Comparative Proteomics Analysis of Phosphine-Resistant and Phosphine-Susceptible *Sitophilus oryzae* (Coleoptera: Curculionidae)

Hyun-Na Koo 1, Seung Ju Seok 1, Hyun Kyung Kim 1, Gil-Hah Kim 1,4 and Jeong Oh Yang 2,*

1 Department of Plant Medicine, College of Agriculture, Life and Environment Science, Chungbuk National University, Cheongju 28644, Korea; hyunnakoo@hanmail.net (H.-N.K.); tmdwn405@naver.com (S.J.S.); nshk0917@hanmail.net (H.K.)
2 Animal and Plant Quarantine Agency (APQA), Gimcheon 39660, Korea
* Correspondence: khkim@chungbuk.ac.kr (G.-H.K.); linae@nate.com (J.O.Y.); Tel.: +82-43-261-2555 (G.-H.K.); +82-54-912-0681 (J.O.Y.); Fax: +82-43-271-4414 (G.-H.K.); +82-54-912-0688 (J.O.Y.)

Abstract: A proteomic method combining two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) and matrix-assisted laser desorption/ionization-time-of-flight/time-of-flight mass spectrometry (MALDI-TOF/TOF) was used to compare the protein expression profiles of phosphine-resistant (PH₃-R) and -susceptible (PH₃-S) strains of *Sitophilus oryzae*. Thirty-nine differentially expressed protein spots were identified between the PH₃-R and PH₃-S strains; 20 protein spots were upregulated, and 19 protein spots were downregulated in the PH₃-R strain compared with their expression in the PH₃-S strain. In particular, cytochrome oxidase subunit I showed 15-fold higher expression in the PH₃-R strain than in the PH₃-S strain. Additionally, citrate synthase 2, delta-1-pyrolline-5-carboxylate dehydrogenase, and triose-phosphate isomerase were highly expressed in the PH₃-R strain. In summary, our study has improved understanding of the molecular mechanisms of phosphine resistance in the rice weevil.

Keywords: 2D-PAGE; phosphine resistance; *Sitophilus oryzae*

1. Introduction

Phosphine (PH₃) is now probably the most widely used fumigant in the world at present to disinfest stored products [1] after the decreased use of methyl bromide, which depletes the ozone layer [2,3]. Phosphine can also be applied directly to a commodity, is easy to obtain, easy to use, and relatively inexpensive [4,5]. Additional positive attributes of phosphine use include its residue-free nature when used as a treatment and effectiveness against insect pests at all developmental stages, including eggs; however, phosphine requires longer exposure periods (within commercial reality) than other disinfectants to exhibit toxicity, particularly against highly PH₃-resistant insects [6]. Resistant populations are divided into two groups depending on the phenotype: a “weak resistance” group and a “strong resistance” group. Strong resistance to phosphine occurs because of two loci that work in synergy, the *rph1* (resistance to phosphine) and *rph2* genes, which are responsible for phosphine resistance in *Rhyzopertha dominica* [7,8], *Tribolium castaneum* [9] and *Sitophilus oryzae* [10,11]. Among these, in particular, high-level resistance to phosphine in *S. oryzae* has been reported in India, China, Morocco, Brazil, and Australia [10,12–14]. *Sitophilus oryzae* causes damages to stored products and its contamination of grain products has become a major problem in cereal market. Resistance to phosphine in *S. oryzae* had increased to 75% in developing countries by 2000 [10], requiring the management of resistance. To replace or reduce the usage of insecticides, environmentally friendly alternatives have been developed. One of them is the modified atmosphere (MA) technology [15–17]. Levy-
De la Torre et al. evaluated that heat shock protein and ATP synthase β subunit were overexpressed in *R. dominica* exposed to MA by LC MS/MS [17].

