Toxicity of *Melaleuca alternifolia* essential oil on mitochondrion and NAD+/NADH dehydrogenase in *Tribolium confusum*

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Background. In our previous study, acting on the mitochondrial respiratory chain is considered the insecticide action of *Melaleuca alternifolia* essential oil. However, its mode of action is not fully understood.

Methods. In this study, we investigated the insecticidal efficacy of *M. alternifolia* essential oil against another major stored-product pest, *Tribolium confusum* Jacquelin du Val. Then, rarefaction and vacuolization of the mitochondrial matrix were evident in the oil-fumigated *T. confusum* adults.

Results. Alterations to the mitochondria confirm the insecticidal effect of *M. alternifolia* essential oil, which may act on mitochondrial respiratory. Furthermore, comparative transcriptome analysis of *T. confusum* using RNA-seq indicated that most of the differentially expressed genes were involved in insecticide detoxification and mitochondrial function. The biochemical analysis showed that the intracellular NAD+/NADH ratio is involved in the differential effect of the *M. alternifolia* essential oil.

Discussion. These results led us to further conclude that NAD+/NADH dehydrogenase may be the prime target site of EOs in insects, leading to the blocking of the mitochondria respiratory chain.

Toxicity of *Melaleuca alternifolia* essential oil on mitochondrion and NAD^+/NADH dehydrogenase in *Tribolium confusum*

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Abstract

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Discussion. These results led us to further conclude that NAD^+/NADH dehydrogenase may be the prime target site of EOs in insects, leading to the blocking of the mitochondria respiratory chain.

Keywords: *Melaleuca alternifolia* essential oil; *Tribolium confusum*; Transcriptome; NAD^+/NADH; Transmission electron microscopy

INTRODUCTION

Essential oils (EOs) obtained from aromatic plants by steam distillation are regarded as a new and safe alternative to conventional insecticides because of their bioactive potential and high volatility (Bai et al. 2015; Liang et al. 2016). The volatile nature of plant essential oils reduces concerns regarding residues of their constituents on stored grains, which mitigates environmental contamination and effects on non-target organisms (Li et al. 2013a; Polatoglu et al. 2016). The potential for synergistic or additive effects between the volatile secondary metabolites of essential oils, which act on insects via various targets and mechanisms, is also an advantage (Abdelgaleil et al. 2015).

The mechanisms underlying the toxicity of EOs have been explored for decades. To date, most of the studies focused on enzyme inhibition or induction (Kavitha et al. 2013; Li et al. 2013b; Matthews et al. 2010; Zhang et al. 2013). The main targets of EOs are neurotoxic target enzymes such as acetylcholinesterase and a variety of detoxifying enzymes such as glutathione *S*-transferase (GST) and carboxylesterase (CarE). EOs have lethal and sublethal effects on pests that attack stored grains, and they are frequently applied via fumigation by stored-grain managers (Peixoto et al. 2015; Suthisut et al. 2011). Thus, the activity of essential oils may cause abnormal respiration, which is similar to the effect of octopamine (Enan 2005); however, relatively little is known about the underlying mechanisms, particularly the mitochondrial electron transport chain.

Recently, transcriptome profiling analysis has increased our understanding of insect response to various stressors (Chen et al. 2016; Du et al. 2016). RNA-seq is an effective tool for studying the extensive regulation at transcriptional levels (Clements et al. 2016; Hamisch et al. 2012), and

it can be used for characterizing the complexity of mitochondrial transcriptomes (Stone & Storchova 2015). EOs are mixtures of volatile secondary metabolites, mainly monoterpenes and sesquiterpenes; therefore, they have various modes of action in insects. It is difficult to separate and purify an active substance to study its mechanisms. Moreover, the insecticidal activity of an essential oil can be attributed to the synergistic effects of its major components (Wu et al. 2015); therefore, all the major components need to be considered while assessing the mechanism of an essential oil. In our previous study, we had reported, for the first time, a comprehensive transcriptome analysis of the maize weevil, *Sitophilus zeamais*, to identify the genes and pathways that are likely to be changed upon exposure to the essential oil obtained from *Melaleuca alternifolia*(Liao et al. 2016b). In the study, many differentially expressed genes (DEGs), such as cytochrome P450s, CarEs, GSTs, complex I to IV, and ATP synthesis-related proteins, were associated with respiration and metabolism of xenobiotics on the basis of Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway annotations. Our findings suggested that the mitochondrial electron transport chain is a likely target in insects. Such information contributes to new insights on the biological response of insects to EOs and helps us in understanding the molecular mechanisms underlying the insecticidal activity of plant EOs.

M. alternifolia is derived from an Australian plant, *Melaleuca leucadendron*, and it was developed to meet increasing demands for its monoterpene-rich EOs (Bustos-Segura et al. 2015). Notably, the major component, terpinen-4-ol, showed distinct fumigant toxicity against the pests of stored grains: 6.78 mg/L air of median lethal concentration (LC_{50}) for *S. zeamais* (Liao et al. 2016b). Terpinen-4-ol has also been found in the most EOs reported in previous studies and should

be studied further (Abdelgaleil et al. 2015; Brahmi et al. 2016; Yeom et al. 2013).

To obtain information on the fumigant toxicity of EOs of *M. alternifolia* and their chemical compounds against stored grain insects and identify a better chemotype, while focusing on the standardization of natural insecticides, we studied the toxicity of *M. alternifolia* EO against the confused flour beetle (*Tribolium confusum* Jacquelin du Val.), which is closely related to the flour beetle *Tribolium castaneum* (Herbst) (Golestan et al. 2015). To expand on the applicability of our previous transcriptomic analysis and provide a clearer picture of the mode of action of natural insecticides, we also performed RNA-seq analysis of the *T. confusum* transcriptome to investigate changes in the abundance of mitochondrial transcripts after exposure to the *M. alternifolia* EO. To verify the reliability of the RNA-seq data, we tested the inhibitory effects of the EO on NAD^+/NADH dehydrogenase, which is a possible insecticidal target. Subsequently, we assessed the action of the *M. alternifolia* EO in degrading the mitochondria in the cells obtained from oil-fumigated *T. confusum*. To our knowledge, no studies on the molecular events underlying the response of *T. confusum* to plant EOs have been performed or published.

MATERIALS AND METHODS

EO and chemicals

The EO was purchased from Fujian Senmeida Biological Technology Co., Ltd (China). Terpinen-4-ol (40.09%), γ-terpinene (21.85%), α-terpinene (11.34%), α-terpineol (6.91%), and 1,8-cineole (1.83%) were the major compounds.

Insect culture

A culture of *T. confusum* was maintained in the laboratory, and the insects were not exposed to

97 any insecticides. The insects were reared on sterilized whole wheat at 28 ± 1 °C and 68 ± 5 °C
98 relative humidity under complete darkness. Approximately, 2-week-old post-emergence adults
99 were used in the subsequent experiments

100 **Fumigant toxicity assay**

101 The fumigant toxicity of *M. alternifolia* EO against *T. confusum* was determined according to our
102 previous protocol (Liao et al. 2016b). For oil exposure, 30 adults were exposed to serial dilution
103 doses in sealed gas-tight 300-mL glass jars and incubated for 24, 48, and 72 h at 28 °C. For the
104 doses, drops of the oil or compounds were applied to a piece of filter paper (2 × 3 cm) by using a
105 microinjector, and the filter paper was attached to the undersurface of the jar lid. Equivalent groups
106 of control adults were treated similarly, but without exposure to the oil. Three biological replicates
107 were maintained for each treatment. In addition, *T. confusum* specimens exposed to LC_{50} (6.37
108 mg/L air) of oil for 12, 24, 36, 48, 60, and 72 h were collected and washed twice or three times
109 with pre-cooled saline, flash-frozen in liquid nitrogen, and stored at -80 °C for the subsequent
110 bioassays.

