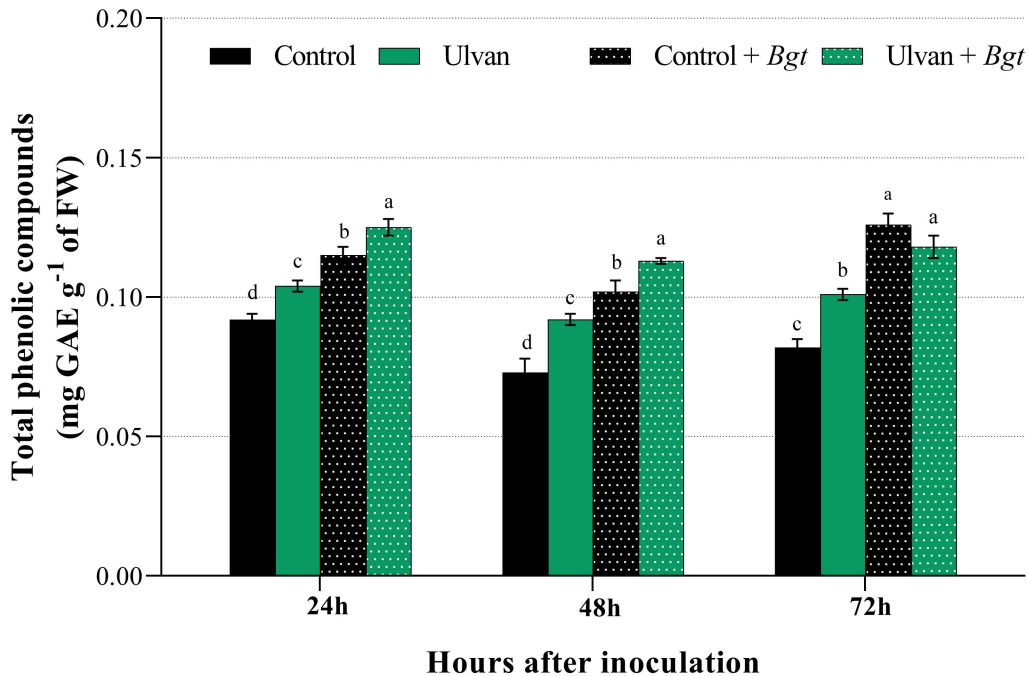












