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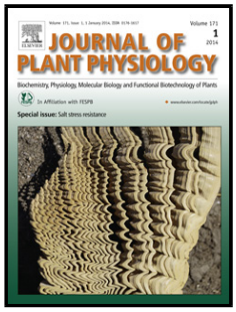

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Lignin biosynthesis as a key mechanism to repress *Polystigma amygdalinum*, the causal agent of the red leaf blotch disease in almond

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Abstract

The red leaf blotch (RLB) of almond, caused by the fungus *Polystigma amygdalinum*, is considered as one of the most important leaf diseases of this fruit tree. Differential cultivar susceptibility to the RLB has been described based on field observations, while its molecular and biochemical bases remain unknown to date. We aimed to explore the plant

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defence mechanisms related to the cultivar susceptibility by identifying some relevant physical and chemical strategies for the pathogen control. Thus, we studied the regulation of seven defence-related genes as well as the lignin deposition in two almond cultivars with highly differential response to RLB: the highly tolerant 'Mardía' and the susceptible 'Tarraco' cultivars. 'Mardía' displayed an up-regulation of the *CAD* and *DFN1* genes at early stages of RLB symptom expression, with further lignin deposition in the fungal-colonized area that was visualized by microscopy. Thus, 'Mardía' uses both physical and chemical responses to effectively repress the pathogen. In contrast, 'Tarraco' triggered the up-regulation of *HQT* and *LDOX* genes, related to chlorogenic acid and anthocyanin biosynthesis pathways, respectively, while lignin deposition was not clearly noticed. This strategy recorded in 'Tarraco' at later stages of RLB symptoms failed to control the fungal infection and colonization. Our results suggested a major role of the phenylpropanoids pathway in the defence response against RLB, by showing that an early production of lignin might be a major mechanism to control the spread of *P. amygdalinum* within the host leaf tissues.

Abbreviations:

β-TUB, β-tubulin; C4H; cinnamate 4-hydroxylase; CAD, cynnamoyl alcohol dehydrogenase; DFN, defensin; HQT, hydroxycinnamoyl CoA quinate hydroxycinnamoyl transferase; LDOX, leucoanthocyanidin dioxygenase; LOX, lipoxygenase; PR protein, pathogenesis-related protein; RE, relative expression; RLB, red leaf blotch; RT-qPCR, reverse-transcription quantitative PCR; SA, salicylic acid; SLU7, pre-mRNA splicing factor 7; TEF1, translation elongation factor 1α

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***Keywords**:*

defence genes; lignin; *Polystigma amygdalinum*; *Prunus dulcis*; red leaf blotch; varietal susceptibility

1. Introduction

The red leaf blotch disease (RLB) of almond (*Prunus dulcis*), caused by the ascomycete *Polystigma amygdalinum*, was firstly reported in 1970 in Shiraz, Iran (Ghazanfari and Banihashemi, 1976), and appears to be widely distributed in the Mediterranean region and the Middle East (Cannon, 1996). The RLB is considered one of the most important leaf diseases of almond, since it reduces photosynthetic capacity of the host and may induce an early defoliation of the tree (Kranz, 1962). A subsequent decrease in fruit production has also been reported as caused by the disease (Miarnau and Vargas, 2013; López-López et al., 2016). Leaf infections occur in spring, when ascospores of *P. amygdalinum* are released from previous-year fallen leaves under favorable environmental conditions (Banihashemi, 1990). RLB symptoms are characterized at early stages by diffuse, roundish discoloured blotches (1–2 cm) on the leaves, which expand and turn subsequently into reddish and purplish dark necrosis, as the fungal stromata develop along the season. The typical leaf symptoms appear after a latent period lasting 30–35 days (Banihashemi, 1990; Saad and Masannat, 1997). The symptomatic leaves fall at the end of summer, earlier than usual and overwinter in the ground in order to produce mature perithecia and ascospores in the fungal stromata. The fungus fails to grow in artificial media and sporadic germination of ascospores are

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observed with no further mycelium development (Habibi and Banihashemi, 2015), which confirms its biotrophic nature.

Little is known about the RLB disease and specifically about the host-pathogen interaction. However, works focused on the disease cultivar sensitivity have reported differential susceptibility of almond cultivars commonly planted in Spain. Thus, most of the settled cultivars in 1980's were classified as susceptible or most susceptible to *P. amygdalinum* by Egea et al. (1984), who checked 81 almond cultivars for RLB susceptibility. In the last decade, different field surveys were carried out to report on the susceptibility of newly generated cultivars to RLB; visual observations have confirmed high susceptibility of 'Guara' and 'Tarraco' and high tolerance of 'Vairo' and 'Mardía' (Vargas et al., 2011; Marimon et al., 2012; Ollero et al., 2016). Ollero et al. (2016) suggested that differential susceptibility among cultivars could be due to the genetic background of varieties. However, the molecular bases of the biology of cultivar tolerance to RLB are currently unknown.

A plethora of plant signaling pathways are induced by biotic stress composing intricate and cross-talked networks in order to activate the appropriate defence responses (Anderson et al., 2004). The phenylpropanoids pathway, with important secondary-derived compounds related to plant-pathogen interactions, plays a crucial role in plant defence (Dixon et al., 2002). Lignin is one of these compounds whose accumulation is considered an active resistance mechanism of plants against pathogens and pests (Maher et al., 1994; Mauch-Mani and Slusarenko, 1996; Wuyts et al., 2006; Tronchet et al., 2010). Pathogen attack induces deposition of lignin in the walls of host cells and this reinforced structure enhances resistance against pathogen invasion (Agrios, 2005; Bhuiyan et al., 2009). Anthocyanins, also derived from the phenylpropanoids pathway, have been secondarily related to biotic stress (Gould, 2004; Reveles-Torres et al., 2018;

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Wang et al., 2017). One of the most extensively studied host-defence compounds is the salicylic acid (SA) that is synthetized from multiples pathways including the phenylpropanoids pathway (Dixon et al., 2002). The SA-dependent signaling pathway leads to the expression of certain pathogenesis-related (PR) proteins that are toxic to invading fungal pathogens (Sherif et al., 2012). In addition to PR proteins, small antimicrobial peptides (generally called defensins) work as host defence compounds relevant in the induced defence system (Wilmes et al., 2011). In peach (*Prunus persica*), a defensin 1 (DFN1) has been described as inductor of destabilization and permeabilization of fungal membranes (Nanni et al., 2013). DFN1 production coupled with the synthesis of SA and phenolics is the main response activated in *P. persica* against *Taphrina deformans* (Svetaz et al., 2017). All these pathogen-induced compounds could be generally described as chemical weapons which function is to combat pathogenic agents (Van Loon *et al*., 2006).