111 **RNA sequencing**

112 Total RNA was extracted from oil treatment and control groups (collected at 72 h) with TRIzol
113 reagent (Kangwei Century Biological Co., Ltd., China), according to the manufacturer's
114 instructions, and treated with DNase I (Sangon Biotech, Shanghai, China). The RNA quality was
115 checked with a 2100 Bioanalyzer (Agilent Technologies, USA). Library construction and Illumina
116 sequencing were performed at BGI-Tech (Wuhan, China). For cDNA library construction, 5 μg of
117 RNA per sample from three biological replicates were combined and used. Two cDNA libraries

were constructed for the oil treatment and control groups. For Illumina sequencing, which was followed the protocol of the Illumina TruSeq RNA Sample Preparation Kit (BGI-Tech, Wuhan, China), and 2 × 100-bp paired-end reads were sequenced using Illumina HiSeq™ 4000 (Illumina, Inc. USA). The reads were submitted to the NCBI Sequence Read Archive (SRA; accession number, SUB2742979).

Bioinformatic analyses

The reads for the treatment and control groups were mapped to the 165.944-Mb *T. castaneum* reference genome obtained from NCBI (BioProjects: PRJNA12540) by using TopHat v.2.08 (Kim et al. 2013a), with quality aware alignment algorithms (Bowtie v.2.2.5) (Langmead et al. 2009).

The raw RNA-seq reads were assessed for quality with FastQC (version 0.11.4; Babraham Bioinformatics, Cambridge, UK) and saved as FASTQ files with default parameters (Cock et al. 2010). Then, *de novo* assembly of the clean reads was performed using the Trinity method (version 2.0.6) (Grabherr et al. 2011). All the unique Trinity contigs were analyzed using BlastX (E-value $< 10^{-5}$) against the protein databases Nr (Agarwala et al. 2016), Nt (Agarwala et al. 2016), COG (Tatusov et al. 2000), KEGG (Kanehisa & Goto 2000), Swiss-Prot, and InterPro using InterProScan5 [33] with default parameters. To annotate the assembled sequences with GO terms, Nr Blast results were imported into Blast2GO.(Conesa et al. 2005)

Transcript abundance was calculated as FPKM for each sample (Li & Dewey 2011). Differential gene expression analysis (fold changes) and related statistical significance in pair-wise comparison were performed using the DESeq program (http://doi.org/10.1186/gb-2010-11-10-r106) (Anders & Huber 2010). The DEGs were identified using a false discovery rate (FDR)

139 threshold ≤ 0.001 and absolute value of log2Ratio ≥ 1 (Hao et al. 2016). Genes with an adjusted
140 P-value were used for controlling FDR, and those with a threshold < 0.05 were classified as
141 di□erentially expressed (Ma et al. 2015).

142 For each DEG, GO and KEGG enrichment analyses were conducted using the DESeq R
143 package (http://www.geneontology.org/ and http://www.genome.jp/kegg/, respectively). The
144 GOslim annotations results were then classified into three main classes: molecular function,
145 biological process, and cellular component. The KEGG database was used to identify significantly
146 enriched metabolic pathways or signal transduction pathways.

147 **Quantitative real-time PCR**

148 qRT-PCR was used to further validate and quantify the RNA levels for 20 selected genes that
149 encode NADH or NAD^+ by using the iCycler iQ Real-time Detection System (Bio-Rad, Hercules,
150 CA, USA). Gene-specific primers were designed using Primer Premier 5, and the sequences are
151 listed in Table S1. The house-keeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH)
152 was used as the reference gene, as proposed by Prentice et al (Pan et al. 2015). For the qRT-PCR
153 analysis, cDNA templates were diluted 20-fold in nuclease-free water. Then, mRNA levels were
154 measured in triplicate (technical repeats) with qPCR by using the SYBR Green Master Mix
155 (Vazyme Biotech Co., Ltd, Nanjing, China), according to the manufacturer's instructions. PCR
156 amplification was performed in a total volume of 20.0 μL containing 10.0 μL of the SYBR Master
157 Mix, 0.4 μL of each primer (10 μM), 2.0 μL of cDNA, and 7.2 μL of RNase-free water. The
158 amplification procedure was composed of an initial denaturation step at 95 °C for 5 min, followed
159 by 40 cycles of 95 °C for 10 s and 60 °C for 30 s and the melting curve step at 95 °C for 15 s, 60

°C for 60 s, and 95 °C for 15 s. Gene expression was quantified (mean ± SD) as relative fold change by using the $2^{-\Delta\Delta CT}$ method (Schmittgen & Livak 2008).

Measurement of intracellular NAD^+/NADH ratio

Both oxidized and reduced forms of intracellular NAD were determined using an NAD(H) quantification kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). Briefly, 0.1 g of the test insects were collected at 12, 24, 36, 48, 60, and 72 h and extracted with 1 mL of NAD^+/NADH extraction buffer in three freeze/thaw cycles. The samples were centrifuged at 10000 × *g* for 5 min at 4 °C. Then, 0.5 mL of the extracted NADH or NAD^+ supernatant was transferred to a centrifuge tube and neutralized with an equal volume of the opposite extraction buffer. The samples were centrifuged at 10000 × *g* for 10 min at 4 °C and then used for the subsequent bioassays. NADH or NAD^+ cycling mix was prepared according to the manufacturer's protocol (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). Finally, absorbance was measured at 570 nm. In addition, the concentration of the total protein was determined using the total protein quantitative assay (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). Three replicates were used for each treatment, and each replicate was determined three times.

Statistical analysis

The mortality rates observed in the toxicity bioassays were corrected for the control group by using Abbott's formula (Abbott 1925). All data are expressed as mean ± SE values of three independent experiments and analyzed using one-way nested analysis of variance and unpaired sample *t*-test. A significant difference was accepted at a p value < 0.05. An extremely significant difference was accepted at p value < 0.01. The LC_{50} values were evaluated using probit analysis (Fong et al. 2016),

and corresponding confidence intervals at 95% probability were obtained using IBM SPSS Statistics 22.0 (SPSS, USA). Figures depicting the effects of the EO on enzymatic activities and the qRT-PCR results were created using Origin Pro 9.0 (Origin Lab Corporation, USA).

RESULTS

Fumigant toxicity of *M. alternifolia* EO and constituents

To investigate the toxicity of the *M. alternifolia* EO against *T. confusum* adults, we performed the fumigation assay. Toxicity similar to that against *S. zeamais* (Liao et al. 2016b) was observed, providing evidence that the *M. alternifolia* EO has potent fumigant toxicity (Fig. 1a). We also found that the effect of fumigation gradually increased over time (24 h, 48 h, and 72 h); the corresponding LC_{50} values were 7.45, 7.09, and 6.37 mg/L air, respectively (Fig. 1b). The largest dose of 11.97 mg/L air EO caused 91.11%, 97.78%, and 98.86% mortality, respectively, in the *T. confusum* adults. For the same dose, increased effects of fumigation were observed over 24–48 h, as compared with those over 48–72 h.

