Application of citral to control postharvest diseases of oranges

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Alhamdulillahhirrabbilalamin…

Kupersembahkan thesis ini kepada ibunda tercinta Hj. Sk. Aisyah, yang dengan kasih sayangnya tidak pernah lelah mendoakanku,
kepada suami tercinta Wuryatmo dan anak-anakku,: Ika, Luqman and Galuh, atas kasih sayang, doa dan kesabaran kalian mendukung sehingga thesis ini dapat terwujud

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Abstract

Green mould, blue mould and sour rot, caused by the fungi *Penicillium digitatum*, *P. italicum* and *Geotrichum citri-aurantii*, are postharvest diseases which cause significant losses to the citrus industry worldwide. Current control of the diseases raises some problems, such as development of fungicide resistance, concerns about residues harmful to humans, and also restrictions on the use of certain fungicides. Those problems have led to a need to develop alternative fungicides, including exploitation of some natural products such as essential oils.

Application of the essential oil, citral (3,7-dimethyl-2,6-octadienal) to control the fungi and the diseases was assessed in this study. *In vitro*, citral incorporated into agar at 2%, 6% and 15% prevented germination of spores of the fungi, and no mycelial growth was observed by microscopic observation after 17 days of incubation. When citral was applied as a solution on agar, spore germination of *P. digitatum* and *G. citri-aurantii* was inhibited at concentrations of 6% and 15%. However, germination of *P. italicum* spores was not affected. Vapour of citral and its individual isomers, geranial and neral, generated from 6 and 15% aqueous solutions, inhibited spore germination and growth of the three pathogens. Vapour generated from 15% aqueous solutions of citral and geranial were fungicidal to *P. digitatum* and *G. citri-aurantii*, and fungistatic to *P. italicum*, while neral was fungicidal to *G. citri-aurantii* and fungistatic to the other two fungi. The result suggested that method of application and citral concentration affected the efficacy of

citral in controlling the fungi. In the three methods of applications examined, citral was effective in controlling *G. citri-aurantii*, especially at high concentration.

As an α, β-unsaturated aldehyde, citral may be degraded over time due to oxidative reactions, resulting in change in its composition, and this may affect its antifungal activity. Storage of citral may result in the oxidation of neral and geranial to produce neric acid and geranic acid. GC/MS results showed that neral, geranial, neric acid and geranic acid were detected, while the related compounds, nerol, geraniol, citronellal, citronellol and citronellic acid were not detected either for citral stored at 5^{o}C or at room temperature. At room temperature, geranial and neral content declined more quickly than at 5^{o}C.

The effect of citral on the incidence of disease on fruit was studied by applying citral as a fumigant. Wounded oranges inoculated with spore suspension (10^{6} spores mL^{-1}) of the fungi were placed in 5-litre plastic boxes, fumigated with 2, 6, or 15% citral, and incubated at 5°C or room temperature. Fumigation of oranges with citral in this closed system delayed the onset of sour rot at room temperature by 7 – 10 days and at 5°C, by 13 – 30 days, suggesting that volatile citral controlled *G. citri-aurantii* on fruit as well as *in vitro*. The effects of fumigation with citral on green and blue mould were more variable. Fumigation delayed the onset of green mould and blue mould at 5°C by 2 days at the higher concentrations (6 and 15%) tested, while at room temperature, spoilage was not delayed even at the highest concentration tested. Measurement of citral in the headspace of boxes containing fruit and citral-soaked pads showed that the concentration above the fruit was higher than that measured

below the fruit both at 5°C and at room temperature. Phytotoxicity symptoms were observed on the upper surface of some fruit that was close to or in direct contact with the citral-soaked pad at concentrations of 6% and 15%, suggesting that phytotoxicity may have been associated with high volatile citral concentration. However, citral residue was not detected in oranges irrespective of treatment with citral, which suggested that little citral had penetrated into the peel. During storage the citral content decreased due to oxidation of geranial and neral to produce geranic acid and neric acid both at 5°C and room temperature. This may have had an impact on the efficacy of citral against the pathogens.

Findings may contribute to a better understanding of the efficacy of citral when applied to the pathogens *in vitro* and to the development of effective control methods when applied on fruit. The possibility of combining citral treatment with other commonly used practices is also worthy of consideration. For example, citral could be combined with heat treatment to increase the volatility of the citral. In addition, incorporation of citral in a wax formulation may allow a low concentration of citral to be used in direct contact with the pathogens on fruit. Fumigation of fruit with citral may offer potential as a means to control development of sour rot of oranges, and its effects on fruit quality, flavour and nutritional aspects require further investigation.

Declaration

NAME: ERMINAWATI WURYATMO (WATI). PROGRAM: PhD

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Publications and conference proceedings

The following publications and abstracts in conference proceedings were produced during the PhD candidature;

Wuryatmo, E., Klieber, A. and Scott, E. (2001) The effect of citral components on citrus postharvest fungal spoilage in culture. In: Australasian Postharvest Horticulture Conference Proceedings. Oral Presentation *Australasian Postharvest Horticulture Conference*, Adelaide, September 23-27, 2001.

Klieber, A., Scott, E., and Wuryatmo, E. (2002) Effect of method of application on antifungal efficacy of citral against post-harvest spoilage fungi of citrus in culture. *Australasian Plant Pathology,* **31**, 329-332.

Wuryatmo, E., Klieber, A., and Scott, E. (2003) Inhibition of citrus postharvest pathogens by vapor of citral and related compounds in culture. *Journal of Agricultural and Food Chemistry*, **51**, 2637-2640.

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Abbreviations

^{o}C	degree Celsius
2 ,4 D	2,4 Dichlorophenoxy
ANOVA	analysis of variance
a_w	water activity
$Ca(OH)_2$	calcium hydroxide
$C_2 H_2$	acetylene
$C_2 H_4$	ethylene
cfu	colony forming units
CH_2Cl_2	dichloro methane
CO_2	carbon dioxide
CuCl	cuprous chloride
DBAD	di-tert butyl azo dicarboxilate
FID	flame ionisation detector
kg, g, mg, µg	kilogram, gram, milligram, microgram
Gcd	*Geotrichum candidum* (*Geotrichum citri-aurantii*)
GC	gas chromatography
GC/MS	gas chromatograph/mass spectrometry
h	hours
H_2O	water (dihydrogen oxide)
KPa	kilo Pascal
K_2CO_3	potassium carbonate
L, mL, µL	litre, millilitre, microlitre

LSD	least significant difference
m, mm, μm	metre, millimetre, micrometre
min	minutes
n	number of mole present
NDY	Neutral-Dox Yeast
Pdg	*Penicillium digitatum*
Pit	*Penicillium italicum*
P	pressure
PDA	potato dextrose agar
π	phi
R	gas constant (8.3143 Joules/Moles/K)
r	radius
SARDI	South Australian Research and Development Institute
SPME	solid-phase microextraction
T	temperature
TFE	tetrafluoroethylene
TLC	thin layer chromatography
V	volume
v/v	volume per volume

Glossary of terms used in this study:

2% citral	In Petri dishes (*in vitro* experiment) : 2 μL citral diluted to 100 μL with 400 μL L^{-1} aqueous TritonX solution In box (*in vivo* experiment) : 131 μL citral diluted to 6550 μL with 400 μL L^{-1} aqueous TritonX solution
6% citral	In Petri dishes (*in vitro* experiment) : 6 μL citral diluted to 100 μL with 400 μL L^{-1} aqueous TritonX solution In box (*in vivo* experiment) : 393 μL citral diluted to 6550 μL with 400 μL L^{-1} aqueous TritonX solution
15% citral	In Petri dishes (*in vitro* experiment) : 15 μL citral diluted to 100 μL with 400 μL L^{-1} aqueous TritonX solution In box (*in vivo* experiment) : 983 μL citral diluted to 6550 μL with 400 μL L^{-1} aqueous TritonX solution
100% disease incidence	The time required for all fruit to show disease
Δ value	The time course for disease development, as a measure of the efficacy of citral in delaying the onset of disease

Chapter One
General Introduction

Citrus cultivation is an important commercial and industrial agronomic activity worldwide. Citrus fruit is widely consumed, both as fresh fruit and as juice, not only for its flavour but also because of its vitamin C and antioxidant content.

Citrus is the main fresh fruit exported by the Australian horticultural industry, with fruit mainly grown in New South Wales (NSW), Queensland, South Australia and Victoria. The main markets for Australian citrus are fresh fruit (domestic and export), fresh juice (domestic and export) and drinks manufactured from frozen concentrated juice. In 2002/2003 record export volumes of 167,000 tonnes were achieved, with an estimated gross value production of AUD$201 million (Australian Citrus Limited, 2009). In particular, South Australia exported fresh oranges to the value of $58.1 million in 2007 (Primary Industries and Resources SA, 2010).

In general, Australia has potential marketing advantages due to its location. Australia can export high quality fresh citrus fruit to northern hemisphere countries (USA and Canada) when they are out of season, also to Asian markets including Japan, Hong Kong, Malaysia, and Indonesia (Australian Citrus Limited, 2009). Export involves a need for extended storage during transport from Australia to these destination countries. Therefore, effective control of postharvest diseases, which extends storage time, is especially important for Australia to compete in the citrus world export market, and to provide high quality fresh citrus fruit that are unaffected by fungal spoilage.

Postharvest fungal diseases result in significant economic losses in the citrus industry. The most common postharvest fungal diseases affecting citrus fruits worldwide are green mould, blue mould and sour rot, which are caused by the filamentous fungi *Penicillium digitatum* (Pers.:Fr.) Sacc., *Penicillium italicum* Wehmer and *Geotrichum citri-aurantii* Link ex Pers. respectively (Pitt and Hocking, 1997; Plaza *et al.*, 2003; Cunningham and Taverner, 2007; Droby *et al.*, 2008). These pathogens may infect fruit on the tree, in the packing-house, in transit, in storage and in the market. Current postharvest disease control relies on synthetic chemical fungicides, which can be applied by a variety of methods including dipping in soak tanks or as aqueous solutions sprayed over brushes, mixed with wax or as fumigant. However, this practice is raising consumer concern about the risk of residues on human health, and the impact on the environment of fungicide disposal after use in packing-houses. In addition, sour rot is particularly problematic because the control method for the Australian domestic market is to use fungicides containing guazatine, while use of this chemical for export markets is restricted (Cunningham and Taverner, 2007). These problems have led to a need to develop alternatives to synthetic chemical fungicides for controlling postharvest diseases. One possible alternative is to use natural chemicals.

Several researchers have attempted to protect fruit from fungal pathogens through the use of the natural defence system of the fruit, by applying citrus essential oils (Stange *et al*., 2002; Fisher and Philip, 2006: Chutia *et al.*, 2008; Droby *et al.*, 2008; Viuda-Martos *et al.* 2008). Citrus essential oil contains several components including citral, geranial, neral, citronellal, citronellol and citronellic acid (Shaw, 1977; Erman, 1985), all of which may have antifungal property. Of the citrus

essential oils, citral appears to be the most effective in inhibiting a range of microorganisms, including *P. digitatum, P. italicum* and *G. citri-aurantii* (Rodov *et al*., 1995; Wannisorn *et al*., 1996; Suprapta *et al*., 1997; Pattnaik *et al*., 1997; Dunn, 1998; Caccioni,, *et al*., 1998). However, the method of fungicide application can affect efficacy in controlling the pathogens (Dunn, 1998). Therefore, in this study three methods of application of citral were tested to examine how citral could best control citrus spoilage fungi.

Citral contains isomers and oxidation/reduction products. Citral, as a natural mixture of a trans-isomer (geranial) and a cis-isomer (neral), is susceptible to reactions such as oxidation and reduction. According to Erman (1985), geranial and neral are the biosynthetic oxidation products of geraniol, which then undergo reduction to citronellal and further oxidation to citronellic acid, while citronellal is the biosynthetic oxidation product of citronellol. The activity of the individual citral isomers, geranial and neral, and the related compounds citronellal, citronellol and citronellic acid against *P. digitatum*, *P. italicum* and *G. citri-aurantii* has not been documented. Results of *in vitro* studies showed that volatile phase appeared to have potential as a commercial antifungal treatment of citrus fruit. Therefore, the activity of the citral isomers as well as their related compounds in the vapour phase against spores of *P. digitatum*, *P. italicum* and *G. citri-aurantii* was examined *in vitro* to determine if differences in citral composition are likely to influence its antifungal activity.

In vitro results suggested that volatile citral has potential to control citrus postharvest fungal spoilage, and application of citral in the vapour phase as a

fumigant may minimise its phytotoxic effect on the fruit. In addition, fumigation can easily be applied for relatively short periods in storage chambers or continuously over the long term within packages (Williams *et al.*, 2000). Several fumigants have been used to control postharvest disease of citrus due to *P. digitatum* (Roistacher *et al.*, 1958; Eckert and Kolbezen, 1963; Eckert and Rahm, 1983; and Chu *et al.*, 2000). However, there has been little research on fumigation of citrus with citral to control postharvest diseases of oranges due to *P. digitatum*, *P. italicum* and *G. citri-aurantii*. In this study fumigation of oranges with citral to control postharvest fungal diseases of oranges was examined.

Experimental work reported in this thesis was conducted from 1999 to 2002, but completion of the thesis was interrupted due to personal circumstances. Thesis writing resumed in October 2008. In relation to this study, experimental data presented in chapters 4 and 5 were published in the following papers:

Klieber, A., Scott, E., and Wuryatmo, E. (2002) Effect of method of application on antifungal efficacy of citral against post-harvest spoilage fungi of citrus in culture. *Australasian Plant Pathology*, **31**: 329-332.

Wuryatmo, E., Klieber, A., and Scott, E. (2003) Inhibition of citrus postharvest pathogens by vapor of citral and related compounds in culture *Journal of Agricultural and Food Chemistry*, **51**: 2637-2640.

Chapter Two

Literature review

2.1. Citrus

Citrus is a non-climacteric fruit, therefore it has a relatively long shelf-life. Its cultivation is an important commercial agronomic activity worldwide. Citrus fruits are the most valuable fruit crop in international trade, and include; mandarins, sweet oranges, limes and lemons, pomelos and grapefruit.

The common sweet oranges (*Citrus sinensis* (L. Osbeck) are the most widely grown and commercially important, and they constitute about two-thirds of total orange production worldwide. Sweet oranges are the principal member of the orange group. Four types of sweet oranges are recognized; common or round orange, acidless orange, pigmented or blood orange, and navel orange (Hodgson, 1989; Mukhopadhyay, 2004). The common or round orange comprises a large number of cultivars including Hamlin, Valencia, Natal, Shamauti and Pineapple. The common cultivars of acidless orange are; Lima, Succari and Anliucheng. Some common cultivars of pigmented, or blood orange are Double Fina, Maltaise, Sanguinelli, Taroccom and Moro. The navel orange includes; Washington, Lane Late, Navelina, Navelate, Leng, Palmer, Robyn, Caracara and Bahia (Mukhopadhyay, 2004).

In Australia, citrus is one of the largest horticultural industries, being about 20% of the total value of horticultural production (South Australian Research and Development Institute /SARDI, 2000). Citrus is grown mainly in New South Wales (NSW), Queensland, South Australia (SA) and Victoria; the largest growing areas

are the Riverland, the Riverina and Sunraysia districts along the Murray Valley (Australian Citrus Growers /ACG, 2009) (Figure 2.1).

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This figure is included on page 6
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Figure 2.1 Major citrus growing regions in Australia (Source: Citrus Australia, 2010a)

In 2007, oranges accounted for 49% of the export share of Australian horticulture fruit and nuts, with the majority exported between June to November (Figure 2.2).

Australia lies between 10°S and 40°S latitude (southern hemisphere), and is characterized by winter during March – October and summer during November – February. Australia can market fresh citrus fruits during March – October, to northern hemisphere countries in their off session. The availability of oranges during the year in Australia is shown in Figure 2.3.

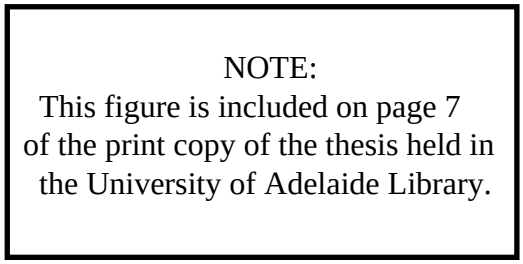

Figure 2.2 Australian export of oranges 2006/2007 (Source: Australian Export Statistics, 2009)

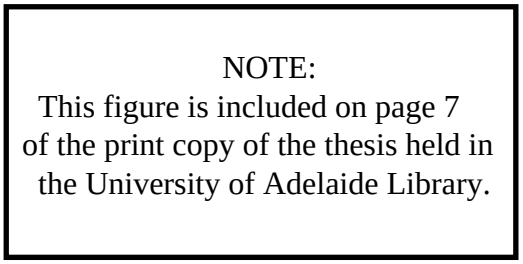

Figure 2.3 The availability of oranges during the year in Australia (Source: Australian Citrus Grower, 2009).

About 25% of Australian citrus is produced in The Riverland of SA (Ladaniya, 2008). SA is characterised by a Mediterranean climate (cool winter, warm summer without excessive rainfall), and until recently adequate supply of irrigation water from the River Murray. Citrus species, particularly sweet navels and mandarin, grow well because they are suited to the climate. Annual South Australian fresh citrus exports for 2007 included 32,500 tonnes of navel oranges, 15,800 tonnes of Valencia oranges, 5,300 tonnes of mandarins, 317 tonnes of lemons, 130 tonnes grapefruit and 116 tonnes of other orange varieties, to a value of $58.1 million (Primary Industries Resources of South Australia/PIRSA, 2007).

The fruit of oranges is a type of berry sometimes called a hesperidium. The fruit is composed of the rind (pericarp), juice sacs, seed and central axis (Schneider, 1968). The general structure of the citrus fruit is presented in Figure 2.4. The rind is the first line of defence of the fruit. Wounded or damaged rind provides an entry point for decay pathogens. The flavedo or exocarp of the rind consists of the outermost tissue layers of the fruit, composed of the cuticle-covered epidermis layer, and laterally flattened cells above the oil glands in mature fruit (Schneider, 1968). The epidermis is composed of tubular parenchyma cells, guard cells, accessory cells and oil-gland-cover cells. Within this parenchymatous tissue occur crystal idioblasts and oil glands. The mesocarp or albedo occurs between the exocarp and endocarp and resembles the spongy mesophyll of the leaf. The endocarp is the inner side of the pericarp and a portion of the locular membranes, and is the edible portion of the fruit (Schneider, 1968). A transection of the rind of mature orange fruit is presented in Figure 2.5. Furthermore, the oil gland contains essential oil which contributes to the natural aroma of citrus fruit and plays important role in defending fruit against pathogenic and pest attack (Ben-Yehoshua *et al.*, 1998).

The first oil glands are formed in the ovary wall of the flower, and new glands form continually during enlargement of the fruit, by the process of schizogeny (Knight, 2002).

Cold-pressed citrus peel oil is obtained commercially by rupture of the glands during juice extraction. The oil consists of mixtures of hydrocarbon groups such as

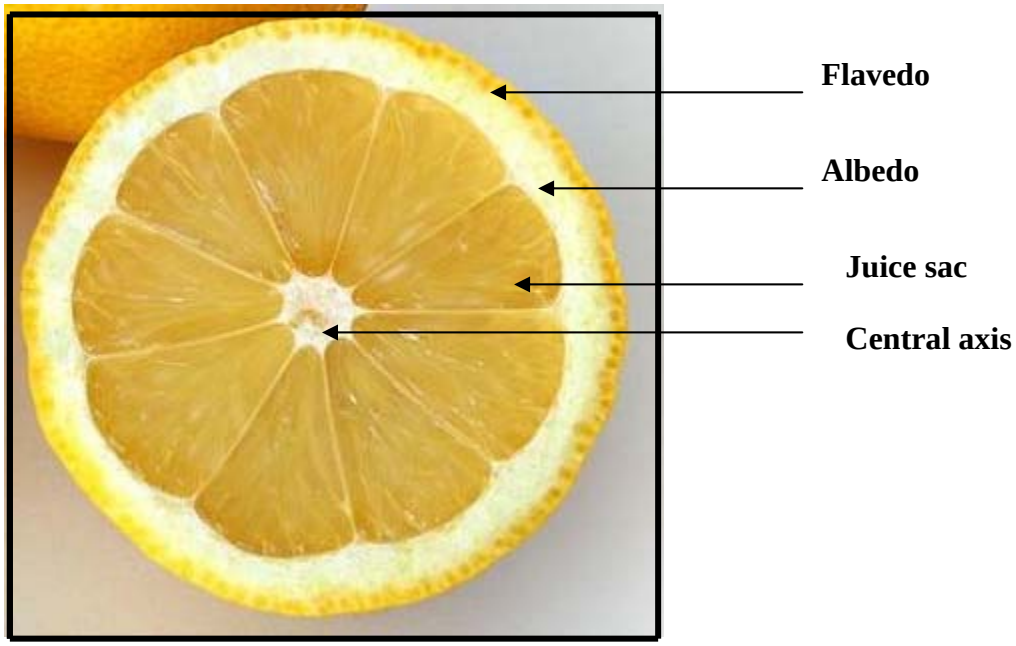

Figure 2.4 General structure of orange fruit.

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of the print copy of the thesis held in
the University of Adelaide Library.

Figure 2.5 Transection of rind of mature orange fruit.
Al, albedo; FL, flavedo; and OG, oil gland
(Source: Baker and Scott, 1947)

limonene, and oxygenated compounds, including alcohols and aldehydes. Limonene is the largest hydrocarbon component of the oil, comprising 90-95% by weight of the oil (Shaw, 1977). The oxygenated compounds are highly odoriferous and are the principal odour carriers. The concentration of the oxygenated compounds is about 2-6% of the oil (Owuso *et al*., 1986); for example, linalool contributes 0.3 – 5.3% and citral about 3% (Shaw, 1977). Furthermore, citral has been shown to exhibit significant antifungal activity, whereas limonene has been reported to enhance the development of *Penicillium digitatum* in yellow lemons (Ben-Yehoshua *et al*., 2008).

During the postharvest stage, oranges are susceptible to attacked by pathogens, especially wound pathogens that might attack the fruit during handling, transport, storage and marketing. Diseases causes by pathogens can affect the fruit quality and causing significant economic losses.

This chapter briefly reviews postharvest diseases of oranges (green mould, blue mould and sour rot), their control by means of synthetic chemicals, biocontrol and natural chemicals; and with particular emphasis on application of citral as an alternative to synthetic chemical fungicides.

2.2 Postharvest fungal pathogens of oranges

Postharvest diseases limit the storage life of the fruit and cause significant economic losses for fruit exporting industries worldwide. The most economically important postharvest diseases affecting citrus production worldwide are green and blue moulds (Eckert and Eaks, 1989), and to lesser extent sour rot. Green mould, blue

mould and sour rot are caused by *Penicillium digitatum*, *P. italicum* and *Geotrichum candidum* citrus race (syn. *G. candidum* var. *citri-aurantii*), respectively. In general, the susceptibility of citrus fruit to green mould and blue mould increases with maturity (Stange *et al.*, 2002). Oranges affected by green mould, blue mould, and by sour rot are shown in figure 2.6; 2.7 and 2.8, respectively. These diseases are predominantly related to harvesting and handling of fruit. These pathogens may attack the fruit on the tree, in the packing-house, in transit, in storage and in the market. During these stages, citrus fruits can be affected by environmental conditions that favour the development of the diseases, such as storage temperatures (cold or ambient conditions) or rain. In ambient conditions, green mould develops more rapidly than blue mould. Blue mould is more common in fruit held in cold storage during the summer; it can spread through packed cartons more easily than green mould, producing a 'nest' of decayed fruit. Furthermore, the incidence of sour rot increases during the wet season and when harvesting occurs after abundant rainfall (Plaza *et al*., 2003).

2.2.1. Green mould

Green mould, caused by *P. digitatum* (Sacc.), is the most common and serious postharvest disease of citrus worldwide. This pathogen is responsible for 90% of citrus losses due to diseases during storage, and it causes serious commercial loss (Eckert and Eaks, 1989). *P. digitatum* grows on the surface of citrus fruits after harvest producing a characteristic powdery, green olive-coloured lesion. Symptoms of green mould (Figure 2.6) are, initially, the appearance of a soft watery spot, followed by the production of white mycelium on the lesion surface and then, when

the lesion diameter enlarges to approximately 2-3 cm, olive-green spores are produced in the centre. The sporulation area then is surrounded by a broad zone of white mycelium, and the rind in the outer region of the lesion becomes soft. Masses of olive-green spores soon encompass the whole fruit, and these can be dispersed by air currents. *P. digitatum* infects the fruit through injuries or wounds, and all types of citrus fruits can be infected. The injuries or wounds may be due to rupture of oil glands, chilling injury, mechanical damage, or stem-end breakdown. The fungus survives in the field on soil debris and produces asexual spores (conidia), which are carried by wind currents to the surface of injured fruit in the tree canopy. Wounds formed during harvesting and handling release nutrients and moisture that favour spore germination. When oil glands of the fruit are ruptured, they provide an avenue for infection by the fungus (Bates, 1933). Conidia of *P. digitatum* lie dormant on the fruit surface and commence active growth only if an injury is perceived via exposure to citrus fruit volatiles (Stange *et al.*, 2002).

Figure 2.6 Orange with green mould (Source: Droby, 2009)

The germination and growth of *P. digitatum* is affected by temperature and water activity (Plaza *et al.*, 2003). The development of green mould occurs most rapidly at temperatures near 25^{o}C and much more slowly above 30^{o}C and below 10^{o}C. The rot

is almost completely inhibited at 1^{o}C (Pitt and Hocking, 1997; Timmer *et al.*, 2000). The minimum water activity (a_w) for germination of *P. digitatum* conidia at 25^{o}C is 0.90 (Plaza *et al.*, 2003).

2.2.2 Blue mould

Blue mould, caused by *P. italicum* Wehmer is less prevalent than green mould. Blue mould occurs primarily on fruit in cold storage. The fungus can grow slowly at low temperatures, and causes decay after extended storage. The disease cycle and the symptoms of blue mould (Figure 2.7) are similar to those of green mould and sour rot. The decayed area appears as a soft watery spot, and white mycelium is formed when the lesion diameter enlarges to 2-6 cm, and then blue spores are produced. The lesion differs from that caused by *P. digitatum* in having a narrow band of white mycelium surrounding the sporulating area, which is encompassed by a definite band of water-soaked rind.

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This figure is included on page 13 of the print copy of the thesis held in the University of Adelaide Library.

Figure 2.7 Orange with blue mould (Source: Droby, 2009)

Similar to *P. digitatum*, the germination and growth of *P. italicum* is also affected by temperature and water activity (Plaza *et al.*, 2003). Growth of *P. italicum* on oranges occurs between –3 and 32-34^{o}C, with an optimum at 22-24^{o}C; however, *P. italicum*

grows better than *P. digitatum* below 10°C and may predominate over green mould in cold storage (Pitt and Hocking, 1997; Trimmer *et al.*, 2000). The minimum a_w for germination is 0.87 and the pH range for growth is 1.6 to 9.8 (Pitt and Hocking, 1997; Plaza *et al.*, 2003).

2.2.3. Sour rot

The third most common postharvest disease of citrus is sour rot, caused by *Geotrichum citri-aurantii* (Snowdon, 1990; Pitt and Hocking, 1997).

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This figure is included on page 14 of the print copy of the thesis held in the University of Adelaide Library.

Figure 2.8 Orange with sour rot (Source: Taverner *et al.*, 2001)

G. citri-aurantii is a significant pathogen of citrus fruit during postharvest storage, with growth favoured at temperatures of 25-30°C and at a_w of 0.95 (Plaza *et al.*, 2003). This fungus penetrates the fruit only through particularly deep injuries that extend into the albedo. Sour rot is often associated with green mould and is stimulated by its presence (Brown, 2003).

Sour rot first appears as a water-soaked lesion, light to dark yellow in colour. Then

the lesion is covered with a yeast-like mycelium. Compared with green and blue moulds, the cuticle in the lesion is more easily removed from the epidermis, and the decayed fruit disintegrates into a slimy and watery mass. The name sour rot is due to its characteristic sour odour, which attracts fruit flies that, in turn, spread the fungus to other injured fruit (Pitt and Hocking, 1997).

Plaza *et al.* (2003) found that at 30^{o}C, spores of *P. digitatum* and *P. italicum* showed a similar germination rate at both 0.99 a_w and 0.95 a_w, whereas germination of *G. citri-aurantii* was over three times higher at 0.99 a_w than at 0.95 a_w.

2.3. Control of postharvest fungal diseases of oranges

Control of these diseases is achieved by careful handling of the fruit, careful harvesting to prevent injuries and avoiding contact with soil. Careful handling of fruit during harvesting, transport and storage is aimed at preventing wounding, while contact with soil must be avoided because spoilage organisms occur in the soil. Currently, in the citrus industry, postharvest diseases are commonly controlled by applying fungicide treatments. One of the main aims of fungicide application in citrus postharvest is to destroy inoculum of *P. italicum*, *P. digitatum* and *G. citri-aurantii* on fruit arriving in the packing shed (Cunningham, 2005).

In general, fungicides can be applied as a preharvest spray (for example Benlate), with bin drenchers as an initial treatment of fruit on arrival and on the packing-house line (for example thiabendazole or TBZ) (Ritenour *et al.*, 2006**).** Types of fungicides and method of application used in the packing sheds vary and the latter

may include dipping/drenching, inline flood, inline controlled droplet application (CDA) and mixing with wax (Cunningham, 2005).

2.3.1. Synthetic chemical fungicides

Several chemicals have been used to control postharvest fungal diseases of oranges, including fungicides such as thiabendazole, imazalil and guazatine (Pitt and Hocking, 1997). A list of fungicides registered in Australia for postharvest use on citrus is provided in Table 2.1.

