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Management of Postharvest Diseases of Horticultural Crops using Australian Essential Oils

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Management of Postharvest Diseases of Horticultural Crops using Australian Essential Oils

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Foreword

The project outlined in this report evaluated the antifungal activity of essential oils extracted from lemon myrtle (*Backhousia citriodora*), anise myrtle (*Syzigium anisatum*) and tea tree *(Melaleuca alternifolia*) essential oils and two standards, citral and trans-anethole against the postharvest pathogens of the horticulture industry *Botrytis cinerea*, *Monilinia fructicola*, *Fusarium spp*. and *Geotrichum candidum.* Previous research has shown that some essential oils have exhibited antifungal properties against tested postharvest pathogens and that there may be potential for the development of new safe, biocidal postharvest products for fresh horticultural produce.

This preliminary study provided an assessment of the value of continued work in this area. Both the essential oils industry and industries producing soft, perishable horticultural products could receive considerable financial benefit from this work, particularly in areas where organic or 'green' markets are important. However, for this research to be adopted it will be important that potentially benefitting horticultural industries be included in a well targeted communication strategy.

In-vitro trials revealed that each of the tested pathogens exhibited a different level of sensitivity to each essential oil/standard with preliminary results suggesting that the essential oils/standards provided excellent control of some pathogens with respect to mycelium growth and spore germination at very low concentrations, whereas for other pathogens, higher concentrations than would be economically viable were needed to significantly reduce mycelium growth and spore germination. The most effective treatments were evaluated further in *in-vivo* trials for their antifungal activity via fumigation using inoculated nectarines and tomatoes. *In-vivo* trials indicated that the oil and the standard exhibited antifungal activity, to some extent, with some difference in activity between oil's concentrations against tested fungal pathogens.

Twenty-five untrained self selected NSW Industry and Investment employee panellists were involved in conducting sensory evaluation trials. Results demonstrated that lemon myrtle essential oil treatment effect on sensory value in this experiment was not significant. The association between lemon myrtle essential oil treatment and intention to purchase was also not significant.

Cost benefit analysis indicated that lemon myrtle essential oil is a viable alternative to conventional citral. The main objectives of this study were to evaluate the antimicrobial properties of the essential oils and their main components, and therefore evaluation of the optimal parameters such as concentration, formulation, time of application, number of oil applications remain to be evaluated in order to determine the most economical treatment. Other factors including consumer's acceptability of using synthetic chemicals for postharvest disease control, pathogen resistance to fungicides that are currently commercially available and the limitation of registered chemicals for treatments on organically grown crops also need to be considered.

This project was funded from RIRDC Core Funds, which are provided by the Australian Government.

This report, an addition to RIRDC's diverse range of over 2000 research publications, forms part of our Essential Oils and Plant Extracts R&D program which aims to provide the knowledge and skills base for industry to provide high, consistent and known qualities in their essential oils and plant extracts products that respond to market opportunities and enhance profitability.

Most of RIRDC's publications are available for viewing, free downloading or purchasing online at www.rirdc.gov.au. Purchases can also be made by phoning 1300 634 313.

Craig Burns
Managing Director
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Executive Summary

What the report is about

This report describes the evaluation of the effectiveness of essential oils extracted from Australian native plants, lemon myrtle (*Backhousia citriodora*), anise myrtle (*Syzigium anisatum*) and tea tree (*Melaleuca alternifolia*) and their active constituents against the important postharvest pathogens of the horticulture industry *Botrytis cinerea*, *Monilinia fructicola*, *Geotrichum candidum* and *Fusarium* species.

Who is the report targeted at?

The report is targeted at current industry participants including horticultural producers, exporters, essential oil producers, and the scientific community.

Background

The postharvest losses of fruit and vegetables are estimated world-wide to range from 10 to 50% of the saleable volume of the crop. One of the key contributors to postharvest crop loss is fungal infection. It is therefore important for growers to have an effective means of controlling postharvest fungal pathogens. In most cases this involves using a registered synthetic fungicide and low temperature storage and /or controlled atmosphere storage. It may be possible to use essential oils as an alternative to some synthetic fungicides.

Essential oils, (Generally Regarded as Safe, GRAS) may provide "greener" alternative fungicides for horticultural industries. Fungi may also be less likely to develop resistance to essential oil treatments because of their very complexity (made up of many constituents) which makes it very difficult for organisms to evolve coping mechanisms. This compares favourably to the synthetic fungicides currently in use which often have only a few constituents. *In-vitro* studies have demonstrated the antifungal activity of essential oils against a range of postharvest pathogens including *B. cinerea*, *M. fructicola*, *G. candidum*, and *Fusarium spp*. A potential advantage of using an essential oil treatment is their volatile nature which implies that no or little residue is left on the produce after treatment.

Little information is currently available in relation to the antimicrobial properties of essential oils extracted from Australian native plants and their use in treating postharvest pathogens. It has been reported that citral, the main component of lemon myrtle (*Backhousia citriodora*), was a very effective antimicrobial against some postharvest pathogens. Comparison of lemon myrtle chemical composition with other citral-rich commercial oils indicated that lemon myrtle is richer in citral than any other lemon scented botanical such as lemon grass (*Cymbopogon flexuous*) or May Chang (*Litsea cubeba)*. Both the significant activity and the recognition of the compound as a safe food additive by the US Food and Drug Administration supports the assumption that lemon myrtle essential oil has potential for inclusion in foods as a natural antimicrobial and for postharvest use. Based on their antifungal properties other oils that might have potential in this respect are anise myrtle (*Syzigium anisatum*) and tea tree (*Melaleuca alternifolia*). This lack of information currently available provided an opportunity to investigate and evaluate the antifungal effect of Australian native essential oils and the potential for their use as natural fungicides for postharvest purposes.

Aims/objectives

The objectives of the project were to:

- compare the antifungal properties of lemon myrtle oil against citral, and aniseed myrtle oil against trans-anethole on selected horticultural postharvest pathogens *in-vitro*;
- evaluate the optimal application methods for the selected oils/components *in-vivo* against inoculated and non-inoculated products;
- establish the efficacy of the oils/components necessary to achieve at least 50% reduction in postharvest disease;
- trial whether treatment with the selected oils/components under these conditions taints the taste of the product;
- provide a cost benefit analysis comparing the use of essential oil based treatments, with active constituents (citral) and then comparing those treatments with the currently registered postharvest fungicide dip, Rovral.

Methods used

Essential oils extracted from the Australian native plants lemon myrtle (*Backhousia citriodora*), anise myrtle (*Syzigium anisatum*) and tea tree (*Melaleuca alternifolia*) were evaluated *in-vitro* against postharvest pathogens *Botrytis cinerea, Monilinia fructicola, Geotrichum candidum* and *Fusarium spp*. All *in-vitro* and *in-vivo* testing was carried out utilising the essential oils in the vapour phase as a fumigant rather than by direct contact as this application method seemed to be more applicable for postharvest disease management. The treatments that were most effective in controlling the individual pathogens were then evaluated further in *in-vivo* trials for their antifungal activity. Tainting can be a difficulty when essential oils are applied to fresh produce, therefore a sensory evaluation trial was conducted to assess the effect of lemon myrtle essential oil on nectarines.

Results/key findings

The results of this research demonstrated that lemon myrtle and tea tree oil exhibited antifungal activity against the tested postharvest pathogens via fumigation. Although this is a preliminary study and not all of the factors contributing to the *in-vivo* efficacy have been investigated, the data provided here demonstrated that lemon myrtle in particular has potential as an antifungal agent to control postharvest pathogens.

In-vitro trials indicated that each of the four tested pathogens exhibited a different level of sensitivity to each essential oil and its main component, with results suggesting that the essential oils and their main constituents provide excellent control of some of these pathogens at very low concentrations. For other pathogens, higher concentrations than would be economically viable were needed to significantly reduce mycelium growth and spore germination. Lemon myrtle essential oil and citral were the most effective treatments to control mycelium growth of *M. fructicola* and *G. candidum*. Lemon myrtle essential oil also inhibited *M. fructicola* spore germination.

Based on the essential oils efficacy results of the *in-vitro* experiments, only *M. fructicola* and *G.candidum* were selected for further testing in *in-vivo* trials. Stone fruit are susceptible to *M. fructicola* while tomato fruit are susceptible to *G.candidum*, hence each pathogen was tested *in-vivo* using these fruit. Lemon myrtle, citral, anise myrtle and transanethole were tested against *M. fructicola* while lemon myrtle and citral only were tested against *G.candidum*.

Lemon myrtle oil and citral exhibited antifungal activity to some extent against *M. fructicola*, with differences in activity levels dependent upon the concentration of the oil. Fumigation of nectarines following inoculation significantly ($P<0.05$) reduced the incidence of brown rot in comparison to the inoculated control treatment. Citral was ineffective at a concentration of 0.6% against *M. fructicola*. Tomato fruit had lower sour rot (*G.candidum*) disease incidence following exposure to lemon myrtle essential oil and citral compared to the untreated control when stored at 10°C.

Results from the sensory evaluation trials indicated that lemon myrtle essential oil treatment effect on sensory value in this experiment was not significant. The association between lemon myrtle essential oil treatment and intention to purchase was also not significant.

Implications for relevant stakeholders:

Preliminary results from this project provide evidence which may support the use of essential oils as a component of management strategies for the control of postharvest diseases in horticulture. Their use would ease the current reliance on conventional pesticides for disease control. The Australian horticultural industry is highly reliant on synthetic fungicides. Given consumer and legislative concerns over the use of synthetic fungicides, essential oils may be viable given comparable efficacy and cost.

Recommendations

The primary output of the first year of the project consisted of evaluating only the antimicrobial properties of certain essential oils derived from Australian native plants against four common postharvest pathogens. Consistently, results indicated that lemon myrtle essential oil was the most effective against the tested pathogens. However, because of the preliminary nature of this work it is recommended that further research into the efficacy of lemon myrtle essential oil, needs to be conducted in order to collect scientifically reliable data on the potential use of essential oils for postharvest disease control.

It is also recommended to further examine the use of lemon myrtle essential oil as a potential for organic horticulture where no chemical control for postharvest disease management is available. In this case economic losses experienced by the growers may be reduced significantly. Growers may have to turn to this new strategy to protect their crop after harvest in the future.

Results from this study lead to the recommendation to further investigate or develop combinations of lemon myrtle and other essential oils that might act synergistically to control postharvest pathogens and also demonstrate effectiveness that is comparable to commercially available synthetic fungicides already used for postharvest disease control.

1. Introduction

Fresh fruit and vegetables increase in value as they are moved through the supply chain from the field to the consumer. During this period postharvest pathogens can develop rapidly and losses can reach staggering proportions, in particular in developing countries, or in regions where tropical weather and poorly developed infrastructure amplify the problem. The estimated annual losses can be significant reportedly ranging from 10 to 50 % (Wills *et al.*, 1998; Cook, 2002; Kader, 2002). Postharvest losses due to fungal decay are influenced by the perishability of the commodity, the environmental temperature and relative humidity which determine the natural course of decay, the length of time between harvesting and consumption, and practices of postharvest handling, storage and processing (FAO, 1981). On top of losses due to wastage, further economic loss can occur if the market requirements necessitate sorting and repacking of partially contaminated consignments (Wills *et al.*, 1998). It is therefore important for agricultural supply chains to have effective means of controlling postharvest fungal pathogens. In most cases this involves using a synthetic fungicide and low temperature storage.