The same toxicity pattern was also observed for the major compounds (Liao et al. 2016b). In particular, terpinen-4-ol was the most potent toxicant with an LC_{50} value of 3.83 mg/L air (Fig. 1c). In the *M. alternifolia* EO, terpinen-4-ol was the main component (40.09% of the EO), indicating that terpinen-4-ol is the major contributor to the fumigant toxicity of the EO. In addition, α-terpinene and γ-terpinene exhibited weaker fumigant toxicity (LC_{50} = 28.52 and 44.53 mg/L air, respectively) against *T. confusum*, showing that the oxygen-containing compounds could cause a remarkable change in bioactivity. Kim et al. (Kim et al. 2013b) described a similar structure–activity relationship among oil constituents with aldehyde, ketone, and alcohol groups and

hydrocarbons against rice weevil adults. Thus, the fumigant toxicity of the *M. alternifolia* EO may be attributable to a synergistic effect of the activities of the oil constituents, and the constituents may have different modes of action.

Transmission electron microscopy (TEM) of mitochondria

An ultra-structural examination of the morphology of the mitochondria from untreated and oil-fumigated *T. confusum* larvae is shown in Fig. 2. In the untreated *T. confusum* larvae, the mitochondria have highly electron-dense cristae, membranes, and matrix. However, the mitochondria in the columnar and regenerative nidi cells from the thorax of the oil-treated *T. confusum* larvae had undergone ultra-structural changes detected by the vacuolization of the mitochondrial matrix. The vacuolization increased with time after the oil treatment and, in severe cases, caused fragmentation of the mitochondria. The results were consistent with those obtained in a previous study in which allylisothiocyanate oil and PH_3 were used (Mansour et al. 2012).

Illumina sequencing and *de novo* assembly

To obtain a global, comprehensive overview of the *T. confusum* transcriptome, RNA was extracted from the treatments and control groups. A total of 126,280,032 paired-end reads (100 bp) were generated from the samples by using the Illumina HiSeq™ 4000 platform. Then, 89,342,546 clean reads were obtained by preprocessing and filtering the reads (low-quality sequences were removed; Table 1). Subsequently, the clean reads were subjected to transcriptome assembly by using the Trinity software package (Grabherr et al. 2011), and 28,885 assembled unigenes were generated using overlapping information from high-quality reads, which accounted for 36,998,010 bp (Table 1). Of the assembled unigenes, approximately 38.54% were ≤600 bp and 61.46% were

>500 bp. The average length of the unigenes was 1,280 bp, with an N50 length of 2,097 bp and mean length of 1280 bp. The length distribution of the unigenes is shown in Fig. 3a.

Functional annotation of *T. confusum* transcripts

All the assembled unigenes were aligned against seven public databases, namely, Non-redundant (Nr) (Agarwala et al. 2016), Nucleotide (Nt) (Agarwala et al. 2016), Cluster of Orthologous Groups (Tatusov et al. 2000), Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway (Kanehisa & Goto 2000), Swiss-Prot (UniProt 2015), InterPro (Mitchell et al. 2015), and Gene Ontology (GO) (Ashburner et al. 2000) databases (Table 1). Of the 28,885 assembled unigenes, 23,160 (80.18%) exhibited sequence similarity to a sequence within the Nr database; 23,571 (81.60%) unigenes were annotated in at least one database, indicating that just a few unigenes (18.40%) could not be identified. The homologous genes that showed the best match (54.24%) were from *T. castaneum* (91.82%). On the basis of the Nr annotation, GO functional analysis of the unigenes was performed. A total of 6,333 (21.92%) unigenes were assigned to the biological process, molecular function, and cellular component categories, including 57 GO terms (Fig. 3b). In addition, 18,074 (62.57%) unigenes were divided into 42 subcategories and 295 KEGG pathways by using the KEGG annotation system with default parameters to predict the metabolic pathways (Fig. 3c).

Differential expression analysis and pathway enrichment

The sequence analysis and annotation of all the unigenes in *S. zeamais* fumigated by the *M. alternifolia* EO provided some valuable information for analyzing the *T. confusum* transcriptome. From the 23,571 unigenes identified in the analysis, we chose to focus on transcripts encoded by

the genes associated with known mechanisms to cope with xenobiotic compounds, including quantitative or qualitative changes in major detoxification enzymes and transporters to decrease exposure (pharmacokinetic mechanisms) or changes in target site sensitivity (pharmacodynamic mechanisms) (Bajda et al. 2015). Specifically, changes in the expression levels of four classes of enzymes and proteins (GST, CarE, cytochrome P450 monooxygenases, and mitochondrial respiratory chain-related proteins) were investigated to determine whether patterns emerged in the upregulation or downregulation of specific transcripts. The transcriptome of *T. confusum* showed that the largest and most abundant group was ATPase transporters, followed by cytochrome P450s; some of them may be involved in insecticidal mechanisms. The transcriptome also showed five possible NAD^+/NADH dehydrogenase transcripts, which may be the main targets for the EO.

For comparison, the fragments per kilobase of exon per million fragments mapped (FPKM) of each transcript was calculated to estimate the expression levels between the oil-fumigated and oil-free samples. The important DEGs (999 upregulated and 1,209 downregulated) were identified on the basis of threshold FDR < 0.01 and fold change 2 between the oil-fumigated and oil-free samples. To annotate these DEGs, both GO and KEGG functional analyses were performed.

The GO annotation analysis classified 632 DEGs into three GO categories and 339 terms (Fig. S1a). In the molecular function category, 560 DEGs were classified into 11 terms, namely, antioxidant activity, binding, catalytic activity, electron carrier activity, enzyme regulator activity, guanyl-nucleotide exchange factor activity, molecular transducer activity, nucleic acid binding transcription factor activity, receptor activity, structural molecule activity, and transporter activity.

Among the DEGs, 1,180 unigenes were mapped to 287 different KEGG pathways and five

265 categories (Fig. S1b). According to the threshold of Q value < 0.05, 22 pathways were significantly
266 enriched (Table S2). Many DEGs were significantly enriched in the metabolism pathways
267 associated with respiration and metabolism of xenobiotics, suggesting that abnormal respiration
268 and metabolic disorders occurred in the *T. confusum* adults after fumigation with the *M.*
269 *alternifolia* EO. In addition, 92 possible insect hormone biosynthesis transcripts, some of which
270 are known targets of chlorbenzuron, were detected (Xu et al. 2017).

271 To verify the expression patterns of the DEGs involved in metabolism, 20 genes were selected
272 for qRT-PCR analysis. As shown in Fig. 4, similar trends of upregulation/downregulation of the
273 selected DEGs were observed between the qRT-PCR and transcriptome data, confirming the
274 accuracy of our transcriptome profiling.

275 **NAD^+/NADH ratio in *T. confusum* fumigated with the *M. alternifolia* EO**

276 NAD^+ is a biological oxidizing agent for many metabolic reactions, and tNOX oxidizes
277 hydroquinones and NADH, converting the latter to the oxidized NAD^+ form (Titov et al. 2016) .
278 The *M. alternifolia* EO probably directly affects the hydrogen carrier to block the electron flow,
279 and interference with energy synthesis has been previously proposed for *S. zeamais*. This
280 suggestion prompted us to further investigate whether the EO acts on NAD^+/NADH, and, if so, by
281 what mechanism.

