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► To cite this version:

Aurélie Backes, Sophie Charton, Sébastien Planchon, Qassim Esmaeel, Kjell Sergeant, et al.. Gene expression and metabolite analysis in barley inoculated with net blotch fungus and plant growth-promoting rhizobacteria. *Plant Physiology and Biochemistry*, 2021, 10.1016/j.plaphy.2021.10.027. hal-03405616

HAL Id: hal-03405616

<https://hal.univ-reims.fr/hal-03405616v1>

Submitted on 5 Jan 2024

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1 Gene expression and metabolite analysis in 2 barley inoculated with net blotch fungus and 3 plant growth-promoting rhizobacteria

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19 Abstract

20 Net blotch, caused by the ascomycete *Drechslera teres*, can compromise barley production.
21 Beneficial bacteria strains are of substantial interest as biological agents for plant protection in
22 agriculture. Belonging to the genus *Paraburkholderia*, a bacterium, referred to as strain B25, has
23 been identified as protective for barley against net blotch. The strain *Paraburkholderia phytofirmans*
24 (strain PsJN), which has no effect on the pathogen's growth, has been used as control. In this study,
25 the expression of target genes involved in cell wall-related processes, defense responses,
26 carbohydrate and phenylpropanoid pathways was studied under various conditions (with or without
27 pathogen and/or with or without bacterial strains) at different time-points (0-6-12-48 h). The results
28 show that specific genes were subjected to a circadian regulation and that the expression of most of
29 them increased in barley infected with *D. teres* and/or bacterized with the strain PsJN. On the
30 contrary, a decreased gene expression was observed in the presence of strain B25. To complement
31 and enrich the gene expression analysis, untargeted metabolomics was carried out on the same
32 samples. The data obtained show an increase in the production of lipid compounds in barley in the
33 presence of the pathogen. In addition, the presence of strain B25 leads to a decrease in the
34 production of defense compounds in this crop. The results contribute to advance the knowledge on
35 the mechanisms occurring at the onset of *D. teres* infection and in the presence of a biocontrol agent
36 limiting the severity of net blotch in barley.

37 **Additional keywords:** Barley, Biocontrol, *Drechslera teres*, Beneficial bacteria, RT-qPCR,
38 Metabolomics.

1. Introduction

Barley (*Hordeum vulgare* L.) is one of the most important cereal crops cultivated in the world (Ferdous et al., 2015). Due to its worldwide distribution and use, barley has a significant economic impact. Caused by an ascomycete, *Drechslera teres*, net blotch is the foliar disease resulting in the most severe damages on barley (Backes et al., 2021a). It is a disease whose symptoms quickly appear in the form of dark spots. These spots will then enlarge to give way to necrotic lesions followed by a chlorotic halo around the zone of penetration. The disease is responsible for an important loss in barley yields (up to 40%) in different cultivation areas and represents a serious threat for the brewing sector.

The use of plant growth-promoting rhizobacteria (PGPR) to induce the plant resistance is one of the alternatives considered to protect crops against the damage caused by cryptogamic diseases (Prasad et al., 2019). *Paraburkholderia phytofirmans* strain PsJN, a well-known PGPR, has been isolated from surface-sterilized onion roots (Nowak, 1998). PsJN is able to colonize a large variety of plants, such as tomato, potato and grapevine. In addition, this bacterium improves the tolerance against biotic stress (Miotto-Vilanova et al., 2016). According to previous results, PsJN has no antagonistic effect against *D. teres* in barley (Backes et al., 2020). Therefore, this bacterium was used as a bacterization control in this study. Unlike PsJN, another bacterium, belonging to the genus *Paraburkholderia* was able to limit significantly the development of *D. teres*. This is a new species of *Burkholderia* after a comparison against all genomes of typical strains available in the Microbial Genomes Atlas (MiGA) webserver and TYGS database. Therefore, *Paraburkholderia* sp. is the name proposed for the beneficial bacterium having an antagonist effect against net blotch and referred to as strain B25 in the present investigation.

Biotic stresses induce physiologic, transcriptomic and metabolomic changes in plants. The different experiments discussed in this present study were designed with the goal of improving the knowledge about the changes induced at the genes' and metabolites' level after infection by *D. teres* and application of beneficial bacteria on barley.

When the pathogen attacks the plant, defense mechanisms are quickly activated. More particularly, barley-pathogen interactions can lead to compatible response (infection) or incompatible (resistance). In the case of infection, pathogens elicit a set of localized responses around the site of infection. Oxidative burst is one of the early responses, which generally leads to programmed cell death through the hypersensitive response (HR). Reactive oxygen species (ROS) contribute to oxidative stress and have a toxic effect in plants and field crops. Barley has developed protective mechanisms to counteract oxidative stress: among them, glutathione *S*-transferase (*GST*), superoxide dismutase (*SOD*) and catalase (*CAT*) are well-known antioxidant enzymes (Lightfoot et al., 2017). Phytohormones, such as jasmonic acid (*JA*), also play a major role in the signaling networks involved in plant responses to biotic stress. Studies have shown the enhanced synthesis of this phytohormone during fungal pathogens' attack (Qi et al., 2016). Additionally, cereals activate systemic defense responses in uninfected plant parts in parallel with local reactions. These reactions involve pathogenesis-related (*PR*) genes, which enhance the resistance to infection.

The plant cell wall is a dynamic and complex structure and represents the first physical barrier against pathogens (including virus, bacteria, fungi, nematodes and other pests). The cell wall is composed of a network of cellulosic and non-cellulosic polysaccharides, which can be impregnated by the hydrophobic aromatic polymer lignin (Yadav et al., 2020). The plant cell wall is constantly

82 involved in interactions between plants and their environment: for instance, the cell wall
83 composition and structure change to limit the further spread of fungal pathogens.

84 Moreover, enzymes belonging to the phenylpropanoid biosynthetic pathway are involved in
85 the changes in cell wall composition and in the production of antimicrobial compounds in response
86 to attacks by different pathogens. In plants, the phenylpropanoid pathway is a rich source of
87 metabolites derived from phenylalanine. This pathway is required for the biosynthesis of lignin and
88 serves as a starting point for the production of many other important compounds, such as flavonoids,
89 coumarins and lignans. Many biotic stress-induced phenylpropanoids are classified as phytoalexins.
90 They include isoflavanes, stilbenes, flavonols (e.g. quercetin, kaempferol) and coumarins (Yadav et
91 al., 2020). The levels of these compounds increase around the site of infection to concentrations that
92 are toxic to pathogens (virus, bacteria, fungi, nematodes and other pests) in infected plants. Being a
93 leaf disease, net blotch also negatively affects the physiology of barley and notably photosynthesis
94 (Backes et al., 2021b).

95 To decipher the physiological and molecular responses of barley inoculated with *D. teres* in
96 the presence and absence of beneficial bacteria, we here aimed to reveal the mechanisms
97 established at two levels: gene expression and metabolites' production. In order to better
98 understand the interaction among barley, *D. teres* and bacteria, we analyzed genes involved in
99 defense (*GST*, *SOD*, *CAT*, *PR8* and *AOC*), in the phenylpropanoid (*PAL*, *CHS* and *F3'H*) as well as
100 carbohydrate pathways (*SUS2*, *RcaA2*) and in cell wall biosynthesis (*GSL* and *CSLF6*). Metabolomic
101 was previously applied to different pathosystems and provided valuable information about the
102 plant/pathogen interaction and allowed the identification of compounds playing a major role in plant
103 innate immunity. Consequently, we combined targeted gene expression analysis to metabolomics to
104 identify similarities or differences in gene transcript levels and metabolic pathways in barley
105 inoculated with *D. teres* in the presence or absence of beneficial bacteria.

106 **2. Materials and Methods**

107 **2.1 Plant material, growth conditions and treatments**

108 The cultivar Siberia is a six-row winter barley, which is the most susceptible to net blotch and
109 was therefore chosen to perform the experiments in this study. Barley seedlings were grown in
110 controlled conditions in incubators (Aralab, Portugal) following a cycle of 23 °C day / 22 °C night, 80
111 % relative humidity, 14 h/10 h day/night photoperiod. Barley seeds were sown in Gramo Flor non-
112 sterile soil without any treatment. A bacterial suspension (strain B25 and PsJN) in PBS at a
113 concentration of 10⁹ bacteria/mL was sprayed on the barley plants leaves at stages 11-12 according
114 to the Zadoks scale. In parallel, for the control condition, barley leaves were sprayed only with PBS
115 solution. Three days later, spores of the *D. teres* pathogen diluted in sterile water were also sprayed
116 on barley leaves at a concentration of 4 000 spores/mL. At the same time, control barley plants
117 without *D. teres* were sprayed with sterile water. Sampling of barley plants leaves at stages 12-13
118 according to the Zadoks scale took place at the following time points: 0, 6 h, 12 h and 48 h post-
119 infection (hpi). Samples from four biological replicates per treatment were obtained and each sample
120 was a pool of 12 plants.

121 **2.2 RNA extraction and cDNA synthesis**

122 Barley leaves were crushed to a fine powder in liquid nitrogen using a mortar and a pestle.
123 Seventy-five mg of finely-ground material were weighed and total RNA was isolated using the TRIzol
124 reagent (Extract'All, Eurobio), according to the manufacturer's instructions. RNA concentration and
125 purity were determined using a Nanodrop ND-1000 spectrophotometer (Thermo Scientific, Villebon-
126 sur-Yvette, France). All RNA samples had an A260/230 ratio between 1.8 to 2.0 and an A260/280
127 ratio of approximately 2.0. RNA integrity was checked with a Bioanalyzer (Agilent, Santa Clara, CA,
128 USA). All RNAs displayed a RIN above 7. One microgram of RNA was retro-transcribed using the
129 SuperScript II cDNA Synthesis kit (Invitrogen), following the manufacturer's instructions.

130 **2.3 Choice of reference genes, target genes and primer design**

131 Eight reference genes were selected: elongation factor 1 *EF1* (AJ277799.1), glyceraldehyde-3-
132 phosphate dehydrogenase *GAPDH* (AK359500.1), actin *Act* (AY145451.1), ribosomal protein S4 *RP_S4*
133 (NC_042692.1), ubiquitin *UBC* (AK248472.1), ubiquitin *UBC 9* (AK249228.1), cyclophilin *CYP*
134 (AK253120.1) and S-adenosyl-L-methionine-dependent methyltransferase superfamily protein *SALM*
135 (AK355689.1).

