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Ethyl formate and phosphine fumigations on the two-spotted spider mite, *Tetranychus urticae* and their biochemical responses

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Abstract

Two spotted spider mite, *Tetranychus urticae*, is a polyphagous pest to a variety of plants and they are hard to be controlled due to occurrence of resistance to acaricides. In this study, biochemical evaluation after ethyl formate (EF) and phosphine (PH_3) fumigation towards *T. urticae* might help officials to control them in quarantine purposes. PH_3 fumigation controlled eggs (LC_{50}; 0.158 mg/L), nymphs (LC_{50}; 0.030 mg/L), and adults (LC_{50}; 0.059 mg/L) of *T. urticae*, and EF effectively affected nymphs (LC_{50}; 2.826 mg/L) rather than eggs (LC_{50}; 6.797 mg/L) and adults (LC_{50}; 5.836 mg/L). In a longer exposure time of 20 h, PH_3 fumigation was 94.2-fold more effective tool for control of *T. urticae* than EF fumigant. EF and PH_3 inhibited cytochrome *c* oxidase (COX) activity differently in both nymphs and adults of *T. urticae*. It confirmed COX is one of target sites of these fumigants in *T. urticae* and COX is involved in the respiratory chain as complex IV. Molecular approaches showed that EF fumigation completely down-regulated the expression of *cox11* gene at the concentration of LC_{10} value, while PH_3 up-regulated several genes greater than twofold in *T. urticae* nymphs treated with the concentration of LC_{50} value. These increased genes by PH_3 fumigation are *ndufv1*, *atpB*, *para*, and *ace*, responsible for the expression of NADH dehydrogenase [ubiquinone] flavoprotein 1, ATP synthase, and acetylcholinesterase in insects, respectively. Lipidomic analyses exhibited a significant difference between two fumigants-exposed groups and the control, especially an ion with 815.46 m/z was analyzed less than twofold in the fumigants-treated group. It was identified as PI(15:1/18:3) and it may be used as a biomarker to EF and PH_3 toxicity. These findings may contribute to set an effective control strategy on *T. urticae* by methyl bromide alternatives such as EF and PH_3 because they have shared target sites on the respiratory chain in the pest.

Keywords: *Tetranychus urticae*, Ethyl formate, Phosphine, Fumigation, Gene expressions, Lipidomics

Introduction

Two spotted spider mites, *Tetranychus urticae*, is a polyphagous herbivore and quickly abundant in host plant fields because of short life span and some favorable growth conditions. They are one of major crop pests, which are caused loss of crop production and quality [1]. Therefore, a variety of control methods including natural acaricides are developed to reduce *T. urticae* attack on cultivated vegetables.

For the control of two-spotted mites, *T. urticae*, a variety of disinfection methods have introduced in the agricultural market. Promising control methods are employed predators as a biological control agent, attacking eggs, larva, pupae and adult stages of *T. urticae* [2–4]. Recently, a physical control, ultraviolet-B radiation was used to suppress population of *T. urticae* on strawberry in a greenhouse [5]. However, chemical control of *T. urticae* has been used worldwide and a variety of acaricides are introduced in the agricultural market [6].

In the control of *T. urticae*, developmental stage should be considered because different efficacy of each acaricide

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has been suggested [7]. Pokle and Shukla [7] tested seven acaricides such as wettable sulphur, fenazaquin, propargite, chlorfenapyr, diafenthiuron, triazophos, and fenpyroximate for measuring their hatching rates and mortalities on *T. urticae* under laboratory conditions. Among them, diafenthiuron suppressed strongly hatching rates (92.61%) and exhibited the highest mortality of 92.15% in mobile phase of *T. urticae*.

Other considerable issue in *T. urticae* disinfection is resistance occurred in *T. urticae* and it has been well documented against acaricides including bifenazate, cyenopyrafen, and SYP-9625 [8]. Interestingly, bifenazate shows strong inhibitory effect on complex III, while cyenopyrafen and SYP-9625 exhibit their inhibitory effects on complex II. Even field-evolved resistance in *T. urticae* against these three acaricides is low, cross-resistance between cyenopyrafen and SYP-9625 is clear, indicating that a strategy to control resistance should be developed using different mode of acaricidal action on the pest [8].

In trades, methyl bromide is still used to manage insect infestation, but there is a need to prepare alternatives to methyl bromide. Recently, Kim et al. [9] used phosphine (PH_3) gas for quarantine uses of imported plants and flowers to control *T. urticae* populations and they demonstrated PH_3 gas was considered an alternative for methyl bromide. Interestingly, Lee et al. [1] studied ethyl formate (EF) efficacy on *T. urticae* for imported sweet pumpkin, and they reported that EF fumigation for 4 h accomplished 100% mortality on both adults and eggs at the temperature of 10 °C. PH_3 fumigation was not effective to control *T. urticae*. They also treated EF with PH_3 to understand their synergistic effects to control *T. urticae*, but they did not observe synergism.

In other words, Lee et al. [1] reported *T. urticae* control using EF and 1-methylcyclopropene (MCP) for quarantine issues of exported sweet persimmons. As the previous report mentioned that EF fumigation is effective towards *T. urticae*, MCP needs to treat for anti-ethylene effect for post-harvest fruits [1]. After the concurrent application of these two different chemicals, complete control for *T. urticae* was obtained with delaying color changes and softening [1].