In recent years consumers and regulatory agencies have expressed increasing concern over the health hazards of using synthetic chemicals for postharvest disease control. For some pathogens, however, no alternative control is available, as pathogens have developed resistance to chemicals that are currently commercially available (Rosenberger & Meyer, 1981; Viňas *et al*., 1991; Gullino *et al*., 1994) of which only a very few are still registered for postharvest treatment. To reduce the dependency on synthetic fungicides, more natural methods of postharvest disease control are currently being investigated (Ogawa & Manji, 1984; Jeger & Jeffries, 1988; Wilson *et al.*, 1999; Janisiewicz & Korsten, 2002). Biological control of postharvest diseases has only recently developed as a practical effective strategy (Janisiewicz & Korsten, 2002).While there are potentially a number of biological control agents that may be useful for controlling postharvest diseases, identification and commercial development are at a relatively early stage. It is highly unlikely that any one of these alternative methods alone will be as effective as fungicides; however, the development of strategies combining several of these methods may be a commercially acceptable approach. Currently, promising alternatives such as biological control and natural antimicrobial volatiles, alone, and in various combinations, are being investigated for their potential as successful postharvest decay control strategies in countries such as the USA, Israel and the EU. If chemical control is no longer a viable option, due either to pathogen resistance or legislative restriction caused by health and environmental concerns, growers may have to turn to these new strategies to protect fruit after harvest in the future.

The use of some essential oils as an alternative ‘Generally Regarded As Safe’ (GRAS) chemical treatment (Gorris *et al.*, 1994) has attracted considerable interest from postharvest scientists over the past years. Essential oils may be seen to be "greener" alternatives for consumers and fungi are less likely to develop resistance to essential oils which is often a problem with synthetic fungicides currently used. *In-vitro* studies have demonstrated the antifungal activity of essential oils against a range of postharvest pathogens including *Botrytis cinerea* (Wilson *et al.*, 1997; Walter *et al.*, 2001; Bouchra *et al.*, 2003; Ben Arfa *et al.*, 2006), *Penicillium spp.(* Arras *et al.*, 1995; Ben – Yehousha & Rodov, 2006) *Monilinia spp.* (Caccioni & Guizzardi,1994; Chu *et al.*, 2001; Tsao & Zhou, 2001; Liu *et al.*, 2002; Lazar & Jobling, 2009), *Geotrichum candidum* (Szczerbanik *et al.*, 2006) *Fusarium spp.* (Gorris *et al.*, 1994; Vaughn & Spencer, 1994; Oosterhaven *et al.*, 1996; Daferera *et al*., 2003) . A potential advantage of essential oil treatments is their volatile nature which implies that no or little residue is left on the produce after treatment. Patents have been issued or are pending on these technologies.

In recent years there has been an increasing interest in using essential oils for postharvest applications in Australia and there are many opportunities for developing new markets for plant extracts from both native and exotic species. The Australian essential oils industry is made up of around 150 commercial producers, with a wholesale value of the processed product of about $6 million and production

dominated by a few larger firms (RIRDC, 1995). Australia produces commercial quantities of lavender, parsley, dill, boronia, blackcurrant bud, fennel, tea tree, eucalyptus and peppermint essential oils (RIRDC, 2003).

Although antimicrobial properties of tea tree oil against human and food-borne pathogens have already been investigated (Carson & Riley 1995), to date there is little information on the antimicrobial properties of essential oils extracted from other Australian native plants against postharvest pathogens (Szczerbanik *et al*., 2006; Lazar & Jobling, 2009). Research into the chemical composition of Australian plants has found that the active ingredients are often in higher proportions compared with other similar species with similar chemical structures. For example, a comparison of the chemical composition of lemon myrtle to other citral-rich commercial oils found that lemon myrtle was higher in citral than other lemon scented botanical species such as lemon grass(*Cymbopogon flexuous*) or May Chang(*Litsea cubeba*) (Archer, 2004). Other Australian essential oils that might have potential for postharvest disease control include anise myrtle (*Syzigium ani satum*).

Preliminary work for the Australian native species, *Backhousia citriodora* and *Melaleuca alternifolia*, has highlighted an opportunity to investigate and evaluate the antifungal effect of Australian native essential oils and the potential for their use as natural fungicides for postharvest disease control.

2. Objectives

The objectives of this research were to:

- compare the antifungal properties of lemon myrtle oil against citral and anise myrtle oil against trans-anethole on selected horticultural postharvest pathogens *in-vitro*
- evaluate the optimal application methods for the selected oils/components *in-vivo* against inoculated and non-inoculated products.
- establish the efficacy of the oils/components *in-vivo* - treatments necessary to achieve at least 50% reduction in postharvest disease.
- trial whether treatment with the selected oils/components under these conditions taints the taste of the product
- provide a cost benefit analysis comparing the use of essential oil based treatments, with active constituents (citral) and then comparing those treatments with the currently registered postharvest fungicide dip, Rovral.

3. Methodology

Origin of the oil extracts

The essential oils used in this trial (lemon myrtle, anise myrtle and tea tree) were donated as commercial preparations of 100 % purity from Australian Rainforest Products and Telmont Essentials Pty Ltd., respectively. The origin and the purity of the oils were certified by quality certificates supplied by the company. The GC/ MS analysis corresponds to the Australian standard for these specific oils. The two standards, citral and trans-anethole, were supplied by Sigma – Aldrich Pty Ltd. (Sydney, Australia).

Pathogens

Pure fungal isolate of *Monilinia fructicola* was obtained from the Deciduous Fruit Pathology Laboratory, Orange Agricultural Institute, Australia. *Fusarium oxysporum, Geotrichum candidum* and *Botrytis cinerea* were isolated from symptomatic fruit, purified and preserved on plates for immediate use. Prior to each trial each pathogen was subcultured to new plates. Potato Dextrose Agar (PDA) (Difco Laboratories, BD, Sparks, MD, US) was used as the base medium to maintain cultures.

In-vitro experiments

Culture preparation

Isolates of each of the four pathogens were subcultured by placing a 0.8cm plug of growth onto new Potato Dextrose Agar (PDA) (Difco Laboratories, BD, Sparks, MD, US) media for *Fusarium*, *Geotrichum* and *Botrytis*, and quarter acid potato dextrose agar using: PDA 9.75g l^{-1} Agar 9 g l^{-1}; SDW (sterile distilled water) 1L; lactic acid stock solution (25%v/v : lactic acid (76%) 6.6mL; SDW 13.4mL). for *Monilinia*. Nine centimetre plastic Petri dishes (Bacto PDS 9014G) were used throughout the experiment. The sub-cultured isolates were then incubated at 25˚C in the dark for 7 days, except *Monilinia*, which was incubated for 10 days. These cultures were then used for all subsequent parts of the *in-vitro* trials.

Calculating volume of oil required

Using the molecular weight and density of each essential oil, lemon myrtle (*Backhousia citriodora*), anise myrtle (*Syzigium anisatum*) and tea tree (*Melaleuca alternifolia*) plus the standards citral and transanethole, a conversion factor for the expansion of each liquid to a gas was obtained. The volume of headspace in a 9cm Petri dish containing 20ml of agar was calculated. Using these values, the volume of oil required to achieve the desired volatile concentration in the headspace for use in the experiment was calculated. As citral makes up 95.3% of lemon myrtle essential oil, and trans-anethole makes up 83% of anise myrtle essential oil, calculations needed to be adjusted to ensure that the concentration of these main compounds were the same in each treatment.

Determination of inhibitory activity of volatiles against mycelial growth

An 8 mm diameter agar plug containing mycelia and spores of the fungus was collected from the edge of each actively growing fungal colony per species after 7 days, except *Monilinia* where mycelia and spores were harvested after 10 days. The agar plugs were transferred to the centres of plates containing fresh PDA or quarter acid potato dextrose agar using a sterile cork borer and scalpel blade.

In all cases the agar disk was inverted so that the inoculums' fungal growth was in direct contact with the agar. PDA media was divided equally (20 ml) into each Petri dish by using a liquid dispenser (OminispensePlus®, Wheathon Science Products, Millvile, USA). Pre-sterilised lids of centrifuge tubes (Bacto Laboratories # ART 00298-00) were placed at the edge of the plate on top of the agar and oil/volatile standards dispensed into the lid using a Rainin 10µl Positive Displacement pipette. Plates were then sealed immediately using Parafilm (Pechiney, Chicago, IL) and incubated at 25˚C in the dark for 7 days, except for *Monilinia* were incubation took place for 10 days. After 7 days and 10 days respectively for *Monilinia*, the plates were assessed for mycelial growth (mm), using digital calipers (Absolute Digimatic-Mitutoyo Corp. Japan).

The five treatments and controls were replicated three times using a randomised completed block design; each treatment unit consisting of 3 Petri dishes. The mycelial growth was assessed by measuring two radii at right angles to each other, after 3 and 7 days, except for *Monilinia*, where the assessment was after 2 and 12 days. Mycelial growth on control Petri dishes was assessed in parallel without the further addition of the essential oils. The mycelial growth was measured for each essential oil at five concentrations, 1.5, 2.0, 2.5, 3.0 and 3.5%. Data was expressed as percentage inhibition of mycelial growth where,

dc=mycelial growth diameter in control sets;

dt= mycelial growth diameter in treatments sets (Shahi *et.al*,2003)

Determination of inhibitory activity of volatiles against spore germination

The effect of the essential oils on spore germination of selected pathogens was tested *in-vitro*. Spores were harvested from an isolate after 10 days growth for *M. fructicola* and 7 days growth for *B. cinerea, F. oxysporum, G. candidum* and suspended in sterile distilled water (SDW). The resulting suspensions were filtered through sterile muslin to remove mycelia. The spore concentration was measured using a haemocytometer (Neubauer Superior, Germany) and adjusted to a final concentration of 10^6 spores/ ml^{-1}. Three independent replicate conidia suspensions were prepared and assigned to a complete block of treatments in order to avoid pseudoreplication. 100µl of spore suspension was then pipetted and spread onto PDA or quarter acid potato dextrose agar, and oil was added at the same rates as for the mycelial growth experiment. Plates were then sealed immediately using Parafilm and incubated at 25˚C for 18-20 hours in the dark before being assessed for spore germination. Each Petri dish was assigned one of the seven treatments (5 concentrations and 2 controls) for each oil tested. The treatments were arranged in a randomised complete block design with 3 replicates. Conidia germination was assessed under a compound microscope (10x20 magnification). One hundred spores/plate were assessed and the number of germinated spores was recorded. Each pathogen was evaluated in a separate experiment.

In-vivo trials

Based on the results of the *in-vitro* experiments, only *G.candidum* and *M. fructicola* were selected for further testing in *in-vivo* trials. *G.candidum* commonly infects tomatoes, while *M. fructicola* commonly infects stone fruit, hence each pathogen was tested *in vivo* using these fruit. Lemon myrtle and citral only were tested against *G.candidum*, while lemon myrtle, citral, anise myrtle and transanethole were tested against *M. fructicola* - these being the most effective oils in the in vitro trials.

