MANAGEMENT OF POSTHARVEST DISEASES OF APPLES USING ESSENTIAL OILS

by

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SUMMARY

Grey mould, blue mould and bull's eye rot, caused by *Botrytis cinerea*, *Penicillium expansum* and *Neofabraea alba*, respectively, are postharvest diseases which cause significant losses to the apple industry in South Africa and other parts of the world. Current control methods of the diseases raises some concerns, such as development of fungicide resistance, residues harmful to humans, and increasing public health restrictions on the use of certain fungicides. These public concerns over synthetic pesticides in foods and the environment has spurred interest among researchers and the global public to find effective and safe non-fungicide means of controlling postharvest pathogens. The aim of this study was therefore to evaluate the effect of lemongrass and citrus essential oils in combination with cold storage regimes to control *B. cinerea*, *P. expansum* and *N. alba,* both *in vitro* and *in vivo*.

Volatile composition of essential oils of lemon (*Citrus limon*), lime (*Citrus aurantifolia*) and lemongrass (*Cymbopogan citratus*) were analysed using gas chromatography-mass spectrometry (GC-MS). A total of 44 volatile compounds were detected and identified in *C. limon*, with limonene (58.52%) and γ-terpinene (19.80%) as the major compounds; *C. aurantifolia* showed 20 components with limonene (79.00%) and γ-terpinene (11.22%) as the abundant compounds; while *C. citratus* revealed 26 components with geranial (48.14%) and neral (38.32%) as the major compounds.

Direct contact and vapour phase methods were used to test the *in vitro* antifungal activity of citrus and lemongrass oils against *B. cinerea*, *P. expansum and N. alba*. Direct contact phase of lemongrass oil, mixtures of lemon + lemongrass and lime + lemongrass oils exhibited the strongest toxicity and completely inhibited the mycelial growth and spore germination of all three tested pathogens, at concentrations of 1.50-100.00% at 20°C (95% relative humidity), regular atmosphere (-0.5°C, air), and controlled atmosphere (5%O_2 + 10% CO_2, -0.5°C). Lemongrass oil, mixtures of lemon + lemongrass and lime + lemongrass oils were fungicidal against the three postharvest pathogens both in direct contact and vapour phase of 1.50% and 0.13%, respectively, at 20°C, regular atmosphere and controlled atmosphere. Lemon and lime oils also showed antifungal activity, but were much less effective than lemongrass essential oil. In the vapour phase method, all essential oil treatments significantly ($p < 0.05$) controlled all three pathogens at concentrations of 0.02-1.00% compared to control treatments. Mycelial growth of fungal pathogens was inhibited by the tested essential oils in a dose-dependent manner.

The mechanisms of antifungal action of lemongrass oil against *B. cinerea* was investigated. Light microscopy and scanning electron microscopy observations indicated that *B. cinerea* hyphae exposed to lemongrass oil undergo morphological damage such as vesiculation, cytoplasmic disruption and collapsed hyphae. Glucose-induced reduction in

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external pH of *B. cinerea* was inhibited by lemongrass oil in a time and concentration dependent manner. *Botrytis cinerea* spores treated with lemongrass oil showed strong propidium iodide fluorescence in the cytosol. Lemongrass oil significantly altered the plasma membrane, the release of cell constituents, and the total lipid content of *B. cinerea*. These observations indicate that the antifungal activity of lemongrass oil can be attributed to the disruption of the cell membrane integrity and membrane permeability.

Application of lemongrass and citrus essential oils through fumigation, thermal fogging and dipping were tested *in vivo* for their potential to inhibit postharvest decay caused by *B. cinerea*, *P. expansum* and *N. alba* on 'Granny Smith', 'Golden Delicious' and 'Pink Lady' apples. Treated fruit were stored at controlled atmosphere ('Granny Smith' and'Pink Lady': 1.5% O_2 + 1% CO_2,-0.5°C and 'Golden Delicious': 1.5% O_2 + 2.5% CO_2,-0.5°C) for 28 days followed by 7 days at 20°C. After storage, lesion diameter was measured and expressed as percentage inhibition relative to control treatment. Application of citrus and lemongrass essential oils through fumigation, dipping and thermal fogging showed some potential to inhibit *B. cinerea*, *P. expansum* and *N. alba in vivo*. Postharvest control of *B. cinerea*, *P. expansum* and *N. alba* by essential oils through dipping and thermal fogging in combination with controlled atmosphere indicated that a mixture of lime + lemongrass oil was the most effective, followed by a mixture lemon + lemongrass oil, lemongrass oil, lime oil and lemon oil.

The effects of citrus and lemongrass essential oil treatments on quality parameters of apples was evaluated after controlled atmosphere storage. Citrus and lemongrass essential oils tested had no significant effect on fruit firmness of 'Granny Smith' and 'Pink Lady' apples. Significant differences were observed in total soluble solids, titratible acidity, pH and total soluble solids: titratible acidity ratio of both cultivars treated with essential oils compared to control treatments. Citrus and lemongrass essential oil exerted a positive influence in postharvest and quality of 'Granny Smith' and 'Pink Lady' apples.

The results presented in this thesis highlight the potential of essential oils in combination with cold storage regimes as an alternative control strategy against grey mould, blue mould and bull's eye rot in the apple industry. This suggest the application of essential oils by incorporating the oil into wax or coating treatments that are being used in the packing-line and the possibility of fumigation and thermal fogging during the storage period.

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OPSOMMING

Grys-, blou- en teikenvrot word veroorsaak deur die patogene *Botrytis cinerea* , *Penicillium expansum* en *Neofabraea alba*, onderskeidelik, en is na-oes siektes wat beduidende verliese in die appel-bedryf van Suid-Afrika en ander wêrelddele veroorsaak. Huidige beheer metodes van die siektes bring 'n paar bekommernisse; soos weerstand ontwikkeling teen swamdoders, residue nadelig vir die mens en toenemende beperkings as gevolg van openbare gesondheidbewustheid op die gebruik van sekere swamdoders. Die openbare bekommernis oor sintetiese plaagdoders in voedsel en die effek van dit op die omgewing het navorsers aangespoor om meer doeltreffende en veiliger alternatiewe middels vir na-oes pathogene te ontwikkel. Dus was die doel van hierdie studie om die *in vitro* en *in vivo* effek van sitroengras en sitrus essensiële olies tesame met koelkamer sisteme op *B. cinerea, P. expansum* en *N. alba* te ondersoek.

Die vlugtige samestelling van essensiële olies van suurlemoen (*Citrus limon*), lemmetjie (*Citrus aurantifolia*) en sitroengras (*Cymbopogan citratus*) is ontleed met behulp gaschromatografie-massaspektrometrie (GC-MS). Altesaam is 44 vlugtige verbindings opgespoor en geïdentifiseer in *C. limon*, met limoneen (58,52 %) en γ-terpineen (19,80 %) as die belangrikste verbindings. *Citrus aurantifolia* het 20 komponente bevat waarvan limoneen (79,00 %) en γ-terpineen (11,22 %) die oorvloedigste verbindings was. Van *C. citratus* se 26 komponente was geraniaal (48,14 %) en neraal (38,32 %) as die belangrikste verbindings beskou.

Direkte kontak en dampfase metodes is gebruik om die *in vitro* antifungale aktiwiteit van sitrus en sitroengras olies teen *B. cinerea, P. expansum* en *N. alba* toets. Die direkte kontak van sitroengras olie, mengsels van suurlemoen+ sitroengras en lemmetjie + sitroengras olie was die mees toksiese olies en het miseliumgroei en spoor-ontkieming vir al drie pathogene geïnhibeer. Inhibisie was verkry by konsentrasies van 1,50-100,00% teen 20°C (95% relatiewe humiditeit), normale atmosfeer (-0,5 °C, lug), en gereguleerde atmosfeer (5% O2 + 10% CO2, -0,5 °C). Sitroengras olie, mengsels van suurlemoen + sitroengras en lemmetjie + sitroengras olies het swamdodende aktiwiteit getoon vir al drie na-oes patogene in direkte kontak en damp fase van 1,50% en 0,13%, onderskeidelik, by 20°C in normale asook gereguleerde atmosfeer. Suurlemoen en lemmetjie olies het ook antifungale aktiwiteit getoon, maar was minder doeltreffend as sitroengras essensiële olie.

Met die dampfase metode het al die essensiële olie behandelings aansienlike beheer ($P < 0.05$) getoon vir al drie patogene by konsentrasies van 0,02-1,00% in vergelyking met die kontrole behandeling. Die swamme se miseliumgroei was geïnhibeer deur die essensiële olies in 'n dosis-afhanklike wyse te toets.

Die meganisme van sitroengras olie se antifungale werking teenoor *B. cinerea* was ondersoek met behulp van ligmikroskopie en skandering elektronmikroskopie. Die hifes van *B.cinerea* was blootgestel aan sitroengras olie en morfologiese skade soos vesikulasie, sitoplasmiese ontwrigting en ineenstorting is waargeneem. Sitroengras olie het glukose-geïnduseerde vermindering in die eksterne pH van *B. cinerea* geïnhibeer op 'n tyd- en konsentrasie-afhanklike wyse. *Botrytis cinerea* spore wat behandel is met sitroengras olie het sterk propidium jodied fluoressensie in die sitosol getoon. Sitroengras olie veroorsaak aansienlike veranderinge in die plasma membraan, uitlating van sel inhoud en die totale lipied inhoud van *B. cinerea*. Hierdie waarnemings dui daarop dat die antifungale aktiwiteit van sitroengras olie toegeskryf kan word aan die ontwrigting van die selmembraan se integriteit en deurlaatbaarheid.

Die potensiaal van sitroengras en sitrus essensiële olies om na-oes bederf, wat veroorsaak word deur *B. cinerea, P. expansum* en *N. alba*, is *in vivo* getoets as beroking, termiese verneweling en doopmiddel op 'Granny Smith', "Golden Delicious" en " Pink Lady' appels. Behandelde vrugte is gestoor in gereguleerde atmosfeer. Vir 'Granny Smith' en 'Pink Lady': 1,5% O2 + 1% CO2, -0,5°C en vir 'Golden Delicious': 1,5% O2 + 2,5% CO2, -0,5°C vir 28 dae gevolg deur 7 dae by 20°C. Na opberging was letsel deursnee gemeet en weergee as persentasie inhibisie relatief tot die kontrole behandeling. Toepassing van sitrus en sitroengras essensiële olies in die vorm van beroking, doop en termiese verneweling beskik oor die potensiaal om *B. cinerea, P. expansum* en *N. alba* te inhibeer *in vivo*. Die behandeling van *B. cinerea, P. expansum* en *N. alba* met essensiële olies as 'n doop en termiese verneweling gekombineer met gereguleerde atmosfeer het getoon dat 'n mengsel van lemmetjie+ sitroengras olie die mees doeltreffende na-oes beheer gehad het. Dit is gevolg met 'n mengsel van suurlemoen + sitroengras olie, sitroengras olie, lemmetjie olie en suurlemoen olie.

Die effek van behandeling met sitrus en sitroengras essensiële olies op die kwaliteit van appels is geëvalueer na opberging in gereguleerde atmosfeer. Sitrus en sitroengras essensiële olies het geen wesenlike uitwerking op die fermheid van 'Granny Smith' en 'Pink Lady' appels gehad nie. Beduidende verskille is waargeneem in die totale oplosbare vastestowwe, titreerbare suur, pH en totale oplosbare vastestowwe. Die titreerbare suurverhouding van beide kultivars behandel met essensiële olies was vergelyk met die kontrole behandelings. Sitrus en sitroengras essensiële olie het 'n positiewe invloed op die na-oes beheer en gehalte van 'Granny Smith' en 'Pink Lady' appels.

Die resultate wat verkry is in hieride tesis beklemtoon die potensiaal van essensiële olies in kombinasie met koelkamer stelsel as 'n alternatiewe beheerstrategie teen grys vorm Grys-, blou- en teikenvrot in die appelbedryf. essensiële olies kan dus moontlik

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geinkorporeer word in was- en filmlaag behandelings van paklyne, beroking en en termiese verneweling tydens die opbergingsfase.

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CHAPTER 1

CURRENT MANAGEMENT STRATEGIES FOR POSTHARVEST DECAY ON APPLES AND PROSPECTS FOR ESSENTIAL OIL TREATMENTS – A REVIEW

INTRODUCTION

The Food and Agriculture Organization (FAO) of the United Nations has estimated that by 2050 the world population will reach 9.1 billion. Nearly all of this population increase will occur in developing countries, and about 70% of the global population will be urban. To feed this larger, more urban, and richer population, food production must increase by 70% and it will need to be moved from the areas where it is produced to the areas where it is consumed (FAO, 2009).

Postharvest decay of fruits is a major challenge that affects food availability throughout the world. Postharvest diseases caused by pathogenic fungi and bacteria result in great losses to the fruit industry, and in particular the deciduous fruit industry (Sholberg and Conway, 2004; El-Ghaouth *et al*., 2004; Droby, 2006; Zhu, 2006; Singh and Sharma, 2007; Sharma *et al*, 2009). This is a loss incurred at the end of all the farming activities, from land preparation to maturity management, harvesting, packing, transport and storage management. Thus, there is a great demand for effective measures to reduce postharvest losses and improve fruit quality. The FAO estimated that with respect to the total amounts of fruit and vegetables produced globally, 15-50% is lost at the postharvest stage, before even reaching the tables of the consumers (FAO, 2011). The highest losses were recorded in the developing countries of Africa and Asia, which lack the necessary technologies to prolong the storage life of fresh produce (FAO, 2011).

Fruit are highly perishable, and the causes of postharvest losses can generally be ascribed to physiological deterioration, pathological breakdown, pathophysiological injury and physical injury (Feliziani, 2012). In many instances, these causes are interrelated; i.e., mechanical injury can be associated with postharvest decay from many causes. Fruit are very prone to microbial spoilage because of their succulent nature. Frequently, infection by microorganisms that cause postharvest decay can occur before harvest at the field stage, and can remain latent until storage where the environmental conditions are favorable for disease development. The rate of postharvest deterioration also depends on several external factors including storage temperature, relative humidity, air speed, atmospheric composition (concentrations of oxygen, carbon dioxide, and ethylene), and sanitation procedures (Kader, 2005). Postharvest pathogens of fruit can develop very rapidly from

rotted fruit next to the healthy fruit, causing extensive breakdown of the commodity, and sometimes spoiling entire lots. Moreover, aside from direct economic considerations, diseased produce poses a potential health risk, as some fungal genera are known to produce mycotoxins under certain conditions, such as *Penicillium* spp., *Aspergillus* spp. and *Alternaria* spp.

The correct choice of fruit maturity at harvest, and careful handling and use of technologies that delay fruit ripening during storage are fundamental for decay control (Mari *et al.,* 2009). At the same time, the application of synthetic fungicides remains the most common method to control postharvest rot of fruits. In South Africa, fungicides such as Iprodione (Rovral^{TM}), Fungazil (Imazalil^{TM}), Mertect (Thiabendazole^{TM}) and Scholar (Fludioxinol^{TM}) are some of the primary means of controlling postharvest diseases of most fruits (Eckert and Ogawa, 1985; Kupferman, 1998). However, continuous use of fungicides has faced major challenges, including public growing concerns for human health and environmental pollution associated with pesticide usage in orchards, persistence of residues on treated fruits (Legard *et al.,* 2001, 2005; Rabolle *et al.,* 2006), development of resistance of fungal pathogens to fungicides and high development costs of new chemicals (Spotts and Cervantes, 1986; Eckert, 1990; Eckert *et al.*, 1994; Yourman and Jeffers, 1999; Dianez *et al.,* 2002). Therefore, these challenges have motivated the search for alternative control methods (Wilson and Wisniewski, 1994; Kovach *et al.*, 2000). Essential oils have been proven to be inhibitory against a wide range of postharvest diseases of fruits (Paster *et al.,* 1995; Inouye *et al.,* 1998; Hammer *et al.,* 1999) and food spoiling microorganisms (Tajkarimi *et al,* 2010). Application of essential oils have become more important since they are perceived as being environmentally safer and more acceptable to the general public (Janisiewicz and Korsten, 2002).

This review will include an overview of three postharvest diseases caused by *Botrytis cinerea* Pers.: Fr., *Penicillium expansum* Link. (Thom) and *Neofabraea alba* (E.J. Gutrie) on apples, an economic important crop in South Africa and globally. The disease cycle and management strategies to control *B. cinerea, P. expansum* and *N. alba* found on apple fruit is discussed. Furthermore, an overview is provided on the use of essential oils and cold storage regimes to control postharvest diseases.

THE APPLE – ECONOMIC IMPORTANCE AND QUALITY ASPECTS

Apple (*Malu*s *domestica* Borkh.) is a member of Rosaceae family and considered one of the most economically important fruit tree crop (Jackson, 2003; Martinelli *et al.,* 2008). It is primarily grown for the fresh fruit market (Salunkhe and Desai, 1984). Apples are one of the

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most widely consumed fruits, due in part to their wide-range of beneficial effects on human health (Bokhari, 2002). A high intake of apples has been shown to reduce the risk of lung cancer, asthma, type-2 diabetes, thrombotic stroke, and ischemic heart disease (Hansen *et al.,* 2009; Chai *et al.,* 2011). These benefits are associated with the large content of structural cell walls and polysaccharides (Sun-Waterhouse *et al.,* 2008), as well as various phytochemical antioxidants (Devic *et al.,* 2010).

Fruit must have good quality and meet market specifications in order to meet export requirements. Factors that contribute to fruit quality include: a) pre-harvest and harvest factors (cultivar; soil conditions; climatic condition; pesticides application and harvesting practices; b) postharvest factors (fruit physiology, pathology and cold chain management practices). Apples are susceptible to a number of postharvest diseases that cause significant losses during the marketing of fresh fruit. Blue mould (*Penicillium expansum*), grey mould (*Botrytis cinerea*) and bull's eye rot (*Neofabraea alba*) are major diseases of apples (Sholberg and Conway, 2004). Postharvest spoilage affects the quality of economically important crops resulting in reduced shelf life and major economic loss to the fruit industry (Marek *et al.,* 2003).

Two principal factors namely, high water content and wounding, make fresh produce more susceptible to spoilage. Optimal conditions for pathogen invasion are created by high water content, while wounds which occur during harvesting and postharvest handling are an easy infection route (Opara and Pathare, 2014). Due to improved storage conditions, apples can be retained for up to 9 months, before released to the market, thereby enabling retailers to more effectively manage stock flow and price control (Jackson, 2003). In South Africa, these opportunities have resulted in major growth for the industry, particularly in terms of export potential, to the extent that up to approximately 80% of apples grown are exported (Groenewald, 2000). Long term storage has implications for losses due to infection by latent pathogens which cause calyx-end decay and bull's eye rots/ lenticel rot in apples.

Consumer satisfaction, which is the main objective of production, handling, storage and distribution of fresh fruits including apples, is mainly related to product quality (Hoehm *et al.*, 2005). A definition of fruit quality varies along the supply chain, depending on the intended end use (Kader, 2002). A lack of understanding of different perspectives may be the most limiting factor in maintaining the quality of fresh fruit as delivered to consumers (Hoehm *et al.*, 2005). To be an acceptable apple fruit, it must be free from mechanical damage, physiological and pathological disorders (Opara and Pathare, 2014). However, the importance of non-visual characteristics such as flavour, texture and nutritional value on the acceptance of fresh fruits in the market has increased in recent years (Opara, 2013a, b).

SCOPE OF THE APPLE INDUSTRY IN SOUTH AFRICA

Apples are an important export commodity for the South African market (Snowdon, 1990), with roughly half of the apples produced being exported (Mogala, 2012). Apple cultivars produced in South Africa for export include 'Big Chief', 'Braeburn', 'Fuji', 'Golden Delicious', 'Granny Smith', 'Pink Lady' or Cripp's Pink', 'Royal Gala', 'Sundowner' and 'Top Red' or 'Starking' (Jooste, 2000). The total pome fruit crop for 2012 season was 40 845 303 cartons, 3.00% (1.20 million cartons), and 44 224 936 cartons, 3.10% (1.30 million cartons) in 2013 season. The pome fruit industry is the front-runner in developing Africa (PPECB 2012-2013). In 2012-2013, this industry successfully exported 26.00% of its total apple export crop to Africa. The cultivars that contributed to the increased volume were two apple cultivars, 'Royal Gala' (5.00%) and 'Cripps Pink' (24.00%). The pear export crop increased by 2.20% compared to the previous season, due mainly to increase in the 'Forelle' (8.00%) and 'Williams Bon Chretien' (30.00%) varieties (PPECB 2012-2013). Despite the season's challenges, 40.80 million pome fruit cartons were exported to international markets. Apples were primarily exported to the United Kingdom (30.00%), the Far East (26.00%) and Africa (26.00%), while the European Union (47.00%) had the biggest demand for pears, followed by The Far East (20.00%) and The Middle East (16%) (PPECB 2012-2013). There is still a major need to improve fruit quality and solve issues affecting global trade of apples in order to meet current consumer demands (M Laing, pers. commun.). One of the major factors hindering trade in apples is their susceptibility to postharvest diseases during postharvest storage. This chapter reviews the most economically important postharvest diseases affecting apple supply chain worldwide (Fig. 1) and the management strategies.

MAJOR POSTHARVEST DISEASES OF APPLES

Grey mould

Causal organism

Grey mould is caused by *B. cinerea* (teleomorph *Botryotinia fuckeliana* (de Bary) Whetzel). *Botrytis cinerea* has a white cottony appearance, which turns light grey with age on potato dextrose agar (PDA). The young hyphae are thin, hyaline, and 8-16 µm wide, and become brown and septate with age. Conidiophores are light brown, septate with slightly enlarged tips bearing small pointed sterigmata bearing 1-2 celled, hyaline, oval conidia forming clusters (Coley-Smith *et al.,* 1980). Characteristics and sporulation of *B. cinerea* depend on, and vary with, nutrient medium, temperature, and ecological factors (Xiao, 2006). Isolates recovered from decayed apple and pear fruit vary greatly in sporulation on potato dextrose

agar (PDA). Some isolates sporulate abundantly, whereas others produce only sclerotia or sclerotia with scarce conidia. Mycelium of *B. cinerea* grows at temperatures as low as -2ºC and conidia can germinate at 0ºC (Xiao, 2006). Grey mould is a disease of mature fruit which is of minor importance in well managed orchards. *Botrytis* diseases are probably the most common and widely distributed diseases of vegetables, ornamentals, fruit, and even field crops throughout the world (Agrios, 1997). According to Rosslenbroich *et al.* (1998), the economic importance is higher when considering the fact that these cash crops is not only endangered in the field but also during transport and storage. *Botrytis* diseases commonly appear as blossom blights and fruit rots. Other diseases caused by this fungus are damping off, stem cankers and rots, leaf spots, and tuber, corm, bulb, and root rots (Gonsalves and Ferreira, 1994).

Symptoms

Grey mould originates from infection of wounds such as bruises and punctures that are created at harvest and during postharvest handling. Rotted fruit has a pleasant fermented odour (Coley-Smith *et al.,* 1980; Beattie *et al.,* 1989; Xiao, 2006). Stem-end grey mould is also common on pears and also occurs on apples. *Botrytis cinerea* also invades floral parts of fruit and causes calyx-end rot. The decayed area appears light brown to dark brown and colour is similar across the decayed area. The decayed area is spongy, and diseased tissue is not separable from the healthy tissue, which is different from blue mould. Under high relative humidity, grey spore masses and/or fluffy white to grey mycelia may appear on the decayed area (Xiao, 2006). Generally, grey mould does not have a distinct odour, but in advanced stages decayed apples may have a cedar-like smell. In advanced stages, the entire decayed fruit may appear baked and eventually may turn softer than in the early stage. Sclerotia may form on the lesion surface of an advanced decayed fruit. Fruit-to-fruit spread of grey mould also results in nesting of decayed fruit in storage containers (Jijakli and Lepoivre, 2004). The internal decayed flesh appears light brown to brown at the margin area (Coley-Smith *et al.,* 1980; Beattie *et al.,* 1989; Xiao, 2006).

Epidemiology

The major source of inoculum is in the orchard where the fungus lives on plant debris (Coley-Smith *et al.,* 1980; Xiao, 2006). Conidia of the fungus are dispersed mainly by air currents and water splash. Infection occurs mainly through wounds and skin injuries that are created at harvest and during the handling processes or at bloom/ blossom time when floral infection occur. The fungus develops faster at cool storage temperatures than blue mould (Coley-Smith *et al.,* 1980; Sommer, 1985; Beattie *et al.,* 1989). *Botrytis cinerea* is able to

grow at pear storage temperatures of -0.5 to 0ºC, move through the stem of the fruit to reach the fruit flesh after a period of time in cold storage, and then cause decay. The fungus can also invade stems of 'Golden Delicious' apples and cause stem-end rot (Xiao, 2006). Spores of *B. cinerea* are carried in orchard soil and produced on decaying plant material brought into storage in bulk bins and other containers. Additional inoculum is provided by fruit that decays during storage. Infection spreads to adjacent fruit in cartons or bins causing nests or pockets of infection (Sommer, 1985; Beattie *et al.,* 1989). Secondary infection of fruit through fruit-to-fruit contact during storage is commonly seen after a long period of storage and can cause significant losses. Mycelial growth and secondary infection through fruit-to-fruit spread is enhanced by high relative humidity (Sommer, 1985; Xiao, 2006). Fruit infected by grey mould due to secondary infection in storage bins may not have visible symptoms or lesions are very small at the time of packing, and thus infected fruit may be packed but symptoms develop on packed fruit during storage or transit (Sommer, 1985; Xiao, 2006).

Management

Orchard sanitation to remove decayed fruit and organic debris on the orchard floor helps reduce inoculum levels of *B. cinerea* in the orchard (Xiao, 2006). Good harvest management to minimize punctures and bruises on the fruit skin helps avoid decay from wound infections. Pre-harvest fungicides such as thiram and ziram applied near harvest provide some control of grey mould (Coley-Smith *et al.,* 1980; Xiao, 2006). The vast majority of *B. cinerea* isolates from apple-related sources are sensitive to thiabendazole (Xiao, 2006). A postharvest drench treatment with Mertect™ (thiabendazole) applied prior to storage is effective to control grey mould, particularly for those that originate from infected wounds. In 2004, two new postharvest fungicides, Penbotec™ (pyrimethanil) and Scholar™ (fludioxonil), were registered as postharvest treatments for control of postharvest diseases of pome fruits in the U.S. Both fungicides are labelled for use as either drench treatments or online sprays. It has been shown that both fungicides applied as pre-storage treatments are effective to control grey mould from wound infections (Coley-Smith *et al.,* 1980; Xiao, 2006).

Blue mould

Causal organism

Blue mould rot, caused by *Penicillium expansum,* is one of the most common and destructive rots of harvested apples and pears, but it can also be found on sweet cherries and other commodities such as apricots, grapes, blueberries, peaches, strawberries, walnuts, pecans and hazelnuts (Andersen *et al.*, 2004; Murphy *et al.*, 2006). Blue mould is a

worldwide severe disease, even in production areas where the most advanced storage technologies are available. Several other *Penicillium* species, including *P. chrysogenum, P. commune*, *P. regulosum*, *P. solitum* and *P. verrucosum*, have also been reported to cause decay on apples and pears (Rosenberger and Sugar, 1990). *Penicillium expansum* can grow at temperatures as low as -3ºC and conidia can germinate at 0ºC. According to Rosenberger and Sugar (1990), this disease is an economic concern not only to the fresh fruit industry, but also to the fruit processing industry because some strains of *P. expansum* produce the mycotoxin patulin, which can rise to unacceptable levels and thus affect the quality of apple juice and baby food products.

Symptoms

Blue mould is easily recognized by the mass of blue conidia produced on infected fruit. Blue mould originates from infection of wounds such as punctures and bruises on fruit (Rosenberger and Sugar, 1990; Sugar and Spotts, 1999). The fruit at first shows soft, pale-brown, watery spots with the decayed portions completely separable from healthy tissue (Beattie *et al.,* 1989). The decayed tissue is soft and watery and the lesion has a very sharp margin between diseased and healthy tissues (Rosenberger and Sugar, 1990; Sugar and Spotts, 1999). Spots enlarge rapidly and under favourable conditions may completely envelope the fruit. Under warm, moist conditions, the fungal growth is at first white, and then pale blue and finally blue-green powdery clumps of fungal spores develop on the surface of the lesions (Persley, 1993). The fruit eventually becomes a soft, watery mass with a characteristic musty smell. Spread from fruit to fruit infection results in nests of infection (Beattie *et al.*, 1989).

Epidemiology

Fruit, soil and bulk bins are the major sources of blue mould spores (Beattie *et al.*, 1989). Blue mould fungi infect damaged fruit on the orchard floor. Spores develop on the surfaces of these fruit, contaminate the soil and are blown by wind throughout the orchard to fruit on the trees (Beattie *et al.*, 1989). Conidia are also present in the air and on the surface of fruit. In the packinghouse facility, fungicide-drench solutions, flume water and dump-tank water are common sources of *Penicillium* spores for fruit infection during the handling and packing processes. Spores of *P. expansum* are also commonly present in the air and on the walls of storage rooms (Rosenberger and Sugar, 1990). During harvest, this contaminated soil is transported with the fruit in bulk bins to packaging sheds and loaded into fruit dump tanks, dips or recirculating drench solutions. Rejected fruit in the packing sheds produce masses of spores which are spread with dust onto the grading machinery surfaces (Beattie *et al.*,

1989). *Penicillium expansum* is essentially a wound pathogen. The fungus enters the fruit through a wound or an open calyx cavity. Blue mould develops slowly at cool storage temperatures and in cool, dry conditions, the spore masses usually do not appear. However, warm humid conditions favour blue mould development (Persley, 1993). Fruit dump tanks, dips and drenches provide ideal situations for infection. Fruit with wounds can be inoculated with spores of thiabendazole-resistant isolates of *P. expansum* during postharvest drenching with DPA and Mertect™ (thiabendazole). As a result, fruit rot can occur even in healthy fruit during storage and marketing (Beattie *et al.*, 1989; Xiao, 2006).

Management

Orchard sanitation to remove decayed fruit and organic debris on the orchard floor helps reduce inoculum levels of *Penicillium* spp. in the orchard. Good harvest and handling management to minimize punctures and bruises on the fruit helps prevent the fruit from infection at wounds by *P. expansum* and other *Penicillium* species (Rosenberger and Sugar, 1990; Sugar and Spotts, 1999). Thiabendazole™ is commonly used as either a pre-storage drench treatment or a line spray to control grey mould and blue mould (Rosenberger and Sugar, 1990; Sugar and Spotts, 1999; Xiao, 2006). Thiabendazole™ is effective to control grey mould but is not effective to control TBZ-resistant *Penicillium*. Two new postharvest fungicides, fludioxonil (Scholar™) and pyrimethanil (Penbotec™), can be used as drenches, dips or line sprays and have been reported to be effective to control blue mould originating from wound infections. Biocontrol agent BioSave 110 (*Pseudomonas syringae*) applied on the packing line helps control blue mould from infection of wounds (Rosenberger and Sugar, 1990; Sugar and Spotts, 1999). Sanitizing dump-tank and flume water is an essential practice to reduce infection of fruit by *Penicillium* spp. during the packing process. Fruit bins and storage rooms can harbour TBZ-resistant isolates. Bin and storage room sanitation may be beneficial in reducing TBZ-resistant populations in the packing facility (Rosenberger and Sugar, 1990; Sugar and Spotts, 1999).

Bull's eye rot

Causal organism

Bull's eye rot of pome fruit is caused by several fungi including *Neofabraea alba* (Guthrie) *Neofabraea (Pezicula) malicortis* (Jacks.) Nannf. And *Neofabraea (Pezicula) perennans* (Kienholz) (Gariepy, 2002; Henriquez *et al.,* 2004). Bull's eye rot occurs sporadically in South Africa on late maturing apple varieties (den Breeyen, 2012). In South Africa, den Breeyen (2012) harvested asymptomatic 'Cripps Pink' fruit from November 2010 to April

2011 and using genus-specific primers, it was determined whether *N. alba* could be detected on orchard fruit throughout the growing season. Inoculum was detected on orchard fruit as early as four months before harvest (den Breeyen, 2012). *Neofabraea alba* is one of the most frequent and damaging diseases occurring in stored apples in Italy and other European apple growing countries (Lockhart and Ross, 1961; Bompeix and Cholodowski-Faivre, 1998). In Washington State, bull's eye rot is more commonly seen on 'Golden Delicious', particularly on apples from orchards with perennial canker problems on trees. Bull's eye rot also occurs in Europe and some other fruit-growing regions (De Jong *et al.,* 2001; Henriquez *et al.,* 2004; Spotts, 1990).

Symptoms

Infection in the orchard can occur at any time during the growing season and incipient infections remain latent until symptom development after three to five months in storage (Snowdon, 1990; Neri *et al.*, 2009; den Breeyen, 2012). Fruit lesions are flat to slightly sunken, brown and often with a lighter brown centre (Snowdon, 1990; Spotts, 1990; Henriquez *et al.,* 2004). The rotten tissue are relatively firm and acervuli are frequently present in old lesions under humid conditions (Neri *et al.,* 2009). Cream-coloured spore masses in the aged decayed area may appear. Decayed tissue is firm and does not readily separate from healthy tissue (Spotts, 1990; Henriquez *et al.,* 2004). Bull's eye rot commonly originates from infected lenticels, but stem-end bull's eye rot is commonly seen on 'Golden Delicious' and 'Gala' apples, particularly on the fruit from orchards with overhead irrigation (Henriquez *et al.,* 2004). Calyx-end bull's eye rot has also been observed on 'Golden Delicious' fruit (Spotts, 1990; Henriquez *et al.,* 2004).

Epidemiology

The fungus is considered a weak parasite (Bompeix and Bondoux, 1974) to purely saprophytic (Bompeix, 1988; Edney, 1956). It survives as a saprophyte on dead bark, pruning snags and leaves of pome fruit (Burchill and Edney, 1961; Tan and Burchill, 1972; Bompeix and Bondoux, 1974). Saprophytic growth of *N. alba* on apple leaves has also been observed. The fungus grew epiphytically and colonised both leaf surfaces and sporulated only when growing on dead and damaged leaf tissues (Tan and Burchill, 1972).

Fruit is susceptible to infection throughout the growing season from petal fall to harvest, and its susceptibility increases as the growing season progresses (Edney, 1958; Spotts, 1985). The pathogen arrests its growth and remains quiescent until the fruit reaches a certain stage of ripeness, when it can invade fruit tissues. Although histological studies on the infection process of the genus *Neofabraea* are scanty and mainly regard *Neofabraea*

perennans, it is expected that latent infections of *N. alba* survive in lenticels as germinated conidia or hyphae. Fruit maturity results in a reduction of the mechanical resistance of the fruit, an increase in pH, which activates fungal pectolytic enzymes and a reduction of phenolics and other compounds (Verhoeff, 1974; Bompeix, 1978; Lattanzio *et al.*, 2001). Conidia are dispersed from acervuli by water throughout the year, but particularly during the autumn in Europe, and infect unripe fruit typically through the lenticels. Germinated conidia are unable to penetrate the cortical tissue surrounding the substomatal cavity of lenticels in unripe fruit (Edney, 1958 and 1964). Bull's eye rot does not spread from fruit to fruit in storage. However, spread of conidia could happen with any processing which involves drenching or dipping of the fruit in water (Bompeix, 1988).

Management

There is limited research information on the management of *Neofabraea* in South Africa. *In vitro* studies of plant volatiles against *N. alba* have shown promising results for decay control on apples (Neri *et al.*, 2009). The fungicides pyrimethanil, thiophanate-methyl, thiabendazole and pyroclostrobin plus boscalid has shown effectiveness for the control of bull's eye rot caused by *N. alba* on pear fruit (Spotts *et al.*, 2009). In the Pacific Northwest, Ziram™ is a recommended fungicide applied in orchards within two weeks before harvest for control of bull's eye rot (Henriquez *et al.,* 2004). Ziram™ or captan sprays at petal fall and before harvest and benomyl dip treatments after harvest have shown promise in controlling bull's eye rot (Grove, 1990). Postharvest treatments have the advantage of allowing better control of treatment conditions. Dipping or drenching fruits with thiabendazole is the most common postharvest treatment to reduce storage rots in pome fruits (Bompeix, 1988). Burchill and Edney (1972) reported that thiabendazole reduced the incidence of bull's eye rot when applied as a postharvest fruit dip of 'Cox's Orange Pippin' apples. Postharvest fungicide application is effective for control but may not reach the fungus if it is established deep in lenticels as result of early-season infections (Grove, 1990).

CONTROL METHODS OF POSTHARVEST DISEASES

Fungicides

Currently, in the deciduous fruit industry, postharvest diseases are commonly controlled by applying fungicide treatments. One of the main aims of fungicide application is to destroy inoculum of *B. cinerea* and *P. expansum* on fruit arriving in the packing shed (Cunningham, 2005). Fungicides are commonly applied pre-harvest as field sprays to control fruit diseases in orchards and to prolong cold chain management practices to prevent and/or control

quiescent fungal infections of fruits. Despite the use of fungicides, the losses of up to 20% of the harvested product are still recorded in countries even with advanced cold storage facilities (Cappellini and Ceponis, 1984). In South Africa, Iprodione™ is used as a postharvest fungicide treatment of apples and pears. In developing countries, where the disease management practices and proper handling of postharvest commodities are poor, postharvest losses of fruits are rated to about 50% (Eckert and Ogawa, 1985). To minimize losses and improve the shelf life of fruits, the application of good pre- and postharvest practices including sanitation, careful harvesting and effective cold chain management practices is crucial. Fungicide residue levels in fruit have also become problematic. Residue levels in fruit are affected by the fungicide concentration, exposure time and temperature, and method of applications (heated immersion, ambient immersion, spray, dip or in wax). Schirra *et al.* (1997) found that when imazalil was applied at 50°C (heated immersion) there was a linear relationship between the residue concentration in lemon fruit and the amount of the fungicide employed. This suggests that a combination of increased temperature (from 22°C to 50°C) with fungicide application may allow a decrease in fungicide usage. This increased control is believed to be due to increased mobility of fungicides in the fruit epicuticular wax, but also may be due to thermal inactivation of pathogens; hot water treatment may decrease infection by removing spores from wounds and inducing defence mechanisms in the outer layers of the fruit epicarp (Schirra *et al.,* 1997). However, McDonald *et al.* (1991) found that thiabendazole and imazalil treatments applied at 5°C reduced chilling injury and inhibited decay of grapefruit more effectively than when these fungicides were applied at room temperature, suggesting that the relationship between fungicide activity and temperature may be complex.

Several modes of action have been suggested to explain the activity of fungicides. The mode of action of fungicides include impacting membranes, nucleic acids and protein synthesis, signal transduction, respiration, mitosis and cell division, and multisite activity, as well as on their side effects on non-target organisms (Yang *et al.,* 2011). The cell membrane is a selectively permeable wall that separates the cell content from the outside environment. Membranes perform many biological functions in all living cells. They preclude the passage of large molecules, provide the shape of the cell, maintain cell water potentials, and are involved in signal transduction (Alberts *et al.,* 2002).

Problems with fungicide residues on fruit, concern for human health and the environment and development of fungicide resistance have led to a need to develop alternative methods for controlling postharvest diseases (Wilson *et al*., 1993; Timmer and Duncan, 1999). Possible alternatives include using biological control and naturally-occuring antifungal compounds.

Biological control agents (BCAs)

Awareness of the health and ecological risks associated with increased synthetic fungicide usage in agriculture dictates the search for natural, safe and environmentally friendly means of disease control (Campbell, 1989). Biocontrol of plant diseases has been defined by Baker (1987) as, "the reduction in the amount of inoculum or the disease-producing activity of a pathogen accomplished by or through one or more organisms other than man". Wilson (1997) also defines biological control as the control of a plant disease with a natural biological process or the product of a natural biological process, and includes bio-chemicals delivered by and extracted from living organism, and also host resistance.

Biological control of postharvest diseases using microbial antagonists is considered a desirable alternative to synthetic fungicide (El-Ghaout *et al.*, 2000). Biocontrol agents have been widely investigated and promising results have been achieved (Janisiewicz and Korsten, 2002). Biological control agents such as bacteria, yeast, and filamentous fungi have been used effectively to protect a number of fresh fruit against pathogens (Chalutz and Wilson, 1990). They have been selected because of their antagonistic activity, suitable mode of action and their harmlessness to human beings and to the environment (Arras *et al.*, 1999). Many successful laboratory trials have been reported on apples, stone fruit, citrus, grapes, and other fruit. Microbial antagonists such as bacteria, yeast, and filamentous fungi play an important role in the natural control of numerous postharvest pathogens of fruits (Janisiewicz, 1987; Wilson and Chalutz, 1989; Roberts, 1990; Lima *et al.*, 1997). Some semi-commercial scale trials in pack houses have been carried out with success using biological control agents (El-Ghaouth *et al.*, 2000). Research on postharvest decay of pome fruit has led to the registration of YieldPlus®, the first South African produced and registered biological control agent for the control of postharvest decay on pome fruit (Vero *et al.*, 2002).

A potential microbial antagonist should have certain desirable characteristics to make it an ideal biocontrol agent (Wilson and Wisniewski, 1989; Barkai-Golan, 2001): the antagonist should be: (a) genetically stable; (b) effective at low concentrations; (c) not fastidious in its nutritional requirements; (d) capable of surviving under adverse environmental conditions; (e) effective against a wide range of the pathogens and different harvested commodities; (f) resistant to pesticides; (g) a non-producer of metabolites harmful to human; (h) non-pathogenic to the host; (i) preparable in a form that can be effectively stored and dispensed; and (j) compatible with other chemical and physical treatments. In addition, a microbial antagonist should have an adaptive advantage over specific pathogens (Wilson and Wisniewski, 1989).

Several modes of action have been suggested to explain the biocontrol activity of microbial antagonists. Competition for nutrients and space between the pathogen and the antagonist is considered the major mode of action by which microbial agents control

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pathogens causing postharvest decay (Droby *et al.,* 1992; Wilson *et al.*, 1993; Filonow, 1998; Ippolito *et al.,* 2000; Jijakli *et al.*, 2001). In addition, production of antibiotics (antibiosis), direct parasitism, and possible induced resistance are other modes of action of the microbial antagonists by which they suppress the activity of postharvest pathogens on fruits and vegetables (Janisiewicz *et al.*, 2000; Barkai-Golan, 2001; El-Ghaouth *et al.*, 2004).