In this study, for improving efficacy of EF and PH_3 fumigation towards *T. urticae* and to proper understanding the mode of fumigation action, inhibitory effects on acetylcholinesterase (AChE), carboxylesterase (CE), glutathione-*S*-transferase (GST) activities, and cytochrome *c* oxidase (COX, equivalent for complex IV in the respiratory chain) activity after EF and PH_3 treatments were evaluated according to the developmental stages. Expression levels of six genes in *T. urticae* were also determined to understand effects of EF and PH_3 treatments based on the developmental stages. Finally, lipid biosynthesis was analyzed in *T. urticae* adults after the fumigant exposure to determine the effect on lipid profile. Through these studies, comparison of the efficacy between EF and PH_3 fumigation towards *T. urticae* was reported in relation to molecular effects on protein activities, gene expression, and lipid profiles.

Materials and methods

Chemicals

Phosphine (PH_3; ECO_2Fume^{TM}; 2% PH_3 + 98% CO_2) was obtained from Cytec (Sydney, Australia). Ethyl formate (EF; 97% purity), acetylthiocholine iodide (ATChI), bovine serum albumin (BSA), cytochrome *c*, and 5,5′-dithiobis (2-nitrobenzoic acid) were purchased from Sigma-Aldrich (St. Louis, Mo, USA). The DEPC-treated water was purchased from Biosesang (Seongnam, South Korea). The Maxima First Strand cDNA Synthesis Kit with dsDNase was purchased from Thermo Fisher Scientific (Waltham, MA, USA). TRIzol® Reagent was purchased from Ambion (Austin, TX, USA) and Luna® Universal qPCR Master Mix was purchased from New England Biolabs (Ipswich, MA, USA).

Insect strain and breeding

The two-spotted spider mite (*Tetranychus urticae*) was placed on kidney bean leaves (*Phaseolus vulgaris*), which were grown to a 5 to 7 cm leaf length, for feeding and breeding. Kidney beans were maintained in a glass greenhouse at 27 ± 2 °C and a relative humidity of 50%. The *T. urticae* were bred on kidney beans in a metal tray (36 × 32 × 5 cm) at 23 ± 2 °C and a relative humidity of 50%.

Fumigation bioassay of PH_3 and EF

A fumigation bioassay of PH_3 and EF was performed with *T. urticae* placed in 12-L desiccators (Duran, Mainz, Germany) for 20 h for PH_3 and 1 h for EF at a concentration of 0.01 to 1.0 mg/L and 20 °C. The *T. urticae* was classified according to their developmental stages (eggs, nymphs, adults), 30 *T. urticae* were used for each fumigation bioassay in triplicate. A 12-L desiccator was equipped with a lid fitted with a septum injection system (Alltech Crop Science, Nicholasville, KY, USA) and sealed with glass stoppers containing a septum of filter paper. The actual volume of each desiccator was measured by weighing the amount of water at 20 °C. A magnetic bar was placed at the bottom of each desiccator to stir the fumigant. The concentrations of the fumigants were monitored at time intervals and used to calculate the Ct (concentration × time) values using Eq. (1).

$$Ct = \sum (C_i + C_{i+1})(t_{i+1} - t_i)/2, \quad (1)$$

where C is the concentration of the fumigant (mg/L); t is the time of exposure (h); i is the order of measurement; and Ct is the concentration × time (mg h/L).

PH_3 and EF toxicity against *T. urticae* was described as the mortality of > 30 *T. urticae* of each developmental stages (eggs, nymphs, and adults) for at least three different Ct values. Ct values were calculated to 10% (Ct 10) and 50% mortality (Ct 50), as well as the time values for 10 and 50% mortality due to PH_3 and EF fumigation by the probit analysis using the SPSS statistics software (version 23.0).

Measurement of fumigant concentrations

To monitor the fumigation concentration in the 12-L desiccator, 50-mL gas samples were drawn with a syringe from the chamber and stored in 1-L Tedlar® gas sampling bags (SKC, Dorset, United Kingdom) and analyzed within 10 min of sampling. The concentration of PH_3 was monitored at 10 min and 1, 3, 6, and 20 h, while that of EF was monitored at 10, 30, and 60 min. The subsequent concentration was determined using an Agilent GC 7890A equipped with a flame photometric detector (FPD) and HP-PLOT/Q (30 m × 530 μm × 40 μm; Agilent, Santa Clara, CA, USA) operating in split mode (10:1). The concentrations of PH_3 and EF were calculated based on peak areas against external standards.

Protein extraction and enzyme activities

Each group of *T. urticae* after exposure to LC_{10} and LC_{50} of each fumigant was immediately frozen at −70 °C. *T. urticae* with leaf was washed with PBS buffer (pH 7.4) to separate *T. urticae* from the leaf and spin down briefly. Each group was homogenized with Tris-buffer containing 500 mM sucrose (pH 7.4) using pencil-type homogenizer. The homogenized solution was centrifuged at 600×*g* and 4 °C for 10 min to remove cell debris. The supernatant was centrifuged at 10,000×*g* and 4 °C for 15 min to collect mitochondria fraction. The supernatant and the precipitate were used for enzyme activities, respectively. Protein quantification was performed using the Bradford Assay with the Protein Assay Dye Reagent Concentrate (Bio-Rad, Hercules, CA, USA), and the protein standard curves were constructed using varying concentrations of bovine serum albumin (BSA) according to the manufacturer's recommendations. The supernatant was used for acetylcholinesterase (AChE), carboxylesterase (CE), and glutathione-*S*-transferase (GST) activities, while the pellet was resuspended and used for cytochrome *c* oxidase (COX) activity. Activities of AChE, CE, GST, and COX were determined using the methods reported previously by Ellman et al. [10], Mackness et al. [11], Habig and Jakoby [12], and Tyler and Nathanailides [13], respectively. Each assay was performed in triplicate. Enzyme activities were expressed as μmol/mg protein × min.

