

RESEARCH ARTICLE

# Rosellinia necatrix infection induces differential gene expression between tolerant and susceptible avocado rootstocks

Adela Zumaquero<sup>1</sup>, Elsa Martínez-Ferri<sup>2</sup>, Antonio J. Matas<sup>3</sup>, Bianca Reeksting<sup>4,5</sup>, Nicholas A. Olivier<sup>5,6</sup>, Fernando Pliego-Alfaro<sup>3</sup>, Araceli Barceló<sup>1</sup>, Nöelani van den Berg<sup>4,5</sup>, Clara Pliego<sup>1\*</sup>

**1** Department of Genomics and Biotechnology, Fruticultura Subtropical y Mediterránea (IFAPA) Unidad Asociada de I+D+i al CSIC, Málaga, Spain, **2** Department of Crop Ecophysiology, Fruticultura Subtropical y Mediterránea (IFAPA) Unidad Asociada de I+D+i al CSIC, Málaga, Spain, **3** Department of Plant Physiology, Instituto de Hortofruticultura Subtropical y Mediterránea “La Mayora” (IHSM-UMA-CSIC), Unidad Asociada IHSM-IFAPA, University of Malaga, Málaga, Spain, **4** Department of Biochemistry, Genetics and Microbiology, University of Pretoria, Pretoria, South Africa, **5** Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa, **6** Department of Plant and Soil Science, University of Pretoria, Pretoria, South Africa

\* [mclara.pliego@juntadeandalucia.es](mailto:mclara.pliego@juntadeandalucia.es)



**OPEN ACCESS**

**Citation:** Zumaquero A, Martínez-Ferri E, Matas AJ, Reeksting B, Olivier NA, Pliego-Alfaro F, et al. (2019) *Rosellinia necatrix* infection induces differential gene expression between tolerant and susceptible avocado rootstocks. PLoS ONE 14(2): e0212359. <https://doi.org/10.1371/journal.pone.0212359>

**Editor:** Anil Kumar Singh, ICAR-Indian Institute of Agricultural Biotechnology, INDIA

**Received:** October 25, 2018

**Accepted:** January 31, 2019

**Published:** February 14, 2019

**Copyright:** © 2019 Zumaquero et al. This is an open access article distributed under the terms of the [Creative Commons Attribution License](https://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

**Data Availability Statement:** The data set from this study are available from the NCBI Gene Expression Omnibus on accession number GSE119140.

**Funding:** This research was supported by grants RTA2017-00040-00-00 (INIA-AEI) to CPP, AVA201601.14 to EMF (20% Junta de Andalucía, 80% FEDER) and AGL 2014-52518-C2-1-R (Ministerio de Ciencia e Innovación), Spain to FPA. MC. Pliego is currently supported by an INIA-CCAA

## Abstract

*Rosellinia necatrix* is the causal agent of avocado white root rot (WRR). Control of this soil-borne disease is difficult, and the use of tolerant rootstocks may present an effective method to lessen its impact. To date, no studies on the molecular mechanisms regulating the avocado plant response towards this pathogen have been undertaken. To shed light on the mechanisms underpinning disease susceptibility and tolerance, molecular analysis of the gene’s response in two avocado rootstocks with a contrasting disease reaction was assessed. Gene expression profiles against *R. necatrix* were carried out in the susceptible ‘Dusa’ and the tolerant selection BG83 avocado genotypes by micro-array analysis. In ‘Dusa’, the early response was mainly related to redox processes and cell-wall degradation activities, all becoming enhanced after disease progression affected photosynthetic capacity, whereas tolerance to *R. necatrix* in BG83 relied on the induction of protease inhibitors and their negative regulators, as well as genes related to tolerance to salt and osmotic stress such as aspartic peptidase domain-containing proteins and gdsI esterase lipase proteins. In addition, three protease inhibitors were identified, glu protease, trypsin and endopeptidase inhibitors, which were highly overexpressed in the tolerant genotype when compared to susceptible ‘Dusa’, after infection with *R. necatrix*, reaching fold change values of 52, 19 and 38, respectively. The contrasting results between ‘Dusa’ and BG83 provide new insights into the different mechanisms involved in avocado tolerance to *Phytophthora cinnamomi* and *R. necatrix*, which are consistent with their biotrophic and necrotrophic lifestyles, respectively. The differential induction of genes involved in salt and osmotic stress in BG83 could indicate that *R. necatrix* penetration into the roots is associated with osmotic effects, suggesting that BG83’s tolerance to *R. necatrix* is related to the ability to withstand osmotic imbalance. In addition, the high expression of protease inhibitors in tolerant BG83 compared

contract, co-financed by INIA (20%) and FEDER (80%).

**Competing interests:** The authors have declared that no competing interests exist.

to susceptible 'Dusa' after infection with the pathogen suggests the important role that these proteins may play in the defence of avocado rootstocks against *R. necatrix*.

## Introduction

Avocado (*Persea americana* Mill.), a member of the Lauraceae, is an important fruit crop in over 50 countries. The documented health benefits of avocado consumption are numerous, and avocado is considered one of the top 15 healthiest foods according to surveys across the United States and Western Europe (<http://www.medicalnewstoday.com/articles/245259.php>). There has been an increase in consumption worldwide during the last decade, bringing a certain concern about the difficulties of satisfying the demand in the near future. In this regard, efforts to increase avocado production by decreasing the incidence of avocado diseases are important. Significant losses in avocado production result from root rots caused by soilborne pathogens such as *Phytophthora cinnamomi* (*Phytophthora* root rot; PRR) and *Rosellinia necatrix* (white root rot; WRR). While *P. cinnamomi* affects avocado plantations worldwide, infections caused by *R. necatrix* have a considerable impact on avocado orchards in temperate regions, particularly Europe and Asia; however, recent out-breaks of this pathogen in other areas, such as California, México, Korea and South Africa, have increased the interest in gaining basic knowledge of its interaction with avocado.

To date, most investigations have focused on the control of PRR, and at present, an integrated approach involving the use of phosphate, tolerant rootstocks, and proper field management is giving positive results [1, 2]. Currently, there are commercial rootstocks with partial tolerance/resistance to *P. cinnamomi* ('Thomas', 'Duke 7' and 'Dusa'), and significant efforts are in progress to select material with enhanced tolerance and other agronomic traits [3]. In addition, investigations are underway to gain a better knowledge of the avocado-*P. cinnamomi* interaction with the objective of developing tools that could be used to speed up avocado rootstock breeding and selection programmes. Along this line, Reeksting et al. [4] evaluated the transcriptomic responses through microarray analysis of the commercial rootstock 'Dusa' to *P. cinnamomi* and flooding, reporting the expression of many defence-related transcripts related to *P. cinnamomi* tolerance.

In contrast to PRR, the control of WRR disease remains a complex and difficult task due to the pathogen's features, including resistance to drought, survival capacity in acidic soils, colonization of numerous hosts, deep penetration into the soil, and immunity to various common fungicides [5–7]. *R. necatrix* simultaneously invades avocado at several points within the roots, and invasion is followed by the proliferation of hyphal strands in one or several directions, colonizing both epidermal and cortical cells and finally, collapsing the vascular system of the plant [8].

Current control approaches involve the use of physical and chemical methods [9], although none have proven to be fully effective. In this sense, the utilization of tolerant/resistant rootstocks may represent an effective approach to lessen the impact of this soil-borne pathogen. To date, there are no commercial tolerant avocado rootstocks to *R. necatrix*, and tolerant selections to PRR, such as 'Dusa' or 'Duke 7', have been shown to be highly susceptible to this pathogen under artificial inoculation [5]. To overcome this problem, the biotechnology group at IFAPA-Málaga has been involved since 1995 in a breeding programme aimed at obtaining material with tolerance/resistance to WRR. In this programme, seeds collected from different provenances worldwide as well as asymptomatic trees located in areas affected by the pathogen

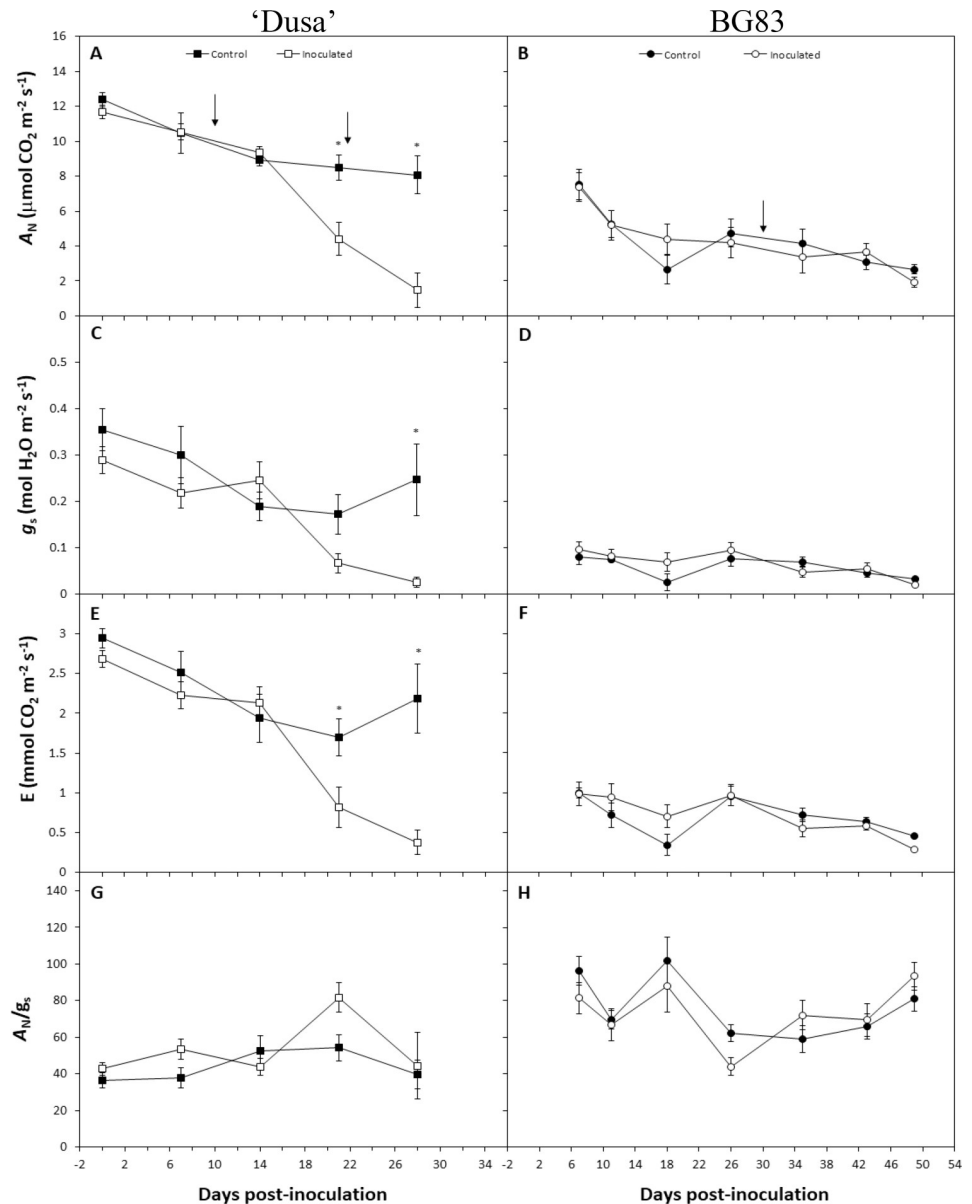
have been evaluated [10]. To date, several selections showing tolerance to this fungus have been identified, and they are currently being tested under field conditions prior to commercialization. These selected genotypes provide a very useful tool for the study of the mechanisms involved in the avocado tolerant response to *R. necatrix*. In a previous study, Martinez-Ferri et al. [11] reported the physiological changes that occur during the initial stages of *R. necatrix* infection on avocado roots prior to the appearance of visible aboveground symptoms. While leaf photochemistry was affected at early stages of disease development in 'Dusa' (tolerant to PRR, but susceptible to WRR), even when no aerial symptoms were visible, no changes could be detected in the tolerant BG83 genotype (one of the selections from IFAPA-Málaga breeding programme tolerant to WRR), despite the presence of the fungus in the root system. The absence of changes at the photosynthetic level in the BG83 genotype suggested that the tolerance to *R. necatrix* could be associated with mechanisms conferring a higher robustness to the photosynthetic machinery of the plant.