136 The target genes chosen are: phenylalanine ammonia lyases *PAL* and *PAL2* (BAJ88975.1 and
137 Z49145.1), glucan synthases *GSL1* and *GSL3* (AY177665.1 and FJ853601.1), chalcone synthase *CHS*
138 (X58339.1), flavonoid 3'-hydroxylase *F3'H* (AK363912.1), cellulose synthase-like gene *CsIF6*
139 (AB621305.1), glutathione S-transferase d2 *GSTd2* (BAJ99765.1), cytosolic superoxide dismutase *SOD*
140 (KU179438.1), pathogenesis-related protein 8 *PR8* (KAE8773798.1), allene oxide cyclase *AOC*
141 (CAC83766.1), catalase *CAT2* (AAC17730.1), sucrose synthase *SUS2* (CAZ65725.1) and RuBisCO
142 activase *RcaA2* (M55447.1).

143 Primers were designed using Primer3Plus ([http://www.bioinformatics.nl/cgi-](http://www.bioinformatics.nl/cgi-bin/primer3plus.cgi)
144 [bin/primer3plus.cgi](http://www.bioinformatics.nl/cgi-bin/primer3plus.cgi)) and checked using the OligoAnalyzer 3.1 tool from Integrated DNA technologies
145 (<http://eu.idtdna.com/calc/analyser>). Primer efficiencies were determined by RT-qPCR using six serial
146 dilutions of cDNA (10, 2, 0.4, 0.08, 0.016, 0.0032 ng/μL). The list of reference and targeted genes is
147 given in Table 1 with the primer sequences, amplicons' length, melting temperature and primer
148 efficiency.

149 **2.4 Quantitative real-time PCR and statistical analysis**

150 For the RT-qPCR analysis, reactions were performed in 384-well plates using a liquid handling
151 robot (epMotion 5073, Eppendorf, Hamburg, Germany). The cDNA was amplified using the Low Rox
152 SYBR MasterMix dTTP Blue Kit (Eurogentec, Liège, Belgium). The RT-qPCR reactions were set up and
153 run according to Backes et al. (2020) in technical triplicates and repeated on four independent
154 biological replicates on a ViiA 7 Real PCR System (Thermo Scientific, Villebon-sur-Yvette, France). A
155 melt curve analysis was performed at the end to check the specificity of the amplified products.
156 Genes expression was calculated using qBasePLUS (version 3.2, Biogazelle, Zwijnaarde, Belgium). *Act*
157 and *RP_S4* were identified as the most stable genes and as sufficient for normalization in the
158 experimental set up chosen.

159

160

161 **Table 1.** List of primers used in the study with details on the amplicons' length, T_m (°C), PCR
 162 amplification efficiency (in %) and regression coefficient.

Name	Sequence (5'→3')	Amplicon Length (bp)	Amplicon T _m (°C)	PCR Amplification Efficiency (%)	Regression Coefficient (R ²)
Act Fwd	GGAATCCACGAGACGACCTACA	130	82.7	90.6	0.996
Act Rev	CTTGCTCATACGGTCAGCGATA				
EF1 Fwd	ATGGCATCAAGAAGCTCCAG	131	82.7	99.6	0.989
EF1 Rev	GAAGGCAACAATGTACAGC				
GADPH Fwd	TGAGGGTTTGATGACCACTG	122	83.9	92.1	0.992
GADPH Rev	CAGTGTGCTTGAATGATG				
RP_S4 Fwd	CGATTGGGTATGGCTTCAAC	107	80.0	89.8	0.998
RP_S4 Rev	GGTTTGAACGAAAATTGG				
UBC Fwd	TCAATCCCAGCAGTATCC	101	81.1	97.2	0.991
UBC Rev	TCCAGGCATATTCACCAAGTC				
CYP Fwd	GCTCCCAGTTCTCATCTGC	112	87.0	102.4	0.992
CYP Rev	CCCACCTTCTCGATGTTCTT				
UBC9 Fwd	TGCTCCTTCAATCTGCTCTC	110	85.0	102.5	0.994
UBC9 Rev	CGCTGTGGACTCATACTTCG				
SALM Fwd	GGGAGATTGGCTCGAAAT	110	79.0	104.8	0.969
SALM Rev	GCCTCTGGGTGTGGTTTAG				
PAL Fwd	ACAATGGTCTGCCTCCAAC	113	86.0	107.8	0.962
PAL Rev	CCCAAGAATTGAAGCTCAG				
GSTd2 Fwd	GTCGGAAGGTGAAAGAAACG	131	83.0	101.0	0.997
GSTd2 Rev	ATCAAGGTCCAGGACGGATAC				
PR8 Fwd	ACAACAAGGTGAACGGGAAG	100	85.7	95.3	0.991
PR8 Rev	CAGTCCGACACGATGTATCC				
AOC Fwd	ATGTACTTCGGCGACTACGG	146	88.5	103.7	0.997
AOC Rev	TTGAAGGGGAAGACGATCTG				
CAT2 Fwd	TCTTCAACGAGAACGAGCAG	149	89.5	89.9	0.992
CAT2 Rev	GGGAGCATCAGGTAGTTTG				
GSL1 Fwd	ATTGGATATGGCTGGTCTGG	126	82.9	93.8	0.985
GSL1 Rev	TTCCTTCACCTTCTGCAC				
GSL3 Fwd	CAGGGGCTTGTGTCTTTC	138	82.9	101.4	0.985
GSL3 Rev	ATGGTCGAGTGGGAAGTTTG				
CHS Fwd	TGGAACCTCGTCTTCTGGATAG	141	88.0	92.7	0.997
CHS Rev	ACATGCACTGGACATGTTGC				
F3'H Fwd	GCCAGGGAGTTCAAGGACA	168	90.4	86.0	0.942
F3'H Rev	CTCGCTGATGAATCCGTCCA				
PAL2 Fwd	AGAAAATGCGTGCGGTTT	82	85.7	110.3	0.990
PAL2 Rev	TTGGCAAACACTGACGTCTC				
CsIF6 Fwd	CCGTGCTCTACATCAACATCC	92	83.9	98.1	0.988
CsIF6 Rev	TGTGTCGACCAACTTCTTGC				
SOD Fwd	TGACCTCGGAAATGTGACAG	142	84	89.5	0.996
SOD Rev	ACCCTTGCCAAGATCATCAG				
SUS2 Fwd	ACGAACTCAACGTGCAACAG	127	85.7	90.6	0.990
SUS2 Rev	GGGATTAAGGCAGTGAATGG				
RcaA2 Fwd	ACACCGTCAACAACCAAGATG	142	86.5	89.6	0.984
RcaA2 Rev	AGCGTCGAGAAATCGTTACC				

163

164

165 Statistics were performed on log₂ transformed data using a one-way ANOVA with a Tukey's
166 post-hoc test after having checked the normal distribution of the data and homogeneity with a
167 Shapiro-Wilk's and a Levene's tests, respectively, using IBM SPSS Statistics V19 (IBM SPSS, Chicago, IL,
168 USA). A hierarchical clustering using uncentered absolute correlation and complete linkage was used
169 to generate groups with similar expression patterns in Cluster 3.0 (correlation coefficient threshold =
170 0.83). The heat map was visualized with Java TreeView available at
171 <http://jtreeview.sourceforge.net/>.

172 The principal component analysis (PCA) was performed with ClustVis (available online at
173 <https://biit.cs.ut.ee/clustvis/>). To visualize the expression pattern of genes related to the
174 phenylpropanoid and cell wall biosynthetic pathways, the barley eFP Browser
175 (<http://bar.utoronto.ca/efpbarley/cgi-bin/efpWeb.cgi>) was queried. To obtain the contig number
176 necessary as input in the eFP Browser, the FASTA sequence of the gene was blasted on PLEXdb (Plant
177 Expression Database; <http://www.plexdb.org>). The expression levels of the genes are shown as a
178 heatmap with yellow and red colors indicating low and high expression, respectively, in various
179 tissues.

180 **2.5 Metabolites extraction and analysis**

181 **2.5.1 Freeze-drying and weighing**

182 Metabolomics was performed on the samples corresponding to 12 hpi and 48 hpi. Three
183 biological replicates were analyzed. Samples ground to fine powder were freeze-dried during 48 h.
184 Then, 10 ± 0.2 mg of freeze-dried plant material were weighed and stored at -80 °C until extraction.

185 **2.5.2 Extraction**

186 Leaf samples were extracted with 998 µL of methanol/water (4:1, v/v) and 2 µL of
187 chloramphenicol at 5 mg/mL as internal standard (Sigma-Aldrich). This mixture was homogenized
188 using a vortex during 1 min, and shaken during 4 h at 14 000 rpm and room temperature in a
189 Thermomixer (Eppendorf, Hamburg, Germany), then vortexed again during 30 sec. After
190 centrifugation at 20 000 g for 30 min at 4 °C, 750 µL of supernatant were collected and evaporated to
191 dryness under a nitrogen flow (TurboVapLV, Biotage, Sweden). Samples were resuspended in 375 µL
192 of methanol: water (5:95, v/v) and filtered through 0.22 µm PTFE syringe filter (Millex-LG, Merck
193 KGaA, Darmstadt, Germany).

194 **2.5.3 Untargeted metabolomics analysis with UHPLC-DAD-ESI-MS/MS**

195 Extracts were analyzed using an Acquity UPLC I-class ultra-high pressure liquid
196 chromatography (UHPLC) system equipped with a diode array detector (DAD) (Waters, Milford, MA,
197 USA) coupled to a hybrid quadrupole-time of flight mass spectrometer (TripleTOF 6600, SCIEX,
198 Framingham, MA, USA) in positive and negative ionization modes. Five µL of the samples in random
199 order were separated on a reverse-phase Acquity UPLC BEH C18 column (2.1 x 100 mm, 1.7 µm
200 particle size) (Waters, Milford, MA, USA). The eluents were 0.1 % (v/v) formic acid in water (A) and
201 0.1 % (v/v) formic acid in acetonitrile (B). The gradient was as follows: 0 min, 1 % B; 4 min, 1 % B;
202 16 min, 5 % B; 35 min, 40 % B; 45 min, 100 % B; 50 min, 100 % B; 54 min, 1 % B; 60 min, 1 % B. The
203 flow rate was 0.5 mL/min and the column temperature was set to 50 °C. UV-visible spectra were also
204 acquired between 190 and 800 nm at a rate of 10 points/sec.

