Transcriptome analysis of the fungal pathogen *Rosellinia necatrix* during infection of a susceptible avocado rootstock identifies potential mechanisms of pathogenesis

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**Abstract**

**Background:** White root rot disease caused by *Rosellinia necatrix* is one of the most important threats affecting avocado productivity in tropical and subtropical climates. Control of this disease is complex and nowadays, lies in the use of physical and chemical methods, although none have proven to be fully effective. Detailed understanding of the molecular mechanisms underlying white root rot disease has the potential of aiding future developments in disease resistance and management. In this regard, this study used RNA-Seq technology to compare the transcriptomic profiles of *R. necatrix* during infection of susceptible avocado ‘Dusa’ roots with that obtained from the fungus cultured in rich medium.

**Results:** The transcriptomes from three biological replicates of *R. necatrix* colonizing avocado roots (RGA) and *R. necatrix* growing on potato dextrose agar media (RGPDA) were analyzed using Illumina sequencing. A total of 12,104 transcripts were obtained, among which 1937 were differentially expressed genes (DEG), 137 exclusively expressed in RGA and 160 in RGPDA. During the root infection process, genes involved in the production of fungal toxins, detoxification and transport of toxic compounds, hormone biosynthesis, gene silencing and plant cell wall degradation were overexpressed. Interestingly, 24 out of the 137 contigs expressed only during *R. necatrix* growth on avocado roots, were predicted as candidate effector proteins (CEP) with a probability above 60%. The PHI (Pathogen Host Interaction) database revealed that three of the *R. necatrix* CEP showed homology with previously annotated effectors, already proven experimentally via pathogen-host interaction.

**Conclusions:** The analysis of the full-length transcriptome of *R. necatrix* during the infection process is suggesting that the success of this fungus to infect roots of diverse crops might be attributed to the production of different compounds which, singly or in combination, interfere with defense or signaling mechanisms shared among distinct plant families. The transcriptome analysis of *R. necatrix* during the infection process provides useful information and facilitates further research to a more in-depth understanding of the biology and virulence of this emergent pathogen. In turn, this will make possible to evolve novel strategies for white root rot management in avocado.

**Keywords:** Ascomycete, Effectors, *Persea americana*, Virulence, White root rot

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Background
Rosellinia necatrix is a soilborne ascomycete, belonging to the order Xylariales, which causes white root rot (WRR) disease in a wide range of commercially important crops and ornamental plants. It has been reported that R. necatrix can infect over 170 plant species from 63 genera and 30 families [1], listed in 344 R. necatrix-host combinations by the United States Department of Agriculture [2]. This pathogen has a worldwide distribution being able to survive in temperate, tropical and subtropical climates [3–6].

In the Mediterranean region of Spain, WRR is especially damaging due to the co-occurrence of favorable environmental conditions for the development of the fungus and susceptible hosts such as avocado (Persea americana Mill.) and mango (Mangifera indica L.) [7, 8]. Nowadays it is considered as one of the most important threats affecting avocado productivity [7].

Affected avocado trees show rotten roots and are characterized by a yellowing of the leaves that eventually wilt and ultimately, results in death of the tree. R. necatrix root invasion usually occurs by the formation of mycelial aggregates over the root surface which penetrate the root tissues among epidermal and cortical cells and finally, collapse the vascular system of the plant [9]. Neither chemical nor physical methods have proven to be fully effective to control this disease due to the capacity of the fungus to survive in acidic soils as well as to colonize numerous hosts; in addition, the pathogen is quite resistant to drought [4, 7]. Nowadays, the obtaining of tolerant rootstocks appears as the most promising approach to control this disease and efforts are underway to reach this goal [10]. To add future developments in disease resistance, systematic analysis of pathogenic fungi’s genomes and transcriptomes has become a top priority. Thus, in recent years, many researchers have addressed transcriptomics studies of plant pathogenic fungi/host interactions [11–13]. The analyses of gene expression profiles associated with the fungal infection provides key sources for understanding fungal biology, leading to the identification of potential pathogenicity determinants [11, 14–17]. Recently, Shimizu et al. [13] provided a 44-Mb draft genome sequence of R. necatrix virulent strain W97, in which 12,444 protein encoding genes were predicted. The transcriptome analysis of the hypovirulent strain W97, infected with the megabirnavirus 1 (RNmbv1), revealed that primary and secondary metabolism, as well as genes encoding transcriptional regulators, plant cell wall-degrading enzymes (CWDE), and toxin production such as cytochalasin E, were greatly disturbed in the hypovirulent strain. In another study, the transcriptome analysis of the virulent R. necatrix strain (KACC40445) identified 10,616 full-length transcripts among which, pathogen related effectors and CWDE encoding genes were predicted [12]. Data presented in both transcriptomics studies are a valuable resource of genetic information; however, to get a deep insight into pathogenesis of R. necatrix a comprehensive transcriptomic analysis of a virulent R. necatrix strain interacting with its host is necessary. With this aim, this research addresses the comparison of the transcriptomic profiles of R. necatrix during infection of susceptible ‘Dusa’ roots (RGA) and in vitro growth on PDA (Potato Dextrose Agar) media (RGPDA) using RNA-Seq technology. Functional classification based on assignments to publicly available datasets was conducted, and potential pathogenicity genes related to R. necatrix virulence were identified providing a better understanding of the WRR disease.