Physical methods alone or in combination with other control practices

Physical measures include heat treatment, UV light, treatment at pressures higher or lower than atmospheric pressure, and exposure to modified or controlled atmospheres or to ozone, among other measures. Physical treatments can have dual effects on the fruit, as these are active against the pathogen and at the same time they can induce host defense responses (Wilson *et al.,* 1994). The antagonistic activity of some biocontrol agents in controlling postharvest decay can be increased by some chemical compounds, such as carbonate and bicarbonate (Palou *et al.,* 2001). According to Lima *et al.* (2007), a number of studies have shown that a multi-component approach can provide enhanced or synergistic effects to biocontrol and noted that it should be able to totally control the development of postharvest infections. The multi-component approach has been successfully applied to apples in order to control *P. expansum* (Link) Thom. optimum control of postharvest fungal infection has been achieved by combining heat treatment, calcium chloride and the antagonist *P. syringae* (Conway *et al.,* 1999) or a biocontrol mixture, applied with a heat treatment and/or sodium bicarbonate (Conway *et al.,* 2005). The combination of more persistent activity of biocontrol agents with heat has the potential to offer effective longer term disease control (Obagwu and Korsten, 2003). Karabulut *et al.* (2002) reported that a postharvest treatment combination of hot water brushing at 60°C followed by a yeast antagonist (*Candida* spp.) as a postharvest treatment was very successful in controlling natural infections of *Monilinia fructicola* (G. Winter) Honey. and *P. expansum* (Link) Thom. of peach and nectarine, compared with the hot water treatment alone. Tian *et al.* (2007) reported that a postharvest treatment combination of sodium silicate with yeast antagonists (*Cryptococcus laurentii* Kufferath C.E. Skinner. and *Rhodotorula glutinis* Harrison.*)* provided synergistic effects in controlling postharvest diseases caused by *P. expansum*, *M. fructicola* and *Alternaria alternata* in sweet cherry, peach and jujube fruit. Obagwu and Korsten (2003) reported that the use of *Bacillus subtilis* alone to control the incidence of both green and blue moulds on artificially inoculated ‘Valencia’ and ‘Shamouti’ oranges was less effective than the fungicide treatment (quazatine plus imazalil), and they observed a significant increase in biocontrol activity when they combined *B. subtilis* with sodium bicarbonate, and when *B. subtilis* was applied following hot water treatment, it gave 100% control. This suggests that a combination of biocontrol with

other alternative methods may provide additive or synergistic effects and enhance the efficacy.

Defence mechanisms in the outer layers of the fruit epicarp may be induced after hot water treatment, which also decrease infection by removing spores from wounds (Teixido *et al.,* 2001). High temperatures and chemical compounds alone offer only short-term inhibitory activity against fruit storage rots, whereas biocontrol agents can persist for long periods and protect fruit from re-infection (Teixido *et al.,* 2001). Application of biological control agents (i.e yeast, bacteria, fungi) alone to control postharvest diseases of fruits faces limitation. Some factors limiting commercial interest in biocontrol is that they not effective when applying them alone, high cost of production due to high cost of substrate, low biomass productivity, or limited economies of scale (Fravel, 2005). Therefore, optimizing or improving the performance of biocontrol agents by combining them with other control methods is essential (Karabulut *et al*., 2002).

Naturally-occuring antifungal compounds

A wide variety of plant and animal-derived compounds are known to be fungicidal and some have shown to be effective in reducing postharvest decay of fruit and vegetables (El-Ghaouth and Wilson, 1995; Isman, 2000; Mansingh, 2004; Cao *et al.,* 2004; Antunes and Cavaco, 2010). Essential oils, volatile substances, and extracts from various plants have been shown to inhibit radial growth of major postharvest pathogens such as *B. cinerea, P. expansum, Monilinia fructicola Wint*, *Rhizoctonia solani, Fusarium moniliforme, Sclerotinia sclerotiorum*, *F. oxysporum*, *Aspergillus niger*, *P. digitatum, F. solani, Pythium ultimum, Colletotrichum lindemuthianum*, *Alternaria padwickii* and *Bipolaris oryzae* (Wilson *et al*., 1987; Shimoni *et al.,* 1993; Muller *et al.,* 1995; Arras *et al.,* 1995; Paster *et al.,* 1995; Carta *et al.,* 1996; Cutler *et al.,*1996; Zambonelli *et al*., 1996; Bowers and Locke, 2000; Daferera *et al.,* 2000; Nguefack *et al*., 2007). Reduction of decay by extracts and volatiles from plants have been reported in strawberry, raspberry and peach (Sholberg and Shimizi, 1991), apple and citrus fruit (Pesis and Avissar, 1990; Matheis and Roberts, 1993).

Control of postharvest decay was also reported with natural antifungal compounds redived from microbial fermentation (Pusey and Wilson, 1984) and animal- by-products (El-Ghaouth *et al*., 1992b). The antimicrobial iturin and pyrrolnitrin were shown to be effective in reducing decay on peaches, apple, pear and strawberry (Pusey *et al.,* 1988; Janisiewicz *et al*., 1991). However, their potential as food preservative will depend on whether their use in agriculture could promote the development of antibiotic-resistance strains of animal and plant pathogens. Control of postharvest decay was also reported with chitosan (El-Ghaouth *et al*., 1992a). Inorganic salts have been shown to be active antimicrobial agents against a range of phytopathogenic fungi, and among these agents, bicarbonates have been proposed

as safe and effective alternative means to control postharvest rot of fruit and vegetables. Also, as well as these salts being nontoxic and having minor environmental impact at effective concentrations, they are inexpensive (Sanzani *et al.*, 2009). Several sanitizers classified as 'generally recognized as safe' (GRAS) have been applied to extend the postharvest storage of various produce, including acetic acid, electrolyzed oxidizing water, and ethanol (Romanazzi *et al.,* 2012).

ESSENTIAL OILS

Essential oils (EOs) are made up of many different volatile compounds and the processes of extracting the essential oils are quite different between plant 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 are extracted from different parts of the aromatic plant (leaf, flower, seed or bark, fruit peels) using various techniques, including water or steam distillation, solvent extraction, expression under pressure, supercritical fluid or sub-critical water extractions (Burt, 2004; Wang *et al.,* 2004). Essential oils can have fungicidal or fungistatic effect on plant pathogens, or they can provide conditions favorable for the establishment and increase of antagonistic microorganisms on host plants (Scheuerell and Mahafee, 2002). Essential oils and their main components possess a wide range of biological activity, which may be of great importance, and the main advantage of essential oils is that they are considered GRAS (Burt, 2004).

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.

The chemical structures of terpenes were established in 1887 by Wallach (Reineccius, 1994). Terpenes in essential oils are made up of the isoprene units. Each isoprene contains five 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 such as champhene, nerol, pinene, myrcene, limonene and citral are the most common in nature (Buckle, 2004). Some examples of sesquiterpenes are cardinal and farnesol. Examples of diterpenes are phytol and Vitamin A1. 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 (Tan *et al.,* 1999; Jasicka-Misiak *et al.,* 2004).

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*. Geraniol is known to have antifungal activity (Saikia *et al.,* 2001; Carson and Riley, 1995; Buckle, 2004). Terpinen-4-ol is effective against *Pseudomonas aeruginosa* (Budhiraja *et al.,* 1999). 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 *Microsporum gypseum* followed by citronellal.

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 in lemongrass, geranial in lemon eucalyptus and neral in lemon verbena (Reineccius, 2004). Citral is known to have strong antiseptic and antibacterial properties (Buckle, 2004). Citronellal is known to have antifungal properties (Saikia *et al.,* 2001). Saikia *et al.* (2001) found that citronellal is active against *Microsporum gypseum*. Esters are a combination of acid and alcohol and often have fruity odours (Buckle, 2004). Some examples of esters are linalyl acetate found in lavender, and genaryl acetate found in sweet marjoram (Clarke, 2002). Some have antifungal properties (Buckle, 2004; D' Auria *et al.,* 2005).

Interaction between components of essential oils

The antimicrobial properties of EOs have been reported in several studies (Clark *et al*., 2003; Huang *et al*., 2004). In many cases the activity results from the complex interaction between the different classes of compounds such as phenols, aldehydes, ketones, alcohols, esters, ethers or hydrocarbons found in EOs (Streif *et al*., 2003; Isidoro and Almeida, 2006). In some cases, the bioactivities of EOs are closely related with the activity of the main components of the oils (Mahovic *et al*., 2007). Several studies have found that a number of these compounds exhibited significant antimicrobial properties when tested separately (Clark *et al*., 2003; Lee and Bostock, 2007; Amorim *et al*., 2008).

It has been reported that EOs containing aldehydes or phenols, such as cinnamaldehyde, citral, carvacrol, eugenol or thymol as major components showed the highest antibacterial activity, followed by EOs containing terpene alcohols. Other EOs, containing ketones or esters, such as β-myrcene, α-thujone or geranyl acetate had much weaker activity. While volatile oils containing terpene hydrocarbons were usually inactive (Clark *et al*., 2003; Tripathi and Dubey, 2004). Different terpenoid components of essential oils can interact to either reduce or increase antimicrobial efficacy (Giovannoni, 2001). The interaction between essential oil compounds can produce four possible types of effects: indifferent, additive, antagonistic, or synergistic effects (Gagliardi *et al*., 2003; Espinosa-Medina, 2006). An additive effect between the components of essential oils is observed when the combined effect is equal to the sum of the individual effects. Antagonism is observed when the effect of one or both compounds is less when they are applied together than when individually applied. Synergism is observed when the effect of the combined substances is greater than the sum of the individual effects while the absence of interaction is defined as indifference (Burt, 2004). Some studies have concluded that whole essential oils have a greater antibacterial activity than the major components mixed (Mourey and Canillac, 2002), which suggests that the minor components are critical to the activity and may have a synergistic effect. For example, the two structurally similar major components of oregano essential oil, carvacrol and thymol, were found to give an additive effect when tested against *S. aureus* and *Pseudomonas aeruginosa* (Lambert *et al*., 2001). Ultee *et al.* (2000) indicated synergism between carvacrol and its biological precursor p-cymene against *Bacillus cereus* vegetative cells. It appears that p-cymene swells bacterial cell membranes to a greater extent than carvacrol. By this mechanism p-cymene probably enables carvacrol to be more easily transported into the cell so that a synergistic effect is achieved when the two are used together (Ultee *et al*., 2000). Fractions of cilantro, coriander, dill and eucalyptus essential oils (each containing several components), when mixed in various combinations, resulted in additive, synergistic or antagonistic effects (Delaquis *et al*., 2002). A mixture of cinnamaldehyde and eugenol respectively inhibited growth of *Staphylococcus* sp., *Micrococcus* sp. *Bacillus* sp. and *Enterobacter* sp. for more than 30 days completely, whereas the substrates applied individually did not inhibit growth (Moleyar and Narasimham, 1992). Zaridah *et al.* (2003) reported that combining citronella (*Cymbopogon nardus*), lemon (*Citrus limon*), rose (*Rosa damascena*), and lavender and basil essential oils with one litre of distilled water was effective toward indoor insect pests (Zaridah *et al*., 2003).

Generally compounds with similar structures exhibit additive rather than synergistic effect. The occurrence of additive interaction of some essential oils has been related to their main phenolic compounds (carvacrol and thymol) (Clark *et al*., 2003; Isidoro and Almeida, 2006; Himathongkham *et al*, 2007). Antagonistic effect has been attributed to the interaction

between non-oxygenated and oxygenated monoterpene hydrocarbons (Hammer *et al*., 1999; Goni *et al*., 2009).

Mode of action of essential oils

Although the antimicrobial properties of essential oils and their components have been reviewed in the past (Shelef, 1983; Nychas, 1995), the mechanism of action has not been studied in great detail (Lambert *et al*., 2001; Holley and Patel, 2005). Considering the large number of different groups of chemical compounds present in essential oils, it is most likely that their antibacterial activity is not attributable to one specific mechanism but to several targets in the cell (Carson *et al*., 2002). Examples of selected crude essential oils and their identified sites and mode of action are shown in Table 1.

An important characteristic of essential oils and their components is their hydrophobicity, which enables them to partition in the lipids of the bacterial cell membrane and mitochondria, disturbing the structures and rendering them more permeable (Sikkema *et al*., 1994). Leakage of ions and other cell contents can then occur (Carson *et al*., 2002). Although a certain amount of leakage from bacterial cells may be tolerated without loss of viability, extensive loss of cell contents or the exit of critical molecules and ions will lead to death (Denyer and Hugo, 1991). The outer membrane of *Escherichia coli* and *Salmonella typhimurium* disintegrates following exposure to carvacrol and thymol (Helander *et al*., 1998) and major thickening and disruption of the cell wall, together with increased roughness and lack of cytoplasm have been reported on *Listeria monocytogenes* on treatment with thyme essential oil (Rasooli *et al.,*2006). Similar findings have been made with *E. coli* O157:H7 and *L. moocytogenes*, in the presence of oregano and cinnamon, respectively (Oussalah *et al.,* 2006). Generally, the essential oils possessing the strongest antibacterial properties against food borne pathogens contain a high percentage of phenolic compounds such as carvacrol, eugenol [(2-methoxy-4-(2-propenyl) phenol)] and thymol (Cosentino *et al*., 1999; Lambert *et al*., 2001). Their mechanism of action would therefore be similar to other phenolics; this is generally considered to be the disturbance of the cytoplasmic membrane, disrupting the proton motive force (PMF), electron flow, active transport and coagulation of cell contents (Sikkema *et al*., 1995; Davidson, 1997; Hammer *et al.,* 2004; Parveen *et al.,* 2004).

The chemical structure of the individual essential oil components affects their precise mode of action and antibacterial activity (Dorman and Deans, 2000). The importance of the presence of the hydroxyl group in phenolic compounds such as carvacrol and thymol has been confirmed (Dorman and Deans, 2000; Ultee *et al*., 2002). The relative position of the hydroxyl group on the phenolic ring does not appear strongly to influence the degree of antibacterial activity; the action of thymol against *B. cereus*, *Staphylococcus aureus* and *Pseudomonas aeruginosa* appears to be, for example, comparable to that of carvacrol

(Lambert *et al.*, 2001; Ultee *et al.*, 2002). However, in one study carvacrol and thymol were found to act differently against gram-positive and gram-negative species (Dorman and Deans, 2000). Ultee *et al* (2002) reported that, the significance of the phenolic ring is demonstrated by the lack of activity of menthol compared to carvacrol.

Components of essential oils also appear to act on cell proteins embedded in the cytoplasmic membrane (Knobloch *et al.*, 1989). Enzymes such as ATPases are known to be located in the cytoplasmic membrane and to be bordered by lipid molecules. Two possible mechanisms have been suggested whereby cyclic hydrocarbons could act on these. Lipophilic hydrocarbon molecules could accumulate in the lipid bilayer and distort the lipid-protein interaction; alternatively, direct interaction of the lipophilic compounds with hydrophobic parts of the protein is possible (Juven *et al.*, 1994; Sikkema *et al.*, 1995). Carvacrol has been shown to increase membrane fluidity and cause leakage of protons and potassium ions, resulting in a collapse of membrane potential and inhibition of ATP synthesis (Ultee *et al.,* 1999; Ultee *et al.,* 2002). This has also shown to be the case of tea tree oil against *Staphylococcus aureus*, which affected the cell wall and produces a loss of 260 nm nuclear material, K+ ions and salt tolerance. The presence of mesosome-like structures is observed and inhibition of glucose-dependant respiration occurs (Halcon and Milkus, 2004). Terpenes have the ability to disrupt and penetrate the lipid structure of cell wall of bacteria, leading to denaturing of proteins and destruction of cell membrane leading to cytoplasmic leakage, cell lysis and eventually cell death. The total amount of K^+ ions lost in *S. aureus* increased with increasing concentration of tea tree oil. The decrease in pH that occurs due to this cell membrane disruption means that control of cellular processes such as DNA transcription; protein synthesis and enzyme activity is lost (Oussalh *et al.,* 2006). The essential oils do not only penetrate the cell membrane but they also penetrate the mitochondrial membrane leading to greater permeability of the organelle and the same ion leakage process (Raybaudi- Massilia *et al.,* 2006).

Some essential oils such as garlic oil, onion oil, oregano oil, and thyme oil have been found to stimulate the growth of pseudomycelia (a series of cells adhering end-to-end as a result of incomplete separation of newly formed cells) in certain yeasts (Conner and Beuchat, 1984). This could be an indication that essential oils act on the enzymes involved in the energy regulation or synthesis of structural components. For, instance cinnamon oil and its components have been shown to inhibit amino acid decarboxylases in *Enterobacter aerogenes* through binding of proteins (Wendakoon and Morihiko, 1995).

Undesirable physiological effects of essential oils

Although a considerable number of essential oil components are GRAS and approved food flavourings, some research data indicate possible irritation and toxicity, (Burt, 2004). Major

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components such as phenols and lactones in essential oils can cause irritation of skin tissues. For example, eugenol and thymol have been known to cause irritation of mouth tissues (Burt, 2004). Some essential oils have been shown to be toxic to plant products, with the phytotoxic effects caused by essential oils on fruit and vegetable varying according to the produce treated, application methodologies, concentrations of oils and adaptive duration. For instance, dips of thyme, oregano, cinnamon and clove essential oils used on orange can cause peel injury in the fruit (Burt, 2004) while thymol was effective as a postharvest treatment with no apparent phytotoxicity in treated apricot and plums with no effect on desirable fruit attributes (Liu *et al.,* 2002). Moreover, the strong aroma of some essential oils may affect the original flavour of some products.

There is a physical advantage in applying essential oils as postharvest treatments. As they can be applied as a vapour, essential oil treatments can reduce costs as fumigation requires less oil and labour in postharvest handling (Wuryatmo *et al,* 2003). The vapour phase for postharvest application limits the tainting of original organoleptic attributes of fruit as well as the risk of phytotoxic effects on fruit skin. The volatile constituents of some aromatic plants including lemongrass, lemon, lime, savory, red thyme, geranium, tea tree, clove, basil and caraway are well-known for their antifungal properties. Cold treatments may also be applied in combination with chemical fumigation, thus reducing the amount of fumigant required (Wuryatmo *et al,* 2003). The essential oils assessed in the current study were lemongrass oil, lemon oil, and lime oil.

Lemongrass and citrus essential oils

Cymbopogon is a genus of about 55 species, which are indigenous in tropical and semi-tropical areas of Asia and are cultivated in South and Central America, Africa and other tropical countries (Shah *et al.,* 2011). Lemongrass (*Cymbopogon citratus*) grows in dense clumps that can grow to 1.8 m in height and about 1.2 m in width, with a short rhizome. It contains 1-2% 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, 2012).

Cymbopogon citratus essential oil has shown significant inhibitory activities against a number of filamentous fungi, namely *Alternaria alternata*, *Aspergillus niger*, *Fusarium oxysporum* and *Penicillium roquefortii* (Irkin and Korukluoglu, 2009). A study conducted by Saikia *et al.* (2001) found that lemongrass oil is highly effective at inhibiting the growth of *M. gypseum.* The oil has been found to possess bactericidal and antifungal properties, which is comparable to penicillin in its effectiveness (Lutterodt *et al.,* 1999; Shahi *et al.,* 2003; Saikia *et al.,* 2001). It is an oral anti-tumor drug for cancer and in combination with cyclodextrin lengthened the survival time (Oshiba *et al.,* 1991; Parekh and Chanda, 2007). Lemongrass

essential oil contains high percentage of Vitamin C, which is an essential nutrient for humans (Parekh and Chanda, 2007). Lemongrass oils show activity towards the phyto-pathogenic fungi. Lemongrass essential oil is also effective against human and domestic animal pathogens (Kisaki and Yama, 1998).

Lemongrass is composed of monoterpene compounds. Chemical structure of the major constituents of lemongrass essential oil is shown in Fig. 2 (Schaneberg and Khan, 2002). The most abundant compound in lemongrass oil is citral, composed of neral and geranial isomers (Ming *et al.,* 1996). Lemongrass oil also consists of myrcene, geraniol, geranyl acetate (Bonada de Silva *et al.,* 2008). Limonene is found at a low level (2.9%) as well as myrcene, cis-β-ocimene, borneol, α-terpineol and β-caryophyllene. The chemical composition of the essential oil of *C. citratus* varies according to the geographical origin, the compounds as hydrocarbon terpenes, alcohols, ketones, esters and mainly aldehydes have constantly been registered (Abegaz *et al.,* 1983; Trease, 1996).

Another genus of interest is the *Citrus* spp. with approximately 16 species in the family Rutaceae and are mainly cultivated in subtropical regions (Fisher and Phillips, 2008). Most of the species are cultivated in India for their fruits, including *Citrus limon* (Linn.) Burm. F. and *C. aurantifolia* (Christm.) Swingle, commonly known as lemon and lime, respectively (Singh and Singh, 2002). Citrus fruit EOs are extracted from the peel of fresh fruits by using a cold press system extraction. Since citrus EOs are mainly located in the fruit peel, their extraction is economically sustainable, because the fruit peel constitutes a waste for the fruit juice industry (Tirado *et al.,* 1995; Espina *et al.,* 2011). Citrus EOs have been industrially applied in many products, including food and beverages (e.g. as flavouring agents), cosmetics and medicines (Uysal *et al.,* 2011) and the antimicrobial activities against some of the most important foodborne pathogens (e.g. *Campylobacter jejuni*, *Escherichia coli*, *Listeria monocytogenes, Bacillus cereus* and *Staphylococcus aureus*) have been shown (Kim *et al.,* 1995; Fisher and Phillips, 2006).

Lemon oil (*Citrus limon*) is an evergreen tree which grows up to 6 m in height and has dark green serrated oval leaves with pink/white flowers that are highly perfumed content (The Herbs Place, 2012). The trees have thorns and fruit that turn from green to yellow on ripening. Lemon oil has a sharp, fresh smell, pale greenish-yellow in colour and is watery in viscosity. It is used in aromatherapy, fragrances, household cleaners and disinfectants content (The Herbs Place, 2012). Lemon oil is extracted from the fresh fruit peel by cold pressing. This essential oil have the ability to stop bleeding, kill infection, activate white blood and red blood cell production to fight anemia (Foster, 2012). It is also claimed to have powerful antiseptic, antibacterial, antifungal and antiviral activities due to its limonene content (The Herbs Place, 2006).

Chemical structure of the major constituents of lemon essential oil is shown in Fig. 3 (Schaneberg and Khan, 2002). The main chemical components of lemon oil are a-pinene, camphene, b-pinene, sabinene, myrcene, a-terpinene, linalool, b-bisabolene, limonene, trans-a-bergamotene, nerol and neral (Foster, 2012). Gas chromatography analysis of lemon oil by Ojeda de Rodriguez *et al.* (1998) revealed a total of 51 compounds. 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 Rodriguez *et al.,* 1998). 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 Rodriguez *et al.,* 1998).

Lime oil *(Citrus aurantifolia*) is an evergreen tree growing up to 4.5 m high with smooth, green leaves, stiff sharp spines and small, white flowers. Lime essential oil is extracted from *Citrus aurantifolia* of the Rutaceae family (Tisserand and Balacs, 1995). Lime oil has a sharp, citrus peel smell and a pale yellow to light olive in colour. Lime oil is used to flavour cold drinks and also used in the perfume industry. Lime oil can be extracted by cold expression or steam distillation. Lime oil is beneficial to the immune system, easing infection in the respiratory tract and relieving pain in muscles and joints, refresh a tired mind and helps with depression. Chemical structure of the major constituents of lime essential oil is shown in Fig. 3 (Schaneberg and Khan, 2002). The main constituents in lime oil are a-pinene, b-pinene, myrcene, limonene, terpinolene, 1,8-ceneole, linalool, borneol, citral and traces of neral acetate and geranyl acetate (Foster, 2012). Several researchers have reported that citrus essential oils and their potential antifungal components can significantly inhibit spore germination and mycelial growth of *P. italicum* and *P. digitatum* (Caccioni *et al.,* 1998; Sharma and Tripathi, 2006; Droby *et al.,* 2008).

EFFECT OF CONTROLLED ATMOSPHERE STORAGE ON FRUIT QUALITY AND DEVELOPMENT OF PATHOGENS

Controlled atmosphere (CA) storage can be defined as the storage of a commodity under an atmosphere that is different to air. The atmosphere is monitored and maintained through the term of storage. Usually controlled atmosphere storage of fruits employs a lower O_2 concentration and higher CO_2 concentration than normally found in air (Brecht, 1980). Ideally, the rate of respiration of fruits held under CA storage is lower than it would be under air (Shewfelt, 1986). CA storage is a widely used technique for long-term storage of freshly picked fruits and vegetables. Historically, CA storage has been the primary method for the long-term storage of apples. Through a biological process called respiration, apples take in

oxygen and generate carbon dioxide, water, and heat. CA storage is an entirely natural process that reduces the effects of respiration to a minimum by controlling the environmental conditions surrounding the stored fruit. CA storage makes it possible to buy crisp, juicy apples year round. Many cultivars of apples can be preserved for a period of 9-12 months in CA storage, as opposed to only 2 -3 months if using refrigerated storage. Varying atmosphere conditions during storage, where either the O_2 or CO_2 concentration is changed for a short period with subsequent return to the initial concentration set point, have been reported to improve fruit quality and storability of some apple cultivars such as 'Gala' apples (Mattheis *et al.,* 1998).

Apple fruits are kept in cold storage after harvest to preserve their quality. Low temperature plays main role in slowing the degradation of apple fruit quality during storage, depending on the sensitivity of particular cultivars to chilling injury. The storage of fruit under modified and controlled atmospheres has been used directly or indirectly to reduce postharvest decay (Sommer, 1982). The growth of many organisms is retarded in CA storage, but the mixture of gases that provides the best results in extending the life of fruit in storage is not necessarily the best to reduce the development of storage rots. Some pathogens are not affected at all, and others are even favored by CA storage (Lockhart, 1969). Low temperatures slow down the growth of fungi causing postharvest decay including *B. cinerea, P. expansum, Mucor piriformis* and *Alternaria alternata*, but growth is resumed as soon as the fruit are transferred to a high temperature (Sommer, 1985). Similar to fruit, postharvest pathogens also require O_2 for respiration. Low O_2 and CO_2 concentrations can inhibit fungal growth but the concentrations generally used for CA storage have a negligible effect (Combrink, 1974). CA storage delays fruit ripening and a fruit retains its natural resistance to infection for a longer time (Sommer, 1985).

Montgomery (1958) reported that rot development by *N. alba* was reduced in CA storage but the same treatment did not affect rot development by *N. perennans*. Sommer (1982) showed that, the growth of *N. alba in vitro* was reduced by decreasing concentrations of oxygen, but stimulated with the addition of CO_2. Mycelial growth of *N. alba in vitro* was greater at 10% than at 5% CO_2. Lockhart (1967) also reported that the incidence of postharvest decay on 'McIntosh' apples, caused by *Neofabraea* sp. was reduced in the presence of 5% CO_2 compared to 0% CO_2. Apples are stored at -0.5˚C and the gas regimes for CA storage vary depending on apple cultivar. El-Goorani and Sommer (1979) studied the effect of CO added to air and to CA (2.3% O_2 + 5% CO_2) on *Botrytis* rot of strawberries and observed that 9% CO + CA reduced the rate of rot development 80-90%.

Advantages of controlled atmosphere

Under CA storage, apples and pears of a high quality are available for longer periods than in the past and the quality is much better than after regular atmosphere (RA) storage for shorter periods. This is beneficial both to producers and consumers. The major benefit of CA storage is the prevention of ripening by retardation of the process regulating ripening and senescence (Kader, 1980). Most of the enzymatic processes occurring in fruit after harvest are O_2 dependent and lowering the O_2 content around the fruit reduces the activity of the enzymes (Weichman, 1986). Ethylene plays an important role in ripening (Burg and Burg, 1965) and CA storage prevents the autocatalytic production of ethylene. By delaying ripening, the onset of the climacteric is delayed and the storage life of the fruit is extended. The more rapidly the O_2 concentration in the store is decreased, the better the retention fruit firmness and texture (Lau and Looney, 1982; Lau, 1983). However, the rate of CA establishment becomes less critical if the fruit is cooled rapidly (Van Eeden *et al.,* 1988). The reduction in the rate of softening is probably due to the accumulation of polymanes which inhibit the activity of polygalacturonase, the enzyme which degrades the cell wall (Kramer *et al.,* 1989). The advantage of ultra-low O_2 storage is that the incidence of fruit disorders including superficial scald and bitter pit, are reduced (Truter *et al.,* 1982; Little, 1985; Johnson *et al.,* 1985). Initial O_2 stress is induced by keeping fruit at 0.3% O_2 for 10 days prior to conventional CA storage also reduces the development of superficial scald (Little, 1985; Van der Merwe *et al.,* 1997; Van der Merwe and Combrink, 2001). CA storage reduces changes in colour by reducing the rate at which chlorophyll is metabolised (Ginsburg *et al.,* 1969; Weichman, 1986). Low O_2 levels slow down the decomposition of carbohydrates. Losses in total sugars are smaller, the lower the O_2 concentration in the storage atmosphere (Weichman, 1986). Increasing the CO_2 content of the storage atmosphere slows down the process even further. Some apple cultivars are sensitive to low temperatures. The storage life of these fruits can be extended by CA storage at temperatures that can be tolerated by the fruit.

Each different commodity and in some cases different cultivars have different responses to low O_2 and high CO_2. In South Africa, the recommended gas regimes for CA storage of 'Granny Smith', 'Golden Delicious', and 'Pink Lady' apple cultivars are 1.5% O_2 + 1.0 % CO_2, 1.5% O_2 + 2.5% CO_2 and 1.5% O_2 + 1.0 % CO_2, respectively. Under these conditions, 'Granny Smith', 'Golden Delicious', and 'Pink Lad' apple cultivars will have a storage life of up to 11 months, 9 months and 6 months, respectively (Van der Merwe, 1996). Controlled atmosphere has an indirect influence on reducing water loss and reducing consequences of physical injuries, and indirect effect on pathogen growth (Kader, 1997 and 1986). Another important benefit of controlled atmosphere is handling the product at temperatures lower than the optimum storage temperature, with special interest to chilling

sensitive products. Controlled atmosphere storage with carbon dioxide concentrations above 2.8% reduced the development of lesions incited by *B. cinerea, P. expansum* and *Pezicula malicorticis* in 'McIntosh', 'Delicious', and 'Golden Delicious' apples (*Malus domestica)* kept for 61 days at 0˚C (Sitton and Patterson, 1992). A low O_2 pre-treatment in avocado fruit for 24 hr at 17 ºC reduced chilling injury symptoms after storage at 2 ºC for 3 weeks (Pesis *et al*., 1994).

Disadvantages of controlled atmosphere

Fruits may also experience compositional and developmental changes, physiological disorders and increased susceptibility to decay when stored at CA (Isenberg, 1979; Kader, 1986; Kader *et al.,* 1989). Physiological disorders, such as brown stain (a form of CO_2 injury) on lettuce, internal browning and surface pitting of pome fruits and blackheart in potatoes (Kader *et al*., 1989) may be induced. Pears kept in long term CA storage may not develop the volatiles required to attain a good aroma (Kader, 1989; Lidster *et al.,* 1981; Streif and Bangerth, 1988). Transferring the fruit to air may regenerate volatile production, but not completely. CO_2 injury, brown core are different terms to characterize the core and flesh browning of pears under elevated CO_2 (Kader *et al*., 1989). Exposure of fresh fruits to O_2 levels below their tolerance limits or to CO_2 levels above their tolerance limits may hazard fruits and decrease its storage life. Low levels of O_2 may increase anaerobic respiration and the consequent accumulation of ethanol and acetaldehyde causing off-flavours (Kader *et al*., 1989). 'Granny Smith' apples are highly susceptible to core flush. Controlled atmosphere storage is one of the factors that may aggravate this disorder (O'Loughlin, 1985). Its incidence can be decreased by increasing the storage temperature (Dalton *et al.,* 1982) and keeping the CO_2 concentration low, especially at low temperatures (Ginsburg *et al.,* 1982). Carlin *et al.* (1990) observed high potassium ion leakage and high lactic acid bacteria growth in fresh grated carrots when exposed to anaerobic conditions.

CONCLUSION

Apple production has a great economic importance, but it is affected by several preharvest and postharvest diseases. Despite the use of modern storage facilities, losses from 5 to 25% of apples are still being recorded. Fungal pathogens such as *B. cinerea*, *P. expansum* and *N. alba* are responsible for the main economical losses. After harvest, apples are stored at low temperature (0-1°C) to maintain quality and to minimize spoilage. However, development of fungal diseases, caused mainly by *B. cinerea, P. expansum* and *N. alba*, cannot be avoided. Control measures are still principally based on the protection of fruits

from prehaverst and postharvest infection by using fungicide treatment. Synthetic fungicides such as Iprodione (Rovral™), Thiabendazol (TBZ™) and Benomyl (Benlate™) are still used for control of postharvest diseases. However, in the context of consumer reluctance to accept chemical residues in food and of public concern for environmental safety, there is an increasing demand to develop alternative disease control methods. This becomes a critical issue with respect to deregistration of effective and widely used fungicides and the development of fungicide resistant strains of postharvest pathogens. In this context, alternative methods for plant disease control, including the use of microbes, their metabolites and products derived from plants, are highly desirable considering that the number of biological products available on the market for postharvest diseases control is still very limited. Some antagonists have been commercialized for control of postharvest diseases of fruit such as those registered in South Africa for fruit disease control i.e. *Bacillus subtilis* (Avogreen) for the control of prehaverst and postharvest disease of avocado and *Cryptococcus albidus* (Yieldplus®) for the control of postharvest diseases of apples and pears. Other commercial products such as *Pseudomonas syringae* (BioSave 110 and 111) to control *Geotrichum candidum* on pome fruit and citrus; *Candida oleophila* (Aspire™) to control *Penicillium* decay on citrus and pome fruits have been registered by Ecogen Inc. in the USA (Shachnal *et al*., 1996). The search for new products is, however, a continuous process and one can accept a significant growth in this market as new and more effective biocontrol agents are accepted onto the market.

However, biological control on its own is often less effective compared to commercial fungicides (Leverentz *et al*., 2003) or provides inconsistent levels of control. Therefore, to achieve a similar and consistent level of efficacy, the use of microbial antagonists integrated with commercial chemicals (Droby *et al*., 1998), hot water (Obagwu and Korsten, 2003), chloride salts (Wisniewski *et al*., 1995), carbonate salts (Obagwu and Korsten, 2003), natural plant extracts (Obagwu, 2003) and other physical treatments such as curing and heat treatments (Ikediala *et al*., 2002) have been used. Volatile chemicals (Poswal, 1996; Obagwu, 2003; Dudareva *et al*., 2004;), and essential oils (Plaza *et al.* 2004) from plant extracts were successful in controlling microbial diseases of some agricultural crops, stored fruits, vegetables and food commodities. Essential oils of plants and herbs are important natural sources of bioactive substances. In fact, there are increasing scientific evidence that support the use of essential oil against human and plant diseases

This review of the literature has shown that the potential of using essential oils in combination with controlled atmosphere regimes to control postharvest diseases of apples has not been investigated. Therefore, the aim of this study was to evaluate the antifungal efficacy of citrus and lemongrass essential oils in combination with controlled atmosphere storage regimes to control pathogenic fungi *B. cinerea*, *P. expansum* and *N. alba,* both *in*

vitro and *in vivo*. In Chapter 2 the chemical composition of citrus and lemongrass essential oils was determined using gas chromatography-mass spectrometry (GC-MS). The *in vitro* antifungal activity of citrus and lemongrass essential oils through direct contact and vapour phase methods on mycelial growth and spore germination of *B. cinerea, P. expansum* and *N. alba* were evaluated in Chapter 3. In Chapter 4 the mechanism of antifungal action of lemongrass essential oil on *B. cinerea* was investigated. In Chapter 5 the *in vivo* effects of citrus and lemongrass essential oils against *B. cinerea*, *P. expansum* and *N. alba* on apples applied as fumigation, dipping and thermal fogging treatments was investigated. The effect of citrus and lemongrass essential oil treatments on quality parameters of 'Granny Smith' and 'Pink Lady' apples after controlled atmosphere storage was investigated in Chapter 6. It is hoped that the results of this study will make a significant contribution to the South African apple fruit industry in helping to reduce postharvest decay in apples caused by *B. cinerea, P. expansum* and *N. alba*. Moreover, the results of this study will probably aid future research to the apple fruit industry to meet the demand for fresh fruit in the world apple fruit market and to reduce postharvest losses during exportation.

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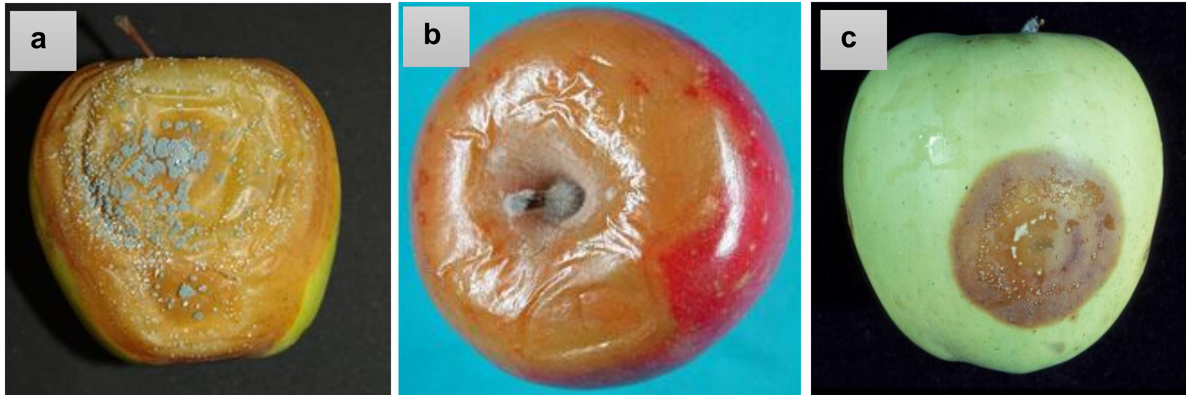

Figure 1. Postharvest fruit rots in apples caused by *Penicillium expansum* (a), *Botrytis cinerea* (b) and *Neofabraea alba* (c) (photos were taken from http://entomology.tfrec.wsu.edu/cullage_site/diseases.html).

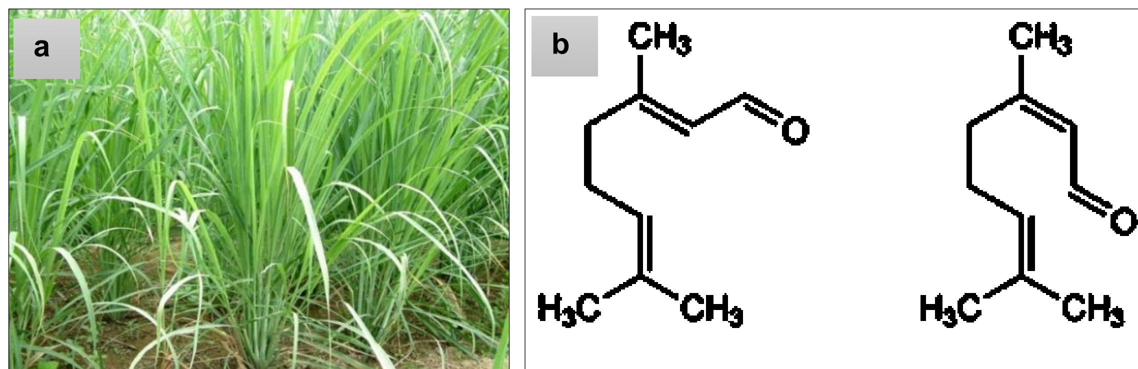

Figure 2. Lemongrass (*Cymbopogan citratus*) (A) and chemical structures of its major compounds, geranial and neral, respectively (Schaneberg and Khan, 2002).

Figure 3. Lemon (*Citrus limon*) (A), lime (*Citrus aurantifolia*) (B), and their chemical structure of its major compound, limonene (B) (Schaneberg and Khan, 2002).

Table 1. Examples of crude essential oils and their identified target sites and mode of action.

