Article

Synergistic Effects and Toxic Mechanism of Phosphine with Ethyl Formate against Citrus Mealybug (*Planococcus citri*)

Kyeongnam Kim ^{1,†}, Min-Goo Park ^{2,†}, Yong Ho Lee ³, Hwang-Ju Jeon ¹, Tae Hyung Kwon ⁴, Chaeun Kim ⁵, Jungeun Park ⁵, Byung-Ho Lee ⁶, Jeong Oh Yang ^{7,‡} and Sung-Eun Lee ^{1,5,*,‡}

¹ Department of Applied Biosciences, Kyungpook National University, Daegu 41566, Korea; kn1188@knu.ac.kr (K.K.); jeonhj@knu.ac.kr (H.-J.J.)

² Plant Quarantine Technology Center, Animal and Plant Quarantine Agency, Gimcheon 39660, Korea; pmg@korea.kr

³ Institute of Ecological Phytochemistry, Hankyong National University, Ansong 17579, Korea; yonghoyongho@hanmail.net

⁴ Division of Applied Life Science (BK21+Program), Institute of Life Science, Gyeongsang National University, Jinju 52828, Korea; xoxogudgud@naver.com

⁵ Department of Integrative Biology, Kyungpook National University, Daegu 41566, Korea; dkrkek01@knu.ac.kr (C.K.); pjestj211@knu.ac.kr (J.P.)

⁶ Institute of Agriculture and Life Science, Gyeongsang National University, Jinju 52828, Korea; byungholee@gnu.ac.kr

⁷ Yeongnam Regional Office, Animal and Plant Quarantine Agency, Busan 48943, Korea; joyang12@korea.kr

* Correspondence: selpest@knu.ac.kr

† These authors contributed equally to this paper as first author.

‡ The authors equally contributed to this paper as corresponding authors.

Citation: Kim, K.; Park, M.-G.; Lee, Y.H.; Jeon, H.-J.; Kwon, T.H.; Kim, C.; Park, J.; Lee, B.-H.; Yang, J.O.; Lee, S.-E. Synergistic Effects and Toxic Mechanism of Phosphine with Ethyl Formate against Citrus Mealybug (*Planococcus citri*). *Appl. Sci.* **2021**, *11*, 9877. <https://doi.org/10.3390/app11219877>

Academic Editor:
Alessandra Durazzo

Received: 21 August 2021

Accepted: 19 October 2021

Published: 22 October 2021

Publisher's Note: MDPI stays neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Copyright: © 2021 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (<https://creativecommons.org/licenses/by/4.0/>).

Featured Application: The objective of the work is to assess alternatives to methyl bromide such as ethyl formate and phosphine for the control of insect pests in the quarantine process.

Abstract: Methyl bromide (MB) has been used in a wide range of applications, but since it was determined to be an ozone-depleting compound, it has only been used for pre-shipment and quarantine purposes in trade. Phosphine (PH₃) is currently the ideal fumigant as an MB alternative worldwide. However, the development of PH₃ resistance in the target insect pest and longer PH₃ fumigation treatment times raise questions about the continued use of PH₃. This study attempted to shorten treatment time via combination treatment with ethyl formate (EF). *Planococcus citri* was used as the main quarantine pest in Korea, and the acute toxicity of EF, PH₃, and EF + PH₃ was determined at every developmental stage. EF treatment at 4 h showed LCT₉₉ values of 45.85–65.43 mg·h/L, and PH₃ treatment at 20 h showed that of 0.13–0.83 mg·h/L depending on the developmental stage. The efficacy of PH₃ decreased after reducing the treatment time, but synergistic effects were observed at all stages of development of *P. citri* when both fumigants were used simultaneously for 4 h. After combined treatment, dihydrolipoyl dehydrogenase expression and the production of two phospholipids, PI(O-16:0) and PC(18:2), were significantly reduced in treated *P. citri* adults compared with the control. Therefore, combined treatments might be key to reducing the treatment time and resistance of PH₃ in the field.

Keywords: quarantine pest; methyl bromide alternatives; anti-resistance strategy; dihydrolipoyl dehydrogenase; phospholipids

1. Introduction

When importing and exporting grains, fruits, vegetables, and plants in the trade between countries, fumigation agents are normally used against quarantine pests [1–3]. Methyl bromide (MB) is the most versatile, and it has an excellent control efficacy against various insect pests, fungi, weeds, and nematodes [4,5]. However, MB has been confirmed

to contribute to ozone layer depletion, a major cause of global warming; therefore, its use has been banned and phased out from the market [6]. Although MB is still used in the quarantine and pre-shipment stages during trade, alternative fumigants are urgently needed as its use may be wholly prohibited soon [1,3,7].

Many alternatives to MB have been tested, from physical control (e.g., heat, cold, and aridity), fumigant alternatives (e.g., phosphine, carbonyl sulfide, sulfuryl fluoride, carbon dioxide, ethyl or methyl formate, and so on), contact insecticides, and combination treatments (e.g., fumigation with low temperature, multiple fumigants) [8]. Based on previous studies of alternatives to MB, phosphine (PH_3) is the most ideal fumigant in the world, but it has several drawbacks, including slow activity and increased insect resistance [8]. The emergence of PH_3 resistance is a serious problem worldwide, especially in stored-grain insect pests [9,10]. High resistance factors in the range of 60- to 600-fold based on median lethal concentration (LC_{50}) values were reported in major stored-grain pests [11]. Therefore, studies on PH_3 -related resistance mechanisms, discovering the optimum concentration of PH_3 for pest fumigation, and the development of combination fumigation methods with PH_3 and EF have been widely conducted [11–13]. These various trials for solving the resistance problem are essential for prompt pest quarantine applications of PH_3 .

Ethyl formate (EF) is a natural substance with relatively low toxicity to animals [14,15] and has fast-acting properties similar to MB [16]. The resistance of insect pests to EF has not been reported, but EF has a relatively higher application concentration than PH_3 [1]. There is currently no single alternative to MB's broad range of activities and benefits [8]. Therefore, the evaluation of various combination treatments and integrated pest management methods is required, along with toxicity mechanism studies to completely replace MB.

The combination treatment of two fumigants has been reported from a variety of applications due to the complementary features; EF acts quickly at high doses and PH_3 acts slowly at low doses [7,17,18]. To apply the combinational application in quarantine, pineapples and sweet pumpkins were used for controlling two-spotted spider mites and citrus mealybugs in Korea [18,19]. *Planococcus citri* Risso (Hemiptera: Pseudococcidae) has been considered an economically important insect pest of various crops worldwide [20,21]. In particular, it is registered in each country as a pest to be controlled in plant quarantine; thus, quarantine management should be systematically established for EF and PH_3 fumigation. However, an understanding of the toxicity mechanism for EF and PH_3 combination treatment against *P. citri* has not been sufficiently studied.

Thus, this study aimed to evaluate the efficacy of EF and PH_3 treatments (alone and in combination) on all developmental stages of *P. citri*. The mode of action of combination treatment of EF and PH_3 was verified using biochemical and molecular studies, including proteomics and lipidomics techniques.

2. Materials and Methods

2.1. Insects and Breeding Conditions

Stock colonies of *P. citri* were provided by Professor Gil-Hah Kim from Chungbuk National University (Cheongju, Korea) in 2018. *P. citri* was continuously reared on potatoes provided twice a month, at 25 ± 1 °C with 60% relative humidity and a photoperiod of 16:8 h (light:dark) at the Plant Quarantine Technology Center, Animal and Plant Quarantine Agency (Gimcheon, Korea).

