Assessment of ethanol, honey, milk and essential oils as potential postharvest treatments of New Zealand grown fruit

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Statement of Originality

‘I hereby declare that this submission is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person (except where explicitly defined in the acknowledgements), nor material which to a substantial extent has been submitted for the award of any other degree or diploma of a university or other institution of higher learning.’

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Abstract

Brown and *Penicillium* rot (blue and green mould) are the most common postharvest diseases in New Zealand, causing significant postharvest fruit losses. Current practice uses fungicides to control the postharvest diseases; however there are perceived health risks associated with the use of such chemicals. Recently, there has been substantial interest in chemicals that are considered Generally Regarded as Safe or GRAS and natural products as alternative postharvest treatments to replace currently used fungicides.

In this study, ethanol (GRAS chemical) and the natural products honey, milk and essential oils (lemon, lemongrass, manuka and orange) were assessed as potential alternative treatments to replace the currently used fungicides on both peaches and oranges. In pilot studies ethanol was applied to the fruit by either vapour or dipping (30 seconds or 1.5 minutes). Honey, milk and essential oils were applied by dipping at 30 seconds. Essential oils were also tested using a microtiter assay.

Exposing fruit to ethanol vapour proved effective at inhibiting fungal growth, but impacted negatively on fruit quality. Peaches that were exposed to 70% to 100% ethanol vapour were protected against fungal infection for up to 30 days when stored at either 4ºC or room temperature. This is compared to two days for untreated peaches and three days for fungicide –treated peaches. However, the ethanol-treated peaches suffered from severe browning. In contrast, 20% ethanol protected peaches for ten days when stored at 4ºC and two days at room temperature. The fruit that were exposed to 20% ethanol did not brown as a result of the treatment. Oranges that were exposed to 20%, 50%, 70% and 100% ethanol vapour were protected from fungal inhibition for 30 days at both 4ºC and room temperature, but they too suffered from severe browning.

Dipping was not as effective as vapour at protecting against fungal infection, but had a little effect on fruit quality. Peaches dipped in 20% to 100% ethanol were completely rotten by ten days when stored at room temperature, but the peaches experienced little to no browning. Untreated and fungicide-treated fruit were protected for one day and two days, respectively.

Milk and honey do not appear to have potential as postharvest treatments. Peaches that were treated with 20%, 50% and 100% whole milk and 50% manuka honey showed greater degree of fungal infection compared to untreated peaches after both room temperature and 4ºC storage. At room temperature, peaches that were exposed to 20%, 50% and 100% milk were completely rotten at eight days, compared with ten days for untreated peaches. In contrast, at 4ºC, peaches that were treated with 100% milk were completely rotten at 30 days, while only a slight fungal infection observed on untreated fruit. Similar to milk, honey-treated peaches were also completely rotten at 30 days at 4ºC storage.

I*n vitro* (microtiter) assay of the essential oils showed that orange and manuka oils appeared to be effective only at high concentrations. In contrast, lemongrass and lemon oils appeared to be effective even at low concentrations. Of the essential oils tested in the *in vivo* assay, lemongrass and lemon oils have the greatest potential. Oranges that were exposed to 0.05% lemongrass oil, 0.25% and 0.5% lemon oil were protected for 30 days when stored at 4ºC or room temperature. They provided the best antifungal activity compared to the other concentrations of all four essential oils tested as well as fungicide treatment for 30 days.

Of all the treatment tested, 0.05% lemongrass oil, 0.25% and 0.5% lemon oil appeared to be the most promising treatments. However, these treatments need to be tested for antifungal effects, fruit quality, flavour and nutritional effects in large scale experiments before they can be applied as replacements to currently used fungicides. Also, essential oils are complex compounds; therefore it would be of interest to determine the active compound(s) of the lemongrass and lemon oils.

Table of Contents

Methods and Materials

Results

Discussion

Introduction

Fruit are high-value commodities that are vulnerable to disease during the postharvest period. Postharvest diseases limit the storage life of fruit and cause significant economic losses for fruit exporting industries worldwide. Estimated losses due to postharvest diseases are 5 to 10% with application of fungicides or greater than 50% without application of fungicide) (Lurie *et al.*, 1995).

In New Zealand, brown rot caused by *Monilinia fructicola* is a major postharvest fungal pathogen. It occurs regularly on stone fruit, causing significant problems for producers. It causes tree damage and both pre- and postharvest fruit losses, especially in peaches (Tate and Wood, 2000).

Both green mould and blue mould, also known as *Penicillium* rots, are caused by different species of *Penicillium*. They are one of the most common postharvest diseases, especially on citrus fruits. They cause up to 90% of citrus fruit losses in transit, storage and after sales (Agrios, 2005).

Several good packaging methods such as controlled atmosphere packaging and vacuum packaging have been found to be effective in delaying fruit from ripening and thereby slowing down the development of pathogens (Mari and Guizzardi, 1998).

Application of synthetic fungicides is the usual practice to control pre and postharvest disease; however, there are limitations. Synthetic fungicides have often proven ineffective in the long-term as the pathogens often develop resistance to the products (Agrios, 2005). Additionally, synthetic fungicides have been limited with public perception of residues in fruit and food products that may potentially be harmful and caused side effects on humans (Lingk, 1991; Unnikrishnan & Nath, 2002 as quoted in Sharma and Tripathi, 2006).

Due to these perceived health risks associated with the use of such chemicals, there is an increasing public concern on their use as postharvest treatments. Consequently, scientists have been prompted to find alternative treatments.

Recently, there has been increased interest in biological control agents, chemicals that are considered Generally Regarded as Safe or GRAS (Litcher *et al.*, 2002; Chervin, 2005) and natural products (e.g. essential oils) as alternative treatments (Jobling, 2000).

The aim of this project was to discover effective, safe and economical treatments to reduce the number of postharvest fruit losses in New Zealand. Ethanol, a GRAS chemical was used as a non-biological agent to treat stone and citrus fruit. This study also involved the assessment of the natural/biological products: honey, milk and essential oils. They were assessed as replacement(s) for the currently used, potentially hazardous chemicals and fungicides.

1.1 Ascomycota

Ascomycota or “sac fungi” comprise approximately 30,000 described species, including a number of familiar and economically important fungi. They are present in a wide range of environments, such as soil, dung and marine and fresh waters. Many grow as saprophytes on dead plants and animals (Sharma, 2005). Others are plant pathogens (Sharma, 2005), including blue, green and brown mould fruit diseases (*Penicillium spp.* and *Monilinia spp.*), (Taylor *et al.*, 1996). The yeast, *Saccharomyces cerevisiae*, also belongs to the Ascomycota (Taylor *et al.*, 1996).

Ascomycota are normally distinguished by their vegetative state (unicellular or septate hyphae), cell wall and mode of asexual and sexual reproduction (Sharma, 2005).

Sexual reproduction in Ascomycota involves the formation of an “ascus” (plural: asci) i.e. a sac-like cell in which two matched haploid nuclei of different mating types come together and fuse to form a diploid nucleus, followed by meiosis to produce haploid sexual spores called ascospores (Deacon, 2006). Ascus formation usually occurs within a fruiting body composed of tightly interwoven hyphae called an "ascocarp". Most ascocarps are macroscopic.

An ascocarp may be open and more or less cup-shaped ("apothecium", Fig.1), closed and spherical in shape (“cleistothecium", Fig.1), or flask-shaped, with a small pore through which the ascospores escape ("perithecium", Fig.1). The layer of asci within the ascocarp is called the "hymenium", or hymeneal layer (Simmons, 2006a).

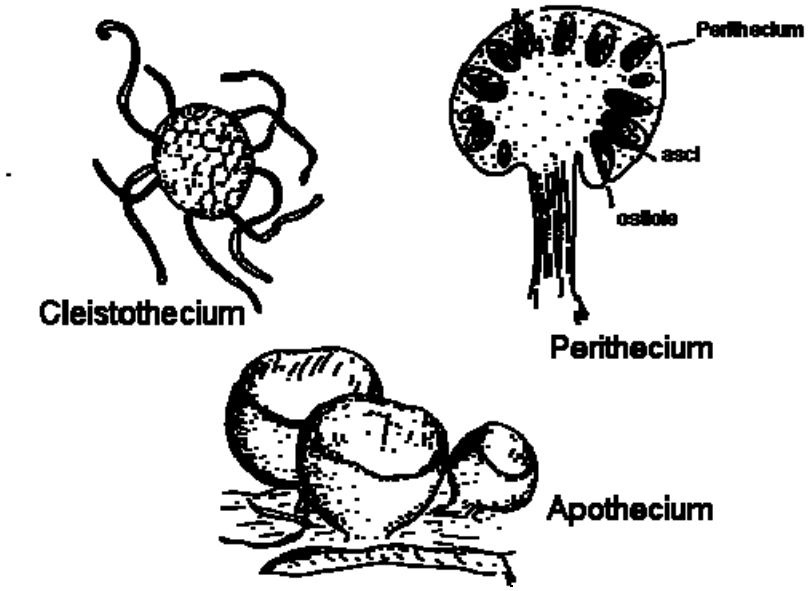


Figure 1. Some typical ascocarps found in the division Ascomycota (taken from Simmons, 2006a).

In most Ascomycetes, an ascus is formed as a result of the fertilisation of the female sex cell, called the ascogonium, which then fuses with the multinucleate male cell, the antheridium (Fig. 2) (Agrios, 2005; Simmons, 2006a).

Sexual reproduction (Fig. 2) begins with plasmogamy, the union of two protoplasts bringing the opposite nuclei close together within the same cell (Sharma, 2005). The nuclei which migrated in from the antheridium pair up with the nuclei of the ascogonium during plasmogamy, but they do not fuse immediately (Sumbali, 2005).

Ascogenous hyphae then start to grow out from the ascogonium (Sumbali, 2005) and the pairs of nuclei travel into the ascogenous hyphae, leading to simultaneous mitotic divisions in both hyphae and ascogonium (Simmons, 2006a). These mitotic divisions produce "dikaryotic cells" (i.e. contain two haploid nuclei, one from each strain). The dikaryotic hyphae grow together to form an ascocarp. Asci are formed at the tip of each developing dikaryotic hypha (Agrios, 2005). The two nuclei in the ascus of the dikaryotic hypha fuse and become a diploid nucleus. The ascus then elongates and the diploid nucleus divides by meiosis, forming four haploid nuclei (Simmons, 2006a). Most of the time, the haploid nucleus from the meiotic division usually divides again by mitosis, resulting in a total of eight haploid nuclei. These haploid nuclei are then cut off in segments of the cytoplasm to form ascospores (Fig. 2) (Sumbali, 2005; Deacon, 2006).

The majority of Ascomycota also reproduce asexually by mitosis (Fig. 2). Typically asexual reproduction occurs by the formation of specialised spores, known as conidia (singular conidium) or conidiospores, which are cut off from the tips of modified hyphae called conidiophores (Simmons, 2006a). These conidia are genetically identical to the parent, and the number of nuclei present can be more than one. They are also called “mitospores” due to the way they are generated through the cellular process of mitosis (Deacon, 2006). These “mitospores” are very common and function in dispersal (Deacon, 2006).

In general, asexual reproduction occurs in fungi mostly when nutrients and water are abundant while sexual reproduction occurs when nutrients or water becomes scarce (Johnson, 2006).

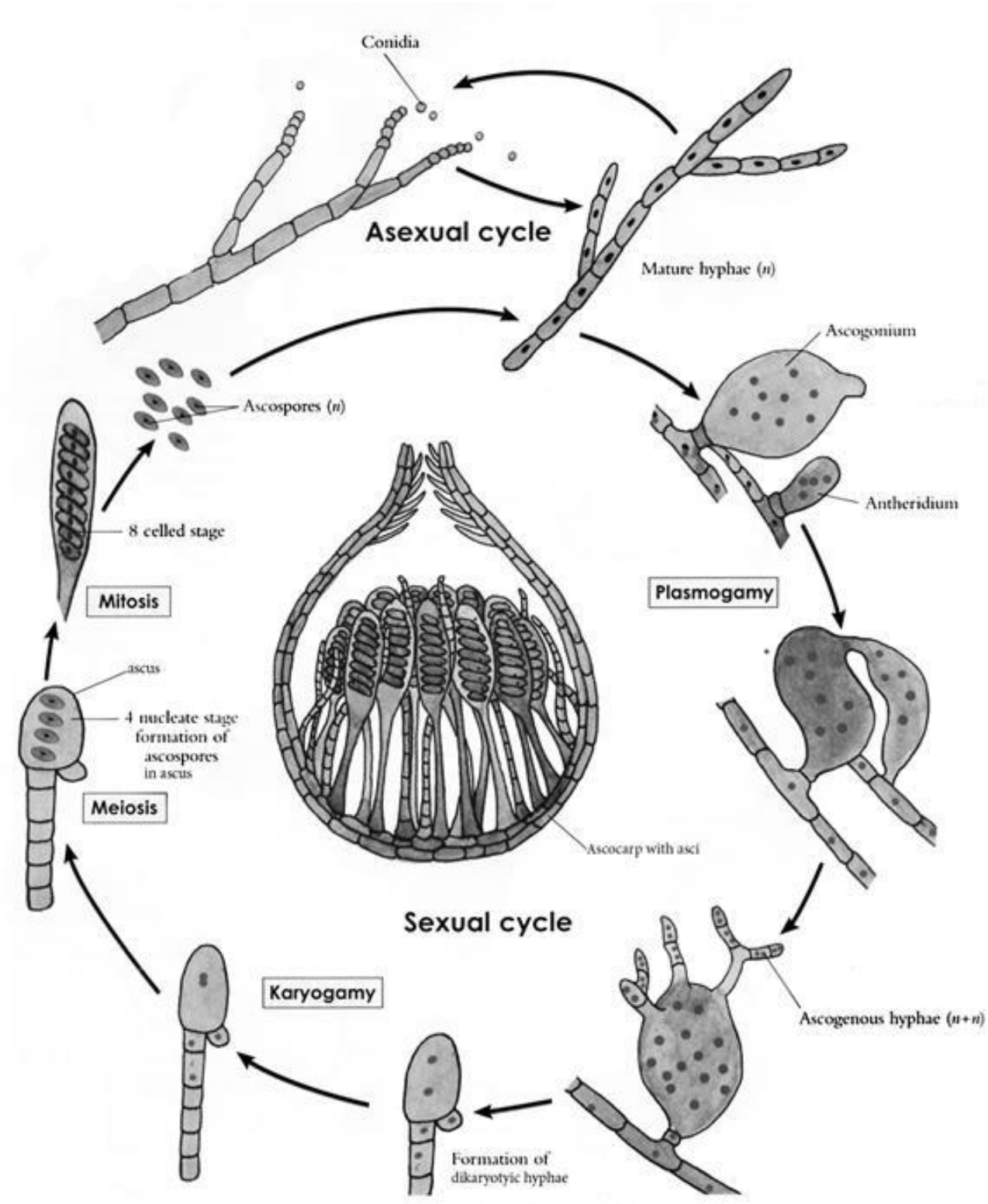


Figure 2. The life cycle of Ascomycota (taken from Simmons, 2006b)

1.2 Postharvest disease: Brown rot

Brown rot disease causes severe pre- and postharvest losses of stone fruits worldwide, affecting peaches, cherries, plums, apricots and almonds (Agrios 2005). Elmer *et al.* (2002) did a study on brown rot incidence in nectarine and peach orchards in New Zealand. They found losses in individual orchards could be very severe, ranging from 0 to 92% with an average of 37%. In Central Otago, more than 50% of cultivars of peach, nectarine and plum trees die within the first five years without any application of fungicide (McLaren and Fraser, 1994). Significant losses have been reported in North American of peaches, cherries and plums (Biggs, 1997). Peach losses of one million AUD occurred in 1969 in the Murrumbidgee area, Australia (European and Mediterranean Plant Protection Organisation, 2006), and heavy losses have also been reported of apricots in Tasmania (Smith *et al.*, 1992). Up to 50 to 75% losses from brown rot occur during fruit rotting in the orchard, but serious losses (25-50%) may also appear during transit and marketing of the fruit (Agrios, 2005).

1.2.1. The fungal pathogen: *Monilinia fructicola*

Monilinia fructicola belongs to the group Ascomycota and the family of *Sclerotiniaceae* (Batra, 1991). The life cycle of Ascomycota is shown in Figure 2. In *M. fructicola*, asexual reproduction occurs by formation of conidia (Fig. 3) on hyphal branches and arranged in sporodochia (tuft). The fungus also produces spermatia (microconidia) on a chain of condiophores. The spermatia may produce new mycelia and are also involved in sexual reproduction (Fig. 4) (Sharma, 2005). The sexual stage in *M. fructicola* results in formation of an apothecium that forms in mummified fruit buried partly or wholly in the soil (Fig. 4) (Agrios, 2005). More than 20 apothecia may form on one mummy. The inside or upper surface of the apothecium is lined with thousands of asci (Fig. 4). Each ascus contains eight single-celled spores (Agrios, 2005).

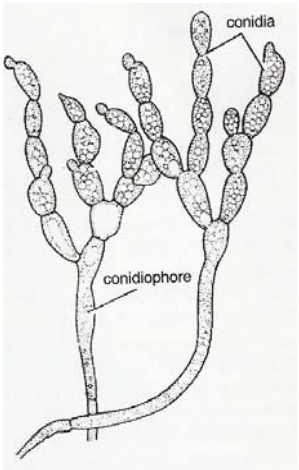


Figure 3. Reproductive structure of *M. fructicola* (taken from Sharma, 2005)

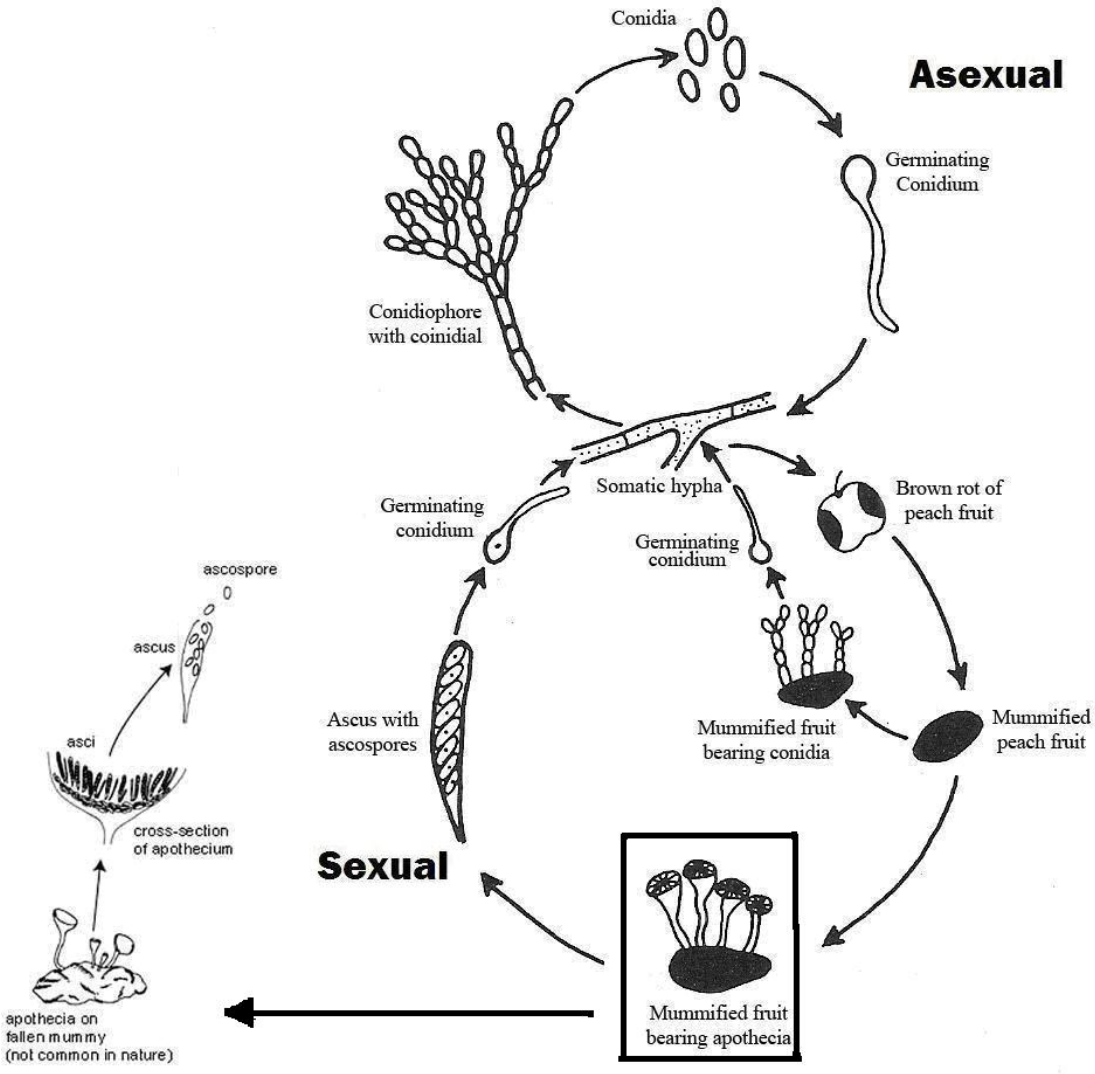


Figure 4. Disease cycle of brown rot of stone fruits caused by *M. fructicola* (taken from Sumbali, 2005). For clarity, a cross section of an apothecium on mummified fruit is shown at the bottom left (Ritchie, 2006).

1.2.2. Disease cycle and symptoms

Typical symptoms of brown rot induced by *M. fructicola* are blossom and twig blight (Fig. 5 A), cankers (Fig. 5 B) and fruit rot (Fig. 5 C), (Biggs, 1997). *M. fructicola* overwinters as mycelia in mummified fruit on the tree (Fig 5 D) or in cankers of affected twigs or rarely as pseudosclerotia (dark masses of melanised fungal and host tissue) in mummies on the ground. During winter and early spring, the mycelia in mummified fruit on the tree and in twig cankers produces new conidia (Sumbali, 2005), whereas the pseudosclerotia produce apothecia, which later produce asci and ascospores (Fig. 4), (Agrios, 2005).

The first indication of the disease is the rapid death of the blossom and may involve the entire flower and its stem. Blossom blight (Fig. 5 A) may be caused by infection from apothecia or from the conidia that were dispersed by wind, rain, water splash or insects and germinate rapidly under favourable conditions (Sumbali, 2005). Disease incidence and severity are strongly dependent on temperature and wetness duration (Agrios, 2005). In humid conditions, the infected flowers produce many greyish-brown conidial tufts, and later shrivel and dry up with the rotting mass adhering to the twigs (Agrios, 2005).

Following blossom, the mycelia can spread rapidly into the flower peduncles and leaf petioles and into the fruit spurs and the twigs, where a reddish-brown canker forms (Fig. 5 B). Shoot blight symptoms will occur if the fungus encircles the infected shoot which has leaves that turn brown and remain adhered for several weeks (Biggs, 1997). The cankers that form appear as brownish, sunken areas that are soon covered with conidial tufts. The conidia act as inoculum for fruit infection later in the season when the fruit begin to ripen (Sumbali, 2005).

Insects, twig punctures or hail can be important as vectors of the fungus during fruit ripening (Biggs, 1997). In some cases the conidia can penetrate through stomata or directly through the cuticle. The fungus grows intercellularly at first and secretes enzymes, causing maceration and browning of the infected tissue (Agrios, 2005).

Brown rot on fruit (Fig. 5 C) begins as small, circular, brown spots that spread rapidly from the lesion throughout the entire fruit (Sumbali, 2005). It then proceeds with the production of masses of buff-coloured tufts of conidia which break through the skin of the infected areas and appear on the fruit surface (Pscheidt, 2006). One large or several small rotten areas may be present on the fruit, which can become completely rotten within a few days. It then either remains hanging on the tree or falls to the ground (Ritchie, 2006). Fruit on the ground can disintegrate through the action of saprophytic fungi and bacteria. Infected fruit on the tree lose moisture, shrivel and become dry, distorted mummies (Fig. 5 D) which serves as a source of mycelium and apothecia to begin the cycle again (Fig. 4), (Agrios, 2005).

Fruit infection can also take place after harvest during storage, transit and marketing. Infected fruit continue to rot after harvest while the healthy fruit can be attacked by direct contact with the infected fruit (Agrios, 2005)

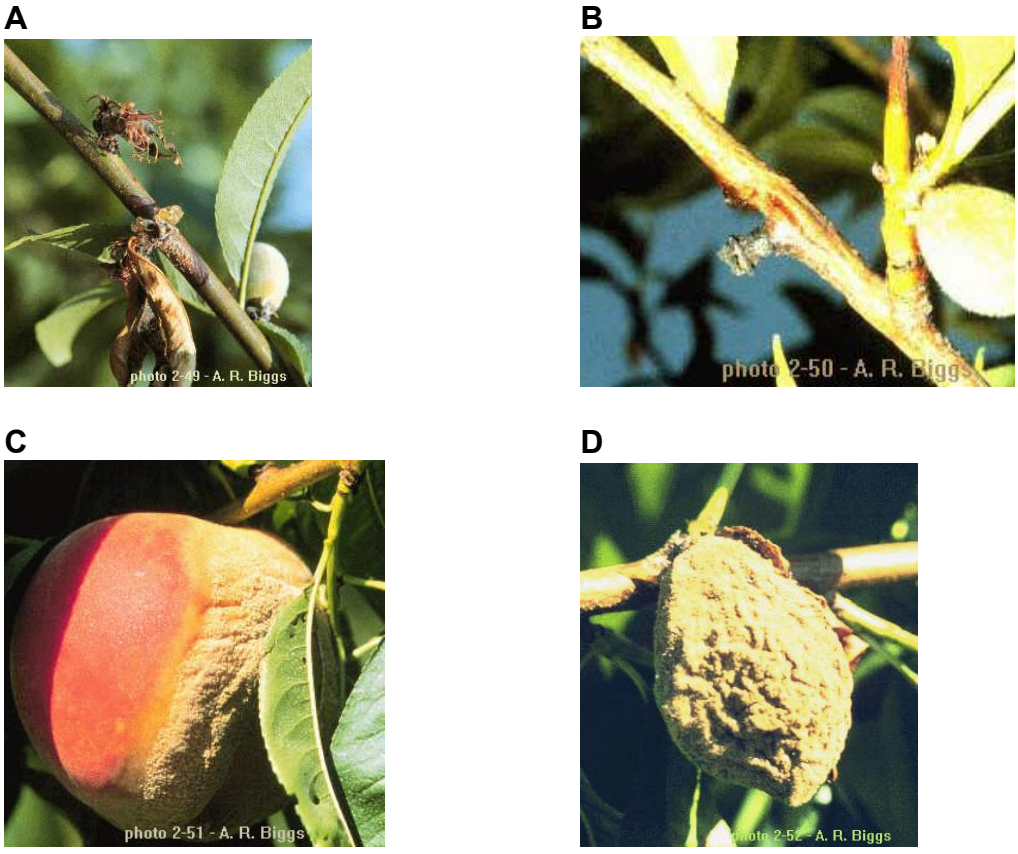


Figure 5. Different symptoms caused by *M.* fructicola. **A)** Blossom and twig blight **B)** Cankers **C)** Fruit rot **D)** Mummified fruit (Photos taken from Biggs, 1997).

1.2.3. Control

Brown rot can be controlled by controlling the blossom blight phase of the disease. This is normally done by chemical spraying several times during the growing season (Agrios, 2005). Several fungicides such as vinclozolin (Brackmann *et al.*, 1994), iprodione and triforine (Harman and Beever, 1987) and bitertanol (Takamura and Ochiai, 1989) have been reported to be very effective against the fungus (Smith *et al.*, 1992). However, several chemical applications during the growing period may lead to a build up of fungicide resistance in *M. fructicola*. Studies in New Zealand showed that some strains of *M. fructicola* are resistant to most of the commonly used fungicides (Elmer and Gaunt, 1986). These include the first major systemic fungicides, the benzimidazoles with “Benlate” as the forerunner (Beresford, 1994).

The dicarboximide fungicides, which include chlozolinate (Serinal), iprodione (Rovral), procymidone (Sumisclex) and vinclozolin (Ronilan), have activity against a limited number of fungi contained in nine genera (Elmer and Gaunt, 1986). These dicarboximide fungicides are the standard fungicides applied to control brown rot in stone fruits. They have low phytotoxicity and high fungicidal activity (Beresford, 1994).

Dicarboximide resistance has been most studied in New Zealand, especially for *Botrytris spp.* (Beresford, 1994) and *M.fructicola* (Elmer and Gaunt, 1986). When the use of dicarboximides stops, the fungal resistance frequency decreases. It was reported that loss of disease control has been more obvious in glasshouse situations than in the outdoors, but there is evidence that high resistance is associated with a loss of disease control. It was also reported that there is cross resistance among all the dicarboximide fungicides (Beresford, 1994).

According to Ministry of Agriculture and Forestry (MAF) Policy Technical Paper (Holland and Rahman, 1999), the use of dicarboximide fungicide in New Zealand has declined due to the increase in fungal resistance as a result, scientists have been working towards alternative treatments.

1.3 Postharvest disease: *Penicillium* rots

Blue and green moulds, also known as *Penicillium* rots, are common postharvest diseases of citrus (Agrios, 2005). It was estimated that green mould recently destroyed 5% of fresh citrus fruit in the USA, amounting to an annual loss of $USD 30 – 50 million (KES Science and Technology Inc., 2006). It was found that without fungicides, the sales of citrus in Florida would be reduced by at least 50%, resulting in a minimum of $USD 250 million reduction in sales (Ismail and Zhang, 2004).

1.3.1. The fungal pathogen: *Penicillium spp.*

Penicillium spp. belongs to the group Ascomycota and the family of *Eurotiaceae* (Talbot, 1971). *Penicillium* is one of the most common genera of fungi present in the air (Sharma, 2005). The genus, *Penicillium*, is ubiquitous and most of the species are saprophytes (Talbot, 1971). The mycelia consist of septate and highly branched hyphae. The conidiophore in *Penicillium* is simple, erect, long, and branches at the apex to form a brush-like structure (Fig. 6) known as the "penicillus" (Sumbali, 2005; Carlile *et al.*, 2001). Each branch of the conidiophore ends in a group of phialides that bear long conidial chains (Sumbali, 2005). The penicillus is known as "monoverticillate" when the conidiophore ends in a spiral of phialides and "biverticillate" if the branching takes place at two (or sometimes more) levels at the apex of the conidiophore before the level of phialides is reached (Talbot, 1971).

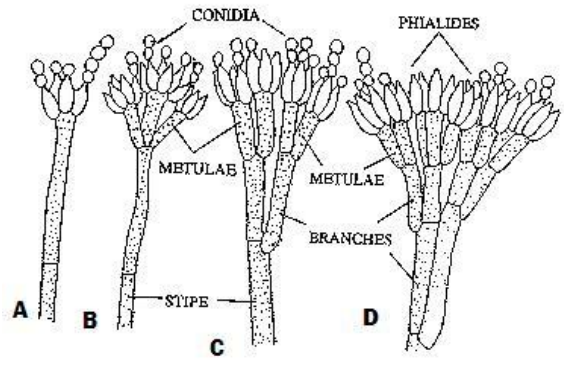


Figure 6. Morphological structures and types of conidiophore branching in *Penicillium*. **A)** Simple; **B)** One-stage branched; **C)** Two-stage branched; **D)** Three-stage branched (Samson *et al.*, 1984 as found in Ellis, 2006).

Sexual reproduction in most of the *Penicillium* species does not occur (Sumbali, 2005). Those that do undergo sexual reproduction, have been placed in two genera of *Eurotiaceae*, i.e. *Eupenicillium* and *Talaromyces* (Carlile *et al.*, 2001; Sumbali, 2005; Talbot, 1971). Sexual reproduction in some species of these genera occurs by functional gametangia, whereas in most of the species, the antheridia are not functional (Sumbali, 2005). Both *Penicillium digitatum* and *Penicillium italicum* belong to *Eupenicillium* (Department of Environment and Heritage, 2005).

In *Eupenicillium*, the cleistothecia (i.e. close spherical ascocarp, Fig. 1) are yellow or light brown (Sumbali, 2005). It begins as a sclerotium-like mass of thick-walled tissue that reaches a definite size and then starts forming ascogenous hyphae and asci from the centre out (Talbot, 1971). The asci form on short branches of the ascogenous hyphae (Sumbali, 2005).

In *Talaromyces*, the cleistothecia are colourless or yellow. The cleistothecial wall is a loose thread of hyphae and the cleistothecium grow continuously even after the first ascospores have reached maturity (Talbot, 1971). The ascospores are unicellular and they are released by the decay of cleistothecial wall, germinate by germ tube and eventually grow into a mycelium (Sumbali, 2005).

1.3.2. Disease cycle and symptoms

Penicillium enters tissues through wounds, but it can also spread from infected fruit by contact with healthy fruit through the uninjured skin (Agrios, 2005). Under humid conditions, the initial symptoms of *Penicillium* rots is the appearance of a soft, watery, slightly discoloured spot of varying size (approximately 0.5 cm-1.5 cm in diameter) on any part of the fruit (Olsen *et al.*, 2000). The discoloured spots enlarge from 2.5 cm to 5 cm in diameter after 1 to 2 days at 25 °C (Olsen *et al.*, 2000). Soon after, white mycelia appear on the surface of the fruit, near the centre of the spot and start producing blue or olive green spores. Soon, the entire fruit surface is rapidly covered with the spores, which are easily spread if the fruit is handled or exposed to air currents (Olsen *et al.,* 2000).

In cool conditions, surface mould is rare, even when fruit interior is completely decayed. Decayed fruit has a musty odour and it may shrink and become mummified in dry conditions. In humid conditions, secondary fungi or yeasts also enter fruit, which is then reduced to a wet, soft mass (Agrios, 2005).

The information regarding the disease cycle of *Penicillium spp.* is more limited than *M. fructicola*. In citrus fruit, green mould is caused by *P. digitatum*, whereas blue mould is caused by *P. italicum*. These species of *Penicillium* are ubiquitous to all citrus growing regions (Brown, 2003a). The spores of these fungi are airborne and extensively produced from infected fruit due to bad harvesting technique or rough handling (Zhang and Swingle 2005). The extensive spore production enhances contamination during packing until the time of consumption (Ismail and Zhang, 2004).

Blue mould disease (Fig. 7 A) is relatively less vigorous compared with green mould in the United State of America (Ismail and Zhang 2004). Blue mould is able to develop slowly at cold temperature, hence it is more common in cold conditions. At room temperature, green mould infects fruit much more rapidly than blue mould (Brown 2003b).

Fruit infected with green mould (Fig. 7 B) produces quite a large amount of ethylene gas which promotes respiration, senescence and premature colour development. *P. digitatum* can cause a condition known as “soilage” when masses of spores from infected fruit are dispersed to the surface of healthy fruit.

The extensive spore production ability of this fungus enables it to rapidly develop strains with resistance to chemical fungicides (Brown, 2003a).

A **B**

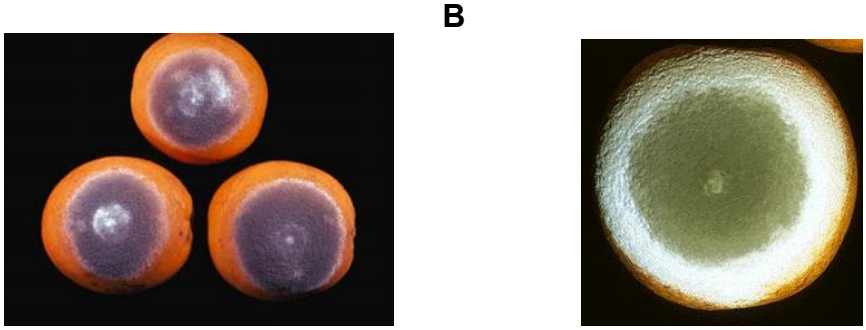


Figure 7. *Penicillium* rots on oranges. **A)** Blue mould (on oranges **)** caused by *P. italicum* (Brown, 2003b). **B)** Green mould (on an orange) caused by *P. digitatum* (Brown, 2003a)

1.3.3. Control

Green and blue mould can be prevented by careful harvesting and handling to minimise the risk of injury of the fruit (Brown, 2003a and b). Pre-harvest fungicide can reduce the incidence of green and blue mould. In most packing houses, citrus fruits are treated with sodium *o*-phenylphenate, imazalil or thiabendazole to control decay (Holmes and Eckert, 1999).

Thiabendazole is a systemic benzimidazole fungicide used to control a variety of fruit and vegetable moulds, blights, rots and stains caused by various fungi (Extension Toxicology Network, 1993). Thiabendazole was first registered as a pesticide in the U.S. in 1969 by Merck and Company, Inc. (Environmental Protection Agency, 2002). It was registered as a dip or spray on citrus fruits, apples, pears, bananas, mangos, papaya, plantain, carrots, avocados, peas, and potatoes (Environmental Protection Agency, 2002).

o-Phenylphenol, also known as 2-phenylphenol, is an organic compound that consists of two linked benzene rings and a phenolic hydroxyl group. It is a biocide used as a preservative under the trade names Dowicide, Torsite, Preventol, Nipacide and many others. *o*-Phenylphenol is primarily used as a fungicide, for example in citrus fruit (Wikipedia, 2006a).

Thiabendazole and *o*-phenylphenol have been used regularly on citrus fruits over the past 3 decades, resulting in a serious resistance problem to these two fungicides (Holmes and Eckert, 1999). The resistance is due to the large population of resistant *Penicillium spp* biotypes. Besides the resistance, high doses of these two compounds also cause effects on the cardiovascular system, gastrointestinal tract, kidney, liver and lungs (Environmental Protection Agency, 2002). However, even at the recommended doses, concern remains.

Applications of *o*-phenylphenol and thiabendazole have been reduced in New Zealand because of their associated health problems (Holland and Rahman, 1999). Recently, scientists have been working towards safer alternative treatments, such as biological control agents, non-biological control agents and natural products.

1.4 Treatment: non-biological control

All the perceived health concerns and genuine limitations (resistance) with fungicide application that were described earlier have led to many studies to find alternative treatments.

Recently, there has been substantial interest in non-biological control agents as well as biological control agents to replace the existing chemical applications. Non-biological control involves chemicals that are GRAS such as ethanol (Karabulut *et al.*, 2004), sodium bicarbonate (Smilanick *et al.*, 1995) and calcium salts (Saftner *et al.*, 2003) as alternative treatments.

1.4.1. Ethanol

Ethanol is used commercially in a lot of products, such as perfumes, paints and alcoholic beverages. It is also used as a disinfectant due to its antibacterial properties. Ethanol is used as a disinfectant at a concentration of about 62% by weight. The peak of disinfecting power of ethanol occurs around 70%; solutions stronger than 70% ethanol have less ability to disinfect. Low concentrations of ethanol (16% and below) do not have good antiseptic properties (Wikipedia, 2006b). Generally, ethanol kills organisms by denaturing their proteins and dissolving their lipids and is effective against most bacteria and fungi (Wikipedia, 2006b).

Ethanol is known to have antifungal properties. External application of ethanol can inhibit the ripening of some fruits (Podd and Van Staden, 1998), reduce postharvest fungal diseases (Karabulut *et al.*, 2004) and kill insect pests (Dentener *et al.*, 1998 as mentioned in Chervin *et al.*, 2005).

Previous studies assessed the effectiveness of ethanol as a postharvest treatment of table grapes (Litcher *et al*, 2002; Chervin *et al.*, 2005; Karabulut *et al.*, 2004; Smilanick *et al.*, 1995). Dipping or exposing the fruit to ethanol vapour improved the storage life by limiting postharvest rot development on this fruit.

Karabulut *et al.* (2004) did a study on immersion in ethanol or hot water alone or in combination to control grey mould on table grapes. The research was also done to determine any adverse reaction of these treatments on the quality of the grapes. Fruit was dipped in either water alone or in 20%, 30%, 40%, 50% and 60 % ethanol for 30 seconds at 24 °C prior to packaging and storage. The results showed 30%, 40%, 50% or 60 % alcohol reduced the number of berries infected by 50% while 20% ethanol was not significantly different from the untreated berries. Grapes inoculated with *Botrytris cinerea* were also treated. The inoculated grape bunches were warmed at 24°C for one hour before treatment and the ethanol solution was heated for most treatments. As a result, addition of ethanol to the water, a longer immersion period and increasing temperature seemed to improve the control of decay of the grapes.
Ethanol seemed to be more effective compared to hot water alone. Immersion of inoculated grapes in 10% ethanol at 60°C was more effective than immersion in 30% ethanol at 30°C. Highest efficacy was achieved by 10% ethanol treatment at 55 and 60°C for 30 seconds and water treatment at 60°C for 30 seconds. None of these treatments had an adverse affect on the quality parameters of the grapes.

Recently, Chervin *et al*. (2005) did a similar study on ethanol vapours as a substitute for SO_2 fumigation of table grapes. The grapes picked from a local vineyard were packed in wooden boxes wrapped with polyethylene bags prior to four to seven weeks of storage at 0ºC. The experiment involved three treatments which were use of a SO_2 pad, 2mL/kg or 4mL/kg of ethanol. A control was also used. The ethanol vapour was generated by separately pre-soaking newspaper sheets in both concentrations of ethanol in a sealed plastic bag for two hours. A plastic sheet was placed between the newspaper sheet and the grapes to prevent direct contact. At the end of the storage period, the percentage of infected berries was counted and a sensory evaluation was performed.

This study showed a significant reduction of berries infected when treated with SO_2 and both of the ethanol treatments in comparison to the control. There was no significant difference between SO_2 treatment and the ethanol treatments. Moreover, SO_2 and low concentrations of ethanol were effective at reducing stem browning. In sensory evaluation, SO_2 was found to be less acceptable due to the unpleasant taste created.

Both of these studies confirmed that ethanol has the potential to improve postharvest shelf life of table grapes. However, both studies had limitations. For example, ethanol when applied on wet fruit was less effective than when applied on dry fruit, therefore washing the fruit prior to treatment became a problem. Current industry practice involves washing of fruit prior to sorting to remove soil and debris (Fig. 8), since soil and debris can impair the treatments (Dimsey, 1995). Karabulut *et al.* (2004) also observed that wet treatment, such as ethanol can cause fruit to crack unless they are dried promptly. Therefore, if the ethanol treatment were implemented commercially, controlled drying would be needed.

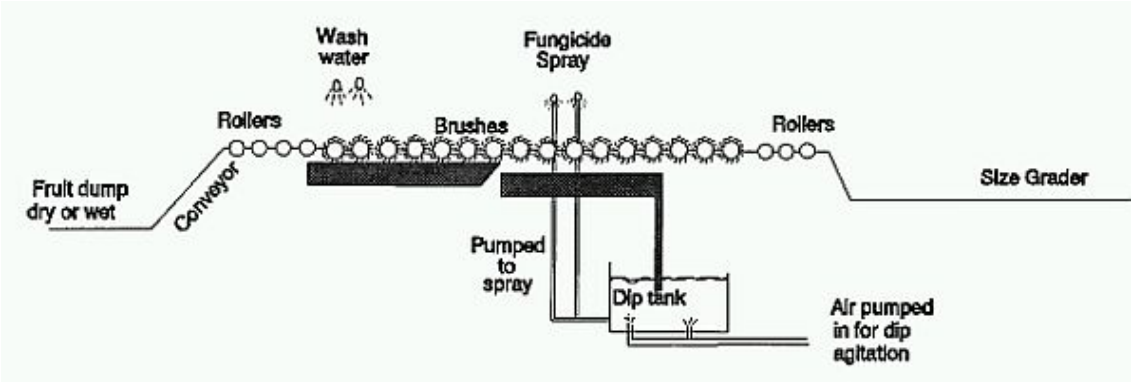


Figure 8. Inline spray or bulk dip part of postharvest handling (Dimsey, 1995).

Another limitation was observed. As applied only once on the fruit after harvesting, ethanol was effective for up to one month storage. However, if the fruit many latent infections, the ethanol could not control these.

The above studies concluded that ethanol is an effective postharvest treatment on table grapes, but none of these studies was done on other types of fruit. Also, no studies have been done with ethanol as a postharvest treatment in New Zealand and on fruit that is grown in New Zealand. Therefore, one aim of this project was to assess the effect of ethanol (dipping and vapour) as postharvest treatment for New Zealand grown fruit.

1.5 Treatment: biological control

One way of controlling postharvest diseases in fruits that has been used recently is biological control i.e. the use of one organism to control the growth of another.

Over the past few decades, biological control of plant pathogens has developed as a feasible disease control strategy (Harman, 2000 as quoted in Elmer *et al.*, 2005). The increasing interest in biological control is due to several factors, such as the health hazard associated with the application of fungicides (White, 1998 as quoted in Elmer *et al.*, 2005), increased regulatory restrictions (Janisiewicz and Korsten, 2002) and pathogen resistance to commonly used fungicides (Rosslenbroich and Stuebler, 2000 as quoted in Elmer *et al.*, 2005).

The mechanism by which biological control agents work varies, including the induction of plant resistance by elicitors, the interference of pathogen infection pathways by antagonistic microorganisms and direct suppression of pathogens with antimicrobial compounds derived from the biological control agent (Elmer *et al.*, 2005).

Many studies have been done assessing biological control. In New Zealand, there is an increasing interest in biological control (Pyke *et al.*, 1994; Elmer *et al.*, 2005). For example, a company called Botry-Zen was formed in April 2001 specialising in the development and commercialisation of biological control agents.

The first biological control agent launched by Botry-Zen was a product called BOTRY-Zen which was specifically developed for the effective control of *Botrytris cinerea* fungal infection (grey mould or bunch rot) in grapes (Botry-Zen Limited, 2001).

However, there are limitations to the use of biological control agents. These limitations occur in formulation, registration and commercialisation of the organisms (Janisiewicz and Korsten 2002). Most of the previous studies on biological control agents have been done on a small scale and under controlled conditions. It is important to be able to implement the methods on a larger scale and in commercial situations. Also, biological control tends to cost more than chemical fungicides and effectiveness depends on the biological agent used, crop and the land area to be treated (Wawrzynski and Ascerno, 2006).

1.6 Treatment: natural products

Natural or biological products such as honey, milk and some essential oils have been previously reported to have antibacterial activity. Several compounds present in honey and milk are known to have antibacterial activity (Waikato Honey Research Unit 2005; Losnedahl *et al*., 1996), but there is little known about the antifungal activity. Also, several chemical constituents in essential oils are believed to have antibacterial and antifungal activity (Goubran and Holmes, 1993; Oxenham, 2003).

1.6.1. Honey

Antibacterial activity in honey was first recognised in 1982 by van Ketel, initiating several studies to analyse this activity (Dustmann, 1979). Honey has been used as traditional medicine in some cultures for decades (Ransome, 1937). Nowadays, researchers have proven that honey can be efficaciously used in medicine due to its antibacterial activity (Molan, 1992).

Honey is a saturated solution of sugars consisting of a mixture of fructose and glucose (84%) and 15-21% water by weight (Wikipedia, 2006c). These sugar molecules strongly interact with the water molecules, and therefore leave very few water molecules available for micro-organisms to grow (Healing Honey, 2005). Honey is fairly acidic which can also be inhibitory to some pathogens and micro-organisms. In diluted honey (Wikipedia, 2006c), the pH would not be acidic, reducing its effectiveness as an antibacterial agent compared to undiluted honey. Apart from its acidity, hydrogen peroxide that is produced enzymically by glucose oxidase (GOX) in honey has been found to be the major antibacterial agent (Airborne Honey, 2003).

1.6.1.1. Glucose oxidase and hydrogen peroxide

Glucose oxidase (GOX) breaks down glucose to form gluconic acid and hydrogen peroxide (H_2O_2) (Fig. 9) (Meyer and Wohlfahrt, 2006). H_2O_2 is the main agent responsible for the antibacterial activity in most types of honey (Airborne Honey, 2003). The GOX activity is usually measured by the production of H_2O_2 and is highly variable between different types of honey (Airborne Honey, 2003). Glucose oxidase activity can be reduced by heat and light, room temperature and a small amount of visible light (Airborne Honey, 2003).

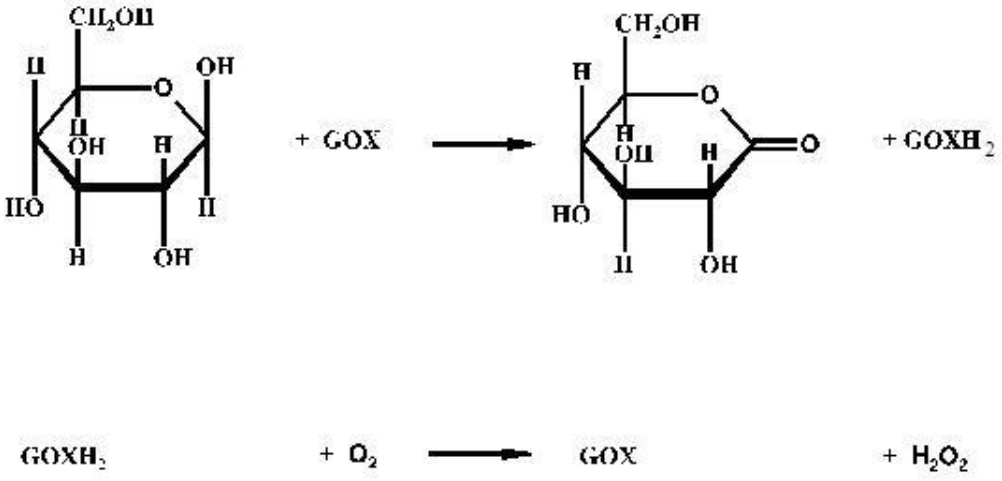


Figure 9. The enzymatic reaction catalysed by glucose oxidase (Meyer & Wohlfahrt, 2006)

Hydrogen peroxide (H_2O_2) has been used as an antiseptic and antibacterial agent for many years due to its strong oxidising properties (Wikipedia, 2006d). Iron present in honey oxidises the oxygen free radicals released by the hydrogen peroxide (Wikipedia, 2006c).

1.6.1.2. Gluconic acid

Honey contains a number of acids, such as amino acids (0.05-0.1%) and organic acids (0.57%). Honey is characteristically quite acidic with its pH being between 3.2 and 4.5 (National Honey Board, 2006). The major organic acid found in honey is gluconic acid ($C_6H_{12}O_7$) that is formed by the oxidation of the first carbon of glucose (National Honey Board, 2006). Gluconic acid (Fig. 10) occurs naturally in fruit, honey and wine and is used in foods as an acidity regulator (Wikipedia, 2006e).

Honey is known to have antibacterial activity due to its acidity level. It can inhibit most bacteria, such as *Escherichia coli* (optimum growth pH: 7.2-7.4), *Salmonella spp* (pH 4), *Pseudomonas aeruginosa* (pH 4.4) and *Streptococcus pyogenes* (pH4.5) (National Honey Board, 2006). There is little to no information available on the antifungal activity of honey. Therefore, one aim of this project was to investigate if honey is effective against fungal pathogens.

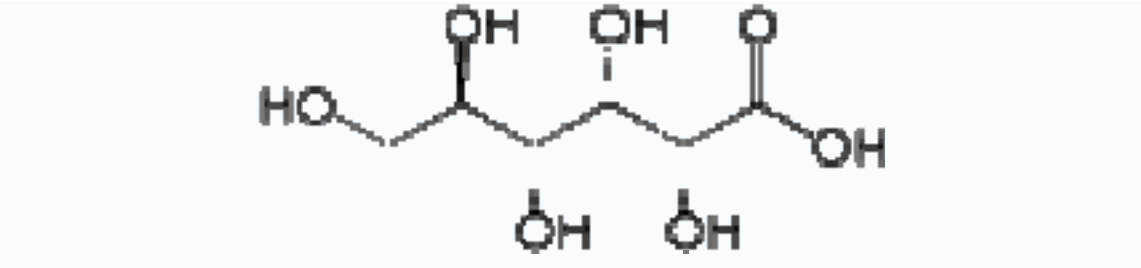


Figure 10. The chemical structure of D-Gluconic acid (Wikipedia 2006e).