Application method

Phytotoxicity can be a concern when using essential oils as a treatment. To determine the best application method of the tested essential oils and their main constituents and evaluate what effect

volatiles would have on the fruit, a preliminary trial was undertaken. Two application methods, the direct contact method such as dipping and the indirect contact method such as fumigation were evaluated**.**

Tomatoes (*Lycopersicon esculentum*) and nectarines (*Prunus persica)* were treated by dipping or fumigating with each oil and component. Aqueous solutions of lemon myrtle, anise myrtle oil, citral and trans-anethole were prepared at a concentration of 2%. Concentration of the volatiles had been selected as the maximum concentration to be cost effective for the growers. Fruit was dipped in solutions for 30 and 10 seconds, then stored in sealed plastic containers and kept at room temperature for 24 hours. Based on previous research (Lazar, unpublished data) fumigation with selected volatiles was carried out in 40L plastic containers also at 2% concentration for 8 hours.

Fruit was assessed for changes in quality which would affect consumer acceptance. Preliminary results indicated that for all volatiles, fruit which had been treated by direct contact method presented a decrease in quality. Tomatoes had lost turgidity, whilst nectarines suffered skin colour changes showing a strong phytotoxic effect when assessed after 24 hours. Fruit treated by fumigation did not show any perceptible changes in quality after 24 hours. Figures 1a and 1b present the effect of the lemon myrtle oil on the fruit after being dipped in a 2% solution for 30 seconds. The phytotoxic effect was similar for all treatments.

Figure 1a Tomatoes dipped in aqueous solution of lemon myrtle oil at 2% for 30 seconds

Figure 1b Nectarines dipped in aqueous solution of lemon myrtle oil at 2% for 30 seconds; Treated fruit bottom left and right, control fruit, centre

Based on the above results, direct contact methods such as dipping or spraying were determined to be non-viable methods of applying essential oils for the control of postharvest diseases for the nectarines and tomatoes. Therefore, only fumigation was evaluated further.

Determination of inhibitory activity of volatiles against *G. candidum*

Tomato fruit (*Lycopersicon esculentum*) cultivar *Amoroso Petite Truss tomatoes* were picked at commercial maturity from Pacific Hydroponics (NSW, Australia) at three different times, in 2008. No postharvest fungicides or sanitizers were applied to the fruit. Fruit was then stored at 10ºC until needed. Fruits of similar size without any deterioration were selected and were surface sterilized with 98% alcohol, followed by rinses with distilled water. The *in-vivo* experiments were carried out in 40L plastic drums, fitted with a suspended mesh container, a battery operated fan, and gas sampling outlets

in the lid (Fig.2a). Lemon myrtle and citral were the most effective treatments *in vitro*, and so only these compounds were tested in the *in vivo* trials, each at 3 concentrations.

Fig 2a Plastic containers used for *in-vivo* trials

The nine treatments were arranged following DiGGer design (Coombes 2002). Each treatment unit consisted of 7 fruit. Each sample fruit was inoculated using a Terumo 23G needle fitted to a Terumo 1ml tuberculin syringe, by puncturing to a depth of 3 mm. Each fruit was wounded on the side once and left to dry in the laboratory. Fifty µl of spore suspension (10^6 spores/ ml) was added to the wound and allowed to air dry at room temperature for three hours. The treatment was added to an open glass Petri dish which was also placed inside the container base (Fig. 2b).The fruit was placed inside the suspended mesh container and the fan switched on before the container was sealed (Fig.2c).

Fig 2 b,c Plastic containers used for *in-vivo* trials for nectarines

The gas sampling ports were sealed for 24 hours, after which time one of the ports was left unsealed to prevent CO_2 build up inside the container (which would negatively affect the growth of the pathogens), and every 3rd day the gas was sampled for CO_2 to ensure levels did not rise. The tomato fruit was then incubated in a temperature controlled environment at either 10 or 20°C until the control sample was sufficiently developed. The essential oils were used at the same rates per volume as for the *in-vitro* experiments. Tomatoes were checked daily for symptoms and decayed fruit were removed to avoid secondary infection. Each unit of fruit was scored on day 12 according to the following scale: 0=no decay; 1=1-5 % decay; 2=6-15 % decay; 3=16-25 % decay; 4=26-50 % decay; 5=>50 % decay.

Determination of inhibitory activity of volatiles against *M. fructicola*

Nectarines (*Prunus persica* (L) Batsch var. nectarina (Aiton.) Maxim.) cultivar Summer Blush were picked at commercial maturity from one orchard (Orange, NSW, Australia) in 2008. No postharvest fungicides or sanitizers were applied to the fruit. The same design as described above was used to investigate the effect of volatiles against *M.fructicola*, except that there were eleven treatments and one temperature. The eleven treatments were lemon myrtle essential oil and citral at concentrations of 0.6 and 1%; anise myrtle essential oil and trans-anethole at concentrations of 0.8 and 1.2% and three controls. As tea tree oil required a much higher concentration to achieve the same level of fungal inhibition as the other oils, it was not tested *in vivo*. Each treatment unit consisted of 5 fruit. Each fruit was wounded on the side once and left to dry in the laboratory. Ten µl of spore suspension (10^6spores /ml) was added to the wound. Nectarines were treated in the plastic containers for 24 hours at 20°C, after which the fruit were removed and placed into individual humidified containers loosely wrapped in a plastic bag. These individual containers were then held for 14 days at 12°C before being assessed and scored for disease incidence.

Nectarine sensory evaluation

Fumigation

Nectarines cultivar Summer Blush were taken out from a 0°C cool room in the afternoon and stored at 20°C overnight. After that, fruit were subjected to the following treatments: untreated (control), lemon myrtle 0.6% and lemon myrtle 1%. All treatments were carried out at 20°C. Fruit were fumigated following the same protocol as for *in-vivo* trials. After 20 hours of treatment, the nectarines were removed from the drums and immediately transferred to a new cool room for storage at 20°C. The two treatments were arranged in five randomised complete blocks. Each treatment unit consisted of nine fruit.

Panellist Assessment

Twenty-five untrained self selected NSW Industry and Investment employees were invited to assess the treated nectarines. Each one was presented with three nectarine samples. The panellists and samples were ordered according to the Latin square design. The effects of the panellists and order of presentation was accounted for with this design. As some treatments affected the colour of the fruit skin, all nectarines samples had their skin removed before being cut into 8 pieces and assessed.

Every panellist was provided with a different order of samples according to the experimental design. Panellists were asked to taste the samples one by one in the stated order and take some water between each sample to refresh their palate. According to panellists' degree of liking or disliking, they evaluated the flavour of each sample by marking an 'X' on a line scale. Evaluators were also asked to decide which fruits they would be likely to purchase and to state the reason for their decision. Sensory value was assessed by measuring the position of the 'X' as marked on the line scale.

Statistical analysis

Experimental Design

Each pathogen was investigated in a separate experiment. Three constant temperature cabinets were used with one replicate of the 27 treatments (5 oils x 5 concentrations + 2 controls) placed in each cabinet. Each cabinet had 3 shelves and each shelf accommodated 9 experimental units. The experimental unit was 3 Petri dishes

For the mycelial growth experiments, designs which accounted for possible differences between the 3 replicate cabinets and shelves within each cabinet were generated using DiGGer (Coombes 2002) for each pathogen. A split-plot-in-time analysis which assumes that the correlations between the residuals at each time are equal, was used.

Statistical analysis

The data for each pathogen was analysed separately and in a similar manner using the ASReml (Gilmour *et al.* 2002) statistical package. Treatment effects were assessed for significance and treatment means compared using the least significant difference (l.s.d.) technique at the 5% level.

The mycelial growth data was initially analysed using a mixed effects linear regression model which allowed the random effects of replicate and shelf within replicate to be estimated in addition to the fixed effects of oil type, concentration, time and their interactions. Since the variance component for shelf-within-replicate effect was negligible, it was ignored in the analysis. For *Fusarium oxysporum*, some treatments had all replicates with mycelial growth extending to the edge of the Petri dishes (40cm).This data was omitted from the analysis as its true value cannot be known. These included all replicates of the positive and negative control at time 2, TTO at concentrations of 1.5, 2.0 and 2.5 %

at time 2. A factorial treatment structure was created which included the treatments with incomplete combinations as well as the treatments with all combinations present.

A generalised linear mixed model with logit link function and binomial errors was initially fitted to the spore germination data. In a similar manner to the growth data the shelf-within-replicate random effect was negligible. Treatment effects were examined for significance and treatment means were separated on the logit scale then back-transformed for presentation.

Individual fruit score observations were averaged prior to analysis. The mean scores were assumed normally distributed and analysed using ANOVA. Means were then converted to a disease incidence percentage (DI %) for presentation.

The effect of treatment on the sensory value data was assessed using analysis of variance. Errors were assumed to follow the normal distribution and inspection of diagnostic plots supported this assumption.. The experimental design was originally planned to be a set of eight 3x3 Latin Squares for 24 panellists so that the effect of panellist and order of tasting could be included in the analysis. Instead, 25 panellists were recruited which led to an unbalanced design requiring the use of the ASReml (Gilmour *et al.*, 2002) statistical package to perform a mixed linear regression analysis. Panellist and tasting order were classed as random effects and treatment as a fixed effect.

Economic analysis

A basic cost effectiveness analysis was provided using $10m^3$ of nectarines as an example. As this project evaluated the use of essential oils as a postharvest treatment only, the main difference is the actual costs of the products. The analysis compares the costs of lemon myrtle oil, and it's main component citral against the current postharvest fungicide dip, Rovral.

4. Results

In-vitro trials on antifungal activity of volatiles against postharvest pathogens

Effect of various essential oils and volatile compounds on colony growth

The origin and the purity of the oils were certified as evidenced by quality certificates supplied by the company. The major compounds of lemon myrtle (*Backhousia citriodora*), aniseed myrtle (*Anetholea anisata*), and tea tree (*Melaleuca alternifolia)* oils, and the ratio (given as a percentage) of these compounds within each oil as determined by Gas Chromatography (GC) are presented in Table1.

Table 1 Essential oils tested and their main constituents as determined by GC analysis

Essential oil	Components	Percentage (%)
Lemon myrtle*	geranial	49.7
	neral	39.7
	trans-iso-citral	3.3
	cis-iso-citral	2.3
	exo-iso-citral	0.6
	citronellal	0.8
	linalool	0.6
	6-methyl-5-hepten-2-one	1.4
	2,3-dihidro-1,8-cineole	0.3
	myrcene	0.6
Aniseed myrtle	Trans - anethole	83.0
	methil charvicol	11.2
Tea tree	α- pinene	2.6
	sabinene	0.1
	α – terpinene	9.4
	limonene	1.1
	p – cymene	2.4
	1, 8 cineole	3.3
	γ terpinene	21.0
	terpinolene	3.4
	terpinen - 4 ol	40.8
	α – terpineol	3.1
	aromadendrene	1.2
	ledene	0.8
	δ- cadinene	0.9
	globulol	0.3
	viridiflorol	0.2

Total citral* (geranial, neral, trans-iso-citral, cis-iso-citral, exo-iso-citral) in lemon myrtle essential oil is 95.6%

Fusarium oxysporum

Evaluation of *in-vitro* bioactivity of essential oils and standards were undertaken in the gaseous phase. Table 2 shows the growth in mycelium of *F. oxysporum* at 25°C after exposure to volatiles. The average radial growth of colonies on unamended (control) PDA media plates was 40.0 mm after 7 days incubation at 25°C. Exposure to lemon myrtle essential oil and citral at all the concentrations

tested, 1.5-3.5%, resulted in significant inhibition of mycelial growth compared to untreated colonies (Table2). Colonies grew more slowly on media amended with the other volatiles tested – anise myrtle essential oil, trans-anethole and tea tree essential oil (Fig 3). Differences in the radial growth rate of the fungus did vary ($P<0.05$) between these treatments.