2.2. Chemicals

EF (97% purity, analytical grade) was purchased from Sigma-Aldrich (St. Louis, MO), and phosphine (PH_3 , 2% PH_3 + 98% CO_2) was obtained as $\text{ECO}_2\text{Fume}^{\text{TM}}$ from Cytec (Sydney, Australia). For enzyme assays, acetylthiocholine iodide (ATChI), bovine serum albumin (BSA), 1-chloro-2,4-dinitrobenzene (CDNB), cytochrome c, 5,5'-dithio-bis-(2-nitrobenzoic acid), Fast Blue B salt, reduced L-glutathione, and 1-naphthyl acetate (α -NA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). For the gene expression experiment, DEPC-treated water was purchased from Biosesang (Seongnam, Korea). TRIzol[®]

reagent was purchased from Ambion (Austin, TX, USA), and Maxima First Strand cDNA Synthesis Kits with dsDNase were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Luna[®] Universal qPCR Master Mix was purchased from New England Biolabs (Ipswich, MA, USA).

2.3. Fumigation Bioassay and Calculation of Lethal Concentration-Time (LCT)

For each developmental stage, thirty *P. citri* individuals were used in triplicate and placed in 12 L desiccators (Duran, Germany) sealed with glass stoppers containing a septum of filter paper for bioassay of each fumigant. The concentration of fumigant was monitored at timed intervals over the exposure period using an Agilent GC 7890A with each detector. The analysis conditions for each fumigant were as below.

- EF: EF fumigation was performed in a concentration range of 1.5 to 40.57 mg/L for 4 h. Gas was sampled at 0.5, 1, 2, and 4 h post fumigation and analyzed using a flame ionization detector.
- PH₃: PH₃ fumigation was performed in a concentration range of 0.005 to 0.102 mg/L for 20 h. Gas was sampled at 0.5, 1, 2, 4, 18, and 20 h post fumigation and analyzed using a flame photometric detector.

The lethal concentration × time (LCT, mg·h/L) value of each fumigant toward *P. citri* was calculated using Equation (1) [22]. The data represent an average of CT and SE (standard error) based on a probit analysis using IBM SPSS Statistics for Windows version 23.0 (IBM Corp, Armonk, NY). The LCT values were converted to lethal concentration (LC_x, mg/L) values to compare biological responses after exposure to each fumigant (Table S1).

$$LCT = \Sigma(C_i + C_{i+1})(t_{i+1} - t_i)/2 \quad (1)$$

where 'C' is the fumigant concentration (mg/L), 't' is the exposure time (h), and 'i' is the order of measurement.

2.4. Synergistic Effect of EF and PH₃

The exposure time of the combination treatment was set for 4 h to evaluate the synergistic effect of EF and PH₃ by correcting the different exposure times of the two fumigants. Therefore, in the case of PH₃, bioassays were additionally performed on a 4 h basis at 20 °C. Synergistic ratios were evaluated using Equation (2) according to the previously reported method [23].

$$SR = (EF + PH_3 \text{ mortality}) / (EF \text{ mortality} + PH_3 \text{ mortality}) \quad (2)$$

where SR = 1 describes additive action, SR < 1 describes antagonism, and SR > 1 describes synergism.

2.5. RNA Extraction and Real-Time Quantitative PCR (RT-qPCR)

P. citri was exposed to the LC₁₀ and LC₅₀ of each fumigant for 4 h (EF) or 20 h (PH₃) for each *P. citri* developmental stage; then, *P. citri* was collected independently and stored at −70 °C. The sample was rinsed twice with DEPC-treated water, homogenized with 1 mL of TRIzol reagent, and frozen at −70 °C for 1 day. The frozen homogenates were incubated on ice for 20 min, and total RNA was extracted according to the manufacturer's protocol. The RNA quality was determined by checking the ratio of absorbance (260 nm/280 nm) and the images of agarose gel electrophoresis. Primers were designed using Primer-BLAST [24] to compare the gene expression response of fumigants; this information is listed in Table S2. The qPCR was performed in triplicate using a QuantStudio 3 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) with Luna[®] Universal qPCR Master Mix (New England Biolabs, Ipswich, MA), according to the manufacturer's instructions. Cycling conditions were as follows: 95 °C for 10 min; followed by 40 cycles of 95 °C for 10 s, 60 °C for 15 s, and 72 °C for 20 s; then gradual heating from 72 °C to 95 °C. The results were

calculated using the $2^{-\Delta\Delta C_t}$ method [25], followed by one-way ANOVA with post hoc Tukey's tests in IBM SPSS Statistics for Windows version 23.0 (IBM Corp.).

2.6. Protein Extraction and Enzyme Assay

P. citri was exposed to the LC₁₀ and LC₅₀ of each fumigant for each developmental stage; then, *P. citri* was collected independently and stored at $-70\text{ }^{\circ}\text{C}$ and was homogenized with Tris buffer containing 500 mM sucrose (pH 7.4) and centrifuged at $600\times g$ at $4\text{ }^{\circ}\text{C}$ for 10 min. The supernatant was centrifuged at $10,000\times g$ at $4\text{ }^{\circ}\text{C}$ for 15 min to collect the pellet as the 'mitochondria fraction' for cytochrome c oxidase (COX) enzyme assay. The supernatant as cytosolic fraction was used for acetylcholinesterase (AChE), carboxylesterase (CE), and glutathione-S-transferase (GST) enzyme activities. Proteins were quantified using the Bradford assay [26], and bovine serum albumin was used as the standard. Each enzyme assay was conducted according to the previously reported methods [27–30]. The enzyme activities were normalized using protein concentration, and the normalized results were analyzed for mean comparison by one-way ANOVA with post hoc Tukey's tests in IBM SPSS Statistics for Windows version 23.0 (IBM Corp.). A heatmap was constructed using Log2 fold change between the control and treated groups.

2.7. Proteomic Analysis

Adults of *P. citri* were exposed to different combinations (EF only, 10 mg/L; PH₃ only, 0.1 g/m³, combined treatment of EF and PH₃) for 4 h. Then, 20 individuals were collected regardless of whether they were alive or dead. The samples were stored at $-70\text{ }^{\circ}\text{C}$ until they were used for proteomic and lipidomic analyses. Proteins for proteomic analysis were extracted using the same procedure for enzyme assay. The proteins were denatured for 3 h at room temperature using 50 mM ammonium bicarbonate buffer (pH 7.8) containing 6 M urea. They were then incubated with 10 mM dithiothreitol for 2 h at room temperature to reduce their disulfide bonds. The reduced protein extracts were reacted with iodoacetamide (IAA) for 1 h and then with trypsin at $37\text{ }^{\circ}\text{C}$ for 18 h. Proteomic analysis of *P. citri* samples was performed using a Thermo Scientific Q Exactive Hybrid Quadrupole-Orbitrap (Thermo Fisher Scientific Inc., Waltham, MA, USA) with a Dionex U 3000 RSLC nano high-performance liquid chromatography system. The mass data were matched with the *P. citri*, mosquitoes *Anopheles* sp., and fruit flies *Drosophila* sp. protein databases, and statistical analysis was performed based on the ANOVA test using the Scaffold [31] version 4.8.4 (Proteome Software, Portland, OR, USA).