Table 2.1 Fungicides registered in Australia for postharvest use on citrus

Source: Australian Citrus Grower, 2004

In May 2010, the Australian Pesticides and Veterinary Medicines Authority (APVMA) granted registration of Scholar Fungicide (active constituent fludioxonil)

for postharvest treatment of citrus (Taverner, 2010), while the use of carbendazim on citrus in Australia suspended in July 2010 (Citrus Australia, 2010b). Fungicides containing benomyl, carbendazim, imazalil, fludioxonil and thiabendazole are only effective against green and blue moulds. On the other hand, fungicides containing guazatine are very effective in the control of green mould, blue mould and sour rot especially when applied at least 24 hours after harvest, and guazatine can be used most effectively as a bulk dip when fruit first come into the packingshed (Cunningham, 2005). However, some countries apply limitations for fungicide usage in imported fruit, for example, Panoctine (guazatine) is not permitted in the USA, Canada and Japan (SARDI, 2001; Cunningham and Taverner, 2007; Cunningham, 2008), while the fungicide is widely used for Australian domestic market. Effort to find a replacement for dealing with sour rot is considered to be a future need of the citrus industry (Droby, 2009; Taverner, 2009). Fungicides permitted for use in export destination for Australian citrus are presented in Table 2.2 below.

Table 2.2 Fungicides permitted for use in export destinations for Australian citrus

Active constituent	USA	Canada	Japan	Singapore	Malaysia	Indonesia	Hongkong
Panoctine	X	x	x	✓	✓	✓	✓
Carbendazim	✓	✓	x	✓	✓	✓	✓
Thiabendazole	✓	✓	✓	✓	✓	✓	✓
Imazalil	✓	✓	✓	✓	✓	✓	✓

✓ = permitted, and x = not permitted
Source: SARDI, 2001; Cunningham and Taverner, 2007; Cunningham, 2008

Furthermore, due to intensive and continuous use, the efficacy of fungicides has decreased because of the development of resistant strains of the pathogens. The development of resistance by *P. digitatum* and *P. italicum* to thiabendazole, imazalil

and benomyl has been reported (Dave *et al*., 1989; Eckert *et al*., 1994; Pitt and Hocking, 1997).

Fungicide residue levels in fruit have also become problematic. Residue levels in fruit are affected by the fungicide concentration, exposure time and temperature, and method of applications (heated immersion, ambient immersion, spray, dip or in wax). Schirra *et al*. (1997) found that when imazalil was applied at 50^{o}C (heated immersion) there was a linear relationship between the residue concentration in fruit and the amount of the fungicide employed. In addition, the fungicide concentration needed to control pathogens and the residues in fruit were lower for application at 50^{o}C than at 22^{o}C. This suggests that a combination of increased temperature (from 22^{o}C to 50^{o}C) with fungicide application may allow a decrease in fungicide usage. This increased control is believed to be due to increased mobility of fungicides in the fruit epicuticular wax, but also may be due to thermal inactivation of pathogens; hot water treatment may decrease infection by removing spores from wounds and inducing defence mechanisms in the outer layers of the fruit epicarp (Schirra *et al*., 1997). However, McDonald *et al*. (1991) found that thiabendazole and imazalil treatments applied at 5^{o}C reduced chilling injury and inhibited decay of grapefruit more effectively than when these fungicides were applied at room temperature, suggesting that the relationship between fungicide activity and temperature may be complex.

Problems with fungicide residues on fruit, concern for human health and the environment and development of fungicide resistance have led to a need to develop

alternative methods for controlling postharvest diseases. Possible alternatives include using biological control and natural chemicals.

2.3.2. Biological control

Awareness of the health and ecological risks associated with increased synthetic fungicide usage in agriculture dictates the search for natural, safe and environmentally friendly means of disease control.

In the search for alternatives to synthetic chemical treatment of postharvest diseases, biological control has been investigated. According to Baker and Cook (1983), biological control is the reduction in the amount of inoculum or disease-producing activity of a pathogen accomplished by or through one or more organisms other than man. More inclusively, Wilson (1997) defines biological control as the control of a plant disease with a natural biological process or the product of a natural biological process, and includes bio-chemicals delivered by and extracted from living organism, and also host resistance (constitutive and elicited). Mercier and Smilanick (2005) reported that volatile compounds produced by the fungus *Muscodor albus* when used to fumigate lemons for 24 -72 h controlled green mould.

Biological control of postharvest diseases using microbial antagonists is considered a desirable alternative to synthetic fungicides (El-Ghaouth *et al*. 2000). Antagonists that have been investigated for biological control of *Penicillium* rots include a yeast, *Debaryomyces hansenii* (Chalutz, 1988; Droby and Chalutz, 1989) and a Gram-positive bacterium, *Bacillus pumilus* (Huang *et al*., 1992). *B. pumilus* showed a

strong inhibitory effect *in vitro*, producing large and distinct inhibition zones in the mycelium of *P. digitatum* surrounding the colony, and it was reported to be more active than imazalil and guazatine (Huang *et al*., 1992). Also *B. pumilus* at 1.6×10^{10} - 1.6×10^{12} cfu mL^{-1} gave strong antagonistic activity against *P. digitatum* on Valencia orange, which was as effective as 500 μg mL^{-1} imazalil and was significantly better than 500 μg mL^{-1} benomyl. The biological control activity of *D. hansenii* was found to be dependent on both pathogen and antagonist concentration, and was greatly affected by nutrient levels at the wound site. In culture, at 10^9 cfu mL^{-1} *D. hansenii* gave significant inhibition of spore germination and hyphal growth of *P. digitatum.* Competition for nutrients is believed to be the mechanism by which *D. hansenii* exerts control of *P. digitatum* (Droby and Chalutz, 1989). Biocontrol of postharvest rots of citrus has been achieved using Aspire, a product of the yeast *Candida oleophila*, which is commercially registered. Droby *et al*. (1998) reported that Aspire is highly efficacious against sour rot caused by *Geotrichum citri-aurantii.*

The antagonistic activity of some biocontrol agents in controlling postharvest decay can be increased by some chemical compounds, such as carbonate and bicarbonate (Palou *et al*. 2001). The combination of more persistent activity of biocontrol agents with heat treatment has the potential to offer effective longer term disease control (Obagwu and Korsten 2003). Obagwu and Korsten (2003) reported that the use of *Bacillus subtilis* alone to control the incidence of both green and blue moulds on artificially inoculated ‘Valencia’ and ‘Shamouti’ oranges was less effective than the fungicide treatment (quazatine plus imazalil), and they observed a significant increase in biocontrol activity when they combined *Bacillus subtilis* with sodium

bicarbonate, and when *B. subtilis* was applied following hot water treatment, it gave 100% control. This suggests that combination of biocontrol with other alternative methods may provide additive or synergistic effects and enhance the efficacy. Defence mechanisms in the outer layers of the fruit epicarp may be induced after hot water treatment, which also decrease infection by removing spores from wounds. High temperatures and chemical compounds alone offer only short-term inhibitory activity against fruit storage rots, whereas biocontrol agents can persist for long periods and protect fruit from re-infection (Teixid *et al*. 2001).

A combination of Aspire with the fungicide thiabendazole (200 µg mL^{-1}) reduced the incidence of decay caused by *P. digitatum* and *P. italicum* as effectively as a conventional fungicide treatment (Droby *et al*, 1998). However, the application of biological agents alone to control postharvest diseases of oranges faces limitation. One factor limiting commercial interest in biocontrol is the high cost of production for most biocontrol agents due to high cost of substrate, low biomass productivity, or limited economies of scale (Fravel, 2005).

2.3.3. Natural chemical fungicides

Another possible approach to control citrus postharvest diseases involves the application of natural chemicals. Several researchers have reported antimicrobial activity of plant extracts and essential oils which have potential to meet the requirement for non-hazardous eco-friendly treatments to control citrus postharvest diseases. Essential oils and their main components possess a wide spectrum of biological activity, which may be of great importance, and the main advantage of

essential oils is that they are natural and are considered ‘generally recognized as safe’ (GRAS).

In recent years, essential oils have received much attention as resources of potentially useful compounds which possess a wide spectrum of biological activity. Essential oils are natural, complex, multi-component systems composed mainly of terpenes, in addition to some non-terpene components. The essential oils are extracted from different parts of the aromatic plant (flower, leaf, seed or bark) using various techniques, including water or steam distillation, solvent extraction, expression under pressure, supercritical fluid or sub-critical water extractions.

Davis and Ward (2003) reported that citral-containing essential oils, Nepalese lemon grass oil, lemon scented eucalyptus (*Eucalyptus citrodora*) oil and lemon scented tea tree (*Leptospermum petersonii*) oil were the most active at controlling the *in vitro* growth of *Ascosphaera apis*, causal agent of chalkbrood of bees.

Citrus essential oils and their major components limonene, citral and linalool are designated GRAS and have been found to be inhibitory both in direct oil and vapour form against a range of both Gram-positive and Gram-negative bacteria (Fisher and Phillips, 2006). Citrus essential oil also appears to have the potential to inhibit fungal spoilage and shows a number of advantages. Fisher and Phillips (2006) reported that essential oil of lemon, sweet orange and bergamot and their components, linalool and citral, exerted antimicrobial effects both in direct oil and vapour form against *Bacillus cereus, Campylobacter jejuni, Escherichia coli* O157, *Listeria monocytogenes* and *Staphylococcus aureus*, *in vitro* using a paper disc

diffusion method in brain heart infusion agar medium for *B. cereus* and *S. aureus*; *Campylobacte*r agar base medium for *C. jejuni*; nutrient agar for *E. coli*, and *Listeria* selective agar medium for *L. monocytogenes*. The essential oils of *Citrus sinensis*, *C. aurantium, C. deliciosa, C. paradises* and *C. limon* were effective against *P. digitatum* and *P. italicum* at doses of 2180, 1015.4, 713.3, 910.3, 1056.4 ppm, respectively (Caccioni, *et al.,* 1998). Furthermore, naturally occurring citrus volatiles, acetaldehyde and ethanol, are effective in controlling citrus postharvest diseases. Prasad (1975) reported that in agar media, 10 % (v/v) acetaldehyde vapour at 21 ^{o}C for 10 min was fungitoxic to *P. italicum*, *Alternaria citri, A. tenuis, Colletotrichum gloeosporioides* and *Glomerella cingulata*.

Citral, as a natural component of citrus fruit, has potential therefore, in management of postharvest spoilage and is discussed in the following sections.

2.4. Citral

2.4.1. Chemistry of citral

The name citral is derived from *Backhousia citriodora*, the plant species from which the oil was originally isolated (Erman, 1985). The primary use of citral is as lemon flavouring in foods, beverages, biscuits, candies and oil. It is also used to provide lemon fragrance in soaps, shampoos, perfumes and other toiletries, in the pharmaceutical industry, medicine, household chemicals, and in production of vitamin A and ionones. Citral is the most applied flavour compound with consumption of 1200 tonnes in 1996 (FAO/WHO, 2003). It has been declared as a GRAS compound for food use, with an acceptable daily intake (ADI) of 0 – 0.5 mg

kg^{-1} day^{-1}, there is no evidence to suggest that it has mutagenic activity, carcinogenic potential or poses a health risk in food (FAO/WHO, 2003).

Citral is commonly prepared by isolation of citral-containing oils or by chemical synthesis. Naturally, citral occurs mainly in lemon myrtle oil, distilled from the leaf of *B. citriodora* (90-95% content), lemongrass oil, distilled from the leaf of *Cymbopogon flexuosus* (80-85% content), and litsea cubeba oil, distilled from the fruits of *Litsea cubeba* (70-75% content), and several other plant oils. Synthetically, citral can be produced by the reaction of acetylene and acetone (Erman, 1985).

As an acyclic monoterpene aldehyde, citral is a type of isoprenoid compound. The chemical name of citral is 3,7-dimethyl-2,6-octadienal; it is a mobile, pale yellow liquid with strong lemon odour; boiling point of 229^{o}C, specific gravity of 0.891 – 0.897 (15^{o}C); a refractive index of 1.4860-1.4900 (20^{o}C); is not optically active; is combustible and non-toxic (Hawley, 1981). Moreover, as a monoterpene aldehyde, citral is a natural mixture of the geometric trans- and cis-isomers, geranial and neral, respectively; both occur concurrently in nature. Commercial citral typically contains 60% geranial and 40% neral (Wolken *et al.*, 2002). Citral contains α, β-unsaturated carbons with the chemical structure shown below (Figure 2.9, Robert and Caserio, 1965).

Furthermore, the properties of the individual isomers vary. Geranial has a boiling point of 77^{o}C, a refractive index of 1.4898 (20^{o}C), and a maximum ultraviolet absorbency at 236 nm, while neral has a boiling point of 76.5^{o}C, a refractive index of 1.4868-1.4869 (20 ^{o}C), and a maximum ultraviolet absorbency at 234 nm (Venuto *et al*., 1964).

Figure 2.9 Structure of citral isomers, geranial and neral and the position of α– and β-carbons (Robert and Caserio, 1965)

Thermodynamically, neral is unstable and will undergo isomerisation to the stable form, geranial. They are difficult to separate, but are readily obtained by oxidation of their corresponding alcohols, geraniol and nerol (Whittaker, 1972). In condensation with acetone, they give pseudoionone A and B, for geranial and neral, respectively (Whittaker, 1972); correspondingly the pseudoionones readily cyclise in acid to give a mixture of α and β-ionone (Figure 2.10).

Aldehydes are an intermediate form between primary alcohols and carboxylic acids. Therefore, aldehydes are easily oxidised to the corresponding carboxylic acids (Kotz and Purcell, 1991).

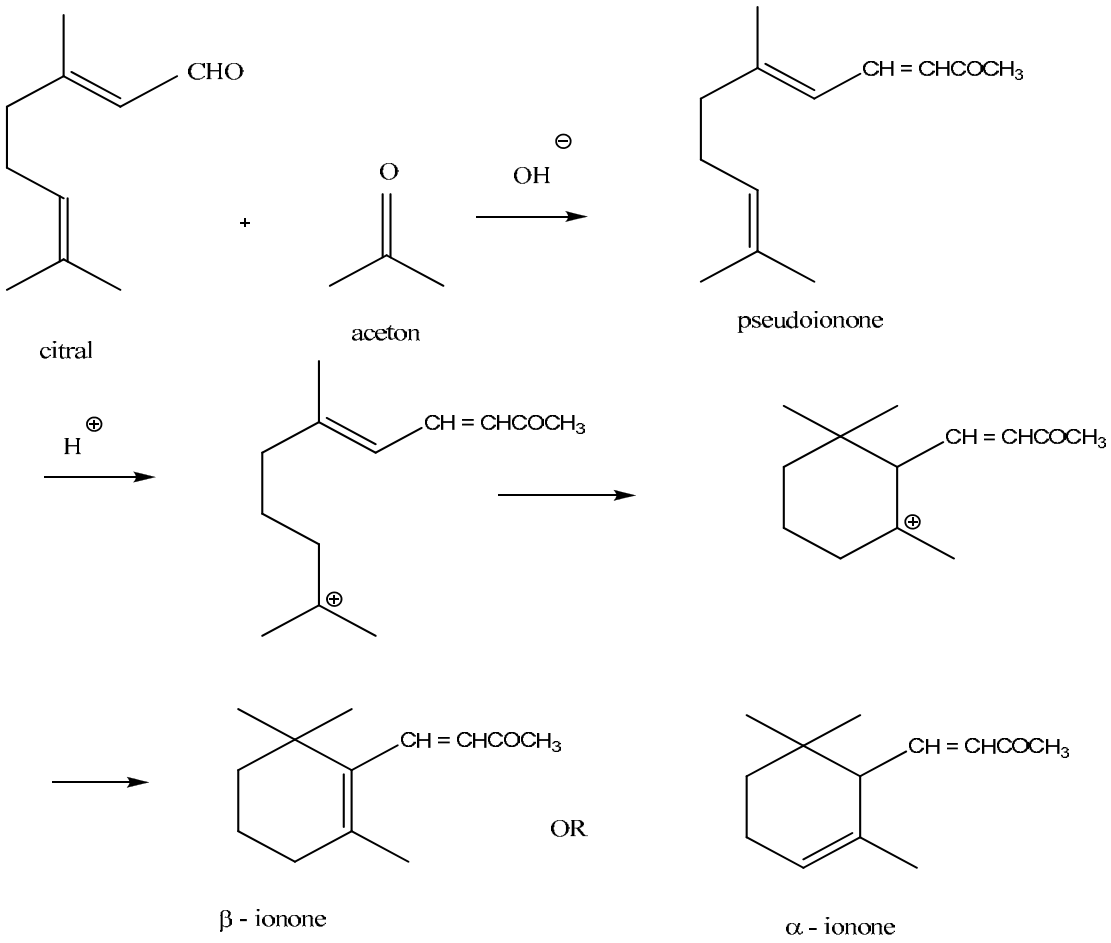


Figure 2.10 Condensation of citral with acetone produces α– ionone and β-ionone (Whittaker, 1972)

The general biotransformation reaction scheme of oxidation of simple aldehydes is (Feron *et al*., 1991):

$$R\text{-}CHO + NAD^{+} + H_2O \text{ ----- } R\text{-}COOH + NADH + H^{+}$$

Feron *et al*. (1991) also stated that the reduction to an alcohol is reversible, while oxidation to carboxylic acids is essentially irreversible.

As an α, β-unsaturated aldehyde which contains two C=C double bonds and one carbonyl group, citral is susceptible to several reactions, such as oxidation and reduction, and even cyclisation reactions. According to Erman (1985), geranial and neral are the biosynthetic oxidation products of geraniol, which undergoes reduction to citronellal. Citronellal is the biosynthetic oxidation product of citronellol. This suggests that citral is related to citronellal, citronellol, citronellic acid, and even cyclocitral. All of these compounds will be termed citral-related compounds in this review (Figure 2.11). Stashenko *et al*. (1996) found that citral (trans- and cis-isomers) could be converted to citronellal and dihydrocitronellal during catalytic hydrogenation of orange oil.

2.4.2. Citral in citrus

Citral in certain plants, particularly in citrus species, is formed through conversion of geraniol to geranial (citral isomers) in cell-free systems by the enzyme geraniol dehydrogenase (Erman, 1985). In citrus fruit, citral is located inside oil glands (cavities) in the flavedo layer of the peel, and is not distributed uniformly throughout the fruit surface (Rodov *et al.,* 1995).

The characteristic flavour and aroma of citrus fruit are associated with their volatile constituents. Citral and linalool are thought to be the most potent aroma compounds of Colombian citrus peel oils (lemon, mandarin and orange), compared to other components of the oils (Tirado *et al.*, 1995). Chida *et al.* (2006) reported that citral was the main aroma character of lemon oil, and considered it to be a key aroma compound of lemon fruit.

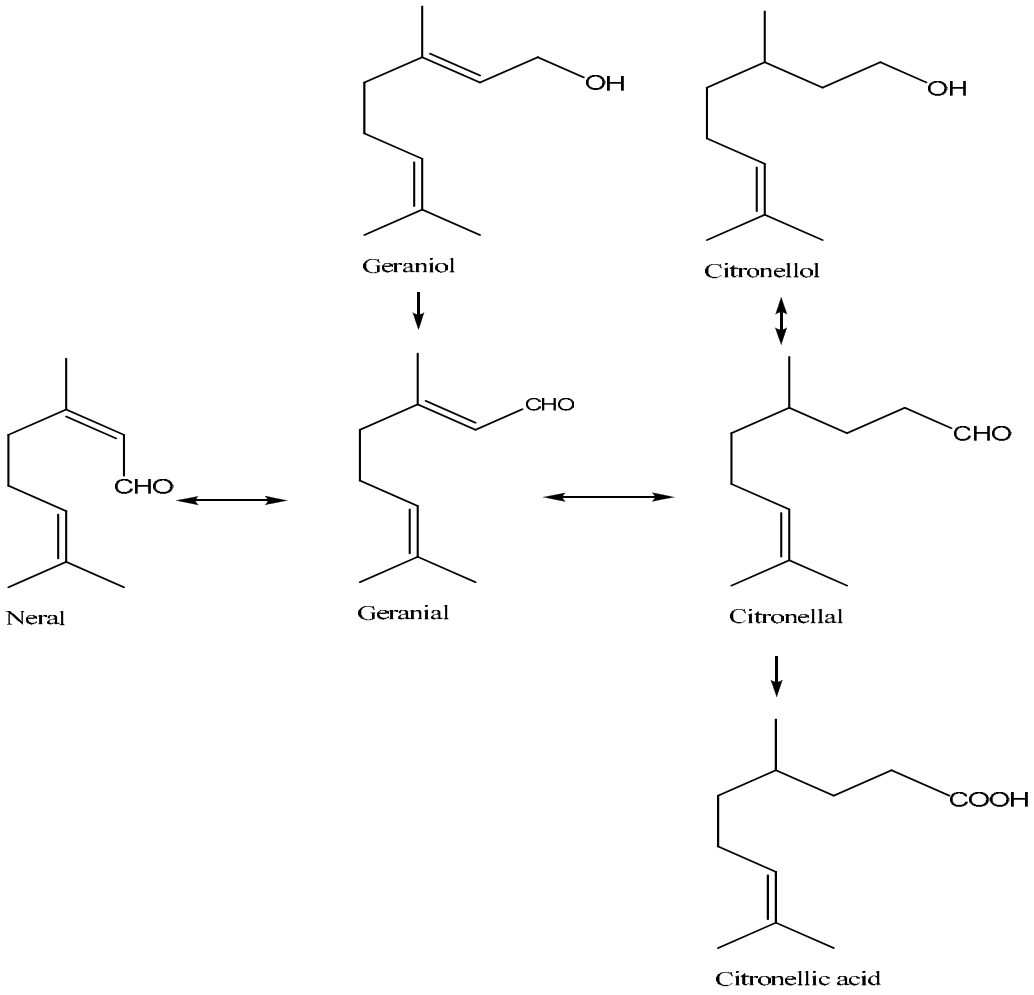


Figure 2.11 Biogenesis of monoterpenes (adapted from Erman, 1985)

Citral has been shown to be present in the essential oil of lemon (0.1%), sweet orange (3.0%) and bergamot (0.7%), often in the form of the stereoisomers neral and geranial (Moufida and Marzouk, 2003). More recently, Ladaniya (2008) reported the citral content of peel oil of some citrus fruits to be; 0.05-0.2 % in oranges, 0.06% in grapefruits, 1.9-2.6% in lemons, and 3.1-5.3% in limes. Therefore, different varieties of citrus fruit contain different amounts of citral and, in turn, different methods of extraction will also affect the amount obtained. To take as an example sweet oranges (*Citrus sinensis* (L) Osbeck), Caccioni *et al*. (1998) reported that when fresh rind

tissue of fruit was subjected to steam distillation, rind of Washington navel consisted of 94.81, 0.32, 0.06 and 0.07% of limonene, citral (geranial + neral), citronellal and citronellol (geraniol + nerol), respectively, whereas rind of Valencia late consisted of 94.95, 0.17, 0.03 and 0.11% limonene, citral (geranial + neral), citronellal and citronellol (geraniol + nerol), respectively. Furthermore, Dongmo *et al.* (2002) reported that hydrodistillation extracts of rind of Valencia late consisted of 82.36% limonene, 0.76% citral and 0.59% citronellol. Sawamura *et al*. (2005) reported that limonene, neral and geranial were the major peel oil components of four different cultivars of *C. sinensis* Osbeck var. Hongjian, var. Anliu, var. Sihui and var. Washington navel, which were isolated by cold-pressed method and investigated by Gas Chromatography (GC) and Gas Chromatography/Mass Spectrometry (GC/MS); peel oil of these cultivars contained (for Hongjian, Anliu, Sihui Washington navel, respectively), 93.6; 94.2; 93.6 and 94.4% for limonene 0.2; 0.2; 0.2 and 0.1% for neral and 0.3; 0.2; 0.2; 0.2 and 0.2% for geranial, respectively. Additionally, for mandarin (*C. reticulata* Blanco), the citral (geranial and neral contents) is higher while limonene content is lower than those of *C. sinensis*; as Chutia *et al*. (2008) identified the major components of essential oil of peel of fully mature fruits of mandarin isolated by hydro-distillation and analyzed by GC/MS to be limonene (46.7%), geranial (19.0%), neral (14.5%), geranyl acetate (3.9%), geraniol (3.5%), b-caryophyllene (2.6%), nerol (2.3%) and neryl acetate (1.1%).

Rodov *et al*. (1995) reported that the citral content in the flavedo decreased with fruit maturity, as did resistance to decay. Furthermore, they also found that decreased citral content with maturity was related to an increased concentration of neryl acetate. Neryl acetate is an oxygenated monoterpene, which is formed from

neral, the unstable isomer of citral. In addition, neryl acetate is a very poor inhibitor of citrus fruit decay caused by *P. digitatum* (Rodov *et al*., 1995).

2.4.3. Analysis of citral

Much research has concerned the analysis of citrus oil components, yet little research has focussed on analysing individual citral components, either qualitatively or quantitatively. Many researchers have carried out qualitative analysis of cold–pressed citrus essential oils. Shaw (1979) has critically reviewed the qualitative and quantitative analysis of cold-pressed citrus essential oils. GC analysis has been the main technique used to quantify essential oil components, as well as citral. Quantitative gas chromatographic analysis of cold-pressed citrus oils using preliminary separation steps to separate its volatile and non-volatile components has been reported (Stanley *et al*., 1961; Lifshitz *et al*., 1970). Fisher and Philips (2006) analysed the limonene, citral and linalool contents of oils of lemon (*Citrus limon*), sweet orange (*C. sinensis*) and bergamot (*C. bergamia*) using gas chromatography (GC) and a flame ionization detector. Recently, Rauber *et al*.(2005) reported for the first time quantification of citral in volatile oil by high performance liquid chromatography (HPLC) with UV detection method.

Very little research has been reported on qualitative and quantitative analysis or separation of citral into its isomers, geranial and neral, using gas chromatographic methods. Naves (1952) and Venuto *et al*. (1964) separated citral into geranial and neral by fractional distillation. Sacks *et al*. (1983) separated the citral isomers using vacuum spinning band distillation. However, in these reports the complete

separation of citral was not achieved and, therefore, pure geranial or neral were not obtained.

Marko *et al.* (1998) reported that alcohols under mild conditions were oxidised to their corresponding carbonyl derivative aldehydes and ketones with high yield and with the release of water as the only by-product. In their experiment, geranial and neral were obtained separately through oxidation of their corresponding alcohols, geraniol and nerol, with 83 and 73% yields, respectively. No double bond isomerisation took place under these conditions.

Because citral comprises the two isomers, geranial and neral, and citral is also susceptible to oxidation/reduction reaction (due to its C=C bonds), determination of the precise composition of citral prior to its application in research may be important as the various products may have different individual effects on fungi.

2.4.4 Bioactivity of citral

Considerable research has been undertaken on the antibacterial and antifungal properties of citral. The toxicity of citral have been described in the literature; in term of medically important bacteria, Onawunmi (1989) reported that citral at 10% (v/v) dilution showed antibacterial activity against *Staphylococcus aureus, Bacillus subtilis*, *Escherichia coli* and *Pseudomonas aeruginosa.* Citral and perillaldehyde had minimum bactericidal concentration (MBC) of 100 μg mL^{-1} against *Viellonella vulnificus* and at 500 μg mL^{-1} completely killed *Escherischia coli, E. coli O157:H7* and *Salmonella typhimurium* (Kim *et al*., 1995). Furthermore, Pattnaik *et al*. (1997)

observed that citral could inhibit 14 bacteria, including Gram-positive cocci and rods, and Gram-negative rods and proved to be an effective antifungal compound for 12 fungi, three of which were yeast-like and nine filamentous. Furthermore, citral has been shown to inhibit growth of various fungal pathogens of humans; for example Viollon and Chaumont (1994) found that citral inhibited the yeast-like pathogen *Cryptococcus neoformans* in vitro and Bona de Silva *et al.* (2008) reported that citral at concentration range of 25-200 μg mL^{-1} in 4% Sabouraud dextrose agar (SDA) medium inhibited the mycelial growth of *Candida albicans.* Wannisorn *et al.* (1996) reported that citral inhibited and killed four dermatophytes, *Trichophyton mentagrophytes, T. rubrum, Epidermophyton floccusom* and *Microsporum gypseum.*

There has been little research reported on the bioactivity of individual citral components. Park *et al.* (2007) reported that geranial and neral isolated from tea tree (*Leptospermum petersonii* Bailey) oil tested using diffusion agar method on SBA showed antifungal activity against *Microsporum canis, T. mentagrophytes* and *Microsporum gypseum*. At a concentration of 0.1 mg mL^{-1}, geranial was fungicidal, whereas neral was fungistatic.

2.5. Application of citral to control postharvest fungal pathogens of oranges

There are very few reports on the application of citral to control the citrus postharvest fungal diseases, green mould, blue mould and sour rot. Citral has been reported to be a preformed antifungal material in lemon peel, exerting high inhibitory activity against *P. digitatum* (Ben-Yehoshua *et al.*, 1992; Rodov *et al.*, 1995). Initially, Ben-Yehoshua *et al*. (1992), detected citral and limettin as

antifungal materials contained in the extract of lemon flavedo, and in vitro using agar diffusion assay citral inhibited germtube elongation and spore germination of *P. digitatum*. Then Rodov *et al*. (1995) reported that a Thin Layer Chromatography (TLC) bioassay of lemon flavedo extract revealed the presence of citral in the largest inhibitory spot (Figure 2.12), suggesting that citral exhibited strong antifungal activity.

In the case of toxicity of citral to *P. digitatum*, Wolken *et al.* (2002a) for the first time studied the difference in susceptibility of mycelium and spores of *P. digitatum* to citral by assessing the spore and mycelium viability. Spores were induced by adding 0.4 μL mL^{-1} citral of spore suspension, and incubating for 11 h at 25°C with shaking, washed with buffer, diluted with sterile buffer, plated on malt extract agar (MEA) plates, and incubated at 20°C, colonies were counted after 3 days. Induced mycelium was obtained by adding 0.2 μL mL^{-1} citral to growing mycelium culture (2 days old), incubating for 15 h at 30°C with shaking, washed with buffer, diluted with sterile buffer, plated on MEA plates, and incubated at 20°C, colonies were counted after 2 days. They found that spores were more resistant to citral than mycelium of *P. digitatum*.

