Final Report

Effect of Sulphur Dioxide and Cold on Survival of Insects During Storage of Table Grapes

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Effect of Sulphur Dioxide and Cold on Survival of Insects During Storage of Table Grapes – TG15003

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Summary

Australian table grape exports have increased from 30,000 to 88,000 tonnes in the last 5 years and are the largest Australian fresh fruit export sector by value. Major markets include China and Indonesia, with strong demand in Japan, the Philippines and the Middle East. In order to maintain and grow export markets, the Australian industry must maintain phytosanitary access. Pre-export inspections of Australian table grapes have found live insects and mites in some consignments. Tolerance for live insects in most export markets is zero, and insects that are missed at inspection and survive the journey may pose a threat to the viability of the table grape export industry. Overseas research has shown that exposure to cold and sulphur dioxide (SO_2) can kill some insects. Sea freight export of table grapes to closer markets such as Indonesia and Singapore takes 2 – 3 weeks, while it takes 3 – 4 weeks or longer to export to China, Japan and South Korea. Grapes are generally stored at 1°C during transit and all cartons are treated with a SO_2 generator sheet to control rots. In these experiments, the survival of six recorded contaminants of table grape exports, long-tailed mealybug (*Pseudococcus longispinus*), ladybird beetle (*Chilocorus* sp.), European earwig (*Forficula auricularia*), two-spotted spider mite (*Tetranychus urticae*), Argentine ant (*Linepithema humile*) and Carpophilus or dried fruit beetle (*Carpophilus hemipterus*) was tested under conditions similar to those used during sea freight export of table grapes to China and other Asian export markets. Insects and mites were transferred to grape berries in a vented container and placed in cartons of table grapes, with or without a SO_2 generator sheet and stored for up to 8 weeks at 1°C to simulate sea freight export.

After approximately 2 weeks storage, all mealybugs, ladybird beetles and Carpophilus beetles were dead, regardless of whether they had been treated with SO_2 or not. Further experiments with these organisms with more frequent removals confirmed 100% mortality and showed that the SO_2 treatment increased mortality substantially in the first week of storage. It took 8 weeks of storage for 100% mortality of two-spotted spider mites, although approximately 95% in both treatments were dead after 4 weeks. There was no significant difference in mortality when mites were exposed to cold plus SO_2. European earwigs were the most tolerant organism tested with only 29.2% mortality in the cold alone treatment after 8 weeks of storage. Exposure to SO_2 increased mortality substantially with 91.7% of earwigs dead after 4 weeks. However, mortality remained at this level for the remainder of the 8 week storage period. Total mortality of three insect species after 2 weeks storage is a promising result as sea transport to overseas markets generally takes 2 – 4 weeks. These results indicate that current treatments for sea freight export of table grapes reduce the risk of the insect contaminants tested here surviving the journey to Asian export markets.

Research to improve the SO_2 delivery system to ensure higher and more uniform SO_2 levels throughout the carton is warranted as this may increase insect mortality. In addition, further research is recommended to test the survival of other insects of concern to export markets, particularly the light brown apple moth, as well as other recorded contaminants of grape export consignments.

Keywords

Export, disinfestation, long-tailed mealybug, Argentine ant, Chilocorus, Carpophilus, European earwig, two-spotted spider mite.

Introduction

Australian table grape exports have increased from 30,000 to 88,000 tonnes in the last 5 years and are the largest Australian fresh fruit export sector by value. Major markets include China and Indonesia with strong demand in Japan, the Philippines and the Middle East. In order to maintain and grow export markets, the Australian industry must maintain phytosanitary access. Pre-export inspections of Australian table grapes have found live insects and mites in some consignments. Tolerance for live insects in most export markets is zero, and insects that are missed at inspection and survive the journey may pose a threat to the viability of the table grape export industry. To minimise the risk, a comprehensive systems approach to prevent contamination has been adopted by table grape growers and packers as a compulsory prerequisite for approval to export table grapes. Despite these measures, live arthropods occasionally find their way into export grape cartons. Industry has asked if they will survive the journey to market under cold storage and the standard sulphur dioxide (SO_2) treatment that is used to prevent rots.

A small number of studies have examined the effect of low temperature and SO_2 on the survival of arthropods. A review of postharvest treatments for disinfestation of fruits in South Africa claims that exposure to 0°C for 3 weeks can control Pseudococcidae (Pryke and Pringle 2008). A study in California showed that storage of ruby seedless grapes infested with grape mealybug (*Pseudococcus maritimus* Ehrhorn) at 0°C and approximately 1 $\mu l.l^{-1}$ SO_2 for 2 to 5 weeks resulted in greater than 92% mortality (Yokohama et al. 2001). New Zealand research demonstrated that storage of Royal Gala apples infested with the obscure mealybug (*Pseudococcus viburni* Signoret) at 0°C resulted in 100% mortality after 6 weeks (Hoy and Whiting, 1997). These results indicate that low temperatures with or without SO_2 can be effective in killing mealybugs, although the efficacy appears to vary between species. Furthermore, there does not appear to be any published studies on the effect of low temperature and SO_2 on the survival of the long-tailed mealybug, the insect of concern here.

Even so, there has been some work done on the survival of other pest species in table grapes in response to low temperature and SO_2. Complete control of 2nd instar omnivorous leaf roller larvae (*Platynota stultana*) was achieved at a storage temperature of between 1.1 and 2.1°C and a SO_2 concentration of 0.3 to 1.0 $\mu l.l^{-1}$ after 3 weeks. It is interesting to note that complete control of the same instar larvae was not achieved with low temperature alone after 6 weeks storage (Yokoyama et al. 1999). Further small scale tests at 0.4 to 1.7°C with SO_2 levels between 0.2 and 1.6 $\mu l.l^{-1}$ resulted in over 93% mortality of grape mealybug after 5 weeks and 100% mortality after 6 weeks (Yokoyama et al. 1999). Western flower thrips (*Frankliniella occidentalis*) were completely controlled after 1 week, and 98 – 99% control of pacific spider mite (*Tetranychus pacificus*) and two-spotted spider mite (*Tetranychus urticae*) was achieved after 6 weeks storage (Yokoyama et al. 2001). Queensland fruit fly eggs on artificial diet and enclosed in a plastic bag with a commercial SO_2 pad were killed over time at room temperature in preliminary studies by Jessup and De Lima (2014).

