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Research

Stored-Product

Ethanedinitrile as a Fumigant for *Lasioderma serricorne* (Coleoptera: Anobiidae), and *Rhyzopertha dominica* (Coleoptera: Bostrichidae): Toxicity and Mode of Action

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Abstract

This study evaluated the fumigant ethanedinitrile (EDN) against the cigarette beetle, *Lasioderma serricorne*, and phosphine-resistant and susceptible lesser grain borer, *Rhyzopertha dominica*, life stages under laboratory conditions. Eggs of both species were the most susceptible stage to EDN. EDN is, therefore, a promising alternative because eggs are generally tolerant to most common fumigants. *Lasioderma serricorne* eggs were the most susceptible with an LC_{50} estimated of 50.4 ppm, followed by adults, pupae and larvae with LC_{50} values of 160.2, 192.5, and 446.6 ppm, respectively, after 24-h exposure at 25°C. Eggs of phosphine-susceptible (LC_{50} = 11.2 ppm) and resistant (LC_{50} = 12.0 ppm) *R. dominica* strains were more susceptible to EDN than were adults of both strains, with LC_{50} values of 27.7 and 36.0 ppm, respectively. *Lasioderma serricorne* mixed life stage cultures were completely controlled at concentrations ≥2,000 ppm at 24 h. Fumigation with 600 ppm was enough to suppress adult emergence in the case of the phosphine-susceptible *R. dominica* strain (USDA), while an average of only 4.0 adults emerged from the phosphine-resistant *R. dominica* strain (Belle Glade) compared with 514.3 adults in the control. *Lasioderma serricorne* was more tolerant to EDN than both *R. dominica* strains. EDN caused 61.8 and 68.2 % inhibition of *R. dominica* (USDA) cytochrome c oxidase activity at concentrations of 0.0038 and 0.0076 mM in vitro, respectively, and it did not inhibit its activity in the case of an in vivo assay. These results suggest that cytochrome *c* oxidase may not be the main target for EDN toxicity.

Key words: stored product, chemical control, grains

Fumigants are the most effective management tools for stored product protection. Methyl bromide and phosphine are the most common fumigants worldwide due to their high efficiency toward a wide range of stored product pests and storage structures. Unfortunately, methyl bromide is banned in many countries due to its detrimental environmental impact as an ozone depleting substance according to the Montreal Protocol (United Nations Environment Programme, UNEP 1996). Phosphine remains widely used because of having many advantages compared with other fumigants, such as its relative ease of use, its versatility across commodities, its relative low cost, and its low resulting residue levels in the treated commodities (Collins et al. 2004). Unfortunately, many stored product pests have evolved resistance to phosphine due to the repetitive applications (e.g., Sağlam, Afful et al. 2018, Nayak et al. 2015). Phosphine is very corrosive to metals that limits it use in buildings with valuable electrical systems, and other approved stored product fumigants such as sulfuryl fluoride and carbon dioxide also have limitations in efficacy (Phillips et al. 2012). The lack of promising alternative fumigants are major threats to current pest management programs in stored products.

When considering the limitations for fumigants currently used, there is a need to research and develop new products for stored product insect management. In fact, numerous studies have been carried out to develop new alternative fumigants for stored product protection and several fumigants, such as sulfuryl fluoride, propylene oxide, carbonyl sulfide, ethyl formate, hydrogen cyanide, and methyl iodide have been studied for pest control (Haritos et al. 2006, Opit et al. 2016, Isikber et al. 2017). Ethanedinitrile (EDN) shows potential for controlling stored product pests, and limited studies point to its insecticidal activity against a wide range of stored product pests (Hooper et al. 2003).

EDN, also known as cyanogen and oxalonitrile, has the molecular formula $(CN)_2$ (also as C_2N_2) and is a colorless gas with

an almond like odor. The Commonwealth Scientific and Industrial Research Organization (CSIRO 1996) patented EDN with the chemical name of cyanogen. The threshold limit value (TLV) of 10 ppm (v/v) compares favorably with either methyl bromide or phosphine with TLVs of 5 and 0.3 ppm, respectively. EDN is considered environmentally friendly because it is not an ozone-depleting or greenhouse gas (Ren et al. 2005). Few studies have reported the potential for EDN as alternative to methyl bromide in timber fumigation (Ren 2001; Ren et al. 2005, 2011; Park et al. 2014) and soil fumigation (Ren et al. 2002, Rosskopf 2007). Its ability for quick penetration through the wood during log fumigation shows that EDN may have a potential as methyl bromide alternative for logs that need disinfestation from insects and nematodes prior to international shipment.