205 Electro spray ionization (ESI) was performed on analytes using the following parameter values
206 for positive and negative modes: source temperature 650 °C; ion spray voltage of 4.5 and -4.5 kV,
207 respectively, curtain gas (nitrogen) of 30 psi, nebulizer gas (air) of 55 psi and turbine gas (air) of 50
208 psi. The declustering potential was set up at 60 V and -60 V in positive and negative mode,
209 respectively. The precursor charge state selection was set at 1. For information-dependent
210 acquisition in high sensitivity mode, survey scans were acquired in 175 msec. In addition, the 10 most
211 abundant products ion scans were collected during 200 msec if exceeding a threshold of 100
212 counts/sec, thus leading to a total cycle time of 2.225 sec. A sweeping collision energy of 15 V below
213 and above 15 and -15 V, for the positive and negative modes, respectively, was applied to all
214 precursor ions. The dynamic exclusion was set for two seconds after three occurrences before the
215 precursor could be fragmented again. Full high-resolution MS spectra were recorded between 100
216 and 2000 mass-to-charge ratio (m/z) for MS1, whereas MS2 scans were recorded between 50 and
217 2000 m/z , in profile mode.

218 **2.5.4 Data processing and statistical analysis**

219 Data were processed using Progenesis Q1 (v 2.3, Nonlinear Dynamics, Waters, Newcastle,
220 UK). Briefly, the software automatically extracts features (combinations of isotopic peaks and
221 adducts ions at a given retention time) between 0 and 50 min and aligns them. A normalization to all
222 compounds was carried out. For each comparison of modalities two by two, only features with MS2
223 data and both fold-change above 1.5 and p -value below 0.05 in Progenesis's ANOVA were kept for
224 the identification step, which consisted in the use of Metascope, ChemSpider and National Institute
225 of Standards and Technology (NIST) plugins, for the search in an in-house database, in CHEBI 3-star,
226 in MassBank for the first plugin, in PubChem for the second and in NIST MS/MS database for the
227 third. A manual review of the output data was then carried out, in combination with the software
228 PeakView (v 1.2, SCIEX, Framingham, MA), LipidMaps (<http://www.lipidmaps.org/>), Metlin
229 (<https://metlin.scripps.edu/index.php>), MassBank of North America (MoNA)
230 (<https://mona.fiehnlab.ucdavis.edu/>) and PubChem databases (<https://pubchem.ncbi.nlm.nih.gov>),
231 as well as literature data for structure elucidation.

232 To reduce the number of compounds to be identified and further sharpen the analysis,
233 different comparisons were made according to several variables. Indeed, comparisons 1 to 6 were
234 focused on time as variable (12 hpi vs 48 hpi), while comparisons from 15 to 20 were dedicated to
235 the pathogen's absence or presence as variable. Finally, only comparisons from 7 to 14, with the
236 presence of either bacteria B25 or PsJN as variable, were further investigated for this study, since we
237 were interested in knowing the effects on barley metabolism of these bacteria used as a biocontrol
238 agent. More specifically, comparisons from 7 to 14 consisted in: control vs strain B25 without
239 pathogen at 12 hpi ([comparison 7](#)), control vs strain B25 with pathogen at 12 hpi ([comparison 8](#)),
240 control vs strain B25 without pathogen at 48 hpi ([comparison 9](#)), control vs strain B25 with pathogen
241 at 48 hpi ([comparison 10](#)), control vs strain PsJN without pathogen at 12 hpi ([comparison 11](#)), control
242 vs PsJN strain with pathogen at 12 hpi ([comparison 12](#)), control vs PsJN strain without pathogen at 48
243 hpi ([comparison 13](#)), control vs PsJN strain with pathogen 48 hpi ([comparison 14](#)).

244 Annotations and identifications were classified in accordance with the levels of the
245 Metabolomics Standards Initiative (MSI), as described in Sumner et al. (2007). Compounds in class 2
246 were identified based on exact mass, retention time, MS2 fragmentation pattern and UV-visible
247 spectrum by comparison with data in databases and/or literature. Class 3 was assigned to

248 compounds with the same information as class 2 when they allowed only chemical class
249 determination, typically when the molecule identified is a fragment of a bigger, not fully determined
250 molecule. Class 4 was allocated to compounds for which only a part of the molecule is elucidated,
251 with no complete molecular formula generated.

252 **3. Results and Discussion**

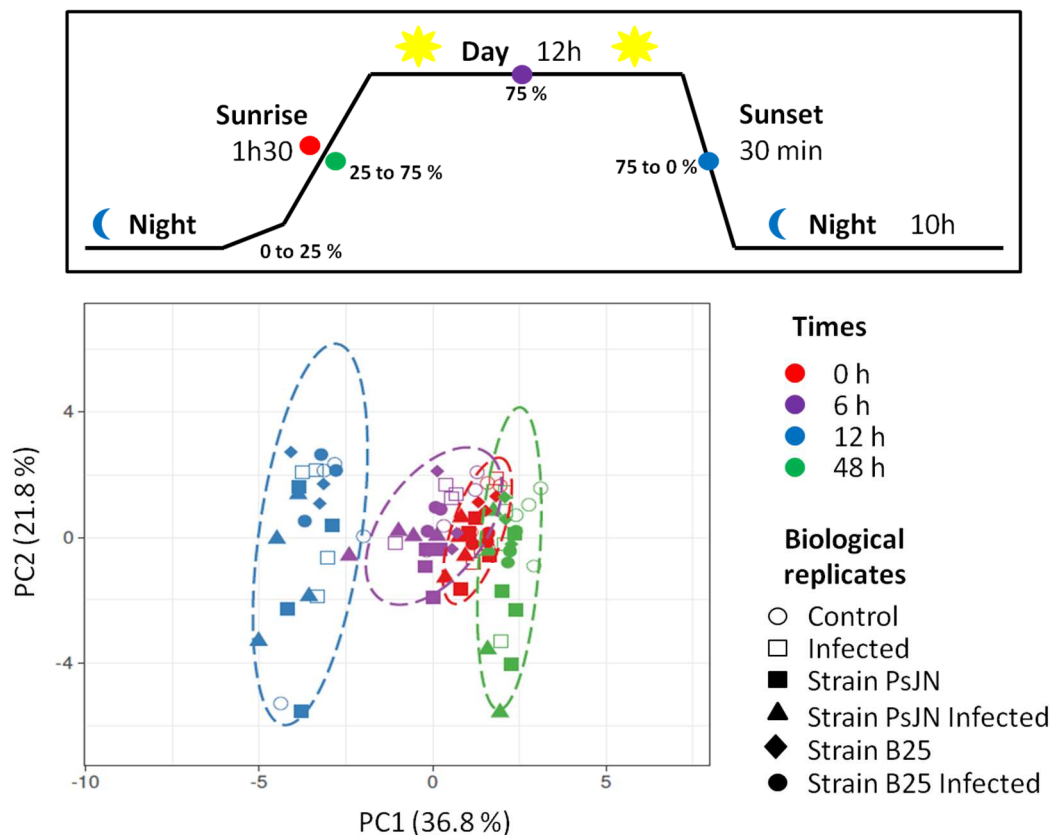
253 **3.1. Gene expression depends on the circadian rhythm and the experimental** 254 **conditions**

255 As a first step towards understanding the molecular effects of *D. teres* infection and PGPR
256 application on barley leaves, the expression of a selection of genes was analyzed. We performed an
257 *in silico* analysis of the expression values across barley tissues at different developmental time-
258 points, by searching the barley eFP Browser. The goal was to verify that the selected genes were
259 expressed in the leaves.

260 Genes involved in stress response [glutathione *S*-transferase d2 (*GSTd2*), pathogenesis-
261 related protein 8 (*PR8*), allene oxide cyclase (*AOC*), catalase 2 (*CAT2*), cytosolic superoxide dismutase
262 (*SOD*)], cell wall biosynthesis [glucan synthases (*GSL1* and *GSL3*), cellulose synthase-like (*Cs/F6*)],
263 carbohydrate metabolism [sucrose synthase 2 (*SUS2*), RuBisCO activase (*RcaA2*)] and the
264 phenylpropanoid pathway [phenylalanine ammonia lyases (*PAL* and *PAL2*), chalcone synthase (*CHS*),
265 flavonoid 3'-hydroxylase (*F3'H*)] were selected, as they provide information on the changes occurring
266 in pathways activated at the onset of biotic stress. Hereafter, we will present the results of a PCA to
267 provide an overview of the data clustering in the experimental conditions and the different time-
268 points studied. We will then report and discuss the expression data for all the genes analyzed.

269 According to the PCA, four groups, corresponding to the different time-points are visible
270 (Figure 1). The first includes barley plants sampled at T0 (red color). This sampling time corresponds
271 to the start of the day with a light intensity from 0 to 75 %. The second group includes samples taken
272 at 6 hpi (purple color, corresponding to midday, i.e. 75 % light intensity). The third group includes
273 leaves sampled 12 hpi and corresponding to the end of the day with a light intensity decreasing from
274 75 to 0 % (blue color). The last group includes barley leaves sampled 48 hpi and corresponding again
275 to the start of the day (green color). The distinction of these groups is mainly due to the sampling
276 time-points. A clear grouping of the samples following the time points is evident, hence denoting a
277 dependence on the circadian rhythm (Figure 1). Light affects the host response, as well as the
278 virulence of the bacterial and fungal pathogens. Recent reports have shown direct light effects on the
279 defense response in the host plant. One of the earliest studies providing indications about a
280 connection between circadian rhythm and defense responses was on a pathogen-responsive gene
281 coding for a glycine rich protein (GRP) in barley. Two GRPs (*Hvgrp2* and *Hvgrp3*) were previously
282 shown to be rapidly induced upon fungal pathogens' attack (Sharma and Bhatt, 2015). Defense genes
283 show a peak in expression at midnight and at dawn, the time points coinciding with the time of
284 sporulation and spore dissemination, respectively (Sharma and Bhatt, 2015). Subsequently, other
285 studies have shown modulations of some genes with the circadian cycle (Luna et al., 2005; Dao et al.,
286 2011; Khan et al., 2010). For instance, the level of a catalase-*CAT1* transcript was regulated by the
287 circadian clock and persisted in continuous darkness, whereas *CAT2* expression decreased by night in
288 wheat (Luna et al., 2005). In *Arabidopsis*, *CHS* expression was also regulated by the circadian clock
289 (Dao et al., 2011). Several enzymes involved in cell wall biosynthesis were found to be regulated by

290 the circadian rhythm in maize (Khan et al., 2010). To achieve cell wall stiffening, cinnamoyl-CoA
 291 reductase (CCR) and cinnamyl alcohol dehydrogenase (CAD) catalyze the final steps in the
 292 phenylpropanoid pathway; results have demonstrated that transcripts encoding both enzymes have
 293 cyclic expression, peaking at dawn in maize (Khan et al., 2010).