RNA extraction and RT-qPCR

Tetranychus urticae in each group were independently collected after exposure to LC_{10} and LC_{50} of each fumigant and frozen immediately at −70 °C. *T. urticae* were rinsed twice with DEPC-treated water and homogenized using pencil-type homogenizer with 1 mL of Trizol reagent. The total RNA was extracted according to the manufacturer's protocol. The quality of total RNA was determined by measuring its A260/280 nm ratio (1.8–2.0) and checked by agarose gel electrophoresis. The Complementary DNA (cDNA) was immediately synthesized using The Maxima First Strand cDNA Synthesis Kit with dsDNase (Thermo Fisher Scientific Inc., Waltham, MA) and stored at −20 °C. The qPCR was performed on a QuantStudio 3 Real-Time PCR System (Applied biosystems, Foster City, CA, USA) using Luna® Universal qPCR Master Mix (New England Biolabs, Ipswich, MA), according to the manufacturer's instructions. All qPCRs were performed in triplicate. The primers for *T. urticae* were designed using Primer-BLAST [14] and are listed in Table 1. The Ribosomal protein L13A and glyceraldehyde-3-phosphate were used to normalize the expression level of the gene of interest (GOI) and gene expression levels were expressed using the ΔΔCt method [15]. All experiments were independently performed in triplicate.

Lipidomics analysis after exposure to PH_3 and EF

Tetranychus urticae in each fumigant-treated group with the concentration of LC_{50} value were randomly collected and stored at −70 °C until the use. Each group was collected and added 500 μL methanol–chloroform (1:2, v/v) solution. The sample was homogenized using pencil-type homogenizer and shaken at room temperature for 20 min. After incubation, it was centrifuged at 2000 rpm for 10 min to remove the cell debris. The supernatant was added 200 μL of 0.9% sodium chloride and vortexed briefly. The mixture was centrifuged at 2000 rpm for 10 min to separate the two phases. The lower chloroform phase containing lipids was used for lipidomics analysis. For lipid profiling after exposure to fumigants in *T. urticae*, 1 μL of lipid extract was spotted on the MTP 384 ground steel target and allowed to dry completely at room temperature. Saturated amino acid solution (2-propanol/acetonitrile, 60:40, v/v) was used as a matrix solution. All samples were analyzed by MALDI-TOF MS and the results were confirmed by lipid mass analysis using a lipid database (http://www.lipidmaps.org).

Table 1 Primer list for the determination of gene expression level in *Tetranychus urticae* after ethyl formate and phosphine fumigation

Gene name	Symbol	Function	Size (bp)	Primer sequence (5′-3′)
Ribosomal protein L13A	*rpl13*	Housekeeping gene	104	F) GCTCACAGCCTATGAAGGTATT
				R) GAACTTACGACCTCCTTGTAATC
Glyceraldehyde-3-phosphate dehydrogenase	*gapdh*	Housekeeping gene	99	F) CGATGCGCCTATGTTTGTTATG
				R) GGAGCAAGACAGTTGGTTGTA
NADH dehydrogenase [ubiquinone] flavoprotein 1	*ndufv1*	Mitochondrial electron transport	105	F) CGATGAAGGAGAACCTGGCA
				R) CAGGCACCCATAGCCTTACC
Cytochrome *c* oxidase assembly protein COX11	*cox11*	Mitochondrial electron transport	101	F) TCAGTCCTGGGGAGACAACA
				R) AGCTTCGAACGGCAGAACAG
ATP synthase subunit beta	*atpB*	ATP synthesis	115	F) TTGCTGGTGTCGGAGAAAGA
				R) TGACCGTATACAAGGGCGAC
Voltage-gated sodium channel alpha subunit	*para*	Regulation of ion transmembrane transport	95	F) GCCGGTCTTGCCAAAATTCA
				R) ACCCTCATCCCTTTAGACCGA
Acetylcholinesterase-like	*ace*	Acetylcholine catabolic process	104	F) CGATGAAGGAGAACCTGGCA
				R) CAGGCACCCATAGCCTTACC
Septin-11-like	*sept11*	Cell cycle and division	107	F) CCGGACCATTAGAGAAGGC
				R) CATCGGGAAACTTGGTGTTGA

Statistic analysis

All data of enzyme assay and RT-qPCR are reported as mean ± standard deviation (SD). Statistical significance was determined by one-way analysis of variance (ANOVA) and Tukey's post hoc test using SPSS statistics version 23.0.

Results

Fumigation toxicities of EF and PH_3

Lethal concentrations (LCs) after EF and PH_3 fumigation towards *T. urticae* are expressed in Table 2. LC values were obtained based on the developmental stages including eggs, nymphs, and adults. For the EF fumigation, LC_{99} values were very similar up to about 10 mg/L, but LC_{50} values showed that nymphs were susceptible about 2.5 times lower in EF treatment in comparison to eggs and adults. For PH_3 treatment, eggs were much tolerable with at 2 times higher LC values than nymphs and adults. In this study, EF treatment needed 1 h exposure time towards *T. urticae*, while PH_3 needed 20 h exposure time. Therefore, higher concentrations of EF needs to control *T. urticae* populations because of lower exposure time when compared to PH_3.