2.8. Lipid Extraction and Lipidomics Analysis Using MALDI-TOF MS

Total lipids were extracted following a slightly modified version of the Folch method [32]. Briefly, *P. citri* was homogenized using a pencil-type homogenizer with 500 μL methanol-chloroform (1:2, *v/v*) solution. The homogenate was shaken at room temperature for 20 min and centrifuged at 2000 rpm for 10 min. The supernatant was transferred; then, 200 μL of 0.9% sodium chloride was added, and the mixture was vortexed for 10 s. The mixture was incubated for 5 min and centrifuged at 2000 rpm for 10 min. The lower chloroform phase containing lipids was used for lipidomic analysis using a matrix-assisted laser desorption ionization time-of-flight mass spectrometer (MALDI-TOF MS; Voyager-DE™ STR Workstation, Applied Biosystems, Foster City, CA). The results were statistically analyzed with the Kruskal–Wallis test and the cut-off value set as *p*-value < 0.001 using IBM SPSS Statistics for Windows version 23.0 (IBM Corp.). A heatmap was constructed using the Log2fold change between the control and treated groups. The mass results of lipids were matched using negative ion mode ($[\text{M}-\text{H}]^{-}$) in the LIPID MAPS (accessed on 18 July 2021 at www.lipidmaps.org). The whole scheme of the methodology used in this study is summarized in Figure 1.

Figure 1. A graphical scheme of the study approach.

3. Results

3.1. Individual Efficacy of EF and PH₃ against *P. citri*

The 10%, 25%, 50%, and 99% LCT values of EF for 4 h were 9.49, 13.13, 18.84, and 65.43 mg·h/L for the egg stage; 6.83, 9.41, 13.44, and 45.85 mg·h/L for the nymph stage; and 14.06, 17.23, 21.57, and 46.90 mg·h/L for the adult stage, respectively (Table 1). The 10%, 25%, 50%, and 99% LCT values of PH₃ for 20 h were 0.20, 0.26, 0.33, and 0.83 mg·h/L for the eggs; 0.07, 0.07, 0.08, and 0.13 mg·h/L for the nymph stage; and 0.14, 0.17, 0.20, and 0.36 mg·h/L for the adult stage, respectively (Table 1). The relative sensitivity of the developmental stages to two fumigants was high in nymphs and adults, but low in eggs. The 4 h LCT values of PH₃ for the egg stage were not calculated because it represented a 60% mortality at the maximum applicable concentration of PH₃, 4 mg/L (Table S3). The 10%, 25%, 50%, and 99% LCT values of PH₃ for the nymph and adult stages were 0.14, 0.25, 0.36, and 1.32 mg·h/L (Table 1).

Table 1. Lethal concentration time (LCT) of *P. citri* in adult, nymph, and egg stages exposed to ethyl formate (EF) or phosphine (PH₃) for 4 h or 20 h at 20 ± 1 °C (*n* = 30, triplicate).

Fumigant	Time (h)	Stages	LCTx (mg·h/L, 95% CL ^a)				Slope ± SE	df	χ ²
			LCT ₁₀	LCT ₂₅	LCT ₅₀	LCT ₉₉			
EF	4	Egg	9.49 (5.24–12.78)	13.13 (8.63–16.62)	18.84 (14.43–23.12)	65.43 (46.43–132.60)	4.30 ± 0.52	5	8.29
		Nymph	6.83 (0.23–12.05)	9.41 (0.82–15.05)	13.44 (3.09–20.33)	45.85 (28.23–627.17)	4.37 ± 1.28	7	13.77
		Adult	14.06 (6.91–18.03)	17.23 (10.39–20.91)	21.57 (15.98–25.19)	46.90 (37.04–91.26)	6.90 ± 1.13	5	0.44
PH ₃	20	Egg	0.20 (0.14–0.25)	0.26 (0.20–0.31)	0.33 (0.28–0.42)	0.83 (0.60–1.71)	5.92 ± 0.77	5	2.55
		Nymph	0.07 (0.01–0.11)	0.07 (0.01–0.12)	0.08 (0.02–0.13)	0.13 (0.05–0.18)	12.67 ± 2.15	5	0.00
		Adult	0.14 (0.07–0.20)	0.17 (0.09–0.22)	0.20 (0.12–0.25)	0.36 (0.29–0.41)	8.95 ± 0.97	5	0.10
PH ₃	4	Egg	- ^b	-	-	-	-	-	-
		Nymph and Adult	0.14 (0.10–0.16)	0.25 (0.21–0.28)	0.36 (0.32–0.40)	1.32 (1.05–1.82)	4.12 ± 0.42	22	9.01

^a CL denotes the confidence limit. ^b Not analyzed. Details in Supplementary Materials Table S3.

3.2. Enzymatic Responses at Each Stage of Development by Individual Treatment of EF and PH₃

In the case of EF fumigation, AChE activity was not significantly affected at any of the developmental stages (Figure 2a); however, COX activity was observed to decrease during the LC₅₀ treatment of the egg stage, whereas it was measured to increase more than two-fold during the nymph stage (Figure 2a). EF fumigation decreased GST activity in the adult stage only with the LC₁₀ treatment, while it did not change significantly through other developmental stages, including the adult stage with the LC₅₀ treatment (Figure 2a).

Figure 2. Heatmap of enzyme activities after exposure to ethyl formate (EF) for 4 h or phosphine (PH₃) for 20 h on *P. citri* (adults, nymphs, and eggs). Responses of four different enzymes, acetylcholinesterase (AChE), cytochrome c oxidase (COX), glutathione S-transferase (GST), and carboxylesterase (CE), after exposure to lethal concentration LC₁₀ and LC₅₀ values of EF (a) and PH₃ (b). A heatmap was constructed using the Log₂ enzyme activity ratio between the control and treatment groups. Asterisks indicate significant differences (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$) and no mark means 'not significant'.

CE activity significantly decreased by more than two-fold in eggs at the two different treatments and increased three-fold in the EF-treated nymphs treated with the LC₅₀ value (Figure 2a). Therefore, COX and CE activities in eggs may be susceptible to EF fumigation, whereas these two enzyme activities were dramatically elevated in the nymph stage, especially at the LC₅₀ treatment.

In the case of PH₃ fumigation, AChE activity was not dramatically affected at any developmental stage (Figure 2b). However, COX activity was observed to decrease significantly in both the LC₁₀ and LC₅₀ level PH₃-treated adults, whereas no significant increase or decrease in the COX activity was observed in the egg and nymph stages (Figure 2b). GST activity was not changed by the PH₃ treatment in all stages (Figure 2b). Unlike EF fumigation (Figure 2a), the CE activity in the PH₃-exposed eggs was five-fold higher at the two different LC₁₀ and LC₅₀ treatments than that of the control (Figure 2b). No significant difference in CE activity was found in other developmental stages.

3.3. Gene Expression Responses

The measured genes were chitin synthase (chs), UDP-N-acetylglucosamine enolpyruvyl transferase (murA), UDP-N-acetylenolpyruvylglucosamine reductase (murB), homo-

cysteine S-methyltransferase (hmt), V-ATPase (atp), and diaminopimelate decarboxylase (dapdc). The expression of the six genes in the egg developmental stage was not affected by EF treatment (<2-fold, Figure 3a). Only the *chs* gene showed three-fold and two-fold differences in expression in the EF-treated concentrations in the nymph stage, corresponding to the LC₁₀ and LC₅₀ levels, respectively (Figure 3b). There was no difference in expression in the six genes measured in the adult stage (Figure 3c).

Figure 3. Gene expression levels of exposure to lethal concentration LC₁₀ and LC₅₀ values of ethyl formate (EF) for 4 h on *P. citri* developmental stages. Relative gene expressions of eggs (a), nymphs (b), and adults (c) were normalized using the expression level of glyceraldehyde-3-phosphate. Gene abbreviations were as follows: chitin synthase (*chs*), UDP-N-acetylglucosamine enolpyruvoyl transferase (*murA*), UDP-N-acetylenolpyruvoylglucosamine reductase (*murB*), homocysteine S-methyltransferase (*hmt*), V-ATPase (*atp*), and diaminopimelate decarboxylase (*dapdc*). Data were analyzed using one-way ANOVA, followed by post hoc Tukey's tests. Different lowercase letters indicate significant differences among groups; a > b > c.