Table 2 Mycelial growth (mm) and growth inhibition (GI%) of Fusarium oxysporum at 25°C after exposure to volatiles.

The mycelium radius l.s.d.($P=0.05$) value to compare between days and treatments is 0.98. Values are the means of readings from 3 replicates of 3 colonies.

Treatment	Concentration (%)	Incubation time (days)				Average radius (mm)
		2		7		
		GI (%)	Radius (mm)	GI (%)	Radius (mm)	
Lemon myrtle	1.5	70	7.8	49	20.4	14.1
	2.0	72	7.2	50	19.1	13.2
	2.5	72	6.9	50	20.1	13.5
	3.0	80	5.2	57	17.3	11.2
	3.5	82	4.7	62	15.2	9.9
Citral	1.5	80	5.1	54	18.3	23.4
	2.0	79	6.1	55	18.0	12.0
	2.5	80	5.8	55	17.9	11.9
	3.0	82	4.8	59	16.5	10.6
	3.5	83	4.5	65	13.9	9.2
Anise myrtle	1.5	16	21.9	13	34.6	28.2
	2.0	19	21.0	18	32.9	27.0
	2.5	22	20.4	12	35.3	27.8
	3.0	37	15.6	12	35.1	36.0
	3.5	39	16.0	19	32.2	24.1
Trans-anethole	1.5	30	18.4	16	33.7	26.0
	2.0	31	18.0	20	32.0	25.0
	2.5	31	18.0	20	32.0	25.0
	3.0	31	18.0	22	31.3	24.6
	3.5	36	16.6	22	31.9	24.2
Tea tree	1.5	15	22.1	-	40.0*	31.0
	2.0	15	22.1	-	40.0	31.0
	2.5	17	21.6	-	40.0	30.7
	3.0	22	20.3	15	34.0	27.1
	3.5	20	21.0	14	34.3	27.6
Control 1[a]	0	-	26.1	-	40.0	33.0
Control 2 [a a]	0	-	26.3	-	40.0	33.1

[a] Control 1 represents plates inoculated with pathogen when no treatment is applied; it includes the dish the oils were placed into on the agar

[a a] Control 2 represents plates inoculated with pathogen when no treatment is applied

*Data for treatments that had all replicates with mycelial growth extending to the edge of the Petri dishes (40cm) was omitted from the statistical analysis as its true value cannot be known.

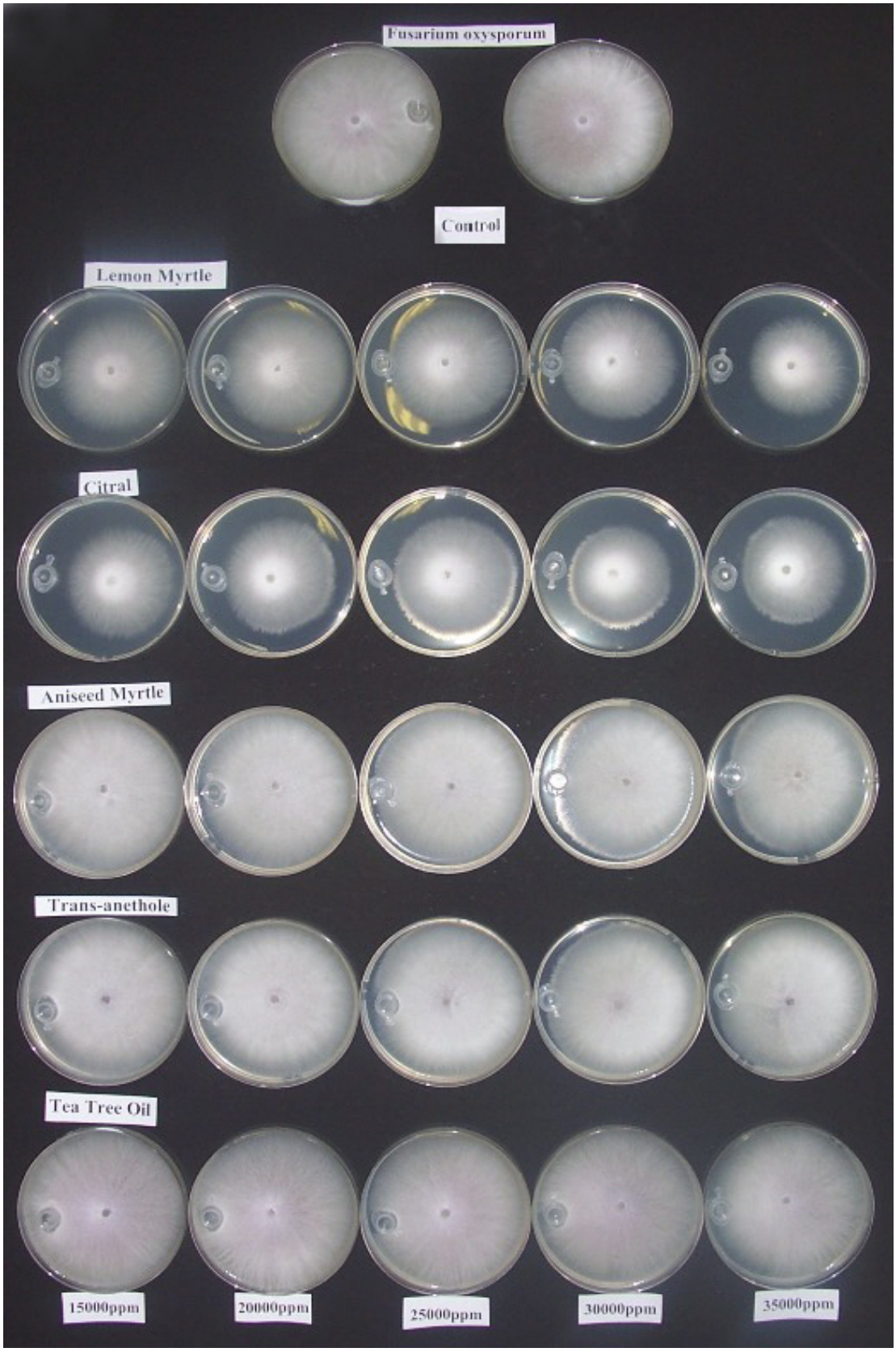

Fig 3 Colonies of *Fusarium oxysporum* after exposure to vilatiles

Monilinia fructicola

Complete inhibition of mycelium growth of *M. fructicola* was observed at all concentrations tested, 0.4-1.2 %, after 2 days exposure to lemon myrtle essential oil, citral, anise myrtle essential oil and trans-anethole (Table.3, Fig 4). After 12 days exposure the four volatiles were still effective, while a decrease in inhibitory activity of volatiles was observed. Tea tree oil exhibited a maximum mycelium growth inhibition of 61% after 2 days and 56% after 12 days exposure respectively. Although all tested volatiles oils reduced mycelial growth significantly ($P<0.05$) compared with fungal growth on the unamended control, the most effective were citral, and trans- anethole. Citral and trans-anethole demonstrated the greatest mycelial growth inhibition of 77% at a concentration of 3.5% at day 12 assessment.

Table 3 Mycelial growth (mm) and growth inhibition (GI%) of Monilinia fructicola at 25°C after exposure to volatiles.

The mycelium radius l.s.d.($P=0.05$) value to compare between days and treatments is 2.3. Values are the means of readings from 3 replicates of 3 colonies.

Treatment	Concentration (%)	Incubation time (days)				Average Radius (mm)
		2		12		
		GI (%)	Radius (mm)	GI (%)	Radius (mm)	
Lemon myrtle	0.4	100	0[3]	45	18.5	18.5
	0.6	100	0	47	22.4	22.4
	0.8	100	0	51	17.1	17.1
	1.0	100	0	59	14.4	14.4
	1.2	100	0	59	14.5	14.5
Citral	0.4	100	0	59	14.4	14.4
	0.6	100	0	71	10.1	10.1
	0.8	100	0	74	9.0	9.0
	1.0	100	0	76	8.5	8.5
	1.2	100	0	77	8.0	8.0
Anise myrtle	0.4	100	0	41	20.9	20.9
	0.6	100	0	45	18.5	18.5
	0.8	100	0	55	15.7	15.7
	1.0	100	0	60	14.0	14.0
	1.2	100	0	73	9.5	9.5
Trans-anethole	0.4	100	0	67	11.5	11.5
	0.6	100	0	69	11.0	11.0
	0.8	100	0	70	10.3	10.3
	1.0	100	0	70	10.9	10.9
	1.2	100	0	77	7.9	7.9
Tea tree	1.5	46	5.5	40	21.1	23.9
	2.0	53	4.8	44	19.6	12.1
	2.5	61	4.0	50	15.1	9.6
	3.0	61	3.9	50	15.5	9.7
	3.5	61	3.9	56	15.6	9.8
Control 1 [a]	0	-	10. 3	-	35.1	22.7
Control 2 [a a]	0	-	10.8	-	34.6	22.7

[a] Control 1 represents plates inoculated with tested pathogen with no treatment applied; it includes the dish the oils were placed into on the agar

[a a] Control 2 represents plates inoculated with tested pathogen with no treatment applied

