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Controlled atmosphere treatment for control of grape mealybug, *Pseudococcus maritimus* (Ehrhorn) (Hemiptera: Pseudococcidae), on harvested table grapes

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ABSTRACT

Controlled atmosphere (CA) treatments with ultralow oxygen (ULO) alone and in combinations with 50% carbon dioxide were studied to control grape mealybug, *Pseudococcus maritimus* (Ehrhorn) on harvested table grapes. Two ultralow oxygen levels, 30 and <0.01 $\mu L\,L^{-1}$, were tested in both ULO and ULO + 50% CO_2 treatments. The ULO treatments with the lower oxygen level were more effective than the ULO treatments at the higher oxygen level. The ULO + 50% CO_2 treatments were more effective than the ULO treatments. Grape mealybug eggs were significantly more tolerant of ULO and ULO + CO_2 treatments than nymphs and adults. A 14 day ULO treatment with 30 $\mu L\,L^{-1}$ O_2 at 2 °C did not achieve 100% mortalities of any life stage. In the presence of 50% CO_2, the 14 d treatment achieved complete mortality of all life stages of the grape mealybug. A 3 d ULO treatment with <0.01 $\mu L\,L^{-1}$ O_2 at 2 °C resulted in 93.3% mortality of nymphs and adults. The 3 d ULO treatment in combination with 50% CO_2 treatments, however, achieved complete control of grape mealybug nymphs and adults and caused 70.5% relative egg mortality. Complete egg mortality was achieved in a 10 d ULO + 50% CO_2 treatment with <0.01 $\mu L\,L^{-1}$ O_2 at 2 °C. Both the 14 d CA treatment with 30 $\mu L\,L^{-1}$ O_2 and 50% CO_2 and the 10 d CA treatment with <0.01 $\mu L\,L^{-1}$ O_2 and 50% CO_2 were tested on table grapes and grape quality was evaluated after two weeks of post-treatment storage. The CA treatments did not have a significant negative impact on grape quality and were safe for table grapes. The study indicated that CA treatments have potential to be developed for postharvest control of grape mealybug on harvested table grapes.

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1. Introduction

Grape mealybug, *Pseudococcus maritimus* (Ehrhorn) (Hemiptera: Pseudococcidae), is a quarantined pest on exported Californian table grapes for overseas markets such as Australia (AQIS, 2000). The insect has the potential to cause significant damage to grape production and economic losses. It not only damages plants through feeding and secretion of sugary deposits on which mold grows, but also transmits grape leafroll virus (Golino et al., 2002). Grape mealybug develops two generations per year and overwinters as eggs or crawlers. In the spring, crawlers move onto expanding green tissues and mature during May and June. Females then return to old wood to lay eggs. The eggs hatch during June and July, and most crawlers move to feed on fruit and foliage. It is the second brood that causes most of the fruit damage. During August and September, females are often found on the fruit and can lay eggs in berry clusters (Flaherty et al., 1992; Varela, 2005).

Grape mealybug on exported table grapes is currently controlled with methyl bromide fumigation. However, such reliance on methyl bromide fumigation will likely be unsustainable because of the global phase out of methyl bromide production as mandated by the Montréal Protocol. Methyl bromide fumigation also reduces grape quality as stored grapes need to be warmed up to 15.6–20.6 °C for methyl bromide fumigation (Mitcham et al., 2005). Therefore, developing a safe and effective alternative control for the pest is important for retaining and expanding export markets of U.S. table grapes.

Controlled atmosphere (CA) treatments have been studied for the last 30 years for postharvest pest control with various outcomes (Carpenter and Potter, 1994; Mitcham et al., 2001; Liu, 2010). CA treatment for pest control typically uses low oxygen and/or increased carbon dioxide. There can be synergistic effects between reduced O_2 and elevated CO_2 on insect mortality (Calderon and Navarro, 1979). However, antagonistic effects between elevated