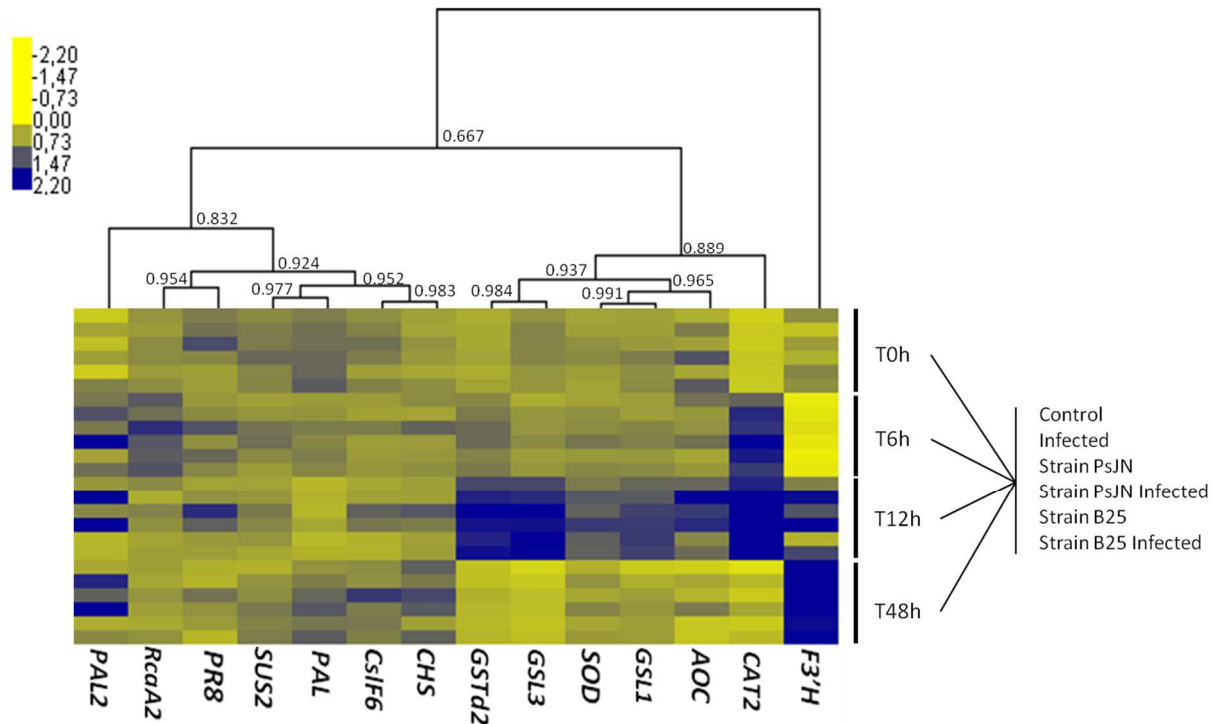


294
 295 **Figure 1.** Principal component analysis of the gene expression data. The data are log₂-transformed
 296 and correspond to the sampling time-points. Four biological replicates are used under the different
 297 experimental conditions.

298 **3.2. Gene expression analysis in *D. teres*-infected barley leaves in the**
 299 **absence/presence of PGPR**

300 We will hereafter report the gene expression results by presenting first the changes triggered
 301 by *D. teres* and then by focusing on the effects of PGPR addition.

302 According to the heat map hierarchical clustering (Figure 2), three different expression
 303 patterns can be identified in response to *D. teres*. The first group is composed by *PAL2*, *RcaA2*, *PR8*,
 304 *SUS2*, *PAL*, *CsIF6* and *CHS* and shows no significant variation in gene expression under the
 305 experimental conditions. The second group, comprising *GSTd2*, *SOD*, *GSL1*, *GSL3*, *AOC* and *CAT2*,
 306 shows a tendency towards an increase at 6 hpi and 12 hpi (Suppl. Table 1). The last group consists
 307 only of *F3'H*, which shows a higher expression in the presence of *D. teres* at 12 hpi and 48 hpi. The
 308 expression level of this gene is dependent on the sampling time-points. Indeed, its expression tends
 309 to increase at 12 hpi and 48 hpi with *D. teres*. In addition, *F3'H* is repressed at 6 hpi, regardless of the
 310 experimental condition. On the other hand, the expression of *F3'H* is modulated according to the
 311 experimental condition. For example, this is clearly visible at 48 hpi when its expression increases.



312

313 **Figure 2.** Heatmap hierarchical clustering of genes related to cell wall biosynthesis, defense response,
 314 carbohydrate and phenylpropanoid pathway in *H. vulgare* in the absence/presence of the pathogen
 315 and/or in combination with strain PsJN or strain B25. The lines refer to the order of the six
 316 experimental conditions for each time point. The heat map hierarchical clustering was generated
 317 using a Pearson's correlation in complete linkage (the numbers close to the branches indicate the
 318 coefficients). The normalized relative quantities and statistics are indicated in Suppl. Table 1.

319 The interaction between plant and different pathogens induces an accumulation of ROS and
 320 therefore the expression of genes coding for antioxidant enzymes, such as CAT2, GST and SOD. In the
 321 presence of *D. teres* (6, 12 and 48 hpi), the transcripts encoding the antioxidant enzymes CAT2 and
 322 GSTd2 have a higher expression compared to SOD. The attack of the insect *Nilaparvata lugens* on
 323 rice, another monocot, resulted in increased CAT and GST expression, while SOD gene was repressed
 324 (Jannoey et al., 2017). According to Lightfoot et al. (2017), *HvCSD1*, a cytosolic SOD, was up-regulated
 325 during the interaction between barley and the pathogen causing net blotch.

326 Jasmonic acid is a phytohormone synthesized from lipids through the actions of several
 327 enzymes including AOC during the acclimation to biotic stresses. At 12 hpi, AOC shows a higher
 328 expression in the presence of *D. teres* in barley (Figure 2). The difference is not statistically significant
 329 (Suppl. Table 1), but it indicates a tendency in the gene expression pattern. According to Maucher et
 330 al. (2004), the same result was reported in barley infected with the powdery mildew agent.

331 In this study, *GSLs* and *CsIF6* were chosen for their roles in callose deposition and mixed-
 332 linkage β -glucan synthesis, respectively. Our results show that *CsIF6* does not vary significantly in
 333 expression in the presence of the pathogen (Suppl. Table 1). On the contrary, *GSL1* and *GSL3* have a
 334 slight increase in expression at 12 hpi in the presence of *D. teres*. Similar results were obtained by
 335 Singla et al. (2020), indicating that the β -glucan levels are maintained in barley infected with
 336 *Puccinia*.

337 The genes coding for enzymes belonging to the phenylpropanoid pathway, namely *PAL* and
338 *F3'H*, modulate their expression in the presence of the pathogen (Huang et al., 2010). Phenylalanine
339 ammonia lyase (*PAL*) catalyzes the deamination of phenylalanine to produce *trans*-cinnamate, which
340 is the first step in the phenylpropanoid pathway. Several studies have shown that *PAL* gene
341 expression is responsive to a variety of environmental stimuli, including pathogen infection by
342 viruses, bacteria, fungi, nematodes and other biotic stressors. Flavonoids, including anthocyanins,
343 flavones and flavonols are ubiquitous secondary metabolites of plants. From a biological perspective,
344 flavonoid biosynthesis plays important roles in the plant's defense against various biotic stresses. Our
345 results show that the expression of *PAL* increases from 12 hpi only in the presence of the pathogen.
346 Comparable results were published during a barley - *Erysiphe graminis* interaction, showing an
347 increase in *PAL* expression at 6 hpi and between 12 hpi and 15 hpi (Shiraishi et al., 1995). In the same
348 way, an increase in *PAL* gene expression was demonstrated 6 hpi by *Blumeria graminis* in barley
349 (Jawhar et al., 2017). A recent RT-qPCR analysis showed an increase in *PAL* expression 6 hpi with *D.*
350 *teres* in barley, which is in agreement with our results (Arabi et al., 2020).

351 Two genes involved in photosynthesis were also investigated: RuBisCO activase (*RcaA2*) and
352 sucrose synthase (*SUS2*). During a compatible host-fungal pathogen interaction, like barley and *D.*
353 *graminea*, RuBisCO activity declined 2 hpi (Goel and Kumar Paul, 2020). Therefore, the pathogen can
354 directly affect photosynthetic mechanisms by affecting the host tissues. Alternatively, the pathogen
355 may act indirectly by inhibiting RuBisCO activase (*Rca*). *Rca* allows the activation of RuBisCO by
356 facilitating the ATP-dependent removal of several inhibitory sugar phosphates from the RuBisCO
357 activase site. This action is necessary for the spontaneous carbamylation of RuBisCO active sites *in*
358 *vivo* and is thus essential for photosynthetic CO₂ assimilation. According to our results, *RcaA2*
359 expression tends to increase at 6 hpi with normalized relative expression values shifting from 0.9 to
360 ca. 1.5 (Suppl. Table 1).

361 Sucrose is the end product of photosynthesis and is transported from source to sink tissues.
362 Sucrose catabolism implies the following main enzymes: invertase (*INV*) catalyzing the irreversible
363 hydrolysis of sucrose into its hexose monomers (Stein and Granot, 2019) and sucrose synthase (*SUS*)
364 catalyzing the reversible transformation of sucrose and nucleotide diphosphate (*NDP*) into nucleotide
365 diphosphate glucose (*NDP*-glucose) and fructose. During plant-fungal pathogen interaction, *SUS*
366 expression was induced in *Arabidopsis* roots infected with *Plasmodiophora brassicae* (Siemens et al.,
367 2006). Under stress conditions, sucrose functions as messenger resulting in the activation of signaling
368 enzymes such as mitogen-activated protein kinases (*MAPKs*). *SUS* expression is associated with the
369 response of plants to various environmental stresses. According to Figure 2, *SUS2* is slightly over-
370 expressed in the presence of *D. teres* compared to the control condition. Barrero-Sicilia and
371 collaborators (2011) have demonstrated that the expression of genes encoding *SUS* from barley is
372 modulated by different abiotic stimuli, including water deprivation and cold temperature.

373 In the previous paragraphs, the gene expression analysis highlighted the impact of *D. teres* in
374 barley (Figure 3). We then aimed at providing an answer to the following question: are there similar
375 or different effects during PsJN or strain B25 application with or without pathogen? We will hereafter
376 present the results relative to the bacterization with PsJN first and then with strain B25 for each
377 studied gene.

