Evaluation of the Efficacy of the Methyl Bromide Fumigation Schedule Against Mexican Fruit Fly (Diptera: Tephritidae) in Citrus Fruit

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ABSTRACT Methyl bromide fumigation is widely used as a phytosanitary treatment. Mexican fruit fly, Anastrepha ludens (Loew) (Diptera: Tephritidae), is a quarantine pest of several fruit, including citrus (Citrus spp.), exported from Texas, Mexico, and Central America. Recently, live larvae have been found with supposedly correctly fumigated citrus fruit. This research investigates the efficacy of the previously approved U.S. Department of Agriculture-Animal and Plant Health Inspection Service treatment schedule: 40 g/m^3 methyl bromide at $21-29.4^\circ$ C for 2 h. Tolerance of A. ludens to methyl bromide in descending order when fumigated in grapefruit (*Citrus* × paradisi Macfad.) is third instar > second instar > first instar > egg. Two infestation techniques were compared: insertion into fruit of third instars reared in diet and oviposition by adult A. ludens into fruit and development to the third instar. Inserted larvae were statistically more likely to survive fumigation than oviposited larvae. When fruit were held at ambient temperature, $0.23 \pm 0.12\%$ of larvae were still observed to be moving 4 d postfumigation. Temperatures between 21.9 and 27.2°C were positively related to efficacy measured as larvae moving 24 h after fumigation, pupariation, and adult emergence. Coating grapefruit with Pearl Lustr 2–3 h before fumigation did not significantly affect the proportion of third instars moving 24 h after fumigation, pupariating, or emerging as adults. In conclusion, fumigation with 40 g/m^3 methyl bromide for 2 h at fruit temperatures $> 26.7^{\circ}$ C is not found to be inefficacious for A. *ludens*. Although a few larvae may be found moving >24 h postfumigation, they do not pupariate.

KEY WORDS Anastrepha ludens, phytosanitary, quarantine, commodity treatment

Methyl bromide fumigation is used as a phytosanitary treatment against Mexican fruit fly, *Anastrepha ludens* (Loew) (Diptera: Tephritidae), for citrus (*Citrus* spp.) fruit grown in southern Texas and Mexico. Before this research was conducted the treatment schedule against Mexican fruit fly for Texas citrus fruit (T101-j-2-1) consisted of fumigation with 40 g/m³ methyl bromide at fruit temperatures between 21 and 29.4°C for 2 h (APHIS 2010).

Although methyl bromide has been implicated as a stratospheric ozone-depleting substance and is therefore subject to regulation to restrict its use under the 1987 Montreal Protocol on Substances that Deplete the Ozone Layer, indefinite exemptions have been granted for certain postharvest phytosanitary applications (Heather and Hallman 2008).

This research was conducted to explore factors contributing to the efficacy of the methyl bromide treatment schedule for citrus fruit infested with Mexican fruit fly in preventing live larvae from being detected at inspection stations when fumigated fruit are shipped interstate, which could be as little as 24 h after fumigation. The methyl bromide treatment schedule against Mexican fruit fly is based on research done 25 yr ago (Williamson et al. 1986). Here, we present several discrepancies between how that study was done and the results reported versus the implementation and interpretation of the treatment schedule by regulatory agencies. These discrepancies might be at least partly responsible for the finding of live Mexican fruit fly larvae in supposedly properly fumigated citrus fruit. Even if these discrepancies are not the reason for the supposed treatment failure, observed differences between the research and the treatment schedule were sufficient to warrant further investigation.

Fumigations in Williamson et al. (1986) were done at 26.7 \pm 1°C, whereas the schedule allowed for a minimum treatment temperature of 21°C. Efficacy of a fumigant is usually directly proportional to temperature (Heather and Hallman 2008).

Williamson et al. (1986) worked with eggs and early instars, whereas the treatment must be effective against all stages present in fruit, eggs through late instars. Williamson et al. (1986) assumed that there were no significant differences among eggs and all instars inside fruit in susceptibility to methyl bromide, although they stated that "the egg stage may be slightly more tolerant."