Enzyme activities in *T. urticae* after fumigant treatments

Four enzyme activities in relation to two fumigant treatments were determined in *T. urticae*. In this experiment, eggs were not considered because they are too small to collect enough numbers to undertaken enzyme assays. In addition to this regard, enough numbers of samples at the lethal concentration to 99% of tested organisms (LC_{99}) values were not collected, thus LC_{10} and LC_{50} samples were obtained with the control group as shown in Fig. 1. In Fig. 1, COX expression in nymphs was 200

Table 2 Lethal concentrations (LCs) of *Tetranychus urticae* exposed to ethyl formate (EF) for 1 h and phosphine (PH_3) for 20 h

Fumigant	Stages	LC_X (mg/L, 95% CL[a])			Slope ± SE	*df*	χ^2
		LC_{10}	LC_{50}	LC_{99}			
EF	Egg	5.577 (4.189–6.327)	6.797 (5.803–7.349)	9.733 (9.068–11.149)	14.921 ± 2.202	6	0.02
	Nymph	1.364 (0.557–2.179)	2.826 (1.615–3.845)	10.606 (8.751–13.741)	4.051 ± 0.418	5	1.06
	Adult	4.317 (2.684–5.271)	5.836 (4.492–6.612)	10.088 (8.987–12.703)	9.789 ± 1.331	5	0.00
PH_3	Egg	0.112 (0.081–0.140)	0.158 (0.125–0.211)	0.298 (0.221–0.560)	8.452 ± 1.120	5	0.47
	Nymph	0.015 (0.006–0.025)	0.030 (0.016–0.042)	0.103 (0.086–0.125)	4.308 ± 0.446	5	0.23
	Adult	0.042 (0.022–0.058)	0.059 (0.037–0.075)	0.112 (0.096–0.123)	8.468 ± 0.942	5	0.60

CL: confidence limit

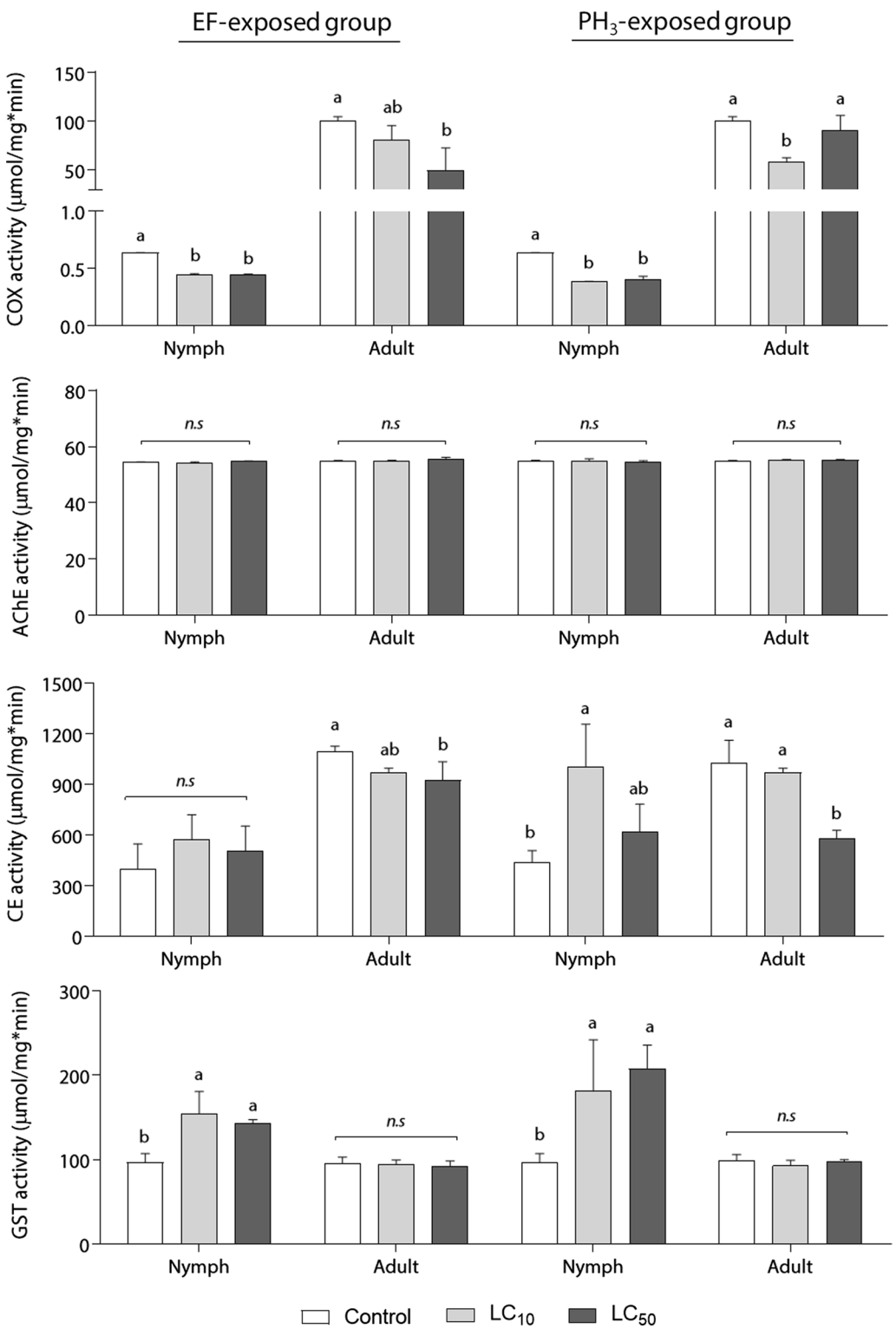

Fig. 1 Enzyme assays in the two different developmental stages of *T. urticae* (nymphs and adults) after ethyl formate (EF) and phosphine (PH_3) treatment, respectively. COX: cytochrome *c* oxidase; AChE: acetylcholinesterase; CE: carboxylesterase; GST: glutathione-*S*-transferase. The enzyme activities were expressed as μmol/mg min of the mite. Different letters on the bars indicate statistical differences between phosphine-treated samples and the control ($p < 0.05$). Treated concentrations were equivalent to LC_{10} (4.32 mg/L for EF; 0.042 mg/L for PH_3) and LC_{50} (5.84 mg/L for EF; 0.059 mg/L for PH_3) values

