Transcriptomic Profiling Identifies Candidate Genes Involved in Polyporus Umbellatus Sclerotial Formation Affected by Oxalic Acid

Yong-Mei Xing

Chinese Academy of Medical Sciences & Peking Union Medical College

Bing Li

Chinese Academy of Medical Sciences & Peking Union Medical College

Xu Zeng

Chinese Academy of Medical Sciences & Peking Union Medical College

Li-Si Zhou

Chinese Academy of Medical Sciences & Peking Union Medical College

Tae-Soo Lee

Incheon National University

Min-Woong Lee

Dongguk University

Xiao-Mei Chen (✉ cxm_implad@163.com)

Chinese Academy of Medical Sciences & Peking Union Medical College

Shun-Xing Guo

Chinese Academy of Medical Sciences & Peking Union Medical College

Research Article

Keywords: Polyporus umbellatus, transcriptomic sequencing, oxalic acid, non-invasive micro-test technology, Ca²⁺, H₂O₂

Posted Date: December 4th, 2020

DOI: <https://doi.org/10.21203/rs.3.rs-115257/v1>

License: © ⓘ This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Version of Record: A version of this preprint was published at Scientific Reports on August 30th, 2021. See the published version at <https://doi.org/10.1038/s41598-021-96740-7>.

Abstract

Polyporus umbellatus is a precious medicinal fungus. The whole transcriptome of *P. umbellatus* exposed to different concentrations of oxalic acid was performed and analyzed using RNA-seq based on sequencing technology. Reactive oxygen species (ROS) detection of *P. umbellatus* mycelia was visually conducted and using non-invasive micro-test technology (NMT), the net Ca^{2+} and H_2O_2 fluxes of *P. umbellatus* were measured. Totally, 22,523 unigenes were generated by De novo assembly of reads and there are 1223 differentially expressed genes (DEGs) between the control group and the high oxalic acid complemented (PD_S) group and 459 DEGs between the control group and the low concentration oxalic acid additive (PU_SM) group. The transcriptomic analysis indicated DEGs encoding enzymes related to oxidative stress, energy metabolism and so on. Compared to that of the control group, the biomass of the sclerotia in the PU_SM group increased 66%, however, no sclerotia formed in the PD_S group. The low concentration of oxalic acid could increase Ca^{2+} and H_2O_2 influx, while the high concentration of oxalic acid presented slight H_2O_2 efflux. There is a great significant positive correlation between the net Ca^{2+} and H_2O_2 fluxes. Different concentrations of exogenous oxalic acid affected *P. umbellatus* sclerotial formation in different ways.

Introduction

In fungi and plants, as one of the most simplest dicarboxylic acids, oxalic acid plays important roles all through the life of the creatures¹. In plant pathogenic fungus of *Sclerotinia sclerotiorum*, oxalic acid played double roles during the pathogenic interactions with the host plant^{2,3}, thus, oxalic acid was shown to induce ROS and it can also inhibit ROS as well.

Polyporus umbellatus (Pers.) Fr., a valuable medicinal fungus, the sclerotium of which has been used as diuretic and antidote for more than two thousand of years in China. Steroids and polysaccharides are two principal groups of chemicals in *P. umbellatus* sclerotia⁴. Among these bioactive components, sterone compounds exhibited nephroprotective activity on renal interstitial fibrosis against aristolochic acid or adenine induced nephrotoxicity in rats⁵. In the previous study in our laboratory⁶, a HPLC method was performed for simultaneous quantitative determination of five sterone compounds including polyporusterone A and polyporusterone B which were isolated from the wild sclerotia of *P. umbellatus*. HPLC fingerprint was used to compare the wild sclerotia, the mycelia and sclerotia under artificial conditions. The results showed that the cultured sclerotia possessed the similar spectrum with the wild sclerotia, while the mycelia only shared chromatographic peak of a few sterone compounds with the wild sclerotia, thus, it is necessary to conduct experiments to induce sclerotia directly from hyphae effectively. In the previous study, it has been elucidated that *P. umbellatus* sclerotial formation is in close relationship with oxidative stress state and we have found that oxalic acid served as an antioxidant during *P. umbellatus* sclerotial formation⁷. However, whether oxalic acid affecting *P. umbellatus* sclerotial formation in a “low promotion and high suppression” way deserves further study. Based on the previous study, we are trying to find whether exogenous oxalic acid especially the minimum and the most suitable

concentration to promote and the concentration of the oxalic acid to just completely inhibit *P. umbellatus* sclerotial formation, thus different concentrations of oxalic acid on sclerotial biomass was further studied in detail in the present study. According to the previous study, it was demonstrated that calcium antagonists could inhibit *P. umbellatus* sclerotial formation⁸. Calcium ion and ROS are always stimulated by each other and function coordinately during signal transduction⁹. Due to stress, it was induced Ca^{2+} influx and activate ROS production¹⁰. However, during *P. umbellatus* sclerotial formation exposed to different concentrations of oxalic acid, detailed molecular information about calcium signalling pathway and the real-time dynamic changes as well as the relationship between ROS and calcium ion is not clear.

RNA sequencing (RNA-seq), also named sequence-based assays of transcriptomes has been widely used these years and has become a relatively accurate tool for instantaneous gene expression analyses¹¹. To better understand the molecular process of *P. umbellatus* sclerotial formation under different concentrations of oxalic acid conditions, we performed RNA-seq transcriptomic analysis. In this study, DEGs between mycelia of *P. umbellatus* subjected to exogenous oxalic acid were described overall.

As an emerging detection, NMT has been widely used in many fields of research, such as recording H^+ , Ca^{2+} , NO_3^- and H_2O_2 fluxes in plant systems¹². In order to confirm whether different concentrations of oxalic acid can affect intracellular ROS in *P. umbellatus* mycelia, fluorescence staining assay was conducted in this study. Furthermore, the real-time Ca^{2+} and H_2O_2 fluxes in *P. umbellatus* mycelia by treatment of different concentrations of oxalic acid were detected by NMT. Thus, the aims of this study on the one hand was to identify the DEGs involved in the oxidative stress and calcium signal transduction as well as the profiles of their expression patterns based on high-throughput data. On the other hand, the relationship of the fluxes of ROS and calcium ion was also analyzed by Pearson correlation analysis. The study will provide certain genetic evidence for the involvement of oxalic acid during *P. umbellatus* sclerotial formation.