1.6.1.3. Osmotic effect

Honey is a saturated mixture of the two monosaccharides: glucose and fructose. This mixture has a low osmotic pressure since most of the water molecules bind with the sugars leaving few available for microorganisms. Therefore, honey is a poor environment for their growth (Wikipedia, 2006c).

1.6.2. Manuka Honey

Manuka honey is collected by honey-bees from the manuka tree (*Leptospermum scoparium)*, a New Zealand native plant well-known for its medicinal properties (Honey New Zealand, 2006).

The H_2O_2 antibacterial property of other honeys has lower effectiveness compared to active manuka honeys (Molan, 1992). A survey was done by Allen *et al.* (1991) on 345 samples of New Zealand honeys from 26 different floral sources.

It was found that when catalase was added to any of the honeys to destroy the H_2O_2, only manuka honey had a significant amount of antibacterial activity left (Allen *et al.*, 1991), indicating that manuka honey has an additional biological activity that is not present in other honeys.

This additional activity is called Unique Manuka Factor (UMF) which is very stable towards light, heat and body enzymes (Honey New Zealand, 2006) and it is also resistant to heat (Waikato Honey Research Unit, 2005). UMF was first discovered by Dr. Peter Molan from the Waikato Honey Research Unit (Healing Honey, 2005). UMF activity can exist in addition to the antibacterial properties of H_2O_2. Together, these two factors behave synergistically (Waikato Honey Research Unit, 2005). It was proven that UMF is very effective at inhibiting the growth of a wide range of bacteria, such as *Helicobacter pylori, Staphylococcus aureus, E. coli* and *S. pyogenes* (API Health Ltd, 2006).

Theunissen *et al.* (2001) did a study on the antifungal action of three single samples of South African honey (wasbessie, bluegum and fynbos) against *Candida albicans*, a diploid sexual yeast. It belongs to the phylum of Ascomycota, family *Saccharomycetaceae*, and causes oral and vaginal infections in humans. Various concentrations of honey ranging from 0 to 25% were tested for antifungal activity compared with various sugars as controls. This study concluded that the sugars and honey concentrations stimulated the growth of *C. albicans* with optimal growth between 2.5% and 5%.

Increased honey concentrations reduced the fungal growth; for example, 25% wasbessie honey reduced the fungal growth by 29.4% while the control, bluegum and fynbos honey only had partial inhibition. This study reported on the inhibition effect of 25% wasbessie honey, but the antifungal activity of this honey and the concentration at which the growth was fully inhibited was not reported.

Other studies have reported that honey has antibacterial activity against *Staphylococcus aureus*, isolated from infected wounds (Cooper *et al.*, 1999; Molan and Russell, 1988; Russell, 1983). This antibacterial activity appeared to be due to the hydrogen peroxide activity present in honey. However, it is known that the concentration of hydrogen peroxide in honey varies, resulting in major variations seen in overall antibacterial activity of honey (National Honey Board, 2006).

Many studies have been done on the antibacterial activity of honey on several bacteria, but not many on antifungal activity. One aim of this project was to assess if similar antifungal activity can be observed in honey against fungi pathogenic to plants.

1.6.3. Milk

It was found that milk has antimicrobial activity due to some of the proteins present (Losnedahl *et al*., 1996). These proteins are known as lactoferrin, lactoperoxidase, lysozyme and N-acetyl-β-D-glucosaminidase (NAGase) (Losnedahl *et al*. 1996). There is little known antifungal activity in milk against plant pathogenic fungi, therefore an aim of this project was to assess this activity.

1.6.3.1. Lactoferrin

Lactoferrin (Fig. 11) is an iron binding glycoprotein that acts as an iron transporter (Lonnerdal and Lyer, 1995). It was originally isolated from bovine milk and contains approximately 703 amino acids with a molecular mass of 80 kilodalton (kDal) (Lonnerdal and Lyer, 1995). Milk contains about 20 to 200 μg/mL of lactoferrin (Linden and Lorient 1999).

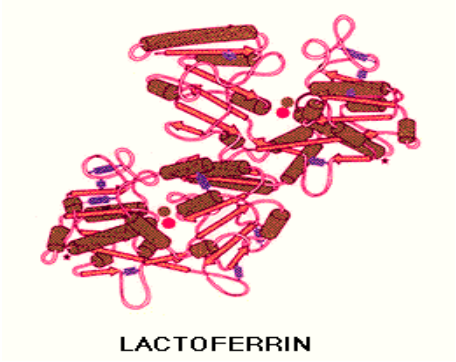


Figure 11. The structure of lactoferrin (raw-milk-facts.com, 2006)

Lactoferrin has antibacterial (Losnedahl *et al*., 1996), antifungal (Samaranayake *et al.*, 2001) and antiviral activities (Swart *et al.*, 1998). These activities seem to be derived from the ability of lactoferrin to bind iron (Lonnerdal and Lyer, 1995). It is very resistant to proteolysis and is stable down to pH 2 (Linden and Lorient, 1999).

Lactoferrin's antibacterial and antifungal activity depends on its capability of inhibiting microbial growth by depriving them of iron. The effectiveness of the antimicrobial activity of lactoferrin depends on the iron requirement of the organisms, the availability of exogenous iron and the concentration or degree of iron saturation (Losnedahl *et al*., 1996). Lactoferrin is effective against several strains of *Escherichia coli* (Lonnerdal and Lyer, 1995) and *C. albicans* (Samaranayake *et al.*, 2001). Lactoferrin has also been shown to be effective against viruses, such as those that are responsible for AIDS (Swart *et al.*, 1998), herpes (Jenssen, 2005) and polio (Van Der Strate *et al.*, 2001). Lactoferrin appears to interfere with the virus's ability to attach to target receptors on the cell surface (Swart *et al.*, 1998).

1.6.3.2. Lactoperoxidase

Lactoperoxidase (Fig. 12) is a glycoprotein with a molecular weight of 78 kDal (Harper, 2000) with each molecule containing one iron atom. Lactoperoxidase has been identified as an antibacterial agent in milk, saliva and tears (Losnedahl *et al*., 1996; Harper, 2000), especially against *Listeria monocytogenes* (Gaya *et al.*, 1991), *E. coli, Pseudomonas spp., Salmonella spp.* (Reiter, 1976) and *Streptococcus spp* (Thomas *et al.*, 1983).
It also plays a role in protection against bacterial growth on the mammary gland (Losnedahl *et al*., 1996).

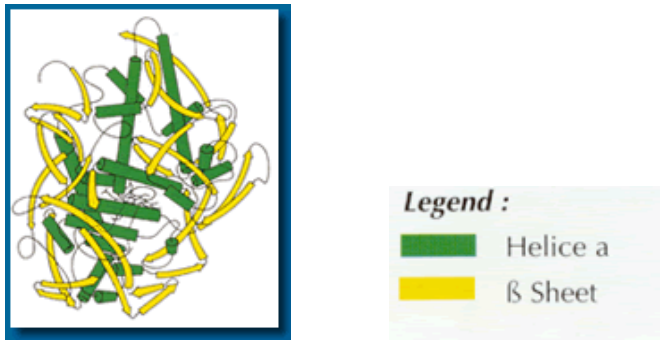


Figure 12. Structure of lactoperoxidase (Biopole, 2006)

Lactoperoxidase kills bacteria by an oxidation mechanism (Harper, 2000). Lactoperoxidase, on its own, has no antibacterial activity. However, it forms a powerful natural antibacterial system together with hydrogen peroxide and thiocyanate (Fig. 13). “This mechanism is called the lactoperoxidase system and the antibacterial effect of the lactoperoxidase system is mediated by the reaction of hydrogen peroxide and thiocyanate under lactoperoxidase catalysis and the resultant generation of short-lived hypothiocyanate, which is thought to be a major antibacterial substance” (Losnedahl *et al*., 1996).

The source of antibacterial properties in this system is the inhibition of vital bacterial metabolic enzymes due to the oxidation of hypothiocyanate (Losnedahl *et al*., 1996). There is little known antifungal activity of lactoperoxidase, to confirm the existence of antifungal activity for this enzyme.

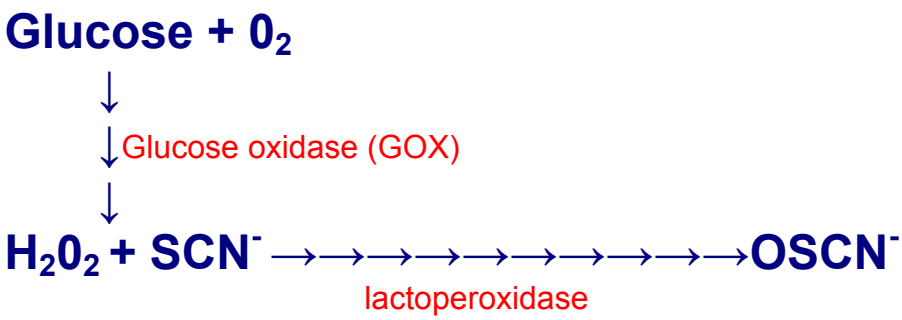


Figure 13. Lactoperoxidase system (Biopole, 2006)

1.6.3.3. Lysozyme

Lysozyme (Fig. 14) is an enzyme that lyses certain bacteria by hydrolysing the β(1,4) linkage between muramic acid and N-acetylglucosamine of bacterial peptidoglycans (Fox and McSweeney, 1998), which constitute the major part of the bacterial cell wall of Gram negative bacteria (Carter and Carter, 2000). Lysozyme is present mostly in milk and egg white (Mullan, 2001). It is a basic protein that is also found in human skin, tears, saliva and nasal secretions (Nishiyama *et al.*, 2001).

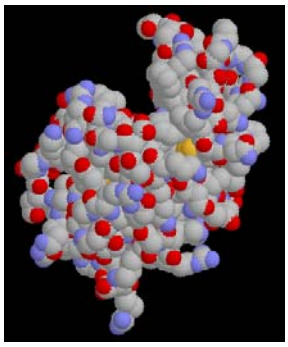


Figure 14. Structure of lysozyme (Birkbeck College, 1995)

Lysozyme has antibacterial activity and normally functions in association with lactoferrin or immunoglobin. The susceptibility of different bacteria to lysozyme depends on the accessibility of the substrate and the ionic environment (Mullan, 2001). Lysozyme is effective against Gram negative bacteria, such as *E. coli* and *Salmonella spp.* (Losnedahl *et al*., 1996), since they have a simple cell wall consisting of 90% peptidoglycan (Mullan, 2001). In contrast, it is not effective against Gram positive bacteria, such as *Staphylococci* due to the teichoic acid that is present in their cell walls (Mullan, 2001).

While the antibacterial activity of lysozyme is well documented, only a few studies have been done on the action of lysozyme against fungal cells. Lysozyme is known to be fungicidal against *C. albicans* (Samaranayake *et al.*, 2001; Marquis *et al.*, 1991 and Nishiyama *et al.*, 2001). A constant quantity of lysozyme caused damage to *C.albicans* cells. Most of the cells were swollen and deteriorated and some were completely destroyed (Kamaya, 1970).

Nishiyama *et al.* (2001) used microscopy to show that 50µg/mL lysozyme affected the viability of *C. albicans*. Cell growth was inhibited within eight hours of exposure to lysozyme (Fig. 15 A and B) with alterations in the composition of cell walls observed. Some swollen cells were observed under scanning electron microscopy or SEM (Fig. 15 C and D).

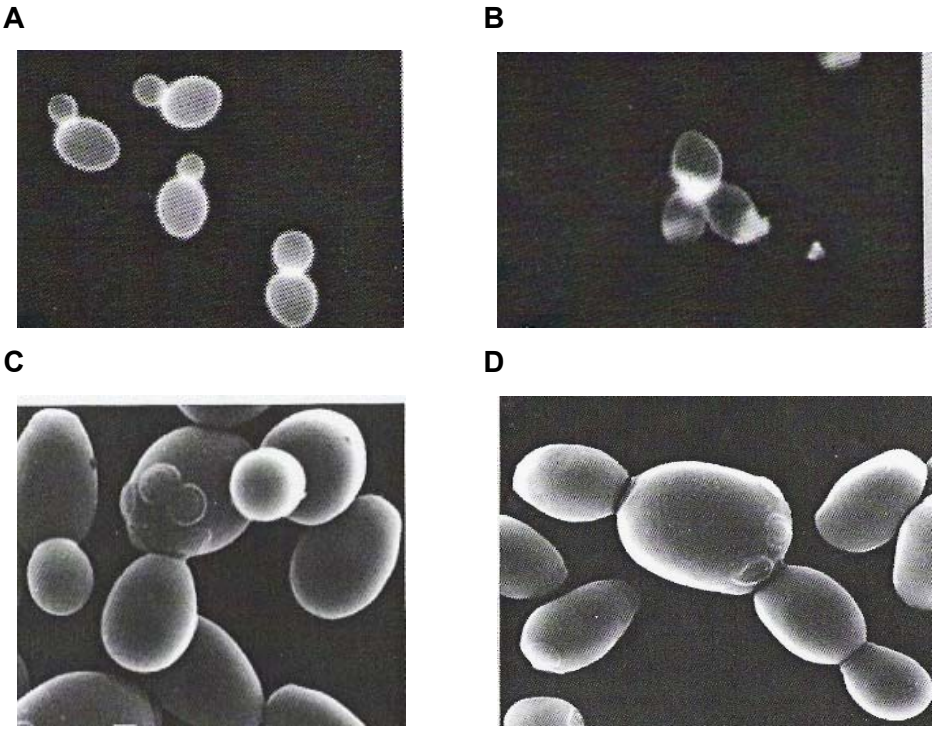
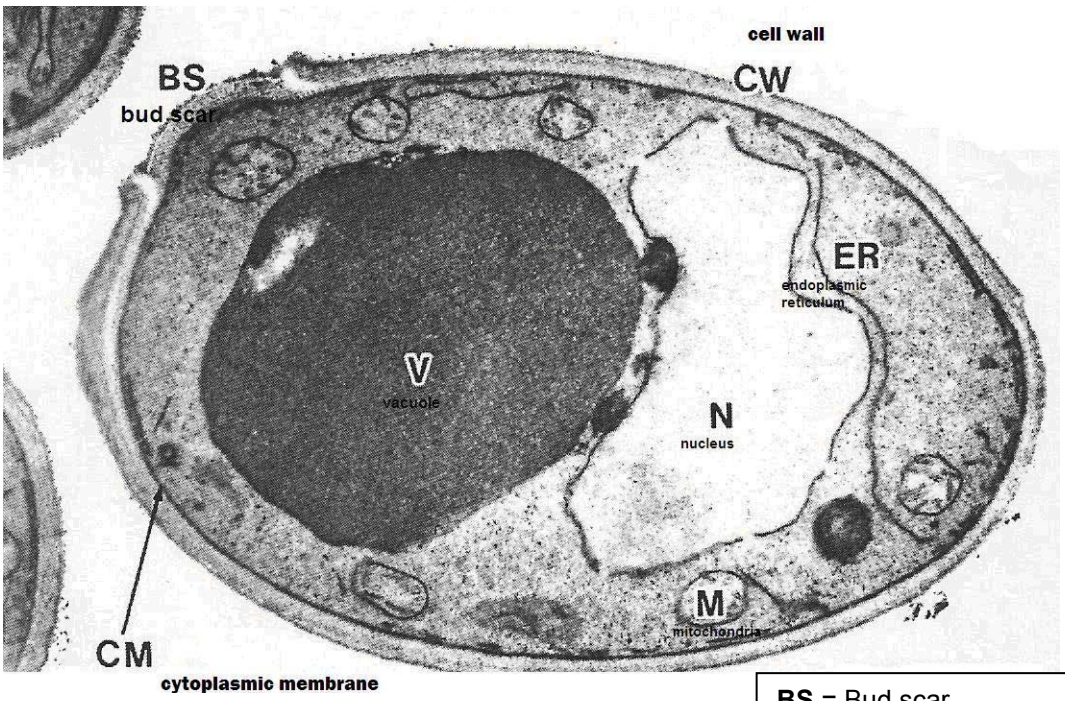


Figure 15. Cell morphology of *C. albicans* grown for 8 hours. **A and B** showing the fluorescent images of the cells stained with Fungiflora Y, **A)** Without lysozyme **B)** With 50µg/mL lysozyme. **C and D** showing the Scanning electron microscope (SEM) image of cells **C)** Without lysozyme **D)** with 50µg/mL lysozyme (Nishiyama *et al.*, 2001).

Transmission electron microscopy (TEM) showed that in untreated cells, the cytoplasmic membrane was attached to the cell wall (Fig. 16 A).
In contrast, lysozyme-treated cells showed localised accumulation of “cell wall-like” materials in the periplasmic space (Fig. 16 B) which caused swelling of the cell and a rupturing of the cell wall.

A



BS = Bud scar
CW = Cell wall
N = Nucleus
ER = Endoplasmic reticulum
V = Vacuole
CM = Cytoplasmic membrane

B

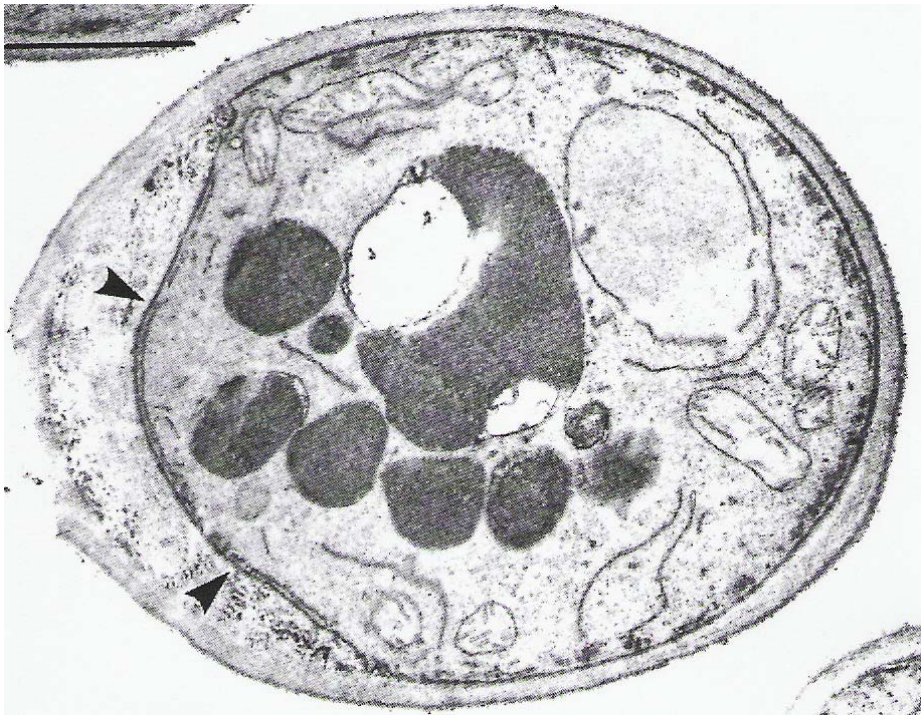


Figure 16. Trasmission electron microscope (TEM) images of *C. albicans* grown for 8 hours, from Nishiyama *et al.*, 2001. **A)** Control cell **B) C**ell treated with 50µg/mL lysozyme (arrowhead indicating the accumulation of wall-like materials within the space between the cell wall and cytoplasmic membrane).

This study concluded that lysozyme appeared to affect cell wall formation by interrupting the normal structure and integration of cell wall components (Fig. 15 and Fig. 16).

Antifungal effects of lysozyme have been tested only against *C.albicans* to date. *C. albicans* belongs to the phylum Ascomycota, therefore lysozyme may have a similar antifungal effect on the plant pathogenic fungi, *M. fructicola* and *Penicillium spp.*

1.6.3.4. N-Acetyl-β-D-glucosaminidase (NAGase)

"N-Acetyl-β-D-glucosaminidase (NAGase) (Fig. 17) hydrolyse terminal, non reducing N-acetyl-β-D-glucosamine residues from glycoproteins" (Fox and McSweeney, 1998). It is an enzyme whose activity has been implicated as an indicator of tissue damage during mastitis, a common bacterial infection among dairy cows (Lonnerdal and Lyer, 1995). NAGase is secreted in large quantities in the mammary gland during inflammation.

The specific function of NAGase in the mammary gland is not known, however, recent studies have discovered that NAGase may reduce the growth of some pathogens (Losnedahl *et al.*, 1996). It has been found that there is a relationship between the presence of pathogens in the udder and NAGase levels in milk (Lonnerdal and Lyer, 1995).

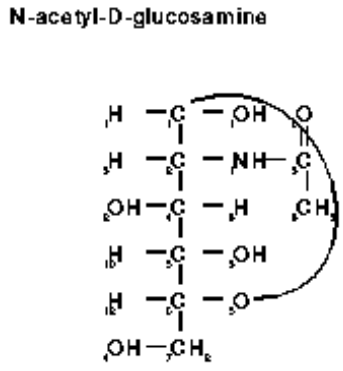


Figure 17. Structure of N-acetyl-D-glucosaminidase (Biocheminfo.org, 2006)

Since NAGase had been found in uterine fluids, it was suggested that NAGase may have a role in the bactericidal function of the uterus (Lonnerdal and Lyer, 1995). The bactericidal effect of NAGase on several bacterial pathogens that are commonly found to infect the cow uterus was studied (Lonnerdal and Lyer, 1995). It was found that NAGase inhibits pathogens such as *Staphylococcus aureus*, *Streptococcus agalactiae*, and *Pseudomonas aeruginosa*, but it does not inhibit pathogens, such as *E.coli* and *Enterobacter aerogenes*.

In contrast to the antibacterial property, no antifungal activity has been reported for NAGase.

1.6.4. Essential Oils

An essential oil is a concentrated, hydrophobic liquid containing volatile aromatic compounds extracted from plants. They can be produced by distillation, expression, or solvent extraction (Wikipedia, 2006f). Many essential oils are believed to provide plants with a defence mechanism against pests and pathogens (Goubran and Holmes, 1993; Oxenham, 2003).

There is evidence that essential oils were used by the priests and alchemists thousands of years ago to heal the sick. They are the oldest type of medicines and cosmetics known (Hauck, 2006). Nowadays, essential oils are generally used in perfumery, aromatherapy, cosmetics, incense and flavours. They have also been used in medicine, household cleaning products and as food grade materials (Wikipedia, 2006f). They are presumed to be effective as an alternative antifungal and antibacterial treatment for fresh produce, however this has not been fully explored (Jobling, 2000).

Essential oils are made up of many different volatile compounds and the processes of isolating the oils are quite different between species (Jobling, 2000). The complexity of essential oils is due to terpene alcohols, aldehydes, ketones, acids and esters (Wijesekara *et al.*, 1997). The essential oils assessed in this study were sweet orange oil, lemon oil, lemongrass oil and manuka oil.

1.6.4.1. Chemistry of essential oils

The chemicals found in essential oils are classified as hydrocarbons (i.e. terpenes), derivatives of the hydrocarbons (oxygenated terpenoid compounds), aromatic compounds (benzenoid structures) and compounds containing nitrogen or sulphur (Reineccius, 1994). The amount of chemical components present in essential oils vary depending on which part of plant it is isolated from, the area where the plant is grown and how it is processed.

A. Terpenes

The chemical structures of terpenes were established in 1887 by Wallach (Reineccius, 1994). Terpenes in essential oils are made up of the isoprene units or building blocks. Each isoprene contains 5 carbon atoms with one of them attached by a double bond (Buckle, 2004). The general formula of a terpene is $(C_5H_8)n$, where n is the number of isoprene units. Monoterpenes have two isoprene units, sesquiterpenes have three, diterpenes have four, triterpenes have six and tetraterpenes have eight isoprene units (Reineccius, 1994).

Monoterpenes (Fig. 18 A) such as champhene, nerol, pinene, myrcene, limonene and citral are the most common in nature (Buckle, 2004). Some examples of sesquiterpenes (Fig. 18 B) are cardinal and farnesol. Examples of diterpenes are phytol and Vitamin A_1. Squalene is a good example of a triterpene and carotene is a tetraterpene (Kirste, 1994).

Sesquiterpenes are less volatile than monoterpenes because of their larger structure. They have stronger odours, are anti-inflammatory and have antibacterial properties (Buckle, 2004). There is some evidence that they also have antifungal activity (Jasicka-Misiak *et al.*, 2004; Tan *et al.*, 1999; Krauze-Baranowskaa *et al.*, 2002).

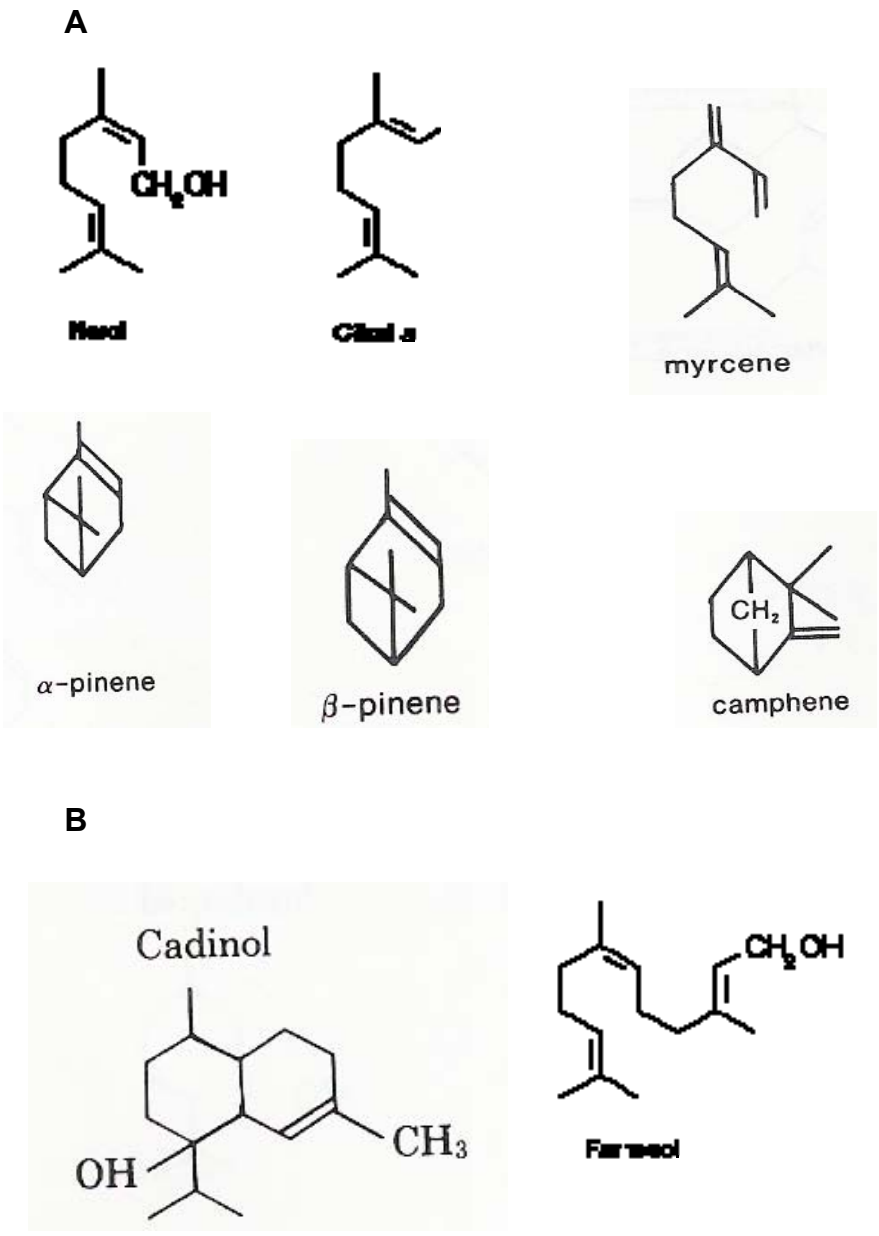


Figure 18. Formule for several examples of terpenes (Reineccius, 1994). **A)** Monoterpene alcohols. **B)** Sesquiterpene alcohols.

Santolinylol (isolated from *Artemisia* genus) is an example of a monoterpene with antifungal activity against *C. albicans* (Tan *et al.*, 1999). Generally in fungal cells, there is a predominant lipid molecule that functions to regulate membrane fluidity, permeability and the activity of many membrane-bound enzymes (Parveen *et al.*, 2004). This predominant lipid molecule is assumed to play an important role in cellular growth. Terpenes are thought to induce alterations in the cell permeability by disrupting lipid packing and causing changes to membrane properties and functions (Parveen *et al.*, 2004; Hammer *et al.*, 2004).

B. Oxygenated terpenoid compounds

Oxygenated derivatives of terpene hydrocarbons include alcohols, aldehydes, ketones and esters. These compounds are responsible for the distinctive odours and flavours in essential oils (Reineccius, 1994).

Alcohols or terpenic alcohols can be found in many essential oils. In structure, they have a hydroxyl group attached to one of their carbon atoms. Monoterpenic alcohols (monoterpenols) are believed to be good antiseptics with some antibacterial and antifungal properties (Buckle, 2004). Some examples of terpenic alcohols are linalool in *Lavandula angustifolia*, geraniol in *Cymbopogon martini* and terpinen-4-ol in *Melaleuca alternifolia* (Fig. 19). Geraniol is known to have antifungal activity (Carson & Riley, 1995 as quoted in Buckle, 2004; Saikia *et al.*, 2001) Terpinen-4-ol is effective against *Pseudomonas aeruginosa* (Budhiraja *et al.*, 1999 as quoted in Buckle, 2004). Saikia *et al.* (2001) analysed four components present in lemongrass oil (geraniol, citronellol, citronellal and citral) for their comparative activity against *Microsporum gypseum* (a fungus that causes hair and scalp infection on humans). Out of the four components, geraniol was the most active towards inhibiting the growth of *M. gypseum* followed by citronellal.

Tan *et al.* (1999) isolated a compound called pinitol from *Artemisia* that inhibits the growth of *C. albicans, Aspergillus flavus, A. niger, Geotrichun candidum, Trichophyton rubrum* and *Epidermophyton floccosum*.

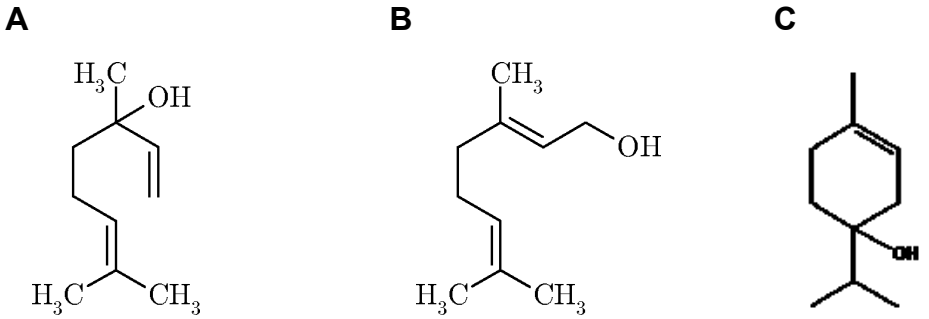


Figure 19. Example of terpenic alcohols and their structures, and they are: **A)** linalool, **B)** geraniol and **C)** terpinen-4-ol (Buckle, 2004; Wikipedia, 2006g; Wikipedia, 2006h).

An aldehyde has an oxygen atom double bonded to a carbon atom at the end of a carbon chain, with hydrogen atom on the fourth bond (Bowles, 2000). Examples of aldehydes are citral found in lemon balm, citronellal (Fig. 20) in lemongrass, geranial (Fig. 20 B) in lemon eucalyptus and neral (Fig. 20 B) in lemon verbena (Reineccius, 2004). Citral is known to have strong antiseptic and antibacterial properties (Onawunmi and Oguniana, 1981 as quoted in Buckle, 2004). Citronellal is known to have antifungal properties (Saikia *et al.,* 2001; Hmamouchi *et al.*, 1990 as quoted in Buckle, 2004). Saikia *et al.* (2001) found that citronellal is active against *M. gypseum*.

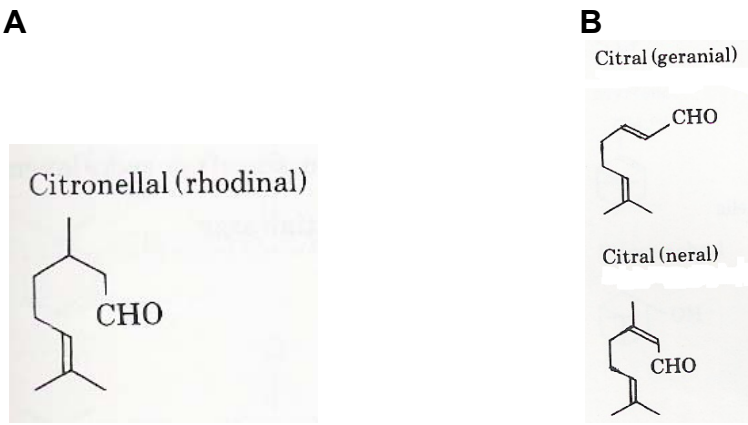


Figure 20. Examples of aldehydes and their structures. **A)** citronellal **B)** geranial and neral (citral) (Reineccius, 2004).

Esters are a combination of acid and alcohol and often have fruity odours (Buckle, 2004). Some examples of esters are linalyl acetate (Fig. 21 A) found in lavender and genaryl acetate (Fig. 21 B) found in sweet marjoram (Clarke, 2002). Some have antifungal properties (Buckle, 2004; D'Auria *et al.*, 2005). D'Auria *et al*. (2005) investigated lavender oil with the main components linalool and linalyl acetate against 50 isolates of *C. albicans*. The growth inhibition, killing time and inhibition of germ tube formation was evaluated. It was found that linalool was more effective than linalyl acetate. These two components inhibited hyphal elongation of *C. albicans (*about 50% inhibition). In this study the lavender oil was found to be fungistatic and fungicidal against *C. albicans* strains and at lower concentrations, it reduced the fungal progression. These antifungal activities were presumably due to linalool and linalyl acetate (D'Auria *et al*., 2005).

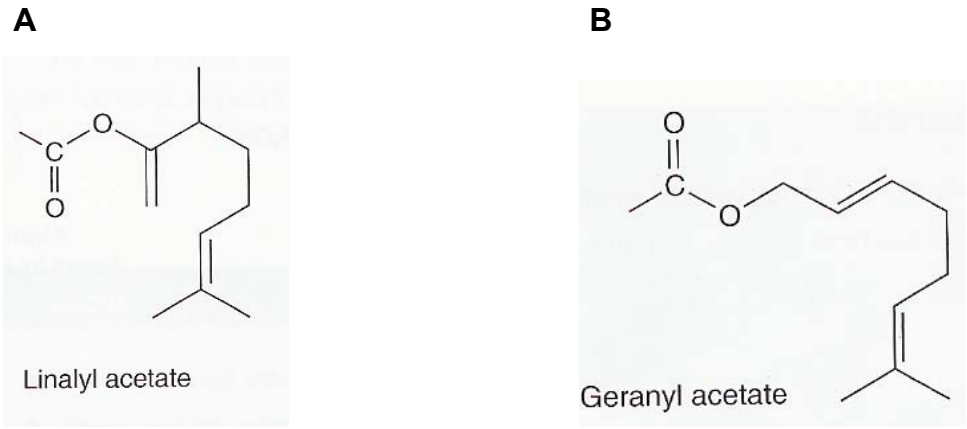


Figure 21. Example of esters and their structures, **A)** linalyl acetate and **B)** geranyl acetate (Clarke, 2002).

1.6.4.2. Sweet orange oil

Essential oil from sweet orange or *Citrus sinesis* (Fig. 22) is usually extracted from the orange peel by cold pressing with a yield from 0.3% to 0.5% (Jensen, 2006).

Figure 22. Sweet orange or *Citrus sinesis* (Jensen, 2006)

Orange oil is generally used in many types of liqueur, food and drink flavouring, confectionery, household cleaners and furniture polish (Wikipedia, 2006i). It has also been used therapeutically as an antiseptic, antidepressant, antispasmodic, anti-inflammatory agent among other roles. Sweet orange oil was found to have antibacterial property against Gram negative and Gram positive bacteria (Fisher and Phillips, 2006) while commercial orange oil is claimed to have antibacterial and antifungal activity (Rainforest Organics Natural Soap Co., 2006). There is little in the scientific literature to support this claim.

Forty-two components of orange oil were identified and quantified by Ojeda de Rodríguez *et al.* (2003). Table 1 shows the summary of some of the major compounds present in orange oil, the monoterpene limonene being the major component (94.55%). Aldehydes were the next major components (1.55% in total) followed by alcohols (0.84%) and sesquiterpenes (0.46%).

Apart from limonene, among all of the compounds, mycerene (1.22%), α-pinene (0.51%), decanal (0.45%), linalool (0.48%) were the most abundant. Esters were found in low quantities (0.29%) and no ketones were identified.

1.6.4.3. Lemon oil

Lemon oil from *Citrus limon* (Fig. 23) is primarily used in aromatherapy, fragrances, household cleaners and disinfectants. Lemon oil is extracted from the fresh fruit peel by cold pressing (Esoteric Oils CC and Sallamander Concepts Ltd, 1999). Commercial lemon oil is claimed to have the ability to stop bleeding, kill infection, activate white blood and red blood cell production to fight anemia (Starthealing.com, 2005). It is also claimed to have powerful antiseptic, antibacterial, antifungal and antiviral activities due to its limonene content (The Herbs Place, 2006). Again, there is little in the scientific literature to support these claims.

Figure 23. Lemon (*Citrus limon*) (Jensen, 2006)

Gas chromatography analysis of lemon oil by Ojeda de Rodríguez *et al.* (1998) revealed a total of 51 compounds (Table 1). The majority (28) of the compounds were found to be mono and sesquiterpene hydrocarbons, eight aldehydes, ten alcohols, three esters, one ketone and one oxide (Ojeda de Rodríguez *et al.,* 1998). As for orange oil, limonene was found to be the primary compound present in lemon oil (65.65%).

Aldehydes were the next most abundant compounds (2.71%) followed by alcohol (0.52%) and esters (0.57%) (Ojeda de Rodríguez *et al.,* 1998). Similar to orange oil, no ketones were identified (Table 1).

1.6.4.4. Lemongrass oil

Lemongrass (Fig. 24) belongs to the genus *Cymbopogon* (aromatic grasses). It contains 1 to 2% essential oil on a dry weight basis (Carlson *et al.*, 2001). Lemongrass oil is extracted from the fresh or partly dried leaves by steam distillation and is generally used in perfumery, as flavourings and herbal medicine (Jensen, 2006).

Figure 24. Lemongrass, *Cymbopogon citratus* (Jensen, 2006)

Commercial lemongrass is considered to be an effective antiseptic. It can be used as a treatment for acne, muscle aches, scabies, insect bites and athlete's food (Answers.com, 2006). Lemongrass has also been shown to have antifungal activity (Shahi *et al.*, 2003; Saikia *et al.*, 2001). A study done by Saikia *et al.* (2001) found that lemongrass oil is highly effective at inhibiting the growth of *Microsporum gypseum*.

The chemical composition of lemongrass oil is highly variable, depending on the genetic diversity, habitat and agronomic treatment of the culture (Paviani *et al.*, 2006; Clarke, 2002). While the absolute amount of each constituent can vary, the relative abundance is maintained i.e. neral and geranial are always the most abundant. In contrast to orange and lemon oils, the most abundant compound is the adehyde citral (composed of neral and geranial isomers). Limonene is found at a low level (2.9%) as well as mycrene, cis- β-ocimene, borneol, α-terpineol and β-caryophyllene (Table 1).

In comparison to orange and lemon oil, the ester content especially linalyl and geranyl acetate was present in greater abundance in lemongrass oil. Similar to orange and lemon oil, no ketones were identified in lemongrass oil (Table1).

1.6.4.5. Manuka oil

Manuka is the Maori name for *Leptospermum scoparium,* an abundant New Zealand shrub (Porter, 2001) (Fig. 25) that belongs to the *Myrtaceae* family. It ranges from 4m to 8m in height and is able to grow throughout New Zealand, from lowland to sub-alpine (Porter, 2001).

Figure 25. Manuka flowers (Crop & Food Research, 2000).

The Maori have traditional uses for manuka. The leaves can be used in vapour baths and to scent toilet oil while the pulped seed capsules can be applied as wound dressings. Also, the infusion from the leaves can be drunk as a tea substitute (Crop & Food Research, 2000). There is growing interest in the use of manuka oil due to its activity against Gram positive bacteria, including the antibiotic resistant strains (Douglas *et al.*, 2004).

There are major variations in the chemical composition of manuka oils, depending on the ecotype harvested, which can affect the aroma and biological activity of the oil (Crop & Food Research, 2000). These variations can lead to potential confusion in the marketplace (Douglas *et al.*, 2004).

Crop & Food Research (2000) reported a study on three major manuka oil chemotypes (i.e. races of plants with different chemical constituents) throughout New Zealand. In far north New Zealand, the manuka oil had high pinene content, whereas in the East Cape region, the oil contained a high proportion of triketone.

The breakdown of the chemical components of New Zealand (East Cape) manuka oil is shown in Table 1. In contrast to lemon, orange and lemongrass oil, a ketone (leptospermone) is one of the major constituents. The other is the terpene trans-calamenene (Douglas *et al.* 2004). It contains a very small proportion (or sometimes none) of α and ß- pinene and myrcene as sometimes these compounds are removed as it is fractionated (Douglas *et al.*, 2004). There are no aldehydes, such as geranial and neral in manuka oil. There was also no limonene present in manuka oil, whereas limonene was present in orange, lemon and lemongrass oil (Table1).

Table 1. Some of the major compounds present in orange, lemon, lemongrass and manuka oil (Ojeda de Rodriguez *et al.*, 2003; Ojeda de Rodriguez *et al.*, 1998; Paviani *et al.*, 2006; Clarke, 2002; Douglas *et al.*, 2004). **x** means the compound is not present.

Group	**Major compounds found**	**Orange oil**	**Lemon oil**	**Lemongrass oil**	**Manuka oil**
Terpenes :		(%w/w)	(%w/w)	(%w/w)	(%w/w)
(Monoterpenes	limonene	94.55	65.65	2.9	**X**
and	mycrene	1.22	1.01	1.7	0.3
Sesquiterpenes)	α-pinene	0.51	1.88	**X**	0.7
	ß-pinene	**X**	11	**X**	0.3
	terpinene	**X**	9.01	**X**	**X**
	Ylangene + α-copaene	**X**	**X**	**X**	5.7
	ß- caryophyllene	0.12	0.25	2.1	1.5
	trans-calamenene	**X**	**X**	**X**	15.6
	ß-elemene	0.13	**X**	**X**	**X**
	trans-α-bergamotene	**X**	0.41	**X**	**X**
	ß-bisabolene	**X**	0.40	**X**	**X**
	germacrene	**X**	0.11	**X**	**X**
	Cis-ß-ocimene	**X**	**X**	1.5	**X**
	Cadinene	**X**	**X**	**X**	0.2
	α-humulene	**X**	**X**	**X**	3.6
	α-amorphene	**X**	**X**	**X**	2.6

Group	Major compounds found	Orange oil	Lemon oil	Lemongrass oil	Manuka oil
Aldehydes:		(%w/w)	(%w/w)	(%w/w)	(%w/w)
	neral	**X**	0.87	34.1	**X**
	decanal	0.45	**X**	**X**	**X**
	geranial	0.23	1.44	44.5	**X**
	citronellal	**X**	0.14	**X**	**X**
	nonanal	**X**	0.12	**X**	**X**
	octanal	0.23	**X**	**X**	**X**
Alcohols:					
	linalool	0.48	**X**	**X**	**X**
	α-terpineol	0.13	0.17	1.7	0.1
	citronellol	0.11	0.04	**X**	**X**
	terpinene-4-ol	**X**	0.06	**X**	0.1
	hydrate	**X**	0.16	**X**	**X**
	geraniol	**X**	**X**	**X**	**X**
	ß- eudesmol	**X**	**X**	**X**	0.8
	borneol	**X**	**X**	1.6	**X**
Esters:					
	octyl acetate	0.07	**X**	**X**	**X**
	decyl acetate	0.07	**X**	**X**	**X**
	citronellyl acetate	0.05	**X**	**X**	**X**
	neryl acetate	0.05	0.35	**X**	**X**
	geranyl acetate	**X**	0.22	5.0	**X**
	linalyl acetate	**X**	**X**	4.8	**X**
	citronellyl formate	**X**	**X**	**X**	0.2
Ketones:					
	Leptospermone	**X**	**X**	**X**	16.6
	Isoleptospermone	**X**	**X**	**X**	6.2
	1,8-cineole	**X**	**X**	**X**	0.7

Commercial manuka oil is known as a powerful antibacterial, antifungal and insecticidal agent (Gluyas, 2006). It is claimed that some manuka oil has antimicrobial activity against specific organisms (Table 2) (Manukaoil.com), but there is little in the scientific literature to support these claims.

Table 2 shows that manuka oil was mostly effective against Gram positive, but not Gram negative bacteria. It was also effective against a few fungi, but not yeast (Manukaoil.com, 2006).

Table 2. Typical antimicrobial activities of manuka oil (Manukaoil.com, 2006).

Organism	Organisms type	Maximum concentrations of oil required to effect a complete kill
Staphylococcus aureus	Gram positive	1:1500
Staphylococcus faecalis	Gram positive	1:2000
Streptococcus pyogenes	Gram positive	1:2000
Bacillus subtilis	Gram positive	1:750
Propionibacterium acnes	Gram positive	1:700
Listeria monocytogenes	Gram positive	1:2000
Klebsiella pneumoniae	Gram negative	1:10
Pseudomonas aeruginosa	Gram negative	1:10
Escherichia coli	Gram negative	1:10
Candida albicans	Yeast	1:30
Trichophyton mentagrophytes	Fungi	1:1250
Aspergillus niger	Fungi	1:50
Microsporum canis	Fungi	1:1000

It is believed that the antimicrobial property of manuka oil is due to the triketone chemotype (Douglas *et al.*, 2004). There has been growing interest in triketones due to their ability to inhibit the growth of most of Gram positive bacteria (Christoph *et al.*, 2000 as quoted in Douglas *et al.*, 2004). A study done by Douglas *et al.* (2004) found that the high triketone chemotype of manuka was located in the East Cape district. A study on the effectiveness of East Cape manuka oil against some bacteria associated with acne vulgaris and underarm odour was conducted by the University of Otago, New Zealand (Gluyas, 2006). The responsible bacterial species were *S. aureus, Staphylococcus epidermidis, Propionibacterium acnes, Peptostretococcis spp., Bacteriodes spp, Corynebacterium spp, Micrococcus luteus* and *Staphylococcus homini*. The concentrations of manuka oil used were from 0.03% to 10% v/v. The study found that 0.03% manuka oil did not completely inhibit bacterial growth of all tested species, but 0.07% was enough to achieve complete growth inhibition. It was concluded that East Cape manuka oil is effective against some species of bacteria even at low concentrations (Douglas *et al.* 2004).

Comparison of the oil constituents for the four oils used in this study showed that myrcene and ß- caryophyllene are present in all four oils (Table 1). Both orange oil and lemon oil have high limonene and citral (neral and geranial) contents with no ketone present (Table 1). Lemongrass oil has the highest citral (neral and geranial) and ester (geranyl acetate and linalyl acetate) content compared to the other oils (Table 1). Manuka oil has the highest ketone level while ketone is not present in the other three oils. It is also high in some terpenes, such as ylangene, α-copaene and trans-calamenene which are not present in the other three oils (Table 1).

1.7 Research Objectives

The aim of this research was to discover effective, safe and economical treatments to reduce the number of postharvest fruit losses in New Zealand, especially on stone fruit (smooth skin/ soft fruit) and citrus fruit (thick skin fruit).

Previous studies showed that ethanol is an effective postharvest treatment on table grapes, but no studies have been reported for other fruit. Also, no studies have been done on assessing ethanol as a postharvest treatment in New Zealand and on fruit that are grown in New Zealand. Therefore, one aim of this project was to assess the effect of ethanol (dipping and vapour) as a postharvest treatment of New Zealand grown fruit. Ethanol is produced cheaply in bulk in New Zealand from dairy whey.

Natural or biological products such as honey and milk have been reported to have antibacterial activity. Specific compounds present in honey and milk are known to have antibacterial activity (Waikato Honey Research Unit 2005, and Losnedahl *et al*. 1996) but none of these compounds have been tested for antifungal activity. In this project, milk and honey were analysed for any antifungal activity that may be used as postharvest treatment on fruits. Natural products, such as honey and milk are also produced cheap in New Zealand.

While essentials oils are also believed to have antibacterial activity, some commercial essential oils are also thought to have antifungal activity. However, there is currently little scientific literature on the use of commercial essential oils as postharvest treatment on New Zealand grown fruit. Therefore, in this project, essential oils at low concentrations were tested as postharvest treatment on New Zealand grown fruit. It is also known that essential oils are generally used as food additives, therefore they maybe more acceptable to customers and less hazardous than other chemicals.

Methods and Materials

2.1 Fungal Organisms

2.1.1. *Monilinia spp*

A wild type (REB 102-1) strain of *Monilinia fructicola* was obtained from Dr. Ross Beever, Landcare Research, Auckland, New Zealand. A culture was maintained on Potato Dextrose Agar (PDA) plates prepared as in section 2.1.3.

2.1.2. *Penicillium spp*

Wild type *Penicillium italicum* (REB 315-1) and *Penicillium digitatum* (REB 315-2) were obtained from Dr. Ross Beever, Landcare Research, New Zealand. These cultures were provided as pure cultures on PDA plates. These cultures were maintained on PDA plates prepared as in section 2.1.3.

2.1.3. Maintenance of cultures

PDA plates were prepared as follows: 10.26 g of PDA powder (Difco) was dissolved in 400mL of deionised water, autoclaved and cooled to room temperature. About 15mL of the cooled medium were poured into each Petri dish and left to set at room temperature for 24 hours.

After 24 hours, the centre of each PDA plate was inoculated with *Monilinia* or *Penicillium* spores scraped from the pure culture plates using a sterile inoculation needle. The plates were incubated at 25°C in the dark for five to seven days.

2.2 Fruit

2.2.1. Peaches

Peaches used for the initial study of storage treatment were obtained from the supermarkets New World and Foodtown. For all other experiments, organically grown peaches were used. These were obtained from Mrs Jones Orchard, Otago, New Zealand.

2.2.2. Oranges

Organic navel oranges were obtained from Kerimere Orchard, Kerikeri, New Zealand.

2.3 Microtiter assay

2.3.1. Preparation of *Monilinia* inoculum

Mycelial fragment (which contains spores) of *Monilinia* (Wild type 102-1) were used as the inoculum. A suspension was made using the brown mycelia scraped from a *Monilinia* plate that had been grown on PDA for seven days (as section 2.1.3). The mycelia were suspended and mixed in 10mL sterile distilled water until the water turned brown. 50 µL of this suspension were used as the inoculum in the microtiter assay.

2.3.2. Preparation of *Penicillium* inoculum

1 x Potato Dextrose Broth (PDB, Difco) was made according to the manufacturer's instruction as follows: 12 g of PDB powder was dissolved in 500mL of deionised water, autoclaved and cooled to room temperature. 0.1x PDB was made by diluting 1x PDB with sterile water.

Cork borer No.5 was used to cut hyphal discs from the edge of *Penicillium* culture plates that had been grown for seven days on PDA (as described in section 2.1.3.). Each disc was placed into 25mL sterile full strength PDB and incubated at 25 ºC for three days in the dark. 50 µL of this mycelial fragment (which contains spores) were used as the inoculum in the microtiter assay.

