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A Comparative Transcriptome Analysis of Avocado Embryogenic Lines Susceptible or Resistant to *Rosellinia necatrix* Exudate

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Abstract: Avocado embryogenic cultures were selected for resistance to the culture filtrate (CF) of *Rosellinia necatrix*, the causal agent of White Root Rot disease. A resistant callus line was obtained through recurrent selections in progressively increasing concentrations of fungal CF (from 60% to 80%). RNA sequencing (RNA-Seq) technology was used to compare the transcriptomic profiles of the avocado embryogenic-callus-resistant line L3 (capable to survive in the presence of 80% CF) and control line AN-9 (not exposed to CF), after 24 h of growth in a medium containing 40% CF. A total of 25,211 transcripts were obtained, of which 4,918 and 5,716 were differentially expressed in the resistant and control line, respectively. Interestingly, exposure of embryogenic callus lines to 40% of *R. necatrix* exudates induced genes previously reported to be related to avocado defense against fungal diseases (lignin biosynthesis, Pathogenesis Related (PR) proteins, WRKY (WRKYGQK) Transcription Factor (TF), NAC (NAM, ATAF1/2, and CUC2) TF, proteinase inhibitors and Ethylene Response Transcription Factor (ERF), among others), which were accumulated in greater amounts in the resistant line in comparison to the susceptible one. This research will contribute to the understanding of avocado defense against this pathogen, thereby aiding in the selection of resistant avocado rootstocks.

Keywords: avocado; embryogenic culture; *Rosellinia necatrix*; resistant genotypes; transcriptomic analysis

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1. Introduction

Avocado (*Persea americana* Mill.), a member of the *Lauraceae* family, is an economically important fruit crop consumed in more than 60 countries. Worldwide, the production and harvested area of avocado have shown average increases of ~7.91% and ~6.84%, respectively, over the last few years [1]; however, avocado crop yields are rising at a slower rate (~1.07% increase per year) [1]. This difference could be explained by the incidence of avocado diseases. Two of the most important soil-borne pathogens affecting avocado orchards in Spain are the oomycete *Phytophthora cinnamomi* Rands, causing *Phytophthora* Root Rot (PRR), and the ascomycete *Rosellinia necatrix* Prill, causing White Root Rot disease (WRR) [2]. *P. cinnamomi* affects avocado orchards worldwide, but the disease caused by *R. necatrix* has a higher incidence in temperate regions [2]. Nevertheless, the fact that *R. necatrix* can colonize over 170 different plant hosts [3], some of them of economic importance, and the presence of this fungus in other areas such as California, México, Korea, South Africa and, particularly, Spain, have increased the interest in this pathogen [4].

Avocado trees affected by *R. necatrix* can show aerial and root symptoms, both because of damaged roots when hyphae invade and penetrate the primary and secondary xylem and due to the release of toxic compounds inside the vascular system [2]. As mechanical actions, enzymatic and toxigenic activities also appear to be important in *R. necatrix* infection [5] and could be related to aerial symptoms observed in plants [6]. This pathogen produces different metabolites with phytotoxic effects such as rosellichalasin [7,8] diketopiperazines, rosellic acid and rosnecatrone [7], and cytochalasin E, which has a direct effect on photosynthesis [9], although their roles in pathogenicity need to be clarified [10]. Zumaquero et al. [11], in their analyses of the *R. necatrix* transcriptome during infection of susceptible avocado, observed the up-regulation of genes related to the production of fungal toxins, two of them previously related to the biosynthesis of aflatoxin, a very toxic and carcinogenic mycotoxin.

Fungal features complicate the control of WRR disease; e.g., *R. necatrix* shows resistance to drought and soil acidity, is capable of penetrating deeply into the soil, and is tolerant to several common fungicides [4,5]. Current control approaches involve the use of chemicals such as the fungicide fluazinam [5]; physical control through solarization; biological control with some bacteria, especially species of *Agrobacterium* and *Pseudomonas,* which have shown an antagonistic effect against this fungus [12]; and cultural methods to prevent fungal infection. Nevertheless, an integrated approach using biological control and tolerant rootstocks is highly recommended for better and sustainable control of this pathogen [12,13]. However, there are currently no tolerant selections available to growers. To overcome this problem, IFAPA-Málaga has been involved for over a decade in a breeding program to obtain avocado rootstocks tolerant to WRR, and some selections are being evaluated under field conditions (A. Barceló-Muñoz, IFAPA-Málaga, personal communication, February 2023).

Avocado breeding programs require a long time to obtain the genotype of interest; hence, attempts are being made to help in the selection of trees, such as molecular studies of avocado/pathogen interaction [13–16] to identify markers that can speed up the selection process.

In vitro cell culture has been shown to be a valid tool to elucidate plant defense mechanisms against fungi. In *Cicer arietinum*, cell cultures of resistant and susceptible cultivars showed different behavior following exposure to an *Ascochyta rabiei*-derived elicitor, with a highly inducible protein drastically increased in the resistant cultivar. Moreover, this cultivar also accumulated a higher amount of phytoalexins [17]. Using grape calli, Ramirez-Suero et al. [18] demonstrated that extracellular compounds produced by *Neofusicoccum parvum* were more aggressive than those of *Diplodia seriata*; among these compounds, proteins seem to play a key role and could be involved in the induction of more defense genes in calli exposed to *N. parvum* [19]. Using an autoclaved biomass fungal extract of *Phaemoniella chlamydospora* to elicit grape cell cultures, Lima et al. [20] found the overexpression of classes 6 and 10 pathogenesis-related (PR) genes in these cells, concluding that this system was a reliable approach to study this interaction.

The in vitro cell culture system is generally associated with the occurrence of somaclonal variation [21]. The selection of cell lines through recurrent selection pressure, such as exposure to gradually increased concentrations of fungal culture filtrate, phytotoxins, or secreted elicitors, has been used to obtain cell lines and regenerated plants with disease resistance [22–25]. During this process, genetic changes have occurred, which will be inherited by the offspring. According to Lestari [26], this approach has the advantage of being more accurate for obtaining resistance to a specific type of stress; in addition, it shortens the time required to select for desirable traits [27].

This investigation attempted to obtain an avocado cell line resistant to *R. necatrix* filtrate through recurrent exposures to the filtrate. Afterward, a comparative transcriptomic study was carried out to evaluate the response of resistant vs susceptible cells, after a new exposure to the fungal filtrate. Obtained responses were compared to those observed on susceptible and tolerant genotypes following inoculation with this pathogen under greenhouse conditions. This study will help to evaluate the usefulness of avocado cell

cultures to study avocado–*R. necatrix* interaction and will shed light on the role played by extracellular compounds on the pathogenicity of this fungus and subsequent response at the cell level.

2. Materials and Methods

2.1. Plant Material

The avocado embryogenic callus line AN-9, derived from an immature zygotic embryo of cv. "Anaheim", susceptible to *R. necatrix*, was used in this study. This line had been established in Murashige and Skoog (MS) medium [28] supplemented with 30 g L^{-1} sucrose and 0.1 mg L^{-1} picloram (MSP medium) and solidified with 6 g L^{-1} agar in darkness at 25 ± 1 °C [29]. Callus was maintained in the same medium used for culture initiation with subcultures at 4-week intervals. The medium pH was adjusted to 5.74 before autoclaving at 0.1 MPa and 121 °C for 20 min.