Methods

P. Fungal growth conditions and exogenous oxalic acid treatments in cultivation experiments

umbellatus was isolated from Guxian, Shanxi Province of China and incubated on solid wheat bran medium at 25 °C in the dark. After inoculation, the petri dishes were placed in the dark at 25 °C. The modified maltose medium contains maltose 20 g·L⁻¹, peptone 4 g·L⁻¹, $\text{K}_2\text{HPO}_4 \cdot 3\text{H}_2\text{O}$ 1 g·L⁻¹, KH_2PO_4 0.5 g·L⁻¹, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.5 g·L⁻¹, CaCl_2 0.005 g·L⁻¹, vitamin B1 0.05 g·L⁻¹ and agar 12 g·L⁻¹. Before inoculation, different concentrations of the exogenous sterile oxalic acid were added to the sterilized growth medium through a 0.22-μm Millipore filter (Merck, Germany). The final concentrations of oxalic acid in the maltose medium were 0 mg ml⁻¹, 0.005 mg ml⁻¹, 0.01 mg ml⁻¹, 0.05 mg ml⁻¹, 0.10 mg ml⁻¹ and 1.10 mg ml⁻¹. The group without oxalic acid (0 mg ml⁻¹) was treated as the control group in the

experiments. After cultivation for 30 days, the colony morphology of sclerotia and mycelia were observed. The experiment was repeated three times, with 30 replicates in each group.

Fungal Growth Conditions and Treatments for Transcriptome Sequencing

The mycelia cultivated for 30 days in the optimal concentration of exogenous oxalic acid (PU_SM group) that promote sclerotial formation of *P. umbellatus* and the minimum exogenous oxalic acid that thoroughly inhibit sclerotial development (PD_S group) and the control group without oxalic acid named as PU_S (CK) were performed transcriptome sequencing. Each sample of the mycelia from different groups was collected respectively and immediately frozen in liquid nitrogen for total RNA extraction.

Transcriptome Sequencing

Transcriptome sequencing and analysis was performed by Beijing Novogene Co. Ltd (Headquarters). In order to obtain the transcriptome of *P. umbellatus* mycelia subjected to different concentrations of exogenous oxalic acid, the total RNA was isolated from the frozen tissues of the mycelial samples of the three groups of *P. umbellatus* (PU_SM, CK and PD_S), with three biological replicates in each group, using the RNA plant mini kit with column DNase digestion (Qiagen, Hilden, Germany) following the manufacturer's instructions. Then, RNA concentration was detected and the RNA integrity and purity were assessed. Then, 3 µg RNA per sample was used as input material for the RNA preparations. Finally, nine samples with RNA integrity number (RIN) values above 8 were used for libraries construction. Sequencing libraries were generated and index codes were added to attribute sequences to each sample. Briefly, mRNA was purified and the fragmentation was carried out using divalent cations under elevated temperature in NEB Next First Strand Synthesis Reaction Buffer. First strand cDNA and the second strand cDNA synthesis were in succession synthesized. To prepare for hybridization, NEBNext Adaptor with hairpin loop structure was ligated. Subsequently, the library fragments were purified to select cDNA fragments of preferential 150~200 bp in length. After that, 3 µl USER Enzyme (NEB, USA) was added at 37 °C for 15 min, followed by 5 min at 95 °C prior to PCR. Then PCR was conducted with Phusion High-Fidelity DNA polymerase, Universal PCR primers and Index (X) Primer. At last, the PCR products were purified (AMPure XP system) and the library quality was assessed on the Agilent Bioanalyzer 2100 system. The clustering of the index-coded samples was performed on a cBot Cluster Generation System using TruSeq PE Cluster Kit v3-cBot-HS (Illumina). Then the library preparations were sequenced using Illumina Hiseq 2500 platform and paired-end reads of 125 bp were produced¹¹.

Sequence Annotation

Raw data of fastq format were firstly processed through in-house Perl scripts. During the process, clean data were obtained by removing reads containing ploy-N, adapter and low quality reads from raw data. Meanwhile, Q20, Q30, GC-content and sequence duplication level were also calculated. The DEGs analysis was based on the clean data with high quality. All the reads were then assembled using Trinity 50. Gene function was annotated based on the following databases: the non-redundant protein (NR) database, the nonredundant nucleotide (NT) database, Protein family (Pfam), Swiss-Prot (a manually

annotated and reviewed protein sequence database), Kyoto Encyclopedia of Genes and Genomes (KEGG), Clusters of Orthologous Groups (COG) and Gene Ontology (GO), using BLASTX with a significance threshold of $E\text{-value} < 10^{-5}$.

Differential expression analysis of transcripts

The nine samples of the total RNA extracted from *P. umbellatus* mycelial tissue samples were used to perform three independent sequencing libraries. To better obtain the mechanism of *P. umbellatus* sclerotial formation affected by different concentrations of oxalic acid, it is important to identify the DEGs among the three groups. Then, to determine the functional annotations of the genes, the clean tags in each cDNA library were mapped to the all-unigenes data from the transcriptome sequencing using the short reads alignment program²⁹. Data from the two biological replicates of each group were merged, and the transcript abundance of each gene was determined by the reads per kilobase of exon model per million mapped read (RPKM) values.

To identify genes responding to oxalic acid and sclerotial formation-related DEGs, the log₂ ratio of gene transcript abundance among the sequencing libraries for the mycelial tissue of the CK group and the oxalic acid- complemented groups were calculated. Differential expression analysis of the three groups was conducted using the DESeq R package (1.10.1). Based on the negative binomial distribution model, the statistical routines for determining differential expression in digital gene expression data were provided by DESeq³⁰. The statistical significance of the differential expression level for each gene was determined by evaluating the probability. For controlling the false discovery rate, the adjusted p values were used according to the Benjamini and Hochberg's approach. The false discovery rate (FDR) was used to determine the threshold of the adjusted p -value. A gene with an adjusted p -value < 0.05 and the absolute value of log₂ ratio > 1 was termed as a DEG.

Validation of DEGs expression using Real-Time Quantitative PCR (qRT-PCR)

All the primers were designed using Primer 3.0 software, assessed by AmplifX1.5.0 and synthesized by Genewiz Company (China). The 18S rRNA gene (EU442272) of *P. umbellatus* was used as a reference control¹¹. All the primers detected were PCR-amplified in the cDNA of the samples of the CK, PU_SM and PD_S groups respectively. All the randomly selected 9 genes from the RNA-seq data exhibited >2 folds higher or down-regulated <0.5 -fold lower expression in the *P. umbellatus* in response to oxalic acid. Using qRT-PCR method, these DEGs were conducted to validate the expression patterns. The method was performed using the SYBR® Premix ExTaq™ (TaKaRa, Dalian, China) on the Roche Light Cycler 480. The reaction was conducted as follows: denaturated at 95 °C for 30 s for the first step, followed by 40 cycles of amplification at 95 °C for 5 s and 60 °C for 34 s. Each detected gene was repeated three times in independent runs. Gene expression was evaluated by the $2^{-\Delta\Delta Ct}$ method.