Examining avocado gene expression profiles in contrasting disease reactions will contribute to a better understanding of the molecular interactions underlying tolerance and susceptibility to *R. necatrix* and would allow the identification of new genes that could be used as markers in avocado rootstock breeding programmes. Therefore, the aim of this work was to analyse the transcriptome responses of tolerant (BG83) and susceptible ('Dusa') avocado rootstocks to *R. necatrix* by using an Agilent array in which genes involved in the incompatible response to *P. cinnamomi* and flooding are represented. Since some of the upregulated genes in the avocado-*P. cinnamomi* interaction have already been identified [12, 13], this study will also shed light on the differences at the transcriptome expression level between the pathogens *P. cinnamomi* and *R. necatrix* when infecting avocado.

## Results and discussion

### Post-infestation development of physiological and aerial symptoms

The primary mechanisms of woody plants in response to root pathogen infections are associated with the impairment of water relations and the limitation of photosynthesis [14, 15]. In a previous study, Martinez-Ferri et al. [11] showed that *R. necatrix* infection in susceptible avocado rootstocks caused a decrease in photosynthesis at the photochemical level, suggesting that pathogen infection could be associated with stomatal limitations of photosynthesis. In this regard, the effects of *R. necatrix* on photosynthetic performance during the first stages of disease progression by gas exchange measurements were analysed. The results showed an early and significant decrease in CO<sub>2</sub> assimilation and a decline in stomatal conductance in the susceptible genotype 'Dusa' after 22 days post-inoculation prior to the appearance of any aerial symptoms. In this genotype, net assimilation rates ( $A_N$ ) decreased concomitantly with stomatal closure ( $g_s$ ) and transpiration rates ( $E$ ), resulting in no variation in the instantaneous water use efficiency (Fig 1A, 1C, 1E and 1G). In contrast, no significant variation in any of the gas exchange parameters was observed during disease progression in the tolerant BG83 genotype (Fig 1B, 1D, 1F and 1H). This result indicates an effective ability of this genotype, either at the root or at the leaf levels, to counteract the pathogen's effect on water relations, avoiding photosynthetic decline. This BG83 tolerant response to *R. necatrix* is different from the 'Dusa' tolerant response to *P. cinnamomi*, in which tolerance was associated with a greater ability to restore both  $A_N$  and  $g_s$  at similar levels as those seen in the non-inoculated control [16]. In this sense, it is remarkable that BG83 displayed lower values of  $A_N$ ,  $g_s$ , and  $E$  than 'Dusa' but higher  $A_N/g_s$ , suggesting a behaviour of avoidance of water losses [17], which is consistent with the higher robustness of the photosynthetic machinery previously reported by Martinez-Ferri et al. [11].

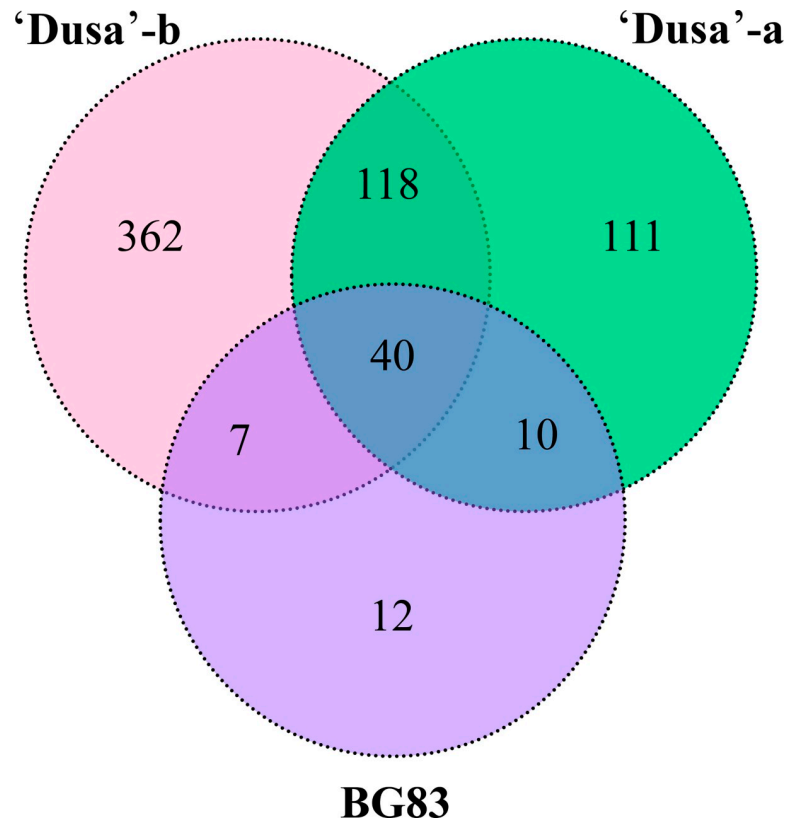


**Fig 1. Post-infestation development of physiological and aerial symptoms.** Variation in net assimilation rates ( $A_N$ ; A and B), stomatal conductance ( $g_s$ ; C and D), transpiration rate ( $E$ ; E and F) and intrinsic water use efficiency ( $A_N/g_s$ ; G and H) in 'Dusa' and BG83 avocado plants at stage 1 (no aerial symptoms) after inoculation with *R. necatrix*. The asterisks indicate significant differences between inoculated and control treatments ( $P < 0.05$ ). Each data point represents the mean ( $\pm$ SE;  $n = 8$  to 18). The arrows indicate root sampling for RNA extractions.

<https://doi.org/10.1371/journal.pone.0212359.g001>

### Transcriptome responses of avocado

To analyse the avocado response to *R. necatrix* infection in tolerant and susceptible avocado, we used a targeted cDNA microarray containing transcripts from *de novo* sequencing of a *Phytophthora* tolerant 'Dusa' rootstock in response to flooding and *P. cinnamomi* infection [12]. Root samples of the susceptible 'Dusa' genotype infected with *R. necatrix* were collected before ('Dusa'-b) and after ('Dusa'-a)  $CO_2$  assimilation rates in the leaves significantly decreased (8 and 22 days post-inoculation, respectively). In contrast, root samples from the BG83 genotype



**Fig 2. Venn diagram of differentially expressed genes.** Numbers of common and specific differentially expressed genes (DEGs) obtained in the microarray analysis of susceptible 'Dusa'-b, 'Dusa'-a and tolerant BG83 avocado rootstocks after infection with *R. necatrix*. Shared transcripts are illustrated where the circles meet, while unique DEGs are shown in only one of the three circles. A higher number of both specific and shared DEGs, were observed in the susceptible 'Dusa' than in the tolerant BG83 rootstock.

<https://doi.org/10.1371/journal.pone.0212359.g002>

were collected in asymptomatic infected plants eight days after the observation of a decrease in the net assimilation rate in the susceptible 'Dusa' genotype (30 days post-inoculation). The hybridization percentages of the microarrays were similar for the three samples at 55.6% for 'Dusa'-b, 70.8% for 'Dusa'-a and 61.9% for BG83.

The total number of genes on the array with significantly altered expression elicited by *R. necatrix* infection was 527 (80.4% induced and 19.6% repressed), 279 (76.6% induced and 23.4% repressed) and 69 (85.5% induced and 14.5% repressed) transcripts in 'Dusa'-b, 'Dusa'-a and BG83, respectively (Adj. P-value < 0.05). As shown in Fig 2, 362 genes were specific to 'Dusa'-b and 111 to 'Dusa'-a, while 12 genes were exclusively deregulated in BG83 (Fig 2). The lower number of genes with significantly altered expression observed in tolerant plants seems to be a consequence of a better performance during *R. necatrix* infection in comparison to sensitive plants and could be linked to a higher number of constitutively expressed genes in the tolerant genotype when compared to the susceptible one, in accordance with results obtained in other plant-pathogen interactions; e.g., olive genotypes susceptible to *Verticillium dahliae* showed significantly more upregulated differentially expressed genes (DEGs) than tolerant plants upon infection [18]. Similar results have also been observed in tolerant and susceptible sugarcane and rice genotypes used in other stress studies, e.g., water stress-sensitive plants activated their response mechanisms to water deficit earlier than the tolerant ones, showing a larger number of stress-responsive transcripts [19, 20].

## Validation of the microarray

Differences found in gene expression profiles between susceptible and tolerant rootstocks to *R. necatrix* were further verified by performing a real-time quantitative qPCR (qRT-PCR) assay on total cDNA samples from roots of three biological replicates. Fifteen unigenes showing contrasting expression patterns among Dusa-b, Dusa-a and BG-83 were analysed. This selection included three genes potentially involved in the tolerance of BG to *R. necatrix* (Pa\_contig02540; Pa\_contig05213; Pa\_contig04097). Negative controls were used to confirm the absence of contamination. *Actin* was used as a reference gene for data normalization. The expression levels of these genes amplified by qRT-PCR are shown in Table 1. Although higher expression values were obtained by qRT-PCR than those observed on the microarray, the results corroborated the overall differences found among the three samples ('Dusa'-b, 'Dusa'-a, BG83) in the microarray analysis.

## Tolerant and susceptible avocado rootstocks show divergent differentially expressed gene (DEG) patterns during root infection with *R. necatrix*

Studies of different gene expression profiles occurring in roots of tolerant and susceptible avocado genotypes are crucial to explain the tolerance or susceptibility to *R. necatrix* in avocado rootstocks. In this study, gene expression profiles taking place in roots of the susceptible versus tolerant genotype were analysed and compared. Principal component analysis (PCA) was performed on all transcripts significantly modified by infection. The scores plot of the PCA showed a stronger relationship between 'Dusa'-b and 'Dusa'-a with BG83 being the most dissimilar sample (Fig 3).

Hierarchical clustering (HCL) was performed on DEGs according to the expression profiles obtained from the microarray (Fig 4); 'Dusa'-b and 'Dusa'-a showed closer expression patterns than those observed for the tolerant BG83 genotype in which a high percentage of the genes were repressed (red colour). Genes were clustered into five groups according to the expression pattern observed after *R. necatrix* infection (S1 Table). Group 1 (red in Fig 4) included DEGs that were predominantly induced in 'Dusa'-b but not in BG83. This is the largest group, including 361 DEGs. The biological processes most commonly identified with GO-term-enriched analysis ( $P < 0.025$ ) comprised, among others, oxido-reduction processes, heme binding, recognition of pollen, hydrolase activities and iron ion binding (S1 Fig). Genes included in this group showing higher induction values were pathogenesis-related protein PR-4-like (Pa\_contig07140), thaumatin-like protein 1 (Pa\_contig01450), glucan endo-beta-glucosidase-like (Pa\_contig03461) and beta-glucanase (Pa\_contig00542). These genes, except for the one encoding the PR-4-like protein, are included in the top10 genes showing higher induction in 'Dusa'-b and 'Dusa'-a (Table 2), decreasing their expression once alterations of net assimilation rates at the leaf level were observed ('Dusa'-a) and not detectable in BG83. These genes have been described as putative defence-related genes in 'Dusa'/*P. cinnamomi* interactions, when 'Dusa' behaves as a tolerant rootstock [4, 12, 13], suggesting that they play an important role in the tolerance against *P. cinnamomi* but not against *R. necatrix* infection. This group also includes the top10 repressed genes in BG83 (Table 2), with a gene encoding a defensin-like protein (Pa\_contig04185) showing the highest repression. This gene is also repressed in the susceptible cultivar but at lower levels. Although plant defensins are a group of pathogenesis-related defence mechanism proteins [21], their high repression in the tolerant cultivar indicates that they do not play a key role in tolerance to *R. necatrix*.