2.2. Preparation of Crude Filtrate from Rosellinia necatrix

The virulent CH53 fungal strain of *R. necatrix*, isolated at Almuñecar (Granada, Spain) [2] was cultured on potato dextrose agar (PDA; OXOID) in darkness at 25 °C. To obtain the fungal filtrate, six 0.5 × 1 cm fragments of PDA with fungal mycelium were incubated on the surface of 200 mL of Potato Dextrose Broth (PDB; DifcoTM, Líbano, 2025) in 2 L Erlenmeyer flasks over 21 days at 25 °C in darkness. This medium was filtered out through 2 layers of filter paper to eliminate hyphae and debris from the fungus; the medium obtained, containing the fungal exudates, was sterilized through Whatman ® 0.2 µm filters (LLG Labware, Germany, 2026), frozen in liquid nitrogen, and stored at −80 °C until use.

2.3. Obtainment of a Callus Line Resistant to Fungal Filtrate

For this assay, a small fraction of the embryogenic culture was used. This fraction was obtained following incubation of 0.4 g embryogenic callus into 40 mL of liquid MSP medium for 9 days at 120 rpm and 25 °C in darkness; thereafter, the culture was sequentially sieved through a 2 mm and then a 1 mm pore mesh, selecting the retained fraction over the 1 mm pore.

Initially, an experiment to test the effect of the fungal culture filtrate (CF) and of the medium used to grow the fungus (PDB) on avocado embryogenic callus proliferation was carried out. 200 milligrams of AN-9 embryogenic callus were incubated for 1 week in 20 mL of liquid MSP supplemented with 0, 20, 40, 60, and 80% (*v*/*v*) of PDB or *R. necatrix* CF, both sterilized through 0.2 µm filters at 120 rpm and 25 °C in darkness, followed by 1 month of culture on solid MSP medium. Afterward, the increase in callus weight was recorded. Three replicates were used for each treatment.

To obtain a cell line resistant to *R. necatrix* CF, 400 mg of this fine fraction was incubated in 40 mL MSP medium supplemented with 60% (*v*/*v*) *R. necatrix* CF for 1 week at 120 rpm and 25 °C in darkness; afterward, the embryogenic callus was cultured on solid MSP medium without fungal filtrate for cell recovery. The callus was recultured monthly (3 or 4 times) until a sufficient amount of newly grown callus was obtained; afterward, a small fraction of this callus was obtained and further exposed to 60% CF with subsequent culture in solid MSP medium, as previously indicated. The small callus fraction of the newly grown callus was further exposed twice to a higher concentration of CF (80%), as previously indicated, for the 60% CF treatment. The callus growing after the second exposure to 80% CF was selected as a resistant embryogenic line (L3). The experiment was initiated with 6 flasks. Figure 1a shows the workflow followed to obtain the resistant embryogenic line.

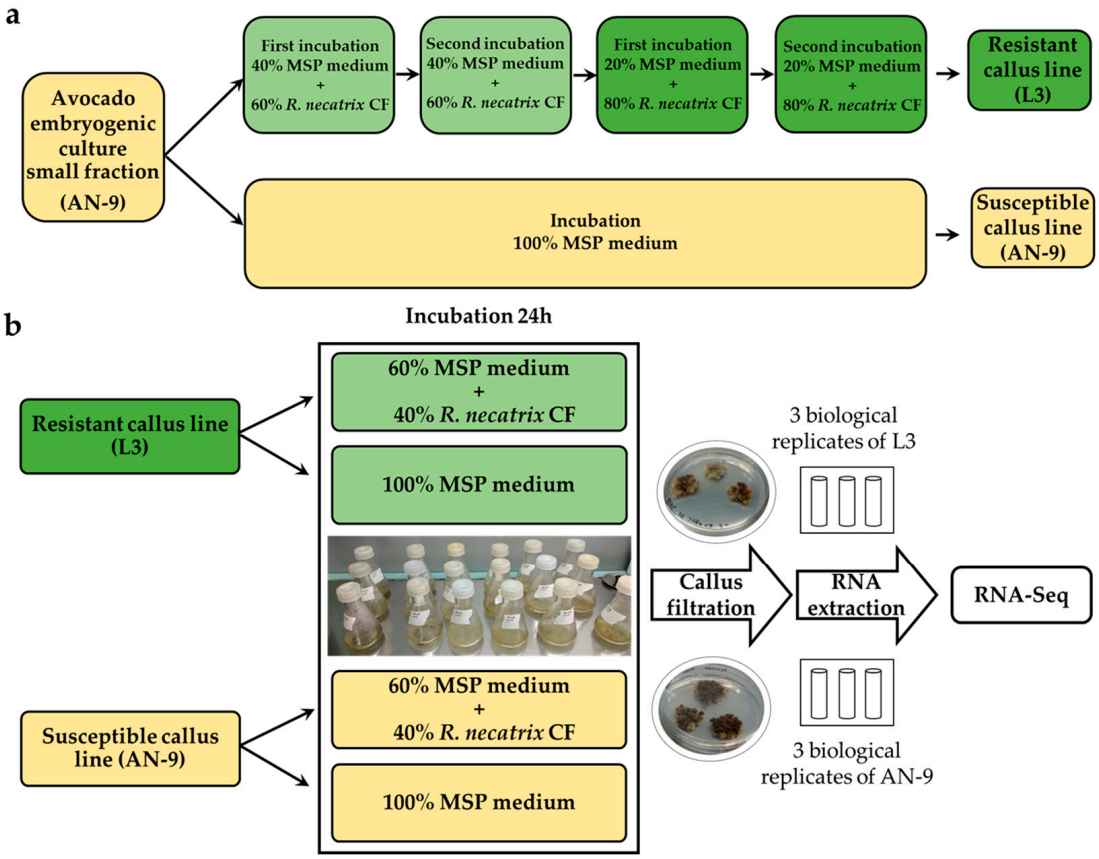

Figure 1. (**a**) Workflow to obtain a resistant avocado embryogenic line to *R. necatrix* culture filtrate (CF). The line was considered resistant after being exposed twice to Murashige and Skoog medium with 0.1 mg L^{-1} picloram (MSP) supplemented with 60% (*v*/*v*) CF, followed by two exposures to MSP supplemented with 80% (*v*/*v*) CF. A small 1–2 mm fraction of embryogenic callus was used throughout the process. (**b**) Schematic representation of the transcriptome analysis carried out in resistant (L3) and susceptible (AN-9) avocado callus lines after 24 h of growth in MSP medium and MSP medium containing 40% of *R. necatrix* CF.

2.4. Experimental Design

To evaluate differential gene expression between susceptible (AN-9) and resistant (L3) lines to *R. necatrix* CF, embryogenic calli of both lines were incubated in an MSP liquid medium containing 40% of *R. necatrix* filtrate for 24 h at 120 rpm and 25 °C in darkness. Controls were obtained by growing the callus of both lines in the MSP liquid medium without fungal filtrate. After the incubation period, the embryogenic calli were removed from the medium, weighed, and frozen in liquid nitrogen. The experiment was laid out in a randomized block design with three biological replicates per treatment in which each biological replicate consisted of three embryogenic callus groups that were 0.4 g each (Figure 1b).

2.5. RNA Extraction

For RNA extraction, the embryogenic calli from L3 and AN-9 exposed and not exposed to fungal exudates were macerated with liquid nitrogen using a pestle, and 0.1 g of frozen powder was collected in a 2 mL Eppendorf. Afterward, RNA was extracted using a Spectrum plant RNA kit (Sigma Aldrich, St. Louis, MI, USA) following the manufacturer's instructions.