The table grape industry wants to continue the growth in exports to high value markets but needs to be sure that the grape consignments do not contain live insects. At present, SO_2 releasing sheets are included in all export cartons for disease control. Experiments were established to investigate the effect of cold and SO_2 on the survival of insects and mites found to contaminate table grape shipments. Organisms selected for testing were the long-tailed mealybug (*Pseudococcus longispinus*), ladybird beetle (*Chilocorus* sp.), European earwig (*Forficula auricularia*), two-spotted spider mite (*Tetranychus urticae*), Argentine ant (*Linepithema humile*) and Carpophilus or dried fruit beetle (*Carpophilus hemipterus*). The results of experiments to determine whether these organisms can survive the low temperature and SO_2 environment used for sea freight export of table grapes are reported here.

Methodology

Experiments

Full detailed experimental methodology is provided in Appendix A.

Three experiments were conducted. In the first, long-tailed mealybugs (*Pseudococcus longispinus*) and ladybird beetles (*Chilocorus* sp.) were stored with Thompson seedless grapes for 17 days at 1°C ± 0.5°C with or without a Uvasys® green dual release SO_2 generating sheet (Grapetek Pty Ltd, Cape Town South Africa; a.i. 970 g kg^{-1} Sodium metabisulphite). Earlier studies indicated that both species died within 2 weeks storage at 1°C both with and without SO_2 treatment (Tomkins and Yen 2016). To validate this result and to study the dynamics of insect mortality more closely, insects were removed for assessment at day 3, 7, 10, 14 and 17. In the second experiment, European earwigs (*Forficula auricularia*), two-spotted spider mites (*Tetranychus urticae*), Argentine ants (*Linepithema humile*) and Carpophilus or dried fruit beetles (*Carpophilus hemipterus*) were stored for 8 weeks at 1°C in Crimson seedless table grape cartons for 8 weeks. Boxes of grapes were removed from storage and the insects and mites were examined after 2, 4, 6 and 8 weeks storage. All the ants and Carpophilus were dead after 2 weeks, therefore a third experiment was conducted to study the dynamics of insect mortality more closely in response to cold alone and cold + SO_2. In this experiment, grape boxes were removed from storage at day 3, 7, 10 and 14, and the insects examined to test if they were alive or dead. SO_2 concentration at three different positions within cartons was measured at regular intervals throughout the experiment.

Grape material

Cartons (10 kg) of white table grapes (*Vitis Vinifera*, L 'Thompson seedless') and red table grapes (*Vitis Vinifera*, L 'Crimson seedless') were obtained directly from a specialist grape wholesaler. Fruit was harvested by hand at commercial maturity and packed directly into 1 kg bunch bags with 10 bunch bags packed into each carton. Cartons were then transported within 3 h of harvest to a forced air cooling facility and stored at 2°C overnight before transport under refrigeration to the wholesaler. The next morning the fruit were delivered under refrigeration to the laboratory where they were stored at 1°C until used. On arrival, fruit were in field fresh condition with green stems and firm berries with an attractive waxy bloom. Grape quality was assessed at regular intervals throughout the experiment.

Insects

For the first experiment, a colony of long-tailed mealybugs was reared in a commercial facility (Biocontrol Services, Langwarrin South, Victoria) from insects collected from infested table grape bunches grown in a vineyard at Mildura, Victoria. Insects were reared on grape vines grown in pots in a plant growth chamber set at 25°C and normal daylight hours. A colony of mealybugs on their food host plants were delivered to the laboratory on the morning before the experiment commenced. Ladybird beetles (*Chilocorus* sp.) were reared in a commercial facility (Bugs for Bugs Pty Ltd, Mundubbera, Queensland). Adult beetles were shipped to the laboratory by overnight courier the day before the experiment commenced. Ladybird beetles were shipped with adequate food and liquid for the journey and arrived healthy and active. The day before the experiments commenced, eight ladybirds or a leaf containing 10 to 31 mealybugs were transferred to vented containers with a bunch comprising two grapes. The next morning, containers were checked to ensure the mealybugs and beetles were still alive prior to application of experimental treatments.

For the second and third experiments, two-spotted spider mites (*Tetranychus urticae*) were reared in a commercial facility (Bugs for Bugs Pty Ltd, Mundubbera, Queensland). Mite colonies on detached bean leaves were shipped to the laboratory by overnight courier the day before the experiment commenced. Mites were shipped with adequate food for the journey and arrived healthy and active. Adult dried fruit beetles (*Carpophilus hemipterus*) were obtained from a laboratory colony started from adults caught in pheromone traps in orchards in the Goulburn Valley, Victoria and reared on peaches or artificial diet in a

controlled environment room at 25°C and 12 h photoperiod. Adult European earwigs (*Forficula auricularia*) were obtained from a wild population in an almond orchard at Mildura, Victoria and Argentine ants (*Linepithema humile*) were collected from wild populations in suburban Melbourne. Ants and earwigs were kept at room temperature in purpose built enclosures with adequate water and food available at all times until used. The day before the experiments commenced, four earwigs, five ants, five Carpophilus beetles or a piece of bean leaf with two to ten adult mites were transferred to separate vented containers with a bunch comprising two grapes. The next morning containers were checked to ensure the insects were still alive and active.

Outputs

An industry article has been published and we have discussed the project results with CEO ATGA, and with exporters and growers, and received feedback on future research requirements.

Tomkins, Bruce and Alan Yen (2016). SO_2 trials well under way for table grapes. The Vine 12(3):30-31.