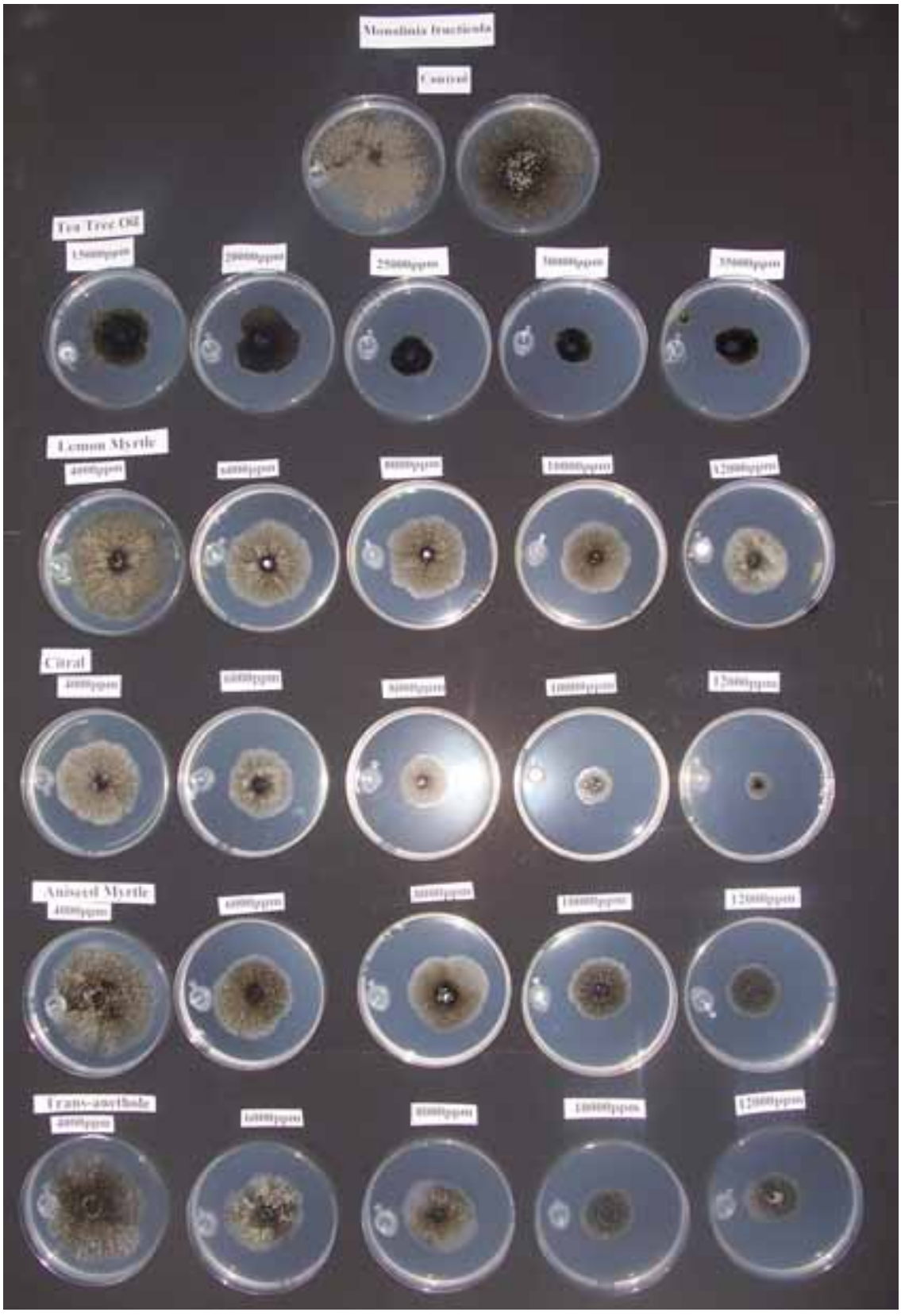

Fig 4 Colonies of *Monilinia fructicola* after exposure to volatiles

Geotrichum candidum

Results in Table 4 and Fig 5 indicate colony growth of *Geotrichum candidum* after exposure to vapours of tested substances. Lemon myrtle essential oil and citral were the most effective treatments at all the concentrations tested. Mycelial growth inhibition of *G. candidum* for lemon myrtle essential oil was 75-76%, while citral showed a mycelium growth inhibition between 78-86% respectively at day 2 assessment. After 7 days incubation, these compounds still exhibited a strong antifungal activity against *G.candidum*. The inhibitory activity of these compounds did not differ significantly from each other ($P<0.05$). A maximum of 11.9% and 10.9% mycelium growth inhibition was observed for anise myrtle, tea tree essential oils and trans-anethole respectively. In contrast Szczerbanik *et al*., 2006, found that tea tree oil gave a ~85% reduction in mycelial growth. This discrepancy could be due, however, to the differences in the experimental approaches used in the two studies. We applied the volatiles only once at the beginning of the experiment simulating the method used for insect fumigants as a practical approach. Szczerbanik *et al*., 2006, used multiple applications of the tea tree oil to control *G.candidum* growth. While the latter approach did result in maintaining a constant concentration of the essential oil in vapour phase, practicality of this method needs to be assessed further.

Table 4 Mycelial growth (mm) and growth inhibition (GI%) of Geotrichum candidum at 25°C after exposure to volatiles.

The mycelium radius l.s.d.($P=0.05$) value to compare between days and treatments is 0.54 Values are the means of readings from 3 replicates of 3 colonies.

Treatment	Concentration (%)	Incubation time (days)				Average Radius (mm)
		2		7		
		GI (%)	Radius (mm)	GI (%)	Radius (mm)	
Lemon myrtle	1.5	75	3.2	71	9.5	6.3
	2.0	76	2.9	77	7.3	5.1
	2.5	76	2.9	80	6.3	4.6
	3.0	75	3.2	80	6.3	4.7
	3.5	75	3.2	82	5.7	4.4
Citral	1.5	78	2.7	73	8.8	5.8
	2.0	80	2.5	79	6.7	4.6
	2.5	80	2.5	82	5.7	4.1
	3.0	85	1.9	85	5.0	3.4
	3.5	86	1.7	86	4.7	3.2
Anise myrtle	1.5	8	11.5	5	30.9	21.2
	2.0	8	11.4	5	30.9	21.1
	2.5	8	11.0	6	30.5	20.8
	3.0	8	11.4	10	29.3	20.4
	3.5	8	11.5	10	29.0	20.3
Trans-anethole	1.5	13	10.9	8	30.0	20.4
	2.0	13	10.9	9	29.6	20.2
	2.5	16	10.6	9	29.6	20.1
	3.0	17	10.4	9	29.5	20.0
	3.5	16	10.6	10	29.4	20.0
Tea tree	1.5	12	11.1	8	29.6	20.4
	2.0	13	10.9	9	29.2	20.0
	2.5	15	10.6	10	29.1	19.9
	3.0	18	10.2	12	28.5	19.3
	3.5	25	9.4	11	28.6	19.0
Control 1 [a]	0	-	12.5	-	32.3	22.4
Control 2 [a a]	0	-	12.4	-	32.6	22.5

[a] Control 1 represents plates inoculated with tested pathogen with no treatment applied; it includes the dish the oils were placed into on the agar

[a a] Control 2 represents plates inoculated with tested pathogen with no treatment applied

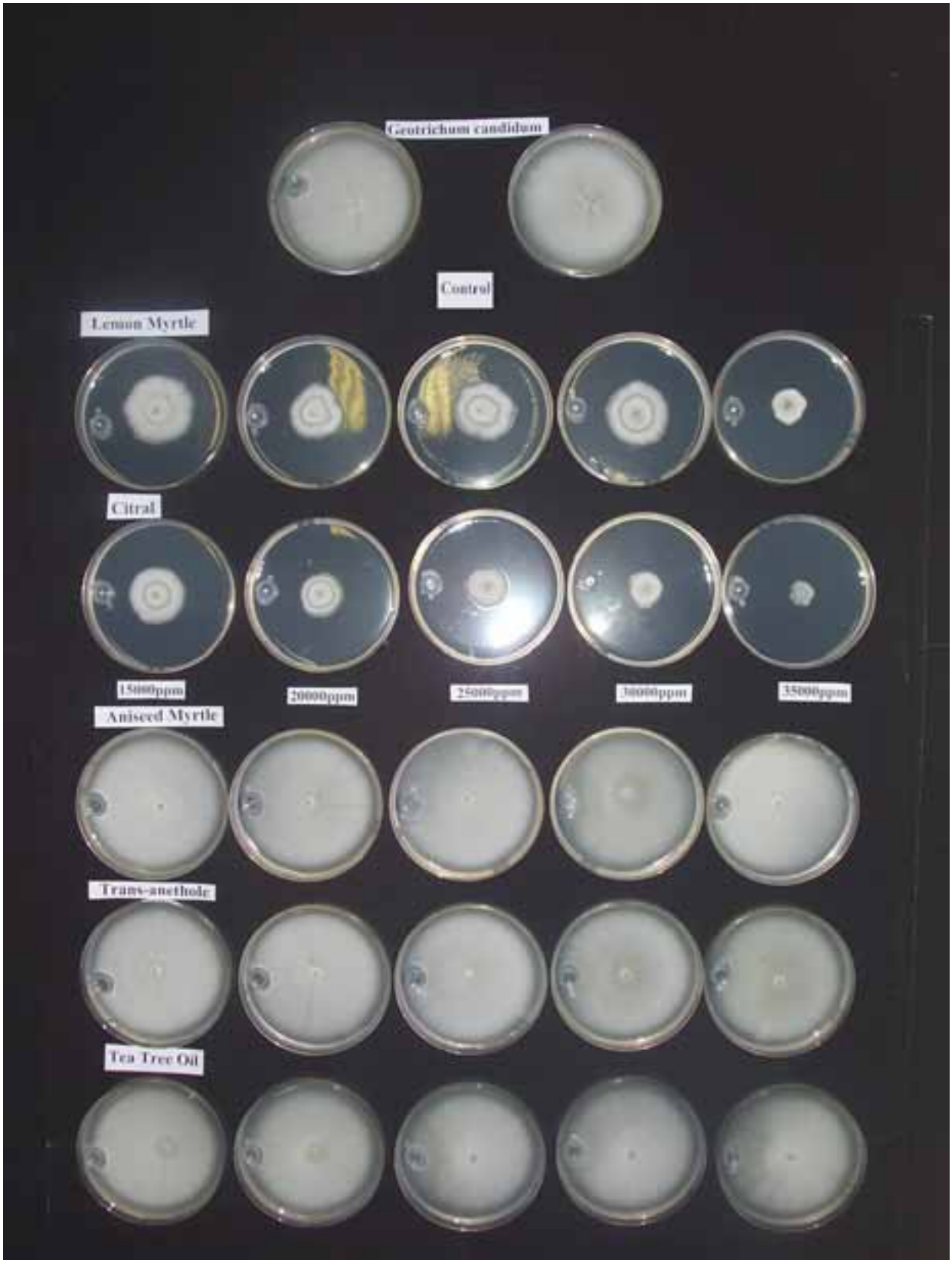

Fig 5 Colonies of *Geotrichum candidum* after exposure to volatiles

Botrytis cinerea

The average radial growth of *Botrytis cinerea* colonies on unamended (control) PDA media plates was 21 mm after 7 days incubation at 25°C (Table 5, Fig 6). Where colonies were grown on media amended with lemon myrtle essential oil and citral at all tested concentrations, growth was reduced in comparison to the unamended control treatment. This reduction was significant ($P<0.05$) following incubation for 2 and 7 days. Colonies also grew more slowly on media amended with the other volatiles tested – anise myrtle, trans-anethole and tea tree essential oil. Preliminary results from this study are in agreement with those of Plotto *et al*., 2003, who reported that citral showed complete growth inhibition of *Botrytis cinerea*. Szczerbanik *et al*., 2006 found that tea tree oil was also effective against *B. cinerea* with a reduction in mycelial growth of 99.6%.

Table 5 Mycelial growth (mm) and growth inhibition (GI%) of Botrytis cinerea at 25°C after exposure to volatiles.