Information on antifungal activity of citral to *G. candidum* is very limited. Suprapta *et al*. (1997) reported that *in vitro* at 1.0 μl mL^{-1} citral inhibited both germination and mycelial growth of *G. candidum in vitro* by more than 50%.

Dunn (1998) found that citral in culture medium was more effective than limonene, linalool or commercial citrus rind oil in inhibiting *P. digitatum, P. italicum* and *G.*

candidum (*G.citri-aurantii*). He reported that citral could inhibit spore germination and mycelial growth of the fungi at 3000 ppm in neutral dox yeast (NDY) agar at room temperature, and at 5^{o}C inhibition was achieved at 500 ppm.

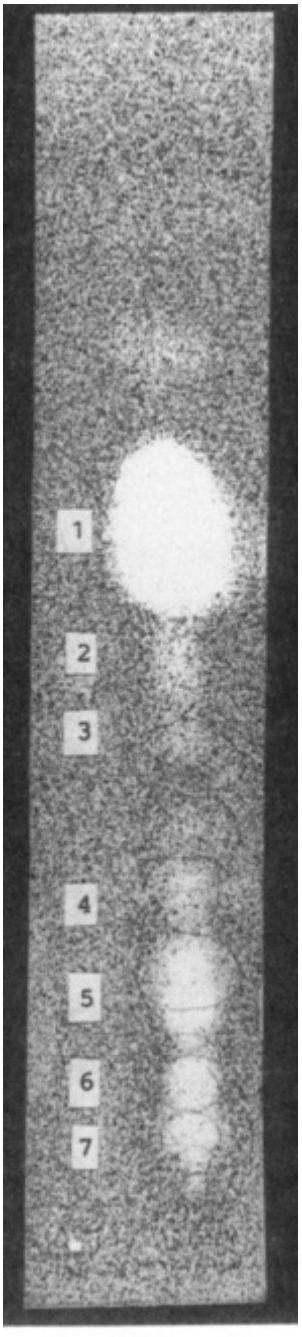


Figure 2.12
Detection of antifungal compounds in lemon flavedo essential oil using TLC.
1, citral; 3, 5-geranoxy-7-methoxycoumarin; 4, isopimpinellin; 5, limettin; 6, unidentified psoralen derivative; 2 and 7, unidentified.
(Source: Rodov *et al*. (1995)

Several researchers have reported methods of citral application *in vitro* and *in vivo*. French *et al.* (1978) reported that nonanal and citral could stimulate spore

germination of *P. digitatum* when applied in a 1% water agar without addition of emulsifier. They observed 17.8 and 13.5% germination of conidia of *P. digitatum*, respectively, when treated with 500 μL L^{-1} nonanal and citral. However, no germination was observed when treated with 1000 μL L^{-1} of citral. Bioactivity of citral the more than likely is dependent on concentration. Droby *et al.* (2008) reported that citral strongly stimulated germination of *P. digitatum* at concentrations equivalent to 0.06–0.15 μL L^{-1} but completely inhibited germination at higher concentrations. *P. italicum* was stimulated over a wider range of concentrations (from 0.06 to 1.5 ppm) and complete inhibition was not evident until 15 ppm. Additionally, since citral is insoluble in water and is volatile, without emulsifier it may be not be well distributed or may be adsorbed in the surface of the Petri dish, therefore its exact concentration that affects the pathogen may be lower than its applied concentration. Meanwhile, Suprapta *et al.* (1997) reported that citral and nonanol in 25 % ethanol mixed in PDA medium inhibited growth of *G. candidum.* Dunn (1998) tested two methods of application of citral in culture, namely, direct mixing with Neutral Dox Yeast (NDY) agar before inoculation, and surface flood application of citral solution + emulsifier after inoculation. Of these two, surface application resulted in less control of spore germination. This was probably because, with flood application, citral dissipated during the subsequent drying of the surface, while citral mixed in the medium continued to volatilise and therefore interact with the spores. The above suggests that method of application may affect the efficacy of citral in inhibiting *Penicillium* spp. and *G. citri-aurantii* for control of the diseases they cause.

There are several reports to the effect that citral has potential to damage the citrus fruit. Rupture of oil glands releases their oil content and, since some oils are phytotoxic (Wild, 1992, Sun and Petracek, 1999), this causes plasmolysis with typical cell collapse and pitting. This is followed by the appearance of greenish-brown areas on an orange or yellow background of just degreened fruits, or a pitted area of lighter colour on fruit that were damaged when orange in colour. This phenomenon is termed oleocellosis (Fawcett, 1916; Whiteside *et al.*, 1988; Shomer and Erner, 1989). Applying the essential oil alone without dilution to the fruit peel will also cause oleocellosis. Ben-Yehoshua *et al*. (1992) dipped fruit for 2 minutes in aqueous emulsion of citral (0.05 – 1.0% v/v with addition of L-77 detergent, 0.05% v/v). They found that 1 % citral emulsion reduced the decay of inoculated fruit from 100% to 9%, but resulted in phytotoxic damage to the peel. However, the incidence of damage did not exceed 7% at 0.5% citral and at lower concentrations the damage was negligible. Knight (2002) reported that direct contact of pure citral with rind of Washington Navel oranges resulted damage to the rind. Dunn (1998) found that application of 6000 ppm citral (aq) for 1 minute to navel oranges did not cause oleocellosis or rind blemish. From this point of view it is worth considering the effect of concentration of citral components, length of exposure, and temperature of the treatment on the fungi and the fruit.

Most research has involved dipping fruit in a citral solution (Ben-Yehoshua *et al*., 1992; Rodov *et al*. 1995; Dunn, 1998), and observing differences due to the time of dipping and citral formulation. Little is known about application of citral as a fumigant to control postharvest spoilage fungi on citrus. The application of citral in volatile form would allow continuous or temporary fumigation of spores without

direct contact with the fruit peel, for relatively short periods in storage chambers or during continuous fumigation over the long term within packages (Williams *et al.*, 2000). In practice, it is necessary for a fumigant to be distributed homogeneously throughout the fruit being fumigated, and this is affected by temperature (Bond, 2002). Temperature controls the release rate of the fumigant and speed of penetration to the fruit, as increasing the temperature increases the volatility of the fumigant (Bond, 2002).

2.6. Summary

In summary, this review highlights the need for further study to develop alternatives to synthetic chemical fungicides to protect citrus fruits from rot due to *P. digitatum*, *P. italicum*, and *G. citri-aurantii*.

The essential oil citral is a natural component of citrus fruit and has potential for use as an alternative option for control of citrus postharvest fungal spoilage (green mould, blue mould and sour rot).

The method of application of citral and its components can affect their efficacy in controlling the pathogens in culture; therefore examination of several application methods in order to find the best method which is applicable to industry is required.

Citral, geranial, neral, citronellal, citronellol and citronellic acid are known to be components of citrus essential oils. However, limited research has been conducted on the effect of the vapour phase of citral components on *P. digitatum*, *P. italicum*

and *G. citri-aurantii*. The activity of the citral isomers as well as their related compounds in the vapour phase against spores of *P. digitatum, P. italicum* and *G. citri-aurantii,* and whether the activity differs, needs to be investigated.

On the basis of results obtained from application of citral in culture, fumigation of oranges with citral to control postharvest fungal diseases (green mould, blue mould, and sour rot) was examined.

2.7 Aims of the study

The aim of this research was to assess methods for application of citral and its components in controlling the main postharvest fungal diseases of oranges in culture and in fruit.

To achieve this, the following strategies were adopted;

1. Examination of citral application methods to control *P. digitatum, P. italicum* and *G. citri-aurantii* in culture, in order to find the best citral application method.

2. Use the method identified above to examine the activity of the citral isomers as well as their related compounds against spores of *P. digitatum, P. italicum* and *G. citri-aurantii.*

3. Assess the ability of citral in volatile phase through fumigation of oranges to control green mould, blue mould and sour rot.

The information gained from this study is expected to contribute to development of citral as an alternative to synthetic chemical fungicides in protecting citrus fruits from *P. digitatum*, *P. italicum* and *G. citri-aurantii*.

Chapter Three

General materials and development of methods

3.1.Introduction

The materials and methods which are common to several experiments reported in this thesis are described in this chapter. Specific materials and methods of particular experiments will be described in the relevant chapters.

3.2. Fungal cultures

3.2.1. Isolation, identification and maintenance of the fungi

Cultures of *P. digitatum, P. italicum* and *G. citri-aurantii* were isolated from naturally infected fungicide-free lemon fruit supplied by Dr Andreas Klieber (12 November 1999, from his garden), cultured by aseptic transfer of conidia to 90 mm diameter plates of potato dextrose agar (PDA, DIFCO Laboratories, USA), and maintained also on PDA. The plates were incubated at room temperature (approximately 22°C) in natural daylight.

Pure cultures were identified on the basis of colony morphology and conidial shape and size, using the description by Pitt and Hocking (1997). Four isolates were established in pure culture. Colonies of isolate L1 were powdery green to grey in colour and the conidia were elliptical, and their length 6 – 8 μm was within the range for *P. digitatum* of 6 – 8 (-15) μm according to Pitt and Hocking (1997). The morphology of the colonies and conidia of isolate L3 also conformed to the

description of *P. digitatum*. Colonies of isolate L2 were watery white in colour and the conidia were cylindrical (2 x 8 μm) and within the range for *G. citri-aurantii* (2–5 x 5-8 μm) according to Pitt and Hocking (1997). Colonies of isolate L4 were powdery blue in colour and the conidia were small and almost round (2.9 – 3.6 μm), within the range for *P. italicum* (3 – 5 μm) according to Pitt and Hocking (1997).

3.2.2. Pathogenicity tests

The pathogenicity of the four isolates was confirmed by inoculation of citrus fruit in the laboratory. Mature lemons and Navel oranges obtained from Waite Campus orchard, not previously exposed to fungicide, were washed with fruit detergent (Decco Fruit and Vegetable Kleen, Cerexagri, Australia) then surface sterilised with bleach solution (Milton Antibacterial solution 1% active chlorine w/v, Proctor & Gamble Australia) containing 0.4% active chlorine for 10 minutes and rinsed with sterile reverse osmosis water.

After drying in a laminar flow cabinet the fruit were wounded with a sterile scalpel in one of three ways, through the oil glands, between the oil glands and down into the juice sacs. A drop of spore suspension of each isolate in sterile water (concentration was not determined) was applied to each wound. The fruit were left to dry in a flow of sterile air, placed in sterile plastic containers with wet, autoclaved paper towels to maintain high humidity, and incubated at room temperature in natural daylight. Symptoms were observed every day for 7 days.

Decay occurred most readily and fastest in fruit wounded into the juice sacs, followed by those wounded into the oil glands and then those wounded between the oil glands.

There was no difference observed between the two isolates of *P. digitatum* used (L1 and L3). Both formed white mycelia on the fruit and then produced green conidiophores, resulting in green mould. *P. italicum* caused the peel to soften, and then produced white mycelia and then blue spores, typical of blue mould. *G. candidum* caused the peel to soften and then watery white colonies were observed. The fungi were re-isolated aseptically from the lemons and the Navel oranges onto PDA plates, and given codes as follows; nPdg L1, nPit L4 and nGcd L2 were isolated from Navel oranges and lPdg L1, lPit L4 and lGcd L2, were isolated from lemon. There were no obvious differences between isolates obtained from the two hosts.

As oranges were the focus of this study, single spore-derived cultures were established from Navel oranges using the streak plate method. The resulting cultures were used throughout this study (nPdg L1 for *P. digitatum*, nPit L4 for *P. italicum* and nGcd L2 for *G. citri-aurantii*). The cultures were stored at 5°C and subcultured on potato dextrose agar (PDA) periodically.

3.2.3. Culture media and inoculum production

The medium used in all experiments involving fungal cultures was Neutral-Dox Yeast agar (NDY) (Warcup, 1950; Dunn, 1998), comprising 15 g L^{-1} agar (BiTek,

Difco Laboratories, Spark, MD, USA); 30 g L^{-1}sucrose (APS Finechem Ltd, NSW, Australia); 2 g L^{-1} $NaNO_3$ (Ajax Chemicals Ltd, Sydney, Australia), 1.0 g L^{-1} KH_2PO_4 (Ajax Chemicals Ltd, Sydney, Australia) and 0.5 g L^{-1} each of yeast extract (SigmaAldrich Pty Ltd, Castle Hill, Australia), KCl (BDH Merck Pty Ltd, Kilsyth, Australia) and $MgSO_4$ (Ajax Chemicals Ltd, Sydney, Australia).

Spore suspensions were prepared by suspending conidia from 10 to 14 day old cultures on potato dextrose agar (PDA) in sterile reverse osmosis water and one drop of Tween 80 (DIFCO Laboratories, West Molesey, UK) added as a wetting agent. The concentration of spores was estimated using an improved Neubauer haemocytometer (Improved Neubauer, BS74B, Weber, England) and adjusted to the required concentration by adding sterile reverse osmosis water.

Spore suspension, 100 μL, was spread on NDY agar using a glass spreader, and the plates were incubated at room temperature (22°C) in natural daylight for 14 days then colony forming units (cfu) were counted daily.

3.3. Chemicals and analysis

3.3.1. Chemicals used

Chemicals used routinely in experimental work are listed in Table 3.1.

TritonX-100 was used as emulsifier in preparation of aqueous solution of citral, and throughout this study citral aqueous solution was prepared in 400 μL L^{-1} TritonX.

Table 3.1 Description and source of chemicals used in experiments

Chemical name	[w]CAS No.	Brief description	Supplier
(R)-citronellal 3,7-dimethyl-2,6-octadienal	5392-40-5	95%, [x]FW 152.25, [y]bp 229°C, [z]d 0.891	Sigma Aldrich, Castle Hill, Australia
(R)-citronellal 3,7-dimethyl-6-octenal	2885-77-5	96%, FW 154.25 bp 207°C, d 0.851	Sigma Aldrich, Castle Hill, Australia
(S)-citronellal 3,7-dimethyl-6-octenal	5949-05-3	96%, FW 154.25 bp 207°C, d 0.851	Sigma Aldrich, Castle Hill, Australia
(R)-(+)β–citronellol 3,7-dimethyl-6-octenol	1117-61-9	98%, FW 156.27 bp 112-113°C, d 0.857	Sigma Aldrich, Castle Hill, Australia
(S)-(-) β–citronellol 3,7-dimethyl-6-octenol	7540-51-4	99%, FW 156.27 bp 225-226°C, d 0.856	Sigma Aldrich, Castle Hill, Australia
(R)-citronellic acid 3,7-dimethyl-6-octenoic acid	18951-85-4	98%, FW 170.25, bp 119°C, d 0.926	Sigma Aldrich, Castle Hill, Australia
(S)-citronellic acid (S)-(-)-3,7-dimethyl-6-octenoic acid	2111-53-7	98%, FW 170.25, bp 115-120°C, d 0.926	Sigma Aldrich, Castle Hill, Australia
Geranic acid 3,7-dimethyl-2,6-octadienoic acid	459-80-03	85%, FW 168.24, bp 250°C, d 0.97	Sigma Aldrich, Castle Hill, Australia
Geraniol *trans*-3,7-dimethyl-2,6-octadien-1-ol	106-24-1	98%, FW 154.25, bp 229-230°C, d 0.879	Sigma Aldrich, Castle Hill, Australia
Nerol *cis*-3,7-dimethyl-2,6-octadien-1-ol	106-24-2	97%, FW 154.25, bp 103-105°C, d 0.876	Sigma Aldrich, Castle Hill, Australia
TritonX-100	9002-93-1	d 1.070	Ajax Laboratory, Philadelphia. USA

Note: [w]: Chemical Abstracts Service number, [x]: Formula weight/molecular weight, [y]: boiling point and [z]: specific gravity

3.3.2. GC-analysis of citral

The composition of citral, which is liquid at room temperature was analysed by means of gas chromatography using a Shimadzu GC 14A (Kyoto, Japan) equipped with a BP20 capillary column (50 m long, thickness 0.25 μm, internal diameter of 0.43 mm, SGE Scientific Pty Ltd, Australia) and flame ionisation detector (FID). The carrier gas used was nitrogen with a head pressure of 123 KPa, the injection temperature was 225°C with an initial column temperature of 60°C for 2 minutes, temperature ramping to 190°C at 6°Cmin^{-1} and holding at 190°C for an additional 20 minutes. The detection temperature was programmed at 225°C.

3.4. Synthesis of geranial and neral

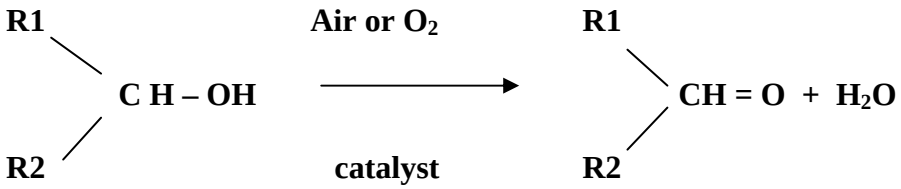
The method of Marko *et al*. (1998) was used in the present study, with the specific procedure for oxidation of geraniol and nerol given by Marko (pers. comm., 2000).

Geranial and neral with high purity and stability were synthesised using an aerobic catalytic oxidation method with the general reaction shown in Figure 3.1. The synthesis was conducted at the Chemistry Department, North Terrace Campus, The University of Adelaide.

The experimental procedure was as follows:

Fluorobenzene (100 mL, Sigma Aldrich, Castle Hill, Australia), 160 mg CuCl (Sigma Aldrich, Castle Hill, Australia) and 292 mg phenanthroline (BDH Merck, Kilsyth, Victoria, Australia) were added into a 200 mL two-necked flask fitted with a reflux condenser and an air inlet tube.

Reaction:



Catalyst = CuCl-Phenanthroline, DBAD (di-tert butyl azo dicarboxylate)

R1, R2 = alkyl, aryl or H

Figure 3.1 Aerobic catalytic oxidation of alcohols to aldehydes (Marko *et al*., 1998)

The mixture was stirred for 15 minutes at room temperature, then 1.1 g of K_2CO_3 (BDH Merck, Kilsyth, Victoria, Australia), and 373 mg DBAD (di-tert butyl azo dicarboxylate, Sigma Aldrich, Castle Hill, Australia) were added to the mixture and stirred for another 5 minutes. In a separate flask, 5 mL of geraniol or nerol (Sigma Aldrich, Castle Hill, Australia) was mixed with 60 mL of fluorobenzene and then added into the first mixture over a 5 minutes period. The mixture was placed in a pre-heated oil bath (90-95°C) and a gentle flow of oxygen was passed through the reaction mixture and refluxed for 2-3 h.

The reaction progress was monitored by means of thin layer chromatography (TLC). The reaction mixture was sampled and separated every 30 minutes on TLC plates using silica gel as the stationary phase and CH_2Cl_2 as the mobile phase until only one spot (product) was observed (Figure 3.2). Then, after cooling to room temperature, the black reaction mixture was filtered through a pad of Ciligel, a mixture of 15 g celite and 5 g of chromatographic grade silica gel (both from Sigma Aldrich, Castle Hill, Australia). The Cilegel was washed four times with 100 mL of CH_2Cl_2 (Sigma Aldrich, Castle Hill, Australia). The solvent was evaporated under reduced pressure and the crude product was rapidly purified by silica gel column chromatography. The yield was calculated as the percentage of aldehyde obtained compared to alcohol used as starting material (w/w).

The purity of the synthesised geranial and neral was then analysed by means of gas chromatography, with conditions similar to those for analysis of liquid citral (see Section 3.3.2).

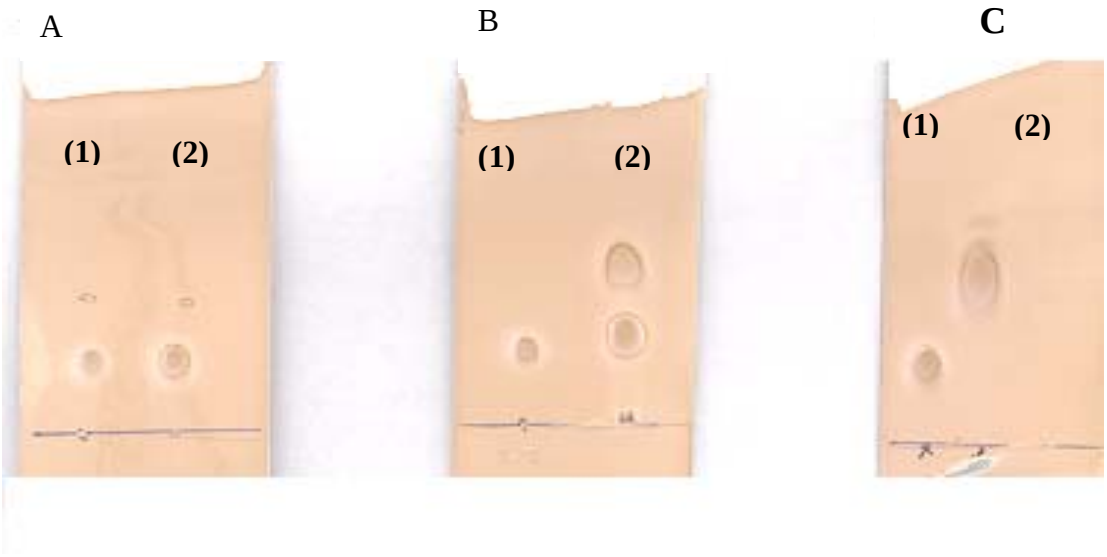


Figure 3.2 Separation of reaction mixture on TLC plates
(A) Initial reaction mixture with (1) the starting material (geraniol) as reference and (2) the reaction mixture.
(B) Reaction in progress with geraniol still observed in lane 2.
(C) All geraniol in reaction mixture has been converted into geranial.

3.5. SPME-GC analysis of volatile citral

The solid-phase microextraction (SPME) technique was developed by the Pawliszyn group in early 1990 (Song *et al.,* 1998). The technique was initially used for the analysis of air, soil and water, and was then applied to analyse flavour in fruit (Song *et al.,* 1998), vegetable oils (Yang and Peppard, 1994) and orange juice (Jia *et al.*, 1998). SPME is a technique based on absorption of gaseous or liquid samples onto an absorbent-coated fused silica fibre for a fixed time. The SPME fibre is then inserted directly into a GC injection port for thermal desorption (Chen and Pawliszyn, 1995). The advantage of the method is that it allows direct sampling of the vapour phase in equilibrium with the matrix headspace, provided that a suitable fibre is available. The technique reduces the time required for analysis, because no preliminary extraction is required; it is inexpensive since it can be performed with an ordinary gas chromatograph without any modification; it is solvent-free because the analytes are extracted directly from solution or headspace; it is sensitive due to the concentration factor achieved by the fibre; it is selective because of the different coating materials that can be used and it is simple to operate and allows rapid sampling of volatiles (Bicchi *et al.*, 1997).

The SPME method is based on the equilibrium of analytes among the phases involved in the system. These are the phases of the original sample, the headspace and the polymeric liquid coating of the fused silica fibre used. Since the analysis is measuring the analytes in the gas phase, the equilibrium partition of the analytes

between the headspace of the sample bottle and the SPME coated fibre is mainly affected by temperature, sample bottle volume and sample concentration. Once equilibrium has been reached, the concentration of the analytes can be considered constant in all phases involved.

The SPME device combines concentration and extraction procedures and directly transfers the absorbed compounds into the GC injector. In the concentration process, chemical equilibrium is allowed to establish between the compound in the initial phase (aqueous or liquid) and the headspace. The extraction process occurs when the fused silica fibre coated with a thin layer of selected organic polymer is inserted into the headspace of the system and absorbs the analytes from the headspace. The quality of measurement is highly affected by equilibrium and extraction time, therefore equilibrium and extraction times have to be determined experimentally first.

The experimental procedures were as follows:

General SPME procedure

The SPME device was purchased from Supelco Co (Bellefonte, PA, USA), with a fused silica fibre coated with poly(dimethylsiloxane) stationary phase of 100 μm thickness. An aliquot of 50 μL liquid citral was left to equilibrate in a sealed 20 mL vial for various times to establish the equilibrium time. This is the time needed for citral in the system to equilibrate in the headspace at a given environmental temperature, producing the maximum peak area of the chromatogram. Then the fibre was inserted for various times into the vial to extract the maximum volatile amount that can be adsorbed by the fused silica fibre, as determined by the time needed to

produce the maximum peak area of the chromatogram. After the set extraction time the fibre was introduced into the GC injector in splitless mode.

A Shimadzu GC 14A (Kyoto, Japan) equipped with a BP20 capillary column (50 m long, thickness 0.25 μm, internal diameter of 0.43 mm, SGE Scientific Pty Ltd, Australia) with on column injection (Shimadzu AOC-17) fitted with regular split/splitless injector and flame ionisation detector (FID) was used. The carrier gas used was nitrogen with a head pressure of 123 kPa, the fibre was injected manually and injection temperature was 225°C with an initial column temperature of 60°C for 2 minutes, temperature ramping to 190°C at 6°C min^{-1} and holding at 190°C for an additional 20 minutes. The detection temperature was programmed at 225°C.

For thermal desorption, the SPME fibre was retained in the injector for 5 minutes and was then retracted.

Calculation of the theoretical concentration

At a given temperature, the amount of analytes adsorbed on the SPME fibre is dependent on the sample volume of a pure compound until saturation of the headspace is achieved (Yang and Peppard, 1994). Therefore, it is essential to maintain a large sample volume in order to establish constant SPME adsorption.

The concentration of citral in the headspace at equilibrium is constant. Using the ideal gas equation:

$$PV = nRT \quad (1)$$

Then, the concentration of citral at a certain temperature and vapour pressure can be calculated by modifying the equation as follows:

$$\text{Concentration} = n/V = P/RT \quad (2)$$

where P = pressure, V = volume, n = number of mole present, T = temperature and R = gas constant (8.3143 Joules/Moles/K). Citral vapour pressure (P) at different temperatures (T) was calculated according to Yamamoto (2001).

The theoretical headspace citral concentration was calculated using equation 2.

Determination of equilibrium and extraction time

Equilibrium time and extraction condition were established prior to measurement

A 20 mL vial (Alltech, Australia) sealed with a rubber cap was used as a sample container. The vial was filled with 50 μL citral and the fused silica fibre was injected through the cap.

Calibration graph construction

A calibration graph for calculating the citral headspace concentration was constructed by measuring the peak area of 50 μL liquid citral in a 20 mL vial under the established equilibrium and extraction conditions. Considering that oranges are stored or shipped at 5°C and marketed at room temperature, the calibration temperatures chosen were -18°C (freezer), 0°C (ice in water), 22°C (room temperature) and 40°C (hot water bath).

Headspace citral concentration measurements

In experiments involving fungal cultures and volatile citral, liquid citral (2, 6 or 15 μL) was diluted to 100 μL in 400 ppm aqueous TritonX and applied to inverted NDY agar plates (90 mm diameter). A small hole was made in the side of the Petri dish and covered with a TFE silicone liner (Alltech/Adelab Scientific, Australia) to allow the introduction of the silica fibre for extraction of the citral (Figure 3.3); then the Petri dish was sealed with Parafilm™ (American Natural, Chicago, USA).

The measurement was conducted at 22°C and at 5°C. At 22°C, the Petri dish was left for 3 h to allow the citral to equilibrate, and the fused silica fibre was injected into the Petri dish through the silicone liner and left for 4 h to extract citral in the headspace. At 5°C, the equilibration time was 2 h and the extraction time was 4 h. Analysis was carried out in duplicate and the concentration was calculated based on the calibration graph.

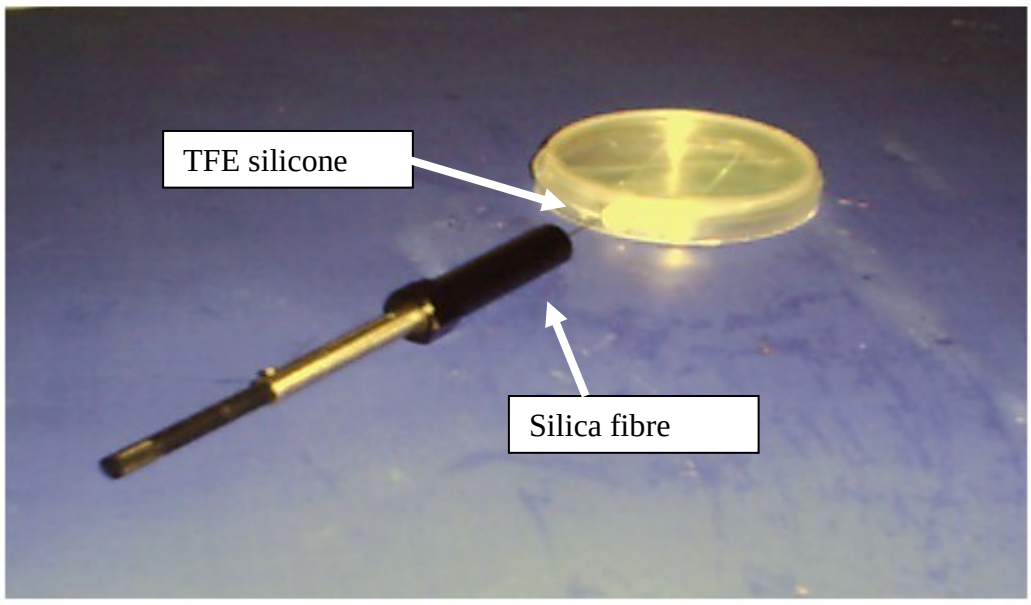


Figure 3.3 Arrangement of Petri dish for headspace measurement of citral

In fumigation experiments, Supesorp pads (DRI-LOC DLS 25 White E86-Sookerpad, Cryovac Australia, Pty Ltd, Victoria) were arranged in five positions in a 5 L Handy Storer plastic box (Quadrant Melbourne, Australia) filled with six oranges; then citral solution in 400 ppm TritonX was injected into the pads.