ROS detection in *P. umbellatus* Mycelial Cells

ROS measurement was conducted using a GMS10010.7 kit protocol. *P. umbellatus* mycelia of the PU_SM, PD_S and the control groups were respectively picked off using scotch tape. The fluorescence staining was prepared by mixing 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H₂DCFDA) with the dilution at the volume ratio of 1:20 on ice to avoid quenching of the fluorescent dye in the dark. Subsequently, 20 µl of the prepared staining fluid was dropped onto the glass slide and then the samples adhered to scotch tapes were placed down to closely contact the staining fluid on the slide. The samples were then incubated at 28°C for 1h in the dark. Finally, using an AxioCan HRc Zeiss microscopy, the labelled ROS in the mycelial cells from each sample was visualized with the excitation wavelength of 490nm and an emission wavelength of 530nm, according to the previous study³¹.

Real-time Ca²⁺ and H₂O₂ fluxes measurement of *P. umbellatus* mycelia affected by different concentrations of oxalic acid using NMT

Ca²⁺ and H₂O₂ was measured on the surface of *P. umbellatus* mycelia in different groups after cultivation for 30 days as mentioned above using NMT System (NMT100 Series, Younger USA LLC, Amherst, MA, USA). Ca²⁺-sensitive and ROS-sensitive microsensors were purchased from NMT Service Center, Xuyue Beijing Sci. & Tech Co. Ltd. The experimental voltage is +600 mV. The primary position (M1) of Ca²⁺ or ROS-sensitive microsensor was placed 30 µm from *P. umbellatus* mycelial surface, and the further away position (M2) is 60 µm (Sample detection images were shown in Supplement Figure 1).

Statistical Analysis

The data were analyzed with double-factor Pearson correlation analysis and all statistical analyses were performed using SPSS 17.0 (SPSS, Chicago, IL, USA). Data were presented as means±SD from at least three independent experiments. *P* values<0.05 were considered a significant difference.

Results

Colony morphology of *P. umbellatus* under exogenous different concentrations of oxalic acid

From the concentrations of the exogenous oxalic acid below 1mg mL⁻¹, it was found that the oxalic acid with 0.05mg·mL⁻¹ served as the most suitable in *P. umbellatus* sclerotial formation (Fig. 1B), compared to that of the control group (Fig. 1A), *P. umbellatus* biomass in this group increased significantly (*P*<0.05). In addition, the minimum concentration of oxalic acid that completely inhibit *P. umbellatus* sclerotial development was 1.10 mg·mL⁻¹ (Fig. 1C).

Transcriptome sequencing and *de novo* Assembly and sequence annotation

To obtain the *P. umbellatus* mycelial transcriptome expression profile treated by oxalic acid, two libraries with different concentrations of oxalic acid and one library without oxalic acid as control were respectively constructed. Illumina sequencing data of *P. umbellatus* could be obtained in the NCBI

BioProject and the Sequence Read Archive (SRA) database with the ID of PRJNA669949. Totally 284190976 raw reads were generated (Table 1). There were 276265498 clean reads left after removing adaptor sequences, ambiguous nucleotides and low-quality sequences. Assembly of clean reads brought about 22,523 unigenes in the range of 201~19,256 bp with a N50 length of 2834 bp (Fig. 2).

After the repeated and the short-length sequences were deleted, 22,523 non-redundant unigenes were subjected to similarity analysis according to the 7 public databases (Table 2). Among them, 12,864 (57.11%) unigenes had the highest matches in the NT database, followed by 11,236 (49.88%) unigenes in the SwissProt database. Specifically, 10,914, 10,645, 10,571, 6934 and 4063 unigenes had functional annotations in the other five databases (NR, GO, PFAM, KOG and KO), respectively accounting for 48.45%, 47.26%, 46.93%, 30.78% and 18.03%, as shown in Table 2.

According to GO, 10,645 unigenes were classified into three major functional ontologies, including biological process, cellular component and molecular function. For biological process, 'cellular process' and 'metabolic process' comprise huge proportions. For cellular component, 'cell' and 'cell part' account for considerable parts. In addition, 'binding' and 'catalytic activity' had great percentages in the molecular function part (Fig. 3).

Identification of DEGs in the mycelia of *P. umbellatus* treated with different concentrations of oxalic acid

Due to the analysis of the DEGs, RPKM values were calculated and the Pearson's correlation coefficients (R^2) were high between the biological replicates in the same group ranging from 0.902 to 1. The R^2 of the samples between the different groups ranged from 0.705 to 1.816 (Fig. 4). The DEGs between the CK and PU_SM, the CK and PD_S were analyzed and the DEGs with higher expression levels in PU_SM and PD_S of both groups were denoted as 'up-regulated', while those with lower expression levels in PU_SM and PD_S were 'down-regulated' (Fig. 5). There are 1223 DEGs between the control group without oxalic acid and the high oxalic acid group and among them, 679 were up-regulated and 544 were down-regulated. There are 459 DEGs between the control group and the low oxalic acid group and among them, 228 were up-regulated and 231 were down-regulated (Supplement Figure 2).

DEGs Related to oxidases and dehydrogenases

In comparison to that of the control (PU_S) group, *c43_g1* and *c159_g1* encoding cytochrome c oxidase in the control group decreased 158.80 and 189.27 folds respectively in the PD_S group; NADPH oxidase encoded by *c3315_g1* and *c8851_g1* in the PD_S group was down-regulated 1.54 and 2.03 folds. *c7390_g2* encoding galactose oxidase in the control group increased 2.16 folds than that of the PD_S group. In addition, D-amino-acid oxidase encoded by *c7941_g1*, L-gulonolactone/D-arabinono-1,4-lactone oxidase and so on encoded by *c7166_g1* decreased in the PD_S group. Compared to that of the control group, alcohol oxidase encoded by *c8987_g1* and *c8020_g1* increased by 7.31 and 2.78 folds in the PU_SM group, however, *c8176_g4* encoding alcohol oxidase in the PD_S decreased 2.20 folds.