Common name	Species	Major constituents	Model organism	Mode of action	Reference
Cinnamon	*Cinnamomum verum*	E- cinnamaldehyde (73.35 %), β-caryophyllene (4.09 %), linalool (3.555 %), cinnamyl acetate (2.96 %), eugenol (2.68 %)	*Campylobacter jejuni* *Enterobacter aerogenes* *E. coli* *Listeria monocytogenes* *Pseudomonas aeruginosa* *S. enteritidis*	Released cellular content; reduced intracellular pH; affected membrane integrity	Wendakoon and Morihiko (1995), Smith-Palmer *et al.* (1998), Bouhdid *et al.* (2010)
	Cinnamomum cassia	Cinnamaldehyde (73.3 %), coumarin (10.6 %), cinnamic alcohol (3 %)	*S. aureus* *B. cereus* *S. aureus* *E coli* *Salmonella infantis* *L. monocytogenes*	Released cellular content; reduced intracellular pH; affected membrane integrity	Alzoreky and Nakahara (2003), Oussalah *et al.* (2006)
Cloves	*Syzytgium aromaticum*	Eugenol (64 %), eugenyl acetate (16.3 %), caryophyllene (14.5 %)	*C. jejuni* *E. coli* *E. aerogenes* *S. aureus*	Inhibited histidine decarboxylase (*E. aerogenes*)	Wendakoon and Morihiko (1995), Smith-Palmer *et al* (1998)
Coriander	*Coriandrum sativum*	Linalool (25.9-64.4 %), (E)-decenal (0-20.2 %), decanol (0.1 4-8.4 %), (E)-2-decen-1-o-l (0 7.9 %)	*Candida* species *L. monocytogenes*	Damage cytoplasmic membrane; released cellular content	De *et al.* (1999), Gill *et al.* (2002), Silva *et al* (2001)
Cumin	*Cuminum cyminum* L (seed)	Cumin aldehyde (29 %), α-terpinen-7-al (20.7 %), γ-terpinene (12.9 %), p-cymene (8.6 %)	*B. cereus* *B subtilis*	Mild changes in cytoplasm; cell wall envelope intact	De *et al.* (1999), Pajohi (2011)
Garlic	*Allium sativum*	Allicin (70 %)	*C. albicans* *E. coli* *L. monocytogenes* *S. typhi* *S. aureus*	Induced leakage from *E. coli* cells	Hughes and Lawson (1991), Kumar and Berwal (1998), Ankri and Mirelman (1999), Perry *et al.* (2009),
Kaffir lime	*Citrus hystrix*	Citronellol (10.7 %), limonene (7.3 %), linalool (5.8 %)	*A. flavus* *A. parasiticus*	Reduced aflatoxin production	Rammanee and Hongpattarakere (2011)
Lemongrass	*Cymbopogon citratus*	Geranial (45.7%), myrcene (3.9%). 6-methylhept-5-en-2-one (2.7%)	*L. innocula* *L. monocytogenes* *S. aureus*	Permeabilized membrane	Baratta *et al.*, (1998); Nguefack *et al.*, 2004
Lime	*Citrus aurantifolia*	Limonene (69.1%), *p*-cymene (12.8%)	*A. flavus* *A. parasiticus* *E. coli* *S. typhi*	Reduced aflatoxin production; extra- and intracellular damages to the cells	Rammanee and Hongpattarakere (2011)

Menthol	*Mentha longifolia*	Menthol (32.5 %), menthone (20.7 %), pulegone (17.8 %), 1, 8-cineole (5.6 %), terpineol-4 (4.9 %)	*S. typhimurium* *E. coli* *Micrococcus luteus* S. aureus	Damaged cell wall	Hafedh *et al* (2010)
Oregano	*Origanum compactus*	Carvacrol (36.5 %), thymol (29.7 %), *p*-cymene (24.3 %), γ-terpinene (1.1%)	*P. aeruginosa* *S. aureus*	Dissipated potassium gradient; depolarized membranes; permeabilized membranes, inhibited cell respiration; affected cell structure: coagulated cytoplasmic material; liberation of membrane vesicles; mesosome-like structures	Bouhdid *et al.* (2009), Babili *et al* (2001)
Rosemary	*Rosmarinus officinalis*	Carnosic acid, carnosol, rosmadial, genkwanin, rosmarinic acid	*C. albicans* *S. cerevisiae* *B. subtilis* *E. coli* *S. aureus*	Membrane-rigidifying effects; affected lipid polymorphism	Panizzi *et al.* (1993), Smith-Palmer *et al.* (1998), Perez-Fons *et al.* (2006)
Savory	*Satureja montana*	Thymol (29 %), *p*-cymene (12 %), linalool (11 %), carvacrol (10.7 %)	*C. albicans* *S. cerevisiae* *B. subtilis* *E. coli* *S. aureus*	Increased extracellular ATP, reduced intracellular pH, affected membrane integrity; structural damages; and cell lysis	Panizzi *et al.* (1993), Ousalah *et al.* (2006, 2007), De Oliveria *et al.* (2011)
Thyme	*Thymus vulgaris*	Thymol (31.4%), *p*-cymene (17%), carvacrol (12.4%), γ-terpinene (11.1%)	*E. coli* *Camphylobacter jejuni* *C. albicans* *B. subtilis* *S. aureus* *S. cerevisiae* *Erwinia amylovora*	Permeabilized membrane; caused changes in outer membrane protein profile of *Erwinia* strains	Panizzi *et al.* (1993), Smith-Palmer *et al.* (1998), Nguefack *et al.* (2004), Horvath *et al.* (2009)

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CHAPTER 2

VOLATILE COMPOSITION OF CITRUS AND LEMONGRASS ESSENTIAL OILS

Abstract

Volatile composition of essential oils of lemon (*Citrus limon*), lime (*Citrus aurantifolia*) and lemongrass (*Cymbopogan citratus*) were analysed using gas chromatography-mass spectrometry (GC-MS). *Citrus limon* showed 20 terpenes, 10 aldehydes, 10 alcohols, 2 esters and 2 unknown compounds; *C. aurantifolia* showed 8 terpenes, 5 aldehydes, 4 alcohols, 2 esters and 1 unknown compound; and *C. citratus* showed 8 terpene hydrocarbons, 9 aldehydes, 4 alcohols, 1 ketone, 2 esters and 2 unknown compounds. A total of 44 volatile compounds were detected and identified in *C. limon*, with limonene (58.52%) and γ-terpinene (19.80%) as the major compounds; *C. aurantifolia* showed 20 components with limonene (79.00%) and γ-terpinene (11.22%) as the abundant compounds. *Cymbopogan citratus* revealed 26 components with geranial (48.14%) and neral (38.32%) as the major compounds. *C. limon* and *C. aurantifolia* had the highest composition of monoterpenoid hydrocarbons, while *C. citratus* mainly consisted of carbonyl compounds and other hydrocarbons. These results will be instrumental in improving understanding of the chemistry and antifungal activity of *C. limon*, *C. aurantifolia* and *C. citratus* essential oils.

Keywords: *Citrus limon*, *Citrus aurantifolia*, *Cymbopogan citratus*, gas chromatography-mass spectrophotometry, volatile compounds, essential oils.

Introduction

Lemongrass (*Cymbopogon citratus* (DC.) Strapf) belongs to the order Poales and the family Poaceae (Shah *et al.,* 2011). It is an aromatic perennial tall grass with rhizomes and densely tufted fibrous root. It has short underground stems with ringed segments, coarse, green slightly leathery leaves in dense clusters (Carlin *et al.*, 1986). The plant is a native herb from India and is cultivated in South and Central America, Africa and other tropical countries (Figueirinha *et al.,* 2008; Shah *et al.,* 2011). 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, 2012). Several studies have reported on the antimicrobial activities of lemongrass oil (Saikia *et al.,* 2001; Appendini and Hotchkiss, 2002; Plotto *et al.,* 2003; Serrano *et al.,* 2005). Saikia *et al.* (2001) investigated the effect of three distinct

genotypes of *Cymbopogon* spp., namely *C. martini* (Roxb.) Will. Watson; *C. flexuosus* (Nees ex Steud.) Will. Watson and *C. winterianus* Jowitt ex Bor for *in vitro* antifungal activity against *Microsporum gypseum, Aspergillus niger, Candida albicans and Sporothrix schenckii*. The authors observed that the antifungal activity of *C. flexuosus* oil was most active followed by *C. martini* oil and *C. winterianus* oil (Saikia *et al.,* 2001).

Another genus of interest is the *Citrus* spp. with approximately 16 species in the family Rutaceae and are mainly cultivated in subtropical regions (Fisher and Phillips, 2008). Most of the species are cultivated for their fruits, including *Citrus limon* (Linn.) Burm. F. and *C. aurantifolia* (Christm.) Swingle, commonly known as lemon and lime, respectively (Singh and Singh, 2002). Citrus fruit essential oils (EOs) are extracted from the peel of fresh fruits by using a cold press system extraction. Since citrus EOs are mainly located in the fruit peel, their extraction is economically sustainable, because the fruit peel constitutes a waste for the fruit juice industry (Tirado *et al.,* 1995; Espina *et al.,* 2011). Citrus EOs have been industrially applied in many products, including food and beverages (e.g. as flavouring agents), cosmetics and medicines (Uysal *et al.,* 2011) and the antimicrobial activities against some of the most important foodborne pathogens (e.g. *Campylobacter jejuni*, *Escherichia coli*, *Listeria monocytogenes, Bacillus cereus* and *Staphylococcus aureus*) have been shown (Kim *et al.,* 1995; Fisher and Phillips, 2006).

In recent years there has been an increase interest in using natural substances (EOs and extracts) derived from plants for different industrial purposes, e.g. food seasoning and natural medicine (Isman, 2000; Cao *et al.,* 2004), and also pest management in agriculture (Mansingh, 2004). The main advantage of EOs is that they are considered 'generally recognised as safe' (GRAS) (Burt, 2004). The EOs are extracted from different parts of the aromatic plant (leaf, flower, seed or bark, fruit peels) using various techniques, including water or steam distillation, solvent extraction, expression under pressure, supercritical fluid or sub-critical water extractions (Burt, 2004). Essential oils are natural, complex, multi-component systems composed mainly of terpenes, in addition to some non-terpenes components (Delaquis *et al.,* 2002; Djenane *et al.,* 2011; Espina *et al.,* 2011). In view of the multiple applications of EOs, their characterization based on their chemical profiles, is of great importance. Components of essential oils are important as their qualitative and quantitative composition determines the characteristics of the oil, which in turn could have an influence on the antimicrobial activity/ efficacy of the EO (Dugo *et al*., 2000; Inouye *et al.,* 2001; Burt, 2004).

Qualitative and quantitative analysis of cold-pressed citrus EOs has been studied extensively (Shaw, 1979). Gas chromatography (GC) analysis has been the main technique used to quantify essential oil components. Quantitative GC analysis of cold-pressed citrus oils using preliminary separation steps to separate its volatile and non-volatile components

has been reported (Stanley *et al.,* 1961; Lifshitz *et al.,* 1970). For instance, Fisher and Philips (2006) and Anaruma *et al.* (2010) extracted and analysed EOs from *C. limon*, sweet orange (*C. sinensis*), bergamot (*C. bergamia*) and *C. citratus* using GC. The results indicated that limonene was more abundant than citral or linalool in the oils tested, while bergamot contained 15% linalool and sweet orange 3% citral; *C. citratus* showed geranial (47.52%) and neral (35.54%) as the major compounds. Regardless of chemical composition/volatiles being reported for other essential oils, no research has reported on South African lemon, lime and lemongrass essential oil. The objective of this study was to determine the chemical composition of essential oils extracted from lemon [*Citrus limon* (Linn.) Burn], lime [*Citrus aurantifolia* (Christm.) Swingle] and lemongrass [*Cymbopogon citratus* (DC.) Strapf] in order to identify which compound(s) contribute to the essential oil antifungal activity.

Materials and methods

Essential oils

Essential oils of lemon (*Citrus limon*), lime (*Citrus aurantifolia*) and lemongrass (*Cymbopogon citratus* (DC.) Strapf] were obtained from Groenkop Farm (Melmoth, KwaZulu-Natal, South Africa) and stored at 4°C until used. Moreover, raw oils of lemon and lime were originally extracted from the fresh fruit peel by steam distillation, while lemongrass oil was extracted from fresh or partly dried leaves by steam distillation.

Gas chromatography-mass spectrometry analysis of essential oils

The gas chromatography-mass spectrometry (GC-MS) analysis of the essential oils was performed on an Agilent 6890N GC (Agilent, Palo Alto, CA) gas chromatograph coupled with an Agilent 5975B MS (Agilent, Palo Alto, CA) mass selective detector. The ZB 274305 SemiVolatiles column (30 m x 0.25 mm id x 0.25 μm) was used for the separation of components. Helium was used as a carrier gas at a constant pressure of 60.1 kPa. Sample of individual essential oil was diluted in hexane (1:10000). Approximately 1 μL of the mixture was thereafter injected into the column with a split ratio of 10:1. The oven temperature was maintained as follows: 70 °C for 0.5 min, and then ramped up to 100 °C at the rate of 5 °C/min; then ramped up to 240 °C at the rate of 5 °C/min, finally ramped up to 300 °C at a rate of 25 °C/min, and kept at that temperature for 3 min. Mass spectra were analysed in the SCAN mode over the range of 35 to 600 m/z. Under the previously mentioned GC-MS conditions, the main components of essential oils were analysed and the total ion currents were obtained. The individual peaks were identified by comparing their mass spectra with

National Institute of Standards and Technology (NIST) and also comparison with those published in the literature. For quantification, the percentage relative abundances was used. There were three replicates for each oil.

Results

Chemical composition of essential oils

Qualitative and quantitative analytical results of *C. limon*, *C. aurantifolia* and *C. citratus* are shown in Figs. 1-3 and Table 1. A total of 44 components were identified in lemon essential oil, representing 99.45% of the detected components. The essential oil of lemon was dominated by monoterpene hydrocarbons (79.90%) with limonene (58.52%) followed by γ-terpinene (19.80%) as the major components; while minor components observed were β-bisabolene (3.25%), geranial (3.07%), E,E-α-farnesene (2.20%), neral (1.86%), neral acetate (1.85%) and β-caryophyllene (1.01%). Other components were present in amounts less than 1.00%.

On the other hand, lime essential oil exhibited 20 components, representing 100.00% of the detected components. Lime essential oil was dominated by monoterpene hydrocarbons (90.81%) with limonene (79.00%) followed by γ-terpinene (11.22%) as the major components. The minor compounds presented in significant percentages in lime oil investigated were geranial (2.02%), β-bisabolene (1.33%) and neral (1.19%). Other components were present in amounts less than 1.00%.

A total of 26 components were identified in lemongrass essential oil, accounting for 98.24% of the detected components. Lemongrass essential oil was dominated by carbonyl compounds (88.39%) with geranial (48.14%) followed by neral (38.32%) was the major components. The minor compounds presented in significant percentages in lemongrass essential oil investigated were bicyclo[4.1.0]heptane, 3-methyl- (1.80%) and geraniol (1.34%). Other components were present in amounts less than 1.00%.

Discussion

Essential oils obtained from plants have many potential applications, including their use as food additives, preservatives, in perfumes and in pharmaceuticals. Volatiles found in essential oils are classified as hydrocarbons (terpenes), derivatives of the hydrocarbons (oxygenated terpenoid compounds), aromatic compounds (benzenoid structures) and compounds containing nitrogen or sulphur (Reineccius, 1994). In this study, different compositional ratios of these classes of volatiles for lemon, lime and lemongrass essential

oils were found. As previously reported by Shaw (1979) and Lancas and Cavicchiolo (1990), limonene, a monoterpene hydrocarbon, is the major component of lime and other related citrus essential oils. This is consistent with the findings from this study. Higher percentage of limonene was detected in lime essential oil (79.0%) compared to lemon and lemongrass essential oils (58.52% and 0.18%, respectively). Similarly, Di Vaio *et al*. (2010) reported limonene as the main component in the lemon essential oil, accounting for 72.5-76.4%, followed by β-pinene (11.6-18.7%). Furthermore, Bialon *et al.* (2014) recently reported higher amounts of limonene in lemon essential oils of the German company ETJA (48.3%), Avicenna-Oil (42.0%) and the Italian company Croce Azzurra (38.5%). The essential oil of *C. aurantifolia* (lime) has the major constituents, limonene (58.4%), β-pinene (15.4%), γ-terpinene (8.5%), and citral (4.4%) (Spadaro *et al*., 2012). These findings are similar to data provided by Yadav *et al.* (2004) where limonene (37.8%) was the major component followed by b-pinene (16.0%) g-terpinene (9.5%), nerolidol (7.1%) and a-terpineol (6.7%).

In this study, lemon and lime essential oils consist of monoterpene hydrocarbons (79.9% and 90.8%, respectively) with limonene as the major compound (58.52% and 79.0%, respectively). These results are contrary to the major component reported by Vekiari *et al.* (2002), Ahmad *et al.* (2006), Hosni *et al*. (2010) and Espina *et al.* (2011). Hosni *et al.* (2010) reported limonene (85.50%) as the major compound of Citrus EOs. The *Citrus* essential oils consist mainly of monoterpene hydrocarbons (97.59-99.3%), with limonene (92.52-97.3%) and β-pinene (1.37-1.82%) being the major constituents (Hosni *et al*., 2010). Espina *et al.* (2011) reported limonene (85.50%) and *α*-terpineol (0.36%) as chief component of *Citrus* essential oils. The major constituents of *Citrus* peel oils investigated by Vekiari *et al.* (2002) were limonene, neral, geranial, *β*-pinene, *β*-caryophyllene and neryl acetate. Ahmad *et al.* (2006) stated limonene (86.27%), *γ*-terpinene (2.11%) and *α*-pinene (1.26%) in Grapefuit peel essential oil and limonene (76.28 %), *β*-pinene (5.45%), linalool (2.32%), citral (1.74%) and *α*-pinene (1.26%) in Mousami peel oil.

The essential oil of lemongrass contains mainly geranial and neral as the major compounds (Schaneberg and Khan, 2002; Huynh, 2008). This is similar to the findings observed in this study. Lemongrass essential oil was dominated by carbonyl compounds (88.39%) with geranial (48.14%) followed by neral (38.32%) as the major components. Components such as geranial, neral, geraniol, limonene and β-myrcene have been found as major compounds in many other *Cymbopogon* species (Luiz *et al*., 2001; Huynh, 2008). Other isolated components, such as β-myrcene, ocimene, β- ocimene, linalool, citronellal, caryophyllene and β-pinene, were present as minor components (Torres and Ragadioa, 1996). Koba *et al.* (2009) analysed chemical composition of lemongrass essential oil from Togo and observed that geranial (45.2%), neral (32.4%) and myrcene (10.2%) were the main components of this essential oil. Similar observation was reported by Choi *et al.* (2012),

with geranial (50.04%) and neral (36.20%) as major constituents of lemongrass essential oil. Other studies reported variations of 20-50% of geranial and 30-40% of neral in lemongrass volatile composition (Nath *et al.,* 1994; Weiss, 1997; Pandey *et al*., 2003; Chandrashekar and Joshi, 2006). Tyagi and Malik (2010) analysed lemongrass oil and found geranial (36.2%), neral (26.5%), nerol (5.1%), limonene (4.2%), neryl acetate (4.0%) and 5-hepten-2-one (2.9%). In this study, the amount of geranyl and neral was higher, however, limonene content (0.18%) was lower.

The monoterpene hydrocarbons represent 79.0% and 90.81% of the total composition of lemon and lime oils, respectively, while only 0.74% of lemongrass oil. On the other hand, carbonyl compounds represent more than 85% of lemongrass oil and only 5% and 3.6% of lemon and lime oils, respectively. Volatile classes of essential oils are responsible for the superior aroma of the oil (Baser and Buchbauer, 2010). Variations in the chemical composition of essential oils are also due to the differences in the methods of extraction of the volatile oils (Peter, 2003), chemotypes, different geographical location, and seasonal variations (Njoroge *et al.,* 1994; Nannapaneni *et al.,* 2009; Nejad Ebrahimi *et al*., 2010; Pereira and Meireles, 2010; Wanner *et al*., 2010; Nejad Ebrahimi *et al*., 2010; Demuner *et al.,* 2011; Paibon *et al.,* 2011).

In conclusion, results reported in this study confirmed previous observations that citrus and lemongrass essential oils are mainly composed of monoterpenes hydrocarbons and carbonyl compounds, respectively, of which, limonene, neral and geranial were the main components.

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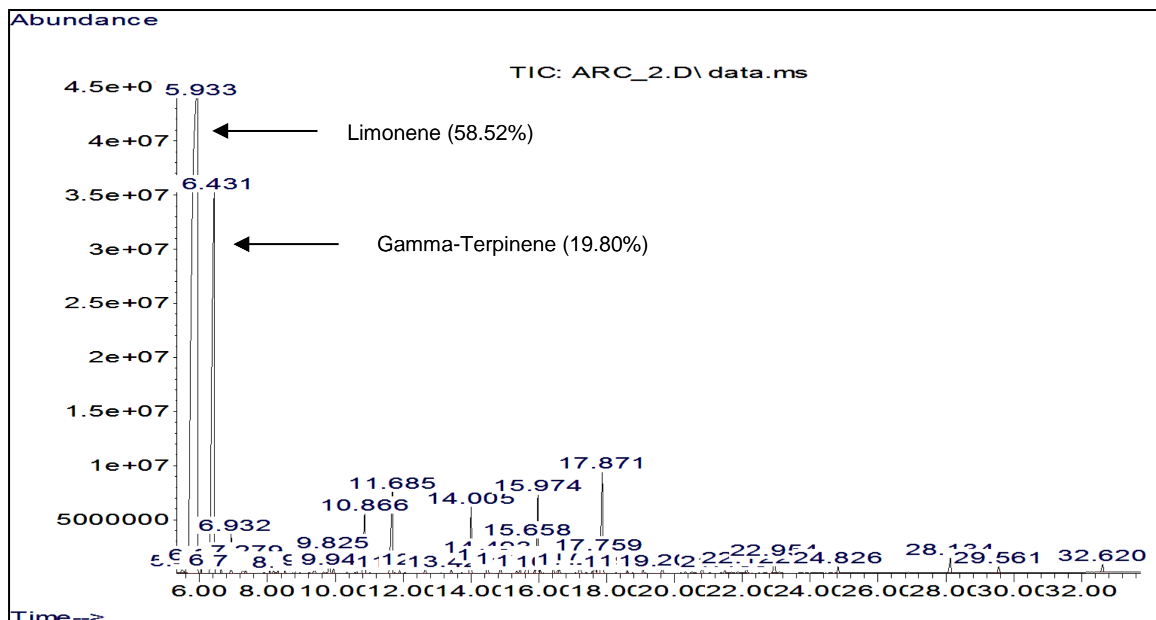

Figure 1. GC-MS chromatogram showing the peak areas of compounds present in lemon essential oil in relation to retention times. A ZB 274305 SemiVolatiles column was used.

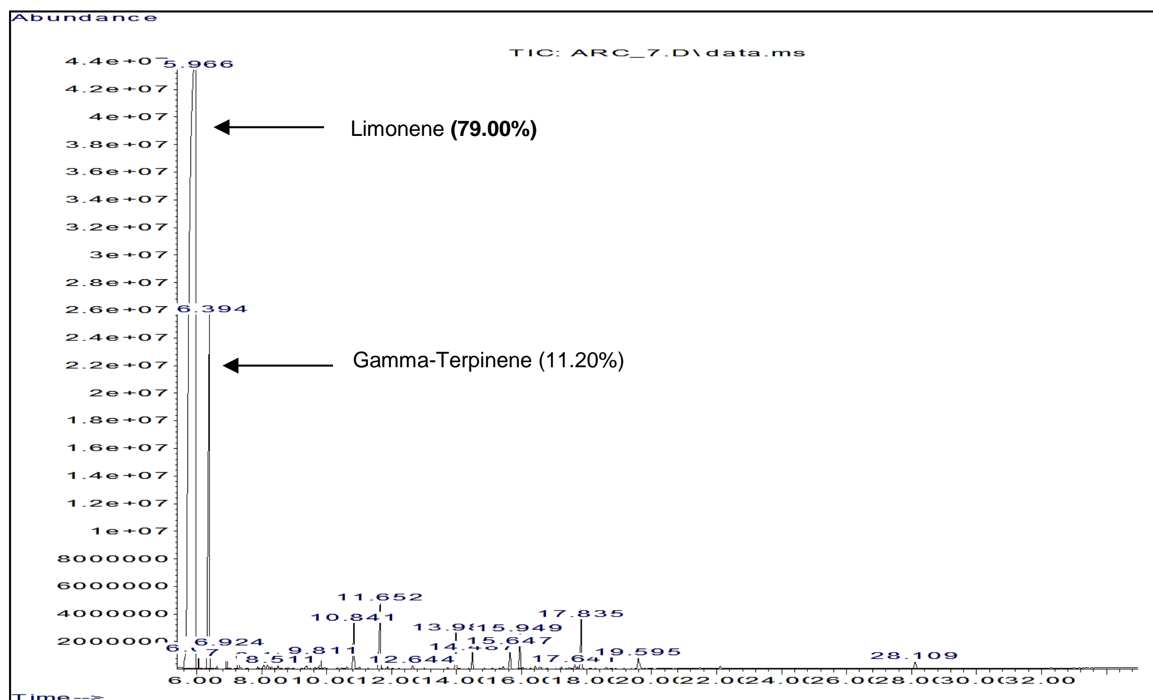

Figure 2. GC-MS chromtogram showing the peak areas of compounds present in lime essential oil in relation to retention times. A ZB 274305 SemiVolatiles column was used.

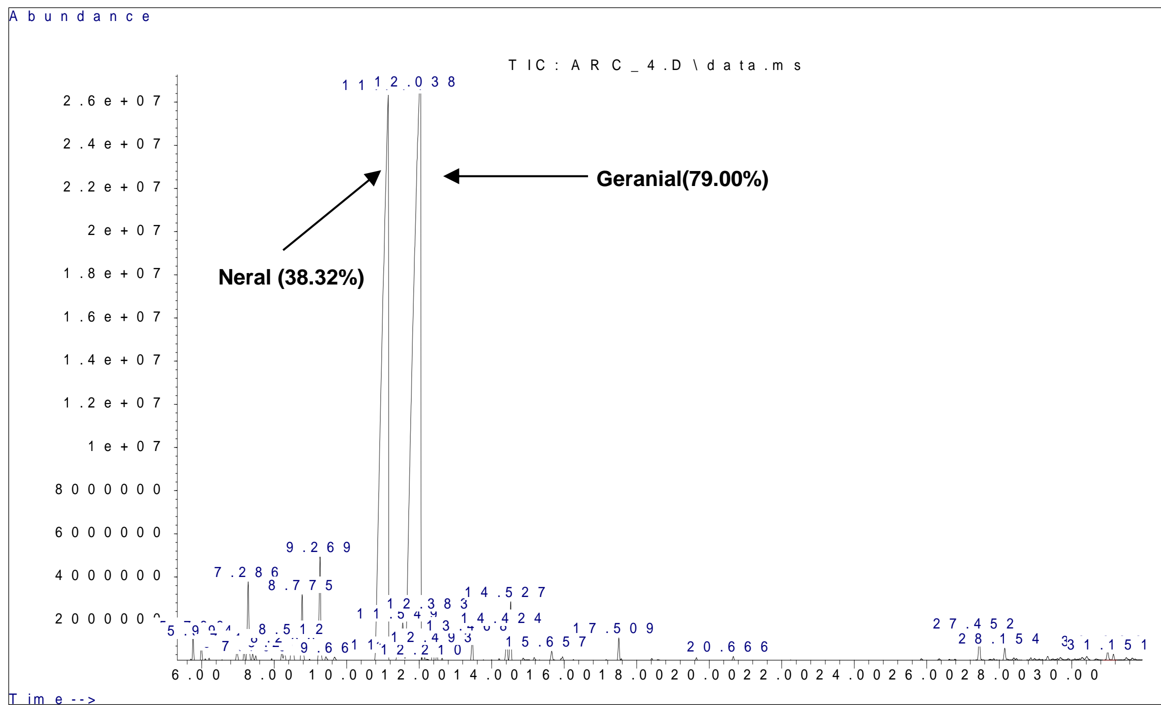

Figure 3. GC-MS chromatogram showing the peak areas of compounds present in lemongrass essential oil in relation to retention times. A ZB 274305 SemiVolatiles column was used.

Table 1. Chemical composition of lemon, lime and lemongrass essential oil by GC-MS.

Group	Components	Relative abundance* (%)		
		Lemon	**Lime**	**Lemongrass**
Terpenes (monoterpenes and sesquiterpene)	(+)-2-Carene	0.20	x	x
	(+)-β-funebrene	0.23	x	x
	β-elemene	0.26	x	x
	β-bisabolene	3.34	1.33	x
	β-caryophyllene	1.01	0.63	0.17
	β-santalene	0.09	x	x
	cis-β-farnesene	x	x	0.20
	cis-ocimene	x	x	0.31
	cis-α-bisabolene	0.09	x	x
	δ-elemene	0.09	x	x
	E,E-α-farnesene	2.20	0.85	x
	Farnesene	0.52	x	x
	Germacrene B	0.10	x	x
	Limonene	58.52	79.00	0.18
	trans-β-farnesene	0.19	x	x
	trans-α-bisabolene	0.40	0.11	x
	trans-β-ocimene	0.15	0.21	0.25
	α-bergamotene	0.16	x	x
	α-humulene	0.10	x	x
	γ-elemene	0.09	x	x
	γ-terpinene	19.80	11.22	x
	1,5-Heptadiene, 3,3-dimethyl-, (E)-	x	x	0.24
	α-terpinolene	0.81	0.38	x
	3-Octyne, 2,2,7-trimethyl-	x	x	0.18
	Bicyclo[4.1.0]heptane, 3-methyl- (CAS)	x	x	1.80
Aldehydes	2-Caren-10-al	x	x	0.22
	2H-1-Benzopyran-2-one, 7-methoxy- (Herniarine)	0.48	x	x
	3-(4-methyl-3-pentenyl)-furan (perillene)	x	x	0.19

	Bergapten (Xanthotoxin)	0.23	x	x
	cis-limonene oxide	0.09	0.20	x
	Dodecanal	0.08	x	x
	Geranial	3.07	2.02	48.14
	1-Undecyne	x	x	0.51
	Isopimpinellin	0.28	x	x
	Neral	1.86	1.19	38.32
	Perillaldehyde	0.07	x	x
	Piperitone	x	x	0.99
	trans-chrysanthemal	x	x	0.14
	trans-limonene oxide	0.13	0.17	x
	2H-1-Benzopyran-2-one, 5,7-dimethoxy-	0.20	0.24	x
	(+)-Carvotanacetone	x	x	0.39
	(6E,10E)-2,6,12,15-tetramethylhexadeca-2,6,10,14-tetraen-8-yne	x	x	0.44
Alcohols	*cis*-sabinene hydrate	0.10	x	x
	trans-thujan-4-ol	0.40	x	x
	Terpinen-4-ol	0.14	x	x
	α-terpineol	0.60	0.33	x
	Caryophyllene oxide	0.12	0.43	x
	Espatulenol	0.16	x	x
	Ledol	0.09	x	x
	α-bisabolol	0.19	x	x
	Geraniol	x	x	1.34
	β-citronellol	x	x	0.09
	Citronellal	0.06	0.08	0.34
	Linalool L	0.31	0.23	0.98
Esters	Geranyl acetate	0.45	0.40	0.82
	Neryl acetate	1.85	0.86	x
	Geraniol formate	x	x	0.17
Ketones	2-undecanone	x	x	0.57
Unknown	Unknown 1	0.14	0.12	0.10

	Unknown 2	0.17	x	1.16
	Total identified	**99.45%**	**100.00**	**98.24**
	Terpenes	88.35	93.73	3.33
	Aldehydes	6.49	3.82	89.34
	Alcohols	2.17	1.07	2.75
	Esters	2.30	1.26	0.99
	Ketones	x	x	0.57
	Unknown	0.14	0.12	1.26

*The relative abundance represents the average of three analyses. x means the compound is not present.

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CHAPTER 3

IN VITRO EFFECTS OF CITRUS AND LEMONGRASS ESSENTIAL OILS ON *BOTRYTIS CINEREA*, *PENICILLIUM EXPANSUM* AND *NEOFABRAEA ALBA*

Abstract

The use of essential oils to control pathogen growth has gained significance due to the resistance acquired by pathogens towards a number of widely-used fungicides. The aim of this study was to test *in vitro* antifungal activity of citrus and lemongrass essential oils against *Botrytis cinerea, Penicillium expansum* and *Neofabraea alba*. Direct contact and vapour phase methods were used to test the antifungal activity of these essential oils against *B. cinerea*, *P. expansum* and *N. alba,* the causal agents of postharvest decay of apples. Direct contact phase of lemongrass essential oil, mixtures of lemon + lemongrass and lime + lemongrass essential oils exhibited the strongest toxicity and completely inhibited the mycelial growth and spore germination of *B. cinerea*, *P. expansum and N. alba,* at concentrations of 1.5-100% at 20°C (95% relative humidity), regular atmosphere (-0.5°C, air), and controlled atmosphere (5%O_2 + 10% CO_2, -0.5°C). Lemongrass essential oil was fungicidal against *B. cinerea*, *P. expansum* and *N. alba* both in its direct contact and vapour phase of 1.5% and 0.125%, respectively, at 20°C, regular atmosphere and controlled atmosphere. Similar results were observed for mixtures of lemon + lemongrass and lime + lemongrass essential oils. Lemon and lime essential oils alone also showed antifungal activity, however were much less effective than lemongrass essential oil. In the direct contact method, mixtures of lemon + lime did not show any inhibitory activity against *B. cinerea* at 20°C, regular atmosphere and controlled atmosphere. In the case of vapour phase method, all essential oil treatments significantly (P < 0.05) inhibited all three pathogens at concentrations of 0.016-1.0% compared to control treatments. Mycelial growth of fungal pathogens was inhibited by the tested essential oils in a dose-dependent manner. This study has demonstrated that *C. limon*, *C. aurantifolia* and *C. citratus* essential oils (alone and in combination with cold storage regimes) have a potential for the control of postharvest pathogens of apples caused by *B. cinerea*, *P. expansum* and *N. alba*.

Keywords: Antifungal activity, cold storage regimes, essential oils, postharvest pathogens.

Introduction

Fungal infections are the main source of postharvest rots of fruits and vegetables during storage and transport and cause significant economic losses during postharvest handling (Chan and Tian, 2005; Gatto *et al.,* 2011). Grey mould, blue mould and bull's eye rot caused by *Botrytis cinerea* Pers.:Fr., *Penicillium expansum* (Link) Thom., *Neofabraea alba* (E.J. Gutrie) Verkley, respectively, are important postharvest diseases of apples (Solaimani *et al.,* 2009; Tripathi *et al.,*2008). *Botrytis cinerea* and *P. expansum* are considered among the most serious postharvest diseases affecting various types of fruit including apples (Ten-Have *et al*., 1998; Tripathi *et al.,* 2008; Kader, 2002; Jijakli and Lepoivre, 2004; Kim and Xiao, 2008). *Botrytis cinerea* is a ubiquitous fungus that infects a wide range of horticultural and field crops. Conidia are abundant and colonize organic matter on the ground (Xiao, 2006). They are readily produced on dead infected plant organs and are dispersed by water splash and air currents. *Penicillium expansum* can grow at temperatures as low as -3ºC and conidia can germinate at 0ºC (Rosenberger and Sugar, 1990). *Neofabraea alba* is considered a weak parasite (Bompeix and Bondoux, 1974) to purely saprophytic (Bompeix, 1988; Edney, 1956). Infection in the orchard can occur at any time during the growing season and incipient infections remain latent until symptom development after three to five months in storage (Snowdon, 1990; Neri *et al*., 2009; den Breeyen, 2012). Current postharvest disease control strategies rely on registered fungicides. Continuous use of fungicides has faced two major challenges; increasing public concern regarding contamination of fruits and vegetables with fungicidal residues, and development of resistance in pathogen populations (Eckert *et al*., 1994; Holmes and Eckert, 1999; Tripathi and Dubey, 2004). These challenges have led to a need to develop alternative control methods, including adoption of natural plant products with fungicidal properties such as essential oils, plant extracts, antagonistic microorganisms such as yeast and bacteria (Sholberg and Conway, 2004; Conway *et al*., 2005; Soylu *et al.,* 2005; Droby, 2006).

Several researchers have reported antimicrobial activity of plant extracts and essential oils which have potential to meet requirements for non-hazardous eco-friendly treatments to control postharvest diseases of fruits (Paster *et al.,* 1995; Inouye *et al.,* 1998; Hammer *et al.,* 1999; Janisiewicz and Korsten, 2002). Essential oils and their main components possess a wide range of biological activity, which may be of great importance, and the main advantage of essential oils is that they are considered 'generally recognized as safe' (GRAS) (Burt, 2004). Antifungal activities of certain essential oils or their components have also been assessed and found effective for *B. cinerea* and *Monilinia fructicola* (Wilson *et al.,* 1987), *Rhizoctonia solani, Fusarium moniliforme* and *Sclerotinia sclerotiorum* (Muller *et al.,* 1995), *Aspergillus niger* (Paster *et al.,* 1995), *Penicillium* spp. (Caccioni and Guizzardi,

1994; Arras and Usai, 2001), *P. digitatum* (Daferera *et al.,* 2000) and *F. solani, Pythium ultimum* and *Colletotrichum lindemuthianum* (Zambonelli *et al*., 1996). The aim of this study was to evaluate the *in vitro* antifungal activity of lemon [*Citrus limon* (Linn.) Burm], lime [*Citrus aurantifolia* (Christm.) Swingle] and lemongrass [*Cymbopogon citratus* (DC.) Strapf] essential oils in combination with cold storage regimes through direct contact and vapour phase methods on mycelial growth and spore germination of *B. cinerea*, *P. expansum and N. alba.*

Materials and methods

Pathogen isolation

Three fungal pathogens, *N. alba* (Accession number: DOK7-SUN) isolated from infected apples, *B. cinerea* (Accession number: PPRI 7338) and *P. expansum* (Accession number: PPRI 5944) isolated from infected plums and pears, respectively, were used. All the isolates were tested for pathogenicity on apples and pure isolates were prepared (single spore). *Neofabraea alba* was cultured on acidified potato dextrose agar(aPDA) (pH 3.5, Merck, Johannesburg, South Africa) for 1 month at 25°C. *P. expansum* and *B. cinerea* were cultured on potato dextrose agar (PDA, pH 5.6, Merck, Johannesburg, South Africa) at 25°C for 3 days for mycelial plugs and 7 days for the production of spores. The cultures of *B. cinerea, P. expansum* and *N. alba* were maintained on PDA slants at 4°C. Conidia were harvested from the medium surface with sterile distilled water together with Tween 80 (0.05% W/V), and gentle agitating the plates to dislodge the spores. The final inoculum concentration was adjusted to 1×10^4 conidia mL^{-1} for each pathogen using a haemocytometer.

Essential oils

Essential oils of lemon (*Citrus limon*), lime (*Citrus aurantifolia*) and lemongrass (*Cymbopogon citratus*) were obtained from Groenkop Farm (Melmoth, KwaZulu-Natal, South Africa) and stored at 4°C until used. Moreover, raw oils of lemon and lime were originally extracted from the fresh fruit peel by steam distillation, while lemongrass oil was extracted from fresh or partly dried leaves by steam distillation.

Direct contact effects of essential oils on mycelial growth and conidial germination

The inhibitory effects of three essential oils were tested individually and in combinations against mycelial growth and spore germination of *B. cinerea*, *P. expansum* and *N. alba.* Potato dextrose agar was autoclaved (121°C) and cooled in a water bath at 40°C. Different

concentrations of essential oils ranging from 1.5-60% were prepared by adding the requisite amount of the essential oil to ethanol (40%, v/v) to make up a volume of 2.8 mL, which was then added to 140 mL of PDA medium (pH 5.6). The amended PDA was poured into 90 mm sterile Petri plates (20 mL/plate). Discs (3 mm diameter) were excised from the edge of 5-day old *B. cinerea* and *P. expansum* cultures, or from 10-day old *N. alba* cultures and placed at the centre of each amended PDA plates. The pure essential oils (100% concentration) were also tested. In control sets, equal amounts of sterilized distilled water (positive control) and 40% ethanol (negative control) were amended with PDA. Inoculated Petri plates were incubated at (a) 20°C (95% relative humidity) for 7 days, (b) 7 days at regular atmosphere (-0.5˚C, air) followed by 5 days at 20°C, (c) 7 days at controlled atmosphere (5%O_2 + 10% CO_2, -0.5°C) followed by 5 days at 20°C. Radial growth of *B. cinerea, P. expansum* and *N. alba* cultures was measured using a vernier calliper (± 0.01 mm). The mean growth values were obtained and then converted into the inhibition percentage of mycelial growth in relation to control treatment by using the formula, MGI (%) = ((dc-dt)/dc) x 100, where dc = mycelial growth diameter in control sets (sterile distilled water), dt = mycelial growth diameter in treatment sets. Determination of *in vitro* antifungal activities of essential oils on conidial germination was examined by spreading 100 µL of the pathogens spore suspension (10^4 spore mL^{-1}) on the amended PDA medium. Plates were incubated as described above. Germination of spores was observed with a microscope after incubation and recorded as conidia germinated (+) and non-germinated (-). The experiment was performed in three replications and repeated twice.

Vapour phase effects of essential oils on mycelial growth and conidial germination

Disposable Phytatray containers (175(L) x 150(W) x 65(H), Zibo containers (Pty) LTD, Kuilsrivier, South Africa) with sterilized lid were used as a chamber containing the essential oil and the fungus. Discs (3 mm diameter) were excised from the edge of 5-day old *B. cinerea* and *P. expansum* cultures, or from 10-day old *N. alba* cultures and placed at the centre of PDA plates (65 mm). From direct contact method it was evident that essential oils were effective and it was decided for vapour phase method to use lower concentrations. The effect of 10-30% ethanol on pathogen growth was tested in its vapour phase, therefore 20% ethanol was selected. Different concentrations of essential oils ranging from 0.016-1.5% were prepared. Potato dextrose agar was poured into 65 mm sterile Petri plates (20 mL/plate). A volume of 5 mL of the requisite concentration of the oil treatments were introduced into 65 mm glass sterile Petri dish. Both inoculated agar plates and glass sterile Petri plate were placed in Phytatray containers. The control sets were prepared similarly using equal amounts of sterilized distilled water or ethanol (20%, v/v) in place of the oil. Inoculated Petri plates were incubated at (a) 20°C (95% relative humidity) for 7 days, (b) 7

days at regular atmosphere (-0.5˚C, air) followed by 5 days at 20°C, (c) 7 days at controlled atmosphere (5%O_2 + 10% CO_2, -0.5°C) followed by 5 days at 20°C. Percentage of mycelial growth inhibition (MGI) was calculated as previously described. Inhibition of conidial germination was examined by spreading 100 µL of the pathogens spore suspension (10^4 spore mL^{-1}) on PDA plates (65 mm) and exposing inoculated plates to vapours of essential oils as described above. Petri plates were incubated as described above. Germination of spores was observed with a microscope after incubation and recorded as conidia germinated (+) and non-germinated (-). The experiment was performed in three replications and repeated twice.

Determination of antimicrobial properties of essential oils

The minimum concentration of essential oils required to give complete control or the minimum inhibitory concentration (MIC) for pathogen growth was determined. The MIC of each of the essential oil was classified as fungicidal (permanent inhibition) or fungistatic (temporary inhibition) in its effect. The agar discs of the pathogens which failed to grow were either transferred onto PDA media without oils (for contact phase effect of oils) or onto Phytatrays containing distilled water without oil (for volatile phase effect of oils). Petri plates were incubated for 5 days at 20°C (95% relative humidity). Activity of the MIC of the various oils was considered fungicidal if the pathogen did not grow or fungistatic if the pathogen growth occurred.

Statistical analysis

For *in vitro* trials, the experimental design was a split plot with essential oils at different concentrations as main plot treatments and storage regime as split plot factor. The main plot design was completely random with three replications for each treatment at each concentration prepared at random. Data for the two trials was combined and Univariate analysis of variance was performed on all data accessed using GLM (General Linear Models) Procedure of SAS statistical software version 9.2 (SAS Institute Inc., Cary, NC, USA). Shapiro-Wilk test was performed to test for normality (Shapiro, 1965). Fisher's least significant difference was calculated at the 5% level to compare treatment means (Ott, 1998). A probability level of 5% was considered significant for all significance tests.