Group 2 (yellow in Fig 4) consisted of 60 DEGs predominantly upregulated in 'Dusa'-b and BG83 but not in 'Dusa'-a. Among the genes included in this cluster, the most noticeable behaviour was for a glu-protease inhibitor like (Pa\_contig 05213), a gene with much higher

**Table 1. qRT-PCR and microarray expression data of selected contigs from susceptible ‘Dusa’-b, ‘Dusa’-a and tolerant BG83 avocado rootstocks infected with *R. necatrix*.**

Name	Annotation	qRT-PCR FC ‘Dusa’-b	Microarray FC ‘Dusa’-b	qRT-PCR FC ‘Dusa’-a	Microarray FC ‘Dusa’-a	qRT-PCR FC BG83	Microarray FC BG83
Pa_contig 00651	12-oxophytodienoate reductase 11	<b>387.99</b>	<b>14.49</b>	<b>403.39</b>	<b>10.57</b>	<b>26.85</b>	2.83
Pa_contig 03380	Conserved hypothetical protein	<b>379.27</b>	<b>6.11</b>	<b>137.06</b>	<b>3.76</b>	7.06	1.23
Pa_contig 00931	Receptor protein kinase ZmPK1	<b>137.29</b>	<b>7.94</b>	<b>97.86</b>	<b>6.71</b>	<b>5.75</b>	1.84
Pa_contig 02033	Glutamine amidotransferase	122.54	5.34	<b>50.73</b>	4.55	21.11	<b>4.96</b>
Pa_contig 03267	Peroxidase 5-like	117.16	<b>20.81</b>	18.15	<b>4.75</b>	5.35	ND
Pa_contig 05854	Protein hothead-like	20.12	<b>7.86</b>	<b>340.76</b>	<b>23.88</b>	367.94	<b>6.03</b>
Pa_contig 01404	Putative plant syntaxin	25.55	<b>5.20</b>	<b>57.32</b>	<b>12.26</b>	<b>4.08</b>	ND
Pa_contig 05917	Germin-like protein	<b>14.42</b>	<b>10.35</b>	<b>54.46</b>	<b>25.17</b>	0.50	ND
Pa_contig 02874	Germin-like protein subfamily 1 member 20-like	8.61	<b>17.28</b>	<b>22.88</b>	<b>30.04</b>	-0.49	ND
Pa_contig 00951	Kynurenine formamidase-like	<b>10.24</b>	ND	<b>12.85</b>	<b>12.98</b>	12.27	<b>6.13</b>
Pa_contig 05213	Glu proteasa inhibitor	13.94	<b>16.19</b>	<b>32.85</b>	ND	<b>52.07</b>	<b>43.66</b>
Pa_contig 02540	Tumor related protein	<b>28.67</b>	<b>8.18</b>	<b>31.86</b>	<b>22.06</b>	<b>38.31</b>	<b>16.01</b>
Pa_contig 04097	Trypsin inhibitor	6.18	<b>5.19</b>	-4.17	<b>2.75</b>	<b>18.80</b>	<b>33.80</b>
Pa_contig 00456	Methionine gamma-lyase-like	3.30	ND	<b>3.37</b>	ND	<b>4.44</b>	<b>6.43</b>
Pa_contig 04185	Defensin j1-2-like	-11.04	<b>-6.40</b>	-73.48	<b>-6.35</b>	-431.88	<b>-33.12</b>

The data are displayed as fold changes (FC), calculated by comparing plants inoculated with *R. necatrix* with control plants (non-inoculated). The expression data are the mean of three biological replicates. The numbers in bold indicate statistically significant results (*t*-test,  $P < 0.05$ ). ND: not detected.

<https://doi.org/10.1371/journal.pone.0212359.t001>

expression in BG83 when compared to ‘Dusa’, (FC BG83 = 43.66 and FC ‘Dusa’-b = 16.19 in the microarray analysis and FC BG83 = 52.07 and FC ‘Dusa’-b = 13.94 in qRT-PCR experiments). The role of protease inhibitors in plant defence against phytophagous insects and microorganisms has been extensively reported [22, 23]. Interestingly, eight out of the top10 repressed transcripts selected for ‘Dusa’-a are included in this group (Table 2); the metallothionein-like protein (Pa\_contig04303) repressed in ‘Dusa’ after *R. necatrix* infection (Pa\_04303) has been recently included within the top 25 avocado transcripts induced in an incompatible avocado/ *P. cinnamomi* interaction, highlighting the important role of this gene in tolerance to this oomycete [24], while its expression is not detected in the BG83 genotype, tolerant to *R. necatrix*. Genes whose expression was increased as the infection progressed in the susceptible genotype (repressed or slightly induced in ‘Dusa’-b and highly induced in ‘Dusa’-a) comprised groups 3 and 5 (green and purple, respectively, in Fig 4). These groups include 102 and 88 DEGs, respectively. The biological processes most commonly identified with GO-term-enriched analysis ( $P < 0.025$ ) in group 3 comprised mainly cell wall macromolecule catabolic processes, chitinase activity, chitin binding and chitin catabolic processes (S2 Fig). This is in

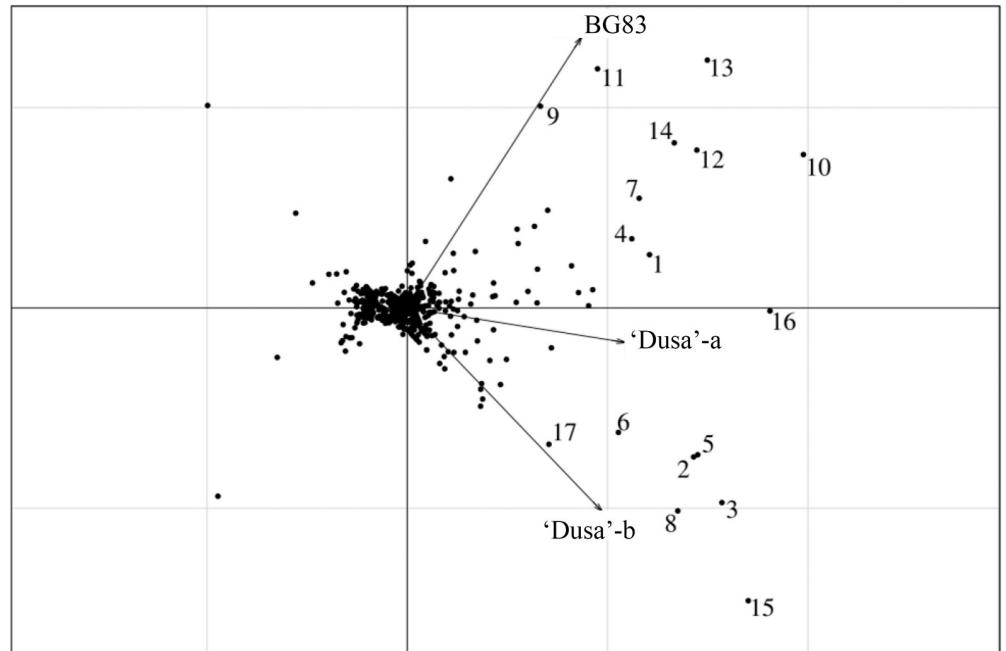
**Table 2. Top 10 avocado transcripts differentially regulated by R. necatrix in 'Dusa'-b, 'Dusa'-a and BG83.**

'Dusa'-b			'Dusa'-a			BG83		
Name	Description	FC	Name	Description	FC	Name	Description	FC
Pa_Contig07140	Pathogenesis-related protein pr-4-like.	119.12	Pa_Contig04808	Protein hothead-like.	78.74	Pa_Contig06358	—NA—	45.98
Pa_Contig03461	Glucanendo-β-glucosidase-like.	79.64	Pa_Contig07184	Protein hothead-like.	74.12	Pa_Contig05213	Glu protease inhibitor-like	43.66
Pa_Contig00542	Beta-β-glucanase.	72.50	Pa_Contig01014	Basic endochitinase-like partial.	72.48	Pa_Contig04808	Protein hothead-like	37.54
Pa_Contig07403	Pathogenesis-related protein	56.50	Pa_Contig00535	Chitinase 1-like.	69.84	Pa_Contig06808	Basic 7s globulin-like (aspartic peptidase containing protein)	37.51
Pa_Contig01014	Basic endochitinase-like partial.	49.89	Pa_Contig00542	Beta-glucanase.	63.30	Pa_Contig04097	Trypsin inhibitor	33.80
Pa_Contig00535	Chitinase 1-like.	46.60	Pa_Contig00205	Protein hothead-like.	58.11	Pa_Contig06176	Protein hothead-like	29.39
Pa_Contig01450	Thaumatococcus-like protein.	45.00	Pa_Contig01450	Thaumatococcus-like protein.	47.88	Pa_Contig02817	Basic 7s globulin-like (aspartic peptidase containing protein)	21.66
Pa_Contig07184	Protein hothead-like.	43.16	Pa_Contig03461	Glucanendo-β-glucosidase-like.	41.21	Pa_Contig00559	—NA—	21.52
Pa_Contig01608	Cytochrome p450 89a2-like.	42.84	Pa_Contig06015	Beta-D-galactosidase.	41.00	Pa_Contig07184	Protein hothead-like	20.44
Pa_Contig01569	Peroxidase n1-like.	38.37	Pa_Contig06358	—NA—	40.20	Pa_Sin_HA66E9C01BVIYG	—NA—	19.41
Pa_Sin_GI32N0T02IWL2D	Hypothetical protein LEMA_P014390.1	-82.05	Pa_Contig02823	Uncharacterized protein LOC104613500	-12.62	Pa_Contig04185	Defensin j1-2-like	-33.12
Pa_Contig02835	Probable nucleoredoxin 2	-39.33	Pa_Contig04303	Metallothionein-like protein type 2	-10.73	Pa_Contig06047	—NA—	-12.59
Pa_Contig00540	Protein exordium-like 2	-19.30	Pa_Contig07016	Chalcone synthase	-10.25	Pa_Contig00062	Polyphenol chloroplastic-like	-7.46
Pa_Contig01573	Cytochrome p450 714c2-like	-18.58	Pa_Contig06047	—NA—	-9.53	Pa_Contig04951	Gibberellin-regulated protein 3	-6.95
Pa_Contig02105	Protein walls are thin 1-like	-13.96	Pa_NA_RC_Contig06966	—NA—	-9.12	Pa_Sin_HA66E9C01AIWJ3	Heat shock protein 21	-6.95
Pa_Contig03065	Protein gast1-like	-12.11	Pa_Contig01429	Dihydroflavonol 4-reductase	-7.87	Pa_Contig01098	21 kda protein	-6.06
Pa_Contig06047	—NA—	-9.32	Pa_NA_F_contig06354	—NA—	-7.79	Pa_NA_F_contig07407	—NA—	-5.90
Pa_Contig05365	Starch branching enzyme isoform 3	-8.19	Pa_Contig04354	Anthocyanidin reductase	-7.67	Pa_Contig00751	Xyloglucanendotransglucosylasefamilyprotein	-5.74
Pa_Contig02780	Uncharacterized loc101208739 isoform 1	-7.99	Pa_Contig03042	Gibberellin-regulated protein 6	-7.64	Pa_Sin_GI32N0T02GTAO6	Glucose-1-phosphate adenylyltransferase large subunit 1-like	-5.43
Pa_Contig01427	Tubulin alpha chain	-7.90	Pa_Contig03065	Protein gast1-like	-7.31	Pa_Contig01026	Polyphenol chloroplastic-like	-3.28

NA: non annotated. FC: Fold Change.