2.3.3. Control treatments

The positive control used in this assay was the fungicide, Saprol (manufactured by Watkins) was purchased from Palmers, New Zealand. The Saprol has an active ingredient of 65g/L triforine in the form of an emulsifiable concentrate, A working solution of Saprol was made by diluting 75µL in 25mL sterile PDB.

The negative control used in the microtiter assays was either full strength PDB or 0.1x PDB (full strength PDB diluted in 10x sterile distilled water).

2.3.4. Effect of ethanol on fungal growth

Ethanol (UNIVAR Analytical Reagent) was added to 5mL of either full strength or 0.1x PDB to final concentrations of 0.01%, 0.1%, 0.5%, 1%, 2%, 5% and 10%. Each of the individual wells of a 96 well flat bottomed microtiter plate (Kartell) were filled with 200µL of each ethanol solution, or the negative and positive control solutions, and inoculated with 50 µL of the fungal spore suspensions. This assay was done on both *Monilinia* and *Penicillium* inoculum,

Plates were incubated at 25 ºC in the dark. Growth was monitored at 0, 1, 2, 3, 4, 5 and 6 days by measuring the absorbance aseptically at 540 nm using a MUREX MRX microtiter plate reader.

The optimal ethanol concentration to ensure miscibility of essential oils in fungal growth medium without affecting fungal growth was also determined with this following difference: the inoculum used was a hyphal suspension of *Penicillium* instead of *Monilinia* spore suspension.

2.3.5. Effect of essential oils on fungal growth

Sweet orange, lemon, lemongrass or manuka essential oils (Dolphin Clinic Ltd.), (0%, 0.01%, 0.05%, 0.1%, 0.25%. 0.5%, 0.75%, 1% and 2%) were added to 5mL of sterile 0.1x PDB containing 1% ethanol. These concentrations of essential oils were tested on hyphal suspensions of *Penicillium* along with positive and negative controls as described in section 2.3.3. The effect on *Monilinia* growth was not tested in this assay.

200µL of each oil solution were added to each of the individual wells of 96 well flat-bottomed microtiter plates (Kartell) and inoculated with 50 µL of the *Penicillium* hyphal suspension. Plates were incubated at 25 ºC in the dark. Growth was monitored at 0, 1, 2, 3, 4, 5 and 6 days by measuring the absorbance aseptically at 540 nm using a MUREX MRX microtiter plate reader.

2.3.6. Analysis of results

The effects of the different treatments on fungal growth were determined by calculating the mean for all wells treated identically at the various time points. These mean values were then plotted against time. Error bars were calculated as standard errors of each mean.

2.4 Assessment of treatments on fruit

Preliminary studies were carried out to determine if any of the treatments that showed fungal growth reduction on the microtiter assay can potentially be used as postharvest treatments on fruit.

2.4.1. Determining optimal post – treatment storage conditions

A pilot experiment was carried out to determine the appropriate storage method for the fruit following treatment and the time taken for the fruit to go completely rotten. Fruit were placed onto individual Petri dishes and stored in either an enclosed plastic container, a tray covered with a sealed plastic bag or in an uncovered tray. Three pieces of fruit were placed in each storage treatments. Paper towels were placed on the bottom of each container and saturated with water. The fruit were left at room temperature, away from the sun and monitored for infection on a regular basis until the fruit appeared completely rotten.

2.4.2. Control treatments

Fruit were treated either with sterile distilled water or the fungicide, Saprol (manufactured by Watkins) and made up according to the manufacturer's instruction: 0.3% solution in sterile distilled water.

2.4.3. Vapour treatment

Twenty sheets of Hygenex Royale paper towel (14 x 21cm) were placed on the bottom of each of the plastic containers. The towels were saturated with 250mL of ethanol at 20%, 50%, 70% or 100% made up in sterile distilled water. Three pieces of fruit in each plastic container were exposed per treatment and compared with fruit dipped in sterile distilled water or fungicide. Following treatment, each fruit was placed onto individual Petri dishes to prevent direct contact between the fruit and the wet paper towel and stored in an enclosed plastic container.

2.4.4. Dipping treatment of unwounded fruit

Whole fruit were dipped in 500mL of each treatment solution in a 1L beaker. Three pieces of fruit per plastic containers were treated with 20%, 50%, 70% or 100% of ethanol (for 30 seconds or 1.5 minutes); 20%, 50% and 100% of milk (30 seconds) or 50% of honey (30 seconds). These solutions were made as follows:

- Ethanol: UNIVAR Analytical Reagent ethanol was diluted with sterile distilled water.
- Milk: "Anchor" blue top milk (3.3g/100mL) was diluted with sterile distilled water.
- Honey: 250 g "Signature Range" manuka honey was diluted with 250mL sterile distilled water.

The fruit pieces were also dipped in essential oils. Sweet orange, lemon, lemongrass or manuka essential oils were diluted in 5mL of 0.1x PDB containing 1% ethanol to the final concentrations as follows:

- Sweet Orange: 0.5% and 2%
- Lemon: 0.05%, 0.5%, 0.75% and 2%
- Lemongrass: 0.05%, 0.25%, 0.5% and 2%
- Manuka: 0.5% and 2%

Three pieces of fruit were dipped in sterile distilled water or fungicide as the control treatments. Fruit was dipped in the control and essential oil treatments for 30 seconds.

Following treatment, fruit were stored in plastic containers containing a paper towel (14 x 21 cm) placed on the bottom. The towels were saturated with sterile distilled water to ensure humidity was maintained. Three fruit pieces were placed in one plastic container. Containers were stored at room temperature (25°C) or in a cool room (4°C) and fruit were monitored for infection on a regular basis for up to 30 days. Following treatment, each fruit was placed onto individual Petri dishes to prevent direct contact between the fruit and the wet paper towel and stored in an enclosed plastic container.

2.4.5. Dipping treatment of wounded fruit

The effect of ethanol and essential oils on protecting wounded fruit from infection was also assessed. Fruit were surface sterilised by dipping in 70% ethanol for 30 seconds and left to air dry for half an hour. Spores of *Penicillium* REB 315-1 and REB 315-2 that had been grown on PDA plates for seven days were transferred into 9mL of sterile water and mixed well. The fruit were dipped into each treatment according to the method described in section 2.4.4. Further, the spore suspension (5µL) that was prepared earlier was dropped onto the surface of treated fruit. A sterile needle was poked through the centre of the spore droplet to penetrate the fruit skin by approximately 30 mm.

2.5 Assessment of infection and fruit quality

2.5.1. Monitoring infection of inoculated fruit

The size of any soft lesions present after a defined period of time was measured using a ruler and photographed. Fruit was monitored everyday for infection, including the number and size of the soft lesions for up to 30 days.

2.5.2. Assessments of the effect of ethanol on fruit quality

A small study was carried out to assess the quality of the fruit that were treated with various concentrations of ethanol. Both peaches and oranges were studied. The experiment was first prepared as described for the ethanol vapour experiment (as in section 2.4.3.) before analysis. Due to the limited time available to complete this study, the analyses were only carried out for ten days.

2.5.2.1. Analysis of liquid accumulation and flesh quality

It was previously found that high ethanol concentrations can damage cell walls of fruit (Podd and Staden, 2004). Previous experiments that were carried out in this study observed that some liquid accumulated on the Petri dishes after a certain period of storage before the fruit turned brown. Hence this experiment was done to determine if the liquid accumulation related to cell wall damage caused by high concentration of ethanol and, also, if the ethanol penetrated through the skin to the flesh of the fruit.

Ten peaches and oranges were exposed to 20%, 50%, 70% or 100% ethanol by vapour (as described in section 2.4.3.). The fruit were stored at room temperature and one piece of fruit was taken out with the Petri dishes from the storage container for analysis everyday for up to ten days. The liquid that accumulated on the Petri dishes was poured into a beaker and weighed. The fruit were cut into half, photographed and monitored for flesh browning.

Results

3.1 Post – treatment storage

A pilot experiment was carried out to determine the optimal storage method for fruit following treatment and the time taken for fruit to become rotten i.e. to determine the period over which infection should be assessed. Fruit were untreated and monitored every day for up to 14 days.

Figure 26 shows that all untreated fruit were completely rotten by day 12, regardless of the storage conditions. However, the rate of infection development differed for the different storage methods. Fruit that was stored covered rotted fastest (Fig 26). After two days, fruit that were stored in a tray covered with a plastic bag showed a 35mm soft lesion (Fig. 26 A), whereas, fruit that were stored in an enclosed plastic container showed nine soft lesions (ranging from 3 mm to 15 mm, Fig. 26 B). In contrast, fruit stored uncovered showed no infection at this stage (Fig. 26 C). After four days, fruit stored in trays covered with a plastic bag and fruit that were stored uncovered were partly infected, whereas fruit that were stored in an enclosed plastic container were badly infected with fungal growth on the whole surface of the fruit. By day 12 all fruit were completely rotten regardless of the storage method.

As untreated fruit were completely rotten by day 12, it was assumed that treated fruit would take a longer time to rot than untreated fruit. Therefore, 30 days was chosen as the storage time for any experiments involving any potential antifungal treatments.

From these results, storing fruit in enclosed plastic containers was considered to be the best storage method as the fruit rotted at a rapid rate and the level of infection was high. It would be possible to tell very quickly if any of the treatments tested would have any impact on fungal infection.

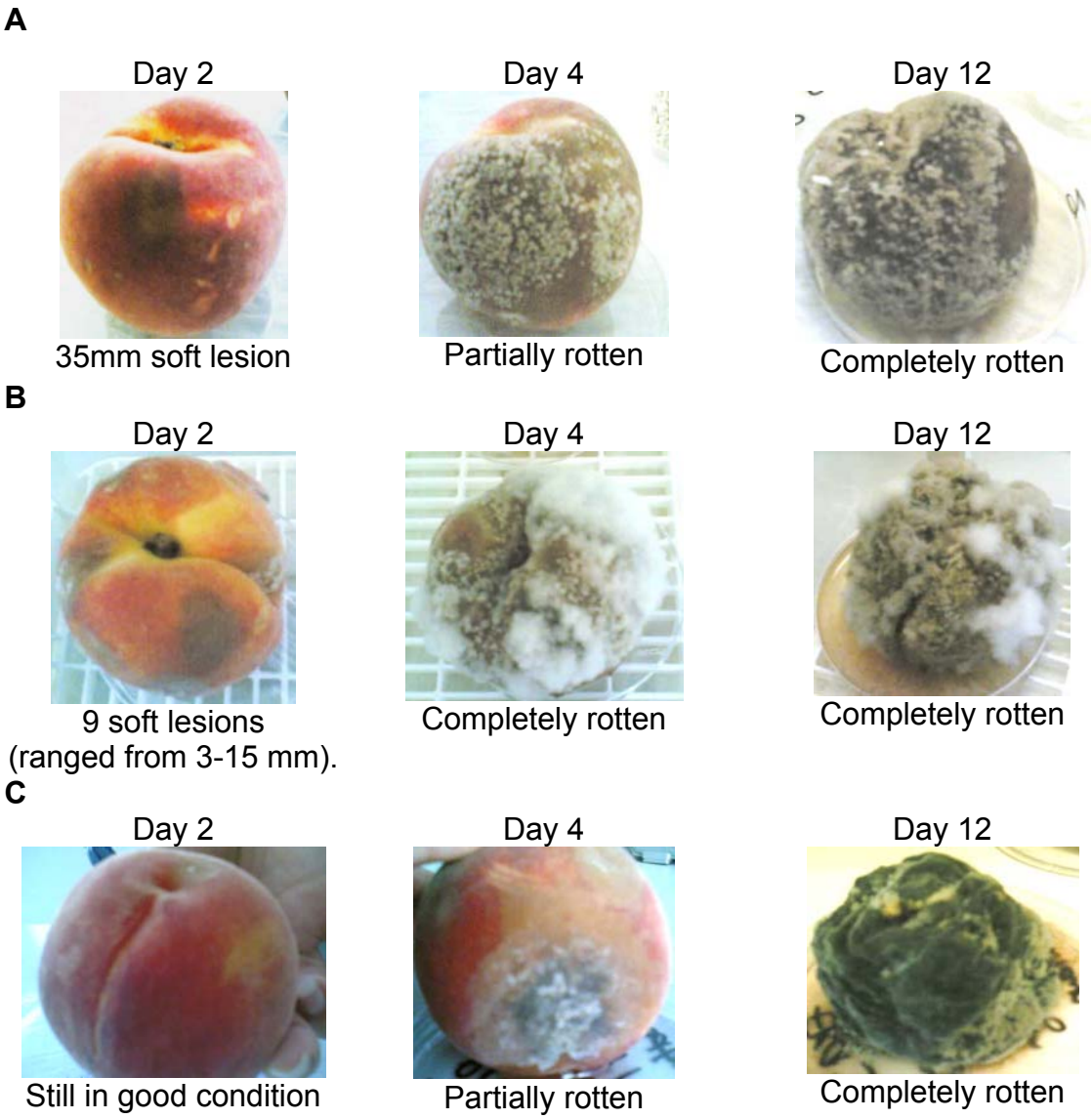


Figure 26. Example results, comparing post-treatment storage methods and infection period. **A)** Fruit stored in a tray sealed with a plastic bag. **B)** Fruit stored in an enclosed plastic storage container. **C)** Fruit stored in an uncovered tray. Fruit shown are examples of the level of infection seen at days 2, 4 and 12 for each treatment.

3.2 Ethanol treatment

The inhibition effect of ethanol on *Penicillium spp.* and *M. fructicola* spores was investigated. Different ethanol concentrations were assessed for their ability to inhibit growth *in vitro* using a microtiter assay and *in vivo* using treated fruit. Results were compared with fruit treated with sterile distilled water (negative control) or fungicide (positive control).

3.2.1. Microtiter assay

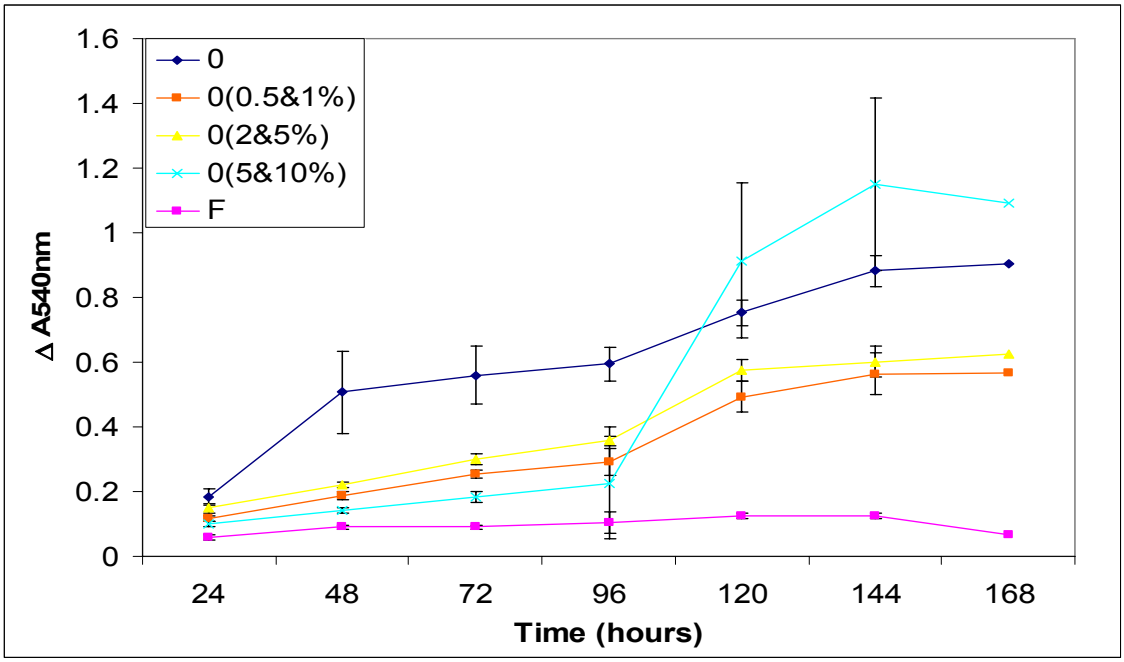
3.2.1.1. Interference of zero values

Since ethanol gives off a vapour, it was thought that any vapour rising out of any well containing ethanol may affect the surrounding wells. To test this, the microtiter plates were set up so that wells with no ethanol added were interspersed with wells containing ethanol of increasing concentrations. If ethanol vapour was having an effect, a reduced fungal growth was expected to be observed for those wells in proximity of wells containing increasing ethanol concentrations. This was indeed the case. Figure 27 A shows that the untreated samples located near the low and moderate ethanol concentrations ($0^{0.5.\&1}$ and $0^{2\&5}$) had significantly less growth than the untreated sample 0. The untreated sample $0^{5\&10}$ inhibited *Penicillium* growth for the first 96 hours and after that the fungus appeared to recover and increase its rate of growth.

Figure 27 B shows the comparison between the fungicide and the mean of all untreated samples $0^{0.5.\&1}$, $0^{2\&5}$ and $0^{5\&10}$ ("0"). While there was a significant difference overall between the untreated and fungicide treated samples, the growth of the untreated samples was cleary reduced.

The effect of the growth media was also tested. Cultures were grown in 0.1x PDB and full strength (1x) PDB (see section 3.2.1.2.). While the amount of growth observed in 0.1 x PDB was lower overall than 1x PDB, the effects of the different ethanol concentrations on the untreated wells was the same (data not shown).

A



B

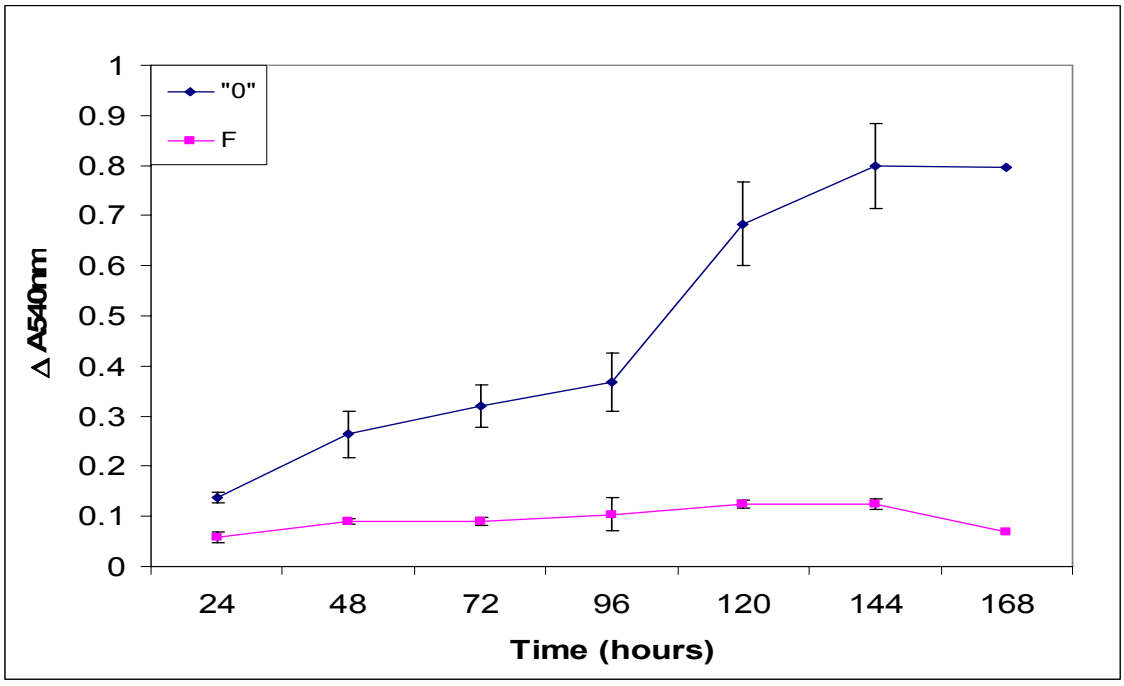


Figure 27. Representative graphs for comparison of growth between untreated and fungicide treated *Penicillium* REB 315-1 in full strength PDB over 168 hours as measured using a microtiter growth assay. The amount of growth was measured as the A_{540nm} at day 0 subtracted from the absorbance at each subsequent time point (ΔA_{540nm}). Each point represents the mean of four samples, error bars represent the standard error of the mean. **A)** The amount of growth for all of the untreated samples. 0 refers to untreated samples placed next to the lowest concentration of ethanol used in the assay i.e. 0.01%, 0(0.5&1%) refers to untreated samples placed between samples treated with 0.5% and 1% ethanol (referred to as $0^{0.5.\&1}$ in the text), 0(2&5%) refers to untreated samples placed between samples treated with 2% and 5% ethanol (referred to as $0^{2\&5}$ in the text), 0(5 & 10%) refers to untreated samples placed between samples treated with 5% and 10% ethanol (referred to as $0^{5\&10}$in the text). **B)** The amount of growth for the mean of all untreated samples compared with fungicide. "0" refers to the mean of all untreated samples (0, $0^{0.5.\&1}$, $0^{2\&5}$ and $0^{5\&10}$) in the assay. F refers to samples treated with fungicide.

Since the untreated samples ($0^{0.5.\&1}$, $0^{2\&5}$ and $0^{5\&10}$) had been affected by the surrounding wells, these samples could not be considered to be truly untreated. Presumably, ethanol volatiles had affected the samples, therefore comparative analyses were not done using these samples. For consistency, the untreated sample of 0 was used for all comparisons with ethanol concentrations.

It was also observed that ethanol from surrounding wells can impact the growth of untreated *M. fructicola*. Similar to what was observed for *Penicillium* growth, this effect increased as the ethanol concentration increased (data not shown). Again, the untreated wells closest to the higher concentrations of ethanol could not be considered truly untreated.

3.2.1.2. Effect of ethanol treatment on *Penicillium* REB 315-1 growth

The effect of varying ethanol concentrations on *Penicillium* REB 315-1 growth was assessed using full strength and 0.1x PDB. This was done to determine if any effects would be more obvious in one medium over the other. Full strength PDB is quite a rich medium potentially providing an environment for the fungus to withstand assault from candidate antifungals. It was considered that 0.1x PDB would provide less protection in this way and therefore any antifungal effects may be more obvious.

This assay discovered that the effect of varying concentrations of ethanol was quite consistent between both full strength and 0.1x PDB. In both full strength and 0.1x PDB, the 0.01% and 0.5% ethanol showed no inhibition effect towards *Penicillium* growth (Fig. 28 A and C and Fig. 29 A and C). In contrast to full strength PDB, 0.1% ethanol in 0.1x PDB showed no growth inhibition, whereas it showed growth inhibition in full strength PDB (Fig. 28 B and Fig. 29 B). Ethanol at these concentrations had less inhibitory effect compared to the fungicide, appearing to have lower antifungal activity than the fungicide for the duration of the assay.

It appeared that 1% and 2% ethanol showed a slightly greater degree of inhibition in full strength PDB than in 0.1x PDB. In full strength PDB, 1% and 2% ethanol were inhibitory for the duration of the assay.

In contrast, 1% and 2% ethanol in 0.1x PDB was inhibitory only up to 96 hours, after which the growth rate increased, allowing the fungus to recover (Fig. 28 D and E and Fig. 29 D and E).

5% ethanol in 0.1x PDB did not inhibit fungal growth. Indeed, it appeared to be stimulatory to fungal growth from 96 hours (Fig. 28 F and Fig. 29 F). In contrast, 5% ethanol in full strength PDB appeared to be inhibitory within the first 96 hours before the fungus began to recover.

10% ethanol inhibited fungal growth for the first 96 hours in both growth media. This ethanol concentration seemed to have better antifungal activity than fungicide for the first 48 hours. However, the fungus appeared to overcome the antifungal activity and appeared to be stimulated by the 10% ethanol after 96 hours of exposure (Fig. 28 G and Fig. 29 G).

In summary, 0.1x PDB gave less fungal growth overall compared to full strength PDB. Additionally, it appeared that most of the ethanol concentrations gave slightly better antifungal activity in full strength PDB compared to 0.1x PDB. The fungicide was fungicidal in 0.1x PDB as well as in full strength PDB. The opposite result was expected as it was thought that full strength PDB may protect the fungus from the treatment. This did not appear to be the case.

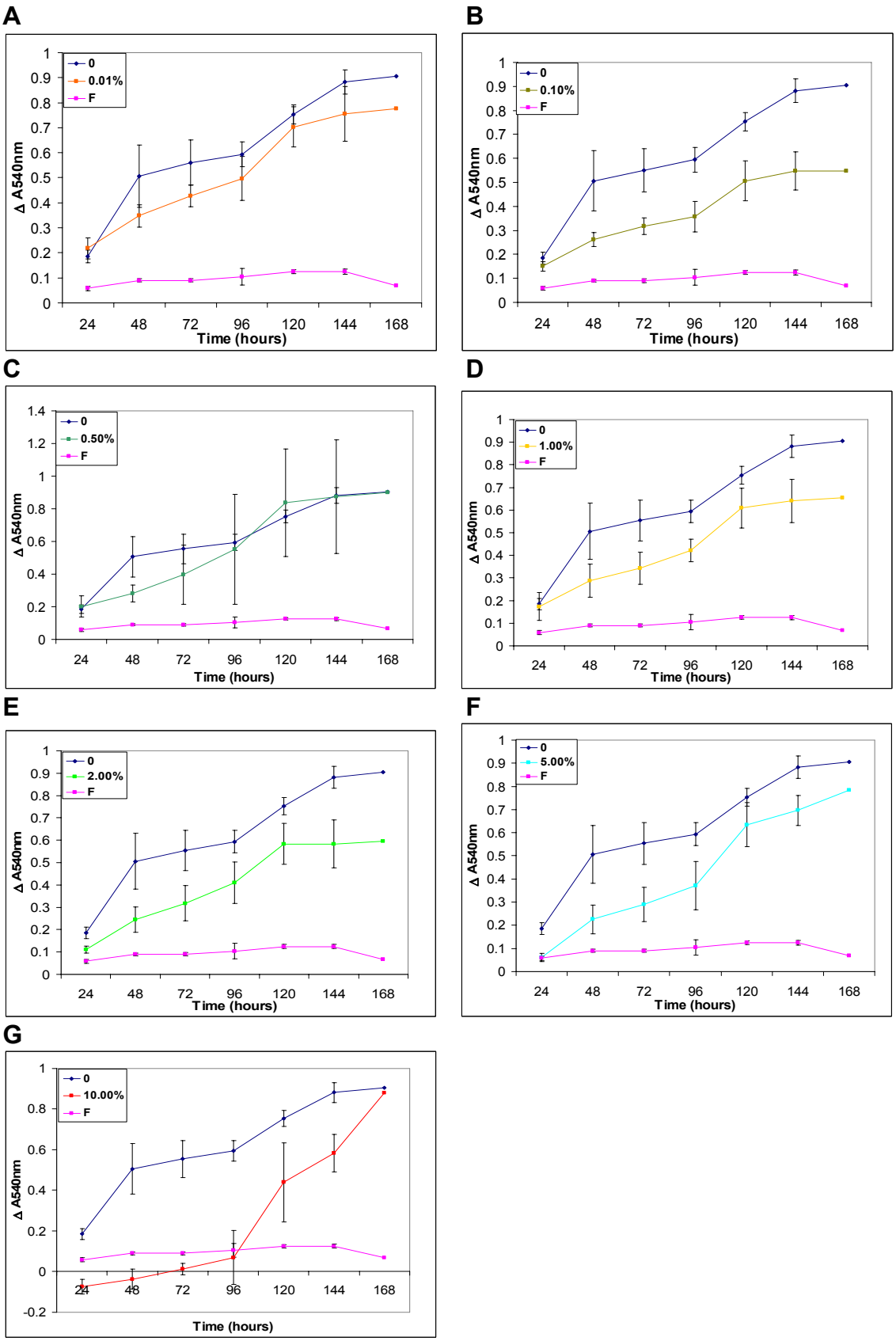


Figure 28. Inhibition effect of ethanol towards the growth of *Penicillium* REB 315-1 in 1xPDB over 168 hours as measured using a microtiter growth assay. Each graph shows normalised data: the amount of growth measured as the A_{540nm} at day 0 was subtracted from the absorbance at each subsequent time point (ΔA_{540nm}). The graphs refer to the ethanol concentration of: **A)** 0.01% **B)** 0.1% **C)** 0.5% **D)** 1% **E)** 2% **F)** 5% **G) 10%.** 0 refers to untreated samples placed next to the lowest concentration of ethanol used in the assay i.e. 0.01% and F refers to samples treated with fungicide. Error bars represent the standard error of the mean.

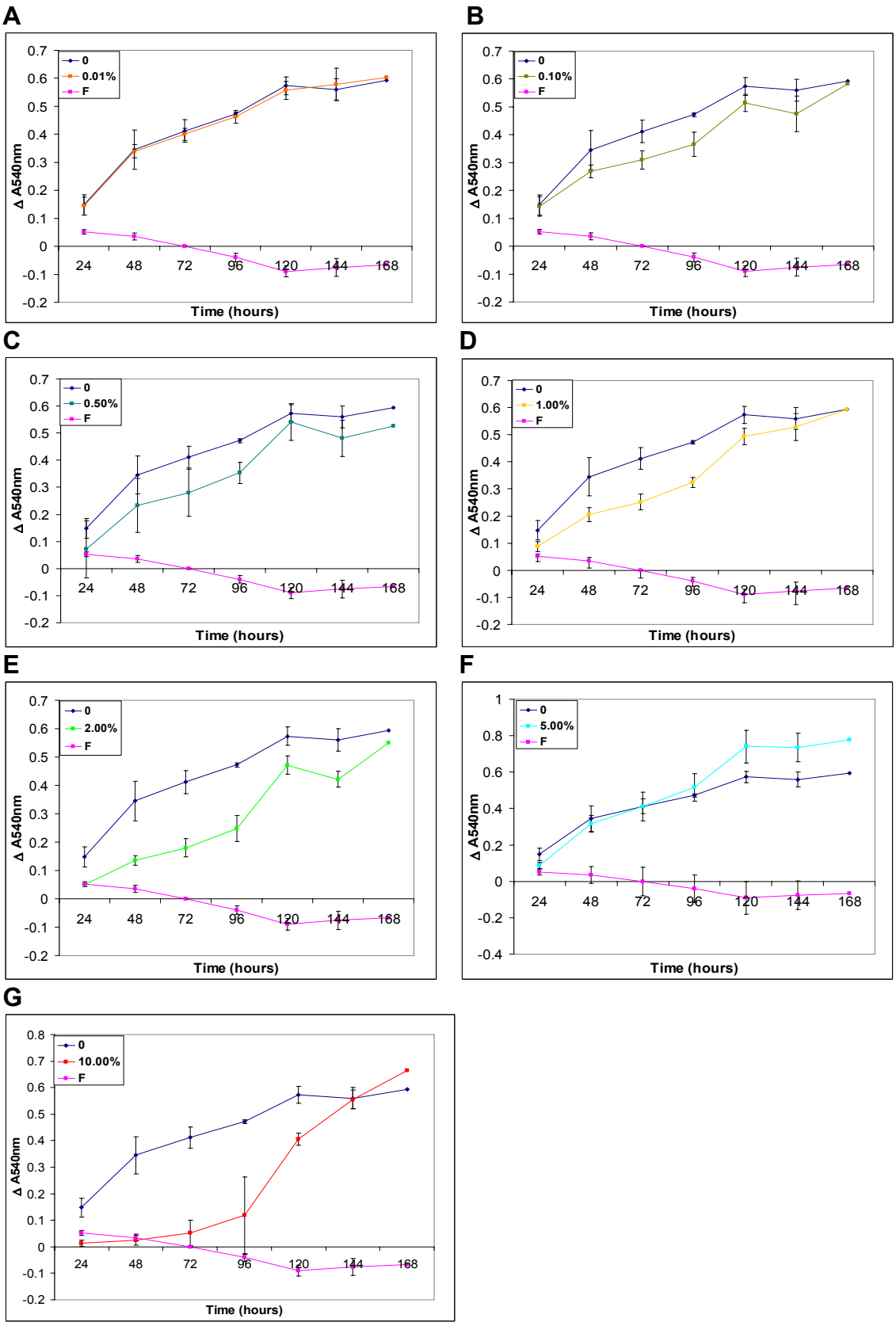


Figure 29. Inhibition effect of ethanol towards the growth of *Penicillium* REB 315-1 in 0.1xPDB over 168 hours as measured using a microtiter growth assay. Each graph shows normalised data: the amount of growth measured as the A_{540nm} at day 0 was subtracted from the absorbance at each subsequent time point (ΔA_{540nm}). The graphs refer to the ethanol concentration of: **A)** 0.01% **B)** 0.1% **C)** 0.5% **D)** 1% **E)** 2% **F)** 5% **G) 10%.** 0 refers to untreated samples placed next to the lowest concentration of ethanol used in the assay i.e. 0.01% and F refers to samples treated with fungicide. Error bars represent the standard error of the mean.

3.2.1.3. Effect of ethanol treatment on growth of *M. fructicola*

The effect of varying ethanol concentrations on *M.fructicola* growth was also assessed using full strength and 0.1x PDB. This was done to determine if ethanol would be a potential antifungal treatment for *M. fructicola*. Data for treatment in 0.1x PDB only is shown (Fig. 30).

Ethanol at all concentrations tested appeared to have an effect on *M. fructicola* growth (Fig. 30). The ethanol concentrations of 0.01% (Fig. 30 A), 0.1% (Fig. 30 B), 0.5% (Fig. 30 C) and 1% (Fig. 30 D) initially reduced *M. fructicola* growth for the first 48 hours following which growth was similar to the negative control. 0.01% ethanol was no longer effective at inhibiting growth by 168 hours, since the amount of fungus reached that of the untreated control. In contrast to 0.01% ethanol, *M. fructicola* exposed to 0.1%, 0.5% and 1% ethanol recovered somewhat after 168 hours treatment but growth did not reach that observed for the untreated control (Fig. 30 A to D).

Unlike the previous ethanol concentrations above, fungal growth in the presence of 2% and 5% ethanol steadily increased until 120 hours. Growth in 2% ethanol then slowed whereas growth in 5% ethanol increased further until the end of the assay (Fig. 30 E and F).

Interestingly, 10% ethanol seemed to have the best antifungal activity compared to the other concentrations. Fungal growth was very low for most of the assay time; however, the fungus began to recover after 144 hours exposure (Fig. 30 G).

Both *Penicillium spp.* and *M. fructicola* responded differently to the various ethanol concentrations in the microtiter assay. Ethanol at all concentrations tested on *M. fructicola* appeared to inhibit growth, whereas some ethanol concentrations failed to inhibit *Penicillium* growth.

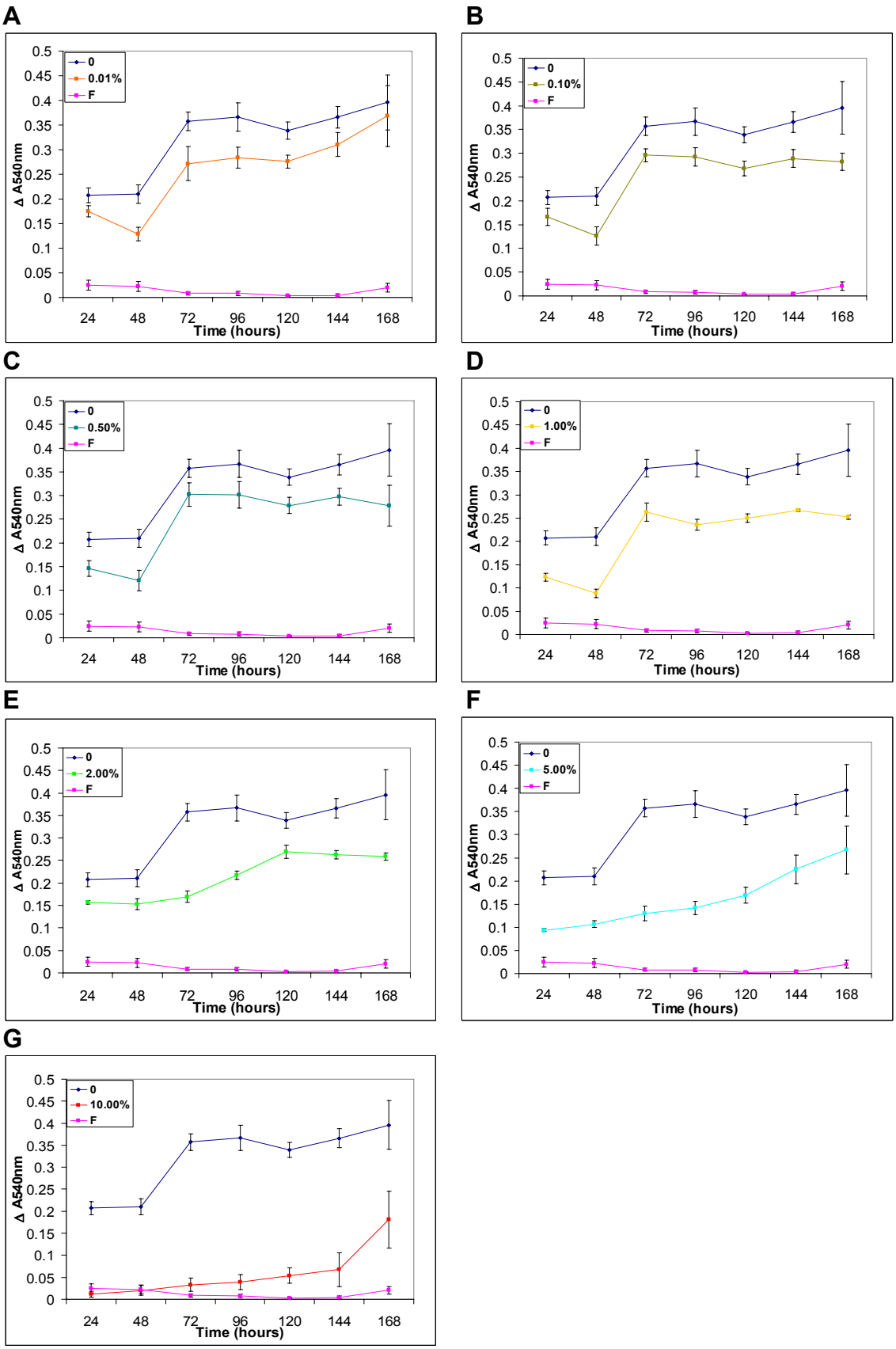


Figure 30. Inhibition effect of ethanol towards the growth of *M. fructicola* in 0.1xPDB over 168 hours as measured using a microtiter growth assay. Each graph shows normalised data: the amount of growth measured as the A_{540nm} at day 0 was subtracted from the absorbance at each subsequent time point (ΔA_{540nm}). The graphs refer to the ethanol concentration of: **A)** 0.01% **B)** 0.1% **C)** 0.5% **D)** 1% **E)** 2% **F)** 5% **G) 10%.** 0 refers to untreated samples placed next to the lowest concentration of ethanol used in the assay i.e. 0.01% and F refers to samples treated with fungicide. Error bars represent the standard error of the mean.

3.2.2. Ethanol vapour treatment of fruit

3.2.2.1. Vapour treatment on peaches

The effect of varying concentration of ethanol vapours was assessed on peach fruit. The fruit that were exposed to the various ethanol vapour concentrations were monitored everyday for any infection. The ethanol-treated fruit were compared with fungicide-treated fruit and untreated (water-treated) fruit.

A. Room temperature storage

The effect of various ethanol vapour concentrations as postharvest treatment on peaches was assessed. Fungal growth was rated as slight growth, moderate growth and heavy growth. Rotted fruit was when the whole fruit was covered in fungal growth or when the cell wall ruptured with the cell contents leaked out. Figure 31 shows that untreated fruit (0) had greater fungal growth and rotted faster compared with treated fruit. The fruit were maintained in good condition with no infection for the first three days of storage (Fig. 32 A). After four days, seven soft lesions ranging from 3mm to 20mm appeared on the fruit (Fig. 32 B). Further, after ten days, half of the fruit was badly infected and half-covered with fungal growth (Fig. 32 C). From 14 up to 30 days, the fruit became completely rotten as the whole fruit became covered with fungal growth (Fig.32 D and E).

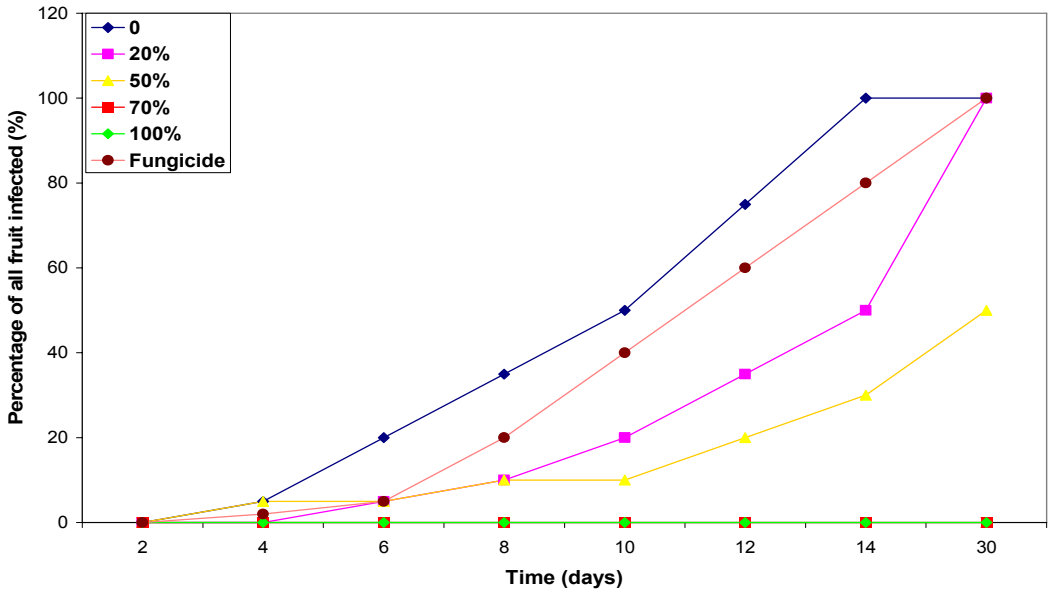


Figure 31. The effect of different concentrations of ethanol vapour on peaches, showing the development of fruit infection over time. Fruit were stored at room temperature for 30 days.

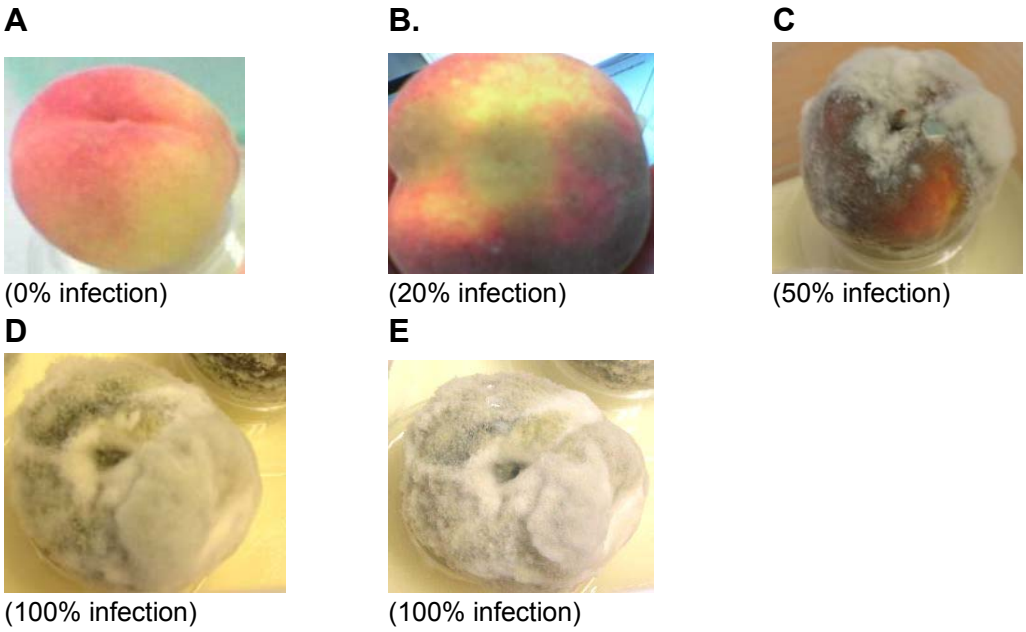


Figure 32. The development of fungal infection over time on untreated peaches, stored at room temperature. **A)** Fruit stored for 1-2 days. **B)** Fruit stored for 4 days. **C)** Fruit stored for 10 days. **D)** Fruit stored for 14 days. **E)** Fruit stored for 30 days. The percentage of fungal infection on each fruit is indicated inside the brackets.

At room temperature, fungicide-treated fruit showed no inhibition of fungal growth (Fig. 31). Similar to untreated fruit, the fungicide-treated fruit maintained good condition and no infection for the first three days of storage (Fig. 33 A). However, after ten days, the fruit was badly infected and covered with fungal growth (Fig. 33 B). From 14 up to 30 days, the fruit became completely rotten. It appeared that cell walls had begun to rupture as the cell contents appeared to leak out (Fig. 33 C and D).

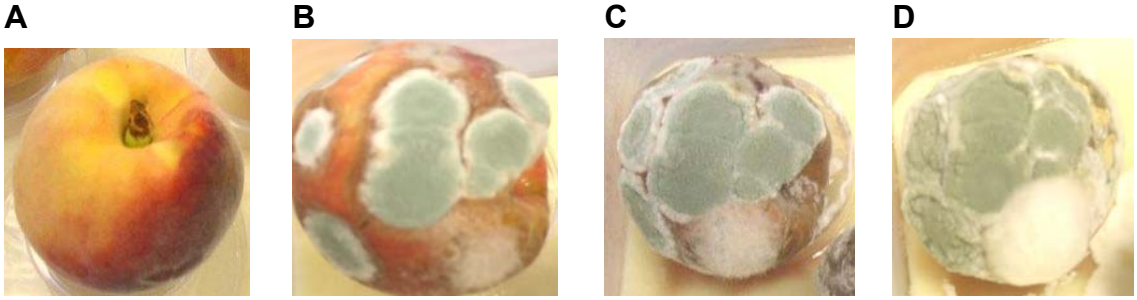


Figure 33. The development of fungal infection over time on fungicide-treated peaches after: **A)** 3 days **B)** 10days **C)** 14 days and **D)** 30 days of storage at room temperature.

Figure 31 shows that 20% and 50% ethanol reduced the percentage of fruit infected compared with the fungicide. It appeared that 20% ethanol-treated fruit were in good condition for the first four days of storage (Fig. 34 A). At day five, three small soft lesions started to appear on the fruit (Fig. 34 B), which started to grow bigger at day ten. 50% ethanol treated fruit were maintained in good condition for up to three days (Fig. 35 A), before fruit browning occurred (Fig. 35 B to D), which will be discussed later (in this section).

After ten days of storage, 50% of untreated fruit were infected and 40% of fungicide treated fruit, whereas only 10% of fruit treated with 50% ethanol vapour were infected.

It appeared that two out of three ethanol-treated fruit were completely covered with fungal growth and one fruit was partially covered with fungal growth at day 14 (Fig. 34 C). From 14 up to 30 days the fruit became completely rotten (Fig. 34 D). After 14 days of storage, thicker white growth was observed and some liquid was found to leak out of the top of the 50% ethanol-treated fruit (Fig. 35 D). The surface of all of the three fruit was covered with white growth after 30 days of storage in room temperature (Fig. 35 E).

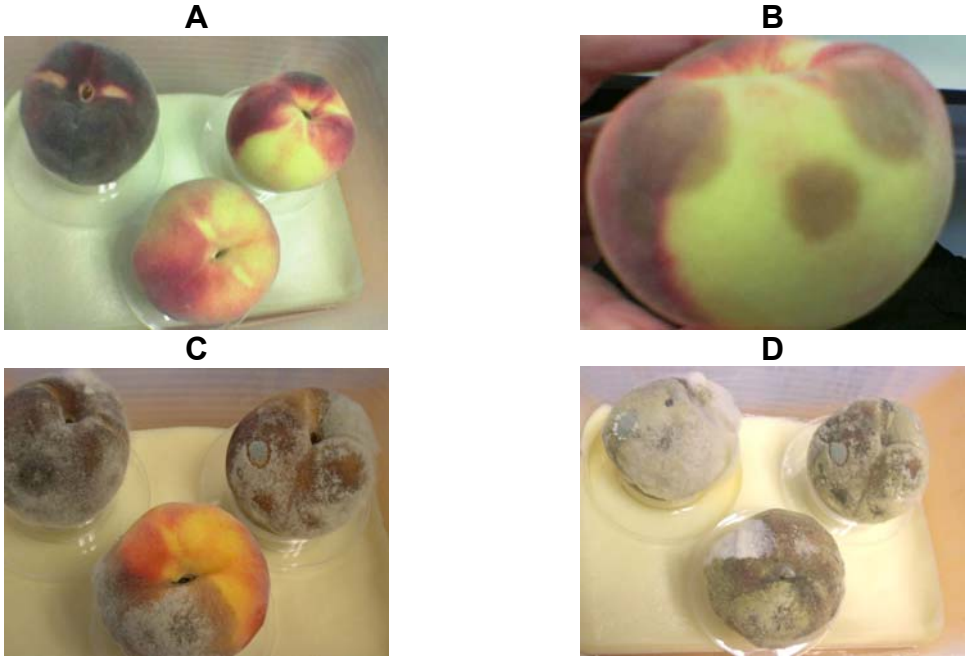


Figure 34. The development of infection over time of 20% ethanol-treated fruit. Fruit were stored in room temperature for: **A)** 0 day; **B)** 5 days; **C)** 14 days; **D)** 30 days.

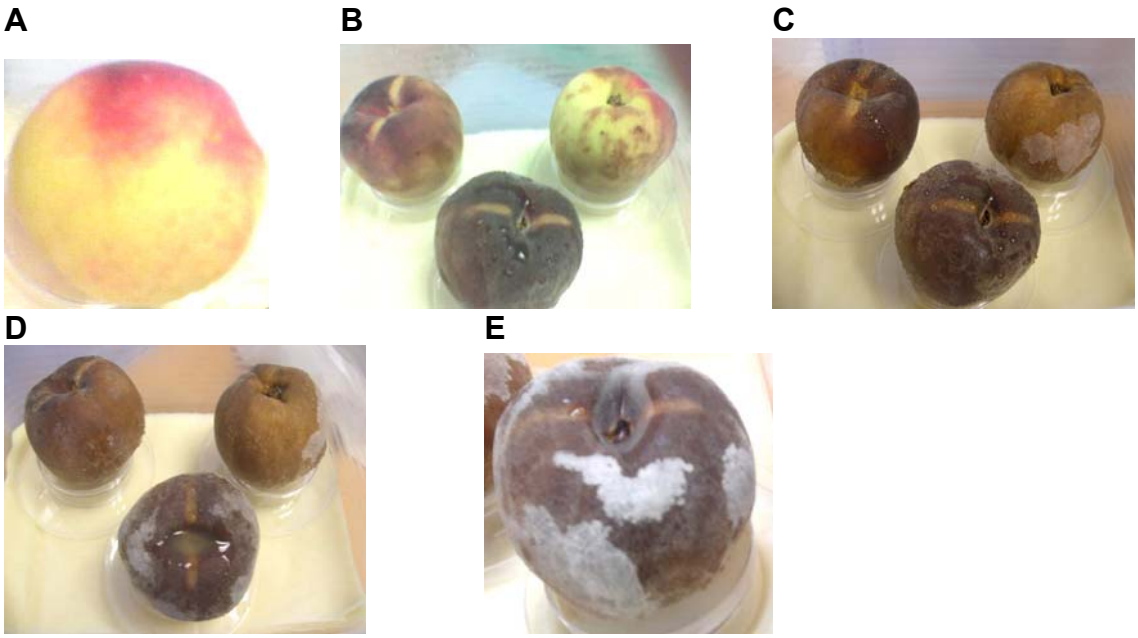


Figure 35. The development of infection over time on 50% ethanol treated fruit. The fruit were stored at room temperature for: **A)** 0 day. **B)** 5 days. **C)** 10 days. **D)** 14 days. **E)** 30 days.