Fumigants are thought to have different modes of toxic action when compared with contact insecticides. Certain toxic gases are known to inhibit activity of the cytochrome c oxidase enzyme, including carbon monoxide (D'Amico et al. 2006), hydrogen cyanide (Leavesley et al. 2008), hydrogen sulfide (Volpato et al. 2008), phosphine (Price and Dance 1983, Jian et al. 2000), and nitric oxide (Cooper et al. 2002). Cytochrome *c* oxidase is the terminal enzyme of the mitochondrial electron transport chain. The function of this enzyme is to receive an electron from each of four cytochrome *c* molecules to convert one oxygen molecule into two water molecules with the concomitant synthesis of ATP. The inhibition of this enzyme by the inhibitors suppresses the respiration of the mitochondria by preventing the transport of electrons to the oxygen molecule leading to cell asphyxiation and death. However, it is still unclear to propose that cytochrome *c* oxidase is the main target for these fumigants as most of these gases showed inhibition of cytochrome *c* oxidase in vitro and did not inhibit the enzyme in vivo (Price and Dance 1983, Jian et al. 2000, Cooper et al 2008). To the best of our knowledge, the mode of action of EDN has not been reported previously. However, some studies have hypothesized that EDN may be converted to hydrogen cyanide (HCN) during the fumigation process (Waterford et al. 2004, Brash et al. 2013). Meanwhile, Hall et al. (2018) have shown that no HCN was produced as a result of logs fumigation with EDN or the concentration of HCN was undetectable. Based on the available information in literature, we hypothesized that inhibition of cytochrome *c* oxidase by EDN could be the primary cause of insect toxicity.

In the literature, there are no reported studies on the fumigant toxicity of EDN against *Rhyzopertha dominica* (F.) (Coleoptera: Bostrichidae), known as the lesser grain borer and *Lasioderma serricorne* (F.) (Coleoptera: Ptinidae), the cigarette beetle, except Hooper et al. (2003) who mentioned that EDN was highly toxic to *R. dominica*. Also, the toxicology of EDN for insects has received no research attention up to now. Additionally, both *R. dominica* and *L. serricrone* have evolved resistance to phosphine gas, the most commonly used fumigant for these pests, and alternatives to phosphine are needed (Afful et al. 2018, Sağlam et al. 2015). Therefore, the present study was undertaken to evaluate the fumigant toxicity of EDN against the life stages of *L. serricorne*, and both a strong phosphine-resistant (Belle Glade, Afful et al. 2018) and a susceptible (USDA) strains of *R. dominica*, under laboratory conditions. A validation study using mixed life stages cultures exposed to the recommended dose for complete control of all life stages of both species was carried out. In addition to the work on toxicity we also studied the impact of EDN on cytochrome *c* oxidase activity in *R. dominica* (USDA strain) both in vivo and in vitro in the present study.

Materials and Methods

Insects

A laboratory culture of *L. serricorne* adults was reared as per the methods of Sağlam et al. (2015) on a diet mixture of 95 % organic whole wheat flour (*Triticum aestivum*) with 5% brewer's yeast added (w/w). Cultures of two strains of *R. dominica* were reared with methods like those reported by Afful et al. (2018) and maintained on 100% organic soft white winter wheat kernels with a moisture content between 12 and 13%. Both species were incubated at 28°C, relative humidity of 50–70% and a photoperiod of 16:8 (L:D) h in incubator under laboratory conditions.

Chemicals

EDN (cyanogen, ≥ 99.90 %) for fumigation trials was supplied by Draslovka Services Pty Ltd., Sydney, Australia. A cytochrome *c* oxidase kit for enzyme analyses was purchased from Sigma Aldrich, Louis, MO. d-Mannitol, 98.0% (Acros Organics), sucrose (crystalline, Certified ACS, Fisher Brand), ethylenebis (oxyethylenenitrilo) tetra-acetic acid (EGTA; crystalline, certified ACS, Fisher Brand), essentially fatty acid-free bovine serum albumin (BSA; BIO World), 4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid (HEPES), and 99.0% (Alfa Aesar) were purchased form Thermo Fisher Scientific.

EDN Toxicity Against Adults

EDN was evaluated against adults of *L. serricorne* (1- to 7-d old) and *R. dominica* (1–3 wk old). Fifty adults of *R. dominica* strains or *L. serricrorne* were transferred separately to a clean small glass vials (10 ml) containing about 1 g of whole wheat kernels (*R. dominica*) or wheat flour (*L. serricorne*). The glass vials were covered using muslin cloth and rubber ring. All glass vials were transferred into fumigation chambers (one vial per each chamber). Three replicates were used for each concentration and control treatment (air only). The fumigation chambers were tightly closed and the appropriate EDN volumes were injected in to each fumigation chamber through the gas introduction port using the gas tight syringe after the same volume of air was removed to obtain the target concentrations which ranged between 10 and 80 ppm for *R. dominica*, and between 100 and 350 ppm for *L. serricorne*. After injection, EDN concentrations in the headspace of the fumigation chambers were measured using GC-MS (see below) and then all fumigation chambers were kept in an incubator at 25°C and a photoperiod of 16:8 (L:D) for 24 h. After 24-h exposure, the fumigation chambers were aerated in the fume hood for 1 h and all glass vials were transferred to the incubator and kept at the same conditions for three additional days for insect recovery or delayed mortality inspection. Insects were considered dead when legs or antennae did not move or respond after being touched with a fine needle under the microscope.