NADH dehydrogenase encoded by *c1494_g1*, *c17518_g1* and *c6419_g1* in the PD_S group decreased by 109.18, 114.90 and 2.06 folds respectively, in comparison to that in the control group. In addition, the expression of *c524_g1*, *c1758_g1* and *c2305_g1* encoding NADH dehydrogenase was up regulated in the control group, while they did not express in the PD_S group at all.

c7742_g1, *c6369_g1* and *c7145_g1* encoding alcohol dehydrogenases in the PD-S group increased respectively 2.33, 1.85 and 2.09 folds compared to that in the control group (Table 3, Supplement Table S2).

DEGs Associated with Reductases and glutathione S-transferase

Aldo/keto reductase encoded by *c8217_g1* decreased 2.43 folds in the PU-SM group, compared to that of the control group. Meanwhile, in the PD_S group, *c9378_g1* encoding aldo/keto reductase decreased 2.15 folds less than that of the control group. The same enzyme encoded by *c9881_g2* in the control group expressed 3.27 folds more than that of the PU_SM group. In addition, *c8932_g1* encoding quinone reductase and nitrite reductase encoded by *c10001_g1* down-regulated in the PU_SM group, compared to that of the control group. *c16064_g1* encoding cytochrome c reductase was up regulated in the PD_S group, while it did not express in the control group at all. Cytochrome-b5 reductase encoded by *c4751_g1*, ribonucleotide reductase alpha subunit and gamma-glutamyl phosphate reductase encoded by *c4563_g1* and *c5317_g1* all down-regulated expressed in the control group, in comparison to that of the PD_S group.

Compared to the control group, *c9468_g4* and *c7959_g1* encoding glutathione S-transferase in the PD_S group increased 19.30 and 1.93 folds respectively. The same enzyme encoded by *c6055_g1* decreased 6.92 folds in the PU_SM group, in comparison to that of the control group (Supplement Table S3).

DEGs Related to Peroxidases and Catalase

Compared to that of the control group, *c5235_g1* encoding heme peroxidase increased 1.85 folds in the PD_S group. Manganese peroxidase encoded by *c6600_g1* of the mycelia in the control group decreased by 2.46 times compared to the PD_S group, in addition, *c6769_g1* encoding the same protein decreased by 6.15 folds in the PU_SM group, compared to that of the control group. In the control group, *c6329_g1* encoding catalase decreased by 2.30 folds compared to the PD-S group. Glutathione peroxidase encoded by *c4316_g1* was specifically expressed in the PD_S group but did not express in the PU_S group (Supplement Table S3).

DEGs related to energy metabolism

In comparison to the the control group, isocitrate lyase encoded by *c7209_g1* increased by 34.67 folds in the PU_SM group. The expression of the same gene increased 56.85 folds in the low oxalic acid group, compared to the high oxalic acid group. Regard to the DEG (*c6742_g2*) encoding fatty acid hydroxylase, the gene in the mycelia exposed to the PU_SM group was up-regulated about 29.21 times that of the control group. Due to fatty acid synthase encoded by *c3295_g1* and *c4529_g1*, it was down-regulated

1.74 and 1.62 folds in the PD_S group, in comparison to the control group. In addition, *c9487_g1* encoding fatty acid desaturase (delta-12 desaturase) expressed in the PD-S group 3.41 folds less than that in the control group. *c8540_g1* encoding NADP⁺-dependent malic enzyme (1.1.1.40) in the PD_S group was increased 2.84 folds than that of the control group.

In comparison to that of the control group, *c4703_g1* encoding L-rhamnose 1-dehydrogenase was up-regulated 12.38 folds in the mycelia of the PU_SM group, while the same gene was down-regulated 12.80 folds in that of the PD_S group. *c7929_g2* encoding L-threo-3-deoxy-hexylosonate aldolase was down-regulated 3.55 folds in the PU_SM group, compared to that of the control group. Rhamnogalacturonase encoded by *c4388_g1* in the PD_S was down-regulated 3.36 folds less than that of the CK group, in addition, *c4271_g1* encoding rhamnogalacturonan acetylerase was up-regulated 6.43 folds (Table 4). Compared with the high oxalic acid group, HMG-CoA reductase encoded by *c8652_g1* and C-4 methyl sterol oxidase encoded by *c8791_g2* were both up-regulated in the control group.

DEGs related to Calcium signaling pathway

The expression of *c8739_g2* encoding calmodulin in the PU_SM group increased 2.66 folds than that of the control group. In addition, *c2043_g1* and *c3257_g1* encoding calmodulin expressed in the control group, while it also did not express in that of the PD_S group. Calmodulin-binding motif encoded by *c4558_g1* expressed in the PU_SM group while it did not express at all in the control group. *c8133_g1* encoding calmodulin-binding motif in the control group increased 2.13 folds than that of the PD_S group. Furthermore, *c9830_g2* which participated in calcium ion transport in the PU_SM group expressed 2.71 folds more than that of the control group. In the PD_S group, *c3191_g1* and *c6291_g1* took part in cellular calcium ion homeostasis and calcium ion transport was down-regulated 5.62 and 1.91 times in comparison to that of the control group (Table 5).

The ROS content in *P. umbellatus* mycelia affected by different concentrations of oxalic acid

In the control group without oxalic acid, the ROS content of *P. umbellatus* mycelia has accumulated to a certain extent after cultivation for 30 days as shown in Fig. 6A, while in the PU_SM group, ROS level was more than that of the control group (Fig. 6B), however, in the PD_S group, the ROS content was much lower than that of the control group (Fig. 6C).

Validation of the DEGs using qRT-PCR.

The 9 unigenes were randomly selected and examined at the transcriptional level by qRT-PCR, to validate the reliability of the RNA-Seq data (Fig. 7). All the unigenes were shown that the expression patterns were consistent with the RNA-Seq data, indicating the validity of the RNA-seq sequencing results.

Net Ca²⁺ and H₂O₂ fluxes during *P. umbellatus* growth affected by different concentrations of oxalic acid using NMT

In the PD_S group, the Ca^{2+} influx less were shown with the net fluxes ranging from -54.74~63.15 ($\text{pmol}\cdot\text{cm}^{-2}\cdot\text{s}^{-1}$) than that of the control group and the PU_SM group (Fig. 8A), but the H_2O_2 in the PD_S group represented efflux slightly with the net fluxes ranging from 4.05 to 9.77 $\text{pmol}\cdot\text{cm}^{-2}\cdot\text{s}^{-1}$ (Fig. 8B), in comparison, the Ca^{2+} performed influx more in the control group and much more in the PU_SM group (Fig. 8A), however, H_2O_2 influx a litter more in the control group and the PU_SM group than that of the PD_S group (Fig. 8B). The significant positive correlationship with the Pearson's correlation coefficient ($r=0.991$, $P<0.01$) between the fluxes measurement of Ca^{2+} and H_2O_2 was performed by correlationship analysis using SPSS and the scatter plot was shown in Supplement Figure 3.