282 We found that treatment with 6.37 mg/L EO significantly increased NAD^+ (Fig. 5a) but
283 decreased NADH (Fig. 5b) levels at 12–48 h; however, the opposite trend was observed after 60
284 h. The ratio of NAD^+/NADH in *T. confusum* from 12 to 60 h after treatment decreased
285 (significantly in 24–48 h) and increased after 60 h, but not effective enough (Fig. 5c).

DISCUSSION

In this study, the *M. alternifolia* EO displayed strong fumigant insecticidal activity on the stored-grain insect pest *T. confusum*. The study also demonstrated that the toxicity of the *M. alternifolia* EO differed according to the insect species (8.42 and 7.45 mg/L air LC_{50} at 2 h for *S. zeamais* and *T. confusum*), duration of exposure, and different concentrations used. Moreover, terpinen-4-ol demonstrated the most potent biological activity and accounted for 40% of the EO content. We deduced that the terpinen-4-ol chemotype is the main insecticidal active component. The amount of terpinen-4-ol directly affects the insecticidal activity of the EO, according to the fumigant toxicities of the constituents of the EO. Terpinen-4-ol is also found in many reported EOs (Du et al. 2014; Liang et al. 2017). Thus, we suggest that the chemotypes of oils rich in terpinen-4-ol should be explored as potential natural insecticides.

EOs have produced remarkable results; however, several barriers stand in the way of their application in agriculture. Their unclear mode of action is one of the most significant barriers. An EO is a well-known mixture of volatile secondary metabolites that operate via several modes of action. In insects, octopamine (Enan 2005) and GABA receptor (Enan 2001) are considered targets for EO activity. We showed that the *M. alternifolia* EO has sub-lethal behavioral effects on insects by blocking the mitochondrial electron transport chain (Liao et al. 2016a), as suggested in our previous study. Inouye et al. also showed the respiration-inhibitory effects of EOs on filamentous fungi (Inouye et al. 1998). Similarly, the TEM analysis showed that fumigation with the *M. alternifolia* EO affected the mitochondria in the thorax, where the mitochondria became enlarged and swollen. This led to respiratory failure and energy deficiency in the insect body. Prates et al.

(Prates et al. 1998) reported that terpenoids had lethal effects on rice weevils because they affected the respiratory and digestive systems. The main components of the *M. alternifolia* EO are terpinen-4-ol (40.09%), followed by γ-terpinene (21.85%), α-terpinene (11.34%), α-terpineol (6.91%), and α-pinene (5.86%), which are all terpene compounds (Liao et al.). The findings of this study are also supported by the morphological alterations, represented by matrix rarefaction and vacuolization, observed in the mitochondria.

Moreover, our comparative transcriptome analysis revealed that many genes associated with mitochondrial functions were differentially expressed (Table S3). The qRT-PCR analysis validated the expression of selected DEGs detected using RNA-seq. Interestingly, we found that five transcripts encoding the subunits of NAD^+/NADH dehydrogenase in complex I were significantly upregulated. Our biochemical analysis showed that the *M. alternifolia* EO caused pronounced inhibition of NADH but increased NAD^+ level from 12 to 60 h and then subsequently inhibited it. Complex I is the gatekeeper of the respiratory chain and catalyzes the first step of NADH oxidation. NAD^+ is a biological oxidizing agent for many metabolic reactions, and tNOX oxidizes NADH to NAD^+ (Chueh 2000). It elevates the NAD^+/NADH ratio and translocates protons across the inner mitochondrial membrane, which ultimately leads to energy production. To increase energy production in response to oil interference, *T. confusum* probably coverts NADH excessively to the oxidized NAD^+, resulting in an increase in NAD^+ levels. However, *T. confusum*'s own regulatory mechanism is destroyed with time, resulting in a significant reduction in NAD^+ levels. Therefore, *T. confusum* recovers the activity of NADH by upregulating the expression of *NADH* genes; however, the NADH levels have been reduced because of excessive

conversion. This might explain our observation that the transcripts encoding the subunits of NAD^+/NADH dehydrogenase were significantly upregulated at 24 h.

In addition, another reason why the EOs identified to date are primarily effective against stored-product insects but their unstable and slow effects hamper their applications is that terpenes are very important components of EOs and they was prone to in *vivo* metabolism by GST, CarE, and P450s in the insect body (Patra et al. 2015). Miyazawa et al. (Miyazawa & Kumagae 2001) and Haigou et al. (Haigou & Miyazawa 2012) also showed that terpinen-4-ol was prone to in *vivo* metabolism. Experience of the participates metabolic genes would be intended for the future agricultural application. Based on the GO and KEGG annotations, many DEGs, such as cytochrome P450s, CarE, and GST, were mapped to the metabolism pathways in the KEGG pathway database. In insects, GST, CarE, and P450s have been found to play an important role in insect response to various stressors. However, to date, there are few studies on global gene expression profiles for pest insects in response to plant EOs. In our study, the *T. confusum* transcriptome revealed 54 transcripts that encode cytochrome P450s, with 18 differentially expressed more than 2-fold and 33 significantly increased ($p < 0.05$) under oil exposure (Table S4). These genes mainly belong to the CYP6 family. This might explain why terpinen-4-ol can be metabolized by P450s (Haigou & Miyazawa 2012). Most of the genes that encode CarEs and GSTs were also significantly downregulated upon oil exposure (Table S4). It is possible that the redundant components may bind to the site of the enzyme, resulting in the disturbance of the activity. When the conjugated xenobiotics are translated into innocuous substances, the bound enzymes are damaged. This result is consistent with that observed in a previous study (Liao et al.

2016a). Overall, *T. confusum* probably uses these enzymes in combination to catalyze and improve the transformation and degradation of exogenous compounds, resulting in the enhancement of the immune system of the insect. Silencing the upregulated gene expression may contribute to increasing the insecticidal activities of the EO.

Overall, NAD^+/NADH dehydrogenase may be the prime target site of EOs in insects, leading to the blocking of the mitochondria respiratory chain. This results in a dysfunctional energy system, damage to the mitochondria, and death.

CONCLUSIONS

To further clarify the applicability of the findings of our previous study, we investigated the action of the *M. alternifolia* EO in degrading mitochondria of *T. confusum*. Alterations to the mitochondria confirm the insecticidal effect of *M. alternifolia* essential oil, which may act by damaging the mitochondria. To better understand the insecticidal mechanism of the *M. alternifolia* EO, comparative transcriptome analysis of *T. confusum* by using RNA-seq identified a total of 2,208 DEGs in response to oil fumigation. The biochemical analysis showed that the intracellular NAD^+/NADH ratio is involved in the differential effect of the *M. alternifolia* essential oil. Thus, we deduced that NAD^+/NADH dehydrogenase appears to be a prime target for pest control.

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Conflict of interest: The authors declare that they have no conflict of interest.

Supporting Information

Fig. S1. GO (a) and KEGG (b) pathway analysis of DEGs of *S. zeamais* after oil- fumigation.

Table S1. qRT-PCR primers and primer efficiency.

Table S2. Top 22 enriched KEGG pathways between the oil-fumigated and control samples.

Table S3. Differentially expressed genes that encode respiration-related enzymes.