In contrast to 20% and 50% ethanol, both 70% and 100% ethanol completely inhibited the development of fungal infection on the fruit for the 30 days observations were made (Fig. 31). However, the fruit began to brown (Fig. 36) with liquid accumulation on the bottom of each fruit and development of an “off” odour after three days of storage. Stronger “off” odour developed after four to five days and all fruit had gone completely brown by day five (Fig. 37 B).

While the higher concentrations of ethanol appeared effective at inhibiting fungal growth, 50%, 70% and 100% ethanol-treated fruit started to go brown after two days (Fig. 37). High concentrations of ethanol, such as 70% and 100% turned the peaches completely brown after four to five days (Fig. 37 B). Fruit that were treated with 50% ethanol turned completely brown after 10 days (Fig. 35 C).

Low concentration of ethanol (20%), as well as 0% ethanol and fungicide did not result in any fruit browning (Fig. 36).

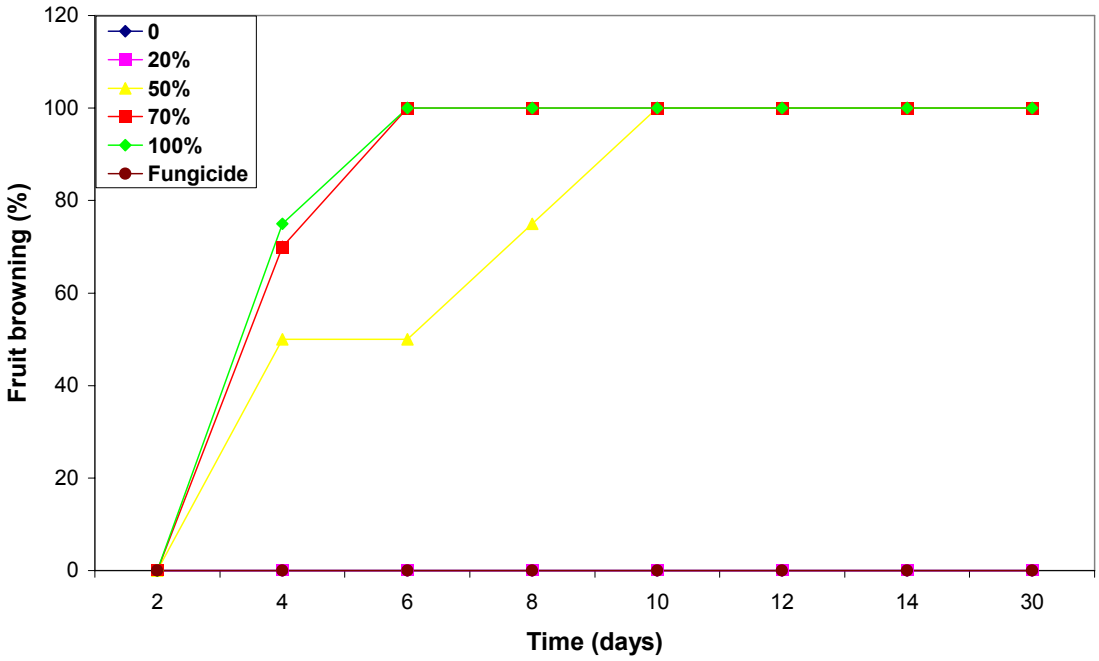


Figure 36. The effect of different concentration of ethanol vapour on peaches browning over time. The fruit were stored at room temperature for 30 days.

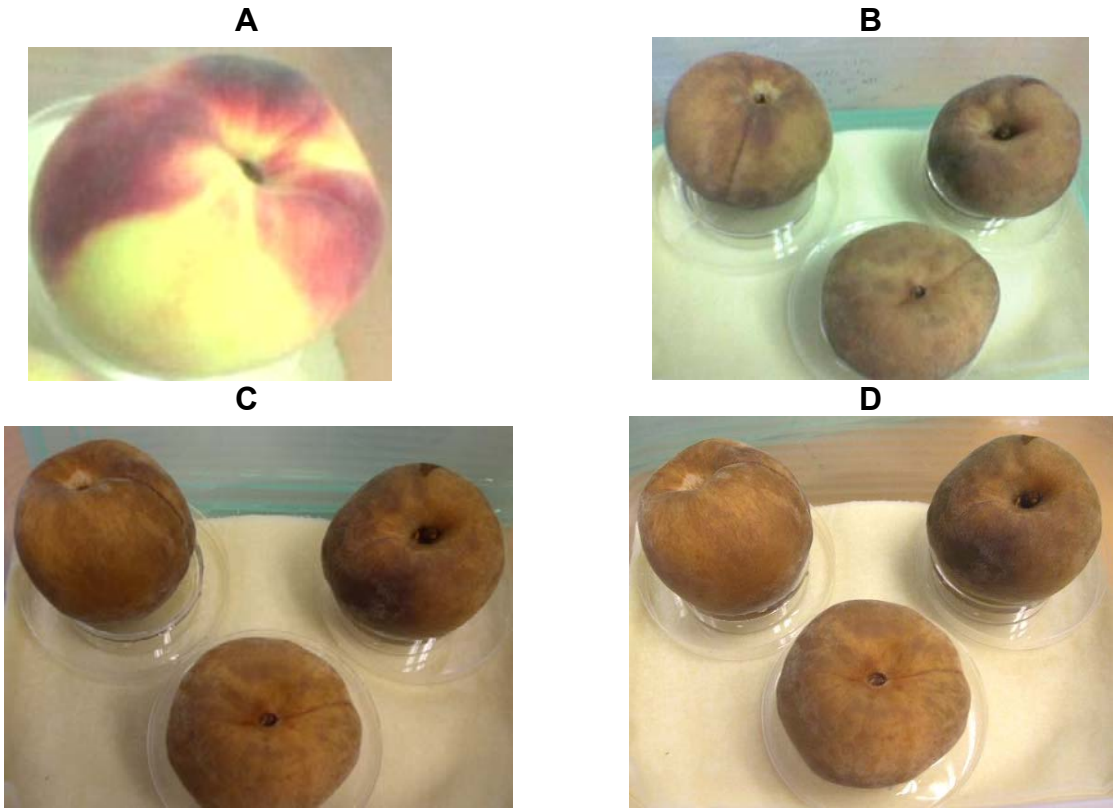


Figure 37. The development of infection over time on 70% ethanol treated fruit, stored in room temperature. **A)** Peach before storage. **B)** Peaches stored for 4 days. **C)** Peaches stored for 10 days, showing a 100% browning. **D)** Peaches stored for 30 days. These pictures also represent the 100% ethanol, since the fruit reacted in the same way with these two concentrations.

As summary, at room temperature, fungicide-treated peaches had slightly less fungal growth than the untreated peaches (Fig. 32 and 33). The infection started to develop at day four for both untreated and fungicide-treated fruit. The untreated fruit became completely rotten at day fourteen while fungicide-treated fruit became completely rotten at 30 days. Therefore, fungicide slowed down the development of infection, but it was not effective at protecting the fruit from ever developing infection.

Ethanol slowed down the development of infection on peaches compared to untreated peaches and fungicide-treated fruit. The higher the ethanol vapour concentration used, the greater the inhibition of fungal infection achieved. However, it was also found that high ethanol vapour concentrations (50%, 70% and 100%) resulted in fruit browning. The higher the ethanol vapour used, the faster the browning occurred. 70% and 100% ethanol completely inhibited fungal infection and protected the fruit from having no infection for 30 days. However, the fruit turned brown after six days.

From these results, it appeared that 20% ethanol was as effective as fungicide at delaying infection for 6 days with no browning of the fruit. Effects on flavour would need to be tested.

B. Cool temperature storage

In general, cool temperature storage slowed the development of fungal growth on fruit compared to room temperature storage. Water treated fruit had no soft lesions or fungal growth for up to 20 days (Fig. 38). This is in contrast to four days observed for fruit stored at room temperature (Fig. 38). A slight white growth began to appear on cold-stored, water treated fruit after 30 days at 4ºC (Fig.39 A). Fungicide-treated fruit did not develop any infection for up to 30 days (Fig. 39 B). Fruit treated with vapour from 50%, 70% and 100% ethanol also had no infection for up to 30 days (Fig.39 D). A slight white growth appeared on fruit treated with 20% ethanol vapour after 30 days (Fig. 39 C).

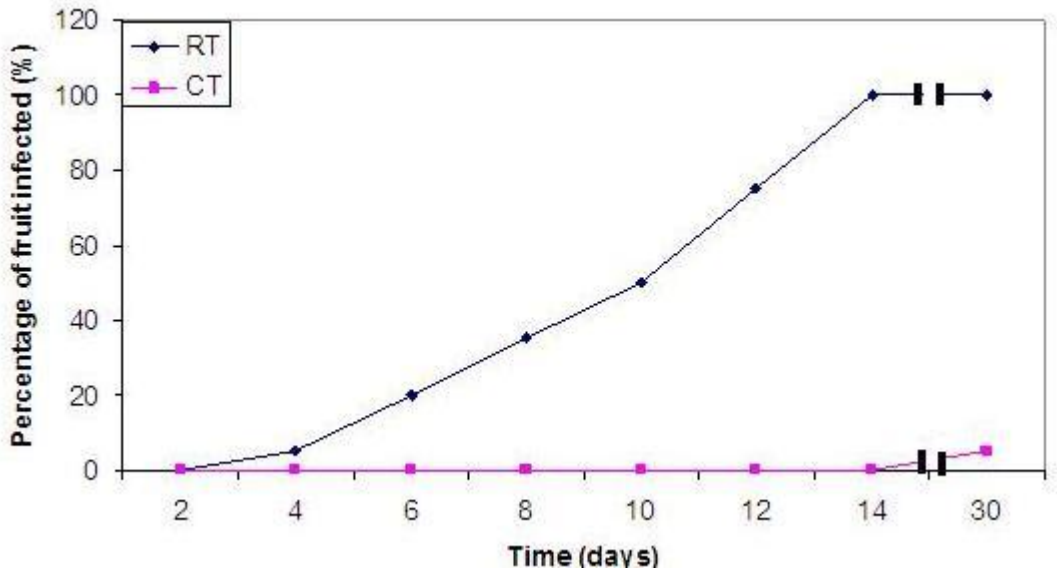


Figure 38. The development of fungal infection over time of untreated peaches with different storage temperature. RT refers to room temperature storage and CT refers to cool temperature storage (4°C).

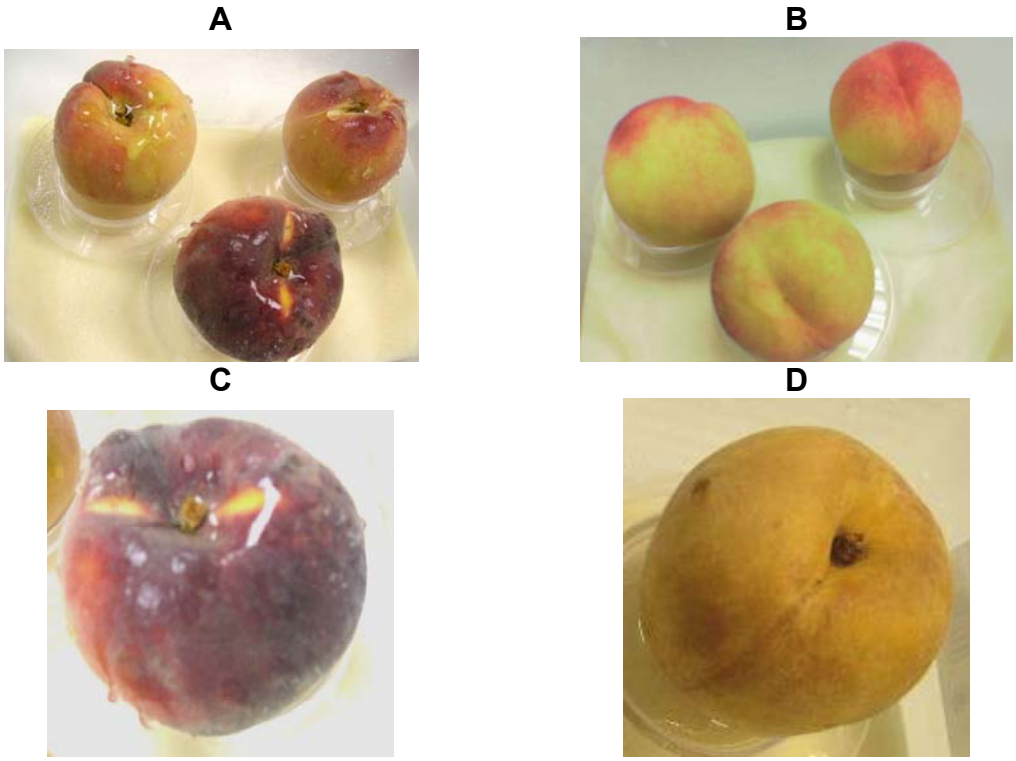


Figure 39. The development of infection over time for fruit that were stored at 4ºC. **A)** Untreated peaches after 30 days of storage. **B)** Fungicide treated peaches after 30 days. **C)** 20% ethanol treated peach after 30 days of storage. **D)** 50% ethanol treated peach after 30 days of storage.

Fruit browning was not affected by different storage temperatures. Both room temperature and cool temperature storage showed the same result for fruit browning (Fig. 40). Similar to room temperature storage, fruit treated with vapour from 50%, 70% and 100% ethanol and stored in cool temperature started to go brown after two days. High concentrations of ethanol, such as 70% and 100% turned the peaches completely brown after six days. The browning process due to 100% ethanol vapour is shown in Fig. 41. Fruit that were treated with 50% ethanol vapour turned completely brown after eight days. As for room temperature storage, low concentration of ethanol (20%), as well as 0% ethanol and fungicide treated fruit stored in cool temperature did not develop any browning (Fig 40).

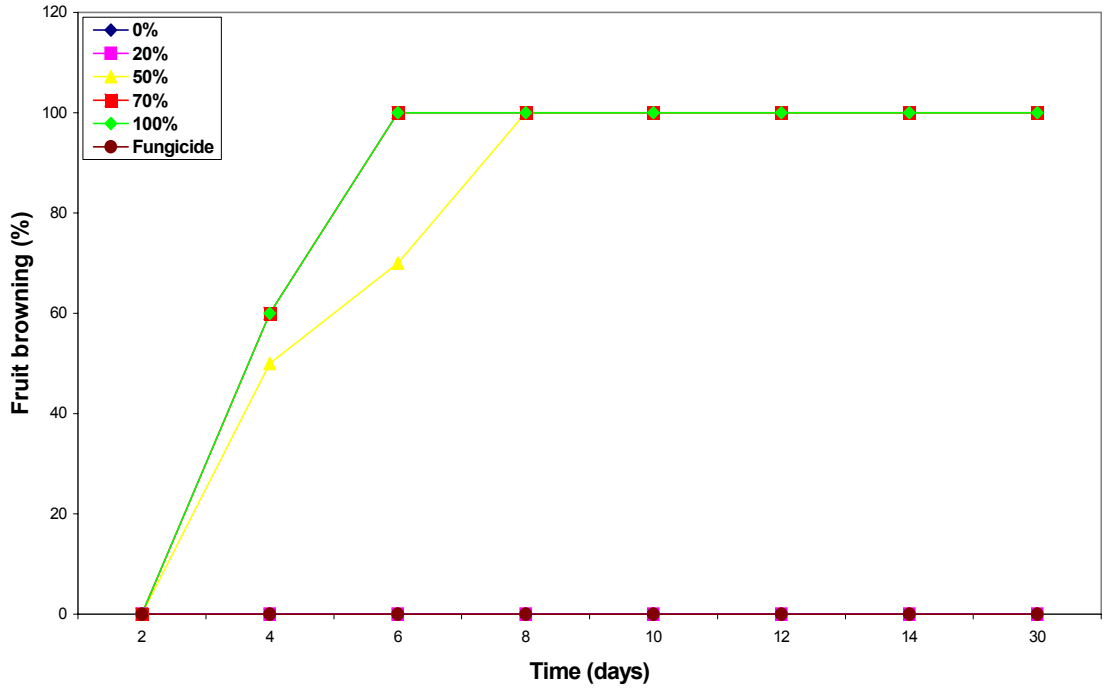


Figure 40. The effect of different concentrations of ethanol vapour on fruit (peaches) browning over time. The fruit were stored at 4 ºC.

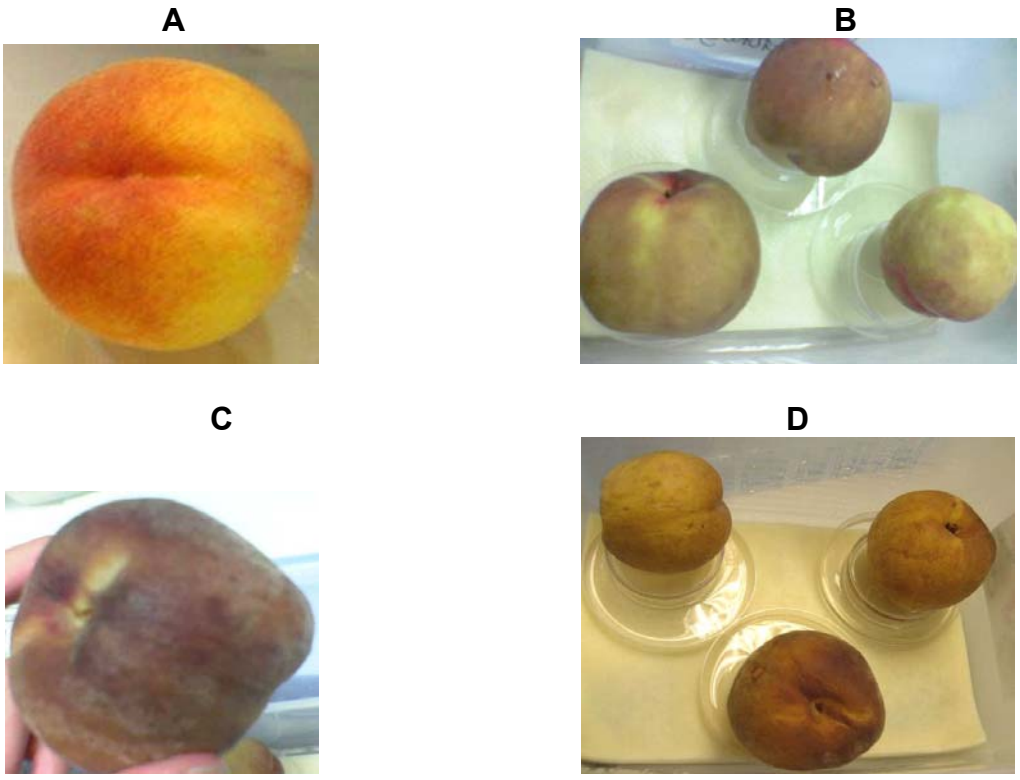


Figure 41. The development of fruit browning on 100% ethanol treated fruit stored at 4 ºC for **A)** 0 days **B)** 3 days **C)** 5 days and **D)** 30 days.

In summary, it appeared that 20% ethanol inhibited fungal growth to the same degree as the fungicide. It failed to protect the peach fruit from infection for a long period of storage (30 days) at room temperature, but when 20% ethanol was combined with 4ºC storage, it managed to protect the fruit from fungal infection for up to 20 days (Fig. 39 C).

3.2.2.2. Vapour treatment of oranges

Vapour treatment of oranges was assessed on fruit that were unwounded and wounded to determine if ethanol vapour would be more effective on healthy over the injured fruit. The inhibitory effect of 20%, 50%, 70% and 100% ethanol vapour on fungal growth was compared between the injured fruit and non-injured fruit at room temperature and cool temperature storage. The ethanol vapour-treated fruit were also compared with untreated fruit and fungicide-treated fruit.

A. Unwounded fruit

Untreated and unwounded fruit stored at room temperature were still in good condition for up to 20 days before a very slight white growth appeared on the bottom of the fruit (Fig. 42). In contrast, 20%, 50%, 100% ethanol vapour-treated fruit had no infection for up to 30 days, therefore it appeared that ethanol delayed the fungal infection on the oranges. Fungicide treated fruit were still in good condition for up to 30 days.

Figure 42. Untreated oranges after 20 days of storage at room temperature, showing a slight fungal growth on the bottom of the fruit.

Ethanol vapour treated fruit showed discolouration or fruit browning at room temperature storage (Fig. 43 and Fig. 44). The level of browning was different for each concentration of ethanol vapour (Fig. 43). Ethanol vapour concentrations of 20% and 50% gave the worst discolouration compared to 70% and 100%. In contrast to peaches, browning started to occur on 20% and 50% ethanol vapour treated fruit after six days of storage and turned completely brown after 30 days of storage. Treatment with 100% ethanol vapour resulted in a slight glossy bright orange appearance on the skin of the fruit. Untreated fruit and fungicide treated-fruit showed no browning up to 30 days of storage in room temperature. No other changes in the external appearance were observed for those fruit.

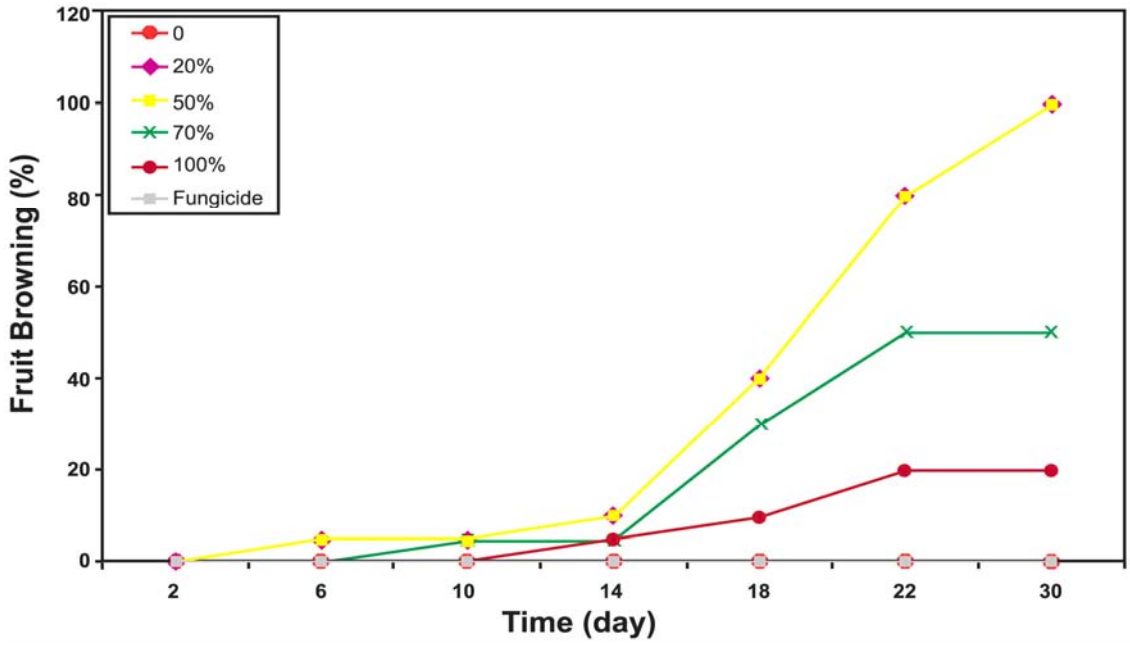


Figure 43. The effect of different concentrations of ethanol vapour on fruit (orange) browning over time. The fruit were stored at room temperature.

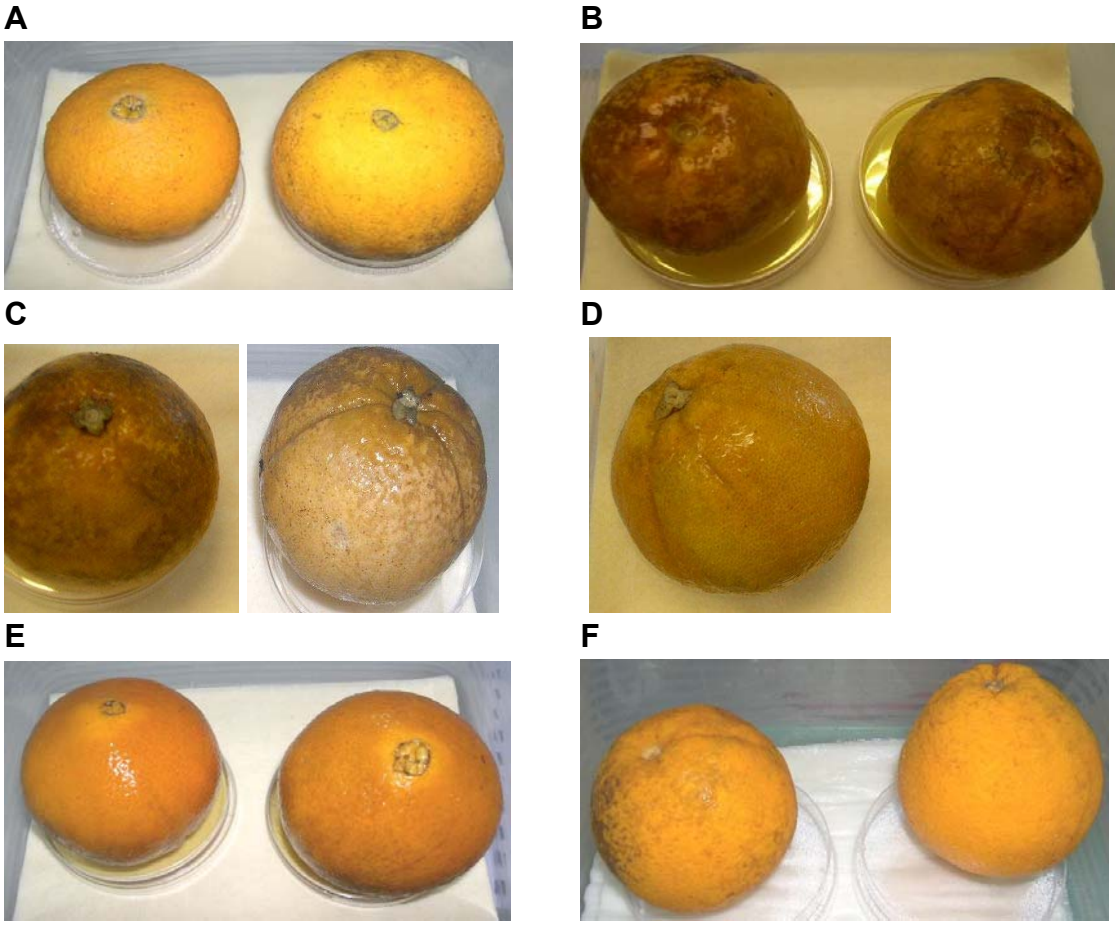


Figure 44. Comparison of oranges that were treated with varying concentrations of ethanol vapour with untreated and fungicide-treated oranges. Fruit was stored for 30 days at room temperature. Fruit were treated with **A) 0% B)** 20% **C)** 50% **D)** 70% **E)** 100% ethanol vapour and **F)** fungicide.

Unwounded fruit that were stored at 4°C were still in good condition for up to 30 days (Fig. 45). Ethanol vapour-treated fruit stored at 4°C showed the same pattern of discolouration or fruit browning as those stored at room temperature (Fig. 45). Similar to room temperature storage, treatment with 20% and 50% ethanol vapour and then stored at 4°C gave the worst discolouration compared to 70% and 100%. 70% ethanol vapour gave slight fruit discolouration. It was very hard to notice the browning that occurred in fruit that were treated with 100% ethanol vapour since it gave a slight glossy bright orange appearance on the fruit. Untreated fruit and fungicide-treated fruit showed no browning or any other external changes for up to 30 days of storage.

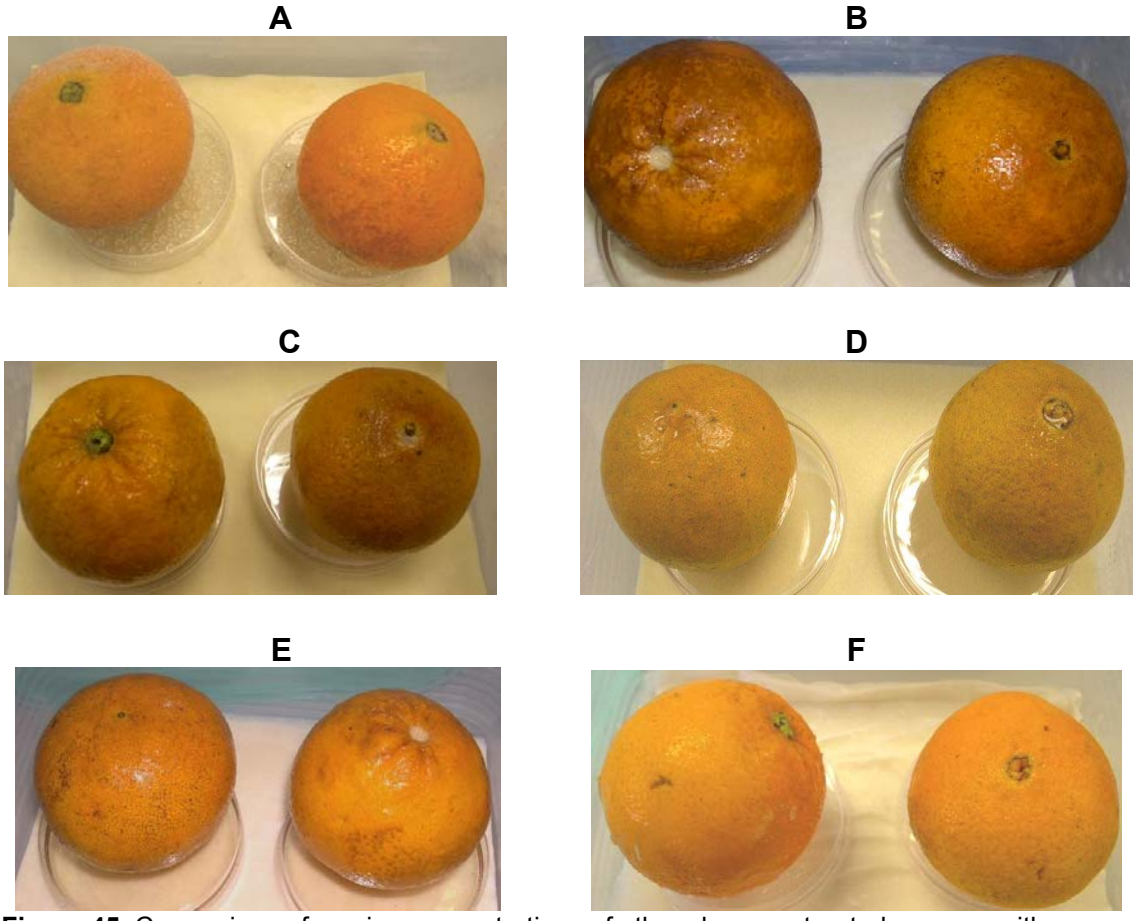


Figure 45. Comparison of varying concentrations of ethanol vapour treated oranges with untreated and fungicide treated oranges. Fruit was stored for 30 days at 4°C. Fruit were treated with **A) 0% B)** 20% **C)** 50% **D)** 70% **E)** 100% ethanol vapour and **F)** fungicide.

B. Wounded fruit

In this experiment, the oranges were wounded and inoculated with *Penicillium spp.* prior to exposure to the various treatments. This was done, as in general, oranges take a longer time to rot compared to peaches. The aim of this experiment was to analyse the inhibitory effect of 20%, 50%, 70% and 100% ethanol vapour towards *Penicillium* growth on inoculated fruit. Untreated fruit in this context refers to the fruit that were wounded and treated with water.

As expected, wounded fruit that were stored at room temperature rotted faster than unwounded fruit. The wounded areas for both *Penicillium* REB 315-1 and REB 315-2 showed soft lesions of 2 cm and 5 cm, respectively, after four days of storage at room temperature on untreated fruit and fungicide treated fruit. In seven days, the fungal growth was noticeable as either a green mould or blue mould depending on the inoculum (Fig. 46). The green mould spread faster than the blue mould in both untreated fruit and fungicide treated fruit. The fruit were completely rotten after ten days (Fig. 46 E). No soft lesions or any fungal growth occurred on fruit treated with 20%, 50%, 70% and 100% ethanol vapour. However, the same fruit browning was observed as in unwounded fruit.

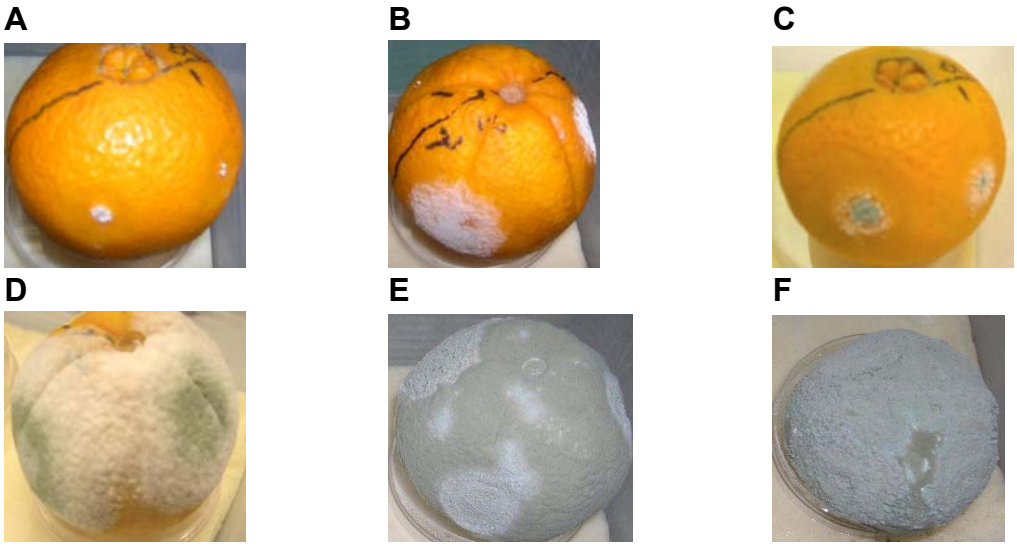


Figure 46. Development of fruit infection on wounded untreated oranges. Each fruit was inoculated at different locations with drops of spore suspension, containing either *Penicillium* REB 315-1 or *Penicillium* REB 315-2 each and wounded with a sterile needle. The orange were inoculated with **A)** *Penicillium* REB 315-1 **B)** *Penicillium* REB 315-2 **C)** *Penicillium* REB 315-1 **D)** *Penicillium* 315-2 **E)** *Penicillium REB* 315-1 and REB 315-2. **F)** *Penicillium REB* 315-1 and REB 315-2. The oranges were then stored at room temperature for **A)** and **B)** 4 days **C)** and **D)** 7 days **E)** 10 days and **F)** 21 days.

At room temperature, ethanol vapour not only changed the skin colour of the fruit, it also affected the flesh of the fruit. There was discolouration on the flesh of the fruit from an orange colour to yellow (Fig 47). There was no obvious difference in the flesh appearance between 20%, 50%, 70% and 100% ethanol vapour-treated fruit.

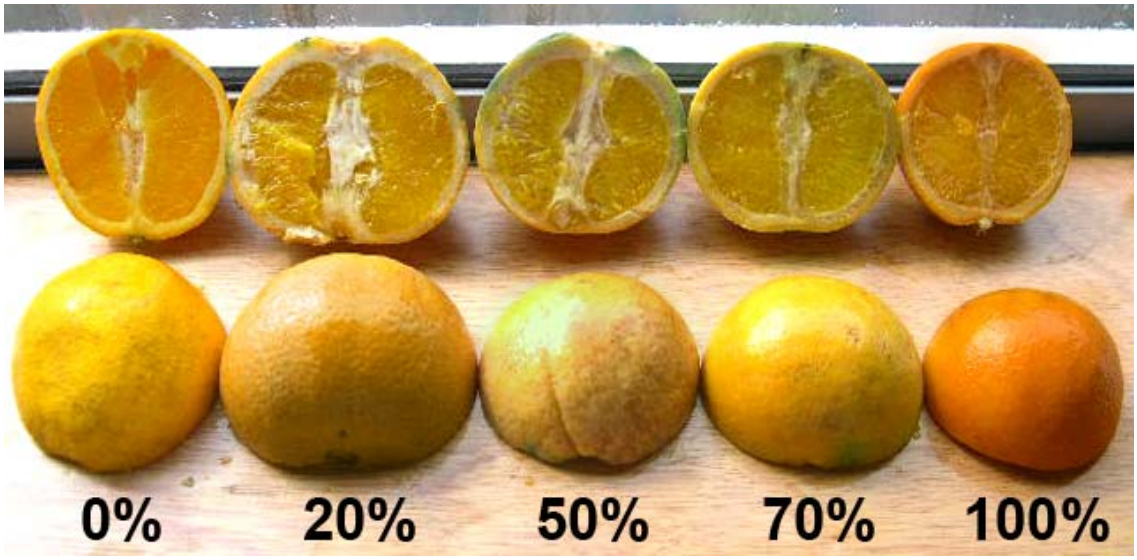


Figure 47. Fruit discolouration due to ethanol vapours. 20%, 50%, 70% and 100% ethanol vapour were used as the treatment and the fruit were stored for 21 days at room temperature. Fruit treated with varying concentrations of ethanol vapour were compared with untreated fruit.

Wounded fruit that were stored at 4ºC showed similar infection levels as unwounded fruit (Section 3.2.2.2 A, Page 70). No infection or any soft lesions were observed in untreated fruit, fungicide or fruit treated with 20%, 50%, 70% and 100% ethanol vapour (Fig. 48). Similar to room temperature storage, treatment with varying ethanol concentrations caused skin discolouration on the oranges (Fig. 48).

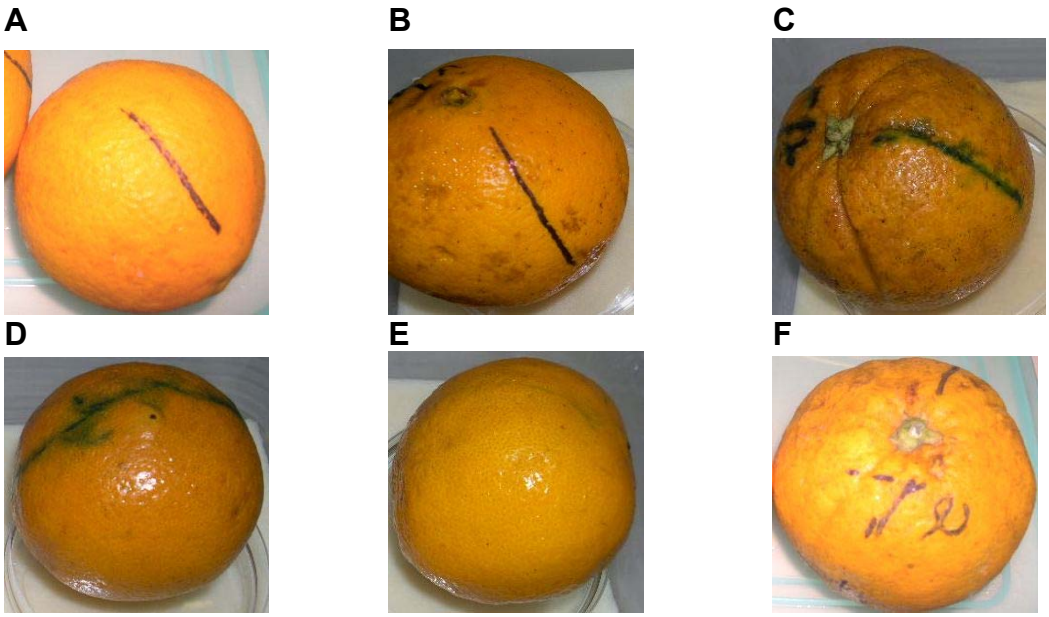


Figure 48. Comparison of untreated oranges with oranges treated with varying concentrations of ethanol vapour. These oranges were also compared with fungicide-treated oranges. All the oranges were wounded and stored for 30 days at 4°C. Fruit were treated with **A) 0% B)** 20% **C)** 50% **D)** 70% **E)** 100% ethanol vapour and **F)** fungicide.

3.2.3. Dipping Treatment

This experiment was carried out to investigate the effectiveness of 20%, 50%, 70% and 100% ethanol as postharvest treatment on peaches by dipping in the ethanol solution for 30 seconds or 1.5 minutes. Comparisons were made between ethanol dipping and ethanol vapour treatment. Treatments were compared with sterile distilled water (negative control) and fungicide (positive control).

3.2.3.1. Room temperature storage

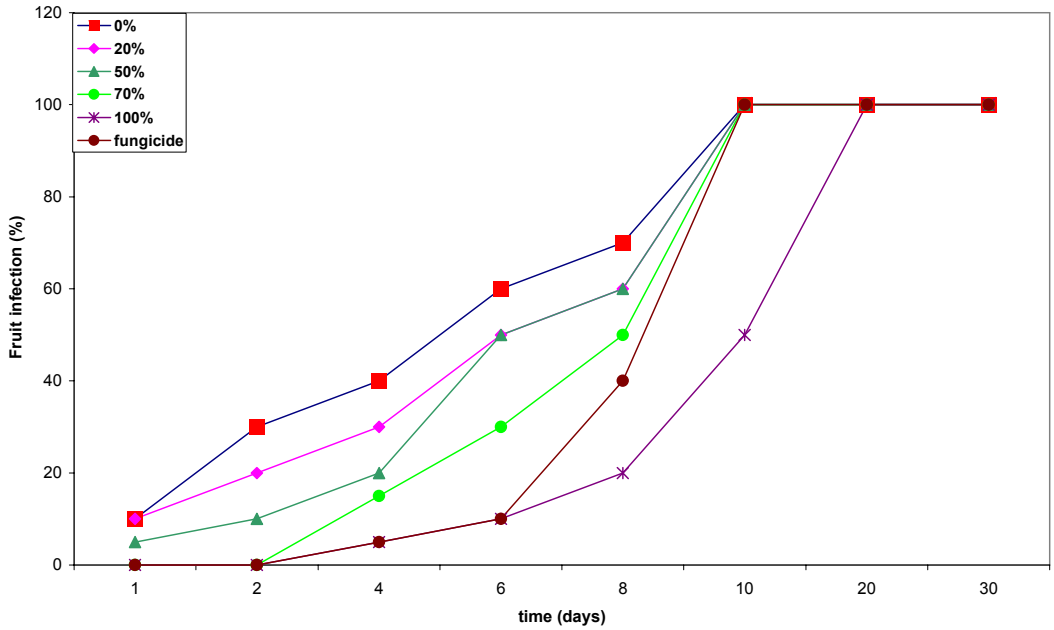
The development of fruit infection did not correlate with time of exposure of the peaches to the treatment. Fruit dipped for 30 seconds for each treatment gave similar results compared to fruit dipped for 1.5 minutes (Fig. 49 A, Fig. 49 B and Fig. 50). Water treated fruit and fruit dipped in 20%, 50% ethanol solution for both 30 seconds and 1.5 minutes developed infection with two or three small soft lesions sized 4 to 8 mm after one day, which later grew into bigger soft lesions after two days and three days.
Fungal growth developed on half of the fruit after six days. This growth spread out until the whole fruit turned completely rotten by ten days.

Fungicide-treated fruit and fruit that were dipped into 70% and 100% ethanol solution remained in good condition for up to two days. Most of the infection started after two days of storage with whole fruit completely rotten after ten days for (70% ethanol) or 20 days (100% ethanol) (Fig.51).

In contrast to vapour treatment, ethanol dipping treatment did not result in any discolouration on the skin of the fruit as was observed for ethanol vapour treatment, except for 100% ethanol. Fruit dipped in 100% ethanol for 1.5 minutes showed slight discolouration of the fruit's skin after seven days (Fig. 52).

It appeared that peaches that were exposed to various concentrations of ethanol by dipping were more susceptible to fungal infection compared to ethanol vapour treatment. Fruit that were exposed to 70% and 100% ethanol vapour showed complete fungal inhibition, whereas fruit that were dipped in 70% and 100% ethanol for 30 seconds were completely rotten by day ten. Ethanol dipping treatment did not turn the fruit brown.

A



B

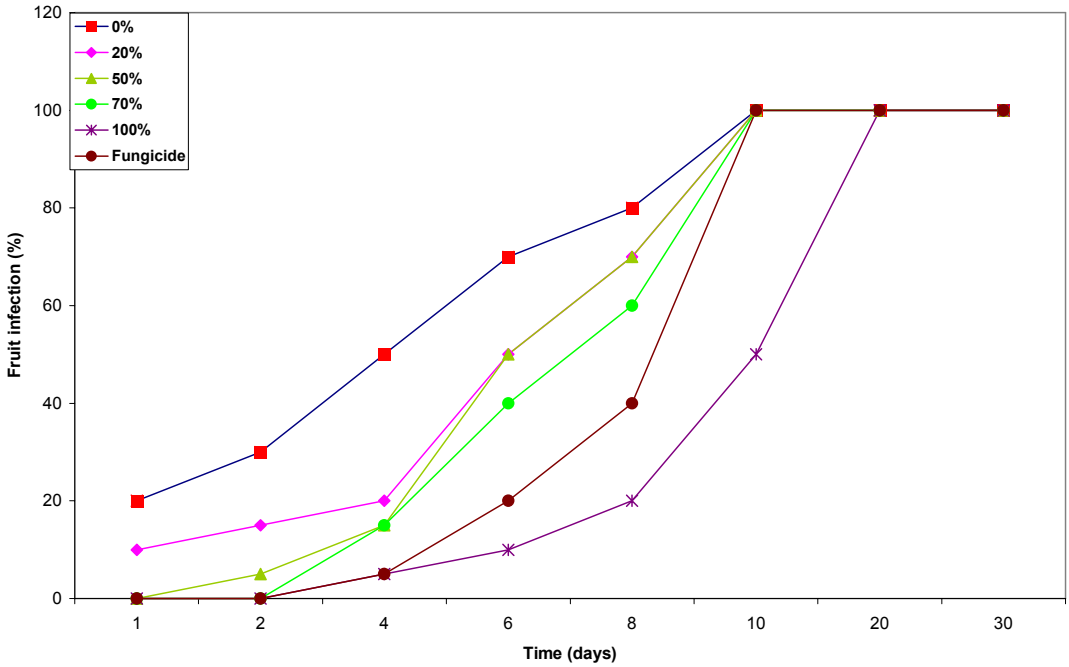


Figure 49. The development of fruit infection over time on peach fruit, dipped in ethanol solution for: **A)** 30 seconds; **B)** 1.5 minutes. The fruit were stored in room temperature for 30 days.

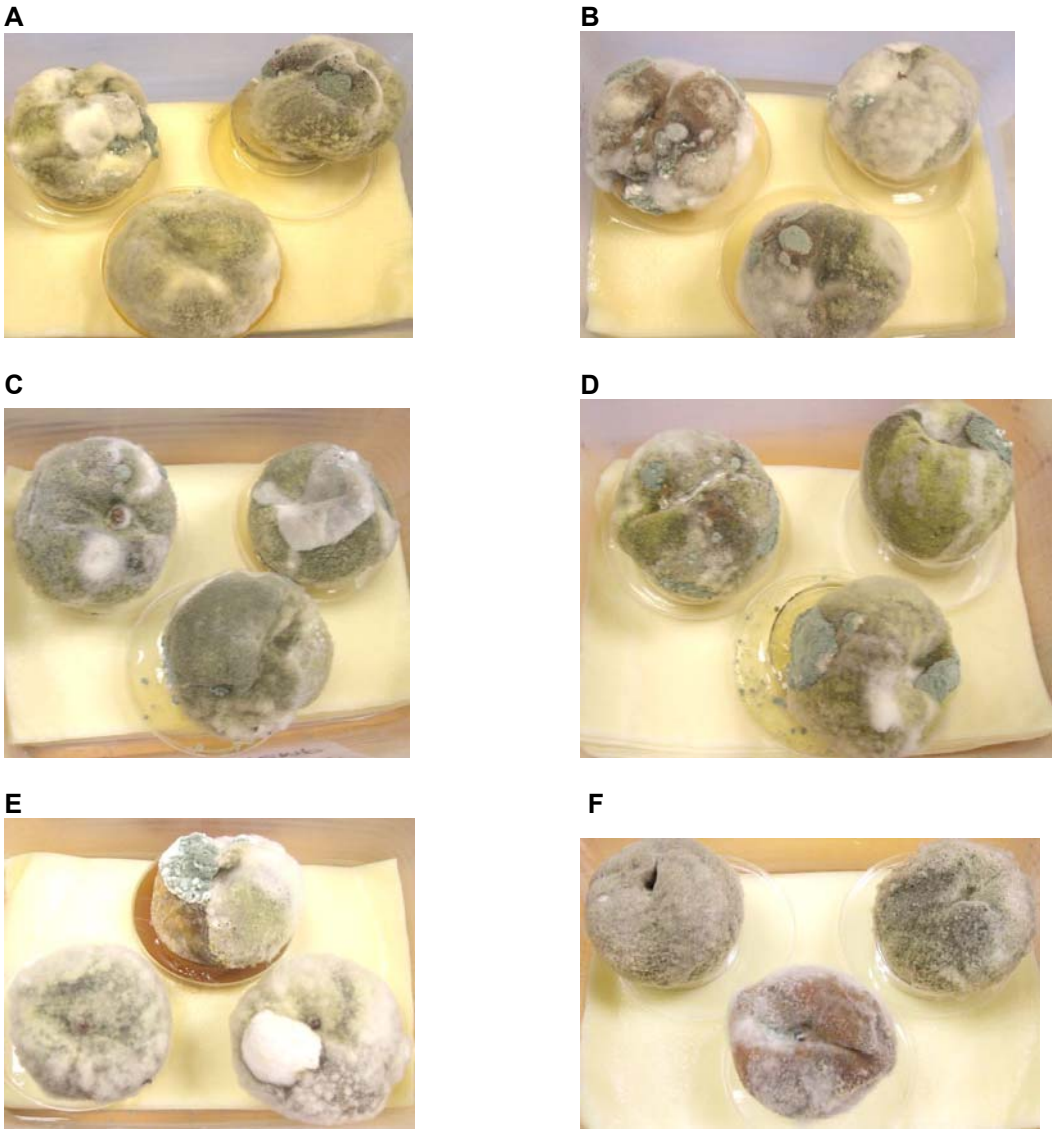


Figure 50. Fruit dipped in sterile distilled water for **A)** 30 seconds and **B)** 1.5 minutes. Fruit dipped in 20% ethanol for **C)** 30 seconds and **D)** 1.5 minutes. Fruit dipped in 50% ethanol for **E)** 30 seconds and **F)** 1.5 minutes.