<https://doi.org/10.1371/journal.pone.0212359.t002>





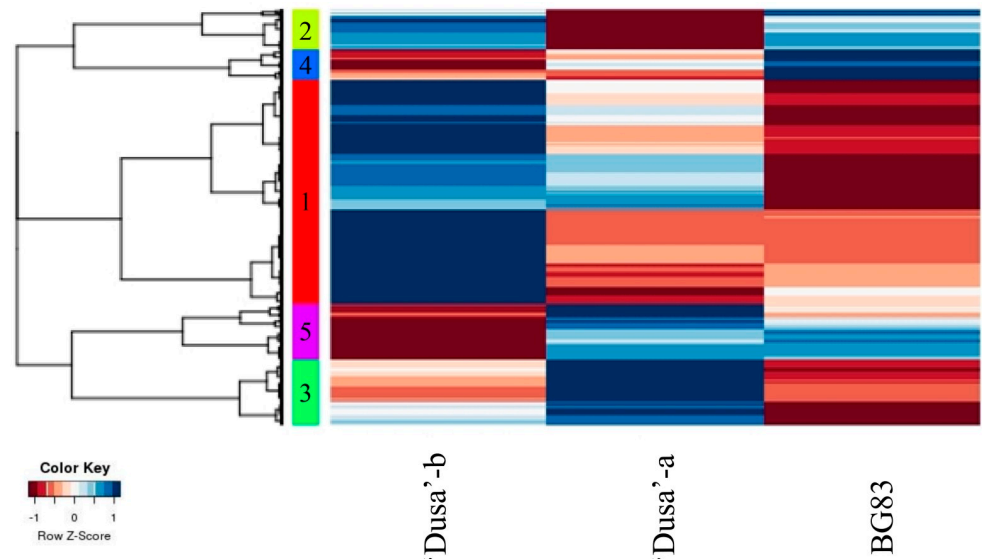
**Fig 3. Principal component analysis (PCA) of differentially expressed transcripts (DEGs).** PCA of DEGs obtained in the microarray analysis of susceptible 'Dusa'-b, 'Dusa'-a and tolerant BG83 avocado rootstocks after infection with *R. necatrix*. PCA of DEGs present in at least one of the samples, in which the first two dimensions explain up to 88.74% of the variance. Samples from the tolerant BG83 avocado rootstock showed the largest differences with those from the susceptible 'Dusa' ('Dusa'-b, 'Dusa'-a), which were grouped together in the biplot. Numbers are as follows: 1: protein hothead-like (Pa\_Contig00205); 2: chitinase 1-like (Pa\_Contig00535); 3: beta-glucanase (Pa\_Contig00542); 4: non-annotated (NA) (Pa\_Contig00559); 5: basic endochitinase-like partial (Pa\_Contig01014); 6: thaumatin-like protein (Pa\_Contig01450); 7: basic 7s globulin-like (Pa\_Contig02817); 8: glucan endo-beta-glucosidase-like (Pa\_Contig03461); 9: trypsin inhibitor (Pa\_Contig04097); 10: protein hothead-like (Pa\_Contig04808); 11: glu protease inhibitor-like (Pa\_Contig05213); 12: protein hothead-like (Pa\_Contig06176); 13: NA (Pa\_Contig06358); 14: basic 7s globulin-like (Pa\_Contig06808); 15: pathogenesis-related protein pr-4-like (Pa\_Contig07140); 16: protein hothead-like (Pa\_Contig07184); 17: NA (Pa\_Contig07403).

<https://doi.org/10.1371/journal.pone.0212359.g003>

accordance with the top10 differentially expressed transcripts selected for 'Dusa'-b (Table 2), in which five of the genes are involved in cell wall catabolic processes (basic endochitinase Pa\_contig01014, chitinase1 Pa\_contig00535, beta-glucanase Pa\_contig00542, glucan endo-beta-glucosidase Pa\_contig03461 and beta-d-galactosidase Pa\_contig06015). In contrast to glucanases, chitinases increase their expression as the disease progresses, and their role as defence-related proteins from the attack of pathogens has been widely described [25]. Expression of chitinase genes was also observed in tolerant rootstocks but at lower levels, suggesting constitutive rather than induced resistance mechanisms, as previously described in the *Humulus lupulus-Verticillium albo-atrum* interaction [26].

The smaller group comprising 50 genes, Group 4 (blue in Fig 4), includes those mainly induced in BG83. Many of these genes are related to an oxidation-reduction response and include defence-related genes such as cytochrome *p450*, protease inhibitors, and btb poz and taz domain-containing protein 1-like genes, which are known to play a role in plant defence [27–29].

Subsequently, to identify processes and functions over-represented in the different genotypes, all differentially expressed genes were functionally enriched and categorized based on blast sequence homologies and GO annotations using Blast2GO software ( $P < 0.025$ ) (Fig 5). In relation to the susceptible genotype, we observed commonly responsive genes before and after



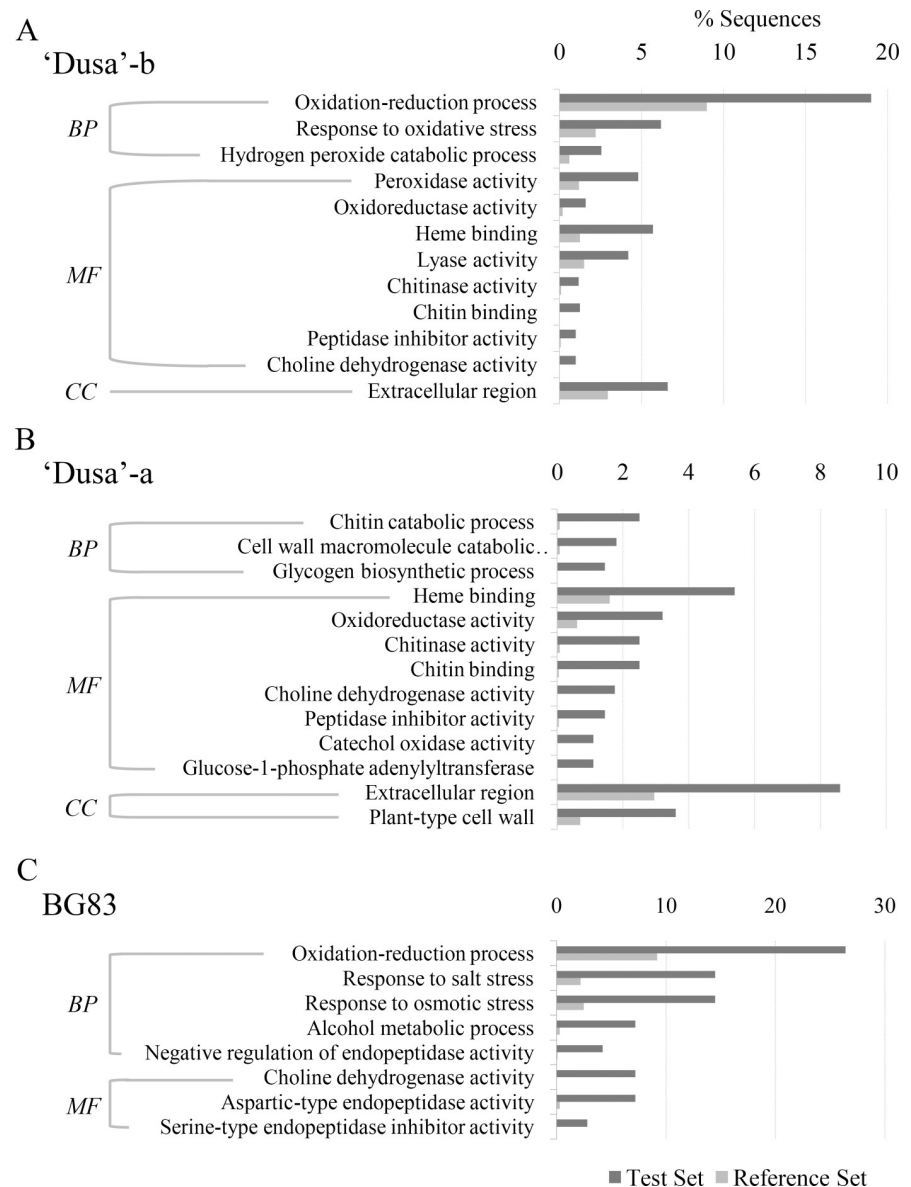
**Fig 4. Hierarchical clustering (HCL) of differentially expressed genes (DEGs).** HCL of DEGs in at least one of the samples of 'Dusa'-b, 'Dusa'-a and tolerant BG83 avocado rootstocks after infection with *R. necatrix*, clustered by hclust according to their expression profiles (Pearson's values correlation analysis). The five colours on the left correspond to the five groups with different expression profiles.

<https://doi.org/10.1371/journal.pone.0212359.g004>

a significant decrease in the CO<sub>2</sub> assimilation rate in the leaves was detected; these genes were related to oxidoreductase activity, chitinase activity, chitin and heme binding, choline dehydrogenase activity and peptidase inhibitor regarding molecular function activity and associated with the extracellular region regarding cellular components. Some GO terms were specific to 'Dusa'-b, such as oxidation-reduction processes, response to oxidative stress, hydrogen peroxide catabolic processes within biological process and peroxidase and lyase activities regarding molecular functions (Fig 5A). All of them had been induced in response to the reduction-oxidation status of the host cells as a result of pathogen invasion. In contrast, the most significant GO terms found solely in 'Dusa'-a were related to cell wall macromolecule catabolic processes, chitin catabolic processes and glycogen biosynthetic processes included in biological processes; glucose-1-phosphate, adenylyl transferase and catechol oxidase activities included in molecular functions, and plant cell wall-type activities included in cellular components (Fig 5B), most of them probably related to cell wall degradation as a consequence of disease progression [26].

Seven GO terms were enriched in DEGs from the BG83 genotype, and two were also found in the susceptible genotype (oxidation-reduction process and choline dehydrogenase). Among the GO terms found solely in the tolerant genotype were the following: response to salt and osmotic stresses, alcohol metabolic process and negative regulation of endopeptidase activity, in biological processes; and aspartic-type endopeptidase activity and serine-type endopeptidase inhibitor activity, in molecular function.

The differential induction of genes involved in salt and osmotic stress in BG83, such as aspartic peptidase domain-containing proteins (Pa\_contig06808, Pa\_contig04419, Pa\_contig02817, Pa\_contig05393) [30], gdsI esterase lipase protein (Pa\_contig00520) [31] and btb pzo and taz domain-containing protein (Pa\_contig00582) [32], could indicate that *R. necatrix* penetration into the roots is associated with osmotic effects, suggesting that BG83's tolerance to *R. necatrix* is related to the ability to withstand osmotic imbalance. This is in agreement with the



**Fig 5. Gene Ontology (GO) enrichment analysis of differentially expressed genes (DEGs).** GO enrichment analysis of DEGs in susceptible 'Dusa'-b, 'Dusa'-a and tolerant BG83 avocado rootstocks after infection with *R. necatrix*. Enrichment GO terms were obtained by Blast2GO using a cut-off of 0.025. (BP) biological process; (MF) molecular function; (CC) cellular component.

<https://doi.org/10.1371/journal.pone.0212359.g005>

collapse of the vascular system and the diminished water flow towards the aerial parts of the plant reported after *R. necatrix* invasion of root epidermal and cortical cells [8, 11].

Other GO terms expressed solely in BG83 seem to be associated with controlled protein degradation, which has been reported to be essential for the growth and development of plants and for survival under abiotic and biotic stresses [33]. To date, several protease and protease inhibitors have been confirmed to have functions in plant defence against pathogens [34, 35]. In addition, other studies reported that protease inhibitors also play a role in tolerance to abiotic stress, such as salt stress. Li et al. [36] showed that **transgenic plants** expressing a cysteine protease inhibitor exhibited increased salinity resistance, better growth performance, lower

malondialdehyde contents, higher anti-oxidase activity, and higher [cell viability](#) under salinity stress. Hence, it appears that tolerance to salt and osmotic stress, control of protein degradation and tolerance to *R. necatrix* share common pathways which merit further investigations.