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After fumigation Williamson et al. (1986) waited for surviving Mexican fruit flies to finish larval development, naturally emerge from fruit, and complete development to the eclosed adult stage. Under that scenario, it is possible that some Mexican fruit flies were alive for some time in the fruit after fumigation. Some larvae could have emerged from the fruit but did not complete development to the eclosed adult. Inspectors finding moving larvae upon cutting open fruit would consider that shipment in violation of regulatory standards. This could result in regulatory action being taken, such as refumigation, returning the fruit to the origin, redirection to another market, shutting down the packinghouse, or other aspects of the fruit production and shipping operation until the supposed failure could be remedied (Heather and Hallman 2008).

Even if prevention of eclosed adults was accepted as the measure of efficacy of the treatment, Williamson et al. (1986) did not satisfy the most liberal published norm of efficacy accepted by some regulatory agencies for fruit flies, which would be no survivors among 30,000 treated (Heather and Hallman 2008). Williamson et al. (1986) reported an average of approximately one eclosed adult per 30,000 insects treated. However, under circumstances of low risk, such as the Mexican fruit fly situation in southern Texas (Thomas et al. 1999), a more liberal level of required efficacy could be argued (Heather and Hallman 2008).

We also examine the effect of fruit coatings on efficacy. Fruit is fumigated in field boxes or after it is packed. Therefore, they may be coated before or after fumigation. Fruit coatings interfere with gaseous exchange (McHugh and Krochta 1994). In a preliminary study, coating mangoes infested with 4–7-d-old Caribbean fruit fly, *Anastrepha suspensa* (Loew), 1 d before fumigation seemed to reduce efficacy of methyl bromide fumigation relative to noncoated, fumigated fruit (Hallman 1996). However, an unpublished study cited in that publication stated that the coating did not appreciably affect methyl bromide uptake and loss in guavas (*Psidium guajava L.*).

The objectives of this research were to 1) determine the most methyl bromide-tolerant Mexican fruit fly stage that can be found in fruit (egg through larva); 2) test whether an easier to use artificial infestation technique could substitute for natural infestation in methyl bromide phytosanitary research; 3) determine the extent of moving larvae, pupariation and adult emergence after fumigation; 4) determine the effect of temperatures between 21 and 27°C on efficacy; and 5) measure the effect of a commercial fruit wax on efficacy.

Materials and Methods

Source of Mexican Fruit Fly. Most of the A. ludens used in this research was from a colony maintained by the U.S. Department of Agriculture–Animal and Plant Health Inspection Service (USDA–APHIS), Mexican Fruit Fly Rearing Facility in Mission, TX. At the time of this research (2005–2006), this colony was used in the sterile insect technique population suppression program in southern Texas (Thomas et al. 1999). In some of the experiments, A. ludens was collected in naturally infested oranges [Citrus \times sinensis (L.) Osbeck[from Montemorelos, Nuevo León, Mexico.

Methyl Bromide Fumigation Chamber and Dosimetry. Fumigations were done in a small but commercial-scale 3.4-m³ steel chamber (FumaVac, John Muir & Sons, Chicago, IL) at the USDA-APHIS Plant Inspection Station at Los Indios, TX. The proper amount of fumigant was measured in a liquid state in a calibrated glass cylinder and introduced into the chamber through a copper coil passing through a hot water bath to ensure that the methyl bromide enters in a gaseous state. A fan inside the chamber kept the atmosphere in motion throughout the treatment. The concentration of methyl bromide was monitored constantly by drawing air through a thermal conductivity meter (Fumiscope, Key Chemical and Equipment Co., Clearwater, FL), which was the standard used by industry at the time of this research. The meters used were calibrated with known standard concentrations at various occasions during the research period (Scott-Martin, Riverside, CA). The air sample was passed though a CO2 adsorbent (Ascarite II, Arthur H. Thomas Co., Swedesboro, NJ) before entering the meter because CO₂ could artificially increase the estimation of methyl bromide concentration.

An atmospheric pressure of \approx 97.1 kPa was set inside the chamber with a vacuum pump at the start of each fumigation as a check for leaks and monitored continuously (Ashcroft, Duragauge, Stratford, CT) during the fumigation. This pressure results in a slight vacuum equivalent to \approx 380 m above sea level. The altitude of Los Indios is 17 m above seal level.

Temperatures were recorded with three #36 gauge (0.13-mm-diameter wire) type T thermocouples placed at the top, middle, and bottom of loads and read every 10 min with an electronic thermometer (model 4021, Control Company, Friendswood, TX) calibrated with an ASTM-certified glass mercury thermometer (model 1003-FC, ERTCO, W. Paterson, NJ).