Results
Comparative transcriptome analysis of R. necatrix growing on avocado roots vs PDA medium
A transcriptome analysis was carried out to capture genes expressed during R. necatrix growth on susceptible ‘Dusa’ avocado roots and on PDA medium, in order to compare their expression profiles (Fig. 1). The RNA-Seq data including the raw reads from three biological replicates of R. necatrix CH53 virulent strain colonizing avocado roots (RGA1; RGA2 and RGA3) and growing on culture medium (RGPDA1; RGPDA2 and RGPDA3) were processed. A total of 12,104 transcripts were obtained, among which 11,807 were present in both conditions, while 137 and 160 transcripts were exclusively expressed in either RGA or RGPDA, respectively (Fig. 2). Total transcripts were subjected to statistical analysis to evaluate differential gene expression between RGA vs RGPDA test situations. Analyses resulted in 1937 differentially expressed genes (DEG), 61.9% induced and 38.1% repressed (−2 > fold change (FC) > 2; P-value < 0.05) (Fig. 3). A heat map of DEGs showed consistence in expression patterns among RGA1, RGA2 and RGA3 and among RGPDA1, RGPDA2 and RGPDA3, supporting the reliability of the RNA-Seq data (Fig. 4).

Validation of the RNA-Seq analysis
Differences found in gene expression profiles between RGA vs RGPDA were further verified through a quantitative real time PCR (qRT-PCR) assay on total cDNA samples from mycelia of three biological replicates. For this, five randomly selected genes over-expressed in RGA vs RGPDA and with different FC, were analyzed. Actin gene was used as 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 RNA-Seq, results corroborated the overall differences found between the two samples (RGA and RGPDA) in the RNA-Seq analysis.

Functional annotation and pathways analysis of differentially expressed genes (DEGs)

To better understand the infection process of *R. necatrix* colonizing susceptible avocado roots, all differentially expressed genes were functionally enriched and categorized based on blast sequence homologies and gene ontology (GO) annotations using Blast2GO software \[18\] (*P* < 0.05), selecting the NCBI blast Fungi as taxonomy filter and default parameters. DEGs were significantly grouped into the regulation of eight molecular function (MF), such as heme binding (GO:0020037), iron ion binding (GO:0005506), oxidoreductase activity acting on CH-OH group of donors (GO:0016614), flavin adenine dinucleotide binding (GO:0050660), cellulose binding (GO:0030248), NADP binding (GO:0050661), peroxidase activity (GO:0004601) and N,N-dimethylaniline monooxygenase activity (GO:0004499), and three biological process (BP), such as carbohydrate transport (GO:0008643), cellular oxidant detoxification (GO:
0098869) and mycotoxin biosynthesis (GO:0043386) (Fig. 5a). To identify processes and functions over-represented in *R. necatrix* during infection, GO term enrichment analysis was also applied to the Top 100 over-expressed genes (Fig. 5b). The functions of these DEGs were significantly enriched in the regulation of five BP, such as oxido-reduction process (GO:0055114), cellulose catabolic process (GO:0030245), mycotoxin biosynthesis (GO:0043386), glucose import (GO:0046323) and response to hydrogen peroxide (GO:0042542), and 13 MF (Fig. 5b) among which activities related to plant cell wall degradation, including glucosidase activity (GO:0015926); endo-1,4-β-xylanase activity (GO:0031176); cellulose 1,4-beta-cellobiosidase activity (GO:0016162); xyloglucan-specific exo-β-1,4-glucanase activity (GO:0033950) and arabinogalactan endo-1,4-β-galactosidase activity (GO:0031218) were found. To investigate the metabolic pathways affected in *R. necatrix* during avocado root infection, a KEGG pathway analysis was performed with Blast2go [18]. For the total of 1937 DEGs, 100 metabolic pathways that involved 208 genes were identified (*P*-value < 0.05). The metabolic pathways were reorganized into eleven categories (Table 2) being the nucleotides metabolism the one with the highest number of genes (n = 64). Interestingly, metabolic pathways involved in antibiotic and drug metabolism were also affected, in accordance with GO enrichment analysis results, where mycotoxin biosynthetic process was one of the molecular functions over-represented.

**Candidate genes involved in the pathogenesis of *R. necatrix***

At least 69 transcripts showing homology to genes previously reported to be involved in fungal infection were identified among the 1937 DEGs. These include homologs to genes involved in the production ofCWDE (Table 3), proteases, fungal toxins, detoxification and transport of toxic compounds, gibberellin biosynthesis and gene silencing (Table 4) as well as gene effectors (Table 5). Out of the 69 selected genes, 30 were associated with cell wall hydrolysis, among which 16 showed fold change (FC) values above 50, with three of them (SAMD00023353_0503130, SAMD00023353_6500680 and SAMD00023353_4001240) allocated in the top20 over-expressed genes in *R. necatrix* during avocado root-colonization (Table 3 and Additional file 1). Five genes were identified as proteases, two aspartic proteases and three serine proteases, with the contig SAMD00023353_1500930 expressed over 411 times in RGA vs RGPDA (Table 4). Five contigs showed homology to genes encoding fungal toxins, among which the contig SAMD00023353_5500610 encoding the putative aflatoxin B1 aldehyde reductase member 2 showed the higher transcript abundance with a FC value of 18.65 (Table 4).