### Differential expression of defence-related genes in 'Dusa' and BG83 avocado rootstocks after infection with *R. necatrix*

Genes previously reported to be important for conferring resistance in the 'Dusa'-defence response against *P. cinnamomi* were shown to be upregulated during 'Dusa'-*R. necatrix* interaction ([Table 3](#)). Some of them, such as the protein PR-4-like, cytochrome p450, thaumatin-like protein, glutathione S-transferase and chitinases, were even included in the list of the 20 genes showing the highest expression in 'Dusa' after infection with *R. necatrix* ([S2 Table](#)).

Five endochitinases were induced in 'Dusa'-b, two of them (basic endochitinase-like partial and acidic endochitinase-like) showing FC values of 49.89 and 11.34, respectively. Only one endochitinase (endochitinase pr4-like) was expressed in the tolerant rootstock BG83 ([Table 3](#)). An increase in the transcript levels of endochitinases was reported in tolerant avocado rootstocks, including 'Dusa', during the first 24h of infection with *P. cinnamomi* [[13](#)]. Chitinases belong to the PR protein families PR3, 4, 8 and 11 and are involved in the plant defence response against pathogens. The higher upregulation of chitinase genes in 'Dusa' compared to BG83 could be a consequence of a higher fungal colonization of susceptible plant cells and subsequent activation of fungal cell-wall degradation.

Inoculation of 'Dusa' and BG83 avocado plants with *R. necatrix* resulted in an accumulation of P450-like transcripts. Cytochrome P450 monooxygenases (P450s) are ubiquitous enzymes that catalyse the oxidation of many substrates by the activation of molecular oxygen and are involved in biotic and abiotic environmental responses as well as in the HR response to infection [[37](#), [38](#), [39](#)]. Different P450-like proteins were induced in susceptible and tolerant genotypes, e.g., while seven P450-like proteins were highly induced in the 'Dusa' genotype, only two were upregulated in BG83 ([Table 2](#)).

Expression of thaumatin-like protein (PR5) was only observed in 'Dusa' after infection with *R. necatrix*. PR5 has been reported to be associated with the salicylic acid (SA) pathway, and it has also been described to be significantly upregulated in 'Dusa' 48h after *P. cinnamomi* infection [[13](#)]. The fact that PR5 is upregulated in both 'Dusa'-*R. necatrix* and 'Dusa'-*P. cinnamomi* interactions, suggests that its induction must be important for tolerance to *P. cinnamomic* but not to *R. necatrix*. These results agree with previous studies reporting that SA signalling is mostly associated with defence against biotrophs, while regulation by ethylene and jasmonic pathways is a major defence response against necrotrophic pathogens [[37](#), [40](#)]. This is consistent with the significant overexpression of genes regulated by jasmonate, such as *btb poz* and *taz* domain-containing protein 1-like, observed in the tolerant rootstock BG83 ([Table 2](#)). Future studies addressing the transcriptome analysis of *R. necatrix*-tolerant rootstock interaction will be needed to confirm the expression of genes associated with the jasmonate or ethylene pathways.

Peroxidases are PR9 proteins induced in plant tissues upon pathogen infection and are known as defence-related proteins. In avocado roots, they seem to play a role in counteracting massive production of reactive oxygen species, which are induced when *P. cinnamomi* invades root cells [[41](#)]. In this study, *R. necatrix* infection induced 21 peroxidase genes in susceptible 'Dusa' and only two in the tolerant BG83 genotype. Low peroxidase expression in tolerant genotypes has also been described in other studies, i.e., rice genotypes in response to *Fusarium fujikuroi* [[37](#), [42](#)], which has been attributed to a reduced spread of the pathogen.

Plant protease inhibitors are extensively studied for their role in defence against pests and pathogens due to their ability to inhibit specific proteases of the intruder [[43](#)] and are used to

**Table 3. Defence-related genes overexpressed in ‘Dusa’-b, ‘Dusa’-a and BG83 avocado rootstock following *R. necatrix* infection.**

Contig ID ‘Dusa’-b	Contig ID ‘Dusa’-a	Contig ID BG83	Annotation
Pa_contig00582	Pa_contig00582	Pa_contig00582	Btb poz and taz domain-containing protein 1-like
Pa_contig02574			Universal stress protein a-like protein
Pa_contig00410			Phenylalanine ammonia-lyase (PAL)
Pa_Sin_GI32N0T02IMW			Lipoxygenase (LOX)
Pa_contig01014, Pa_contig06246	Pa_contig00472, Pa_contig01014, Pa_contig01395, Pa_contig06246, Pa_contig00472	Pa_contig01395	Endochitinase
Pa_contig01422, Pa_contig01568		Pa_contig01568	Glutathione S-transferase
Pa_contig01288, Pa_contig01450, Pa_contig01462	Pa_contig01288, Pa_contig01450, Pa_contig01462	Pa_contig01462	Thaumatococcus-like protein 1
Pa_contig01608, Pa_contig02847, Pa_contig03623, Pa_contig05603, Pa_contig06747, Pa_contig07325, Pa_contig07667	Pa_contig02847	Pa_contig01652, Pa_contig07667	Cytochrome p450
Pa_contig01236, Pa_contig05917	Pa_contig01236, Pa_contig02874, Pa_contig05917		Germin-like protein
Pa_contig00371, Pa_contig00528, Pa_contig01007, Pa_contig01569, Pa_contig03234, Pa_contig04589, Pa_contig05459, Pa_contig06331, Pa_Sin_FZ03KKT01A7ZOH, Pa_Sin_GI32N0T02F7BG6, Pa_Sin_GI32N0T02GDB8Q, Pa_Sin_GI32N0T02GS0JQ, Pa_Sin_GI32N0T02H6JSO, Pa_Sin_GI32N0T02HAYGM, Pa_Sin_GI32N0T02HMZMX, Pa_Sin_GI32N0T02HQGBT, Pa_Sin_GI32N0T02I1757	Pa_contig00371, Pa_contig03890, Pa_contig05459, Pa_Sin_GI32N0T02HAYGM, Pa_Sin_GI32N0T02HMZMX, Pa_Sin_GI32N0T02HQGBT, Pa_Sin_GI32N0T02I1757, Pa_Sin_GI32N0T02JURYY, Pa_Sin_HA66E9C01BY5PD	Pa_contig01569, Pa_contig05459	Peroxidase
Pa_contig02540, Pa_contig04097, Pa_contig05213, Pa_Sin_HA66E9C01AKZ67	Pa_contig02540, Pa_contig04097, Pa_Sin_HA66E9C01AKZ67	Pa_contig02540, Pa_contig04097, Pa_contig05213	Protease inhibitor
Pa_contig6278			Pathogenesis-related protein 4 (PR-4)
Pa_contig05982, Pa_contig07140, Pa_Sin_HA66E9C01BLTDJ	Pa_contig01063, Pa_contig05982, Pa_contig07140		Pathogenesis-related protein (PR)

<https://doi.org/10.1371/journal.pone.0212359.t003>

develop transgenic plants resistant to pathogens [44]. In this study, we identified three protease inhibitors (Pa\_contig05213; Pa\_contig02540; Pa\_contig04097) (Table 1) that were highly overexpressed in the tolerant BG83 genotype when compared to susceptible ‘Dusa’, after infection with *R. necatrix*, reaching FC values of 44, 34 and 16, respectively, in microarray analysis and FC values of 52.7, 18.8 and 38.31, respectively, in qRT-PCR experiments. Two of these protease inhibitors (Pa\_contig05213 and Pa\_contig02540) have been shown to be upregulated in

tolerant 'Dusa' after 18 h post-inoculation with *P. cinnamomi*, suggesting an important role of these proteins in the tolerance of avocado to the oomycete [24].

Interestingly, trypsin inhibitor overexpressed in buckwheat seeds suppressed spore germination and mycelial growth in the phytopathogenic fungi *Alternaria alternata* and *Fusarium oxysporum* [45]. The expression of these protease inhibitors is more than 20 times higher in tolerant BG83 compared to susceptible 'Dusa' after infection with the pathogen, suggesting the important role that these proteins could play in the defence of avocado rootstocks against *R. necatrix*. Since protease inhibitors are widespread in plant tissues and highly active with respect to proteases of pathogenic insects, bacteria and fungi [35, 44], they should be taken into consideration in plant breeding programmes aiming to improve plant resistance.

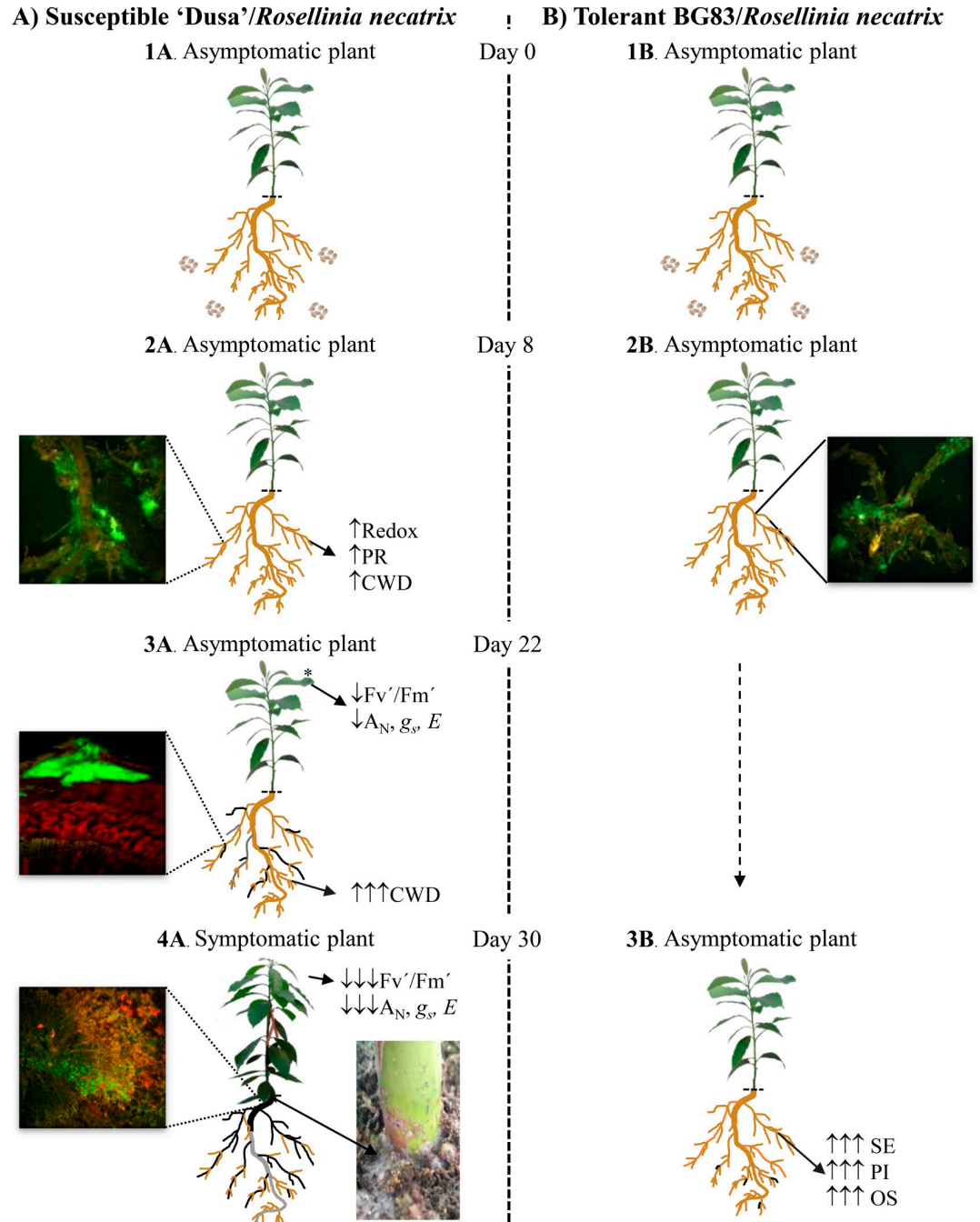
### Comparative model of *Rosellinia necatrix* infection on susceptible 'Dusa' and tolerant BG83 genotypes