At the end of the fumigation time interval, the atmosphere inside the chamber was forcibly evacuated to the outside of the building through a 5-cmdiameter duct. Within 10 min after initiation of evacuation, methyl bromide could not be detected via the thermal conductivity meter. The chamber was opened 30 min after evacuating the chamber.

Fruit Infestation. Mexican fruit fly-infested citrus fruit were achieved in three ways. Naturally infested oranges were collected in Mexico, 'Rio Red' grapefruit were placed in a screen infestation cage (1.2 by 0.8 by 0.5 m) with thousands of Mexican fruit fly adults for 1–5 h, or a core (1 cm in diameter) was bored to the center of Rio Red grapefruit, 10 diet-reared feeding third instars were inserted to the center, and the core was replaced and sealed with hot melt glue. Mean orange and grapefruit weights in the studies were 143 \pm 6.6 and 337 \pm 11.0 g, respectively.

Simulated infestation with diet-reared insects inserted into fruit is often used as a more manageable alternative to developing phytosanitary treatments than infesting fruit with eggs and allowing them to develop naturally (Heather and Hallman 2008). Among problems associated with the latter are unknown and variable infestation rates, compromised ability to control infestation rate, and degradation of fruit before the insects are of the desired stage for treatment. The chief advantage for infesting fruit via oviposition is that it is the closest to the natural state and thus more likely to vield a realistic efficacy response. The ideal infestation scenario involves the use of naturally infested fruit with the proper stage of feral insects from the field, but a ready supply of naturally infested fruit is usually not available. In all of the studies, ≈15% of infested fruit were used as nonfumigated controls to verify normal behavior and development, and the chamber was filled to $\approx 80\%$ capacity with noninfested grapefruit. Fruit was in standard corrugated cardboard grapefruit boxes (25 by 29 by 45 cm) when fumigated.

Determination of Most Methyl Bromide-Resistant Stage. Rio Red grapefruit were placed in the infestation cage for 1-4 h; removed; wiped clean; and placed in a chamber at 26.5 ± 0.5 °C and $78 \pm 10\%$ RH until insects were at the proper stage for fumigation. Development is slower in grapefruit than in diet (Levva et al. 1991). Fruit were opened periodically to check insect development. Twenty fruit each with predominantly first, second, or third instars were fumigated together with 20 fruit infested with 1-d-old eggs at 24 g/m^3 for 2 h at $\approx 23^{\circ}$ C. This dose was chosen as a discriminatory dose that would yield some survivors in some of the stages to identify differences among stages. Several control fruit with each stage of insects in them were not fumigated. Approximately 490 individuals were treated at each stage.

Twenty-four hours after fumigation, grapefruit containing third instars was opened, and larvae were removed and placed in small plastic containers with moist vermiculite and stored at $26.5 \pm 0.5^{\circ}$ C and $78 \pm$ 10% RH. Numbers of larvae, stage, and mobility were recorded. Every 1–3 d, they were checked for further development and mobility. After 1 mo, adults emerging from any puparia were counted. Grapefruit containing the other stages were opened when late third instars began to emerge from the fruit. A two-tailed *t*-test was used to determine whether the difference in pupariation and adult emergence between fumigated second and third instars was statistically significant.

Infestation via Oviposition Versus Insertion of Third Instars. Grapefruit infested with Mexican fruit fly third instars by placement in a cage with ovipositing adults and held until late third instars developed were fumigated (40 g/m³ for 2 h at \approx 23°C) together with grapefruit infested by insertion of third instars reared on diet into holes bored into the fruit. Fruit were cut open 24 h after fumigation and examined for larvae, which were placed in plastic containers with moist vermiculite at 26.5 ± 0.5°C and 78 ± 10% RH. Development was subsequently monitored daily. Percentage of pupariation and adult emergence were analyzed with two-tailed paired *t*-tests. There were seven replicates of 20 fruit per treatment done on different dates, with a total of $\approx 1,400$ third instar per treatment.