Results

Direct contact effects of essential oils on mycelial growth and conidial germination

The direct contact effect of different concentrations of essential oils on mycelial growth of *B. cinerea*, *P. expansum* and *N. alba* is shown in Tables 1-3. Table 1 shows that mycelial

growth of *B. cinerea* was inhibited by lime essential oil in a dose-dependent manner at 20°C. Lemon essential oil exhibited complete inhibition of *B. cinerea*, *P. expansum* and *N. alba* at concentrations of 20-100%, while lime essential oil showed complete inhibition at concentrations of 35-100%. Lemongrass essential oil was more inhibitory to *B. cinerea, P. expansum* and *N. alba* compared to lemon and lime essential oils. Mycelial growth of the three test pathogens was completely inhibited (100%) by lemongrass essential oil at concentrations of 1.5-100%. A combination of lemon + lemongrass essential oils, lime + lemongrass essential oils was more inhibitory (100%) to *B. cinerea, P. expansum* and *N. alba* compared to lemon + lime essential oil combination. A combination of lemon + lime essential oils did not inhibit mycelial growth of *B. cinerea* at all concentrations tested and it was not significantly different ($P < 0.05$) from the water control. Mycelial growth of *P. expansum* and *N. alba* was slightly inhibited by a combination of lemon + lime essential oils, however it was not significantly different from the water control.

Mycelial growth of *B. cinerea, P. expansum* and *N. alba* was completely inhibited by all essential oil treatments in combination with regular atmosphere (-0.5°C, air) and controlled atmosphere (5%O_2 + 10% CO_2, -0.5°C) cold storage regimes after 7 days including control treatments (data not shown). Therefore, Petri plates were further incubated at 20°C for 5 days and the results are shown in Tables 2-3. Mycelial growth inhibition of *B. cinerea*, *P. expansum* and *N. alba* increased with increasing concentrations of lemon and lime essential oils. Lemon essential oil exhibited complete inhibition of *B. cinerea*, *P. expansum* and *N. alba* at concentrations of 20-100%, while lime essential oil showed inhibition at concentrations of 35-100%, for both cold storage regimes (Tables 2 and 3). Lemongrass oil exhibited complete inhibition (100%) of *B. cinerea* at all concentrations tested. Essential oil combinations of lemon + lemongrass, lime + lemongrass showed 100% inhibition of *B. cinerea*, *P. expansum* and *N. alba* at all concentrations tested, compared to lemon + lime combination (Tables 2 and 3).

The effect of different concentrations of direct contact phase of essential oils on spore germination of *B. cinerea, P. expansum* and *N. alba* is shown in Tables 4-6. Complete germination inhibition of all three pathogens by lemon and lime essential oils was observed at concentrations of 20-100% and 35-100% respectively, while lemongrass oil showed complete germination inhibition at concentrations of 1.5-100% (Table 4). A combination of lemon + lemongrass, lime + lemongrass completely inhibited spore germination of *B. cinerea, P. expansum* and *N. alba* at concentrations of 1.5-100% and not significantly different from lemongrass essential oil. Lemon + lime essential oil combination exhibited positive spore germination of *B. cinerea, P. expansum* and *N. alba* at all concentrations tested (Table 4).

Spore germination of all three pathogens was completely inhibited by all essential oil treatments in combination with regular atmosphere (-0.5°C, air) or controlled atmosphere (5%O_2 + 10% CO_2, -0.5°C) cold storage regimes after 7 days including the control treatments (data not shown). Therefore, Petri plates were then further incubated at 20°C for 5 days and the results are shown in Tables 5 and 6. Complete germination inhibition of *B. cinerea, P. expansum* and *N. alba* by lemon and lime essential oils alone was observed at concentrations of 20-100% and 35-100%, respectively (Tables 5 and 6). Lemongrass showed complete germination inhibition of all three pathogens at concentrations of 1.5-100%. Essential oil combinations of lemon + lemongrass, lime + lemongrass completely inhibited spore germination of *B. cinerea, P. expansum* and *N. alba* at concentrations of 1.5-100%. Lemon and lime essential oil combination exhibited positive spore germination of all three pathogens at all concentrations tested

Vapour phase effects of essential oils on mycelial growth and conidial germination

The vapour phase effect of different concentrations of essential oils on mycelial growth inhibition of *B. cinerea, P. expansum* and *N. alba* is shown in Tables 7-9. At 20°C, all the essential oils effectively controlled mycelial growth of *B. cinerea, P. expansum and N. alba* and were significantly different ($P<0.05$) from the control treatments. Increasing concentration of essential oil vapours of lemon, lime, lemongrass, essential oil combinations of lemon + lemongrass and lime + lemongrass increases mycelial growth inhibition of *B. cinerea, P. expansum* and *N. alba* (Table 7). Lemongrass essential oil showed complete inhibition of *B. cinerea* at concentrations of 0.125-1.0%. These results were not significantly different from essential oil combinations of lemon + lemongrass, lime + lemongrass at concentrations of 0.125-1.0% (Table 7). It was observed that mycelial growth of *B. cinerea, P. expansum* and *N. alba* was completely inhibited by all essential oil treatments in combination with regular atmosphere (-0.5°C, air) and controlled atmosphere (5%O_2 + 10% CO_2, -0.5°C) cold storage regimes after 7 days including control treatments (data not shown). Therefore, Petri plates were further incubated at 20°C for 5 days and the results are shown in Tables 8 and 9. All essential oil treatments in combination with either regular atmosphere or controlled atmosphere reduced mycelial growth of *B. cinerea, P. expansum* and *N. alba* with increasing concentrations. Lemongrass oil was also more inhibitory to *B. cinerea, P. expansum* and *N. alba* compared to lemon and lime essential oils, and it showed complete inhibition at concentrations of 0.125-1.0% (Tables 8 and 9).

Vapour phase effect of essential oils on spore germination of *B. cinerea, P. expansum* and *N. alba* is shown in Tables 10-12. Complete spore germination inhibition of *B. cinerea, P. expansum* and *N. alba* by essential oil of lemongrass was observed at concentrations 0.125-1.0%, while lemon and lime essential oils did not inhibit spore

germination (Table 10). Essential oil combinations of lemon + lemongrass, and lime + lemongrass completelty inhibited spore germination of the three pathogens at concentrations of 0.125-1.0% (Table 10). Spore germination of *B. cinerea, P. expansum* and *N. alba* was completely inhibited by all essential oil treatments in combination with regular atmosphere (-0.5°C, air) or controlled atmosphere (5%O_2 + 10% CO_2, -0.5°C) cold storage regimes after 7 days including the control treatments (data not shown). Therefore, Petri plates were further incubated at 20°C for 5 days and the results are shown in Tables 11 and 12. Complete germination inhibition of *B. cinerea, P. expansum* and *N. alba* by essential oil of lemongrass + lime was observed at concentrations of 0.125-1.0%, while lemon + lime essential oils exhibited positive spore germination (Tables 11 and 12). Essential oil combinations of lemon + lemongrass, and lime + lemongrass completelty inhibited spore germination of *B. cinerea, P. expansum* and *N. alba* at concentrations of 0.125-1.0%.

Antimicrobial properties of essential oils

The antimicrobial properties of direct contact and volatile phase minimum inhibitory concentration varied according to the essential oils tested. Essential oils of lemon and lime in their contact phase were fungistatic since the previously exposed mycelial discs grew when transferred to medium without essential oil. Both contact and volatile phase of essential oils of lemongrass, mixtures of lemon + lemongrass, and lime + lemongrass were found to be fungicidal at the respective MIC. Lemongrass essential oil was fungicidal against *B. cinerea, P. expansum* and *N. alba* both in its direct contact and vapour phase of 1.5% and 0.125%, respectively. Similar results were observed for essential oil mixtures of lemon + lemongrass and lime + lemongrass essential oils.

Discussion

Essential oils are volatile substances obtained from several plant organs through fermentation, extraction, and steam distillation. They play several essential functions for the plant survival including defence against invader microorganisms (Cowan, 1999). In this study essential oils were tested individually, mixtures and in combination with cold storage regimes for their effect on mycelial growth and spore germination of *B. cinerea, P. expansum* and *N. alba.* The results from this study indicated that essential oils of lemon, lime, lemongrass and mixtures of lemon + lemongrass and lime + lemongrass possess antifungal activity against *B. cinerea, P. expansum* and *N. alba.*

Lemongrass essential oil was fungicidal against *B. cinerea, P. expansum* and *N. alba* both in its direct contact and vapour phase of 1.5% and 0.125%. Combination of lemongrass+ lime or lemongrass+lemon did not have additive or synergistic effect on

mycelial growth or spore germination of *B. cinerea, P. expansum* and *N. alba* at 20°C, regular atmosphere or controlled atmosphere. Low temperatures create a postharvest environment unfavourable to the growth and reproduction of microorganisms. This is a very important physical method of postharvest wastage control, and all other methods can be considered as supplements to low temperature (Wills *et al.,* 2007). Temperature is considered to be one of the most important environmental factors that seriously affects growth and germination of pathogens *in vitro,* but also the initiation and developmental stages of plant pathogens *in vivo* (Spotts and Cervantes, 1991). Tian and Bertolini (1995) reported that low temperatures (-2°C and -4°C) significantly inhibited mycelial growth, delayed spore germination, and limit the disease incidence and the development of *Botrytis allii*, which causes bulb rot in garlic and onion.

Tzortzakis and Economakis (2007) reported that lemongrass oil showed antifungal activity against several plant pathogens, such *B. cinerea,* and *R. stolonifer*. In vapour phase method, it was observed that essential oils of lemongrass, mixtures of lemon + lemongrass and lime + lemongrass completely inhibited mycelial growth and spore germination of *B. cinerea* at concentrations of 0.125-1.0%. Similar results were observed when essential oil treatments were combined with regular atmosphere or controlled atmosphere cold storage regimes. The effect of essential oils on spore germination and sporulation may reflect the effect of volatiles emitted by essential oils on surface mycelial development (Tzortzakis and Economakis, 2007).

Both direct contact and vapour phase methods indicated that lemongrass oil and mixtures of lemon + lemongrass and lime + lemongrass exhibited the highest antifungal activity against *B. cinerea.* Inhibition of mycelial growth and spore germination of postharvest pathogens by essential oil treatments could make a major contribution in limiting the spread of the pathogen by lowering the spore load in storage environments and on surfaces. Volatile phase of essential oils of different plants were also reported to possess antimicrobial activity against plant pathogenic fungi and bacteria (Edris and Farrag, 2003; Soylu *et al.,* 2005). The results obtained both in direct contact and vapour phase methods revealed that increasing the concentration of essential oils increases mycelial growth inhibition of *B. cinerea.*

Oil extracts from marjoram (*Oreganum syriacum*), lavender (*Lavandula angustifolia*), lemongrass (Shimoni *et al.,* 1993), thyme (*Thymus capitatus*) (Arras *et al.,* 1995), sage (*Silva officinalis*) (Carta *et al.,* 1996), tea tree (*Melaleuca alternifolia*), Melissa (*Melissa officinalis*), peppermint (*Mentha piperita*), penny royal (*Mentha pulegium*), jasmine (*Jasminum grandiflorum*), neroll (*Citrus aurantium* sub.*aurantum*), wintergreen (*Gaultheria procumbens*) and hyssop (*Hyssopus officinalis*) (Cutler *et al.,*1996), all inhibited *in vitro* mycelial growth of *B. cinerea.* It appeared that lemon, lime and lemongrass essential oils

have different capacities to inhibit fungal growth and spore germination, which is believed to be due to the variation of compounds present in each of the oils. Volatile composition of citrus and lemongrass essential oils (Chapter 2) showed that lemon and lime oils are dominated by monoterpene hydrocarbons limonene and α-terpinene; while lemongrass composed of carbonyl compounds neral and geranial. Sokovic and van Griensven (2006) reported that different essential oils have different efficacy and the modes of action of essential oils are different for bacterial and fungal species. Also, there seemed to be a correlation between chemical structures of the essential oil constituents with the antifungal activity. Dorman and Deans (2000) stated that the antifungal activity of the essential oil would be expected to be related to the composition of the oil, the structural configuration of the constituent components of the oil and their functional groups. Although major active compounds are known to be responsible for the antimicrobial activity displayed by essential oils, some studies reported that minor compounds might also have synergistic or additive effects (Tyagi and Malik, 2011).

Essential oils may inhibit the growth of fungi either permanently (fungicidal) or temporarily (fungistatic). The antimicrobial and antifungal activity of essential oils is well documented (Deans and Ritchie, 1987; Zambonelli *et al.,* 1996; Ouattara *et al.,* 1997; Meepagala *et al.,* 2002; Utama *et al.,* 2002), and only few studies have been reported on their effect on postharvest pathogens (Bishop and Thompdon, 1997; Tripathi and Dubey, 2004; Neri *et al.,* 2006). Ultee *et al.* (1999) reported that essential oils have the ability to change permeability of the cell membranes of pathogens for cation exchange and affect the ion gradients which will affect the metabolic processes in the pathogens cells and ultimately results in cell death. In direct contact method, it was observed that essential oils of lemongrass, lemon + lemongrass, lime + lemongrass exhibited fungicidal inhibition against *B. cinerea* at concentrations of 1.5-100%. This was proven by the absence of radial mycelial growth on *B. cinerea* after transferring previously exposed discs onto freshly poured PDA medium. The effect of lemon and lime oils alone (20-100% and 35-100%, respectively) on mycelial growth of the tested pathogens was fungistatic and *B. cinerea* started to grow 3 days after transferring previously exposed mycelial plugs to the fresh freshly poured PDA medium. In vapour phase, lemongrass oil, mixtures of lemon + lemongrass and lime + lemongrass showed fungicidal inhibition (MIC = 0.125%). It is known that the cell wall of pathogens is the main target of phenolic compounds and these compounds may disrupt the permeability barrier of cell membrane and inhibit respiration. Hydrophobic nature of essential oils and their components enables these compounds to penetrate lipid of fungal cell membrane and mitochondria as a result disturbing their structure and these compounds accumulate in the cell membrane of pathogen causing energy deletion (Cox *et al.*, 2000). In addition, in some studies, it is reported that the essential oils may affect the metabolic

pathways of microorganisms. Nychas (1995) found that phenolic compounds in low concentration disrupt proteins and in high concentrations damaged the enzymes outbreak in production of energy.

Inouye *et al.* (2001 and 2006) and Inouye (2003) evaluated the inhibition of *Aspergillus fumigatus* and *A. niger* by cinnamon and lemongrass essential oil and documented that these essential oils were effective against mycelial growth of both pathogens (Lopez *et al.,* 2005 and 2007). Mixtures of cinnamon and clove essential oils against *A. flavus* were tested at ranges of 1.0-2.0% and results indicated that the antifungal activity of essential oils depends on the experimental assay used. The inhibitory effects of essential oils were greater in the vapour phase than in a direct contact phase (Guynot *et al.,* 2003; Matan *et al.,* 2006; Tullio *et al.,* 2006). The effect of thyme vapour phase strongly suppressed the sporulation of *A. niger* at concentrations of 1.0% (Segvic-Klaric *et al.,* 2006).

In conclusion, the volatile phase of citrus essential oils was more potent than direct contact phase, while lemongrass essential oil, mixtures of lemon + lemongrass and lime + lemongrass were potent in both direct contact and vapour phase. It was noted that the efficacy of the tested essential oils against *B. cinerea*, *P. expansum* and *N. alba* was not affected by regular atmosphere or controlled atmosphere and application could be used in combination. Our study indicated the potentiality of citrus and lemongrass essential oils as eco-friendly antifungal agents against postharvest pathogens *B. cinerea*, *P. expansum* and *N. alba.*

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Table 1. Direct contact effect of essential oils on mycelial growth of *B. cinerea, N. alba* and *P. expansum* incubated at 20°C for 7 days.

Treatments	Concentration (%)	Mycelial growth inhibition (%)		
		B. cinerea	***N. alba***	***P. expansum***
Control		0.0d	0.0f	0.0f
Ethanol	40	0.0d	0.23f	0.6ef
Lemon	1.5	0.0d	0.37f	0.7ef
	2.5	0.0d	0.37f	0.7ef
	5	0.0d	0.37f	0.7ef
	7.5	0.0d	0.37f	0.7ef
	12.5	0.0d	0.37f	0.7ef
	20	100.0a	100.0a	100.0a
	35	100.0a	100.0a	100.0a
	60	100.0a	100.0a	100.0a
	100	100.0a	100.0a	100.0a
Lime	1.5	0.0d	0.37f	0.7ef
	2.5	0.0d	0.37f	0.7ef
	5	2.3d	2.9ef	2.5e
	7.5	14.5c	21.7d	9.0d
	12.5	69.4b	74.8c	65.4c
	20	71.3b	83.4b	81.6b
	35	100.0a	100.0a	100.0a
	60	100.0a	100.0a	100.0a
	100	100.0a	100.0a	100.0a
Lemongrass	1.5	100.0a	100.0a	100.0a
	2.5	100.0a	100.0a	100.0a
	5	100.0a	100.0a	100.0a
	7.5	100.0a	100.0a	100.0a
	12.5	100.0a	100.0a	100.0a
	20	100.0a	100.0a	100.0a
	35	100.0a	100.0a	100.0a
	60	100.0a	100.0a	100.0a
	100	100.0a	100.0a	100.0a
Le + Lg	1.5	100.0a	100.0a	100.0a
	2.5	100.0a	100.0a	100.0a
	5	100.0a	100.0a	100.0a
	7.5	100.0a	100.0a	100.0a
	12.5	100.0a	100.0a	100.0a
	20	100.0a	100.0a	100.0a
	35	100.0a	100.0a	100.0a
	60	100.0a	100.0a	100.0a
	100	100.0a	100.0a	100.0a

Li + Lg	1.5	100.0a	100.0a	100.0a
	2.5	100.0a	100.0a	100.0a
	5	100.0a	100.0a	100.0a
	7.5	100.0a	100.0a	100.0a
	12.5	100.0a	100.0a	100.0a
	20	100.0a	100.0a	100.0a
	35	100.0a	100.0a	100.0a
	60	100.0a	100.0a	100.0a
	100	100.0a	100.0a	100.0a
Le + Li	1.5	0.0d	0.37f	0.2ef
	2.5	0.0d	0.37f	0.7ef
	5	0.0d	0.37f	0.7ef
	7.5	0.0d	0.37f	0.7ef
	12.5	0.0d	0.37f	0.7ef
	20	0.0d	0.37f	0.7ef
	35	0.0d	0.37f	0.7ef
	60	0.0d	0.37f	1.4ef
	100	0.0d	0.37f	1.8ef
Significance (Treatment x Concentration)		***P* < 0.0001**	***P* < 0.0001**	***P* < 0.0001**

Means followed by the same letter in the column for each pathogen are not significantly different according to Fisher's least significant difference test at $P \leq 0.05$. Colony diameter was measured after seven days at 20°C and expressed as percentage inhibition. Le: Lemon; Lg: Lemongrass; Li: lime.

Table 2. Direct contact effect of essential oils on mycelial growth of *B. cinerea, N. alba* and *P. expansum* incubated at regular atmosphere (-0.5°C, air) for 7 days followed by 5 days at 20°C.

Treatments	**Concentration (%)**	**Mycelial growth inhibition (%)**		
		B. cinerea	***N. alba***	***P. expansum***
Control		0.0d	0.0h	0.0f
Ethanol	40	0.0d	0.0h	0.0f
Lemon	1.5	0.0d	24.0f	0.0f
	2.5	0.0d	24.0f	0.0f
	5	0.0d	24.0f	0.0f
	7.5	0.0d	24.0f	0.0f
	12.5	0.0d	24.0f	0.0f
	20	100.0a	100.0a	100.0a
	35	100.0a	100.0a	100.0a
	60	100.0a	100.0a	100.0a
	100	100.0a	100.0a	100.0a
Lime	1.5	0.0d	9.3g	0.0f
	2.5	0.0d	24.0f	7.9e
	5	0.0d	34.9e	9.3e
	7.5	21.8c	68.7d	17.7d
	12.5	76.5b	84.4c	65.4c
	20	78.2b	90.2b	89.9b
	35	100.0a	100.0a	100.0a
	60	100.0a	100.0a	100.0a
	100	100.0a	100.0a	100.0a
Lemongrass	1.5	100.0a	100.0a	100.0a
	2.5	100.0a	100.0a	100.0a
	5	100.0a	100.0a	100.0a
	7.5	100.0a	100.0a	100.0a
	12.5	100.0a	100.0a	100.0a
	20	100.0a	100.0a	100.0a
	35	100.0a	100.0a	100.0a
	60	100.0a	100.0a	100.0a
	100	100.0a	100.0a	100.0a
Le + Lg	1.5	100.0a	100.0a	100.0a
	2.5	100.0a	100.0a	100.0a
	5	100.0a	100.0a	100.0a
	7.5	100.0a	100.0a	100.0a
	12.5	100.0a	100.0a	100.0a
	20	100.0a	100.0a	100.0a
	35	100.0a	100.0a	100.0a
	60	100.0a	100.0a	100.0a
	100	100.0a	100.0a	100.0a

Li + Lg	1.5	100.0a	100.0a	100.0a
	2.5	100.0a	100.0a	100.0a
	5	100.0a	100.0a	100.0a
	7.5	100.0a	100.0a	100.0a
	12.5	100.0a	100.0a	100.0a
	20	100.0a	100.0a	100.0a
	35	100.0a	100.0a	100.0a
	60	100.0a	100.0a	100.0a
	100	100.0a	100.0a	100.0a
Le + Li	1.5	0.0d	24.0f	0.0f
	2.5	0.0d	24.0f	0.0f
	5	0.0d	24.0f	0.0f
	7.5	0.0d	24.0f	0.0f
	12.5	0.0d	24.0f	0.0f
	20	0.0d	24.0f	0.0f
	35	0.0d	24.0f	0.0f
	60	0.0d	24.0f	0.0f
	100	0.0d	24.0f	0.0f
Significance (Treatment x Concentration)		***P* < 0.0001**	***P* < 0.0001**	***P* < 0.0001**

Means followed by the same letter in the column for each pathogen are not significantly different according to Fisher's least significant difference test at $P \leq 0.05$. Colony diameter was measured after seven days at 20°C and expressed as percentage in hibition. Le: Lemon; Lg: Lemongrass; Li: lime.

Table 3. Direct contact effect of essential oils on mycelial growth of *B. cinerea, N. alba* and *P. expansum* incubated at controlled atmosphere (-0.5°C, 5% O_2 + 10% CO_2) for 7 days followed by 5 days at 20°C.

Treatments	**Concentration (%)**	**Mycelial growth inhibition (%)**		
		B. cinerea	***N. alba***	***P. expansum***
Control		0.0d	0.0e	0.0f
Ethanol	40	0.0d	1.3e	1.3ef
Lemon	1.5	0.0d	25.0d	1.3ef
	2.5	0.0d	25.0d	1.3ef
	5	0.0d	25.0d	1.3ef
	7.5	0.0d	25.0d	1.3ef
	12.5	0.0d	100.0a	1.3ef
	20	100.0a	100.0a	100.0a
	35	100.0a	100.0a	100.0a
	60	100.0a	100.0a	100.0a
	100	100.0a	100.0a	100.0a
Lime	1.5	0.0d	0.9e	0.9ef
	2.5	0.0d	25.0d	1.3ef
	5	0.0d	27.7d	3.2e
	7.5	19.2c	54.3c	9.7d
	12.5	74.2b	90.7b	78.5c
	20	76.1b	92.2b	91.5b
	35	100.0a	100.0a	100.0a
	60	100.0a	100.0a	100.0a
	100	100.0a	100.0a	100.0a
Lemongrass	1.5	100.0a	100.0a	100.0a
	2.5	100.0a	100.0a	100.0a
	5	100.0a	100.0a	100.0a
	7.5	100.0a	100.0a	100.0a
	12.5	100.0a	100.0a	100.0a
	20	100.0a	100.0a	100.0a
	35	100.0a	100.0a	100.0a
	60	100.0a	100.0a	100.0a
	100	100.0a	100.0a	100.0a
Le + Lg	1.5	100.0a	100.0a	100.0a
	2.5	100.0a	100.0a	100.0a
	5	100.0a	100.0a	100.0a
	7.5	100.0a	100.0a	100.0a
	12.5	100.0a	100.0a	100.0a
	20	100.0a	100.0a	100.0a
	35	100.0a	100.0a	100.0a
	60	100.0a	100.0a	100.0a

	100	100.0a	100.0a	100.0a
Li + Lg	1.5	100.0a	100.0a	100.0a
	2.5	100.0a	100.0a	100.0a
	5	100.0a	100.0a	100.0a
	7.5	100.0a	100.0a	100.0a
	12.5	100.0a	100.0a	100.0a
	20	100.0a	100.0a	100.0a
	35	100.0a	100.0a	100.0a
	60	100.0a	100.0a	100.0a
	100	100.0a	100.0a	100.0a
Le + Li	1.5	0.0d	25.0d	1.3ef
	2.5	0.0d	25.0d	1.3ef
	5	0.0d	25.0d	1.3ef
	7.5	0.0d	25.0d	1.3ef
	12.5	0.0d	25.0d	1.3ef
	20	0.0d	25.0d	1.3ef
	35	0.0d	25.0d	1.3ef
	60	0.0d	25.0d	1.3ef
Significance (Treatment x Concentration)		***P* < 0.0001**	***P* < 0.0001**	***P* < 0.0001**

Means followed by the same letter in the column for each pathogen are not significantly different according to Fisher's least significant difference test at $P \leq 0.05$. Colony diameter was measured after seven days at 20°C and expressed as percentage in hibition. Le: Lemon; Lg: Lemongrass; Li: lime.

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Table 4. Direct contact effect of essential oils on spore germination of *B. cinerea, N. alba* and *P. expansum* incubated at 20°C for 7 days.

Treatments	Concentration (%)	Spore germination		
		B. cinerea	***N. alba***	***P. expansum***
Control		+	+	+
Ethanol	40	+	+	+
Lemon	1.5	+	+	+
	2.5	+	+	+
	5	+	+	+
	7.5	+	+	+
	12.5	+	+	+
	20	-	-	-
	35	-	-	-
	60	-	-	-
	100	-	-	-
Lime	1.5	+	+	+
	2.5	+	+	+
	5	+	+	+
	7.5	+	+	+
	12.5	+	+	+
	20	+	+	+
	35	-	-	-
	60	-	-	-
	100	-	-	-
Lemongrass	1.5	-	-	-
	2.5	-	-	-
	5	-	-	-
	7.5	-	-	-
	12.5	-	-	-
	20	-	-	-
	35	-	-	-
	60	-	-	-
	100	-	-	-
Le + Lg	1.5	-	-	-
	2.5	-	-	-
	5	-	-	-
	7.5	-	-	-
	12.5	-	-	-
	20	-	-	-
	35	-	-	-
	60	-	-	-
	100	-	-	-
Li + Lg	1.5	-	-	-
	2.5	-	-	-
	5	-	-	-
	7.5	-	-	-
	12.5	-	-	-
	20	-	-	-
	35	-	-	-
	60	-	-	-
	100	-	-	-
Le + Li	1.5	+	+	+
	2.5	+	+	+
	5	+	+	+
	7.5	+	+	+
	12.5	+	+	+
	20	+	+	+
	35	+	+	+
	60	+	+	+
	100	+	+	+

Spore germination was evaluated on inoculated PDA plates after seven days at 20°C. Le: Lemon; Lg: Lemongrass; Li: lime. conidia germinated (+) and non-germinated (-).

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Table 5. Direct contact effect of essential oils on spore germination of *B. cinerea, N. alba* and *P. expansum* incubated at regular atmosphere (-0.5°C, air) for 7 days followed by 5 days at 20°C.

Treatments	Concentration (%)	Spore germination		
		B. cinerea	***N. alba***	***P. expansum***
Control		+	+	+
Ethanol	40	+	+	+
Lemon	1.5	+	+	+
	2.5	+	+	+
	5	+	+	+
	7.5	+	+	+
	12.5	+	+	+
	20	-	-	-
	35	-	-	-
	60	-	-	-
	100	-	-	-
Lime	1.5	+	+	+
	2.5	+	+	+
	5	+	+	+
	7.5	+	+	+
	12.5	+	+	+
	20	+	+	+
	35	-	-	-
	60	-	-	-
	100	-	-	-
Lemongrass	1.5	-	-	-
	2.5	-	-	-
	5	-	-	-
	7.5	-	-	-
	12.5	-	-	-
	20	-	-	-
	35	-	-	-
	60	-	-	-
	100	-	-	-
Le + Lg	1.5	-	-	-
	2.5	-	-	-
	5	-	-	-
	7.5	-	-	-
	12.5	-	-	-
	20	-	-	-
	35	-	-	-
	60	-	-	-
	100	-	-	-
Li + Lg	1.5	-	-	-
	2.5	-	-	-
	5	-	-	-
	7.5	-	-	-
	12.5	-	-	-
	20	-	-	-
	35	-	-	-
	60	-	-	-
	100	-	-	-
Le + Li	1.5	+	+	+
	2.5	+	+	+
	5	+	+	+
	7.5	+	+	+
	12.5	+	+	+
	20	+	+	+
	35	+	+	+
	60	+	+	+
	100	+	+	+

Spore germination was evaluated on inoculated PDA plates after seven days at regular atmosphere (-0.5°C) followed by five days at 20°C. Le: Lemon; Lg: Lemongrass; Li: lime. conidia germinated (+) and non-germinated (-).

Table 6. Direct contact effect of essential oils on spore germination of *B. cinerea, N. alba* and *P. expansum* incubated at controlled atmosphere (-0.5°C, 5% O_2 + 10% CO_2) for 7 days followed by 5 days at 20°C.

Treatments	Concentration (%)	Spore germination		
		B. cinerea	*N. alba*	*P. expansum*
Control		+	+	+
Ethanol	40	+	+	+
Lemon	1.5	+	+	+
	2.5	+	+	+
	5	+	+	+
	7.5	+	+	+
	12.5	+	+	+
	20	-	-	-
	35	-	-	-
	60	-	-	-
	100	-	-	-
Lime	1.5	+	+	+
	2.5	+	+	+
	5	+	+	+
	7.5	+	+	+
	12.5	+	+	+
	20	+	+	+
	35	-	-	-
	60	-	-	-
	100	-	-	-
Lemongrass	1.5	-	-	-
	2.5	-	-	-
	5	-	-	-
	7.5	-	-	-
	12.5	-	-	-
	20	-	-	-
	35	-	-	-
	60	-	-	-
	100	-	-	-
Le + Lg	1.5	-	-	-
	2.5	-	-	-
	5	-	-	-
	7.5	-	-	-
	12.5	-	-	-
	20	-	-	-
	35	-	-	-
	60	-	-	-
	100	-	-	-
Li + Lg	1.5	-	-	-
	2.5	-	-	-
	5	-	-	-
	7.5	-	-	-
	12.5	-	-	-
	20	-	-	-
	35	-	-	-
	60	-	-	-
	100	-	-	-
Le + Li	1.5	+	+	+
	2.5	+	+	+
	5	+	+	+
	7.5	+	+	+
	12.5	+	+	+
	20	+	+	+
	35	+	+	+
	60	+	+	+
	100	+	+	+

Spore germination was evaluated on inoculated PDA plates after seven days at controlled atmosphere (-0.5°C, 5% O_2 + 10% CO_2) followed by five days at 20°C. Le: Lemon; Lg: Lemongrass; Li: lime. conidia germinated (+) and non-germinated (-).

Table 7. Vapour phase effect of essential oils on mycelial growth of *B. cinerea, N. alba* and *P. expansum* incubated at 20°C for 7 days.

Treatments	Concentration (%)	Mycelial growth inhibition (%)		
		B. cinerea	***N. alba***	***P. expansum***
Control		0.0^{m}	0.0^{j}	0.0^{l}
Ethanol	20	0.0^{m}	0.0^{j}	0.0^{l}
Lemon	0.0157	14.6^{l}	16.9^{i}	13.4^{k}
	0.0313	19.3^{kl}	21.5^{i}	16.9^{k}
	0.125	33.8^{hi}	49.7^{g}	37.1^{hi}
	0.5	41.3^{fg}	73.7^{e}	58.9^{f}
	1.0	52.0^{de}	92.6^{ab}	78.8^{c}
Lime	0.0157	25.2^{jk}	33.7^{h}	26.7^{j}
	0.0313	28.1^{ij}	37.9^{h}	30.3^{ij}
	0.125	46.2^{ef}	63.4^{f}	50.9^{g}
	0.5	51.9^{de}	89.5^{bc}	72.4^{cd}
	1.0	62.9^{b}	96.7^{ab}	91.8^{b}
Lemongrass	0.0157	33.3^{hi}	46.4^{g}	36.9^{hi}
	0.0313	58.9^{bc}	83.6^{cd}	67.5^{de}
	0.125	100.0^{a}	100.0^{a}	100.0^{a}
	0.5	100.0^{a}	100.0^{a}	100.0^{a}
	1.0	100.0^{a}	100.0^{a}	100.0^{a}
Le + Lg	0.0157	25.2^{jk}	33.1^{h}	26.2^{j}
	0.0313	43.9^{f}	62.6^{f}	49.9^{g}
	0.125	100^{a}	100.0^{a}	100.0^{a}
	0.5	100^{a}	100.0^{a}	100.0^{a}
	1.0	100^{a}	100.0^{a}	100.0^{a}
Li + Lg	0.015	35.9^{gh}	49.8^{g}	36.6^{h}
	0.0313	54.7^{cd}	75.6^{de}	63.2^{ef}
	0.125	100.0^{a}	100.0^{a}	100.0^{a}
	0.5	100.0^{a}	100.0^{a}	100.0^{a}
	1.0	100.0^{a}	100.0^{a}	100.0^{a}
Significance (Treatment x Concentration)		***P* < 0.0001**	***P* < 0.0001**	***P* < 0.0001**

Means followed by the same letter in the column for each pathogen are not significantly different according to Fisher's least significant difference test at P < 0.05. Colony diameter was measured after seven days at 20°C Le: Lemon; Lg: Lemongrass; Li: lime.

Table 8. Vapour phase effect of essential oils on mycelial growth of *B. cinerea, N. alba* and *P. expansum* incubated at regular atmosphere (-0.5°C, air) for 7 days followed by 5 days at 20°C.

Treatments	Concentration (%)	Mycelial growth inhibition (%)		
		B. cinerea	***N. alba***	***P. expansum***
Control		0.0^{j}	0.0^{i}	0.0^{k}
Ethanol	20	0.0^{j}	0.0^{i}	0.0^{k}
Lemon	0.0157	23.5^{i}	18.4^{h}	13.7^{j}
	0.0313	28.1^{i}	23.2^{h}	17.9^{ij}
	0.125	42.4^{fg}	50.8^{f}	39.7^{fg}
	0.5	53.2^{e}	80.6^{d}	62.7^{d}
	1.0	61.0^{d}	88.1^{bcd}	84.3^{b}
Lime	0.0157	33.7^{h}	35.8^{g}	25.1^{hi}
	0.0313	38.6^{gh}	39.5^{g}	32.4^{gh}
	0.125	53.1^{e}	66.0^{e}	54.1^{de}
	0.5	71.7^{b}	92.0^{abcd}	74.4^{c}
	1.0	63.9^{cd}	95.0^{a}	91.8^{ab}
Lemongrass	0.0157	42.2^{fg}	50.8^{f}	38.9^{fg}
	0.0313	68.8^{bc}	91.1^{bc}	71.9^{c}
	0.125	100.0^{a}	100.0^{a}	100.0^{a}
	0.5	100.0a	100.0^{a}	100.0^{a}
	1.0	100.0^{a}	100.0^{a}	100.0^{a}
Le + Lg	0.0157	34.0^{h}	35.8^{g}	27.5^{h}
	0.0313	52.7^{e}	68.2^{e}	53.2^{e}
	0.125	100.0^{a}	100.0^{a}	100.0^{a}
	0.5	100.0^{a}	100.0^{a}	100.0^{a}
	1.0	100.0^{a}	100.0^{a}	100.0^{a}
Li + Lg	0.015	44.7^{f}	54.2^{f}	42.1^{f}
	0.0313	63.3^{cd}	83.6^{cd}	60.4^{de}
	0.125	100.0^{a}	100.0^{a}	100.0^{a}
	0.5	100.0^{a}	100.0^{a}	100.0^{a}
	1.0	100.0^{a}	100.0^{a}	100.0^{a}
Treatment x Concentration		**$P < 0.0001$**	**$P < 0.0001$**	**$P < 0.0001$**

Means followed by the same letter in the column for each pathogen are not significantly different according to Fisher's least significant difference test at $P < 0.05$. Colony diameter was measured after seven days at regular atmosphere (-0.5°C) followed by five days at 20°C. Le: Lemon; Lg: Lemongrass; Li: lime.

Table 9. Vapour phase effect of essential oils on mycelial growth of *B. cinerea, N. alba* and *P. expansum* incubated at controlled atmosphere (-0.5°C, 5% O_2 + 10% CO_2) for 7 days followed by 5 days at 20°C.

Treatments	Concentration (%)	Mycelial growth inhibition (%)		
		B. cinerea	***N. alba***	***P. expansum***
Control		0.0^{j}	0.0^{m}	0.0^{k}
Ethanol	20	0.0^{j}	0.0^{m}	0.0^{k}
Lemon	0.0157	19.9^{j}	19.4^{kl}	15.6^{j}
	0.0313	24.7^{i}	24.3^{jk}	19.7^{ij}
	0.125	39.1^{fg}	47.1^{g}	42.9^{g}
	0.5	47.6^{e}	72.8^{e}	62.8^{ef}
	1.0	57.4^{d}	83.6^{cd}	85.3^{bc}
Lime	0.0157	30.6^{h}	34.9^{hi}	31.1^{h}
	0.0313	35.5^{gh}	40.1^{gh}	35.2^{gh}
	0.125	49.9^{e}	64.3^{f}	58.0^{f}
	0.5	58.1^{d}	86.7bc	78.2^{cd}
	1.0	67.9^{b}	92.7^{ab}	91.9^{ab}
Lemongrass	0.0157	38.6^{fg}	46.9^{g}	39.9^{gh}
	0.0313	63.9^{bc}	82.8^{cd}	77.2^{cd}
	0.125	100.0^{a}	100.0^{a}	100.0^{a}
	0.5	100.0^{a}	100.0^{a}	100.0^{a}
	1.0	100.0^{a}	100.0^{a}	100.0^{a}
Le + Lg	0.0157	30.4^{h}	13.2^{l}	30.2^{hi}
	0.0313	49.3^{e}	62.6^{f}	57.3^{f}
	0.125	100.0^{a}	100.0^{a}	100.0^{a}
	0.5	100.0^{a}	100.0^{a}	100.0^{a}
	1.0	100.0^{a}	100.0^{a}	100.0^{a}
Li + Lg	0.015	41.5^{f}	28.6^{ij}	45.7^{g}
	0.0313	59.9^{cd}	77.8^{de}	72.5^{de}
	0.125	100.0^{a}	100.0^{a}	100.0^{a}
	0.5	100.0^{a}	100.0^{a}	100.0^{a}
	1.0	100.0^{a}	100.0^{a}	100.0^{a}
Significance (Treatment x Concentration)		$P < 0.0001$	$P < 0.0001$	$P < 0.0001$

Means followed by the same letter in the column for each pathogen are not significantly different according to Fisher's least significant difference test at $P < 0.05$. Colony diameter was measured after seven days at controlled atmosphere (-0.5°C, 5% O_2 + 10% CO_2) followed by five days at 20°C. Le: Lemon; Lg: Lemongrass; Li: lime.

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Table 10. Vapour phase effect of essential oils on spore germination of *B. cinerea, N. alba* and *P. expansum* incubated at 20°C 7 days.

Treatments	Concentration (%)	Spore germination		
		B. cinerea	***N. alba***	***P. expansum***
Control		+	+	+
Ethanol	20	+	+	+
Lemon	0.0157	+	+	+
	0.0313	+	+	+
	0.125	+	+	+
	0.5	+	+	+
	1.0	+	+	+
Lemongrass	0.0157	+	+	+
	0.0313	+	+	+
	0.125	-	-	-
	0.5	-	-	-
	1.0	-	-	-
Lime	0.0157	+	+	+
	0.0313	+	+	+
	0.125	+	+	+
	0.5	+	+	+
	1.0	+	+	+
Le + Lg	0.0157	+	+	+
	0.0313	+	+	+
	0.125	-	-	-
	0.5	-	-	-
	1.0	-	-	-
Li + Lg	0.0157	+	+	+
	0.0313	+	+	+
	0.125	-	-	-
	0.5	-	-	-
	1.0	-	-	-

Spore germination was evaluated on inoculated PDA plates after seven days at 20°C. Le: Lemon; Lg: Lemongrass; Li: lime. conidia germinated (+) and non-germinated (-).

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Table 11. Vapour phase effect of essential oils on spore germination of *B. cinerea, N. alba* and *P. expansum* incubated at regular atmosphere (-0.5°C, air) for 7 days followed by 5 days at 20°C.

Treatments	Concentration (%)	Spore germination		
		B. cinerea	***N. alba***	***P. expansum***
Control		+	+	+
Ethanol	20	+	+	+
Lemon	0.0157	+	+	+
	0.0313	+	+	+
	0.125	+	+	+
	0.5	+	+	+
	1.0	+	+	+
Lemongrass	0.0157	+	+	+
	0.0313	+	+	+
	0.125	-	-	-
	0.5	-	-	-
	1.0	-	-	-
Lime	0.0157	+	+	+
	0.0313	+	+	+
	0.125	+	+	+
	0.5	+	+	+
	1.0	+	+	+
Le + Lg	0.0157	+	+	+
	0.0313	+	+	+
	0.125	-	-	-
	0.5	-	-	-
	1.0	-	-	-
Li + Lg	0.0157	+	+	+
	0.0313	+	+	+
	0.125	-	-	-
	0.5	-	-	-
	1.0	-	-	-

Spore germination was evaluated on inoculated PDA plates after seven days at 20°C. Le: Lemon; Lg: Lemongrass; Li: lime. conidia germinated (+) and non-germinated (-).