Taking into consideration previous studies [7, 8, 11] together with results presented in this investigation, a scheme of early disease progression in susceptible and tolerant avocado/*R. necatrix* interactions is proposed integrating molecular and physiological data as well as Confocal Laser Scanning Microscopy (CLMS) observations (Fig 6). Plants were inoculated with wheat grain infected with *R. necatrix* (Day 0). Eight days after inoculation, *Rosellinia necatrix* spread through the soil, reaching avocado roots (visualization under a fluorescence microscope Leica Microsystems using a derivative strain of *R. necatrix* expressing the green fluorescent protein, *R. necatrix-gfp*) [8, 11]. Leaf photochemistry was not affected, and no aerial symptoms were observed in any of the genotypes. Molecular data revealed that infected susceptible 'Dusa' showed an upregulation of genes mainly related to oxidoreduction activity, cell wall degradation (chitinases and glucanases) as well as potential genes involved in defence to *P. cinnamomi* (pathogenesis-related protein PR-4-like (Pa\_contig07140), thaumatin-like protein 1 (Pa\_contig01450), glucan endo-beta-glucosidase-like (Pa\_contig03461) and beta-glucanase (Pa\_contig00542)). After 22 days of inoculation, although no visible symptoms were observed in any of the genotypes, variation in trapping efficiency of photosystem-II ( $F_v'/F_m'$ ) [11], net assimilation rates ( $A_N$ ), stomatal conductance ( $g_s$ ) and transpiration rate ( $E$ ) were observed in susceptible 'Dusa'. At the root level, genes related to cell wall macromolecule catabolic processes, chitinase activity, chitin binding and chitin catabolic processes became more relevant. CLSM observations using *R. necatrix-gfp* derivative strain, revealed that once leaf assimilation rates were affected in susceptible cultivars, external colonization of *R. necatrix* mycelia and penetration of mycelial aggregates into the root system followed by hyphal proliferation can be observed, although the root collar of the plant was not yet affected [11]. At thirty days post-inoculation, changes in net assimilation rates and leaf photochemistry became more pronounced, and visible symptoms, such as mild wilting of the leaves, started to appear in 'Dusa'. A profuse invasion of the vascular system and fungal colonization of the root collar was observed under CLSM studies, severely obstructing water flow to aerial parts of the plant [8].

At this stage, the tolerant genotype BG83 did not show either physiological changes or visible symptoms, although the fungus was present in the roots and penetration had occurred (visible and fluorescence microscopy observations). Molecular studies revealed an induction of protease inhibitors and their negative regulators, as well as salt- and osmotic stress-related genes.

### Conclusions

This study represents the first report on gene expression analysis for assessing the response of tolerant and susceptible avocado rootstocks to *R. necatrix*. The microarray analysis showed differential gene expression against this pathogen in tolerant and susceptible avocado rootstocks.



**Fig 6. Comparative model of *Rosellinia necatrix* infection on susceptible 'Dusa' (A) and Tolerant BG83 genotypes (B).** 1A. Inoculation of asymptomatic 'Dusa' plant with wheat grains infected with *R. necatrix*. 2A. 8 days post inoculation. Visualization of *R. necatrix* derivative strain CH53-*gfp* mycelia colonizing the root surface of avocado plantlets without aerial symptoms. The green fluorescent emitted from *R. necatrix-gfp* was visualized using a fluorescence microscope. Upregulation of genes mainly related to oxidoreduction activity (Redox), cell wall degradation (CWD) as well as potential genes involved in defence to *P. cinnamomi* (PR) was observed. 3A. 22 days post inoculation. The image shows a radial section of susceptible roots infected with *R. necatrix-gfp*; penetration and proliferation of *R. necatrix* mycelia through the root cortex was observed by Confocal Laser Scanning Microscopy (CLSM) concomitant with a decrease in leaf photochemistry (Fv'/Fm') and gas exchange parameters (A<sub>N</sub>, g<sub>s</sub>, E). Expression of genes related with cell wall degradation enzymes (CWD) mainly, chitin related enzymes, become more relevant. 4A. 30 days post inoculation, mild-wilting symptoms of the leaves start to appear in susceptible genotype. CLSM images of radial sections of the roots revealed a profuse invasion of *R. necatrix*, collapsing vascular vessels. 1B. Inoculation of asymptomatic BG83 plant with wheat grains infected with *R. necatrix*. 2B. 8 days post inoculation. Visualization of *R. necatrix* derivative strain CH53-*gfp* mycelia colonizing the root surface of avocado plantlets

without aerial symptoms. **3B.** 30 days post inoculation. Asymptomatic tolerant BG83 genotype showing upregulation of genes related to osmotic (OE) and salt stress (SE) as well as genes encoding protease inhibitors (PI).

<https://doi.org/10.1371/journal.pone.0212359.g006>

In the susceptible genotype, the early response was mainly related to oxido-reduction processes and cell-wall degradation activities, which became more noticeable after the photosynthetic capacity was affected by disease progression. In contrast, in addition to oxido-reduction processes, tolerance to *R. necatrix* in BG83 relies on the induction of protease inhibitors and their negative regulators, as well as salt and osmotic stress-related genes.

Furthermore, this study provides new insights into the different mechanisms involved in avocado tolerance responses to *P. cinnamomi* and *R. necatrix*. Interestingly, *R. necatrix* induced in susceptible ‘Dusa’ a set of general defence-related transcripts (i.e., universal stress protein, thaumatin-like protein and cytochrome p450) that are also induced in the tolerant response of ‘Dusa’ to *P. cinnamomi*. Some of these genes with enhanced expression are related to salicylic acid-dependent pathways involved in the defence mechanism against hemi-biotrophic pathogens such as *P. cinnamomi*. These genes were not noticeable in BG83. However, in BG83, a high induction of protease inhibitors, which have been extensively associated with plant defence against pathogens and abiotic stresses such as water stress [43], was observed. These results could indicate that these protease inhibitors play an important role in avocado tolerance against *R. necatrix*. Since protease inhibitors have been reported to be induced by water stress, in future studies, we will address the possibility of enhancing avocado tolerance to *R. necatrix* by priming with water stress. In addition, further transcriptomic analysis of the tolerant rootstocks would be a valuable tool to decipher the basis of *R. necatrix* tolerance in this species.

## Methods

### Plant material and experimental design

Experiments were carried out on one-year old plants (50–60 cm height) of two avocado rootstocks, BG83 and ‘Dusa’, with contrasting disease reactions to *R. necatrix*. The genotype BG83 was identified as tolerant in the ongoing breeding programme at IFAPA-Málaga, while ‘Dusa’ has been previously described as susceptible to *R. necatrix* [5]. BG83 explants were established *in vitro* and micropropagated according to protocol [46], while plants of ‘Dusa’ were vegetatively propagated by Brokaw nursery (Spain) using a modified Frohlich method [47]. To carry out the inoculation assay with *R. necatrix*, plants of each rootstock (BG83 and ‘Dusa’) were grown in a greenhouse under day light illumination and semi-controlled conditions of air temperature (T) and relative humidity (RH). Photosynthetic photon flux density (PPFD), T and RH conditions inside the greenhouse were continuously registered by a quantum sensor (Apogee SQ-110, USA) and by a T/RH U23-001 HOBO Pro v2 logger (Onset Computer Corporation, USA). Maximal midday values of PPFD varied between 440 and 1012  $\mu\text{mol m}^{-2} \text{s}^{-1}$ , and daily T was allowed to fluctuate according to external weather conditions, but its variation range inside the greenhouse was maintained between  $20 \pm 10^\circ\text{C}$  by an automatic cooling system and heating when necessary. The RH values inside the greenhouse were always over 40%. *R. necatrix* inoculum was produced on wheat seeds according to [48]. Briefly, seeds were soaked for 12 h in 250-ml Erlenmeyer flasks filled with distilled water. The flasks, each containing 100 ml of seeds, were subsequently autoclaved after excess water had been drained off. After sterilization, four fungal disks of a 2-week-old culture of *R. necatrix* grown on PDA were placed aseptically in each flask and incubated at  $24^\circ\text{C}$  in the dark for three weeks until wheat grains were homogeneously covered by *R. necatrix* mycelium.



From each rootstock, nine plants were used as non-inoculated (control plants), and eighteen plants were inoculated with *R. necatrix*. To ensure the spread of the inoculum, it was placed in eight points scattered around the stem (~3.5 cm apart) and introduced at two depths (~5 cm and ~15 cm). Plants were inoculated with 3.75 g of colonized wheat seeds per litre of substrate and monitored during disease progression until the appearance of aerial symptoms. The experiment was laid out in a randomized block design with three biological replicates per rootstock/time-point, in which each biological replicate consisted in three plants.

### Gas exchange variables

To test the effects of *R. necatrix* on photosynthetic performance during the first stages of disease development, gas exchange measurements were taken periodically (~4–7 d) at midmorning (1000–1100 h) on the first two fully expanded mature leaves from the top of each plant. These leaves were labelled at the onset of the experiment for repeated measurements. An open gas exchange system Li-6400 (LICOR Inc., USA) equipped with a LED-light source (6400-02B) and with a CO<sub>2</sub> mixer (6400-01) to modify the incoming air's CO<sub>2</sub> concentrations was used. The operating flow rate was 500 mL min<sup>-1</sup>, and the CO<sub>2</sub> partial pressure was 400 μmol CO<sub>2</sub> mol<sup>-1</sup> air. All measurements were performed at 22°C with a relative humidity of 50% and at a saturating photosynthetically active photon flux density (PPFD) of 1000 μmol quanta m<sup>-2</sup> s<sup>-1</sup>. Net CO<sub>2</sub> assimilation rates ( $A_N$ ), stomatal conductance ( $g_s$ ), and transpiration rates ( $E$ ) were estimated with established equations [49]. Intrinsic water use efficiency ( $A_N/g_s$ ) was calculated as the ratio between  $A_N$  and  $g_s$ .

The effects of *R. necatrix* inoculation on the gas exchange variables of the avocado rootstocks during disease progression were evaluated by one-way repeated measures analysis of variance (r-ANOVA). To analyse single sampling dates, one-way ANOVA was used. Significant differences were considered at the 5% probability level unless otherwise stated. Prior to ANOVA, normality and homogeneity assumptions were tested using the Kolmogorov-Smirnov and Cochran's C tests, respectively. When significant differences were observed, Fisher's least significant difference (LSD) test was used to compare the mean values. Statistical analysis was performed using STATISTICA 7.0 analytical software (Statsoft Inc., USA).

### RNA extraction

The infected roots of each 'Dusa' plant were harvested at eight and 22 days post-inoculation, before ('Dusa'-b) and after ('Dusa'-a) the observation of a decrease in net assimilation rates, although the plants appeared to be healthy. Since no significant variation in any of the gas exchange parameters was observed for the tolerant genotype, infected root samples were collected at 30 days post-inoculation eight days after the observation of a decrease in the net assimilation rate in the susceptible 'Dusa' genotype. RNA from ground root tissue of three independent plants was extracted using the CTAB extraction method [50], a simple and efficient method for isolating RNA from pine trees, with slight modifications. The chloroform:isoamyl alcohol step was repeated 3–5 times, depending on the stability of the interphase and colour of the sample. 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., Montchanin, USA) spectrophotometer. RNA integrity was confirmed by the appearance of ribosomal RNA bands and lack of degradation products after separation on a 2% agarose gel and red safe staining.