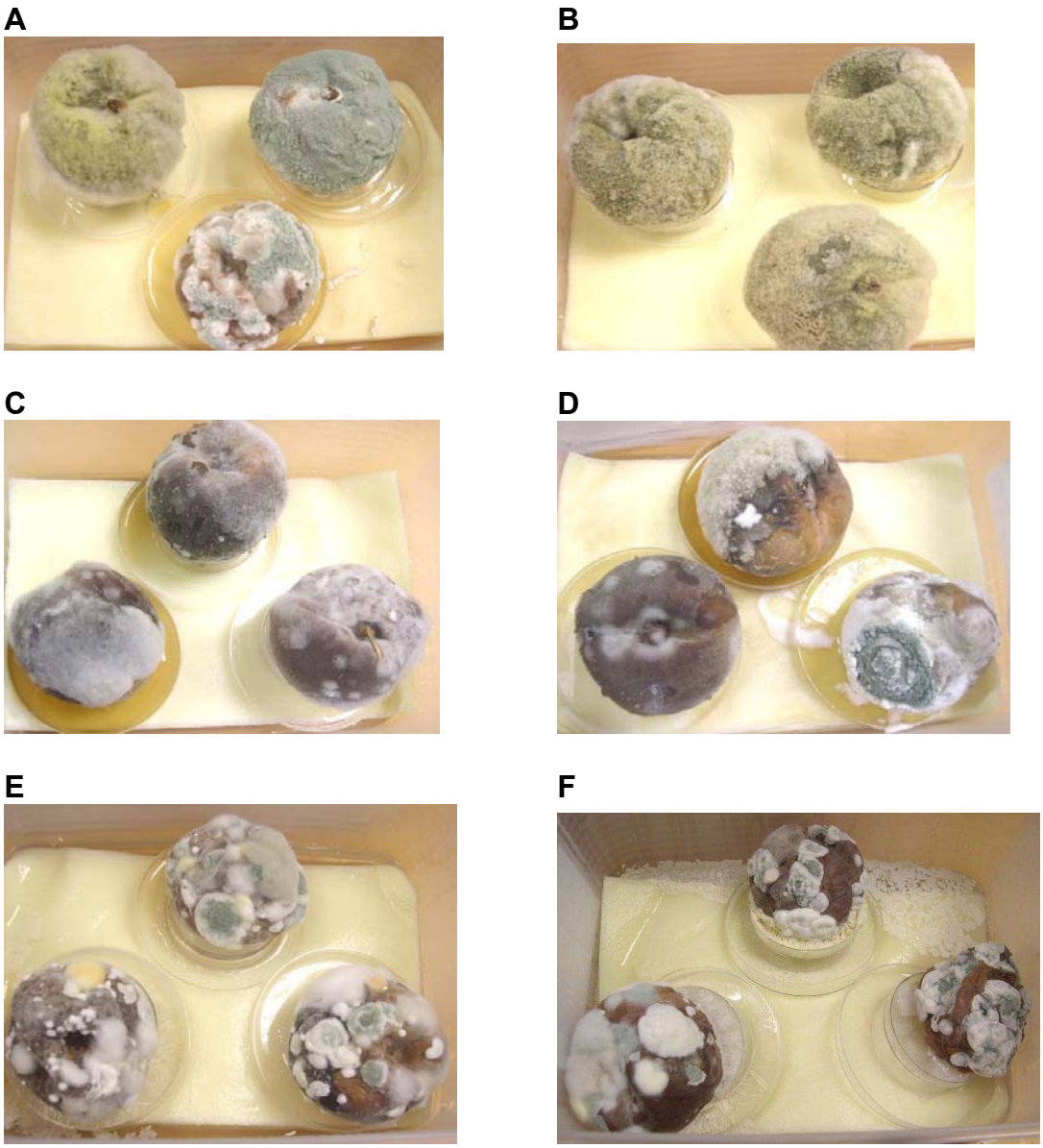


Figure 51. Figure 50. Fruit dipped in 70% ethanol for **A)** 30 seconds and **B)** 1.5 minutes. Fruit dipped in 100% ethanol for **C)** 30 seconds and **D)** 1.5 minutes. Fruit dipped in fungicide for **E)** 30 seconds and **F)** 1.5 minutes.

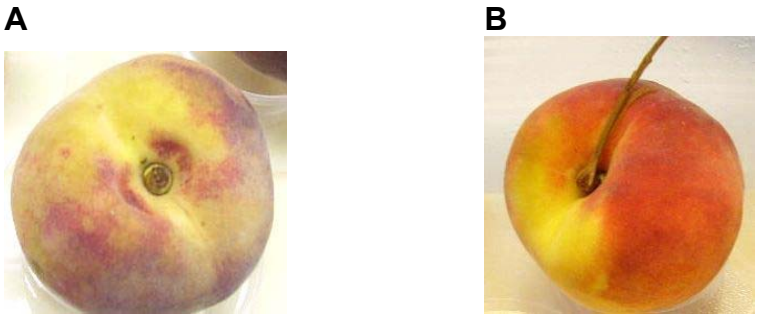


Figure 52. A) Fruit that was exposed to 100% ethanol for 1.5 minutes and stored for 7 days at room temperature, showing discolouration on the skin. **B)** Water treated fruit with the original skin colour.

3.2.3.2. Cool temperature storage

Similar to fruit stored at room temperature, at cool temperature storage, the development of fruit infection did not correlate with time of exposure of the fruit to the treatment.

However, as expected, the rate of infection was slowed at 4ºC compared to room temperature. Figure 53 shows the appearance of the fruit after 30 days of storage at 4ºC. In contrast to room temperature storage (Fig. 50 and Fig. 51), no heavy fungal growth was observed on fruit stored at 4ºC after 30 days of storage (Fig. 53).

Water treated fruit and fungicide-treated fruit were covered with light fungal growth after twenty days, which then became more obvious after 30 days (Fig. 53 A and B). Fruit that were treated with 20% and 100% ethanol showed tiny droplets on the skin of the fruit after 20 to 30 days (Fig. 53 C and F). 50% and 70% ethanol treatment followed by storage at 4 ºC inhibited fungal growth for up to 30 days (Fig. 53 D and E).

There were no obvious soft lesions or fungal growth on the fruit, even after 30 days of storage at 4 ºC. Fruit dipped in 100% ethanol for 30 seconds and 1.5 minutes showed slight discolouration on the skin colour of the fruit, which became more obvious after longer storage (Fig. 53 F).

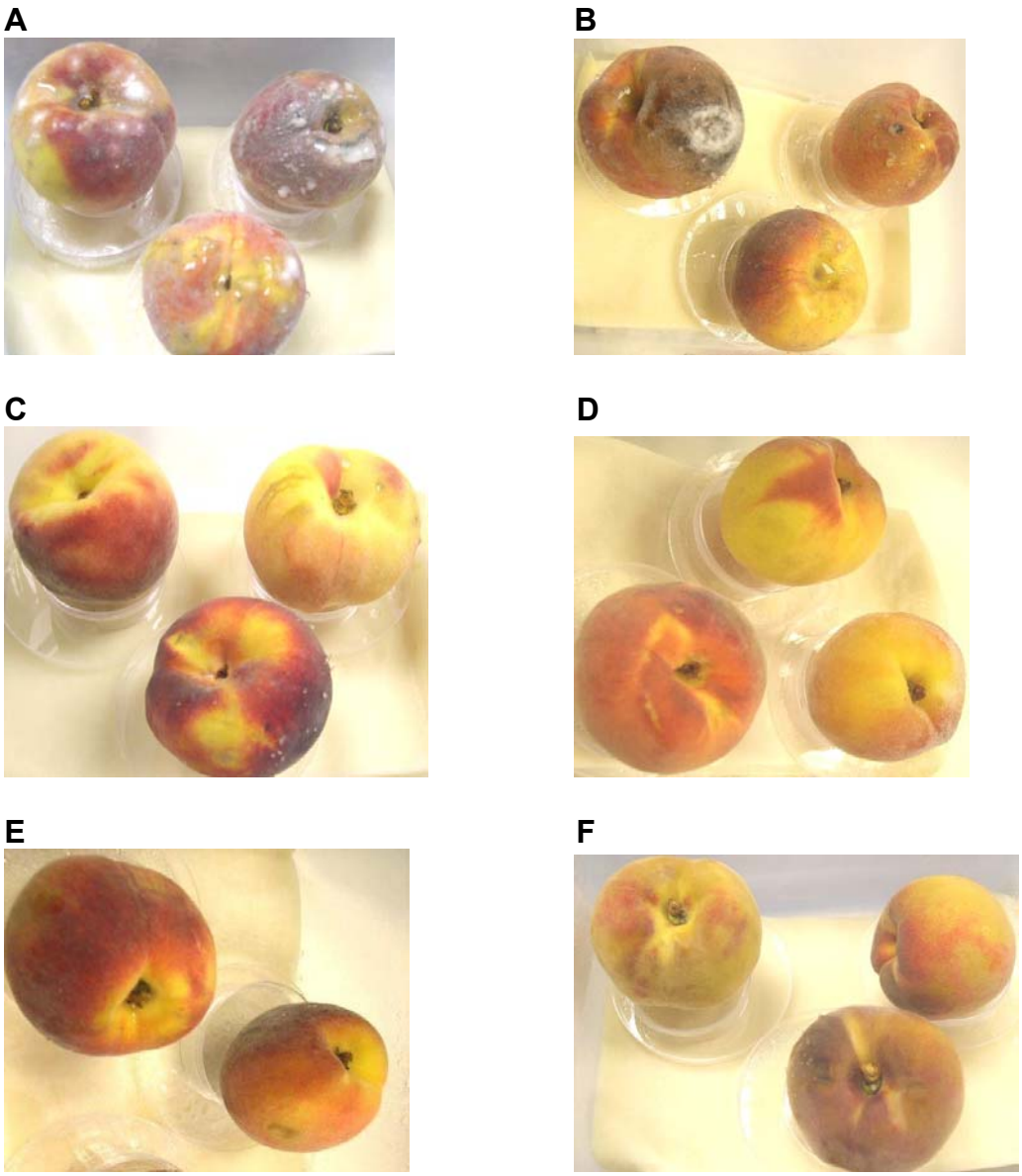


Figure 53. Peach fruit dipped in 20%, 50%, 70% and 100% ethanol and fungicide solutions for 30 seconds and stored at 4ºC for 30 days. **A)** Fruit dipped in fungicide **B)** Untreated fruit **C)** Fruit dipped in 20% ethanol **D)** Fruit dipped in 50% ethanol **E)** Fruit dipped in 70% ethanol **F)** Fruit dipped 100% ethanol.

Tables 3 to 6 summarise the observations made for ethanol treated fruit. It appears that peach fruit was more susceptible to fungal infection than oranges. Lower concentrations (20% and 50%) of ethanol vapour inhibited fungal growth to some degree when peach fruit were stored at room temperature. However, they were not able to protect the whole fruit from fungal infection for the full storage period. In contrast, high concentrations of ethanol (70% and 100%) completely inhibited fungal growth and gave the best protection for up to 30 days. The downside of these high concentrations of ethanol was fruit browning that occurred from an early storage time. Fruit browning on peaches also occurred in 50% ethanol vapour, but it was not as extensive as 70% and 100%.

Fungicide reduced the fungal infection, but it only protected the fruit from infection for a very short period of time (up to two or three days of storage).

No fruit browning occurred on fruit dipped in 20%, 50% and 70% ethanol. However there was slight browning that appeared on fruit dipped in 100% ethanol. The downside of the ethanol dipping method was this method inhibited fungal growth poorly. Fruit that were dipped in 20% and 50% ethanol were completely rotted after ten days of room temperature storage, whereas fruit that were exposed to 20% and 50% ethanol had just the began the fungal infection at that time. Further, 70% and 100% ethanol vapour gave complete protection from fungal infection for 30 days, but fruit that were dipped in 70% and 100% ethanol solution were only protected from fungal infection for the first two days of storage. Fungal infection was slowed on ethanol dipped peaches, but this method was not as effective as the ethanol vapour method.

Unwounded oranges maintained good condition for approximately 30 days of storage at both storage temperatures. Oranges were more susceptible to fungal infection if they were injured prior to treatment. Ethanol vapour of 20%, 50%, 70% and 100% protected the wounded fruit from infection for up to 30 days at both storage temperatures. In contrast, fungicide failed to give good protection to oranges at room temperature storage. Fruit browning also occurred on these oranges. Oranges treated with 20% and 50% ethanol gave the worst browning compared to 70% and 100%. Ethanol not only turned the skin brown, but it also decolourised the flesh of the oranges.

Table 3. Summary table of the development of infection on untreated (0%), 20%, 50%, 70% and 100% ethanol-treated; and fungicide-treated peaches. Each of the numbers represents the percentage of the fungal infection of all infected fruit with 100 as the highest and – as no fungal infection observed.

Storage temperature	**Room temperature (25ºC)**											
Treatments / **Time (day)**	**Vapour**						**Dipping**					
	0	**20**	**50**	**70**	**100**	**F**	**0**	**20**	**50**	**70**	**100**	**F**
0	-	-	-	-	-	-	-	-	-	-	-	-
2	-	-	-	-	-	-	30	20	10	-	-	-
4	10	-	-	-	-	-	40	30	20	10	-	10
6	20	5	5	-	-	5	60	50	40	30	10	20
8	30	10	10	-	-	20	80	70	60	50	30	40
10	50	20	10	-	-	40	100	100	100	100	50	80
12	70	40	20	-	-	60	100	100	100	100	60	100
14	100	50	30	-	-	80	100	100	100	100	70	100
20	100	70	40	-	-	100	100	100	100	100	100	100
30	100	100	50	-	-	100	100	100	100	100	100	100
	Cool temperature (4ºC)											
	0	**20**	**50**	**70**	**100**	**F**	**0**	**20**	**50**	**70**	**100**	**F**
0	-	-	-	-	-	-	-	-	-	-	-	-
2	-	-	-	-	-	-	-	-	-	-	-	-
4	-	-	-	-	-	-	-	-	-	-	-	-
6	-	-	-	-	-	-	-	-	-	-	-	-
8	-	-	-	-	-	-	-	-	-	-	-	-
10	-	-	-	-	-	-	-	-	-	-	-	-
12	-	-	-	-	-	-	-	-	-	-	-	-
14	-	-	-	-	-	-	-	-	-	-	-	-
20	-	-	-	-	-	-	10	-	-	-	-	10
30	10	10	-	-	-	-	30	10	-	-	10	20

Table 4. Summary of the fruit browning on water treated (0%), 20%, 50%, 70% and 100% ethanol-treated and fungicide-treated peaches. Each of the numbers represents the percentage of the fruit browning of all fruit tested with 100 as the highest and – as no fruit browning observed.

Storage temperature	**Room temperature (25ºC)**											
Treatments / Time (day)	**Vapour**						**Dipping**					
	0	**20**	**50**	**70**	**100**	**F**	**0**	**20**	**50**	**70**	**100**	**F**
0	-	-	-	-	-	-	-	-	-	-	-	-
2	-	-	-	-	-	-	-	-	-	-	-	-
4	-	-	50	60	70	-	-	-	-	-	-	-
6	-	-	50	100	100	-	-	-	-	-	-	-
8	-	-	80	100	100	-	-	-	-	-	-	-
10	-	-	100	100	100	-	-	-	-	-	-	-
12	-	-	100	100	100	-	-	-	-	-	-	-
14	-	-	100	100	100	-	-	-	-	-	-	-
20	-	-	100	100	100	-	-	-	-	-	-	-
30	-	-	100	100	100	-	-	-	-	-	40	-
	Cool temperature (4ºC)											
	0	**20**	**50**	**70**	**100**	**F**	**0**	**20**	**50**	**70**	**100**	**F**
0	-	-	-	-	-	-	-	-	-	-	-	-
2	-	-	-	-	-	-	-	-	-	-	-	-
4	-	-	50	60	60	-	-	-	-	-	-	-
6	-	-	70	100	100	-	-	-	-	-	-	-
8	-	-	100	100	100	-	-	-	-	-	-	-
10	-	-	100	100	100	-	-	-	-	-	-	-
12	-	-	100	100	100	-	-	-	-	-	-	-
14	-	-	100	100	100	-	-	-	-	-	-	-
20	-	-	100	100	100	-	-	-	-	-	-	-
30	-	-	100	100	100	-	-	-	-	-	40	-

Table 5. Summary table of the development of infection on untreated (0%), 20%, 50%, 70% and 100% ethanol-treated; and fungicide-treated oranges. Each of the numbers represents the percentage of the fungal infection of all fruit infected with 100 as the highest and – as no fungal infection observed.

Storage temperature	**Room temperature (25ºC)**											
Treatments / Time (day)	**Unwounded**						**Wounded**					
	0	**20**	**50**	**70**	**100**	**F**	**0**	**20**	**50**	**70**	**100**	**F**
0	-	-	-	-	-	-	-	-	-	-	-	-
2	-	-	-	-	-	-	-	-	-	-	-	-
4	-	-	-	-	-	-	10	-	-	-	-	10
6	-	-	-	-	-	-	60	-	-	-	-	60
8	-	-	-	-	-	-	80	-	-	-	-	80
10	-	-	-	-	-	-	100	-	-	-	-	100
12	-	-	-	-	-	-	100	-	-	-	-	100
14	-	-	-	-	-	-	100	-	-	-	-	100
20	5	-	-	-	-	-	100	-	-	-	-	100
30	10	-	-	-	-	-	100	-	-	-	-	100
	Cool temperature (4ºC)											
	0	**20**	**50**	**70**	**100**	**F**	**0**	**20**	**50**	**70**	**100**	**F**
0	-	-	-	-	-	-	-	-	-	-	-	-
2	-	-	-	-	-	-	-	-	-	-	-	-
4	-	-	-	-	-	-	-	-	-	-	-	-
6	-	-	-	-	-	-	-	-	-	-	-	-
8	-	-	-	-	-	-	-	-	-	-	-	-
10	-	-	-	-	-	-	-	-	-	-	-	-
12	-	-	-	-	-	-	-	-	-	-	-	-
14	-	-	-	-	-	-	-	-	-	-	-	-
20	-	-	-	-	-	-	-	-	-	-	-	10
30	-	-	-	-	-	-	-	-	-	-	-	20

Table 6. Summary of the fruit browning on water treated (0%), 20%, 50%, 70% and 100% ethanol-treated and fungicide-treated oranges. Each of the numbers represents the percentage of the fruit browning of all fruit with 100 as the highest and – as no fruit browning observed.

Storage temperature	**Room temperature (25ºC)**											
Treatments / Time (day)	**Unwounded**						**Wounded**					
	0	**20**	**50**	**70**	**100**	**F**	**0**	**20**	**50**	**70**	**100**	**F**
0	-	-	-	-	-	-	-	-	-	-	-	-
2	-	-	-	-	-	-	-	-	-	-	-	-
4	-	-	-	-	-	-	-	-	-	-	-	-
6	-	5	5	-	-	-	-	5	5	-	-	-
8	-	5	5	-	-	-	-	5	5	-	-	-
10	-	10	10	5	-	-	-	10	10	5	-	-
12	-	10	10	5	-	-	-	10	10	5	-	-
14	-	30	30	5	5	-	-	30	30	5	5	-
20	-	60	60	30	10	-	-	60	60	30	10	-
30	-	100	100	50	20	-	-	100	100	50	20	-
	Cool temperature (4ºC)											
	0	**20**	**50**	**70**	**100**	**F**	**0**	**20**	**50**	**70**	**100**	**F**
0	-	-	-	-	-	-	-	-	-	-	-	-
2	-	-	-	-	-	-	-	-	-	-	-	-
4	-	-	-	-	-	-	-	-	-	-	-	-
6	-	5	5	-	-	-	-	5	5	-	-	-
8	-	5	5	-	-	-	-	5	5	-	-	-
10	-	10	10	5	-	-	-	10	10	5	-	-
12	-	10	10	5	-	-	-	10	10	5	-	-
14	-	30	30	5	5	-	-	30	30	5	5	-
20	-	60	60	30	10	-	-	60	60	30	10	-
30	-	100	100	50	20	-	-	100	100	50	20	-

In conclusion, high concentrations of ethanol gave the best protection compared to the other concentrations, but the tendency of fruit browning was also high.

Storage at 4ºC slowed down the infection following both ethanol dipping and ethanol vapour treatment of peaches. At this temperature, oranges maintained very good condition for up to 30 days. It appeared that this storage temperature successfully protected the fruit from fungal infection for longer periods of time compared to room temperature storage. However, the same fruit browning appeared as in the room temperature storage for both peaches and oranges.

3.2.4. Assessment of the effect of ethanol on fruit quality

3.2.4.1. Analysis of liquid accumulation

This experiment was an initial study done to investigate further if high concentrations of ethanol could damage the cell wall of the fruit and cause the cell contents to leak out. Due to the limited time available for this study, this experiment was carried out over ten days rather than 30.

As the ethanol concentration increased, so did the amount of liquid released from both peaches and oranges (Table 7; Fig. 54 and 55). Treatment with 100% ethanol resulted in the greatest damage since the highest amount of liquid released was observed - up to 21.5g after six days for peaches and 10.5g after eight days for oranges. In contrast, treatment with 20% ethanol appeared not to damage either peaches or oranges as no liquid accumulated. Peaches and oranges responded differently to the ethanol. It took longer for the oranges to begin leaking liquid (four days vs two days) and the amount of liquid released by oranges was approximately half that from peaches (Table 7).

Table 7. The weight (g) of liquid accumulated at different times following exposure of peaches and oranges to various concentrations of ethanol. Fruit were stored at room temperature.

Time (days) / **Ethanol Concentrations (%)**	0	2	4	6	8	10
Peaches:						
20	0	0	0	0	0	0
50	0	0.18	2.58	7.67	11.96	13.64
70	0	3.12	4.77	11.41	14.90	16.57
100	0	5.83	14.81	21.54	15.40	14.80
Oranges:						
20	0	0	0	0	0	0
50	0	0	0	0.41	0.55	1.15
70	0	0	0.22	3.12	3.02	3.27
100	0	0	0.42	7.12	10.55	9.00

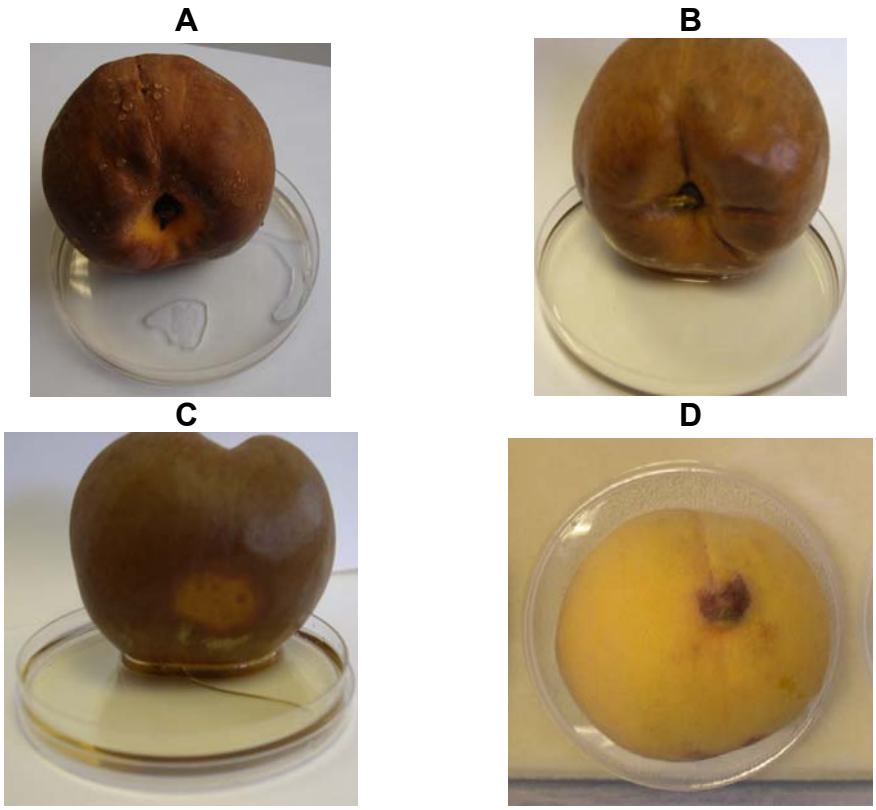


Fig 54. The effect of ethanol on fruit quality, showing browning with some leakage from peaches that were exposed to high ethanol concentrations. The peaches were stored at room temperature for 4 days and were treated with: **A)** 50% ethanol **B)** 70% ethanol **C)** 100% ethanol **D)** 20% ethanol.

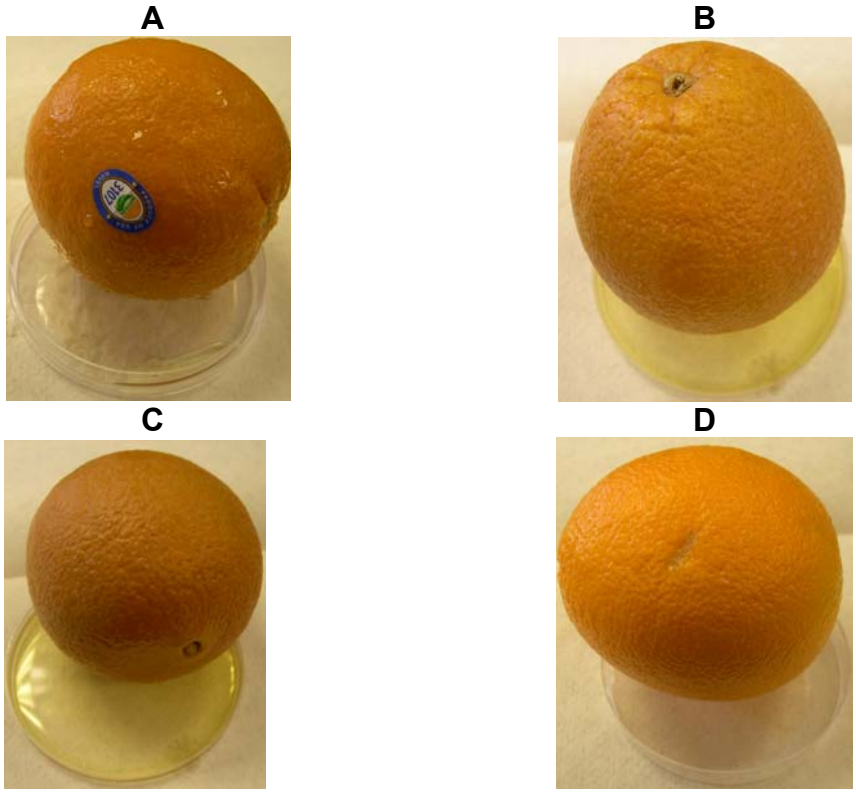


Fig 55. The effect of ethanol on fruit quality, showing browning with some leakage from oranges that were exposed to high ethanol concentrations. The oranges were stored at room temperature for 4 days and were treated with: **A)** 50% ethanol **B)** 70% ethanol **C)** 100% ethanol **D)** 20% ethanol.

3.2.4.2. Analysis on the quality of the flesh

This experiment was an initial study done to investigate further if ethanol at various concentrations can penetrate the skin and turn the flesh brown. Again, due to the time limit of this study, this experiment was done for a period of ten days.

It appeared that ethanol at high concentrations could penetrate the skin to the centre part of the fruit and not only turned the skin of peaches to brown, but also the flesh (Fig. 56 A and B). After four days of room temperature storage, the amount of flesh browning were 1.5 cm for 100% and 70% ethanol-treated peaches and reduced to 1cm for 50% ethanol-treated peaches. There was no sign of flesh browning on fruit that were treated with 20% ethanol. In contrast, the inside of the oranges did not turn brown for ten days; however, colour changes were noticed in the flesh and the peel by ten days (Compare Fig. 56 D with A, B and C).

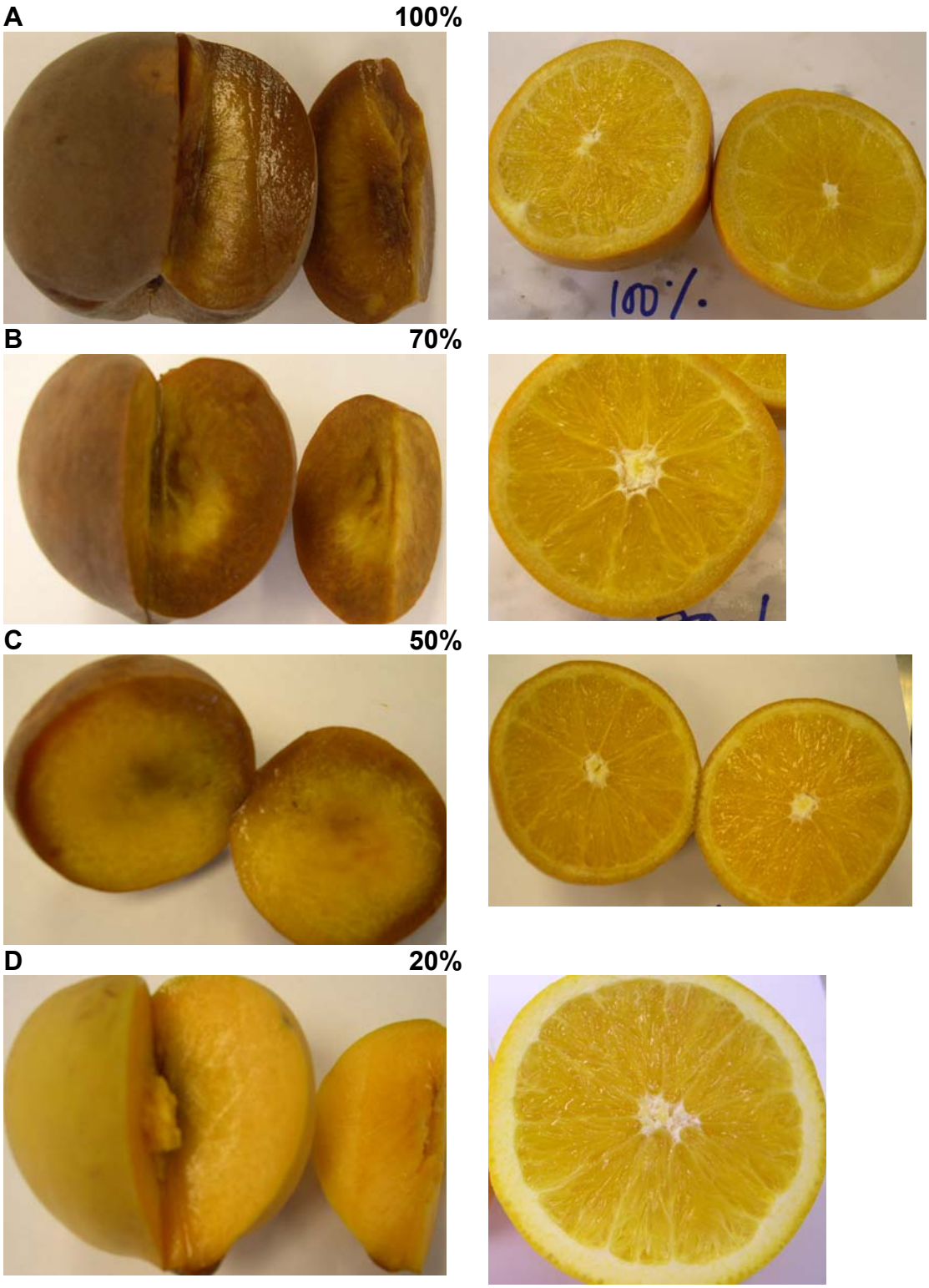


Fig. 56. The effect of various concentrations of ethanol on fruit quality - browning on the skin and the flesh of the fruit. Each fruit were treated with different concentrations of ethanol and stored at room temperature. The peaches were stored for four days, whereas the oranges for ten days. The ethanol concentrations were: **A)** 100% **B)** 70% **C)** 50% **D)** 20%.

3.3 Milk treatment

The effectiveness of diluted and undiluted whole fat milk as a postharvest treatment on peaches was investigated. The fruit were dipped for 30 seconds and monitored everyday for up to 30 days. The results were compared with a negative (sterile distilled water) and a positive control (fungicide). The negative control (water treated) is referred to as untreated or 0%. Due to fruit availability, this experiment was only done on peaches.

3.3.1. Room temperature storage

In contrast to ethanol, milk had no antifungal activity (Fig. 57 and Fig. 58). Fruit that were treated with milk and stored at room temperature had more growth and rotted faster compared to untreated fruit (Fig. 57 and 58).The higher the concentration of milk used as treatment, the greater the amount of fungal growth was observed. Further, all of the fruit treated with diluted and undiluted milk and stored at room temperature were completely infected after eight days, compared with ten days for untreated fruit and 30 days for fungicide-treated fruit (Fig. 57). Figure 58 shows photographs that are representative of what was observed. The infection began as small brown lesions on both untreated peaches and treated peaches after three days of room temperature storage, but additionally, a fungal growth that appeared as grey tufts developed over the surface of the fruit on treated peaches. As the lesions expanded at day six, untreated fruit developed a fungal growth (gray to brown tufts) at the surface of the fruit, while fruit treated with milk were completely covered with brown and white tufts. After two weeks of storage, untreated peaches were completely rotted as they turned very soft and the whole surface of the fruit was covered with brown tufts. A similar observation was found with treated peaches, except the whole fruit were covered with white tufts rather than of brown tufts.

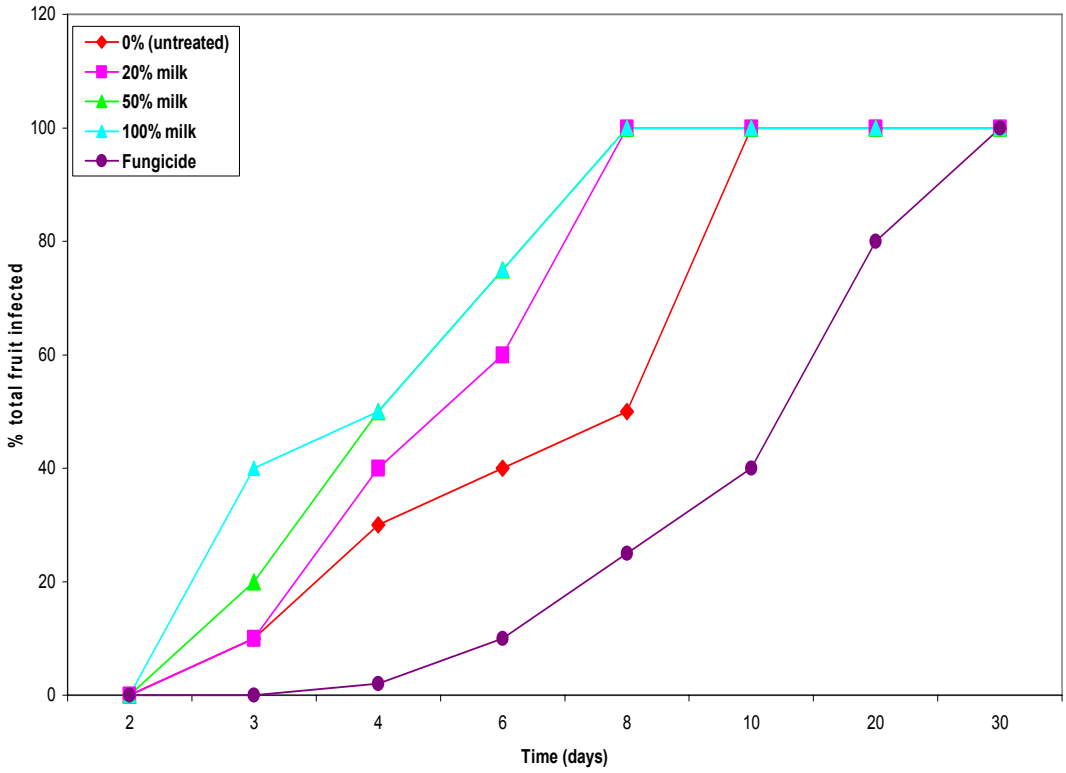


Figure 57. The development of infection over time on peaches treated with milk. The peaches were treated with varying concentrations of milk as indicated and stored at room temperature for 30 days.

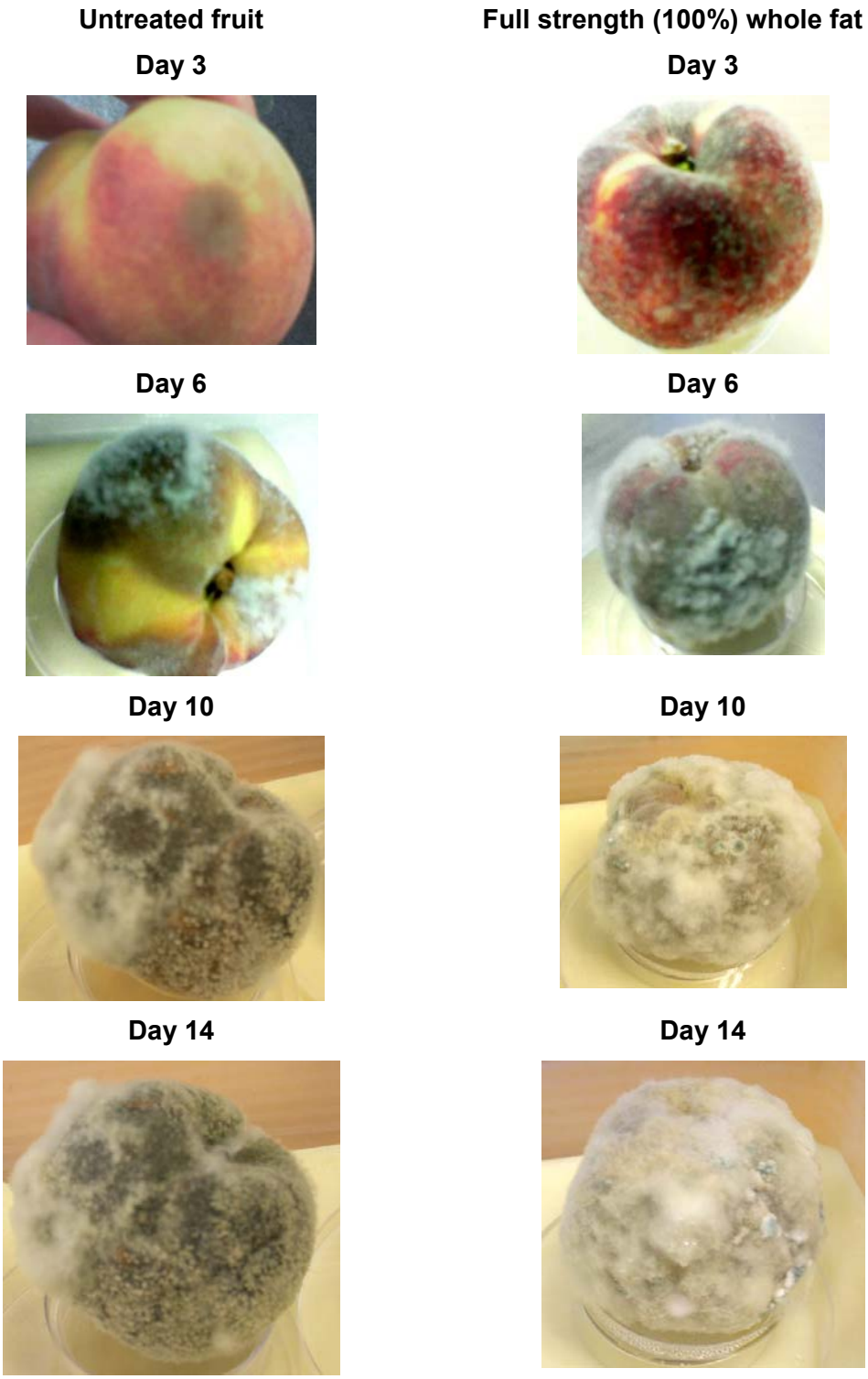


Figure 58. Comparison of fungal infection of untreated peaches with peaches treated with undiluted milk. Fruit was stored at room temperature. The days at which each photograph was taken is indicated above the photos. These photos are representative of all fruit analysed.

3.3.2. Cool temperature storage

As seen with the ethanol treatment, cool temperature storage (4ºC) slowed the development of fungal growth on fruit compared to room temperature storage, regardless of the treatment. However, similar to room temperature storage, fruit that were treated with milk and stored at 4ºC had a greater and a faster degree of fruit infection compared to untreated fruit (Fig. 59 and 60). Further, the higher the concentration of milk used as treatment, the greater the degree of infection observed. It can be seen in figures 59 and 60 that the amount of infection at day 30 was greater for fruit treated with undiluted milk compared with diluted milk.

It appeared that undiluted milk (100%) and half strength (50%) milk enhanced the fungal growth in the early stages (Fig. 59). The infection began to develop after day three for fruit treated with both of these concentrations. Additionally, the infection began to develop after day eight for fruit that were treated with 20% milk, compared with day 14 for untreated fruit. In contrast, no sign of infection was observed on fungicide treated fruit after 30 days of storage at 4ºC.

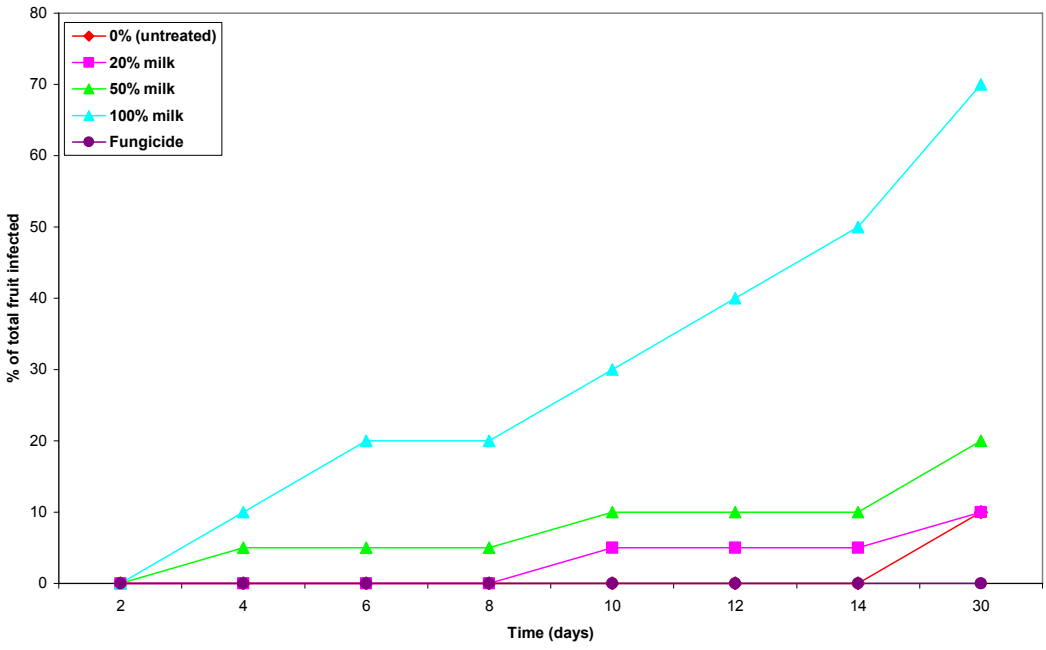


Figure 59. The development of infection over time on peaches treated with milk. The peaches were treated with varying concentrations of milk as indicated and stored at 4ºC for 30 days.

The infection at 4ºC was quite different from that observed on fruit stored at room temperature (Compare Fig. 58 with Fig. 60). It appeared to be much slower growing with no soft lesions present due to the temperature storage. There was no change on the appearance of untreated fruit after 14 days of cool temperature storage, however, a very light white growth that appeared as white powder developed on some areas of the fruit surface after 30 days of storage. There was also no sign of any soft lesions present on fruit that were treated with 20% and 50% milk, however, the type of fungal growth was similar to that seen on untreated fruit. The only difference was the fungal infection on 20% and 50% milk-treated fruit developed faster compared to untreated fruit.

In contrast to all of the above treatments, undiluted milk had the greatest degree of infection. However, similar to all diluted milk, the fungal growth appeared as white powder on some areas of the fruit surface after 4 days of storage at 4ºC. The growth then continued as white tufts and some soft lesions developed on the fruit. After 30 days of storage in 4ºC, the whole fruit were covered with grey tufts, but the fungal growth was not as extensive as that found on fruit stored at room temperature.

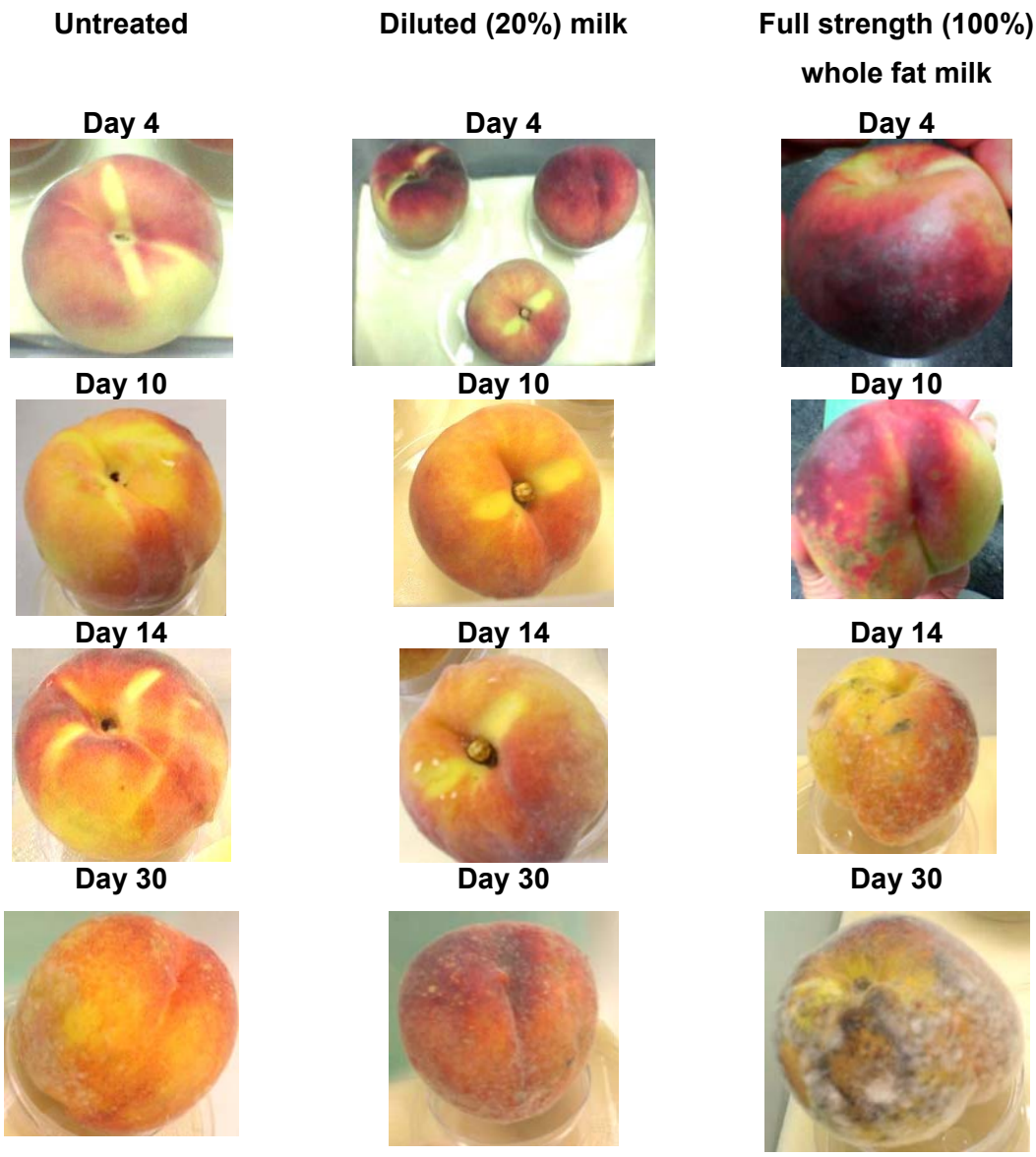


Figure 60. Comparison of fungal infection of peaches treated with diluted and undiluted milk. Fruit was stored at 4ºC. The days at which each photograph was taken is indicated above the photos. These photos are representative of all fruit analysed.

3.4 Honey treatment

The effectiveness of honey as a postharvest treatment on peaches was investigated. The fruit were dipped for 30 seconds in varying concentrations of honey and monitored every day for 30 days. Fruit treated with sterile distilled water and fungicide was compared with honey-treated fruit. Due to the fruit seasons, this experiment was only done on peaches.

3.4.1. Room temperature storage

Honey appeared to have a mild effect on fungal growth. Peaches treated with honey developed infection more slowly at room temperature compared with water-treated controls. Water-treated fruit were completely infected by day ten whereas fruit treated with 50% honey were infected by day 14 – a delay of 4 days (Fig. 61). In contrast, peaches that were treated with fungicide showed greater inhibition of fungal growth compared to untreated and honey-treated fruit, since 100% infection was not seen until day 24. The development of fruit infection on honey-treated fruit is shown in Figure 62.

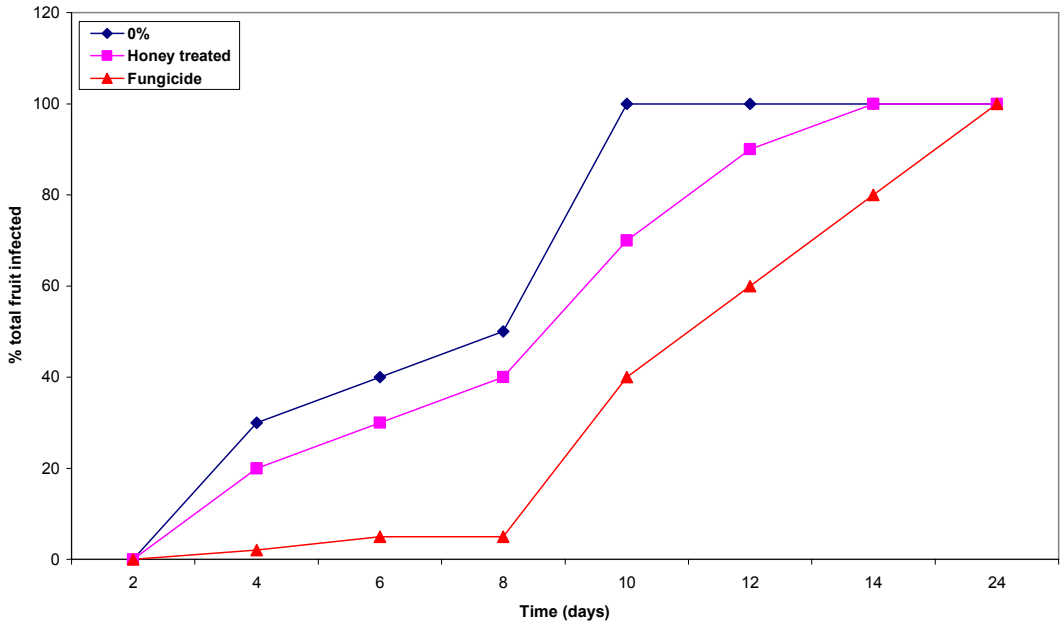


Figure 61. The development of infection over time on peaches treated with honey. The peaches were treated with 50% honey and stored at room temperature for 30 days. Peaches treated with honey were compared with untreated peaches and fungicide-treated peaches.

Figure 62 shows representative photographs of what was observed. The type of infection appeared to be different between the peaches that were treated with the controls and with the honey. Infection on water-treated peaches and fungicide-treated peaches appeared as a brown rot. The infection began as soft lesions, which then developed relatively slowly over time. The fungal growth then spread as grey to brown tufts, covering the whole fruit, completely infecting the fruit.

Infection that occurred on honey-treated milk also started with soft lesions, developing into a fungal growth that scattered over the whole surface of the fruit. The appearance of the fungus growth was different on the fruit treated with honey compared with the controls. The fungus appeared as a white growth with a round, thick and fluffy appearance. There was also surface browning that occurred on fruit that were treated with honey. Honey was either inhibitory to brown rot or provided a suitable environment for this honey-specific fungus that was not provided on the control fruit. Since there were different types of fungal growth on honey-treated and untreated peaches, the growth of those fungi would be different and therefore difficult to compare.