The amount of citral used was calculated to scale up from Petri dishes (diameter = 90 mm = 0.9 dm and height = 12 mm = 0.12 dm) to 5 L Handy Storer plastic boxes as follows:

$$(3) \quad \text{Scale up factor} = \frac{\text{Volume of Handy Storer (L)}}{\text{Volume of Petri dish (L)}}$$

$$= \frac{V\ (L)}{\pi \times r^2 \times h}$$

$$= \frac{5L}{\pi \times (0.45\ dm)^2 \times (0.12\ dm)}$$

$$= 65.5$$

where, V is volume of box, r is radius of Petri dish and h is height of Petri dish. Accordingly, the amount of citral was scaled up to 131, 393 and 983 μL in 6550 μl 400 ppm TritonX in the 5 L Handy Storer plastic boxes, corresponding to 2, 6 and 15 % treatments in Petri dishes, respectively.

The citral headspace concentration in the Handy Storer boxes was measured at room temperature (22°C) and at 5°C. The equilibrium times were 3 or 2 h respectively, while the extraction time was 4 h in both cases. Analysis was carried out in duplicate and the concentration was calculated based on the calibration graph as described earlier.

3.6. Statistical analysis

Statistical analysis was conducted using Genstat version 5.1 (Genstat 5, Release 4.1, 4th edition, Lawes Agricultural Trust, 1998), and GenStat Release 11.1 (PC/Windows; Copyright 2008, VSN International Ltd, UK).

A general analysis of variance with one-way analysis was used. The treatment means were compared using least significant difference at $P \leq 0.05$ unless stated otherwise. Each fungus was evaluated separately. Regression analysis was also performed to test specific relationships, as described in the relevant chapters.

Chapter Four

Methods of citral application to control pathogens in culture

4.1. Introduction

Citral, a natural mixture of geranial and neral, has been reported to exert antifungal activity (French *et al.,* 1978; Onawunmi, 1989; Rodov *et al.*, 1996; Dunn, 1998; Section 2.5). Ben-Yehoshua *et al*. (1992) found citral to be a preformed antifungal material in lemon peel, exerting high inhibitory activity against *P. digitatum*; Dunn (1998) reported that citral can inhibit the growth of *P. digitatum, P. italicum* and *G. citri-aurantii in vitro.*

In his study, Dunn (1998) tested two methods of application of citral to spores of *P. digitatum* in culture, by incorporating citral into NDY culture medium before inoculation, and by flooding the surface of the agar with citral solution after inoculation of the culture medium. Of these two methods, incorporation of citral into the culture medium resulted in the greater reduction of spore germination.

In this study, three different methods of citral application were tested to examine how citral could best control citrus spoilage pathogens in a commercial setting. The first method was direct mixing of citral components with the medium to allow comparison with previous studies; the second was exposure of spore suspensions to citral in solution to simulate commercial dipping of fruit and the third was exposure of spores to volatilised citral to simulate continuous fumigation.

4.2. Materials and methods

Spores of *P. digitatum* (nPdg L1), *P. italicum* (nPit L4) and *G. citri-aurantii* (nGcd L2) were suspended to concentration range of 5.3 – 7.1 x 10^3 spores mL^{-1} and 100 μL was inoculated onto NDY agar as previously described in Section 3.2.

In order to simulate the textured surface of the fruit peel, where some spores may lodge in recesses, the agar surface was scratched in all experiments before inoculation in a crosswise pattern with five scratches of approximately 15 mm apart and 1 mm depth in each direction, using a sterile needle of about 0.5 mm diameter.

The plates were incubated at 22°C for 14 days or at 5°C for 6 weeks. For plates showing no obvious growth, microscopic evaluation of spores for germination was carried out using a compound microscope at 400 X magnification by staining the agar with lactoglycerol cotton blue, applying a cover slip and observing *in situ*.

4.2.1. Preliminary experiment

A preliminary experiment was conducted to compare the efficacy of citral and imazalil and carbendazim, the fungicides commonly used on citrus at the time of the experiment was conducted (2000) using spore suspension and agar plug methods as follows.

NDY agar was autoclaved at 121°C for 20 minutes, allowed to cool slowly to 50°C, then aqueous solutions of citral in 400 $\mu L L^{-1}$ TritonX, imazalil (Farmoz Pty Ltd, St

Leonards, NSW) or carbendazim (Rhone-Poulenc Rural Australia Pty Ltd, Wagga Wagga, NSW) were added aseptically to give a final concentration of 3000, 500 and 500 μLL^{-1}, respectively, for citral, imazalil and carbendazim. The agar was immediately poured into Petri dishes and left to set in a laminar flow hood. For the spore suspension method, 100 μL of the spore suspension was spread onto the plates using a glass spreader. For the agar plug method, initially 100 μL of the spore suspension was spread onto plates using a glass spreader, and then incubated at 22°C for 10 days. Agar plugs of 1 cm diameter containing mycelia and spores were transferred to fresh plates of amended with citral, imazalil or carbendazim.

The experiment was carried out in two replicate plates for each solution and for each isolate.

4.2.2. Exposure to citral in culture medium

NDY agar was autoclaved at 121°C for 20 minutes, allowed to cool slowly to 50°C, then citral solution in 400 μL L^{-1} TritonX was added aseptically to give a final concentration of 3000 μL L^{-1}, 6000 μL L^{-1}, and 15000 μL L^{-1}in the medium. The agar was immediately poured into Petri dishes and left to set in a laminar flow hood. Then 100 μL of the spore suspension was spread onto the plates using a glass spreader. The experiment was carried out in five replicates per citral concentration for each isolate, and controls were NDY agar only and NDY agar mixed with 400 μLL^{-1} aqueous TritonX. This method was modified from that reported by Dunn (1998).

4.2.3. Exposure to citral solution

Spore suspensions of final concentration of 10^4 spores mL^{-1} were incubated in aqueous citral solutions of 3000, 6000 or 15000 μL L^{-1} in 400 μL L^{-1} TritonX for 60 minutes and continually agitated. Then 100 μL of the suspension was spread onto each NDY plates using a glass spreader. The experiment was carried out in five replicates per citral concentration per isolate. Controls were spore suspensions inoculated directly onto NDY agar and spores that were suspended in 400 μL L^{-1} aqueous TritonX before plating. This method was modified from that reported by Brown *et al*. (1999).

4.2.4. Exposure to volatile citral

Initially, 100 μL of the spore suspension was spread onto each NDY agar plate using a glass spreader. Aqueous citral solution was applied as five equidistant drops of 20 μL aliquots on glass slides on the inside of the lid, with the dish placed upside down. The solutions consisted of 2, 6 or 15 μL citral each diluted to 100 μL with 400 μL L^{-1} aqueous TritonX solution (2, 6 and 15%). The experiment was carried out in five replicates with one control of water instead of citral and another of 400 μL L^{-1} aqueous TritonX applied on the glass slides in the plate lid. Plates were sealed with Parafilm™ (American Natural, Chicago) to minimise escape of volatile citral from the plates.

Considering that oranges are stored or shipped at 5°C, an additional experiment was conducted in which spores were exposed to volatile citral at 5°C. The procedure was

as above, except that the solutions consisted of 0.5, 2, 6 or 15 μL citral each diluted to 100 μL with 400 μL L^{-1} aqueous TritonX (0.5, 2, 6 and 15%).

4.2.5. SPME-GC analysis of headspace citral concentration inside NDY plates

The headspace volatile citral concentration of each plate was measured by means of Solid Phase Micro Extraction (SPME)-Gas Chromatography as previously described in Section 3.5. The citral headspace concentration was measured at 22°C and at 5°C. The equilibrium times were 3 or 2 h respectively, while the extraction time was the same at 4 h in both cases. Analysis was carried out in duplicate and the concentration was calculated based on the calibration graph.

4.2.6. Statistical analysis

The number of colony forming units (cfu) on each plate was counted daily for 14 days, except for the experiment incubated at 5°C, which was observed daily for 6 weeks as the pathogens grew slowly at this temperature. Analysis of variance (ANOVA) among treatments was performed using Genstat version 5.1 (Genstat 5, Release 4.1, 4th edition, Lawes Agricultural Trust, 1998), using one-way ANOVA (no blocking). Data for each of the pathogens and each method were analysed separately.

4.3. Results

The preliminary experiment to compare the efficacy of citral, imazalil and carbendazim showed that citral could prevent mycelial growth and spore

germination of the three pathogens. Carbendazim did not inhibit the mycelial growth and spore germination of *G. citri-aurantii* (Table 4.1).

Table 4.1 Comparison study of the efficacy between citral and fungicides to the three pathogens

Components	*P. digitatum*		*P. italicum*		*G. citri-aurantii*	
	M	S	M	S	M	S
Citral, 3000 μL L^{-1} in 400 μL L^{-1} TritonX	*	*	*	*	*	*
Carbendazim, 500 μL L^{-1}	*	*	*	*	X	X
Imazalil, 500 μL L^{-1}	*	*	*	*	*	*

M: Mycelial growth S: spore germination *: inhibited X: not inhibited

4.3.1. Effect of citral incorporation in culture medium

The development of *P. digitatum* was completely inhibited in all citral treatments. No mycelial growth was observed, even under microscopic observation after 17 days of incubation. Exposure of the spores to 400 μL L^{-1} TritonX appeared to have no significant effect on cfu compared to the control (Table 4.2).

Similarly, citral treatments in this experiment also completely inhibited the development of *P. italicum*; no mycelial growth was observed after 17 days of incubation. However, exposure of the spores to TritonX at 400 μL L^{-1} reduced the number of cfu by about 41%.

Likewise, citral treatments completely inhibited the development of *G. citri-aurantii* (nGcd L2) and no mycelial growth was observed after 17 days of incubation. Furthermore, TritonX at 400 μL L^{-1} reduced the number of cfu by about 30%.

Table 4.2 Effect of citral incorporated into NDY agar on germination and growth of fungal spores incubated at 22°C for 14 days

Treatment applied in NDY agar		Number of cfu[X]		
TritonX, μL L^{-1}	Citral, μL L^{-1}	*P. digitatum*	*P. italicum*	*G. citri-aurantii*
0	0	63[Y]	87	119
400	0	64	61	70
400	3,000	0	0	0
400	6,000	0	0	0
400	15,000	0	0	0
LSD at P <0.01		9	13	11

[X] cfu = colony forming units. [Y]The data shown represent the means of five replicates

It was observed that the scratches on the agar did not provide a refuge for the pathogens (data not shown).

4.3.2. Effect of citral solution

Compared to the control, citral treatments in solution showed some inhibition of the three pathogens at all three concentrations tested (Table 4.3). Exposure of *P. digitatum* spores to citral solutions for 1 h before inoculation onto NDY agar medium resulted in complete inhibition of fungal development at a citral

concentration of 15000 μL L^{-1}. The number of cfu was reduced by 94 and 90%, respectively, at concentration of 3000 and 6000 μL L^{-1}. Treatment with 400 μL L^{-1} TritonX did not significantly reduce the number of cfu.

P. italicum was not completely inhibited at any of the concentrations tested, with cfu reductions of 77, 73 and 68% respectively, when the spores were exposed to 3000, 6000 and 15000 μL L^{-1} citral solutions for 1 h before being inoculated onto NDY agar. Furthermore, 400 μL L^{-1} TritonX reduced the cfu by 14% compared to the control.

Citral treatments in this experiment reduced the cfu of *G. citri-aurantii* by 92% when the spores were exposed to 3000 μL L^{-1} citral solutions and completely inhibited development when 6000 and 15000 μL L^{-1} citral solutions were used. Furthermore, 400 μL L^{-1} TritonX reduced the cfu by 53% compared to the untreated control.

4.3.3. Effect of volatile citral

4.3.3.1. Exposure of spores to volatile citral at 22°C

The relatively small cfu counts of *P. digitatum* in this experiment were due to counting difficulties as colonies that were observed had merged. However, in all treatments a consistent trend was observed.

Exposing *P. digitatum* spores to volatile citral at 22°C resulted in complete inhibition of their development in the presence of 6 μL and 15 μL citral diluted to

100 μL in aqueous 400 μL L^{-1} TritonX (Table 4.4). In the presence of 2 μL citral diluted to 100 μL in aqueous 400 μL L^{-1} TritonX, the number of cfu was reduced by 90%, while 400 μL L^{-1} TritonX only reduced the cfu by 10% compared to the untreated control.

Table 4.3 Effect of exposure of fungal spores to citral solution for 1 hour before being inoculated onto NDY agar then incubated at 22°C (room temperature)

Treatment applied in NDY agar		Number of cfu[X]		
TritonX, μL L^{-1}	Citral, μL L^{-1}	*P. digitatum*	*P. italicum*	*G. citri-aurantii*
0	0	49[Y]	22	62
400	0	46	19	29
400	3000	3	5	5
400	6000	4	6	0
400	15000	0	7	0
LSD at P <0.01		8	4	6

[X] cfu = colony forming units, [Y] The data shown represent the mean of five replicates

The effect of volatile citral treatments on the growth of *P. italicum* appeared similar to that on *P. digitatum,* in that there was complete inhibition of development in the presence of 6 μL and 15 μL citral diluted to 100 μL in aqueous 400 μL L^{-1} TritonX. Furthermore, in the presence of 2 μL citral diluted to 100 μL in aqueous 400 μL L^{-1} TritonX the number of cfu was reduced by 70%, while 400 μL L^{-1} TritonX only reduced the number of cfu by 3%.

A similar effect was also observed for *G. citri-aurantii*; in the presence of 6 μL and 15 μL citral diluted to 100 μL in aqueous 400 μL L^{-1} TritonX, development was completely inhibited and in the presence of 2 μL citral diluted to 100 μL in aqueous 400 μL L^{-1} TritonX the cfu was reduced by 40%, while 400 μL L^{-1} TritonX alone reduced the number of cfu by 13%.

Table 4.4 Effect of volatile citral on fungal spores inoculated onto NDY agar

Then incubated at 22°C for 14 days

Treatment applied on glass slide in Petri dish	Number of cfu[X]		
	P. digitatum	*P. italicum*	*G. citri-aurantii*
Water only	10[Y]	113	131
TritonX only	9	122	114
2% citral in 400 μL L^{-1} TritonX	1	34	78
6% citral in 400 μL L^{-1} TritonX	0	0	0
15% citral in 400 μL L^{-1}TritonX	0	0	0
LSD at P <0.01	2	23	17

[X] cfu = colony forming units, [Y] The data shown represents the means of five replicates

4.3.3.2. Exposure of spores to volatile citral at 5°C

When the fungal spores were exposed to volatile citral and incubated at 5°C, the conidia of *P. digitatum* were completely unable to germinate and produce colonies in the presence of 2, 6 and 15 μL citral diluted to 100 μL in aqueous 400 μL L^{-1} TritonX. However, in the presence of 0.5 μL citral the number of cfu produced was only reduced by 89% compared to untreated control (Table 4.5). The number of cfu was reduced by 37% in the presence of 400 μL L^{-1} TritonX.

For *P. italicum*, the citral treatment reduced the number of cfu by 48 and 55 %, respectively, in the presence of 0.5 and 2 μL citral. Development was completely prevented in the presence of 6 and 15 μL citral, and 400 μL L^{-1} TritonX did not reduce the number of cfu.

Furthermore, for *G. citri-aurantii*, a similar effect to that on *P. digitatum* was observed, where volatile citral completely prevented development in the presence of 2, 6 and 15 μL citral. At 0.5 μL, the number of cfu was reduced by 83%, whereas 400 μL L^{-1} TritonX reduced the number of cfu by 20%.

Again, in this experiment it found that the scratches on the agar did not provide a refuge for the pathogens, as scratching the agar had no significant effect on the growth of the pathogens (data not shown).

Table 4.5 Effect of volatile citral on fungal spores inoculated onto NDY agar and Then incubated at 5°C for 6 weeks

Treatment applied on glass slide in Petri dish	Number of cfu[X]		
	P. digitatum	*P. italicum*	*G. citri-aurantii*
Water only	144[Y]	126	117
TritonX only	91	126	94
0.5% citral in 400 μL L^{-1} TritonX	15	65	20
2% citral in 400 μL L^{-1} TritonX	0	57	0
6% citral in 400 μL L^{-1} TritonX	0	0	0
15% citral in 400 μL L^{-1} TritonX	0	0	0
LSD at P <0.01	9	14	12

[X] cfu = colony forming units, [Y]The data shown represent the means of five replicates

4.3.3.3 Citral headspace concentration in NDY plates

The headspace concentration of citral in the NDY plates was measured using gas chromatography and calculated by means of a calibration graph as previously described in section 3.5. Based on the calibration curve constructed from headspace concentrations of citral at -18°C, 0°C, 22°C and 40°C (Appendix 1), the headspace citral concentrations in plates generated from 2, 6 and 15% solutions were 12496, 16143 and 40990 μL L^{-1}, respectively at 22°C; and 604, 1550 and 6069 μL L^{-1}, respectively at 5°C. The headspace citral concentration generated from 0.5% solution at 5°C was not measured because the response was very low.

4.4. Discussion

4.4.1. Effect of citral incorporation into culture medium

All citral treatments applied using this method resulted in complete inhibition of all three pathogens (Table 4.2). No spore germination or mycelial growth was evident. Under microscopic observation after 17 days of incubation only debris was observed in the agar medium. These results suggested that the spores, which were in direct contact with the citral in the agar medium, may have been killed or that germination was inhibited.

Dunn (1998) reported that when citral was incorporated into NDY culture medium at 3000 μL L^{-1}, *P. digitatum, P. italicum* and *G. citri-aurantii* spores were unable to germinate and grow. Therefore, results of this study support those of Dunn (1998), and this method was considered a means of screening chemicals for antifungal activity in the laboratory.

4.4.2. Effect of citral solution

Exposure of the spores to aqueous citral solutions for 1 h resulted in varying inhibition of the three pathogens (Table 4.3). The growth of *P. digitatum* was completely inhibited at citral concentration of 150 mLL^{-1} and *G. citri-aurantii* was inhibited at citral concentrations of 60 and 150 mLL^{-1}. However, *P. italicum* was not completely inhibited at any of the concentrations tested. The incomplete inhibition may be due to 1 h exposure was insufficient for citral to control the pathogens, especially at low concentration. Citral is a non-polar liquid, which needed emulsifying agent TritonX to suspend it in water. Citral molecules would be suspended in small droplets surrounded by a layer of TritonX emulsifier, and at low aqueous citral concentration there may be reduce the opportunity for citral to interact with fungal spores in water to have an effect (Kotz and Purcell, 1991). In addition, French (1978) reported that aqueous citral at lower concentration could stimulate spore germination, especially in water agar.

This study revealed that at the highest concentration of 15% citral (150 mL L^{-1}) the growth of the three pathogens was not completely inhibited, suggested that concentration higher than 150 mL L^{-1} needed to control the pathogens. However, the high levels of citral needed to achieve even partial control would damage the fruit. Dunn (1998) found that dipping Navel oranges in 150 mL L^{-1} aqueous citral with 30 mL L^{-1} TritonX for one minute caused intense red blemishes and Ben-Yehoshua *et al.* (1992) also found that dipping lemons in 1% (10 mL L^{-1}) aqueous citral for 2 minutes resulted in phytotoxic damage to the peel. Dipping of citrus fruits is normally for brief periods only, and this would be insufficient to eliminate

blue mould. Therefore, this method has little potential for commercial application on its own. Since *P. italicum* is a major post-harvest citrus pathogen and, as aqueous citral solutions used in this study did not control this pathogen, dips of fruit in aqueous citral alone in commercial situations may be of limited value.

4.4.3. Effect of volatile citral

At 22°C, exposure of the spores to volatile citral completely inhibited the growth of all three pathogens in the presence of 6 μL and 15 μL citral diluted to 100 μL in aqueous 400 μL L^{-1} TritonX (Table 4.4). TritonX alone did not significantly reduce cfu of any of the pathogens. This means that citral at the above levels was effective in controlling the pathogens in culture. However, volatile citral generated from 2 μL citral (headspace concentration of 2113 μL L^{-1}) appeared to be insufficient to inhibit the fungal growth in culture.

When the fungal spores were exposed to volatile citral and incubated at 5°C, development of *P. digitatum* and *G. citri-aurantii* was completely inhibited in the presence of 2, 6 and 15 μL citral diluted to 100 μL in aqueous 400 μL L^{-1} TritonX; while 0.5 μL citral did not fully inhibit any of the pathogens (Table 4.5). At lower temperature cells metabolise at slower rates due to slowing enzyme function. However, *P. italicum* grew in the presence of 2 μL citral at 5°C and this may reflect the ability of, *P. italicum* to grow at low temperature (Domsch *et al.*, 1980; Pitt and Hocking, 1997; Wardowski and Brown, 1993).

The results of this experiment appeared to suggest that at 22°C, citral vapour generated from 6 and 15 μL solutions was effective to control the three pathogens on NDY agar, while at 5°C, the reduced headspace concentration from 2 μL solution inhibited the growth of *P. digitatum* (nPdg L1) and *G. citri-aurantii* (nGcd L2) but not *P. italicum* (nPit L4). This suggested that *P. digitatum* (nPdg L1) and *G. citri-aurantii* (nGcd L2) were more sensitive than *P. italicum* (nPit L4) to citral at 5^{o}C.

4.5. Summary

Incorporating citral into the culture medium at all treatments gave total inhibition of all the pathogens. Due to citral in agar medium is likely to be retained in direct contact with the pathogens, in which case maximum effect would be obtained. This result supported the previous study by Dunn (1998).

Exposure of the spores to aqueous citral solutions for 1 h failed to prevent germination and growth of the pathogens. This suggested that concentrations greater than these tested might be needed to control the pathogens.

Exposure to volatile citral generated from 6 and 15 μL citral diluted to 100 μL in aqueous 400 μL L^{-1} TritonX effectively controlled the growth of the pathogens both at 5°C and 22°C. The application of citral in the volatile phase showed the greatest potential as a commercial antifungal treatment of citrus fruit.

Continuous volatile exposure exerted effective control of the fungi, while reducing the risk of phytotoxic damage. Citral could easily be applied as a volatile for relatively short periods in storage chambers or over the long term within packages (Williams *et al.,* 2000). The short period applications could be applied in storage chamber by applying high citral concentrations to ensure that the treatment kills the spores before they have a chance to germinate. However, application of high citral concentrations is more likely to cause phytotoxic damage to the fruit peel. Long term applications could be carried out by continuous fumigation within packages, whereby the citral concentration applied could be possibly lowered. Research to determine appropriate concentrations of citral and techniques for application to fruit in storage is required, so that effective control can be achieved.

In addition, residual levels of citral in the fruit and their effect on consumer acceptability need to be examined; although major concerns are unlikely considering that citral is also found on the fruit peel inside the oil glands (Rodov *et al.*, 1995).

Chapter Five

Inhibition of citrus post-harvest pathogens by vapour of citral and its related compounds in culture

5.1. Introduction

Citral is a natural mixture of the geometric trans- and cis-isomers, geranial and neral, respectively (Section 2.4.1). Citral is susceptible to oxidation and reduction, and is degraded during storage and thermal processing (Robert and Caserio, 1965; Djordjevic *et al.*, 2008; Gallardo *et al.*, 2008; Nguyen *et al.*, 2009). Aldehydes are an intermediate form between primary alcohols and carboxylic acids, and are easily oxidised to the corresponding carboxylic acids (Kotz and Purcell, 1991). Citral readily forms isomers, is sensitive to exposure to air and light, and can react with alkalis and strong acids (Robert and Caserio, 1965). Citral, geranial, neral, citronellal, citronellol and citronellic acid are components of citrus essential oils (Shaw, 1977; Moshonas *et al.*, 1972; Erman, 1985).

Tsao and Zhou (2000), in their evaluation of the antifungal activity of 22 naturally occurring monoterpenoids against *Botrytis cinerea* and *Monilinia fructicola,* found that vapours of citral, citronellol and citronellal were good inhibitors of growth. In addition, acetaldehyde vapour has been used to inhibit the growth of *P. digitatum, P. italicum, Alternaria citri, A. tenuis, Glomerella cingulata* and *Colletotrichum gloeosporioides* in culture (Prasad, 1975).

The activity of the individual citral isomers, geranial and neral, and the related

compounds citronellal, citronellol and citronellic acid, against *P. digitatum, P. italicum* and *G. citri-aurantii* has not been documented, and the antifungal activity of citral against these pathogens seems to be variable (Rodov *et al*., 1995; Wannison *et al*., 1996; Pattnaik *et al*., 1997; Dunn, 1998).

Experiments reported in Chapter 4 suggested that application of citral in the volatile phase showed potential as a commercial antifungal treatment for citrus fruit. Furthermore, exposure of spores to citral in the volatile phase completely prevented growth of *P. digitatum, P. italicum* and *G. citri-aurantii* when generated from 15% aqueous solutions in sealed Petri dishes (Chapter 4). Therefore, the experiments reported in this chapter were undertaken to examine the activity of the citral isomers as well as their related compounds in the vapour phase against spores of *P. digitatum, P. italicum* and *G. citri-aurantii*, and to examine the change in citral composition over time.

5.2. Materials and methods

5.2.1. Synthesis of geranial and neral

Geranial and neral were synthesised separately according to the procedure of Marko *et al.* (1998) and Marko (pers. com., 2000) as previously described in Section 3.4. Yields were calculated as % of moles of aldehydes obtained from the synthesis after silica gel column purification divided by moles of alcohols used as starting material.

In order to examine the purity of the product, gas chromatographic analysis was also performed on the synthesised geranial or neral according to the method described in

Section 3.3, with acetone as the solvent.

5.2.2. Comparison of citral with its isomers, and with its related compounds

Cultures of *P. digitatum, P. italicum* and *G. citri-aurantii,* spore suspensions and the medium used for the experiment were maintained and prepared as described in Section 3.2. The procedure used was similar to the procedure for exposing spores to volatile citral as described in Section 4.2. Firstly, the effect of volatile citral on the germination of spores of the pathogens was compared to that of volatile geranial and neral. The solutions of citral, geranial and neral used consisted of 2, 6 or 15 μL diluted to 100 μL with 400 μL L^{-1} aqueous TritonX solution (2, 6 or 15% solutions). Petri dishes were sealed with Cellotape (Scotch^{TM}, China) to minimise gaseous exchange and incubated as described in Section 4.2, then colonies counted. Secondly, the effect of volatile citral on spore germination was compared to that of vapour from R-citronellal, S-citronellal, R-citronellol, S-citronellol, R-citronellic acid or S-citronellic acid.

In order to allow the classification of the vapour of citral, geranial or neral as fungistatic or fungicidal, the following procedure was adopted. The glass slides and solutions on them were removed from the Petri dishes after 14 days, and the Petri dishes were then resealed and incubated at 22°C for another 8 weeks to determine if any viable spores were present, but had not germinated. In addition, agar plugs of 1 cm diameter bearing spores (confirmed microscopically) were transferred to fresh NDY plates to eliminate any citral absorbed by the medium and these plates were

incubated at 22°C for another 2 weeks. The growth was assessed as percentage of the agar surface with mycelium.

5.2.3. Citral degradation analysis

Because citral is a unsaturated compound which is susceptible to oxidation and reduction reactions and the application of citral occurred over a prolonged period in this study, the composition of the citral may have changed. Therefore, a preliminary experiment was conducted to determine the degradation products of citral in the liquid phase. Solutions of citral, R-citronellal, S-citronellal, R-citronellol, S-citronellol, R-citronellic acid, S-citronellic acid and cyclocitral were made to the required concentration in 2% (v/v) ethanol, with 2% (v/v) tetradecane as the internal standard. Citral solution was left at room temperature for 12 days before analysis by gas chromatography and mass spectrometry (GC/MS). The analysis was performed with a GC System Hewlett Packard (HP) 6890 series equipped with an HP 5973 mass spectrometer. A BP20 capillary column (50 m long, thickness 0.25 μm, internal diameter of 0.43 mm(SGE Scientific Pty Ltd, Australia) was used as a stationary phase.

The conditions were as follows: 1.0 μL split injection, split ratio 50:1, flow rate of 20 mL min^{-1} for the carrier gas (helium) at 23.52 psi head pressure, injector temperature of 200°C. The temperature program was as follows: the column was held at 60°C for 2 minutes and then programmed to reach 220°C at 6°C min^{-1} , which was held for 20 minutes.

Constituents observed were identified by comparison of their retention time and mass spectra with standard compounds (if available) or compared with mass spectra of compounds from the National Institute of Standards and Technology (NIST05) library using Agilent G1701A Revision E.02.00 ChemStation software. The mass spectra (MS) of observed constituents were identified at > 40 mHz.

In this preliminary experiment the degradation of citral stored at room temperature for 12 days was assessed. However, experiments with oranges were to be conducted at 5°C and room temperature for longer than 12 days. Therefore, degradation of citral was examined again in September 2009, using GC/MS to assess the change in composition of citral that might have been expected to occur during the course of experimentation involving fumigation of oranges. The experiment was conducted in The Australian Wine Research Institute - Metabolomics Australia Laboratory, Waite Campus.

The experimental procedure was as follows:

Standard solutions of citral, citronellal, citronellol, geranic acid (all 2% v/v) were made in ethanol and tetradecane (2% v/v) as internal standards.

Citral solution was left at 22°C (room temperature) for 4, 8, 12 and 16 days, and at 5°C for 4, 16, 32 and 40 days prior to analysis. A control consisted of an original sample that was freshly prepared prior to analysis. The GC/MS analysis was performed on an Agilent 7890 gas chromatograph equipped with a Gerstel MPS2 multi-purpose sampler and coupled to an Agilent 5975C VL mass selective detector.