Outcomes

Cold storage for two weeks at 1°C provided an effective disinfestation treatment for the long-tailed mealybug, Chilocorus ladybird beetle and Carpophilus beetle in cartons of export table grapes. It took 8 weeks for 100% mortality of two-spotted spider mites and 100% mortality of earwigs was not achieved. The combination of cold + SO_2 increased the mortality of earwigs. After 8 weeks, 29.2% of earwigs were dead in the cold alone treatment and 83.3 % were dead in the cold + SO_2 treatment. However, over 90% of both two-spotted mites and earwigs from the cold + SO_2 treatment were dead at the 4 week examination.

The high levels of mortality after 4 weeks is a promising result and will reduce the risk of live insect and mite contaminants in table grape exports reaching markets. Furthermore there was 100% mortality of three species after only two weeks. If the results observed here are reflected in commercial shipments, it will substantially reduce the risk of live insect detections in export markets. The outcome will be increased confidence of growers and exporters shipping to phytosanitary markets such as China, Japan and South Korea, and increased sustainability and viability of Australian table grape exports and the table grape industry.

Evaluation and Discussion

Insect mortality

Experiment 1
All mealybugs and ladybird beetles were dead after 17 days exposure to cold + SO_2 (Figure 1A-B). There was a significant interaction between storage treatment and storage time on mortality of mealybugs ($P < 0.001$) and ladybird beetles ($P < 0.001$), where insects in the cold + SO_2 treatment had greater mortality than those in the cold alone treatment. In particular, the sulphur treatment had a greater effect on mortality of the ladybird beetle at day 3, and of the mealybugs at days 3, 7 and 10. At later sampling dates, the mortality of insects from both treatments was similar, and the effect of SO_2 was non-significant. This indicates that cold alone was effective at increasing mortality as the storage time progressed.

Experiment 2
All argentine ants and Carpophilus beetles exposed to both storage treatments were dead at the first removal after 2 weeks storage (data not shown).

There was a significant interaction between storage treatment and storage time on mortality of earwigs ($P < 0.001$), where insects in the cold + sulphur treatment had greater mortality than those in the cold alone treatment, particularly at later sampling days (Figure 1C). After 4 weeks storage, 91.7% of earwigs from the cold + SO_2 treatment were dead and all the earwigs from the cold alone treatment were still alive. Survival in the cold alone treatment remained relatively high with 70.8% of earwigs still alive after 8 weeks storage compared to 16.7% alive in the cold + SO_2 treatment.

In contrast, there was no significant effect of SO_2 treatment on the mortality of the two-spotted mite ($P = 0.921$), although mortality increased significantly with storage time ($P < 0.001$, Figure 1D). Approximately 95% of mites in both treatments were dead after 4 weeks storage, and 100% mortality was achieved in both treatments after 8 weeks. Although susceptible to cold, there was no additive effect of SO_2 on the mortality of two-spotted mites.

Experiment 3
There was a significant effect of cold + SO_2 treatment on mortality of both the Argentine ant and Carpophilus beetle. After 3 days, all ants in the SO_2 treatment were dead, but only 3.3% were dead in the cold alone treatment. The cold alone treatment achieved 50% mortality of ants by day 14. After 10 days, there was 100% mortality of Carpophilus beetles in the SO_2 treatment and 83.3% in the cold alone treatment. 100% mortality of Carpophilus beetles was achieved in the cold alone treatment by day 14. This confirms that SO_2 substantially increases the mortality of these species in the first week of storage.