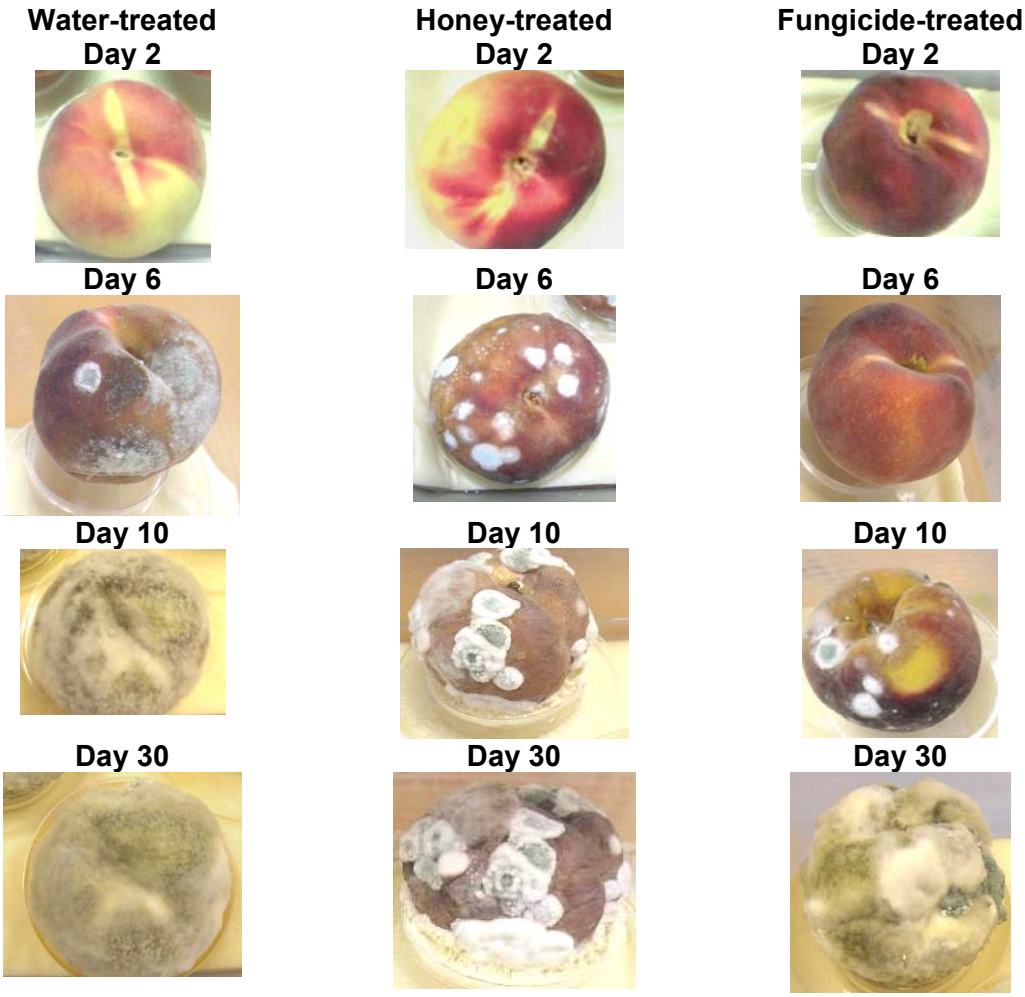


Figure 62. The development of infection on honey-treated, fungicide-treated and water-treated peaches. Fruit was stored at room temperature. The days at which each photograph was taken is indicated above each photo. These photos are representative of all fruit analysed.

3.4.2. Cool temperature storage

As for all other treatments, cool temperature storage of control and honey treated fruit delayed infection. Interestingly, in contrast to milk treatment, honey treatment showed no enhancement of fungal growth in the early stages. At 4ºC, the honey-treated peaches were maintained in good condition for the first eight days, before fungal infection occurred. It appeared that honey enhanced fungal growth over the time, since the fruit were badly infected compared to fungicide-treated fruit and untreated fruit at day 30. Further, the untreated fruit stayed in good condition for the first 14 days before any sign of fruit infection was seen. In contrast, fungicide-treated fruit stayed in good condition with no sign of fungal infection for up 30 days (Fig. 63).

Further, similar to room temperature storage, the type of infection observed at 4ºC storage was different on honey-treated fruit compared with water-treated fruit. The fungal growth on untreated peaches appeared as white powder on the fruit surface while the fungus that grew on honey-treated peaches appeared as thicker white powder scattered around the fruit (Fig. 64). Again, since there were different types of fungus growth on honey-treated and untreated peaches, the growth of those fungi would be different and therefore difficult to compare.

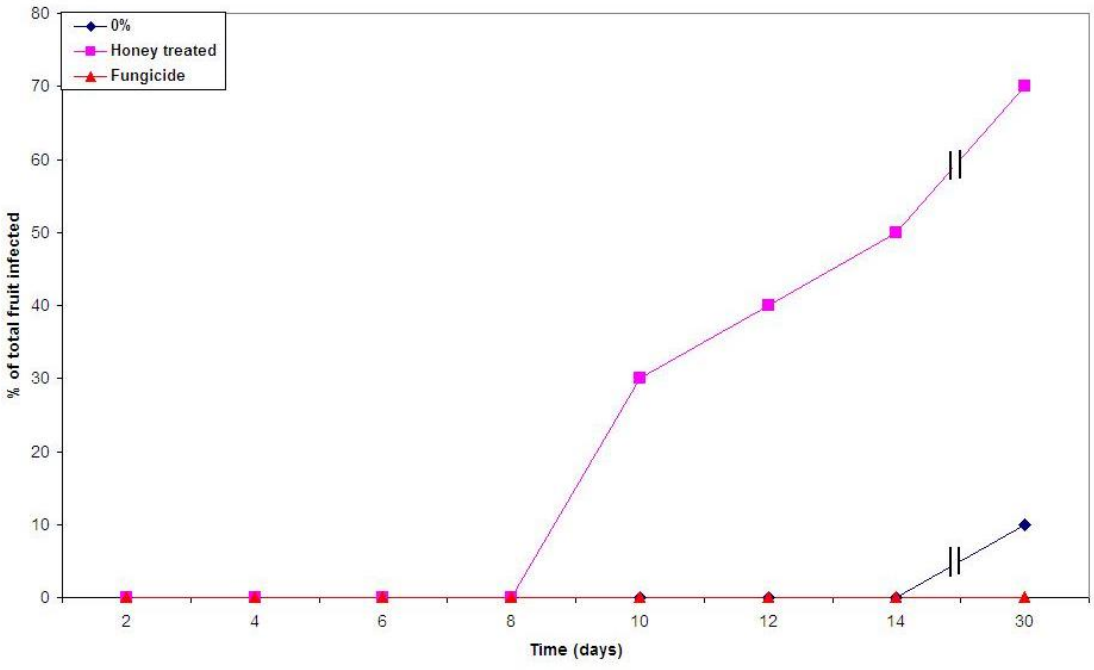


Figure 63. The development of infection over time on peaches treated with honey. The peaches were treated with 50% honey and stored at 4ºC for 30 days. Peaches treated with honey were compared with untreated peaches and fungicide-treated peaches.

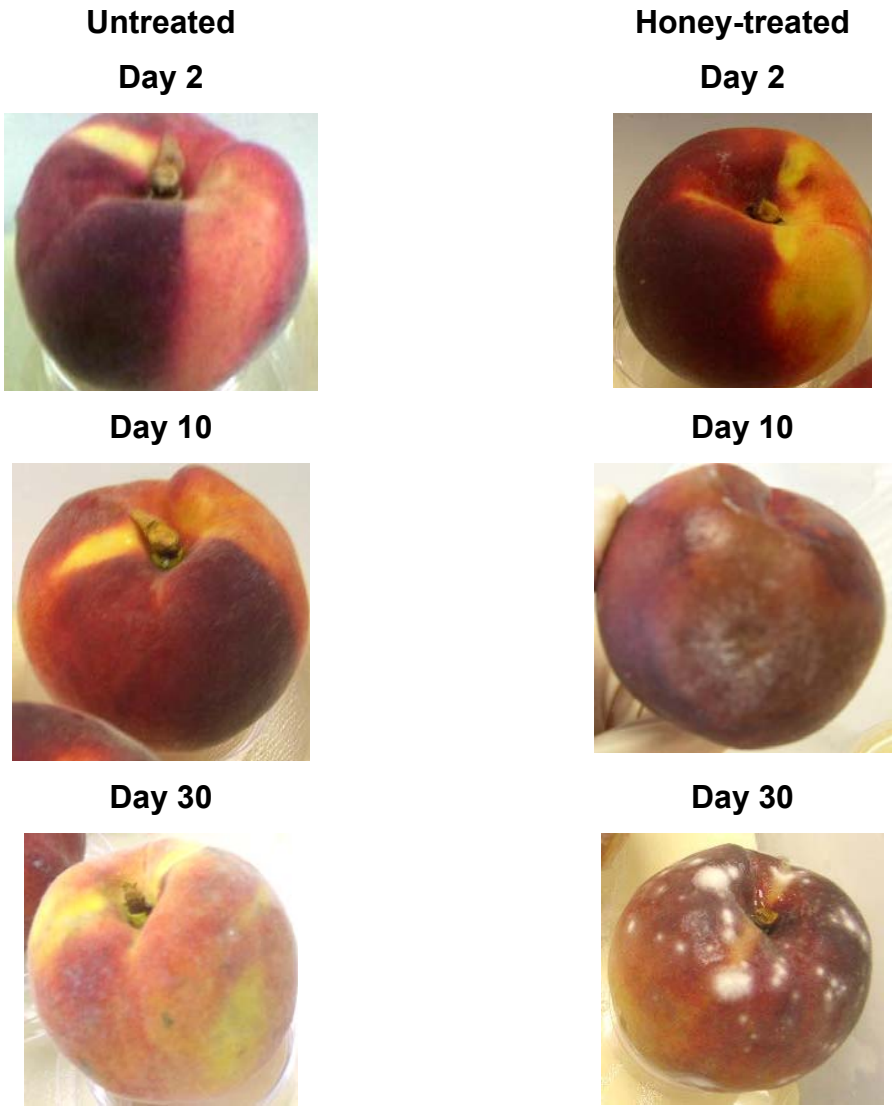


Figure 64. The development of infection on honey-treated and untreated peaches. Fruit was stored at 4ºC. The days at which each photograph was taken is indicated above each photo. These photos are representative of all fruit analysed.

In summary, milk and honey appeared not to have a significant antifungal activity on peaches. Peaches that were treated with varying concentrations (20%, 50% and 100%) of milk and 50% manuka honey showed a greater degree of infection compared to untreated and fungicide treated fruit at room temperature and 4 ºC.

Experiments were not performed on oranges as it was considered, given the above results, that milk and honey treatments would be ineffective.

3.5 Essential Oil treatment

The effectiveness of different concentrations of essential oils (manuka, lemongrass, lemon and orange oil) as postharvest treatments on fruit was assessed. Concentrations of essential oils with antifungal activity against *Penicillium spp.* were first identified using a microtiter assay. Fruit were then dipped in these concentrations of essential oils and the antifungal activity determined. Fruit dipped in the concentrations of essential oils was compared to fruit dipped in sterile distilled water and fungicide.

3.5.1. Orange oil

3.5.1.1. Microtiter assay

The effectiveness of varying essential orange oil concentrations on *Penicillium spp* was assessed using 0.1x PDB. This was done to determine if any antifungal activity would be more obvious in one concentration over the others.

This assay was not done using 1x PDB as it was previously found that the effect of varying ethanol concentrations was quite consistent between both 1x and 0.1x PDB (Section 3.2.1.2.). Further, this assay was not assessed against *M. fructicola* due to the lack of time to finish this research.

In contrast to ethanol, there appeared to be no effect of surrounding wells within the microtiter assay plate for the zero values (Fig. 65 A). Fungal growth was not affected by increasing orange essential oil concentrations in nearby wells of the plate. When the mean and standard error of the mean were taken for all the negative control values to create one set of numbers representing all negative control samples, no significant difference was seen between fungicide and no treatment (Fig. 65 A). For consistency with other oils, the untreated sample (0) located next to the lowest oil concentration was used for all comparison with orange oil.

For clarity, figure 65 B shows the comparison between the untreated sample (0) and the fungicide. The fungicide was fungistatic rather than fungicidal as fungus grew somewhat during the assay but the amount of growth was significantly less than no treatment. Initially the fungal growth inhibited for the first 24 hours but then the fungus appeared to recover and to double at the same rate as the no treatment controls. However, due to the initial growth inhibition, the overall growth was significantly less in the presence of fungicide than where there was no treatment.

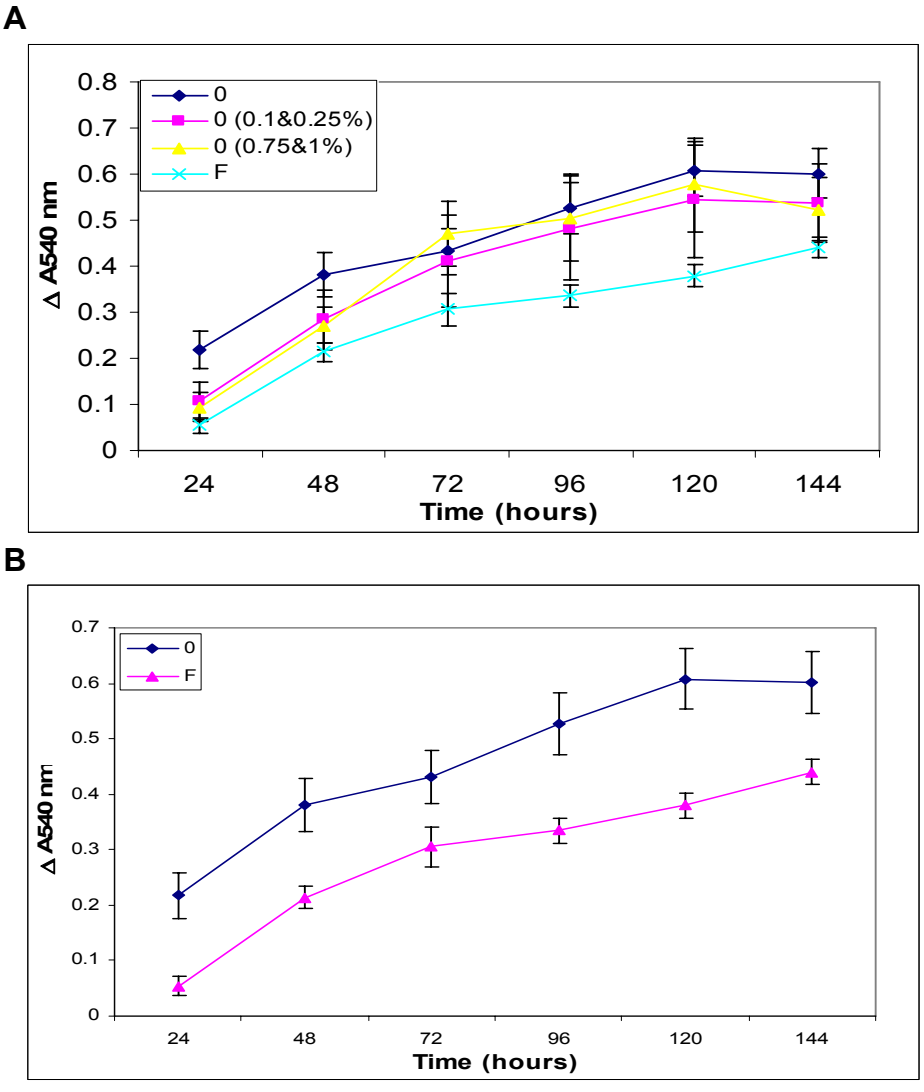


Figure 65. The amount of *Penicillium* REB 315-1 growth measured as the A_{540nm} at day 0 subtracted from the absorbance at each subsequent time point (ΔA_{540nm}). Each point represents the mean of four samples, error bars represent the standard error of the mean. **A)** Growth of untreated and fungicide treated spores grown near microtiter wells containing orange oil. 0 refers to untreated samples placed next to the lowest concentration of orange oil used in the assay i.e. 0.01%, 0(0.1&0.25%) refers to untreated samples placed between samples treated with 0.1% and 0.25% orange oil (referred to as $0^{0.1\&0.25}$ in the text), 0(0.75&1%) refers to untreated samples placed between samples treated with 0.75% and 1% orange oil (referred to as $0^{0.75\&1}$ in the text). F refers to samples treated with fungicide. **B)** Comparison of growth between untreated (0) and fungicide treated spores

Orange oil at 0.01%, 0.05%, 0.1% and 0.25% did not appear to inhibit fungal growth overall (Fig. 66 A to D). Indeed, oil at 0.01% appeared to be stimulatory (Fig. 66 A). While 0.05% and 0.25% appeared to inhibit growth for the first 24 hours, this was not sustained for the duration of the assay (Fig 66 B and D). In contrast, orange oil at higher concentrations (0.5% to 2%) did appear to be as effective overall as that observed for treatment with fungicide (Fig. 67 A to D).

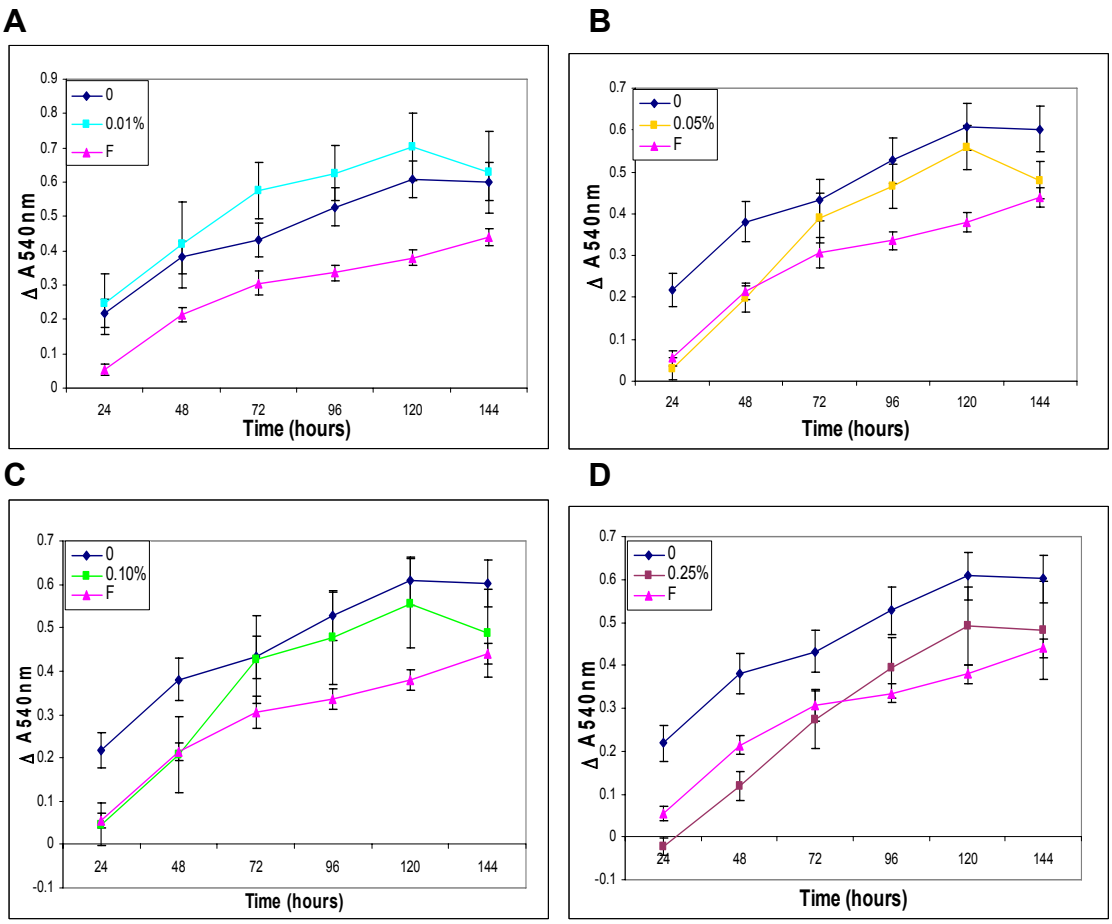


Figure 66. Inhibition effect of orange oil towards the growth of *Penicillium* REB 315-1 in 0.1x PDB over 144 hours as measured using a microtiter growth assay. The graphs represent the amount of growth measured as the A_{540nm} at day 0 subtracted from the absorbance at each subsequent time point (ΔA_{540nm}). 0 refers to untreated samples placed next to the lowest concentration of orange oil used in the assay i.e. 0.01% and F refers to samples treated with fungicide. Error bars represent the standard error of the mean. These graphs represent orange oil treated samples at **A)** 0.01% **B)** 0.05% **C)** 0.01% **D)** 0.25%.

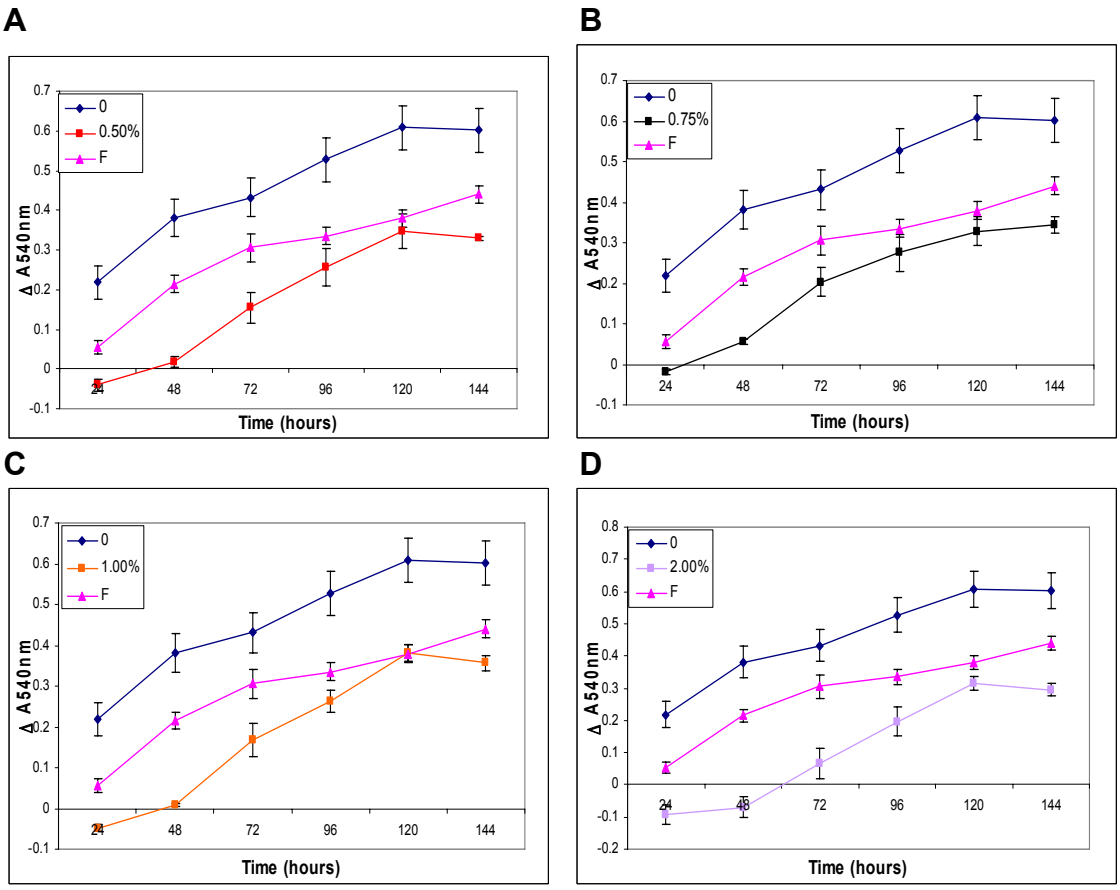


Figure 67. Inhibition effect of orange oil towards the growth of *Penicillium* REB 315-1 in 0.1x PDB over 144 hours as measured using a microtiter growth assay. The graphs represent the amount of growth measured as the A_{540nm} at day 0 subtracted from the absorbance at each subsequent time point (ΔA_{540nm}). 0 refers to untreated samples placed next to the lowest concentration of orange oil used in the assay i.e. 0.01% and F refers to samples treated with fungicide. Error bars represent the standard error of the mean. These graphs represent orange oil treated samples at **A)** 0.50% **B)** 0.75% **C)** 1% **D)** 2%.

For all of the concentrations of orange oil that showed antifungal activity, this activity appeared to be greatest in the first 48 hours of the assay. After this time period, the fungus appeared to recover, having a faster growth rate than in the presence of fungicide. By the end of the assay both fungicide and the orange oil appeared to have a similar activity, with the oil having its greatest impact earlier in the assay while the antifungal activity of the fungicide was steady throughout the assay.

It appeared that concentrations of orange oil from 0.5% and above inhibited *Penicillium* growth, therefore 0.5% and 2% were the concentrations that were tested for their antifungal activity on oranges. The highest concentration of orange oil used in the microtiter assay was 2% and it showed effective fungal inhibition. 0.5% orange oil was also chosen, since it appeared to be equally effective as fungicide in the microtiter assay.

3.5.1.2. Orange oil dipping treatment

A. Unwounded fruit

All of the unwounded untreated oranges at room temperature appeared to be completely infected after 30 days of storage at room temperature (Fig. 68 A), whereas only one out of three fungicide-treated oranges was completely infected by this time (Fig. 68 B).

In contrast to the microtiter assay result, neither 0.5% nor 2% orange oil showed very good inhibition when applied on oranges (Fig. 68 C). Of the three oranges that were each treated with 0.5% and 2% orange oil, two of them became infected after 30 days of room temperature storage. Therefore the inhibition observed *in vitro* by the microtiter assay was not supported *in vivo* by the fruit dipping experiment. In the microtiter assay, 0.5% and 2% orange oil inhibited *Penicillium* growth and therefore was believed to have antifungal activity. In contrast, oranges that were treated with 0.5 and 2% orange oil appeared to have more growth compared to fungicide-treated fruit (Fig. 68).

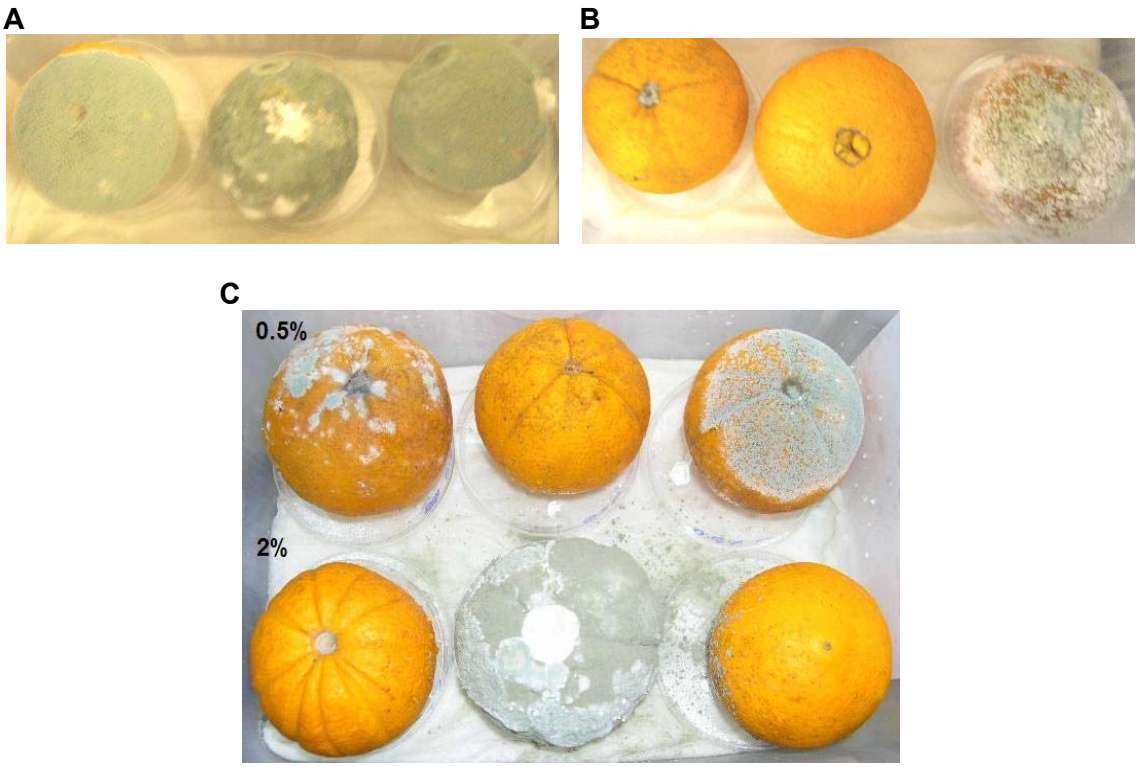


Figure 68. Comparison of fungal infection of untreated oranges with oranges treated with orange oil or fungicide. Fruit was stored at room temperature for 30 days, unwounded. **A)** Untreated fruit **B)** Fungicide-treated fruit. **C)** Fruit treated with 0.5% and 2% orange oil.

In contrast to room temperature, fruit that were treated with 0.5% and 2% orange oil and incubated at 4ºC were maintained in good condition with no fungal infection after 30 days of incubation (Fig. 69). Further, there was also no sign of fungal infection on untreated oranges and fungicide-treated oranges.

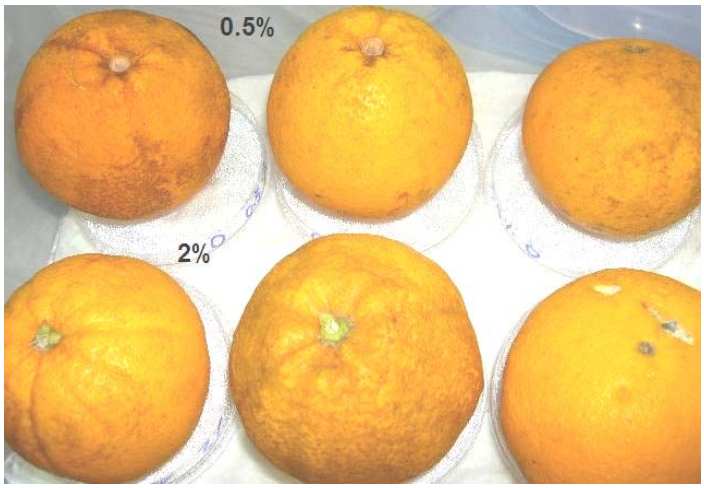


Figure 69. Unwounded oranges that were treated with 0.5% and 2% orange oil. The oranges were stored at 4ºC for 30 days.

B. Wounded fruit

Oranges that were treated with 0.5 and 2% orange oil were each wounded with a sterile needle and inoculated with *Penicillium* spore suspensions. These oranges were completely rotten after ten days of incubation at room temperature (Fig. 70 A). Similar observations were made of the wounded oranges that were treated with water and fungicide prior to wounding. These oranges were also completely rotten after ten days of storage at room temperature (Fig. 70 B and C). Interestingly, when the oranges were wounded, there appeared to be no difference in the fungal growth inhibition between untreated, fungicide-treated or orange oil-treated fruit.

In contrast to room temperature, untreated, fungicide-treated and orange oil-treated oranges were maintained in a very good condition for 30 days at 4ºC storage. There was no sign of fungal infection on the oranges. It appeared that there was no fungal infection on both wounded and unwounded oranges when stored at 4ºC (Fig. 71).

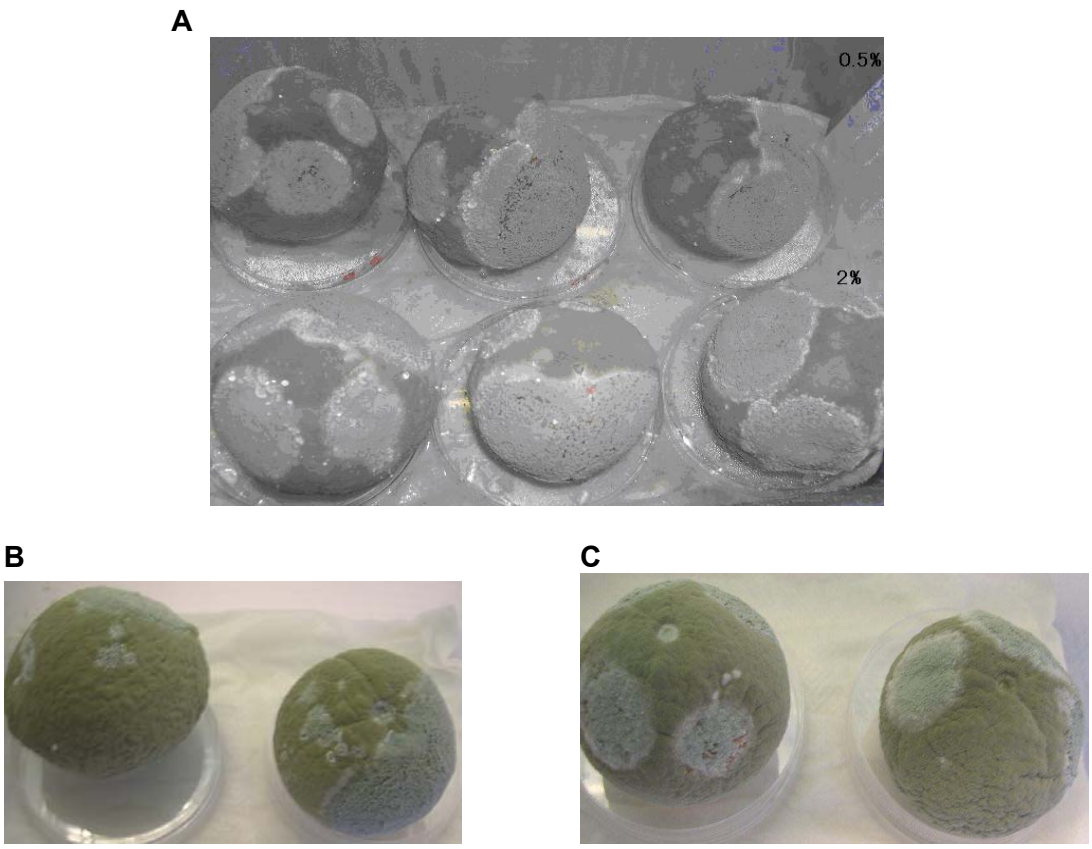


Figure 70. Wounded oranges after 10 days incubation at room temperature, showing green and blue mould caused by *Penicillium spp.* **A)** Untreated orange **B)** Fungicide-treated orange **C)** 0.5% and 2% orange oil-treated oranges

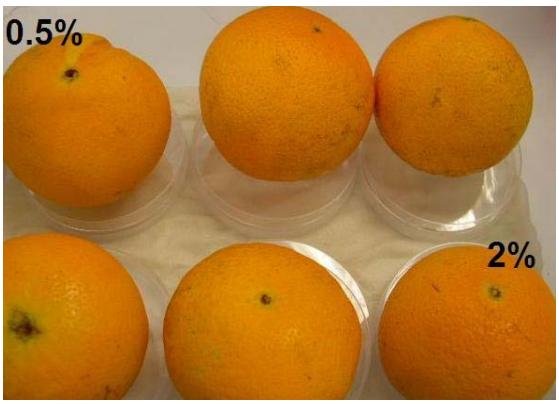


Figure 71. Wounded oranges that were treated with 0.5% and 2% orange oil. The oranges were stored at 4ºC for 30 days.

It appeared that no fungal infection could take place at 4ºC. Oranges that were treated with 0.5% and 2% orange oil and stored at 4ºC were still in good condition for up to 30 days. In contrast, at room temperature, oranges that were wounded with sterile needle failed to inhibit fungal growth with any treatments. Neither of the 0.5% and 2% orange oil managed to completely inhibit fungal growth for 30 days. However, the fungal infection appeared to be slower on orange oil and fungicide treated fruit compared to water treated fruit.

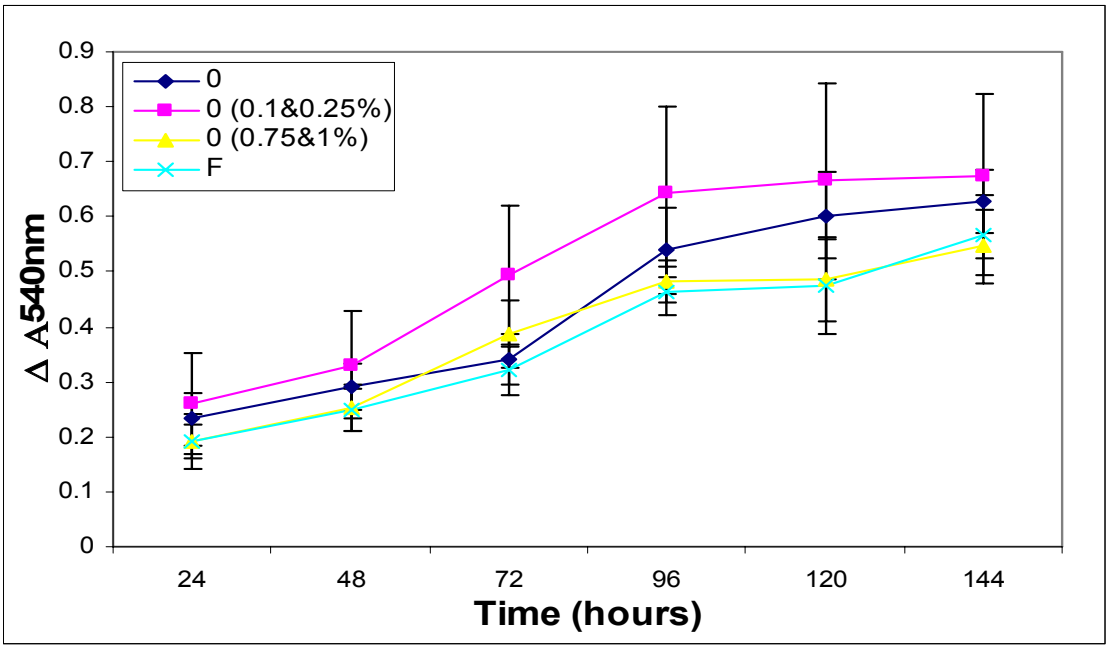
3.5.2. Manuka oil

3.5.2.1. Microtiter assay

Similar to orange oil, there was no effect of surrounding wells within the microtiter plate of all the zero values on the manuka oil (Fig. 72 A). Fungal growth was not affected by increasing essential manuka oil concentrations in nearby wells of the plate. Further, the untreated samples $0^{0.1\&0.25}$ and $0^{0.75\&1}$ of manuka oil appeared to have no fungal growth inhibition. When the means and standard errors of the means were taken for all the negative control values to create one set of numbers representing all negative control samples then no significant difference is seen between fungicide and no treatment (Fig. 72 A). For consistency with other oils, the untreated sample (0) located next to the lowest oil concentration was used for all comparison with the manuka oil. For clarity, figure 72 B shows the comparison between the untreated sample (0) and the fungicide.

The fungicide treatment in this microtiter assay gave a different result to the fungicide treatment used in the microtiter assay of orange oil. It appeared that the fungicide inhibited fungal growth in the orange oil microtiter assay, whereas there was no significant difference between fungicide and the untreated sample in the manuka oil microtiter assay. Therefore, it is believed that the fungicide was not effective in the presence of manuka oil.

A



B

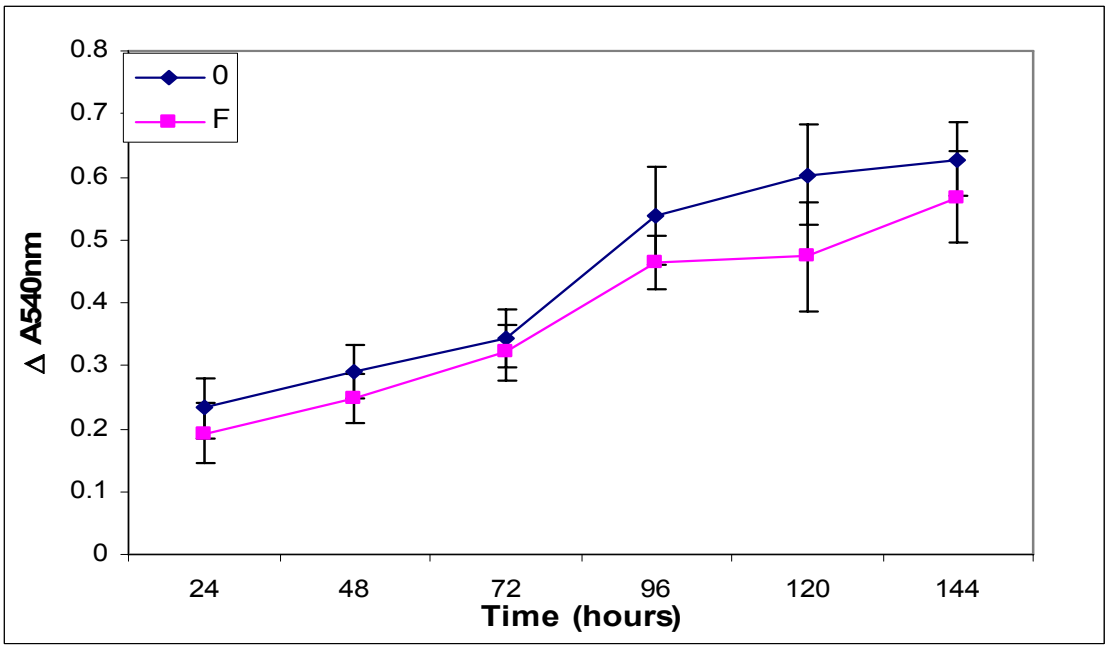


Figure 72. The amount of *Penicillium* REB 315-1 growth measured as the A_{540nm} at day 0 subtracted from the absorbance at each subsequent time point (ΔA_{540nm}). Each point represents the mean of four samples, error bars represent the standard error of the mean. **A)** Growth of untreated and fungicide treated spores grown near microtiter wells containing manuka oil. 0 refers to untreated samples placed next to the lowest concentration of manuka oil used in the assay i.e. 0.01%, 0(0.1&0.25%) refers to untreated samples placed between samples treated with 0.1% and 0.25% manuka oil (referred to as $0^{0.1\&0.25}$ in the text), 0(0.75&1%) refers to untreated samples placed between samples treated with 0.75% and 1% manuka oil (referred to as $0^{0.75\&1}$ in the text). F refers to samples treated with fungicide. **B)** Comparison of growth between untreated (0) and fungicide treated spores

The fact that the fungicide treatment was not effective in this assay (Fig. 73) means the effect of manuka oil can only be compared to the untreated controls. For clarity, figure 74 shows the comparison between untreated control and the manuka oil in the absence of fungicide.

No significant differences were observed between 0.01%, 0.05% and 0.1% manuka-treated and water-treated spores (Fig. 74 A to C). In contrast, manuka oil at 0.25% appeared to be inhibitory for the duration of the assay (Fig. 74 D). Interestingly, 0.5% manuka oil inhibited fungal growth initially in the first 24 hours, but the fungus then appeared to recover from the exposure to the oil at a fast rate with the growth slowing in the presence of oil between 120 and 144 hours (Fig. 74 E). Further, 0.75% manuka oil inhibited fungal growth initially up to 48 hours before the fungus recovered from the oil exposure and similar to 0.5%, the growth also slowed in between 120 and 144 hour (Fig. 74 F).

Manuka oil at 1% and 2% appeared to be effective in inhibiting fungal growth (Fig. 74 G and H). Similar to 0.25% manuka oil, both of these concentrations inhibited fungal growth for the duration of the assay.

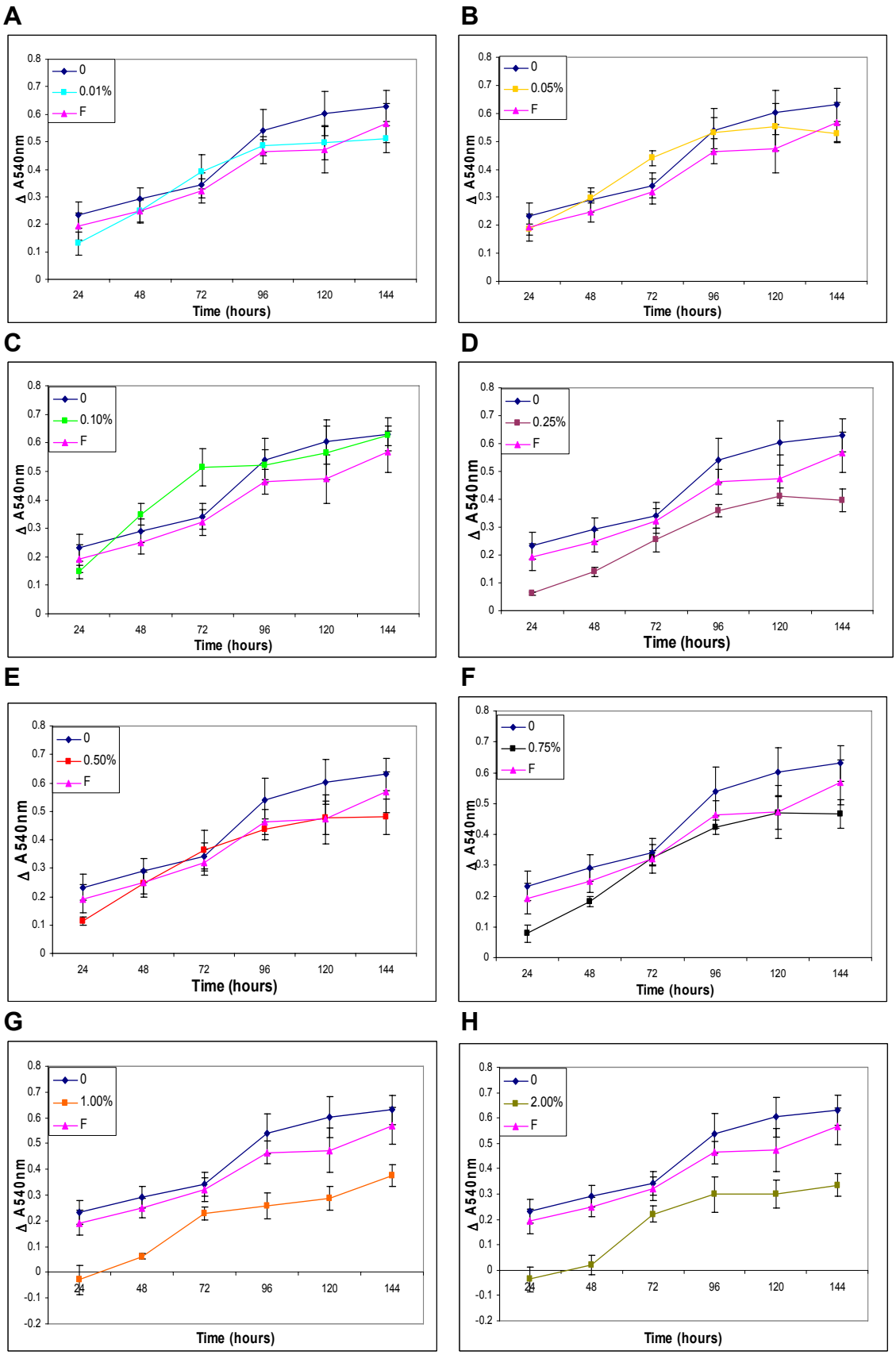


Figure 73. Inhibition effect of manuka oil towards the growth of *Penicillium* REB 315-1 in 0.1x PDB over 144 hours as measured using a microtiter growth assay. The graphs represent the amount of growth measured as the A_{540nm} at day 0 subtracted from the absorbance at each subsequent time point (ΔA_{540nm}). 0 refers to untreated samples placed next to the lowest concentration of manuka oil used in the assay i.e. 0.01% and F refers to samples treated with fungicide. Error bars represent the standard error of the mean. These graphs represent manuka oil treated samples at **A)** 0.01% **B)** 0.05% **C)** 0.01% **D)** 0.25% **E)** 0.50% **F)** 0.75% **G)** 1% **H)** 2%.

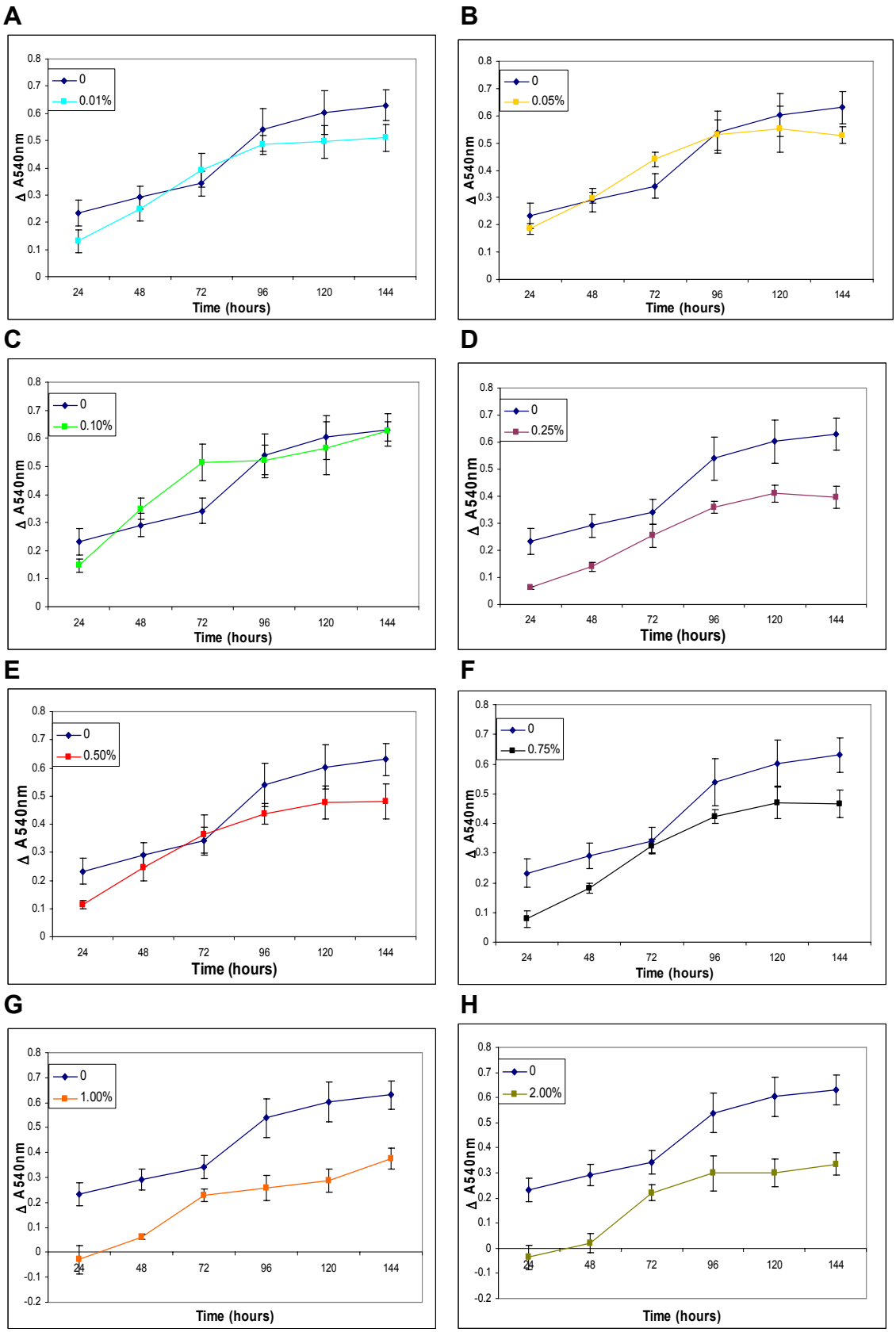


Figure 74. The effect of different concentrations of manuka oil on *Penicillium* REB 315-1 spores in 0.1x PDB over 144 hours. These graphs are similar to what is shown in figure 70, but the fungicide values were taken off for a clearer view on the oil. **A)** 0.01% **B)** 0.05% **C)** 0.01% **D)** 0.25% **E)** 0.50% **F)** 0.75% **G)** 1% **H)** 2%.