times lower than adults, and COX levels decreased significantly after EF and PH_3 fumigation. In adults, EF and PH_3 fumigation did not exhibit considerable inhibitory effects on COX enzyme. AChE enzymes mediate acetylcholine breakdown after neurological signaling, but their activities were not affected by the fumigation (Fig. 1).

CE and GST are exerted to prevent cells from xenobiotics such as pesticides. In this study, their levels after the fumigation were determined and PH_3 fumigation was more effective than EF fumigation within the two developmental stages of *T. urticae*. In nymphs, CE levels at the two different treated concentrations of PH_3 were elevated about twofold when compared to the control group, but in adults CE levels were decreased according to the increasing LC values. With GST activities, adults showed no changes after two fumigant treatments, while nymphs increased about twofold after PH_3 fumigation.

Gene expression after fumigant treatments

In EF treatments, levels of *cox11* expression COX enzyme in nymphs were significantly down-regulated about 10 times and about 2 times lower than the control group after the fumigation at the LC_{10} and LC_{50} concentrations, respectively. The levels of *para* and *ache* genes were 2 times down-regulated after EF fumigation at LC_{10} concentration, but they were recovered to the similar levels to the control (Fig. 2). Voltage-gated sodium channel and acetylcholinesterase are expressed by *para* and *ace* genes. There were no changes in both nymphs and adults after EF fumigation in the expression of *ndufv1* for NADH dehydrogenase [ubiquinone] flavoprotein 1, *atpB* for ATP synthase, and *sept11* for septin 11-like.

For PH_3 fumigation (Fig. 3), *T. urticae* adults exhibited less changes than nymphs, and the expression levels of *ndufv1*, *atpB*, *para*, and *ace* were significantly enhanced. Among them, para gene was dramatically increased in relation to PH_3 treatment at LC_{50} concentration.

With these results, para might be a possible target site of EF and PH_3 fumigation in *T. urticae* nymphs in conjunction with an inhibitory effect of COX activity.

Lipid profiles in adults after fumigation

Figure 4 shows lipid profiles in *T. urticae* adults after EF and PH_3 fumigation using MALDI-TOF MS/MS. Table 3 exhibits lipid list which was differently produced after fumigation with the matched chromatograms as Fig. 4a for control group, Fig. 4b for EF-fumigated group, and Fig. 4c for PH_3-fumigated group. In these chromatograms, two major peaks were differently produced after fumigation as m/z ratios of 815.46 and 859.54 as shown in Fig. 3d. The peak at 815.46 m/z was 3.4-fold decreased after PH_3 fumigation, while the peak at 859.54 m/z was