Discussion

It was found that oxalic acid accumulation led to lowering ambient pH and favored *Sclerotinia sclerotiorum* sclerotial development¹³. Recently, it has also been reported that *S. sclerotiorum* sclerotial formation was inhibited by exogenous oxalic acid of 25mM and higher¹⁴. Thus, oxalic acid played dual roles in sclerotial differentiation. In the present study, low concentrations of exogenous oxalic acid (ranging from 0.05 mg ml^{-1} to 0.1 mg ml^{-1}) promote but high concentrations of oxalic acid (1.10 mg ml^{-1} and higher) completely inhibit *P. umbellatus* sclerotial formation (Fig. 1C). We presented detailed molecular information about effects of exogenous oxalic acid on *P. umbellatus* sclerotial formation and found that oxalic acid affected *P. umbellatus* sclerotial formation, with performance in the special "low promotion and high suppression" way.

It has been well documented that during *S. sclerotiorum* infection, initially oxalic acid inhibits ROS-mediated plant defense responses, but later increases ROS production in *Nicotiana benthamiana* followed by programmed cell death. Therefore, oxalic acid has dual opposing roles in *Sclerotinia* pathogenesis².

Oxalic acid (10mM) application suppressed H_2O_2 and O_2^- contents and increased ascorbic acid concentration in lotus root slices¹⁵. In addition, oxalate can lead to ROS production in renal epithelial cells¹⁶.

ROS are unstable and highly-reactive chemical species¹⁷. Since NADPH oxidase activation is a key enzymatic source of ROS formation¹⁸, and in eukaryotes, it is an important source of ROS including H_2O_2 ¹⁹. According to the high-throughput data, two DEGs encoding this enzyme was downregulated in the PD_S group, compared to that of the control group, indicating the weak oxidizability of the mycelia of the group complemented with high concentrations of oxalic acid. Other oxidases play important roles in maintaining oxidation-reduction balance in fungi, for example, alcohol oxidase, homooctameric flavoprotein, is capable of catalyzing the oxidation of methanol to formaldehyde and hydrogen peroxide. This protein is involved in the pathway methane degradation, which is part of energy metabolism of certain yeasts²⁰. In the present study, the DEGs of *c8176_g4*, *c8987_g1* and *c8020_g1* encoding alcohol

oxidase in both of the control group and the PU_SM group contribute to the high oxidative stress in the mycelia of *P. umbellatus*, which will compel sclerotial formation. Galactose oxidase can catalyze D-galactose and O₂ to form D-galacto-hexodialdose and H₂O₂. In this study, *c7390_g2* encoding this gene was highly expressed in the control group, indicating it contributed to produce ROS and will be more beneficial to sclerotial formation, compared to that of the PD_S group.

The functions of reductases and other ROS-scavenging enzymes are also of the essence. For instance, acetoin reductase encoded by *c9034_g4* was relatively up regulated in the control group, but it was relatively down regulated in the PU_S group, indicating the relative reducibility of the mycelia in the control group, therefore, it was much easier for the mycelia to form sclerotia in the PU_SM group. Glutathione S-transferases (GSTs) have been reported to catalyze reactions involving the conjugation of glutathione to electrophilic compounds and to remove ROS^{21,22}. Based on the transcriptomic data, the expression of *c9468_g4* and *c7959_g1* encoding glutathione S-transferase in the PD_S group increased, meanwhile, the same enzyme encoded by *c6055_g1* decreased in the PU_SM group, compared to the control group. This indicated that in comparison to that of the control group, the reductivity of the *P. umbellatus* mycelia of the PD_S group was higher but the reductivity of the *P. umbellatus* mycelia of the PU_SM group was lower. As one of the antioxidant enzymes, catalase was upregulated in the mycelia of the PD_S group in comparison to that of the control group, which will help to maintain oxidative stress level in the latter group.

NADPH could convert H₂O₂ into H₂O by glutathione peroxidase and this enzyme stands for antioxidant ability²³. In the present study, *c4316_g1* encoding glutathione peroxidase was only expressed in the PD_S group but did not express in the PU_S group, which indicated that the mycelia in the PD_S group expressed stronger antioxidative activity than that in the mycelia of the control group. Glutathione transferase and glutathione peroxidase possesses the similar characteristics in *P. umbellatus* mycelia of different groups. It has been reported that thiol redox state (TRS)-related antioxidant enzymes glutathione reductase, glutathione-S-transferase and glucose 6-phosphate dehydrogenase peaked in the undifferentiated hyphae, indicated TRS-associated high oxidative stress was in close relationship with sclerotial formation in phytopathogenic fungi²⁴.

Unsaturated fatty acids is the intrinsic reason for lipids oxidation²⁵. That *c3295_g1* and *c4529_g1* encoding fatty acid synthase was down-regulated in the PD_S group compared to that of the control group, which indirectly reflects that lipid oxidation is lower in the PD_S group. This speculation has been verified in the previous study, which indicated that exogenous oxalic acid reduced the lipid peroxidation in a concentration-dependent manner⁷.

In this study, with CM-H₂DCFDA probe for visually detection, the ROS content in *P. umbellatus* mycelia of the PU_SM group was the most, followed by that of the control group. The fluorescence intensity of the ROS level in the PD_S group was the slightest. In addition, using NMT technique, in the PD_S group, H₂O₂ represented efflux slightly, while in the control and the PU_SM group, H₂O₂ influxes were demonstrated in

this study (Fig. 8B), and it is conceivable that the intracellular H_2O_2 decreased in the mycelia of *P. umbellatus* in the PD_S group, in comparison to both of the other groups. The result is consistent with the ROS content in *P. umbellatus* mycelia affected by different concentrations of oxalic acid (Fig. 6). Thus, these results have further confirmed that the high oxidative stress has been formed in the control group and the PU_SM group and facilitates *P. umbellatus* sclerotial formation, while lower ROS content in the PD_S group cannot.

NADPH oxidases require different redox cofactors and are regulated either through regulatory subunits or by calcium ions, and calcium-dependent regulation has been verified via EF-hand motifs by experiments²⁶. NADPH oxidases belong to the respiratory burst oxidase homolog (RBOH) family. When the activity of the Ca^{2+} and phosphorylation-dependent RBOHD is upregulated, ROS production is caused to increase during plant defense^{18,27}.