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Table 12. Vapour phase effect of essential oils on spore germination of *B. cinerea, N. alba* and *P. expansum* incubated at controlled atmosphere (-0.5°C, 5% O_2 + 10% CO_2) for 7 days followed by 5 days at 20°C.

Treatments	Concentration (%)	Spore germination		
		B. cinerea	*N. alba*	*P. expansum*
Control		+	+	+
Ethanol	20	+	+	+
Lemon	0.0157	+	+	+
	0.0313	+	+	+
	0.125	+	+	+
	0.5	+	+	+
	1.0	+	+	+
Lemongrass	0.0157	+	+	+
	0.0313	+	+	+
	0.125	-	-	-
	0.5	-	-	-
	1.0	-	-	-
Lime	0.0157	+	+	+
	0.0313	+	+	+
	0.125	+	+	+
	0.5	+	+	+
	1.0	+	+	+
Le + Lg	0.0157	+	+	+
	0.0313	+	+	+
	0.125	-	-	-
	0.5	-	-	-
	1.0	-	-	-
Li + Lg	0.0157	+	+	+
	0.0313	+	+	+
	0.125	-	-	-
	0.5	-	-	-
	1.0	-	-	-

Spore germination was evaluated on inoculated PDA plates after seven days at controlled atmosphere (-0.5°C, 5% O_2 + 10% CO_2) followed by five days at 20°C. Le: Lemon; Lg: Lemongrass; Li: lime. conidia germinated (+) and non-germinated (-).

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CHAPTER 4

INVESTIGATING THE MECHANISM OF ANTIFUNGAL ACTION OF LEMONGRASS (*CYMBOPOGON CITRATUS* (DC.) STRAPF) ESSENTIAL OIL ON *BOTRYTIS CINEREA*

Abstract

The essential oil of lemongrass (*Cymbopogon citratus* (DC.) Strapf) was demonstrated in our previous studies as a potential antifungal agent; however, the antifungal effects on the morphology and biochemistry of *Botrytis cinerea* has not been documented. This study investigated the antifungal mechanisms of lemongrass oil against *B. cinerea,* one of the main postharvest pathogens of apples. Light microscopy and scanning electron microscopy observations indicated that *B. cinerea* hyphae exposed to lemongrass oil undergo morphological damage such as vesiculation, cytoplasmic disruption and collapsed hyphae. Glucose-induced reduction in external pH of *B. cinerea* was inhibited by lemongrass oil in a time and concentration dependent manner. *Botrytis cinerea* spores treated with lemongrass oil showed strong propidium iodide fluorescence in the cytosol. Lemongrass oil significantly altered plasma membrane, the release of cell constituents, and the total lipid content of *B. cinerea*. These observations indicate that the antifungal activity of lemongrass oil can be attributed to the disruption of the cell membrane integrity and membrane permeability.

Keywords: Essential oil, lemongrass oil, *Botrytis cinerea*, membrane integrity, membrane permeability.

Introduction

Lemongrass belongs to the genus *Cymbopogon* (aromatic grasses) (Shah *et al.,* 2011). It has short underground stems with ringed segments, coarse, green slightly leathery leaves in dense clusters (Carlin *et al*., 1986). It contains 1 to 2% essential oil on a dry weight basis (Carlson *et al*., 2001). The plant is a native herb from India and is cultivated in South and Central America, Africa and other tropical countries (Figueirinha *et al.,* 2008; Shah *et al.,* 2011). 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, 2012). Several studies have reported on the antimicrobial activities of lemongrass oil (Saikia *et al.,* 2001; Appendini and Hotchkiss, 2002; Plotto *et al.,* 2003; Serrano *et al.,* 2005). Saikia *et al.*

(2001) investigated the effect of three distinct genotypes of *Cymbopogon* spp., namely *C. martini*; *C. flexuosus* and *C. winterianus* for antifungal activity. Four oil components, namely geraniol, citronellol, citronellal and citral were simultaneously assayed for comparative activity. The authors observed that the antifungal activity of lemongrass oil was most active followed by palmarosa oil and citronella oil (Saikia *et al.,* 2001). Previous research (Chapter 3) also indicated that lemongrass oil was more inhibitory than lemon oil and lime oil against *Botrytis cinerea* in *in vitro* experiments. Although the antimicrobial properties of essential oils and their components have been reviewed in the past (Shelef, 1983; Nychas, 1995), the mechanism of action has not been studied in great detail (Lambert *et al.*, 2001; Holley and Patel, 2005). Considering the large number of different groups of chemical compounds present in essential oils, it is most likely that their antibacterial activity is not attributable to one specific mechanism but to several targets in the cell (Carson *et al.*, 2002). The aim of this study was to investigate the potential antifungal mechanisms of lemongrass essential oil on *B. cinerea*. No studies have been carried so far with regard to precise target of lemongrass on *B. cinerea*.

Materials and methods

Fungal pathogen and essential oil

The isolate of *Botrytis cinerea* (Accession number: PPRI 7338) was obtained from Agricultural Research Council – Plant Protection Research Institute, Pretoria, South Africa. The isolate was cultured on potato dextrose agar (PDA) (pH 5.6, Merck, Cape Town, South Africa) and maintained on PDA slants at 4°C. Essential oil of lemongrass (*Cymbopogon citratus* (DC.) Strapf) was obtained from Groenkop Farm (Melmoth, KwaZulu-Natal, South Africa) and stored at 4°C until further used. Moreover, lemongrass oil was extracted from fresh or partly dried leaves by steam distillation.

Light microscopy

Potato dextrose agar (PDA, pH 5.6, Merck, Johannesburg, South Africa) was autoclaved and cooled in a water bath to 40°C. A concentration of 0.13% of lemongrass oil was prepared by adding the requisite amount of the essential oil to ethanol (20%, v/v) to make up a volume of 2.8 mL, which was then added to flasks with 140 mL molten PDA (pH 5.6), and gently swirled to form a homogenous mixture. The amended PDA was poured into 90 mm sterile Petri plates (20 mL/plate). Potato dextrose agar discs carrying fungal mycelia (3 mm diameter) were excised from the edge of 4-days old *B. cinerea* culture and placed in an inverted manner at the centre of each amended PDA plates, and incubated at 20°C for 3

days. Samples without any oil treatment were considered as controls. Thin layers (1 mm) of agar blocks containing mycelia were cut off from the growing edges for examination by light microscopy. The blocks were placed in a drop of 50% glycerol on a microscope glass slides, covered with a cover slide and examined using a light microscope to observe recognizable cytological changes in comparison to the control treatment. Images were photographed with a digital camera. Each treatment was replicated 3 times. The experiment was conducted twice.

Scanning electron microscopy

The 4-day-old fungal cultures on PDA amended with lemongrass essential oil at 0.125% concentration were used for all SEM observations (Helal *et al.*, 2007; Yahyazadeh *et al.*, 2008). Mycelial discs of *B. cinerea* (6 mm in diameter) exposed to 0.125% lemongrass oil were cut at the periphery of the colony from the cultures growing at each concentration on the amended PDA plates. Mycelial discs were promptly placed in vials containing 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) and fixed for 12 hr at 4°C. The samples were washed two times for 10 min with 0.1 M phosphate buffer (pH 7.2) to remove the glutaraldehyde, and then dehydrated in an ethanol series (30%, 50%, 70%, 80%, 90%, and three times in 100%) for 15 min in each series. The samples were then transferred into critical point drier baskets under 100% ethanol and placed in critical point drier (Polaron Critical Point Dryer, Quaram Technologies). The fixed material was then mounted on aluminum stubs using double-sided carbon tape and coated with gold in a S15OA Sputter in a high-vacuum chamber for 150 s at 9 mA. Finally, the samples were examined with the LEO 1450VP scanning electron microscope (SEM) (ZEISS, Freiburg, Germany) at an accelerating voltage of 7.0 kV. Each treatment was replicated 3 times. The experiment was conducted twice.

Determination of acidification of external medium

The proton pumping activity of *B. cinerea* was detected by monitoring the glucose-induced acidification of the external medium by detecting the pH according to the method used by Manavathu *et al.* (1999) with slight modifications. A conidial suspension of *B. cinerea* was prepared from 3-day-old cultures, by adding 5 mL PBS with 2% (w/v) D-glucose (PBS-2%G) to each Petri dish and gently scraping the surface three times with a sterile L-shaped spreader to dislodge the spores. The conidial suspension of *B. cinerea* was adjusted by a haemocytometer. The conidial suspension containing 1 x 10^4 spores/mL concentration in PBS-2%G (100 µL) was added in flasks containing 20 mL of potato dextrose broth (PDB, pH 3.5, Merck, Johannesburg, South Africa). The flasks were then incubated for 48 hr at 25°C.

Cultures containing mycelia after 48 hr of incubation were filtered through a Whatman™ filter paper (GE Healthcare Life Sciences, Freiburg, Germany) and then washed twice with distilled water. Approximately 0.5 g wet weight of the washed mycelia was suspended in 40 mL of 50 mM KCl solution. The suspensions were then incubated at 4°C for 18 hr for glucose starvation. Lemongrass oil was added to the suspensions to achieve final concentrations of 0.125%, 0.25%, 0.5% and 1%. The volume was adjusted to 45 mL with the addition of 50 mM KCl solution. Samples without any oil treatment were considered as controls. The mixtures were incubated for 10 min at room temperature, and the mycelia were filtered through a Whatman™ filter paper (GE Healthcare Life Sciences, Freiburg, Germany). A 10% glucose solution of 20 mL was added to the mycelia to induce medium acidification. The value of the external pH was checked using a digital pH meter (pH meter 3510, Lasec, South Africa) at 0, 30, 60, 90 and 120 min. Each treatment was replicated 3 times. The experiment was conducted twice.

Assay of plasma membrane integrity

The studies on membrane damage were tested following the procedure of Pinto *et al.* (2009) and Tian *et al.* (2012) but with slight modifications. Propidium iodide (PI) is commonly used for identifying dead cells in a population and as a counterstain in multicolor fluorescent techniques (Moore *et al.,* 1998). Conidial concentration of *B. cinerea* was freshly prepared by filtering the culture grown in PDA through two layers of cheesecloth with distilled water amended with Tween 20 (Sigma-Aldrich, St. Louis, MO, USA) at a concentration of 0.01 mL L^{-1} and adjusted to 1×10^4 spores/mL. The suspension of *B. cinerea* was then added into test tubes containing 10 mL of PDB. A requisite amount of the lemongrass oil was added in the tubes to obtain 0.125%, 0.25%, 0.5% and 1% concentrations. Samples without any oil treatment were considered as controls. All flasks were incubated for 12 hr at 25°C in an incubator shaker (150 rpm). Spores in PDB were collected by centrifugation at 4000 *g* for 15 min at 25°C, and washed three times with 0.1 mM sodium phosphate buffer (pH 7.2) to remove residual medium. The suspensions were stained with 10 µg/mL propidium iodide (PI) for 10 min at a room temperature. Spores were then collected by centrifugation, and washed twice with the buffer to remove residual dye. The spores were observed under an Olympus IX8 fluorescent microscope (Carson Scientific Imaging Group, Ontario, Canada). Three fields of view from each cover slip were chosen randomly, and the number of spores in bright-field was defined as the total number. Membrane integrity (MI) was calculated according to the formula:

MI (%) = [(a-b) /a] x 100; where a = total number of spores, b = number of stained spores. Each treatment was replicated 3 times. The experiment was conducted twice.

Determination of release of cellular material

The release of cell constituents into the suspension was measured according to the method of Paul *et al.* (2011). Three 6 mm-diameter mycelial plugs of *B. cinerea* were taken from the edge of 3-day-old culture and placed in flasks contining 50 mL of potato dextrose broth (PDB, pH 3.5, Merck, Johannesburg, South Africa). The flasks were incubated at 25°C with gentle shaking (150 rpm), and mycelia was harvested by centrifugation at 4000 *g* for 15 min, washed three times and suspended in 20 mL phosphate buffer (0.1 M, pH 7.2). After 1 hr incubation with 0.125%, 0.25%, 0.5% and 1% concentrations of lemongrass oil, samples were centrifuged at 4000 *g* at 4°C for 15 min. To determine the concentration of the released constituents, 1 mL of the supernatant was used to measure the absorbance at 260 nm with a UV-Visible spectrophotometer (Thermo Scientific Technologies, Madison, Wisconsin). Each treatment was replicated 3 times. The experiment was conducted twice.

Determination of total lipid content

Total lipid content of *B. cinerea* cells incubated with the lemongrass oil at concentrations of 0.125%, 0.25%, 0.5% and 1% were determined using the phosphovanillin method (Helal *et al.*, 2007). Three 6 mm-diameter mycelial plugs of *B. cinerea* were taken from the edge of 3-day-old cultures and placed in flasks with 50 mL of potato dextrose broth (PDB, pH 3.5, Merck, Johannesburg, South Africa). The flasks were incubated at 25°C for 2 days with gentle shaking (150 rpm), and mycelia was harvested by centrifugation at 4000 *g* for 15 min. The samples were dried in a vacuum freeze drier for 4 hr. Approximately 0.1 g of dry mycelia was homogenised with liquid nitrogen and total lipid content extracted with 4.0 mL of methanol chloroform water mixture (2:1:0.8, v/v/v) in a clean dry test tube with vigorous shaking for 30 min (150 rpm). The tubes were centrifuged at 4000 *g* for 15 min. The lower phase containing lipids was thoroughly mixed with 0.2 mL saline solution and centrifuged at 4000 *g* for 10 min. Then, an aliquot of 0.2 mL chloroform and lipid mixture was transferred to a new tube and 0.5 mL sulphuric acid were added, before being heated for 10 min in a boiling water bath (100°C). After that, 3 mL phosphovanillin were added and shaken vigorously (150 rpm), and incubated at room temperature for 10 min. The absorbance at 520 nm was read and used to calculate total lipid content from the standard calibration curve using cholesterol as a standard. Each treatment was replicated 3 times. The experiment was conducted twice.

Statistical analysis

All data collected were expressed as the mean ± standard deviation. Statistica software, Statistica Version 11.0 (StatSoft Inc., Tulsa, OK, USA) was used to run one-way ANOVA on

all data collected. Where ANOVA was significant, treatment means were separated using Duncans Multiple Range Test (DMRT) at 5% level of significance

Results

Light microscopy

The observations of *B. cinerea* obtained under the light microscope at 100 x magnification, after treatment with lemongrass oil are presented in Fig. 1. Morphological damage was detected in the hyphae exposed to lemongrass oil compared to the hyphae in the control treatments. The control hyphae grown in the absence of lemongrass oil had normal, tubular, regular and homogenous hyphae, typical features of *B. cinerea* (Fig. 1a, b). After exposure to lemongrass oil, morphological changes in the hyphaea, such as vesiculation (Fig. 1c), cytoplasmic disruption (Fig. 1d) and collapsed hyphae were visible (Fig. 1e).

Scanning electron microscopy

The observations of *B. cinerea* obtained under scanning electron microscopy, after treatment with lemongrass oil are presented in Fig. 2. The SEM micrographs showed morphological damage due to lemongrass oil (Fig. 2c-e). Collapsed hyphae (Fig. 2c), vesiculation (Fig. 2d), shrinkage and formation of extruded material (Fig. 2e) was observed on *B. cinerea* treated with lemongrass oil compared to control hyphae (Fig. 2a-b).

Acidification of external medium

Lemongrass oil inhibited the glucose-induced reduction in external pH of *B. cinerea* in a time-dependent and concentration-dependent manner (Fig. 3). Medium acidification by *B. cinerea* was significantly inhibited by lemongrass oil at 0.125% concentration after incubation for 30 min. The medium acidification was completely inhibited after 60 min incubation at concentrations of 0.25, 0.5 and 1.0%. Furthermore, at 1.0% the medium acidification was completely inhibited after 90 and 120 min incubation.

Plasma membrane integrity assay

The plasma membranes of spores of *B. cinerea* were damaged by lemongrass oil ($P < 0.05$) (Fig. 4). Membrane integrity of *B. cinerea* spores declined with the increase of incubation time in PDB containing different concentrations of lemongrass oil (0.125, 0.25, 0.5, and 1.0%). Furthermore, membrane integrity of control spores incubated in PDB without lemongrass oil remained at 98% with the increase of incubation time. In addition, fluorescence microscopy indicated that spores of *B. cinerea* showed strong propidium iodide

fluorescence in the presence of lemongrass oil compared to the control treatment (Fig. 5). This finding indicated damaged plasma membrane of *B. cinera* by lemongrass oil.

Release of cellular material

The release of cell constituents was determined by measuring the absorbance (260 nm) of *B. cinerea* suspension exposed to different concentrations of lemongrass oil. According to Fig. 6, treating *B. cinerea* hyphae with lemongrass oil at different concentrations for 120 min significantly increased the release of cell constituents ($P < 0.05$). The OD_{260} value in *B. cinerea* suspensions with 0.125, 0.25, 0.50 and 1.00% concentrations of lemongrass essential oil for 30 min were 0.230, 0.355, 0.443 and 0.509, respectively. These were significantly higher ($P < 0.05$) than the control treatment (0.100).

Total lipid content

The effect of different concentrations of lemongrass oil on the total lipid content of *B. cinerea* cells is shown in Fig.7. The total lipid contents of *B. cinerea* cells significantly decreased after 30 min exposure to different concentrations of lemongrass oil ($P < 0.05$). The total lipid contents of *B. cinerea* cells after 30 min of incubation in 0.125, 0.25, 0.5 and 1.0% of lemongrass oil concentrations were 254.50, 192.40, 155.00 and 120.00 mg/g dry weight, respectively, which were significantly lower than that of the control (278.00 mg/g dry weight) ($P < 0.05$). Furthermore, the total lipid contents of *B. cinerea* cells treated with 1.0% lemongrass oil remained stable after 60, 90 and 120 min of exposure.

Discussion

There has been an increase interest in using natural substances such as essential oils and extracts derived from plants for different industrial purposes, e.g. food seasoning, natural medicine and agriculture (Isman, 2000; Mansingh, 2004; Cao *et al.*, 2004; Bakkali *et al.*, 2008). The main advantage of essential oils is that they are considered 'generally recognised as safe' (GRAS) (Burt, 2004). The chemical composition of lemongrass oil used in the present study was previously determined using gas chromatography-mass spectrometry (Chapter 2). The analysis showed that lemongrass essential oil was dominated by carbonyl compounds (88.39%) with geranial (48.14%) followed by neral (38.32%) as the major components. The aldehyde constituent of citral is a mixture of two isomers; geranial and neral, which demonstrates significant antifungal activity against postharvest pathogens (Wuryatmo *et al.*, 2003). Furthermore, it was also speculated that the antifungal activity showed by lemongrass oil might be attributed to the major components.

Essential oils inhibits postharvest pathogens mainly due to their effect on the mycelial growth of the pathogens and spore germination by affecting the cellular metabolism of the pathogens (Serrano *et al.*, 2005; Tzortzakis, 2007; Regnier *et al.*, 2010). The potential mechanisms underlying the antimicrobial activity of plant essential oils are not fully understood. Light microscopy and SEM observations in this study indicated that *B. cinerea* hyphae exposed to lemongrass oil undergo morphological damage such as vesiculation, cytoplasmic disruption and collapsed hyphae. These findings were in agreement with those previously reported by Aiemsaard *et* al. (2011), Park *et al.* (2009), Tyagi and Malik (2010a, b; 2011). Scanning electyron microscopy analysis showed shrinkage of *C. albicans* cells treated with lemongrass oil (Tyagi and Malik, 2010a). According to Mendoza *et al.* (1997), the mode of action of essential oils without phenolic groups such as lemongrass oil could be due to membrane disruption by the lipophilic compounds. The lipophilicity of essential oils enable them to partition from an aqueous phase into membrane structures of the fungi, resulting in membrane expansion, increased membrane fluidity and permeability, disturbance of membrane-embedded proteins, inhibition of respiration, alteration of ion transport processes in fungi and induced leakage of ions and other cellular contents (Beckman, 2000; Burt, 2004; Oonmetta-aree *et al.*, 2006; Khan *et al.*, 2010; Fadli *et al.*, 2012). These findings indicate that the antifungal activity of lemongrass oil against *B. cinerea* could be through membrane disruption.

The plasma membrane plays a vital role in maintaining a homeostatic environment, exchanging materials, and transferring energy and information in the cell to keep cells healthy and alive (Tian *et al.*, 2012). Membrane permeability parameters, including loss of 260 nm absorbing materials, change in extracellular pH, and leakage of potassium ions are commonly used to indicate gross and irreversible damage to the cytoplasmic and plasma membranes (Turgis *et al.*, 2009; Souza *et al.*, 2010; Paul *et al.*, 2011; Bajpai *et al.*, 2013; Shao *et al.*, 2013). The results of PI staining and microscopy showed that membrane integrity of *B. cinerea* declined in PDB containing lemongrass oil. The release of cell constituents in the fungal suspensions increased with increasing lemongrass concentrations, with higher exposure times causing greater cell leakage. The proton-translocating ATPase of fungi has been considered by several investigators to be a possible target in the development of antifungal agents (Talkoff-Rubin and Rubin, 1992; Monk *et al.*, 1999). The proton-pumping ability of fungi mediated by the ATPase at the expense of energy is crucial for the regulation of the internal pH of a fungal cell (Manavathu *et al.*, 1999). According to Manavathu *et al.* (1999), when fungal cells depleted of their carbon sources are exposed to glucose, the sugar is rapidly taken up by the cells by the proton motive force generated by the proton gradient due to the pumping out of intracellular protons. The extrusion of intracellular protons to the surrounding medium will acidify it, and the resulting alteration of

the pH of the external medium can be measured with the help of a pH electrode. This study showed that lemongrass oil inhibited glucose-induced reduction in external pH of *B. cinerea* in a time-dependent and concentration-dependent manner. Therefore, these findings support the hypothesis that the cytoplasmic accumulation of the essential oil compounds membrane causes immediate loss of integrity and increases permeability to ions, which could be responsible for the antifungal activity of the essential oils.

Heaton and Randall (2011) reported that lipids are one of the main components of biological membranes and have many important functions, including adjusting the fluidity of the membrane, increasing membrane stability, reducing the permeability of water-soluble materials. The decrease in lipid content usually suggests a reduction in membrane stability and an increase in permeability to water-soluble materials (Prashar *et al*., 2003; Helal *et al*., 2007). According to Prashar *et al*. (2003) volatile compounds such as terpenes reportedly disrupt or penetrate the lipid structures of cells by increasing the saturation of the cell membrane. Furthermore, Helal *et al*. (2007) reported that treatment of *Aspergillus flavus* cells with *Cymbopogon citratus* essential oil significantly decreased their total lipid content compared with control cells. In the present study, different concentrations of lemongrass oil significantly decreased the lipid contents of *B. cinerea*. This finding indicates that lemongrass oil can affect the cell membrane structure and inhibit the growth of *B. cinerea*.

In conclusion, the present study has indicated that the antifungal activity of *C. citratus* essential oil can be attributed to the disruption of cell membrane integrity and membrane permeability. This finding suggests that the application of *C. citratus* essential oil might be a good natural fungicide for biological control of postharvest diseases of apples.

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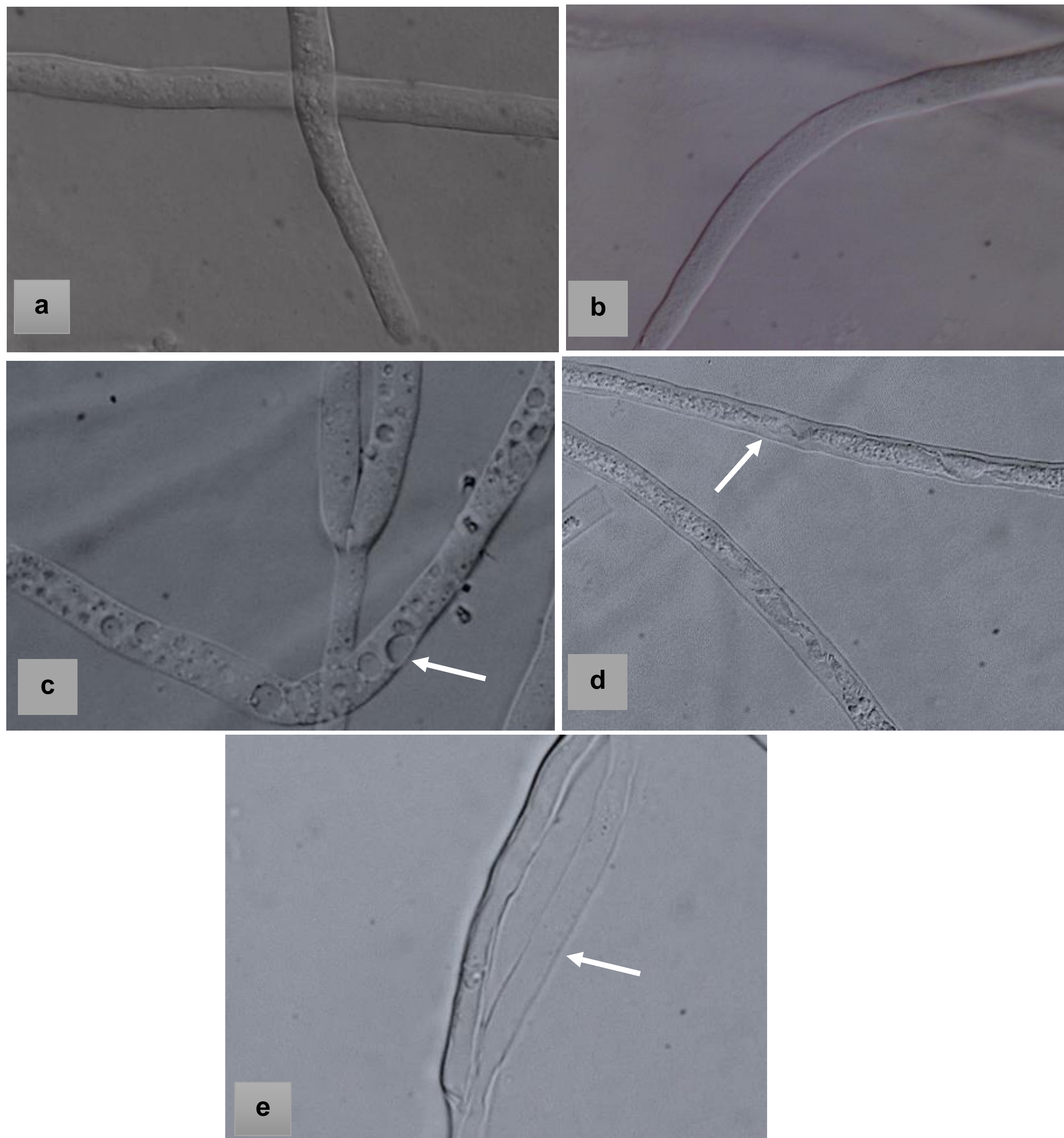

Figure 1. Light micrographs of *Botrytis cinerea* mycelium grown on potato dextrose agar with or without lemongrass oil after 4 days of incubation at 25°C. Normal hyphae of untreated (control) *B. cinerea* (a); normal hyphaea of *B. cinerea* treated with 20% ethanol (b); *B. cinerea* treated with lemongrass oil (c-e). Arrows refer to the morphological changes in the hyphae, such as vesiculation (c), cytoplasmic disruption (d) and collapsed hyphae (e).

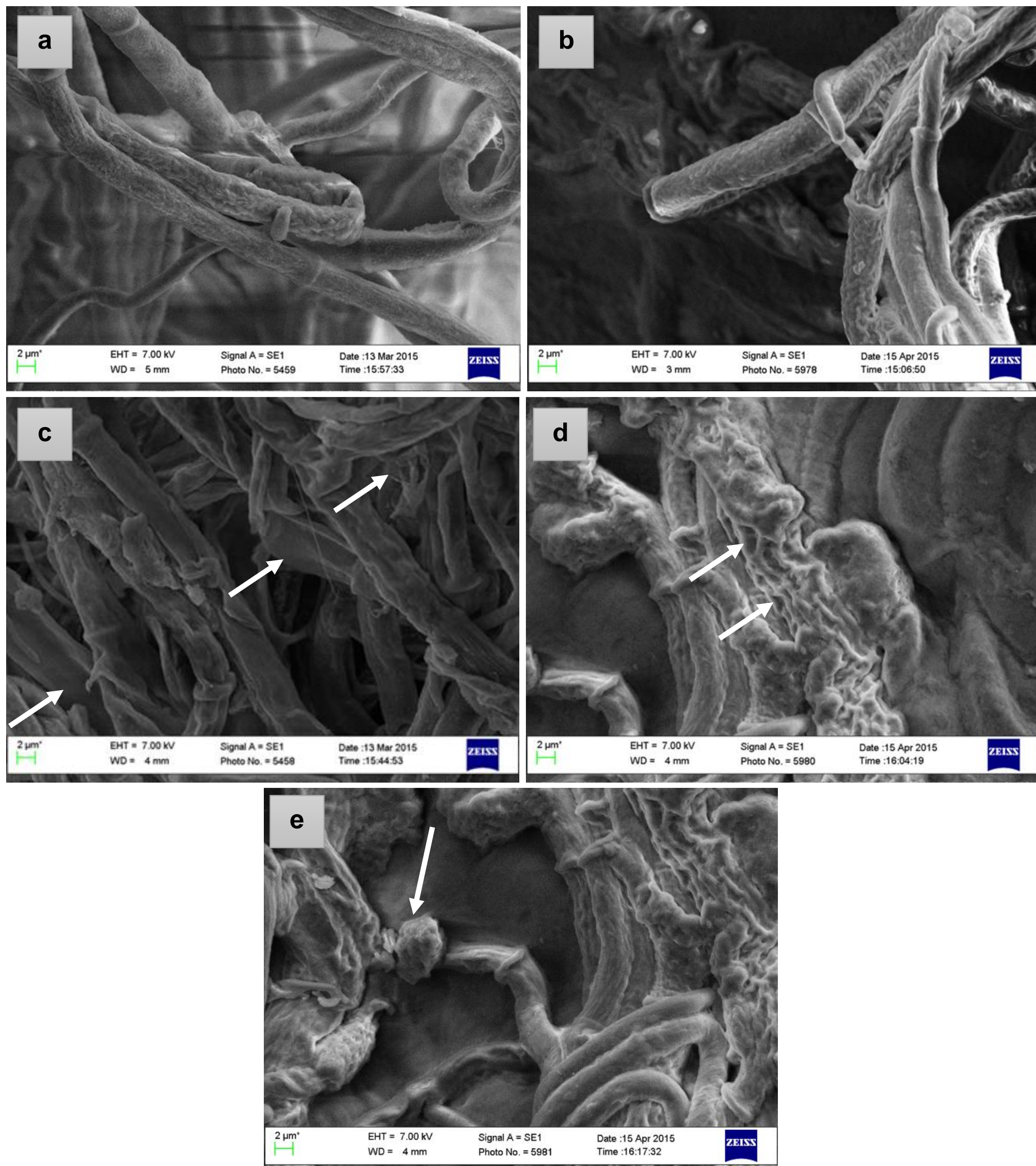

Figure 2. Scanning electron micrographs of *Botrytis cinerea* mycelium grown on potato dextrose agar with or without lemongrass oil after 4 days of incubation at 25°C. Normal hyphae of untreated (control) *B. cinerea* (a); normal hyphaea of *B. cinerea* treated with 20% ethanol (b); *B. cinerea* treated with lemongrass oil (c-e). Arrows refer to the morphological changes in the hyphae, such as collapsed hyphae (c), vesiculation (d), shrinkage and formation of extruded material (e).

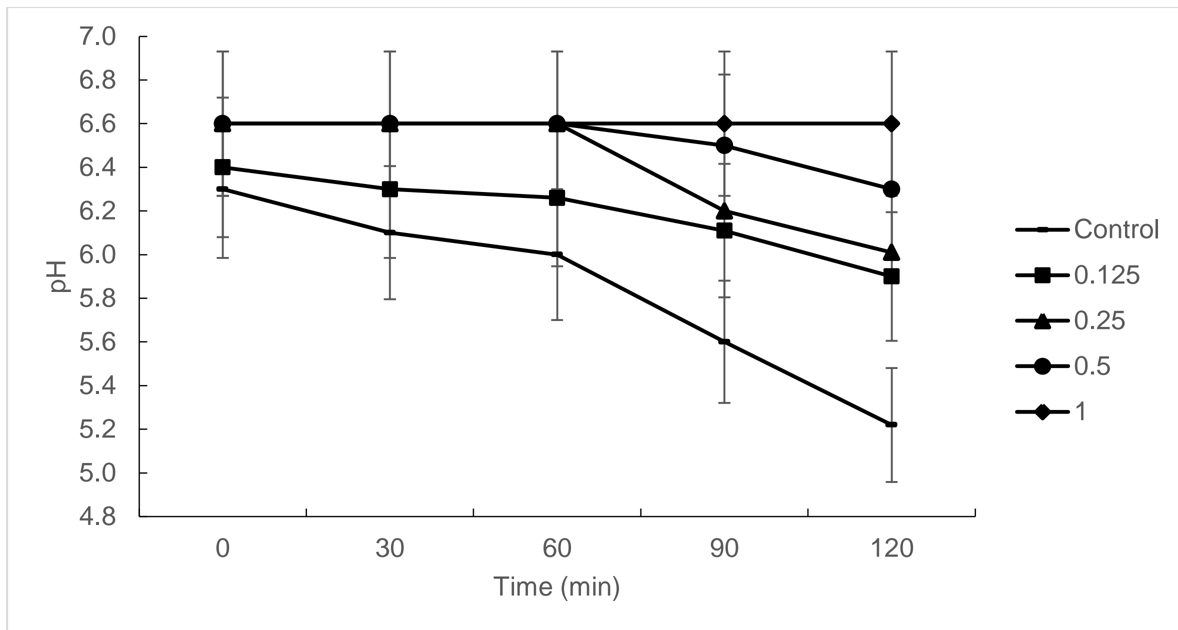

Figure 3. Efficacy of different concentrations of lemongrass oil on the glucose-dependent acidification of medium with *Botrytis cinerea*. Error bars indicate the standard deviations (n = 3).

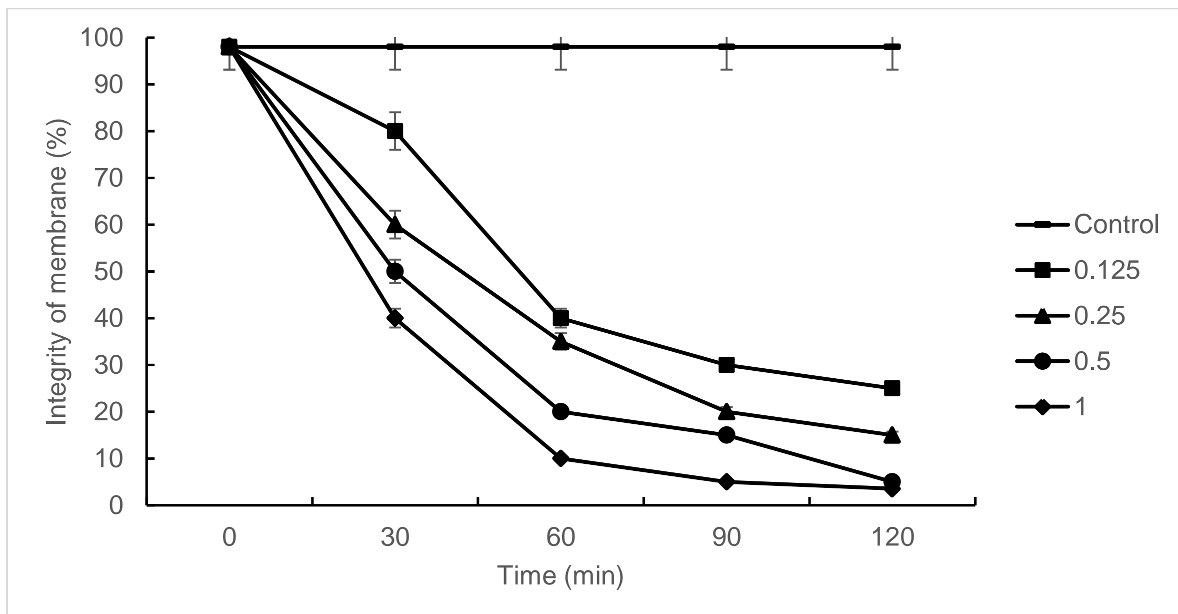

Figure 4. Effect of lemongrass oil on plasma membrane integrity of the spores of *Botrytis cinerea*. Error bars indicate the standard deviations (n = 3).

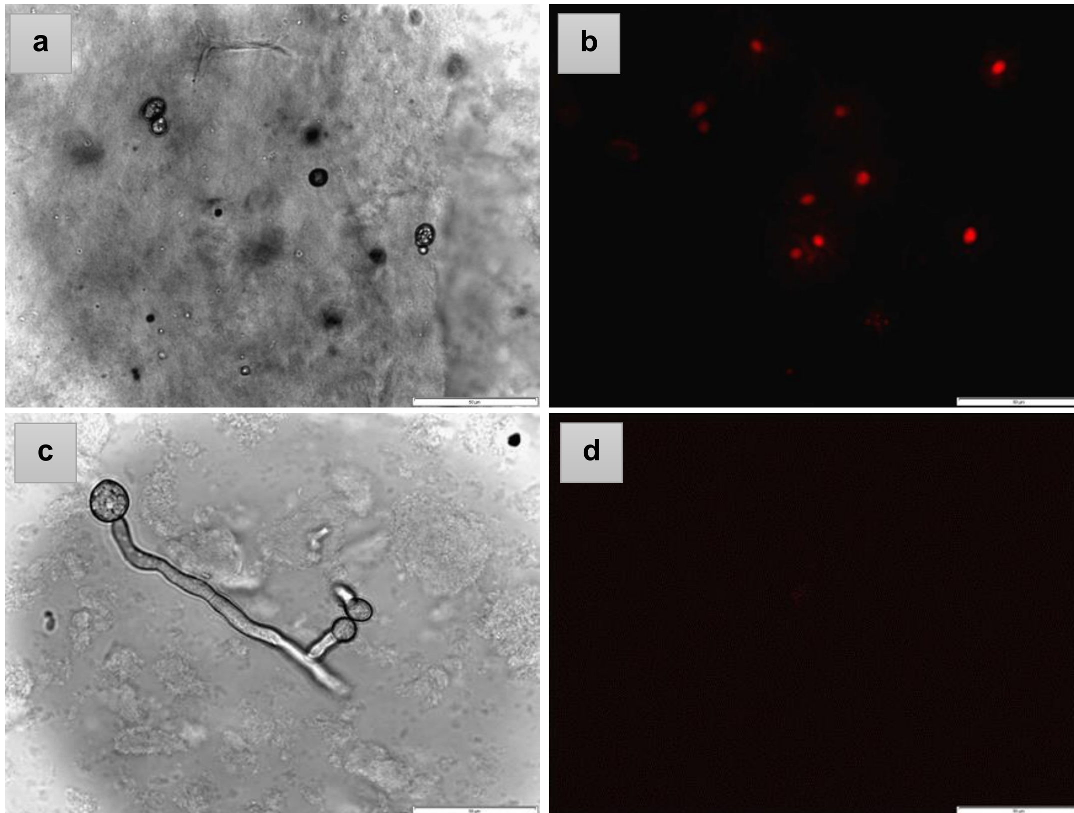

Figure 5. Fluorescence microscopy of *Botrytis cinerea* treated with PI (40× magnification) from the treatments with lemongrass oil (1.0%) and observed with differential interference contrast (a); treated with lemongrass and observed with fluorescence (b); spores without lemongrass addition and observed with DIC (c); and spores without lemongrass addition and observed with fluorescence (d). Bar = 50μm.

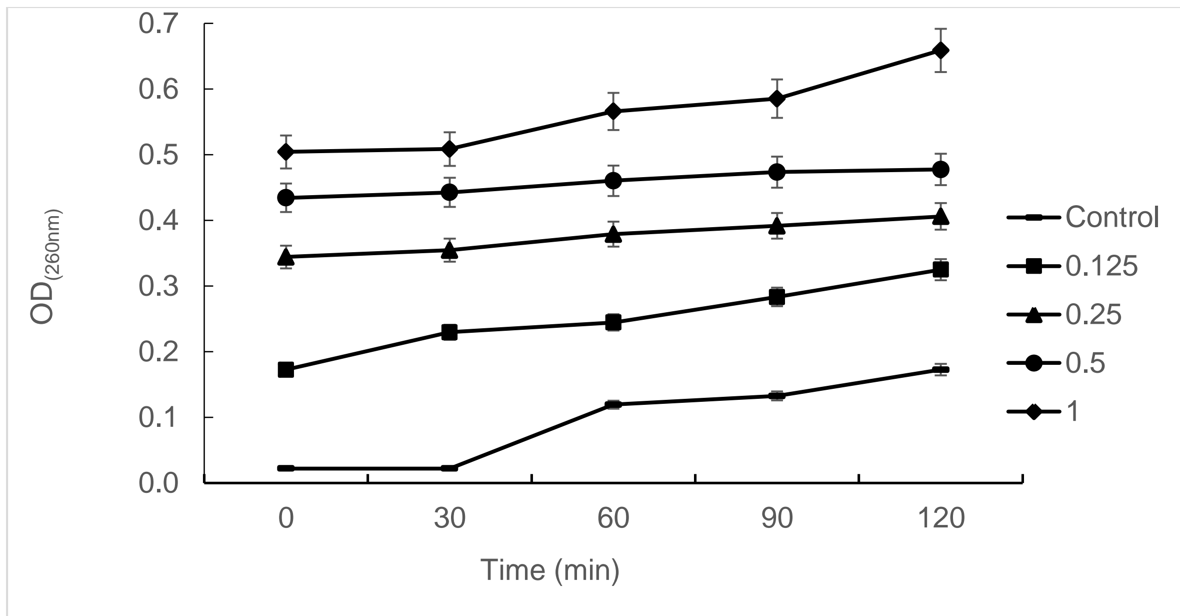

Figure 6. Effect of lemongrass essential oils on the 260 nm-absorbing material release of *Botrytis cinerea*. Data presented are the means of pooled data. Error bars indicate the standard deviations (n = 3).