Table S4. Differentially expressed genes that encode xenobiotic detoxification-related enzymes.

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Figure 1(on next page)

Fumigant toxicity of *M. alternifolia* essential oil (a) and its constituents (c) against *T. confusum* adults and the corresponding regression analysis (b)

Results are reported as mean ± SE (calculated from three independent experiments). The LC_{50} values were subjected to probit analysis. (Fong et al. 2016) Different lowercase letters at the top of the columns mean significant differences at a *p* value of 0.05. The error in Figure 3c represents the 95% fiducial limits.

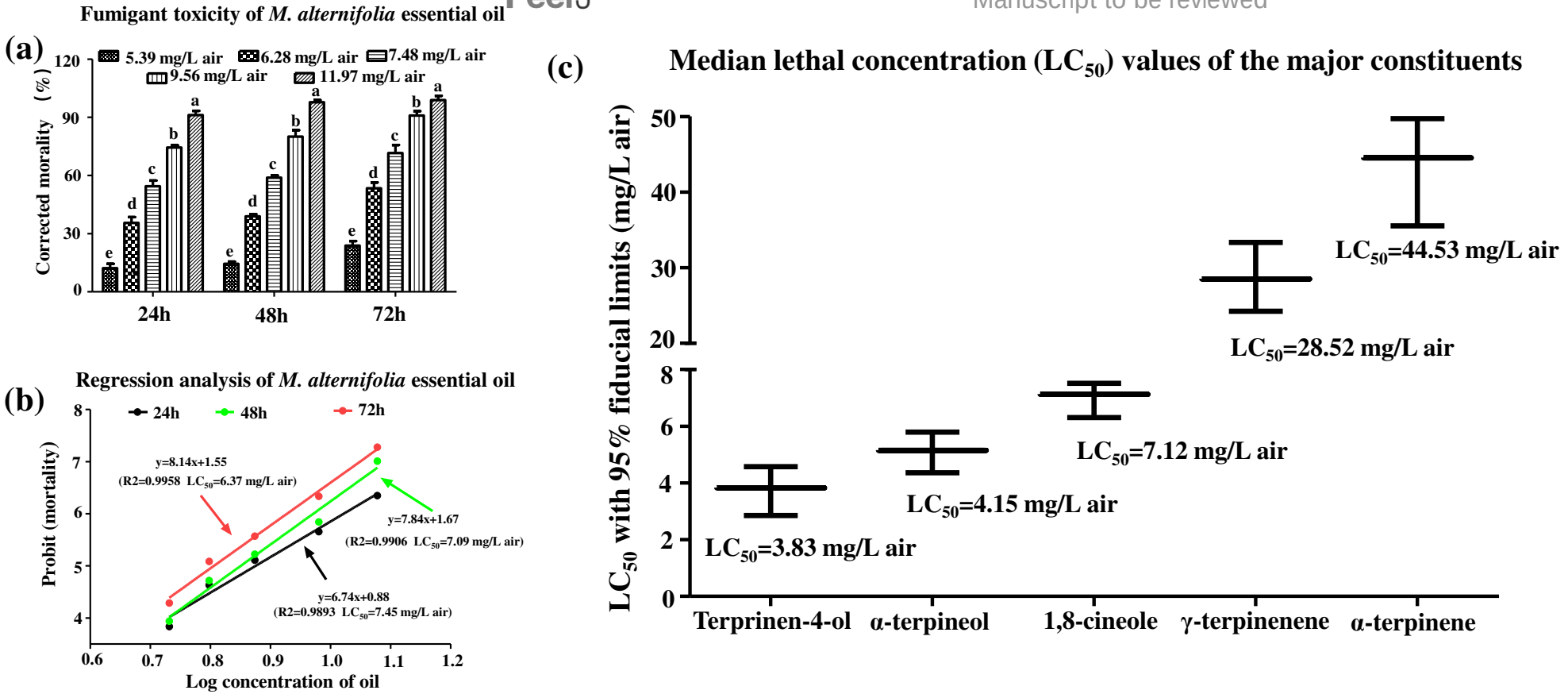

Fumigant toxicity of M. alternifolia essential oil
(a)
5.39 mg/L air
6.28 mg/L air
7.48 mg/L air
9.56 mg/L air
11.97 mg/L air
Corrected morality (%)
24h
48h
72h
Regression analysis of M. alternifolia essential oil
(b)
24h
48h
72h
y=8.14x+1.55
(R2=0.9958 LC50=6.37 mg/L air)
y=7.84x+1.67
(R2=0.9906 LC50=7.09 mg/L air)
y=6.74x+0.88
(R2=0.9893 LC50=7.45 mg/L air)
Probit (mortality)
Log concentration of oil
(c)
Median lethal concentration (LC50) values of the major constituents
LC50 with 95% fiducial limits (mg/L air)
LC50=3.83 mg/L air
LC50=4.15 mg/L air
LC50=7.12 mg/L air
LC50=28.52 mg/L air
LC50=44.53 mg/L air
Terprinen-4-ol
α-terpineol
1,8-cineole
γ-terpinenene
α-terpinene

Figure 2(on next page)

Ultra-structure of the mitochondria from the thorax of non-fumigated (a, c, and e) and fumigated (b, d, and f) *T. confusum* adults.

A: The normal structure of the mitochondrion with many highly electron-dense cristae. B: A part of the thorax and ultra-structural changes in the mitochondria represented by vacuolization (Vm) and rarefaction (Rm) of the mitochondrial matrix (arrow). C: The vacuolization aggravated 24 (b), 48 (d), and 72 h (f) after oil treatment. Scale bar = 2.0 μm.