378 Classified as an endochitinase III, *PR8* breaks down the fungal cell wall, resulting in cell lysis
379 and death (Singla et al., 2020). Our results show no modulation in *PR8* expression when barley is
380 bacterized with PsJN at 6 hpi and 12 hpi with normalized relative expression values close to 0.95 and
381 1.36, respectively (Suppl. Table 1). In addition, the expression of *PR8* does not vary in barley
382 bacterized by strain B25. Other results have shown the modulation of the expression of this gene
383 during a plant-pathogen interaction. For instance, *PR8* is induced in wheat 6 hpi with *Fusarium*
384 *graminearum* (Wu et al., 2014). *PR8* is also over-expressed in barley in the presence of the fungal
385 pathogen *Puccinia* sp. (Singla et al., 2020) and *E. graminis* f. sp. *hordei*, the causal agent of powdery
386 mildew (Ignatius et al., 1994). During pathogens' attack, a group of proteins is produced by the
387 infected plant including "Pathogenesis-Related" (PR) proteins. PR proteins accumulate in the infected
388 tissues and protect plants against biotic stress. Classified into 17 families, PR proteins have several
389 functions, including antifungal, glucanase and chitinase activities, thaumatin-like proteins,
390 peroxidases and protease inhibitors, endoprotease, ribonuclease, defensin, thionin, lipid-transfer
391 proteins and oxalate oxidase.

392 In addition to PR proteins, jasmonic acid signaling molecules are also involved in activating
393 the plant's defenses against pathogens. ROS can damage the lipids and, more particularly, the lipid
394 peroxidation products of unsaturated fatty acids lead to cell membrane damage. For instance, α -
395 linolenic acid draws attention since it represents a substrate for the synthesis of the phytohormone
396 jasmonic acid. This metabolic pathway involves the enzyme allene oxide synthase (AOS) followed by
397 the enzyme allene oxide cyclase (AOC). In our case, the expression of AOC tends to increase in the
398 presence of the pathogen *D. teres* compared to the control, with normalized relative expression
399 values close to 0.75 and 0.41 at 48 hpi, respectively. In the same way, AOC tends to increase with the
400 pathogen and strain PsJN compared to the control, with normalized relative expression values close
401 to 1.13 and 0.63 at 48 hpi, respectively (Suppl. Table 1). On the contrary, AOC expression seems to
402 decrease in the presence of *D. teres* in combination with strain B25 compared to the values of the
403 infected plants at 12 and 48 hpi. In grapevine, the presence of PsJN modulates slightly the AOC
404 transcript levels. However, *Botrytis* alone induces a significant increase in *PR5* and AOC gene
405 expression levels (Miotto-Vilanova et al., 2016). Grapevine plantlets treated with PsJN show a higher
406 expression of PR genes (*PR1*, *PR2* and *PR5*) in response to the pathogen. Therefore, in grapevine,
407 PsJN primes the simultaneous induction of the jasmonic acid-related genes (Miotto-Vilanova et al.,
408 2016). In the presence of the *F. oxysporum* pathogen, *PR1* is induced in tomato and its expression is
409 higher when the plant is not treated with *Pseudomonas chlororaphis* used as biocontrol agent
410 (Kamou et al., 2020).

411 In the presence of *D. teres* and/ or strain PsJN in barley, the antioxidant enzymes *CAT* and
412 *GSTd2* have a higher expression compared to *SOD* (Figure 2). In addition, these three antioxidant
413 enzymes show no significant variations in the presence of strain B25 and/or in combination with the
414 pathogen.

415 *PAL* is the first enzyme in the phenylpropanoid pathway, which synthesizes specialized
416 metabolites from L-phenylalanine, including lignin building blocks, flavonoids and phytoalexins
417 (Peltonen and Karjalainen, 1995; Yadav et al., 2020). The induction of PAL activity is considered as a
418 useful indicator of the activation of defense-related responses. In this study, two isoforms of
419 phenylalanine ammonia lyase (*PAL* and *PAL2*) were studied. According to the heat map hierarchical
420 clustering, *PAL* and *PAL2* show different expression patterns and are thus in different clusters. *PAL2* is

421 induced not only in the presence of the pathogen alone, but also in barley infected by *D. teres* in
422 combination with strain PsJN (Figure 2). *PAL* shows no significant difference in the presence of strain
423 B25. In contrast, *PAL2* appears to be less expressed in the presence of strain B25 or in combination
424 with *D. teres*, though differences are not statistically significant (Suppl. Table 1). According to
425 Chandrasekaran et al. (2017), *PAL* expression is significantly higher in tomato plantlets bacterized
426 with *Bacillus subtilis* compared to non-bacterized plantlets 72 hpi after inoculation with
427 *Xanthomonas campestris* pv. *vesicatoria*. Similarly, Peltonen and Katjalainen (1995) have
428 demonstrated a two- to five-fold increase in *PAL* transcript level in barley and wheat leaves following
429 infection with *Bipolaris sorokiniana*, a necrotrophic cereal pathogen. There is a strong correlation
430 between an increase in *PAL* transcript level and fungal infections in the *Gramineae*, indicating that
431 the synthesis of phenolic compounds is part of their defense reactions (Peltonen and Karjalainen,
432 1995). The increase in *PAL* transcript level was reported to restrict the infected area, by inducing
433 lignification (Peltonen and Karjalainen, 1995).

434 Flavonoids play central roles in plant defense against biological and environmental stresses,
435 such as fungal infection. Stress frequently induces flavonoid biosynthesis in plant tissues. In this
436 study, the expression of genes coding for the two flavonoid biosynthetic enzymes, chalcone synthase
437 *CHS* and flavanone-3'-hydroxylase *F3'H*, is analyzed in barley leaves with or without pathogen and/or
438 PGPR.

439 *CHS* is the first enzyme involved in the flavonoid pathway and synthesizes naringenin
440 chalcone from three molecules of malonyl-CoA and one molecule of coumaroyl-CoA. According to
441 Figure 2, *CHS* expression tends to increase with strain PsJN. On the contrary, *CHS* expression tends to
442 decrease with strain B25 without significant differences (Figure 2 and Suppl. Table 1). During
443 pathogens' attack, *Sorghum* leaves change their color from light to dark brown (Mizuno et al., 2016).
444 The tan-colored *Sorghum* plants accumulate relatively higher levels of flavones (apigenin and
445 luteolin) than red/purple ones. Indeed, pigments are accumulated in response to infection with a
446 fungus (Mizuno et al., 2016). Many studies have shown that the expression of *CHS* can be induced in
447 response to viruses, bacteria, fungi, nematodes and other pests attacking plants, resulting in the
448 enhanced production of flavonoids (Dao et al., 2011). In barley, two isoforms of *CHS* are present,
449 *CHS1* and *CHS2*, which are different in terms of their affinity with the hydroxycinnamoyl-CoA
450 substrate (Christensen et al., 1998). The expression of *CHS2* in barley infected by *B. graminis* is
451 strongly induced, while *CHS1* is expressed at very low levels (Christensen et al., 1998).

452 In the flavonoid pathway, flavonoid 3'-hydroxylase (*F3'H*) catalyzes the hydroxylation of
453 naringenin and dihydrokaempferol (DHK) at the 3' position and belongs to the CYP75B subfamily in
454 the cytochromes P450-dependent monooxygenase superfamily. Like *CHS*, *F3'H* is induced when
455 barley is infected with *D. teres* alone and also in combination with PsJN at 48 hpi. PsJN can be
456 perceived as a pathogenic agent by barley, which triggers defense mechanisms including flavonoids
457 production and, consequently, induces the genes involved in this metabolic pathway. In addition,
458 *F3'H* expression tends to decrease with strain B25 without significant differences (Figure 2 and Suppl.
459 Table 1).

460 According to Figure 2, two glucan synthase genes show a similar expression pattern and
461 belong to the same cluster. Our results show that *GSL1* and *GSL3* are induced at 12 hpi with *D. teres*.
462 Their expressions are then repressed at 48 hpi. We expected to observe a decrease in *GSL* expression
463 in infected barley bacterized with strain B25, since this beneficial bacterium limits the development

464 and the symptoms number of net blotch in barley. However, in the presence of PsJN or strain B25,
465 *GSL1* or *GSL3* expressions do not vary.

466 Many members of the *GSL* family are involved in callose biosynthesis in specific tissues, at
467 certain developmental stages and under different stress conditions. For instance, β -1,3-D-glucan
468 callose is rapidly synthesized upon microbial attack.