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CO_2 and reduced O_2 on insect mortality were also observed (Ali-Niazee, 1971; Mitcham et al., 1997). Grape mealybug subjected to a 13 d treatment with 45% CO_2 at 0 °C only achieved 60% (mortality Mitcham, 2003). More recent studies indicated that CA treatments with ultralow oxygen (ULO) levels of $\leq$30 $\mu L\,L^{-1}$ O_2 were effective against some insects and successful controls of several pests were achieved on perishable commodities (Liu, 2007, 2008a, 2008b, 2010, 2011, 2012; Liu et al., 2008, 2010). A ULO treatment was also developed to control vine mealybug (*Planococcus ficus*) on grape benchgrafts (Liu et al., 2008). The lower oxygen levels correspond with increased insect mortalities as demonstrated with western flower thrips (Liu, 2008a). In this study, ULO treatments with 30 $\mu L\,L^{-1}$ and <0.01 $\mu L\,L^{-1}$ O_2 alone and in combinations with 50% CO_2 were tested on all life stages of grape mealybug to determine effects on insect survival and postharvest quality of table grapes.

2. Materials and methods

2.1. Insect

Grape mealybug was reared on potted grape plants in a greenhouse at 10–30 °C under natural lighting. Grape plants were grown in potting soil in 18.9 liter containers and were watered and fertilized periodically. Leaves and vines infested with grape mealybug were removed and sealed in 0.95 liter glass jars with paper towels for treatment. Grape mealybug egg sacs were collected from the grape plants and placed on Kim-wipe tissue. Eggs in the egg sacs were mixed in cotton-like secretion and therefore, could not be accurately counted without causing damage to eggs. Instead, egg sacs were open and inspected to estimate numbers of eggs in each egg sac and divided into groups with about the same number of eggs. The groups were randomly assigned to treatments, and each group had 2–3 egg sacs on a piece of Kim-wipe tissue. Each group of egg sacs was placed in a plastic vial and sealed with a screened lid lined with Kim-wipe tissue for treatment.

2.2. Treatment procedures

Grape mealybug nymphs, adults, and eggs were subjected to both ULO treatments and ULO plus 50% CO_2 treatments for different durations at 2 °C. Two oxygen levels were used: 30 $\mu L\,L^{-1}$ and <0.01 $\mu L\,L^{-1}$. ULO and ULO + CO_2 treatments with 30 $\mu L\,L^{-1}$ oxygen level were conducted in box chambers (56 cm × 41 cm × 25 cm). ULO and ULO + CO_2 treatments with <0.01 $\mu L\,L^{-1}$ oxygen were conducted in 7.6 liter chambers modified from pressure cookers. All treatment chambers were held in a temperature cabinet equipped with a circulation fan and an external temperature controller to maintain accurate temperatures (±0.5 °C).

For the ULO and ULO + CO_2 treatments with 30 $\mu L\,L^{-1}$ O_2, table grapes of 4 different varieties were included in some of tests and treatment duration was 14 d based on results from preliminary tests (Liu unpublished). The ULO treatment was established and maintained using the same procedures as described by Liu et al. (2010). The chambers were first flushed with bottled nitrogen gas. The oxygen level in the chamber was monitored continuously using an oxygen analyzer (Series 800, Illinois Instruments, Johnsonburg, IL). Once O_2 level reached 30 $\mu L\,L^{-1}$, N_2 was released into the chamber at a reduced rate of 8 mL s^{-1} continuously. When the oxygen level fell below the threshold of 30 $\mu L\,L^{-1}$ (30 ppm), generated nitrogen with about 0.2% (2 mL L^{-1}) O_2 from a nitrogen generator (Balston 7507820, Parker Hannifin Co., Tewksbury, MA) was added to the inlet gas stream at a flow rate of 2 mL s^{-1} controlled by a digital flow controller (16 Series, Alicat Scientific, Tucson, AZ) and a relay alarm of the oxygen analyzer. A circulation fan was also used in the treatment chamber to circulate air continuously. CO_2 gas was metered and released into the chamber to maintain 50% CO_2 in the ULO + CO_2 treatment using a second digital flow controller. CO_2 levels in the chambers were monitored periodically with a CO_2 analyzer (CO_2 Analyzer 2820, Bacharach, New Kensington, PA). Each treatment was replicated 7–12 times.