In the present study, real-time Ca^{2+} fluxes measurement showed that Ca^{2+} influxes in the PU_SM group was the most, followed by that of the control group, and Ca^{2+} influxes in the PD_S group was the least among these three groups. The more Ca^{2+} uptake in the control group and the PU_SM group causes intracellular Ca^{2+} concentration to rise and facilitates Ca^{2+} spiking and interaction with other moleculars in calcium transduction signalling pathway²⁸. Therefore, the intracellular H_2O_2 and Ca^{2+} increased in the PU_SM group but decreased in the PD_S group, compared to that of the control group. In other words, in the control group and PU_SM groups that rendered mycelia to differentiate into sclerotia, the elevated ROS levels accompanied with increase in calcium ion levels. Meanwhile, the transcriptomic analysis showed that the DEGs about calmodulin, calmodulin-binding motif, calcium ion transport and so on were up-regulated in the control group and the PU_SM group, which was different from that of the PD_S group. This implied that DEGs related to calcium signal transduction and ROS cooperatively plays an important role in *P. umbellatus* sclerotial formation.

In conclusion, the low concentration of oxalic acid induces ROS production by H_2O_2 influx, on the other hand, the high concentration of oxalic acid inhibits *P. umbellatus* sclerotial differentiation and oxidative stress by H_2O_2 efflux. The significant positive correlationship was shown between the fluxes measurement of calcium ion and H_2O_2 . Further investigation is required to determine the mechanism of the oxalic acid affecting oxidation-reduction during *P. umbellatus* sclerotial formation.

Declarations

Acknowledgements: This research was financially supported by the CAMS Innovation Fund for Medical Sciences (CIFMS) (2017-I2M-3-013), National Natural Sciences Foundation of China (NSFC81973425, 81773843, 81803666) and Peking Union Medical College Discipline Construction Project (Tsinghua 211-201920100901).

Author contributions

S.X.G. and X.M.C.: experimental design and manuscript review; Y.M.X.: manuscript writing, manuscript review and performed the experiments; B.L.: performed part of the experiments and manuscript review; X.Z. and L.S.Z.: data analysis and raw data uploading; T.S.L. and M.W.L.: manuscript review;. All authors have read and approved the manuscript.

Competing interests

The authors declare no competing interests.

References

1. Magnuson, J.K. & Lasure, L.L. Organic acid production by filamentous fungi. *Advances in Fungal Biotechnology for Industry, Agriculture and Medicine*. Kluwer Academic/Plenum Publishers. 307-340 (2004).
2. Williams, B., Kabbage, M., Kim, H.J., Britt, R. & Dickman, M.B. Tipping the Balance: *Sclerotinia sclerotiorum* Secreted Oxalic Acid Suppresses Host Defenses by Manipulating the Host Redox Environment. *PLoS. Pathog.* **7**(6), e1002107 (2011).
3. Kabbage, M., Williams, B. & Dickman, M.B.. Cell Death Control: The Interplay of Apoptosis and Autophagy in the Pathogenicity of *Sclerotinia sclerotiorum*. *PLoS. Pathog.* **9**(4), e1003287 (2013).
4. Zhao, Y.Y. Traditional use, photochemistry, pharmacology, pharmacokinetics and quality control of *Polyporus umbellatus* (Pers.) Fries: A review. *J. Ethnopharmacol.* 149, 35-48 (2013).
5. Zhao, Y.Y., Shen, X., Cheng, X.L., Wei, F., Bai, X. & Lin, R.C. Urinary metabonomics study on the protective effects of ergosta-4,6,8(14),22-tetraen-3-one on chronic renal failure in rats using UPLCQ-TOF/MS and a novel MSE data collection technique. *Process. Biochem.* **47**, 1980–1987 (2012).
6. Zhou, W.W. Studies on the constituents of sclerotia and fermented mycelia of *Polyporus umbellatus* and the quality analysis. Thesis of Doctor's degree, Chinese Academy of Medicinal Sciences and Peking Union Medical College. 113-124 (2008).
7. Xing, Y.M., Yin, W.Q., Liu, M.M., Wang, C.L. and Guo, S.X. Oxalic acid and sclerotial differentiation of *Polyporus umbellatus*. *Sci. Rep.* **5**, 10759 (2015).
8. Liu, Y.Y. & Guo, S.X. Involvement of Ca^{2+} channel signaling in Sclerotial formation of *Polyporus umbellatus*. *Mycopathologia.* **169**, 139–150 (2010).
9. Demidchik, V. & Shabala S. Mechanisms of cytosolic calcium elevation in plants: the role of ion channels, calcium extrusion systems and NADPH oxidase-mediated ROS- Ca^{2+} Hub. *Funct. Plant. Biol.* **45**, 9-27 (2018).
10. Jha. S.K., Sharma, M. & Pandey, G.K. Role of cyclic nucleotide gated channels in stress management in plants. *Curr Genomics.* **17**, 315-329 (2016).
11. Liu, M.M., Xing, Y.M., Zhang, D.W. & Guo, S.X.. Transcriptome analysis of genes involved in defence response in *Polyporus umbellatus* with *Armillaria mellea* infection. *Sci Rep.* **5**, 16075 (2015).