Effect of Temperature on Efficacy. Temperature could not be precisely controlled in the fumigation facility, and different temperatures occurred during the period of the study. Nine trials of a total of 360 grapefruit containing \approx 9,600 third instars in total were fumigated with 40 g/m³ between mean temperatures of 21.9 and 27.2°C. The number of larvae moving ≈ 24 h after fumigation, the number pupariating, and the number emerging as adults were recorded. Data were analyzed in two different ways for comparison: percentage of larvae moving 24 h after fumigation versus temperature was analyzed with a sigmoidal doseresponse equation (Prism 4, GraphPad Software, San Diego, CA), and the number not moving after 24 h versus temperature was analyzed with probit analysis (Proc Probit, SAS Institute, Cary, NC).

Extent of Moving Larvae Post-Treatment. Larvae removed from fumigated (40 g/m³ for 2 h at 24.7 \pm 0.6°C) and nonfumigated (control) orange and grapefruit were placed in plastic containers with moist vermiculite at 26.5 \pm 0.5°C and 78 \pm 10% RH and observed for movement daily for 8 d. This simple observation is used by inspectors to determine whether larvae found in shipments are alive. There were two replicates of a total of 214 oranges naturally infested from Mexico (303 third instars in total) and seven replicates of a total of 280 grapefruit infested via oviposition (4,712 third instars in total). Puparia from which adults did not emerge were opened to examine how far the insects had developed using puparial nomenclature of Thomas and Hallman (2000).

Effect of Fruit Coating on Efficacy. Coating grapefruit may result in mortality to internal Mexican fruit fly larvae after several days by restricting gaseous exchange (Hallman 1997). Grapefruit were infested with Mexican fruit fly by using the cage method, held until third instars were present, and then half of the grapefruit were coated (Pearl Lustr, DECCO, Monrovia, CA) at the recommended rate (1.0–1.1 ton of fruit per liter) spread by hand and air-dried the day before fumigation (40 g/m³ for 2 h at 24.5 \pm 1°C). There were four replicates of 20 grapefruit per treatment, with 990 third instars in total per treatment. The percentages of larvae moving, pupariating, and emerging as adults were analyzed with two-tailed paired *t*-tests.

Results

Methyl Bromide Dosimetry. The methyl bromide meters frequently required rezeroing during monitoring. Methyl bromide concentrations during fumigation and after rezeroing are shown in Fig. 1. Although we acknowledge that some uncertainty exists in Fig. 1, it shows that methyl bromide concentrations gradually decreased during fumigation but did not drop below the desired treatment concentration of 40 g/m^3 . This is to be expected because methyl bromide typi-



Fig. 1. Mean \pm SEM methyl bromide concentration (40 g/m³) in headspace versus time after initiation of 2-h fumigation of grapefruit at 40 g/m³.

cally does not penetrate fruit completely in the time used for fumigation, leaving the headspace with a higher concentration of the fumigant. The slight vacuum was not lost during fumigation, giving confidence that methyl bromide concentration was not decreasing due to leakage.

This study contributed to evidence that a more precise fumigant monitor was needed for the fumigation industry to address the issues of instrument stability, drift, and CO_2 and volatile interference. Since then, APHIS has worked with a company (Spectros Instruments, Hopedale, MA) to develop a fast and accurate infrared-based monitor (ContainIR) that is immune to CO_2 and other interfering volatiles.

Determination of Most Methyl Bromide-Resistant Stage. No larvae were found in grapefruit infested with Mexican fruit fly eggs that had been fumigated with 24 g/m³ methyl bromide the day after oviposition, although >400 third instars were found in nonfumigated controls. Any first instars present would probably not be found, but most third and some second instars present would have been found, indicating that Mexican fruit fly development from eggs in grapefruit fumigated with that dose did not develop far. A mean of $0.5 \pm 0.23\%$ Mexican fruit flies fumigated while first instars were subsequently found as late instars and none pupariated, although again, >400 third instars were observed in nonfumigated controls. Of second and third instars, respectively, fumigated with 24 g/m^3 methyl bromide in grapefruit, 1.0 ± 0.73 and $4.8 \pm 1.3\%$ pupariated (t = 2.51, df = 6, P = 0.046) and 0.78 ± 0.78 and $2.6 \pm 0.48\%$ (t = 2.00, df = 6, P = 0.093) emerged as adults. Therefore, tolerance of Mexican fruit fly to methyl bromide in descending order when fumigated with methyl bromide in grapefruit and measured as completing the same stage of development can be considered third instar > second instar > first instar > egg.