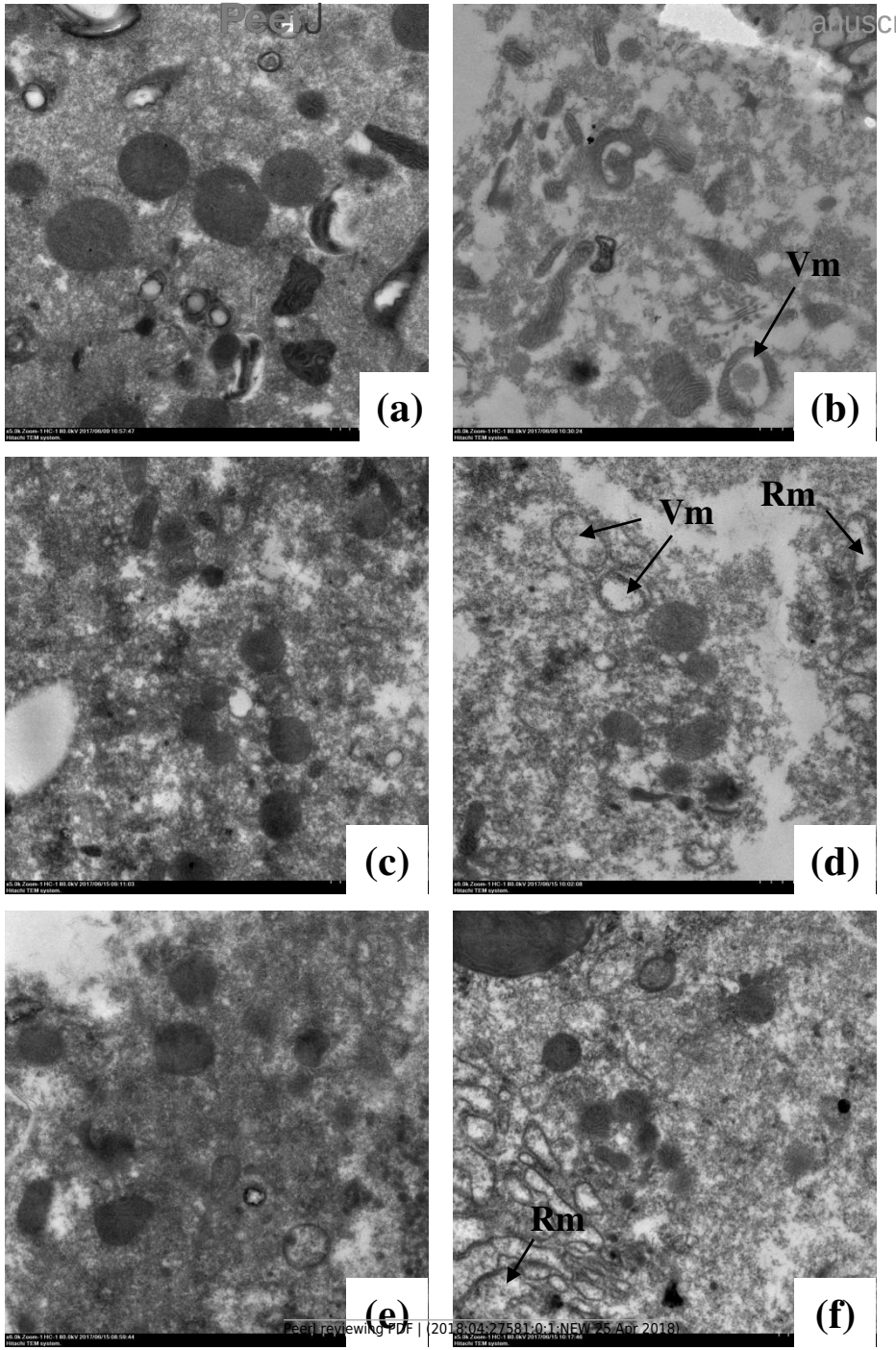
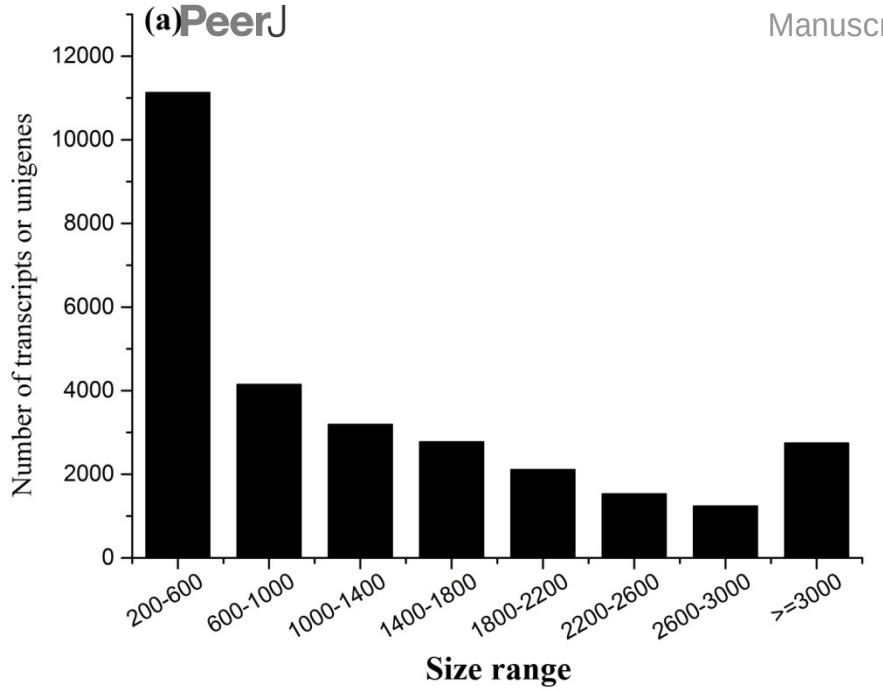
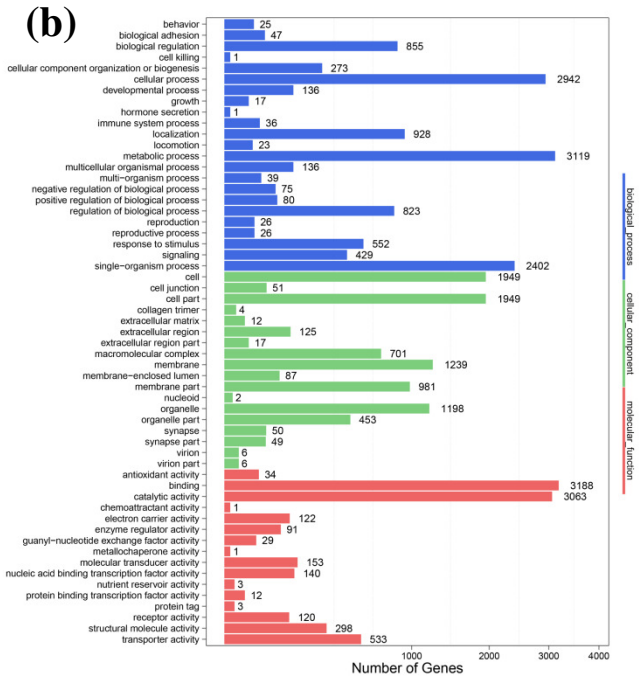


Figure 3(on next page)

Length distribution of assembled sequences (a) and GO (b) and KEGG (c) functional classifications of assembled unigenes of *T. confusum*.

The reads from four libraries were assembled into 2,8885 transcripts.


Number of transcripts or unigenes
12000
10000
8000
6000
4000
2000
0
200-600
600-1000
1000-1400
1400-1800
1800-2200
2200-2600
2600-3000
>=3000
Size range


(b)
behavior 25
biological adhesion 47
biological regulation 855
cell killing 1
cellular component organization or biogenesis 273
cellular process 2942
developmental process 136
growth 17
hormone secretion 1
immune system process 36
localization 928
locomotion 23
metabolic process 3119
multicellular organismal process 136
multi-organism process 39
negative regulation of biological process 75
positive regulation of biological process 80
regulation of biological process 823
reproduction 26
reproductive process 26
response to stimulus 552
signaling 429
single-organism process 2402
cell 1949
cell junction 51
cell part 1949
collagen trimer 4
extracellular matrix 12
extracellular region 125
extracellular region part 17
macromolecular complex 701
membrane 1239
membrane-enclosed lumen 87
membrane part 981
nucleoid 2
organelle 1198
organelle part 453
synapse 50
synapse part 49
virion 6
virion part 6
antioxidant activity 34
binding 3188
catalytic activity 3063
chemoattractant activity 1
electron carrier activity 122
enzyme regulator activity 91
guanyl-nucleotide exchange factor activity 29
metallochaperone activity 1
molecular transducer activity 153
nucleic acid binding transcription factor activity 140
nutrient reservoir activity 3
protein binding transcription factor activity 12
protein tag 3
receptor activity 120
structural molecule activity 298
transporter activity 533
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cellular_component
molecular_function