The expression of the six genes in the egg developmental stage was not affected by PH₃ fumigation treatment (<two-fold, Figure 4a); specifically, the expression of the *murA* gene was reduced by about two-fold. The nymph stage was not affected by the PH₃ fumigation treatment (<2-fold, Figure 4b). There were no differences in the expression of the five genes in the adult stage (Figure 4c), except for an approximately two-fold difference in *dapdc* gene expression in the LC₁₀-level treatment.

Figure 4. Gene expression levels of exposure to lethal concentration LC₁₀ and LC₅₀ values of phosphine (PH₃) for 20 h on *P. citri* developmental stages. Relative gene expressions of eggs (a), nymphs (b), and adults (c) were normalized using the expression level of glyceraldehyde-3-phosphate. Gene abbreviations were as follows: chitin synthase (chs), UDP-N-acetylglucosamine enolpyruvyl transferase (murA), UDP-N-acetylenolpyruvylglucosamine reductase (murB), homocysteine S-methyltransferase (hmt), V-ATPase (atp), and diaminopimelate decarboxylase (dapdc). Data were analyzed using one-way ANOVA, followed by post hoc Tukey's tests. Different lowercase letters indicate significant differences among groups; a > b > c.

3.4. Synergistic Effect of EF and PH₃ against *P. citri*

The synergistic ratio was obtained at the highest value of 1.17-fold in the combined treatment of PH₃ LCT₂₅ and EF LCT₁₀ (Table 2). However, mortality was more than 85% in the EF (LCT₅₀) + PH₃ (LCT₂₅) group. In the 4 h fumigation treatment, since PH₃ did not exhibit fumigation toxicity to eggs, the treatment concentration of PH₃ was set to 1 mg/L, and the treatment concentrations of EF were set differently as LCT₁₀, LCT₂₅, and LCT₅₀ concentration levels. The synergistic ratio was 1.33-fold in EF (LCT₁₀) + PH₃ (1 mg/L). Additionally, the mortality value reached 98% or more in the EF (LCT₅₀) + PH₃ (1 mg/L) combined treatment group (Table 2).

3.5. Omics Analysis Using Proteomics and Metabolomics Techniques

Thirty proteins with increased and decreased expression determined by LC-MS/MS are listed in Table 3. During EF fumigation, about eight proteins in adults showed at least 2-fold different expression.

Table 2. Evaluation of synergistic effects with different combinations of ethyl formate (EF) and phosphine (PH₃) on *P. citri* adults, nymphs, and eggs under 4 h exposure at 20 °C (*n* = 20, triplicate).

Combination No.	Chemicals	LCTx ^a	Dose ^b	CTP	Mortality (Mean ± SE)	SR ^c
Adults and Nymphs						
1	Control	-	0	0	3.3 ± 3.3	1.17
	EF	LCT ₁₀	6	21.3	14.7 ± 2.6	
	PH ₃	LCT ₂₅	0.1	0.29	32.9 ± 4.8	
	EF + PH ₃	LCT ₁₀ + LCT ₂₅	6 + 0.1	19.7 + 0.3	56.0 ± 7.8	
2	Control	-	0	0	0.0 ± 0.0	1.15
	EF	LCT ₂₅	10	27.70	25.0 ± 5.0	
	PH ₃	LCT ₂₅	0.1	0.29	30.0 ± 7.6	
	EF + PH ₃	LCT ₂₅ + LCT ₂₅	10 + 0.1	28.3 + 0.3	63.3 ± 7.3	
3	Control	-	0	0	3.3 ± 1.7	1.13
	EF	LCT ₅₀	17	44.1	46.7 ± 6.0	
	PH ₃	LCT ₂₅	0.1	0.28	28.3 ± 4.4	
	EF + PH ₃	LCT ₅₀ + LCT ₂₅	17 + 0.1	41.6 + 0.3	85.0 ± 2.9	
Eggs						
4	Control	-	0	0	0.0 ± 0.0	1.33
	EF	LCT ₁₀	5	16.1	13.2 ± 3.4	
	PH ₃	1 mg/L	1	3.4	42.9 ± 7.1	
	EF + PH ₃	LCT ₁₀ + 1 mg/L	5 + 1	16.5 + 3.2	75.0 ± 5.8	
5	Control	-	0	0	0.0 ± 0.0	1.24
	EF	LCT ₂₅	10	30.2	26.4 ± 3.4	
	PH ₃	1 mg/L	1	3.4	41.7 ± 1.7	
	EF + PH ₃	LCT ₂₅ + 1 mg/L	10 + 1	31.6 + 3.4	84.7 ± 3.4	
6	Control	-	0	0	1.7 ± 1.7	1.07
	EF	LCT ₅₀	15	38.4	45.0 ± 2.9	
	PH ₃	1 mg/L	1	3.4	46.7 ± 4.4	
	EF + PH ₃	LCT ₅₀ + 1 mg/L	15 + 1	40.6 + 3.4	98.3 ± 1.7	

^a Lethal concentration time (LCT). ^b Dose unit for EF (mg/L) and PH₃ (g/m³). ^c Synergistic effect ratio (SR) = EF + PH₃ mortality / EF mortality + PH₃ mortality; SR = 1 describes additive action, SR < 1 describes antagonism, SR > 1 describes synergism.

Among them, the decreased proteins are putative dihydrolipoyl lysine-residue acetyltransferase, putative 50S ribosomal protein L11, mitochondrial aconitase 1 (isoform B), calmodulin, dihydrolipoyl dehydrogenase (DLD), putative 50S ribosomal protein L9, and putative phosphoribosyl formimino-5-aminoimidazole carboxamideribotide isomerase, and only one protein, putative heat shock protein IbpA, was elevated.

In PH₃ fumigation, 17 proteins with two-fold lower expression were pyruvate kinase, putative dihydrolipoyl lysine-residue acetyltransferase, putative 50S ribosomal protein L11, ATP synthase subunit alpha, mitochondrial aconitase 1 isoform B, DLD, 26S proteasome regulatory subunit 8, 30S ribosomal protein, putative 50S ribosomal protein L9, putative 50S ribosomal protein L7/L12, putative phosphoribosyl formimino-5-aminoimidazole carboxamideribotide isomerase, 26S proteasome regulatory subunit 4, calcium pump, histone H4 replacement isoform A, vacuolar H⁺-ATPase 55kD subunit isoform A, and ATP-dependent chaperone protein ClpB. Only two proteins, acetohydroxyacid isomeroreductase and putative heat shock protein IbpA, were elevated after the PH₃ fumigation. When these two fumigants were treated simultaneously, putative dihydrolipoyl lysine-residue acetyltransferase, putative 50S ribosomal protein L11, DLD, homocysteine S-methyltransferase, putative 50S ribosomal protein L9, putative phosphoribosyl formimino-5-aminoimidazole carboxamideribotide isomerase, and calcium pump were significantly decreased when compared to the control group and both single treatments of EF and PH₃. On the other hand, acetohydroxyacid isomeroreductase and putative heat shock protein (IbpA) in the combinational treated group were up-regulated when compared to the control.

Spectrums of MALDI-TOF MS analysis and lipids differentially generated in *P. citri* adults are reported in Figure 5 and Table 4. Eleven phospholipids were significantly

reduced in EF+PH3-treated samples compared to the control. The highest reduction was a 100-fold decrease in the phospholipid PI (O-16:0).