Infestation via Oviposition Versus Insertion of third Instars. Survival of Mexican fruit fly third instars reared on diet and placed near the center of grapefruit the day before fumigation (artificial infestation) and removed from the fruit the day after fumigation was significantly higher than survival of third instars infesting grapefruit from the egg stage (cage infestation) when measured as pupariation and adult emergence. Percentage of Mexican fruit fly pupariation was $0.17 \pm$



Fig. 2. Percentage of Mexican fruit fly third instars moving 24 h after fumigation with methyl bromide (40 g/m^3) in grapefruit at different temperatures and percentage that pupariated and developed to emerged adults. One point not showing on the graph is 24.1°C, which had no movement or pupariation. The numbers of larvae observed in increasing order by temperature are 506, 979, 346, 150, 363, 3,960, 5,012, 131, and 151.

0.12 and 7.1 \pm 1.9%, respectively, for cage and artificially infested fruit (t = 3.71, df = 6, P = 0.010). Percentage of adult emergence was 0.057 \pm 0.031 and 3.8 \pm 1.3%, respectively, for cage and artificially infested fruit (t = 2.87, df = 6, P = 0.028).

Effect of Temperature on Efficacy. There was an inverse relationship between temperature and the proportion of larvae moving 24 h after fumigation, pupariation, and adult emergence (Fig. 2). The percentage of larvae moving 24 h after fumigation (Y) had an r^2 of 0.93 when correlated to the sigmoidal dose-response equation:

$$Y = 0.0775 + 7.191/1 + 10^{23.19 - X}$$

where *X* is degrees Celsius. Therefore, at the minimum allowed treatment temperature of 26.7°C, 0.08% of larvae are expected to be moving 24 h after fumigation. At the minimum treatment temperature formerly allowed (21°C), 7% of larvae would have been expected to be moving 24 h after fumigation. Number of larvae failing to move after 24 h did not fit the probit model (Pearson $\chi^2 = 71.1$, df = 7, probability <0.0001).

Extent of Moving Larvae Posttreatment. Larvae that moved after removal from fumigated fruit were always less active than larvae from nonfumigated fruit. For the most part, the former larvae did not crawl but often simply moved their heads. Larvae from nonfumigated fruit actively crawled until they prepared to pupariate. Percentage of larvae moving postfumigation from grapefruit infested via oviposition was directly proportional to the time span between fumigation and removal (Fig. 3). One larva was found moving 7 d postfumigation, although it was not observed to move when checked five and 6 d postfumigation and died by 8 d. A few larvae that were not moving when removed from grapefruit moved later or pupariated without ever having been observed to move.

Movement of larvae from the two fumigations of oranges naturally infested from Mexico was relatively high 24 h after fumigation. In the first fumigation, 12.0, 4.2, and 6.4% of the larvae were moving 1, 2, and 5 d after fumigation; 3.1% (n = 4) pupariated and all died in the puparia (three as coarctate larvae and one as a



Fig. 3. Mean percentage (plus 95% CI) of a total of Mexican fruit fly third instars moving up to 7 d after fumigation with methyl bromide (40 g/m³) in grapefruit. No larvae were found moving on days 5 and 6.

cryptocephalic pupa). In the second fumigation, 31.8 and 0% of larvae were moving 1 and 3 d after fumigation, and none pupariated.

The patterns of intrapuparial development and death for insects reared on grapefruit infested via oviposition were similar for fumigated (n = 14) and control (n = 21) puparia (Table 1). Insufficient fumigated insects pupariated for statistical analysis.

Effect of Fruit Coating on Efficacy. The coating (Pearl Lustr) did not significantly affect the proportion of third instars moving after fumigation, pupariating, or emerging as adults when grapefruit were infested by exposure to adult Mexican fruit flies in cages (Table 2).

Discussion

This study noted the discrepancy between the temperature (26.7°C) used in the original research that supported the T101-j-2-1 schedule (Williamson et al. 1986) and the original minimum fumigation temperature required for the schedule (21°C). This information plus supporting data (Fig. 2) showing no pupariation at fumigation temperatures >24.3°C encouraged APHIS (2010) to raise the minimum fumigation temperature from 21 to 26.7°C (70–80°F). However, this modification still does not prevent moving larvae from being found a day or more after fumigation when the fruit would be checked in inspection ports, although it reduces the likelihood.