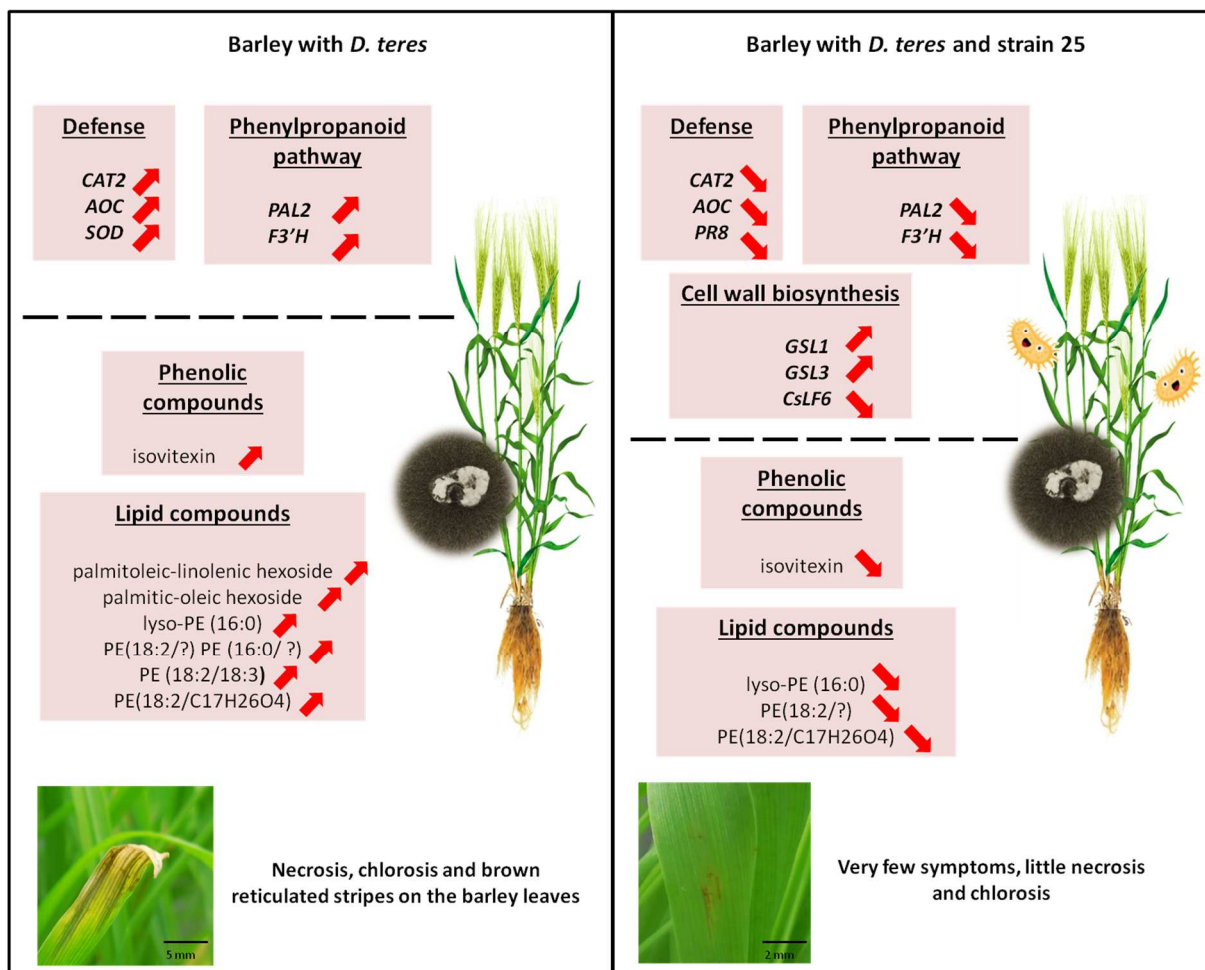
469 A characteristic of cereals and grasses is the presence of (1,3;1,4)- β -D-glucans in the cell wall
470 (Burton et al., 2011). The biosynthesis of this polymer is catalyzed by the members of three cellulose
471 synthase-like (*CsIF/H/J*) subfamilies. *CsIF6* shows a slight increase in expression in the presence of
472 PsJN alone (Figure 2). Additionally, *CsIF6* displays a lower expression at 12 hpi in the presence of
473 strain B25 alone or in combination with the pathogen. Indeed, *CsIF6* expression decreases in the
474 presence of strain B25 compared to PsJN in infected barley, with normalized relative expression
475 values close to 0.65 and 1.17 at 12 hpi, respectively (Suppl. Table 1). The *CsI* gene family is large and
476 has been divided into subgroups, i.e. *CsIA* - *CsIH*. *HvCsIF6* was shown to be directly involved in
477 (1,3;1,4)- β -D-glucan biosynthesis. For instance, the over-expression of *HvCsIF6* in transgenic barley
478 resulted in an increase in (1,3;1,4)- β -D-glucan content by more than 40 % in the grain, with even
479 larger increases in vegetative tissues (Burton et al., 2011). The cell wall is a dynamic and complex
480 structure involved in interactions between plants and their environment, notably to limit the further
481 spread of pathogens. According to the results obtained, in the presence of *D. teres*, a barley plant
482 bacterized with strain B25 shows a decreased expression of these genes related to cell wall synthesis.

483 The role of carbohydrates is well known in the activation of defense mechanisms against
484 pathogens of any kind in plants. However, little information is available concerning the response of
485 plants to beneficial bacteria and in the presence of the pathogen in terms of carbon metabolism. The
486 ability of some beneficial bacteria to improve stress tolerance in different plants by regulating the
487 expression of genes involved in carbohydrate metabolism has been shown (Miotto-Vilanova et al.,
488 2016; Su et al., 2015; Riva and Ribaudó, 2020). According to the results obtained in this study, PsJN
489 does not modulate the expression of the selected genes. On the contrary, strain B25 slightly
490 decreases the expression of *RcaA2* and *SUS2* (Suppl. Table 1). In other studies, strain PsJN, modulates
491 the carbohydrate metabolism-related genes and thus increases cold tolerance in grapevine (Miotto-
492 Vilanova et al., 2016; Su et al., 2015). In addition, *Pseudomonas pseudoalcaligenes* changes both the
493 sugar metabolism and the plant defense, leading to an improved tolerance against *Sclerotium rolfsii*
494 in tomato (Riva and Ribaudó, 2020). These results may indicate that bacteria manipulate the
495 expression of defense genes to ensure the establishment of an alert state, thus enabling an efficient
496 and quick response to pathogens.

497 In our study, PsJN was used as a “bacterization control” since this strain has no effect on the
498 development of the agent responsible for net blotch. Therefore, we can hypothesize that PsJN can
499 induce defense mechanisms in the plant by increasing gene expression without limiting the growth of
500 *D. teres* and without reducing stress symptoms on barley leaves. These results can be explained by
501 the fact that the beneficial bacteria are initially recognized as plant pathogens and, therefore, they
502 elicit the expression of defense-related genes (Kamou et al., 2020).

503 With strain B25, the expression of some genes shows a tendency towards repression
504 compared to the control (Figure 3). We may therefore infer that strain B25 does not protect barley
505 through the induction of defense-related genes since their expression decreases. However, strain B25

506 may protect its host plant by implementing one of two possible mechanisms: firstly, the strain could
 507 produce a biofilm preventing the pathogen from accessing the barley cell wall; secondly, strain B25
 508 could secrete antifungal molecules that might stop the development of the pathogen. These
 509 hypotheses must be checked with future experiments.



510
 511 **Figure 3.** Schematic overview representing the impact of *D. teres* in the absence and presence of
 512 strain B25 on barley leaves. In the presence of the fungus alone, barley induces the expression of
 513 genes related to defense response and the phenylpropanoid pathway. In the context of biological
 514 control, strain B25 acts as an antagonist of *D. teres*.

515 When comparing the data obtained for PsJN and strain B25, results are very distinct. This
 516 difference can be explained by their antagonistic effects against net blotch. Indeed, barley plants
 517 infected with *D. teres* and bacterized with PsJN showed similar symptom levels as those infected only
 518 with *D. teres*. In contrast, plants infected and bacterized with strain B25 had a lower number of
 519 symptoms on the leaves. This decrease in the number of brown spots has great importance on the
 520 primary and secondary metabolisms of barley. For instance, a barley leaf with symptoms develops
 521 more defense mechanisms. In addition, the reduction in healthy leaf area has a direct impact on
 522 photosynthetic performance and carbohydrate assimilation.

523 3.3. Untargeted metabolomics on barley leaves infected by *D. teres* in the 524 absence/ presence of PGPR

525 Our objective was to conduct a combined gene expression and metabolomic analysis to gain
526 information concerning the biological mechanism underlying the increased defense to net blotch in
527 the presence of beneficial bacteria. An untargeted metabolomics study was performed using the
528 same samples as for RT-qPCR analysis. Time-points 12 h and 48 h were selected based on the results
529 obtained by RT-qPCR. Indeed, gene expression shows more differences at 12 hpi and 48 hpi and,
530 therefore, significant differences could also be observed in the accumulation of metabolites. The goal
531 was to enrich and complement the gene expression data and to attempt to identify metabolites
532 spotted as differentially abundant between modalities during the tripartite interaction barley - *D.*
533 *teres* - PGPR. According to the results obtained, barley produces different metabolites in the
534 presence of the pathogen with/without bacteria while 12 hpi is the time-point where the differences
535 between modalities are the most significant (Table 2). Metabolomics results are consistent with RT-
536 qPCR results, 12 hpi also being the time-point at which several genes related to defense, the
537 phenylpropanoid pathway and cell wall biosynthesis are over-expressed in all the experimental
538 conditions.

539 In total, 25 metabolites were putatively identified among the differentially abundant ones
540 according to the experimental conditions. The identified metabolites belong mainly to the following
541 categories: nucleotides, phenolic compounds, fatty acyls (FA) and glycerophospholipids (PL) (Table 2).

542 Among the phenolic compounds, the first identified metabolite is galloyl glucose (n° 2). This
543 compound plays a role in cell wall thickening and also as a phytoalexin. Our results show that galloyl
544 glucose is present in higher abundance in barley leaves bacterized with strain B25 at 48 hpi
545 compared to control plants (Table 2).

546 *N-p-coumaroylhydroxydehydroagmatine* (n° 3, 5 and 7) and *N-p-coumaroylhydroxyagmatine*
547 (n° 4 and 6) are hydroxycinnamic acid amides found in cereals. These compounds are present at
548 higher levels in barley leaves infected with *D. teres* and bacterized with PsJN at 12 hpi compared to
549 barley leaves infected solely with *D. teres* at the same time-point (Table 2). A peroxidase catalyzes
550 the reaction to obtain hordatine from *p*-hydroxycinnamoylagmatine. Hordatines, as well as *p*-
551 coumaroylagmatine and its hydroxylated form, act as preformed defense compounds in barley grains
552 or as inducible defense compounds at the onset of pathogens' attack in barley plants. Hordatin A is a
553 dimer of *p*-coumaroylagmatine, while hordatin B is a conjugate analogous to *p*-coumaroyl and
554 feruloylagmatins. According to the results obtained with RT-qPCR, *AOC* expression tends to increase
555 in the presence of the pathogen and in combination with PsJN strain compared with control at 12 hpi
556 (Figure 2). We can therefore hypothesize that *AOC* induction increases JA production, leading to an
557 increase of *N-p-coumaroylhydroxyagmatine*.

558 The third identified phenolic compound is isovitexin (n° 8), a C-glycosylated flavone belonging
559 to the phenylpropanoid pathway. Previous studies have demonstrated that barley leaves contain a
560 large number of flavonoids including C-glycosylated flavones, isovitexin-7-*O*-glucoside (saponarin)
561 and isoorientin-7-*O*-glucoside (lutonarin). In our study, isovitexin abundance is higher in pathogen-
562 free and bacteria-free leaves at 12 hpi compared with leaves bacterized only with strain B25 at the
563 same time-point (Table 2).