Instrument control and data analysis were performed with Agilent G1701A Revision E.02.00 ChemStation software. A Gerstel PVT (CIS 4) inlet was fitted with a deactivated low volume glass liner (3 mm x 1.0 mm). The inlet was heated to 200°C and maintained at this temperature. The sample, 1 μL, was introduced to the inlet (50.0 μL s^{-1}, penetration 35.0 mm) using split mode (50:1, pressure 27.65 psi). The gas chromatograph was fitted with a 30 m x 0.18 mm Resteck Stabilwax – DA (crossbond carbowax polyethylene glycol) 0.18 μm film thickness that had a 5 m x 0.18 mm retention gap. Helium (Ultra High Purity) was used as the carrier gas with flow-rate 0.7 mL min^{-1} (24.697 psi) in constant flow mode. The oven temperature was started at 60°C, held at this temperature for 2 minutes then increased to 220°C at 6°C min^{-1}, and held at this temperature for 15 minutes. The mass spectrometer quadrupole temperature was set at 150°C, the source was set at 230°C and the transfer line was held at 250°C. Positive ion electron impact spectra at 70eV were recorded in scan mode with a solvent delay of 5.0 minutes. Similar to the preliminary experiment, the constituents observed were then identified by comparison of their retention time and mass spectra with standard compounds (if available) or compared with mass spectra compounds from the National Institute of Standards and Technology (NIST05) library.

5.2.4. Statistical analysis

Data analysis for citral degradation experiments was conducted by quantifying peak area relative to tetradecane (the internal standard). The number of cfu on each plate was counted daily for 14 days. ANOVA for number of cfu was performed using GenStat version 5.1 (GenStat 5, Release 4.1, 4th edition, Lawes Agricultural Trust,

1998), while citral degradation analysis was performed using GenStat Release 11.1 (PC/Windows; Copyright 2008, VSN International Ltd, UK). LSD at P≤ 0.05 was used to determine differences amongst means of treatments.

5.3. Results

5.3.1. Synthesis of geranial and neral

Synthesis of geranial using the method described in Sections 3.4 and 5.2.1 yielded 3.1758 g of geranial, which was then calculated as 64% yield. Furthermore, synthesis of neral resulted in 2.0126 g of neral, which was calculated to be 40% yield.

Quantification of peak area relative to internal standard (tetradecane) from the gas chromatogram of the synthesised geranial showed that it consisted of 94% geranial, 2% neral and the remainder was impurities including the synthesis solvent, CH_2Cl_2 which comprised about 3%. Furthermore, analysis of the synthesised neral showed that it consisted of 92% neral and 2% geranial, with CH_2Cl_2 comprising about 2%. The GC chromatograms of the synthesised geranial and the synthesised neral are presented in Figure 5.1A and 5.1B.

5.3.2. Effects of vapour of citral and related compounds on *P. digitatum*

No growth of *P. digitatum* was observed in the presence of 15% solutions of either citral or its isomers (Table 5.1). In the presence of 2% solutions of these compounds,

the number of cfu of *P. digitatum* was reduced by 66, 65 and 64% for citral, geranial and neral, respectively, compared to the water control; and 62, 60 and 59%,

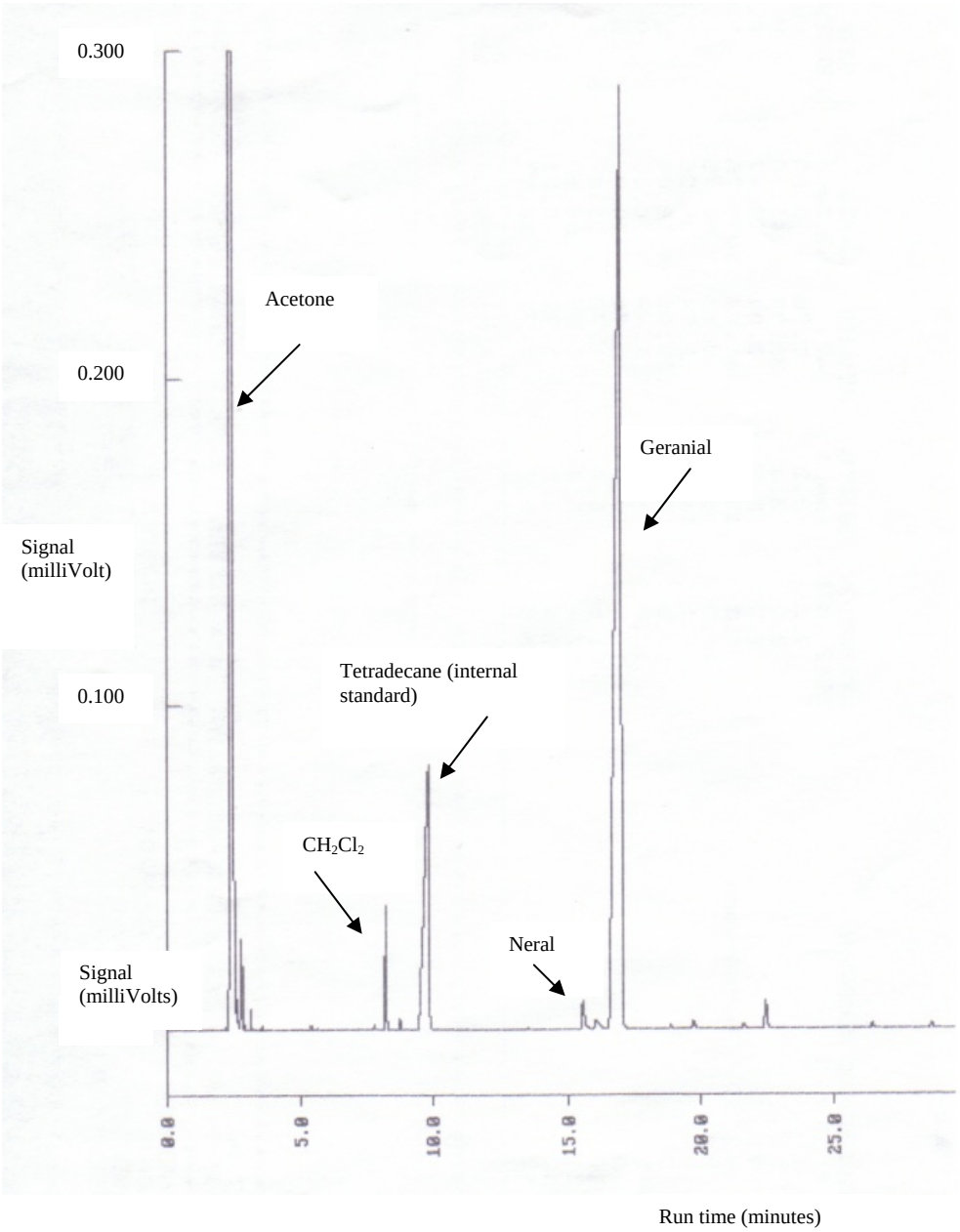


Figure 5.1A The GC chromatogram of synthesised geranial in 2% (v/v) acetone and 2% (v/v) tetradecane as an internal standard

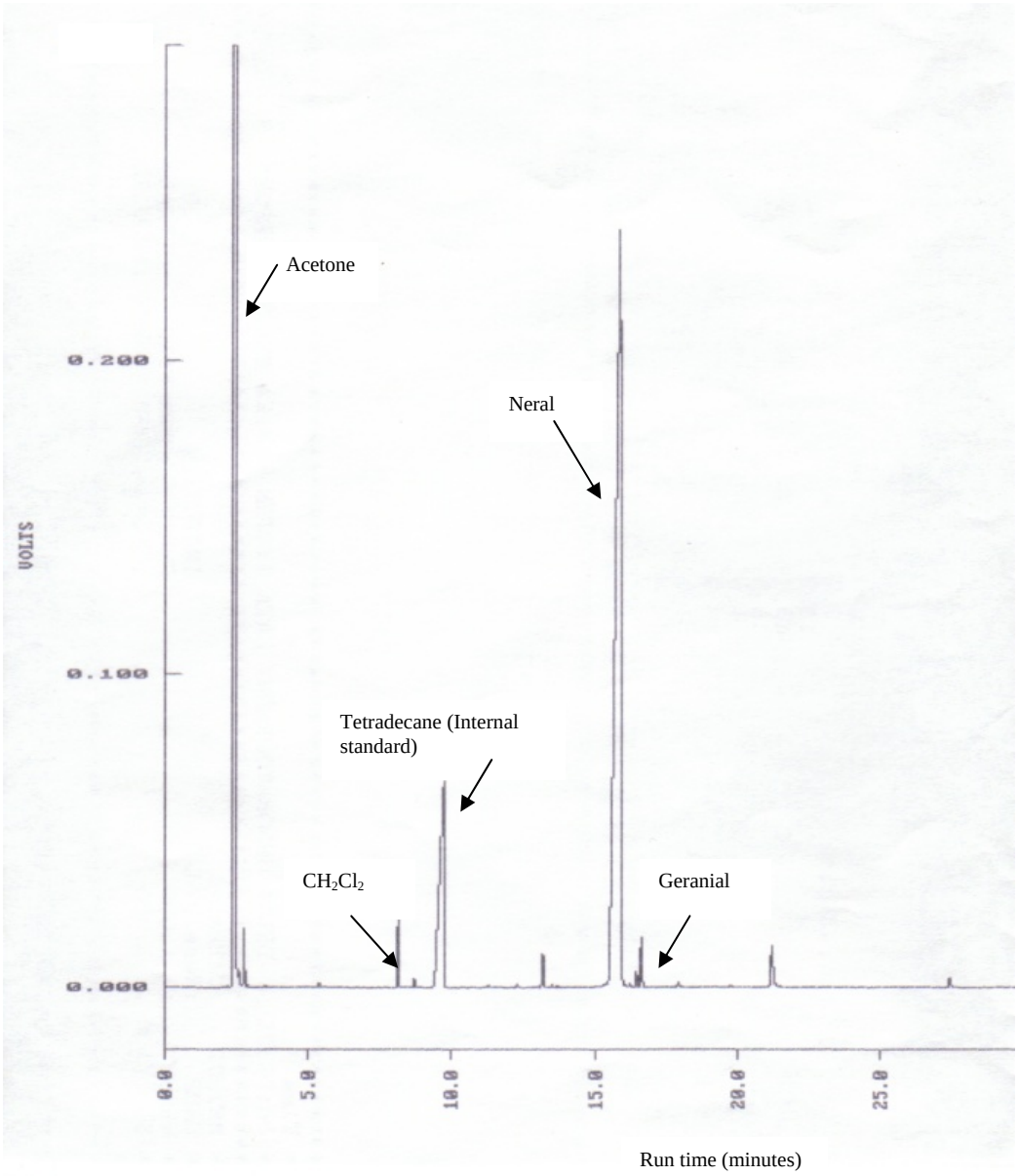


Figure 5.1B The GC chromatogram of synthesised neral in 2% (v/v) acetone and 2% (v/v) tetradecane as an internal standard

respectively, compared to the control of 400 μL L^{-1} TritonX. The vapour of citral, geranial and neral from 6% solutions reduced the number of cfu by 94, 92 and 97% respectively, compared to the water control; and 93, 90 and 96%, respectively, compared to the control of 400 μL L^{-1} TritonX. Exposure to 6% solution of neral was more effective than that of geranial; but it was no different from citral. Exposure to 400 μL L^{-1} aqueous TritonX (control) reduced the number of cfu by 13%.

Vapour generated from 15% solutions of citral, geranial or neral completely inhibited *P. digitatum* during 14 days of incubation. This treatment was used to allow the classification of the vapour of citral, geranial or neral, as fungistatic or fungicidal. Spores of *P. digitatum* previously exposed to vapour of citral and geranial from 15% solutions showed no germination, either from the transferred plug or on the remainder of the original plate. This suggests that citral and geranial at this concentration were fungicidal to *P. digitatum*. However, some spores previously exposed to vapour of neral at this concentration germinated and covered about 50% of the original Petri dish after 8 weeks at 22°C (Table 5.1). Therefore, vapour of neral from 15% solutions was classified as fungistatic for *P. digitatum*.

When comparing the effects of exposure to vapour of citral with related compounds (Table 5.2), citral reduced the number of cfu by 93 and 99% in the presence of 2 and 6% solutions, respectively. Again, vapour from 15% citral solution completely inhibited growth. Citronellic acid provided the least inhibition of growth of *P. digitatum* in the presence of 2% solution, with a 44-48% reduction of cfu when compared to the water control (Table 5.2). In the presence of 6% solutions of citronellic acid, the S isomer was more inhibitory than the R isomer, but there was no difference between the effects of the isomers on cfu number when they were present as 15% solutions. Vapour of citronellal showed the greatest inhibitory effect in the group; compared to the water control, 57-69, 79-81 and 96% reduction of cfu was observed in the presence of 2, 6 and 15% solutions, respectively. The only difference between the citronellal isomers (R and S) was for the 2% solution, where the S isomer was more inhibitory than the R isomer. Furthermore, the reductions in

the number of the cfu were 62-69%, 71-74% and 72-74%, respectively, when the fungus was exposed to volatile citranellol from 2, 6 and 15% solutions. Treatment with 400 μL L^{-1} aqueous TritonX reduced the number of cfu by 8% compared to the water control.

Table 5.1 The effect of vapour of citral and its isomers (in 400 μLL^{-1} TritonX) at different concentrations on the growth of *P. digitatum* at 22°C

Compound/ Concentration	cfu[X] per plate				
	0%	2%	6%	15%	Growth over plate (%) after removal of 15% solution
H_2O	119[Y]	N/A	N/A	N/A	N/A
400 μL L^{-1} TritonX	104	N/A	N/A	N/A	N/A
Citral	N/A	40	7	0	0
Geranial	N/A	42	10	0	0
Neral	N/A	43	4	0	50

Note: N/A = not applicable, [X] cfu = colony forming units, [Y] The data shown represent the mean of four replicates and LSD (at $P \leq 0.05$) = 4

5.3.3. Effects of vapour of citral and related compounds on *P. italicum*

As for *P. digitatum*, total inhibition of *P. italicum* was observed in the presence of 15% solutions of citral, geranial and neral (Table 5.3). In comparison to the water control, treatment with 400 μL L^{-1} aqueous TritonX reduced the number of cfu by 13%. No growth was observed in the presence of geranial at any of the concentrations tested or in the presence of 6 and 15% solutions of citral. The number of cfu of *P. italicum* in the presence of 2% and 6% solutions of neral was reduced by 87% and 93%, respectively, compared with the water control (Table 5.3). The effect of geranial was significantly greater than that of citral at 2%, whereas the

response to neral at 6% was significantly less than the response to citral at this concentration.

Table 5.2 The effect of vapour of citral and its related compounds (in 400 μLL^{-1} TritonX) at different concentrations on the growth of *P. digitatum* at 22^{o}C

Compound/ Concentration	cfu[X] per plate			
	0%	2%	6%	15%
H_2O	131[Y]	N/A	N/A	N/A
400 μL L^{-1} TritonX	120	N/A	N/A	N/A
Citral	N/A	9	1	0
R-citronellal	N/A	56	25	5
S- citronellal	N/A	40	28	5
R-citronellol	N/A	41	38	37
S- citronellol	N/A	50	34	34
R-citronellic acid	N/A	68	48	14
S- citronellic acid	N/A	73	32	18

Note: N/A = not applicable, [X] cfu = colony forming units, [Y] The data shown represent the mean of four replicates and LSD (at P≤ 0.05) = 9

Vapour generated from 15% solutions of citral, geranial and neral completely inhibited the growth of *P. italicum*. However, spores that had been exposed to the vapours germinated and mycelium covered about 30, 10 and 100% of the original Petri dishes after 8 weeks at 22°C, respectively for citral, geranial and neral (Table 5.3). This suggests that citral, geranial and neral vapours from 15% solutions were fungistatic for *P. italicum*.

In the experiments to compare the effect of citral with related compounds, treatment with 400 μL L^{-1} aqueous TritonX reduced the number of cfu by 17% compared to the water control. Of the citral-related compounds tested, vapour of citronellal

showed the greatest inhibitory effect at 15%, where cfu were reduced by 84-88% compared to the water control (Table 5.4). When 2% citronellal solution was used, the S isomer resulted in greater inhibition than the R isomer. Vapour of citronellic acid was least inhibitory and the number of cfu compared to the water control was reduced by 4-17 and 15-28% in the presence of 6% and 15% solutions, respectively. When applied as 6% and 15% solutions, the effect of the two isomers of citronellic acid on *P. italicum* differed significantly, with the S isomer providing more inhibition. For citronellol, no difference between the effects of the isomers on cfu was observed at any concentration tested, and treatment reduced the number of cfu by 23-28%, 66-72% and 57-61% in the presence of 2, 6 and 15% solutions, respectively.

Table 5.3 The effect of vapour of citral and its isomers (in 400 μL L^{-1} TritonX) at different concentrations on the growth of *P. italicum* at 22°C

Compound/ Concentration	cfu[X] per plate				
	0%	2%	6%	15%	Growth over plate (%) after removal of 15% solution
H_2O	102[Y]	N/A	N/A	N/A	N/A
400 μL L^{-1} TritonX	89	N/A	N/A	N/A	N/A
Citral	N/A	14	0	0	30
Geranial	N/A	0	0	0	10
Neral	N/A	13	7	0	100

Note: N/A = not applicable, [X] cfu = colony forming units, [Y] The data shown represent the mean of four replicates and LSD (at $P \leq 0.05$) = 4

Table 5.4 The effect of vapour of citral and its related compounds (in 400 μL L^{-1} TritonX) at different concentrations on the growth of *P. italicum* at 22°C

Compound/ Concentration	Cfu[X] per plate			
	0%	2%	6%	15%
H_2O	109[Y]	N/A	N/A	N/A
400 μL L^{-1} TritonX	90	N/A	N/A	N/A
Citral	N/A	0	0	0
R-citronellal	N/A	100	55	18
S- citronellal	N/A	85	61	13
R-citronellol	N/A	78	31	42
S- citronellol	N/A	84	37	47
R-citronellic acid	N/A	109	105	93
S- citronellic acid	N/A	99	90	79

Note: N/A = not applicable, [X] cfu = colony forming units, [Y] The data shown represent the mean of four replicates and LSD (at $P \leq 0.05$) = 12

5.3.4. Effects of vapour of citral and related compounds on *G. citri-aurantii*

Total inhibition of growth of *G. citri-aurantii* was observed in the presence of 15% citral, geranial and neral (Table 5.5). Treatment with 400 μL L^{-1} aqueous TritonX reduced the number of cfu growth by 29%. In the presence of 2% solutions, the number of cfu was reduced by 96, 95 and 87% for citral, geranial and neral, respectively. The number of cfu was reduced by 98, 96 and 92% in the presence of 6% citral, geranial and neral, respectively.

After removal of 15% solutions of citral, geranial or neral, no germination of spores of *G. citri-aurantii* was observed from transferred plugs or on the remainder of the

original plates (Table 5.5). This suggested that vapour of citral, geranial and neral at this concentration was fungicidal towards *G. citri-aurantii.*

Vapour of citronellic acid had the least inhibitory effect on *G. citri-aurantii* (Table 5.6). Vapour of 15% solution of citral and the two isomers of citronellal inhibited growth of *G. citri-aurantii* completely (Table 5.6). Of the citral-related compounds, vapour of citronellal was most inhibitory to *G. citri-aurantii*, and there were no differences between the two isomers at any of the concentrations tested. Citronellol and citronellic acid did not prevent the growth of *G. citri-aurantii* even at the highest concentration. In the presence of 6% solutions, the number of cfu was reduced by 94-98%, 48-51% and 35-37% for both isomers of citronellal, citronellol and citronellic acid, respectively. In comparison to the water control, treatment with 400 μL L^{-1} aqueous TritonX reduced the number of cfu by 26%.

Table 5.5 The effect of vapour of citral and its isomers (in 400 μL L^{-1} TritonX) at different concentrations on the growth of *G. citri-aurantii* at 22°C

Compound/ Concentration	Cfu[X] per plate				
	0%	2%	6%	15%	Growth over plate (%) after removal of 15% solution
H_2O	112[Y]	N/A	N/A	N/A	N/A
400 μL L^{-1} TritonX	80	N/A	N/A	N/A	N/A
Citral	N/A	4	2	0	0
Geranial	N/A	6	4	0	0
Neral	N/A	12	9	0	0

Note: N/A = not applicable, [X] cfu = colony forming units, [Y] The data shown represent the mean of four replicates and LSD (at $P \leq 0.05$) = 8

Table 5.6 The effect of vapour of citral and its related compounds (in 400 μLL^{-1} TritonX) at different concentrations on the growth of *G. citri-aurantii* at 22°C

Compound/ Concentration	cfu[X] per plate			
	0%	2%	6%	15%
H_2O	81[Y]	N/A	N/A	N/A
400 μL L^{-1} TritonX	60	N/A	N/A	N/A
Citral	N/A	3	1	0
R-citronellal	N/A	38	2	0
S- citronellal	N/A	38	5	0
R-citronellol	N/A	45	42	27
S- citronellol	N/A	49	40	27
R-citronellic acid	N/A	68	51	35
S- citronellic acid	N/A	61	53	34

Note: N/A = not applicable, [X] cfu = colony forming units, [Y] The data shown represent the mean of four replicates and LSD (at P≤ 0.05) = 4

5.3.5. Citral degradation analysis

The results of the preliminary experiment to examine citral degradation products after 12 days of storage are presented in Table 5.7. GC analysis showed that geranial content was reduced from 30.72% (in fresh citral) to 4.87% (in citral stored for 12 days), and neral content from 20.87% (in fresh citral) to 3.10% (in citral stored for 12 days).

Comparison of constituents observed by GC/MS analysis with the retention time of standard compounds and with the mass spectra of compounds present in the NIST

databank showed the only components that matched the signal were neric acid and geranic acid (Table 5.7), while R-citronellal, S-citronellal, R-citronellol, S-citronellol, R-citronellic acid, and S-citronellic acid were not detected. This suggested that storage of citral in the liquid phase probably resulted in the oxidation of neral and geranial to produce neric acid and geranic acid.

Table 5.7 Changes in citral composition after storage for 12 days at room temperature in the preliminary experiment, as determined by GC/MS analysis of citral solution 2% (v/v) ethanol

Retention time (minutes), of major peak observed			Compound	Area %	
Peak No.	Fresh citral	Citral stored for 12 days		Fresh	Stored for 6 days
1.	12.49	12.44	Tetradecane	48.41	58.74
2.	18.50	18.50	Neral	20.87	3.10
3.	19.48	19.41	Geranial	30.72	4.87
4.		23.03	Unknown^{X}	-	1.83
5.		23.64	Unknown^{X}	-	2.65
6.		24.77	Unknown^{X}	0.36	6.71
7.		27.99	Unknown^{X}	0.43	5.61
8.		28.71	Neric acid	-	1.74
9.		29.39	Geranic acid	-	4.46
10.		34.64	2-ethyl cyclohexanone	-	2.79
11.		36.34	Unknown^{X}	-	5.42

Note: Unknown^{X} = not reliably identifiable in database

Results from the subsequent experiment showed that neral, geranial, neric acid and geranic acid were detected after storage of citral for both 40 days at 5°C (Table 5.8) and 16 days at room temperature (Table 5.9). Nerol, geraniol, citronellal, citronellol and citronellic acid were not detected.

Geranial and neral content decreased more quickly at room temperature than at 5°C. At 5°C, storage of citral decreased the content of neral and geranial significantly ($P<0.05$) until day 16, and on storage for 40 days their content appeared to increase slightly (Table 5.8). At room temperature, neral and geranial decreased significantly ($P<0.05$) from day 2 to day 4, and further changes not significant (Table 5.9). The correlation regression equation for geranial and neral content at 5°C was $y = 58.70 - 1.53x$ and $y = 37.14 - 0.97x$; and that at room temperature was $y = 48.7 - 3.231x$ and $y = 31.16 – 2.09x$. In general, from correlation regression analysis, geranial content declined faster than neral content both at 5°C and room temperature.

Neric acid and geranic acid are oxidation products of neral and geranial. At 5°C, a significant increase ($P<0.05$) in geranic acid and neric acid content was observed for citral stored from day 4 to day 16 (Table 5.8). However, neric acid content then decreased significantly ($P<0.05$) for citral stored from day 16 to day 32. Furthermore, at room temperature, the content of geranic acid and neric acid increased significantly ($P<0.05$) for citral stored from day 0 to day 4 (Table 5.9) then neric acid content decreased ($P<0.05$) when citral was stored from day 4 to day 8. In general, the increased content of neric acid and geranic acid was concomitant with the decrease of neral and geranial both at 5°C and room temperature.

5.4. Discussion

5.4.1. Synthesis of geranial and neral

Geranial and neral synthesised in this experiment were 94% and 92% pure, respectively. The main impurity observed was dichloromethane (CH_2Cl_2), the

solvent used during synthesis. Dichloromethane is used in the pharmaceutical industries, as a solvent for chemical reactions for purification and isolation of compounds because of its low miscibility with water, low freezing-point, non-flammability, and ease of removal from reaction products (Euro Chlor, 2003). To my knowledge, dichloromethane has no fungicidal properties, therefore, the amount

Table 5.8 Citral degradation components after storage at 5°C. Data shown are mean content (%) of four replicate measurements (two technical replicates of two biological replicates) calculated relative to tetradecane and LSD at P≤ 0.05

	Content (%) observed after citral stored for					
Component	0 day	4 days	16 days	32 days	40 days	LSD
Geranial	78.7[X]	44.6	13.8	6.9	8.6	9.98
Neral	50.19	27.82	8.7	4.2	5.61	6.39
Geranic acid	1.98	1.8	3.71	3.4	3.37	0.78
Neric acid	0.41	0.46	1.07	0.79	0.94	0.185

Note: [X]The data shown represent the mean content (%) of four replicate measurements (two technical replicates of two biological replicates) calculated relative to tetradecane and LSD at P≤ 0.05

Table 5.9 Citral degradation components after storage at room temperature.

	Content (%) observed after citral stored for					
Component	0 day	4 days	8 days	12 days	16 days	LSD
Geranial	78.7[X]	10.1	2.4	8	15.12	9.86
Neral	50.19	6.61	1.44	4.61	9.42	6.29
Geranic acid	1.98	3.67	2.81	3.33	3.54	1.15
Neric acid	0.41	0.85	0.53	0.44	0.72	0.18

Note: [X]The data shown represent the mean content (%) of four replicate measurements (two technical replicates of two biological replicates) calculated relative to tetradecane and LSD at P≤ 0.05

of dichloromethane obtained in both the geranial and neral synthesised in this experiment, was considered unlikely to have an antifungal effect.

In this study, the yields of geranial (64%) and neral (40%) were less than those obtained by Marko *et al.* (1998), who synthesised geranial and neral through oxidation of the corresponding alcohols, geraniol and nerol, with 86 and 80% yields respectively. The differences between this experiment and that of Marko *et al*. (1998) may be related to the differences in chemicals used, as some chemicals used in this experiment were not fresh and no attempt was made to check their purity. The experimental conditions may have been different also. In catalytic chemical reactions, the role of the catalyst is important for completeness of the reaction (Kotz and Purcell, 1991). The low yield of neral in this experiment may have been because the catalyst, Cu(I) chloride or CuCl, was used without pre-treatment to remove the Cu^{2+} that may have formed during ageing of the chemical. In the synthesis of geranial, the CuCl was first treated with Na_2SO_4 solution, then filtered under nitrogen and dried in order to reduce Cu^{2+} to Cu^{+}. This may explain why the yield obtained for geranial was higher than neral. Nevertheless, sufficient quantities of geranial and neral were obtained for the purposes of the experiments conducted in this project.

5.4.2. Effects of the treatments on post-harvest pathogens

The effects of citral vapour, its isomers and its related compounds on *P. digitatum*, *P. italicum* and *G. citri-aurantii* varied. In both assays, the efficacy of citral in reducing the number of cfu compared to the water control was consistent when applied at 6% and 15% (Tables 5.1-5.6). However, inconsistent results were

observed when conidia of *P. digitatum* and *P. italicum* were exposed to vapour from 2% citral. This may be because it was the lowest concentration tested and inhibition may have been marginal.

Vapour of citral, geranial and neral from 15% solutions completely inhibited the growth of *P. digitatum, P. italicum* and *G. citri-aurantii* on agar-solidified culture media. Therefore, vapours generated from 15% solutions were sufficient to inhibit the germination of the three pathogens, which supported the results reported in the previous chapter (Chapter 4, Klieber *et al*., 2002). However, complete inhibition of mycelial growth was observed when spores of the pathogens were exposed to volatile citral generated from 6% solutions (Chapter 4). In the present study complete inhibition was achieved only for *P. italicum*, while *P. digitatum* and *G. citri-aurantii* were inhibited by 94 and 98%, respectively. As the experiments reported in this chapter were conducted 6 months after those reported in Chapter 4 and the citral had been stored at 5°C in the intervening time, the effectiveness of citral is likely to have declined over time. This study showed that storage of citral resulted in the decrease of its geranial and neral content both at 5°C and room temperature (as discussed in Section 5.4.3).

Results from the first assay showed that vapours of citral and its isomers, geranial and neral, generated from 15% solutions were fungicidal to *G. citri-aurantii*. Given that citral is a mixture of 60% geranial and 40% neral (Wolken *et al.,* 2002), the result suggests that both of the components might contribute to the inhibitory effect of citral on *G. citri-aurantii*. The action of vapours of citral and geranial generated from 15% solution was classified as fungicidal toward *P. digitatum*, while neral was

classified as fungistatic. However, vapours of citral, geranial and neral generated from 15% solutions were classified as fungistatic toward *P. italicum*. In this case, vapour of geranial exhibited more inhibitory effect than either citral or neral in terms of spore germination on the remainder of the original plate after exposure to 15% solution (Table 5.3). Even at the highest concentration used in this study, the compounds could not prevent the growth of *P. italicum*. Further research is required to determine the contribution of geranial and neral to the inhibitory effect of citral on the pathogens, whether synergistic or antagonistic.