The four table grape varieties were obtained from supermarkets and identified by PLU#. They were: PLU# 4022 (Thompson seedless), PLU# 4023 (Red seedless), PLU# 4056 (Black seedless), and PLU# 4499 (Crimson-Majestic/Red seedless). Grapes were screened to remove berries with obvious defects such as decay, skin defects, or discoloration and divided into groups based the number of treatments. They were placed in perforated plastic bags and all varieties for each treatment were then held in a paper bag and placed in a treatment chamber. The control group was stored at 1 °C in a cooler during the treatment.

For the ULO and ULO + CO_2 treatments with <0.01 $\mu L\,L^{-1}$ O_2, treatment durations for grape mealybug nymphs and adults were 1, 2, and 3 d. Only ULO + CO_2 treatment was tested against eggs and treatment durations for eggs were 3, 5, 7, and 10 d. In each test, 3–4 chambers modified from 7.6 liter pressure cookers were connected in series, and the ULO condition was established by flushing the chambers with pure nitrogen and maintained by continuously flowing pure nitrogen at 8 mL s^{-1} using a digital flow controller. The ULO + CO_2 treatment was maintained by flowing both N_2 and CO_2 at 4 mL s^{-1} using two digital flow controllers into the chambers. Treatments of different durations were accomplished by disconnecting one chamber from the end of the series at the end of each treatment. Separate ULO + CO_2 treatments of 7 and 10 d were conducted to confirm the control of grape mealybug eggs. Each of the 1, 2, and 3 d treatments for mealybug nymphs and adults was replicated 2–12 times. The 3 and 5 d treatments for eggs were replicated 3 times, and the 7 and 10 d treatments were replicated 10 times. The 10 d ULO + CO_2 treatment was further conducted on grape mealybug eggs and table grapes and replicated 3 times. Two varieties of table grapes were tested: PLU# 4022 (Thompson seedless) and PLU# 4499 (Red seedless).

At the end of each treatment, mealybug nymphs and adults in glass jars were held at 15 °C overnight in an environmental chamber and then scored for morality under a microscope. For eggs, yellow sticky cards were placed in the vials after each treatment to catch crawlers hatched from surviving eggs. By placing the yellow cards after treatment, only crawlers hatched from surviving eggs after treatment were caught. If there were crawlers mixed with eggs before treatment, they would have been killed by the ULO treatments as they were much more susceptible to ULO treatment than eggs and would not be caught on the yellow sticky cards placed at the end of a treatment. After treatment, eggs were held in an environmental chamber at 22 °C and 95% RH and 14:10 (L:D) photoperiod for $\geq$4 weeks. Then, the yellow cards were inspected under a microscope to count crawlers to determine egg survivorship.

Grape quality was evaluated after two weeks of post-treatment storage at 1 °C. The grapes were scored for overall quality, berry softness, decay, and discoloration. Total numbers of berries tested and marketable berries for each variety were counted for treatments and controls. The overall quality ranged from 1 (unmarketable) to 4 (perfect) with 2 and 3 representing poor to medium and good. The levels for berry decay and discoloration ranged from 0 (no defect) to 3 (severe) and 1 and 2 represented slight and moderate levels, using the method from Liu et al. (2008).

2.3. Data analysis

The relative mortality rates of grape mealybug eggs were calculated based on egg survivorship from the control and the treatments as indicated by the numbers of crawlers caught on the yellow sticky cards. Numbers of eggs from all treatments were assumed to be

Table 1
Responses of grape mealybug nymphs and adults to ULO and ULO + 50% CO_2 treatments with different levels of oxygen at 2 °C.

O_2 level ($\mu L L^{-1}$)	Treatment	Time (days)	Rep	N	Mortality rate (%) (Mean ± SE)
30	ULO	14	7	8577	99.9 ± 0.1a
	ULO + CO_2	14	6	3901	100a
	Control		5	1504	12.3 ± 8.3b
<0.01	ULO	2	4	2108	69.6 ± 19.6b
		3	4	1433	93.3 ± 3.8ab
	ULO + CO_2	1	2	749	96.1 ± 3.9ab
		2	10	3707	99.3 ± 0.5a
		3	12	3885	100a
	Control		8	2860	3.9 ± 1.1c