12. McLamore, E.S., Jaroch, D., Chatni, M.R. & Porterfield, D.M. Self-referencing optrodes for measuring spatially resolved, real-time metabolic oxygen flux in plant systems. *Planta*. **232**, 1087–1099 (2010).
13. Rollins, J.A. & Dickman, M.B. pH signaling in *Sclerotinia sclerotiorum*: identification of a pacC/RIM1 homolog. *Appl. Environ. Microbiol.* **67**, 75–81 (2001).
14. Atallah, O. & Yassin, S. *Aspergillus* spp. eliminate *Sclerotinia sclerotiorum* by imbalancing the ambient oxalic acid concentration and parasitizing its sclerotia. *Environ Microbiol.* online ahead of print, doi: 10.1111/1462-2920.15213. (2020).
15. Ali, S., Khan, A.S., Anjum, M.A., Nawaz, A., Nawaz, A., Naz, S., Ejaz, S. & Hussain, S. Effect of postharvest oxalic acid application on enzymatic browning and quality of lotus (*Nelumbo nucifera* Gaertn.) root slices. *Food. Chem.* **312**, 126051 (2020).
16. Liu, Y.D., Yu, S.L., Wang, R., Liu, J.N., Jin, Y.S. & Li, Y.F. An RH. Rosiglitazone Suppresses Calcium Oxalate Crystal Binding and Oxalate-Induced Oxidative Stress in Renal Epithelial Cells by Promoting PPAR- γ Activation and Subsequent Regulation of TGF- β 1 and HGF Expression. *Oxid. Med. Cell. Longev.* published online doi: 10.1097/JU.0000000000000997.02 (2020).
17. Im, K.H., Baek, S.A., Choi, J. & Lee, T.S. Antioxidant, Anti-Melanogenic and Anti-Wrinkle Effects of *Phellinus vaninii*. *Mycobiology*. **47**(4), 494–505 (2019).
18. Kadota, Y., Shirasu, K. & Zipfel, C. Regulation of the NADPH oxidase RBOHD during plant immunity. *Plant. Cell. Physiol.* **56**, 1472–1480 (2015).
19. Xing, Y.M., Chen, J., Song, C., Liu, Y.Y., Guo, S.X. & Wang, C.L. Nox gene expression and Cytochemical localization of hydrogen peroxide in *P. umbellatus* sclerotial formation. *Int. J. Mol. Sci.* **14**, 22967–22981 (2013).
20. Waterham, H.R., Russell, K.A., Vries, Y.D. & Cregg, J.M. Peroxisomal Targeting, Import, and Assembly of Alcohol Oxidase in *Pichia pastoris*. *J. Cell. Biol.* **139** (6), 1419–1431 (1997).
21. Labrou, N.E., Papageorgiou, A.C., Pavli, O. & Flemetakis, E. Plant GSTome: structure and functional role in xenome network and plant stress response. *Curr. Opin. Biotechnol.* **32**, 186–194 (2015).
22. Estévez, I.H. & Hernández, M.H. Plant Glutathione S-transferases: An overview. *Plant. Gene*. **23**, 100233 (2020).
23. Islam, T., Ganesan, K. & Xu, B.J. New Insight into Mycochemical Profiles and Antioxidant Potential of Edible and Medicinal Mushrooms: A Review. *Int. J. Med. Mushrooms*. **21**(3), 237–251 (2019).
24. Patsoukis, N. & Georgiou, C.D. Thiol redox state and related enzymes in sclerotium-forming filamentous phytopathogenic fungi. *Mycol. Res.* **112**, 602–610 (2008).
25. Giannakopoulos, E. *et al.* Long-Term Preservation of Total Phenolic Content and Antioxidant Activity in Extra Virgin Olive Oil: A Physico-biochemical Approach. *Free Radicals Antioxidants*. 10(1), 4–9 (2020).
26. Kawahara, T. & Lambeth, J.D. Molecular evolution of Phox-related regulatory subunits for NADPH oxidase enzymes. *BMC. Evol. Biol.* **7**, 178 (2007).

27. Di Palmaa, A.A. *et al.* Nitro-oleic acid triggers ROS production via NADPH oxidase activation in plants: A pharmacological approach. *J. Plant. Physiol.* **153****128**, 246–247 (2020).
28. Xing, Y.M., Zhao, M.M., Guo, L.C., Li, B., Chen, J. & Guo, S.X. Identification and expression of DoCCaMK during *Sebacina* sp. symbiosis of *Dendrobium officinale*. *Sci. Rep.***10**, 9733 (2020).
29. Cui, Y.N. *et al.* Transcriptomic Profiling Identifies Candidate Genes Involved in the Salt Tolerance of the Xerophyte *Pugionium cornutum*. *Genes.* **10**, 1039 (2019).
30. Anders, S. & Huber, W. Differential expression analysis for sequence count data. *Genome. Biol.* **11**, R106 (2010).
31. Xing, Y.M. *et al.* Sclerotial formation of *Polyporus umbellatus* by Low Temperature Treatment under Artificial Conditions. *Plos. One.* **8**(2), e56190 (2013).

Tables

Table 1. Summary of quality evaluation of sequencing output data

Sample	Raw reads	Clean reads	Clean bases	Error (%)	Q20 (%)	Q30 (%)	GC (%)
PU_S1 ^a	48862698	47515246	7.13G	0.02	96.46	91.35	55.30
PU_S2 ^a	55825490	54510292	8.18G	0.02	96.61	91.62	55.54
PU_S3 ^a	45034602	43841868	6.58G	0.02	96.06	90.70	55.78
PU_SM1 ^b	44804502	43382478	6.51G	0.02	96.47	91.51	55.95
PU_SM2 ^b	46263970	44731548	6.71G	0.02	96.13	90.85	55.45
PU_SM3 ^b	47793444	46365222	6.95G	0.02	96.46	91.44	55.51
PD_S1 ^c	43868088	42749076	6.41G	0.02	96.65	91.81	55.14
PD_S2 ^c	44566228	43376858	6.51G	0.02	96.58	91.66	55.13
PD_S3 ^c	47705028	46319972	6.95G	0.02	96.51	91.50	56.66
Total	424814050	412792560	61.93G				

^a PU_S1, PU_S2 and PU_S3: the mycelia of *P. umbellatus* in the control group. ^b PU_SM1, PU_SM2 and PU_SM3: the mycelia of *P. umbellatus* in the low oxalic acid group. ^c PD_S1, PD_S1 and PD_S3: the mycelia of *P. umbellatus* in the high oxalic acid group.

Table 2. Gene function annotation analysis in 7 public databases

	Number of Unigenes	Percentage (%)
Annotated in NR	10914	48.45
Annotated in NT	12864	57.11
Annotated in KO	4063	18.03
Annotated in SwissProt	11236	49.88
Annotated in PFAM	10571	46.93
Annotated in GO	10645	47.26
Annotated in KOG	6934	30.78
Annotated in all Databases	2469	10.96
Annotated in at least one Database	18855	83.71
Total Unigenes	22523	100

Table 3. The DEGs of Oxidase and dehydrogenase (partly).