Genomics reveals what genes are present, while transcriptomics shows how active genes are in different cells. These strategies have contributed tremendously to accelerating the discovery of phosphine resistance-associated genes [18]; however, such advances cannot reveal the complexities of protein biochemistry, including protein abundance. Proteomic analysis can help to analyze different protein synthesis changes at the molecular level [19,20]. Furthermore, understanding PH₃-resistance mechanisms in insects may contribute to providing clues for the development of new chemicals, including fumigants to control PH₃-resistant insects. A proteomic study has shown differences of protein expression in a PH₃-resistant *R. dominica* in comparison to a PH₃-susceptible *R. dominica* and among them dihydrolipoamide dehydrogenase, a protein involved in the Krebs cycle, has been identified [21]. Additionally, Kim et al. reported the ethyl formate fumigation caused lethal effects on *Myzus persicae* nymphs by changing cytochrome c oxidase (COX) activity, acetylcholinesterase (AChE) gene expression, and phospholipid production [22]. They analyzed the lipid content using MALDI-TOF MS/MS. Recently, Tang’s study using RT-PCR for the determination of mitochondrial gene expression in PH₃-resistant *Cryptolestes ferrugineus* has demonstrated that *cox1* expression was not significantly different when compared to a PH₃-susceptible *C. ferrugineus*, while *nad3*, *atp6*, and *cob* genes were down-regulated [23]. This pattern is different from other studies [21]. Therefore, PH₃ resistance is dependent on insect species and associated with the modulation of energy production in mitochondria.

This study aimed to compare differences in the protein expression of the PH₃-R and PH₃-S strains of *S. oryzae* using 2D-PAGE and MALDI-TOF/TOF. The results clarify phosphine resistance mechanism, allowing the development of more reliable, sensitive, and useful molecular markers to monitor rice weevil phosphine resistance.

2. Materials and Methods

2.1. Rice Weevil Strains

The PH₃-susceptible strain (control strains) of *S. oryzae* was obtained from Murdoch University (Perth, Australia) and maintained under insecticide-free conditions. The PH₃-resistant strain of *S. oryzae* was obtained from Plant Quarantine Technology Center (Gimcheon, South Korea). The resistance ratios indicated that the lethal concentration time (LCT)₅₀ value was >56.1-fold higher in the PH₃-resistant strain (16.550 mg·h/L) of *S. oryzae* than in the PH₃-susceptible strain (0.295 mg·h/L) [24]. All the strains of *S. oryzae* were successively cultured on rice grains at the Chungbuk National University (Chungcheongbuk-do, Korea) under controlled conditions (25 ± 1 °C, 80% relative humidity, and 16L:8D photoperiod).

2.2. Chemicals

All electrophoresis grade reagents (ultrapure) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Pharmalyte (pH 3.5–10) was obtained from Amersham Biosciences (Cardiff, UK) and IPG DryStrips (pH 4–10 NL; 24 cm) were purchased from Genomine Inc. (Phohang, South Korea). Modified porcine trypsin (sequencing grade) was obtained from Promega (Madison, WI, USA).

2.3. Protein Preparation

The PH₃-S and PH₃-R strains of rice weevils in each group were randomly selected. One hundred rice weevil adults were homogenized directly using a motor-driven homogenizer (PowerGen 125; Fisher Scientific) in sample lysis buffer containing 7 M urea, 2 M thiourea, 4% (w/v) CHAPS, 1% (w/v) DTT, 2% (v/v) pharmalyte and 1 mM benzamidine. Proteins were extracted for 1 h at RT while vortexing. After centrifugation at 12,000 rpm for 1 h at 25 °C, the insoluble fraction was discarded, and the soluble fraction was used for
2D-PAGE. The protein concentration of the extracts was determined using the Bradford assay [25].

2.4. 2D-PAGE and Gel Staining

IPG DryStrips (4–10 NL IPG; 24 cm; Genomine) were equilibrated for 12 h–16 h in a solution containing 2 M thiourea, 2% CHAPS, 7 M urea, 1% DTT, and 1% pharmalyte and then were loaded with 2 mg of sample. Isoelectric focusing (IEF) was performed at 20 °C using a Multiphor II electrophoresis unit and an EPS 3500 XL power supply (Amersham Biosciences, Piscataway, NJ, USA) following the manufacturer’s instructions. The 2D electrophoresis gels were stained with silver as described by Oakley et al. [26], with the omission of the fixing and sensitization with glutaraldehyde steps. All the experiments were independently performed in triplicate.

2.5. 2D Image Analysis

Quantitative analysis of digitized images was performed using PDQuest (version 7.0; BioRad, Hercules, CA, USA) software according to the protocols provided by the manufacturer. For each spot, the true signal intensity is determined by subtracting the median background value and normalized to the total valid spot intensity. Proteins in which the expression significantly deviated by over two-fold compared with that in the control group were selected for further analysis.