The inhibitory effect of citral against the pathogens can be explained by citral being a member of the α, β-unsaturated aldehyde class, in which the carbonyl group is adjacent to α– and β-carbons. Due to their position, the α– and β-carbons are conjugated with the carbonyl group, making the β-carbon more positively polarised and able to react easily with nucleophiles (nuclephilic attack) (Andersen *et al.*, 1994). According to Witz (1989), the chemical nature of α, β-unsaturated aldehydes and some of their toxicological effects are based on their ability to function as direct-alkylating agents. These alkylating agents are capable of covalent binding to cellular nucleophile groups such as protein sulphydryl and amino groups of the pathogen, which means that they are capable of modifying cellular processes and are potentially toxic to pathogens. As protein provides nucleophilic groups, the sensitivity of the pathogens toward citral may be related to the protein content in the cell wall. Protein content of the cell wall of *G. citri-aurantii* has been reported to be 7% (Sietsma and Wouters, 1971), and that of *P. digitatum* and *P. italicum* to be 3% and 1.5%, respectively (Grisaro *et al.,* 1968). This may explain, in part, the finding that *G. citri-aurantii* was more sensitive to citral than *P. digitatum* and *P. italicum.*

More research is required to see if and how citral interacts with and affects the cell walls of the three pathogens.

This study showed that citral exhibited stronger inhibitory effects than its related compounds; citronellal, citronellol and citronellic acid. Vapour of citronellal isomers from a 15% solution prevented spore germination for *G. citri-aurantii*, and reduced cfu by 96 and 88% for *P. digitatum* and *P. italicum*, respectively. Furthermore, citronellic acid exerted the least inhibitory effect on the pathogens. This may be explained by considering the vapour pressure of the components. Vapour pressure of citral, citronellal, citronellol and citronellic acid was; 3902, 169, 29 and 2 kPa, respectively (Pirika, 2001). The effectiveness of the components generally was higher for components with higher vapour pressure, which would result in a higher headspace concentration. Likewise, Eckert and Kolbezen (1963) reported that there was a positive correlation between vapour pressure of amines and control of fruit decay, where vapour at low pressure did not reach the fruit infection site in sufficient concentration to prevent fungal infection.

Citronellic acid is a carbonyl compound and is an irreversible oxidation product of citronellal, while citronellol is not a carbonyl compound and its oxidation to citronellal is reversible (Erman, 1998; Feron *et al*., 1991). Wolken *et al.* (2002), in their investigation of toxicity of terpenes to spores and mycelium of *P. digitatum*, reported that the toxicity of citral toward *P. digitatum* was higher than its related alcohol (nerol/geraniol) and its related acid (geranic acid). The lesser inhibitory effect of citronellic acid and citronellol compared to citral may also be related to their lesser electrophilic properties, which means less ability to bind or modify the

cellular nucleophilic groups of the pathogens. Andersen *et al*. (1994) reported that the presence of the α, β-unsaturated double bond increases the electrophilic properties of the carbonyl compounds, and the inhibitory properties of the compounds and their effectiveness against *A. alternata* may be increased due to their increased tendency to react with thiols and amino groups of the target pathogens.

5.4.3. Change of citral content during storage

Citral is relatively unstable and is degraded over time (Robert and Caserio, 1965; Djordjevic *et al.*, 2008; Nguyen *et al.*, 2009) producing neric acid and geranic acid (Kotz and Purcell, 1991). This study showed that citral is degraded on storage; it is likely that regardless of storage temperature, citral, neral and geranial were oxidised to produce neric acid and geranic acid. The decrease of neral and geranial was concomitant with the increased content of neric acid and geranic acid both at 5°C and room temperature. The activity of geranic acid and neric acid toward the pathogens was not examined in this study. Study of toxicity of terpenes to spores and mycelium of *P. digitatum* by exposing the spores and mycelium to selected compounds for 30 minutes and plating them on malt extract agar, suggested that geranic acid exhibited weaker antifungal activity than did nerol, geraniol and citral (Wolken *et al.*, 2002). In addition, Gallardo *et al.* (2008) reported that neric acid exhibited weak antifungal activity toward *Ascosphaera apis*. This information suggests that the decreased content of citral, geranial and/or neral in stored citral may affect its antifungal activity against the citrus spoilage pathogens, and supports the difference in efficacy noted above for experiments conducted 6 months apart. Inouye *et al.* (2001) reported that antimicrobial activity by vapour contact was

determined mainly by the maximal concentration of vapour in the early stages of incubation. Although citral declines over time in the absence of the pathogens, micro-organisms may also contribute to degradation of the citral. Micro-organisms, including spores of *P. digitatum*, have been shown to transform citral into geranic acid by aldehyde dehydrogenase (Wolken and van der Werf, 2001). Lyophilised cells of *Rhodococcus* sp. NCIMB11216 and *Steptomyces griseus* DSM40236 also transformed citral to produce geranic acid and neric acid as major products by aldehyde dehydrogenase (Hall *et al.*, 2006). The pathogens in this study may have also degraded citral, thereby affecting its efficacy. Further research is needed to determine the effect of storage and incubation time on efficacy of citral vapour in inhibiting germination and growth of *P. digitatum, P. italicum* and *G. citri-aurantii*.

5.5. Summary

The results of this study showed that vapours of citral and its two isomers generated from 15% aqueous solutions in Petri dishes completely inhibited spore germination and growth of the three pathogens. Furthermore, neral was slightly more effective against *P. digitatum* and least effective against *P. italicum* at concentrations produced by 6% solutions.

The inhibitory effect of vapour of citral and its isomers and the nature of activity (fungistatic or fungicidal) differed among the three pathogens. Inhibitory ability may depend on citral concentration, as well as on the nature of the pathogens themselves.

In this experiment, vapours of citral and geranial generated from 15% solution were

classified as fungicidal to *G. citri-aurantii* and *P. digitatum*, but fungistatic to *P. italicum*, whereas vapour of neral at the same concentration was classified as fungicidal to *G. citri-aurantii*, but fungistatic to *P. digitatum* and *P. italicum*. As vapours of citral and both of its components, geranial and neral, generated from 15% solution exhibited fungicidal effect on *G. citri-aurantii*, this indicates that *G. citri-aurantii* is particularly sensitive to citral. In addition, content of citral decreased over time due to oxidation of geranial and neral to geranic acid and neric acid both at 5°C and room temperature.

Vapours of citral-related compounds were much less effective than vapour of citral, with effectiveness decreasing from citronellal to citronellol and citronellic acid. No overall trend between R and S isomers of these three components was found.

As geranial and neral are not available commercially, using citral to control spoilage fungi seems the best option. The use of citral in the vapour phase may constitute a potential alternative to conventional fungicides. Research is required to determine if citral vapour could be used to fumigate citrus fruit and the concentration that might control green mould, blue mould and sour rot of fruit in storage.

Chapter Six

Fumigation of oranges with citral to control postharvest fungal diseases

6.1. Introduction

In vitro experiments in this study (Chapter 5) showed the potential of using volatile citral as a fumigant to control postharvest spoilage fungi of citrus. Fumigation of fruit with volatile citral may therefore facilitate control of disease in several ways. Fumigation may prevent infection by killing exposed conidia or make the peel of the fruit an unsuitable substrate for germination of conidia if volatile citral is absorbed by and accumulates in the injured peel during fumigation. Furthermore, fumigation of fruit with citral may reduce the risk of phytotoxicity which may occur when liquid citral comes into contact with the citrus rind (Ben-Yehoshua *et al*., 1992; Knight, 2002). In addition, as citral is volatile and occurs naturally in the oil glands of the fruit peel, residual levels of citral and any effect in terms of consumer acceptability may be considered negligible.

Several fumigants have been used to control postharvest disease of citrus fruit. For example, Roistacher *et al.* (1958) used gaseous ammonia to control green and blue moulds. They found that control of green and blue moulds on oranges and lemon was achieved when 100 ppm ammonia was maintained in the atmosphere for 9-10 h. Acetic acid has also been used to control green mould (Sholberg, 1998: Venditti *et al.,* 2009). Sholberg (1998) fumigated *P. digitatum* surface-inoculated citrus fruit

with formic, acetic and propionic acids, and found that the three acids effectively reduced incidence of green mould. When acetic acid fumigation was combined with curing, green mould on *P. digitatum* surface-inoculated mandarin was controlled to a greater extent (Venditti *et al.* 2009). Furthermore, Mercier and Smilanick (2005) reported that fumigation of wound-inoculated lemon for 24-72 h with a volatile compound produced by *Muscodor albus* immediately after inoculation effectively controlled green mould and sour rot. However, sour rot was not controlled when the fumigation was applied 24 h or more after inoculation. Although citral has been shown to affect the growth of various fungi (Ben-Yehoshua *et al*., 1992; Rodov *et al*., 1995; Pattnaik *et al*., 1997; Park *et al.*, 2007; Chapter 5), application of citral as a fumigant to citrus fruit to control disease caused by *P. digitatum, P. italicum* and *G. citri-aurantii* has not been reported. Therefore, the experiments reported in this chapter were undertaken to examine the potential of fumigation of oranges with citral to control these postharvest fungal diseases.

6.2. Materials and methods

The investigation of fumigation of fruit with citral was conducted in three experiments. The first experiment involved fumigation of fruit wounded with a sterilised nail (to mimic a puncture injury) before inoculation and incubation at room temperature (22°C) to simulate market conditions. The second was as above but the fruit were incubated at 5°C to simulate shipping conditions. The third was similar to the second experiment but the fruit were wounded with sterilised sandpaper (to mimic abrasion), and inoculated only with *P. digitatum* and *P. italicum* due to

limited availability of fungicide-free fruit at the time of the experiment. Most of the experiments were conducted between July and October 2001.

6.2.1. Preparation of fruit before treatment

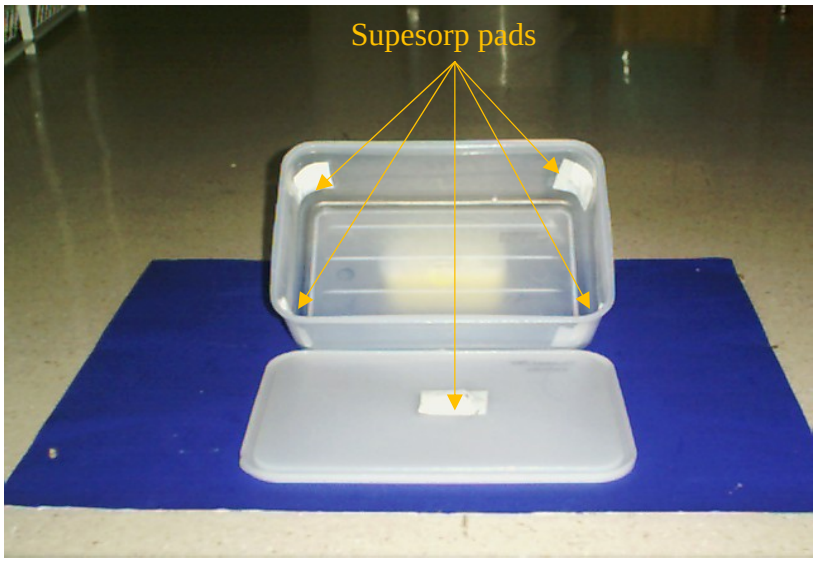
Fruit used in the first, second and third experiments were mature Navel oranges of uniform size and appearance. Fruit were harvested on 12 August 2001 by hand from an orchard on the South Australian Research and Development Institute property at Loxton, South Australia (34°19'S, 140°00'E). Fruit were washed with 0.5% v/v fruit detergent (Decco Fruit and Vegetable Kleen, Cerexagri, Australia), then surface sterilised with bleach solution as described in Section 3.2.2 and left to dry.

Five-litre Handy Storer plastic boxes (Quadrant, Melbourne, Australia) were used as containers for the experiments. Supesorp pads (DRI-LOC DLS 25 White E86-Sookerpad, Cryovac Australia, Pty Ltd, Victoria) were arranged in five positions in each box as shown in Figure 6.1a and 1.31 mL citral solution (2, 6 or 15% in 400 μL L^{-1} TritonX) was applied to each pad. Six inoculated fruit were then randomly assigned to each box (Figure 6.1b). Furthermore, 10 g of calcium hydroxide was placed in each box as a carbon dioxide scavenger to minimise any effect of carbon dioxide produced as a result of respiration (Sitton and Patterson, 1992; Tian *et al*., 2002) and ethylene accumulation was controlled by venting the box for 1 minute every 12 h to avoid stimulation of senescence of the oranges.

6.2.2. Experimental design and treatments

Four wounds were made around the equator of the fruit, either by puncture using a sterilised nail (Bullet & Plate nail 30 mm, Dynaforge, China) to about 3 mm depth or abraded using sterilised sandpaper (Fastcut cabinet paper B115, Carborundum

(a)



(b)

Figure 6.1 Arrangement of (a) Supesorp pads to which citral was applied and (b) fruit in a 5-L Handy Storer container, with Supesorp pad in lid (A), Supesorp pad in box (B) and 10 g of calcium hydroxide as a carbon dioxide scavenger (C)

Abrasives, Labrador, Queensland, Australia) to about 1 cm in diameter. The nail and sandpaper were sterilised in an autoclave for 15 minutes at 121°C using the dry goods cycle before use. Each wound in the fruit was then inoculated with 20 μL of spore suspension (10^6 spores mL^{-1}) of *P. digitatum*, *P. italicum* or *G. citri-aurantii* (prepared as described previously in Section 3.2, except that viability was not assessed). After inoculation the fruit were left to air dry for 30-60 minutes on a rack in a laminar flow cabinet, then arranged in 5-L Handy Storer plastic boxes (as described above). Experiments were conducted with two controls, one consisting of six fruit in a closed box and the other in an open box. There were three replicate boxes of six oranges per treatment. For the controls in each experiment, 1.31 mL of water was applied to each pad instead of citral solution.

The three experiments were as follows:

Experiment 1 Fumigation of fruit with citral at room temperature after inoculation of the puncture wounds. Oranges punctured with a sterilised nail and inoculated with spore suspension of *P. digitatum*, *P. italicum* or *G. citri-aurantii* were incubated at 22°C on the bench of the Postharvest Laboratory, Department of Horticulture, Viticulture and Oenology, University of Adelaide.

Experiment 2 Fumigation of fruit with citral at 5°C after inoculation of the puncture wounds. Oranges punctured with a sterilised nail and inoculated with spore suspension of *P. digitatum*, *P. italicum* or *G. citri-aurantii* were incubated at 5°C in a cold room in the Waite Main Building or in the Plant Research Centre.

Experiment 3 Fumigation of fruit with citral at 5°C after inoculation of sandpaper abrasion sites. Oranges abraded with sterilised sandpaper and inoculated with spore suspension of *P. digitatum* or *P. italicum* were incubated at 5°C, as above.

In addition, in order to interpret the difference between the two controls (open and closed boxes), the relative humidity (RH) was measured using a hand held humidity probe (HM34C, Vaisala, Helsinki) at both 22°C and 5°C in the room, in a representative sealed box with pads containing 15% citral solution (as Figure 6.1a), and in a sealed box with pads containing 15% citral solution and six oranges (as Figure 6.1b). Measurement was conducted four times at 3 h intervals on the same day. However, no attempt was made to measure the RH during the course of fumigation experiments.

6.2.3. Assessment of disease

Fruit were examined for symptoms of disease daily and the effectiveness of the treatments was assessed by determining the time required for the appearance of first symptoms, and time required for all fruit to show disease (100% disease incidence). Furthermore, the time course for disease development, as a measure of the efficacy of citral in delaying the onset of disease, was determined as the time difference between the two categories (Δ value).

Phytotoxicity of citral to the rind was visually evaluated daily for the experiments involving fumigation of fruit with citral after wounding with a nail and subsequent storage at room temperature or 5°C, by examining each orange for the appearance of

phytotoxicity (Knight, 2002). However, since disease development was visible to naked eye and characteristics were comparable to Figures 2.6 – 2.8, no attempt was made to use microscopy to confirm the observations during the course of fumigation experiments.

6.2.4. SPME-GC analysis of headspace citral concentration in box

In order to estimate the volatile citral concentration present in the boxes, a separate experiment was conducted. Headspace citral concentration of boxes containing fruit and citral-soaked pads arranged as per Figure 6.1 was measured at 5°C and 22°C using SPME-Gas Chromatography as described in Section 3.5. To allow citral to equilibrate, measurements were taken 2 h after placing pads and fruit in the box for fruit stored at 5°C, and after 3 h for fruit stored at 22°C. The fibre was then placed in the box for 4 h in both cases, to extract the volatile citral in the system. The headspace concentration was calculated based on the calibration graph (Appendix 1) constructed from known headspace concentration using pure citral as described in Section 3.5. Analysis was carried out in duplicate for two positions of the fibre in the box: above the fruit and below the fruit (Figure 6.2).

Figure 6.2 Fibre position for analysis of headspace concentration of citral in the box at each temperature when (A) positioned above the fruit and (B) positioned below the fruit

6.2.5. Citral residue analysis

Citral residue on treated fruit was analysed by conducting the following experiment. Six fruit were arranged in a box containing pads soaked with 1.31 mL of 15% citral solution or with 1.31 mL water (as a control) (as in Figure 6.1). The experiment was conducted in three replicates (three boxes). The boxes were closed and stored at room temperature for 2 weeks or at 5°C for 6 weeks.

After storage, the rind tissue of each fruit in the box was cut into pieces and albedo tissue was removed and discarded to leave flavedo only. This was because citral is only found in the flavedo (Rodov *et al.,* 1995). Tissue pieces were weighed to a total of 1 g and then placed into a vial containing 4 mL of ethanol containing 200 mg L^{-1} of tetradecane (internal reference standard). These procedures were conducted within less than 5 minutes, to minimise dehydration and avoid oil volatilisation. Samples were stored in the dark at 4°C for 10 days, then at –18°C until the time of oil analysis when samples were filtered. The supernatant was analysed by means of gas chromatography and a final concentration of citral per gram fresh weight of rind tissue was calculated according to the method of Ammon *et al*. (1985). The GC measurements were conducted using a Shimadzu GC 14A (Kyoto, Japan) with injection volume of 5 μL, and the GC conditions as described in Section 3.3.2.

In a separate GC experiment, using similar analytical conditions, a limit of detection of 0.5% v/v citral in ethanol solution was observed using an injection volume of 5 μL, which is equivalent to 25 nL citral. The minimum volume of citral in 4 mL of supernatant was therefore: (4 mL÷5 μL) X (25 $x10^{-3}$ μL) = 20 μL.

Since the specific gravity of citral is 0.888 (Hawley, 1981), then in the 1 g peel sample extracted into 4 mL, the minimum detectable citral content would therefore be: 0.888 μg μL^{-1} X 20 μL = 1.776 μg. Therefore, citral content in fruit peel would be considered as residue if >1.776 μg per gram was detected in a peel sample.

6.2.6. Statistical analysis

Statistical analysis was conducted using GenStat Release 11.1 (PC/Windows; Copyright 2008, VSN International Ltd, UK). The relationship between the appearance of first symptoms, 100% disease incidence and the time between the two events (Δ value) was modelled by using polynomial regression. The response of each fungus in each experiment was evaluated separately using one way analysis of variance. LSD at $P \leq 0.05$ was used to determine differences amongst means of treatments. Furthermore, data for individual treatments in the experiment involving fumigation of fruit inoculated with *P. digitatum* and *P. italicum* following puncture and abrasion at 5°C were compared using student T-tests (Gomez and Gomez, 1984), in order to assess the effect of wounding methods.

6.3. Results

6.3.1. RH measurement to interpret the difference between control in open and closed boxes

RH, measured as an aid to interpret differences between the two controls, is shown in Table 6.1. This experiment was conducted using a closed system which simulated individual storage packages, while uninoculated fruit placed in open boxes were

included in order to see how fumigation might affect fruit stored in a large chamber or room.

At room temperature (22°C), the RH of the room and the sealed box (representing an open and a closed system, respectively) was similar. However, RH in the sealed box containing fruit was 22% and 23% greater than RH of the room and the sealed box without fruit, respectively ($P \leq 0.05$). Meanwhile at 5°C, the RH in the sealed box containing fruit was 6% greater than that of the room (representing open boxes) ($P \leq 0.05$) and was similar to that in sealed box without fruit. Comparison of RH in the sealed box containing fruit and in the sealed box without fruit reflected respiration of the fruit. The result showed that respiration was greater at room temperature than at 5°C.

Table 6.1 Relative humidity (RH) measured at 22°C and 5°C as an aid to interpret differences between the two controls

	RH (%)			
Storage temperature (°C)	Room	Sealed box	Sealed box + fruit	LSD
22	45[X]	44	67	2.61
5	65	72	71	1.30

Note: [X] The data shown represent the mean values of four measurements taken over a 12 h period and LSD (at $P \leq 0.05$)

6.3.2. Fumigation of fruit with citral at room temperature after inoculation of puncture wounds

Data of time required to first appearance of symptoms and 100% disease incidence among fruit inoculated with spoilage fungi via a puncture wound and fumigated with citral at room temperature are shown in Table 6.2.

Sour rot symptoms first appeared on day 9 (mean of 8.90 days) on control fruit inoculated with *G. citri-aurantii* following wounding with a sterilised nail and placement in open boxes at 22°C. By day 38 all fruit were diseased. For control fruit in closed boxes, symptoms were first observed on day 8 and on day 37 all of the fruit were diseased. Statistically, the time to first appearance of sour rot symptoms among citral treatments and both controls was similar (P=0.08). No differences were observed for the time to 100% sour rot incidence between the two controls, nor among citral-fumigated fruit. However, the time to 100% sour rot incidence for all citral-fumigated fruit was 10-11 days longer than that for control fruit placed in closed boxes and 10 days longer than that for control open boxes (P=0.02). Delay in sour rot development (Δ value) was similar for the two set of controls, likewise for all citral treatments. The course of sour rot development on fruit fumigated with 15% citral appeared similar to that of both controls in open and closed boxes and to that of fruit fumigated with 2% and 6% citral. Sour rot development on all citral-fumigated fruit was delayed by 7-10 days compared with control fruit placed in open and closed boxes (P=0.05).

Symptoms of green mould were first observed on control fruit inoculated with *P. digitatum* following wounding with a sterilised nail and placement in open boxes on day 4 (4.10 days) and on day 8 all of the fruit were diseased. For control fruit placed in closed boxes, disease was first observed by day 3 (3.20 days), and on day 7 (6.83 days) all of the fruit were diseased. The time required to the first appearance of green mould symptoms for control fruit in closed boxes was similar to that for fruit exposed to 2% and 6% citral, but shorter for fruit exposed to 15% citral. The time required to the first appearance of green mould symptoms for fruit exposed to 6% citral was 1 day shorter than that for control fruit placed in open boxes (P=0.02). Time required for 100% green mould incidence appeared similar among all citral treatments and control fruit placed in closed boxes, but was faster by 1 day than that of the control fruit placed in open boxes. No significant difference among the treatments and both controls was observed for the delay of green mould development (Δ value) (P = 0.08).

Blue mould symptoms were first observed on day 6 (5.72 days) and day 3 (2.71 days) for control fruit inoculated with *P. italicum* following wounding with a sterilised nail and placed in open and closed boxes at 22°C, respectively; and by day 10 (9.86 days) and day 8 (7.83 days) all such fruit placed in open and closed boxes, respectively, were diseased. The time to first appearance of blue mould symptoms on all citral-fumigated fruit and control fruit placed in closed boxes was similar, but was 3 days shorter than that for control fruit placed in open boxes. The time to 100% blue mould incidence on fruit exposed to 15% citral was 2 days longer than that for control fruit placed in closed boxes, and fruit exposed to 2% and 6% citral. Citral at 15% significantly delayed blue mould development (Δ value) by 1 day compared

with control fruit placed in closed or open boxes, and fruit exposed to 2% and 6 % citral (P=0.02).

At room temperature on day 2, citral phytotoxic symptoms were observed in one of the 18 fruit inoculated with each of *P. digitatum* or *G. candidum* exposed to 15% citral and in contact with the citral pads (Table 6.3, Figure 6.3). Also, two or three of the 18 fruit in each inoculation treatment exposed to 6% and 15% citral and in close proximity to the citral pads exhibited phytotoxic symptoms. Citral phytotoxicity was not observed on fruit exposed to 2% citral (Table 6.3). No phytotoxicity symptoms were observed in controls as expected (data not shown).

Table 6.2 Time (in days) to first appearance of symptoms and 100 % disease incidence among fruit inoculated with spoilage fungi via a puncture wound and fumigated with citral at room temperature

Treatments	*G. citri-aurantii*			*P. digitatum*			*P. italicum*		
	Time (day), for			Time (day), for			Time (day), for		
	The first symptoms	100% disease incidence	Δ^{X}	The first symptoms	100% disease incidence	Δ^{X}	The first symptoms	100% disease incidence	Δ^{X}
Control in open boxes	8.90	38.10^{b}	29.20^{b}	4.10^{a}	8.00^{a}	3.90	5.72^{a}	9.86^{a}	4.14^{b}
Control in closed boxes	8.20	37.10^{b}	28.90^{b}	3.20^{bc}	6.83^{b}	3.63	2.71^{b}	7.83^{c}	5.12^{b}
2% citral	8.60	47.80^{a}	39.20^{a}	3.02^{c}	6.83^{b}	3.81	2.71^{b}	7.83^{c}	5.12^{b}
6% citral	8.80	47.60^{a}	38.80^{a}	3.54^{b}	7.00^{b}	3.46	3.41^{b}	8.49^{b}	5.08^{b}
15% citral	11.20	47.10^{a}	35.90^{ab}	2.61^{d}	6.50^{b}	3.89	3.28^{b}	9.73^{a}	6.45^{a}
P value	0.08	0.02	0.05	0.02	0.005	0.08	0.02	<0.001	0.02
LSD		7.77	8.38	0.38	0.66		1.28	0.66	1.27

Note: $^{X}\Delta$: the time difference between the appearance of first symptoms and 100% disease incidence. Data shown are mean values of three replicate boxes with six fruit in each. Means having the same letter in the same column are not significantly different at 5% level

Table 6.3 Number of fruit (of 18 fruit in total) displaying phytotoxicity symptoms when held at room temperature on day 2 after treatment with different concentrations of citral (2, 6 or 15%)

Treatment	Number of fruit with phytotoxicity symptoms					
	Direct contact with citral-containing pad			Close proximity to citral-containing pad		
	2%	6%	15%	2%	6%	15%
P. digitatum	0	0	1	0	2	2
P. italicum	0	0	0	0	2	2
G. citri-aurantii	0	0	1	0	2	3

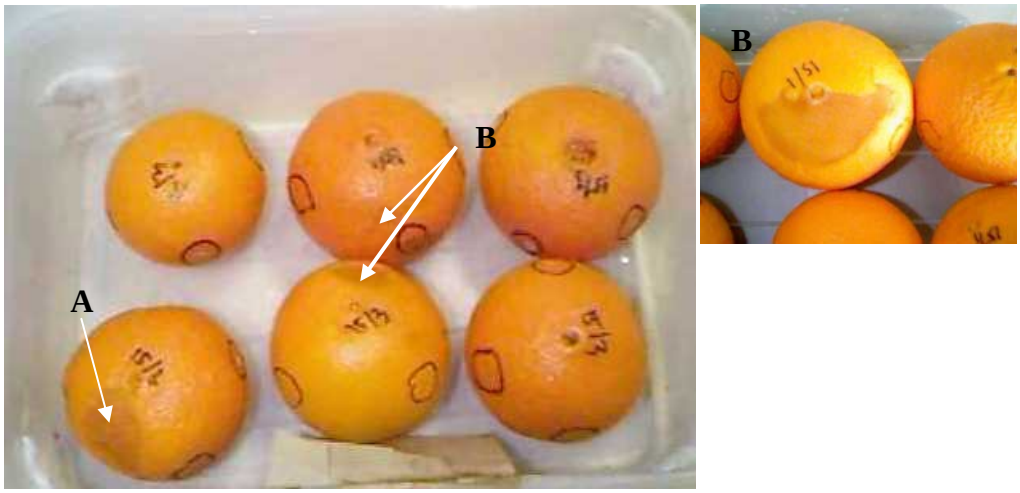


Figure 6.3 Phytotoxicity observed on (A) fruit in direct contact with the citral pad and (B) fruit close to the citral pad attached to the lid of the box at 15% citral

6.3.3. Fumigation of fruit with citral at 5°C after inoculation of puncture wound

Table 6.4 presents the data of time to first appearance of symptoms and 100% disease incidence among fruit inoculated with spoilage fungi via a puncture wound and fumigated with citral at 5°C.

Symptoms of sour rot were first observed on day 58 (57.8 days) for control fruit inoculated with *G. citri-aurantii* placed in open boxes and on day 146 all fruit were diseased. For control fruit placed in closed boxes, the first symptom of sour rot was observed on day 34 (33.90 days) and on day 66 all fruit were diseased. The time to first appearance of sour rot symptoms on fruit fumigated with 6% citral was 6 and 30 days longer, respectively, than that for control fruit placed in open and closed boxes ($P = 0.004$). The time to first appearance of sour rot symptoms on fruit fumigated with 2% and 15% citral was similar to control fruit placed in closed boxes. Time to first appearance of sour rot symptoms on fruit fumigated with 6% and 15% citral was also similar to control fruit placed in open boxes. For the time to 100% incidence of sour rot, that of fruit exposed to all citral treatments was significantly longer than for control fruit placed in closed boxes ($P = 0.02$). The delay of sour rot development (Δ value) for control fruit placed in open boxes was 56 days longer than that of control fruit placed in closed boxes. In terms of citral efficacy, citral at 6% and 15% delayed the development of sour rot by 124 and 21 days, respectively, compared with control fruit placed in closed boxes ($P < 0.05$).