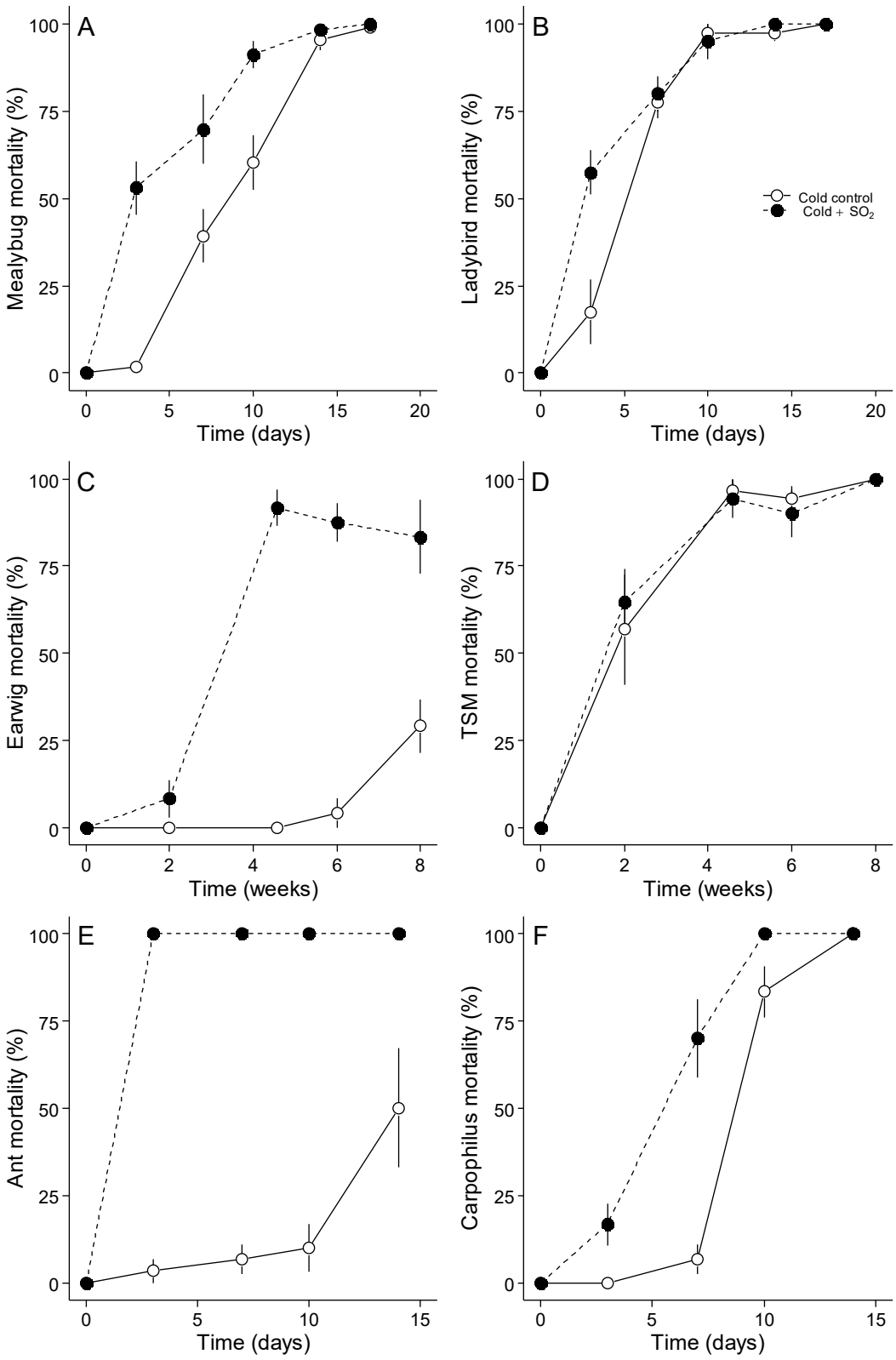

Figure 1. Mortality (%) of (A) long-tailed mealybugs, (B) ladybird beetles, (C) European earwigs, (D) two-spotted mites (TSM), (E) Argentine ants and (F) Carpophilus beetles exposed to a cold treatment or cold and sulphur dioxide treatment (*SO_2*) at 1°C for either 14 – 17 days or 8 weeks. Vertical bars represent the standard error of the mean values (n = 5 – 6). Where no bar is shown it falls within the symbol.

Table 1. Mortality (%) of long-tailed mealybug (*P. longispinus*), ladybird beetle (*Chilocorus* sp), European earwig (*F. auricularia*), two-spotted mite (*T. urticae*), Argentine ant (*L. humile*) and Carpophilus beetle (*C. hemipterus*) stored in cartons of table grapes for 14 – 17 and 28 days at 1°C with or without a SO_2 generator sheet inside the carton. Values represent the mean mortality of 6 replicate cartons.

Organism	**Insect and mite mortality (%)**		
	Treatment	Storage time (days)	
		14 – 17	28
Mealybug	Cold	100	100
	SO_2	100	100
Ladybird beetle	Cold	100	100
	SO_2	100	100
European earwig	Cold	0	0
	SO_2	8.3	91.7
Two-spotted mite	Cold	56.8	96.7
	SO_2	64.6	94.4
Argentine ant	Cold	50	100*
	SO_2	100	100*
Carpophilus beetle	Cold	100	100*
	SO_2	100	100*

*Result from experiment 2

Sea freight of table grapes from Australia to export markets takes 2 – 4 weeks from compilation of the consignment to arrival at the market, depending on the destination. In general, closer markets such as Singapore, Malaysia and Hong Kong which can be reached in 2 – 3 weeks are non phytosanitary markets whilst phytosanitary markets such as China, Japan and South Korea have a shipping time of 3 – 4 weeks or more (D. Minnis Pers Comm). Furthermore, as shipping is required to be more competitive and has to lower freight costs, ships are travelling slower to burn less fuel and reduce overheads. Hence, journeys are getting longer, and shipping times to major phytosanitary markets could be 4 weeks or more.

To ensure that no live organisms are present in consignments on arrival, they should be dead within these time frames. This was achieved for mealybugs, Carpophilus and ladybird beetles with 100% mortality after 14 – 17 days (Table 1). Consequently, cold + SO_2 treatments may provide an effective disinfestation treatment for these insects during sea freight export of table grapes from Australia to Asian markets. However, there was some survival of two-spotted spider mites, European earwigs and Argentine ants at 2 and 4 weeks (Table 1). Cold alone was not effective in killing earwigs with zero mortality after 2 and 4 weeks, whilst SO_2 increased mortality to 8.3% at 2 weeks and 91.7% at 4 weeks. Mortality of earwigs increased in the cold treatment to 29.2% after 8 weeks but did not change much in the SO_2 treatment between 4 and 8 weeks. In experiment 2, after 2 weeks, the cold + sulphur treatment was sufficient to kill all the ants, although the cold alone treatment only achieved 50% mortality. In the cold treatment, 56.8% of two-spotted mites were dead after 2 weeks and 96.7% after 4 weeks. There was no significant effect of SO_2 in increasing mortality ($P = 0.921$) with 64.6% dead in

this treatment after 2 weeks and 94.4% after 4 weeks. Extending the storage period to 8 weeks resulted in 100% mortality of mites in both storage treatments.

Achieving 100% mortality at 4 weeks may be suitable for some longer haul markets or if grapes are stored before shipment to closer markets. However, 100% mortality at 2 weeks is highly desirable and will accommodate all sea freight export markets.

Sulphur dioxide measurements

The SO_2 concentration inside carton liners was highest during the first 24 h of storage, with an average of 193 $\mu l.l^{-1}$ in the top and 172 $\mu l.l^{-1}$ in the centre of the cartons at approximately 24 h after packing (Figure 2). At 48 h, the SO_2 concentration decreased to less than 6 $\mu l.l^{-1}$ at both positions for the remainder of the storage period. SO_2 levels measured in the bottom corner of the cartons were much lower and peaked at 5.6 $\mu l.l^{-1}$ after 24 h and were below the minimum detection level after 48 h for the remainder of the storage period (Figure 2).