The mycelium radius l.s.d.(P=0.05) value to compare between days and treatments is 0.71 Values are the means of readings from 3 replicates of 3 colonies

Treatment	Concentration (%)	Incubation time (days)				Average Radius (mm)
		2		7		
		GI (%)	Radius (mm)	GI (%)	Radius (mm)	
Lemon myrtle	1.5	36	6.4	28	15.1	10.7
	2.0	36	6.7	35	13.7	10.2
	2.5	36	6.4	42	12.2	9.3
	3.0	36	6.6	44	11.7	9.1
	3.5	36	6.4	47	11.1	8.7
Citral	1.5	29	7.1	33	14.0	10.5
	2.0	40	6.0	33	14.0	10.0
	2.5	45	5.5	44	11.8	8.6
	3.0	45	5.5	44	11.7	8.6
	3.5	62	3.8	49	10.7	7.2
Anise myrtle	1.5	32	5.0	12	18.6	11.8
	2.0	35	6.5	11	18.7	12.6
	2.5	35	6.4	21	16.7	11.5
	3.0	35	6.5	21	16.6	9.6
	3.5	35	6.5	22	16.5	11.5
Trans-anethole	1.5	36	6.3	16	17.6	12.0
	2.0	35	6.5	17	16.9	11.7
	2.5	37	6.7	16	17.6	12.1
	3.0	36	6.4	16	17.8	12.1
	3.5	36	6.4	17	17.5	12.0
Tea tree	1.5	16	8.4	19	17.0	10.6
	2.0	27	7.3	16	20.0	13.6
	2.5	32	6.8	16	20.0	13.4
	3.0	37	6.3	17	19.5	12.9
	3.5	43	7.0	17	19.5	13.2
Control 1 [a]	0	-	10.1	-	21.0	15.5
Control 2 [a a]	0	-	10.3	-	21.0	15.6

[a] Control 1 represents plates inoculated with tested pathogen with no treatment applied; it includes the dish the oils were placed into on the agar

[a a] Control 2 represents plates inoculated with tested pathogen with no treatment applied

Fig 6 Colonies of *Botrytis cinerea* after exposure to volatiles

Effect of various essential oils and volatile compounds on spore germination

Monilinia fructicola

The inhibitory effect of volatiles on *M. fructicola* spore germination is presented in Fig 7. A relatively high percentage of spores (71-77%) germinated on PDA media which had not been amended with volatiles. Volatile treatments at all tested concentrations significantly ($P<0.05$) reduced germination rates compared to the untreated controls. Exposure to vapours of lemon myrtle essential oil at 1.2% provided the most inhibitory effect on *M. fructicola* spore germination with only 22% germination rate when compared with unfumigated controls. Anise myrtle essential oil at 0.4% exhibited the least inhibitory activity with the most spores germinated (60%) compared to the untreated control. The effect of citral, trans-anethole and anise myrtle essential oils were all similar, with a spore germination rate of 32, 35 and 34%, respectively at a concentration of 1.2%.

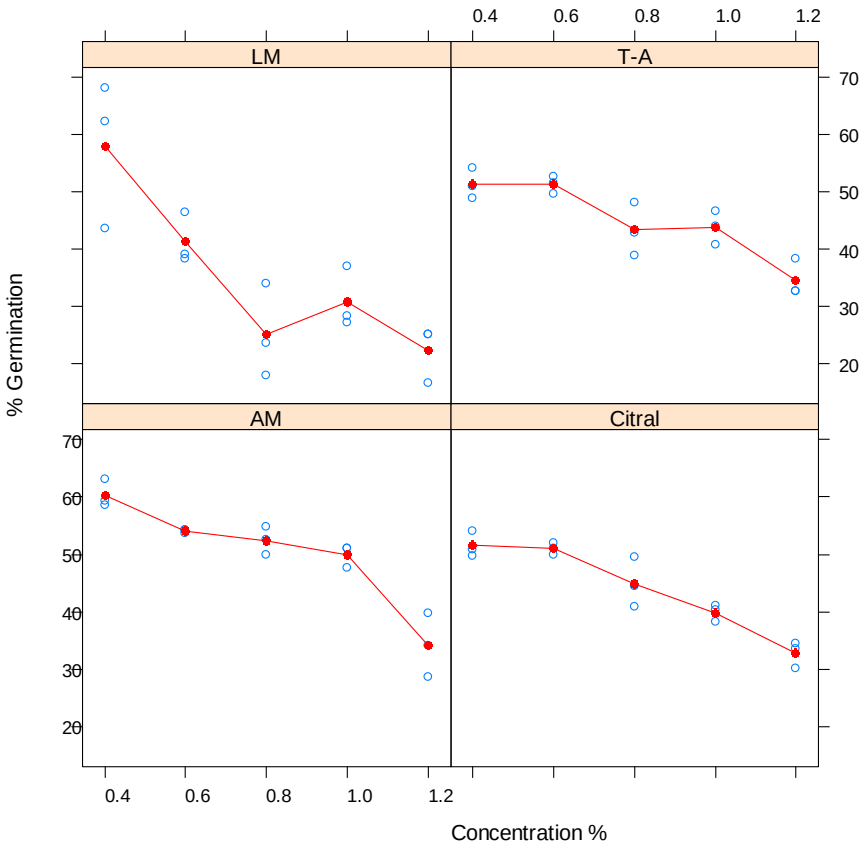

Fig 7 Germination of *M. fructicola* after exposure to volatiles (raw data (blue open circles) and averages (red filled circles) are shown

Vapours of tea tree oil at tested concentrations also reduced spore germination of *M. fructicola* (Fig 8) Fumigation with tea tree oil at all tested concentrations, 0.6-1.2% resulted in spore germination percentage of 45-50%. The addition of tea tree oil at these concentrations resulted in significantly lower ($P<0.05$) germination rates than germination rates in the control treatment.

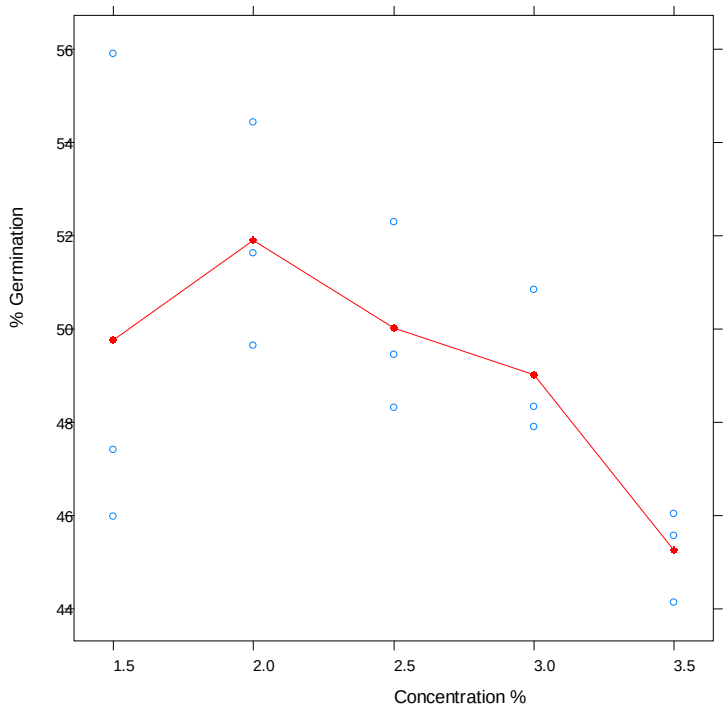

Fig 8 Germination of *Monilinia fructicola* after exposure to tea tree oil volatiles.
(raw data (blue open circles) and averages (red filled circles) are shown

Fusarium oxysporum

Exposure to volatiles at all the concentrations tested, 1.5-3.5% resulted in no significant (P<0.05) reduction of *F.oxysporum* spore germination compared to untreated controls (Fig 9). An average of 95% of spores germinated when exposed to volatiles in comparison with the control (100%). All treatments also failed to inhibit spore germination of *G.candidum.*

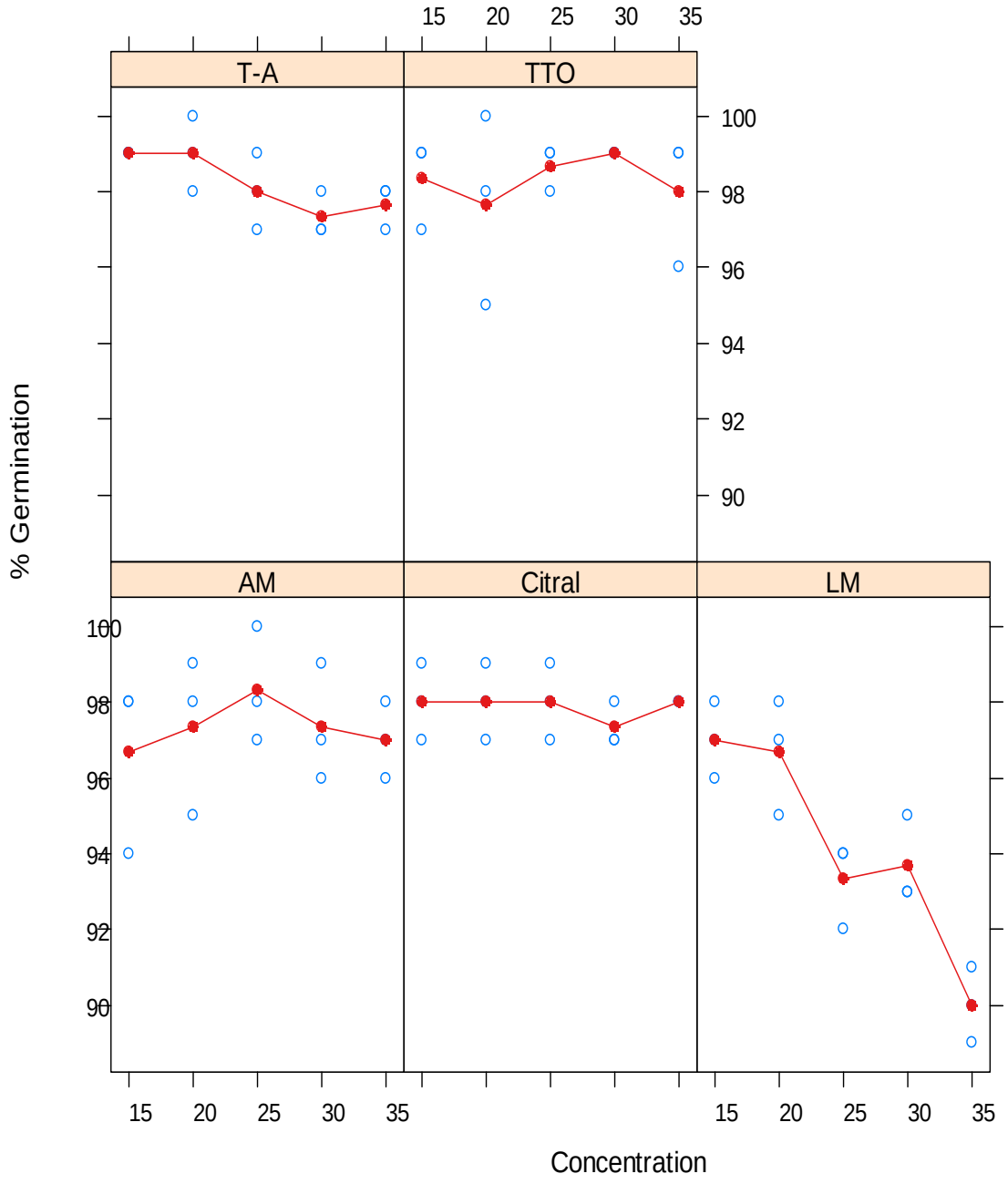

Fig 9 Germination of *Fusarium oxysporum* after exposure to volatiles.
(raw data (blue open circles) and averages (red filled circles) are shown

Botrytis cinerea

The effect of volatiles on the germination rate of *B. cinerea* was determined. The germination of *B. cinerea* spores varied between essential oil volatile treatments (Figure 10). Lemon myrtle essential oil and citral exhibited the greatest inhibitory effect on *B. cinerea* spore germination at all tested concentrations, 1.5-3.5%, with 53% and 55% spore germinated respectively compared to the control (100%).