DNAse I (DNAse I, Thermo, Waltham, MA, USA) treatment was carried out after the extraction process. RNA was purified using a NucleoSpin RNA kit (Macherey-Nagel, Germany) following the manufacturer's instructions.

RNA quantity and quality were determined based on absorbance ratios at 260 nm/280 nm and 260 nm/230 nm using a NanoDrop® ND-1000 (Nanodrop Technologies, Inc., Wilmington, NC, USA) spectrophotometer. RNA integrity was confirmed with the appearance of ribosomal RNA bands and lack of degradation products after separation on a 1.2% agarose gel and Red Safe (Intron Biotechnology, WA, USA) staining.

2.6. Transcriptome Analysis

The integrity and quantitation of RNA were assessed using the RNA Nano 6000 Assay Kit of the Bioanalyzer 2100 system (Agilent Technologies, CA, USA). A total of 1 microgram of RNA from each sample was used for RNA preparations. Sequencing libraries were generated using NEBNext® UltraTM RNA Library Prep Kit for Illumina® (NEB, MA, USA) according to the manufacturer. Index codes were added to attribute sequences to each sample. A cBot Cluster Generation System was employed to perform the clustering of the index-coded samples using the PE Cluster Kit cBot-HS (Illumina) following the manufacturer's instructions. Paired-end reads were generated by sequencing the library preparations on an Illumina platform. Raw data in a FASTQ format were processed using fastp. Raw reads were processed to remove reads containing adapter and poly-N sequences and reads with low quality, and the Q20, Q30, and GC content of the clean data (clean reads) were calculated. High-quality paired-end clean reads were mapped to the reference genome downloaded from the genome website browser (NCBI) using HISAT2 software. Featured counts were used to quantify the read numbers mapped of each gene. Reads Per Kilobase of exon model per Million mapped reads (RPKM) of each gene were calculated based on the length of the gene and the reads count mapped to this gene. Differential expression analysis of three biological replicates per condition was performed using the DESeq2 R package. Benjamin and Hochberg's approach was used to adjust *p*-values for controlling the False Discovery Rate (FDR) and to select genes with an adjusted *p*-value < 0.05 as differentially expressed genes (DEGs). The DEGs were identified using the following conditions: |fold change (FC)| > 2 and *Padjust* < 0.05. The data from this study are available from the NCBI Gene Expression Omnibus under accession number GSE228295.

2.7. Gene Predictions and Annotation

Gene Ontology (GO) and KEGG enrichment analysis of differentially expressed genes were implemented with the cluster Profiler R package. Afterward, the gene length bias was corrected. GO terms were considered significantly enriched by differentially expressed genes with corrected *p*-value < 0.05.

2.8. Quantitative Real Time-PCR

Gene expression levels from transcriptome analysis were validated by qRT-PCR. Single-stranded cDNA was synthesized using the iScript cDNA synthesis kit (BIO-RAD, CA, USA), following the manufacturer's instructions. The concentration and purity of cDNA were determined using a NanoDrop® ND-1000 (Nanodrop Technologies, Inc., Montchanin, DE, USA), and cDNA integrity was confirmed by separation on a 1.2% agarose gel and Red Safe (Intron Biotechnology) staining. The expression of 6 avocado genes was studied. Actin was used as an endogenous control gene for normalization. Primer pairs were chosen to generate fragments between 50 and 150 bp with a melting temperature of 60 °C and designed using Primer 3 software [30–32]. Primers are presented in supplementary Table S1.

Primer specificity was tested, firstly by performing a conventional PCR, and confirmed by the presence of a single melting curve during qRT-PCR. Serial dilutions (1:10, 1:20, 1:50, 1:200) were made from a pool of cDNA from each treatment and time-point, and calibration curves were performed for each gene. For qRT-PCR, the reaction mixture consisted of cDNA first-strand template, primers (500 nmol final concentration), and SYBR Green Master Mix (SsoAdvanced Universal SYBR Green Supermix, Bio-Rad) in a total volume of 20 µL. The PCR conditions were as follows: 30 s at 95 °C, followed by 40 cycles

of 15 s at 95 °C and 30 s at 60 °C, 3 min at 72 °C, and 1 min at 95 °C. The reactions were performed using an iQ5 real-time PCR detection system (Bio-Rad). Relative quantification of the expression levels for the target was analyzed using the ΔΔCt method [33,34]. The expression data are the mean of three biological replicates. Reactions were performed in triplicate. The statistical significance of the data comparing callus exposed to fungal exudates vs control callus not exposed was determined using Student's *t*-test ($p < 0.05$) and statistically significant differences between resistant and susceptible callus lines were studied with one-way ANOVA ($p < 0.05$), carried out with the analytical software STATISTICA 7 (StatSoft, Inc., Tulsa, OK, USA).

3. Results

3.1. Obtaining a Callus Line Resistant to Rosellinia necatrix Filtrate

A preliminary experiment to test the effect of fungal CF and the PDB on embryogenic callus proliferation was carried out. After 1 week of incubation in a liquid medium, no change in callus weight was found; however, the callus exposed to CF showed a dark brown color. After 1 month of culture on solid MSP medium, all embryogenic callus previously cultured in liquid medium supplemented with PDB (from 20% to 80% *v*/*v*) showed the same color and growth as control callus cultured in MSP medium; however, the weight of callus previously exposed to CF decreased as the amount of CF added to the medium increased (76% and 41% of growth in relation to the control at 20% and 40% CF, respectively); however, at higher concentrations, i.e., 60% and 80% CF, callus was necrotic and no new growth could be observed.

To obtain an embryogenic callus line resistant to CF, 6 flasks with 0.4 g of 1–2 mm embryogenic callus were incubated for 1 week in the presence of MSP medium supplemented with 60% (*v*/*v*) of *R. necatrix* CF at 120 rpm in darkness and later transferred for 1 month onto solid MSP medium. After this period, calli from all flasks showed a necrotic aspect, with a black color, and did not show any growth; however, after the second and third months of culture, one out of the six flasks showed new somatic embryos (Figure 2). After several recultures, this callus showed active proliferation allowing its recovery; hence, it was considered a cell line (L3) with putative somatic variations. To confirm its tolerance to 60% (*v*/*v*) CF, it was exposed again to this CF concentration. Afterward, when enough embryogenic callus was obtained, it was incubated in the presence of a higher percentage, 80% (*v*/*v*) CF, and again, after recovery, a new exposure to this concentration of CF was carried out. The L3 line showed noticeable growth after the second exposure to 80% (*v*/*v*) CF; therefore, it was selected as an embryogenic-resistant line to 80% (*v*/*v*) CF and was chosen for use in transcriptomic analysis.