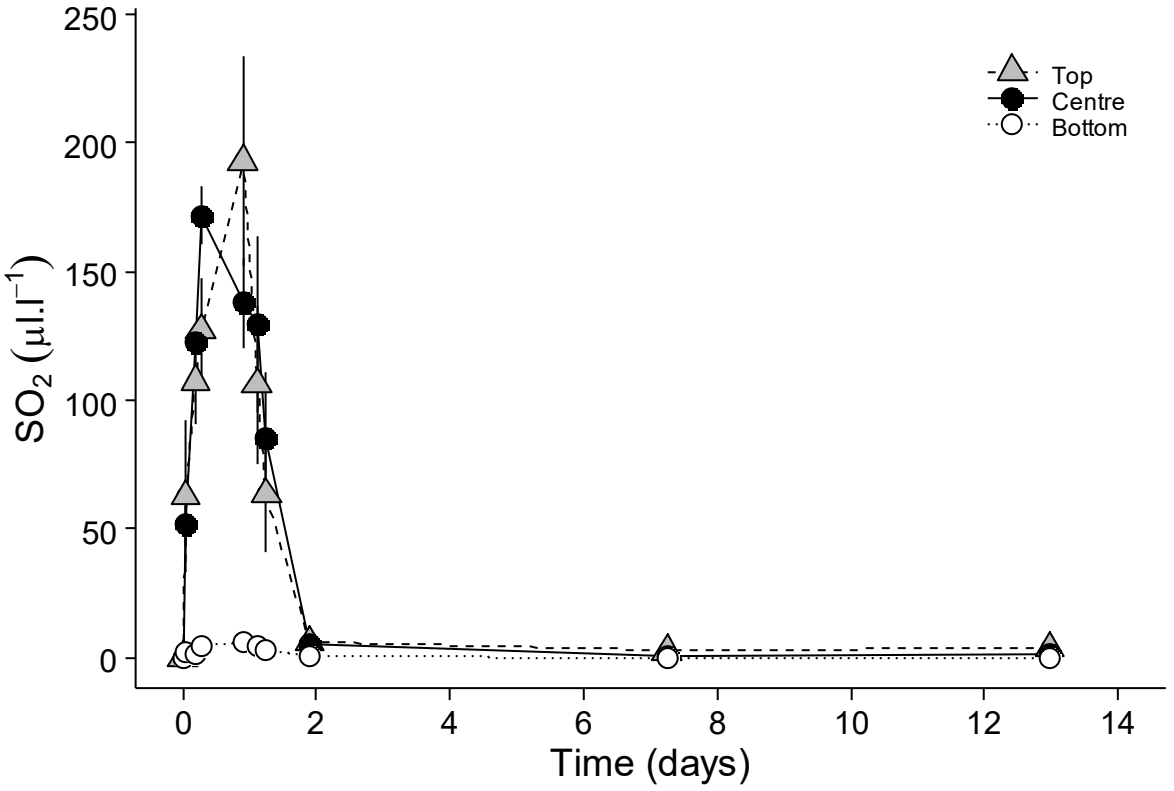

Figure 2. Sulphur dioxide (SO_2) concentration ($\mu l.l^{-1}$) measured inside lined 10 kg cartons of Thompson Seedless table grapes containing a Uvasys® green SO_2 generator sheet. Cartons were held at 1°C and SO_2 was measured in gas samples taken from immediately below the sheet (*top*), in the middle of the carton (*centre*), and in the bottom corner (*bottom*) (n = 6). Vertical bars represent the standard error of the mean values. Where no bar is shown it falls within the symbol.

Grape quality

Due to the high quality of the grapes and a low disease pressure, there was no significant change in grape quality in experiments 1 and 3 due to the relatively short storage period (data not shown). In experiment 2, where grapes were stored for up to 8 weeks, there were small but significant changes in grape quality, particularly rachis browning. There was a significant interaction between storage time and

storage treatment on the Rachis index ($P = 0.006$), where Rachis index was lower (less browning) in grapes within the sulphur treatment compared to those stored in cold alone ($P < 0.001$, Figure 3, Table 2). It has been demonstrated that rachis browning is well correlated with water loss from the stems (Crisosto et al. 2001). In this study, water loss from stems in both treatments would have been similar as bunches from both were packed inside low density polyethylene carton liners which have a low permeability to water vapour. The SO_2 treatment may slow the expression of browning symptoms by inhibiting polyphenol oxidation.

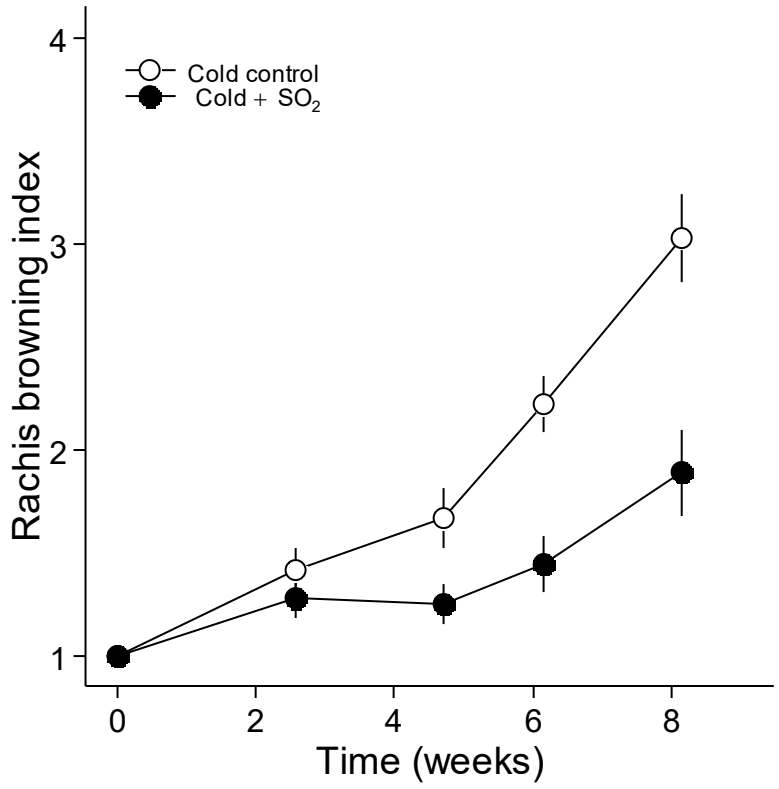

Figure 3. Effect of storage time (weeks) at 1°C with or without sulphur dioxide treatment (SO_2) on the rachis browning index (n = 6) of crimson seedless table grape bunches. Rachis score: 1 = All green, 2 = Most pedicels showing browning, 3 = Pedicels brown and laterals partially brown, 4 = Pedicels and all laterals showing browning, 5 = Whole rachis brown.