Nineteen genes were related to degradation of toxic compounds such as reactive oxygen species (SAMD00023353_5200870), aflatoxins (SAMD00023353_
**Fig. 4** Hierarchical clustering of differentially expressed genes (DEGs). Hierarchical clustering during *R. necatrix* infection on avocado roots (RGA1, RGA2 and RGA3) in comparison with its in vitro growth on Potato Dextrose Agar media (RGPDA1, RGPDA2, RGPDA3). Red and green indicate up- and down regulation, respectively.

| Gene ID                      | Description                                      | RGA vs RGPDA |
|------------------------------|--------------------------------------------------|--------------|
| SAMD00023353_12800020       | Related to psatin demethylase                     | 838.68       |
| SAMD00023353_2901300        | FAD-binding domain-containing protein             | 529.58       |
| SAMD00023353_2901290        | Related to protoporphyrinogen oxidase             | 160.78       |
| SAMD00023353_10000100       | Cytochrome p450                                   | 129.64       |
| SAMD00023353_0800710        | Fungal cellulose binding domain                   | 50.59        |

RGA vs RGPDA

*Data are displayed as fold change (FC), calculated by comparing *R. necatrix* growth on avocado roots (RGA) with *R. necatrix* growth on Potato Dextrose Agar medium (RGPDA). The expression data are the mean of three biological replicates. Bold numbers indicate statistically significant results (t-Test, \( P < 0.05 \)).*
0902760, SAMD00023353_12800020, SAMD00023353_3200110), and antibiotics (SAMD00023353_3600430, SAMD00023353_6600160, SAMD00023353_0702510, SAMD00023353_0100280, SAMD00023353_2201610), among other drugs. 

*R. necatrix* also over-expressed genes related to transport of toxic compounds, in particular, four (SAMD00023353_2601150, SAMD00023353_2501030, SAMD00023353_3000620 and SAMD00023353_6200040) and two contigs (SAMD00023353_10000080 and SAMD00023353_2200710) showed homology with genes encoding ATP-binding cassette (ABC) transporters and major facilitator superfamily (MFS) transporters, respectively. Expression values of genes homologous to ABC transporters were higher (FC values ranging from 5 to 7) than those observed for MFS transporters (ranging from 2 to 3) (Table 4).

Two genes were selected for being associated with hormone biosynthesis (GA₄ desaturase family protein SAMD00023353_10100030 and gibberellin 20-oxidase SAMD00023353_1901120) showing FC values of 38.2 and 2.39 respectively and one gene, the argonaute siRNA chaperone complex subunit Arb1 (SAMD00023353_0801000), postulated to play a role in RNA induced transcriptional silencing (Table 4).
The RNAseq analysis also revealed 137 genes only expressed in *R. necatrix* during its growth on avocado roots. From those contigs, 24 were predicted as candidate effector proteins (CEP) by the CSIRO tool EffectorP2 (a machine learning method for fungal effector prediction in secretomes) [19] with a probability above 60% (Table 5). All CEPs, except for SAMD00023353_2100110, SAMD00023353_2801560, SAMD00023353_3900800, SAMD00023353_11900020 and SAMD00023353_1700590, showed no similarity with proteins in the public database. Out of the 24 CEP, 13 were predicted to be secreted by SignalP3 server and ten were determined to have an apoplastic localization by the CSIRO tool ApoplastP (a machine learning method for predicting localization of proteins) [20] (Table 5).

To test any existing relationship within the candidate effector proteins identified in this study with previously described effectors proteins, the PHI (Pathogen Host Interaction) database was used; i.e., PHI-base is a database of virulence and effector genes that have been experimentally proven via pathogen-host interaction [21]. Blastp was used to match PHI-base with an e-value cutoff of 1E-03 and 30% identity. As result, 3 *R. necatrix* candidate effectors were annotated, SAMD00023353_11900020 encoding a putative glycoside hydrolase, showed the higher percentage of identity with the effector Lysm from *Penicillium expansum* (Identity 44.58%, E-value 9.94 E-53), SAMD00023353_2100110 and SAMD00023353_1700590 showed identity with effectors BEC1040 and Mocapn7 from *Blumeria graminis* (Identity 32.76%, E-value 1.32 E-05) and *Magnaporthe oryzae* (Identity 35.82%, E-value 1.32 E-03), respectively.