Green mould was first observed on day 23 (23.38 days) in control fruit inoculated with *P. digitatum* following wounding with a sterilised nail and placement in open boxes at 5°C, and on day 30 (30.34 days) all of the fruit were diseased. For control fruit placed in closed boxes, symptoms were first observed on day 17 (17.05 days) and on day 26 (26.17 days) all of the fruit were diseased. The appearance of first symptoms was similar among all citral treatments. The time required for the first symptoms on fruit exposed to 2% citral was no different from that of control fruit in closed boxes as well as fruit exposed to 6% and 15% citral. However, the

appearance of first symptoms on fruit exposed to 6% and 15% citral took significantly longer (by 2 days) than that for control fruit in closed boxes ($P<0.001$). There was no difference in time to 100% green mould incidence among the three citral treatments. However, the time to 100% green mould incidence on fruit exposed to 2% citral was 4 days longer than that for control fruit in closed boxes and 3 days longer than that for fruit from the 6 and 15% citral treatments. However, none of the citral treatments differed from the control fruit in open boxes. The Δ value for control fruit in closed boxes appeared to be similar to that for control fruit placed in open boxes. Green mould development was delayed by 4 days when fruit were fumigated with 2% citral when compared to control fruit in closed boxes and with the two other citral treatments ($P = 0.03$).

Symptoms of blue mould were first observed on day 20 (19.68 days) for control fruit inoculated with *P. italicum* following wounding with a sterilised nail and placement in open boxes at 5°C and on day 27 (27.40 days) all of the fruit were decayed, whereas for control fruit in closed boxes spoilage was first observed on day 15 (14.66 days) and on day 24 (23.67) all of the fruit were diseased. Time required for the first appearance of blue mould symptoms was similar for all citral treatments, as was that for control fruit in closed boxes and fruit fumigated with 2% and 15% citral. Likewise, there was no difference in time to 100% blue mould incidence among the three citral treatments and between control fruit in closed boxes with fruit fumigated with 2% and 6% citral. However, the time to 100% blue mould incidence of fruit fumigated with 15% citral was 1 day longer than that of control fruit in closed boxes. Statistically, in terms of Δ value, there were no differences among the treatments and controls.

Control fruit in closed boxes, regardless of which fungus was applied, showed brown discolouration around the wound after 10-11 days (Figure 6.4 A). However, fruit treated with citral did not display this symptom (Figure 6.4 B).

Citral-fumigated fruit stored at 5°C displayed phytotoxic effects after 18 days in fruit directly below the pad. In the treatment where fruit were inoculated with *P. digitatum* and treated with 6% citral, one of the 18 fruit displayed phytotoxic effects. Likewise, two and four of the 18 fruit inoculated with *P. italicum* and *G. citri-aurantii* treated with 15% citral were affected, respectively. Furthermore, contact between fruit and pad for fruit exposed to 6% citral caused one, one and two of 18 fruit inoculated with *P. digitatum, P. italicum* or *G. citri-aurantii* to have symptoms, respectively. For fruit in contact with the pad, two of 18 fruit inoculated with *P. italicum* exposed to 15% citral exhibited phytotoxic symptoms (Table 6.5).

Figure 6.4 Disease development at the wound site of inoculated fruit stored at 5°C; (A) Brown discolouration around the wound site on control fruit in closed boxes inoculated with *P. italicum* stored at 5°C taken 14 days after wounding, and (B) Disease on citral treated fruit due to *P. italicum* stored at 5°C, 14 days after inoculation.

6.3.4. Fumigation of fruit with citral at 5°C after inoculation of abrasion wound

The time required to first appearance of symptoms and 100% disease incidence among fruit inoculated with *P. digitatum* and *P. italicum* after sandpaper abrasion and fumigation with citral at 5°C is presented in Table 6.6.

Symptoms of green mould in this experiment were first observed on day 27 (27.02 days) for control fruit in open boxes and on day 40 (39.81 days) all of the fruit were decayed, whereas for control fruit placed in closed boxes, disease was first observed on day 17 (16.99 days) and on day 31 (30.65 days) all of the fruit were diseased. There was no difference between the time required for the first appearance of green mould symptoms for fruit fumigated with 2% citral and that required for fruit

Table 6.4 Time (in days) to first appearance of symptoms and 100 % disease incidence among fruit inoculated with spoilage fungi via a puncture wound and fumigated with citral at 5^{o}C

Treatments	*G. citri-aurantii*			*P. digitatum*			*P. italicum*		
	Time (day), for			Time (day), for			Time (day) ,for		
	The first symptoms	100% disease incidence	Δ^{X}	The first symptoms	100% disease incidence	Δ^{X}	The first symptoms	100% disease incidence	Δ^{X}
Control in open boxes	57.80 ^{ab}	146 ^{b}	88.20 ^{b}	23.38 ^{a}	30.34 ^{a}	6.96 ^{b}	19.68 ^{a}	27.40 ^{a}	7.72
Control in closed boxes	33.90 ^{b}	66 ^{e}	32.10 ^{d}	17.05 ^{c}	26.17 ^{b}	9.12 ^{b}	14.66 ^{c}	23.67 ^{c}	9.01
2% citral	46.50 ^{b}	80 ^{d}	33.50 ^{d}	17.38 ^{bc}	30.16 ^{a}	12.7 ^{a}	16.13 ^{bc}	24.52 ^{bc}	8.39
6% citral	63.50 ^{a}	220 ^{a}	156.50 ^{a}	19.33 ^{b}	28.78 ^{a}	9.46 ^{ab}	16.59 ^{b}	24.36 ^{bc}	7.77
15% citral	50.90 ^{ab}	105 ^{c}	53.10 ^{c}	19.39 ^{b}	28.79 ^{a}	9.39 ^{b}	16.55 ^{bc}	25.22 ^{b}	8.67
P value	0.004	0.02	0.04	<0.001	0.02	0.03	0.002	<0.001	0.07
LSD	12.70	9.08	8.45	2.03	1.65	3.28	1.93	1.36	

Note: $^{X}\Delta$: the time difference between the appearance of first symptoms and 100% disease incidence. Data shown are mean values of three replicate boxes with six fruit in each. Means having the same letter in the same column are not significantly different at 5% level

Table 6.5 Number of fruit (of 18 fruit in total) displaying phytotoxicity symptoms when held at 5°C after wounding with a sterilised nail. Fruit were counted on day 18 after treatment with different concentrations of citral (2, 6 or 15%)

Treatment	Number of fruit with phytotoxicity symptoms					
	Direct contact with citral-containing pad			Close proximity to citral-containing pad		
	2%	6%	15%	2%	6%	15%
P. digitatum	0	1	2	0	1	0
P. italicum	0	1	5	0	0	2
G. citri-aurantii	0	2	2	0	0	4

fumigated with 6% citral and control fruit placed in closed boxes. The time to 100% green mould incidence was similar for all citral treatments, but 1 day longer for fruit treated with 2 and 6% citral than that for control fruit in closed boxes and 8 days less ($P < 0.001$) than for the control in open boxes. The Δ value for the two sets of controls was not significantly different. The Δ value for fruit fumigated with 6 and 15% citral was 3 and 4 days greater compared with control fruit in closed boxes, respectively ($P<0.001$). Decay started first in fruit fumigated with 15% citral but then advanced more slowly than in other treatments, resulting in a significantly longer delay in disease development when compared to the controls in open and closed boxes (5 and 4 days longer respectively).

Blue mould symptoms first appeared on day 30 (29.82 days) for control fruit in open boxes and on day 44 (44.16 days) all of the fruit were decayed. For control fruit in closed boxes, blue mould was first observed on day 16 (15.90 days) and on day 33 (32.94 days) all fruit were infected. In the citral-treated fruit, blue mould symptoms

were first observed on day 19-21, with no difference among treatments. The first appearance of blue mould symptoms of fruit fumigated with 2% citral and that of control fruit in closed boxes was similar. There was no difference in time to 100% blue mould incidence on fruit exposed to 6 and 15% citral. The time to 100% blue mould incidence on fruit exposed to 2, 6 and 15% citral was about 2, 5 and 7 days longer, respectively, than that for control fruit in closed boxes. The Δ value for all citral treated fruit was also similar (at P = 0.08).

6.3.5. Effect of wounding method on disease incidence

Data from experiments involving fumigation of fruit with citral at 5°C after inoculation of puncture and abrasion wounds, respectively (sections 6.3.2 and 6.3.3) were combined and compared to evaluate the effect of wounding method on disease incidence. Tables 6.7 and 6.8 are presented to show the effect of wounding method, puncture and abrasion, prior to inoculation of fruit with *P. digitatum* and *P. italicum* at 5°C, respectively.

There was no significant effect of wounding method on the time to the first symptoms of green mould for control fruit in closed boxes and fruit exposed to 2% citral treatment (Table 6.7). However, the time to the first symptoms of green mould for control in open boxes following inoculation of puncture wounds was longer by 4 days than for sandpaper abrasion (P=0.013). Furthermore, the first symptoms of

Table 6.6 Time (in days) to first appearance of symptoms and 100 % disease incidence among fruit inoculated with spoilage fungi via abrasion and fumigated with citral at 5^{o}C

Treatments	*P. digitatum*			*P. italicum*		
	Time (day), for			Time (day), for		
	The first symptoms	100% disease incidence	Δ^{X}	The first symptoms	100% disease incidence	Δ^{X}
Control in open boxes	27.02 ^{a}	39.81 ^{a}	12.79 ^{c}	29.82 ^{a}	44.16 ^{a}	14.34
Control in closed boxes	16.99 ^{bc}	30.65 ^{c}	13.66 ^{c}	15.90 ^{c}	32.94 ^{d}	17.04
2% citral	18.24 ^{b}	32.48 ^{b}	14.24 ^{b}	19.36 ^{bc}	35.28 ^{c}	15.92
6% citral	15.36 ^{bc}	32.47 ^{b}	17.11 ^{ab}	20.08 ^{b}	38.17 ^{b}	18.09
15% citral	13.99 ^{c}	31.97 ^{bc}	17.98 ^{a}	21.33 ^{b}	39.57 ^{b}	18.24
P value	<0.001	<0.001	<0.001	<0.001	0.02	0.08
LSD	3.56	1.72	3.45	3.61	2.18	

Note: $^{X}\Delta$: the time difference between the appearance of first symptoms and 100% disease incidence. Data shown are mean values of three replicates boxes with six fruit in each. Means having the same letter in the same column are not significantly different at 5% level.

green mould for fruit fumigated with 6% and 15% citral following a puncture wound appeared 4 and 5 days later than when sandpaper abrasion was used (with $P = 0.001$ and $P<0.001$, respectively).

The time to 100% green mould incidence following sandpaper abrasion and inoculation was significantly longer than that for inoculation of a puncture wound for all treatments and controls (Table 6.7). Control fruit stored in open and closed boxes after inoculation using sandpaper abrasions reached 100% green mould incidence 10 and 5 days later than control fruit inoculated following puncture wound, respectively (both with $P<0.001$). Regardless of citral concentration, citral-treated fruit had reached 100% green mould incidence 2-4 days later following inoculation using sandpaper abrasion than when inoculated using a puncture wound (all with $P<0.001$).

The development of green mould on *P. digitatum*-inoculated fruit fumigated with citral was faster on punctured than on abraded oranges. Green mould development on control fruit stored in open and closed boxes after inoculation using sandpaper abrasion was slower by 5-6 days than control fruit inoculated following puncture wounds (at $P=0.003$ and <0.001, respectively). Furthermore, the green mould development for fruit fumigated with 6% and 15% citral following sandpaper abrasion was delayed, by 7-9 days compared with fruit inoculated via puncture wounds (both with $P<0.001$). However, the development of green mould was not significantly affected by wounding method for fruit fumigated with 2% citral.

Regardless of treatment, the appearance of the first symptoms of blue mould following inoculation of puncture wounds was significantly faster than when sandpaper abrasions were inoculated and fruit were stored at 5°C (Table 6.8). The time to the first symptoms of blue mould for controls in open and closed boxes following a puncture wound was faster by 10 and 1 days, respectively, than that following a sandpaper abrasion (at $P<0.001$ and 0.041). Furthermore, the appearance of the first symptoms of blue mould on all citral-treatments fruit following sandpaper abrasion was delayed by 3-4 days compared with when applied after puncture wound (all with $P\leq0.001$).

The time to 100% blue mould incidence following sandpaper abrasion and inoculation was significantly longer than that of a puncture wound (Table 6.8), regardless of treatment. The time to 100% blue mould incidence on the two sets of controls following sandpaper abrasion was longer by 9-17 days than that following puncture wounds (both at $P<0.001$). In addition, the time to 100% blue mould incidence on all citral-treatment fruit following sandpaper abrasion were longer by 10-15 days than that following puncture wound (all with $P<0.001$).

Likewise, the time course of blue mould development following sandpaper abrasion and inoculation was also longer than that of a puncture wound (Table 6.8), regardless of treatment. Blue mould development was delayed by 6-8 days for both controls, and by 8-9 days for all citral-treatments (all with $P<0.001$).

These results generally showed that fumigation of fruit with citral following sandpaper abrasion and inoculation with *P. italicum* at 5°C was slower in terms of

the appearance of the first symptoms, the 100% incidence and the development of blue mould than for fruit inoculated following puncture wound. This was also observed on the fruit inoculated with *P. digitatum* for the 100% incidence and the development of green mould.

6.3.6. SPME-GC analysis of headspace citral concentration in box

Based on the calibration curve constructed from headspace concentrations of citral (sum of geranial and neral) at -18°C, 0°C, 22°C and 40°C (Appendix 1), the headspace citral concentration generated from 2, 6 and 15% citral aqueous solutions in each box was calculated and is shown in Table 6.9. In general, average headspace concentration increased with temperature and the initial amount of citral in the box. The headspace citral concentration at room temperature was higher than that at 5°C. Furthermore, the distribution of volatile citral in the boxes appeared not to be uniform. The headspace citral concentration was greater at the top position in the box (above the fruit) than that at the bottom position (below the fruit) (Figure 6.2). At 5°C, the headspace citral concentration generated from 2% and 6% citral measured above the fruit was approximately 10 and 13 times higher than that measured below the fruit respectively. However, the headspace citral concentration generated from 15% citral was only about 34 % greater above than below the fruit. At room temperature, the headspace citral concentration generated from 2, 6 and 15% citral at the top position in the box was about three to five times greater than that at the bottom position.

Table 6.7 Comparison on the effect of wounding methods on green mould incidence for fruit inoculated with *P. digitatum* at 5°C

Treatments	Mean value (days), of time								
	For the first symptoms		P value	For 100% green mould incidence		P value	Course for green mould development (Δ)		P value
	Wounding method			Wounding method			Wounding method		
	Puncture	Abrasion		Puncture	Abrasion		Puncture	Abrasion	
Control in open boxes	23.38	27.02	0.013	30.34	39.81	< 0.001	6.96	12.8	0.003
Control in closed boxes	17.05	16.99	0.923	26.17	30.65	< 0.001	9.12	13.66	< 0.001
2% citral	17.38	18.24	0.426	30.16	32.48	< 0.001	12.78	14.24	0.200
6% citral	19.33	15.36	0.001	28.78	32.47	< 0.001	9.46	17.11	< 0.001
15% citral	19.39	13.99	< 0.001	28.79	31.97	< 0.001	9.39	17.98	< 0.001

Note: Time course of green mould development (Δ) is the time difference between the appearance of first symptoms and 100% disease incidence

Table 6.8 Comparison on the effect of wounding methods on blue mould incidence for fruit inoculated with *P. italicum* at 5°C

Treatments	Mean value (days), of time								
	For the first symptoms		P value	For 100% blue mould incidence		P value	Course of blue mould development (Δ)		P value
	Wounding method			Wounding method			Wounding method		
	Puncture	Abrasion		Puncture	Abrasion		Puncture	Abrasion	
Control in open boxes	19.68	29.82	< 0.001	27.4	44.16	< 0.001	7.72	14.35	< 0.001
Control in closed boxes	14.66	15.9	0.041	23.67	32.94	< 0.001	9.01	17.04	< 0.001
2% citral	16.13	19.36	< 0.001	24.52	35.28	< 0.001	8.39	15.93	< 0.001
6% citral	16.59	20.8	0.001	24.36	38.17	< 0.001	7.77	17.37	< 0.001
15% citral	16.55	21.33	< 0.001	25.22	39.57	< 0.001	8.67	18.24	< 0.001

Note: Time course of blue mould development (Δ) is the time difference between the appearance of first symptoms and 100% disease incidence

Table 6.9 Headspace citral (sum of geranial and neral) concentration in 5-L plastic boxes containing six fruit and citral-soaked pads. Samples were taken 2 h after placing pads and fruit in the box for fruit stored at 5°C and after 3 h for fruit stored at 22°C

Treatment at		Headspace citral concentration, µL L^{-1}	
		5°C	22°C
2% citral	Top position	[X]4,806 ± 200	40,087 ± 963
	Bottom position	477 ± 14	8,604 ± 267
	Average	2,642 ± 93	24,346 ± 347
6% citral	Top position	9,777 ± 382	80,057 ± 1455
	Bottom position	754 ± 24	27,199 ± 943
	Average	5,266 ± 203	53,628 ± 255
15% citral	Top position	27,275 ± 237	100,540 ± 2753
	Bottom position	20,407 ± 667	31,210 ± 1128
	Average	23,841 ± 214	65,875 ± 813

Note: [X]Data shown represent the mean values of two replicate measurements of two boxes ± standard error of mean

6.3.7. Residue analysis

Exposing fruit to the highest citral concentration of 15% in boxes at room temperature for 2 weeks resulted in no measurable residue nor did untreated fruit yield detectable residue (Table 6.10). Both the peel oil extracts of the citral-treated oranges and control revealed that only limonene was detectable in significant amounts with the analysis conditions used. Neither geranial nor neral was detected. A representative GC chromatogram of peel oil extract of oranges exposed to 15% citral stored in closed box for 2 weeks at 22°C, control oranges stored in closed box for 2 weeks at 22°C, and citral is presented in Appendix 2.

As citral was not detected in the peel in the experimental treatments at 22°C, the measurement of citral in peel oil of fruit stored at 5°C, where citral has lower volatility, was considered unnecessary.

Table 6.10 Representative chromatogram data of residue analysis of peel extract of fruit stored for 2 weeks at room temperature

Sample	Limonene, %	Geranial, %	Neral, %
Citral	undetected	59.9	30.4
Peel oil of control fruit	65.2	undetected	undetected
Peel oil of treated fruit	88.9	undetected	undetected

6.4. Discussion

6.4.1. Disease development on control fruit

The time to the first appearance of disease symptoms and 100% disease incidence on inoculated fruit not exposed to citral was generally less for fruit incubated at room temperature (22°C) than for those incubated at 5°C, irrespective of the fungus with which fruit were inoculated. Keeping fruit in open boxes at 22°C extended the time to first appearance of disease symptoms by 1-3 days for all of the three fungi compared with that for control fruit in closed boxes. This effect was more marked at 5°C, where appearance of symptoms was extended by 5-24 days compared with that for control fruit in closed boxes, with sour rot symptoms taking longest to appear.

In addition, keeping control fruit in open boxes at 22°C extended the time to 100% disease incidence by 1-2 days compared with that for control fruit in closed boxes, while storage at 5°C extended this by 3-80 days, and again the longest delay was observed for sour rot. The delay in disease development on fruit in open boxes is likely to reflect retarded growth of the pathogens at lower humidity compared to fruit in closed boxes (Pitt and Hocking, 1997). High humidity is known to promote germination of spores of the three pathogens (Sommer *et al.*, 1992; Plaza *et al.*, 2003). This suggestion is supported by the observation that closed boxes containing fruit kept at room temperature showed significantly greater RH than the atmosphere of the room, while at 5°C, the difference was relatively small (Table 6.1), reflecting a higher fruit respiration rate at room temperature which would produce more water vapour than that at 5°C (Kader, 1992).

The Δ value of control fruit reflected the time course of disease development. No difference in Δ value was observed between the two controls both at 22°C and 5°C for green and blue mould development. Likewise, at room temperature, the time course of sour rot development observed for the two controls also similar. This suggested that, in regard to the time course of disease development, results for fumigation in a closed system (simulated by individual storage boxes) might be similar to when fruit are stored in an open system.

At 5°C, brown discolouration around the wound site was observed for control fruit in closed boxes (Figure 6.4A), a phenomenon not observed in citral-treated fruit (Figure 6.4B) nor in control fruit in open boxes. The symptoms were observed

visually and no attempt was made to use microscopy to investigate this further. However, as these symptoms were observed only at low temperature (5°C) this may be related to chilling injury, which generally increases as temperature decreases below 10°C (Cohen *et al.*, 2000; Petracek, 2000; Katina, 2007). As the brown discolouration was not observed on citral-treated fruit, fumigation with citral might have prevented development of this symptom. Further research is needed to elucidate if and how fumigation with citral might affect this symptom, which might be related to chilling injury. Other postharvest treatments have been reported to reduce the development of chilling injury, for example; thiabendazole reduced the incidence of chilling injury on oranges and grapefruit (Schirra and Mulas, 1995; Schirra *et al.*, 2000), and methyl jasmonate reduced the incidence of chilling injury of grapefruit (Mier *et al.*, 1996).

6.4.2. Effect of volatile citral on disease development on fruit

Fumigation with citral delayed the development of sour rot on fruit inoculated with *G. citri-aurantii* by 7-10 days, and the 100% disease incidence was delayed by 10-11 days at room temperature, whereas at 5°C, fumigation with citral delayed the onset of spoilage by 12-20 days, the 100% disease incidence by 14-154 days, and the sour rot development by 21-124 days. The effectiveness of citral in delaying development of sour rot reflected earlier *in vitro* experiments in this study in which volatile citral inhibited *G. citri-aurantii* (Chapter 4 and Chapter 5).

Sour rot is not controlled by imazalil and thiabendazole (Smilanick *et al*., 2005; Liu *et al*., 2009), and is mainly controlled by fungicides containing guazatine, a chemical for which some countries apply limitations in imported fruit (Cunningham and Taverner, 2000; SARDI, 2001). Furthermore, current practice to control sour rot spoilage is through sanitation (Mercier and Smilanick, 2005; Cunningham and Taverner, 2007) which indicates a need to identify fungicides and other strategies capable of controlling sour rot (Droby, 2009; Taverner, 2009; Cunningham, 2010). Therefore, the use of citral may have potential to control sour rot spoilage, and is worthy of further investigation.

Fumigation of fruit with citral failed to delay development of green mould at room temperature (22^{o}C), whereas at 5^{o}C, the onset of spoilage was delayed slightly (2 days) when fruit were fumigated with 6 and 15% citral, and 100% green mould incidence was delayed by 3-4 days at all three concentrations tested. The lack of delay of spoilage due to green mould at 22°C is supported by the nature of *P. digitatum*, which grows well at warmer temperatures (Domsch *et al.*, 1980; Wardowski and Brown, 1993; Pitt and Hocking, 1997).

Fumigation of fruit with citral failed to delay development of blue mould consistently at 5^{o}C. At room temperature, fumigation of fruit with citral only delayed the development of blue mould by 1 day when citral was applied at high concentration (15%) to fruit inoculated with *P. italicum*. This observation is supported by results of *in vitro* experiments in this study, in which *P. italicum* was inhibited only in the presence of high citral concentration (Chapter 4). Droby *et al.*

(2008) also reported that the growth of *P. italicum* was inhibited only at citral concentration of 15 µL per plate, which is similar to the 15% citral concentration in this study.

On punctured fruit, the inoculated pathogens would be present inside the wound. Therefore, citral needs to have penetrated sufficiently into the peel to reach the pathogens to be effective at inhibiting germination. Roistacher *et al.* (1958) reported that green mould penetrated approximately 1 mm into the wound-inoculated fruit rind in 24-30 h when the fruit was incubated at 20°C after inoculation. In addition, study of the effect of ozone exposure on the development of green and blue moulds on artificially inoculated oranges showed that inhibition of germination of both *P. digitatum* and *P. italicum* on the fruit was clearly related to ozone penetration into fruit (Palou *et al*., 2003). Moreover, puncturing fruit with a nail to a depth of 3 mm might provide more nutrients for the pathogens and may favour their growth. However, sandpaper-abrasion of fruit probably causes the rupture of oil glands to release phytotoxic oils (Knight, 2002; Klieber and Knight 2000), including citral, that might retard the growth of the pathogens. In addition, pathogens inoculated onto sandpaper abrasions would be present closer to the surface and this may allow increased contact with headspace citral. Hao *et al.* (2010) in their study using tea saponin to control *P. digitatum, P. italicum* and *G. citri-aurantii*, found that tea saponins were more effective when applied to naturally infected mandarin where the pathogens were present naturally in the surface than to puncture-wounded mandarin. Indeed, this study showed that citral was more effective in protecting from green mould and blue mould fruit inoculated following sandpaper abrasion than fruit

inoculated following nail puncture. This suggested that the method of fumigation applied in this study did not allow volatile citral to reach and inhibit germination of pathogens present inside the wound. However, as a puncture test is the standard method for assessing any commercial application (Eckert and Brown, 1986; Taverner and Cunningham, 2010), further research or modifications to the system are required to ensure volatile citral reaches the pathogens inside the wound.

Temperature, vapour pressure, and other gases or volatiles present in the system may affect the volatility of citral which, in turn, may affect the efficacy of citral to control the diseases. As noted above, wounded fruit may release gases to the atmosphere of the box and limonene, ethylene, ethanol, CO_2 and acetaldehyde were identified by GC as the major volatile compounds around wounded oranges (Eckert and Ratnayake, 1994; and Droby *et al.*, 2008). Limonene, a major component of citrus rind oil (Moufida and Marzouk, 2003; Sawamura *et al*., 2005; Ladaniya, 2008), was the only component detectable in significant amount in peel oil extracts of the citral-treated oranges and controls in this study (Table 6.10). Limonene was shown to induce germination and growth in *P. digitatum* and *P. italicum*; and even at low concentrations (≤6 ppm) a significant stimulation was observed (Droby *et al*., 2008). Stimulation of growth of *Penicillium* species by limonene might explain why the first incidence of green mould and blue mould symptoms in fruit fumigated with 2% citral (the lowest concentration of treatments) did not differ from control fruit placed in closed boxes, both at room temperature and 5°C (Arimoto *et al*., 1995; Droby *et al*., 2008).

Injured or mouldy oranges produce ethylene (Waks *et al*., 1985), and during decay there is an increase in respiration and heat production, so that more ethylene is produced by the fruit and the fungus. Wounded tissue and decaying has been reported to enhance ethylene evolution (Barkai-Golan, 1990). Fruit infected by *P. digitatum* and *P. italicum* released ethylene both from the fungi and the fruit (Marcos, 2005). El-Kazzaz (1983) reported that ethylene in air at concentration of 1-1000 ppm stimulated germination of *P. digitatum* and *P. italicum.* Ethylene also affects citral content on fruit flavedo which in turn affect its antifungal activity. Ben-Yehoshua *et al.* (1995) reported that treatment with ethylene at 25 and 50 ppm for 3 days at 25°C reduced the citral content and antifungal activity in the flavedo of both lemon and grapefruit. The increased fruit senescence may have caused flavedo to release its citral content to the atmosphere. These studies suggested that ethylene released by infected fruit may have not only stimulated germination of *P. digitatum* and *P. italicum* (El-Kazzaz, 1983 and Marcos, 2005) but also reduced internal citral content of fruit (Ben-Yehoshua *et al.*, 1995; Knight *et al.*, 2001) leading to decreased resistance to disease.

To avoid the possible antifungal effects of carbon dioxide produced by fruit respiration, in this experiment, carbon dioxide was removed with calcium hydroxide (Sitton and Patterson, 1992; Tian *et al*., 2002). Calcium hydroxide may have not only captured carbon dioxide but also volatile citral from the atmosphere in the box, which would have decreased the volatile citral concentration and, in turn, would decrease its ability to control disease (S. Ben-Yehoshua, pers. comm., 2000). Chemically, either calcium hydroxide or carbon dioxide may react with citral due to

their oxygen being negatively polarised and may act as nucleophiles. As citral is a member of the α, β-unsaturated aldehyde class, due to their position, the α– and β-carbons are conjugated with the carbonyl group, making the β-carbon more positively polarised and able to react easily with nucleophiles (nucleophilic attack) (Roger *et al.*, 1994).

6.4.3. Effect of citral fumigation on fruit

Citral phytotoxicity and citral residue on the rind may have resulted from fumigation of fruit with citral. In all experiments, symptoms of phytotoxicity appeared on some fruit at positions near the top pad in the box, or where the fruit was in direct contact with the side pads in boxes containing 6 and 15% citral treatments. That the phytotoxic effect was only observed at those positions may be because the citral concentration at that position was higher (Table 6.9). In support of this suggestion, the phytotoxicity appeared earlier at 22°C than at 5°C, and the headspace citral concentration was higher at 22°C than at 5°C (Table 6.9). Ben-Yehoshua *et al*. (1992) found that dipping fruit in 1% citral emulsion reduced the decay of inoculated fruit from 100% to 9%, but resulted in phytotoxic damage to the peel, and Knight (2002) reported that 15 μL of pure citral in 6 mm cardboard discs attached to the surface of Washington Navel oranges damaged the rind and caused oil glands to collapse. These findings suggest that phytotoxicity to the rind is related to high citral concentration. Therefore the phytotoxicity observed in this study was considered to be a limitation of the fumigation method applied, especially with 15% citral treatment. Ensuring homogeneous distribution of volatile citral throughout the

system is necessary in order to avoid phytotoxicity. Further research is needed to develop methods to provide uniform distribution of volatile citral in the system.

The lack of measurable residue of citral in the rind of oranges exposed to 15% citral for 2 weeks may be related to the analytical conditions used or to the physiological condition of the fruit peel surface. This suggests that little citral penetrated the peel during volatile exposure, perhaps due to insufficient amounts of volatile citral present in the box atmosphere, insufficient vapour pressure of volatile citral to penetrate the peel, or due to lack of peel absorptive ability. This may cause lack of direct contact between volatile citral and the pathogens, since the pathogens are usually present inside the peel (in inoculated fruit following both puncture and sandpaper abrasion).