There was no significant effect of the SO_2 treatment on bunch colour, % rots, soluble solids concentration (SSC) or sugars to acid ratio (SSC:TA ratio) (Table 2). Rots (%) increased with storage time ($P = 0.024$) but remained at low levels. After 8 weeks storage only 0.7% of cold treatment berries and 0.4% of SO_2 treated berries had rots. There was a significant effect of storage time on bunch colour ($P < 0.001$) and SSC:TA ($P < 0.001$). After 2 weeks storage, bunches were less red and had a lower SSC:TA ratio. However, the colour was still acceptable after 8 weeks storage with bunches having 73.6 and 78.6% of berries displaying an even red colour in the SO_2 and cold control treatments, respectively. SSC:TA ratio was still acceptable at the 8 week removal, recording 49.8 for SO_2 and 51.3 for cold treated berries. Crimson seedless grapes are considered to be of high eating quality when the SSC:TA ratio exceeds 40 (Jayasena and Cameron 2008). There was no significant effect of storage time on SSC.

Table 2. The effect cold alone and cold + SO_2 storage for up to 8 weeks on bunch colour, rachis browning index, % rots, soluble solids concentration (SSC, °Brix) and sugar to acid ratio (SSC:TA) of Crimson seedless table grapes. Values are the mean of 6 replicate boxes ± the standard error of the difference of the means (SE).

Storage time (weeks)	Treatment	Bunch colour (%)	Rachis index	Rots (% berries affected)	SSC (°Brix)	SSC:TA Ratio
2	Cold alone	83.4 ± 1.6	1.4 ± 0.1	0 ± 0.0	20.2 ± 0.2	55.7 ± 2.2
	Cold + SO_2	85.8 ± 1.0	1.3 ± 0.1	0 ± 0.0	20.9 ± 0.2	54.3 ± 1.3
4	Cold alone	83.9 ± 2.4	1.7 ± 0.1	0.3 ± 0.1	20.6 ± 0.4	50.9 ± 1.3
	Cold + SO_2	82.8 ± 1.8	1.3 ± 0.1	0.2 ± 0.2	20.2 ± 0.3	46.5 ± 1.7
6	Cold alone	73.3 ± 1.9	2.2 ± 0.1	1.0 ± 0.2	20.3 ± 0.1	47.9 ± 1.3
	Cold + SO_2	72.5 ± 1.5	1.4 ± 0.1	0.8 ± 0.4	20.0 ± 0.4	48.1 ± 0.6
8	Cold alone	78.6 ± 2.3	3.0 ± 0.2	0.7 ± 0.6	20.4 ± 0.3	51.3 ± 1.0
	Cold + SO_2	73.6 ± 2.1	1.9 ± 0.2	0.4 ± 0.2	20.2 ± 0.2	49.8 ± 2.0

Recommendations

- Use of SO_2 generator sheets is highly recommended for cold storage and transport of export table grapes, even if time to market is short, such as that experienced by airfreight. The work reported here validates the use of SO_2 to reduce rots and rachis browning and increased mortality of all the insect species tested here (except the two-spotted mite).
- Further large scale tests are warranted to validate these results and provide efficacy data that can be used in negotiations with regulators in phytosanitary markets. With the exception of two-spotted mites, the application of SO_2 increased mortality compared to cold alone.
- Research to improve the SO_2 delivery system to ensure higher and more uniform SO_2 levels throughout the carton is warranted, as this may increase insect mortality and provide increased control of rots and rachis browning.
- Alternative and combined treatments may be required to control tolerant insects such as earwigs and to achieve mortality within 4 weeks. Possible treatments include modified atmospheres in combination with SO_2 and cold, applying the initial SO_2 treatment at a higher temperature and using higher capacity SO_2 generator sheets, particularly for the delivery of a higher pulse at the start of storage.
- Further research is recommended to test the survival of other insects of concern to export markets, particularly the light brown apple moth, as well as other recorded contaminants of grape export consignments.

Scientific Refereed Publications

Journal article

Tomkins, B., Yen, A., Vandegeer, R., Lopresti, J. and Williams, D. (2016). The effect of cold and sulphur dioxide on survival of arthropod contaminants in export table grapes. In preparation.

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Intellectual Property/Commercialisation

No commercial IP generated

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Appendices

Detailed materials and methods TG15003

TG 15003 - Materials and methods.docx

TG 15003 Materials and Methods

Experimentation

Three experiments were conducted. In the first experiment, long-tailed mealybug (*Pseudococcus longispinus*) and Ladybird beetles (*Chilocorus* sp.) were stored with Thompson seedless grapes for 17 days at 1°C ± 0.5°C with or without sulphur dioxide (SO_2) treatment. Earlier studies indicated that both species died within 2 weeks storage at 1°C both with and without SO_2 (Tomkins and Yen, 2016). To validate this result and to study the dynamics of insect mortality more closely, insects were removed for assessment at day 3, 7, 10, 14 and 17. In the second experiment, the two-spotted spider mites (*Tetranychus urticae*), European earwig (*Forficula auricularia*), Argentine ant (*Linepithema humile*) and Carpophilus or dried fruit beetle (*Carpophilus hemipterus*) were stored for 8 weeks at 1°C in Crimson seedless table grape cartons for 8 weeks. Boxes of grapes were removed from storage and the insects and mites were examined after 2, 4, 6 and 8 weeks storage. All the ants and Carpophilus were dead after 2 weeks, therefore a third experiment was conducted to study the dynamics of insect mortality more closely in response to cold alone and cold plus SO_2. In this experiment, grape boxes were removed from storage at day 3, 7, 10, 14 and 17 and the insects examined to test if they were alive or dead.