**Table 2** The KEGG pathway analysis using differentially expressed genes (DEGs)

| Category                              | Sequence number |
|---------------------------------------|-----------------|
| Nucleotides metabolism                | 64              |
| Organic compounds metabolism          | 60              |
| Metabolism of cofactors and vitamins  | 58              |
| Amino acid metabolism                 | 48              |
| Carbohydrate metabolism               | 42              |
| Antibiotics metabolism                | 39              |
| Others                                | 37              |
| Drug metabolism                       | 28              |
| Lipid metabolism                      | 24              |
| Energy metabolism                     | 10              |
| Biosynthesis of other secondary metabolites | 8            |

*The total number of contigs in each category

**Discussion**

Transcriptome analysis of *R. necatrix* strains growing on rich medium, has recently been addressed as an alternative to provide insights into plant pathogenicity mechanisms used by this ascomycete [12, 13]. However, neither of the two studies was carried out using *R. necatrix* directly interacting with a host. This current study fills this gap, obtaining and analyzing the transcriptomes of the virulent CH53 strain during infection of avocado roots and comparing it with that obtained from the fungus cultured in rich medium.

The number of predicted genes (12,104) obtained in this study is congruent with data from previous transcriptomes from *R. necatrix* (10,616 [12]), as well as other plant pathogenic Ascomycota, such as *Fusarium graminearum* (13,332 genes [22]), *Valsa mali* (13,046 genes [11]), or *Magnaporthe oryzae* (11,101 genes [23]). When comparing gene expression profiles between *R. necatrix* infecting avocado roots or growing on PDA medium, a number of transcripts were related with major fungal traits involved in the interaction with the host, among others, CWDE [24], production of toxic compounds and detoxification of those produced by the host, or potential effectors.

Phytopathogenic fungi usually produce numerous extracellular enzymes in order to penetrate the host tissue, being cell wall hydrolases and pectinases the most important ones [25]. The high number of CWDE over-expressed during the infection process correlates with previous visualization studies of *R. necatrix* hyphae that directly penetrate through the avocado root cells [9]. In addition, five putative proteases were also identified. Interestingly, gene expression studies carried out on avocado revealed that three protease inhibitors were highly over-expressed in tolerant rootstocks to *R. necatrix* following inoculation with the pathogen but not in susceptible genotypes [10]. This finding suggests that these proteases, up-regulated in *R. necatrix* during the infection process, could play an important role in degrading basal defense proteins on susceptible avocado roots, however, future experiments need to be carried out to confirm this hypothesis.

Several studies support the idea that *R. necatrix* produce toxins that are likely responsible for the symptoms observed in the aerial parts of the plant [26, 27]. Cytochalasin E and rosanesetrone toxins produced by *R. necatrix* [28, 29] are believed to be involved in the onset of disease symptoms in young apple shoots and detached apple leaves [27]. Shimizu et al., [13], identified the cytochalasin biosynthetic gene cluster, containing fourteen genes, within a 36 kb region of the *R. necatrix* strain W97 genome. In the present study, only one gene (putative aflatoxin B1 aldehyde reductase protein) of the putative cytochalasin cluster was highly up-regulated, while it
was down-regulated in transcriptomic analyses carried out in the hypovirulent \textit{R. necatrix} strain [13] (Additional file 2). Taking this into consideration, this gene could play an important role in the pathogenicity of \textit{R. necatrix} CH53 on avocado roots, however the role of the cytochalasin E in virulence remains unclear as suggested by other authors [30]. Four more genes related with the production of fungal toxins were up-regulated during the infection process, two of them (putative sterigmatocystin 8-O-methyltransferase and the averantin oxidoreductase) had been previously described to be involved in aflatoxin biosynthesis [31]. Aflatoxins are considered as the most toxic and carcinogenic compounds among the known mycotoxins and 25 clustered genes have been reported to be involved in its biosynthesis [31, 32]. Although the expression of other genes potentially involved in aflatoxin biosynthesis was not observed and no aflatoxin production, even at minimum concentration (<1 μg/Kg), was detected in wheat grains infected with \textit{R. necatrix} (data not shown), future studies should address the detection of this compound on infected roots due to its high toxigenic nature.

As other necrotrophic pathogens, \textit{R. necatrix} seems to have adapted mechanisms to detoxify host metabolites that can interfere with its virulence [33]. Nineteen genes potentially involved in detoxification of antimicrobial compounds were significantly over-expressed. Interestingly, SAMD00023353_12800020 and SAMD00023353_