Table 3. Significant protein expressions after exposure to different combinations of ethyl formate (EF) and phosphine (PH₃) on *P. citri* adults for 4 h at 20 °C (*n* = 20, triplicate).

No	Identified Proteins	ANOVA	Average Quantitative Value ¹ (Normalized Total Spectra)				Biological Process
			Con	EF	PH ₃	EF + PH ₃	
1	Pyruvate kinase	<0.0001	6.71 ^a	7.31 ^a	2.59 ^b	3.55 ^b	Glycolysis
2	Putative dihydrolipoyl lysine-residue acetyltransferase	<0.0001	1.35 ^a	0 ^b	0 ^b	0 ^b	Glycolysis
3	Putative 50S ribosomal protein L11	0.0006	1.33 ^a	0.48 ^b	0 ^b	0 ^b	Translation
4	Putative co-chaperonin GroES	0.0009	6.75 ^b	6.35 ^b	10.6 ^a	10.9 ^a	Stress response
5	ATP synthase subunit alpha	0.0012	3.65 ^{ab}	4.82 ^a	1.28 ^c	2.52 ^{bc}	ATP synthesis
6	Homocysteine S-methyltransferase	0.0014	1.35 ^{ab}	0.92 ^{bc}	1.97 ^a	0.28 ^c	Amino acid biosynthesis
7	ATP synthase beta subunit	0.0016	20.8 ^b	21.5 ^{ab}	22.2 ^a	18.9 ^b	ATP synthesis
8	Mitochondrial aconitase 1, isoform B	0.0050	1.12 ^a	0.23 ^b	0 ^b	0.78 ^{ab}	TCA cycle
9	Calmodulin	0.0058	3.99 ^a	1.71 ^b	5.00 ^a	3.02 ^{ab}	Host–virus interaction
10	Tryptophan 2-monooxygenase oxidoreductase	0.0064	25.0 ^a	26.4 ^a	17.9 ^b	20.1 ^{ab}	Tryptophan metabolism
11	Dihydrolipoyl dehydrogenase	0.0077	1.33 ^a	0.46 ^{ab}	0.28 ^b	0 ^b	Glycolysis/TCA cycle
12	26S proteasome regulatory subunit 8	0.0082	1.14 ^a	0.97 ^a	0 ^b	1.22 ^a	Protein catabolic process
13	Acetohydroxyacid isomeroreductase	0.0087	2.43 ^b	3.42 ^b	6.6 ^a	5.19 ^{ab}	Amino acid biosynthesis
14	30S ribosomal protein S7	0.011	1.14 ^{ab}	1.71 ^a	0 ^b	0.82 ^{ab}	Translation
15	Translation elongation factor Tu	0.012	7.86 ^a	7.52 ^{ab}	4.49 ^b	9 ^a	Translation
16	Putative 50S ribosomal protein L9	0.012	0.9 ^a	0 ^b	0 ^b	0 ^b	Translation
17	Putative 50S ribosomal protein L7/L12	0.014	1.13 ^{ab}	0.92 ^{ab}	0.27 ^b	1.65 ^a	Translation
18	Aspartate aminotransferase	0.019	4.25 ^a	3.46 ^{ab}	4.52 ^a	2.18 ^b	Lipid transport
19	Putative phosphoribosyl formimino-5-aminoimidazole carboxamideribotide isomerase	0.027	1.13 ^a	0.25 ^{ab}	0 ^b	0.54 ^{ab}	Amino acid biosynthesis
20	Cysteine synthase	0.028	3.15 ^{ab}	3.64 ^{ab}	2.28 ^b	3.81 ^a	Amino acid biosynthesis
21	Putative heat shock protein IbpA	0.028	0.46 ^b	1.13 ^{ab}	1.71 ^{ab}	2.47 ^a	Stress response
22	26S proteasome regulatory subunit 4	0.028	2.54 ^{ab}	2.77 ^a	0.29 ^b	1.51 ^{ab}	Protein catabolic process
23	Calcium pump	0.029	1.56 ^{ab}	2.07 ^a	0.35 ^b	0.75 ^{ab}	Ion transporter
24	Putative cold shock-like protein CspD	0.031	3.81 ^{ab}	3.00 ^b	3.14 ^{ab}	5.18 ^a	Regulation of transcription
25	Heat shock protein 82	0.032	2.50 ^{ab}	2.50 ^{ab}	2.25 ^b	3.23 ^a	Stress response
26	beta-actin, partial	0.034	15.5 ^b	16.5 ^{ab}	20.3 ^a	16.1 ^{ab}	Skeleton
27	Dihydroxyacid dehydratase	0.037	3.80 ^{ab}	3.44 ^{ab}	3.16 ^b	5.45 ^a	Amino acid biosynthesis
28	Histone H4 replacement, isoform A	0.037	2.14 ^{ab}	1.68 ^{ab}	0.77 ^b	2.25 ^a	Regulation of transcription
29	Vacuolar H ⁺ -ATPase 55kD subunit, isoform A	0.038	1.14 ^{ab}	1.58 ^{ab}	0.24 ^b	1.96 ^a	Ion transporter
30	ATP-dependent chaperone protein ClpB	0.045	2.45 ^{ab}	1.81 ^{ab}	0.59 ^b	1.36 ^a	Stress response

¹ Data were analyzed using one-way ANOVA, followed by post hoc Tukey's tests. Different lowercase letters indicate significant differences among groups; a > b > c.

Figure 5. Mass ions for lipid profiling on *P. citri* adults after exposure to ethyl formate (EF), phosphine (PH₃), and combination treatment (EF + PH₃) for 4 h. (a) Spectrums of MALDI-TOF MS. (b) Pairwise analysis using a heatmap with the Kruskal–Wallis test ($p < 0.0001$). A heatmap was constructed using the Log₂ intensity ratio between the control and treatment groups.

Table 4. List of significant lipid estimated m/z values detected using MALDI-TOF MS with the negative ion mode (Kruskal–Wallis Test ($Pr > \text{Chi-square}$, $p < 0.0001$)).

No	m/z Value	Delta	Assignment [M-H] ⁻	Average Intensity Value			
				Con	PH ₃	EF	EF + PH ₃
1	699.4970	0.000038	PA(O-16:0)	551.7	454.9	151.2	41.4
2	740.5180	0.005587	PC(P-20:0)	569.6	460.0	237.0	50.7
3	742.5392	0.000037	PC(O-20:0)	1618.6	1189.4	678.5	39.8
4	784.5134	0.000017	PC(14:0)	237.8	212.0	81.1	26.0
5	820.5265	0.008047	PS(18:0)	94.1	84.2	48.7	24.5
6	833.4822	0.000022	PG(16:1)	678.2	552.7	245.8	26.0
7	837.4407	0.000008	PI(20:0)	100.6	77.7	41.9	23.4
8	857.4976	0.000167	PG(20:3)	571.8	497.8	294.7	27.0
9	859.5425	0.008287	PI(O-16:0)	3970.8	3389.5	1630.5	34.3
10	860.5447	0.000017	PC(18:2)	2484.6	2088.0	778.9	203.6
11	862.5604	0.000033	PC(18:1)	5549.8	4745.0	1654.6	408.9
12	699.4970	0.000038	PA(O-16:0)	551.7	454.9	151.2	41.4
13	740.5180	0.005587	PC(P-20:0)	569.6	460.0	237.0	50.7
14	742.5392	0.000037	PC(O-20:0)	1618.6	1189.4	678.5	39.8

4. Discussion

There are more than 60 species of mealybug worldwide, and *P. citri* is a major pest of citrus [33]. This pest is currently controlled for the protection of fruits from damage during the cultivation of orange, grapefruit, lemon, and mandarin using chemical insecticides such

as chlorpyrifos, chlorpyrifos-ethyl, methidathion, malathion, imidacloprid, buprofezin, and spirotetramat [34]. Although pest control of fruits using chemical insecticides during cultivation has been successfully performed, there is a presumable hindrance found in post-harvest management using MB and alternatives, including quarantine.