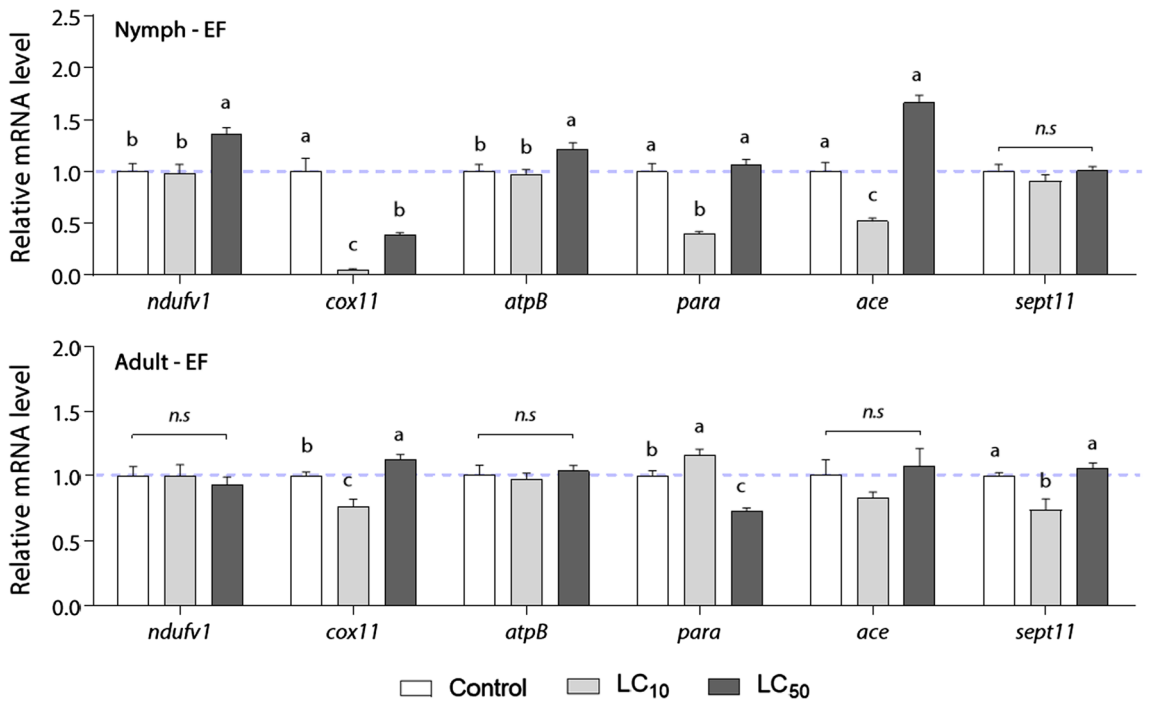

Fig. 2 Gene expressions in the ethyl formate (EF)-treated *T. urticae* determined by RT-qPCR. Six gene primers were listed in Table 1. Different letters on the bars indicate statistical differences between EF-treated samples and the control ($p < 0.05$). EF-treated concentrations were equivalent to LC_{10} (4.32 mg/L) and LC_{50} (5.84 mg/L) values. White, control group; gray, LC_{10} EF-treated *T. urticae*; black, LC_{50} EF-treated *T. urticae*

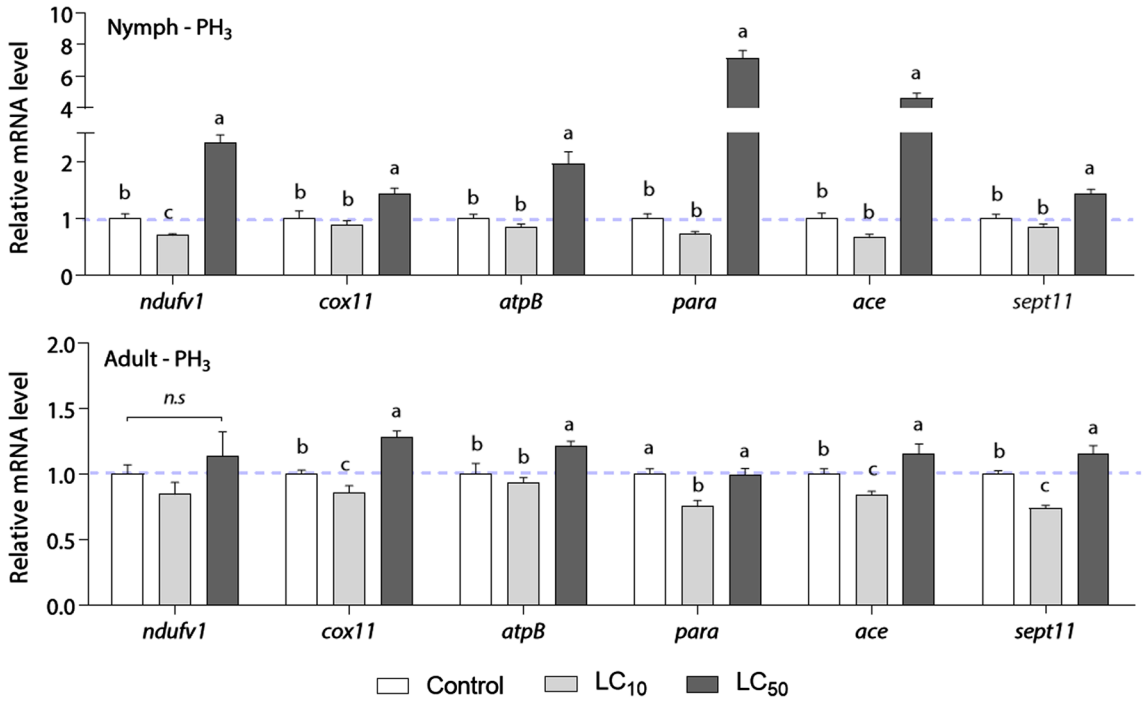

Fig. 3 Gene expressions in the phosphine (PH_3)-treated *T. urticae* determined by RT-qPCR. Six gene primers were listed in Table 1. Different letters on the bars indicate statistical differences between PH_3-treated samples and the control ($p < 0.05$). PH_3-treated concentrations were equivalent to LC_{10} (0.042 mg/L) and LC_{50} (0.059 mg/L) values. White, control group; gray, LC_{10} PH_3-treated *T. urticae*; black, LC_{50} PH_3-treated *T. urticae*

1.4-fold increased after PH_3 fumigation. They were phospholipids identified as PI(15:1/18:3) and PI(18:3/18:0) for 815.46 and 859.54 m/z, respectively. There are 19 phospholipids being differently produced in relation to EF and PH_3 fumigation in *T. urticae* (Table 3). PCA analysis determined a significant difference between the control group and these two fumigants treated groups Fig. 4e.

Discussion

EF and PH_3 are the representative fumigants replaced to methyl bromide in South Korea. These fumigants are used for controlling *Myzus persicae* and synergistically active when they are used together [9]. If used separately, EF needs high concentration in the disinfected area and PH_3 treatment needs longer exposure time up to 20 h (Table 2). Therefore, Kim et al. [9] suggested that low concentration of EF and shorter exposure period (4 h) for PH_3 should control insect pests in quarantine purposes. However, such treatments have been not perfectly applied to control *T. urticae* [1]. One of presumable reasons of this failure is the acaricide resistance developed in *T. urticae*. As mentioned previously, EF and PH_3 show their insecticidal target sites on the respiratory chain, especially complex IV known as cytochrome *c* oxidase (COX) [1, 16]. As shown in Fig. 1, COX activities in *T. urticae* nymphs decreased half by EF and PH_3 treatments. Even adults of *T. urticae* responded a little to the fumigation, COX activities were significantly reduced (Fig. 1). Similarly, after EF treatments, nymphs decreased *cox11* expression level (Fig. 2). With these results, COX might be the target site by these fumigants.