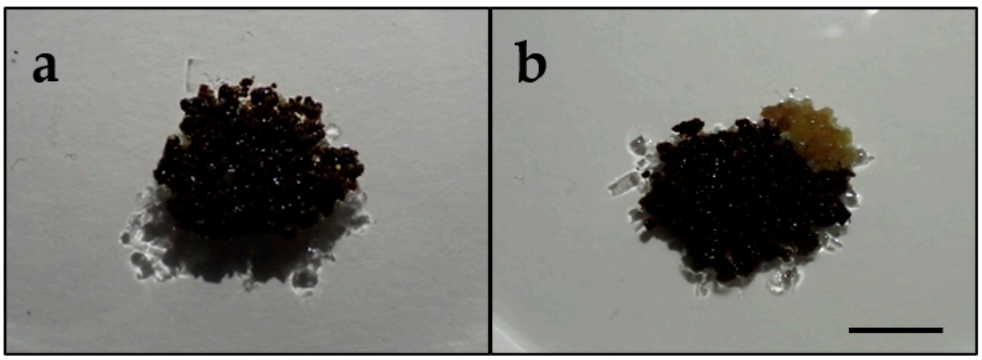

Figure 2. Callus exposed to 60% (*v*/*v*) *R. necatrix* culture filtrate for 1 week followed by culture on solid MSP (Murashige and Skoog supplemented with Picloram medium) for 1 month (**a**) and 2 months (**b**). Bar 1 cm.

3.2. Comparative Transcriptome Analysis of Resistant and Susceptible Callus Lines Exposed to Fungal Filtrate

To analyze the avocado response to *R. necatrix* exudates in resistant and susceptible avocado callus lines, a transcriptome analysis was carried out to compare their expression profiles. The RNA sequencing (RNA-Seq) data were analyzed, including the raw reads from three biological replicates of resistant callus line (L3) exposed (TCLF1, TCLF2, TCLF3) and non-exposed (TCL1, TCL2, TCL3) to fungal filtrate and three biological replicates of the susceptible callus line (AN-9) exposed (SCLF1, SCLF2, SCLF3) and non-exposed to fungal filtrate (SCL1, SCL2, SCL3). A total of 25,211 genes were subjected to statistical analysis to evaluate differential gene expression between TCLF vs TCL and SCLF vs SCL. Analyses resulted in 6,561 differentially expressed genes (DEGs), of which 845 were only found in the resistant line (55.62% induced and 44.38% repressed) and 1,643 in the susceptible (52.77% induced and 47.23% repressed) ($|$fold change (FC)$|$ > 2; p-value < 0.05) (Figure 3). According to the results shown in the Venn diagram, the number of genes with significantly altered expression observed in the susceptible callus line was higher than that of the resistant material L3 line following exposure to fungal filtrate.

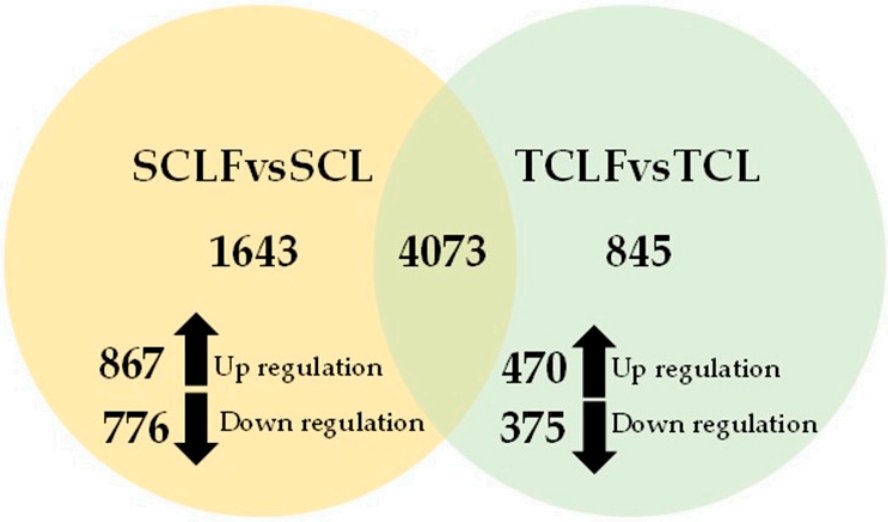

Figure 3. Venn diagram of differentially expressed genes (DEGs) during resistant and susceptible callus growth on medium supplemented, or not, with *R. necatrix* culture filtrate (CF). DEGs obtained in the transcriptome analysis of a resistant callus line exposed and not exposed to fungal CF (TCLF and TCL, respectively) in comparison with a susceptible callus line (AN-9) exposed and non-exposed to CF (SCLF and SCL, respectively). Unique DEGs are shown in only one of the ovals, while shared DEGs are illustrated where the ovals overlap. Arrows show up- and down-regulated DEGs.

A heat map of DEGs shows consistency in expression patterns among treatments, supporting the reliability of the RNA-Seq data (Figure 4).

3.3. RNA-Seq Analysis Validation

Differences found in gene expression profiles between resistant vs susceptible lines were further verified through a quantitative real-time PCR (qRT-PCR) assay on total cDNA samples from the calli of three biological replicates. For this purpose, six randomly selected genes were analyzed. The actin gene was used as a reference gene for data normalization. The expression levels of these genes amplified by qRT-PCR are shown in Table 1. Different expression values were obtained from qRT-PCR than those observed on the RNA-Seq; however, the results corroborated the overall differences found between the two samples (resistant and susceptible) in the RNA-Seq analysis.