Grapes

Cartons (10 kg) of white table grapes (*Vitis Vinifera*, L Thompson seedless) and red table grapes (*Vitis Vinifera*, L Crimson seedless) were obtained directly from a specialist grape wholesaler. Fruit was harvested by hand at commercial maturity and packed directly into 1 kg bunch bags with 10 bunch bags packed into each carton. Cartons were then transported within 3 h of harvest to a forced air cooling facility and stored at 2°C overnight before transport under refrigeration to the wholesaler. The next morning the fruit were delivered under refrigeration to the laboratory where they were stored at 1°C until used. On arrival fruit were in field fresh condition with green stems and firm berries with an attractive waxy bloom.

Insects

For the first experiment, a colony of long tailed mealybugs was reared in a commercial facility (Biocontrol Services, Langwarrin South, Victoria) from insects collected from infested table grape bunches grown in a vineyard at Mildura, Victoria. Insects were reared on grape vines grown in pots in a plant growth chamber set at 25°C and normal daylight hours. A colony of mealybugs on their food host plants were delivered to the laboratory on the morning before the experiment commenced. Ladybird beetles (*Chilocorus* sp.) were reared in a commercial facility (Bugs for Bugs Pty Ltd, Mundubbera, Queensland). Adult beetles were shipped to the laboratory by overnight courier the day before the experiment commenced. Ladybird beetles were shipped with adequate food and liquid for the journey and arrived healthy and active. The day before the experiments commenced, eight ladybirds or a leaf containing 10 to 31 mealybugs were transferred to vented containers with a bunch comprising two grapes. The next morning, containers were checked to ensure the beetles and mealybugs were still alive.

For the second and third experiments, two spotted spider mites (*Tetranychus urticae*) were reared in a commercial facility (Bugs for Bugs Pty Ltd, Mundubbera, Queensland). Mite colonies on detached bean leaves were shipped to the laboratory by overnight courier the day before the experiment commenced. Mites were shipped with adequate food for the journey and arrived healthy and active. Adult dried fruit beetles (*Carpophilus hemipterus*) were obtained from a laboratory colony started from adults caught in pheromone traps in orchards in the Goulburn Valley, Victoria and reared on artificial diet in a controlled environment room at 25°C and 12 h photoperiod. Adult European

earwigs (*Forficula auricularia*) were obtained from a wild population in an almond orchard at Mildura, Victoria and Argentine ants (*Linepithema humile*) were collected from wild populations in suburban Melbourne. Ants and earwigs were kept at room temperature in purpose built enclosures with adequate water and food available at all times until used. The day before the experiments commenced, four earwigs, five ants, five *Carpophilus* beetles or a piece of bean leaf with two to ten adult mites were transferred to separate vented containers with a bunch comprising two grapes. The next morning containers were checked to ensure the insects were still alive and active.

Carton set up

Containers of insects were inserted between the grape bunch bags in the centre of each experimental carton. A Uvasys® green dual release SO_2 generating sheet (Grapetek Pty Ltd, Cape Town South Africa; a.i. 970 g kg^{-1} Sodium metabisulphite) was placed immediately on top of the bunch bags in half the cartons. All cartons were lined with a low density polyethylene carton liner bag which was closed and securely folded over the contents of the carton after the insects and a SO_2 sheet (if applicable) were added.

Cool storage

All cartons were stored on racks in a complete randomised design in a cool room set at 1 ± 0.5°C and 80 ± 5% RH and removed for examination after 3, 7, 10, 14 and 17 days in experiments 1 and 3, and after 2, 4, 6 and 8 weeks storage in experiment 2. Six replicate cartons with or without the SO_2 generator sheet were removed at each sampling date. Tiny Tag Ultra 2 data loggers (Hastings Data Loggers, Port Macquarie, NSW) were used to record room temperature and humidity.

Insect assessments

At the sampling dates, containers of insects were removed from cartons, left at room temperature for at least three hours and examined under a dissecting microscope. Insects were determined to be dead when there was no visual movement after gently tickling with a fine camel hair brush.

Grape assessments

On the day after removal from storage, three to four bunch bags were removed from each carton at random and the grapes assessed for visual quality, rots, soluble solids concentration (SSC) and titratable acid (TA).

Visual quality of berries from cartons with or without SO_2 was compared with respect to berry colour and stem (rachis) browning (Table 1). Grape bunches from within each bunch bag were weighed, laid out on a white plastic tray and scored for rachis browning using a 1 to 5 scoring scale based on Lichter (2016) where 1 = green rachis and 5 = completely brown rachis (Fig. 1). When bunch bags contained multiple bunches, each bunch was scored separately and the average rachis browning score calculated.

Table 1. Description of ratings used to score quality attributes of individual berries and bunches.

Quality attribute	Ratings and Description				
	1 (none)	2 (trace)	3 (slight)	4 (moderate)	5 (severe)
Berry surface browning	None	< 5 berries per bunch bag	5 to 10 berries per bunch bag	11 to 30 berries per bunch bag	> 30 berries per bunch bag
Berry bleaching	None	< 5% surface bleaching	5 to 25% surface bleaching	26 to 50% surface bleaching	> 50% surface bleaching
Berry shrivel	None	Shrivel around pedicel only	Shrivel < 10% of berry surface	Shrivel on 10-25% of berry surface	Shrivel on > 25% of berry surface
Stem (rachis) browning	All green	Most pedicels showing browning	Pedicels brown and laterals partially brown	Pedicels and all laterals showing browning	Whole rachis severely browned