The proportion of larvae moving after fumigation was reduced as time after fumigation increased, but it is evident that moving larvae could be found in fruit

Table 1. Percentage of Mexican fruit fly larvae developing to several puparial stages and then dying after being fumigated as third instars in grapefruit with methyl bromide (40 g/m³ for 2 h at 22 to 27°C) compared with nonfunigated controls

Puparial stage	% in stage		
	Methyl bromide fumigated	Control	
Coarctate larva	21.4	28.6	
Cryptocephalic pupa	50.0	47.6	
Phanerocephalic pupa	21.4	19.0	
Pharate adult	7.1	4.8	

Table 2. Percentage of Mexican fruit fly larvae moving 24 h after fumigation with methyl bromide (40 g/m³ for 2 h at 21 to 25°C) and subsequent percentage of pupariation and adult emergence with or without prior coating (Pearl Lustr applied at the rate of 1,000–1,100 kg grapefruit per liter)

Developmental event	Mean ± SEM (%)		Results of two- tailed <i>t</i> -tests		
	Coated fruit	Noncoated fruit	t	df	Р
Larvae moving	2.1 ± 1.7	4.1 ± 3.7	0.43	3	0.699
Pupariation	0.60 ± 0.60	0.82 ± 0.67	0.21	3	0.848
Adult emergence	0	0.24 ± 0.14	1.73	3	0.182

fumigated according to the approved schedule. At the same time it would be expected that many more nonmoving larvae would be found a couple of days after fumigation. When moving larvae are found upon inspection, but few obviously dead (darkened) larvae are found, contamination or lack of proper treatment might more reasonably be expected to be responsible instead of lack of efficacy of a properly performed treatment itself.

This study found that *A. ludens* in fruit increased in tolerance as it developed when the same endpoint is used for all stages; Williamson et al. (1986) assumed eggs were more tolerant than larvae. Other studies concluding that tephritid eggs were more tolerant of methyl bromide than larvae used different endpoints. For example, Armstrong and Whitehand (2005) concluded that eggs of two other tephritid species were the most tolerant stage, although eggs had only to hatch to be deemed as surviving the fumigation, whereas larvae had to cross several developmental thresholds (first instars had to pupariate and third instars had to emerge as adults).

Although the one coating tested in this study did not have a measurable effect on efficacy of methyl bromide fumigation, a general conclusion about coatings used on fumigated fruit should not be made because of the differences in permeability to gases among available fruit coatings (McHugh and Krochta 1994).

In this study, larvae inserted into fruit were harder to kill with methyl bromide fumigation than those infesting fruit via oviposition. Therefore, although treatments developed using insertion would be efficacious, they would be more severe than needed. The effect of refrigeration on the ability of fumigated larvae to stay alive and moving after treatment should be tested; refrigeration could conceivably extend the period of time that larvae are able to move.

Most methyl bromide fumigation schedules in the APHIS Treatment Manual (APHIS 2010) have minimum required concentrations at 0.5 and 2 h, whereas a number of schedules have no such requirements. The concentrations required in the schedules that require minimums are considerably lower than our readings (Fig. 1). For example, at initial fumigation concentrations of 40 g/m^3 minimum concentrations of 32 and 24 g/m³ are required after 0.5 and 2 h, respectively. These low concentrations allow for considerable loss of methyl bromide during fumigation. That not much methyl bromide was probably lost during our tests suggests the possibility that commercial fumigations might be less efficacious than what we observed.

We note that Williamson et al. (1986) did not report dosimetry and that is probably the reason why minimum concentration requirements are not part of schedule T101-j-2-1. Values in Fig. 1 should not be used to establish minimum concentrations because of lack of sufficient confidence in the meters used to generate those values.

The purpose of our study was not to establish a new phytosanitary treatment; insect numbers treated, temperature control and methyl bromide dosimetry were not at the optimums for supporting a treatment. Rather our research was to explore assumptions made in the original research supporting the treatment (Williamson et al. 1986) and other factors that might affect the efficacy of schedule T101-j-2-1 and the efficacy of methyl bromide fumigation as a phytosanitary treatment in general. Because factors were tested during the same fumigations, hence, under the same conditions each time, we are confident that relative differences found are valid.

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