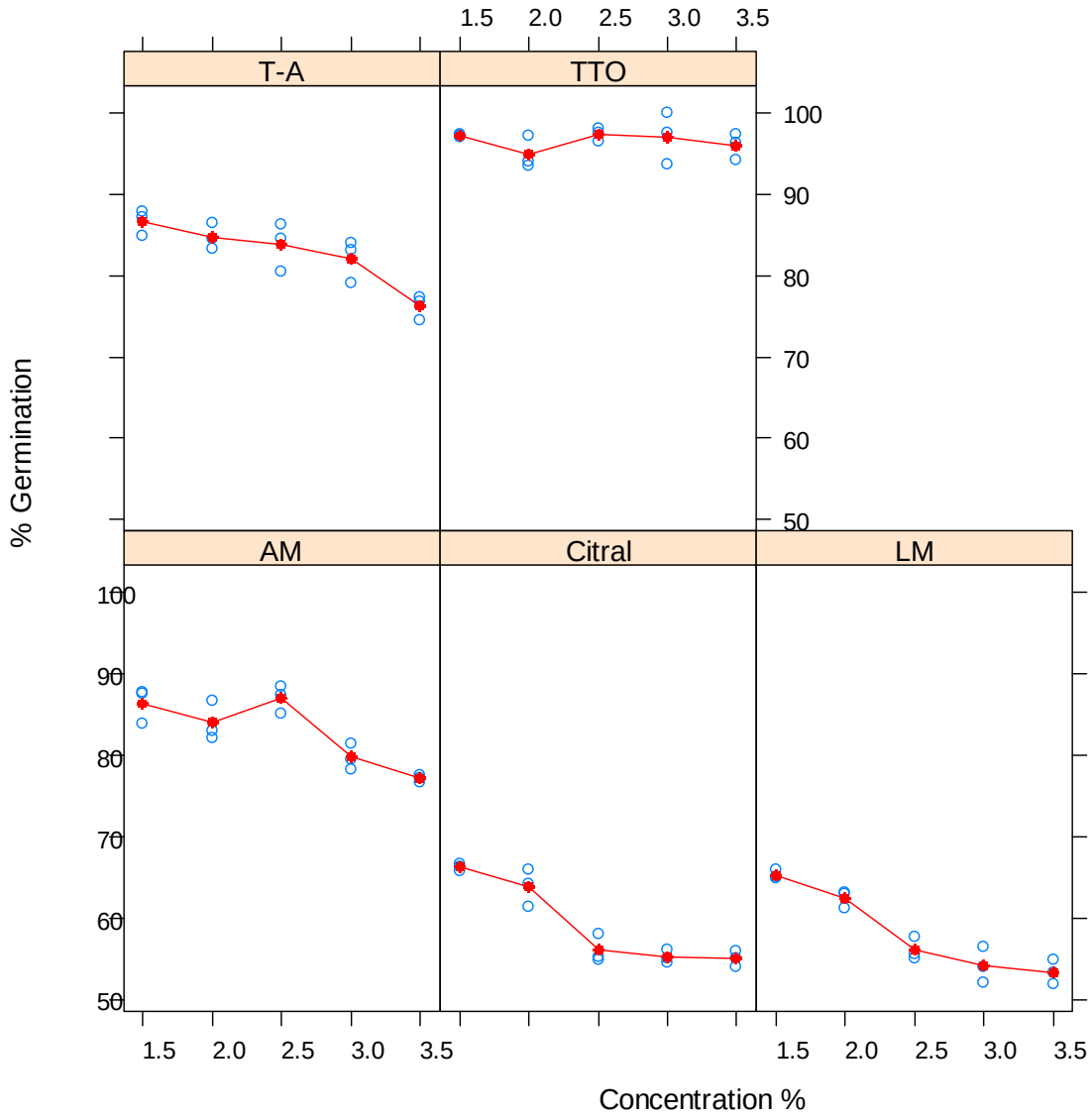

Fig 10 Germination of *Botrytis cinerea* after exposure to volatiles.
(raw data (blue open circles) and averages (red filled circles) are shown

In-vivo trials on antifungal activity of volatiles against postharvest pathogens

Although essential oils can exhibit a strong inhibitory activity in *in-vitro* trials, results can vary considerably when applied *in-vivo*. This is due to the interactions between host tissues, the pathogen and the environment and these can have a major effect on the physiology and metabolism of both the host and the pathogen (Bishop & Regan, 1998). As a result, *in-vitro* results sometimes give only an indication of the potential of essential oils under commercial conditions. Therefore *in-vivo* trials must also be carried out to explore the full potential of essential oils for commercial postharvest use.

Based on the results of the *in-vitro* experiments, only *M. fructicola* and *G.candidum* were selected for further testing in *in-vivo* trials. *M. fructicola* commonly infects stonefruit while *G.candidum* commonly infects tomatoes, hence each pathogen was tested *in-vivo* using these fruit. Lemon myrtle, citral, anise myrtle and transanethole were tested against *M. fructicola* while lemon myrtle and citral only were tested against *G.candidum*

Monilinia fructicola

Results from this study show that where fruit was not fumigated but inoculated, brown rot incidence was 100% after 10 days incubation (Table 6). Fumigation following inoculation did significantly ($P<0.05$) reduce the incidence of brown rot in comparison to the inoculated control treatment, except for citral at a concentration of 0.6%. Disease severity was between 50-66% after exposure to volatiles at all tested concentrations compared to the control. In the case of inoculated fruit, disease development occurred following wounding and disease incidence remained moderate even following fumigation. It is likely that fumigation may be more effective against the superficial infections of naturally occurring spores than against spores which had penetrated the fruit following mechanical inoculation. These results provide an indication that essential oil fumigation is more protectant – rather than curative – in nature. Were this technology ever to reach commercial use these results also provide an indication of the importance of handling fruit so as to minimise wounding. Previous reports (Lazar, unpublished data; Lazar & Jobling, 2009) also indicated reduced brown rot during postharvest treatments with lemon myrtle. Certain differences in experimental results may be caused by variables such as: formulation and concentration of the oils, hydrophobicity, virulence and the inoculum size of the fungal pathogen, incubation time of the pathogen, storage temperature and also the mode of application of the oil, that have an impact on the effectiveness of the oils *in-vivo*.

Table 6 Incidence of brown rot on nectarines following fumigation with citral and volatilised essential oils derived from lemon myrtle.

The diseases score l.s.d.(P=0.05) value is 1.162. Values are the means of readings from 3 replicates of 5 fruit for each treatment

Treatment	Disease severity[1]	Disease incidence (%)[2]
Lemon myrtle 0.6%	**2.5**	**57**
Lemon myrtle 1%	**3**	**50**
Citral 0.6%	**3.9**	**66**
Citral 1%	**2.9**	**57**
Anise myrtle 0.8%	**3**	**60**
Anise myrtle 1.2%	**3**	**59**
Trans anethole 0.8%	**3.2**	**64**
Trans anethole 0.8%	**2.9**	**58**
Control not inoculated, not fumigated 0 %	**0**	**0**
Control inoculated, not fumigated 0 %	**5**	**100**
Control inoculated with SDW, not fumigated 0 %	**0**	**0**

[1]Treatments rating according to the scale: 0=no decay; 1=1-5%decay; 2=6-15%decay; 3=16-25%decay; 4=26-50%decay; 5=>50%

[2]Disease incidence (%) = $\sum$ all disease ratings x100/ total no of ratings x maximum disease grade.

Current findings show that lemon myrtle caused visible phytotoxic effect on nectarines at the tested concentrations, 0.6-1% (Fig.11a,b) while Lazar (unpublished data) using a different method of fumigation and different concentrations (0.025-0.1%) found that lemon myrtle did not exhibit any phytotoxic effect on fruit skin at 0.025, 0.05, 0.1 %. In addition, results from fumigation trials with lemon myrtle at 0.6% for 20 hours (Fig.11c) showed differences in phytotoxic effect on nectarines depending on the presences or absence of an operating fan during the fumigation process. Such comparisons are made difficult by the interaction between essential oil concentrations, exposure time and mode of application of the oil. Notwithstanding these difficulties, phytotoxicity of essential oils needs to be carefully examined to ensure they cause no harmful side effects before an oil is developed for commercial use.

a

b

c

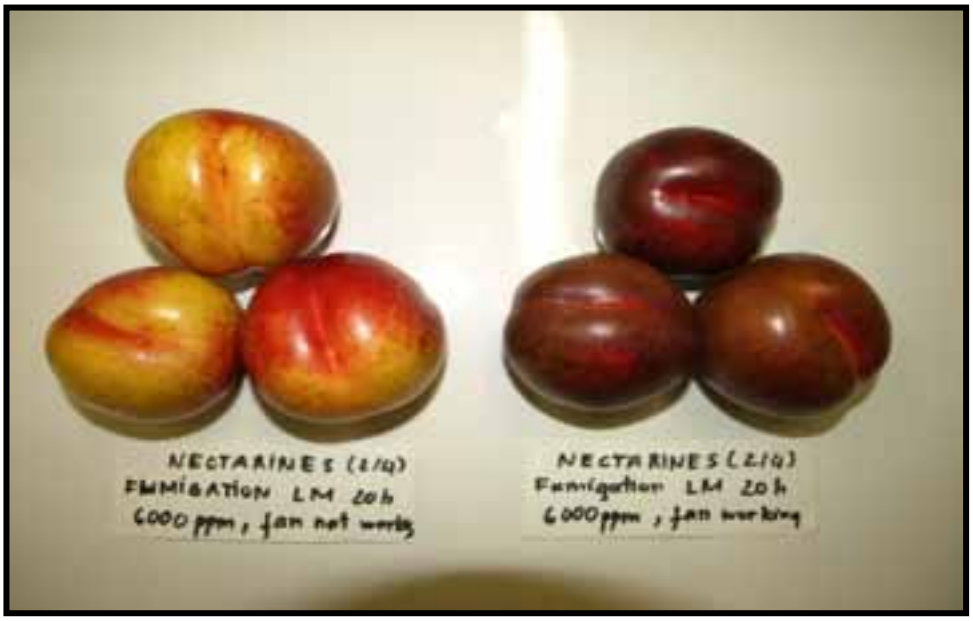

Fig 11 a,b,c Phytotoxic effect after exposure of nectarines to fumigation with lemon myrtle at tested concentrations

Geotrichum candidum

Results presented in Table 7, Fig 12 a,b and Fig 13 a,b indicate disease incidence of sour rot caused by *G. candidum* on tomatoes following fumigation with lemon myrtle and citral at different concentrations and different temperatures. There was a significant effect of temperature ($P<0.05$) and the interaction between temperature and treatment was also significant. Tomato fruit had significantly ($P<0.05$) lower sour rot disease incidence following exposure to volatiles compared to the untreated control when stored at 10°C for 10 days. There was no significant difference in disease severity between any of the volatile treatments at 20°C and 10 days incubation. Volatile treatments at all tested concentrations did not induce phytotoxic effects on tomato fruit. These findings are in agreement with *in-vivo* citral and citral - containing oil vapours where antimicrobial activity was demonstrated against *Geotrichum candidum* (Plotto *et.al*, 2006)

Table 7 Incidence of sour rot on tomatoes following fumigation with citral and volatilised essential oils derived from lemon myrtle.