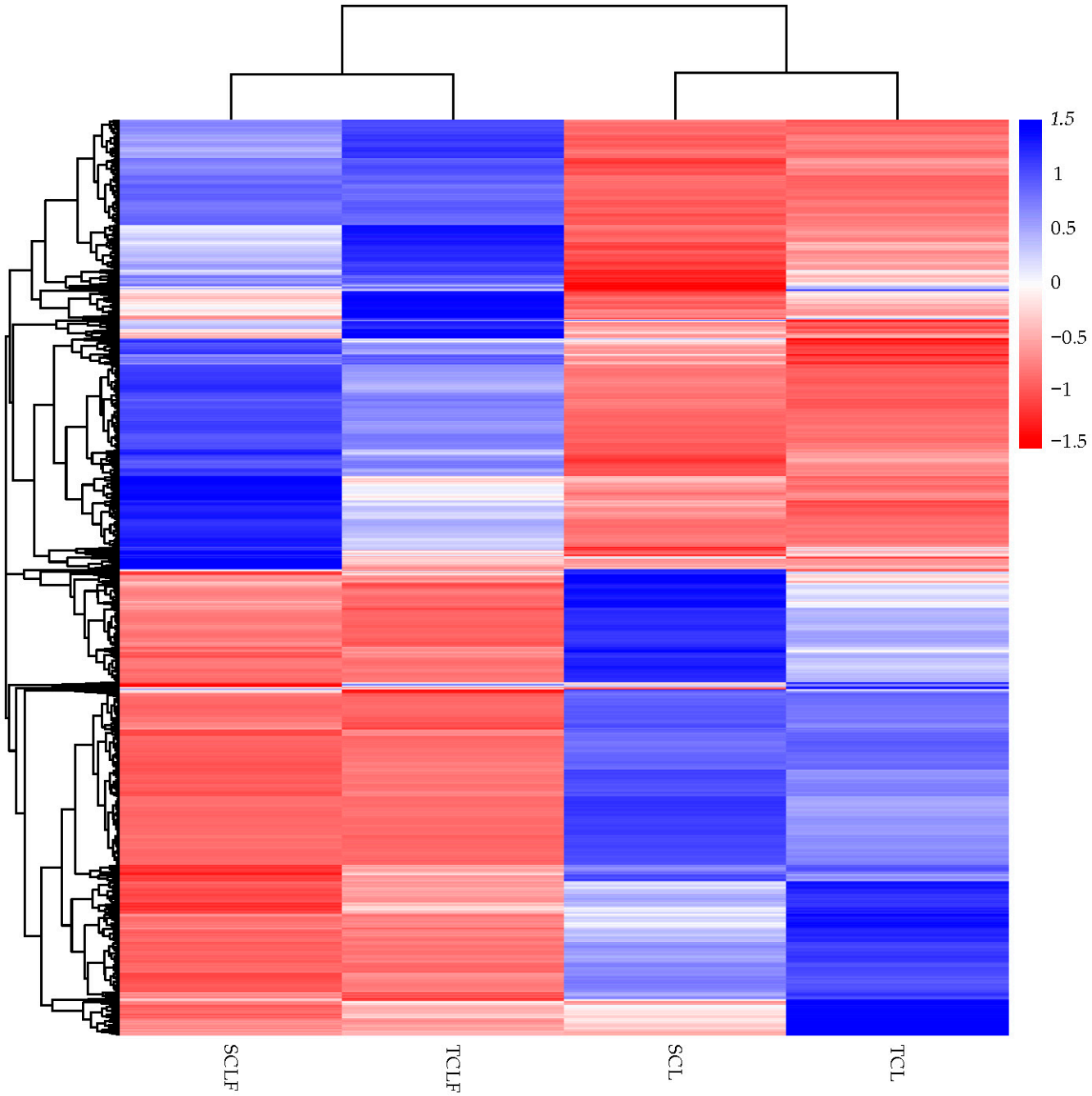

Figure 4. Hierarchical clustering (HCL) of differentially expressed genes (DEGs) obtained in the transcriptome analysis of resistant callus line exposed and not exposed to fungal culture filtrate (TCLF and TCL, respectively) and susceptible callus line (AN-9) exposed and not exposed to fungal culture filtrate (SCLF and SCL, respectively). Blue and red indicate up- and down-regulation, respectively.

Table 1. qRT-PCR expression data of selected contigs from resistant and susceptible callus lines exposed to *R. necatrix* exudates. Data are displayed as fold change (FC), calculated by comparing calli exposed to fungal exudates with control, non-exposed calli. The expression data are the mean of three biological replicates. Bold numbers indicate statistically significant differences (*t*-test, $p < 0.05$), and letters indicate statistically significant differences between resistant and susceptible callus line (ANOVA, $p < 0.05$). ND: Not Detected.

Gene ID	Description	Resistant Callus Line (L3)		Susceptible Callus Line (AN-9)	
		RNA-Seq FC	qRT-PCR FC	RNA-Seq FC	qRT-PCR FC
Pag64949	Proteinase inhibitor	48.50	**38.54 ± 2.00** [a]	22.29	**14.60 ± 1.43** [b]
Pag115909	Tumor related protein	6.79	**11.59 ± 0.40** [a]	5.20	**6.61 ± 0.58** [b]
MSTRG.18398	Pathogenesis-related protein P2	ND	**−1.64 ± 0.19**	ND	**−1.99 ± 0.20**
Pag191108	Lignin-forming anionic peroxidase	78.36	**64.88 ± 3.93** [a]	26.95	**21.30 ± 5.46** [b]
Pag291312	Heat shock transcription factor 30	19.75	**25.15 ± 5.55** [b]	86.46	**62.00 ± 11.23** [a]
Pag275152	Translocon-associated protein subunit beta	−2.88	**−1.76 ± 0.11**	−4.79	**−2.52 ± 0.38**

3.4. Functional Annotation and Pathway Analysis of Differentially Expressed Genes (DEGs)

To better understand the behavior of resistant and susceptible callus lines exposed to the fungal filtrate, Blast2GO software (p-value < 0.05) was used to enrich and categorize DEGs based on blast sequence homologies and gene ontology. DEGs of resistant (Figure 5a) and susceptible (Figure 5b) callus lines were significantly grouped into the regulation of six and eight molecular functions (MF), respectively; six MF were shared among both lines, i.e., catalytic activity (GO:0003824), oxidoreductase activity (GO:0016684, GO:0016491), binding (GO:0020037, GO:0005506, GO:0001071, GO:0043565, GO:0046906, GO:0003700), hydrolase activity (GO:0016788, GO:0008081, GO:0042578), peroxidase activity (GO:0004601), and antioxidant activity (GO:0016209), while molecular functions related to lipase activity (GO:0004630, GO:0004620, GO:0070290, GO:0016298) and kinase activity (GO:0046522, GO:0004674, GO:0004672, GO:0016301) were only found in the susceptible callus line.

Regarding biological processes (BP), response to stimulus (GO:0034605, GO:0009408, GO:0009266, GO:0050896, GO:0006979), transporter activity (GO:0022804, GO:0006865, GO:0006820, GO:0015297, GO:0046942, GO:0098661, GO:0015849, GO:0015711, GO:0015291, GO:0015293, GO:0055085, GO:0022857, GO:0005215, GO:0042910), defense response (GO:0006952), and photosynthesis (GO:0015979) were present in both lines; however, detoxification (GO:1990748, GO:0098869, GO:0098754) was represented only in the resistant line, while catabolic (GO:0046208, GO:0044712, GO:0009395, GO:1901565, GO:0046475, GO:0046503, GO:0042402, GO:0009310) and metabolic processes (GO:0005991, GO:0005985, GO:0044723, GO:0009445, GO:0009446, GO:0006595, GO:0006793, GO:0006796, GO:0009311, GO:0005984, GO:0044262, GO:0006576, GO:0005975, GO:0097164, GO:0006040, GO:0009308) were found only in the susceptible AN-9 line.

3.5. Potential Genes Related to Avocado Embryogenic Callus Defense against Fungal Exudates

Genes previously reported to be important in plant defense against biotic and abiotic stress were shown to be up-regulated in the resistant avocado embryogenic line in comparison with the susceptible one (Table 2). Five genes were involved in cell wall remodeling; in particular, two of them, cinnamoyl-CoA reductase (Pag55749) and a lignin-forming anionic peroxidase (Pag191108), were included in the top 20 induced genes of the L3 line exposed to fungal exudates (Table 3). Interestingly, five genes were related to detoxification and disease resistance proteins and five encoded for PR proteins, among which PR-4 showed a higher induction with an FC value of 18.65 in the resistant line.