### Microarray analysis

The microarray hybridizations were carried out using a custom microarray (GEO accession GPL21856) [12]. For 'Dusa'-b and 'Dusa'-a plants, three biological replicate hybridizations of

infected vs control samples were performed, while two biological replicate hybridizations of treated vs control samples were performed for BG83. qRT-PCR analysis was used to validate gene expression in the third BG83 biological replicate sample. Six micrograms of RNA was used for first strand cDNA synthesis. Single-stranded cDNA was synthesized according to the manufacturer's instructions using Superscript™ III reverse transcriptase (Invitrogen) in a total volume of 30 µl. First strand synthesis was primed with random nonamer (N9, Inqaba-Biotec, Sunnyside, South Africa) and oligo (Dt) (dT23VN, Inqaba) primers. cDNA clean-up was carried out using an RNA clean-up kit (Qiagen RNeasy MiniElute™) to remove hydrolysed RNA. The concentration and purity of cDNA was determined using a Nanodrop ND1000. Sample preparation and labelling for the microarray was carried out as previously described [12]. The yield and specific activity were calculated, and hybridization was performed according to the two-colour microarray-based gene expression analysis protocol (Agilent). Microarray slides were scanned using the Axon GenePix 4000B scanner (Molecular Devices, CA, USA), and Axon GenePix 6.0 software (molecular devices) was used for image assessment. GenePix Array List (GAL) files were generated by Agilent and loaded into GenePix to link information of each printed spot to analyse results. Following automated spot detection using the software, manual feature alignment was performed to validate spot finding. Flagging of features was based on saturation and signal-to-noise ratios (SNR). Features with SNR <2 in both channels were excluded from further analysis, as were features with foreground saturation >20%.

Statistical analysis of the microarray data was performed as previously described [12] using the LIMMA (linear models for microarray data) package in the R version 3.1.0 environment (R Foundation for Statistical Computing). P-values were adjusted for multiple testing using the false discovery rate (FDR) correction. A standard pair-wise Pearson correlation ( $r$ ) was performed using normalized M-values to determine concordance between biological replicates. Targets were defined as differentially expressed genes (DEG) if the  $\log_2$  ratio was greater or equal to 1 or smaller or equal to -1 ( $\log_2$  ratio  $\leq 1$  and  $\log_2$  ratio  $\geq 1$ ) and the adjusted P-value was less than or equal to 0.05 ( $P \leq 0.05$ ). The data from this study are available from the NCBI Gene Expression Omnibus under accession number GSE119140.

### Functional annotation and clustering

Functional annotation, functional enrichment and gene ontology (GO) terms describing biological processes, molecular functions and cellular components were assigned using Blast2GO software (B2G: <http://www.blast2go.com>). Default parameters were used with a cut-off FDR of 0.025. Venn diagrams were drawn using the Venn Diagram package [51]. Hierarchical clustering was performed using the expression values to identify genes with similar profiles across all three biological samples. Pearson correlation and linkage methods were used with the *hclust* function from the stats package [52] and the results plotted with heatmap.2 from the gplots package [53] and R color Brewer [54]. The row (contigs) dendrogram was then divided into distinct clusters, each of which contained genes with a unique expression profile by using the *cutree* function from the stats package [52]. Principal component analysis was performed using FactomineR [55], factoextra [56] and ade4 [57].

### Quantitative real-time PCR

Validation of gene expression levels obtained from the microarray analysis was performed using qRT-PCR.

One microgram of total RNA was treated with DNase RNase-free (Promega, Madison, USA) following the manufacturer's instructions. Single-stranded cDNA was synthesized using

the iScript cDNA synthesis kit (BIO-RAD, California, USA) following the manufacturer's instructions. The cDNA was analysed for genomic DNA contamination by PCR using gene-specific primers F3H-F (5' -TCTGATTTCCGGAGATGACTCGC-3') and F3H-R (5' -TGTAGACTTGGGCCACCTCTTT-3'), which flank an intron of the eflavonone 3-hydroxylase (F3H) gene. PCR amplifications were carried out as previously described by Engelbrecht and van den Berg (2013) using first-strand cDNA as the template.

The expression of fifteen avocado genes was investigated. Two endogenous control genes (actin and 18S) were used for normalization. Primer sequences for endogenous control genes and the fifteen avocado genes are presented in [S3 Table](#). Primer pairs were chosen to generate fragments between 50 and 150 bp with melting temperatures ranging from 55–60°C and designed using Primer 3 software (<http://bioinfo.ut.ee/primer3-0.4.0/>).

Primer specificity was tested by first 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 all treatment groups, and time-points 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 10 s at 95°C and 15 s at 60°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 performed using the comparative Ct method [58]. Three biological replicates of infected vs. control samples were performed for 'Dusa'-b and 'Dusa'-a and BG83, and all reactions were performed in triplicate. Statistical significance of the data was determined by Student's *t*-test carried out with Sigma Stat version 4.0 software (Systat Software GmbH).

## Supporting information

**S1 Table. Annotation and expression values of differentially expressed genes grouped by hierarchical clustering (HCL).**

(XLSX)

**S2 Table. Top 20 overexpressed genes in 'Dusa'-b, 'Dusa'-a and BG83 avocado rootstocks after infection with *R. necatrix*.** Description and features of the top 20 overexpressed genes in the 'Dusa'-b, 'Dusa'-a and BG83 avocado rootstocks after infection with *R. necatrix*.

(DOC)

**S3 Table. qRT-PCR primer sequences used in this study.** Primer sequences used in this study.

(DOC)

**S1 Fig. Gene Ontology (GO) enrichment analysis of group 1 obtained in the hierarchical clustering analysis of Fig 4.** Enrichment of GO terms obtained with Blast2GO using a cut-off of 0.025. (BP) biological process and (MF) molecular function.

(TIF)

**S2 Fig. Gene Ontology (GO) enrichment analysis of group 3 obtained in the hierarchical clustering analysis of Fig 4.** Enrichment of GO terms obtained with Blast2GO using a cut-off of 0.025. (BP) biological process and (MF) molecular function.

(TIF)

## Author Contributions

**Conceptualization:** Antonio J. Matas.

**Data curation:** Elsa Martínez-Ferri, Antonio J. Matas, Nicholas A. Olivier.

**Formal analysis:** Adela Zumaquero, Elsa Martínez-Ferri, Antonio J. Matas, Nicholas A. Olivier.

**Funding acquisition:** Clara Pliego.

**Investigation:** Adela Zumaquero, Bianca Reeksting, Clara Pliego.

**Methodology:** Adela Zumaquero, Bianca Reeksting, Nicholas A. Olivier, Nöelani van den Berg, Clara Pliego.

**Software:** Antonio J. Matas.

**Supervision:** Fernando Pliego-Alfaro, Araceli Barceló, Clara Pliego.

**Validation:** Elsa Martínez-Ferri.

**Visualization:** Elsa Martínez-Ferri, Araceli Barceló.

**Writing – original draft:** Elsa Martínez-Ferri, Fernando Pliego-Alfaro, Nöelani van den Berg, Clara Pliego.

**Writing – review & editing:** Elsa Martínez-Ferri, Fernando Pliego-Alfaro, Nöelani van den Berg, Clara Pliego.

## References

1. Coffey MD. *Phytophthora* root rot of avocado: an integrated approach to control in California. *Plant Dis.* 1987; 71: 1046–1052.
2. Giblin F, Pegg K, Willingham S, Anderson J, Coates L, Cooke T, et al. "Phytophthora revisited". In: Proc. of the New Zealand and Australia Avocado Grower's Conference, Tauranga, New Zealand. Avocado Growers Association. 2005. [http://www.avocadosource.com/Journals/AUSNZ/AUSNZ\\_2005/GiblinFiona2005.pdf](http://www.avocadosource.com/Journals/AUSNZ/AUSNZ_2005/GiblinFiona2005.pdf). Accessed 9 Apr 2018.
3. Rodríguez-Henao E, Caicedo-Arana A, Enríquez-Valencia AL, Muñoz-Florez JE. Evaluation of tolerance to *Phytophthora cinnamomi* Rands in avocado (*Persea americana* Miller.) germplasm. *Acta Agron.* 2017. <https://doi.org/10.15446/acag.v66n1.50705>
4. Reeksting BJ, Coetzer N, Mahomed W, Engelbrecht J, van den Berg N. *De novo* sequencing, assembly, and analysis of the root transcriptome of *Persea americana* (Mill.) in response to *Phytophthora cinnamomi* and flooding. *Plos One.* 2014. <https://doi.org/10.1371/journal.pone.0086399> PMID: 24563685
5. Pérez-Jiménez RM. A review of the biology and pathogenicity of *Rosellinia necatrix*- The cause of white root rot disease of fruit trees and other plants. *J Phytopathol.* 2006. <https://doi.org/10.1111/j.1439-0434.2006.01101.x>
6. López-Herrera CJ, Zea-Bonilla T. Effects of benomyl, carbendazim, fluzinam and thiophanate methyl on white root rot of avocado. *Crop Prot.* 2007. <https://doi.org/10.1016/j.cropro.2007.01.008>
7. Pliego C, López-Herrera C, Ramos C, Cazorla FM. Developing tools to unravel the biological secrets of *Rosellinia necatrix*, an emergent threat to woody crops. *Mol Plant Pathol.* 2012. <https://doi.org/10.1111/j.1364-3703.2011.00753.x> PMID: 22014332
8. Pliego C, Kanematsu S, Ruano-Rosa D, de Vicente A, López-Herrera C, Cazorla FM, Ramos C. GFP sheds light on the infection process of avocado roots by *Rosellinia necatrix*. *Fungal Genet Biol.* 2009. <https://doi.org/10.1016/j.fgb.2008.11.009> PMID: 19100853
9. Pliego C, Ramos C, de Vicente A, Cazorla FM. Screening for candidate bacterial biocontrol agents against soilborne fungal plant pathogens. *Plant Soil.* 2011. <https://doi.org/10.1007/s11104-010-0615-8>
10. Barceló-Muñoz A, Zea-Bonilla T, Jurado-Valle I, Imbroda-Solano I, Vidoy-Mercado I, Pliego-Alfaro F, et al. Programa de selección de portainjertos de aguacate tolerantes a la podredumbre blanca causada por *Rosellinia necatrix* en el Sur de España 2007. [www.avocadosource.com/WAC5/Papers/WAC5\\_p537.pdf](http://www.avocadosource.com/WAC5/Papers/WAC5_p537.pdf). Accessed 9 Apr 2018.