To improve control using citral, further work or modifications to the experimental method are warranted in order to provide sufficient contact between volatile citral and the pathogens inside peel wounds. Modifications may include shorter period fumigation immediately after packing house operations with a higher citral concentration using a circulation fan, or continuous fumigation with citral at low concentration.

6.5. Summary

Fumigation with citral delayed the 100% incidence and development of spoilage of wounded oranges by *G. citri-aurantii* both at room temperature (22°C) and at 5°C,

suggesting that this fungus is sensitive to citral. This result supported findings of the *in vitro* experiments in this study (Chapter 4 and Chapter 5).

Fumigation of fruit with 15% citral at room temperature (22^{o}C) delayed the development of blue mould slightly, but failed to delay development of green mould. At 5^{o}C, fumigation of fruit with citral delayed the onset and of green mould 100% incidence slightly, however failed to delay the development of blue mould.

The method of wounding prior to inoculation appeared to affect the efficacy of volatile citral in delaying the development of disease. Fumigation of fruit with citral appeared to be more effective in protecting fruit wounded by abrasion where the pathogens were present near the fruit surface.

Symptoms of phytotoxicity were observed on some fruit at positions where citral concentration was high, and were observed earlier at 22°C than at 5°C. The phytotoxicity effect was limited to fruit fumigated with high citral concentration. Citral residue was not detected in oranges irrespective of treatment with citral, suggesting little citral penetrated into the peel. This may explain why citral was more effective in protecting fruit wounded superficially by abrasion than more deeply by puncture.

In conclusion, citral may offer a means to control development of sour rot (*G. citri-aurantii*), and this aspect requires further investigation.

Chapter Seven

General discussion

7.1. Research findings

This study has explored the potential of citral as an antifungal agent in the control of three major pathogens of citrus and the diseases they cause: *Penicillium digitatum* (blue mould), *P. italicum* (green mould) and *Geotrichum citri-aurantii* (sour rot). In particular, the aims of this study were achieved as follows:

i) Of three methods of *in vitro* application of citral tested, exposure to volatile citral gave the best performance.

ii) Exposing the pathogens to the vapours of citral isomers and related compounds showed that the vapour of citral, geranial and neral (generated from 15% aqueous solutions) inhibited spore germination and growth of all three pathogens. Citral and geranial at 15% were fungicidal to *G. citri-aurantii* and *P. digitatum*, and fungistatic to *P. italicum*, while neral at 15% was fungicidal to *G. citri-aurantii* and fungistatic to the other two fungi. Furthermore, the inhibitory effect of citronellal was greater than citronellol, which in turn was more effective than citronellic acid.

iii) Fumigation with citral delayed the development of sour rot and the time for all fruit to show symptoms both at room temperature (22°C) and at 5°C. At room temperature, fumigation of fruit with 15% citral delayed the development of blue mould slightly, but failed to delay development of green mould. At 5°C, fumigation of fruit with citral delayed the onset of green

mould and the time for all fruit to show symptoms slightly, however failed to delay the development of blue mould.

7.2. Implications of research findings

Postharvest diseases of citrus have been controlled primarily by the application of conventional fungicides, including carbendazim, imazalil, thiabendazole or mixtures of these compounds (Palou *et al*., 2008). However, concerns about fungicide resistance, effects of fungicide residues on human health and the environment, and restrictions on the use of certain fungicides, have led to the need to explore alternative methods for the control of postharvest diseases of citrus fruit, including the use of essential oils such as citral. To have potential for commercial adoption, non-conventional treatments must be effective at low concentrations and easily fit within current protocols (Cunningham, 2010).

Several studies have investigated both *in vitro* and *in vivo* antifungal activity of essential oils against the pathogens used in this study. Plaza *et al.* (2004) reported that, *in vitro*, thyme, oregano, clove and cinnamon essential oils completely inhibited growth of *P. digitatum* and *P. italicum* either when added into the medium at 0.1% or by vapours generated from 10 μL of the oil in Petri dishes. In *in vivo* experiments, direct application of 15 μL of thyme and cinnamon essential oils at a concentration of 5% to inoculated wounds on 'Salustiana' and 'Valencia' oranges significantly reduced the incidence of green and blue moulds after 7 days storage at 20ºC. However, when these essential oils were combined with citrus wax at 0.1 –

5%, or incorporated into packaging using foam trays soaked with 10 – 500 μL pure essential oils, they failed to reduce the incidence of green and blue moulds and caused severe damage to the rind of treated fruit (Plaza *et al.* 2004). This suggests that the method of application affects the efficacy of essential oils in controlling green and blue moulds. The application of thyme oil has also been shown to control the growth of *G. citri-aurantii* effectively at a concentration of 0.06% *in vitro* while 30 μL of a 0.32% thyme oil solution applied directly to inoculated wounds of 'Satsuma' mandarin effectively reduced sour rot during storage at 20°C (Liu *et al.*, 2009). The treatment did not cause rind damage to the fruit when they were examined after storage at 20°C for 30 days. These two studies (Plaza *et al.*, 2004; Liu *et al.*, 2009) showed that thyme oil may have different phytotoxicity to different citrus cultivars. In addition, Plaza *et al.* (2004) and Liu *et al.* (2009) used a higher concentration of the essential oil to evaluate efficacy on fruit than *in vitro*. The present study also provided evidence that the method of application affects the efficacy of citral in controlling pathogens *in vitro*. However, the concentration of citral used *in vitro* and *in vivo* was similar, and phytotoxicity was observed on fumigation of 'Navel' oranges with citral at relatively high concentration (6% and 15%). The phytotoxicity may be related to high concentration and uneven distribution of volatile citral in the system, as observed from the citral headspace concentration in this study (Chapter 6). Since these experiments were conducted, it has been reported that application of citral aqueous emulsion in a formulation with stabiliser (e.g. 10 – 50% ethanol or > 0.1%Tween20), 0.05 – 0.8% antioxidant (e.g. ascorbic acid, α-tocoferol or β-carotene) and 5 – 100 ppm imazalil to *Penicillium* – inoculated lemons did not cause phytotoxicity to the fruit (Ben-Yehoshua and

Rodov, 2006; Ben-Yehoshua and Kiryat-Ono, 2008). Therefore, it may be possible to formulate citral to minimise or prevent phytotoxicity to fruit. Further research is required to examine the efficacy of citral in controlling the pathogens *in vivo* by using various citrus cultivars, methods and citral formulations.

Recently, the need to identify new fungicides and other strategies capable of controlling sour rot has been identified (Droby, 2009; Taverner, 2009; Cunningham, 2010), as sour rot is not controlled by currently used fungicides (Smilanick *et al*., 2005; Liu *et al*., 2009). This study showed that irrespective of the application method, citral at concentrations of 6% and 15% consistently inhibited germination and growth of *G. citri-aurantii in vitro*. Fumigation with 6% citral delayed sour rot development on wounded oranges inoculated with *G. citri-aurantii* by 10 days at room temperature (22°C) and 124 days at 5°C; and the time for all fruit to spoil was delayed by 11 days at room temperature and 154 days at 5°C (Chapter 6). Therefore, findings reported in the present study provide preliminary information on the potential use of citral for postharvest management of sour rot.

However, for green mould and blue mould, the results obtained *in vivo* only partially confirmed those obtained *in vitro*, as fumigation with citral failed to delay consistently the development of green mould and blue mould. The efficacy of citral in controlling the pathogens *in vivo* might be related to the way in which citral contacts the pathogens. To be effective as a fumigant, vapour of citral has to penetrate into the fruit peel and be absorbed by moist tissues of the injured peel to ensure the peel is an unsuitable substrate for germination of the pathogens. In the

present study, the spores may have been protected by their position inside the peel at the wound site, where nutrients were available for their germination and growth. Citral was not detected in fruit peel exposed to 15% citral in boxes at room temperature for 2 weeks, which suggested that lack of citral accumulation in the peel at the inoculation site may have affected its ability to inhibit the growth of the pathogens. Meanwhile, this study found that at room temperature, citral breakdown quickly; decreased its content and produced geranic and neric acids (Chapter 5), which may explain for undetectable citral in the fruit peel. Geranic acid and neric acid has been reported to exhibit weak antifungal activity toward *P. digitatum* and *A. Apis* (Wolken *et al.*, 2002; Gallardo *et al.*, 2008). However, activity of geranic and neric acids toward the pathogens was not examined in this study; further study to assess its effect to the pathogens is required. Additionally, the fumigation method applied in this study was more effective in inhibiting the growth of pathogens on the surface of fruit than inside the fruit. These observations suggest that this method may be more effective in preventing initial infection than in limiting colonisation after tissue is infected. Nevertheless, the results from the *in vitro* study provide a basis for further examination of the efficacy of citral in controlling these diseases, although other methods of application to fruit need to be examined.

Combination treatments involving the use of fungicides with hot water dips or sprays, or incorporating fungicides into wax or coating treatments are being used in citrus packing-house operations and this is likely to increase (Cunningham, 2010), especially where effectiveness of the treatment is enhanced due to additive effects or synergistic interactions. Likewise, citral treatment alone may not be enough to

control disease, therefore combining application of citral with hot water treatment or wax may enhance its effectiveness in controlling disease.

Application of heated solutions of fungicides as dips and sprays has been effective against citrus postharvest pathogens (Rodov *et al.*, 1995, Schirra *et al.*, 1997). Citrus fruit tolerates hot water up to 60°C for brief periods (Palou *et al*., 2008), and hot water treatment to control decay is often applied before storage because the target pathogens are found on the surface of fruit (Fallik, 2004). In order to control rot caused by *Penicillium* or *Alternaria* spp., immersion of citrus fruit in water at 50–53ºC for 2–3 minutes has been recommended (Schirra *et al.*, 1997; Nafussi *et al.*, 2001). As citral is insoluble in water, addition of emulsifier such as TritonX or Tween 80 would be necessary in the preparation of aqueous citral solution. The addition of Tween 80 has been reported to decrease citral degradation in the aqueous phase appreciably (Choi *et al*., 2010). Furthermore, the solubility of citral may be enhanced when heated, and this would improve contact between citral and pathogens. Heating citral to 53 to 60°C in a quite short time (12–20 minutes) has been shown to increase its antimicrobial effect, for example in inactivating (i) *Escherichia coli* BJ4 *in vitro* after heating for 20 minutes (Somolinos *et al.*, 2010), (ii) a wild strain of *Saccharomyces cerevisiae* in an orange-based soft drink after heating for 15 minutes (Belletti *et al.*, 2010) and (iii) *Listeria innocua* (surrogate for *Listeria monocytogenes*) in orange juice after heating for up to 12 minutes (Char *et al.*, 2010). Given that citral has a boiling point of 229^{o}C (Hawley, 1981), heating citral solution to 61ºC is unlikely to affect its stability. Furthermore, even though citral was degraded during storage (Chapter 5), combining citral with heat treatment

up to 61ºC for a short time period may be effective. As dipping is commonly used in commercial packing-houses, non-conventional heat treatments could be readily applied by installing heating equipment in dipping and recirculating tanks (Cunningham, 2010).

Wax coating of fruit has been shown to extend the postharvest quality by limiting gas exchange and reducing water loss, skin discolouration and fruit deterioration (Hagenmaier and Baker, 1993). Spraying wax supplemented with fungicide such as imazalil has been used in commercial citrus packing-houses (Smilanick *et al*., 1997; Taverner *et al*., 2008; Droby, 2009). Taverner *et al*. (2008) reported that the application of wax supplemented with imazalil at 2000 ppm to 'Late Lane' navel oranges wounded and inoculated with *P. digitatum* reduced green mould incidence to less than 20%. Likewise, incorporation of essential oil into wax coating has resulted in reduced disease incidence, weight loss and respiration rate of fruit; for example, 'Valencia' oranges coated with *trans*-jojoba oil in wax at 20-30% (Ahmed *et al*., 2007), and 'Tomago' oranges wounded and dipped in suspension of spores of *P. digitatum* then sprayed with carnauba wax supplemented with the essential oil of *Lippia scaberrima* at 2500 μLL^{-1} (DuPlooy *et al.*, 2009). When incorporated in wax or another coating, the activity of citral may be similar to that when incorporated into agar, in terms of contact with the pathogens and minimising dissipation of citral from the system. As evident in the present study, citral completely inhibited the growth of the pathogens at concentrations as low as 3% when incorporated into agar (Chapter 4), therefore, the effect of incorporating citral into wax or another coating treatment should be investigated. This would bring citral into direct contact with the

fruit surface and minimise the dissipation which, in turn, may retain its stability and inhibitory effects. Furthermore, preventing sporulation of the pathogens on the fruit surface is also important so that soiling and contamination of the adjacent fruit during shipment and marketing is also reduced (Kanetis *et al*., 2007).

This study suggested that the inhibitory activity of citral against the pathogens was affected by its concentration. *In vitro*, at 15%, vapour of citral, geranial and neral completely inhibited spore germination and growth of the three pathogens. Vapour of citral and geranial was fungicidal to *P. digitatum* and *G. citri-aurantii*, and fungistatic to *P. italicum*, whereas vapour of neral was fungicidal to *G. citri-aurantii* and fungistatic to the other two pathogens. Essential oils, acting as typical lipophiles, have been suggested to disrupt the cell wall and membranes of prokaryotes (Bakkali *et al*., 2008; Fisher and Philip, 2008) and fungi (Kalemba and Kunicka, 2003), increasing cytoplasmic membrane fluidity and permeability, disturb the order of membrane embedded proteins, inhibit cell respiration, and alter ion transport processes leading to lysis (Sikkema *et al*., 1994; Reichling *et al*., 2009). Cellular processes in the pathogen may also be affected by the ability of the α– and β-carbons of citral to function as direct-alkylating agents that affect sulphydryl and amino groups of proteins (Witz, 1989; Andersen *et al.*, 1994). However, the mechanism by which citral inhibits the germination and growth of the pathogens examined in this study is not clear. Citral exhibited different inhibitory effect toward the three pathogens; this may relate to the protein content of the cell wall of the pathogens. As noted in Chapter 5, the protein content of the cell wall of *G. citri-aurantii* was greater than that of *P. digitatum*, which was greater than *P. italicum*

(Grisaro *et al.*, 1968; Sietsma and Wouters, 1971). Further investigation of the mode of action of citral in inhibiting the growth of the pathogens is needed.

As noted in Chapter 3, the boiling point of geranial and neral differed only slightly (i.e. 77°C for geranial and 76.5°C for neral), suggesting that separation of geranial and neral from citral using gas chromatographic methods may be difficult. For the purposes of the experiments conducted in the present study, geranial and neral were synthesised through oxidation of geraniol and nerol using an unpublished method provided by Marko (pers. comm., 2000). This oxidation resulted in high purity products (94% pure geranial and 92% pure neral, respectively) with the solvent used during synthesis (CH_2Cl_2) being the main impurity observed. The method also appeared to be efficient and economical because its only by-product was water, thus alleviating problems associated with the costs of separation and purification of the product (Chapter 5). Synthesis of geranial and neral through oxidation of geraniol and nerol has been reported by other researchers; however, their methods required separation and purification of the products. For example, More and Finney (2002) synthesised geranial through the oxidation of geraniol with *O*-iodoxybenzoic acid (IBX) which gave a yield of 95%, but the product then had to be purified and separated from the IBX by-products by filtration. Recently, Park *et al*. (2007), who synthesised geranial and neral through oxidation of geraniol and nerol with pyridinium dichromate (PDC) oxidation, achieved 85.8% pure geranial (the main impurity was neral at 12.1%) and 75.4% pure neral (the main impurity was geranial at 21.8%), suggesting that further separation of the products was required.

Furthermore, this study introduced a rapid method to measure headspace concentration of volatile compounds in Petri dishes and in boxes using SPME-GC (Chapters 4 and 6). This simple step allowed direct extraction of vapours which were then measured by GC (Chapters 3, 4 and 6). This method could also be used to measure headspace concentrations of other volatile compounds in various types of containers, provided that a suitable fibre is available.

In summary, this study provides information as a basis to develop citral as an alternative to synthetic chemical fungicides for protecting citrus fruits from spoilage due to *G. citri-aurantii,* and perhaps also by *P. digitatum* and *P. italicum.* Citral, as a natural volatile with a lemon aroma, is considered as a 'generally recognised as safe' (GRAS) compound and, as such, is likely to be acceptable to consumers.

7.3. Future research

With regard to the results obtained and the limitations observed in this study, some areas for future research directed to development of citral as an alternative to synthetic chemical fungicides in protecting citrus fruits from *P. digitatum*, *P. italicum* and *G. citri-aurantii* are as follows:

1. Phytotoxicity of citral to fruit appeared to be dependent on the way in which citral came into contact with the fruit and on citral concentration. Phytotoxicity may be avoided by ensuring a homogeneous distribution of volatile citral in the fumigation chamber; therefore, modification of the method used in this study might be a consideration for further research. For example, continuous

fumigation using a lower citral concentration than applied in this study, with the addition of a circulation fan to allow homogeneous distribution of volatile citral in the system and gas chamber equipped with regulator to maintain constant citral concentration over time, might be options for further investigation. Another possible modification that might be considered is a shorter fumigation period using higher citral concentrations than applied in this study to facilitate a maximum concentration in the early stages of treatment to induce an immediate effect (Inouye *et al.,* 2002). Again, this should be done with the use of a circulation fan to allow homogeneous distribution of volatile citral in the system and ensure that volatile citral reaches pathogens inside the wound.

2. Reducing inoculum levels in the air in the packing-house and on the fruit surface through effective sanitation should be considered to minimise the likelihood of disease (Palou *et al*. 2008). In this context, a combination of citral with a heated dip or spray in the packing-house operation should be further researched.
3. Appropriate practices during handling and storage of fruit in order to prevent infection as well as sustaining quality are also important in postharvest disease management (Palou *et al*. 2008). Therefore the incorporation of citral in wax or coating should be investigated to maintain citral in contact with the fruit peel to exert a continuous inhibitory effect on pathogens. Furthermore, the aroma of citral may enhance the sensory quality of the oranges.
4. Finally, in order to provide more information about the suitability of the fumigation method used in this study in preventing spoilage of citrus fruit due to *P. digitatum, P. italicum* and *G. citri-aurantii,* experiments should be conducted

with naturally infected fruit. This would allow assessment not only of the effects of citral application on disease incidence but also the effect of treatment on fruit quality parameters such as acidity, flavour and aroma and sensory evaluation for visual and organoleptic attributes of fruit.

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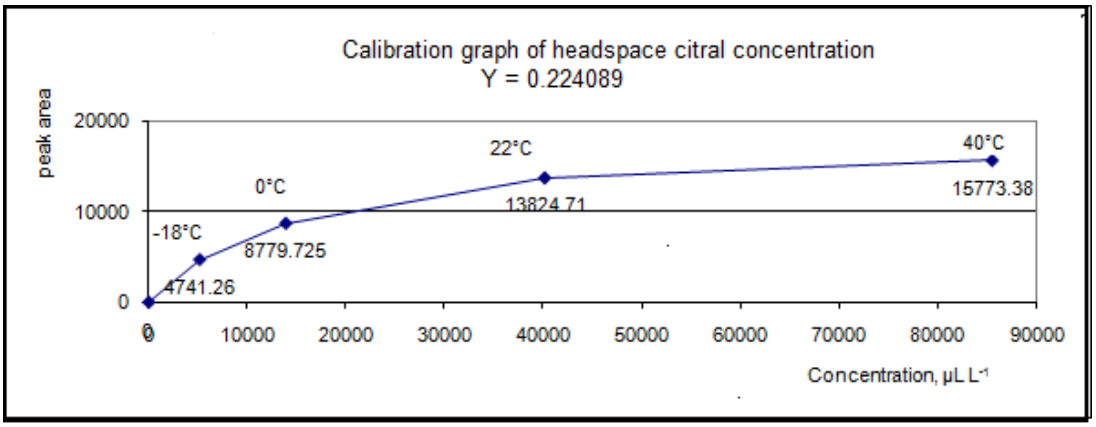
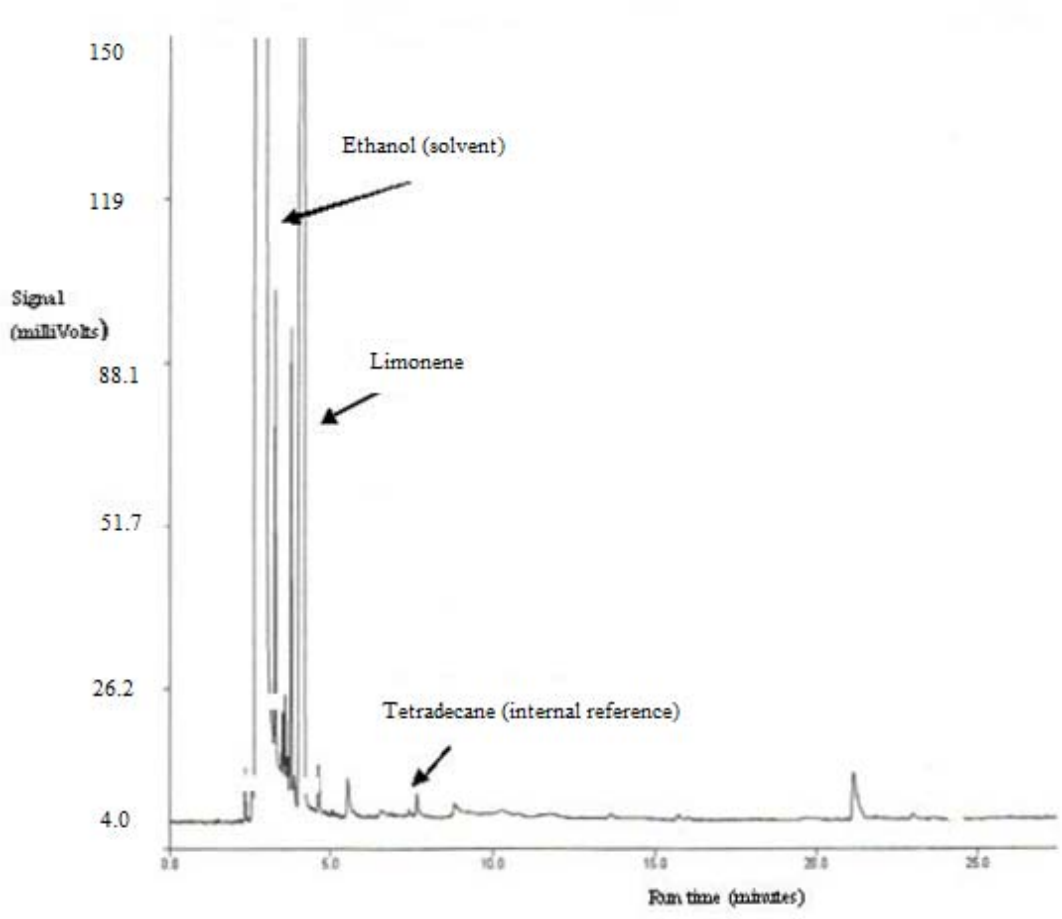
Appendix 1

Calibration curve constructed from headspace concentrations of citral (sum of geranial and neral) at -18°C, 0°C, 22°C and 40°C.

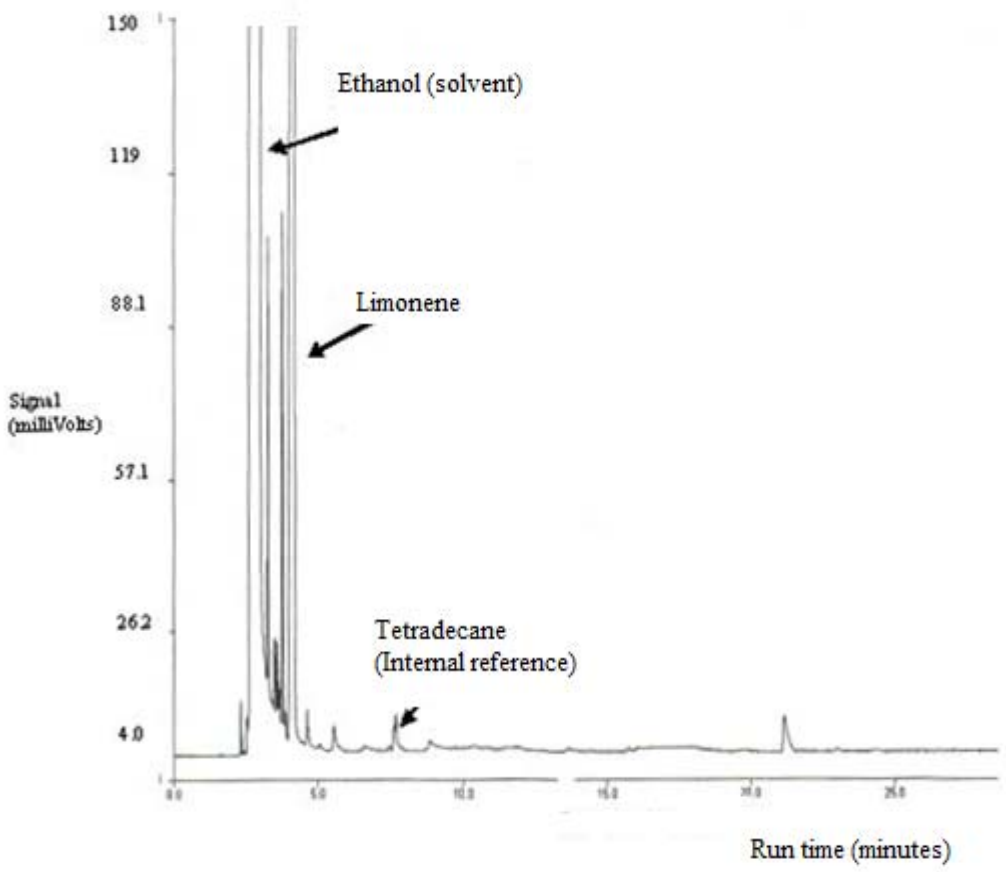


Appendix 2

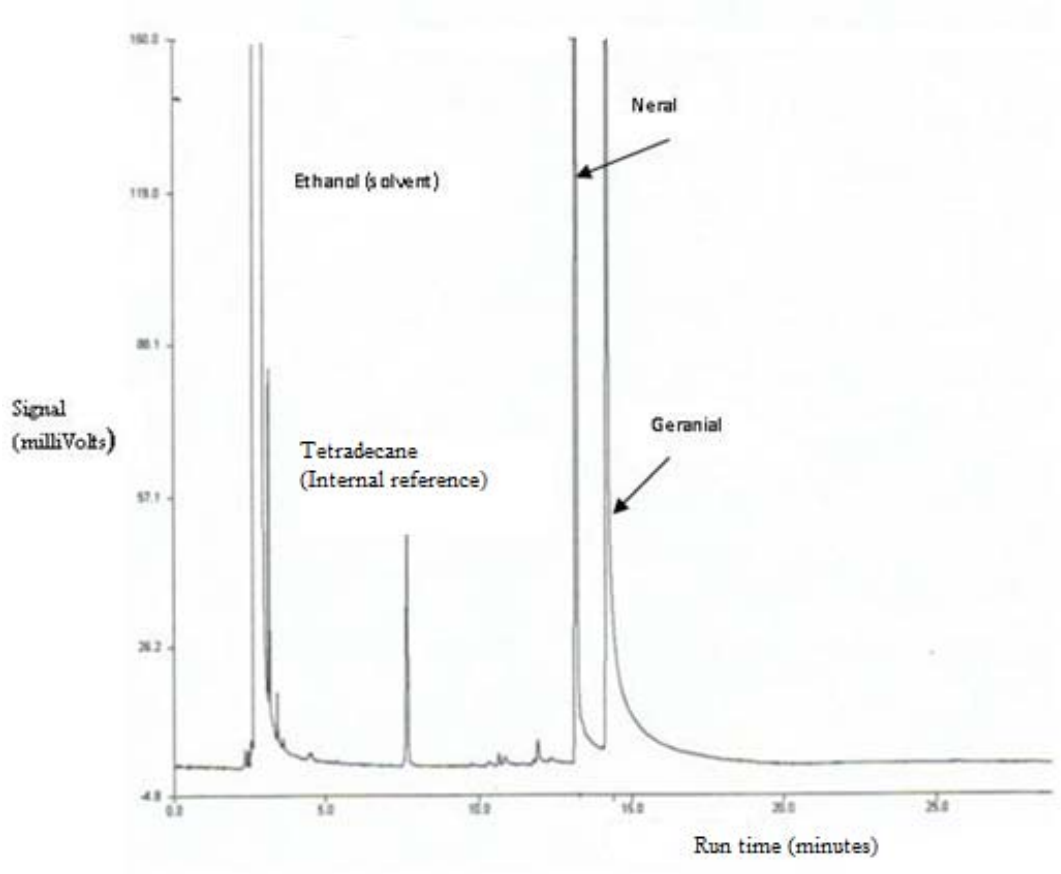
Representative GC chromatograms in 2% (v/v) ethanol and 2% (v/v) tetradecane as an internal standard



Representative GC chromatogram of peel oil extract of oranges exposed to 15% citral stored in closed box for 2 weeks at 22°C



Representative GC chromatogram of control oranges stored in closed box for 2 weeks at 22°C.



Representative GC chromatogram of citral

Appendix 3

Publications and conference proceeding produced during PhD candidature

Wuryatmo., E., Klieber, A. and Scott, E. (2001). 'The Effect of Citral Components on Citrus Postharvest Spoilage Fungi in Culture', in *Proceedings of Australasian Postharvest Horticulture Conference, Adelaide, September 23-27, 2001*.

Klieber, A., Scott, E. and Wuryatmo, E. (2002) Effect of method of application on antifungal efficacy of citral against postharvest spoilage fungi of citrus in culture. *Australasian Plant Pathology, v.31 (4), pp. 329-332, December 2002*

Wuryatmo, E., Klieber, A. and Scott, E. (2003) Inhibition of Citrus Postharvest Pathogens by Vapor of Citral and Related Compounds in Culture.
Journal of Agricultural and Food Chemistry, v.51 (9), pp. 2637-2640, April 2003