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Effects of Nitric Oxide Fumigation on Mortality of Light Brown Apple Moth, *Epiphyas postvittana* (Lepidoptera: Tortricidae)*

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Abstract

Light brown apple moth, *Epiphyas postvittana*, is a quarantine pest in most countries and has established in California and, therefore, has potential to affect export of fresh products from infested areas. There is currently lack of effective treatment especially against eggs. Nitric oxide is a recently discovered fumigant for postharvest pest control and is evaluated in laboratory fumigations to determine its efficacy against different life stages of this pest. Small scale fumigations with nitric oxide at 1.0%, 2.0%, 3.0%, and 5.0% concentrations were conducted under ultralow oxygen conditions at 2˚C against larvae, pupae, and eggs of light brown apple moth. Treatment times ranged from 4 to 24 h depending on nitric oxide concentration and life stage. Complete control of larvae and pupae was achieved in 8 h fumigation with 2.0% NO. Eggs were successfully controlled in 6, 12, and 24 h fumigations with 5.0%, 3.0%, and 2.0% NO respectively. The study demonstrated that nitric oxide fumigation was effective against all life stages of light brown apple moth and, therefore, has potential to be an alternative treatment to methyl bromide fumigation for postharvest control of light brown apple moth.

Keywords

Quarantine Treatment, Nitric Oxide, Fumigation, Light Brown Apple Moth

1. Introduction

Light brown apple moth (LBAM), *Epiphyas postvittana* (Lepidoptera: Tortrici-

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dae), has established in California since its early detection over 10 years ago and could spread to other regions. This pest has a very broad host range of over 500 plant species in >100 families [1] [2] [3]. LBAM has been reported to cause significant losses of crop production [4] [5]. Although LBAM infestations in California mainly occur on nursery plants, it has potential to be a pest on many other crops. LBAM has a very limited distribution and is a quarantine pest in most countries. Fresh products from infested areas, therefore, face both domestic and international quarantine [1] [6]. A postharvest treatment for LBAM control is needed to meet the need of a quarantine treatment for LBAM control on affected fresh commodities.

The current treatment with methyl bromide fumigation is unsustainable due to the global phase out of methyl bromide production and uncertainty of the critical use exception for methyl bromide in the future. Currently, phosphine and sulfuryl fluoride are two major alternative fumigants for postharvest pest control. However, phosphine fumigation typically has long treatment time and treatments of over 10 days may be required to control some stored product insects [7] [8]. Some insects especially at egg or pupa stages have high tolerance to phosphine fumigation and some insects have developed resistance to phosphine [9] [10]. Sulfuryl fluoride fumigation is not effective in controlling insect eggs and is phytotoxic to fresh products [11] [12]. In recent years, ethyl formate fumigation has been tested and used on selective fresh products for postharvest control of some specific pests. But it may have limited applications on fresh products due to its phytotoxicity to most fresh products [13]. Therefore, new alternatives are needed for postharvest control of many pests including LBAM on international traded fresh products. Nitric oxide was recently discovered to be a potent fumigant against a wide variety of insect pests at various life stages and mites [14] [15] but no detailed toxicity data on LBAM has been reported.

Nitric oxide is a ubiquitous cell signal molecule in most organisms including microbes, plants, and animals. It is also produced naturally in fossil fuel combustion and lightning, and manufactured for fertilizer production. It is used in medical fields to treat certain respiratory and cardio vascular conditions [16] [17]. It is also being studied for enhancing postharvest quality and prolonging shelf-life of fresh fruits [18] [19].

Nitric oxide reacts spontaneously with oxygen to produce nitrogen dioxide (NO_2) [20]. Therefore, NO fumigation must be conducted under ultralow oxygen (ULO) conditions to preserve NO. As a fumigant, nitric oxide can kill insects in as short as 2 h at a high concentration of 2% - 5% [14]. The efficacy increases with concentration, treatment time, and temperature. There are also variations in susceptibility among different insect species and life stages [14]. Eggs are more tolerant of NO fumigation than mobile stages. Small soft body insects such as aphid and thrips are very easy to control and can be controlled in 2 - 3 h at low temperatures. Stored product insects such as weevils and beetles are more tolerant but can still be controlled in 72 h with 2% - 3% NO fumigation

[14]. In addition to pest control, NO fumigation can also have desired levels of NO_2 for microbial control by controlling oxygen level before NO injection and NO_2 has been demonstrated to be effective in controlling *Aspergillus flavus* spores on artificial medium and bacteria and fungi on stored almonds and peanuts [15] [21] [22] [23].

Nitric oxide fumigation for fresh products also needs to be terminated by flushing the fumigation chamber with nitrogen to dilute NO before exposing products to ambient air to prevent reaction of NO with O_2 to produce NO_2. The production of NO_2 may cause injuries to fresh products [24]. When terminated properly with N_2 flush, NO fumigation was demonstrated to be safe to postharvest quality of fresh products and even improve postharvest quality as demonstrated on strawberries [24]. In this study, LBAM eggs, larvae, and pupae were fumigated with NO to determine an effective treatment against all of the life stages.