| Table 3 \textit{R. necatrix} genes encoding cell wall degrading enzymes |
|-------------------------------|-------------------|----------------|
| Gene ID                       | Description                   | Fold Change$^a$ |
| Cell Wall Degrading Enzymes   |                                 |                |
| SAMD00023353_0503130          | Putative Glycoside hydrolase family 61 protein | 511.82         |
| SAMD00023353_6500680          | Glycoside hydrolase family 61 protein | 259.37         |
| SAMD00023353_4001240          | Glycosyl hydrolase family 43 protein | 226.29         |
| SAMD00023353_4000040          | Glycoside hydrolase family 128 protein | 201.63         |
| SAMD00023353_5900080          | Putative glycoside hydrolase family 61 | 193.76         |
| SAMD00023353_2700270          | Putative endoglucanase         | 166.44         |
| SAMD00023353_10700130         | Glycoside hydrolase family 128 protein | 162.43         |
| SAMD00023353_3200340          | Glycoside hydrolase family 61 protein | 155.24         |
| SAMD00023353_0105480          | Glycosyl hydrolase family 7    | 132.66         |
| SAMD00023353_11500050         | Celllobiohydrolase II          | 95.38          |
| SAMD00023353_3900390          | Probable endoglucanase         | 88.85          |
| SAMD00023353_1201160          | Glycoside hydrolase family 3 protein | 71.42          |
| SAMD00023353_4000610          | Glycoside hydrolase family 74 protein | 64.97          |
| SAMD00023353_5100270          | Glycoside hydrolase family 5 protein | 56.75          |
| SAMD00023353_3601090          | Putative glycoside hydrolase family 31 | 54.51          |
| SAMD00023353_1700720          | Glycosyl hydrolase family 10   | 52.82          |
| SAMD00023353_0202710          | Glycoside hydrolase family 53 protein | 42.82          |
| SAMD00023353_3700510          | Glycoside hydrolase family 61 protein | 37.07          |
| SAMD00023353_5100760          | Glycosyl hydrodrolase family 61–5 | 36.69         |
| SAMD00023353_0502040          | Glycoside hydrolase family 5 protein | 28.51          |
| SAMD00023353_1901740          | Glycosyl hydrodrolase family 1  | 24.70          |
| SAMD00023353_3000290          | Glycosyl hydrodrolase family 16 | 14.86          |
| SAMD00023353_0204000          | Glycosyl hydrodrolase family 26 | 11.21          |
| SAMD00023353_7600160          | Glycosyl hydrodrolase family 61 | 9.35           |
| SAMD00023353_4000450          | Glycosyl hydrodrolase family 18 | 7.44           |
| SAMD00023353_1601380          | Cutinase                      | 7.15           |
| SAMD00023353_0400070          | Glycosyl hydrodrolase family 76 | 2.77           |
| SAMD00023353_0201450          | Glycosyl hydrodrolase         | 2.76           |
| SAMD00023353_1002100          | Glycosyl hydrolase           | 2.28           |
| SAMD00023353_0101450          | Glycosyl hydrodrolase         | 2.24           |

$^a$RNA-Seq fold change calculated by comparing \textit{R. necatrix} growth on roots vs Potato Dextrose Agar
Table 4 Genes of *R. necatrix* potentially involved in pathogenesis

| Gene ID | Description | Fold Changea |
|---------|-------------|--------------|
| **Genes Related to Proteases** | | |
| SAMD0023353_1509030 | Putative acid proteinase protein | 411.34 |
| SAMD0023353_6300370 | Putative subtilisin-like protein | 13.44 |
| SAMD0023353_3200760 | Putative subtilisin-like protein | 12.19 |
| SAMD0023353_4000440 | Putative aspartyl protease | 4.82 |
| SAMD0023353_0403740 | Related to subtilisin DY | 2.60 |
| **Genes Related to Toxins Production** | | |
| SAMD0023353_5500610 | Putative aflatoxin B1 aldehyde reductase member 2 | 18.65 |
| SAMD0023353_3901210 | Putative averantin oxidoreductase | 13.89 |
| SAMD0023353_1000060 | Putative toxin biosynthesis | 4.81 |
| SAMD0023353_1501590 | Putative toxin biosynthesis protein | 4.30 |
| SAMD0023353_11700090 | Sterigmatocystin 8-O-methyltransferase | 3.29 |
| **Genes Related to Detoxification of Toxic Compounds** | | |
| SAMD0023353_5200870 | *catA*, catalase | 157.78 |
| SAMD0023353_3600430 | Putative cycloheximide resistance protein | 92.12 |
| SAMD0023353_12800020 | Related to pisatin demethylase cytochrome P450 | 90.24 |
| SAMD0023353_7000500 | GMC oxidoreductase | 41.39 |
| SAMD0023353_8000300 | Beta-lactamase family protein | 40.99 |
| SAMD0023353_1100640 | GMC oxidoreductase | 28.74 |
| SAMD0023353_10400180 | Glucose-methanol-choline (GMC) oxidoreductase | 21.40 |
| SAMD0023353_10400170 | Glucose-methanol-choline (GMC) oxidoreductase | 19.54 |
| SAMD0023353_0701730 | Putative multdrug resistance protein frx1 | 12.69 |
| SAMD0023353_6600160 | Beta-lactamase family protein | 11.24 |
| SAMD0023353_0702510 | Putative cicloheximide resistance protein | 7.18 |
| SAMD0023353_0902760 | Putative MFS aflatoxin efflux pump detoxificación | 3.87 |
| SAMD0023353_4900180 | Putative arrestin domain containing protein | 3.40 |
| SAMD0023353_2900030 | GMC oxidoreductase | 2.77 |
| SAMD0023353_0100280 | Putative tetracycline resistance protein TCRI | 2.76 |
| SAMD0023353_11800200 | Putative drug resistance protein | 2.68 |
| SAMD0023353_3200110 | Putative pisatin demethylase | 2.39 |
| SAMD0023353_3500410 | GMC oxidoreductase | 2.19 |
| SAMD0023353_2201610 | Metallo-beta-lactamase superfamily protein | 2.07 |
| **Genes Related to Transport of Toxic Compounds** | | |
| SAMD0023353_2601150 | ABC transporter | 7.37 |
| SAMD0023353_2501030 | ABC transporter | 6.64 |
| SAMD0023353_3000620 | ABC-2 type transporter | 5.38 |
| SAMD0023353_1000080 | Putative MFS multidrug transporter protein | 3.28 |
| SAMD0023353_2200710 | MFS transporter | 2.80 |
| SAMD0023353_6200040 | ABC transporter cdr4 | 2.39 |
| SAMD0023353_7900370 | Drug proton antiporter ykh8 | 2.21 |
| **Genes Related to Gibberelin Biosynthesis** | | |
| SAMD0023353_10100030 | GA4 desaturase family protein | 38.02 |
| SAMD0023353_1901120 | Gibberelin 20-oxidase | 2.39 |
| **Gene Related to Gene Silencing** | | |
| SAMD0023353_0801000 | Argonaute siRNA chaperone complex subunit Arb1 | 2.38 |