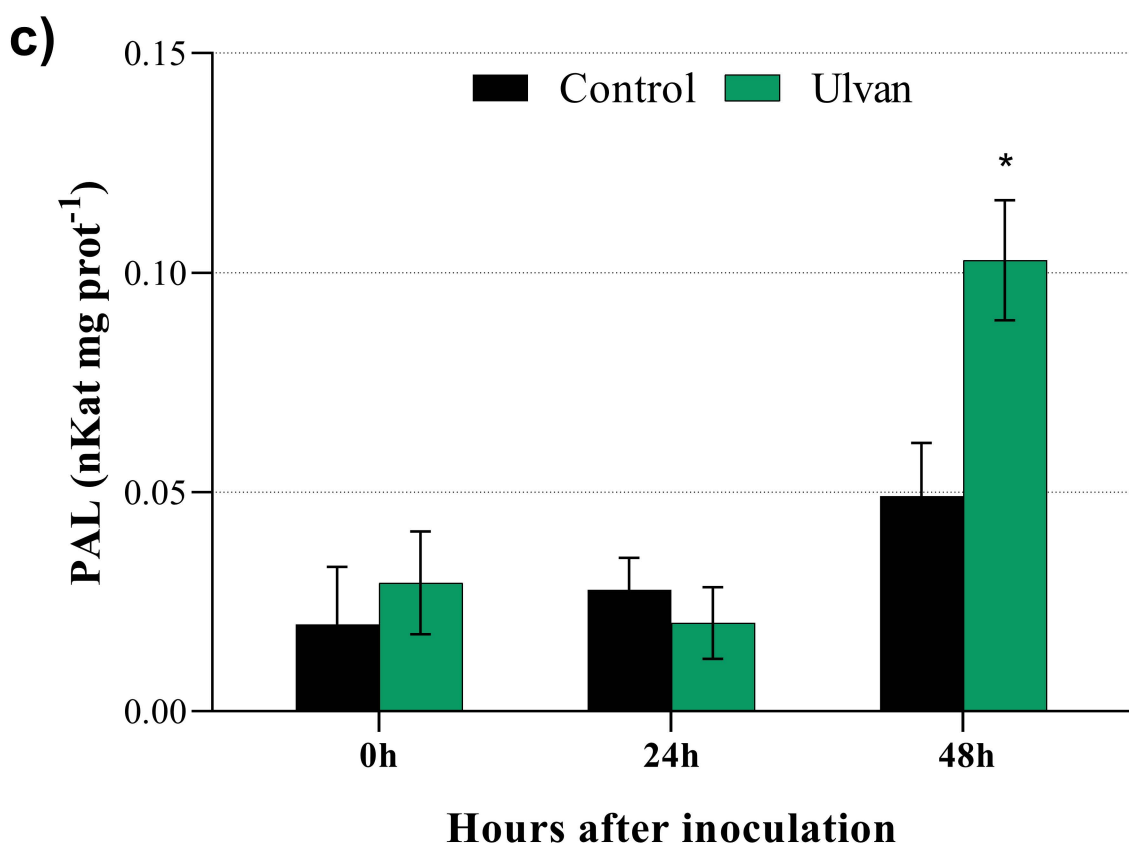
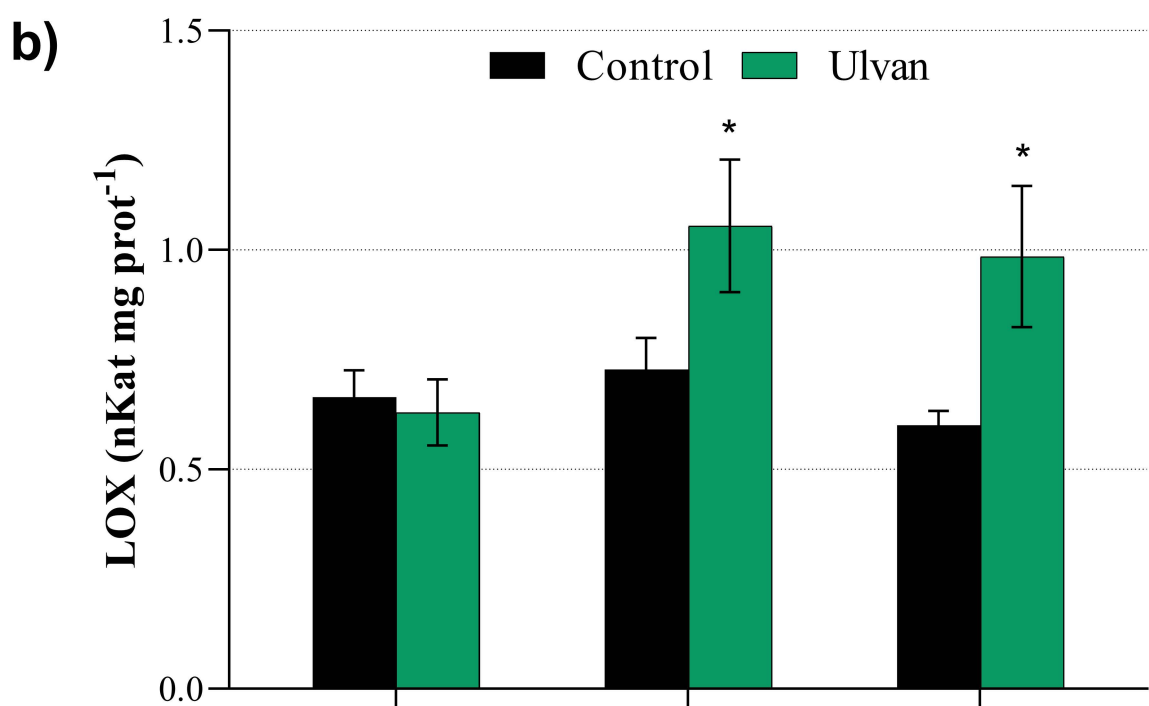