MB can be completely replaced only when a clear combination treatment efficacy and mechanism of action are evaluated. The efficacy of combination treatment of PH_3 with EF has been evaluated in several studies [1,7,35], but there is a lack of understanding of the mechanisms. PH_3 is used as the most global alternative fumigant to MB, but there are still some issues to be solved: a relatively long treatment time with slow activity and high resistance [8]. When the PH_3 treatment time was changed from 20 h to 4 h, the efficacy of *P. citri* on the eggs showed a mortality rate of 60% at the highest concentration of 4 mg/L PH_3 (Table 1 and Table S3). On the other hand, EF has a concentration of 285~1536 times higher than PH_3 based on LC_{50} values (Table 1 and Table S1).

Nevertheless, as *P. citri* is an invasive species, it is an important pest that prohibits transfer between countries. Thus, appropriate quarantine systems should be in operation in each country to prevent their spread. MB is minimally used for quarantine and pre-shipment, but MB substitutes are continuously developed and used selectively in the field [7]. According to Yang et al. (2016), the fumigation control of *P. citri* was reported using EF and PH_3 for quarantine. The LCT_{99} values were obtained using a 55 L fumigation chamber, and EF exhibited the LCT_{99} values 211.04, 49.51, and 124.61 mg·h/L for eggs, nymphs, and adults, respectively [7]. These results are slightly different from those obtained in this study. In our study, the LCT_{99} value of EF was 65.43, 45.85, and 46.90 mg·h/L for eggs, nymphs, and adults, respectively. The differences between these two studies might be considered because of the different temperature conditions of 8 °C [7] and 20 °C in this present study. EF treatment might be dependent on temperature. On the other hand, LCT_{99} values of PH_3 treatment show similar results between Yang et al. (2016) and our study (Table 1).

The EF + PH_3 combined treatment against *P. citri* has been reported in a previous study [7], in which eggs, nymphs, and adults were treated at 8 °C with different treatment times: 2 h and 4 h. However, in our study, the temperature for the combination treatment was set to 20 °C, and the treatment time was set to 4 h. The fumigation toxicity of PH_3 to eggs was not found at 4 h at 20 °C, so it was treated at the level of 1 mg·h/L as other developmental stages, which was the level of LCT_{25} (Table 1). The EF treatment concentration for the combination treatment was simultaneously treated with LCT_{10} , LCT_{25} , and LCT_{50} concentrations, and the treatment time was uniformly applied at 4 h. It was intended to shorten the treatment time because the PH_3 treatment time of 20 h was too long in the quarantine field. With this combined treatment, a synergistic effect was confirmed in eggs, nymphs, and adults (Table 2), and thus there was no need to use EF or PH_3 alone. This combination treatment caused more potent fumigation toxicity than that of the single treatment.

As we determined four different enzyme activities after individual treatment of EF and PH_3 , there was no significant change in AChE and GST activities, but it was confirmed that there were changes in COX and CE activities (Figure 2). Among them, a previous report found that the activity of COX in *Myzus persicae* Sulzer (Hemiptera: Aphididae) nymphs increased in response to EF treatment [36]. This result is similar to our study as EF treatment on *P. citri* nymphs enhanced COX activities (Figure 2a). Interesting molecular-level changes in this study are changes in genes related to chitin biosynthesis, especially in *P. citri* nymphs (Figures 2 and 3). It indicates that treatment with EF and PH_3 affects *P. citri* chitin production, which can be considered as a major route of the mode of fumigant action.

On the other hand, the synergistic effect might be related to the biochemical changes. Through proteomic analysis, protein expression changes after individual treatment of EF and PH_3 were found in *P. citri* adults, and several proteins after both treatments were commonly down-regulated which are involved in mitochondrial proteins related to energy production and protein biosynthesis. Among them, putative dihydrolipoyl lysine-residue

acetyltransferase mediates the conversion of pyruvate to acetyl-CoA and CO₂ as one of the pyruvate dehydrogenase components [37]. Therefore, it is a linker for the glycolytic pathways to the tricarboxylic cycle in living organisms. DLD is another important protein in relation to PH₃ fumigation, because the unique biochemical mechanism for insect pest resistance to PH₃ is a genetic change in DLD. DLD is known to be primarily activated in mitochondria and involved in energy generation. PH₃-resistant insects develop genetically modified DLDs in their amino acid sequences [2,19]. Similarly, the significant reduction in DLD level may produce lower energy in *P. citri* after the combined treatment, indicating that it could be considered as a vital target site. Another down-regulated protein is phosphoribosyl formimino-5-aminoimidazole carboxamideribotide isomerase which is involved in histidine biosynthesis. The decreased expression of this enzyme may reduce histidine production during the fumigation and inhibit the regulation and metabolism of trace elements, and the repair and growth of tissue [38,39]. Therefore, individual fumigation of EF and PH₃ can inhibit similar biochemical processes in *P. citri* adults and they are considered to be their presumable target sites.

Decreased expression of homocysteine S-methyltransferase (HMT) may also be a strong target site of combined EF+PH₃ treatment (Table 3), and this enzyme plays an important role in amino acid (methionine) biosynthesis. It is also primarily linked to the biosynthesis of cysteine, a major component in the production of glutathione [40]. If the homocysteine balance is broken, it affects S-adenosyl methionine production, which acts on DNA methylation [40]. In our study, a dramatic reduction in HMT protein level was found in the combined treatment towards *P. citri* adults (Table 3), indicating a presumable occurrence of homocysteine unbalance. Further study is needed to find a reduction in homocysteine level after the combined EF+PH₃ treatment in *P. citri*, and it can be highlighted as a new target site for those fumigants. However, the gene expression of *hmt* was not different, as shown in Figure 4, and it could be due to the inaccurate timing of sample collection when the response of the gene is already completed.

Interestingly, an up-regulated protein, acetoxyacid isomerase, was obtained after PH₃ and combinational treatment (Table 3). It mediates the conversion of alpha-ketopantoate to pantothenic acid (vitamin B5), which is a component of coenzyme A (CoA) essential for the biosynthesis and metabolism of fatty acids, carbohydrates, and proteins [41]. Therefore, elevation of this enzyme may be a recovery process for the damaged adults after the fumigation.

Additionally, through proteomic studies, it was confirmed that the combined treatment of EF+PH₃ reduced the synthesis of ribosomal proteins that synthesize proteins in *P. citri*, considering another target site of the combinational treatment. Metabolomics analyzed individual lipid levels from the same samples used for the proteomic study, and a significant change in phospholipids was confirmed when EF and PH₃ were simultaneously treated (Table 4 and Figure 5). Inhibition of phospholipid production might have resulted in changes in the cell membrane components, and some of the phospholipids were also detected such that the production was suppressed more than 100-fold (Table 4). EF treatment influenced phospholipid generation more than PH₃ treatment. Interestingly, it was confirmed that the *m/z* values of 859.5425 [PI(O-16:0)] and 860.5447 [PC(18:2)] also changed in *M. persicae* in response to EF treatment, and these were identified as biomarkers in two insects during the future EF treatment [36].