2.6. In-Gel Protein Digestion for MALDI-TOF/TOF

Selected spots were enzymatically digested in gel using modified porcine trypsin (Promega) as previously described [27], with slight modifications. Gel pieces were washed with 50% acetonitrile to remove SDS, salt and stain. Washed and dehydrated gel pieces were then vacuum dried to remove solvent, rehydrated with a trypsin (8–10 ng/µL) solution in 50 mM ammonium bicarbonate, pH 8.7, and incubated for 8 h–10 h at 37 °C. The samples were analyzed using a Bruker Autoflex Speed mass spectrometer and LIFTTM ion optics. Both MS and MS/MS data were acquired using a Smart Beam laser with a 2-kHz repetition rate, and up to 4000 shots were accumulated for each spectrum. The data were collected in the MS/MS mode with 2 keV of collision energy; air was used as the collision gas to achieve nominally single-collision conditions. Although precursor selection had a possible resolution of 200, in studies of known single-component analytes, a resolution of 100 was utilized. Both MS and MS/MS data were acquired using the default instrument calibration without conducting internal or external calibration. MS/MS ion searches were performed using Mascot licensed for in-house use.

2.7. Normalization in PDQuest

Normalization in PDQuest was carried out for each gel in a MatchSet using the following formula:

\[ \text{Normalized spot quantity} = \frac{\text{Raw spot quantity} \times \text{Scaling factor}}{\text{Normalization factor}} \times \frac{1}{(\text{Total quantity in valid spots})} \]

\(a\) Raw spot quantity is the unnormalized quantity (intensity) of each spot; \(b\) Scaling factor is a factor selected to produce a meaningful value; \(c\) Normalization factor is calculated for each gel based on the normalization method.

2.8. Statistical Analysis

The values are expressed as the means ± S.D. of three independent experiments. Differences between PH3-S and PH3-R strains of rice weevil were tested for significance by Student’s \(t\) test. \(p\) values lower than 0.05 were considered evidence for statistical significance.
3. Results and Discussion

3.1. 2D-PAGE Map and Differential Protein Expression

Proteomic techniques help us to understand what happened inside the cells after stress occurred. Previously, we compared global proteome profiles and the expression pattern of defense-related genes in Chinese cabbage when infested by *Myzus persicae* and *Plutella xylostella* [28]. Approximately 1600 protein spots were separated, of which nearly 160 showed reproducible changes in expression. 2D-PAGE (24-cm IPG strips) was combined with silver staining to investigate differences in the proteomes of the PH3-S and PH3-R strains of rice weevil. The relative spot volumes and gray values of the protein spots were detected using PDQuest software (version 7.0). All 2-DE gels showed a broad distribution of spots in a pI range from 4.5–8.5 and a mass range of 14–150 kDa. In total, 1589 protein spots in the PH3-S (Figure 1a) and PH3-R strains (Figure 1b) were identified. Image analysis software was used to identify aligned spots whose mean intensities differed by at least two-fold between the PH3-R and PH3-S strains. Quantitative analysis revealed 39 differentially accumulated protein spots in the PH3-R and PH3-S strains, of which the intensities of 19 protein spots were decreased and those of 20 protein spots were increased in the PH3-R strain of *S. oryzae*.

![Figure 1](image-url)

**Figure 1.** Representative 2-DE maps of proteins extracted from the PH3-S (a) and PH3-R (b) strains of *Sitophilus oryzae*. The spots were visualized by silver staining. Differentially accumulated protein spots are indicated by green sashes and numbers. The 2-DE was repeated at least three times for each group.