EDN Toxicity Against Eggs

The toxicity of EDN was investigated against *L. serricorne* eggs (0- to 24-h old) and *R. dominica* eggs (0- to 48-h old). For obtaining eggs, small glass jelly jars (125 ml) were used containing small amount of pre-sieved wheat flour using a No. 120 mesh standard sieve. About 300 adults of mixed age and sex were released in to each jar and 20 jars were prepared for each insect species. After 24 or 48 h of maintaining *L. serricorne* and *R. dominica* adults, respectively, for laying eggs, the adults were separated by sieving with a No. 30 mesh sieve and the eggs were separated from the flour using 80 mesh sieve. The EDN toxicity was evaluated against eggs of both *R. dominica* strains and *L. serricorne* using the black polystyrene plate (50 wells

per each). Forty eggs of *L. sericorne* and 30 eggs of *R. dominica* were transferred into individual wells of the plates using a microscope and a camel hair brush. Each plate was covered with clear glass slide and held from both sides using clips. Three replicates were fumigated for each concentration and the control treatment (air only). The fumigation chambers were sealed and the desired volumes of EDN were injected through the gas injection port using the gas tight syringe to achieve the target concentrations of 1 to 30 ppm for *R. dominica*, and of 20 to 80 ppm for *L. sericorne*. All fumigation chambers were kept for 24 h at the similar conditions of adult toxicity experiment. After 24 h, the fumigation chambers were aerated in the fume hood and the plates were taken out and kept in incubator at 28°C and 50–70% RH and a photoperiod of 16:8 (L:D) h of for additional 10 d or until complete egg hatching in the control treatment. After 10 d, all plates were examined for egg hatching under the microscope and mortality percentages were determined.

EDN Toxicity Against *L. sericorne* Larvae

The toxicity of EDN was evaluated against the mid-age (21- to 28-d old) larvae of *L. sericorne*. Thirty larvae were separated by sieving the wheat flour containing the insect culture using a No. 30 mesh sieve. The larvae were transferred to clean 10-ml glass vials containing about 1 g wheat flour to prevent insect starvation. The vials were covered with muslin cloth and held by rubber ring. Three replicates were prepared for each concentration and control treatment (air only). Thereafter, all vials were put in the fumigation chambers. The desired volumes of EDN were injected through the gas septum using the gas tight syringe to achieve the target concentrations between 200 and 850 ppm. All fumigation chambers were kept at 25°C and a photoperiod of 16:8 (L:D) h in the incubator for 24 h. After the exposure period, the fumigation chambers were aerated in the fume hood for 1 h and then the glass vials were transferred to an incubator at 28°C, 50–70% RH and a photoperiod of 16:8 (L:D) h for additional 4 d to observe the recovery or delayed mortality. The mortality of larvae was observed under a microscope and they were considered dead if their body did not move after touching with a brush. The mortality percentages were computed.

EDN Toxicity Against *L. sericorne* Pupae

For obtaining the pupae, 30 mid-age (21- to 28-d old) larvae were separated and transferred to clean glass vials containing 3 g of flour. All vials were maintained at 28°C, 50–70% RH and a photoperiod of 16:8 (L:D) h in the incubator for 7 d for pupae emergence. Then, the glass vials were transferred into the fumigation chambers. Three replicates were maintained for each concentration and control treatment. The appropriate volumes of EDN were introduced through the injection port using the gas tight syringe after removing the same volume of air to obtain EDN concentrations ranged from 100 to 600 ppm. The fumigation chambers were kept at 25°C, 50–70% RH and a photoperiod of 16:8 (L:D) h in the incubator for 24 h. After aeration, the glass vials were taken out and kept in the incubator at the same conditions until all pupae in the control treatment were transformed to adults and all pupal cases were checked using the metal probe under microscope for mortality assessment.

Validation Experiment

To validate the recommended dose of EDN against both insect species, the EDN validation study against the mixed life stage cultures of *L. sericorne* and *R. dominica* in the presence of food during the fumigation was evaluated under laboratory conditions. Mixed age cultures of both insects were individually established in 20 g of wheat flour containing 5% brewer yeast in the case of *L. sericorne* and in 20 g wheat kernels in the case of *R. dominica* strains in 125-ml glass jars. About 20 mixed sex adults were added to the food media in the beginning and additional 20 adults were added 7 d later for generation overlapping. The cultures were maintained in the incubator at 28°C and 50–70% RH with photoperiod of 16:8 (L:D) h. Three weeks later, all insects were removed by sieving and the cultures were kept at the same condition. After 5 d of the new adult emergence, all mixed age cultures jars were transferred into 2-liter glass jar for fumigation. The mixed age culture jars that therefore had a mixture of new adults with a range of immature life stages were fumigated with EDN target concentrations of 2,000, 4,000, and 6,000 ppm for *L. sericorne* and 150, 300, and 600 ppm for *R. dominica*. Three replicates for each concentration and control treatment (air only) were carried out. After ventilation in the fume hood for 1 h, all mixed age cultures jars were maintained for 6 wk at the same condition of rearing and all adults were counted each week during 6 wk to detect any emerged adults of both insects in treatments and control.

Fumigation Process

All life stages of both insect species were exposed to EDN concentration in Ball Mason jars, wide mouth (2 liter) equipped with a gas-tight screw-on metal lid and provided with a gas introduction port. EDN (100 %) was taken from the cylinder into a gas sampling bag and diluted with air. The target concentrations for all life stages of both insect species were obtained by injecting the calculated volumes of EDN which represent the desired concentrations using the gas tight syringe through the gas injection port after the same volume of air was removed.