The mortality rates were transformed by arcsine$\sqrt{x}$ prior to statistical analysis. For each oxygen level, mortality rates followed by different letters were significantly different based on Tukey HSD multiple range test using JMP statistical discovery software ($P \leq 0.05$, SAS Institute, 2008).

equal since counting eggs were not practical as eggs were mixed in cotton-like secretion. The relative egg mortalities and mortalities of nymphs and adults were transformed by arcsine$\sqrt{x}$ prior to statistical analysis. Analysis of variance and Tukey HSD multiple range test were used to analyze insect mortality and compare mortalities among the treatments. Grape quality parameters were compared between the treatment and the control using *t*-test. The one-way platform of JMP statistical discovery software was used for all statistical analysis (SAS Institute, 2008).

3. Results

ULO + CO_2 treatments were effective and safe to control grape mealybug on table grapes. Under 30 $\mu L L^{-1}$ O_2, the 14 d ULO and ULO + 50% CO_2 treatment at 2 °C resulted in over 99 and 100% mortalities of nymphs and adults, respectively, and the mortalities were not significantly different (Table 1). Under the reduced oxygen level of <0.01 $\mu L L^{-1}$, mortalities of grape mealybug nymphs and adults increased from 69.6% in the 2 d ULO treatment to 93.3% in the 3 d ULO treatment at 2 °C. The ULO plus 50% CO_2 treatments with <0.01 $\mu L L^{-1}$ O_2 were significantly more effective than the ULO treatments without added CO_2 against grape mealybug nymphs and adults. Mortalities of nymphs and adults increased from 96.1% in the 1 d ULO + 50% CO_2 treatment to 99.3% in the 2 d treatment. Complete control of grape mealybug nymphs and adults was achieved with the 3 d ULO plus 50% CO_2 with <0.01 $\mu L L^{-1}$ O_2 at 2 °C. There were significant differences among the different treatments of ULO and ULO + 50% CO_2 in mortality of nymphs and adults (Table 1).

Eggs of grape mealybug had 90.7% mortality in the 14 d ULO treatment with 30 $\mu L L^{-1}$ O_2. The 14 d ULO + 50% CO_2 treatment with 30 $\mu L L^{-1}$ O_2 achieved 100% egg mortality. Differences in egg mortality were significant between the treatments (Table 2). Under <0.01 $\mu L L^{-1}$ O_2, the 3 d ULO + 50% CO_2 caused 70.5% mortality of grape mealybug eggs and mortalities increased significantly to 94.0 and 99.5% with increased treatment times of 5 and 7 d. Complete egg mortality was achieved in the 10 d ULO + CO_2 treatment. Only ULO + CO_2 treatments with <0.01 $\mu L L^{-1}$ O_2 were tested against grape mealybug eggs because they were more effective than ULO treatments (Table 2).

Both the 14 d ULO + CO_2 treatment with 30 $\mu L L^{-1}$ O_2 and the 10 d ULO + CO_2 treatment with <0.01 $\mu L L^{-1}$ O_2 which controlled all life stages of grape mealybug were safe to table grape quality (Table 3). There was no significant difference between the treatment and the control in visual quality score, percentage of marketable fruit, decay level, or discoloration for any grape variety. No discoloration was evaluated for red grapes. In general, the overall grape quality scores varied between 2.3 and 3.5 among the four varieties. Marketable berries varied between 40.8 and 88.5% and the variations mainly existed among different cultivars. Decay was minimal to none with scores of 0–1.3. Discolorations were also minimal in most cases with scores of between 1.0 and 2.2 (Table 3).

4. Discussion

Grape mealybug is a major pest on table grapes and affects U.S. table grape export to overseas markets where it is quarantined. The results of the current study suggest that it is feasible to control grape mealybug on table grapes using a controlled atmosphere treatment. This was also the first study to demonstrate that a CA treatment was safe and effective in controlling grape mealybug on harvested table grapes.