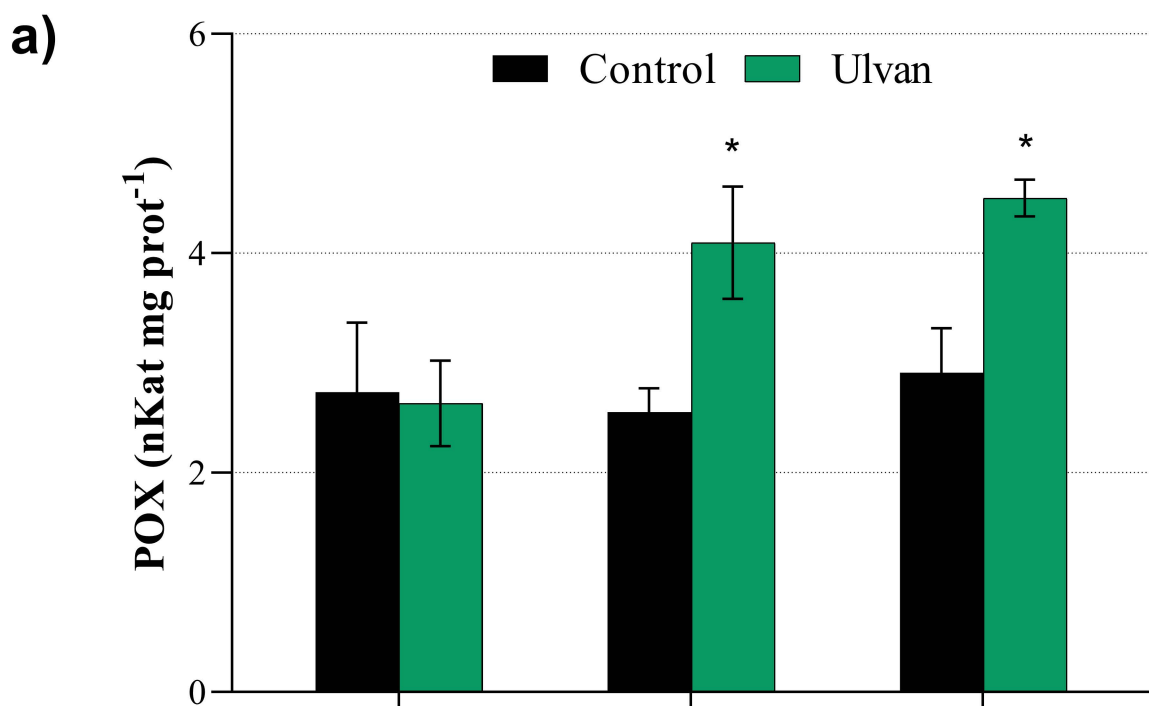