2. Materials and Methods

2.1. Insects

E. postvittana was collected from Santa Cruz County in California in 2007 and reared on artificial pink bollworm diet at the U.S. Department of Agriculture-Animal and Plant Health Inspection Services-Plant Protection and Quarantine-Center for Plant Health Science and Technology (USDA-APHIS-PPQ-CPHST) laboratory in Salinas, California. Moths were held in cages and fed with 10% sucrose solution supplied in saturated cotton rods. Corrugated wax papers were lined along the walls of the cages as substrates to collect eggs. Egg sheets were collected within 24 h and used for fumigation experiments immediately. They were cut into pieces and each piece contained 100 - 200 eggs based on visual estimation. Each piece of egg sheet was placed in a plastic vial (3 cm diam. × 7 cm high). A piece of yellow sticky card was also suspended in each vial to catch neonates after they hatched from surviving eggs.

2.2. Chemicals

Nitric oxide (>99.5% purity) in a compressed cylinder was obtained from a commercial source. It was then released and stored in a foil bag to be used in fumigations tests. Commercial grade nitrogen gas in compressed cylinders from a commercial source was used.

2.3. Effects of Nitric Oxide Fumigation Treatments on Mortality of Eggs

As it is known that insect eggs are more tolerant to NO fumigation than other life stages based on the earlier study [14], LBAM eggs were fumigated first to determine effective treatments than larvae and pupae. LBAM eggs were fumigated with 1.0%, 2.0%, 3.0%, and 5.0% NO in 1.9L jars for 6, 12, and 24 h to determine effective treatments. Egg sheets with <48 h old eggs were cut into pieces each containing 100 - 200 eggs based on visual estimation and placed individually in plastic vials (3 cm diam. × 7 cm high). A piece of yellow sticky card was also suspended in each vial to catch neonates hatched from surviving eggs. In each fumigation test, four vials with eggs in each jar were fumigated with each NO concentration for one treatment time and unfumigated eggs were also included as controls. The fumigation test for each treatment time was replicated four times.

After fumigation, vials with LBAM eggs were incubated in an environmental chamber for over two weeks to allow all viable eggs to hatch and neonates trapped on sticky cards. The sticky cards were then inspected to count all trapped neonates to estimate egg mortality. We assumed that each vial had the same number of eggs and treatment mortality was calculated based on neonates for each treatment and neonates for the controls.

2.4. Effects of Nitric Oxide Fumigation Treatments on Mortality of Larvae and Pupae

LBAM larvae and pupae were fumigated with 2.0% NO in 1.9 L jars under ULO condition of ≤30 ppm O_2 at 2˚C for 4, 6, 8, and 12 h to determine their susceptibility to NO fumigation. The 2.0% NO was selected based on its effects on LBAM eggs in the above experiment. Larvae of medium to large sizes were collected from rearing media in a group of 10 in small screened cages with small amount of diet. Pupae were collected and confined in a group of 10 in screened cages without diet. In each fumigation test, five vials with larvae and five vials with pupae were placed in each jar for each treatment time. Each treatment time was replicated 5 - 10 times. After fumigation, larvae and pupae were incubated in an environmental chamber overnight before being evaluated for mortality. Larvae and pupae that were unresponsive to probes with a pin were classified as dead.

2.5. Data Analysis

Mortalities for each treatment time and each life stage were transformed by arcsine $\sqrt{x}$ before being subjected to one-way ANOVA and Tukey HSD multiple range test to compare mortalities among different NO concentrations. The fit model platform of JMP statistical discovery software was used for all statistical analyses [25].

3. Results and Discussion

Nitric oxide fumigation was effective against larvae, pupae, and eggs of LBAM. Complete control of eggs was achieved in the 6 h with 5.0% NO, 12 h with 3.0% NO, and 24 h with 2.0% NO treatments at 2˚C (**Table 1**). Larvae and pupae were more susceptible to NO fumigation than eggs and complete control of larvae and pupae was achieved in the 8 h fumigation with 2.0% NO at the same low temperature (**Table 2**). In the 6 h fumigations, 2.0% NO treatment had 26.7% egg

Table 1. Effects of nitric oxide fumigations with different combinations of dose and treatment time on mortality of light brown apple moth eggs.