3.2. MALDI-TOF/TOF Analysis and Protein Identification

Differentially expressed protein spots were manually excised from the gels and digested with trypsin, and then analyzed by MALDI-TOF/TOF combined with a database search. Fifteen protein spots were successfully identified, and 24 protein spots could not be detected (Table 1). The identified proteins showed homology to hypothetical proteins from different organisms; however, the observed and theoretical data on pI or molecular weight (MW) were not always in agreement. These differences could be attributed to post-translational modifications, multiprotein isoforms, and different compositions between the *S. oryzae* proteins and homologous known proteins. PH3 toxicity has been focused on the mitochondrial respiration by interaction with cytochrome c oxidase to cause depletion of ATP production [29]. Cytochrome oxidase subunit I (42.5 kDa, pI 7.33), cytochrome P450 (75 kDa, pI 4.96), heat shock protein 70 (HSP70, 66.1 kDa, pI 4.74), triosephosphate isomerase (21.7 kDa, pI 7.95), citrate synthase 2 (37.6 kDa, pI 7.99), delta-1-pyrroline-5-carboxylate dehydrogenase (55.0 kDa, pI 8.07), ATP synthase subunit beta (51.6 kDa, pI 7.21), and glyceraldehyde-3-phosphate dehydrogenase (28.1 kDa, pI 7.36) were overexpressed in the PH3-R strain compared with their expression in the PH3-S strain of *S. oryzae*. 
Cytochrome oxidase is targeted by phosphine [30,31]. In the present study, the protein expression of cytochrome oxidase was markedly higher in the PH$_3$-R strain (18.8-fold) than in the PH$_3$-S strain of _S. oryzae_. The expression of excess cytochrome oxidase in the PH$_3$-R strain compared with that in the PH$_3$-S strain suggests that the PH$_3$-R strain can tolerate the inhibitory effects of phosphine absorbed from functional doses. The upregulation of cytochrome P450 and HSP70 may lead to the detoxification of fumigants [32]. Additionally, HSP70 acts as a molecular sensor of oxidative stress. Phosphine induces oxidative stress in insects, mammalian cells, animals, and humans [33]. The primary mechanism of PH$_3$ resistance in stored-grain insects is to absorb very little PH$_3$ compared to their susceptible strains and an involves additional detoxification process of the fumigant [34]. Triosephosphate isomerase in the glycolysis pathway was upregulated in the PH$_3$-R strain of _S. oryzae_. Additionally, citrate synthase 2, delta-1-pyrroline-5-carboxylate dehydrogenase, ATP synthase subunit beta, and glyceraldehyde-3-phosphate dehydrogenase in the mitochondrial respiratory chain were upregulated in the PH$_3$-R strain compared with their expression in the PH$_3$-S strain of _S. oryzae_. Similar results were found in Levy-De la Torre et al. report where heat shock protein and ATP synthase subunit beta were overexpressed in _R. dominica_ exposed to MA [17]. The accumulation of the protons in the mitochondrial inner space drives the ATP synthase to synthesize ATP molecules from ADP and Pi. However, if O$_2$ intake decreases, the ATP synthase catalyzes the reverse reaction working now as an ATPase [35]. However, Park et al. reported different results from our study. Glyceraldehyde-3-phosphate dehydrogenase and triosephosphate isomerase were dramatically down-regulated in the PH$_3$-resistant strain of _R. dominica_ [21]. In addition, beta-glucuronidase (144.1 kDa, pl 5.19), protein MLP1-like (37.2 kDa, pl 4.8), glycerogen phosphorylase (90.4 kDa, pl 4.74), DNA primase large subunit (39.1 kDa, pl 4.96), and beta-actin (66.8 kDa, pl 4.5) were down-regulated in the PH$_3$-R strain of _S. oryzae_. In summary, we only speculate on the involvement of these regulated genes in phosphine resistance because no phenotypic evidence is directly linked to these genes. Further study to identify the proteins corresponding to the 24 undetected spots is required.

Table 1. Identification of differentially expressed proteins using MALDI-TOF/TOF.