Table 2. List of the compounds identified in barley leaves infected by *D. teres*, in combination with PsJN or strain B25, using UHPLC-DAD-ESI-MS/MS analysis. Metabolites were annotated/ identified based on exact mass, retention time, MS2 fragmentation pattern and UV-visible spectrum by comparison with data in databases and/or literature. The abbreviation of the names of the molecules classes is inspired by LIPID MAPS including fatty acyls (FA) –glycerolipid (GL)- glycerophospholipids (PL)- glycerophosphoethanolamines (PE)- glycerophosphocholines (PC)- glycerophosphoglycerol (PG)

No	RT (min)	Observed m/z	Adduct	Comparison n°	Class	Name	Molecular formula	Theoretical m/z	Mass error (ppm)	MS/MS fragments	λ max (nm)	Highest mean	Lowest mean	Max fold change	Reference	Data bank reference ^a	MSI level ^b
1	1.23	348.0688	[M+H] ⁺	8	nucleotides	adenosine 3'-monophosphate	C ₁₀ H ₁₄ N ₅ O ₇ P	348.0703	-4.64	136.0607, 119.0337, 85.0277, 164.9924, 213.0137	260	Infected T12	Strain B25 infected T12	2.08	/	MID 63401 CID 41211	2
2	2.56	331.0659	[M-H] ⁻	9	phenolic compounds	galloyl glucose	C ₁₃ H ₁₆ O ₁₀	331.0671	-3.54	168.0058, 149.9946, 125.0241, 124.0156, 331.0668, 123.0082, 313.0553	204 255 320	Strain B25 T48	Control T48	2.03	[1] [2]	CID 124021 MoNA PM011302	2
3	4.60	291.1438	[M+H] ⁺	12	phenolic compounds	N-p-coumaroylhydroxydehydroagmatine	C ₁₄ H ₁₈ O ₃ N ₄	291.1452	-4.70	147.0452, 127.0986, 255.1253, 273.1370, 213.1038, 119.0499, 91.0550, 110.0722	193 206 282	Strain PsJN infected T12	Infected T12	2.27	[3]	/	2
	4.62	289.1298	[M-H] ⁻	12	phenolic compounds	N-p-coumaroylhydroxydehydroagmatine	C ₁₄ H ₁₈ O ₃ N ₄	289.1306	-2.84	119.0503	222 283	Strain PsJN infected T12	Infected T12	3.78	[3]	/	2
4	4.62	291.1458	[M-H] ⁻	12	phenolic compounds	N-p-coumaroylhydroxyagmatine	C ₁₄ H ₂₀ O ₃ N ₄	291.1463	-1.72	119.0482, 208.8628, 226.8755	222 283	Strain PsJN infected T12	Infected T12	2.27	[3]	/	2
5	9.14	291.1446 *	[M+H] ⁺	9	phenolic compounds	N-p-coumaroylhydroxydehydroagmatine	C ₁₄ H ₁₈ O ₃ N ₄	291.1452	-1.96	147.0437, 127.0972, 213.1016, 119.0487, 255.1239, 273.1344, 91.0537, 110.0703, 126.0891, 153.0768	216 291 311(sh)	Strain B25 T48	Control T48	2.67	[3]	/	2
	9.14	291.1461 *	[M+H] ⁺	12	phenolic compounds	N-p-coumaroylhydroxydehydroagmatine	C ₁₄ H ₁₈ O ₃ N ₄	291.1452	3.19	147.0448, 255.1258, 127.0981, 273.1359, 213.1034, 119.0498, 91.0545, 110.0717, 126.0900, 153.0774	218 291	Strain PsJN infected T12	Infected T12	3.43	[3]	/	2
6	9.21	291.1457	[M-H] ⁻	12	phenolic compounds	N-p-coumaroylhydroxyagmatine	C ₁₄ H ₂₀ O ₃ N ₄	291.1463	-2.07	119.0498, 208.8656, 226.8741, 100.9340, 150.9163, 132.9036	218 289	Strain PsJN infected T12	Infected T12	2.95	[3]	/	2
7	9.29	289.1297	[M-H] ⁻	12	phenolic compounds	N-p-coumaroylhydroxydehydroagmatine	C ₁₄ H ₁₈ O ₃ N ₄	289.1306	-3.31	119.0499	218 289	Strain PsJN infected T12	Infected T12	5.31	[3]	/	2
8	22.66	431.0962	[M-H] ⁻	7	phenolic compounds	isovitexin	C ₂₁ H ₂₀ O ₁₀	431.0984	-4.96	311.0556, 283.0638, 281.0431, 269.0501, 323.0572, 341.0638	214 339 271	Control T12	Strain B25 T12	2.05	[3]	MID 263495	2

Table 2. (Continued)

No	RT (min)	Observed m/z	Adduct	Comparison n°	Class	Putative name	Molecular formula	Theoretical m/z	Mass error (ppm)	MS/MS fragments	λ max (nm)	Highest mean	Lowest mean	Max fold change	Reference	Data bank reference ^a	MSI level ^b
9	31.99	223.1337	[M-H] ⁻	14	FA	13-oxo-9,11-tridecadienoic acid	C ₁₃ H ₂₀ O ₃	223.1340	-1.23	223.1330, 195.1395, 69.0362 , 59.0154, 179.1451, 167.1112, 153.0937, 161.1324, 205.1224	221	Strain PsJN infected T48	Infected T48	11.52	[4]	CID 11106999 MoNA PR309071	2
10	37.89	518.3221	[M+H] ⁺	7	PL	lyso-PC(18:3)	C ₂₆ H ₄₈ NO ₇ P	518.3241	-3.93	184.0771 , 86.0980, 125.0014, 166.0642, 60.0824, 335.2580, 500.3154, 258.1111, 261.2221	198 221	Strain B25 T12	Control T12	3.81	[5]	CHEBI 133456 LMGP01050038	2
	37.89	518.3221	[M+H] ⁺	11	PL	lyso-PC(18:3)	C ₂₆ H ₄₈ NO ₇ P	518.3241	-3.93	184.0743 , 124.9979, 86.0948, 60.0813, 98.9845, 166.0634, 71.0732, 104.1070, 500.3091, 335.2589, 258.1141	198 221	Strain PsJN T12	Control T12	4.78	[5]	CHEBI 133456 LMGP01050038	2
11	38.11	721.3649	[M-H] ⁻	7	di-fatty acid hexoside	palmitoleic-linolenic hexoside	C ₃₄ H ₅₈ O ₁₆	721.3652	-0.48	277.2179 , 59.0141, 89.0244, 101.0244, 113.0242, 119.0344, 397.1344, 235.0820, 161.0451, 415.1456, 179.0555	198 221	Control T12	Strain B25 T12	2.27	[6]	/	4
12	38.71	520.3375	[M+H] ⁺	7	PL	lyso-PC(18:2)	C ₂₆ H ₅₀ NO ₇ P	520.3398	-4.43	184.0753 , 86.0979, 125.0009, 166.0638, 60.0821, 71.0742, 98.9857	200 222	Strain B25 T12	Control T12	3.34	[5]	CHEBI 28733 LMGP01050035	2
	38.71	520.3375	[M+H] ⁺	11	PL	lyso-PC(18:2)	C ₂₆ H ₅₀ NO ₇ P	520.3398	-4.43	184.0745 , 124.9986, 86.0953, 60.0816, 166.0627, 71.0733, 98.9844, 104.1069, 337.2717, 502.3261, 258.1070, 520.3314	200 222	Strain PsJN T12	Control T12	4.30	[5]	CHEBI 28733 LMGP01050035	2
13	39.46	699.3802	[M-H] ⁻	7	di-fatty acid hexoside	palmitic-oleic hexoside	C ₃₂ H ₆₀ O ₁₆	699.3809	-1.01	255.2329 , 59.0139, 89.0242, 101.0235, 119.0349, 397.1336, 71.0139, 235.0819, 415.1450, 279.2333	222	Control T12	Strain B25 T12	2.58	[6]	/	4
	39.46	699.3802	[M-H] ⁻	11	di-fatty acid hexoside	palmitic-oleic hexoside	C ₃₂ H ₆₀ O ₁₆	699.3809	-1.01	255.2329 , 59.0139, 89.0242, 101.0235, 119.0349, 397.1336, 71.0139, 235.0819, 415.1450, 279.2333	222	Control T12	Strain PsJN T12	2.30	[6]	/	4
	39.46	699.3802	[M-H] ⁻	13	di-fatty acid hexoside	palmitic-oleic hexoside	C ₃₂ H ₆₀ O ₁₆	699.3809	-1.01	255.2310 , 59.0136, 89.0237, 101.0228, 119.0336, 71.0130, 235.0810, 397.1319, 125.0242, 415.1416, 161.0436	222	Strain PsJN T48	Control T48	2.01	[6]	/	4
14	39.50	452.2771	[M-H] ⁻	7	PL	lyso-PE(16:0)	C ₂₁ H ₄₄ NO ₇ P	452.2783	-2.58	255.2333 , 78.9593, 140.0119, 196.0376	222	Control T12	Strain B25 T12	2.27	/	MID 40776 LMGP02050002 CHEBI 73134 NIST 53862-35-4	2
	39.50	452.2771	[M-H] ⁻	13	PL	lyso-PE(16:0)	C ₂₁ H ₄₄ NO ₇ P	452.2783	-2.58	255.2321 , 78.8588, 140.0109, 196.0367	222	Strain PsJN T48	Control T48	2.09	/	MID 40776 LMGP02050002 CHEBI 73134 NIST 53862-35-4	2

Table 2. (Continued)