On the other hand, early adapted *T. urticae* by possessing resistance to respiratory inhibitors as acaricides can overcome EF and PH_3 attack to similar sites in respiratory chains of *T. urticae*. Many of newly introduced acaricides or currently used acaricides have target sites on mitochondrial respiration. For example, bifenazate inhibits complex III (ubiquinone–cytochrome *c* oxidoreductase), suppressing transportation of electrons in cells [17]. It has another target site on GABA-gated chloride channel of *T. urticae* [18]. Authors proved synergistic effect of bifenazate on the subunit of γ-aminobutyric acid (GABA) receptor as the addition of bifenazate at the concentration of 30 μM shifted the EC_{50} values of GABA on the receptor from 24.8 to 4.83 μM. In a recent study, several resistant mechanisms are developed in *T. urticae*, including mitochondrial complex III inhibitors such as bifenazate [19]. This study showed exceedingly 2000-fold of resistance against bifenzate in the resistant strains of *T. urticae* when compared to the susceptible strain, Wasatch strain. Similarly, resistance to bifenazate in *T. urticae* has been found in South Korea via nonsynonymous point

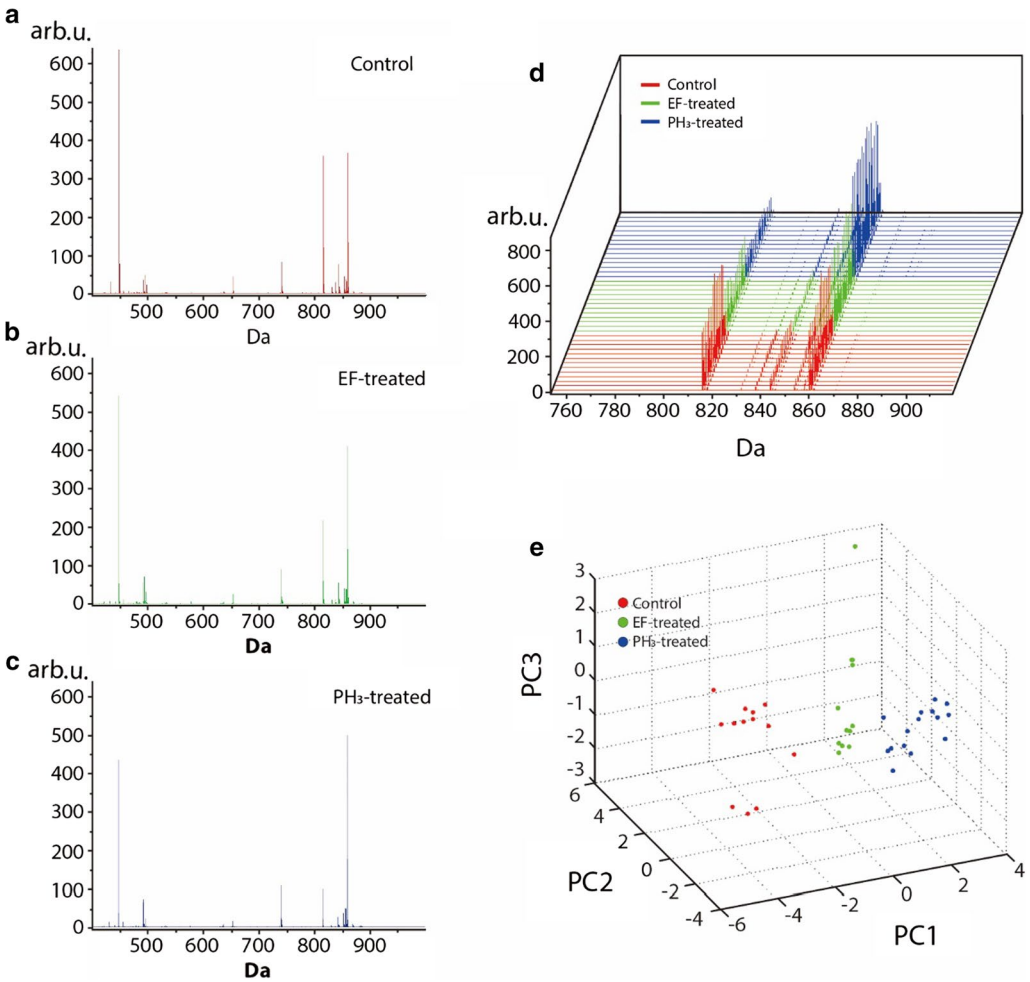

Fig. 4 MALDI-TOF MS/MS chromatograms of lipids of *Tetranychus urticae* adults after ethyl formate (EF) and phosphine (PH_3) fumigation. **a** Control group; **b** EF-treated group; **c** PH_3-treated group. **d** Indicates two major peaks as 815.46 and 859.54 m/z, which are differently produced according to fumigant treatments. **e** Shows PCA analysis on peaks in the chromatogram with the fumigant treatment

mutations [20]. Resistance occurrence in *T. urticae* towards acaricides can change the suppression by EF and PH_3 treatments due to possible modification of electron transport potential in respiratory chains.

In detoxification reaction to acaricides, nine field *Panonychus citri* populations showed that high correlation coefficient ($r = 0.93$) between GST activity and LC_{50} of pyridaben [21]. Some of these field strains possessed enhanced levels of GST activities ranged from 2.5 to 11.6 in relation to resistance to pyridaben when compared to the pyridaben susceptible strain. Therefore, authors demonstrated increased GST activities might be related to the pyridaben resistance in the mite [21]. In our study, GST activities in nymphs were enhanced at least twofold after PH_3 treatment (Fig. 1). Therefore, *T. urticae* exhibits similar detoxification process to remove EF and PH_3 toxicities as well as pyridaben treatment. Inclusion of CE proteins is not easily described in relation to detoxifying EF and PH_3 fumigation because of decreased levels of protein activities in adults (Fig. 1).