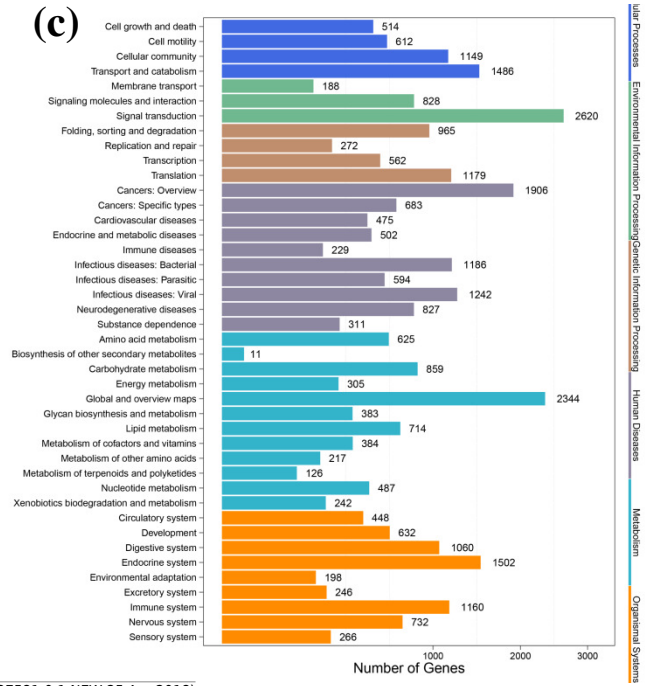

(c)
Cell growth and death 514
Cell motility 612
Cellular community 1149
Transport and catabolism 1486
Membrane transport 188
Signaling molecules and interaction 828
Signal transduction 2620
Folding, sorting and degradation 965
Replication and repair 272
Transcription 562
Translation 1179
Cancers: Overview 1906
Cancers: Specific types 683
Cardiovascular diseases 475
Endocrine and metabolic diseases 502
Immune diseases 229
Infectious diseases: Bacterial 1186
Infectious diseases: Parasitic 594
Infectious diseases: Viral 1242
Neurodegenerative diseases 827
Substance dependence 311
Amino acid metabolism 625
Biosynthesis of other secondary metabolites 11
Carbohydrate metabolism 859
Energy metabolism 305
Global and overview maps 2344
Glycan biosynthesis and metabolism 383
Lipid metabolism 714
Metabolism of cofactors and vitamins 384
Metabolism of other amino acids 217
Metabolism of terpenoids and polyketides 126
Nucleotide metabolism 487
Xenobiotics biodegradation and metabolism 242
Circulatory system 448
Development 632
Digestive system 1060
Endocrine system 1502
Environmental adaptation 198
Excretory system 246
Immune system 1160
Nervous system 732
Sensory system 266
1000
2000
3000
Number of Genes
Environmental Information Processing
Genetic Information Processing
Human Diseases
Metabolism
Organismal Systems

Figure 4(on next page)

Real-time qRT-PCR analysis of DEGs that encode respiration and detoxification-related enzymes in *T. confusum* after oil fumigation.

Gene expression (mean ± SE) was quantified as relative fold change by using the $2^{-\Delta\Delta CT}$ method. The asterisks indicate significant differences in the expression level of DEGs between the oil-treated and no-oil-treated samples (* p value < 0.05 and ** p value < 0.01).

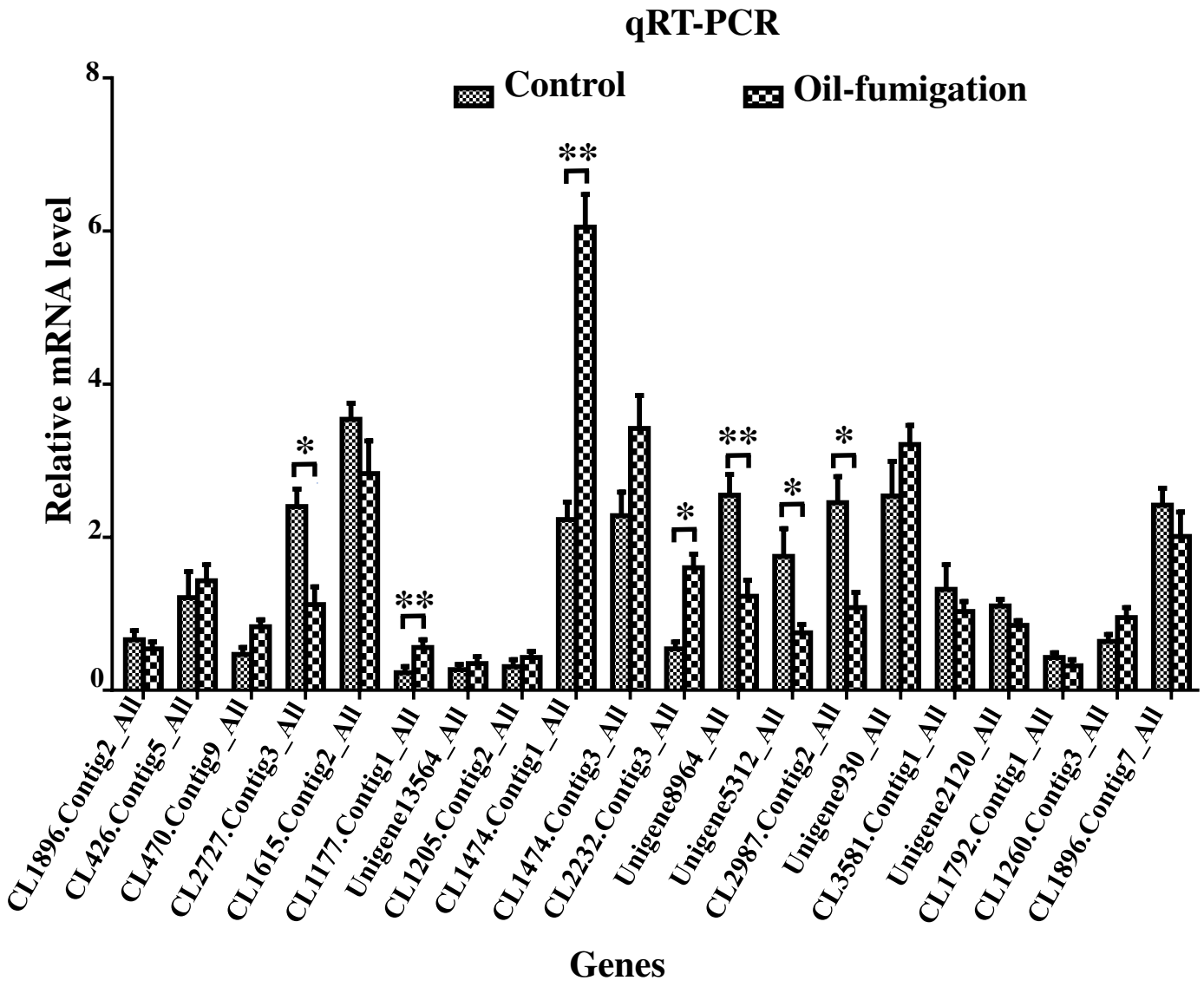
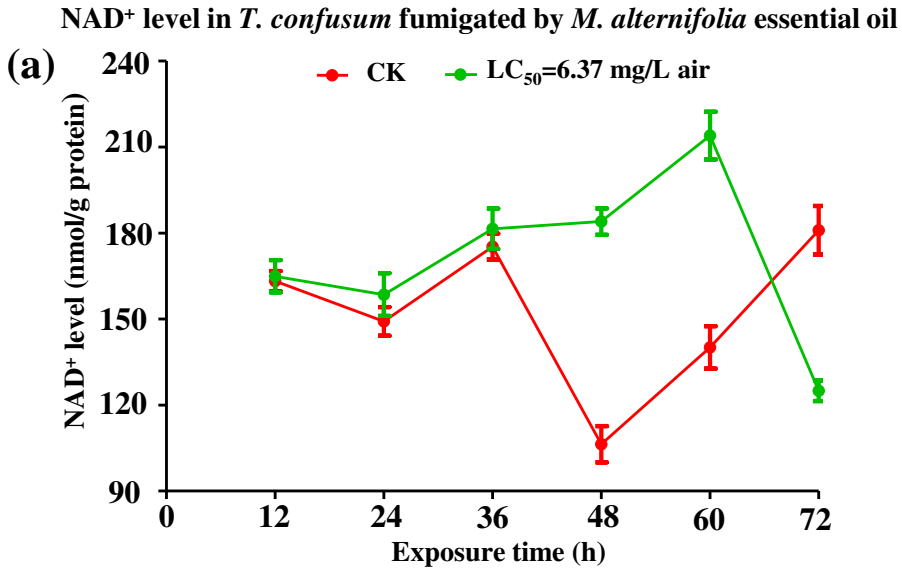
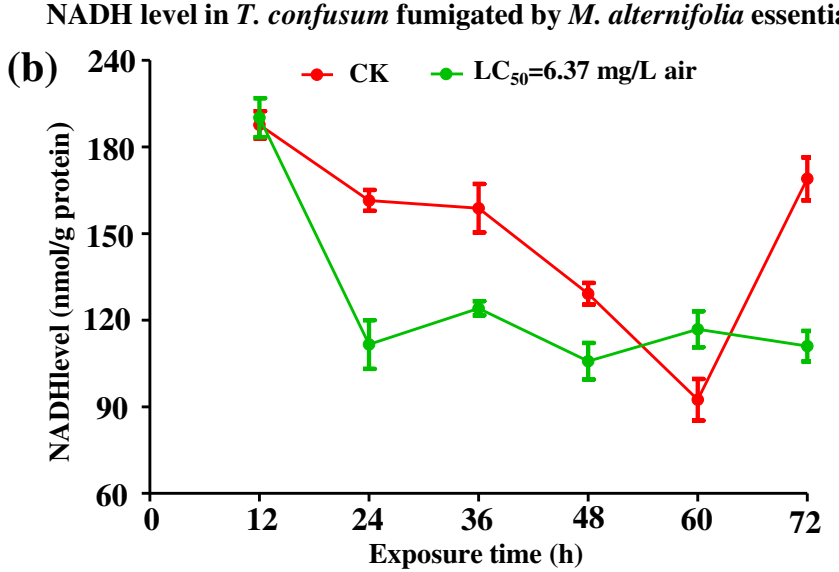