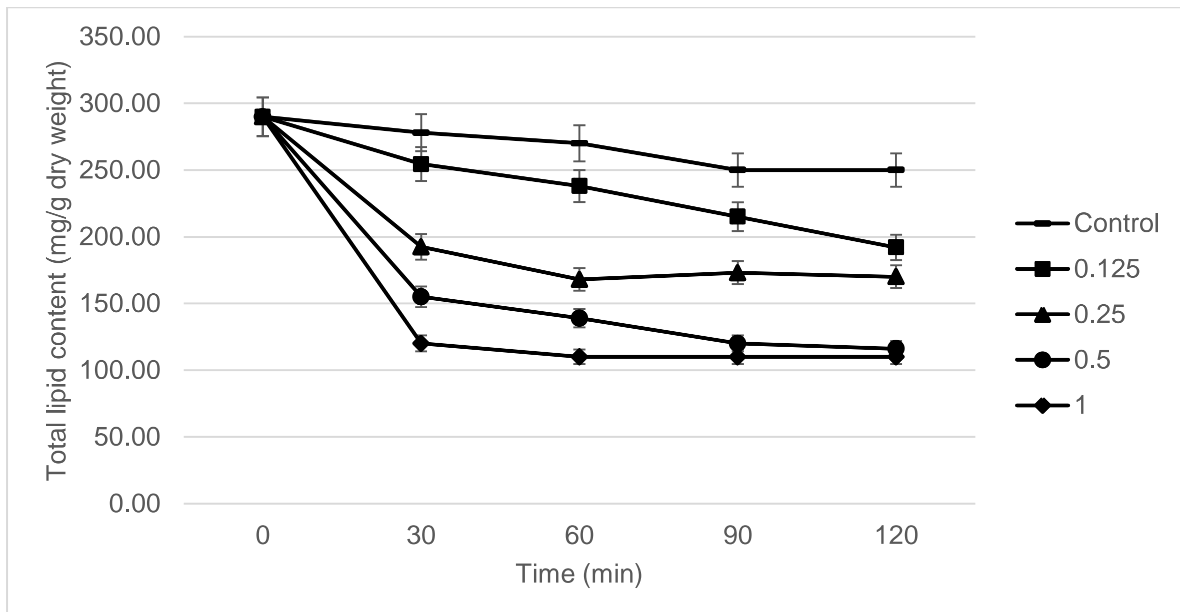

Figure 7. Effect of lemongrass oil on the total lipid content of *Botrytis cinerea* cells. Data presented are the means of pooled data. Error bars indicate the standard deviations (n = 3).

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CHAPTER 5

EFFICACY OF CITRUS AND LEMONGRASS ESSENTIAL OILS ON POSTHARVEST DECAY CAUSED BY *BOTRYTIS CINEREA, PENICILLIUM EXPANSUM* AND *NEOFABRAEA ALBA*

Abstract

Fungal infections are the main source of postharvest rots of fruit during storage, and cause significant economic losses. Increasing consumer concern regarding food safety and demand for organically produced fruit makes it necessary to search for natural environmentally friendly alternative methods for disease control. Preventative and curative application of essential oils through fumigation was tested *in vivo* for their potential to inhibit postharvest decay caused by *Botrytis cinerea*, *Penicillium expansum* and *Neofabraea alba* on 'Granny Smith', 'Golden Delicious' and 'Pink Lady' apples. Treated fruit was stored at 20°C for 7 days, regular atmosphere (-0.5°C, air) or controlled atmosphere ('Granny Smith' and 'Pink Lady' at 1.5% O_2 + 1% CO_2,-0.5°C and 'Golden Delicious' at 1.5% O_2 + 2.5% CO_2,-0.5°C) for 28 days followed by 7 days at 20°C. Preventative application of lemon, lime, lemongrass, lemon+lemongrass and lime+lemongrass essential oils through thermal fogging and dipping were also tested and treated fruit was stored at controlled atmosphere for 28 days followed by 7 days at 20°C. After storage, lesion diameter was measured and expressed as percentage inhibition relative to control treatment. Fumigation of all three apple cultivars with different concentrations of essential oils (curative or preventative) showed antifungal activity against all three tested pathogens at 20°C, regular atmosphere and controlled atmosphere. All essential oil treatments tested showed no phytotoxicity on all three apple cultivars when applied by means of fumigation and thermal fogging, while dipping fruit showed phytotoxic effect at some concentrations. Application of essential oils (alone and mixtures) through thermal fogging inhibited *B. cinerea*, *P. expansum* and *N. alba* on all three cultivars compared to Rovral™, ethanol and control treatments. Essential oil mixtures showed the highest inhibition of *B. cinerea*, *P. expansum* and *N. alba* on all three cultivars compared to single application of essential oils. In case of dipping of fruit, Rovral™ and essential oils provided the best inhibition of *B. cinerea*, *P. expansum* and *N. alba* on each apple cultivar compared to ethanol and control treatments. The results suggest the possibility of using essential oils in combination with controlled atmosphere or regular atmosphere as natural fumigants for controlling postharvest diseases of apples during cold storage.

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Keywords: Postharvest decay, *Botrytis cinerea*, *Penicillium expansum*, *Neofabraea alba*, essential oils, regular atmosphere, controlled atmosphere.

Introduction

Grey mould (*Botrytis cinerea* Pers.: Fr.), blue mould (*Penicillium expansum* (Link) Thom.) and bull's eye rot (*Neofabraea alba* (E.J. Gutrie) Verkley) are the most destructive postharvest pathogens attacking fruit through wounds (Saravanakumar *et al*., 2008; Tripathi *et al.,* 2008; Solaimani *et al.,* 2009; Tolaini *et al*., 2010). Grey mould is a postharvest fungal disease of apples that occurs throughout the marketing chain (Beattie *et al.,* 1989; Elad *et al.,* 2004; Williamson *et al*., 2007; Zhang *et al*., 2007). Infection caused by grey mould results in light brown to dark brown and colour is similar across the decayed area. The decayed area is spongy, and diseased tissue is not separable from the healthy tissue, which is different from blue mould. Under high relative humidity, grey spore masses and/or fluffy white to grey mycelia may appear on the decayed area (Coley-Smith *et al.,* 1980; Beattie *et al.,* 1989; Xiao, 2006). On the other hand, blue mould is easily recognized by the mass of blue conidia produced on infected fruit (Rosenberger and Sugar, 1990; Sugar and Spotts, 1999). The fruit at first shows soft, pale-brown, watery spots with the decayed portions completely separable from healthy tissue (Beattie *et al.,* 1989). The decayed tissue is soft and watery and the lesion has a very sharp margin between diseased and healthy tissues (Rosenberger and Sugar, 1990; Sugar and Spotts, 1999).

In addition, *Neofabraea alba* (E.J. Gutrie) Verkley causal agent of bull's eye rot occurs sporadically in South Africa on late maturing apple varieties (A den Breeyen, pers. commun.). Fruit can be infected in the orchard at any time during the growing season, and spores or appresoria remain dormant until several months of postharvest storage (Edney, 1958; Bompeix, 1978; Snowdon, 1990; Spotts, 1990; Neri *et al*., 2009). *Neofabraea alba* is weakly parasitic to saprophytic on wood of the host trees (Corke, 1956; Bompeix, 1988), it is also weakly parasitic on fruit and starts developing with maturation of the fruit and develops lesions later than the other species in storage (Bompeix, 1978). Fruit lesions are flat to slightly sunken, brown and often with a lighter brown centre (Snowdon, 1990; Spotts, 1990; Henriquez *et al.,* 2004). The rotten tissue are relatively firm and acervuli are frequently present in old lesions under humid conditions (Neri *et al.,* 2009). Cream-coloured spore masses in the aged decayed area may appear. Decayed tissue is firm and does not readily separate from healthy tissue (Spotts, 1990; Henriquez *et al.,* 2004).

The main control strategy of postharvest diseases consists of drench application and/or spray treatment on the packing line using various fungicides. Thiabendazole™, Imazalil™, sodium ortho-phenylphenate™ were the most active ingredients used for decades before the introduction of pyrimethanil (anilinopyrimidine) and fludioxonil

(phenylpyrrole) fungicides to the market. However, following intensive use, resistance to thiabendazole™ has been reported in *Botrytis* and *Penicillium* populations (El-Goorani *et al*., 1984; Leroux and Clerjeau, 1985; Prusky *et al*., 1985; Bus *et al*., 1991; Giraud and Faure, 2000), as well as to IMZ and SOPP (Harding, 1962; Eckert, 1987; Dave *et al.,* 1989). Resistance of pathogens to anilinopyrimidines has been reported in the late 1990s (Chapeland *et al*., 1999) and especially for *B. cinerea* (Latorre *et al*., 2002; Moyano *et al*., 2004). Decreasing efficacy and increasing concern over the adverse environmental effects of synthetic fungicides have brought about the need for the development of new control alternatives and crop protection methods without or with reduced use of conventional fungicides (Soylu *et al*., 2010).

Changing atmosphere conditions during storage, where either the O_2 or CO_2 concentration is changed for a short period with subsequent return to the initial concentration set point, have been reported to improve fruit quality and storability of some apple cultivars such as 'Gala' (Mattheis *et al.,* 1998). The storage of fruit under modified and controlled atmospheres has been used directly or indirectly to reduce postharvest decay (Sommer, 1982). The growth of many organisms is retarded in controlled atmosphere (CA) storage, but the mixture of gases that provides the best results in extending the life of fruit in storage is not necessarily the best to reduce the development of storage rots. Some pathogens are not affected at all, and others are even favored by CA storage (Lockhart, 1969). Low temperatures slow down the growth of fungi causing postharvest decay including *B. cinerea, P. expansum, Mucor piriformis* and *Alternaria alternata*, but growth is resumed as soon as the fruit are transferred to a higher temperature (Sommer, 1985).

Several essential oils have been reported to inhibit postharvest fungi *in vitro* such as *Penicillium* spp. (Caccioni and Guizzardi, 1994; Smid *et al*., 1995; Arras and Usai, 2001), *B. cinerea* and *Monilinia fructicola* (Wilson *et al*., 1987, 1997). The potential of essential oils of citrus and lemongrass, in combination with controlled atmosphere and regular atmosphere as eco-friendly antifungal agents was demonstrated in our previous studies (Chapter 3). It was observed that the efficacy of the citrus and lemongrass essential oils against *B. cinerea*, *P. expansum* and *N. alba* was not affected by regular atmosphere or controlled atmosphere. New alternative control methods alone do not have a wide spectrum of activity under various conditions compared to fungicides and most of them cannot achieve the effectiveness of fungicides even under optimal conditions. Therefore, a combination of promising alternatives must be used to develop a control strategy suitable for commercial application. There is currently no registered fungicide against *N. alba* in South Africa. The objective of this study was to determine the efficacy of essential oils as a postharvest treatments against *B. cinerea*, *P. expansum* and *N. alba* on apples applied by fumigation, dipping and thermal fogging.

Materials and methods

Pathogen isolation

Three fungal pathogens, *N. alba* (Accession number: DOK7-SUN) isolated from infected apples, *B. cinerea* (Accessin number: PPRI 7338) and *P. expansum* (Accession number: PPRI 5944) isolated from infected plums and pears, respectively, were used. All the isolates were tested for pathogenicity on apples and pure isolates were prepared. *Neofabraea alba* was cultured on acidified PDA (pH 3.5, Merck, Johannesburg, South Africa) for 1 month at 25°C. *Penicillium expansum* and *B. cinerea* were cultured on potato dextrose agar (PDA, pH 5.6, Merck, Johannesburg, South Africa) at 25°C for 3 days for mycelial mycelial plugs and 7 days for the production of spores. The cultures of *B. cinerea, P. expansum* and *N. alba* were maintained on PDA slants at 4°C. For *B. cinerea* and *P. expansum*, conidia were haversted from the medium surface with sterile distilled water together with Tween 80 (0.05% w/v), and gentle agitating the plates to dislodge the spores. The final inoculum concentration was adjusted to to 1×10^4 conidia mL^{-1} for each pathogen using a haemocytometer. For *N. alba,* mycelial discs (3 mm diameter) were excised from the edge of 4 weeks old cultures.

Essential oils and fruit

Essential oils of lemon [*Citrus limon* (Linn.) Burm], lime [*Citrus aurantifolia* (Christm.) Swingle] and lemongrass [*Cymbopogon citratus* (DC.) Strapf] were obtained from Groenkop Farm (Melmoth, KwaZulu-Natal, South Africa) and stored at 4°C until used. Lemon and lime EOs were originally extracted from the fresh fruit peel by steam distillation, while lemongrass oil was extracted from fresh or partly dried leaves by steam distillation. Apple (*Malus domestica* Borkh.) cultivars 'Golden Delicious', 'Granny Smith' and 'Pink Lady' were collected from Fruitways (Grabouw, Western Cape, South Africa). Only unblemished, healthy, mature fruit were used in the experiments.

Efficacy of postharvest fumigation of essential oils against **Botrytis cinerea, Penicillium expansum** *and* **Neofabraea alba** *on apples*

Fruit was surface sterilized with 70% ethanol for 1 min and allowed to air dry. Fruit was uniformly wounded, approximately 3 mm deep and 3 mm wide, with a sterile needle at the equatorial side. Wounded fruit was inoculated with 20 µL of spore suspension of *B. cinerea* and *P. expansum*. For *N. alba,* wounded fruit was inoculated with mycelial discs (3 mm diameter) from the edge of 4 weeks old cultures. Phytatray containers (Zibo containers (Pty) LTD, Kuilsrivier, South Africa) were used as containers containing the essential oil and the

inoculated fruits. Lemon, lime, lemongrass, lemon+lemongrass and lime+lemongrass EOs at concentrations of 0.125% and 0.5% were introduced into the phytatray containers by placing 5 mL of the specific concentration in a glass Petri plate inside the phytatray container. Currently used fungicide, Rovral™ (Bayer, South Africa) was also included to compare with essential oils. Five inoculated fruit were then randomly assigned to each box. There were three replicate containers of 5 fruits per treatment. The experiment was conducted twice. Two inoculation methods were tested (1) Preventative treatment and (2) Curative treatment. For the preventative effect of essential oils: fruit was inoculated with the test pathogens and allowed to dry for 3 hr, and thereafter placed in phytatray containers with appropriate treatments. For the curative effect of essential oils: fruit was inoculated with the test pathogens and allowed to dry for 3 hr, inoculated fruit was enclosed with black plastic bags with a piece of wet paper towel to ensure high humidity and promote spore germination for 20 hr at 20°C, and thereafter placed in phytatray containers with appropriate treatments. Phytatray containers were sealed with lids and incubated at (1) 20°C (90-95% RH) for 7 days, (2) regular atmosphere (-0.5°C, air) for 28 days followed by 7 days at 20°C and (3) controlled atmosphere for 28 days followed by 7 days at 20 °C. The controlled atmosphere room for 'Pink Lady' and 'Granny Smith' apples was -0.5°C; 1.5% O_2 + 1% CO_2; and for 'Golden Delicious' was -0.5°C; 1.5% O_2 + 2.5% CO_2. Fruit was evaluated for decay and lesion diameter was measured and expressed as percentage inhibition.

Phytotoxic effects of essential oils on apples

Phytotoxicity can be of concern when using essential oils as a treatment. To determine the best application method of the tested essential oils and their main constituents and evaluate what effect volatiles would have on the fruit, a preliminary trial was undertaken. Two application methods, the direct contact method such as dipping and the indirect contact method such as fumigation were evaluated. Treatments consisted of different concentrations of essential oils (0.125%, 0.25% and 0.5%), Rovral™ 1 mL/L, 20% ethanol (v/v) and water control. Fruit was surface sterilized with 70% ethanol (v/v) for 1 min and allowed to air dry before dip or fumigation. Fruit was dipped for 30 s, 60 s and 90 s in the different test solutions. For fumigation, fruit were exposed in vapours of different test solutions. Treated fruit was stored at regular atmosphere (-0.5°C, air) for 7 days. After storage at regular atmosphere, fruit was transferred to 20°C for 7 days. Each treatment was replicated five times with 15 fruit per replicate. The experiment was conducted twice. After incubation the degree of phytotoxicity on fruit (phytotoxicity score) was rated using a scale of 0-4 (Fig. 1), where 0) no phytotoxicity; 1) 1-25% phytotoxicity; 2) 26-50% phytotoxicity; 3) 51-75% phytotoxicity; 4) 76-100% phytotoxicity.

Efficacy of postharvest dipping of essential oils against* Botrytis cinerea*,* Penicillium expansum *and* Neofabraea alba *on apples

Treatments consisted of 0.125% concentration of essential oils (lemon, lime, lemongrass, lemon+lemongrass and lime+lemongrass), Rovral™ 1 mL/L, Scholar™ 1.3 mL/L, 20% ethanol (v/v) and water control. Fruit was surface sterilized with 70% ethanol (v/v) for 1 min and allowed to air dry before dip. Fruit was uniformly wounded, approximately 3 mm deep and 3 mm wide, with a sterile needle at the equator. For *B. cinerea* and *P. expansum*, different test treatments were mixed with spore suspension for 3 min and thereafter fruit was dipped for 30 sec. After dipping, treated fruit was left to air-dry. For *N. alba*, wounded fruit was dipped for 30 sec at different test treatments and allowed to air-dry, after the wound site had dried, each wound was inoculated with 3 mm mycelial discs obtained from 4 weeks old *N. alba*. Inoculated fruit was enclosed with black plastic bags with a piece of wet paper towel to ensure high humidity and promote spore germination for 20 hr at 20°C, and then stored at controlled atmosphere for 28 days followed by 7 days at 20°C. The controlled atmosphere room for 'Pink Lady' and 'Granny Smith' apples was -0.5°C; 1.5% O_2 + 1% CO_2; and for 'Golden Delicious' was -0.5°C; 1.5% O_2 + 2.5% CO_2. Each treatment was replicated 5 times with 15 fruit per replicate. The experiment was conducted once. After incubation, the degree of phytotoxicity on fruit was inspected and lesion diameter (mm) was measured and expressed as percentage inhibition.

Efficacy of postharvest thermal fogging application of essential oils* against Botrytis cinerea*,* Penicillium expansum *and* Neofabraea alba *on apples

Treatments consisted of 0.125 % concentration of essential oils (lemon, lime, lemongrass, lemon+lemongrass and lime+lemongrass), Rovral™ 1 mL/L, Scholar™ 1.3 mL/L, 20% ethanol (v/v) and water control. Fruit was surface sterilized with 70% ethanol (v/v) for 1 min and allowed to air dry. Fruit was uniformly wounded, approximately 3 mm deep and 3 mm wide, with a sterile needle at the equator. Fruit was sorted and placed in plastic crates and thereafter transferred to a cage supplied by Chempac (Pty) Ltd (Paarl, Western Cape, South Africa) to simulate cold storage rooms. Treatments were introduced into a Falcon fogger (Dyna-Fog Asia LTD) and applied for 30 min. The crates were removed and fruit was inoculated with spore suspension of 10^4 spore/mL (*B. cinerea* and *P. expansum*), or fruit was inoculated with 3 mm mycelial plugs of *N. alba*. Inoculated fruit was enclosed with black plastic bags with a piece of wet paper towel to ensure high humidity and promote spore germination for 20 hr at 20°C and then stored at controlled atmosphere for 28 days followed by 7 days at 20°C. The controlled atmosphere room for 'Pink Lady' and 'Granny Smith' apples was -0.5°C; 1.5% O_2 + 1% CO_2; and for 'Golden Delicious' was -0.5°C; 1.5% O_2 + 2.5% CO_2. Each treatment was replicated 5 times with 15 fruit per replicate. The experiment

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was conducted once. After incubation, the degree of phytotoxicity on fruit was inspected and lesion diameter (mm) was measured and expressed as percentage inhibition.

Statistical analysis

For fumigation trial, the experimental design was completely randomised design (CRD) with interaction between treatment and cultivar for each application method (curative or preventative) for each test pathogen (*B. cinerea*, *P. expansum* and *N. alba*) at each storage (regular atmosphere or controlled atmosphere or 20°C). For dipping and thermal fogging trials, the experimental design was completely randomised (CRD) with interaction between treatment and cultivar for each test pathogen (*B. cinerea*, *P. expansum* and *N. alba*) at controlled atmosphere. Univariate analysis of variance was performed on all data accessed using GLM (General Linear Models) Procedure of SAS statistical software version 9.2 (SAS, 2012). Shapiro-Wilk test was performed to test for normality (Shapiro, 1965). Fisher's least significant difference was calculated at the 5% level to compare treatment means (Ott, 1993). A probability level of 5% was considered significant for all significance tests.

Results

Efficacy of postharvest fumigation of essential oils against* Botrytis cinerea*,* Penicillium expansum *and* Neofabraea alba *on apples

Preventative and curative effect of essential oils against *B. cinerea, P. expansum* and *N. alba* was conducted on apple (*Malus domestica* Borkh.) cultivars 'Golden Delicious', 'Granny Smith' and 'Pink Lady' (Tables 1 - 9). Table 1 shows that all essential oils (single and mixtures) curatively controlled *B. cinerea* on all three apple cultivars in comparison to control and Rovral™ treatments (0.00% respectively). Although not significant ($P = 0.44$), a mixture of lime + lemongrass oils at a concentration of 0.125% showed 96.99% inhibition of *B. cinerea* on 'Golden Delicious'. At a concentration of 0.5%, it was observed that mixtures of lime + lemongrass showed 90.32% inhibition of *B. cinerea* on 'Granny Smith', while a mixture of lemon + lemongrass exhibited 89.84% inhibition on 'Pink Lady'. Ethanol treatments provided some inhibition to 'Golden Delicious', 'Pink Lady' and 'Granny Smith' cultivars (30.43%, 28.10% and 22.32%, respectively). *B. cinerea* was preventatively inhibited by all essential oil treatments on all three apple cultivars compared to control and Rovral™ treatments (Table 1). Although not significant ($P = 0.47$) essential oil mixtures of lime + lemongrass, and lemon + lemongrass at a concentration of 0.5% inhibited *B. cinerea* on preventatively 'Golden Delicious' apples by 95.18% and 93.84%, respectively. Lime essential oil exhibited 94.69% and 93.20% inhibition.

Curative method indicated that single or mixtures of essential oils significantly ($P < 0.0001$) inhibited *B. cinerea* on 'Golden Delicious' and 'Granny Smith' cultivars at both concentrations tested compared to control, ethanol and Rovral™ treatments (Table 2). Lime essential oil showed 98.38% and 87.63% inhibition of *B. cinerea* at concentrations of 0.125% and 0.5% on 'Golden Delicious' and 'Granny Smith' cultivars. *Botrytis cinerea* was significantly inhibited (34.12%) by lime essential oil on 'Pink Lady'. Preventative method showed that all essential oils (single or mixtures) significantly ($P < 0.0001$) inhibited *B. cinerea* on all three apple cultivars compared to control, ethanol and Rovral™ treatments. Lime essential oil exhibited 99.06% and 53.28% inhibition of *B. cinerea* on 'Golden Delicious' and 'Granny Smith' cultivars at a concentrations of 0.125% and 0.5%, respectively and 54.26% inhibition was observed on 'Pink Lady' at a concentration of 0.125%.

It was observed that both preventative and curative effect of all essential oils significantly ($P < 0.0001$) inhibited *B. cinerea* on all three apple cultivars compared to control treatment (Table 3). For curative method, essential oil mixture of lemon + lemongrass at a concentration of 0.125% and 0.5% showed 77.67% and 86.30% inhibition of *B. cinerea* on 'Pink Lady' and 'Granny Smith' cultivars, while lemon essential oil exhibited 83.11% inhibition on 'Golden Delicious' cv. For preventative method, it was observed that lime essential oil exhibited 86.36% and 67.10% inhibition of *B. cinerea* at a concentration of 0.125% on 'Granny Smith' and 'Pink Lady' cultivars, respectively.

Curative and preventative methods showed that all essential oils significantly ($P = 0.0003$) inhibited *P. expansum* on all three apple cultivars compared to control, ethanol and Rovral™ treatments (Table 4). Curative method indicated that single oils of lemon, lime and lemongrass at a concentration of 0.5% inhibited *P. expansum* by 78.87%, 56.48% and 71.66% on 'Granny Smith', 'Pink Lady' and 'Golden Delicious' cultivars, respectively. Preventative method showed that *P. expansum* was significantly inhibited ($P = 0.0003$) by lemongrass oil on 'Granny Smith' and 'Golden Delicious' by 63.38% and 60.87%, respectively, at a concentration of 0.5%, while at a concentration of 0.125% 'Pink Lady' was inhibited by 42.53%.

It is evident from this study that curative and preventative effect of all essential oils significantly ($P < 0.0001$) inhibited *P. expansum* on all three apple cultivars in comparison to control, ethanol, and Rovral™ treatments (Table 5). Curative method indicated that lemongrass oil inhibited *P. expansum* on 'Pink Lady' by 51.71% at a concentration of 0.5%, while the other essential oils (single or mixtures) showed inhibition of ≤ 50% on all three apple cultivars. Preventative method revealed 89.83% and 74.44% inhibition of *P. expansum* by lemongrass and a mixture of lime and lemongrass at a concentration of 0.5% on 'Granny Smith' and 'Pink Lady' cultivars respectively, while lemon oil exhibited 79.28% inhibition on 'Golden Delicious' at a concentration of 0.125%.

Curative method indicated that all essential oil treatments (single or mixtures) significantly (*P* = 0.0370) inhibited *P. expansum* compared to control, ethanol and Rovral™ treatments (Table 6). For curative method, it was observed that 0.5% concentration of lemon oil exhibited 69.31% and 64.17% inhibition of *P. expansum* on 'Golden Delicious' and 'Pink Lady' cultivars, while lemongrass showed 80.36% on 'Granny Smith'. Preventative method showed that all essential oil treatments (single or mixtures) significantly (*P* = 0.0025) inhibited *P. expansum* compared to control, ethanol and Rovral™ treatments. Essential oil of lime and a mixture of lime + lemongrass at a concentration of 0.125% inhibited *P. expansum* by 55.38% and 63.09% on 'Pink Lady' and 'Granny Smith' cultivars, respectively, while lime essential oil showed 65.08% inhibition on 'Golden Delicious' cv.

Curative method showed that all essential oil treatments (alone or mixtures) significantly inhibited *N. alba* (*P* < 0.0001) on 'Golden Delicious', 'Pink Lady' and 'Granny Smith' cultivars compared to control treatments (Table 7). Lime essential oil showed 96.16% and 90.53% inhibition of *N. alba* on 'Golden Delicious' and 'Granny Smith' cultivars, respectively, at a concentration of 0.5%, while at a concentration of 1.125% the inhibition was 89.67% on 'Pink Lady' apples. Rovral ™ showed 43.16% inhibition of *N. alba* only on 'Granny Smith' apples and it was significantly (*P* < 0.0001) different from control and ethanol treatments. For preventative method, it was observed that *N. alba* was significantly inhibited by 89.92% on 'Golden Delicious' and 'Granny Smith' cultivars, respectively, at a concentration of 0.125%; while lemongrass oil showed 83.44% and 61.22% on 'Golden Delicious' and 'Pink Lady' cultivars, respectively, at a concentration of 0.5%.

For both curative and preventative methods, all essential oil treatments were significantly (*P* < 0.0001) different from control treatments (Table 8). Curative method indicated that essential oil of lemongrass inhibited *N. alba* by 69.83% on 'Pink Lady' apples at a concentration of 0.5%; while mixtures of lemon and lemongrass, lime and lemongrass showed 82.78% and 66.87% on 'Golden Delicious' and 'Granny Smith' cultivars, respectively.

It was observed that curative effect of all essential oils significantly (*P* < 0.0001) inhibited *N. alba* on 'Granny Smith', 'Golden Delicious' and 'Pink Lady' cultivars compared to control, ethanol and Rovral™ treatments (Table 9). Lemongrass essential oil inhibited *N. alba* by 85.45% and 70.08% on 'Pink Lady' and 'Golden Delicious' cultivars, respectively, at a concentration of 0.5%; while lemon oil exhibited 69.48% inhibition on 'Granny Smith' apples. Preventative method indicated that lemongrass essential oil inhibited 'Golden Delicious' and 'Granny Smith' by 79.94% and 87.39%, respectively, at a concentration of 0.5%. Furthermore, a mixture of lemon and lemongrass exhibited 78.99% inhibition of *N. alba* on 'Pink Lady' apples, at a concentration of 0.5%.

Phytotoxic effects of essential oils on apples

For fumigation trial, it was observed that exposing 'Golden Delicious' 'Pink Lady' and 'Granny Smith' cultivars to different concentrations (0.125%, 0.25% and 0.5%) of essential oils (single and mixtures) at different exposure times (30 sec, 1 min and 2 min), showed no phytotoxicity (Data not shown). Table 10 shows the phytotoxic effect of essential oils on 'Golden Delicious' 'Pink Lady' and 'Granny Smith' cultivars at different exposure times when applied by dipping. It was observed that all essential oil treatments (alone or mixtures) were not significantly different (*P* < 0.0001) from control treatments at a concentration of 0.125% and exposure time of 30 s. All three apple cultivars were healthy, non-damaged with no skin damage. 'Golden Delicious' and 'Granny Smith' cultivars dipped in 0.5% of lemongrass essential oil and a mixture of lemongrass + lime oil was highly phytotoxic at exposure time of 30 s. A concentration of 0.125% of essential oils and an exposure time of 30 s was therefore selected for further trials (dipping and thermal fogging).

Efficacy of postharvest dipping of essential oils against* Botrytis cinerea*,* Penicillium expansum *and* Neofabraea alba *on apples

Fig. 2 shows the efficacy of essential oils on postharvest decay of apples caused *B. cinerea*, *P. expansum* and *N. alba* on 'Golden Delicious', 'Pink Lady' and 'Granny Smith' cultivars. Fruit were treated with different treatments and stored for 28 days at controlled atmosphere followed by 7 days at 20°C. It was observed that Rovral™ inhibited *B. cinerea* on 'Golden Delicious', 'Pink Lady' and 'Granny Smith' cultivars by 98.6%, 90.37%, and 90.18%, respectively (Fig. 2a). Although not significant (*P* = 0.0834), it was observed that all essential oils (single and mixtures) effectively inhibited *B. cinerea* on 'Golden Delicious', 'Pink Lady' and 'Granny Smith' cultivars compared to control and ethanol, treatments. Single application of essential oils showed that lemongrass oil was the most effective on all apple cultivars compared to lemon and lime oils; and it inhibited *B. cinerea* by 87.08%, 81.16% and 68.93% on 'Golden Delicious', 'Pink Lady' and 'Granny Smith' cultivars, respectively. It was observed that a mixture of lime + lemongrass essential oil performed better than lemon + lemongrass essential oil. Furthermore, a mixture of lime + lemongrass essential oil exhibited 88.54%, 83.17% and 87.17% inhibition of *B. cinerea* on 'Golden Delicious', 'Pink Lady' and 'Granny Smith' cultivars, respectively (Fig. 2a).

All essential oil treatments (single and mixtures) and Rovral™ significantly inhibited (*P* < 0.0001) *P. expansum* on all three apple cultivars compared to control and ethanol treatments (Fig. 2b). Rovral™ inhibited *P. expansum* on 'Golden Delicious', 'Pink Lady' and 'Granny Smith' cultivars by 97.34%, 79.70%, and 82.38%, respectively. It was observed that all essential oils (single and mixtures) effectively inhibited *P. expansum* on all three apple cultivars compared to control and ethanol, treatments. Single application of essential oils

showed that lemongrass oil was the most effective on all apple cultivars compared to lemon and lime oils. Lemongrass essential oil inhibited *P. expansum* by 60.11%, 69.91% and 70.71% on 'Golden Delicious', 'Pink Lady' and 'Granny Smith' cultivars, respectively. It was observed that a mixture of lime + lemongrass essential oil performed better than lemon + lemongrass essential oil. Furthermore, a mixture of lime + lemongrass essential oil exhibited 65.02%, 71.72% and 76.93% inhibition of *P. expansum* on 'Golden Delicious', 'Pink Lady' and 'Granny Smith' cultivars, respectively (Fig. 2b).

It was observed that Scholar™ inhibited *N. alba* on 'Golden Delicious', 'Pink Lady' and 'Granny Smith' cultivars by 96.64%, 83.72%, and 85.58%, respectively (Fig. 2c). Although not significant (P = 0.0714), it was observed that all essential oils (single and mixtures) effectively inhibited *N. alba* on 'Golden Delicious', 'Pink Lady' and 'Granny Smith' cultivars compared to control and ethanol, treatments. Single application of essential oils showed that lemongrass oil was the most effective on all apple cultivars compared to lemon and lime oils; and it inhibited *N. alba* by 80.13%, 75.98% and 56.37% on 'Golden Delicious', 'Pink Lady' and 'Granny Smith' cultivars, respectively. It was observed that a mixture of lime + lemongrass essential oil performed better than lemon + lemongrass essential oil. Furthermore, a mixture of lime and lemongrass essential oil exhibited 82.69%, 79.88% and 64.77% inhibition of *N. alba* on 'Golden Delicious', 'Pink Lady' and 'Granny Smith' cultivars, respectively (Fig. 2c).

Efficacy of postharvest thermal fogging application of essential oils against* Botrytis cinerea*,* Penicillium expansum *and* Neofabraea alba *on apples

Fig. 3 shows the efficacy of essential oils on postharvest decay of apples caused *B. cinerea*, *P. expansum* and *N. alba* on 'Golden Delicious', 'Pink Lady' and 'Granny Smith' cultivars. Fruit were treated with different treatments and stored for 28 days at controlled atmosphere followed by 7 days at 20°C. It was observed that all essential oil treatments significantly ($P <$ 0.001) inhibited *B. cinerea* on 'Golden Delicious' and 'Pink Lady' cultivars compared to control, treatments (Fig. 3a). However, single application of lemon and lime essential oils on 'Granny Smith' apples were not significantly different from control treatments. Single application of essential oils showed that lemongrass oil was the most effective on all apple cultivars compared to lemon and lime oils; and it inhibited *B. cinerea* by 51.77%, 72.50% and 27.65% on 'Golden Delicious', 'Pink Lady' and 'Granny Smith' cultivars, respectively. It was observed that a mixture of lime + lemongrass essential oil performed better than lemon + lemongrass essential oil. Furthermore, a mixture of lime + lemongrass essential oil exhibited 67.60%, 91.24% and 52.34% inhibition of *B. cinerea* on 'Golden Delicious', 'Pink Lady' and 'Granny Smith' cultivars, respectively (Fig. 3a). It was observed that Rovral™ significantly

inhibited *B. cinerea* on 'Golden Delicious', 'Pink Lady' cultivars by 28.49% and 53.77%, respectively, however no inhibition was observed on 'Granny Smith' apples (Fig. 3a).

All essential oil treatments (single and mixtures) significantly inhibited (P < 0.0001) *P. expansum* on all three apple cultivars, compared to control and ethanol and Rovral™ treatments (Fig. 3b). Single application of essential oils showed that lemongrass oil was the most effective on all apple cultivars compared to lemon and lime oils. Lemongrass essential oil inhibited *P. expansum* by 79.23%, 81.24% and 79.70% on 'Golden Delicious', 'Pink Lady' and 'Granny Smith' cultivars, respectively. It was observed that a mixture of lime + lemongrass essential oil performed better than lemon + lemongrass essential oil. Furthermore, a mixture of lime + lemongrass essential oil exhibited 81.39%, 85.69% and 80.50% inhibition of *P. expansum* on 'Golden Delicious', 'Pink Lady' and 'Granny Smith' cultivars, respectively. Rovral™ significantly inhibited *P. expansum* on 'Golden Delicious', 'Pink Lady' and 'Granny Smith' cultivars by 55.19%, 22.13%, and 38.78%, respectively, compared to control treatments (Fig. 3b).

It was observed that all essential oils (single and mixtures) and Scholar™ effectively inhibited *N. alba* on 'Golden Delicious', 'Pink Lady' and 'Granny Smith' cultivars compared to control and ethanol treatments (Fig. 3c). Single application of essential oils showed that lemongrass oil was the most effective on all apple cultivars compared to lemon and lime oils; and it inhibited *N. alba* by 63.90%, 65.12% and 61.65% 'Golden Delicious', 'Pink Lady' and 'Granny Smith' cultivars, respectively. It was observed that a mixture of lime + lemongrass essential oil performed better than lemon + lemongrass essential oil. Furthermore, a mixture of lime + lemongrass essential oil exhibited 79.34%, 75.04% and 67.99% inhibition of *N. alba* on 'Golden Delicious', 'Pink Lady' and 'Granny Smith' cultivars, respectively. It was observed that Scholar™ inhibited *N. alba* on 'Golden Delicious', 'Pink Lady' and 'Granny Smith' cultivars by 62.38%, 55.17%, and 29.77%, respectively (Fig. 3c).

Discussion

The essential oils tested proved their antifungal activity as postharvest treatments against *B. cinerea*, *P. expansum* and *N. alba* on apples. Although essential oils can exhibit a strong inhibitory activity in *in vitro* trials, results can vary considerably when applied *in vivo*. The essential oils tested proved their antifungal activity as postharvest treatments against *B. cinerea*, *P. expansum* and *N. alba* on apples. The efficacy of the essential oils depended on the natural resistance of the apple cultivar to the tested pathogens and application method.

Fumigation of 'Golden Delicious', 'Pink Lady' and 'Granny Smith' cultivars with different concentrations of essential oils (curative or preventative) showed varied antifungal

activity against *B. cinerea*, *P. expansum* and *N. alba* at 20°C, regular atmosphere and controlled atmosphere. Ziedan and Farrag (2008) studied the fumigation of peach fruit with peppermint and sweet basil crude oil under artificial infestation by *Rhizopus stolonifer* (Ehrenberg) Vuillemin and *M. fructicola*. All different concentrations of peppermint and sweet basil essential oils as vapor treatment significantly suppressed decay on peach fruit (Ziedan and Farrag, 2008). Furthermore, they indicated that decay caused by *R. stolonifer* and *M. fructicola* on peach fruit was suppressed in a dose-dependent manner. In addition, Ragab *et al.* (2001) found that the vapour of lemongrass oil at 6 mL/L significantly suppressed postharvest decay of tomato fruit caused by *Fusarium oxysporum*. Schlech emend Snyder Hans, *Alternaria alternata* (Fr) keissler, *B. cinerea*, and *Rhizopus stolonifera*. The results from the present study indicated that all essential oils tested significantly inhibited *B. cinerea*, *P. expansum* and *N. alba* compared to control, fungicides and ethanol treatments at 20°C, regular atmosphere and controlled atmosphere. The inhibitory effect of these essential oils varied among the fungal pathogens on apple cultivars. It is evident that none of the essential oils provided complete inhibition of all three pathogens on apple cultivars at 20°C, regular atmosphere and controlled atmosphere when applied as fumigants. These findings could be at attributed to the alternation of site action of essential oils or alternation in membranes of fungi under *in vivo* condition (Dikbas *et al*., 2008). Furthermore, the interactions between host tissues, the pathogen and the environment can have a major effect on the physiology and metabolism of both the host and the pathogen (Bishop and Regan, 1998). Fumigation of fruit with vapours of essential oils may facilitate control of postharvest diseases in several ways. Fumigation may prevent infection by killing exposed conidia or make the peel of the fruit an unsuitable substrate for germination of conidia if volatile essential oil is absorbed by and accumulates in the injured peel during fumigation. Furthermore, fumigation of fruit with essential oils may reduce the risk of phytotoxicity which may occur when liquid essential oils comes into contact with the fruit skin (Ben-Yehoshua *et al*., 1992; Knight, 2002). In addition, as essential oils are volatile and occurs naturally, residual levels of essential oils and any effect in terms of consumer acceptability may be considered negligible.

Phytotoxicity problems may arise when essential oils are applied to living plant tissues. Some essential oils have been shown to be toxic to plant products, with the phytotoxic effects caused by essential oils on fruit and vegetable varying according to the produce treated, application methodologies, concentrations of oils and adaptive duration (Wuryatmo *et al,* 2003). Our results indicated that, all essential oil treatments tested showed no phytotoxicity on all three apple cultivars when applied by means of fumigation, while dipping fruit in some concentrations showed phytotoxic effect. A concentration of 0.125% of all essential oils, showed no phytotoxicity on all three cultivars at an exposure time of 30 s when applied by dipping. Burt (2004) reported that dips of thyme, oregano, cinnamon and

clove essential oils used on orange caused peel injury in the fruit, while thymol was effective as a postharvest treatment with no apparent phytotoxicity in treated apricot and plums with no effect on desirable fruit attributes (Liu *et al.,* 2002). Moreover, there is a physical advantage in applying essential oils as postharvest treatments. As they can be applied as a vapour, essential oil treatments can reduce costs as fumigation requires less oil and labour in postharvest handling (Wuryatmo *et al,* 2003). The vapour phase for postharvest application limits the tainting of original organoleptic attributes of fruit as well as the risk of phytotoxic effects on fruit skin. Cold treatments may also be applied in combination with chemical fumigation, thus reducing the amount of fumigant required.

Dips of Rovral™ or Scholar™ and essential oils on 'Golden Delicious', 'Pink Lady' and 'Granny Smith' inhibited *B. cinerea*, *P. expansum* and *N. alba* after 28 days at controlled atmosphere followed by 7 days at 20°C, compared to control and ethanol treatments. The fungicides showed the highest inhibition of *B. cinerea*, *P. expansum* and *N. alba*, followed by essential oil mixtures and single oils. Application of essential oils by thermal fogging gave better inhibition of all three pathogens on 'Golden Delicious', 'Pink Lady' and 'Granny Smith' apples compared to fungicides, after 28 days at controlled atmosphere followed by 7 days at 20°C. Ethanol provided some inhibition of *B. cinerea*, *P. expansum* and *N. alba* on some apple cultivars, however, inhibition was less than that of fungicides and essential oils. Gabler *et al*. (2005) observed that ethanol is a volatile compound and quickly evaporated from the berry surface during storage, therefore, it is ineffective against secondary infections. Ethanol treatment efficacy declined after storage indicating the ineffectiveness of ethanol against secondary infections during storage (Lichter *et al*., 2002).

Fungicides are applied in water solution by dipping or drenching the fruit for 30 to 40 sec (Moggia *et al*., 2003). Efficacy of the treatments is based on adequate concentration at the tank solution, and uniform distribution of the chemicals on the fruit surface. Moggia *et al*., (2003) reported that drenching has some disadvantages: fungal diseases can be aggravated by dipping or drenching the fruit, dirt and dust in the chemical solution can reduce the effectiveness of the treatments. It is recommended that essential oils can be applied by thermal fogging as a postharvest treatment against postharvest pathogens. This would allow a uniform distribution of the EO, as well as the use of very small amounts of the EO, and the possibility of several applications during storage period (Moggia *et al*., 2003).