In this study, PH_3 treatments changed expression levels of *ndufv1*, *para*, and *ace* (Fig. 3), responsible for expressing NADH dehydrogenase [ubiquinone] flavoprotein 1 (complex I), voltage-gated sodium channel alpha subunit, and acetylcholinesterase-like (Table 1). This finding may suggest that PH_3 toxicity is related to lower complex I and neuronal toxicity as pyridaben toxicity previously reported [22]. However, in protein level, acetylcholinesterase was not changed after EF and PH_3 treatments in our study (Fig. 1). Similar to our findings, pyridaben and fenazaquin show their inhibitory effects on the complex I in the respiratory chain, NADH–ubiquinone oxidoreductase [22]. Recently, pyridaben has altered mitochondrial dynamics via reduced mitochondrial length and

Table 3 Phospholipids identified from *Tetranychus urticae* adults after ethyl formate (EF) and phosphine gas (PH_3) fumigation

No	Determined m/z	PWKW	Control group	EF-treated group	PH_3-treated group	Identified lipid
1	431.12	0.00000169	31.01	7.62	15.22	LPA(18:3)
2	447.1	0.00459	649.33	475.41	445.21	LPA(O-20:2)
3	491.29	0.00000917	38.64	55.62	67.9	PA(21:1)
4	493.31	0.0000909	51.42	71.16	77.82	PA(21:0)
5	738.48	0.0178	2.55	2.83	3.54	PE(36:4)
6	815.46	< 0.000001	382.91	230.28	111.53	PI(15:1/18:3)
7	831.47	< 0.000001	18.43	10.58	5.84	PI(16:0/18:3)
8	837.44	< 0.000001	32.28	12.63	3.94	PI(34:0)
9	843.49	< 0.000001	84.53	57.94	30.82	PI(17:1/18:3)
10	857.5	0.0000137	40.56	42.21	59.94	PI(36:4)
11	859.54	0.00000188	387.49	437.87	557.02	PI(18:3/18:0)
12	870.56	0.000734	7.18	6.21	9.77	PI(37:4)
13	872.55	0.0000998	0.99	1.12	1.28	PI(37:3)
14	881.52	< 0.000001	1.94	2.3	4.97	PI(38:6)
15	883.53	0.0000087	1.24	1.41	2.04	PI(38:5)
16	885.54	0.0000475	2.4	2.46	3.37	PI(18:1/20:3)
17	887.55	0.000138	1.42	1.51	1.87	PI(38:3)

circularity in a rat dopaminergic neuronal cells (N27 cells) [23]. This report also revealed that pyridaben induced production of reactive oxygen species, which might be related to pesticidal activity. *T. urticae* in South Korea have developed resistance against pyridaben (resistance ratio = 240) via involvement of a mixed function oxidase [24]. With these previously reported findings, early adaption in *T. urticae* against currently used acaricides can interfere proper control by EF and PH_3 as they can share the target site like complex I in the respiratory chain.

These findings are so important to revisit acaricide resistance in *T. urticae* in quarantine disinfestation process. In quarantine issues, chemical treatments provide complete disinfection of *T. urticae* in imported and exported plant products, including plants. In addition to acaricide resistance, use of methyl bromide for quarantine purposes is still acceptable for a set period of time, but alternatives should be prepared. In this regard, several researchers in South Korea have developed alternative fumigation chemicals to methyl bromide to disinfect insect pests in trades [9, 25].

Lipidomic results in this study are very useful to detect EF and PH_3 toxicities as lipid profiles are significantly different from the control group (Fig. 3a–c). In our study, 815.46 m/z ion was dramatically reduced after PH_3 treatment, even EF-treatment affected about 1.7-fold reduction in *M. persicae* (Table 3). It might be used as a biomarker lipid to understand PH_3 toxicity to *T. urticae*. In similar, EF-treated *M. persicae* showed 23 different lipid generation when compared to non-treated *M. persicae* [26]. Among the differently produced lipids, M(IP)2C(d35:1) level in the EF-treated group was 3 times higher than the control group [26]. However, there is no report for comparing lipid profile in insect pests after the PH_3 treatment. PH_3 treatment can generate reactive oxygen species (ROS) because it is a byproduct of respiratory chain [27]. It will react with cellular molecules as lipids finally leading to death [28]. With these findings, EF and PH_3 treatments can result in modification of lipid profiles and failure of proper lipid functions.

Taken together, EF and PH_3 fumigation to control *T. urticae* may be harder because of acaricidal resistance developed in field strains, which target sites are similar to these currently used fumigants, especially on the respiratory chain. Therefore, these biochemical studies might effectively produce a control strategy towards *T. urticae* with an expectation of their defense systems and synergistically available fumigants could be considered with a different mode of fumigation action on *T. urticae* such as naturally occurring chemicals, monoterpenes and volatiles [29–31].

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Authors' contributions
J-OY and S-EL designed experiments as well as wrote the manuscript. KK, YHL, and GK conducted the experiments. KK, B-HL, J-OY, and S-EL conducted result

analysis and interpretation. KK and S-EL inspired the overall work and revised the final manuscript. All authors read and approved the final manuscript.

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Availability of data and materials
The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests
The authors declare that they have no competing interests.

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