| Spot No. | Protein Name | Experimental pI/MW (kDa) | Protein Score | GenBank Accession No. | Regulation in PH$_3$-R | Fold Change |
|----------|--------------|--------------------------|---------------|-----------------------|------------------------|-------------|
| 6602     | cytochrome oxidase subunit I, partial (mitochondrion) [Cordilura albipes] | 7.33/42.48 | 29 | gi|16979442 | Up-regulated | 18.8 |
| 3701     | cytochrome P450 71A1 [Culex quinquefasciatus] | 4.96/75.00 | 17 | gi|170072409 | Up-regulated | 13.0 |
| 2705     | heat shock 70 kDa protein cognate 5 [Tribolium castaneum] | 4.74/66.08 | 74 | gi|91077414 | Up-regulated | 2.7 |
| 8304     | triosephosphate isomerase [Tribolium castaneum] | 7.95/21.72 | 118 | gi|189236533 | Up-regulated | 9.7 |
| 8501     | citrate synthase 2, mitochondrial [Tribolium castaneum] | 7.99/37.55 | 109 | gi|91083623 | Up-regulated | 530.2 |
| 8702     | delta-1-pyrroline-5-carboxylate dehydrogenase, mitochondrial [Tribolium castaneum] | 8.07/55.03 | 61 | gi|91084231 | Up-regulated | 595.9 |
| 6606     | ATP synthase subunit beta, mitochondrial [Hydra vulgaris] | 7.21/51.61 | 26 | gi|221132854 | Up-regulated | 100.3 |
| 7202     | hypothetical protein TcasGA2_TC001380 [Tribolium castaneum] | 7.53/21.68 | 33 | gi|127000262 | Up-regulated | 19.7 |
| 8702     | protein TcasGA2_TC008222 [Tribolium castaneum] | 8.09/143.07 | 15 | gi|1270012119 | Up-regulated | 144.3 |
| 7401     | glyceraldehyde-3-phosphate dehydrogenase, partial [Limenitis reducta] | 7.36/28.13 | 180 | gi|161088548 | Up-regulated | 3.9 |
| 4704     | beta-glucuronidase [Tribolium castaneum] | 5.19/144.10 | 21 | gi|91089483 | Down-regulated | 0.18 |
| 2512     | protein MLP1-like [Tribolium castaneum] | 4.80/37.21 | 22 | gi|642919153 | Down-regulated | 0.73 |
| 2704     | glycerogen phosphorylase [Tribolium castaneum] | 4.74/90.39 | 68 | gi|91081301 | Down-regulated | 0.19 |
| 3503     | DNA primase large subunit [Tribolium castaneum] | 4.96/39.05 | 16 | gi|189241229 | Down-regulated | 0.65 |
| 1705     | beta-actin, partial [Gammarus minus] | 4.50/66.84 | 85 | gi|384402814 | Down-regulated | 0.39 |
4. Conclusions

This research describes for the first time the differences in the protein expression of the PH\textsubscript{3}-R and PH\textsubscript{3}-S strains of \textit{S. oryzae} using 2D-PAGE and MALDI-TOF/TOF. The overexpression of cytochrome oxidase subunit I, cytochrome P450, HSP70, triosephosphate isomerase, citrate synthase 2, delta-1-pyrroline-5-carboxylate dehydrogenase, ATP synthase subunit beta, and glyceraldehyde-3-phosphate dehydrogenase likely contribute to PH\textsubscript{3}-resistant \textit{S. oryzae}. Therefore, we can use this knowledge to clarify phosphine resistance mechanism, allowing the development of more reliable, sensitive, and useful molecular markers to monitor rice weevil phosphine resistance. Taken together, PH\textsubscript{3} toxicity acts as a selection pressure that not only alters cellular metabolism but also modulates energy transduction; this explains the mechanism of PH\textsubscript{3} resistance in \textit{S. oryzae}. To date, PH\textsubscript{3} resistance has become more prominent in stored products' fumigation, so knowing the exact mode of action of phosphine will help us come up with strategies that assist us in fending off resistance.

Author Contributions: Conceptualization, H.-N.K.; methodology, J.O.Y. and S.J.S.; investigation, H.-N.K. and H.K.K.; data curation, H.-N.K. and H.K.K.; writing—original draft preparation, H.-N.K.; writing—review and editing, G.-H.K.; supervision, G.-H.K., and J.O.Y. All authors have read and agreed to the published version of the manuscript.

Funding: This study was supported by the Animal and Plant Quarantine Agency, Plant Quarantine Technology Center (Gimcheon, Korea).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are available on request from the corresponding author.

Acknowledgments: We extend special thanks to Yonglin Ren (Murdoch University, Australia) for sharing the pests.