Measuring the EDN Concentration Using GC-MS

After the desired volumes of EDN were injected to achieve the target concentrations, the EDN concentrations in the headspace of the fumigation chambers were measured at the beginning and just before venting the fumigation chambers. EDN concentrations were measured using a gas chromatograph (Shimadzu –17A QP5050A, Shimadzu Scientific Instruments, Kyoto, Japan) equipped with MS detector in SIM mode with an electron impact (EI) ionization with an ionizing energy of 1.3 kV (Ramadan et al. 2019). The GC was equipped with a J&W Scientific DB-1MS UI column (30.0 ml × 0.250 mm ID × 0.25 µm). The carrier gas used was helium at a flow rate of 1.1 ml/min. The oven temperature was isothermal at 150°C and the injection port temperature was 250°C with split injection mode (1:42 split). The MS detector transfer line temperature was set at 250°C and the most abundant ion (52 m/z) for EDN was selected as the quantifier ion. The concentration of EDN was calculated based on an external standard curve generated prior to fumigation. The standard curve was prepared using Tedlar bags (CEL Scientific Corp., Los Angeles, CA) after decanting the desired volume from EDN cylinder. The standard curve was achieved by the dilution of a precise volume of the 100.0 % EDN into a known volume of air in Tedlar bag. Injections of this standard gas at volumes of 25, 20, 15, 10, and 5 µl were analyzed by GC-MS. In total, 15 µl was drawn from the headspace of each fumigation chamber (3 times per each) using the 25-µl gastight syringe, which was considered as the standard volume, in the case of the standard curve, which was used for calculation of EDN concentrations. The standard curve with the linear equation was calculated and used to estimate the concentration of EDN in the fumigation chamber under study from the area integrated under the GC peak for a given EDN sample. The mean

between the beginning concentrations after injection and the final concentration before venting was used for the average concentration of that treatment for the dose–response estimation of mortality.

Mitochondria Isolation

The isolation of mitochondria was carried out as described by Syromyatnikov et al. (2013). Adults of *R. dominica* were used for studying the inhibitory effect of EDN on cytochrome *c* oxidase as an example for stored product insects and as an enzyme source. Adult *R. dominica* (150 mg) were homogenized in ice-cold isolation buffer (220 mM mannitol, 100 mM sucrose, 1 mM EGTA, 2 mg/ml BSA and 20 mM of HEPES with PH = 7.4) using a tissue homogenizer driven by a CON-TORQUE power unit (Eberbach, model 62400–981). The homogenate was filtered through glass wool and centrifuged at 600 *g* for 5 min at 4°C using a microcentrifuge (Fisherbrand accuSpin Micro 17/Micro 17R). The supernatant was transferred into a 2-ml microcentrifuge tube and centrifuged at 10,000 *g* for 10 min at 4°C. The supernatant was discarded. The pellet was resuspended in 1.5 ml of washing solution containing all isolation buffer components except BSA and centrifuged at 10,000 *g* for 10 min at 4°C. The supernatant was taken out and the pellet was resuspended in 0.2 ml of washing solution and stored in ice (0–4°C). The mitochondria protein content was determined by the Bradford assay (1976) using a kinetic ELISA microplate reader (Molecular Devices, V Max).

In Vivo Cytochrome *c* Oxidase Activity Assay

For determination of cytochrome c oxidase activity, about 1,000 adults of *R. dominica* were fumigated with the LC_{50} of EDN for 24 h at 25°C. After fumigation, the insects were kept in an incubator at 25°C and 55–70% RH. Three replicates were carried out for each treatment and control. From each replicate, 150 mg of adults were taken after different time intervals from fumigation (0, 24, and 48 h) for isolation of mitochondria. The mitochondria preparations were diluted 10-fold using enzyme dilution buffer and were kept at 0–4°C for determination of enzyme activity using the cytochrome *c* oxidase assay kit. The activity of cytochrome *c* oxidase was determined following the decreasing in absorbance at 550 nm at 25°C using a UV-visible spectrophotometer (Pharmacia Biotech, Ultrospec 3000). Data were collected using a kinetic program of 5-s delay and 10-s intervals with a total of six readings per min.

In Vitro Cytochrome *c* Oxidase Activity Assay

Mitochondria contents were isolated from 150 mg of untreated adults of *R. dominica* and diluted 10-fold using enzyme dilution buffer. Thereafter, 950 µl of assay buffer was pipetted into a clean 2-ml GC vial (9-mm screw thread cap with PTFE/silicone septa, Thermo Fisher Scientific) and then 80 µl of enzyme dilution buffer was added. About 20 µl of diluted insect mitochondria preparation were then added to the vial and the vial was sealed. The desired volume of EDN was injected through the vial septa and the gas was bubbled in the solution using the gas tight syringe (1 ml) to get concentrations of 50 and 100 ppm depending on the vial volume (2 ml), which represent 0.0038 and 0.0076 mM of EDN. EDN was incubated with content for 20 min at 25°C. After incubation, the vial content was transferred in to a 1.5-ml spectrophotometer cuvette and 50 µl of reduced ferrocytochrome *c* substrate solution was added, and then, the activity was determined as described earlier for the in vivo assay. Three replicates were carried out for the treatment and control (bubbled with air). The Cytochrome *c* oxidase assay kit was used for determination of enzyme activity using the spectrophotometer. The activity of cytochrome *c* oxidase was calculated using the following equation

$$\mu\text{mole/min} = (\Delta A/\text{min} \times \text{dil.} \times 1.1)/(\text{volume of enzyme (ml)} \times 21.84)$$

where ΔA/min = A/min (sample) – A/min (blank), dil. is the dilution factor of enzyme or sample, 1.1 is the reaction volume in (ml), 21.84 is ΔεmM between ferrocytochrome *c* and ferricytochrome *c* at 550 nm. And then, the specific activity (µmole/min/mg protein) was calculated from the enzyme activity divided by protein concentration in (mg/ml).