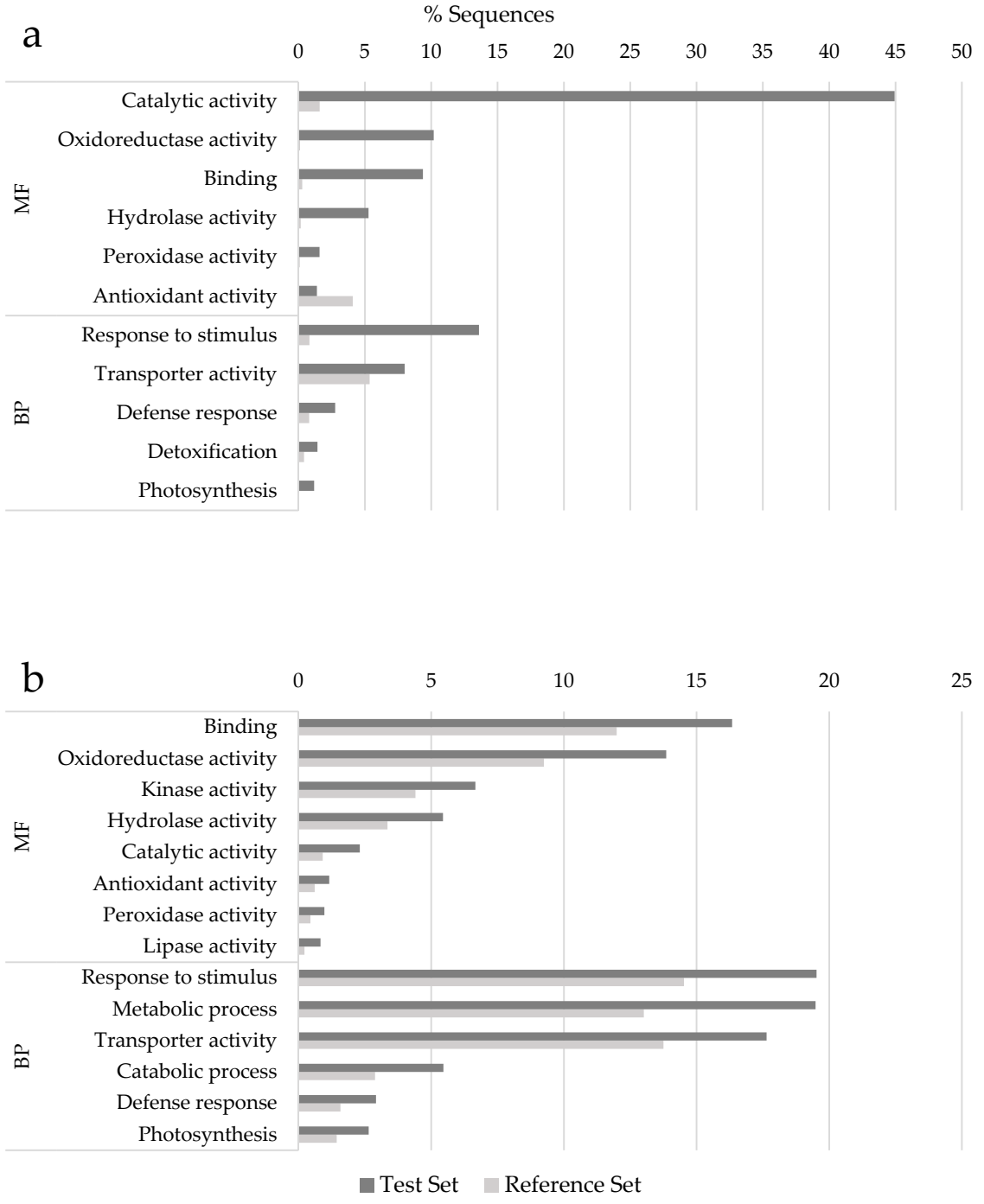

Figure 5. Gene Ontology (GO) enrichment analysis of differentially expressed genes (DEGs). GO enrichment analysis of DEGs in resistant (**a**) and susceptible (**b**) callus lines exposed to *R. necatrix* exudates. Enrichment GO terms were obtained by Blast2GO using a cut-off of 0.05. (MF) molecular function; (BP) biological process.

Table 2. Differentially expressed genes (DEGs) from a resistant callus line exposed and not exposed to fungal culture filtrate (TCLF and TCL, respectively) and a susceptible callus line (AN-9) exposed and non-exposed to *R. necatrix* culture filtrate (SCLF and SCL, respectively). (FC, fold change; ND, Not Detected). PR (pathogenesis related) MYB (myeloblastosis-related proteins), WRKY (WRKYGQK), MYC (Myelocytomatosis), NAC (NAM, ATAF and CUC), ERF (Ethylene-responsive transcription factor).

Gene ID	Description (Blast NCBI)	TCLFvsTCL FC	SCLFvsSCL FC
	Cell wall		
Pag55749	Cinnamoyl-CoA reductase 1	94.23	79.56
Pag191108	Lignin-forming anionic peroxidase	78.36	26.95
Pag66455	Glucan 1,3-beta-glucosidase A-like protein	43.23	29.77
Pag116557	Endonuclease-8-like protein	45.04	8.59
Pag44234	Basic endochitinase-like-protein	2.33	ND
	Detoxification and disease-resistance proteins		
Pag255058	Disease resistance protein RPM1-like	18.05	ND
Pag319424	Pleiotropic drug resistance protein 3-like protein isoform X1	10.08	5.72
Pag279361	Detoxification 49 protein	7.65	4.96
Pag352089	Putative disease resistance protein	4.76	ND
Pag297678	Pleiotropic drug resistance protein 2-like protein	2.06	ND
	Pathogenesis-related proteins		
Pag289080	Pathogenesis-related protein PR-4-like protein	18.65	15.79
Pag154162	Thaumatin-like protein	9.44	6.37
Pag168264	Pathogenesis-related protein STH-21	7.97	4.64
Pag154170	Thaumatin-like protein 1	7.67	5.11
Pag103568	Pathogenesis-related genes transcriptional activator PTI5-like	2.31	ND
	Protease and protease inhibitor activity		
Pag64949	Proteinase inhibitor	48.50	22.29
Pag341255	Subtilisin-like protein protease	41.13	7.70
Pag231368	Cysteine proteinase RD21A-like protein	7.66	4.94
Pag26660	Serine carboxypeptidase-like protein	6.11	ND
Pag136044	Subtilisin-like protein protease SBT5.3	5.72	ND
Pag214427	Subtilisin-like protease SBT5.6	2.60	ND
Pag297773	Serine carboxypeptidase 24	2.00	ND
	Transcription factor MYB, WRKY, MYC and NAC		
Pag51890	Transcription factor MYB1R1-like protein isoform X2	27.15	ND
Pag121997	RWR3 transcription factor MYB108-like protein 1	19.25	ND
Pag345989	Putative WRKY transcription factor 75	17.15	6.68
Pag306446	WRKY64	12.48	5.64
Pag307121	Putative WRKY transcription factor 72 Isoform X1	7.43	4.58
Pag28154	WRKY transcription factor 55	3.22	ND
Pag223376	NAC domain-containing protein 96-like protein	3.22	ND
Pag389501	SANT/Myb domain-containing protein	2.64	ND
Pag46518	WRKY67	2.56	ND
Pag240398	MYB108-like protein	2.56	ND
Pag190993	MYB family transcription factor APL	2.46	ND
Pag201669	NAC domain-containing protein	2.32	ND
Pag276502	SANT/Myb domain-containing protein	2.31	ND
Pag262640	NAC domain-containing protein	2.16	ND
Pag296108	Transcription factor MYC2-like protein	2.01	ND