DEGs NO. Gene ID	Annotation of enzyme	Compared groups	adjusted p- value	Log2 Fold change	Up or down regulated
<i>c3315_g1</i>	NADPH oxidase	CK vs. PD_S	0.036757	0.62	up
<i>c8851_g1</i>	NADPH oxidase	CK vs. PD_S	0.000177	2.02	up
<i>c43_g1</i>	cytochrome c oxidase subunit 1	CK vs. PD_S	1.1009E- 40	7.31	up
<i>c159_g1</i>	cytochrome c oxidase subunit 2	CK vs. PD_S	1.8551E- 20	7.56	up
<i>c13857_g1</i>	cytochrome c oxidase subunit 3	CK vs. PD_S	1.25E-18	7.44	up
<i>c8987_g1</i>	Alcohol oxidase	CK vs. PU_SM	3.22E-10	2.87	down
<i>c8176_g4</i>	Alcohol oxidase	CK vs. PD_S	1.84E-05	1.14	up
<i>c17518_g1</i>	NADH dehydrogenase subunit 1	CK vs. PD_S	9.20E-13	6.82	up
<i>c1494_g1</i>	NADH dehydrogenase	CK vs. PD_S	2.84E-30	6.77	up

Table 4. DEGs associated with energy metabolism

Annotation of enzymes	DEGs NO.	Compared groups	adjusted p-value	Log2 Fold change	Up or down regulated
L-rhamnose 1-dehydrogenase	<i>c4703_g1</i>	CK vs PU_SM	0.00060777	3.63	down
L-rhamnose 1-dehydrogenase	<i>c4703_g1</i>	CK vs PD_S	5.43E-09	3.68	up
L-threo-3-deoxy-hexylosonate aldolase	<i>c7929_g2</i>	CK vs PU_SM	0.00054459	1.83	up
Rhamnogalacturonase	<i>c4388_g1</i>	CK vs PD_S	0.033252	1.75	up
rhamnogalacturonan acetylerase	<i>c4271_g</i>	CK vs PD_S	7.50E-12	2.69	up
Isocitrate lyase	<i>c7209_g1</i>	CK vs PU_SM	1.54E-13	5.12	down
Fatty acid hydroxylase (cytochrome P450)	<i>6742_g2</i>	CK vs PU_SM	3.53E-24	4.87	down
Fatty acid synthase	<i>c3295_g1</i>	CK vs PD_S	0.0034424	0.80	up
Fatty acid synthase	<i>c4529_g1</i>	CK vs PD_S	0.04753	0.70	up
fatty acid desaturase	<i>c9487_g1</i>	CK vs PD_S	0.0019857	1.76	up

Table 5. DEGs related to Calcium signaling pathway

DEGs No.	Annotation of enzyme	Compared groups	Adjusted p-value	Log2 Fold change	Up or down regulated
<i>c8739_g2</i>	calmodulin	CK vs. PU_SM	0.023908	1.413	down
<i>c2043_g1</i>	calmodulin	CK vs. PD_S	0.0001615	Inf	up
<i>c3257_g1</i>	calmodulin	CK vs. PD_S	0.0001615	Inf	up
<i>c4558_g1</i>	calmodulin-binding motif	CK vs. PU_SM	0.00019948	-Inf	down
<i>c8133_g1</i>	calmodulin-binding motif	CK vs. PD_S	0.00031588	1.09	up
<i>c9830_g2</i>	calcium ion transport	CK vs. PU_SM	0.0090109	1.44	down
<i>c3191_g1</i>	cellular calcium ion homeostasis	CK vs. PD_S	3.0275E-26	2.49	up
<i>c6291_g1</i>	calcium ion transport	CK vs. PD_S	0.001211	0.93	up

Figures

Figure 1

Colony morphology of *P. umbellatus* under exogenous different concentrations of oxalic acid A, B and C respectively stand for the colony morphological characteristics of *P. umbellatus* mycelia in the CK, PU_SM and PD_S groups after cultivation for 30d.

Figure 2

The length distribution of the assembled unigenes. The minimum, maximum and the mean length of the assembled unigenes were all showed. N50, N90: The lengths of the splicing transcript sequences were ranked from long to short, and the lengths were accumulated to no less than 50% or 90% of the total length of the splicing transcript sequences were named N50 or N90 respectively.

Figure 3

GO categorization of unigenes. Totally, the 10,645 unigenes were grouped into three parts such as biological process, cellular component and molecular function.

Figure 4

Correlation of RPKM distribution between both biological replicates of each group. Comparisons of estimated RPKM distributions between the three biological replicates for the CK group, the PU_SM group and the PD_S group. Pearson's correlation coefficients (R^2) between the replicates are presented.

Figure 5

Volcano plot of the DEGs between both of the samples of each group. The y-axis represents the mean expression value of $\log_{10}$ (adjusted p-value), and the x-axis stands for the $\log_2$ (fold change) value. The red dots and the green dots show the up regulated and the down regulated expressed transcripts respectively $p < 0.05$, false discovery rate (FDR) $q\text{-value} < 0.05$ among PU_SM and CK, PD_S and CK groups.

Figure 6

The ROS content in *P. umbellatus* mycelia affected by different concentrations of oxalic acid After cultivation for 30 days, the ROS content of *P. umbellatus* mycelia in the CK (Fig. 6A), PU_SM (Fig. 6B) and PD_S group (Fig. 6C) were shown. Images were representatives of three independent experiments ($n=30$). Scale bar, 10 μm .

Figure 7

Validation of the DEGs using qRT-PCR Compared to the CK group, the red columns represent up-regulated, while the blue columns stand for down-regulated. In the upper line, c43_g1, c8987_g1 and c8851_g1 respectively represent DEGs encoding cytochrome c oxidase, alcohol oxidase and NADPH oxidase. In the middle line, c6769_g1, c9881_g2 and c6055_g1 stand for DEGs encoding Manganese peroxidase, aldo/keto reductase and Glutathione S-transferases. In the lower line, c7209_g1, c8739_g2 and c4703_g1 are representative of DEGs encoding Isocitrate lyase, calmodulin and L-rhamnose 1-dehydrogenase. Representative images were from three independent experiments, with 3 replicates in each group.

Figure 8

Real-time Ca^{2+} and H_2O_2 fluxes measurement of *P. umbellatus* mycelia affected by different concentrations of oxalic acid using NMT A, Real-time Ca^{2+} influxes measurement. B, Real-time H_2O_2 fluxes measurement. The negative values stood for influx of Ca^{2+} and H_2O_2 (Fig. 8A and Fig. 8B) and the positive values represented for efflux of H_2O_2 (Fig. 8B). The values of net Ca^{2+} / H_2O_2 fluxes were means $\pm$ SD ($n = 30$). After cultivation for 30d, Ca^{2+} influx was shown in the mycelia of *P. umbellatus* for a period of time in different groups and the net Ca^{2+} uptake in the PU_SM group increased but decreased in the PD-S group, in comparison to that of the control group (Fig. 8A). The net H_2O_2 represented slight excretion in the PD-S group but uptake in the control group and the PU_SM group (Fig. 8B).

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [Supplementaryinformaiton.pdf](#)