Table 1. Percentages of the different developmental stages of *Blumeria graminis* f. sp. *tritici* (*Bgt*) on epidermal leaf cells of both ulvan-treated or water-sprayed (control) wheat plantlets at 24, 48, and 72 h after inoculation. Susceptible plants were sprayed with water or ulvan 48 h before inoculation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

Development stages	Treatments	Hours after inoculation		
		24h	48h	72h
Non-germinated	Control	2.1 ± 1.5 ns	3.1 ± 0.7 ns	2.4 ± 1.0 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^x.

hat^y	<i>PAL</i>	<i>CHS</i>	<i>COMT</i>	<i>ANS</i>	<i>DFR</i>	<i>FLS</i>	<i>LOX</i>	<i>PR1</i>	<i>PR9</i>	<i>PR15</i>
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

^x: 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.

^y: 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^x.

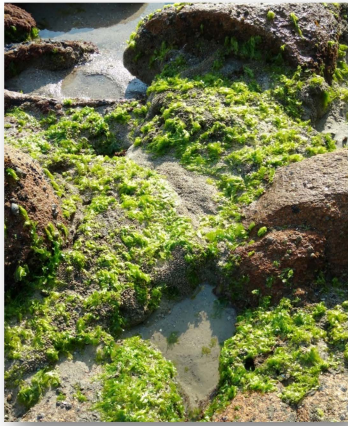
	hai/hat ^y	<i>PAL</i>	<i>CHS</i>	<i>COMT</i>	<i>ANS</i>	<i>DFR</i>	<i>FLS</i>	<i>LOX</i>	<i>PR1</i>	<i>PR9</i>	<i>PR15</i>
<i>Bgt</i>	12 /NT	0.73 NS	0.40 NS	0.35 NS	0.74 NS	0.57 NS	0.27 NS	1.30 NS	7,341 A	1,594 A	58.90 A
	24 /NT	11.60 A	13.73 A	0.94 NS	2.58 A	0.64 NS	0.71 NS	1.78 NS	340 NS	2,332 A	7.70 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 + <i>Bgt</i>	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*
	24 /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
	48 /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

^x: Values shown represent fold up-regulation in leaves of water-sprayed *Bgt* infected and ulvan-treated *Bgt* infected plants compared with water-sprayed 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.

^y: hours after inoculation /equivalent to hours after treatment with ulvan.

NT: Non-treated with ulvan.

Ulva fasciata



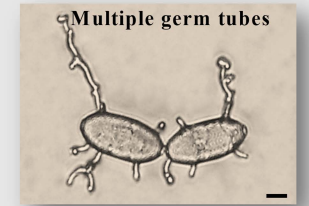
ULVAN



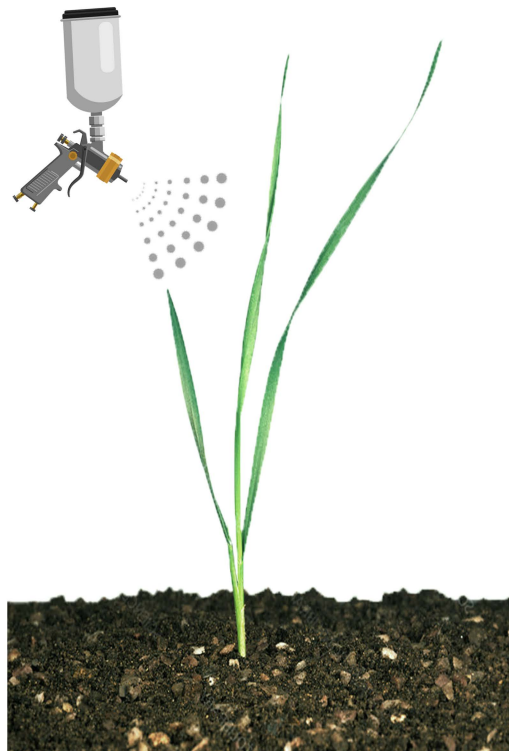
In vitro



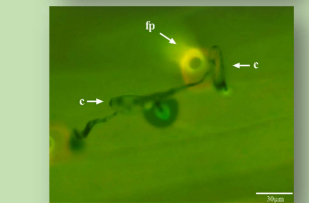
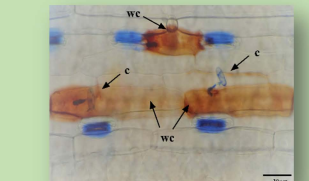
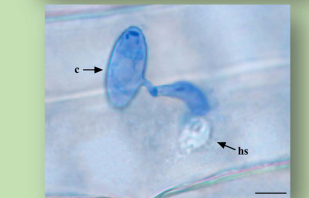
- Conidial germination of *Blumeria graminis* f. sp. *tritici* (Bgt)
- Development of multiple germ tubes



In planta



- Severity of powdery mildew
- Haustorium development
- Accumulation of H₂O₂
- 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