Statistical Analyses

A software program (IBM SPSS V23.0, Statistical Package for Social Sciences) was used to calculate the LC_{50} and LC_{99} values of EDN. Number of adults emerged from the mixed life stage cultures exposed to EDN were subjected to one way analysis of variance (ANOVA) and mean separations were performed by Tukey's HSD test and differences at $P < 0.05$ were considered as significant. The specific activity of cytochrome *c* oxidase (µmole/min/mg) in vitro was subjected to one way ANOVA and mean separations were performed by Tukey's HSD test and differences at $P < 0.05$ were considered as significant. The specific activity (µmole/min/mg) in vivo in treated and untreated insects was performed by *t*-test and differences at $P < 0.05$ were considered as significant.

Results

Insecticidal Activity of EDN Against Susceptible and Resistant Strains of *R. dominica*

EDN was toxic to adults and eggs of both the phosphine-susceptible and highly resistant strains of *R. dominica* after 24 h of exposure period. Toxicity parameters of EDN on these strains exposed to different gas concentrations for 24 h are shown in Table 1. EDN was significantly more toxic to the adults of the phosphine-susceptible strain than the adults of the highly resistant strain with LC_{50} values of 27.7 and 36.0 ppm, respectively. However, EDN caused similar toxicity to the eggs of both *R. dominica* strains. The eggs of both strains were more susceptible to EDN than the adults. The dose–mortality response of both strains to EDN indicated the absence of cross resistance between EDN and phosphine as resistant ratios were 1.3 and 1.1 for adults and eggs, respectively.

Insecticidal Activity of EDN Against Life Stages of *L. serricorne*

Toxicity parameters of EDN on different life stages of *L. serricorne* exposed to different gas concentrations for 24 h are shown in Table 2. The results of the present study indicated that EDN is a promising fumigant showing strong toxicity against all life stages of *L. serricorne*. In general, the susceptibility of life stages to EDN varied significantly. Eggs were the most susceptible stage to EDN with LC_{50} of 50.4 ppm, followed by adult stage (LC_{50} = 160.2 ppm). Larvae were the most tolerant stage and were 10 times more tolerant than eggs with LC_{50} value of 446.6 ppm. Pupae (LC_{50} = 192.5) were four times more tolerant than eggs. Adults were more susceptible than pupae but without significant difference due to the overlapping of 95 % CLs of their LC_{50} values. The order of susceptibility of *L. serricorne* life stages to EDN based on LC_{50} values was eggs > adults>pupae > larvae.

Efficiency of EDN for *R. dominica* and *L. sericorne* Control

Table 3 presents the number of adults (mean ± SE) of *R. dominica* (both strains) and *L. sericorne* emerged from mixed life stages exposed to different concentrations of EDN at 25°C for 24 h and after 6 wk of fumigation. The results indicated that the fumigation of *L. serricorne* mixed life stages with a target concentration of 2,000 ppm (an average of 1,245 ppm measured) was sufficient to kill all life stages of *L. serricorne* population and there were no emerged adults from the fumigated mixed age cultures jars at any fumigation dose. On other hand, the highest concentration applied (600 ppm target; 365 ppm measured) was enough for complete control of *R. dominica* (USDA susceptible) and was sufficient to achieve about 99.2% control for the phosphine-resistant Belle Glade strain with only 4.0 emerged adults compared with 514.3 in control treatment. At 150 and 300 ppm, numbers of emerged adults were significantly less than those of untreated mixed age cultures jars. The results also indicated that all life stages of *L. serricorne* were more tolerant to EDN than *R. dominica*.

Cytochrome *c* Oxidase Activity

The cytochrome *c* oxidase activity in vivo and in vitro is shown in Figs. 1 and 2. The results found no significant differences observed in cytochrome *c* oxidase activity in vivo between treated and untreated insects at different time intervals according to *t*-test. In contrast, ANOVA of in vitro results showed that there were significant differences in the enzyme activity between control (bubbled with air) and treatments (bubbled with EDN concentrations), but there were no significant differences between 0.0076 mM and 0.0038 mM of EDN. Also, the obtained results indicated that cytochrome *c* oxidase activity was decreased by 61.8 and 68.2% in comparison with control treatment at 0.0038 and 0.0076 mM, respectively.

Discussion

The present study showed that EDN caused significant toxicity against all life stages of *L. serricorne* and *R. dominica* and toxicity levels were dependent on insect species, insect strain and life stage. Eggs of both *R. dominica* strains (USDA and Belle Glade) were more susceptible than adults. Our results of EDN toxicity against adults were in agreement with Hooper et al. (2003) who reported that EDN exhibited remarkable toxicity against *R. dominica* adults with LC_{50} value of 74.0 ppm for 6-h exposure period at 25°C. They also mentioned that the minimum tested doses that gave 100 % mortality of *R. dominica* adults and eggs after 24 h of exposure at 25°C were 166.5 and 226.1 ppm. Our results show that adults of *R. dominica* were more tolerant than eggs. However, Hooper et al. (2003) found that *R. dominica* eggs were more tolerant than adults.