Related to the varietal susceptibility in *Prunus* species, differential levels of phenolic compounds in peach varieties showing differences in susceptibility to the brown rot fungus *Monilinia fructicola* have been reported (Bostock et al., 1999; Lee and Bostock, 2007). To date, no comparable information is available for almond, which reflects the lack of knowledge about the gene regulation induced by biotic stress in this tree species.

According to this preliminary scenario, we aimed to characterize the gene-mediated response as well as the lignin deposition in tolerant and susceptible almond cultivars against the infection of *P. amygdalinum.* The relative expression of key genes from different plant defence pathways and the microscopic visualization of lignin in the fungal infected tissues would indicate us the induced strategies (physical and/or chemical) to

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repress the disease. Two Spanish cultivars, 'Mardía' (tolerant) and 'Tarraco' (susceptible) were analyzed along the sequential development of disease symptoms.

2. Materials and methods

2.1. Plant material

'Mardía' and 'Tarraco' almond trees were sampled in an experimental orchard owned by IRTA and located in Les Borges Blanques, Catalonia, Spain (UTM 31T, X = 320870, Y = 4597530). Individual samples consisting of three leaves were obtained from RLB symptomatic and asymptomatic leaves with four trees (replicates) sampled for each cultivar. Leaves were collected at two different sampling dates: period A (samples harvested in July 5, 2016 with low RLB incidence), and period B (samples harvested in July 28, 2016 with higher RLB incidence). Collected leaves were wrapped with aluminium foils conveniently labelled, immediately frozen in liquid nitrogen in the field and brought to the laboratory. Frozen samples were finely pulverized in liquid nitrogen and then stored at –80 ºC until subsequent processing.

2.2. Pathogen quantification and visualization in almond leaves

DNA from about 100 mg of the pulverized leaf samples was extracted using the NucleoSpin® Plant II kit (Macherey-Nagel, Düren, Germany) following manufacturer's instructions. Extracted DNA was quantified and quality checked with a Nanodrop 2000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA). A specific pair of primers for quantification of *Polystigma amygdalinum* was used: I2F4 [5'-GAAGTCCAATCAAGCCGTAG -3'] and I2R2 [5'-GTTTCACTACGCTCAGAGTC-3'] (forward and reverse, respectively) generating a 99 bp fragment (Zúñiga et al., 2018). Fungal quantification was normalized by the *pre-mRNA splicing factor 7* (SLU7) gene of *P. dulcis* used as reference. qPCR was performed on a CFX 384 Real-Time System (Bio-

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Rad, Hercules, CA, USA). Each reaction contained 5 µL of iTaqTM Universal SYBR®Green Supermix (Bio-Rad, Hercules, CA, USA), 1 µL of the sense and antisense primers and 3 µL of a DNA concentration standardized for all samples. The amplification program in all reactions was performed by pre-incubating the DNA for denaturation at 95 °C for 3 min, followed by 40 cycles of 95 ºC for 10 s and then 60 ºC for 30 s. To assess the amplification specificity, melting curve analysis was always performed at the end of the qPCR reaction, by monitoring the fluorescence from 55 to 95 °C, every 0.1 °C. Three technical replicates were used in all reactions. In addition, a negative control without DNA template were also included in each reaction run. The fungal amount was estimated indirectly from the relative quantification of the fungus in all samples corresponding to four trees per variety and sampling period.

Mycelial colonization of *P. amygdalinum* in almond infected leaves, was visualized using Trypan-blue staining as follows. Asymptomatic leaf areas as well as areas with typical RLB symptoms, from both cultivars, were cut in sections with a sterile scalpel (~ 40 µm thickness) and chlorophyll was removed to enable a clear observation according to the protocol of Liberato et al. (2005). For the chlorophyll removal, leaf sections were immersed in 100 % glacial acetic acid and absolute ethanol (1:1, v/v), placed in a water bath at 60 °C (1 h) and, then dyed with Trypan Blue according to the protocol of Toscano-Underwood et al. (2003). Leaves sections, were mounted on microscope slides and observed under a light microscope (Nikon Eclipse E400, Tokyo, Japan) equipped with a digital camera (Nikon).

2.3. RNA extraction and cDNA synthesis

Total RNA was extracted from around 100 mg of frozen pulverized leaves using the Maxwell® RSC plant RNA kit (Promega Corporation, Madison, WI, USA) according to manufacturer's instructions. Extracted RNA was quantified and quality checked as

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described in section 2.2. One microgram RNA was used in a reverse-transcription reaction to obtain cDNA using the cDNA Synthesis kit (Bio-Rad, Hercules, CA, USA) according to manufacturer's instructions.

2.4. RT-qPCR and gene expression analyses

The expression of seven selected genes involved in defence response were analyzed by reverse-transcription quantitative PCR (RT-qPCR). The studied defence genes were selected from previous works (Melan et al., 1993; Beckman, 2000; Dixon et al., 2002; Sherif et al., 2012; Shahzad et al., 2013; Martos et al., 2016). These genes encode for defensin 1 (*DFN1*), pathogenesis-related protein 1 (*PR1*) and the lipoxygenase enzyme (*LOX*), that synthesizes products of fatty acid oxidation called oxylipins (Porta and Rocha-Sosa, 2002).Furthermore, genes involved in the phenylpropanoids pathway were also included, such as cinnamate 4-hydroxylase (*C4H*), hydroxycinnamoyl CoA quinate hydroxycinnamoyl transferase (*HQT*), and cynnamoyl alcohol dehydrogenase (*CAD*). A late structural gene involved in the anthocyanin biosynthesis from the flavonoid pathway, the leucoanthocyanidin dioxygenase (*LDOX*) was additionally selected. The coding sequences for all genes were taken from the Rosaceae database (https://www.rosaceae.org) and sense and antisense primer pairs (Supplementary Table S1) were designed with the aid of the primer-designing tool of GenBank (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). The *PR1* primer pair was designed over the almond genome, whereas other reference-guided genomes used were those of *P. persica* (*CAD, DFN1*, *HQT*, *LDOX*, *LOX*, and *SLU7*) and *P. armeniaca* (*C4H*). The almond *SLU7* gene was use as reference to normalize the relative expression (RE) of all studied genes, as done in previous works (Chan et al., 2014; Yeap et al., 2014). In addition, *SLU7* showed the most constant expression level as compared to other analysed reference genes in preliminary analyses (*TEF1*, translation elongation factor 1α and *β*-