Previous reports indicated reduced fruit decay during postharvest treatments with volatile compounds including raspberry and kiwifruit (Wang, 2003, Williamson *et al*., 2007). Maqbool *et al.* (2010) reported that, cinnamon oil with fungitoxic or fungistatic activity could be considered as a suitable alternative to synthetic fungicides for managing anthracnose in bananas. Feng and Zheng (2007) studied the effects of cassia oil on decay development in

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artificially inoculated and wounds of tomatoes fruits. The results indicate that when wounded cherry tomatoes were treated with cassia oil, all concentrations (except 100 ppm) inhibited *Alternaria alternata* on tomatoes stored at 20°C for 5 days. Dubey *et al.* (2007) found that essential oil from *Eupatorium cannabinum* had an inhibitory effect on pectinase and cellulase, two important enzymes produced by phytopathogenic fungi in disease development.

In conclusion, postharvest control of *B. cinerea*, *P. expansum* and *N. alba* by essential oils through dipping and thermal fogging in combination with controlled atmosphere indicated that a mixture of lime + lemongrass oil was the most effective, followed by a mixture lemon + lemongrass oil, lemongrass oil, lime oil and lemon oil. The use of mixtures of essential oils would allow a reduction of half the cost of essential oils necessary to treat large volume of fruit compared to single use of each type of oil. Treatment with essential oils would cost more than treatment by the tested chemical fungicide. However, the benefits of essential oils (eco-compatible, non-toxic at low doses, biodegradable, and no risk for resistance development) and disadvantages of chemical fungicides on human health and on the environment make essential oils more suitable for apple postharvest treatment.

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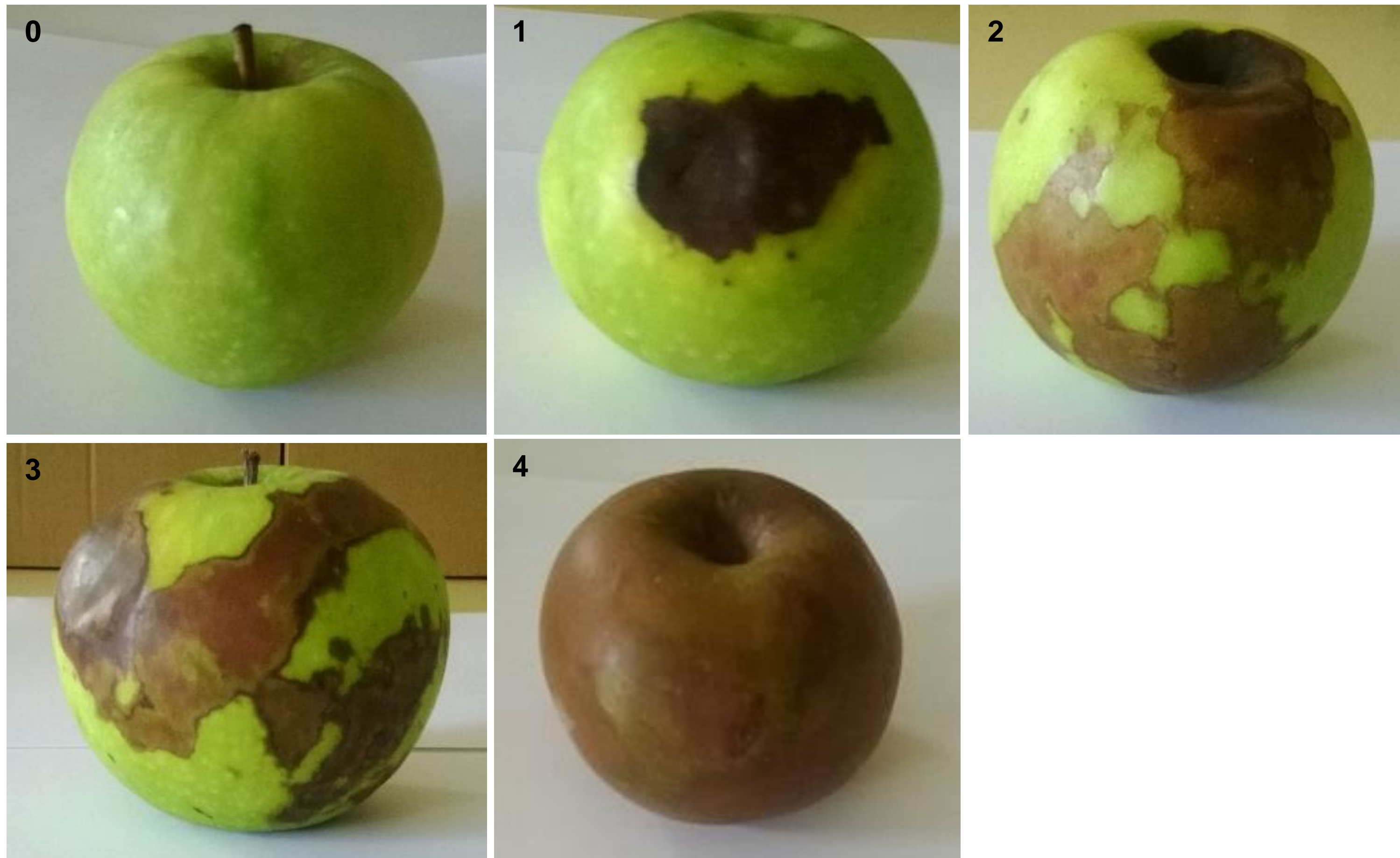

Figure 1. Phytotoxicity effect of dipping essential oils on 'Granny Smith' apple cuticle: 0) no phytotoxicity; 1) 1-25% phytotoxicity; 2) 26-50% phytotoxicity 3) 51-75% phytotoxicity; 4) 76-100% phytotoxicity.

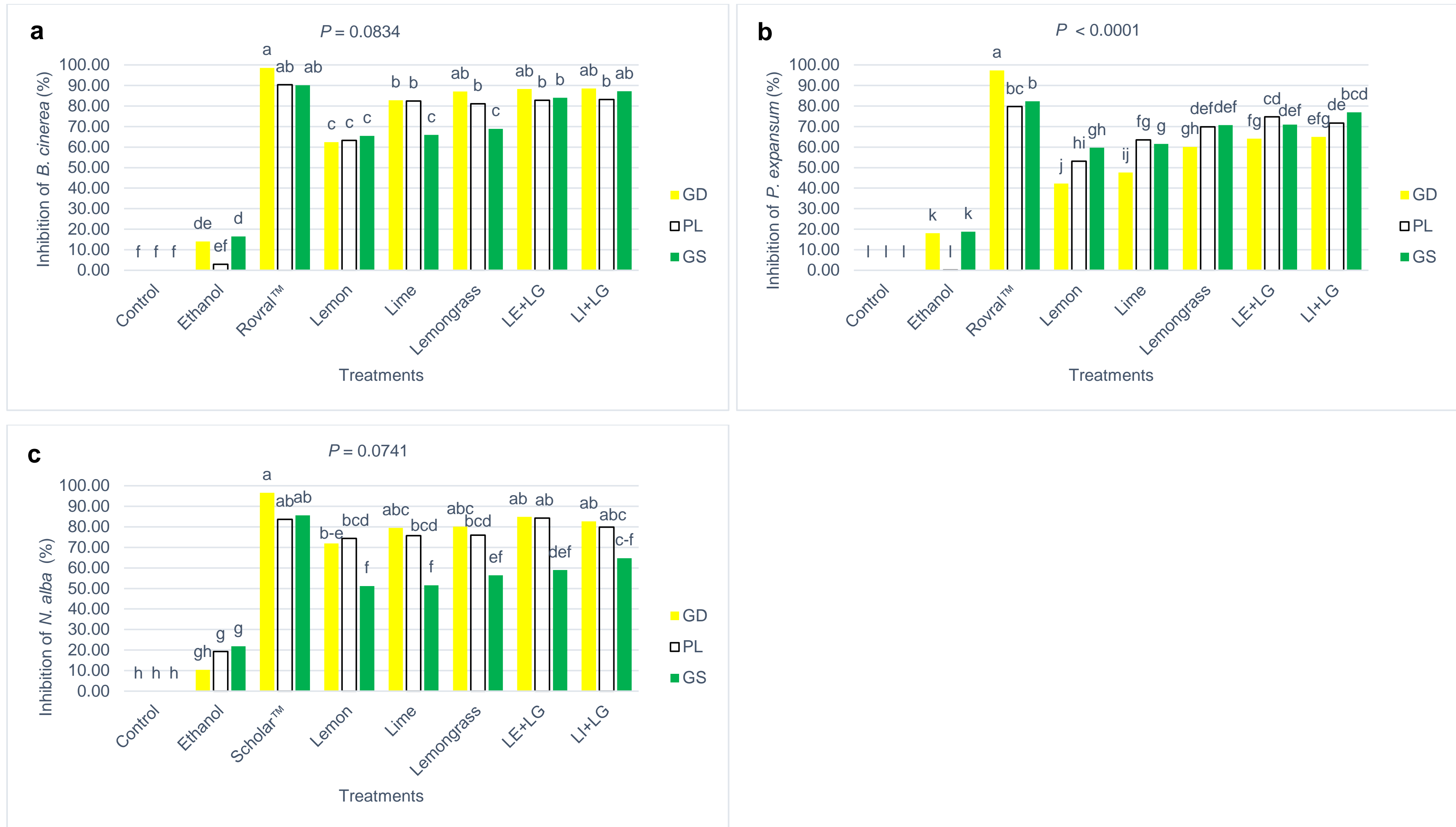

Figure 2. Efficacy of essential oils as a postharvest treatment against *B. cinerea* (a), *P. expansum* (b) and *N. alba* (c) on apples when applied by means of postharvest dipping. Means followed by the same letter between treatments and cultivar are not significantly different according to Fisher's least significant difference test at $P \leq 0.05$. GD: 'Golden Delicious'; PL: 'Pink Lady'; GS: 'Granny Smith'; LE: Lemon; LI: Lime; LG: Lemongrass.

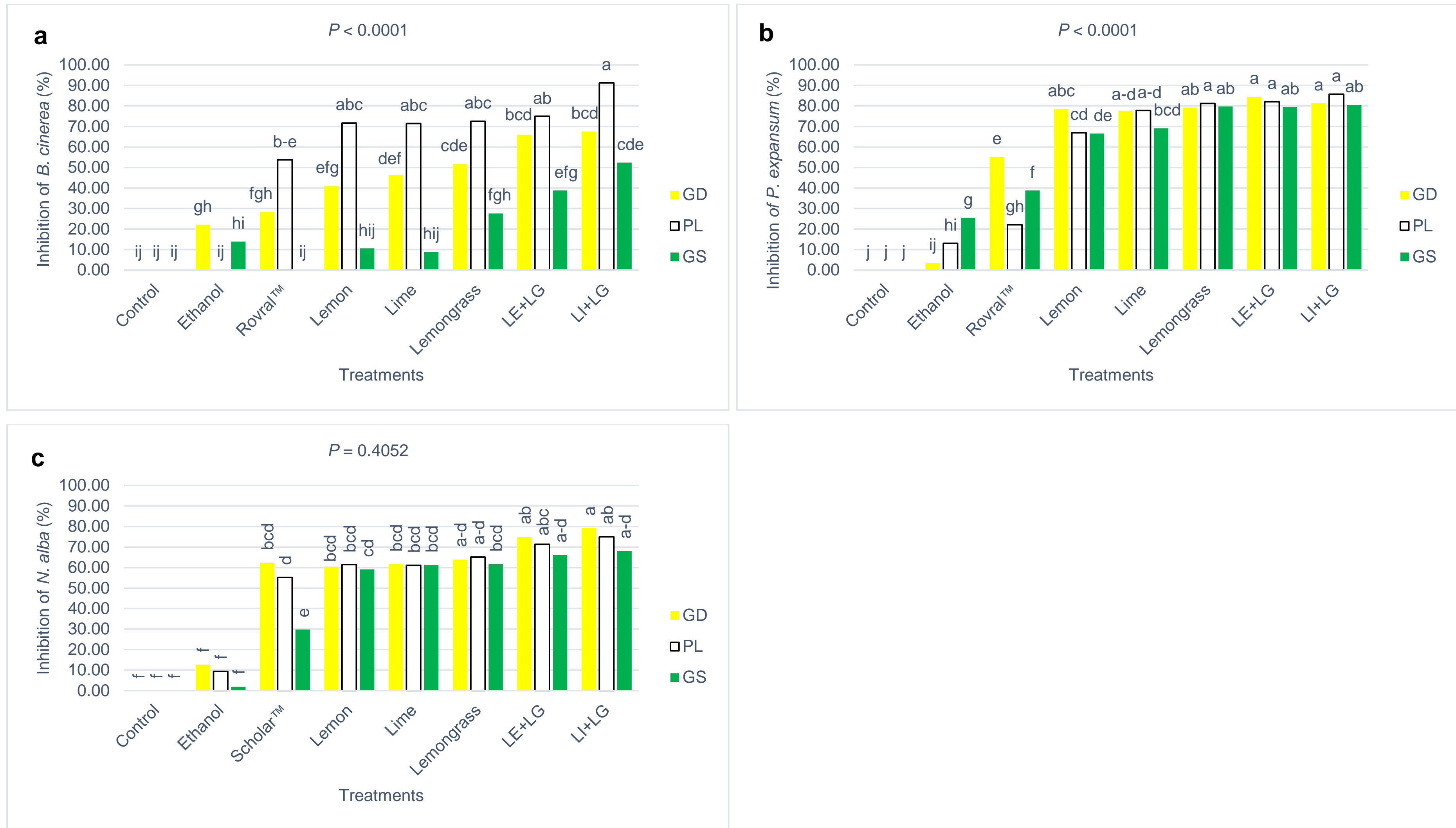

Figure 3. Efficacy of essential oils as a postharvest treatment against *B. cinerea* (A), *P. expansum* (B) and *N. alba* (C) on apples when applied by means of thermal fogging. Means followed by the same letter between treatments and cultivar are not significantly different according to Fisher's least significant difference test at $P \leq 0.05$. GD: 'Golden Delicious'; PL: 'Pink Lady'; GS: 'Granny Smith'; LE: Lemon; LI: Lime; LG: Lemongrass.

Table 1. Efficacy of postharvest fumigation of essential oils on percentage inhibition of *Botrytis cinerea* after 7 days at 20°C.

		Curative			Preventative		
Concentration (%)	**Treatment**	**GD**	**PL**	**GS**	**GD**	**PL**	**GS**
	Control	0.00i	0.00i	0.00i	0.00j	0.00j	0.00j
	Ethanol	30.43gh	28.10gh	22.32h	21.31i	27.00hi	26.79hi
	Rovral™	0.00i	0.00i	0.00i	0.00j	0.00j	0.00j
0.125	LE	93.39ab	80.52b-f	73.59ef	87.75a-f	89.19a-e	92.12abc
	LI	87.91a-e	81.15b-f	68.97f	93.20abc	94.69ab	91.34a-d
	LG	93.57ab	83.52a-f	75.38c-f	92.52 abc	77.98ef	92.93abc
	LE + LG	88.80a-e	69.88f	80.75c-f	91.33a-d	78.84def	82.04b-f
	LI + LG	96.99a	75.37c-f	75.31c-f	91.59a-d	74.76f	92.97abc
0.5	LE	92.55ab	82.45a-f	78.89c-f	88.66a-e	85.15a-f	83.38a-f
	LI	90.17a-d	83.94a-f	69.44f	86.60a-f	84.09a-f	85.54a-f
	LG	89.68a-d	84.30a-f	80.54b-f	90.58a-e	90.53a-e	89.84a-e
	LE + LG	87.34a-e	89.84abc	74.65def	93.84abc	81.37c-f	82.28a-f
	LI + LG	86.83a-e	86.15a-e	90.32abc	95.18a	85.12a-f	91.71a-d
Treatment x Cultivar		$P = 0.44$			$P = 0.47$		
CV		9.90			15.84		
R^2		0.88			0.92		
LSD		15.67			13.08		

There is no significant difference ($P \leq 0.05$) between cultivars with the same letter in the same raw. Means followed by the same letter between treatments and cultivar are not significantly different according to Fisher's least significant difference test at $P \leq 0.05$. GD: 'Golden Delicious'; PL: 'Pink Lady'; GS: 'Granny Smith'; LE: Lemon; LI: Lime; LG: Lemongrass.

Table 2. Efficacy of postharvest fumigation of essential oils on percentage inhibition of *Botrytis cinerea* after 28 days at regular atmosphere (-0.5°C, air) followed by 7 days at 20°C.

Concentration (%)	Treatment	Curative			Preventative		
		GD	PL	GS	GD	PL	GS
0.125	Control	0.00pqr	0.00pqr	0.00pqr	0.00m	0.00m	0.00m
	Ethanol	0.74o-r	10.50m-q	5.28n-r	2.85lm	0.00m	0.00m
	Rovral™	0.00pqr	0.00pqr	0.00pqr	0.00m	0.00m	0.00m
	LE	87.50ab	25.23j-n	62.57c-f	56.06bc	54.26bcd	38.45d-j
	LI	98.38a	34.12i-l	56.23e-h	99.06a	37.56d-i	44.80b-h
	LG	77.87a-d	17.95k-p	36.67h-k	38.14d-j	43.45b-i	32.67g-k
	LE + LG	81.32abc	30.31j-m	20.32j-p	57.08bc	35.62f-j	29.81h-k
	LI + LG	77.10b-e	11.27m-q	54.33f-i	49.24b-g	34.66f-k	27.32ijk
0.5	LE	86.04ab	13.35l-p	70.49b-f	59.37b	43.25b-g	50.66b-f
	LI	86.50ab	21.35j-o	87.63ab	54.29bcd	42.95b-i	53.28b-e
	LG	77.51a-d	25.55j-n	41.24g-j	45.62b-h	43.19b-i	28.46h-k
	LE + LG	58.33d-g	13.13m-p	81.07abc	39.92c-j	36.54e-j	35.38f-k
	LI + LG	75.75b-e	13.41l-p	21.88j-n	34.79f-k	43.34b-i	28.75h-k
Treatment x Cultivar		<0.0001			<0.0001		
CV		46.17			43.47		
R^2		0.84			0.75		
LSD		20.95			17.36		

Means followed by the same letter within raw are not significantly different according to Fisher's least significant difference test at P < 0.05. GD: 'Golden Delicious'; PL: 'Pink Lady'; GS: 'Granny Smith'; LE: Lemon; LI: Lime; LG: Lemongrass.

Table 3. Efficacy of postharvest fumigation of essential oils on percentage inhibition of *Botrytis cinerea* after 28 days at controlled atmosphere followed by 7 days at 20°C.

Concentration (%)	Treatment	Curative			Preventative		
		GD	PL	GS	GD	PL	GS
	Control	0.00o	0.00o	0.00o	0.00o	0.00o	0.00o
	Ethanol	27.23mn	23.45n	48.70kl	21.76op	32.11l-o	59.15c-g
	Rovral™	0.00o	1.17o	0.72o	0.00o	7.23op	37.85j-o
	LE	83.11ab	55.27g-k	69.80b-g	68.64b-f	56.81d-i	75.31a-c
	LI	69.80b-g	66.88d-h	82.21a-c	47.15h-l	67.10b-g	86.36a
0.125	LG	63.28e-k	66.14d-i	70.97b-f	29.58m-o	54.94e-j	67.21b-g
	LE + LG	31.46mn	77.67a-e	48.61kl	32.86l-o	55.71e-i	61.63c-h
	LI + LG	21.73n	56.27f-k	38.93lm	35.04k-o	49.31h-l	73.81a-d
	LE	64.59d-j	57.64f-j	79.58a-d	39.43i-n	44.73h-m	53.54f-i
	LI	54.73h-k	49.63j-l	82.20a-c	32.26l-o	50.96g-k	81.51ab
0.5	LG	54.61h-k	51.25i-l	82.07a-c	86.80a	53.48f-j	53.79f-j
	LE + LG	53.21h-l	55.50g-k	86.30a-c	26.92no	55.20e-j	80.56ab
	LI + LG	67.93c-h	63.04e-k	57.66f-k	32.87l-o	48.02h-l	71.40a-e
Treatment x Cultivar			$P < 0.0001$			$P < 0.0001$	
CV			26.33			32.78	
R^2			0.87			0.80	
LSD			15.05			17.55	

Means followed by the same letter within raw are not significantly different according to Fisher's least significant difference test at P < 0.05. GD: 'Golden Delicious'; PL: 'Pink Lady'; GS: 'Granny Smith'; LE: Lemon; LI: Lime; LG: Lemongrass. The controlled atmosphere room for 'Pink Lady' and 'Granny Smith' apples was -0.5°C; 1.5% O_2 + 1% CO_2; and for 'Golden Delicious' was -0.5°C; 1.5% O_2 + 2.5% CO_2.

Table 4. Efficacy of postharvest fumigation of essential oils on percentage inhibition of *Penicillium expansum* after 7 days at 20°C.

Concentration (%)	Treatment	Curative			Preventative		
		GD	PL	GS	GD	PL	GS
	Control	0.00mn	0.00mn	0.00mn	0.00no	0.00no	0.00no
	Ethanol	23.69l	9.61m	9.69m	18.29lm	12.40mn	23.18k-m
	Rovral™	0.00mn	0.00mn	0.00mn	5.26no	11.08mn	0.00no
	LE	58.84d-h	40.69jk	56.32e-i	47.56c-f	29.45i-l	47.80c-f
	LI	63.02b-f	36.88k	66.41b-e	30.05h-l	36.48e-j	44.90d-g
0.125	LG	66.09b-e	48.12h-k	51.09f-j	41.20d-i	42.53d-h	52.04b-d
	LE + LG	56.18e-i	42.47jk	67.24a-e	33.69g-k	35.71f-k	36.17e-j
	LI + LG	55.15e-i	48.10h-k	59.11d-h	39.47d-j	32.60g-k	27.79j-l
	LE	70.06a-d	45.68i-k	78.87a	51.90b-d	38.25e-j	48.23b-f
	LI	66.66a-e	56.48e-i	61.98c-g	42.84d-g	37.39e-j	48.64b-e
0.5	LG	71.66a-c	49.98g-j	74.98ab	60.87ab	40.99d-i	63.38a
	LE + LG	61.33c-f	50.82f-j	63.13b-f	35.66f-k	32.87g-k	58.73a-c
	LI + LG	65.96b-e	46.95h-k	57.14e-i	41.82d-i	40.65d-i	39.22e-j
Treatment x Cultivar			0.0003			0.0003	
CV			23.50			32.60	
R^2			0.91			0.82	
LSD			12.32			12.67	

Means followed by the same letter within raw are not significantly different according to Fisher's least significant difference test at P < 0.05. GD: 'Golden Delicious'; PL: 'Pink Lady'; GS: 'Granny Smith'; LE: Lemon; LI: Lime; LG: Lemongrass.

Table 5. Efficacy of postharvest fumigation of essential oils on percentage inhibition of *Penicillium expansum* after 28 days at regular atmosphere (-0.5°C, air) followed by 7 days at 20°C.

Concentration (%)	Treatment	Curative			Preventative		
		GD	PL	GS	GD	PL	GS
	Control	0.00op	0.00op	0.00op	0.00s	0.00s	0.00s
	Ethanol	0.00op	14.69k-m	0.00op	7.60rs	15.41r	3.12s
	Rovral™	0.85n-p	9.76l-o	3.88m-p	7.50rs	0.00s	0.00s
	LE	36.93b-d	26.59d-j	11.20l-n	79.28b	61.24g-j	35.08o-q
	LI	31.73c-h	32.94c-g	10.22l-o	75.91bc	57.22i-l	29.12q
0.125	LG	32.48c-h	32.02c-h	10.24l-o	72.67b-e	65.43d-i	40.99n-p
	LE + LG	19.82i-l	29.50d-i	14.64k-m	76.82bc	53.51j-m	32.90pq
	LI + LG	22.88g-k	50.05a	1.64n-p	69.62b-g	58.99h-k	28.51q
	LE	29.37d-i	46.10ab	18.16j-l	78.75b	61.34g-j	49.48k-n
	LI	36.39b-e	22.08h-k	24.77f-k	73.44b-d	62.29f-j	46.99mn
0.5	LG	34.03c-f	51.71a	41.81a-c	75.80bc	67.46c-h	89.83a
	LE + LG	25.77e-j	42.53a-c	28.58d-j	68.45c-h	62.90e-j	28.44q
	LI + LG	32.38c-h	32.78c-h	23.89f-k	71.74b-f	74.44c-d	44.71m-o
Treatment x Cultivar			$P < 0.0001$			$P < 0.0001$	
CV			42.08			18.37	
R^2			0.80			0.93	
LSD			10.82			10.11	

Means followed by the same letter within raw are not significantly different according to Fisher's least significant difference test at P < 0.05. GD: 'Golden Delicious'; PL: 'Pink Lady'; GS: 'Granny Smith'; LE: Lemon; LI: Lime; LG: Lemongrass.

Table 6. Efficacy of postharvest fumigation of essential oils on percentage inhibition of *Penicillium expansum* after 28 days at controlled atmosphere followed by 7 days at 20°C.

Concentration (%)	Treatment	Curative			Preventative		
		GD	PL	GS	GD	PL	GS
	Control	0.00l	0.00l	0.00l	0.00k	0.00k	0.00k
	Ethanol	18.16k	6.96kl	0.00l	0.00k	9.43jk	14.52j
	Rovral™	7.28kl	1.81l	7.30k	8.25jk	4.31jk	3.81jk
	LE	59.07d-j	62.20b-i	54.84f-j	53.51a-g	44.61f-i	54.87a-g
	LI	45.69j	49.54h-j	60.86c-j	51.08b-i	55.38a-f	38.41i
0.125	LG	56.29e-j	53.55f-j	65.02a-h	54.42a-g	41.36g-i	43.41f-i
	LE + LG	65.49a-h	58.12d-j	77.61ab	48.29e-i	54.40a-g	41.40g-i
	LI + LG	67.62a-g	63.05b-h	73.93a-d	49.56d-i	52.48a-h	63.09a-d
	LE	69.31a-f	64.17b-h	57.51e-j	64.66ab	47.30f-i	61.31a-e
	LI	59.37d-j	53.20g-j	76.69a-b	65.08a	45.45f-i	50.87c-i
0.5	LG	68.62a-g	63.21b-h	80.36a	55.75a-f	39.69hi	64.48a-c
	LE + LG	46.35ij	52.73g-j	76.41a-c	52.58a-h	47.79e-i	48.65e-i
	LI + LG	59.86d-j	53.25g-j	71.82a-e	51.38b-i	37.92i	46.48f-i
Treatment x Cultivar			0.0370			0.0025	
CV			28.52			29.79	
R^2			0.86			0.91	
LSD			16.01			13.63	

Means followed by the same letter within raw are not significantly different according to Fisher's least significant difference test at P < 0.05. GD: 'Golden Delicious'; PL: 'Pink Lady'; GS: 'Granny Smith'; LE: Lemon; LI: Lime; LG: Lemongrass. The controlled atmosphere room for 'Pink Lady' and 'Granny Smith' apples was -0.5°C; 1.5% O_2 + 1% CO_2; and for 'Golden Delicious' was -0.5°C; 1.5% O_2 + 2.5% CO_2.

Table 7. Efficacy of postharvest fumigation of essential oils on percentage inhibition of *Neofabraea alba* after 7 days at 20°C.

Concentration (%)	Treatment	Curative			Preventative		
		GD	PL	GS	GD	PL	GS
	Control	0.00m	0.00m	0.00m	0.00n	0.00n	0.00n
	Ethanol	20.23km	13.00lm	9.31m	6.39n	10.67mn	19.41k-n
	Rovral™	20.05k-m	0.30m	43.16h-k	24.02j-n	22.29j-n	23.35j-n
	LE	46.23g-j	88.42a-d	83.05a-e	58.34d-i	24.45j-n	88.43ab
	LI	36.22j-l	89.67a-c	78.30a-f	80.53a-d	23.18j-n	89.92a
0.125	LG	67.65c-h	59.04e-j	41.87i-k	75.61a-f	37.50h-l	77.92a-f
	LE + LG	63.90d-i	59.47e-j	47.22g-j	56.00e-i	20.00k-n	58.36d-i
	LI + LG	67.31c-h	40.42i-k	51.04g-j	83.05a-c	17.70l-n	88.63ab
	LE	82.38a-e	40.88i-k	81.30a-e	56.95d-i	41.38g-l	64.21b-g
	LI	96.16a	34.49j-l	90.53a-c	34.88i-m	43.46g-k	45.40g-j
0.5	LG	93.42ab	48.20g-j	44.04h-k	83.44a-c	61.22c-h	54.92f-i
	LE + LG	82.03a-e	35.73j-l	59.29e-j	42.69g-k	35.51i-l	86.53ab
	LI + LG	63.79d-i	55.14f-j	70.97b-g	57.36d-i	43.62g-k	89.50a
Treatment x Cultivar		<0.0001			<0.0001		
CV		42.88			44.39		
R^2		0.80			0.77		
LSD		25.14			24.46		

Means followed by the same letter within raw are not significantly different according to Fisher's least significant difference test at P < 0.05. GD: 'Golden Delicious'; PL: 'Pink Lady'; GS: 'Granny Smith'; LE: Lemon; LI: Lime; LG: Lemongrass.

Table 8. Efficacy of postharvest fumigation of essential oils on percentage inhibition of *Neofabraea alba* after 28 days at regular atmosphere (-0.5°C, air) followed by 7 days at 20°C.

Concentration (%)	Treatment	Curative			Preventative		
		GD	PL	GS	GD	PL	GS
	Control	0.00r	0.00r	0.00r	0.00r	0.00r	0.00r
	Ethanol	3.27qr	4.89qr	14.44pq	9.86p-r	5.52qr	19.24o-q
	Rovral™	0.00r	14.50pq	19.66op	0.00r	2.61r	0.00r
	LE	18.83op	44.52h-l	86.64a	43.63h-k	56.50d-h	26.77l-o
	LI	38.28k-n	70.86b-d	72.92bc	72.37a-c	60.08c-g	45.58h-k
0.125	LG	43.12i-l	44.04h-l	68.48b-e	63.27c-e	59.71c-g	31.44j-o
	LE + LG	28.58m-o	57.93d-g	62.85b-g	45.07g-j	46.58f-j	46.63
	LI + LG	39.82j-n	75.01ab	57.16e-h	28.76k-o	38.22i-l	52.48e-i
	LE	33.35l-n	42.24i-l	51.99f-j	22.37m-p	36.80j-m	21.57n-p
	LI	41.59j-m	44.04h-l	41.02j-m	56.73d-h	45.18g-j	34.70j-n
0.5	LG	40.47j-n	41.26j-m	60.62c-g	58.86c-g	69.83a-d	61.38c-f
	LE + LG	36.76k-n	35.77l-n	55.20e-i	82.78a	62.58c-e	61.58c-f
	LI + LG	27.55n-p	49.89g-k	64.50b-f	80.97ab	45.36g-j	66.87b-e
Treatment x Cultivar			<0.0001			<0.0001	
CV			29.75			33.81	
R^2			0.86			0.85	
LSD			13.30			15.22	

Means followed by the same letter within raw are not significantly different according to Fisher's least significant difference test at P < 0.05. GD: 'Golden Delicious'; PL: 'Pink Lady'; GS: 'Granny Smith'; LE: Lemon; LI: Lime; LG: Lemongrass.

Table 9. Efficacy of postharvest fumigation of essential oils on percentage inhibition of *Neofabraea alba* after 28 days at controlled atmosphere followed by 7 days at 20°C.

Concentration (%)	Treatment	Curative			Preventative		
		GD	PL	GS	GD	PL	GS
	Control	0.00s	0.00s	0.00s	0.00pq	0.00pq	0.00pq
	Ethanol	15.42qr	16.87qr	18.10qr	2.51o-q	26.96mn	11.24op
	Rovral™	26.67pq	7.18rs	6.55rs	16.51no	8.04op	0.00pq
	LE	49.12k-n	75.47a-c	55.35h-l	47.63g-l	42.12i-l	51.30f-l
	LI	43.27m-o	68.69b-g	45.04l-o	44.89h-l	54.12e-j	42.05j-l
0.125	LG	59.48f-k	69.99b-f	51.14j-n	57.23e-h	59.87d-g	58.27d-h
	LE + LG	51.64j-n	58.13g-k	37.17op	40.79j-m	38.45lm	52.36f-l
	LI + LG	52.84i-m	59.90f-k	41.08no	45.83g-l	39.45k-m	57.07e-h
	LE	66.33c-h	73.59b-d	69.48b-g	63.59c-f	53.54e-k	59.03d-h
	LI	61.83e-j	71.71b-e	59.78f-k	42.26i-l	63.86c-f	67.97b-e
0.5	LG	70.08b-f	85.45a	56.83h-k	79.94ab	77.91a-c	87.39a
	LE + LG	58.07g-k	80.01ab	48.89k-n	56.72e-i	78.99ab	60.19d-g
	LI + LG	66.66c-h	63.49d-i	56.18h-l	62.27d-f	72.40b-d	62.50d-f
Treatment x Cultivar		<0.0001			0.0003		
CV		21.01			28.05		
R^2		0.90			0.86		
LSD		11.66			14.59		

Means followed by the same letter within raw are not significantly different according to Fisher's least significant difference test at P < 0.05. GD: 'Golden Delicious'; PL: 'Pink Lady'; GS: 'Granny Smith'; LE: Lemon; LI: Lime; LG: Lemongrass. The controlled atmosphere room for 'Pink Lady' and 'Granny Smith' apples was -0.5°C; 1.5% O_2 + 1% CO_2; and for 'Golden Delicious' was -0.5°C; 1.5% O_2 + 2.5% CO_2.

Table 10. Phytotoxic effect of dipping essential oils on Golden Delicious', 'Granny Smith' and 'Pink Lady' apple cultivars at different exposure times.

Concentration (%)	Treatment	30 s			1 min			2 min		
		GD	GS	PL	GD	GS	PL	GD	GS	PL
	Control	0.00g	0.00g	0.00g	0.00g	0.00g	0.00g	0.00h	0.00h	0.00h
	Ethanol	0.00g	0.00g	0.00g	0.00g	0.00g	0.00g	0.00h	0.00h	0.00h
	Rovral™	0.00g	0.00g	0.00g	0.00g	0.00g	0.00g	0.00h	0.00h	0.00h
0.125	LE	0.00g	0.00g	0.00g	0.00g	0.00g	0.00g	0.00h	0.00h	0.00h
	LI	0.00g	0.00g	0.00g	0.00g	0.00g	0.00g	5.00h	0.00h	0.00h
	LG	0.00g	0.00g	0.00g	0.00g	3.33g	0.00g	8.33gh	38.33cd	0.00h
	LE + LG	0.00g	0.00g	0.00g	15.00f	25.00e	3.33g	79.17a	84.17a	6.67gh
	LI + LG	0.00g	0.00g	0.00g	0.00g	0.00g	0.00g	5.00h	0.00h	0.00h
0.25	LE	1.67g	0.00g	0.00g	3.33g	0.00g	0.00g	18.33fg	18.33fg	0.00h
	LI	0.00g	3.33g	15.00f	5.00g	0.00g	0.00g	5.00h	8.33gh	0.00h
	LG	25.00e	45.83d	0.00g	78.33b	84.17ab	0.00g	84.17a	90.00a	22.50ef
	LE + LG	15.00f	5.00g	5.00g	15.00f	28.33de	5.00g	62.50b	5.00h	25.00ef
	LI + LG	0.00g	5.00g	0.00g	1.67g	5.00g	0.00g	3.33h	5.00h	0.00h
0.5	LE	1.67g	5.00g	0.00g	35.00d	31.67de	0.00g	45.83c	31.67de	0.00h
	LI	1.67g	0.00g	0.00g	1.67g	1.67g	0.00g	21.67ef	8.33gh	0.00h
	LG	72.50c	78.33b	3.33g	90.00a	90.00a	51.67c	90.00a	90.00a	84.17a
	LE + LG	0.00g	0.00g	0.00g	8.33fg	0.00g	0.00g	38.33cd	6.67gh	0.00h
	LI + LG	78.33b	90.00a	5.00g	90.00a	90.00a	0.00g	31.67de	90.00a	0.00h
Treatment x Cultivar		$P < 0.0001$			$P < 0.0001$			$P < 0.0001$		
CV		41.11			41.03			36.29		
R^2		0.98			0.97			0.96		
LSD		5.74			9.41			12.18		

Means followed by the same letter within raw are not significantly different according to Fisher's least significant difference test at $P < 0.05$. GD: 'Golden Delicious'; PL: 'Pink Lady'; GS: 'Granny Smith'; LE: Lemon; LI: Lime; LG: Lemongrass.

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CHAPTER 6

EFFECTS OF CITRUS AND LEMONGRASS ESSENTIAL OIL TREATMENTS ON QUALITY ATTRIBUTES OF APPLES AFTER CONTROLLED ATMOSPHERE STORAGE

Abstract

This study was conducted to evaluate the effect of citrus and lemongrass essential oils on antioxidant, phytochemical and physicochemical properties of apple (*Malus domestica*) cvs. 'Granny Smith' and 'Pink Lady'. Fruit was evaluated after thermal fogging with lemongrass and citrus essential oils and storage at controlled atmosphere (-0.5°C; 1.5% O_2 + 1% CO_2) for 28 days followed by 7 days at 20°C. Results showed that 'Granny Smith' apples treated with lemon oil alone and lime+lemongrass essential oils retained firmness, while lemon, lime and lemongrass essential oils retained firmness of 'Pink Lady' apples. The titratible acidity of 'Granny Smith' apples treated with essential oils was significantly lower compared to control treatment. However, the titratible acidity of 'Pink Lady' apples treated with essential oils was significantly higher compared to control treatment ($P < 0.05$). The total soluble solid content of both cultivars treated with essential oils was significantly lower compared to control treatments ($P < 0.05$). The total soluble solids: titratible acidity ratio of 'Granny Smith' apples treated with essential oils was high compared to control treatment ($P < 0.05$) but low for 'Pink Lady' apples treated with essential oils compared to control treatment. The pH and ascorbic acid of both apple cultivars were slightly significantly higher compared to control treatments ($P < 0.05$). The total phenolic content of both apple cultivars was higher for all essential oil treatments. Radical scavenging activity of 'Granny Smith' apples treated with lime oil alone, lemon+lemongrass and lime+lemongrass oils was significantly high compared to other essential oils and control treatment ($P < 0.05$), but low for 'Pink Lady' apples. Radical ferric ion reducing antioxidant power of 'Granny Smith' apples was not significantly different from control treatment, while lemon+lemongrass treated 'Pink Lady apples showed no significant difference ($P < 0.05$). The results suggest that essential oils may maintain fruit quality related attributes in addition to the well documented antimicrobial protection during fresh produce storage and transit.

Keywords: Essential oils, controlled atmosphere, antioxidant capacity, phytochemicals, *Malus domestica*.

Introduction

Consumption of fresh fruit helps to prevent a number of chronic diseases such as cancer and cardiovascular diseases (Reddivari *et al.*, 2007). Increased consumer demand for high quality fruit with attractive appearance, high nutritional value, and good taste combined with the reported health benefits from biologically active components such as antioxidants and phenolics (He and Giusti, 2010), has focused attention of researchers on the retention of quality (both flavour and physicochemical) of fresh poroduce. Apple (*Malus domestica* Borkh) fruit are commonly stored for long periods at low temperatures under controlled atmosphere. The most important factor for retaining apple quality in long term storage is the correct maturity of the fruit at harvest (Frasnelli *et al.,* 1996; Wilcke, 1996). After harvest, apples follow a climacteric ripening process, where ethylene is produced and self-regulates the ripening process. During respiration and ripening glucose is converted to CO_2 while its flesh softens, water is lost and heat is produced. Most of the biochemical constituents such as vitamin C, antioxidants, total phenolics and flavour are greatly affected by the cultivar, stage of harvest and the postharvest maturity storage conditions (Pelayo *et al.*, 2003).

All postharvest treatments are aimed at reducing the rate of respiration and water loss, thereby retaining the fruit in an acceptable stage of maturity for consumption. Quality and fruit nutritional value decrease during storage. A number of techniques such as treatment with chemicals (Leverentz *et al.*, 2003), modified atmosphere (Hertog *et al.*, 2001) and heat treatment (Klein and Lurie, 1992) have been used to retard deterioration. However, fungicide residues present on the fruit surface may pose a serious threat to the environment and consumers (Maqbool *et al*, 2011). The development of non-hazardous approaches to control postharvest disease and extend shelf life are required.

The use of natural components such as plant extracts and essential oils have been extensively reported to control postharvest diseases and to prolong the overall quality and storage life of fresh commodities (Dudareva *et al.*, 2004; Tripathi and Dubey, 2004; Martinez-Romero *et al.*, 2004; Serrano *et al.*, 2005; Alikhani *et al.*, 2009; Antunes and Cavaco, 2010; Abdolahi *et al.*, 2010; Mousavizadeh *et al.*, 2011). Tzortzakis (2007) reported that decay was reduced in strawberries and tomatoes by the use of essential oils from eucalyptus and cinnamon, with no effect on fruit firmness. 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). Because of possible application of essential oils as natural antimicrobial and antioxidant agents in fresh horticultural crops, they may be considered as valuable alternatives for preserving physical and chemical properties of food plants. The aim of the present study was to determine the

effects of citrus and lemongrass essential oil treatments on quality parameters of 'Granny Smith' and 'Pink Lady' apples after controlled atmosphere storage.

Materials and methods

Essential oils and fruit

Essential oils of lemon (*Citrus limon* (Linn.) Burm), lime (*Citrus aurantifolia* (Christm.) Swingle) and lemongrass (*Cymbopogon citratus* (DC.) Strapf) were obtained from Groenkop Farm (Melmoth, KwaZulu-Natal, South Africa) and stored at 4°C until used. Lemon and lime EOs were originally extracted from the fresh fruit peel by steam distillation, while lemongrass oil was extracted from fresh or partly dried leaves by steam distillation. Apple (*Malus domestica* Borkh.) cultivars 'Granny Smith' and 'Pink Lady' were collected from Fruitways (Grabouw, South Africa) during 2014 commercial harvesting season. Only unblemished, healthy and mature fruit were used.