5. Conclusions

As a fumigated compound for the control of *P. citri*, EF and PH₃ were treated as a single treatment or a combination treatment as MB alternatives. With reference to the LCT values resulting from the single treatment of EF and PH₃, the treatment amount of PH₃ was set to the level of 1 mg/L for the combination fumigation, and the LCT₁₀, LCT₂₅, and LCT₅₀ values of EF were treated at the same time. Single fumigation toxicities of EF and PH₃ were associated with changes in COX and CE activities and changes in gene expression related to chitin biosynthesis. The synergistic effect of EF + PH₃ was confirmed in *P. citri* adults,

and synergistic effects strongly resulted in decreases in DLD expression and changes in phospholipid production when examined at the biochemical level. Therefore, this study might contribute to the inhibition of excessive use of PH_3 for the control of *P. citri* with reduction in resistance development to PH_3 , and shortening the PH_3 treatment time from 20 h to 4 h. In the future, it is urgently needed to find a new fumigation combination with EF or PH_3 simultaneously to make the use of these substances sustainable.

Supplementary Materials: The following are available online at <https://www.mdpi.com/article/10.3390/app11219877/s1>, Table S1: Lethal concentration of *P. citri* exposed to fumigants at 20 °C in a 12 L desiccator, Table S2: List of primers for the *P. citri*, Table S3: Mortality of phosphine on the eggs of *P. citri* for 4 h exposure at 20 ± 1 °C ($n = 60$, triplicate).

Author Contributions: Conceptualization, J.O.Y. and S.-E.L.; methodology, Y.H.L., H.-J.J. and B.-H.L.; validation, M.-G.P. and T.H.K.; formal analysis, K.K., T.H.K., H.-J.J., C.K. and J.P.; investigation, K.K., M.-G.P. and T.H.K.; data curation, K.K.; writing—original draft preparation, K.K. and S.-E.L.; writing—review and editing, K.K., J.O.Y. and S.-E.L.; visualization, K.K.; supervision, J.O.Y. and S.-E.L.; project administration, S.-E.L.; funding acquisition, S.-E.L. All authors have read and agreed to the published version of the manuscript.

Funding: This study was supported by grants from the Animal and Plant Quarantine Agency of the Ministry of Agriculture, Food, and Rural Affairs of the Republic of Korea (Z-1543086-2017-19-01 and Z-1543086-2020-22-01).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: No data available.

Conflicts of Interest: The authors declare no conflict of interest.

References

- Kim, K.; Lee, Y.H.; Kim, G.; Lee, B.H.; Yang, J.O.; Lee, S.E. Ethyl formate and phosphine fumigations on the two-spotted spider mite, *Tetranychus urticae* and their biochemical responses. *Appl. Biol. Chem.* **2019**, *62*, 1–10. [CrossRef]
- Kim, K.; Yang, J.O.; Sung, J.Y.; Lee, J.Y.; Park, J.S.; Lee, H.S.; Lee, B.H.; Ren, Y.; Lee, D.W.; Lee, S.E. Minimization of energy transduction confers resistance to phosphine in the rice weevil, *Sitophilus oryzae*. *Sci. Rep.* **2019**, *9*, 1–14. [CrossRef]
- Park, M.G.; Park, C.G.; Yang, J.O.; Kim, G.H.; Ren, Y.L.; Lee, B.H.; Cha, D.H. Ethyl Formate as a Methyl Bromide Alternative for Phytosanitary Disinfestation of Imported Banana in Korea With Logistical Considerations. *J. Econ. Entomol.* **2020**, *113*, 1711–1717. [CrossRef]
- De Cal, A.; Martinez-Treceno, A.; Salto, T.; Lopez-Aranda, J.M.; Melgarejo, P. Effect of chemical fumigation on soil fungal communities in Spanish strawberry nurseries. *Appl. Soil Ecol.* **2005**, *28*, 47–56. [CrossRef]
- Wood, J.P.; Wendling, M.; Richter, W.; Lastivka, A.; Mickelsen, L. Evaluation of the Efficacy of Methyl Bromide in the Decontamination of Building and Interior Materials Contaminated with Bacillus anthracis Spores. *Appl. Environ. Microb.* **2016**, *82*, 2003–2011. [CrossRef] [PubMed]
- Gareau, B.J. Lessons from the Montreal Protocol delay in phasing out methyl bromide. *J. Environ. Stud. Sci.* **2015**, *5*, 163–168. [CrossRef]
- Yang, J.; Park, Y.; Hyun, I.H.; Kim, G.H.; Kim, B.S.; Lee, B.H.; Ren, Y. A Combination Treatment Using Ethyl Formate and Phosphine to Control *Planococcus citri* (Hemiptera: Pseudococcidae) on Pineapples. *J. Econ. Entomol.* **2016**, *109*, 2355–2363. [CrossRef] [PubMed]
- Fields, P.G.; White, N.D.G. Alternatives to methyl bromide treatments for stored-product and quarantine insects. *Annu. Rev. Entomol.* **2002**, *47*, 331–359. [CrossRef] [PubMed]
- Opit, G.P.; Phillips, T.W.; Aikins, M.J.; Hasan, M.M. Phosphine Resistance in *Tribolium castaneum* and *Rhyzopertha dominica* From Stored Wheat in Oklahoma. *J. Econ. Entomol.* **2012**, *105*, 1107–1114. [CrossRef] [PubMed]
- Collins, P.J.; Falk, M.G.; Nayak, M.K.; Emery, R.N.; Holloway, J.C. Monitoring resistance to phosphine in the lesser grain borer, *Rhyzopertha dominica*, in Australia: A national analysis of trends, storage types and geography in relation to resistance detections. *J. Stored Prod. Res.* **2017**, *70*, 25–36. [CrossRef]
- Schlipalius, D.I.; Valmas, N.; Tuck, A.G.; Jagadeesan, R.; Ma, L.; Kaur, R.; Goldinger, A.; Anderson, C.; Kuang, J.J.; Zuryn, S.; et al. A Core Metabolic Enzyme Mediates Resistance to Phosphine Gas. *Science* **2012**, *338*, 807–810. [CrossRef]
- Agrafioti, P.; Athanassiou, C.G.; Nayak, M.K. Detection of phosphine resistance in major stored-product insects in Greece and evaluation of a field resistance test kit. *J. Stored Prod. Res.* **2019**, *82*, 40–47. [CrossRef]