11. Martínez-Ferri E, Zumaquero A, Ariza MT, Barceló-Muñoz A, Pliego C. Nondestructive detection of white root rot disease in avocado rootstocks by leaf chlorophyll fluorescence. *Plant Dis*. 2016. <https://doi.org/10.1094/PDIS-01-15-0062-RE> PMID: 30688585
12. Reeksting BJ, Olivier NA, van den Berg N. Transcriptome responses of an ungrafted *Phytophthora* root rot tolerant avocado (*Persea americana*) rootstock to flooding and *Phytophthora cinnamomi*. *BMC Plant Biol*. 2016. <https://doi.org/10.1186/s12870-016-0893-2> PMID: 27658453
13. Engelbrecht J, van den Berg N. Expression of defence-related genes against *Phytophthora cinnamomi* in five avocado rootstocks. *S Afr J Sci*. 2013. <https://doi.org/10.1590/sajs.2013/20120058>
14. Fleischmann F, Koehl J, Portz R, Beltrame AB, Oßwald W. Physiological changes of *Fagus sylvatica* seedlings infected with *Phytophthora citricola* and the contribution of its elicitor 'citricolin' to pathogenesis. *Plant Biol*. 2005. <https://doi.org/10.1055/s-2005-872891> PMID: 16388468
15. Clemenz C, Fleischmann F, Haberle KH, Matyssek R, Oßwald W. Photosynthetic and leaf water potential responses of *Alnus glutinosa* saplings to stem-base inoculation with *Phytophthora alni* subsp. *alni*. *Tree Physiol*. 2008. <https://doi.org/10.1093/treephys/28.11.1703>
16. Reeksting BJ, Taylor N, van den Berg N. Flooding and *Phytophthora cinnamomi*: Effects on photosynthesis and chlorophyll fluorescence in shoots of non-grafted *Persea americana* (Mill.) rootstocks differing in tolerance to *Phytophthora* root rot. *S Afr J Bot*. 2014. <https://doi.org/10.1016/j.sajb.2014.08.004>
17. Martínez-Ferri E, Balaguer L, Valladares F, Chico JM, Manrique E. Energy dissipation in drought-avoiding and drought-tolerant tree species at midday during the Mediterranean summer. *Tree Physiol*. 2000. <https://doi.org/10.1093/treephys/20.2.131>
18. Leyva-Pérez MO, Jiménez-Ruiz J, Gómez-Lama C, Valverde-Corredor A, Barroso JB, Luque F, et al. Tolerance of olive (*Olea europaea*) cv Frantoio to *Verticillium dahliae* relies on both basal and pathogen-induced differential transcriptomic responses. *New Phytol*. 2018. <https://doi.org/10.1111/nph.14833>
19. Rodrigues FA, de Laia ML, Zingaretti SM. Analysis of gene expression profiles under water stress in tolerant and sensitive sugarcane plants. *Plant Sci*. 2009. <https://doi.org/10.1016/j.plantsci.2008.11.007>
20. Walia H, Wilson C, Condamine P, Liu X, Ismail AM, Zeng L, et al. Comparative transcriptional profiling of two contrasting rice genotypes under salinity stress during the vegetative growth stage. *Plant Physiol*. 2005. <https://doi.org/10.1104/pp.105.065961> PMID: 16183841
21. Zainal Z, Marouf E, Ismail I, Fei CK. Expression of the *Capsicum annuum* (Chili) defensin gene in transgenic tomatoes confers enhanced resistance to fungal pathogens. *Am J Plant Physiol*. 2009. <https://doi.org/10.3923/ajpp.2009.70.79>
22. Lawrence PK, Kounda KR. Plant protease inhibitors in control of phytophagous insects. *Electron J Biotechnol*. 2002. <https://doi.org/10.2225/vol5-issue1-fulltext-3>
23. Kim JY, Park SC, Hwang I, Cheong H, Nah JW, Hahm KS, et al. Protease inhibitors from plants with antimicrobial activity. *Int J Mol Sci*. 2009. <https://doi.org/10.3390/ijms10062860> PMID: 19582234
24. van den Berg N, Mahomed W, Olivier NA, Swart V, Crampton BG. Transcriptome analysis of an incompatible *Persea americana-Phytophthora cinnamomi* interaction reveals the involvement of SA- and JA-pathways in a successful defense response. *Plos One*. 2018. <https://doi.org/10.1371/journal.pone.0205705> PMID: 30332458
25. Sharma N, Sharma KP, Gaur RK, Gupta VK. Role of chitinase in plant defense. *Asian J Biochem*. 2011. <https://doi.org/10.3923/ajb.2011.29.37>
26. Mandelc S, Timperman I, Radišek S, Devreese B, Samyn B, Javornik B. Comparative proteomic profiling in compatible and incompatible interactions between hop roots and *Verticillium albo-atrum*. *Plant Physiol Biochem*. 2013. <https://doi.org/10.1016/j.plaphy.2013.03.017> PMID: 23619241
27. Xu J, Wang XY, Guo WZ, Xin-yu W, Wang-Zhen G. The cytochrome P450 superfamily: Key players in plant development and defense. *J Integr Agric*. 2015. [https://doi.org/10.1016/S2095-3119\(14\)60980-1](https://doi.org/10.1016/S2095-3119(14)60980-1).
28. Howell JT, Davis MR. Plant defense mechanisms against fungal pathogens: polygalacturonase inhibitor proteins. *Can J Plant Pathol*. 2005. <https://doi.org/10.1080/07060660509507188>
29. Boyle P, Le Su E, Rochon A, Shearer HL, Murmu J, Chu JY, et al. The BTB/POZ domain of the Arabidopsis disease resistance protein NPR1 interacts with the repression domain of TGA2 to negate its function. *Plant Cell*. 2009. <https://doi.org/10.1105/tpc.109.069971> PMID: 19915088
30. Xia Y, Suzuki H, Borevitz J, Blount J, Guo Z, Patel K, et al. An extracellular aspartic protease functions in *Arabidopsis* disease resistance signaling. *Embo J*. 2004. <https://doi.org/10.1038/sj.emboj.7600086> PMID: 14765119
31. Naranjo MA, Forment J, Roldan M, Serrano R, Vicente O. Overexpression of *Arabidopsis thaliana* LTL1, a salt-induced gene encoding a GDSL-motif lipase, increases salt tolerance in yeast and transgenic plants. *Plant Cell Environ*. 2006. <https://doi.org/10.1111/j.1365-3040.2006.01565.x> PMID: 16930315

32. Guo M, Liu X, Wang J, Li L, Zhang W, Gong B et al. Investigation on salt-response mechanisms in *Arabidopsis thaliana* from UniProt protein knowledgebase. *J Plant Interact*. 2019. <https://doi.org/10.1080/17429145.2018.1551581>
33. Budic M, Sabotic J, Meglic V, Kos J, Kidric M. Characterization of two novel subtilases from common bean (*Phaseolus vulgaris* L.) and their responses to drought. *Plant Physiol Biochem*. 2013. <https://doi.org/10.1016/j.plaphy.2012.10.022> PMID: 23201564
34. Pogány M, Dankó T, Kámán-Tóth E, Schwarczinger I, Bozsó Z. Regulatory proteolysis in *Arabidopsis*-pathogen interactions. *Int J Mol Sci*. 2015. <https://doi.org/10.3390/ijms161023177> PMID: 26404238
35. Dunaevsky YE, Elpidina EN, Vinokurov KS, Belozersky MA. Protease inhibitors in improvement of plant resistance to pathogens and insects. *Mol Biol*. 2005. <https://doi.org/10.1007/s11008-005-0076-y>
36. Li R, Wang W, Wang W, Li F, Wang Q, Xu Y, Wang S. Overexpression of a cysteine proteinase inhibitor gene from *Jatropha curcas* confers enhanced tolerance to salinity stress. *Electron J Biotechn*. 2015. <https://doi.org/10.1016/j.ejbt.2015.08.002>
37. Matic S, Bagnaresi P, Biselli C, Orru L, Carneiro GA, Siciliano I, et al. Comparative transcriptome profiling of resistant and susceptible rice genotypes in response to the seedborne pathogen *Fusarium fujikuroi*. *BMC Genomics*. 2016. <https://doi.org/10.1186/s12864-016-2925-6> PMID: 27515776
38. Coram TE, Wang M, Chen X. Transcriptome analysis of the wheat-*Puccinia striiformis* f. sp. *tritici* interaction. *Mol Plant Pathol*. 2008. <https://doi.org/10.1111/j.1364-3703.2007.00453.x> PMID: 18705849
39. Thomas SW, Glaring MA, Rasmussen SW, Kinane JT, Oliver RP. Transcript profiling in the barley mildew pathogen *Blumeria graminis* by serial analysis of gene expression (SAGE). *Mol Plant Microbe Interact*. 2002. <https://doi.org/10.1094/MPMI.2002.15.8.847>
40. Ali S, Ganai BA, Kamili AN, Bhat AA, Mir ZA, Bhat JA, et al. Pathogenesis-related proteins and peptides as promising tools for engineering plants with multiple stress tolerance. *Microbiol Res*. 2018. <https://doi.org/10.1016/j.micres.2018.04.008> PMID: 29853166
41. García-Pineda E, Benezzer-Benezzer M, Gutiérrez-Segundo A, Rangel-Sánchez G, Arreala-Cortés A, Castro-Mercado E. Regulation of defence responses in avocado roots infected with *Phytophthora cinnamomi* (Rands). *Plant Soil*. 2010. <https://doi.org/10.1007/s11104-009-0225-5>
42. Desjardins AE, Manandhar HK, Plattner RD, Manandhar GG, Poling SM, Maragos CM. *Fusarium* species from nepalese rice and production of mycotoxins and gibberellic acid by selected species. *Appl Environ Microbiol*. 2000. <https://doi.org/10.1128/AEM.66.3.1020-1025.2000>
43. Srinivasan T, Kirti PB. Protease inhibitors and stress tolerance. *Funct Plant Sci Biotechnol*. 2012; 6(1): 59–66.
44. Habib H, Fazili KM. Plant protease inhibitors: a defense strategy in plants. *Biotechnol Mol Biol Rev*. 2007; 2(3): 68–85.
45. Dunaevsky YE, Pavlukova EB, Beliakova GA, Tsybina TA, Gruban TN, Belozersky M. Protease inhibitors in buckwheat seeds: Comparison of anionic and cationic inhibitors. *J Plant Physiol*. 1998. [https://doi.org/10.1016/S0176-1617\(98\)80032-1](https://doi.org/10.1016/S0176-1617(98)80032-1)
46. Pliego-Alfaro F, Barceló-Muñoz A, López-Gómez R, Ibarra-Laclette E, Herrera-Estrella L, Palomo-Ríos E, et al. *Biotechnology*. In: Schaffer B, Wolstenholme N, Whiley AW, editors. *The Avocado: Botany, Production and Uses*. 2nd edition, CAB International. Wallingford; 2013. pp. 268–300.
47. Frolich EF, Platt RG. Use of the etiolation technique in rooting avocado cuttings. *California Avocado Soc*. 1972; 55: 97–109.
48. Szejnberg A, Madar Z. Host range of *Dematophora necatrix*, the cause of white root rot disease in fruit trees. *Plant Dis*. 1980; 64: 662–664.
49. von Caemmerer S, Farquhar G. Some relationships between the biochemistry of photosynthesis and the gas exchange of leaves. *Planta*. 1981. <https://doi.org/10.1007/BF00384257> PMID: 24276943
50. Chang S, Puryear J, Cairney J. A simple and efficient method for isolating RNA from pine trees. *Plant Mol Biol Rep*. 1993. <https://doi.org/10.1007/BF02670468>
51. Chen H. Venn Diagram: Generate high-resolution venn and euler plots. R package version 1.6.17. 2016. Available from: <https://CRAN.R-project.org/package=VennDiagram>. Accessed 9 Apr 2018.
52. R Core Team. R: A language and environment for statistical computing. R Foundation for Statistical Computing. Vienna. 2017. Available from: <https://www.R-project.org/>. Accessed 9 Apr 2018.
53. Warnes GR, Bolker B, Bonebakker L, Gentleman R, Liaw WHA, Lumley T, et al. gplots: Various R programming tools for plotting data. R package version 3.0.1. 2016. Accessed 9 Apr 2018.
54. Neuwirth E. RcolorBrewer: Colorbrewer palettes. R package version 1.1–2. Available from: Available from: <https://CRAN.R-project.org/package=RColorBrewer>. 2014. Accessed 9 Apr 2018.
55. Le S, Josse J, Husson F. FactoMineR: An R package for multivariate analysis. *J Stat Softw*. 2008. <https://doi.org/10.18637/jss.v025.i01>

56. Kassambara A, Mundt F. Factoextra: Extract and visualize the results of multivariate data analyses. R package version 1.0.4. 2017. Available from: <https://CRAN.R-project.org/package=factoextra>. Accessed 9 Apr 2018.
57. Dray S, Dufour AB. The ade4 package: implementing the duality diagram for ecologists. J Stat Softw. 2007. <https://doi.org/10.18637/jss.v022.i04>
58. Pfaffl MW. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res. 2001. <https://doi.org/10.1093/nar/29.9.e45>