*aRNA-Seq fold change calculated by comparing *R. necatrix* growth on roots vs Potato Dextrose Agar*
3200110, both repressed in the hypovirulent \textit{R. necatrix} strain [13], showed homology to genes previously described to be involved in detoxification of phytoalexins. The importance of phytoalexin degradation ability in pathogenesis has been proved through transformation experiments [34]. To date, no phytoalexin production has been reported in \textit{Dusa} avocado rootstocks however, mutation experiments of these two genes would be of great interest to reveal their role in degradation of possible fungal toxic compounds produced by avocado roots.

Other contigs were related to transport mechanisms by which endogenous and exogenous toxicants can be secreted. Two major classes of transporter proteins were represented in \textit{R. necatrix} DEGs such as ABC and MFS transporters. Members of both classes can have broad and overlapping substrate specificities for toxic compounds and have been considered as a "first-line fungus defense barrier" [35].

Some necrotrophs are also able to influence host phytohormone levels or employ their own hormone biosynthesis machinery thereby disrupting defense signaling [24, 36–41]. Two genes involved in gibberellin biosynthesis, GA$_4$ desaturase family protein and Gibberellin 20-oxidase, were up-regulated during the infection process. Role of GAs in plant-pathogen interactions is not well known [42]; i.e., Studt et al. [43] showed the positive relation between GA production and bakanae disease in rice while Manka [44] found no correlation between GA production and pathogenesis of \textit{Fusarium}.

Throughout the infection process, fungi can actively manipulate host cellular machinery in order to suppress defenses and/or aid disease progression throughout the release of the so-called ‘effector’ proteins [45]. These effectors are usually secreted proteins that act at the host cell surface [46] or are taken up by the plant cell and act internally [47]. In this investigation, a total of 23 genes were predicted to be effectors (with probability above 60%), among which 19 encoded for hypothetical proteins and 10 were predicted as apoplastic effectors, being their place of action the interphase between the hyphae and the host cell. One of the predicted effectors, showed homology to the Lysm1 effector of \textit{Penycilium expansum}. Lysm-containing proteins have been proposed to

| Gene ID                  | Description                              | CDS Length | Signal peptide | Effector prediction* | Localization |
|-------------------------|------------------------------------------|------------|----------------|----------------------|--------------|
| SAM00023353_2100110     | SSCP protein                             | 923        | Yes            | 0.936                | Apoplastic   |
| SAM00023353_1002580     | Hypothetical protein                     | 183        | No             | 0.910                | Non-apoplastic |
| SAM00023353_3000810     | Hypothetical protein                     | 594        | Yes            | 0.890                | Apoplastic   |
| SAM00023353_1201650     | Hypothetical protein                     | 400        | Yes            | 0.889                | Apoplastic   |
| SAM00023353_4800590     | Hypothetical protein                     | 292        | No             | 0.865                | Non-apoplastic |
| SAM00023353_1401580     | Hypothetical protein                     | 230        | Yes            | 0.864                | Apoplastic   |
| SAM00023353_5300760     | Hypothetical protein                     | 216        | No             | 0.842                | Non-apoplastic |
| SAM00023353_2801560     | Putative lactoylglutathione lyase         | 630        | No             | 0.835                | Non-apoplastic |
| SAM00023353_7700300     | Hypothetical protein                     | 207        | Yes            | 0.829                | Non-apoplastic |
| SAM00023353_1401720     | Hypothetical protein                     | 189        | No             | 0.819                | Apoplastic   |
| SAM00023353_9200230     | Hypothetical protein                     | 240        | Yes            | 0.815                | Non-apoplastic |
| SAM00023353_6400250     | Hypothetical protein                     | 189        | Yes            | 0.805                | Apoplastic   |
| SAM00023353_0600790     | Hypothetical protein                     | 288        | No             | 0.805                | Non-apoplastic |
| SAM00023353_2100970     | Hypothetical protein                     | 150        | No             | 0.804                | Non-apoplastic |
| SAM00023353_3900800     | Cytochrome P450 monoxygenase             | 360        | No             | 0.802                | Non-apoplastic |
| SAM00023353_1901790     | Hypothetical protein                     | 501        | No             | 0.784                | Non-apoplastic |
| SAM00023353_1330070     | Hypothetical protein                     | 273        | No             | 0.765                | Non-apoplastic |
| SAM00023353_0104930     | Short-chain dehydrogenase reductase      | 195        | No             | 0.764                | Non-apoplastic |
| SAM00023353_0103460     | Hypothetical protein                     | 336        | Yes            | 0.756                | Apoplastic   |
| SAM00023353_11900020    | Glycoside hydrolase                      | 705        | Yes            | 0.753                | Apoplastic   |
| SAM00023353_1700590     | Ankyrin repeat domain-containing S2      | 246        | Yes            | 0.731                | Non-apoplastic |
| SAM00023353_2400240     | Hypothetical protein                     | 585        | Yes            | 0.721                | Apoplastic   |
| SAM00023353_6500130     | Hypothetical protein                     | 1340       | Yes            | 0.615                | Apoplastic   |
| SAM00023353_1000090     | Hypothetical protein                     | 177        | Yes            | 0.603                | Non-apoplastic |