3.5.2.2. Manuka oil dipping treatment

A. Unwounded fruit

All of the unwounded oranges (untreated) at room temperature appeared to be completely infected after 30 days of storage at room temperature (Fig. 75 A), whereas only one out of three oranges that was completely infected with the fungicide treatment (Fig. 75 B). Similar to the untreated oranges, fruit dipped in 0.5% and 2% manuka oil were also completely infected after 30 days (Fig. 75 C).

The untreated and fungicide-treated oranges appeared to be infected by green mould, caused by *Penicillium spp*. Interestingly, fruit that were treated with manuka oil had some reddish brown stains on the peel and were covered with a fungal growth that appeared to be different from a green mould (Fig. 75 and Fig. 76). From the three oranges that were treated with 0.5% and 2% manuka oil, all of them were infected. The infection started as white spots scattered on some areas of the fruit surface at day 10 (Fig. 76 B). The spots then grew into a white, cream and gray tuft which covered the whole fruit after 20 to 30 days of storage, (Fig. 76 B and C). This appearance of fungal growth was quite similar to the infection observed during the honey experiment.

Therefore, neither 0.5% nor 2% appeared to have antifungal activity since all of the fruit were infected. It seems that the infection was not caused by *Penicillium spp.* (Fig. 76). Fruit that were treated with both oil concentrations appeared to have more fungal infection compared to fungicide treated fruit (Fig. 75).

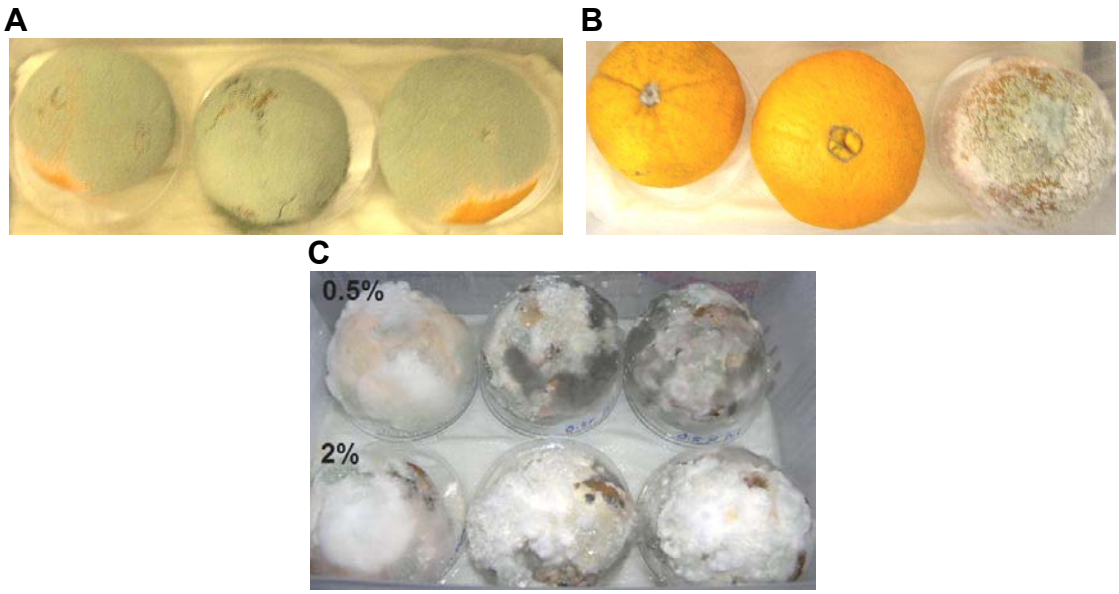


Figure 75. Comparison of fungal infection of untreated oranges with oranges treated with manuka oil or fungicide. Fruit was stored at room temperature for 30 days, unwounded. **A)** Untreated fruit **B)** Fungicide-treated fruit **C)** Fruit treated with 0.5% and 2% manuka oil

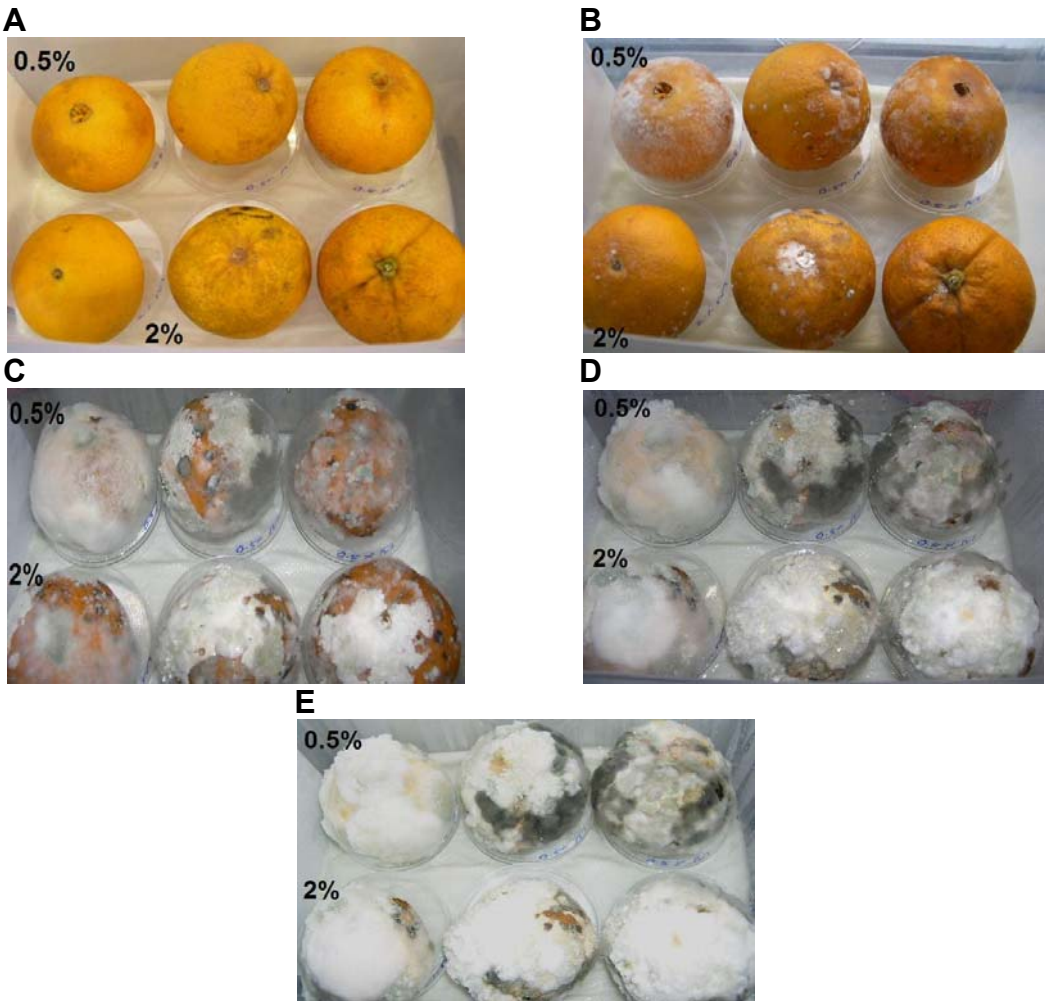


Figure 76. Oranges dipped with 0.5% and 2% manuka oil for 30 seconds and stored at room temperature, showing the development of fungal infection over time. These photos represent the manuka oil treated oranges that were stored for: **A)** Day 0 **B)** 10 days **C)** 20 days **D)** 30 days and **E)** 45 days

In contrast to room temperature, fruit that were treated with 0.5% and 2% manuka oil and incubated at 4ºC were maintained in good condition with no fungal infection after 30 days of incubation (Fig. 77). Further, there was also no sign of fungal infection for the untreated and fungicide-treated oranges.

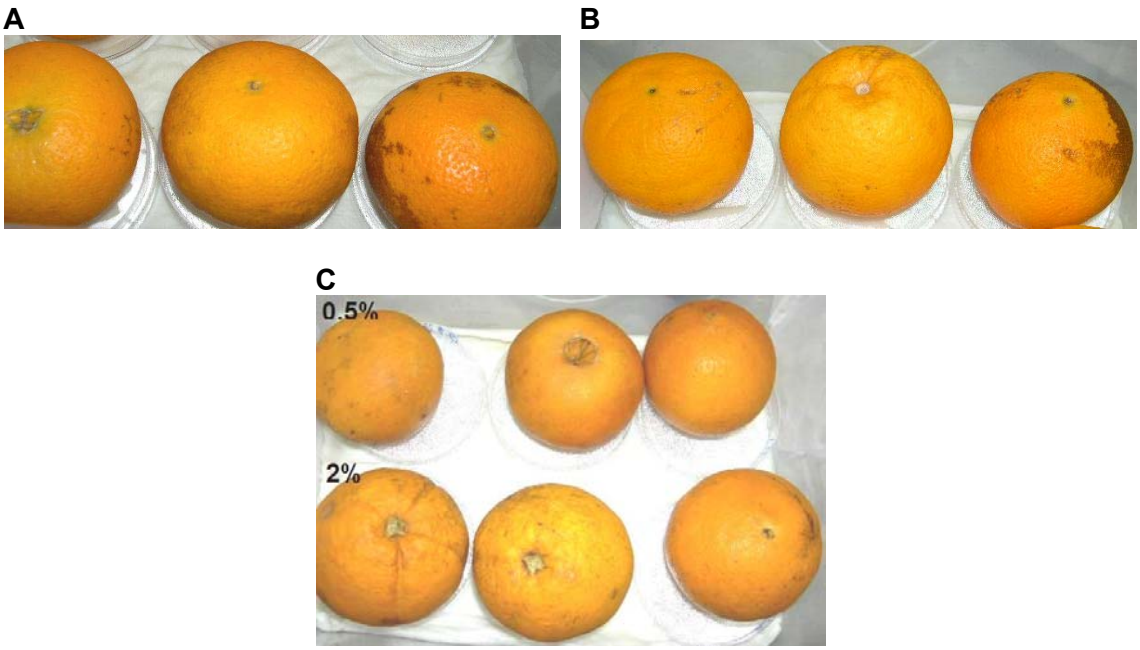


Figure 77. Comparison between unwounded oranges. The fruit were stored at 4ºC for 30 days. **A)** Untreated oranges. **B)** Fungicide-treated oranges **C)** 0.5% and 2% manuka oil-treated oranges.

Manuka oil at 0.5% and 2% seemed to be stimulatory to fungal growth on fruit, therefore this oil does not appear to be a good postharvest treatment.

B. Wounded fruit

Similar to orange oil, fruit that were treated with 0.5% and 2% manuka oil prior to wounding appeared to be completely rotten after ten days of room temperature storage (Fig. 78 A). The same observation was found for both untreated and fungicide-treated wounded oranges (Fig. 78 B and C). There appeared to be no difference in the fungal growth inhibition between untreated, fungicide-treated or manuka oil-treated fruit when the fruit exposed to wounding. Oranges that were treated with manuka oil and wounded also seemed to have fungal growth other than the “typical” *Penicillium* growth. The other growth was previously described in section 3.5.2.2.**A** (Pg. 113).

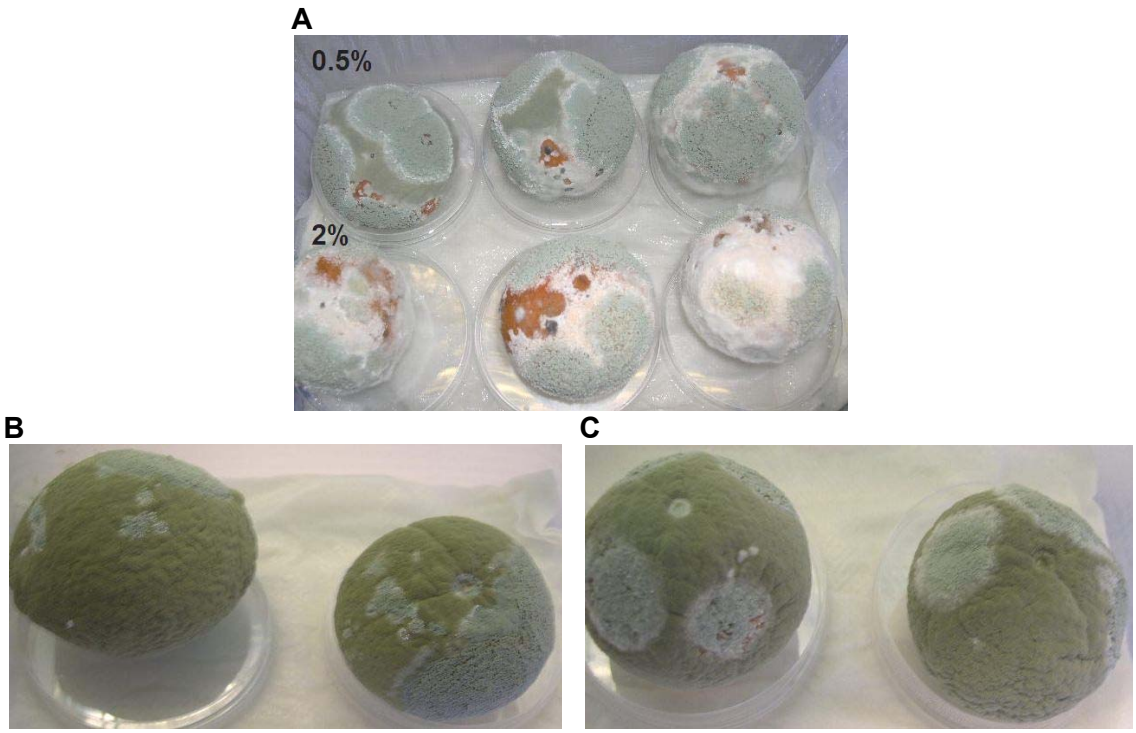


Figure 78. Wounded oranges after 10 days of room temperature storage. **A)** 0.5% and 2% manuka oil-treated oranges. **B)** Untreated oranges. **C)** Fungicide-treated oranges

Similar to figure 77, untreated, fungicide-treated and manuka oil-treated oranges were maintained in a very good condition for 30 days at 4ºC storage. Therefore, there was no fungal infection on both wounded and unwounded oranges when stored at 4ºC.

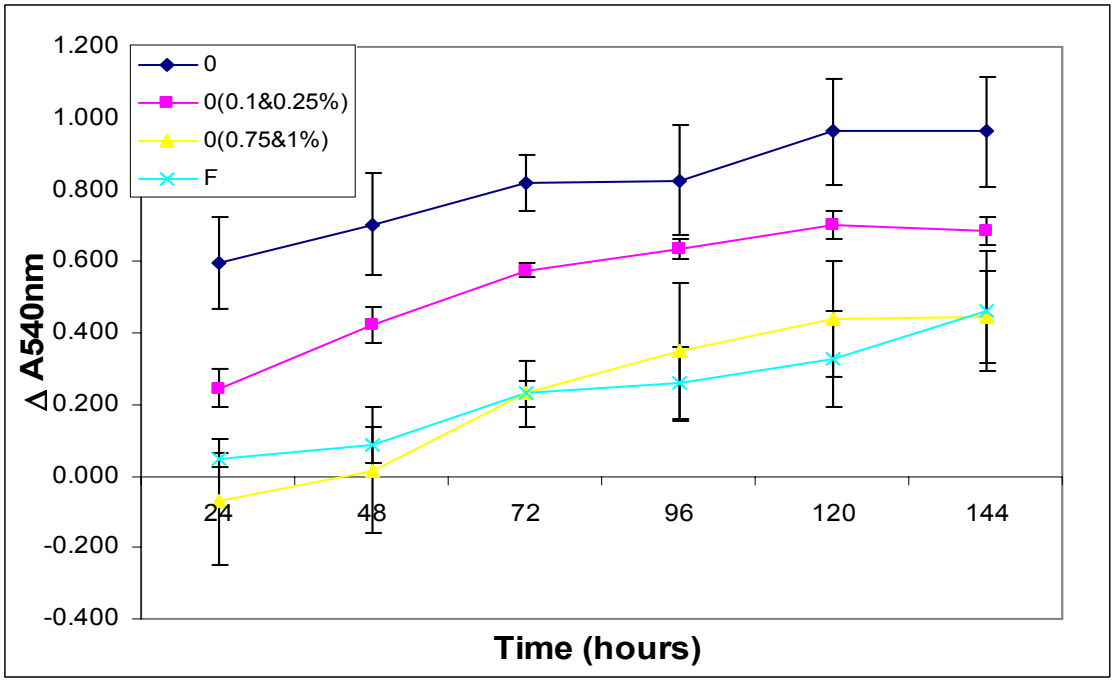
In summary, manuka oil appeared to have a weak antifungal activity, since it did not inhibit any of the fungal growth on *in vivo* (fruit dipping) assay. The observation on *in vivo* assay did not correlate with the *in vitro* assay. Manuka oil appeared to be stimulatory to other fungal growth other than *Penicillium* on both wounded and unwounded fruit at room temperature storage. The identity of this other growth is still unknown. Similar to other treatments, there was no fungal infection on both wounded and unwounded oranges when stored at 4ºC.

3.5.3. Lemongrass oil

3.5.3.1. Microtiter assay

In contrast to orange and manuka oil, there appeared to be an effect of thelemongrass oil to surrounding wells within the assay plate for the zero values. Fungal growth appeared to be affected by increasing lemongrass oil concentrations in nearby wells of the plate. This suggests that volatiles from the oils were impacting on surrounding wells. Those untreated samples placed between $0^{0.75\&1}$ lemongrass oil appeared to have fungal growth inhibited to the same degrees the fungicide treated samples. It would appear that the fungus does not need to be in direct contact with the essential oil for there to be an impact on its growth (Fig 79 A). For consistency with other oils, the untreated sample (0) located next to the lowest oil concentration was used for all comparison with lemongrass oil. For clarity, figure 79 B shows the comparison between the untreated sample (0) and the fungicide.

A



B

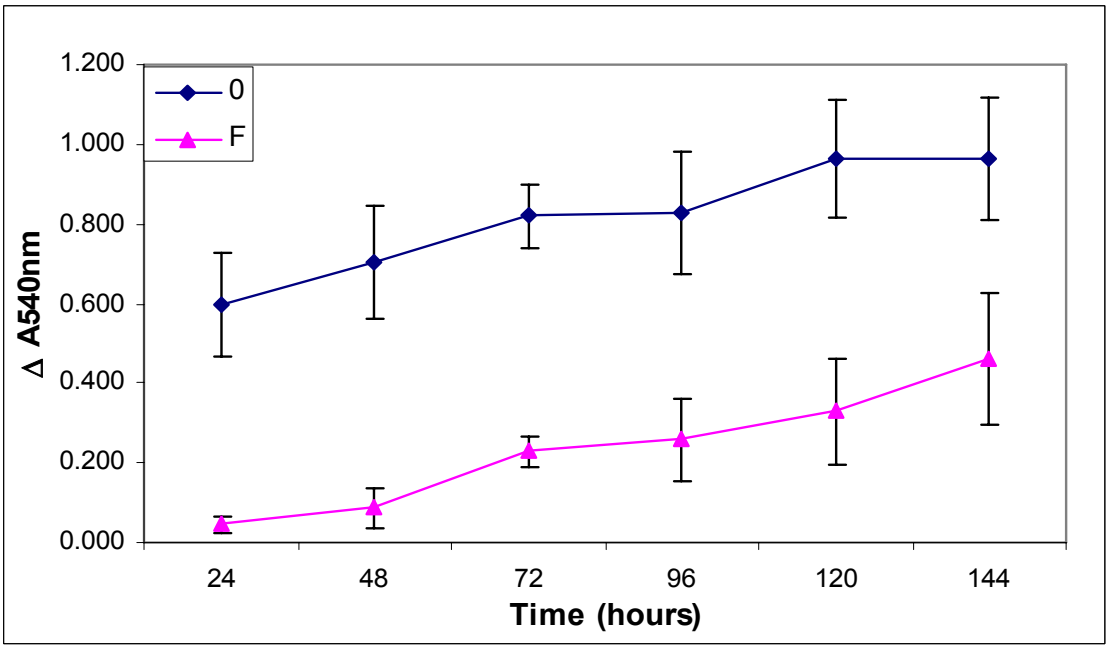


Figure 79. The amount of *Penicillium* REB 315-1 growth measured as the A_{540nm} at day 0 subtracted from the absorbance at each subsequent time point (ΔA_{540nm}). Each point represents the mean of four samples, error bars represent the standard error of the mean. **A)** Growth of untreated and fungicide treated spores grown near microtiter wells containing lemongrass oil. 0 refers to untreated samples placed next to the lowest concentration of lemongrass oil used in the assay i.e. 0.01%, 0(0.1&0.25%) refers to untreated samples placed between samples treated with 0.1% and 0.25% lemongrass oil (referred to as $0^{0.1\&0.25}$ in the text), 0(0.75&1%) refers to untreated samples placed between samples treated with 0.75% and 1% lemongrass oil (referred to as $0^{0.75\&1}$ in the text). F refers to samples treated with fungicide. **B)** Comparison of growth between untreated (0) and fungicide treated spores

Lemongrass oil appeared to be a strong antifungal agent against *Penicillium spp*. (Fig. 80 and 81). Lemongrass oil concentration as low as 0.05% appeared to have antifungal activity, but it was less effective compared to the fungicide. While 0.05% was not quite as effective as fungicide treatment at inhibiting growth (Fig. 80 B), 0.1% and 0.25% oil appeared as effective (Fig. 80 C and D), while higher concentrations appeared more effective (Fig. 81 A to D).

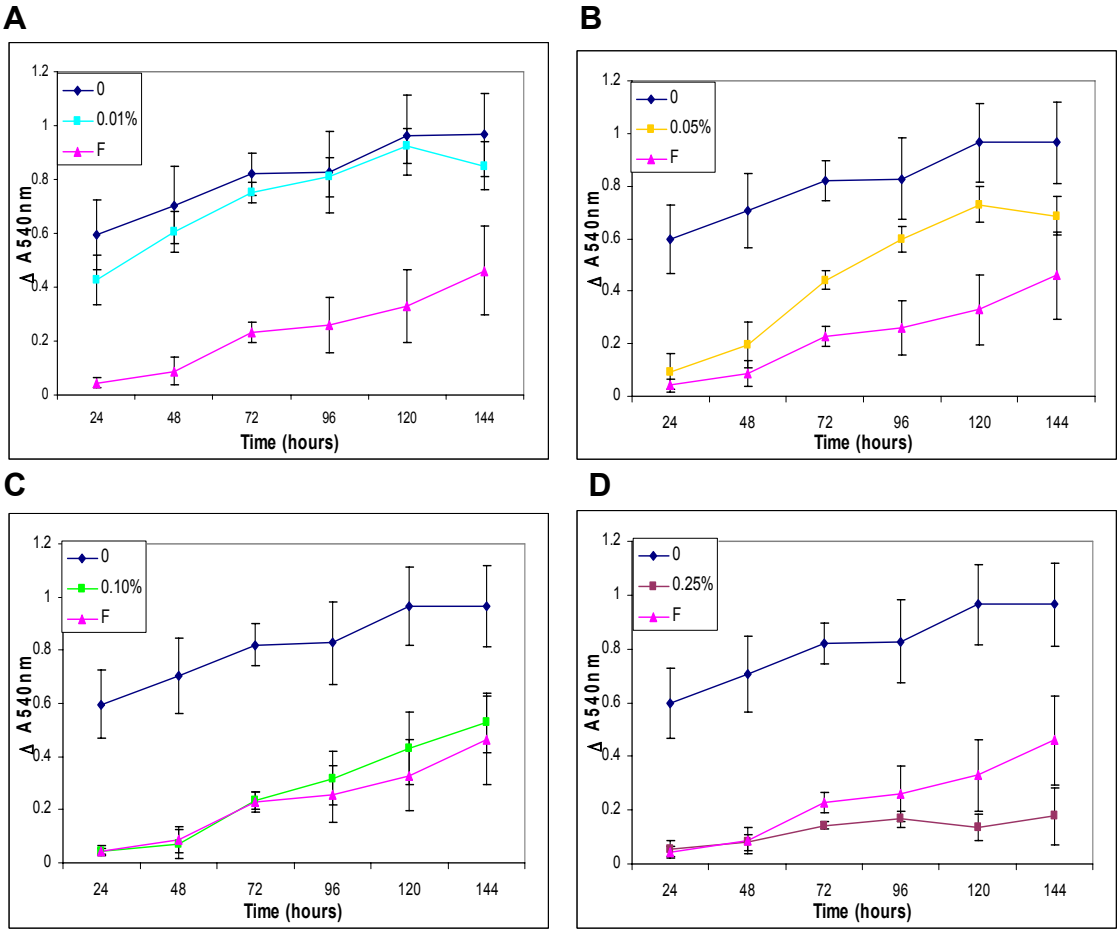


Figure 80. Inhibition effect of lemongrass oil towards the growth of *Penicillium* REB 315-1 in 0.1x PDB over 144 hours as measured using a microtiter growth assay. The graphs represent the amount of growth measured as the A_{540nm} at day 0 subtracted from the absorbance at each subsequent time point (ΔA_{540nm}). 0 refers to untreated samples placed next to the lowest concentration of lemongrass oil used in the assay i.e. 0.01% and F refers to samples treated with fungicide. Error bars represent the standard error of the mean. These graphs represent lemongrass oil treated samples at **A)** 0.01% **B)** 0.05% **C)** 0.01% **D)** 0.25%.

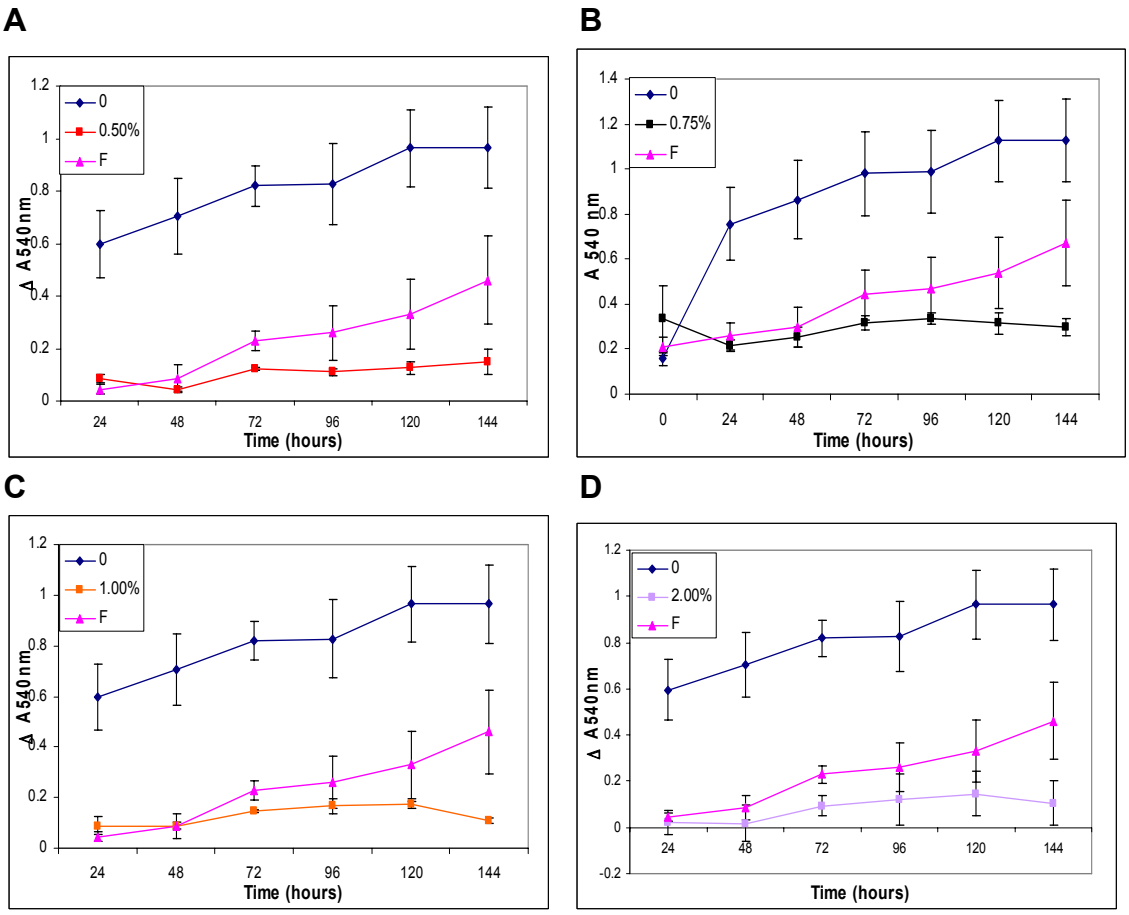


Figure 81. Inhibition effect of lemongrass oil towards the growth of *Penicillium* REB 315-1 in 0.1x PDB over 144 hours as measured using a microtiter growth assay. The graphs represent the amount of growth measured as the A_{540nm} at day 0 subtracted from the absorbance at each subsequent time point (ΔA_{540nm}). 0 refers to untreated samples placed next to the lowest concentration of lemongrass oil used in the assay i.e. 0.01% and F refers to samples treated with fungicide. Error bars represent the standard error of the mean. These graphs represent lemongrass oil treated samples at **A)** 0.50% **B)** 0.75% **C)** 1% **D)** 2%.

Higher concentrations of lemongrass oil appeared to have a greater impact compared to fungicide throughout the assay. It appeared that lemongrass oil inhibited *Penicillium* growth at 0.05% oil concentration and above. Therefore four different oil concentrations were tested for their antifungal activity on oranges. These concentrations were 0.05%, 0.5%, 0.75% and 2%.

3.5.3.2. Lemongrass oil dipping treatment

A. Unwounded fruit

Oranges that were exposed to 0.05% lemongrass oil and unwounded were still in good condition after 30 days of storage at room temperature (Fig. 82 A).

Interestingly, in contrast to the microtiter assay, all of the lemongrass oil concentrations above 0.05% failed to give complete protection to the oranges (Fig. 82 A to D). Two out three oranges that were dipped into 0.5% oil were infected after 30 days of storage. This oil concentration appears to have inhibited fungal infection to the same degree as fungicide (Fig 82 B and E). The worst fruit infection was found on the fruit that were treated with 0.75% and 2% lemongrass oil, since all of the oranges were completely rotten by 30 days of storage at room temperature (Fig. 82 C and D). High concentrations of lemongrass oil seemed to be a suitable environment for not only "typical" *Penicillium* growth, but also for other fungi. Oranges that were exposed to 0.75% and 2% lemongrass oil were covered with fungal growth that appeared as green mould together with additions of white, pink, cream and black colour tufts. These two concentrations also appeared to give an adverse reaction on the skin of the fruit. Pink pigmentation appeared on the skin of the fruit after seven days of incubation (Fig. 83).

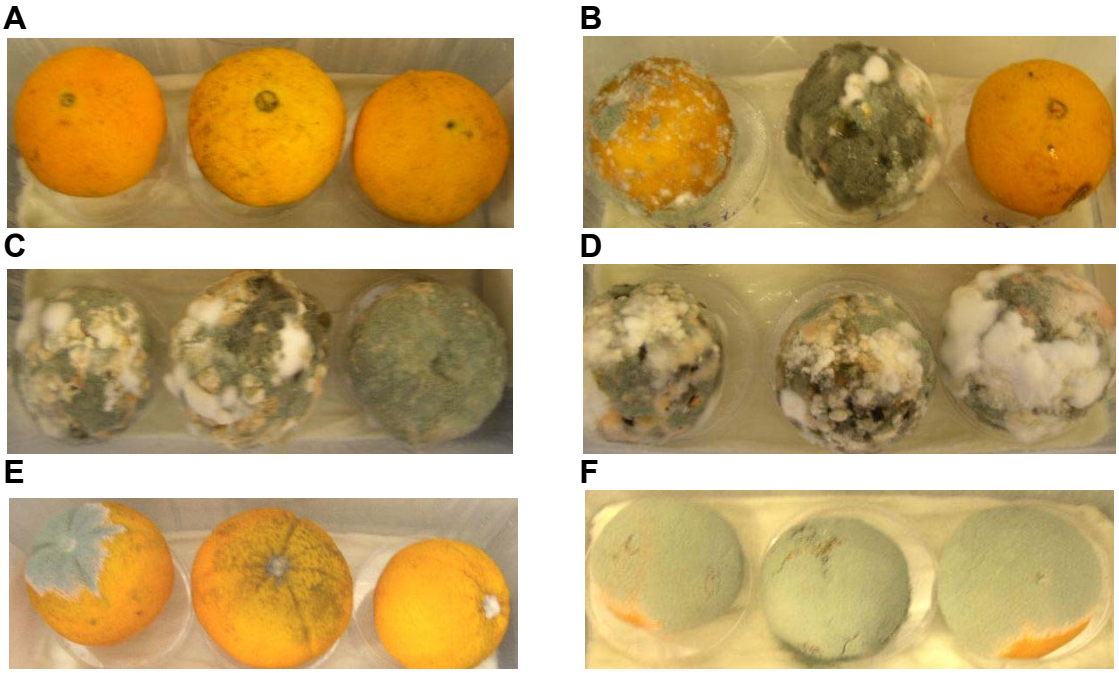


Figure 82. Comparison of oranges that were exposed to 0.05%, 0.5%, 0.75% and 2% lemongrass oil with fungicide-treated oranges and water-treated oranges. The oranges were stored at room temperature for 30 days. The oranges were treated with **A)** 0.05% lemongrass oil **B)** 0.5% lemongrass oil **C)** 0.75% lemongrass oil **D)** 2% lemongrass oil **E)** Fungicide and **F)** Water.

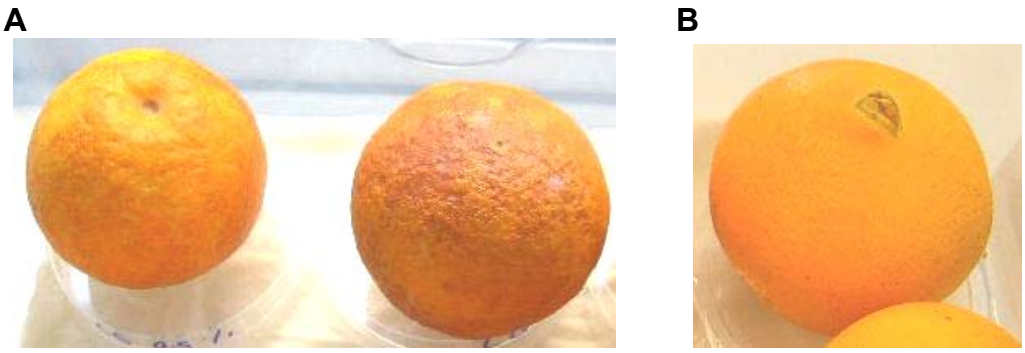


Figure 83. Pink pigmentation appeared on 0.75% lemongrass oil after seven days incubation at room temperature. Oranges that were exposed to 0.75% lemongrass oil **A)** after 7 days of incubation and **B)** before the incubation

In contrast to room temperature storage, all lemongrass oil, fungicide and water treated oranges had no fungal growth after 30 days of storage at 4ºC (Fig. 81). However, oranges that were treated with 0.5%, 0.75% and 2% appeared to have red pigmentation and a leathery look to the peel (Fig. 81).

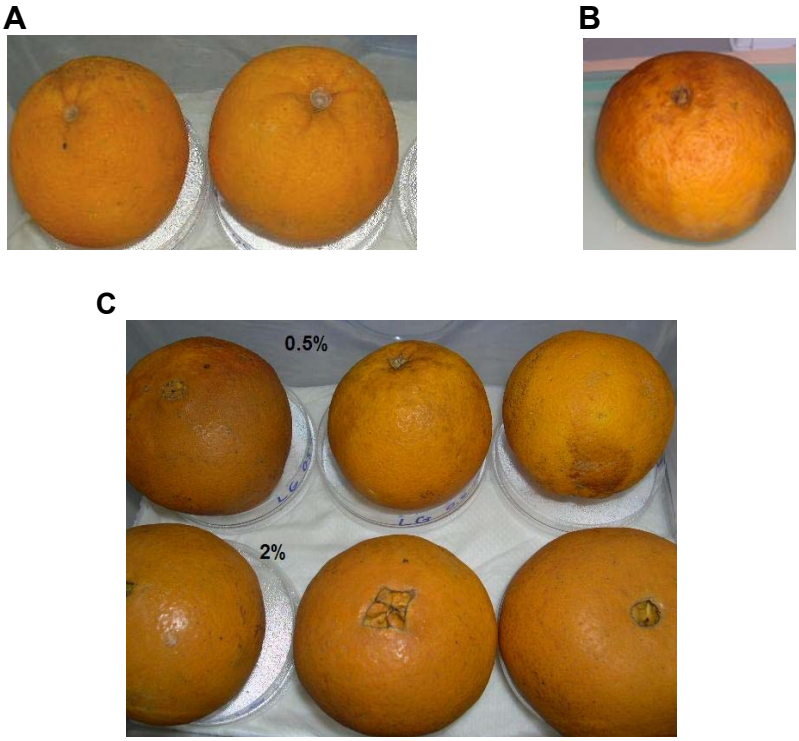


Figure 84. Unwounded oranges that were exposed to 0.05%, 0.5%, 0.75% and 2% lemongrass oil after 30 days of storage at 4ºC. Oranges exposed to **A)** 0.05% **B)** 0.75% **C)** 0.5% and 2% lemongrass oil.

Lemongrass oil at 0.05% could be a good postharvest treatment on oranges against blue and green mould, since it managed to protect the oranges from fungal infection for up to 30 days at both room and 4ºC storage. However, more studies are required to confirm this statement.

B. Wounded fruit

Similar to orange and manuka oils, oranges that were exposed to 0.05%, 0.5%, 0.75% and 2% lemongrass oil and wounded were completely rotten after ten days of storage at room temperature (Fig. 85). The fungal growth was noticeable as either a green mould or blue mould depending on the inoculum. Further, green and blue mould was also found on fungicide treated fruit and water treated fruit after 10 days of storage in room temperature (Fig. 85).

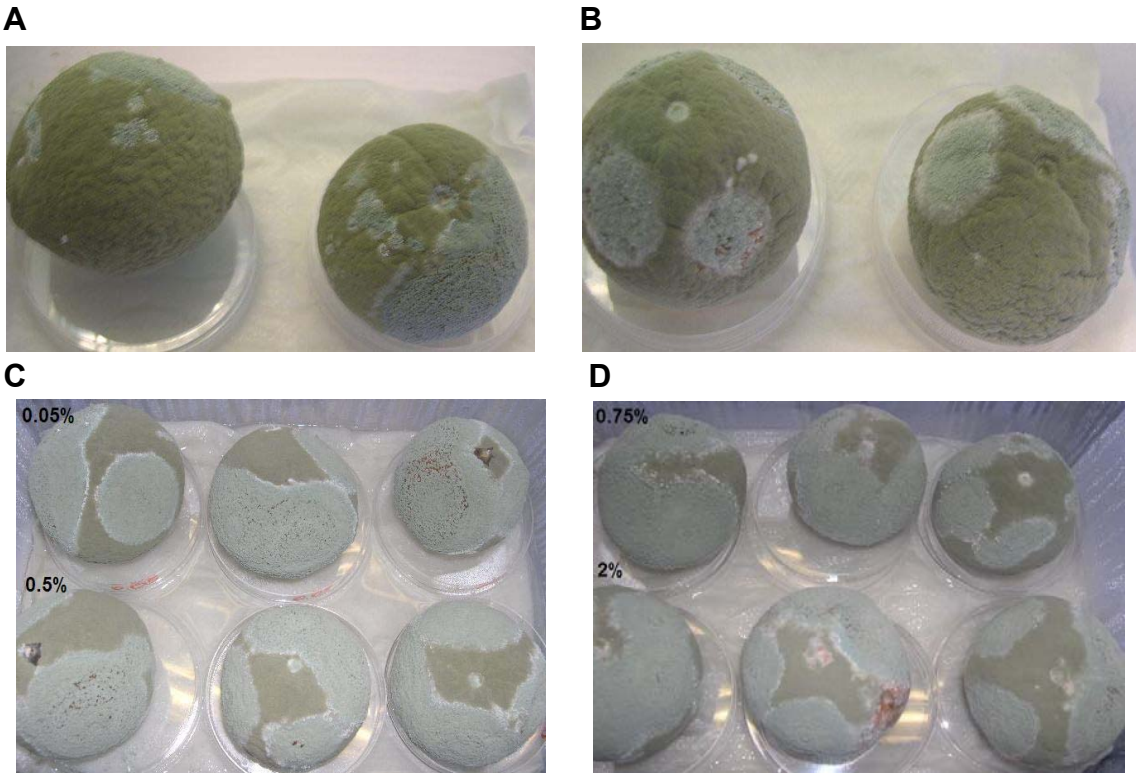


Figure 85. Wounded oranges after 10 days of storage at 4ºC, showing a green and blue mould caused by *Penicillium spp.* **A)** Water treated oranges. **B)** Fungicide-treated oranges. **C)** 0.05% and 0.5% lemongrass oil-treated oranges **D)** 0.75% and 2% lemongrass oil-treated oranges.

As far for all similar experiments, water, fungicide and lemongrass oil treated oranges and wounded were maintained in a very good condition for up to 30 days of storage at 4ºC.

In summary, lemongrass oil appeared to have quite a strong antifungal activity, since low concentrations inhibited any of the fungal growth in both *in vitro* and *in vivo* (microtiter and fruit dipping) assays. In the *in vitro* (microtiter) assay high concentrations of lemongrass oil showed better fungal inhibition compared to lower concentrations. This is in contrast to the *in vivo* assay, where high lemongrass oil concentrations appeared to be stimulatory to other fungal growth on unwounded oranges at room temperature storage. The identity of this other growth is unknown.

Pink pigmentation was observed during the early storage of oranges that were treated with high concentration of lemongrass oil. At room temperature storage this reaction was observed after seven days of storage, before it was covered by infection, whereas it could still be seen after 30 days at 4ºC storage, since there was no fungal infection on both wounded and unwounded oranges when stored at 4ºC.

Lemongrass oil at 0.05% appeared to be the best treatment, since it managed to protect the oranges from fungal infection for up to 30 days at both room temperature and 4ºC storage. There was no red pigmentation observed on oranges that were treated at this concentration.

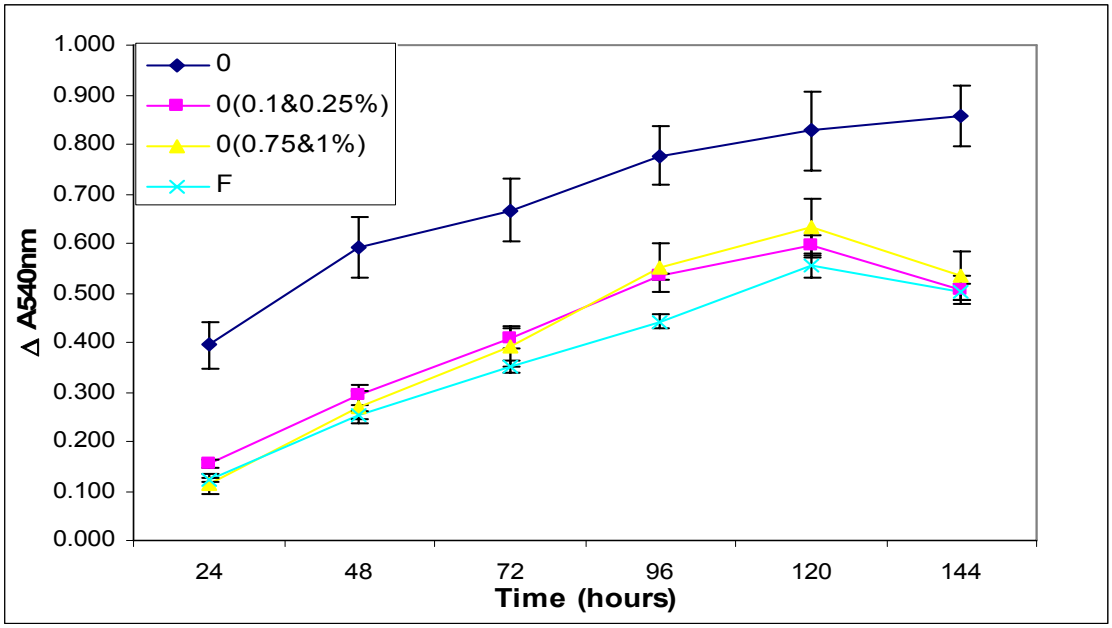
3.5.4. Lemon oil

3.5.4.1. Microtiter assay

Similar to lemongrass oil, there appeared to be an effect of surrounding wells within the assay plate for the zero values on the lemon oil. Fungal growth appears to have been affected by increasing lemon oil concentrations in nearby wells of the plate. This suggests that volatiles from the oils are impacting on surrounding wells. Those untreated samples placed between $0^{0.75\&1}$ lemon oil appear to have fungal growth inhibited to the same degree as the fungicide treated samples. It would appear that the fungus does not need to be in direct contact with the essential oil for there to be an impact on its growth (Fig. 86 A). Lemon appears to be inhibitory in this way at lower concentrations than lemongrass.

For consistency with other oils, the untreated sample (0) located next to the lowest oil concentration was used for all comparison with lemon oil. For clarity, figure 86 B shows the comparison between the untreated sample (0) and the fungicide.

A



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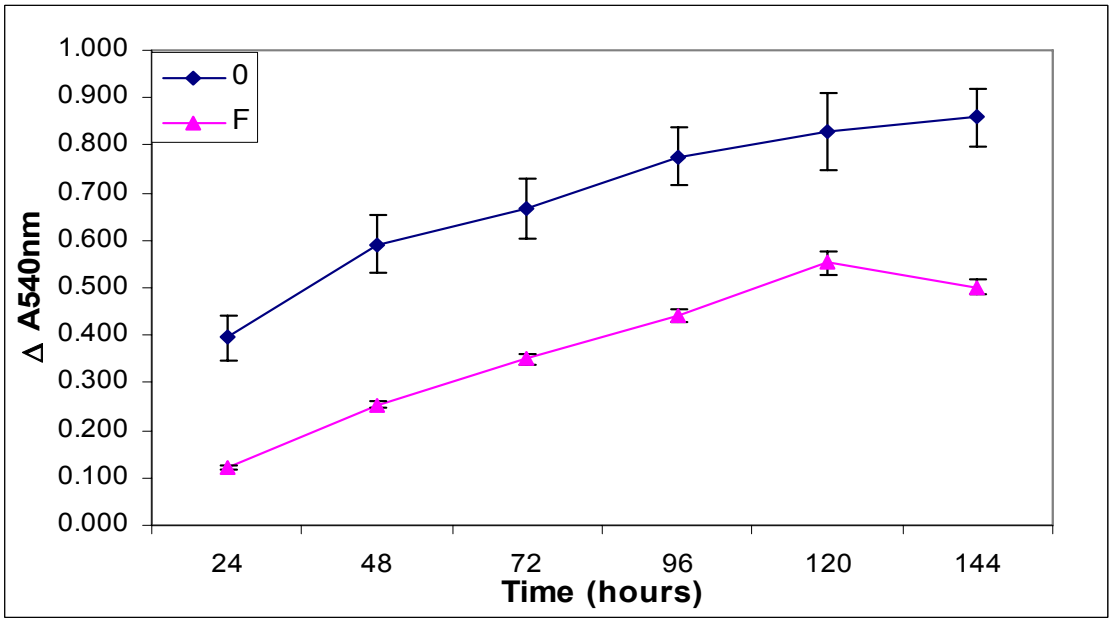


Figure 86. The amount of *Penicillium* REB 315-1 growth measured as the A_{540nm} at day 0 subtracted from the absorbance at each subsequent time point (ΔA_{540nm}). Each point represents the mean of four samples, error bars represent the standard error of the mean. **A)** Growth of untreated and fungicide treated spores grown near microtiter wells containing lemongrass oil. 0 refers to untreated samples placed next to the lowest concentration of lemongrass oil used in the assay i.e. 0.01%, 0(0.1&0.25%) refers to untreated samples placed between samples treated with 0.1% and 0.25% lemongrass oil (referred to as $0^{0.1\&0.25}$ in the text), 0(0.75&1%) refers to untreated samples placed between samples treated with 0.75% and 1% lemongrass oil (referred to as $0^{0.75\&1}$ in the text). F refers to samples treated with fungicide. **B)** Comparison of growth between untreated (0) and fungicide treated spores

Lemon oil appeared to be a stronger antifungal agent against *Penicillium spp.* compared to the other oils, since the antifungal activity was observed at the lowest concentration of the oil used in the assay (i.e. 0.01%). Lemon oil at low concentrations (i.e. 0.01% and 0.05%) appeared to be inhibitory towards the fungal growth (Fig. 87). 0.01% lemon oil appeared to be slightly less effective compared to the fungicide (Fig. 87 A), whereas 0.05% lemon oil inhibited fungal growth to the same degree as fungicide (Fig. 87 B).

Lemon oil at 0.1% concentration also appeared to inhibit fungal growth although to a lesser degree than fungicide (Fig. 87 C). However, 0.25% lemon oil appeared to have a similar impact on fungal growth as the fungicide treatment (Fig. 87 D).

0.5% lemon oil also appeared to have a similar impact on fungal growth as fungicide treatment (Fig. 88 A). Further, 0.75% lemon oil showed that over the first 24 hours of the assay, no difference was noted in the growth of the fungus compared with the fungicide treated control. However, as the assay progressed, a negative impact of the oil on the fungal growth became apparent. It appeared to have a similar effect on growth as the fungicide treatment up until 72 hours after which the fungus recovered (Fig. 88 B).

Both 1% and 2% lemon oil appeared to have an inhibitory effect for up to 72 hours, however the fungus appeared to overcome this and the oil became less effective at inhibiting growth compared with the fungicide (Fig. 88 C and D).