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TUB, β-tubulin) (*results not shown*). RE of targeted genes were obtained through the $2^{-\Delta Ct}$ formula as described by Livak and Schmittgen (2001). RE values for each defence-related gene were averaged on the basis of each variety and sampling period.

2.5. Validation of designed primers used in RT-qPCR

The suitability of primers designed in this study was tested by sequencing the generated amplicons from *P. dulcis* samples and those from field-collected leaves of *P. persica* and *P. armeniaca*. Leaves of *P. persica* and *P. armeniaca* were collected in 2017 in commercial peach and apricot orchards located in Avinyonet del Penedès, Catalonia (Spain), and processed for RNA extraction and cDNA synthesis as explained above.

Conventional PCR followed by nucleotide sequencing were used to identify and characterize the targeted almond defence-related genes. Reactions were performed in a final volume of 20 µL containing 2 µL H_2O HPLC-grade, 10 µL 2× PCR Master Mix (Biotools B&M Labs, S.A., Madrid, Spain), 1.5 µL of each primer, and 5 µL of sample cDNA. The PCR were performed in a GeneAmp® 9700 thermal cycler (Applied Biosystems®, Foster City, California, USA), with the following conditions: initial denaturation at 95 °C for 3 min, followed by 40 cycles of denaturation at 95 °C for 45 s, primer annealing at 60 °C for 60 s, and extension at 72 °C for 60 s, and a final extension at 72 °C for 5 min. Amplicons were purified using the High Pure PCR Product Purification kit (Roche Diagnostics GmbH, Manhein, Germany) and sequenced by GATC Biotech (Köln, Germany). Sequences were aligned in pairs of *P. dulcis* and *P. persica* or *P. armeniaca* with the BioEdit Sequence Alignment Editor (Hall, 1999). The sequenced *P. dulcis* regions were then submitted to the basic local alignment search tool (BLAST) of GenBank (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to search for similarities with deposited reference sequences. The percentage of identity with the first significant alignment for each specific region and plant species were recorded.

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2.6. Histological observation of lignin deposition in almond leaves

The deposition of lignin was studied in RLB-affected leaves of both selected cultivars. Lignin was stained from leaves of both studied cultivars at different stages of the sequential development of RLB symptoms. RLB lesions from leaves with initial yellowish discolorations and further orange-reddish blotches (stages 1, 2 and 3, as described by Zúñiga et al., 2018) were selected. Transverse sections of leaves were prepared and manipulated as explained in section 2.2. for the observation of fungal colonization in leaf tissues. Lignin was dyed with Wiesner staining (Phloroglucinol–HCl) as described by Pradhan Mitra and Loqué (2014) and visualized with an optical microscope. Sixty leaves per cultivar and symptom stage were stained and observed. Asymptomatic almond leaves were also analyzed to determine the basal content of lignin in both cultivars.

2.7. Statistical analyses

Data from the relative expression of defence genes were analyzed using lineal modelling in R (R Core Team, 2017) and a multifactorial design approach. The original data were log-transformed prior to the analyses to ensure the normal distribution and homocedasticity of data and the independency of errors. Previously, means of technical replications were calculated for each individual combination of factors to reduce pseudoreplication. A preliminary analysis was done with a saturated model including the fixed effects of cultivar (1 df), sampling period (1 df), symptom expression (1 df), defence genes (6 df) and all their possible interactions. Posterior analyses were done separately for each defence gene but keeping the multifactorial approach. Depending on the ANOVA outputs, the models obtained for each individual gene were later simplified by stepwise deletion of non-significant factors and interactions using the R `anova` function and the LRT option. Since all factors and their interactions in the individual gene models

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had 1 df, the significance was declared at $P < 0.05$ and no further mean comparisons were done.

3. Results

3.1. Leaf detection of Polystigma amygdalinum

Polystigma amygdalinum was positively detected by qPCR in leaves exhibiting typical symptoms associated to the RLB (Fig. 1). This result confirmed that the specific primers designed by Zúñiga et al. (2018) targeted correctly the fungal DNA in infected almond leaves. The fitted linear model showed statistical significance of the factors cultivar ($P < 0.001$) and symptom expression ($P < 0.001$), along with the interaction cultivar × symptom ($P < 0.001$) on the relative quantification (RQ) of *P. amygdalinum*. However, the period was not found statistically significant including the interactions with the other two factors. The highest fungal amount was quantified in the symptomatic leaves of the susceptible 'Tarraco' as compared to 'Mardía' which RQ values remained close to zero (range: 0.002 to 0.049, despite of the infective stage and period). Mean RQ value in asymptomatic 'Tarraco' leaves were 0.013 and 0.036 at early and late periods, respectively, whereas same figures in symptomatic leaves were dramatically increased, up to 13.229 (early) and 18.902 (late) (Fig. 1). Additionally, the presence of hyphae presumably corresponding to *P. amygdalinum*, in leaves exhibiting RLB typical symptoms, was visualized with the aid of Trypan-blue staining (Fig. 2). The microscope images confirmed that fungal colonization was limited by the lesion boundaries, without hyphae spreading out of the affected area.