Table 2. *Cont.*

Gene ID	Description (Blast NCBI)	TCLFvsTCL FC	SCLFvsSCL FC
	Hormonal regulation		
Pag26291	Ethylene-responsive transcription factor (ERF) 020	60.92	45.99
Pag169786	Abscisic acid-insensitive 5-like protein 6	37.65	ND
Pag204839	Ethylene-responsive transcription factor ERF109-like protein	35.06	ND
Pag204844	AP2/ERF domain-containing protein	33.32	23.44
Pag174105	Abscisic acid 8′hydroxylase 2	22.62	11.51
Pag212858	Gibberellin 20 oxidase	12.74	3.58
Pag120094	Auxin-responsive protein SAUR71-like protein	8.02	3.97
Pag197469	Ethylene-responsive transcription factor 1B-like protein	7.05	2.18
Pag157588	Auxin-induced protein	5.51	ND
Pag70205	Auxin transporte-like protein 5	4.22	ND
Pag101088	AP2/ERF domain-containing protein	3.03	ND
Pag75125	Ethylene-responsive transcription factor RAP2-11 like protein	2.80	ND
Pag46880	Gibberellic acid methyltransferase 2 isoform X2	2.54	ND
Pag227350	Auxin-responsive protein SAUR71	2.26	ND
Pag75115	Ethylene-responsive transcription factor 1A	2.09	ND
Pag362340	Auxin-induced protein AUX22	2.04	ND
	Redox homeostasis		
Pag378827	Cytochrome P450 704B1	35.03	8.60
Pag254946	Peroxidase 68	34.50	ND
Pag236665	Beta-amyrin 28-oxidase-like protein	27.32	ND
Pag268581	Protein DMR6-Like oxygenase 2-like protein	26.89	16.06
Pag364610	Protein DMR6-Like oxygenase 2-like protein	25.61	18.95
Pag264980	Cytochrome P450 71A1	23.62	16.60
Pag323404	P450 domain-containing protein	17.10	ND
Pag260376	Cytochrome P450 94C1-like protein	16.83	14.05
Pag255004	Cationic peroxidase 1-like protein	14.79	8.31
Pag88265	Cytochrome P450 78a5-Like protein	13.07	9.38
Pag171020	Allene oxidase synthase 1	11.22	7.42
Pag281952	Cytocrome B6-f complex iron-sulfur subunit 2.	10.23	ND
Pag171015	Allene oxidase synthase 1	9.80	7.62
Pag34770	Protein DMR6-Like oxygenase 2	8.60	4.90
Pag339287	Plant peroxidase	7.21	ND
Pag236679	Cytochrome P450	6.53	ND
Pag19781	Cytochrome P450	5.24	3.29
Pag357969	NADPH-cytochrome P450 reductase-like	3.86	ND
Pag369777	Cytochrome P450 87A3	2.83	ND
Pag377126	Cytochrome P450 cyp72A219-like protein	2.09	ND

Table 3. Top 20 avocado callus contigs overexpressed in a resistant callus line exposed and not exposed to fungal culture filtrate (TCLF and TCL, respectively) and a susceptible callus line (AN-9) exposed and not exposed to the *R. necatrix* culture filtrate (SCLF and SCL, respectively). The expression data are the mean of three biological replicates (FC, fold change; ND, Not Detected).

Gene ID	Annotation	TCLFvsTCL FC	Gene ID	Annotation	SCLFvsSCL FC
Pag246776	Hypothetical protein	188.08	Pag344126	Protein P21-like protein	644.07
Pag92571	Heme peroxidase	177.29	Pag234412	Hypothetical protein CKAN_00293900	287.37
Pag162432	Photosystem II reaction center W protein	145.81	Pag242850	Nicotianamine synthase	260.95

Table 3. *Cont.*

Gene ID	Annotation	TCLFvsTCL FC	Gene ID	Annotation	SCLFvsSCL FC
Pag21497	Protein chlororespiratory reduction 7	140.12	Pag92571	Heme peroxidase	242.25
Pag147146	Putative transcription factor bHLH041	131.20	Pag108159	Glyco-hydro 9 domain-containing protein	182.57
Pag321196	Phosphoribosylformylglycinamidine cyclo-ligase, chloroplastic	130.83	Pag81308	Small heat shock protein 22K	166.87
Pag275645	Vacuolar amino acid transporter protein	118.48	Pag102350	Sugar/inositol transport protein	155.56
Pag308947	Protein P21-like	113.78	Pag44452	ABC transporter G family member	153.64
Pag127237	Hypothetical protein CKAN_02343900	111.18	Pag246776	Hypothetical protein	143.79
Pag55749	Cinnamoyl-CoA reductase	94.23	Pag102899	F-box protein FBW2	135.27
Pag369420	Omega-hydroxypalmitate O-feruloyl transferase	88.77	Pag308947	Protein P21-like	117.13
Pag102899	F-box protein FBW2	87.72	Pag275645	Vacuolar amino acid transporter protein	112.13
Pag380545	Monocopper oxidase-like protein SKU5	82.92	Pag129297	17.3 kDa class II heat shock protein	103.24
Pag147154	Putative transcription factor bHLH041	79.00	Pag21497	Protein chlororespiratory reduction 7	102.12
Pag191108	Lignin-forming anionic peroxidase	78.36	Pag104432	Transcription initiation factor IIF subunit alpha	100.13
Pag112733	Cysteine-rich receptor-like protein kinase 2	76.61	Pag147146	Putative transcription factor bHLH041	90.97
Pag385754	TOX high mobility group box family member 4-A, Putative isoform	74.68	Pag288015	Alpha carbonic anhydrase 1. chloroplastic	88.85
Pag226385	Ethylene-responsive transcription factor ERF096	63.20	Pag365527	Heat shock factor protein HSF30	88.83
Pag26291	Ethylene-responsive transcription factor ERF020	60.92	Pag47864	22.0 kDa class IV heat shock protein	87.44
Pag48634	Lysine histidine transporter-like 5	60.09	Pag291312	Heat shock factor protein HSF30	86.46

In addition, the exposure of callus lines to fungal filtrate resulted in the accumulation of protease and protease inhibitor proteins, which was much more noticeable in the resistant callus line, i.e., the proteinase inhibitor (Pag64949) and the subtilisin-like protein protease (Pag341255) were induced 2 and 6 times more in the L3 line in comparison with the AN-9 line, respectively.

Transcription factors are key elements in the response to abiotic and biotic stresses; in this study, 15 defense-related transcription factors (TFs) were up-regulated in the L3 line, 5 WRKY (WRKYGQK) (Pag345989, Pag306446, Pag307121, Pag28154, Pag46518,) 6 MYB (myeloblastosis-related proteins) (Pag51890, Pag121997, Pag389501, Pag240398, Pag190993, and Pag276502), 1 MYC (Myelocytomatosis) (Pag296108), and 3 NAC (NAM, ATAF and CUC) (Pag223376, Pag201669, Pag262640). All of them, except for Pag345989, Pag306446, and Pag307121, were overexpressed in the resistant callus line but could not be detected in susceptible AN-9, in which the transcription factor MYB1R1-like protein isoform X2 was the one showing the higher induction with an FC of 27.15.

According to the comprehensive analyses, the hormonal balance was disordered in both resistant and susceptible callus lines, where the genes encoding responsive proteins for ethylene, abscisic acid, gibberellin, and auxin regulation are highlighted. Out of the 36 up-regulated genes, 16 showed higher overexpression in L3 than in the susceptible AN-9 line (Table 2), and 7 genes encoded ERF (ethylene-responsive transcription factors) proteins that were related to ethylene regulation; specifically, the ethylene-responsive transcription factor ERF020 protein (Pag26291) was the one showing the higher expression in both lines (FC of 60.92 in L3; 45.99 in AN-9 line).

Genes involved in the detoxification of harmful reactive oxygen species (ROS) generated during callus exposure to fungal filtrate were also found. The resistant line over-

expressed 60 genes encoding cytochrome P450 (CYT P450), peroxidases, and oxygenase proteins, among which 16 showed higher induction than the AN-9 line (10 CYTP450, 3 peroxidases, and 3 oxygenases). CYT P450 704B1 (Pag378827) and Peroxidase 68 (Pag254946) were the ones showing the higher FC values (FC of 35.03 and FC of 34.50, respectively). The susceptible line responded to the redox environment by deregulating 60 genes, among which cytochrome c oxidase subunit 6a was the one exhibiting the higher FC value (70.32).