*Effector prediction: EffectorP 2 (http://effectorp.csiro.au/), Probability > 60%
be involved in binding and sequestering chitin oligosaccharides in order to prevent elicitation of host immune responses [48] and/or to protect fungal hyphae against chitinases secreted by competitors [49]. In this sense, the expression of this effector during *R. necatrix* infection correlates with previous studies in which the overexpression of chitinases on susceptible avocado rootstocks/*R. necatrix* interaction, was reported [10]. Finally, other contig showed homology with the previously described *Blumeria graminis* effector gene BEC1040, which reduces haustoria formation in barley powdery mildew when silenced [50]. These results confirm previous observations by [12], in which BEC1040 homologous effectors in the virulent *R. necatrix* strain KACC40445 were found.

**Conclusion**

This study revealed, for the first time, several genes potentially associated with *R. necatrix* pathogenesis on avocado roots. The analysis of the full-length transcriptome of *R. necatrix* during the infection process suggests that the success of this fungus to infect diverse crops might be attributed to a number of produced compounds such as CWDE, toxins, antimicrobial detoxification compounds, transporters, effectors which, singly or in combination, likely interfere with defense or signaling mechanisms found on different plant families [24]. These results are revealing the complexity underlying *R. necatrix* pathogenesis being consistent with the difficulty of WRR management.

Functional characterization of these genes could help to understand how the fungus interferes with the host machinery and the development of white root rot disease. Along this line, a genetic manipulation protocol for transformation of *R. necatrix* has been established, although its efficiency needs to be improved [9]. Nevertheless, the transcriptome analysis of *R. necatrix* during the infection process provides useful information and facilitates further research to a more in-depth understanding of the biology and virulence of this pathogen. In turn, this will make possible to evolve novel strategies for white root rot management in avocado.

**Methods**

**Plant material, fungal isolate and inoculation**

Clonal 1 year old ‘Dusa™’ plants, described as susceptible to *R. necatrix* [51] and provided by Brokaw nursery (Brokaw España S.L.), were potted in 1.5 L plastic pots, previously disinfected with hypochlorite solution (2%) with an sterilized substrate consisting in peat, coconut fibre and perlite mixture (10:10:1) supplemented with 12 g osmocote® and placed into a semi-controlled greenhouse conditions (~ 20°C temperature and ~ 60% relative humidity). The virulent CH53 fungal strain, isolated at Almuñecar (Granada, Spain) [52], was used in this study and cultured on potato dextrose agar (PDA; Difco Laboratories, Detroit, USA) at 25 °C.

For transcriptome analysis of *R. necatrix* growing on rich medium, the isolate was cultured on PDA covered with a perforated layer of cellophane and incubated 5 days at 25 °C.

For RNA-Seq analysis of *R. necatrix* during infection, plants were removed from the pot and roots were washed with distilled water to remove soil debris. Roots were cut and placed into 15 cm diameter Petri dishes covered with three layers of filter paper soaked with sterilized distilled water. Three perforated cellophane discs, 6 cm diameter, were placed along the roots (Fig. 1). The inoculation was carried out by placing two wheat grains infected with *R. necatrix* onto each cellophane disc. Petri dishes were closed, sealed with parafilm and incubated in dark for 5 days.

**RNA isolation and sequencing**

For RNA extractions, cellophane discs covered with grown mycelium, were collected and macerated with liquid nitrogen using a mortar and pestle. One g of frozen powder was collected in a 2 ml Eppendorf and resuspended in 1 ml of denaturation solution (guanidine thiocyanate, 4 M, Na-citrate 25 mM sarcosyl, 0.5%) (Fluka; Switzerland) and saturated phenol pH 4.3 (1:1) plus 7 μl of β-Mercaptoethanol. One hundred μl chloroform were added to the mixture; samples were vortexed and incubated 3 min at room temperature and centrifuged at 12,000 g for 10 min at 4 °C. Afterwards, RNA was extracted using NucleoSpin RNA plant kit (Macherey-Nagel, Germany) following manufacturer’s instructions.