Nymphs and adults of grape mealybug were much more susceptible than eggs to the CA treatments and were controlled with ULO at <0.01 $\mu L L^{-1}$ plus 50% CO_2 in 3 d at 2 °C. The complete control of eggs was achieved in 10 d. Grape mealybug develops two generations per year: an overwinter generation and a summer generation. Only females from the summer generation lay eggs on fruit in late August and September (Flaherty et al., 1992; Varela, 2005). Therefore, grapes harvested before August are free of grape mealybug eggs and the 3 d CA treatment would be sufficient to control grape mealybug on table grapes harvest before August. Grapes harvested in the fall, however, would need the 10 d ULO + CO_2 treatment to kill grape mealybug eggs.

CA has been studied for postharvest pest control for many years (Mitcham et al., 2001). However, most early studies did not yield satisfactory results due to either incomplete control of target pests or/and injuries to the subject commodities. Most of those studies employed CA treatments with ≥0.02% (200 $\mu L L^{-1}$ or 200 ppm) O_2. In more recent studies, CA treatments with ≤30 $\mu L L^{-1}$ (0.003% or 30 ppm) O_2 were demonstrated to be effective and safe to control several pests on perishable commodities (Liu, 2010). Oxygen concentrations have significant effects on the effectiveness of

Table 2
Responses of grape mealybug eggs to ULO and ULO + 50% CO_2 treatments with different levels of oxygen at 2 °C.

O_2 level ($\mu L L^{-1}$)	Treatment	Time (days)	Rep (Vial)	Number of crawlers		Relative mortality (%) (Mean ± SE)
				Observed	Expected	
30	ULO	14	7 (21)	154	1684	90.7 ± 2.9b
	ULO + CO_2	14	3 (8)	0	642	100a
	Control		7 (16)	1283	1283	–
<0.01	ULO + CO_2	3	3 (12)	318	974	70.5 ± 4.8c
		5	3 (12)	69	974	94.0 ± 1.8b
		7	7 (21)	12	1705	99.5 ± 0.2a
		10	10 (34)	0	2761	100a
	Control		10 (30)	2436	2436	–

Relative mortalities of eggs were calculated from the numbers of expected crawlers and the actual numbers of crawlers from treated eggs. The mortality rates were transformed by arcsine$\sqrt{x}$ prior to statistical analysis. For each oxygen level, egg mortalities followed by different letters were significantly different based on Tukey HSD multiple range test using JMP statistical discovery software ($P \leq 0.05$, SAS Institute, 2008).

Table 3
Effects of combinations of ULO and 50% CO_2 with 30 and <0.01 $\mu L L^{-1}$ O_2 at 2 °C on the quality of table grapes after 2 weeks of post-treatment storage at 1 °C.

Experiment	Cultivar	Treatment	Number of berry	Quality	Marketable fruit (Mean ± SE)	Decay (Mean ± SE) *(%)	Discoloration
14 d and 30 $\mu L L^{-1}$ O_2	Thompson seedless	ULO + CO_2	727	2.5 ± 0.3a	64.0 ± 16.8a	1.0 ± 0.4a	2.2 ± 0.3a
		Control	693	2.5 ± 0.2a	59.3 ± 13.2a	1.3 ±± 0.4a	1.8 ± 0.3a
	Red seedless	ULO + CO_2	347	3.0 ± 0.0a	88.5 ± 1.7a	0a	1.0 ± 0.0a
		Control	281	3.0 ± 0.0a	81.5 ± 7.3a	0.5 ± 0.5a	1.0 ± 0.0a
	Black seedless	ULO + CO_2	326	3.5 ± 0.5a	88.4 ± 6.0a	0a	1.0 ± 0.0a
		Control	282	2.5 ± 0.5a	71.4 ± 4.5a	1.0 ± 1.0a	1.0 ± 0.0a
	Crimson/Red seedless	ULO + CO_2	376	2.7 ± 0.3a	52.0 ± 18.6a	1.0 ± 0.6a	–
		Control	403	2.3 ± 0.3a	51.1 ± 20.8a	1.0 ± 0.6a	–
10 d and <0.01 $\mu L L^{-1}$ O_2	Thompson seedless	ULO + CO_2	436	3.0 ± 0.6a	51.6 ± 18.8a	1.0 ± 0.0a	1.6 ± 0.9a
		Control	366	2.3 ± 0.7a	40.8 ± 14.6a	1.0 ± 1.0a	2.0 ± 0.0a
	Concord/Red seedless	ULO + CO_2	388	2.7 ± 0.7a	54.0 ± 11.0a	0.7 ± 0.7a	–
		Control	403	2.7 ± 0.3a	44.7 ± 2.4a	1.3 ± 0.7a	–

Treatments for all grape varieties were replicated 3 times except that Thompson seedless (PLU# 4022) was replicated 6 times in the 14 d experiments. The paired values of each parameter for each cultivar followed by the same letter were not significantly different based on least square means difference student *t*-test using fit model platform of JMP statistical discovery software ($P > 0.05$, SAS Institute, 2008).