3.2. Suitability of designed primers

The suitability of primers designed in this study to amplify targeted *P. dulcis* genes was checked by different methods. Firstly, band sizes for the seven *P. dulcis* genes were

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similar to those obtained for the guide genomes of *P. persica* and *P. armeniaca* (*results not shown*). Secondly, the alignments of primers pairs between *P. dulcis* and either *P. persica* or *P. armeniaca* showed a 100% of similarity with the reference-guided genomes (*results not shown*). Finally, BLAST searches at GenBank revealed between 95 and 100% of identity with the same targeted genes belonging to *P. persica* and *P. armeniaca* (Supplementary Table S2). These results confirmed that the designed primers were suitable to be used on *P. dulcis*.

3.3. Gene expression of defence genes

The mean values of the relative gene expression for each defence gene as a combination of all main considered factors are shown in Fig. 3. The preliminary ANOVA on the whole dataset of the relative expression of defence genes showed the significance of the factor symptom expression ($P = 0.001$), and the 2-way interaction gene × cultivar ($P = 0.008$). In addition, the factor defence gene and the interaction gene × symptom were near significance ($P = 0.066$ and $P = 0.055$, respectively). Moreover, no 3-way interactions and the 4-way interaction were found significant. A simplified model was consequently fitted by removing all 3-way interactions and higher and reanalyzing the data. The new model showed the significance of factors gene ($P = 0.001$), and symptom expression ($P < 0.001$), and the interactions gene × cultivar ($P < 0.001$), gene × symptom ($P = 0.002$), gene × period ($P = 0.034$), cultivar × period ($P = 0.016$), and symptom × period ($P = 0.007$). These results led us to run separate analyses for each defence gene. The models were again simplified by removing non-significant factors and interactions. Summarized results of the analyses and the significance of factors are shown in Table 1. The genes *C4H*, *LOX* and *PR1* did not show significant 2-way interactions of the main factors, and in the latter two genes, a significant cultivar effect was detected in the relative gene expression. *CAD*, *DFN1* and *HQT* genes showed 2-way interactions between the

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main factors: cultivar × symptom in case of *DFN1* and *HQT*, cultivar × period in case of *CAD*, and symptom × period in case of *CAD* and *DFN1* (Table 1, Fig. 3). Finally, the gene *LDOX* showed a significant 3-way interaction, with no other significance detected among factors. The relative expression of genes depending on the infection status showed significant differences in the case of *C4H* and *PR1* genes, with consistent higher expression in symptomatic leaves than in the asymptomatic ones. Regarding the sampling period, this factor was shown to be only significant in the relative expression of *PR1*.

Genes *CAD* and *DFN1* showed a similar pattern of expression in 'Mardía', with highest mean values (4.93, 4.23, respectively) observed in symptomatic leaves sampled early in the season, as compared to the remaining values (Fig. 3B and C). On the other side, *C4H*, *HQT* and *LDOX* were highly expressed in 'Tarraco' symptomatic leaves sampled as compared to asymptomatic ones (Fig. 3A, D and E). 'Tarraco' showed a higher relative expression for *LOX* and *PR1* (mean values over periods and infection status: 2.26 and 2.40, respectively) as compared to 'Mardía' (1.38 and 1.23, respectively). In addition, the sampling period and the infection status were statistically significant in *PR1*, with higher expressions for the late period and, separately, in the symptomatic leaves. Finally, a significant effect of the infection status was detected for *CH4* and *PR1*, thus indicating increased RE at symptomatic leaves compared to the asymptomatic ones (mean values: 2.98 and 0.65, respectively) (Fig. 3A and G).

3.4. Lignin deposition in leaf fungal-infected areas

RLB asymptoms leaves did not show red coloration associated to Wiesner staining of lignin in any cultivar (Fig. 4A and E). The positive detection of lignin was observed on earlier sampled leaves of 'Mardía' (Fig. 4B and C). Deposition of lignin was clearly localized in cell walls as it can be observed in Fig. 4C. Similarly, leaves of 'Mardía' with advanced RLB symptoms showed higher lignin accumulation as compared to 'Tarraco'

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(Fig. 4D and H, respectively). On the contrary, 'Tarraco' leaves were determined Wiesner-negative in symptomatic leaves collected at early stages of infection, since red coloration was absent in the observed structures (Fig. 4F and G). On the advanced symptoms stage (Fig. 4H), 'Tarraco' presented red lignin coloration more expanded and less accumulated as compared to 'Mardía'. However, symptomatic leaves of 'Tarraco' were characterized by noticeable orange-reddish blotches at both infection stages. Microscopic observation revealed the diffuse presence of this orange color that could not be designated to any plant structure and remained even after chlorophyll removal (Fig. 4G and H). Additionally, a higher number of swollen and dead cells were found in the RLB symptomatic leaves of 'Tarraco' as compared to 'Mardía'.

4. Discussion

Almond susceptibility to RLB has been determined to date based on field observations of symptom incidence and severity (Egea et al., 1984; Saad and Masannat, 1997; Vargas et al., 2011; Marimon et al., 2012; Ollero et al., 2016). So far, the molecular and biochemical bases of this pathogen-plant interaction had not been studied. This is the first approach to understand these susceptibility differences, by analyzing the relative expression of selected putative defence genes of two almond cultivars, 'Mardía' (tolerant to RLB) and 'Tarraco' (susceptible to RLB), under the hypothesis that changes in their transcriptomes would reflect their differential susceptibility response. The seven selected genes studied here comprise five different plant defence strategies: i) phenylpropanoids pathway represented by *C4H*, *HQT* and *CAD* genes, ii) flavonoids pathway by *LDOX* gene, iii) defensin (*DFN1*), iv) pathogen-related protein (*PR1*) and, v) fatty acid oxidation catalyzed by the lipogenase enzyme (*LOX*). Four of the seven selected genes are involved on the phenylpropanoids and flavonoids pathways deriving into the biosynthesis of lignin

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and anthocyanin, respectively. Thus, the lignin deposition was also visualized, as it is one of the best-known plant mechanism induced after pathogen attack (Maher et al., 1994; Dixon et al., 2002; Tronchet et al., 2010; Xu et al., 2011). The pathogen-induced response is the cell wall lignification of the infection-surrounding cells that creates a reinforced physical barrier more efficient to confine the pathogen expansion (Agrios, 2005).