Our results on EDN toxicity against *R. dominica* were comparable with or exceeded those of other fumigants evaluated against the same insect. For example, Tan et al. (1999) found that carbonyl sulfide caused 100% mortality of *R. dominica* at 7.28 mg/l for 24 h which indicates it was less effective than EDN for which we estimates

Table 1. Probit analyses for toxicity of EDN concentrations against adults and eggs of phosphine-resistant (Belle Glade) and susceptible (USDA) *Rhyzopertha dominica* after 24-h exposure at 25°C

Strain	LC_{50} (ppm) (95% CLs)	LC_{99} (ppm) (95% CLs)	Slope ± SE	Intercept ± SE	χ^2 (df)	*P*-value
PH_3 susceptible						
Adult	27.7 (26.4–29.0)	73.0 (65.5–83.6)	5.52 ± 0.34	−7.97 ± 0.50	6.05 (13)	0.944
Egg	11.2 (9.6–13.7)	62.2 (39.3–143.5)	3.13 ± 0.30	−3.29 ± 0.30	27.75 (13)	0.010
PH_3 resistant						
Adult	36.0 (34.2–37.9)	108.3 (94.9–127.8)	4.86 ± 0.30	−7.57 ± 0.47	9.39 (13)	0.743
Egg	12.0 (10.9–13.2)	49.7 (39.9–67.3)	3.77 ± 0.32	−4.07 ± 0.36	13.59 (10)	0.192

Table 2. Insecticidal activity of EDN against *Lasioderma serricorne* life stages after 24-h exposure at 25°C

Stage	LC_{50} (ppm) (95% CLs)	LC_{99} (ppm) (95% CLs)	Slope ± SE	Intercept ± SE	χ^2 (df)	*P*-value
Egg	50.4 (46.1–56.0)	177.2 (135.9–266.9)	4.26 ± 0.33	−7.26 ± 0.55	22.66 (13)	0.046
Larvae	446.6 (421.0–473.8)	1327.1 (1141.1–1621.3)	4.92 ± 0.37	−13.03 ± 0.99	10.64 (16)	0.831
Pupae	192.5 (165.8–217.3)	1542.2 (1076.7–2713.3)	2.57 ± 0.30	−5.88 ± 0.73	9.82 (13)	0.709
Adult	160.2 (151.9–167.4)	349.6 (320.8–391.9)	6.86 ± 0.54	−15.13 ± 1.22	12.29 (13)	0.504

Table 3. Number of emerged adults (mean ± SE, *N* = 3) of *L. serricorne* and *R. dominica* strains from the mixed age cultures exposed to different EDN concentrations (ppm) for 24 h at 25°C

L. serricorne					
EDN concentration (target ppm)	Air control	2000	4000	6000	
Average of EDN concentration	Air control	1245 ± 12.8	2247 ± 23.4	3273 ± 13.5	
Emerged adults (mean ± SE)	385.6 ± 67.3a	0.0 ± 0.0b	0.0 ± 0.0b	0.0 ± 0.0b	$F(3,8) = 32.78$, $P < 0.01$
R. dominica (USDA)					
EDN concentration (target ppm)	Air control	150	300	600	
Average of EDN concentration	Air control	104.0 ± 4.4	232.0 ± 5.4	365.0 ± 5.3	
Emerged adults (mean ± SE)	478.2 ± 66.8a	173.0 ± 51.4b	1.3 ± 0.3b	0.0 ± 0.0b	$F(3,8) = 28.50$, $P < 0.01$
R. dominica (Belleglade)					
EDN concentration (target ppm)	Air control	150	300	600	
Average of EDN concentration	Air control	95.0 ± 1.0	215.0 ± 9.9	348.0 ± 10.8	
Emerged adults (mean ± SE)	514.3 ± 16.9a	174.0 ± 5.0b	31.6 ± 9.8c	4.0 ± 1.5c	$F(3,8) = 532.5$, $P < 0.01$