13. Hubhachen, Z.; Jiang, H.B.; Schlupalius, D.; Park, Y.; Guedes, R.N.C.; Oppert, B.; Opit, G.; Phillips, T.W. A CAPS marker for determination of strong phosphine resistance in *Tribolium castaneum* from Brazil. *J. Pest. Sci.* **2020**, *93*, 127–134. [\[CrossRef\]](#)
14. Lee, M.J.; Kim, H.Y. A 90-Day Inhalation Toxicity Study of Ethyl Formate in Rats. *Toxicol. Res.* **2017**, *33*, 333–342. [\[CrossRef\]](#)
15. Zaitoon, A.; Lim, L.T.; Scott-Dupree, C. Synthesis and Characterization of Ethyl Formate Precursor for Activated Release Application. *J. Agric. Food Chem.* **2019**, *67*, 13914–13921. [\[CrossRef\]](#)
16. Lee, B.H.; Park, C.G.; Park, M.G.; Roh, G.H.; Kim, D.; Riddick, E.W.; Chen, J.; Cha, D.H. Ethyl formate fumigation for the disinfestation of red imported fire ants *Solenopsis invicta* Buren. *J. Asia-Pac. Entomol.* **2019**, *22*, 838–840. [\[CrossRef\]](#)
17. Kim, B.S.; Shin, E.M.; Park, Y.J.; Yang, J.O. Susceptibility of the Cigarette Beetle *Lasioderma serricorne* (Fabricius) to Phosphine, Ethyl Formate and Their Combination, and the Sorption and Desorption of Fumigants on Cured Tobacco Leaves. *Insects* **2020**, *11*, 599. [\[CrossRef\]](#)
18. Lee, J.S.; Kim, H.K.; Kyung, Y.; Park, G.H.; Lee, B.H.; Yang, J.O.; Koo, H.N.; Kim, G.H. Fumigation Activity of Ethyl Formate and Phosphine Against *Tetranychus urticae* (Acari: Tetranychidae) on Imported Sweet Pumpkin. *J. Econ. Entomol.* **2018**, *111*, 1625–1632. [\[CrossRef\]](#) [\[PubMed\]](#)
19. Yang, J.; Park, J.S.; Lee, H.; Kwon, M.; Kim, G.-H.; Kim, J. Identification of a phosphine resistance mechanism in *Rhyzopertha dominica* based on transcriptome analysis. *J. Asia-Pac. Entomol.* **2018**, *21*, 1450–1456. [\[CrossRef\]](#)
20. Franco, J.C.; Zada, A.; Mendel, Z. *Novel Approaches for the Management of Mealybug Pests*; Springer Netherlands: Dordrecht, The Netherlands, 2009; pp. 233–278.
21. Mansour, R.; Grissa-Lebdi, K.; Suma, P.; Mazzeo, G.; Russo, A. Key Scale Insects (Hemiptera: Coccoidea) of High Economic Importance in a Mediterranean Area: Host Plants, Bio-Ecological Characteristics, Natural Enemies and Pest Management Strategies—A Review. *Plant Prot. Sci.* **2017**, *53*, 1–14. [\[CrossRef\]](#)
22. Bond, E.J.; Food and Agriculture Organization of the United Nations. *Manual of Fumigation for Insect Control*; Food and Agriculture Organization of the United Nations: Rome, Italy, 1984.
23. Plackett, R.L.; Hewlett, P.S. A Unified Theory for Quantal Responses to Mixtures of Drugs: The Fitting to Data of Certain Models for Two Non-Interactive Drugs with Complete Positive Correlation of Tolerances. *Biometrics* **1963**, *19*, 517–531. [\[CrossRef\]](#)
24. Ye, J.; Coulouris, G.; Zaretskaya, I.; Cutcutache, I.; Rozen, S.; Madden, T.L. Primer-BLAST: A tool to design target-specific primers for polymerase chain reaction. *BMC Bioinform.* **2012**, *13*, 134. [\[CrossRef\]](#) [\[PubMed\]](#)
25. Livak, K.J.; Schmittgen, T.D. Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the $2^{-\Delta\Delta CT}$ Method. *Methods* **2001**, *25*, 402–408. [\[CrossRef\]](#) [\[PubMed\]](#)
26. Bradford, M.M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **1976**, *72*, 248–254. [\[CrossRef\]](#)
27. Campian, J.L.; Gao, X.; Qian, M.; Eaton, J.W. Cytochrome C oxidase activity and oxygen tolerance. *J. Biol. Chem.* **2007**, *282*, 12430–12438. [\[CrossRef\]](#) [\[PubMed\]](#)
28. Ellman, G.L.; Courtney, K.D.; Andres, V., Jr.; Feather-Stone, R.M. A new and rapid colorimetric determination of acetylcholinesterase activity. *Biochem. Pharmacol.* **1961**, *7*, 88–95. [\[CrossRef\]](#)
29. Habig, W.H.; Jakoby, W.B. Assays for differentiation of glutathione S-transferases. *Methods Enzymol.* **1981**, *77*, 398–405. [\[CrossRef\]](#)
30. Mackness, M.I.; Walker, C.H.; Rowlands, D.G.; Price, N.R. Esterase activity in homogenates of three strains of the rust red flour beetle *Tribolium castaneum* (Herbst). *Comp. Biochem. Physiol. Part C Comp. Pharmacol.* **1983**, *74*, 65–68. [\[CrossRef\]](#)
31. Searle, B.C. Scaffold: A bioinformatic tool for validating MS/MS-based proteomic studies. *Proteomics* **2010**, *10*, 1265–1269. [\[CrossRef\]](#)
32. Folch, J.; Lees, M.; Sloane Stanley, G.H. A simple method for the isolation and purification of total lipides from animal tissues. *J. Biol. Chem.* **1957**, *226*, 497–509. [\[CrossRef\]](#)
33. Franco, J.C.; Suma, P.; da Silva, E.B.; Blumberg, D.; Mendel, Z. Management strategies of mealybug pests of citrus in mediterranean countries. *Phytoparasitica* **2004**, *32*, 507. [\[CrossRef\]](#)
34. Mansour, R.; Belzunces, L.P.; Suma, P.; Zappala, L.; Mazzeo, G.; Grissa-Lebdi, K.; Russo, A.; Biondi, A. Vine and citrus mealybug pest control based on synthetic chemicals. A review. *Agron. Sustain. Dev.* **2018**, *38*, 1–20. [\[CrossRef\]](#)
35. Ahmed, Q.; Ren, Y.L.; Emery, R.; Newman, J.; Agarwal, M. Evaluation of Ethyl Formate, Phosphine, and Their Combination to Disinfest Harvested Celery against Purple Scum Springtails. *Horttechnology* **2018**, *28*, 492–501. [\[CrossRef\]](#)
36. Kim, K.; Lee, B.H.; Park, J.S.; Yang, J.O.; Lee, S.E. Biochemical mechanisms of fumigant toxicity by ethyl formate towards *Myzus persicae* nymphs. *J. Appl. Biol. Chem.* **2017**, *60*, 271–277. [\[CrossRef\]](#)
37. Cabrera-Brandt, M.; Silva, A.X.; Trionnaire, G.L.; Tagu, D.; Figueroa, C.C. Transcriptomic responses of the aphid *Myzus persicae* *nicotianae* Blackman (Hemiptera: Aphididae) to insecticides: Analyses in the single Chilean clone of the tobacco aphid. *Chil. J. Agric. Res.* **2014**, *74*, 191–199. [\[CrossRef\]](#)
38. Del Duca, S.; Riccardi, C.; Vassallo, A.; Fontana, G.; Castronovo, L.M.; Chioccioli, S.; Fani, R. The Histidine Biosynthetic Genes in the Superphylum Bacteroidota-Rhodothermota-Balneolota-Chlorobiota: Insights into the Evolution of Gene Structure and Organization. *Microorganisms* **2021**, *9*, 1439. [\[CrossRef\]](#) [\[PubMed\]](#)
39. Vázquez-Salazar, A.; Becerra, A.; Lazcano, A. Evolutionary convergence in the biosyntheses of the imidazole moieties of histidine and purines. *PLoS ONE* **2018**, *13*, e0196349. [\[CrossRef\]](#)

-
40. Oppold, A.; Kress, A.; Vanden Bussche, J.; Diogo, J.B.; Kuch, U.; Oehlmann, J.; Vandegehuchte, M.B.; Muller, R. Epigenetic alterations and decreasing insecticide sensitivity of the Asian tiger mosquito *Aedes albopictus*. *Ecotoxicol. Environ. Saf.* **2015**, *122*, 45–53. [[CrossRef](#)]
 41. Hart, R.J.; Abraham, A.; Aly, A.S.I. Genetic Characterization of Coenzyme A Biosynthesis Reveals Essential Distinctive Functions during Malaria Parasite Development in Blood and Mosquito. *Front. Cell Infect. Microbiol.* **2017**, *20*, 260. [[CrossRef](#)]