Statistical analysis of gene expression by RT-qPCR indicated that 'Mardía' and 'Tarraco' were reliable candidates for a comparative molecular analysis of susceptibility to RLB. Our results revealed a cultivar-dependent expression pattern of the selected genes. Up-regulation of *CAD* in the tolerant cultivar 'Mardía' during the early expression of RLB symptoms was relevant. *CAD* belongs to the phenylpropanoids pathway as a structural biosynthetic enzyme involved in the late step of the monolignol synthesis (Cheng et al., 2017). Pathogen infections strongly activate the phenylpropanoids pathway in plants and it has been widely documented for fungal pathogens (Xu et al., 2011; Dhokane et al., 2016). Compounds derived from the penylpropanoids pathway show a broad-spectrum of antimicrobial activity and some of them have been specifically associated to *Prunus* pathogens (Bostock et al., 1999; Lee and Bostock, 2007; Villarino et al., 2011). Several lines of evidence indicate that the reinforcement of plant cell walls, mainly lignification, is associated to increased tolerance to microbial pathogens (Dixon et al., 2002; Xu et al., 2011; Hu et al., 2018). *CAD* inhibition resulted in reduced lignin content and increased disease susceptibility to *Peronospora parasitica* in *Arabidopsis* (Mauch-Mani and Slusarenko, 1996). In our study, the increased induction of *CAD* would indicate a greater lignin accumulation in the tolerant cultivar. In fact, 'Mardía' displayed lignin deposition around cells in the infected area in leaves with early and more advanced RLB symptoms, as confirmed by the lignin staining. On the contrary, 'Tarraco' leaves did not show lignin accumulation surrounding the cells in the early infection stages, and

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weak Wiesner-positive staining was observed only in advanced symptoms. This is in accordance with a higher expression of *C4H* and *HQT* in 'Tarraco' at late infection stages; these genes are involved in first steps of the phenylpropanoids pathway. Accumulation of lignin has been already described in *Prunus* spp. affected by the shot-hole disease (caused by the fungus *Wilsonomyces carpophilus*), specifically in the cells layers nearest the infection (Samuel, 1927). It is suggested that the early intense response in 'Mardía' triggers cell wall lignification that repress the pathogen growth and thus characterize the cultivar with a tolerant profile. Conversely, the accumulation of lignin occurred in advanced infections stages in 'Tarraco', but appeared to be ineffective as the pathogen managed to establish the infection. Furthermore, the increased expression of *DFN1* gene in 'Mardía' could indicate the activation of other defence mechanism such as the production of defensins, peptides with described toxic activity for pathogens (Stotz et al., 2009). Defensins play a role in plant innate immunity displaying antifungal activity against wide spectrum of pathogens (Nanni et al., 2013). It has been repported that transgenic plants overexpressing defensins have been shown as highly resistant against fungal pathogens (Stotz et al., 2009).

The flavonoids pathway is also activated after pathogen attack with a significant contribution to plant resistance (Treutter, 2005). Flavonoids are classified in six major groups, being anthocyanins one of those groups (Falcone Ferreyra et al., 2012). Similar to lignin, anthocyanins are synthesized from Phenylalanine, and both pathways share the first three steps (Shi et al., 2015). Recently, Usenik et al. (2017) showed that *Plum pox virus* (Sharka) promotes the accumulation of anthocyanins among other flavonoid compounds in the necrotic tissues of *Prunus domestica*. A late gen of the anthocyanins pathway is *LDOX*, which several evidences demonstrated its critical role in anthocyanin accumulation in members of the Rosaceae family (Aharoni et al., 2001; Salvatierra et al.,

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2013). This gene showed a higher expression on symptomatic leaves of the susceptible cultivar when the RLB symptoms were advanced. This resulted in an orange-reddish pigment accumulation in infected 'Tarraco' leaves, as it was described by Tanaka et al. (2014) in maize plants challenged by *Ustilago maydis*. Flavonoids were accumulated diffusely in the symptomatic area, not localized to any plant structure, dissimilarly to lignin that is accumulated in cell walls. We hypothesize that an increase in anthocyanin production negatively affected the lignin biosynthetic pathway by reducing the levels of the common precursor *p*-coumaric acid. This was already evidenced by Van der Rest *et al.* (2006) who studied tomato transgenic plants exhibiting reduced lignin content but higher amounts of chlorogenic acid and rutin. The increased activity of *HQT* observed in our study would deviate the *p*-coumaric acid to the synthesis of chlorogenic acid instead of lignin that hence reduced its potential protective effects. It was observed that 'Tarraco' activated the production of lignin later than 'Mardía' and also triggered alternative responses (anthocyanins and phenolic acid synthesis) which, all in all, would have reduced lignin accumulation in infected leaves and thus failed to repress the growth and infection of *P. amygdalinum*. This should be further investigated in order to confirm this hypothesis.

The molecular detection of *P. amygdalinum* in leaves was correlated to the cultivar susceptibility observed on the field. Higher amounts of fungal biomass were estimated in 'Tarraco' symptomatic leaves, while pathogen quantification in 'Mardía' was minimal despite of the infection stage. In both cultivars, *P. amygdalinum* was detected in asymptomatic leaves, although with minimal values. This could be due to: 1) the high sensitivity of the specific primers, as Zúñiga et al. (2018) reported a minimum detection of 7 ascospores, and 2) the extreme long latency period (30-35 days) reported for *P. amygdalinum* (Banihashemi, 1990; Saad and Masannat, 1997) which could allow the

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fungus for a sufficient mycelial accumulation in leaves to be detected by qPCR even in absence of noticeable symptoms.