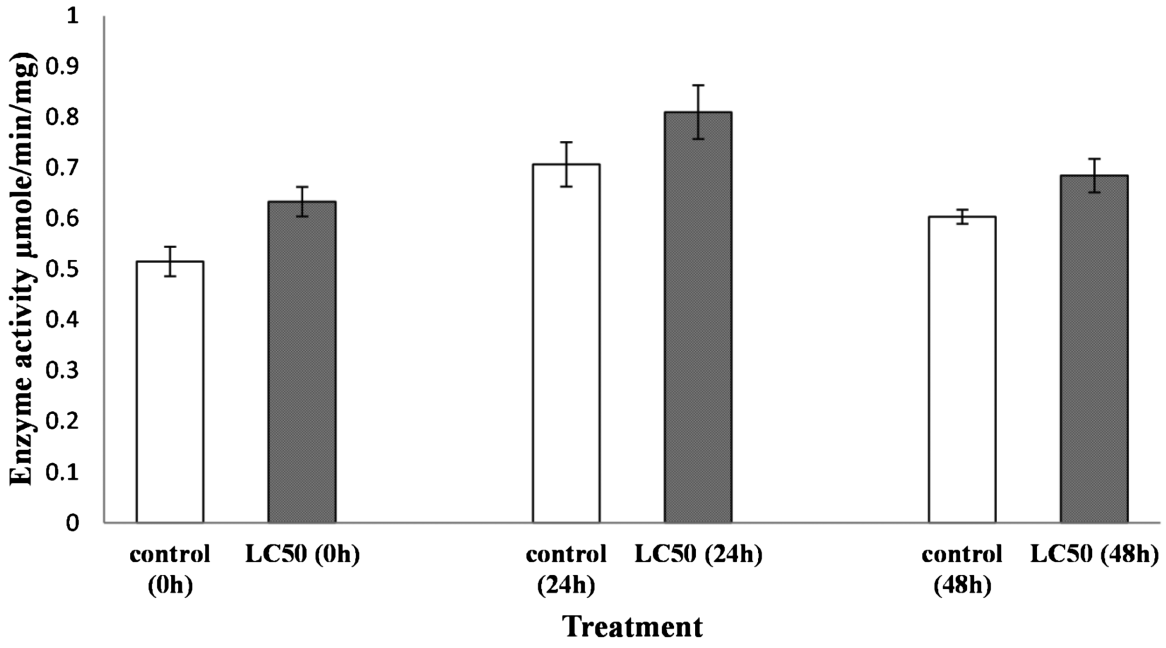

Fig. 1. The specific activity (Mean ± SE, $N = 3$) of cytochrome *c* oxidase of *R. dominica* (USDA strain) from three in vivo experiments. Data followed Student *t* test found no significant difference ($P \geq 0.05$; $n = 3$) between the untreated control and the EDN treatment in any of the time intervals.

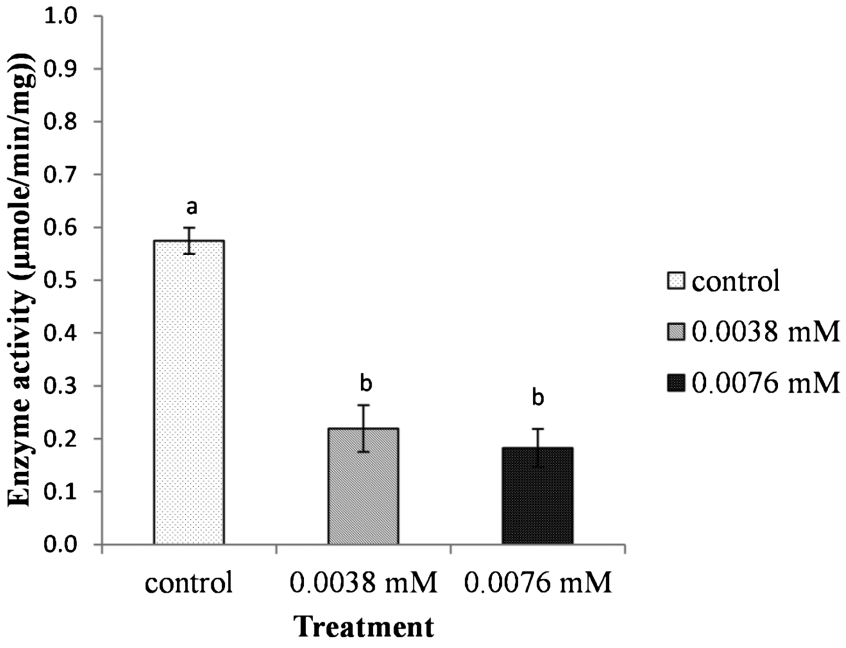

Fig. 2. The specific activity (Mean ± SE, $N = 3$) of cytochrome c oxidase of *R. dominica* (USDA strain) in vitro. Data followed by the same letter show no significant difference ($P \leq 0.05$) according to ANOVA followed by Tukey's HSD test.

an LC_{99} of 108 ppm for phosphine-resistant adults, equivalent to 0.24 mg/liter (following conversion from 462.6 ppm = 1.0 mg/L EDN). Furthermore, our EDN toxicity to phosphine-susceptible *R. dominica* (LC_{95}= 0.12 mg/liter) was much higher than methyl bromide (LC_{95}= 5.5 mg/liter) (Hooper et al. 2003). Jagadeesan et al. (2016) reported that LC_{50} of sulfuryl fluoride against eggs of *R. dominica* was 4.02 mg/liter for 48-h exposure, which was higher than the LC_{50} of EDN against eggs with LC_{50} of 0.024 mg/liter for only 24-h fumigation period, respectively. However, the toxicity of phosphine was higher than EDN against adults of *R. dominica* with LC_{50} of 0.002 mg/liter compared with 0.06 mg/liter for EDN. However, EDN was relatively more efficient against eggs with LC_{50} of 0.024 mg/liter compared with 0.03 mg/liter for phosphine (Rajendran et al. 2001).

To the best of our knowledge, the toxicity of EDN against *L. serricorne* has not been previously reported. Our results showed that EDN offers a promising alternative to phosphine and methyl bromide to control *L. serricorne*. In addition, the susceptibility to EDN varied significantly among life stages of *L. serricorne*. The larvae were the most tolerant stage to EDN, followed by pupae, adults, and eggs. It has been reported that eggs and pupae were the most tolerant stages to most common fumigants, including methyl