No	RT (min)	Observed m/z	Adduct	Comparison n°	Class	Putative name	Molecular formula	Theoretical m/z	Mass error (ppm)	MS/MS fragments	λ max (nm)	Highest mean	Lowest mean	Max fold change	Reference	Data bank reference ^a	MSI level ^b
15	39.58	355.2828	[M+H] ⁺	7	GL	octadecadienoyl-glycerol	C ₂₁ H ₃₈ O ₄	355.2843	-4.33	67.0558, 95.0873, 81.0719, 109.1025, 55.0561, 133.1035, 121.1003, 263.2386, 245.2264, 161.1341, 337.2731, 355.2461	223	Strain B25 T12	Control T12	2.21	/	CHEBI 75457 MID 63032 and 45151	2
	39.58	355.2828	[M+H] ⁺	9	GL	octadecadienoyl-glycerol	C ₂₁ H ₃₈ O ₄	355.2843	-4.33	81.0681, 67.0535, 95.0857, 79.0532, 55.0532, 69.0689, 147.1156, 133.0995, 109.0989, 161.1300	223	Control T48	Strain B25 T48	2.21	/	CHEBI 75457 MID 63032 and 45151	2
	39.58	355.2828	[M+H] ⁺	11	GL	octadecadienoyl-glycerol	C ₂₁ H ₃₈ O ₄	355.2843	-4.33	81.0708, 67.0550, 95.0860, 109.1011, 263.2339, 135.1172, 123.1169, 161.1312, 147.1195, 175.1468, 337.2747, 245.2274	222	Strain PsJN T12	Control T12	2.42	/	CHEBI 75457 MID 63032 and 45151	2
16	41.24	279.2312	[M+H] ⁺	7	FA	octadecatrienoic acid derivative	C ₁₈ H ₃₀ O ₂	279.2319	-2.41	81.0691, 67.0536, 95.0839, 149.0221, 109.0988, 201.0456, 55.0541, 79.0534, 93.06778, 131.0843	223	Strain B25 T12	Control T12	2.41	/	CHEBI 25633	3
17	41.39	676.4167	[M-H] ⁻	7	PL	PE(18:2/?)	/	/	/	279.2333, 199.1334, 140.0120, 78.9592, 96.9698, 171.1390, 196.0378, 122.0012, 211.1337, 476.2727, 476.2892, 458.2592, 458.2744, 613.3834	223	Control T12	Strain B25 T12	3.04	/	/	4
18	41.75	1273.8553	[2M-H] ⁻	11	PL	PE(16:0/?)	/	/	/	201.1497, 255.2338, 636.4292	223	Control T12	Strain PsJN T12	4.36	/	/	4
19	41.87	712.4896	[M-H] ⁻	14	PL	PE(16:0/18:3)	C ₃₉ H ₇₂ NO ₈ P	712.4923	-3.70	277.2184, 255.2334, 140.0118, 196.0387, 669.1875, 452.2789	224	Strain PsJN infected T48	Infected T48	2.64	[7]	LMGP02010041	2
20	42.90	752.4500	[M-H] ⁻	7	PL	PE(18:2/C17H26O4)	C ₄₀ H ₆₈ NO ₁₀ P	752.4508	-1.10	293.1752, 279.2321, 249.1854, 275.1643, 140.0109, 196.0388, 295.2285, 476.2742, 78.9527	224	Control T12	Strain B25 T12	2.62	[7]	/	3
21	43.88	743.4812	[M+H] ⁺	13	PL	PG(34:4)	C ₄₀ H ₇₁ O ₁₀ P	743.4858	-6.11	571.4733, 93.0712, 69.0709, 95.0874, 107.0874, 311.2604, 335.2555, 149.1307, 184.0721, 261.2231	224	Strain PsJN T48	Control T48	2.12	/	LMGP04010213	2
22	44.67	728.5181	[M+H] ⁺	11	PL	PC(32:3)	C ₄₀ H ₇₄ NO ₈ P	728.5225	-6.00	184.0741, 86.0967, 125.0000, 60.0814, 166.0626, 98.9842, 71.0736, 104.1073, 468.3160, 450.2932, 285.2388, 500.3053, 545.4532	225	Strain PsJN T12	Control T12	2.40	/	CHEBI 66847 LMGP01010497	2
23	45.05	736.4910	[M-H] ⁻	11	PL	PE(18:2/18:3)	C ₄₁ H ₇₂ NO ₈ P	736.4923	-1.76	279.2358, 277.2207, 140.0129, 196.0392, 78.9601	225	Control T12	Strain PsJN T12	3.24	[7]	LMGP02010665	2

Table 2. (Continued)

No	RT (min)	Observed m/z	Adduct	Comparison n°	Class	Putative name	Molecular formula	Theoretical m/z	Mass error (ppm)	MS/MS fragments	λ max (nm)	Highest mean	Lowest mean	Max fold change	Reference	Data bank reference ^a	MSI level ^b
24	45.50	756.5505	[M+H] ⁺	11	PL	PC(16:0/18:3)	C ₄₂ H ₇₈ NO ₈ P	756.5538	-4.37	184.0730 , 86.0965, 124.9996, 478.3229, 496.3379, 500.3032, 756.5511	225	Strain PsJN T12	Control T12	4.91	/	CHEBI 84786 LMGP01010598 LMGP01010601	2
25	46.54	449.3403	[M-H] ⁻	13	phyllo-quinones	phylloquinone	C ₃₁ H ₄₆ O ₂	449.3425	-4.85	434.3195 , 238.0983, 223.0771, 449.3442, 185.0609, 209.0614, 237.0918, 171.0460, 339.2018	225	Strain PsJN T48	Control T48	3.11	[8]	/	3

The comparisons shown in the table are differentially abundant (p -value < 0.05 and max fold change > 1.5) in the two experimental conditions of the considered comparisons. Ions in bold are above 50 % relative abundance in MS2. The asterisk (*) indicates that the quantification of compound 5 was made on m/z 273.1340 ([M+H-H₂O]⁺), sh: shoulder.

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^a Metlin ID: MID*, PubChem ID: CID*, Lipid Maps: ID: LMGP*, Chemical Entities of Biological Interest (ChEBI) ID: CHEBI*, National Institute of Standards and Technology (NIST) ID: NIST*, MassBank of North America (MoNA) ID: MoNA*.

^b Metabolite identification level according to Metabolite Standards Initiative recommendation (1- identified metabolites, 2- putatively annotated compounds, 3-putatively characterized compound classes, 4- unknown compounds) (Dunn et al., 2013; Sumner et al., 2007).

615 The synthesis of isovitexin involves several enzymes, including *CHS* and *F3'H* in the phenylpropanoid
616 pathway. The gene expression results show a decrease in *F3'H* expression in the presence of strain
617 B25, in combination or not with the pathogen (Figure 2). Therefore, it is possible to relate a
618 repression of *CHS* and *F3'H* genes with a decrease in the amount of isovitexin in barley leaves.

619 For two compounds (n° 11 and 13), the data comprising the exact mass and retention time
620 obtained are similar to those provided by Pierson et al. (2014). However, from the formula given by
621 Pierson and collaborators, we are unable to find the structure of these compounds identified as
622 palmitoleic-linolenic hexoside (n° 11) and palmitic-oleic hexoside (n° 13) (Pierson et al., 2014). For
623 this reason, the MSI level assigned to these two compounds is 4.

624 Compound n° 25 was identified as phylloquinone. Known as vitamin K1, phylloquinone
625 belongs to the quinone lipids containing a methylated naphthoquinone ring structure and differs in
626 the aliphatic side chain linked to position 3. Phylloquinones are up to three times more abundant in
627 PsJN-bacterized leaves at 48 hpi compared to control ones at the same time-point.

628 The other identified compounds are all classified as lipids, more precisely two fatty acids or
629 derivatives (n° 9 and 16), one glycerolipid (n° 15) and eleven glycerophospholipids (PL). A fatty acid,
630 more precisely, an oxo-tridecadienoic acid (n° 9) is up to 11.52 times more abundant in barley with
631 strain B25 at 12 hpi compared to non-infected barley leaves at the same time-point (Table 2).

632 Based on their molecular structures, lipids can be classified as polar and non-polar. Non-polar
633 lipids include triacylglycerols, sterols and tocopherols. Glycerophospholipids are the most prominent
634 members of polar lipids. Among the glycerophospholipids identified in this study, six compounds are
635 glycerophosphoethanolamines (PE) (n° 14, 17, 18, 19, 20 and 23), four are glycerophosphocholines
636 (PC) (n° 10, 12, 22 and 24), and one is a glycerophosphoglycerol (PG) (n° 21). According to the results
637 obtained, barley leaves in the presence of strain B25 show lower levels of lyso-PE(16:0) and
638 PE(18:2/C17H26O4) at 12 hpi compared to control plants (Table 2 and Figure 3). On the contrary, the
639 relative abundance of other unsaturated species, such as PE(16:0/18:3) and PE(18:2/ 18:3) increases
640 in barley with strain PsJN and *D. teres* at 48 hpi or decreases with PsJN without *D. teres* at 12 hpi,
641 respectively (Figure 3). The regulation of the glycerolipids is important in plants to maintain
642 membrane fluidity and integrity under stress conditions. Lipids also play an important role in the
643 plant's defense response. When exposed to many types of (a)biotic stress, plants sense these
644 exogenous stimuli and transmit the signal through the plasma membrane. The change of the
645 phospholipid composition was studied during the interaction between barley and *Fusarium*. Indeed,
646 the levels of phosphatidylcholine PC(36:4) increased, while lysophosphatidylcholine lyso-PC(16:0),
647 lyso-PC(18:2) and lyso-PC(18:3) decreased in abundance (Reyna et al., 2019).

648 In this study, an untargeted metabolomics analysis identified mainly phenolic compounds
649 and lipids, two classes of compounds known to undergo changes in abundance due to biotic stress.
650 Indeed, flavonoids are molecules produced in response to biotic stress, thus their properties and
651 applications have attracted growing interest. At the same time, lipids are known to be involved in
652 intracellular signaling upon pathogens' attack including virus, bacteria, fungi, nematodes and other
653 biotic stressors. The present results also show the potential protection of strain B25 against net
654 blotch. The presence of *D. teres* and the beneficial bacteria implies changes in the production of
655 metabolites in barley. Lipid compounds are more abundant in plants infected with net blotch. In
656 addition, barley tends to produce fewer defense compounds in the presence of strain B25. A

657 difference in the production of metabolites was also observed when comparing barley plants
658 bacterized by PsJN and strain B25. These differences in transcripts levels and metabolites seem to be
659 correlated to their difference in antagonistic effect towards *D. teres*.

660 **Conclusions and future perspectives**

661 In summary, the infection with *D. teres* causes significant transcriptional and metabolomic
662 changes in barley. The research presented here highlights the molecular changes caused by
663 beneficial PGPR used as biocontrol agents in agriculture. Strain B25 is able to protect barley against
664 net blotch. Additionally, the expression of genes involved in stress response is mitigated and fewer
665 compounds involved in defense mechanisms are produced. These results are likely related to the
666 cultivar used. Future studies, using other cultivars, will validate these results and contribute to
667 identify new markers of susceptibility or tolerance to the disease. The gene expression study and the
668 relative quantification of metabolites performed here represent, to our knowledge, the first study on
669 the tripartite interaction among barley, the biocontrol agent strain B25 and *D. teres*, the pathogen
670 causing barley net blotch.

671 **Acknowledgments:** This work was supported by grand-Reims and Grand-Est region. The authors
672 gratefully acknowledge BAYER SAS Lyon for providing the *D. teres* HE 019, and more specifically
673 Marie-Pascale Latorse, Stéphane Brunet and Catherine Wantier for their technical assistance and
674 participation in this study.

675 **Author Contributions:** A.B., S.C. and G.G. performed the experiments. A.B., S.C. and G.G. analyzed
676 the data and prepared the figures and tables. E.A.B., C.J. and G.G. conceived the experiments. S.C.,
677 G.G., S.P., Q.E., K.S., J-F.H. and J.R. provided feedback on the draft and on the interpretation of the
678 data. E.A.B. and C.J. managed the project. All authors read and approved the final draft.

679 **Funding:** This research did not receive any external funding.

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