The already known biology data recorded for *P. amygdalinum* (Banihashemi, 1990; Cannon, 1996; Habibi and Banihashemi, 2015) combined with the images presented in this study would indicate a hemibiotroph lifestyle of the pathogen. Plant pathogens classified as hemibiotrophs show a multistage infection strategy (O'Connell et al., 2012). In this type of pathogen-plant interactions, several genes and cross-talking signals between pathways are involved, such as the SA signaling pathway (effective against biotrophs) and the jasmonic (JA) and ethylene (ET) signaling pathways (effective against necrotrophs) (Kunkel and Brooks, 2002). The genes studied here (*C4H*, *HQT*, *LOX* and *PR1*) are involved in these pathways but it would be risky to jump onto solid conclusions and more genes should be studied. Our preliminary results opens the door to make future investigations focusing on other defence pathways in order to draw a complete schema of the almond strategies to fight against the RLB disease. Moreover, RNAseq could be a comprehensive approach to discover the set of genes participating in the coordinated plant response to the RLB disease.

5. Conclusion

The RT-qPCR technique may represent a suitable and economic tool to screen almond cultivars for tolerance to RLB. From our results, it can be concluded that 'Tarraco' and 'Mardía' have different molecular and biochemical strategies to face *P. amygdalinum* that could explain their differential susceptibility to the fungal disease. The tolerant cultivar 'Mardía' displays a lignin accumulation in infected leave tissues and likely the production of antifungal compounds (defensins); a dual strategy that would combine physical and chemical responses to repress the fungal spread during the early

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expression of RLB symptoms. On the contrary, the susceptible cultivar 'Tarraco' activated later the lignin synthesis likely together with the production of anthocyanin and phenolic acid, showing to be ineffective in inhibiting fungal growth. The lignin biosynthesis was revealed as a key response to repress the RLB pathogen at early stages of the disease spread.

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Conflict of interest statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interests.

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FIGURE CAPTIONS

Fig. 1. Mean values of *Polystigma amygdalinum* relative quantification (RQ) in two almond cultivars, 'Mardía' and 'Tarraco', under early and advanced infection stages. RQ values are shown with a break in Y-axis because of higher observed RQ values in symptomatic 'Tarraco' leaves. Abbreviations: RQ, relative quantification; Asym, asymptomatic leaves; Symp, symptomatic leaves; A and B, early and late sampling periods, respectively. Error bars show the standard error of the mean.

Fig. 2. Transversal sections of asymptomatic (A) and symptomatic (B) areas after Trypan Blue staining to show the mycelial colonization by *Polystigma amygdalinum* in a representative almond leaf ('Tarraco'). Abbreviations: Ph, *Polystigma amygdalinum* hyphae. Microscope magnification: ×200. Bar = 50 µm.

Fig. 3. Mean values of seven defence genes expression in two almond cultivars, 'Mardía' and 'Tarraco', showing differential susceptibility to *Polystigma amygdalinum*. Abbreviations: RE, relative expression; Asym, asymptomatic leaves; Symp, symptomatic leaves; A and B, early and late sampling periods, respectively. Gene names at the top of each graph. Error bars show the standard error of the mean (N = 4). Least significant difference values shown as bold bars in the top left part of graphics.

Fig. 4. Microscope images of asymptomatic, initial and advanced symptoms caused by *Polystigma amygdalinum* infection in 'Mardía' (A to D) and 'Tarraco' (E to H) leaves, stained with Wiesner staining. Representative images from 60 observed leaves per cultivar and stage of symptomatology. A and E, Non-infected; B, C, F, G, initial symptoms; D and H, advanced symptoms. Transversal sections in A, B, D, E, F and H. Upper view in C and G. Legends: Wp, Wiesner-positive; *, Dead cells. Microscope magnification: ×200 (A, B, C, D, E, G, H); ×400 (F). Bar = 50 µm.

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FIGURES

(all figures in low resolution for reviewing purposes)