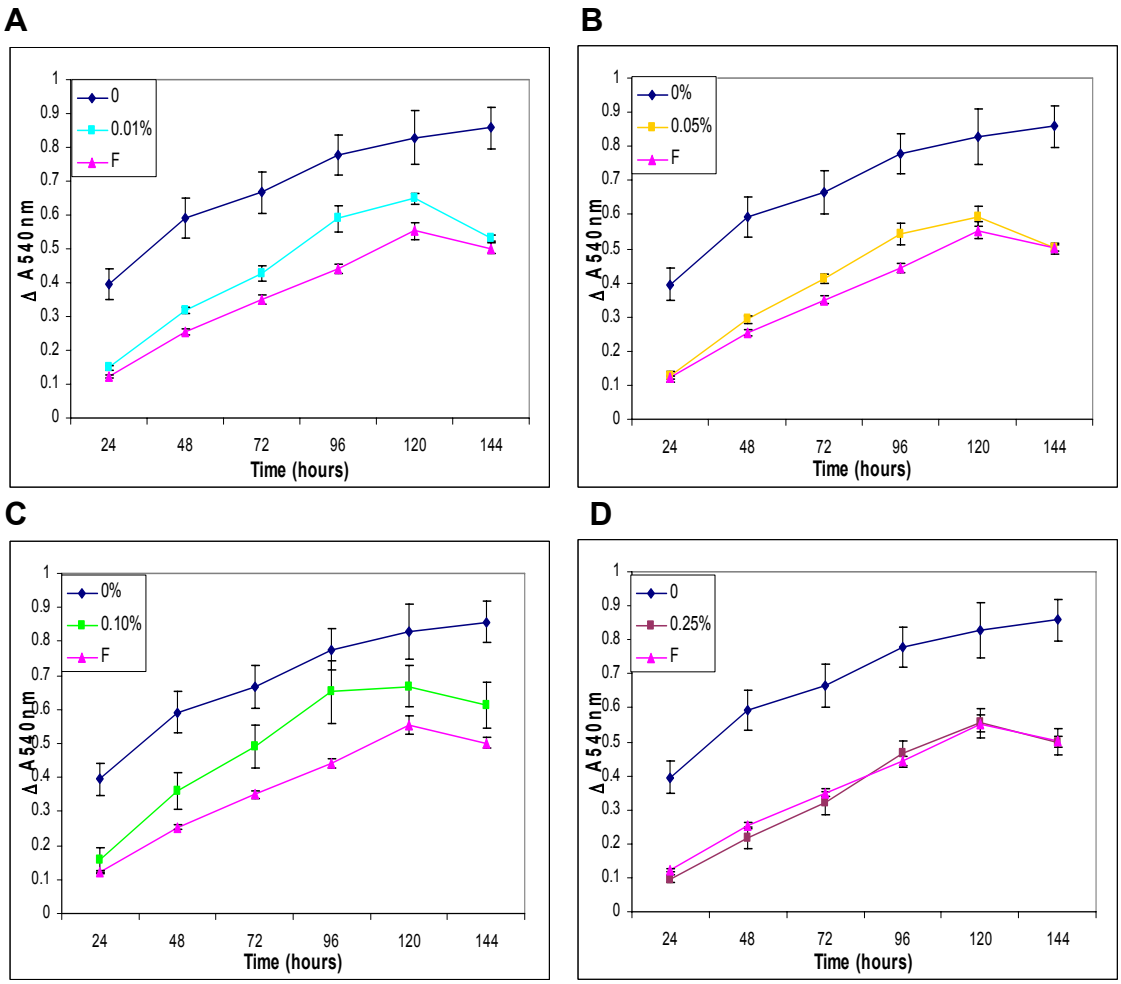


Figure 87. Inhibition effect of lemon oil towards the growth of *Penicillium* REB 315-1 in 0.1x PDB over 144 hours as measured using a microtiter growth assay. The graphs represent the amount of growth measured as the A_{540nm} at day 0 subtracted from the absorbance at each subsequent time point (ΔA_{540nm}). 0 refers to untreated samples placed next to the lowest concentration of lemon oil used in the assay i.e. 0.01% and F refers to samples treated with fungicide. Error bars represent the standard error of the mean. These graphs represent lemon oil treated samples at **A)** 0.01% **B)** 0.05% **C)** 0.01% **D)** 0.25%.

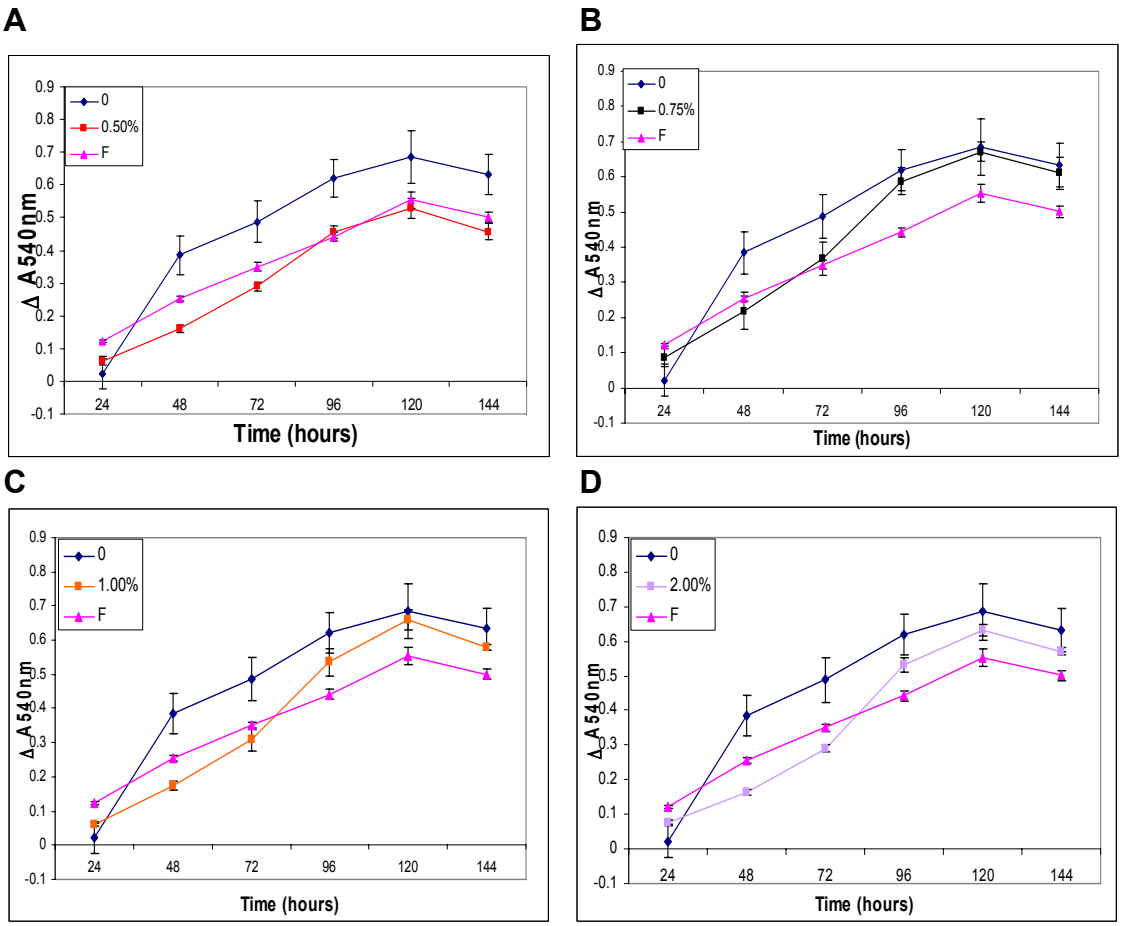


Figure 88. Inhibition effect of lemon oil towards the growth of *Penicillium* REB 315-1 in 0.1x PDB over 144 hours as measured using a microtiter growth assay. The graphs represent the amount of growth measured as the A_{540nm} at day 0 subtracted from the absorbance at each subsequent time point (ΔA_{540nm}). 0 refers to untreated samples placed next to the lowest concentration of lemon oil used in the assay i.e. 0.01% and F refers to samples treated with fungicide. Error bars represent the standard error of the mean. These graphs represent lemon oil treated samples at **A)** 0.50% **B)** 0.75% **C)** 1% **D)** 2%.

Lemon oil at the concentrations of 0.25% and 0.5% appeared to have a similar impact to the fungicide throughout the assay. Therefore, these two concentrations were tested for their antifungal activity on oranges. Lemon oil seemed to be inhibitory at low concentrations, therefore 0.05% was also tested. For comparison with the other oils, 2% lemon oil was also tested.

3.5.4.2. Lemon oil dipping treatment

A. Unwounded fruit

Oranges that were exposed to 0.25% and 0.5% lemongrass oil and left unwounded were still in good condition after 30 days of storage at room temperature (Fig. 89). This was similar to what was found in the microtiter assay. Compared to the microtiter assay, 0.05% and 2% lemon oil failed to give complete protection to the oranges (Fig. 89 A and D). Two out of three oranges that were dipped in these concentrations were infected after 30 days of storage. In contrast, all of the water treated oranges were rotten after 30 days of storage whereas only one out of three fungicide-treated oranges was infected (Fig 89 E and F).

Lemon oil at the concentrations of 0.25% or 0.5% could be good postharvest treatments on oranges against blue and green mould. However, more studies are required to confirm this statement.

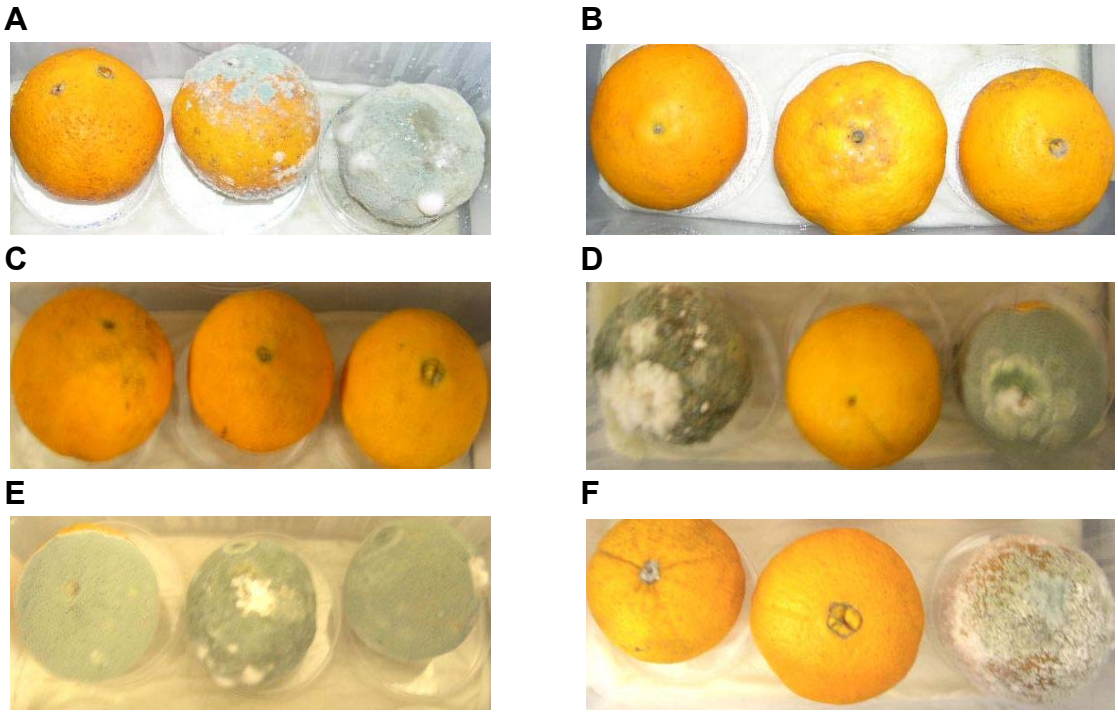


Figure 89. Comparison of oranges that were exposed to 0.05%, 0.25%, 0.5% and 2% lemon oil with fungicide-treated oranges and water-treated oranges. The oranges were stored at room temperature for 30 days. Oranges were treated with **A)** 0.05% lemon oil **B)** 0.25% lemon oil **C)** 0.5% lemon oil **D)** 2% lemon oil **E)** Water-treated fruit and **F)** Fungicide-treated fruit.

Fruit treated with all concentrations of lemon oil and unwounded were still in a very good condition after 30 days storage at 4ºC (Fig. 90).

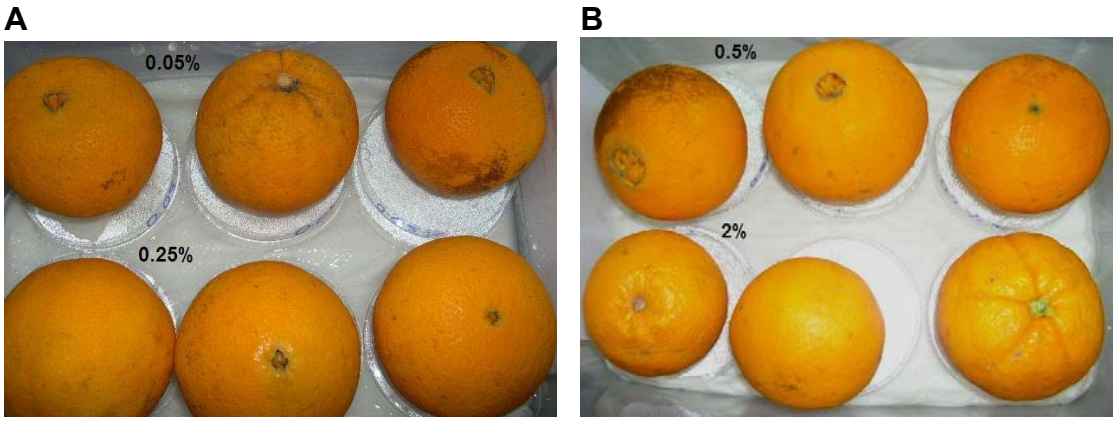


Figure 90. Unwounded oranges that were treated with various concentrations of lemon oil and incubated for 30 days at 4ºC. **A)** Oranges treated with 0.05% and 0.25% lemon oil **B)** Oranges treated with 0.5% and 2% lemon oil.

It appeared that 4ºC slowed down fungal infection for untreated, all concentrations of lemon oil and fungicide treated oranges. Any concentrations of lemon oil would give complete protection from fungal infection when combined with 4ºC storage.

B. Wounded fruit

Similar to all the other oils, oranges that were treated with 0.05%, 0.25%, 0.5% and 2% of lemon oil, wounded and inoculated with *Penicillium* spore suspensions were rotten after ten days of storage at room temperature (Fig. 91 A and B). Further, oranges that were exposed to water and fungicide were also rotten after ten days of storage at room temperature (Fig. 91 C and D).

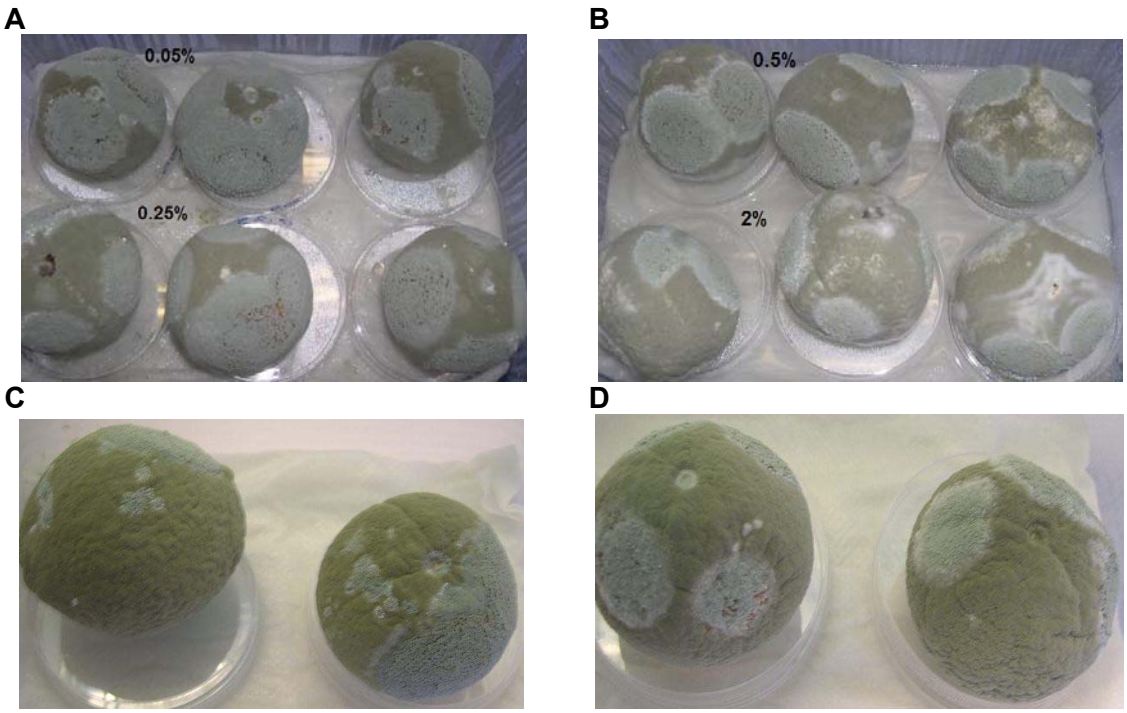


Figure 91. Wounded oranges after ten days of storage at 4ºC, showing a complete fruit rot caused by *Penicillium spp.* **A)** 0.05% and 0.25% lemon oil-treated oranges **B)** 0.5% and 2% lemon oil-treated oranges. **C)** Water-treated oranges **D)** Fungicide-treated oranges.

Similar to figure 90, wounded fruit stored at 4ºC were still in good condition with no fungal infection observed.

In summary, lemon oil appeared to have quite a strong antifungal activity, since low concentrations inhibited fungal growth on both *in vitro* and *in vivo* (microtiter and fruit dipping) assay. In the *in vitro* (microtiter) assay, high concentrations of lemon oil appeared to be less effective at inhibiting fungal growth compared to lower concentrations. Similar observations were seen in the *in vivo* assay, since 2% lemon oil appeared to be less effective at inhibiting fungal growth compared to 0.25% and 0.5% lemon oil at room temperature.

Lemon oil at 0.25% and 0.5% appeared to be the best treatments, since they managed to completely protect the oranges from blue and green mould for up to 30 days at both room temperature and 4ºC storage on unwounded fruit. However, similar to other oils, these two concentrations were ineffective on wounded fruit, since they failed to protect the wounded fruit from fungal infection at room temperature storage.

In summary, 0.05% lemongrass oil, 0.25% and 0.5% lemon oil appeared to provide the best inhibition compared to the other concentrations of the four essential oils (Table 8). No fungal infection, including soft lesions appeared on any of the fruit after 30 days of storage at room temperature. However, the untreated fruit were completely rotten after 30 days of storage at room temperature (Table 9). When the fruit were wounded, they completely rotted in 10 days, regardless of their treatment.

Storage at 4ºC slowed down fungal infection on all of the fruit (wounded and unwounded). None of the oil-treated fruit, fungicide-treated fruit and untreated fruit showed any sign of soft lesions or fungal infections after one month of incubation (Fig. 92). Therefore it appeared that 4ºC storage itself can inhibit fungal growth without any application of essential oils.

Table 8. Comparison of all essential oils tested on unwounded oranges after 30 days storage at room temperature.

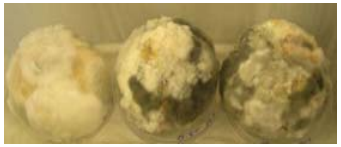
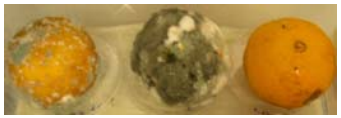
Type of Oils	0.05%	0.25%	0.5%	0.75%	2%
Manuka	N/A	N/A		N/A	
Lemongrass		N/A			
Lemon				N/A	
Orange	N/A	N/A		N/A	

Table 9. Comparison of all control treatments on unwounded oranges after 30 days storage at room temperature.

Negative control (untreated fruit)			
Positive control (Fungicide treated)			

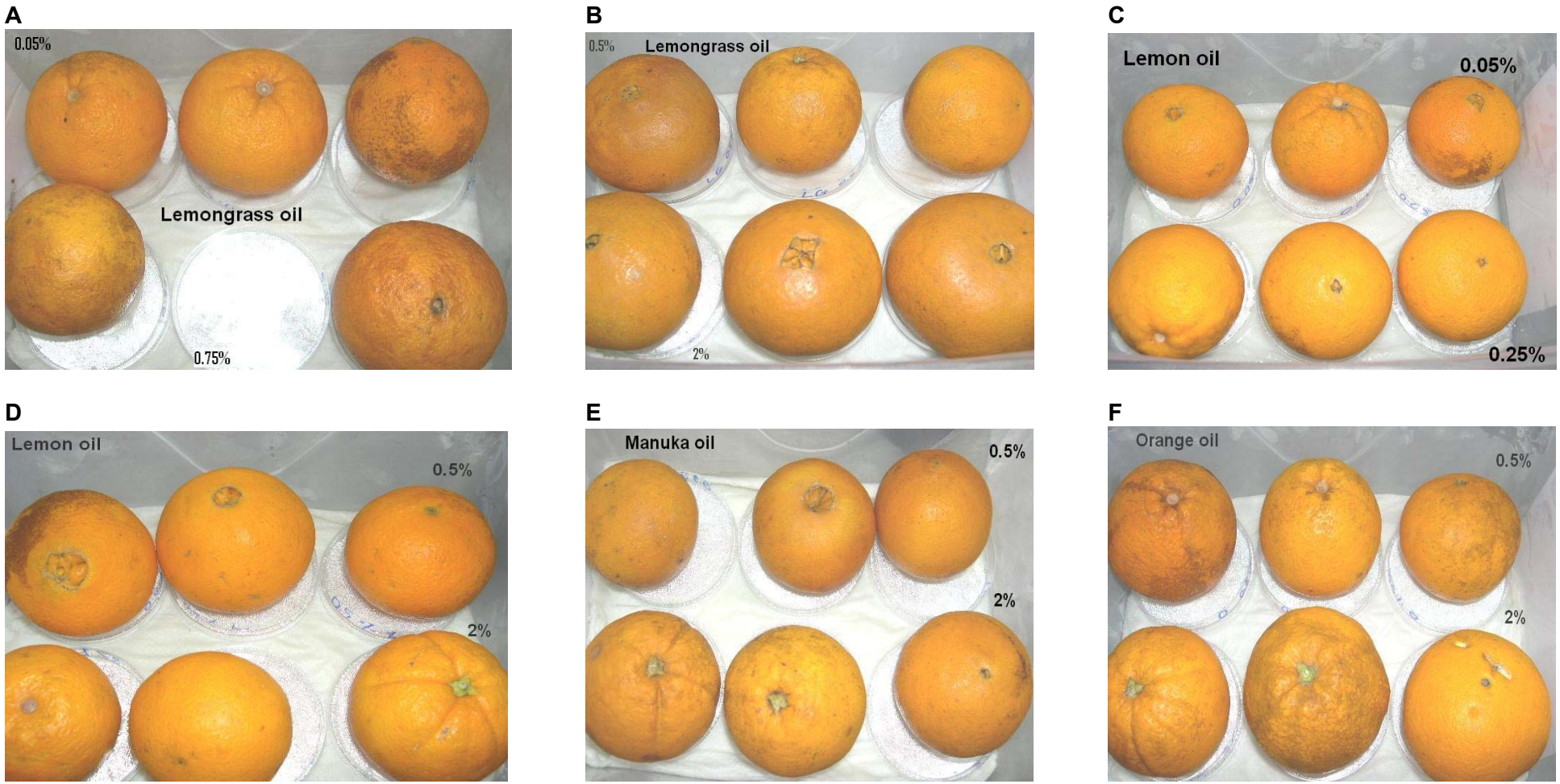


Figure 92. Unwounded oranges that were exposed to varying concentrations of essential oils and stored for 30 days at 4ºC. **A and B** represents oranges that were exposed to lemongrass oil. **C and D** represents oranges that were exposed to lemon oil. **E** represents the oranges that were exposed to manuka oil. **F** represents oranges that were exposed to orange oil.

Discussion

This study was carried out to identify effective, safe and economical treatments to reduce the number of postharvest fruit losses in New Zealand, especially on stone fruit (smooth skin/ soft fruit) and citrus fruit (thick skin fruit). Ethanol (GRAS chemical) and the natural products: milk, honey and essential oils were investigated as postharvest treatment candidates to replace the currently used hazardous chemicals and fungicides.

4.1. Fruit infection

Peach fruit were more susceptible to fungal infection than oranges since untreated peaches started to show fungal infection after two days at room temperature. In contrast, untreated oranges only showed a slight fungal growth after 30 days at room temperature storage. It appeared that soft fruit (peaches) and thick skinned fruit (oranges) had different susceptibilities to postharvest infection. The fact that oranges became infected slowly shows that the orange skin is relatively protective against fungal infection. This could be due to essential oil that is known to be present in orange peel which is believed to have an antimicrobial activity (Rodov *et al.*, 1995; Subba *et al.*, 1967; Fisher and Phillips, 2006). Also, the soft skinned nature of peaches gives no protection against injury during transport or storage, therefore making them more vulnerable to infection. The fungal infection observed in this study arose largely on the damage areas of the peaches. Similar to peaches, injured oranges were more vulnerable to fungal spores than uninjured fruit, since wounded untreated oranges were completely covered with both blue and green mould after ten days at room temperature storage. Taverner *et al.* (2006) reported that the occurrence of blue and green mould on oranges is more likely to occur on late season fruit with damaged rind, since the infections develop on the damaged areas.

4.2. Comparison of assays

The assessments for each of the treatments were done using either an *in vitro* (microtiter) assay or an *in vivo* assay or both. There were limitations with the *in* vitro assay, such as limited amount of information regarding the activity of the varying ethanol concentrations provided. For example, the microtiter assay could not indicate the effect of the ethanol concentrations on the fruit quality. A further limitation is the assay's reproducibility. The sampling was done manually, therefore experimental errors may have occurred in delivery of inoculum. Due to these limitations an, *in vivo* assay was also carried out in this study.

Pilot in vivo experiments were carried out in this study to determine if any of the effective treatments from the microtiter assays may also be effective on fruit. Any candidate treatments would need to be tested on fruit on a much larger scale than was possible in this study and be tested under commercial postharvest treatment conditions. One of the limitations of the pilot in vivo study was that the exact initial health status of each fruit was unknown making analysis difficult.

4.3. Ethanol treatment

4.3.1. Antifungal effect

The main application of ethanol is its use as a disinfectant (Wikipedia, 2006b). The observed antifungal activity of the different concentrations of ethanol in the experiment was due to ethanol's ability to kill microorganisms. It is stated in Wikipedia (2006b) that generally, ethanol kills organisms (including fungi) by denaturing their protein and dissolving their lipids. Studies that have been done on the application of ethanol were focussed only on table grapes (Margosan *et al.*, 1997; Lichter *et al.*, 2002; Karabulut *et al.*, 2004; Smilanick *et al.*, 1995).

In this study, the assessment of ethanol as a potential postharvest treatment was done using an *in vitro* microtiter assay and an *in vivo* assay on peach and citrus fruit. Both *in vitro* and *in vivo* methods showed that increasing ethanol concentration could inhibit fungal growth. Variation in this effect was observed. The degree of inhibition depended on the ethanol concentration used: as the concentration increased, so did the antifungal effect. This finding corroborates a spore mortality experiment done by Karabulut *et al.* (2004). They mixed *Botrytis cinerea* spores with various ethanol concentrations at room temperature and the percentages of germinated spores were counted. They discovered that exposure to 30% or more ethanol completely inhibited spore germination (0% germination), whereas spores that were similarly exposed to 10% or 20% ethanol showed 93 and 78% germination respectively.
In addition, Litcher *et al.* (2002) stated that ethanol was shown to cause a retardation of subsequent mycelial development of ungerminated *B. cinerea* spores (Litcher *et al.*, 2002).

In this study, high ethanol concentrations tended to completely inhibit fungal growth during the initial stages of the microtiter assay after which the fungi started to recover from the inhibition effect caused by the ethanol. It was assumed as the ethanol rapidly killed and reduced the fungal growth, the remaining fungi adapted to the ethanol, hence they recovered and grew. This adaptation could have been due to the induction of alcohol dehydrogenase by the high concentration of ethanol. Flipphi *et al.* (2000) showed that the expression of the structural genes for alcohol dehydrogenase enables the fungus *Aspergillus nidulans* to grow on ethanol. In this study, the level of ethanol needed for induction of the alcohol dehydrogenase appeared to be high based on the microtiter assay of ethanol. The rapid recovery was seen in 10% ethanol. It is believed that when the recovery is rapid at high ethanol concentration, the alcohol dehydrogenase induction is also likely to be more rapid with a concomitant rapid decline in ethanol levels, allowing the fungus to grow.

In the *in vivo* assay, when the fruit were exposed to the high concentrations of ethanol vapours (70% and 100%), the fungal growth was completely inhibited for up to 30 days at both room temperature and 4ºC.

In addition, 50% ethanol vapour seemed to moderately inhibit *M. fructicola* growth on peaches, but completely inhibited *Penicillium spp* growth on oranges. In contrast, 20% ethanol only gave protection to the same degree as the fungicide. Thus, as the ethanol concentration increased, so did the antifungal effect. These findings are supported by the work of Karabulut *et al.* (2004). They found that at ambient temperature, 30% or higher ethanol concentrations resulted in good fungal reduction, while 20% ethanol was ineffective at preventing postharvest decay over a long period of storage time.

This study concluded that 70% and 100% are the best treatments to protect the fruit from fungal infection for 30 days at both room temperature and 4ºC storage. Ethanol at 20% and 50% would provide as much protection as the fungicide for the first 10 days at 4ºC storage, but not at room temperature storage. As 20% ethanol inhibited postharvest decay only during the initial stage of storage, it inhibited the postharvest decay to the same degree as the fungicide. Ethanol is considered to be a GRAS chemical. There are some potential health risks associated with fungicide. This study suggests that ethanol is a viable alternative to fungicide at preventing postharvest treatment on peaches.

4.3.2. Fruit quality

High concentrations of ethanol seemed to had an adverse reaction on the fruit quality of both peaches and oranges. The skin of the peaches that were exposed to 50%, 70% and 100% ethanol concentrations turned brown after two days of storage. A similar reaction was observed on orange peels that were exposed to 20%, 50% and 70% ethanol vapours, with the browning appearing after 14 days of storage. In contrast to peach fruit, 100% ethanol concentration only showed a slight browning of the orange peel.

The difference in time for the browning was likely due to the difference in the skin of the fruit. Peaches may have had some damage from handling due to their soft skin, making them more vulnerable to browning quickly. Alternatively, ethanol may have penetrated quickly into the peaches due to the thin skin.

In contrast to peaches, oranges took a longer time to brown and there was no browning found on the inside of the fruit. This was believed to be due to the thick skin of the oranges that may have delayed the penetration of the ethanol and hence delayed the browning effect. Also, citric acid can be found in abundance in oranges (Seely, 2000; Pinnavaia *et al.*, 2006), which is known as a control for enzymatic browning of fruits (Marshall *et al.*, 2000; Sapers and Miller, 1995). There may be a correlation between the citric acid and the browning: if the citric acid levels decreased as the oranges matured, it is possible that this may have resulted in an increased level of browning over time.

The browning observed following ethanol treatment may have been due to a phytotoxic effect. Chervin *et al.* (2005) commented that phytotoxicity was the likely cause of stem browning on table grapes. They provided no explanations as to why the skin of the fruit turned brown. Podd and Staden (2004) mentioned that if ethanol is present in high concentrations, it can lead to increased membrane permeability and damage to the lipid bilayers. In this study, high ethanol concentrations (50%, 70% and 100%) appeared to damage the cell wall of peaches and oranges causing liquid accumulation in the petri dishes used to store the fruit. This liquid was believed to be cell contents that leaked out due to the cell wall and membrane damage. When the liquid that had accumulated from each treated fruit was weighed, it was found that the higher the ethanol concentration used as treatment, the more liquid accumulated in the Petri dishes. The high concentration of ethanol not only affected the skin of the fruit; it seemed that the ethanol accumulated on the surface of the fruit and penetrated through the skin, causing browning on the inside of peaches and changing the inside colour of oranges.

In general, tissue browning occurs as a result of oxidation of phenolic compounds by polyphenol oxidase and degradation of red pigments (Kader, 2000). Fruit contain an enzyme called polyphenol oxidase that reacts with (both) oxygen and phenol compounds (Helmenstine, 2006). Fruit browning mostly occurs on injured fruit because the damaged cells allow the intracellular oxygen to react with polyphenol oxidase and other chemicals (Helmenstine, 2006).

The polyphenol oxidases would act as terminal oxidases catalysing the oxidation of phenolics involving the breakdown of anthocyanins, resulting in tissue browning (Potter and Hotchkiss, 1995). Possible reactions that led to fruit browning are summarised in a flowchart shown in Figure 93.

Marshall *et al.* (2000) cited that polyphenol oxidases are believed to play key physiological roles both in preventing insects and microorganisms from attacking plants. When phenoloxidase enzymes are produced in fruits or vegetables, they catalyse the production of quinones from their phenolic constituents. Once formed, these quinones undergo a polymerisation reaction, leading to the production of melanin, which provide both antibacterial and antifungal activity to keep the fruit or vegetable in good condition. Therefore, it is likely that high ethanol concentrations used in this study damaged the cell walls of the fruit, causing the enzymatic browning reaction to occur, leading to production of melanin which provided antifungal activity. Hence, no fungal growth was observed. In contrast, Whitehead and de Swardt (1982) thought that ethanol vapours might reduce polyphenol oxidase activity.

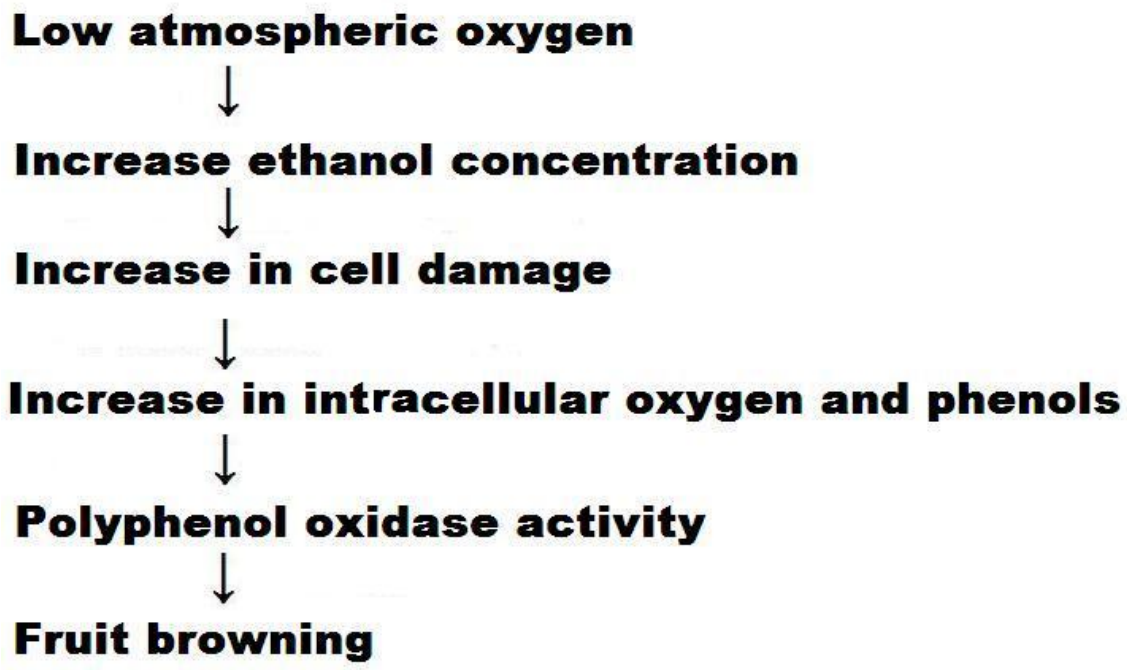


Figure 93. Flowchart to explain the possible explanation on the fruit browning reaction found in this study.

Day (1996) stated that high atmospheric oxygen levels may cause inhibition of polyphenol oxidase, therefore no oxidation of phenolic compounds and no browning can take place. Lu and Toivonen (2000) concluded that apples that had been kept under high pressure of oxygen had a slower rate of browning. Low oxygen can cause fruit injury with loss of flavour and purple/browning of the skin as indications (Ferree and Warrington, 2003); probably via the pathway shown in Fig. 93.

Fielder and North (1971) showed that as the oxygen concentration is lowered, the ethanol level increased. In addition, Norman (1997) stated ethanol accumulation in the fruit can cause severe injuries including browning.

20% ethanol vapour appeared to be effective on inhibiting fungal growth for ten days for peaches stored at 4ºC and 30 days for oranges stored at both room temperature and 4ºC. In addition, 20% ethanol vapour resulted in better fruit quality compared to 50%, 70% and 100% on peaches, since no browning observed. In contrast to peaches, the quality of oranges that were exposed to 20% ethanol vapour was only maintained in good condition for ten days.

This study was an initial study to determine if ethanol, as a GRAS chemical, has potential as a postharvest treatment to replace currently used fungicides. Before ethanol can be applied as postharvest treatment on fruit as an industrial application much more work needs to be done. Each of the ethanol concentrations need to be tested for antifungal effects, fruit quality, flavour and nutritional effects in larger scale experiments in the lab and in an orchard environment. Some other possible future studies that can be done to support this study are assessment of ethanol application together with heat treatments, head space, GC and microscopic analysis of tissue damage. The head space analysis (to measure the oxygen, ethanol and carbon dioxide levels during storage) and the GC analysis would give more detailed information on the fruit browning reaction that was found at exposure to high concentrations of ethanol vapour. The microscopic analysis would provide information about the effect of each ethanol concentration on the cell walls of the fruit.

The assessment of various concentrations of ethanol was also done by dipping. However, dipping did not provide as much protection against fungal growth as did vapour. This could be due to the time of exposure to the ethanol which also may have evaporated during the storage period. Also, as dipping only provides one point of exposure to the treatment, it may require a higher kill rate than constant exposure to vapour. Therefore, if the total kill was not obtained at the time of exposure, little to no protection against fungal growth would be observed.

4.4. Milk and honey treatment

Milk and honey treatment were done on peaches only. This study showed that neither milk nor honey had any inhibitory effect on fungal growth on peaches, therefore the experiments were not extended to oranges.

Milk is rich in nutrients and when applied to the fruit, may have provided a nutritious environment for fungi to grow. The fact that treatment with undiluted milk resulted in more fungal growth than diluted milk supports this.

There are some compounds found in milk that have antibacterial activity, such as lactoferrin, lysozyme and lactoperoxidase. However, they are more likely to be present in raw milk instead of processed milk; these compounds can be removed by heating milk at 50 to 55ºC (Barrett *et al.*, 1999). It is also believed that all the antibacterial activity that was previously found in milk is only effective in maintaining the quality of raw milk. No study on the antifungal activity of raw milk has been reported (Barrett *et al.*, 1999).

Manuka honey was also assessed for its antifungal activity and was found not to inhibit fungal growth. It has been reported that not all honey is effective as antimicrobial agents due to the variability between different batches of honey (Honey New Zealand, 2006).

It appeared that the fungal growth on honey treated peaches was different from that on the controls. The fruit became covered in fungus, however, there appeared to be an absence of brown rot caused by *M. fructicola* on the honey treated peaches. Honey was either inhibitory to brown rot but not other species or provided a suitable environment for this honey-specific fungus that was not provided on the control fruit. Generally, honey is inhibitory to many pathogens due to the low pH in honey that can prevent the growth of many organisms, however this acidity may be neutralised if the honey is diluted. The high sugar content of honey makes the water unavailable for microorganisms, but the more diluted honey becomes, the more species can grow in it (Honey New Zealand, 2006).

The variation in the quality of the honey is presumably because honey from some floral sources contains unidentified substances that increase the sensitivity of some antimicrobial compounds present in honey to breakdown by light (Dustman, 1979). The varying levels of hydrogen peroxide can cause variation in the antimicrobial activity in honey. This variation in hydrogen peroxide level in honey is associated with the catalase levels in the plant sources. Catalase is an enzyme in honey that breaks down hydrogen peroxide (McCarthy, 1995). Processing and handling of honey can also cause variation in the antimicrobial activity in honey. When honey is exposed to light and high temperature, the activity decreases (McCarthy, 1995).

Previous studies have reported that manuka honey also has Unique Manuka Factor (UMF) activity that is very effective at inhibiting the growth of a wide range of organisms (Waikato Honey Research Unit, 2005). The manuka honey used in this study was bought from a local supermarket and may have contained low UMF due to it having been processed. This processing may have reduced its antifungal activity.

Milk and honey were not assessed using the microtiter assay due to the timing of the project and the fruit season. The time required to develop the microtiter assay would have meant that I would not have been able to take advantage of the remainder of the peach season, therefore the milk and honey were tested directly on fruit.

Neither honey nor milk tested in this study was effective as an antifungal agent. Assessment of the antifungal activity of the separate components of unprocessed milk may be a possible future study. By analysing each of the compounds separately, it may eliminate the influence of the whole milk at providing an environment for fungal growth.

Manuka honey that was used in this experiment was a processed honey bought from a local supermarket, therefore this experiment could be repeated with a better quality of unprocessed honey. Unprocessed honey may be of a better quality with more UMF present compared to processed manuka honey.
Also, it might be useful to include different storage conditions such as reduced light effects, since UMF activity is very sensitive to light.

The fungal growth that appeared on the honey-treated peaches was different from brown rot. Honey might be effective against brown rot, since brown rot was not seen on honey treated peaches. To confirm the activity of manuka honey against *M. fructicola* as opposed to other fungi, a simple *in vitro* study could be done to test manuka honey against *M. fructicola* specifically.
Also, this other fungal growth could be identified and cultured to confirm if honey can stimulate its growth.

4.5. Essential oil treatment

In this study, the application of essential orange, manuka, lemon and lemongrass oil was assessed as antifungal compounds to lower the level of postharvest decay on oranges. Assessment of these essential oils was done using an *in vitro* (microtiter) assay and an *in vivo* (fruit) assay. Both methods showed that some concentrations of essential oils could affect fungal growth. Assessment of essential oils as a potential postharvest treatment was done on oranges only due to unavailability of peaches at the time of experimentation.

When the untreated samples of each of the essential oil microtiter plates were compared against fungicide, it was found that the fungal growth was not affected by increasing orange oil or manuka oil in nearby wells of the plate.

However, in contrast, both lemon and lemongrass oil showed fungal growth inhibition with increasing oil concentrations in nearby wells of the plate. This finding was an initial study that showed both lemon and lemongrass oil appeared to have antifungal activity. Inouyea *et al.* (2001) stated that essential oils are highly volatile at room temperature, therefore if the essential oils were effective at inhibiting the fungal spores, it is not surprising that affect was observed on the untreated samples placed nearby.

In the *in vitro* (microtiter) assay, orange and manuka oil appeared to be effective only at high concentrations. In contrast, lemongrass and lemon oil appeared to be effective even at low concentrations. In the *in vivo* assay, lemongrass was the most effective antifungal agent at the lowest concentration of 0.05%. However, it became ineffective at concentrations higher than this. Lemon oil was as effective as lemongrass oil when used at 0.25% to 0.5%. Neither orange oil nor manuka oil were very effective at the concentrations tested.

It appeared that orange, lemon, lemongrass and manuka oils have different capacities to inhibit fungal growth, which is believed to be due to the variation of compounds present in each of the oils. This statement is supported by Sokovic and van Griensven (2006). They indicated that different essential oils have different efficacy and the modes of action of essential oils are different between bacterial and fungal species. Also, there seemed to be a correlation between chemical structures of the essential oil constituents with the antimicrobial activity. Dorman and Deans (2000) also stated that the antimicrobial activity present on each in the volatile oils would be expected to be related to the composition of the oils, the structural configuration of the constituent components of the volatile oils and their functional groups.

Wolken *et al.* (2002) determined the order of toxicity to *Penicillium spp.* from compounds that are present in essential oils. The most toxic compounds were citral (neral/geranial), then geranic acid, methylheptenone and acetaldehyde in that order. Citral is an aldehyde comprised of neral and geranial. Rodov *et al.* (1995) did a study of antifungal compounds on citrus fruit. They discovered that young citrus fruit have lower levels of postharvest decay as compared to older fruit.

This was related to an antifungal compound found in the oil glands of the flavedo (the outer part of the peel), which was then identified as 'monoterpene aldehyde citral' (a mixture of neral and geranial). This compound was reported to be highly inhibitory against *P. digitatum*. During long-term storage of citrus fruit, the level of citral in the flavedo decreased in parallel with the decline of fruit resistance to postharvest decay. Previous studies reported that disease resistance usually declines, especially during the postharvest period (Brady, 1987; Ben-Yehoshua *et al.*, 1988 as cited in Rodov *et al.*, 1995) presumably due to declining citral levels.

Either neral and/or geranial, the components of citral, are present in all the oils tested except for manuka. Palhano *et al.* (2004) stated that citral is the major component of lemongrass oil, composing 70% of the oil. They also discovered that lemongrass significantly reduced the viability of *Colletotrichum gloeosporioides* spores. Lemon oil is also known to have high levels of citral whereas orange oil has only geranial (Ojeda de Rodriguez *et al.*, 2003; Ojeda de Rodriguez *et al.*, 1998; Paviani *et al.*, 2006; Clarke, 2002; Douglas *et al.*, 2004). It appeared that there was a correlation between the relative amounts of citral and the antifungal activity of each of the oils. In manuka oil, where citral is absent, there was no fungal inhibition observed in the *in vivo* assay. Lemongrass oil contains high level of citral and the antifungal activity was seen at a very low concentration in the *in vivo* assay. Lemon oil has less citral, therefore the antifungal activity was seen at higher concentrations compared to lemongrass oil.

Even though flavour was not tested, it is predicted that the market would favour lemon oil-treated oranges over lemongrass oil-treated fruit as it is derived from citrus. Other compounds that are present in the oils might have also contributed to the fungal inhibition of both lemon and lemongrass oil. Palhano *et al.* (2004) believed that compounds such as citronellal, terpineol and limonene might also be toxic to fungal spores. In general, lemon contains more citronellal compared to the other three oils (Ojeda de Rodriguez *et al.*, 2003; Ojeda de Rodriguez *et al.*, 1998; Paviani *et al.*, 2006; Clarke, 2002 and Douglas *et al.*, 2004).

This is believed to be one of the factors that contributes to the antifungal activity in lemon oil. This oil also contains the highest amount of α-pinene and β-pinene compared to the other three oils. These two compounds were found to have considerable antifungal activity with β-pinene showing more (Himejima *et al.,* 1992; Adegoke *et al,.* 2000 as cited in Dorman and Deans, 2000). Therefore, it is possible that the α-pinene and β-pinene in lemon oil may have contributed to lemon oil's ability in inhibiting fungal growth.

Sokovic and van Griensven (2006) observed that several monoterpenes were found to affect the structural and functional properties of the cell membrane of bacteria and yeast, therefore causing the cell contents to leak (Trombetta et al., 2005 as cited on Sokovic and van Griensven, 2006).

Some monoterpenes were also found to inhibit the respiratory enzyme, therefore also inhibiting the microbial oxygen uptake. In addition, monoterpenes are thought to induce alterations in the cell permeability by disrupting lipid packing and causing changes to membrane properties and functions (Parveen *et al.*, 2004; Hammer *et al.*, 2004). According to previous studies done by Ojeda de Rodriguez *et al.* (1998 and 2003); Paviani *et al.* (2006); Clarke (2002); and Douglas *et al.* (2004), lemon oil also appeared to have greater variation of monoterpenes, compared to the other three oils.

Dorman and Deans (2000) suggested that phenolic and non-phenolic alcohol had the strongest inhibitory effects, followed by aldehyde and ketones. Lemon oil contains more variation of the compounds that belong to the alcohol and aldehyde compared to the other three oils. This likely resulted in greater antifungal activity in this study. In addition, orange oil appeared to have more esters compared to the other three oils and manuka oil has ketones that were absent in the other three oils. Ketone is only present in the manuka oil, but manuka oil did not appear to be a strong antifungal agent in these assays, contradicting the statement made by Dorman and Deans (2000). Manuka oil was effective in the microtiter assay at 2% which is high compared to the others, but not effective on fruit at all. This oil may have had antifungal effect against *Penicillium* as indicated in the microtiter assay, but not against the fungus that eventually infected the fruit.

There are quite a lot of complex compounds that can contribute to the antifungal activity observed in lemon and lemongrass oil, making it difficult to determine the active component(s). It would be useful to test each component separately to determine which have the observed activity and to show if there are any synergistic effects between these components.

Manuka oil appeared to be ineffective in this study. This may have been due to different batches of oils potentially having different antifungal activity. A study by Douglas *et al.* (2004) discovered that East Cape manuka oil has an antimicrobial activity, especially against Gram negative bacteria. Therefore, assessing different sources of manuka oils, including East Cape manuka oil, for their antifungal activity would be of interest.
Also, it might be useful to test each ketone that is present in manuka oil in the future separately to determine if ketones have antifungal activity and if one is more effective than the others.

In summary, essential oils appeared to be effective at very low concentrations. The best treatments appeared to be lemon oil at 0.25% and 0.5% and lemongrass oil at 0.05%. Fruit that were exposed to these treatments were in good condition for up to 30 days at both room temperature and 4ºC. However, more work is required to assess this oil before they can be applied as postharvest treatments on fruit as an industrial application. The concentrations of lemon and lemongrass oil that appeared to be effective in this study need to be tested for antifungal effects, fruit quality, flavour and nutritional effects in a larger scale experiment in the lab or in an orchard environment. In addition, orange oil and manuka oil appeared to be ineffective at both 0.5% and 2% concentration in this *in* vivo study. They both might have a strong antifungal activity as lemon and lemongrass oil, but they were not tested at low concentrations in this study. Hence, it would be useful to test orange and manuka oil at low concentrations.

High concentrations of essential oils, especially in lemongrass and lemon oil may have been toxic to the fruit and damaged the cell membrane of the fruit. This would have made it easier for the fruit to be attacked by the fungal pathogens. Hence, these oils were not inhibitory at these levels.

It is known that essential oils are not toxic at low concentration (Cali'ouzos, 1996). The oils studied are also low in cost, therefore would be economically useful. However, it was found that some essential oils are only effective against a limited number of diseases and phytotoxicity can occur on sensitive plant species (Cali'ouzos, 1996). It appeared that high concentrations of oils were not effective in this study. There is evidence from previous literature that some essential oils are toxic to human skin (Hayes and Markovich, 2002; Cox *et al.*, 2000; Hart *et al.*, 2000), therefore it is possible that these oils can be toxic to the fruit.

There appeared to be other fungal growth in the presence of both manuka and lemongrass oil at moderate to high concentrations. The oil at these concentrations may be toxic to the fruit and lead to skin abrasion and injury, which then allowed fungal infection to take place after a certain period of time.

The pink pigmentation and leathery appearance that were observed after seven days of room temperature storage on oranges that were treated with high concentrations of lemongrass oil could possibly be another disease that can take place on oranges. Anthracnose is a disease of oranges that is caused by the fungus *Colletotrichium gloeosporioides*. The symptoms are described as a superficial leathery look and appearance of pink spores under humid conditions (Taverner *et al.*, 2006). This is similar to what was observed on the lemongrass-treated oranges. In addition, oranges that were treated with high concentrations of manuka oil and stored at room temperature showed a reddish brown staining on some areas of the orange peel that was observed after seven days. This could possibly be another disease, such as brush burn, that is caused by damage rind by abrasion. The symptoms are described as superficial red or brown staining of the rind, reddish or brown marks associated with raised surfaces on the rind and scuffing marks can be seen on close examination (Taverner *et al.*, 2006).

Some other possible future studies that can be done to support this study are gas chromatography (GC) analysis of the oils used in this study. The differences in the composition of each of the oils discussed in this study (section 1.6.4.2. to 1.6.4.5. or Table 1, Page 39-40) were taken from other studies and the compositions of the oil used here may not be exactly the same as different batches may slightly differ. Therefore, GC analysis needs to be done on the same oil brands used in this study to confirm the composition on each of the oils. Also, it would be useful to test each component of the oil separately and together for antifungal activity.

The essential oil treatments were compared as wounded and unwounded oranges in the in vivo assay. The oranges were wounded by piercing a needle through on different places following treatments. However, if the needle pierced into the oil gland of the oranges, it could have released the essential oil that was naturally present in the oil gland of the oranges, potentially inhibiting fungal growth. If essential oil was released in one orange but not the others, an entirely different pathological scenario may have resulted. Due to different anatomy of each fruit, it is hard to predict if the needle pierced into an oil gland of the oranges or not and therefore this presented a limitation of this assay. A larger scale experiment would negate such effects.

The greatest impact on fungal infection with no impact on fruit quality occurred when peaches and oranges were stored at 4^{o}C with no treatment. This brings into question the value of using fungicide at all as a postharvest treatment on these fruit. No treatment appeared to be any more effective than fungicide at combating fungal infection when peaches were stored at room temperature. In contrast, 0.05% lemongrass oil, 0.25% and 0.5% lemon oil appeared to be the most promising treatment of oranges. When oranges were exposed to these treatments, they were protected from fungal infection for up to 30 days at room temperature. These treatments were more effective than fungicide at this temperature and warrant further investigation.

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