ULO treatments against western flower thrips (Liu, 2008a, 2012). In the current study, the ULO treatment with <0.01 $\mu L L^{-1}$ O_2 was more effective than the ULO treatment with 30 $\mu L L^{-1}$ O_2, judged by the similar mortality rates from the 14 d ULO treatment with the higher oxygen level of 30 $\mu L L^{-1}$ and 3 d ULO treatment with no oxygen (<0.01 $\mu L L^{-1}$). Given the importance of oxygen concentration in ULO treatments, it can be speculated that the unsatisfactory outcomes of earlier CA studies were likely due to that oxygen levels were not low enough to kill insects within the selected treatment durations. The complete control of grape mealybug with ULO plus 50% CO_2 indicates that CO_2 was an important factor for effective control of grape mealybug. This is consistent with reported synergism between reduced O_2 and elevated CO_2 in controlling insects (Calderon and Navarro, 1979). It is also possible to achieve complete control of grape mealybug with ULO without added CO_2. However, the treatment will likely be much longer.

Compared with most other CA treatments, the CA treatments in this study were more severe due to the combinations of lower oxygen levels, presence of 50% CO_2, and long treatment times. This indicates that grape mealybug is very tolerant of CA treatment. This is consistent with results from another study in which a 13 d CA treatment with 45% CO_2 killed all obscure mealybug (*P. affinis*) but only killed 60% of grape mealybug (Mitcham et al., 2003). It is very likely that the treatment for grape mealybug will also be effective against other pests on table grapes as they are likely less tolerant of CA treatment than grape mealybug. There are many other pests associated with table grapes which are quarantined in overseas markets. For example, there are 19 quarantined pests on exported table grapes from California to Australia (Taylor, 2002). Black widow spiders also need quarantine control on exported table grapes to overseas markets because they pose as a safety hazard to consumers as well as handlers of grapes. Black widow spiders are very susceptible to ULO treatment and can be controlled with one day ULO treatment with 0.5% (5000 $\mu L L^{-1}$ or 5000 ppm) O_2 at 1 °C (Liu et al., 2008). Western flower thrips are also controlled successfully with ULO treatment in 2 and 3 d at 10 and 3 °C, respectively (Liu, 2007, 2008a, 2008b, 2012). The CA treatments for grape mealybug control will also control black widow spiders and western flower thrips. The current CA treatments also control vine mealybug (*Planococcus ficus*), another quarantined pest on exported table grapes to Australia (Liu, unpublished). Pacific spider mites and omnivorous leafroller are also quarantined on exported table grapes (Mitcham et al., 1997). The LT_{99}s for the two pests are 8.1 and 4.3 d respectively under 11.5% O_2 and 45% CO_2 (Mitcham et al., 1997). This implies that they are more susceptible to CA treatment than grape mealybug, and the current CA treatment for mealybug eggs will also likely be effective against them.

In conclusion, grape mealybug was successfully controlled in CA treatments with combinations of ultralow oxygen and 50% carbon dioxide. Egg was more tolerant than nymph and adult. Three day CA treatment with <0.01 $\mu L L^{-1}$ O_2 and 50% CO_2 was sufficient to control grape mealybug nymphs and adults. Ten to 14 d CA treatment was needed to control eggs. The length of treatment varied depending on oxygen level. Table grapes tolerated both 10 and 14 d CA treatments and sustained no injury or quality reduction. The results suggest that it is feasible to control grape mealybug with a CA treatment and more research efforts are warranted to develop CA treatment as an alternative to methyl bromide fumigation for postharvest pest control on table grapes.

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