Figure 1

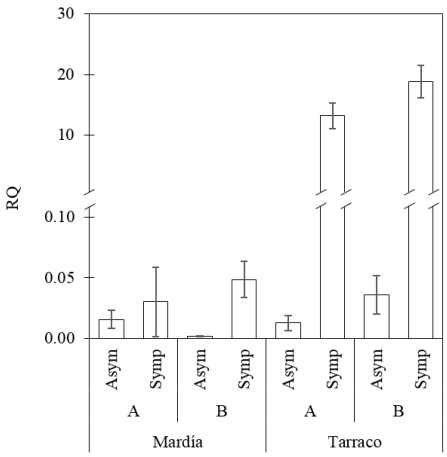

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Figure 2

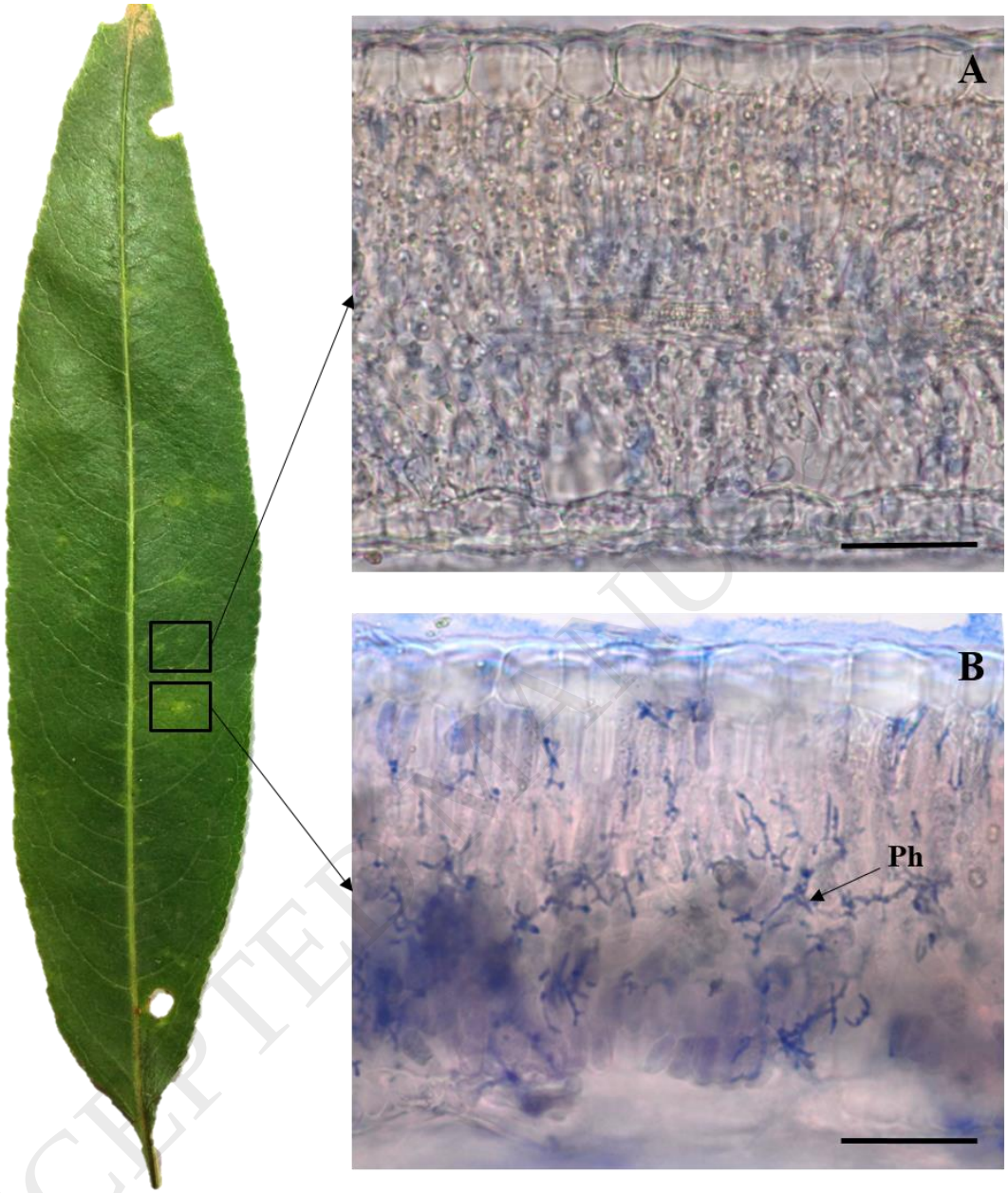

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Figure 3

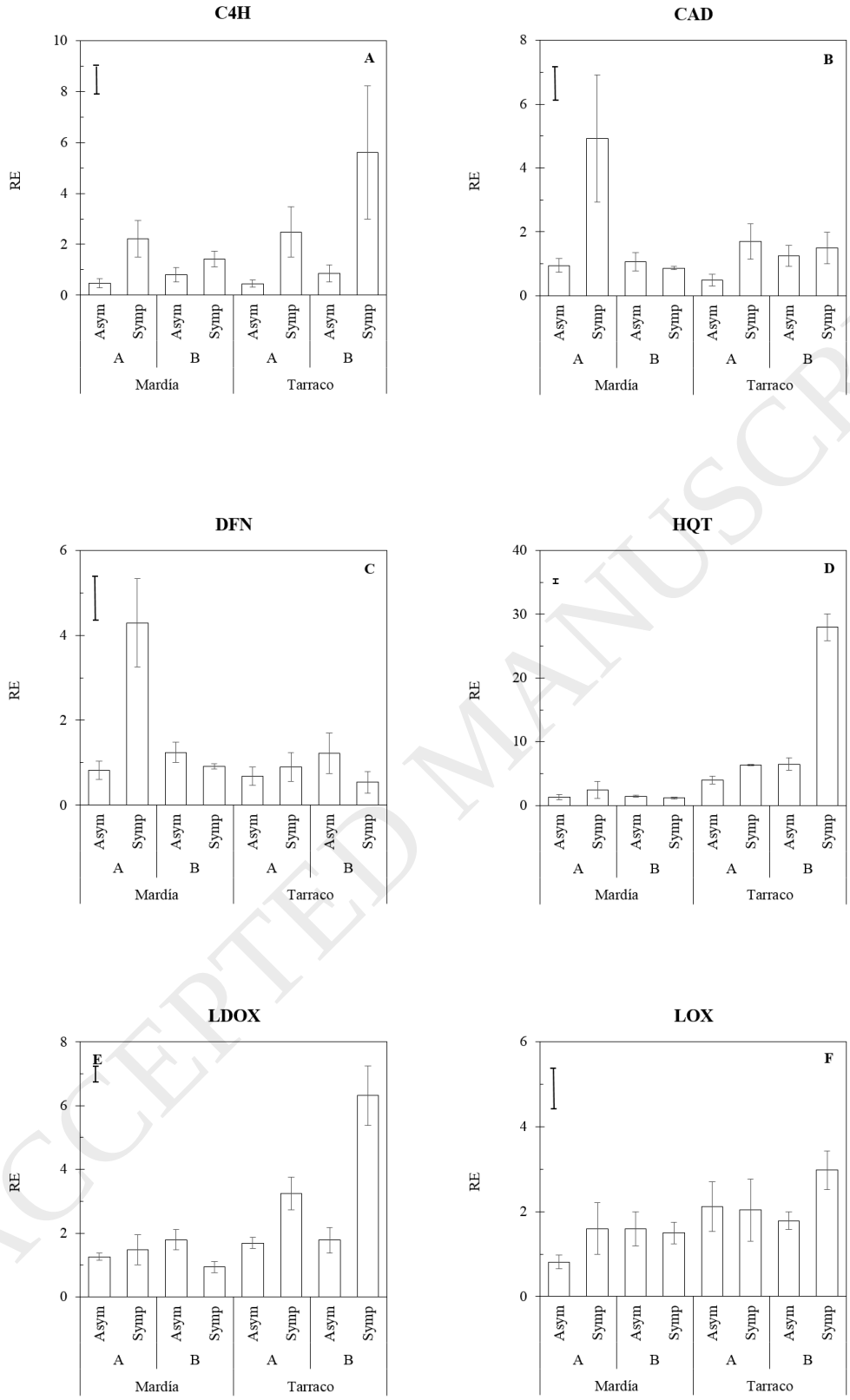

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Figure 3 (cont.)