Time (h)	NO (%)	Total	Mortality (%) (Mean ± SE)	ANOVA
6	1.0	1808	20.07 ± 2.43b	Df = 5, 114
	2.0	1527	26.70 ± 3.76b	F = 153.58
	3.0	1938	93.87 ± 3.62a	P < 0.0001
	5.0	2000	100a	
	C-ULO	1690	19.74 ± 3.61b	
	Control	1820	21.35 ± 3.38b	
12	1.0	1255	62.79 ± 8.49b	Df = 5, 90
	2.0	1567	99.94 ± 0.06a	F = 100.13
	3.0	1571	100a	F < 0.0001
	5.0	1566	100a	
	C-ULO	1174	18.55 ± 4.20c	
	Control	1141	16.60 ± 3.35c	
24	1.0	1181	54.38 ± 6.60b	Df = 5, 84
	2.0	1511	100a	F = 189.90
	3.0	1520	100a	P < 0.0001
	5.0	1496	100a	
	C-ULO	1187	14.83 ± 2.95c	
	Control	1197	13.20 ± 3.39c	

Mortality data were transformed by arcsine $\sqrt{x}$ prior to statistical analysis. For each treatment time, mortalities followed by the same letter were not significantly different at P > 0.05 based on Tukey HSD multiple range test using JMP Statistical Discover Software (SAS Institute 2012).

mortality. In comparison, the same treatment resulted in >99% mortality of larvae and pupae (Table 1 and Table 2). There were no significant differences in mortality between the two types of controls, normal atmosphere and ULO atmosphere (Table 1 and Table 2).

The responses of different life stages of LBAM to NO fumigation are consistent with responses of other insects with mobile life stages more susceptible and eggs most tolerant [14]. Susceptibility of LBAM to NO fumigation is higher as compared with stored product insects and internal feeding insects such as codling moth larvae [14] [26].

It is challenging to find an effective alternative fumigant to control LBAM especially eggs. Cylindered pure phosphine has been used for postharvest pest control on fresh products in recent years [27] [28] [29]. It was also studied for control of LBAM on fresh products. Phosphine fumigation is not very effective

Table 2. Effects of nitric oxide fumigation on mortality of light brown apple moth larvae and pupae.

NO (%)	Time (h)	Larvae		Pupae	
		Total	Mortality (%) (Mean ± SE)	Total	Mortality (%) (Mean ± SE)
2.0	4	300	92.68 ± 1.79b	290	91.00 ± 2.11b
	6	400	99.75 ± 0.25a	398	99.75 ± 0.25a
	8	501	100a	498	100a
	12	250	100a	251	100a
C-ULO		652	4.87 ± 1.15c	638	13.15 ± 2.25c
Control		646	2.18 ± 0.99c	615	9.60 ± 1.79c
ANOVA		Df = 5, 219 F = 1289.244 P < 0.0001		Df = 5, 219 F = 593.372 P< 0.0001	

Mortality data were transformed by arcsine $\sqrt{x}$ prior to statistical analysis. For each treatment time, mortalities followed by the same letter were not significantly different at P > 0.05 based on Tukey HSD multiple range test using JMP Statistical Discover Software (SAS Institute 2012).

against LBAM eggs. Oxygenated phosphine fumigation (fumigation under high oxygen conditions) is more effective than regular phosphine fumigation but still takes 72 h fumigation with high phosphine concentrations to have a complete control of LBAM eggs [30] [31]. The effective control of LBAM larvae, pupae, and eggs suggests that NO fumigation has better efficacy than phosphine fumigation to control LBAM. Therefore, NO fumigation as demonstrated in this study is the only fumigation treatment that is effective against LBAM eggs.

In an earlier study, ten fresh fruit and vegetables were subjected to 4 h fumigations with 1.0% NO at 2˚C. NO fumigations that were terminated by N_2 flush to prevent NO reaction with O_2 to form NO_2 were safe to all of ten fresh fruit and vegetables [24]. Iceberg lettuce was also fumigated with 0.5% NO for 16 h to control lettuce aphid and the treatment was safe to lettuce quality [32]. Strawberries were also fumigated with 3.0% NO for 16 h for control of spotted wing drosophila and the treatment had no negative impact on strawberry quality [33]. In the current study, the 6 and 12 h fumigations with 5% and 3% NO that had complete control of LBAM eggs had a lower CxT product than the 16 h and 3.0% NO fumigation for strawberries [33]. Therefore, the treatments for LBAM eggs are expected to be safe to those previously tested fresh products. LBAM has very broad host range and past infestations mainly occurred on nursery plants [2] [3]. In the past NO fumigation studies, harvested fresh products that had been subjected to cold storage were subjected to NO fumigations [24] [27] [32] [33]. During NO fumigation, there is no oxygen and fresh products are subjected to extreme hypoxia stress. Harvested fresh products that have been subjected to cold storage have slower metabolism and less requirement for oxygen [34] [35]. Therefore, they are expected to be more tolerant to NO fumigation as compared with live plants with active metabolism. For dormant nursery products such as flower bulbs, NO fumigation that is capable to control LBAM has been demonstrated to be safe in an earlier study [36]. However, it is unknow whether NO fumigation is safe to live nursery plants especially those valued for cosmetic appearances and more research is warranted.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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