4. Discussion

Fungal culture filtrate (CF) generally contain non-host-specific toxins causing harmful effects in plant cells [35], e.g., *R. necatrix* is known to exude at least two toxins, cytochalasins E and rosnecatrone [7,36], both believed to be involved in the appearance of symptoms in plants infected with this pathogen [37]. Several studies have proved that released exudates induce defense reactions in plant cells [38]. *Camarosporomyces flavigenus* CF elicited the synthesis of paclitaxel in *Corylus avellana* suspension cultures [39]; this compound is known to have a potent effect on fungi affecting the bark of yew trees [40], while in *Linum album* cell cultures, exposure to *Fusarium graminearum* CF increased PAL (Phenylalanine Ammonia Lyase) activity and synthesis of the antifungal phenolic compound phodophyllotoxin [41]. According to Barz et al. [42], the induced accumulation of these defense compounds merits consideration when studying specific plant–fungi pathosystems.

The present work aims to evaluate differences in gene expression profiles between calli that are resistant and susceptible to *R. necatrix* crude filtrate following exposure to this filtrate; differential gene expression profiles may account for survival or death in this medium. The resistant line could have arisen as a consequence of a somaclonal variation event, as shown in other cell systems following culture in the presence of fungal filtrates [22,23]. Transcriptome analysis revealed a lower number of genes, with significantly altered expression in the resistant callus L3 line in comparison to the susceptible one, AN-9 line, which could be attributed to a better performance of the L3 line during CF exposure. These results agree with molecular studies carried out under greenhouse conditions, in tolerant and susceptible avocado plants upon infection with *R. necatrix*, in which a lower number of DEGs were found to be deregulated in the tolerant BG83 genotype when compared with the susceptible "Dusa" rootstock [13]. Similar results have also been observed in tolerant and susceptible tomato, wheat, olive, and rice genotypes following infection with *Phytophthora parasítica* [43], *Rhizoctonia cerealis* [44], *Verticillium wilt* [45], and *Rhizoctonia solani* [46], respectively.

Gene ontology categories associated with redox activity were significantly enriched in the up-regulated gene sets of both lines and were more noticeable in the susceptible AN-9 line than in resistant L3 line, probably in response to a higher reduction–oxidation status reached in the susceptible line after exposure to CF. Similar results were obtained in a molecular analysis of compatible and incompatible onion/*Alternaria porri* interaction and *Brassica napus*/*Sclerotia sclerotium* interaction [47,48]. In contrast, L3 line showed a much higher catalytic activity compared to the AN-9 line.

The exposure of embryogenic callus lines to 40% *R. necatrix* exudates induced genes previously reported to be related to plant defense against fungal diseases. Interestingly, transcripts of these genes were accumulated in greater amounts in the resistant line in comparison to the susceptible one (Table 2; Supplementary Figure S1). The induction of genes representing enzymes of the phenyl propanoid isoflavonoid pathway, such as the cinnamoyl-CoA reductase and the gene encoding the lignin-forming anionic peroxidase, which are two key enzymes in lignin biosynthesis [49,50], highlights the importance of lignin production for protection against *R. necatrix* exudates. Avocado defense mechanisms against *P. cinnamomi* have also been shown to include structural responses such as strengthening cell walls by depositing callose and lignin [51]. Fungal cell-wall-degrading enzymes such as endochitinases and glucanases have also been detected in the tolerant avocado rootstock "Dusa" following infection with *P. cinnamomi* [16]. In this research, β-1,3- glucosidase proteins and endochitinase-like proteins could be identified. Activities

of these proteins were previously detected in mango embryogenic cultures exposed to *Colletotrichum gloeosporioides* CF [52], while β-glucanase was increased in a cell line of *Cicer arietinum* exposed to CF of *Fusarium oxysporum* [53]. These defense proteins have also been detected in plants regenerated from these cells, and this observation has been correlated with resistance to the pathogen under in vivo conditions [54,55], although this does not always occur, since there are cases in which the expression of resistance to the toxin in in vitro conditions could differ from the reaction observed in vivo [22].

Other PR proteins were also noticeable in avocado embryogenic callus exposed to *R. necatrix* CF. Specifically, transcripts encoding PR-4 (Pag289080), pathogenesis-related protein STH-21 (Pag168264), and thaumatin-like proteins (PR-5) (Pag154162, Pag154170) were induced 1.5 times more in the resistant line compared to the susceptible line. The presence of PR-5 proteins in the medium of grape cells elicited with fungal filtrate of *Elsinoe ampelina* was related to the inhibition of fungal growth in a dual culture system [54]. In the case of avocado, PR-4 and thaumatin-like proteins have not been described to be related to tolerance to either *R. necatrix* or *P. cinnamomi*; however, it has been reported that their early induction after a priming event could represent a benefit for avocado plants in overcoming *R. necatrix* infection [56].

Transcriptome analysis also showed the induction of genes regulating ethylene, abscisic acid, gibberellin, and auxin production. Genes encoding ERF proteins were the most represented in the resistant L3 line compared to the susceptible one. This fact could be related to an improved capacity of the L3 line to integrate hormones and redox signaling in the plant response to stress caused by CF since ERFs are known to have a role in molecular response to pathogen attack by regulating hormone cross-talk and redox signaling [57]. In addition, a significant induction of defense-related transcription factors (TFs) such as MYB, WRKY, MYC, and NAC was observed in embryogenic callus incubated with CF. Interestingly, among the TFs showing higher overexpression in the resistant line compared to the susceptible one are the WRKY and NAC domain-containing proteins, whose role in avocado defense against *P. cinnamomi* and *R. necatrix* has recently been reported [13,16].

The induction of protease inhibitors in plant defense against fungal pathogens has been extensively reported [58]. One important finding of this work was the overexpression of the proteinase inhibitor gene (Pag64949), which was two times higher in the L3 line than in the AN-9 line. The same result was obtained under greenhouse conditions, where the expression of this gene (Pa_Contig05213) was 2.6 times higher in tolerant BG83 avocado rootstock compared to the susceptible "Dusa" after infection with *R. necatrix*, suggesting the important role that these proteins could play in the defense of avocado rootstocks against this pathogen [13,56].

5. Conclusions

This study provides new insights into the different mechanisms involved in avocado tolerance responses to *R. necatrix* CF. In resistant callus line L3, CF induced a set of general defense-related transcripts (genes involved in lignin biosynthesis, PR proteins, WRKY, NAC, proteinase inhibitors, etc.) that are also induced in the tolerant response of avocado rootstocks to *R. necatrix*. Interestingly, the proteinase inhibitor (Pag64949) shared the same coding sequence with the one identified by Zumaquero et al. [13] in greenhouse experiments, in the interaction tolerant BG83 avocado rootstock/*R. necatrix* (Pa_Contig05213), supporting the use of this gene as a potential marker to speed up avocado rootstock breeding programs. This research will contribute to the understanding of avocado defense against this pathogen, thereby aiding in the selection of tolerant avocado rootstocks and supporting the use of in vitro cell culture techniques for studying avocado–*R. necatrix* interaction.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/agronomy13051354/s1. Table S1: qRT-PCR primer sequences used in this study. Figure S1: Comparative model of the exposure of susceptible (AN-9) and resistant (L3) callus lines to 40% of *Rosellinia necatrix* culture filtrate.

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