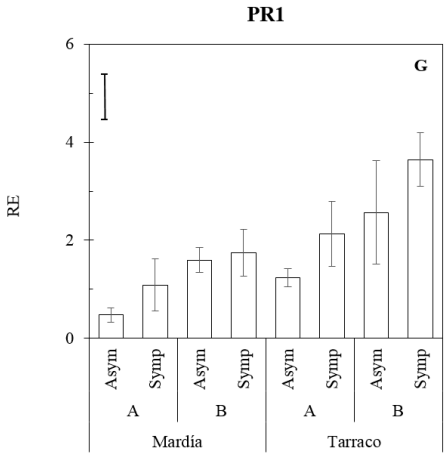

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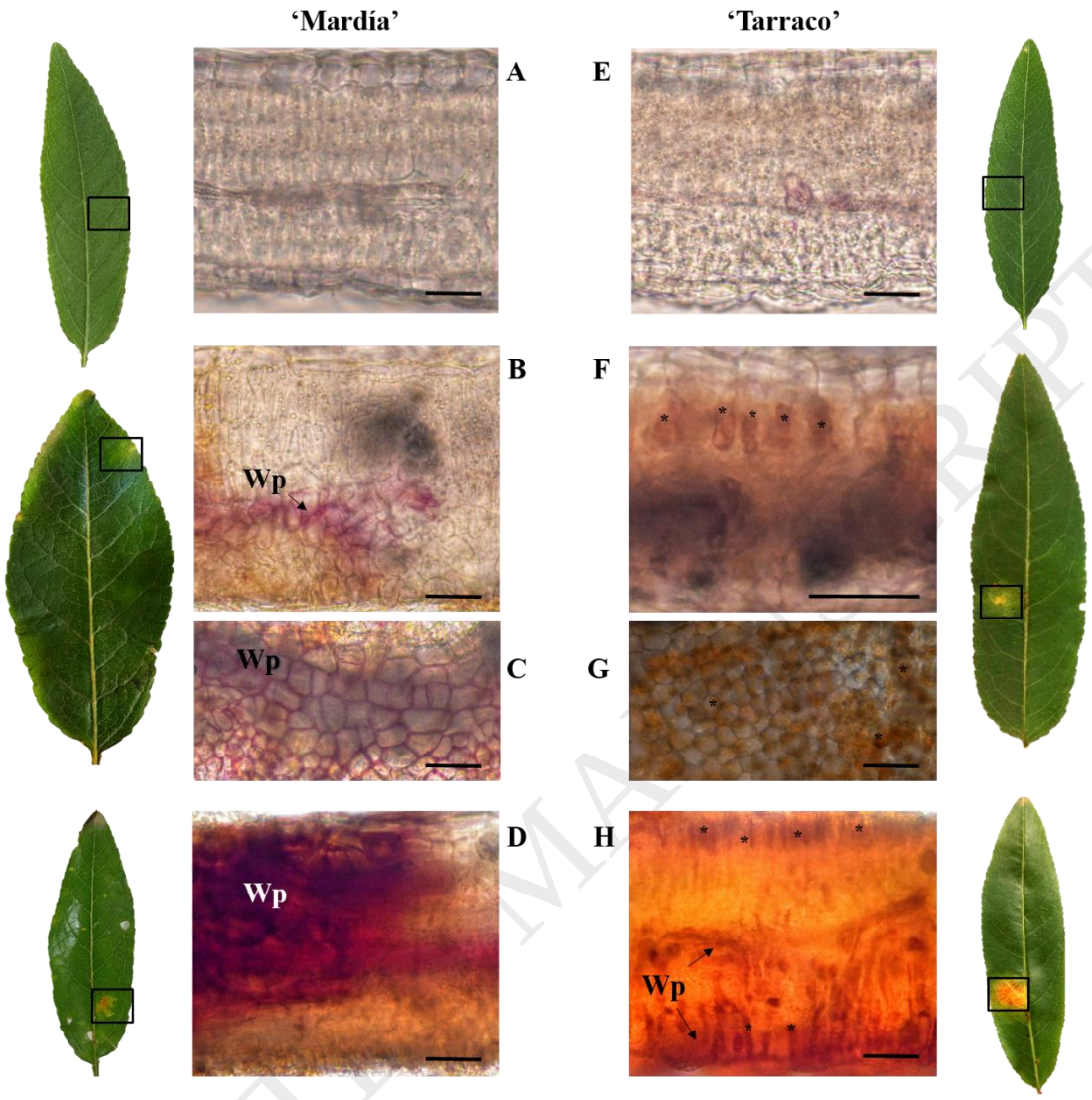
Figure 4



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Tables

Table 1. F-values (F) and significance (*P*) of the fitted models in the study of expression of defense genes in two almond cultivars ('Mardía' and 'Tarraco') showing differential susceptibility to the red leaf blotch pathogen *Polystigma amygdalinum*.

Gene	**Main factors**			**Interactions**				**Model**		
	CV	*Sym*	*Per*	*CVxSym*	*CVxPer*	*SymxPer*	*CxSxP*	*F*	*Res df*	*P*
C4H	F=1.227 ns	F=26.208 *P*<0.001	F=1.935 ns					10.060	27	<0.001
CAD	F=4.629 *P*=0.041	F=8.876 *P*=0.006	F=0.003 ns	F=0.042 ns	F=5.250 *P*=0.031	F=6.938 *P*=0.014		3.544	25	0.011
DFN1	F=1.661 ns	F=10.443 *P*=0.004	F=0.163 ns	F=4.468 *P*=0.045	F=1.311 ns	F=8.921 *P*=0.006		4.722	24	0.003
HQT	F=6.560 *P*=0.018	F=0.004 ns	F=0.027 ns	F=5.081 *P*=0.034	F=3.825 ns	F=0.069 ns		14.24	22	<0.001
LDOX	F=1.231 ns	F=0.005 ns	F=1.497 ns	F=2.545 ns	F=0.668 ns	F=3.149 ns	F=5.596 *P*=0.028	8.652	21	<0.001
LOX	F=6.429 *P*=0.017	F=0.991 ns	F=3.071 ns					3.501	27	0.028
PR1	F=14.906 *P*<0.001	F=4.276 *P*=0.047	F=17.038 *P*<0.001					12.070	28	<0.001

Abbreviations: CV, cultivar; Sym, symptom expression; Per, sampling period; Res df, Residual degrees of freedom.

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