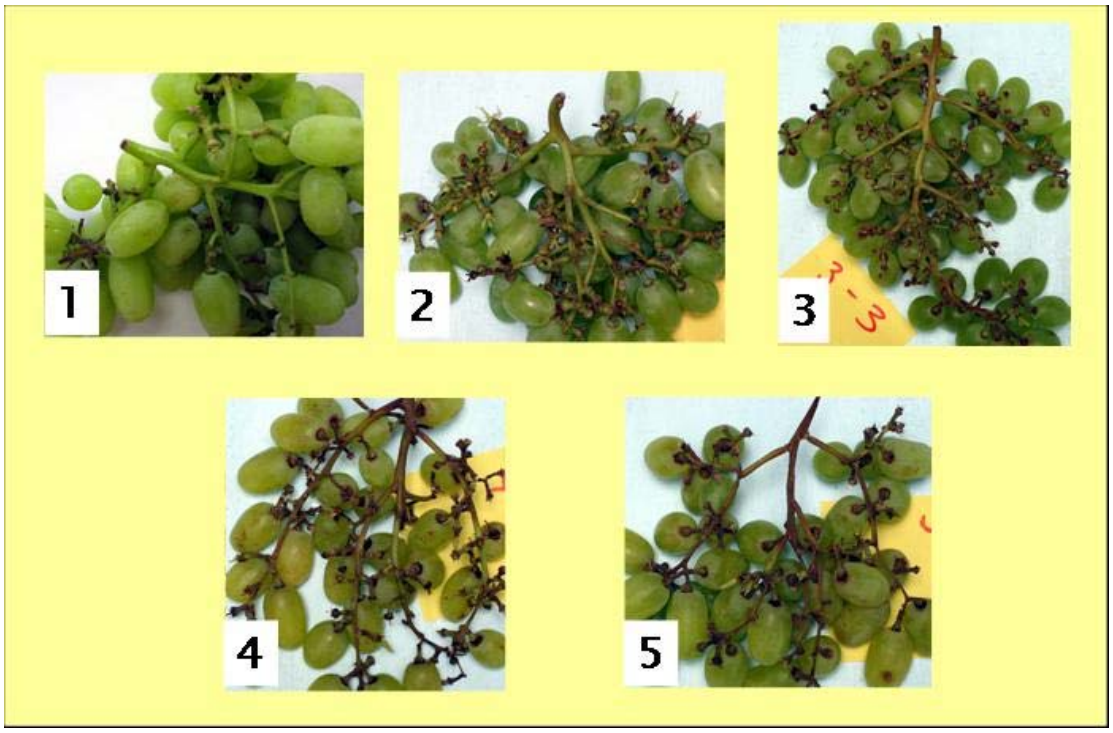

Figure 1. Visual rating scales and descriptions used for scoring of bunch rachis browning

Number of berries with symptoms of Botrytis rot was recorded for each bunch bag both after removal from cool storage and after storage at 18°C for 7 days. Infected berries were weighed and the percent weight of berries with Botrytis within each bunch bag was calculated.

After berries with rots were recorded, 10 berries were removed at random from each bag for SSC and TA analyses. Berries were crushed using a mortar and pestle and the free juice decanted. Juice

was analysed for SSC expressed as °Brix (g sucrose equivalents per 100 g juice) using an Atago® pocket refractometer (Atago, Tokyo, Japan) calibrated with distilled water.

Titratable acidity of the juice was measured using an Accuvin Titratable Acidity Quick Test™ (Accuvin LLC, Napa, CA USA) and expressed as g tartaric acid equivalents per L juice. TA measurements were conducted on a composite 8 ml juice sample collected from three to four bunch bags per replicate carton by placing 91 µl of juice into an Accuvin Eppendorf tube as supplied and comparing the resulting colour change to the colour chart provided. The TA range measured by the kit was 4 – 11 $g.L^{-1}$. Each Eppendorf tube was shaken for 30 sec to ensure complete colour change before assessment.

Sugar to acid ratio (i.e. SSC to TA ratio) was calculated for each bunch bag using individual SSC measurements and average TA measurements based on three to four bunch bags per replicate carton:

$$\text{Sugar to acid ratio} = \frac{\text{SSC} \times 10}{\text{TA}}$$

Sulphur dioxide measurement

Atmospheres in six cartons were sampled regularly during storage through a Teflon tube inserted into spaces immediately below the sheet (top), in the centre of the carton and in the bottom corner of the carton. Samples were drawn through the tube with a gas tight 50ml syringed and injected into the sample port of a DragerSensor®XS EC SO_2 Pac III gas detector (Draeger Safety Inc, Pittsburgh, PA USA). The gas detector was calibrated before each use with an Air Liquide Calgaz containing 5 $\mu l.l^{-1}$ SO_2 with the balance nitrogen (Air Liquide, Sunshine, Victoria). SO_2 concentration in the bags was expressed as $\mu l.l^{-1}$.

Experimental design

The experiment was a complete randomised design. Treatments were cold with or without SO_2 and removal times at 3, 7, 10, 14 and 17 days or 2, 4, 6 and 8 weeks. There were six replicates (10 kg cartons) of each treatment. Representative bunch bags (3 – 4) were removed from each replicate carton for grape quality assessments. Each replicate carton contained a vented container with the insects or mites inside and a small bunch (two berries) of grapes.

Statistical analyses

Genstat software (Version 14, Lawes Agricultural Trust, 2011) was used to statistically analyse the results. The mortality data were not corrected for control mortality because there was no true control treatment available since the experiments all needed to be conducted under cold storage and cold is a known mortality factor for some insects. ANOVA assumptions of homogeneity of variances and normality were satisfied in all cases. The results from using a Generalised Linear Mixed Model approach (with distribution specified as Binomial) were similar to those from the ANOVA based on % data.

For grape quality attributes, a two-way analysis of variance (ANOVA) was conducted to determine the main effect of SO_2 treatment and storage time on bunch quality and any interaction effects between treatment and storage period. Means were compared using 95% confidence intervals, or Fisher's Least Significant Difference (LSD) test with statistical differences between means determined at the 5% significance level ($\alpha = 0.05$).

References

Lichter, A. (2016). Rachis browning in table grapes. Australian Journal of Grape and Wine Research 22:161-168.