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

References

1. Bell, C.H. Fumigation in the 21st century. \textit{Crop Prot.} \textbf{2000}, \textit{19}, 563–569. [CrossRef]
2. Yang, R.S.H.; Witt, K.L.; Alden, C.J.; Cockerham, L.G. Toxicology of methyl bromide. In \textit{Reviews of Environmental Contamination and Toxicology: Continuation of Residue Reviews}; Ware, G.W., Ed.; Springer: New York, NY, USA, 1995; pp. 65–85.
3. Bulathsinghala, A.T.; Shaw, I.C. The toxic chemistry of methyl bromide. \textit{Hum. Exp. Toxicol.} \textbf{2014}, \textit{33}, 81–91. [CrossRef]
4. Kaur, R.; Nayak, M. Developing effective fumigation protocols to manage strongly phosphine-resistant \textit{Cryptolestes ferrugineus} (Stephens) (Coleoptera: Laemophloeidae). \textit{Pest Manag. Sci.} \textbf{2015}, \textit{71}, 1297–1307. [CrossRef] [PubMed]
5. Sciuto, A.M.; Wong, B.J.; Martens, M.E.; Hoard-Fruchey, H.; Perkins, M.W. Phosphine toxicity: A story of disrupted mitochondrial metabolism. \textit{Ann. N. Y. Acad. Sci.} \textbf{2016}, \textit{1374}, 41–51. [CrossRef]
6. Nayak, M.K.; Holloway, J.C.; Emery, R.N.; Pavic, H.; Bartlet, J.; Collins, P.J. Strong resistance to phosphine in the rusty grain beetle, \textit{Cryptolestes ferrugineus} (Stephens) (Coleoptera: Laemophloeidae): Its characterisation, a rapid assay for diagnosis and its distribution in Australia. \textit{Pest Manag. Sci.} \textbf{2013}, \textit{69}, 48–53. [CrossRef] [PubMed]
7. Schlipalius, D.I.; Cheng, Q.; Reilly, P.E.; Collins, P.J.; Ebert, P.R. Genetic linkage analysis of the lesser grain borer \textit{Rhyncopertha dominica} identifies two loci that confer high-level resistance to the fumigant phosphine. \textit{Genetics} \textbf{2002}, \textit{161}, 773–782. [CrossRef]
8. Schlipalius, D.I.; Chen, W.; Collins, P.J.; Nguyen, T.; Reilly, P.E.; Ebert, P.R. Gene interactions constrain the course of evolution of phosphine resistance in the lesser grain borer, \textit{Rhyncopertha dominica}. \textit{Heredity} \textbf{2008}, \textit{100}, 506–516. [CrossRef]
9. Jagadeesan, R.; Fotheringham, A.; Ebert, P.; Schlipalius, D. Rapid genome wide mapping of phosphine resistance loci by a simple regional averaging analysis in the red flour beetle, \textit{Tribolium castaneum}. \textit{BMC Genom.} \textbf{2013}, \textit{14}, 650. [CrossRef] [PubMed]
10. Nguyen, T.T.; Collins, P.J.; Ebert, P.R. Inheritance and characterization of strong resistance to phosphine in \textit{Sitophilus oryzae} (L.). \textit{PLoS ONE} \textbf{2015}, \textit{10}, e0124335. [CrossRef] [PubMed]
11. Nguyen, T.T.; Collins, P.J.; Duong, T.M.; Schlipalius, D.I.; Ebert, P.R. Genetic conservation of phosphine resistance in the rice weevil \textit{Sitophilus oryzae} (L.). \textit{J. Hered.} \textbf{2016}, \textit{107}, 228–237. [CrossRef]
12. Rajendran, S. Phosphine resistance in stored insect pests in India. In Proceeding of the 7th International Working Conference on Stored-product Protection, Chengdu, China, 14–19 October 1999; pp. 14–19.
13. Zeng, L. Development and countermeasures of phosphine resistance in stored grain insects in Guangdong of China. In Proceedings of the 7th International Working Conference on Stored-product Protection, Chengdu, China, 14–19 October 1999; pp. 642–647.

14. Benhalima, H.; Chaudhry, M.Q.; Mills, K.A.; Price, N.R. Phosphine resistance in stored-product insects collected from various grain storage facilities in Morocco. J. Stored Prod. Res. 2004, 40, 241–249. [CrossRef]

15. Carvalho, M.O.; Pires, L.; Barbosa, A.; Barros, G.; Riudavets, J.; Garcia, A.C.; Navarro, S. The use of modified atmospheres to control Sitophilus zeamais and Sitophilus oryzae on stored rice in Portugal. J. Stored Prod. Res. 2012, 49, 49–56. [CrossRef]

16. Cheng, W.; Lei, J.; Ahn, J.E.; Liu, T.X.; Zhu-Salzman, K. Effects of decreased O₂ and elevated CO₂ on survival, development, and gene expression in cowpea bruchids. J. Insect Physiol. 2012, 58, 792–800. [CrossRef] [PubMed]

17. Levy-De la Torre, V.A.; Cinco-Moroyoqui, F.J.; Lopez-Zavala, A.A.; Wong-Corral, F.J.; Martinez-Cruz, O. Mitochondrial response.

18. Galli, G.L.; Richards, J.G. Mitochondria from anoxia-tolerant animals reveal common strategies to survive without oxygen. J. Comp. Physiol. B 2014, 184, 285–302. [CrossRef] [PubMed]