The diseases score l.s.d.(P=0.05) value is 1.07. Values are the means of readings from 3 replicates of 5 fruit for each treatment

Treatment	Disease severity [1]		Disease incidence (%)[2]	
	10°C	20°C	10°C	20°C
Citral 1.5%	**0.7**	**5.0**	**20**	**100**
Citral 2.5%	**0.7**	**5.0**	**13.5**	**100**
Citral 3.5%	**0.7**	**5.0**	**13**	**100**
Lemon myrtle 1.5%	**0.7**	**5.0**	**13**	**100**
Lemon myrtle 2.5%	**0.7**	**5.0**	**13**	**100**
Lemon myrtle 3.5%	**1.0**	**4.4**	**12**	**94.7**
Control not inoculated, not fumigated 0 %	**0**	**0**	**0**	**0**
Control inoculated, not fumigated 0 %	**1.8**	**5.0**	**46**	**100**
Control inoculated with SDW, not fumigated 0 %	**0**	**0**	**0**	**0**

[1]Treatments rating according to the scale: 0=no decay; 1=1-5%decay; 2=6-15% decay; 3=16-25%decay; 4=26-50%decay; 5=>50% .

[2]Disease incidence (%) = $\sum$ all disease ratings x100/ total no of ratings x maximum disease grade.

In this study preliminary results on disease incidence may have been influenced by the fact that a significant proportion of tomato fruit presented a high level of splitting when assessed for decay presence (Fig 12a,b; Fig 13 a,b). This can be explained by the fluctuations in temperature from field to storage temperature which may lead to increased risk of splitting. Physiological damage such as splitting forms locations vulnerable to invasion of wound pathogens and increases disease severity.

Fig 12 a,b Effect of lemon myrtle essential oil on inoculated tomatoes at 20°C

a) inoculated tomatoes without treatment

b) tomatoes fumigated with lemon myrtle at a concentration of 2.5%

Fig 13 a,b Effect of lemon myrtle essential oil on inoculated tomatoes at 10°C

a) inoculated tomatoes without treatment;

b) tomatoes fumigated with lemon myrtle at a concentration of 2.5%.

Sensory evaluation

Tainting may occur following *in-vivo* application of essential oils - some oils can taint food products. Certain fruit and vegetables easily absorb oil vapour resulting in the smell and taste of the oil remaining in the produce after fumigation. The strong aroma of some essential oils may limit their application to fresh produce during storage.

Since lemon myrtle essential oil exhibited a strong antifungal effect against *M. fructicola*, its effect at a concentration of 0.6 and 1%, on the taste of nectarines was investigated. A preliminary sensory evaluation trial was conducted in order to understand consumer preference in terms of sensory attributes (Fig.14 a,b). The results indicated that there was a strong panellist effect (47% of the variability in the data was due to person-to-person variability). The effect of order of tasting on the sensory value was negligible. The lemon myrtle treatment effect on sensory value in this experiment was not significant. The association between treatment and intention to purchase was also not significant. The panellists did accept the eating qualities of nectarines fumigated with lemon myrtle.

Fig 14 a, b Sensory evaluation trial

Economic analysis

The primary output of the project consisted of evaluating the antimicrobial properties of certain essential oils derived from Australian native plants against common postharvest pathogens. As the nature of this work is preliminary it is difficult to provide an accurate cost effectiveness analysis when not all of the factors contributing to the *in-vivo* efficacy have been elucidated.

However a cost - effectiveness analysis of lemon myrtle essential oil and synthetic citral as potential fumigants for postharvest control of *Monilinia fructicola* in nectarine was determined (Table 8). The comparison was also made with Rovral, the only registered fungicide in Australia for postharvest treatment against *Monilinia* in stone fruit.

As the study investigates the use of essential oils as a postharvest treatment only, the main difference is the actual cost of the products. The table below compares the costs of lemon myrtle oil, and it's main component citral against the current postharvest fungicide dip, Rovral, for a known quantity of fruit.

Table 8 Estimated costs for lemon myrtle, citral and Rovral when used as postharvest treatment for nectarines

Treatment:	Rovral	Lemon Myrtle[1] - commercial grade	Citral[1] (synthetic)	Citral[1] (natural)*
Cost / L (Au$)	33.00	104.50	113.22	257.04
Rate of use	100mL/100L	1%	1%	1%
Batch size	$10m^3$	$10m^3$	$10m^3$	$10m^3$
Treatment details	$10m^3$ of fruit dipped in solution.	Headspace in $10m^3$ around fruit	Headspace in $10m^3$ around fruit	Headspace in $10m^3$ around fruit
Volume of treatment. product required	100mL	Assume $5m^3$ headspace = 368mL	Assume $5m^3$ headspace = 350mL	Assume $5m^3$ headspace = 350mL
Final cost of treatment	$3.30	104.50 x 0.368 = $38.46	113.22 x 0.35 = $39.63	257.04 x 0.35 = $89.96

Citral (synthetic) retail price from Sigma-Aldrich website, http://www.sigmaaldrich.com/sigma-aldrich/home.html product W230308-1KG-K

Citral (natural, sourced from May Chang) retail price from Sigma-Aldrich website, http://www.sigmaaldrich.com/sigma-aldrich/home.html product W230316-1KG-K. *Note: this product was not used in our testing.

Lemon Myrtle[1] retail price, from Sydney Essential Oils Co website, http://www.seoc.com.au

Under the experiment parameters, and also using the assumption that the headspace in the treatment packaging is 50% of the total batch volume, the cost of the alternatives, lemon myrtle oil and citral, are significantly more expensive than Rovral for the treatment of *Monilinia* rot of stone fruit. However, optimisation of application technology was not an objective of this study and considerable

potential exists for improving efficiency and economy. If the synthetic form of citral can be used, then either this or lemon myrtle essential oil would provide the most economical alternative to synthetic fungicides. Where natural products only need to be used, i.e. organic industry, lemon myrtle oil is a much more cost-effective treatment than the naturally extracted citral.

The results of this research demonstrate that lemon myrtle in particular, exhibited antifungal activity against tested postharvest pathogens. Although this is a preliminary study and not all of the factors contributing to the *in-vivo* efficacy have been investigated, the data provided here demonstrates that lemon myrtle in particular has potential as an antifungal agent to control postharvest pathogens.

Preliminary results from this project provide evidence supporting the use of essential oils as a component of management strategies for the control of postharvest diseases in horticulture. Their use would ease the Australian horticultural industry's current reliance on synthetic fungicides for postharvest protection. Given consumer and legislative concerns over the use of synthetic fungicides, essential oils should be able to capture reasonable market share given comparable efficacy and cost.

The science and practice of using essential oils for postharvest disease control is at a very early stage, although research progress made in recent years has been remarkable. There is potential to develop commercial postharvest fungicide products using essential oils, but in order to meet industry expectations, large scale trials should take place under commercial and semi-commercial conditions for different commodities, considering all cost, legal, and safety issues.

5. Recommendations

The results presented here were obtained from a preliminary one-year study. Overall, the findings have shown that the commercialisation of some essential oils, particularly lemon myrtle oil, may be realised as the oils were able to control the growth of the fungi. Further research into the formulation of the oil, concentration, hydrophobicity, biostatic time lines of the oils, virulence and the inoculum size of the fungal pathogen, incubation time and treatment temperature also needs to be evaluated in order to collect scientifically reliable data on the antifungal activity of the oils.

The research conducted during this project covered only two types of horticultural produce - tomatoes and nectarines - and only one method of application, fumigation. Preliminary trials on selected fruit had shown that it was more appropriate to use the tested essential oils in their vapour phase for postharvest application. Many oils appear to be more effective against fungal pathogens applied as a fumigant rather than as a solution (Liu *et al.*, 2002; Plotto *et al.*, 2006). When applied as a vapour, less oil is required making the treatment more cost-effective, the residues on the produce are minimised, tainting should be less of a problem and phytotoxic effects on the organoleptic attributes of the fruit avoided. Additional research needs to be carried out on a wide range of horticultural produce to evaluate different formulations of the oils and to identify which horticultural produce do not display phytotoxic effects to essential oils.

The results of this study have shown that different species of fungal pathogens are affected by essential oils to various degrees. Certainly one oil cannot provide maximum control for a large range of fungal pathogens. It has been suggested that the overall antifungal activity of the oils might be due to a synergistic effect between various components (Carson and Riley, 1995), but also to synergism between oils (Levy *et al*., 1986). The presence of a synergistic effect in oils can be considered beneficial in postharvest disease management, because the pathogens are less likely to develop resistance to such a complex mixture of different components with apparently different mechanisms of antimicrobial activity. It is recommended to further investigate or develop combinations of the oils that might act synergistically to control postharvest pathogens and to demonstrate effectiveness that is comparable to commercially available synthetic fungicides already used for postharvest disease control.

The *in-vivo* trials for nectarines were conducted at 20°C. Further work needs to assess the volatility of the lemon myrtle essential oil at storage temperatures. It will be valuable to examine the difference in composition of oil vapour at storage temperatures, as it is likely that fewer components will volatilise than at 20°C.

It would be useful to further examine the use of lemon myrtle oil as a potential biocontrol agent for organic horticulture where no chemical control for postharvest disease management is available. In this case economic losses occurred to the growers may be reduced significantly. Growers, organic and non-organic, may have to turn to this new strategy to protect their crop after harvest in the future.

It is recommended that these results be presented to current industry participants including horticultural producers, exporters and essential oil producers. It is also recommended that these results be published in scientific journals, making data available to the scientific community. In this way, further research based on the recommendations above may be initiated.

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7. Publications Arising From This Project

Journal Articles

E. E Lazar, K. Crampton, L. Spohr (2010)) Potential use of Australian essential oils to control brown rot in nectarines. **Australasian Plant Pathology** (in preparation)

K. Crampton, E.E. Lazar, L. Spohr (2010) The effect of Australian essential oils on the growth of some postharvest pathogens **Plant Protection Quarterly** (in preparation)

Management of Postharvest Diseases of Horticultural Crops using Australian Essential Oils

by Dr Elena E. Lazar, Mrs Kylie Crampton and Mrs Lorraine Spohr

Publication No. 11/036

This report describes the evaluation of the effectiveness of essential oils extracted from Australian native plants, lemon myrtle (*Backhousia citriodora*), anise myrtle (*Syzigium anisatum*) and tea tree (*Melaleuca alternifolia*) and their active constituents against the important postharvest pathogens of the horticulture industry *Botrytis cinerea*, *Monilinia fructicola*, *Geotrichum candidum* and *Fusarium* species.

The report is targeted at current industry participants including horticultural producers, exporters, essential oil producers, and the scientific community.

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