DNase I (DNase I, Thermo, USA) treatment was carried out twice, during and after the extraction process. 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.

The integrity of the RNA samples was further verified using the 2100 Bioanalyzer (Agilent Technologies, Inc., Santa Clara, USA) and submitted to the Centre Nacional d’Anàlisi Genòmica (CNAG, Barcelona, Spain) for sequencing. Two μg RNA from each sample were used for RNA library preparation using the TruSeq RNA Sample Preparation Kit (Illumina Inc) according to the protocols recommended by the manufacturer. Each library was paired-end sequenced (2 x 76 bp) by using the TruSeq SBS Kit v3-HS, in a HiSeq2000 platform. More than 40 million reads were generated for each sample. The RNA-Seq reads from six libraries (three biological
replicates per condition) were processed to remove adaptor sequences, empty reads, low-quality sequences with a Phred score lower than 20 and short reads (<25 bp). Resulting reads were stored in FASTQ format. High quality reads were aligned to the *R. necatrix* reference genome [13] for generation of read counts and differential expression analysis. CH53 RNA-seq reads were mapped to the W97 genome and consensus sequences were made of the mapped reads. The overall rate of base changes in the mapped regions between the CH53 and W97 strains was 0.75%. Raw reads from three biological replicates of *R. necatrix* growing on avocado roots and PDA media, are available from the NCBI Gene Expression Omnibus under accession number GSE134243.

A statistical analysis of the expression data of *R. necatrix* growing on avocado roots (RGA) vs Potato Dextrose Agar (RGPDA) media was performed by the Empirical analysis of DGE (EDGE) in CLC Genomics Workbench 10.0.0 (CLC Bio, Aarhus, Denmark). The DEGs were identified using the following conditions: -2 > fold change > 2 and FDR (P < 0.05). A visual representation of DEGs log10 FDR P-value vs log2 Fold change was plotted in R (version 3.6) with a simple scatterplot color coding the different conditions.

**Gene predictions and annotations**

*R. necatrix* predicted genes were searched against NCBI Fungi databases to assign associated Gen Ontology (GO) annotations using Blast2Go [18]. GO enrichment analysis (Fisher’s Exact test, [53]) and KEGG pathway analyses were carried out by Blast2go 5.2.4. Default parameters were used with a cut-off FDR of 0.05. GO enrichment analysis (Fisher’s Exact test, [53]) describing the enriched biological processes (BP), molecular functions (MF) and cellular components (CC) of DEGs was performed with B2G according to the following parameters: filter mode as P-Value and 0.05 as filter value. Kyoto Encyclopedia of Genes and Genomes (KEGG) annotations [54] of DEGs was performed with B2G.

Genes were clustered using TIGR Multi Experiment Viewer 4.6.1 [55] with Euclidean distances and Average linkage.

**Quantitative real-time PCR**

Validation of gene expression levels obtained from the transcriptome analysis was performed using qRT-PCR. One µg 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 expression of five *R. necatrix* genes was studied. One endogenous control gene, actin, was used for normalization. Primer sequences for endogenous control gene and the five *R. necatrix* genes are presented in Additional file 3. Primer pairs were chosen to generate fragments between 50 and 150 bp with melting temperature of 60 °C and designed using Primer 3 software [57, 58].

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 and calibration curves were performed for each gene. The qRT-PCR 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 [59]. Three biological replicates of RGA or RGPDA vs control samples were performed in triplicate. Statistical significance of the data was determined by a Student’s t-test carried out with Sigma Stat version 4.0 software (Systat Software GmbH).

**Supplementary information**

**Supplementary information** accompanies this paper at https://doi.org/10.1186/s12864-019-6387-5.

**Additional file 1.** Top 20 overexpressed and repressed genes in *R. necatrix* during growth on avocado roots

**Additional file 2.** Genes within the region containing the putative cytochalasin biosynthetic gene cluster in *R. necatrix*

**Additional file 3.** qRT-PCR primer sequences used in this study

**Abbreviations**

BP: Biological process; CWDE: Cell wall degrading enzymes; DEG: Differentially expressed genes; FC: Fold change; GO: Gene ontology; KEGG: Kyoto encyclopedia of genes and genomes; MF: Molecular function; PDA: Potato dextrose agar; PHI: Pathogen host interactions; RGA: *Rosalinia necatrix* growing on avocado roots; RGPDA: *Rosalinia necatrix* growing on PDA; WRR: White Root Rot

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**Authors’ contributions**

CP planned and designed the experiments and obtained the funding. AZ, HN, SK, AM, FPA and CP conducted the experiments, collected and analyzed the
data. AZ, AB, EMF, CLH, FPA, FC and CP prepared the draft. All authors wrote, reviewed and edited the manuscript. All authors read and approved the final manuscript.

Authors’ information

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Availability of data and materials

The data from this study are available from the NCBI Gene Expression Omnibus under accession number GSE134243.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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