bromide (Athanassiou et al. 2015), phosphine (Aulicky et al. 2015), carbonyl sulfide (Plarre and Reichmuth 1997), and sulfuryl fluoride (Athanassiou et al. 2012). In contrast, eggs of both insects were more susceptible to EDN than other life stages. We could relate that to the transformation of EDN to HCN during the fumigation process (Waterford et al. 2004, Brash et al. 2013) and previous work on HCN toxicity against pests indicated that eggs were more susceptible to HCN than other developmental stages. For example, Stejskal et al. (2012) showed that the most tolerant stage of *Tribolium castaneum* (Herbst) (Coleoptera:Tenebrionidae) to HCN was the pupae. Also, Bletchly (1953) indicated that eggs of the wood-infesting pest *Anobium punctatum* (Coleoptera: Anobiidae) were most susceptible to HCN than larvae. This finding is very important because the eggs of most stored product insects were generally the most tolerant stage to the most common fumigants. Our results of EDN toxicity to all life stages of *L. serricorne* compared favorably with other fumigants that have been evaluated before against *L. serricorne*. For example, Maille et al. (2019) reported that LC_{50} values of propylene oxide against *L. serricorne* were 40.98, and 16.04 mg/liter for larvae, and pupae, respectively, which indicated that propylene oxide was less toxic than EDN. Maille (2019) also reported that ethyl formate required 1.88, 15.06, and 16.04 mg/liter for obtaining 50% mortality of adults, larvae and pupae, indicating that EDN was more toxic than ethyl formate to all life stages of *L. serricorne*. In addition, Zettler et al. (1997) reported the toxicity of carbonyl sulfide against *L. serricorne* adults and mentioned that LC_{50} value was 7.72 mg/liter for 24-h exposure, which required higher concentration compared with EDN to achieve the same percent mortality of *L. serricorne* adults. Gautam et al. (2014) reported LC_{50} of mixture of propylene oxide (PPO) and CO_2 (8% PPO: 92% CO_2) on *L. serricorne* eggs to be 2.96 mg/liter after 24-h fumigation, which indicated that EDN was more toxic than PPO. Also, the mixed life stage of *L. serricorne* required 45.0 mg/liter to induce 100.0% mortality after 48-h exposure (Zettler et al. 2003) compared with 4.3 mg/liter for EDN after only 24-h fumigation. In addition, EDN was more efficient than sulfuryl fluoride (SF), since the LC_{50} value of SF were 16.9, 1.83, and 0.88 mg/liter for eggs, larvae, and adults, respectively (Su et al. 1990). Moreover, ethyl formate (EF) was less toxic to *L. serricorne* life stages than EDN, since the LC_{50} value of EF were 31.4, 49.9, and 78.9 mg/liter for adults, pupae, and larvae, respectively (Asimah et al. 2014). On contrary, the toxicity of phosphine to *L. serricorne* adults was about 9.0 times more toxic than EDN with LC_{50} value of 17.8 ppm (Fukazawa et al. 2017).

EDN has been described to exhibit remarkable toxicity against a wide range of target pests. For example, Hooper et al. (2003) reported that the minimum concentrations of EDN to kill 100 % of *Sitophilus granarius* and *S. oryzae* (Coleoptera: Curculionidae) adults were 1.0 and 0.81 mg/liter. They have also reported that *T. castaneum* required 1.1, 0.97, and 1.2 mg/liter for eggs, adults, and larvae, respectively, to achieve 100% mortality, whereas *T. confusum* required 1.3, 0.67, and 1.2 mg/liter for eggs, adults, and larvae, respectively, to achieve complete mortality. Also, EDN possessed a great potential as methyl bromide alternative for timber and soil pest disinfestation. Pranamornkith et al. (2014) demonstrated that EDN showed promising control for the burnt pine longhorn beetle *Arhopalus ferus* (Mulsant) (Coleoptera: Cerambycidae) adult with LC_{99} value of 12.6 mg/liter at 15 C° for 3-h exposure. Moreover, EDN was highly effective against *Anopolophora glabripennis* (Mootchulsky) (Coleoptera: Cerambycidae) larvae which required 4.9 mg/liter at 21.1°C after 6-h exposure (Ren et al. 2006). Lee et al. (2017) have reported that EDN had the potential to replace methyl bromide and metam sodium to control *Monochamus alternatus* (Coleoptera: Cerambycidae) larvae in logs with LC_{50} value of 73.19 mg h/liter.

To the best of our knowledge, there have been no studies reported before on the impact of EDN on cytochrome *c* oxidase activity. The results of cytochrome *c* oxidase activity of *R. dominica* (USDA strain) showed that EDN inhibited the enzyme activity in vitro at both tested concentrations comparing to control treatment and no inhibition was observed in the case of the in vivo assay. These results indicated that cytochrome *c* oxidase may not be the main target for EDN toxicity on insects and further studies are needed to explore the possible modes of toxic action of this gas. Similar findings were observed on inhibitory effects of other fumigants on activity of cytochrome *c* oxidase. For example, Price and Dance (1983) reported that phosphine inhibited cytochrome *c* oxidase activity of *R. dominica* in vitro and it did not inhibit the enzyme in vivo. Also, Jian et al. (2000) found that the inhibition rate of cytochrome c oxidase of *Tyrophagus putrescentiae* (Sarcoptiformes: Acaridae) in vivo was lower than that of in vitro.

In conclusion, based on the results of this study, we suggest that EDN has the potential as a promising alternative to methyl bromide and phosphine to control *L. serricorne* and *R. dominica* in stored products. Nevertheless, additional studies are required, including the following: 1) sorption and desorption of EDN by different commodities to compensate the decrease in the fumigant concentration during the exposure period by applying the gas at a higher initial dose to achieve satisfactory fumigation; 2) estimation of EDN residues in different fumigated commodities, to study the effect of residues on mammalian consumption and to determine the acceptable limit of EDN in the fumigated commodities; and 3) study the effect of EDN on the quality of treated commodities.

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