qRT-PCR
Control
Oil-fumigation
Relative mRNA level
8
6
4
2
0
**
*
**
*
**
*
*
CL1896.Contig2_All
CL426.Contig5_All
CL470.Contig9_All
CL2727.Contig3_All
CL1615.Contig2_All
CL1177.Contig1_All
Unigene13564_All
CL1205.Contig2_All
CL1474.Contig1_All
CL1474.Contig3_All
CL2232.Contig3_All
Unigene8964_All
Unigene5312_All
CL2987.Contig2_All
Unigene930_All
CL3581.Contig1_All
Unigene2120_All
CL1792.Contig1_All
CL1260.Contig3_All
CL1896.Contig7_All
Genes

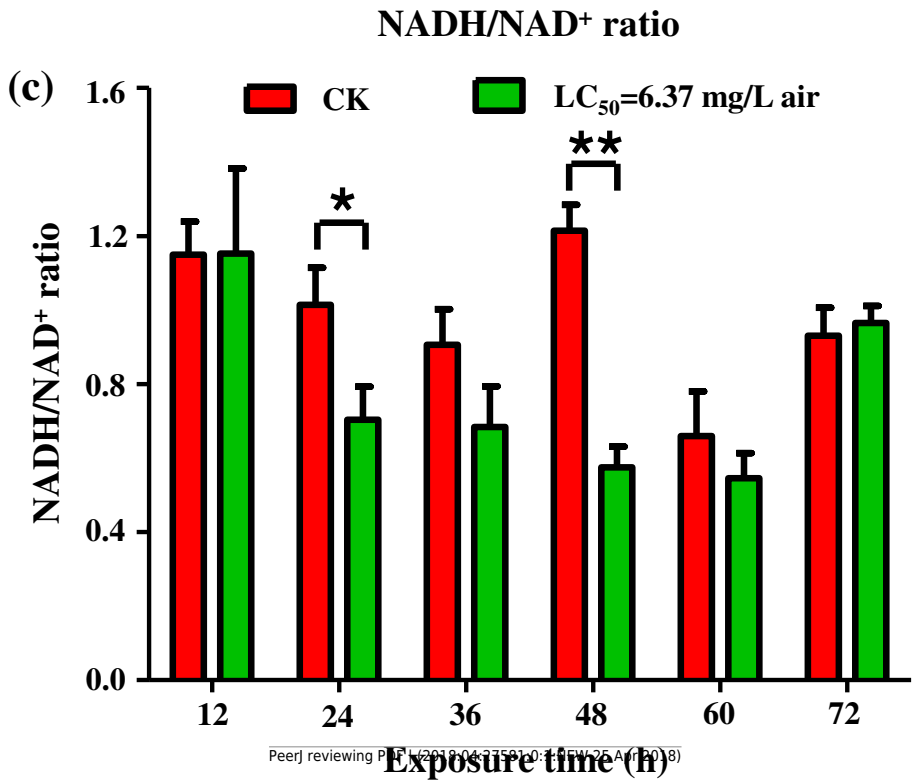
Figure 5(on next page)

NAD$^+$ (a) and NADH (b) in the control and oil-fumigated *T. confusum* extracts were quantified.

Optical density at 450 nm was recorded and used to calculate the NADH/NAD$^+$ ratio (c). Values (mean ± SE) are from three independent experiments: ($*$) $p < 0.05$ and ($**$) $p < 0.001$ for oil fumigation (LC_{50} = 6.37 mg/L air) versus control (CK).


NAD⁺ level in T. confusum fumigated by M. alternifolia essential oil
(a)
CK
LC50=6.37 mg/L air
NAD⁺ level (nmol/g protein)
240
210
180
150
120
90
0
12
24
36
48
60
72
Exposure time (h)


NADH level in T. confusum fumigated by M. alternifolia essential oil
(b)
CK
LC50=6.37 mg/L air
NADHlevel (nmol/g protein)
240
180
150
120
90
60
0
12
24
36
48
60
72
Exposure time (h)


NADH/NAD⁺ ratio
(c)
CK
LC50=6.37 mg/L air
*
**
NADH/NAD⁺ ratio
1.6
1.2
0.8
0.4
0.0
12
24
36
48
60
72
Exposure time (h)