Postharvest thermal fogging application of essential oils

Treatments consisted of 0.125 % concentration of essential oils (alone and in combination), Rovral 1 mL/L, Scholar 1.3 mL/L, 20% ethanol (v/v) and water control. Fruit were surface sterilized with 70% ethanol (v/v) for 1 min and allowed to air dry before thermal fogging. Fruit were sorted and placed in plastic crates and thereafter transferred to a cage supplied by Chempac (Pty) Ltd (Paarl, Western Cape, South Africa) to simulate cold storage rooms. Treatments were introduced into a Falcon fogger (Dyna-Fog Asia LTD) and applied for 30 min. Fruit were stored under controlled atmosphere at -0.5°C; 1.5% O_2 + 1% CO_2 for 28 days followed by 7 days at 20°C. Each treatment was replicated 5 times with 15 fruit per replicate. The experiment was conducted twice. At the end of the storage period, fruit quality parameters were evaluated.

Firmness

Fruit firmness was measured using 12 fruit per cultivar. Measurements were carried out with a texture analyser (Tensilon model UTM-4L, Toyo Measuring Instruments Co., Tokyo) with a 75 mm compression probe. Operating conditions of the instrument were: pre-test speed 1.5 mm s^{-1}, test speed 0.5 mm s^{-1}, post-test speed 10.0 mm s^{-1}, and trigger force 0.20 N. Each fruit was aligned horizontally from the stem end to the calyx, on a smooth holder to prevent slipping and deformation. For each fruit two measurements were taken on opposite sides of the fruit. Fruit firmness (N) of the fruit was taken as the force of compression, which

corresponded to the breakage of the sample (Chen and Opara, 2013a, b; Al-Said *et al.,* 2009).

Chemical properties

Total soluble solids (°Brix) of apple juice was measured using a digital refractometer (Atago, Tokyo). The pH measurements were performed at room temperature with a pH meter (Crison, Barcelona). Titratible acidity (TA), expressed as milligrams of malic acid per millilitre (mg MA mL^{-1}), was measured by titration to an endpoint of pH 8.2 using a Metrohm 862 compact titrosampler (Herisau, Switzerland) (Fawole *et al.,* 2012).

Phytochemical properties and antioxidant capacity

Sample preparation

Two millilitres of apple juice was accurately weighed into centrifuge tubes. The phytochemical and antioxidant contents were extracted by adding 10 mL of 50% methanol, and the sample was vortexed for about 30 s before being cold-sonicated for about 10 min. The apple juice sample was centrifuged at 10 000 rpm for 5 min at 4˚C to precipitate particulates. The extract was carefully collected into test tubes and cold stored at 4℃.

Determination of total phenolics

Total phenolic content in the apple juice was determined in accordance with the method of Makkar *et al.* (2007) and Fawole *et al.* (2012) with slight modifications. Briefly, 450 µL of 50% methanol and 50 µL apple juice were placed into glass test tubes. The total phenolic content concentration was determined spectrophotometrically at 725 nm by adding Folin-Ciocalteu reagent to the juice sample. Gallic acid was used as a standard and results were expressed as milligrams of gallic acid equivalents (mg GAE g^{-1}) of crude sample.

Ascorbic acid

Vitamin C content was measured using the method of AOAC (2000) with slight modifications (Fawole and Opara, 2013). An apple juice sample (1 mL) was mixed with 9 mL of 1% metaphosphoric acid. The mixture was vortexed for about 30 s before being ice-sonicated for about 3 min, and thereafter centrifuged at 10 000 rpm for 5 min at 4 ℃. A sample (1 mL) of diluted apple juice was placed in a glass test tube and 9 mL of 2,6-dichlorophenolindophenol dye (0.0025%) was added. To ensure that only ascorbic acid was measured, the absorbance of the mixture was measured at 515 nm within 30 min of incubation in the dark (Barros *et al.,* 2007). Ascorbic acid content was calculated using the

calibration curve of authentic L-ascorbic acid (0.01–0.1 mg mL^{-1}), and the results were expressed as ascorbic acid equivalents per millilitre crude juice (mg AAE mL^{-1}).

Ferric reducing/antioxidant power assay

Total antioxidant capacity was determined using the ferric reducing/antioxidant power (FRAP) assay of Benzie and Strain (1996) with slight modifications. The FRAP assay measures the ability of antioxidants in the sample to reduce ferric-tripyridyltriazine (Fe^{3+}-TPTZ) complex to the blue-coloured ferrous form (Fe^{2+}), which absorbs light at 593 nm (Khanizadeh *et al*., 2008). In triplicates, diluted apple juice samples (150 mL) were mixed with 2 850 µL FRAP reagent (300 mM sodium acetate buffer (pH 3.6), 10 mM TPTZ solution, 20 mM ferric chloride) and incubated in the dark for 30 min. Absorbance at 593 nm was measured using a spectrophotometer. Total antioxidant capacity was expressed as mean micromoles of Trolox equivalents per millilitre of crude apple juice (mM TE mL^{-1}).

2,2-Diphenyl-1-picrylhydrazyl assay

Apple juice sample was tested against a stable 2,2-diphenyl-1-picrylhydrazyl (DPPH) solution using the method of Wong *et al.* (2006) with some modifications. A solution of 0.1 mM DPPH in methanol was prepared. The initial absorbance of DPPH in methanol was measured at 515 nm and did not change throughout the assay period. In triplicates, a dilute apple juice sample (15 µL) was mixed with methanol (735 µL) and subsequently with DPPH solution (750 µL, 0.1 mM). The change in absorbance at 515 nm was measured after incubation for 30 min. Antioxidant activity based on the DPPH free-radical scavenging ability was expressed as mean millimoles of ascorbic acid equivalents per millilitre of crude apple juice (mM AAE ml^{-1}).

Statistical analysis

Data were subjected to analysis of variance with Statistica software (Statistical 11.0, StatSoft Inc., Tulsa, OK, USA) according to Duncan's multiple range test ($P < 0.05$).

Results

Fruit firmness

There were wide differences among the apple cultivars and treatments in terms of firmness ($P < 0.05$) (Fig. 1). The firmness of 'Granny Smith' apples treated with lemon and lime+lemongrass essential oils, ethanol and Rovral™ were not significantly different from the control treatment ($P < 0.05$). Furthermore, the firmness of 'Granny Smith' apples treated with

lime, lemongrass and lemon+lemongrass essential oils were not significantly different from each other. The firmness of ‘Pink Lady’ apples treated with ethanol, Rovral™, lemon, lime and lemongrass essential oils were not significantly different from the control treatment *(P* < 0.05). Futhermore, the firmness of ‘Pink Lady’ apples lemon+lemongrass and lime+lemongrass were significantly different from the control treatment. The firmness of ‘Granny Smith’ apples treated with lemon and lime+lemongrass essential oils was 72.82N and 76.58 N, respectively. These findings were not significantly different from control, ethanol and Rovral™ treatments. Single oils of lemon, lime and lemongrass showed 42.82, 45.37 and 44.08 N, respectively, on ‘Pink Lady’ apples, and were not significantly different from control treatment.

Chemical properties

Significant differences were observed in total soluble solids, titratible acidity, pH and total soluble solids: titratible acidity ratio of treated ‘Granny Smith’ and ‘Pink Lady’ apples compared to the control treatment (Figs. 2 and 3). Essential oil treatments of ‘Granny Smith’ and ‘Pink Lady’ apples resulted in reduced total soluble solids compared to control treatments (Fig. 2a). Ethanol and Rovral™ treatments increased the total soluble solids of ‘Pink Lady’ apples (13.60°Brix and 13.70 °Brix, respectively) compared to control treatment. Furthermore, these two treatments resulted in reduced total soluble solids on ‘Granny Smith’ apples. The titratable acidity of ‘Granny Smith’ apples was significantly higher than ‘Pink Lady’ apples (Fig. 2b). It was observed that the titratable acidity of all essential oil treatments of ‘Pink Lady’ apples were higher compared to control treatment (0.49 mg MA Ml^{-1}), with lime+lemongrass showing 0.62 mg CA Ml^{-1}. In addition, the titratable acidity of all essential oil treated ‘Granny Smith’ apples was low, except for lime+lemongrass treatment with 1.04 mg MA Ml^{-1} compared to other essential oil treatments. Ethanol and Rovral™ treated ‘Granny Smith’ apples showed 1.10 mg MA Ml^{-1} and 1.14 mg MA Ml^{-1}, respectively, while 0.48 mg MA Ml^{-1} and 0.47 mg MA Ml^{-1} was found on ‘Pink Lady’ apples.

Total soluble solids: titratible acidity ratio was significantly higher on ‘Pink Lady’ apples compared to ‘Granny Smith’ apples (Fig. 3a). The ratio of total soluble solids: titratible acidity of ‘Pink Lady’ apples treated with Rovral™ was 29.39, and significantly higher compared to control and ethanol treatments 28.17 and 28.21, respectively. The total soluble solids: titratible acidity ratio of ‘Pink Lady’ apples treated with lemon essential oil was 23.86, while it was 18.75 for lime+lemongrass essential oil. Furthermore, the total soluble solids: titratible acidity ratio of ‘Granny Smith’ apples treated with lime essential oil was 18.17, while it was 11.61 for lime+lemongrass essential oil. The pH of ‘Pink Lady’ apples was significantly higher than ‘Granny Smith’ apples (Fig. 3b). The pH of both cultivars was higher than control, ethanol and Rovral™ treatments. Furthermore, the pH of Pink Lady’ apples treated

with lemongrass essential oil was 3.78, while it was 3.50 on 'Granny Smith' apples treated with lime essential oil.

Total phenolic content

The total phenolic content of 'Granny Smith' apples treated with Rovral™, lemon oil and lime oil were not significantly different from control treatment, while ethanol, lemongrass oil, lemon+lemongrass and lime+lemongrass oils showed significant differences (Fig. 4a). The total phenolic content of 'Pink Lady' apples treated with lemon, lime+lemongrass, ethanol and Rovral™ treatments were not significant different compared to control treatment. Moreover, lime, lemongrass and lemon+lemongrass treatments exhibited a significant difference in total phenolic content of 'Pink Lady' apples compared to control treatment.

Ascorbic acid

It was observed that ascorbic acid was significantly higher on 'Pink Lady' apples compared to 'Granny Smith' apples (Fig. 4b). Ascorbic acid of both cultivars was higher for all essential oils, ethanol and Rovral™ treatments compared to control treatment. 'Pink Lady' apples treated with ethanol, Rovral™, lemon and lime essential oils were not significantly different from control treatment, while lemongrass, lemon+lemongrass and lime+lemongrass were significantly different from control treatment.

Antioxidant capacity

The radical ferric ion reducing antioxidant power (FRAP assay) value was significantly higher for 'Granny Smith' apples compared to 'Pink Lady' apples (Fig. 5a) after treatment. Although not significant different, the radical ferric ion reducing antioxidant power was higher (145.81 TE µM mL^{-1}) only on 'Granny Smith' apples subjected to lemon+lemongrass compared to control treatment. However, a reduction in radical ferric ion reducing antioxidant power was observed on both apple cultivars treated with essential oils (except for lemon+lemongrass), ethanol and Rovral™ treatments. Antioxidant capacity, based on radical scavenging activity (DPPH assay), showed no significant differences on 'Granny Smith' apples treated with Rovral™, ethanol and all essential oils (except for lemongrass and lemon essential oils), with lemon+lemongrass showing the higher antioxidant capacity (3.91 AAE mM mL^{-1}) (Fig. 5b). 'Pink Lady' apples treated with Rovral™, ethanol and all essential oils (except for lemon+lemongrass essential oils), were not significantly different frpm the control treatment ($p < 0.05$). There was no significant difference between 'Granny Smith' and 'Pink Lady' apples on control treatments.

Discussion

Firmness and soluble solids content are important quality attributes for apples and many other fresh fruits. Our results showed that some of the essential oils tested had no significant effect on fruit firmness after 28 days storage at controlled atmosphere (-0.5˚C; 1.5% O_2 + 1% CO_2) followed by 5 day at 20°C. Serrano *et al.* (2005) and Tzortzakis (2007) showed that use of natural antifungal compounds on sweet cherry storage increased fruit firmness. Conway *et al.* (1987) reported that the loss of firmness due to cell wall carbohydrate metabolism during storage is associated with increased susceptibility to infection by fungal pathogens. Fruit firmness on cherries and grapes was affected after exposure to eugenol, thymol, or menthol vapours (Martinez-Romero, *et al.,* 2005; Serrano *et al*, 2005) whereas acetaldehyde vapour-treated avocado delayed fruit softening (Pesis *et al*., 1998).

Total soluble solids content is a good indicator of sugar content of apples and presumably of sweetness (Hoehn *et al*., 2003; Magwaza and Opara, 2015). Titratable acidity may be an important tool in predicting taste of apples during the assessment of fruit quality, since consumers often have distinct preferences for acid or sweet tasting apples (Daillant-Spinnler *et al*., 1996; Harker *et al*, 2002). Our study showed that the total soluble solids of 'Granny Smith' and 'Pink Lady' apples was significantly lower than control treatments for EO treatments. The total acidity of 'Granny Smith' apples aws significantly lower than control for EO treatments. Reduced total acidity content could be linked to cellular activity in which organic acids serve as substrates that enter Krebs cycle to gain energy for repairing the aging cell and membranes (Taiz and Zeiger, 2002). The results of Anthony *et al*. (2003) study on banana fruit indicated that spraying essential oils of *Cymbopagon nardus*, *Cymbopagon flexuosus* and *Ocimum basilicum* had no effect on total soluble solids during storage after ripening. Furthermore, Ju *et al.* (2000) reported that total soluble solids and titratable acidity of pears treated with emulsions of plant oils, were maintained and depended on the concentration employed. Essential oil vapours of cinnamon and eucalyptus applied on tomatoes and strawberries increased total soluble solids (Tzortzakis (2007). The study by Gnsalez-Aguilar *et al*. (2003) showed that methyl jasminate increased postharvest quality and organic acids of papaya. These results are similar to those reported by Wang (2003) where raspberries treated with natural volatile compounds increased the acidity during storage.

It was observed that ascorbic acid of both cultivars was was high in all fruit treated with essential oils, Rovral™ and ethanol compared to control treatments. This finding is consistent with the results of Fatemi *et al* (2011). Fatemi *et al* (2011) reported that Vitamin C concentration on *Citrus sinensis* cv. was increased by thyme and peppermint essential oil treatments (with concentration of 1000ppm). The beneficial effects of low oxygen

atmospheres in reducing ascorbic acid losses in fruit are well documented in the literature as reviewed by Lee and Kader (2000). In addition, degradation of vitamin C is increased by many factors such as water loss, chilling injury, mechanical injuries and the increase of storage time (Lee and Kader, 2000; Sablani *et al.,* 2005). Tzortzakis *et al.* (2011) reported that ascorbic acid content in tomato fruit was not differentiated during exposure, but tended to increase throughout fruit storage. Vitamin C (ascorbic acid) has a high antioxidant power, providing protection against the presence of free radicals and consequently participating in the prevention of many degenerative diseases as well as it is an essential nutrient for humans (Tzortzakis *et al.*, 2011). Enzymes precede their activities through decomposing and converting complex compounds to simpler substances; and providing the means for cell destruction, enzymes overcome fruit resistance, a process that probably leads to decreased qualitative fruit characteristics such as Vitamin C content. Decreased Vitamin C content might also occur due to increased oxidation from water loss. Decreased generation of ethylene due to the effect of decreased respiration, delayed maturation and senescence might lead to maintaining Vitamin C at high levels.

This study indicated that essential oil treatments increased the total phenolic content on both apple cultivars. Most of the antimicrobial activity in essential oils appears to be associated with interactions between phenolic compounds and the food matrix (Nuchas and Tassou, 2000). Phenolic compounds are plant secondary metabolites which contribute substantially to the antioxidant complement of many fruit and thus play an important role in inhibiting reactions mediated by reactive oxygen species, which are associated with a number of human non-communicable diseases (Horemans *et al*., 2000; Assis *et al*., 2001; Karakaya and Tas, 2001). Several antioxidative parameters such as ascorbic acid and total phenolic compounds, increased following essential oil application in tomato fruits, indicating the induced resistance role of essential oils (Tzortzakis *et al*., 2011; Rousos *et al*., 2013). Beckman (2000) also reported that phenolic content plays a major role in plant resistance and defense mechanism against invasion of plant pathogens.

Antioxidant activity is a complex process usually occurring through several mechanisms. Wang *et al.* (2008) reported that essential oil components such as eugenol revealed an ability to increase antioxidant levels (polyphenols, flavonoids, anthocyanins) and an oxygen absorbance capacity in plant tissues, including in enzymatic and non-enzymatic systems, which support the increase of oxygen radical absorbance and hydroxyl radical scavenging capacity of the fruit tissue. Based on our findings, the radical ferric ion reducing antioxidant power increased on ‘Granny Smith’ apples subjected to lemon+lemongrass essential oil was high. Sivakumar and Bautista-Banos (2013) reported that the influence of essential oil components on increasing the antioxidant capacity and scavenging activity could exert a

significant impact on enhancing the resistance of plant tissues to pathogens and reducing their physiological deterioration.

In conclusion, our study indicated that citrus and lemongrass essential oils tested had no significant effect on fruit firmness of 'Granny Smith' and 'Pink Lady' apples. Essential oils tested in the present study indicated the ability to act as signalling compounds that triggers a signal similar to a mild stress condition in the fruit. In our previous studies (Chapter 5) about *in vivo* antifungal effects of citrus and lemongrass essential oils speculated that efficacy of essential oils in controlling postharvest fungal diseases of apples may be attributed to their potential to initiate defense responses in the fruit. The present study has demonstrated that treating fruit with citrus and lemongrass essential oil exerted a positive influence in postharvest and quality of 'Granny Smith' and 'Pink Lady' apples. The high phenolic content and antioxidant activity of fruit treated with citrus and lemongrass oils indicated that consuming these fruit may impart health benefits and may be regarded as valuable source of beneficial antioxidants.

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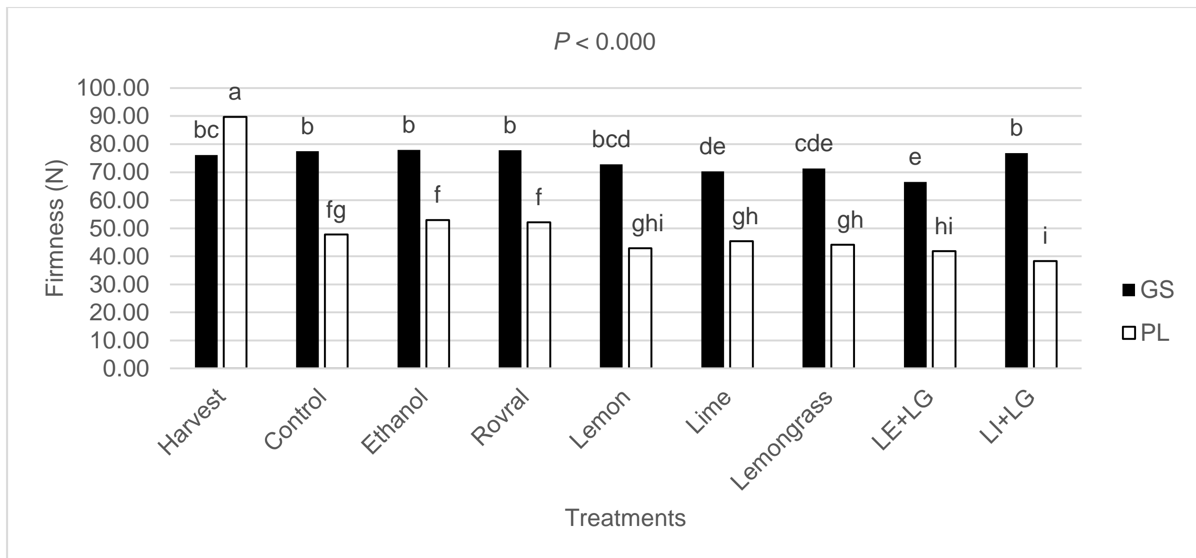

Figure 1. Effect of essential oils on fruit firmness of ‘Granny Smith’ and ‘Pink Lady’ apples stored at controlled atmosphere for 28 days (-0.5°C; 1.5% O_2 + 1% CO_2) followed by 7 days at 20˚C. Different letters on bars represent statistical differences (P < 0.05) using Duncan’s multiple range test. LE = lemon oil; LI = lime oil; LG = lemongrass oil.

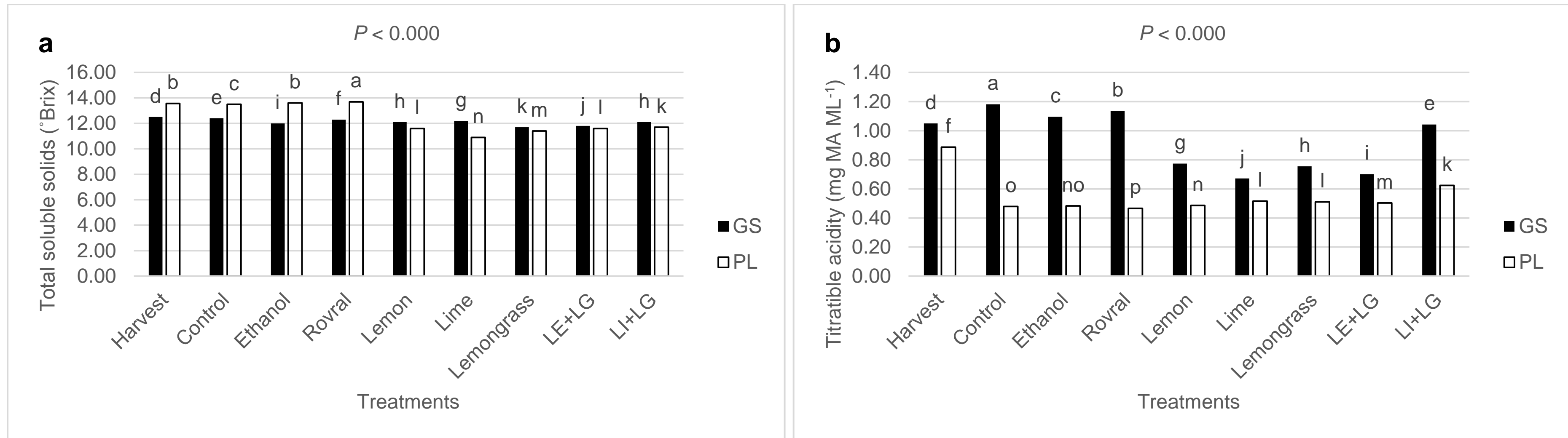

Figure 2. Effect of essential oils on total soluble solids (TSS; a) and titratable acidity (b) of 'Granny Smith' and 'Pink Lady' apples after 28 days storage at controlled atmosphere (-0.5°C; 1.5% O_2 + 1% CO_2) followed by 7 days at 20˚C. Different letters on bars represent statistical differences ($P < 0.05$) using Duncan's multiple range test. LE = lemon oil; LI =lime oil; LG =lemongrass oil.

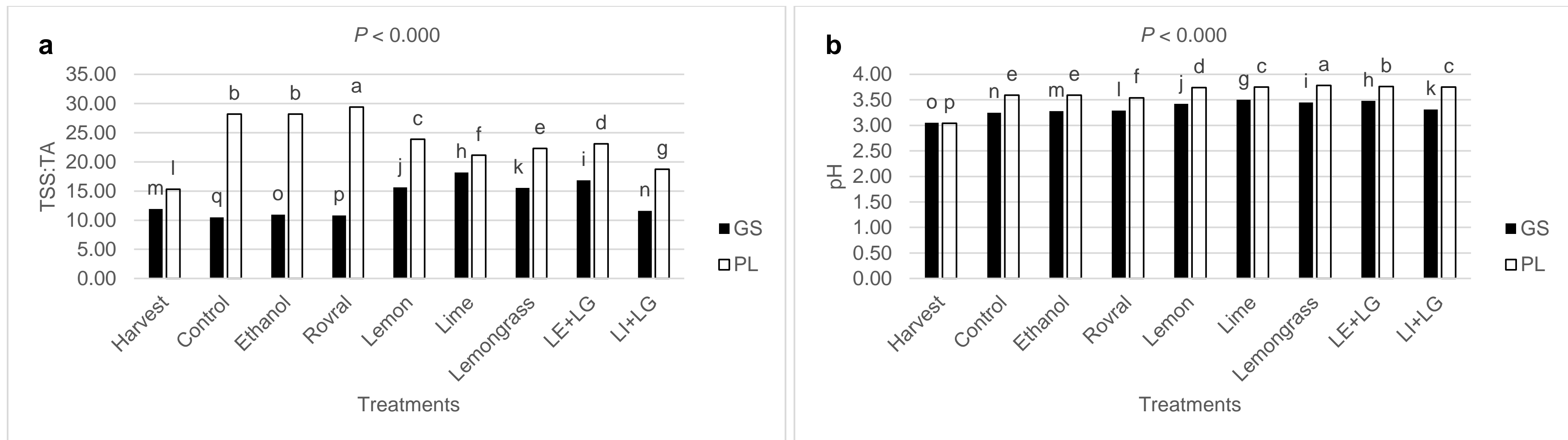

Figure 3. Effect of essential oils on TSS:TA ratio (a) and pH (b) of 'Granny Smith' and 'Pink Lady' apples after 28 days storage at controlled atmosphere (-0.5°C; 1.5% O_2 + 1% CO_2) followed by 7 days at 20˚C. Different letters on bars represent statistical differences (*P* < 0.05) using Duncan's multiple range test. LE = lemon oil; LI =lime oil; LG =lemongrass oil.

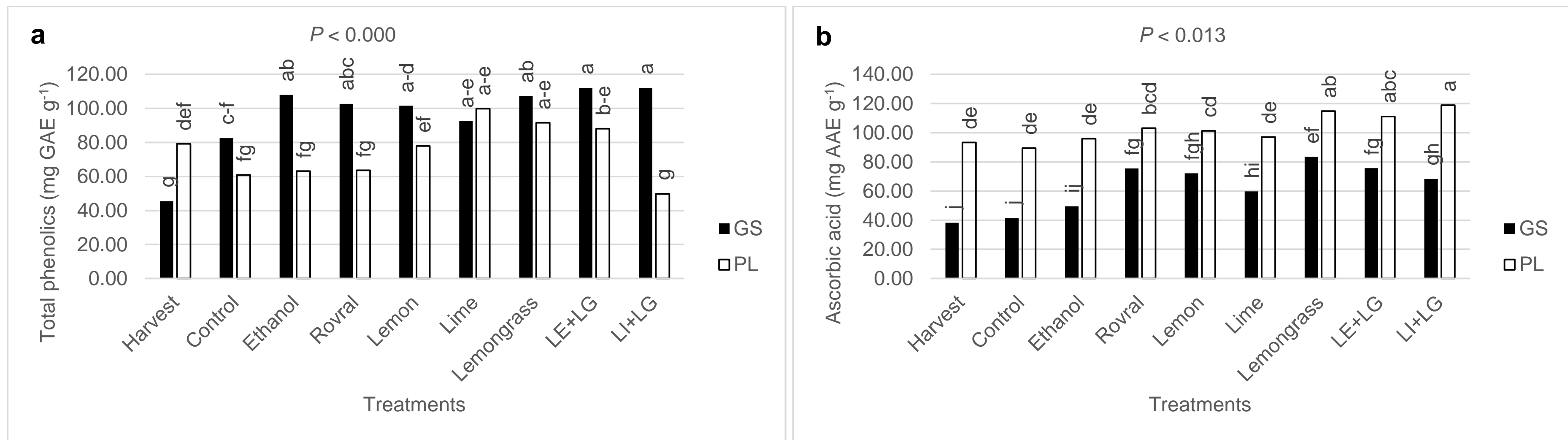

Figure 4. Effect of essential oils on total phenolics (a) and ascorbic acid (b) of 'Granny Smith' and 'Pink Lady' apples after 28 days storage at controlled atmosphere (-0.5°C; 1.5% O_2 + 1% CO_2) followed by 7 days at 20˚C. Different letters on bars represent statistical differences (*P* < 0.05) using Duncan's multiple range test. LE = lemon oil; LI =lime oil; LG =lemongrass oil.

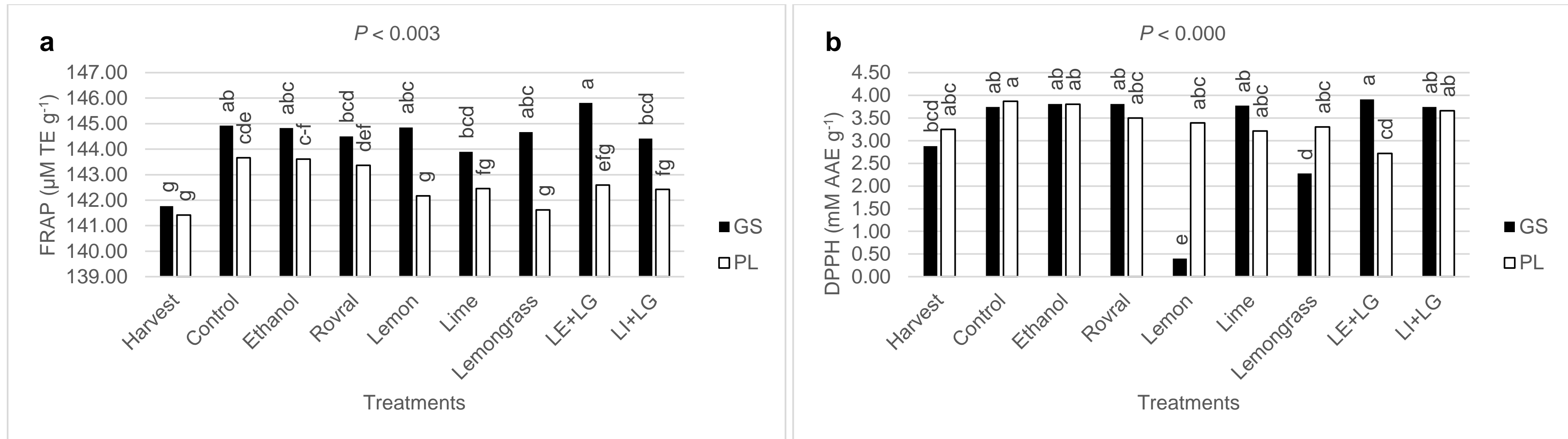

Figure 5. Effect of essential oils on total antioxidants (a and b) of 'Granny Smith' and 'Pink Lady' of 'Granny Smith' and 'Pink Lady' apples after 28 days storage at controlled atmosphere (-0.5°C; 1.5% O_2 + 1% CO_2) followed by 7 days at 20˚C. Different letters on bars represent statistical differences (P < 0.05) using Duncan's multiple range test. LE = lemon oil; LI =lime oil; LG =lemongrass oil.

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CHAPTER 7

GENERAL DISCUSSION, CONCLUSION AND FUTURE PROSPECTS

Postharvest diseases of fruit have been controlled primarily by the application of conventional fungicides, including imazalil, carbendazim, thiabendazle or mixtures of these compounds (Palou *et al*., 2008). However, the application of these fungicides may result in residues on food that may negatively affect human health (Roistacher *et al*., 1960; Houck, 1977; Koeman, 1978; Norman, 1988) and can lead to build up of pathogen resistance or environmental pollution (Janisiewicz, 1987; Wilson and Wisniewski, 1989). Therefore, to meet consumer demand for safe food and to stay in the forefront of global apple export, South Africa is in urgent need of new alternative environmentally friendly products. The use of biocontrol agents and naturally-occuring antifungal compounds to manage postharvest decay of fruit has been explored as alternative methods (Wilson and Wisniewski, 1989; Benbow and Sugar, 1999) and several commercial products are now available (Droby *et al*., 1998; Janisiewicz and Korsten, 2002). This study explored the potential of citrus and lemongrass essential oils as antifungal agents to control postharvest pathogens of apples caused by *Botrytis cinerea, Penicillium expansum* and *Neofabraea alba*.

Essential oils and plant extracts have been used for a wide variety of purposes for many years (Jones, 1996). Recently, essential oils and other extracts from plants have attracted interest as novel sources of natural products for agriculture use. They have also been screened for potential applications as alternative remedies to treat human infectious diseases and preserve commodities. Particularly, the antimicrobial activity of essential oils is considered for industrial applications. Essential oils can be extracted by using several different methods. In this study, we used lemon, lime and lemongrass essential oils which were originally extracted by steam distillation (practical, inexpensive and generally regarded as a safe method). Gas chromatography-mass spectrometry was used to identify the major components of lemon, lime and lemongrass essential oils (Chapter 2). A total of 44 volatile compounds were detected and identified in lemon oil with limonene (58.52%) and γ-terpinene (19.80%) as the major compounds while lime oil showed 20 components with limonene (79.00%) and γ-terpinene (11.22%) as the abundant compounds. These findings were in agreement with Di Vaio *et al*. (2010) study where limonene was found as the main component in the lemon essential oil, accounting for 72.5-76.4%, followed by β-pinene (11.6-18.7%). Lemongrass oil revealed 26 components with geranial (48.14%) and neral (38.32%) as the major compounds. Similar

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observation was reported by Choi *et al.* (2012), with geranial (50.04%) and neral (36.20%) as major constituents of lemongrass essential oil.

Plant essential oils have antimicrobial activity against a variety of plant pathogens and pests. Several studies have explored the potential of essential oils as antifungal agents (Kurita *et al.,* 1981; Grane and Ahmed 1988; Wilson *et al.,* 1997; Cowan, 1999; Abd-Alla *et al.,* 2001; Abdolahi *et al.,* 2010). Most of them have been reported to inhibit postharvest fungi *in vitro* (Hidalgo *et al.,* 2002; Kordali *et al.,* 2005). In Chapter 3, *in vitro* antifungal activity of lemon (*Citrus limon*), lime (*Citrus aurantifolia*) and lemongrass (*Cymbopogon citratus*) essential oils in combination with cold storage regimes through direct contact and vapour phase methods on mycelial growth and spore germination of *B. cinerea*, *P. expansum* and *N. alba* was evaluated. *In vitro* assays showed that direct contact phase of lemongrass oil, lemon+lemongrass and lime+lemongrass oils exhibited the strongest toxicity and completely inhibited the mycelial growth and spore germination of *B. cinerea*, *P. expansum* and *N. alba*, at concentrations of 1.5 - 100% at 20°C (95% relative humidity), regular atmosphere (-0.5°C, air), and controlled atmosphere (5%O_2 + 10% CO_2, -0.5°C). Lemon and lime oils also showed antifungal activity but it was much less effective than lemongrass oil. In the case of vapour phase method, all essential oil treatments significantly ($p < 0.05$) controlled all three pathogens at concentrations of 0.016-1.0% compared to control treatments. Mycelial growth of fungal pathogens was inhibited by the tested essential oils in a dose-dependent manner. Tzortzakis and Economakis (2007) reported that lemongrass oil showed antifungal activity against several plant pathogens, such *B. cinerea,* and *R. stolonifera.* The antifungal activity of essential oils from oregano and thyme showed significant efficacy in apple fruits infected with *B. cinerea* and *P. expansum* (Lopez-Reyes *et al.,* 2010). In addition, the antifungal activity of clove oil in apples was evaluated against *B. cinerea*, *M. fructigena*, *P. expansum* and *P. vagabunda* (Amiri *et al.,* 2008).The volatile phase of the essential oils of lemon and lime were more potent than direct contact phase, while lemongrass essential oil, mixtures of lemon + lemongrass and lime + lemongrass were potent in both direct contact and vapour phase at 20°C (95% relative humidity), regular atmosphere (-0.5°C, air), and controlled atmosphere (5%O_2 + 10% CO_2, -0.5°C). Inhibition of mycelial growth and spore germination of postharvest pathogens by essential oil treatments could make a major contribution in limiting the spread of the pathogen by lowering the spore load in storage facilities and packhouses.

The antimicrobial activity of essential oils could be related to the presence of an aromatic nucleus and OH group that can affect hydrogen bonds of enzymes in microorganisms (Farrag *et al.,* 1989). This would cause a deformation in cell structure and functionality, and permit the loss

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of macromolecules from their interior (Rattanapitigorn *et al*., 2006). Furthermore, each of the essential oil components has its own contribution on biological activity of the oil. For example, neral and geranial were found as the main compounds in lemongrass essential oil, while limonene was found in lemon and lime oils (Chapter 2), and these compounds are known to be responsible for the antimicrobial activity displayed by essential oils (Takayuki *et al*., 2007; Tyagi and Malik, 2011). The potential antifungal mechanisms of lemongrass essential oil on *B. cinerea* was investigated in Chapter 4. Light microscopy and scanning electron microscopy in this study indicated that *B. cinerea* hyphae exposed to lemongrass oil undergo morphological damage such as vesiculation, cytoplasmic disruption and collapsed hyphae. The glucose-induced reduction in external pH of *B. cinerea* was inhibited by lemongrass oil in a time and concentration dependent manner. *Botrytis cinerea* spores treated with lemongrass oil showed strong propidium iodide fluorescence in the cytosol. Lemongrass oil significantly altered plasma membrane, the release of cell constituents, and the total lipid content of *B. cinerea*. These observations indicate that the antifungal activity of lemongrass oil can be attributed to the disruption of the cell membrane integrity and membrane permeability. Moreover, because plant essential oils have low mammalian toxicity, are biodegradable, multifunctional, non-persistent in the environment and cheap to produce, the possibility of developing their use in crop protection is considered an attractive possibility (Abdolahi *et al.,* 2010).

In Chapter 5, the efficacy of essential oils as a postharvest treatment against *B. cinerea*, *P. expansum* and *N. alba* on apples when applied by fumigation, dipping and thermal fogging was investigated. Fumigation of 'Granny Smith', 'Golden Delicious' and 'Pink Lady' apples with different concentrations of essential oils (curative or preventative) showed antifungal activity against *B. cinerea, P. expansum* and *N. alba* at 20°C, regular atmosphere and controlled atmosphere (Chapter 4). All essential oil treatments tested showed no phytotoxicity on all three apple cultivars when applied by fumigation and thermal fogging, while dipping fruit to some concentrations showed phytotoxic effect. Application of essential oils, through thermal fogging, inhibited *B. cinerea*, *P. expansum* and *N. alba* on all three cultivars compared to Rovral™, ethanol and control treatment. Essential oil mixtures showed the highest inhibition of *B. cinerea*, *P. expansum* and *N. alba* on all three cultivars compared to single application of essential oils. Our results suggest the possibility of using essential oils in combination with cold storage regimes as natural fumigants for controlling postharvest diseases of apples. Furthermore, the effects of citrus and lemongrass essential oils was investigated on the quality parameters of 'Granny Smith' and 'Pink Lady' apples after controlled atmosphere storage (Chapter 6). The results indicated that some of the essential oils tested had no significant effect on fruit firmness

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of 'Granny Smith' and 'Pink Lady' apples. Significant differences were observed in total soluble solids, titratible acidity, pH and total soluble solids: titratible acidity ratio of both cultivars treated with essential oils compared to control treatments. Several antioxidative parameters such as ascorbic acid and total phenolic compounds, increased following essential oil application in tomato fruits, indicating the induced resistance role of essential oils (Tzortzakis *et al.*, 2011; Rousos *et al.*, 2013). Citrus and lemongrass essential indicated a positive influence in postharvest and quality properties of 'Granny Smith' and 'Pink Lady' apples which our previous studies (Chapter 4) speculated that efficacy of essential oils may be attributed to their potential to initiate defense responses in the fruit. It can be further suggested that essential oils has the ability to act as signalling compounds that triggers a signal similar to a mild stress condition in the fruit.

In South Africa, apples are harvested and stored at low temperature or controlled atmosphere for up to 9 months to maintain quality and to minimize spoilage. However, development of postharvest fungal diseases is a major challenge. Control measures are still principally based on the protection of fruits from postharvest infection by using fungicide treatments. Synthetic fungicides such as Rovral™ and Sholar™ are recommended for control of postharvest diseases of fruits. Fungicides are applied by dipping the fruit in a fungicide bath for 30-40 sec. However, poor management of fungicide baths can increase postharvest decay; organic matter or dirt and dust can reduce the effectiveness of treatments. In this study, application of citrus and lemongrass essential oils by dipping showed phytotoxic effect on apples, except at a concentration of 0.125% and 30sec exposure time. This suggest the application of essential oils by incorporating the oil into wax or coating treatments that are being used in the packing-line. However, waxing of apples is not currently practised by the SA apple fruit industry. Thermal fogging and fumigation application of citrus and lemongrass essential oils showed no phytotoxicity at a concentration of 0.125% and inhibited fungal growth on apples. These findings recommend the application of essential oils by fumigation or thermal fogging as postharvest treatments in combination with regular atmosphere or controlled atmosphere. Treatments distribution of the oil could be directly applied to the fruit by thermal fogging into storage rooms. This will allow a uniform distribution of the oil, use of very small amounts of the oil and the possibility of several applicatons during the storage period.

In general, results obtained in the present study showed that citrus and lemongrass essential oils have desirable characteristics for postharvest applications to control *B. cinerea*, *P. expansum* and *N. alba*. The commercial use of these essential oils can result in a safe method to protect apples from postharvest decay and could represent a novel postharvest treatment. *In*

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vitro and *in vivo* assays with citrus and lemongrass essential oils showed remarkable control of *B. cinerea*, *P. expansum* and *N. alba*, which may indicate the promising potential for postharvest disease control, especially for the apple fruit industry.

Some areas for future research directed towards the application of essential oils as alternatives to synthetic fungicides in managing of postharvest diseases are as follows. The identification of active components of essential oils and understanding their mechanism of action, in addition to that of the essential oil mixture is essential. In addition, once the active component is known, it is then possible to develop and prepare synthetic analogs. These synthetic components would be better controlled in terms of preparation reproducibility and a higher economical viability. Antifungal activity of essential oils and major compounds should be evaluated on other fruit crops such as citrus, pears, grapes, nectarines, peach and plums. Compatibility of essential oils with other chemicals needs to be explored as well as the legality and safety of application methods. Reducing inoculum levels in the air in the packhouse and on the fruit surface through effective sanitation should be considered to minimise the likelihood of the disease (Palou *et al*., 2008). A combination of essential oil with a heated dip or spray in the packing line operation should be further investigated. Appropriate practices during handling and storage of fruit in order to prevent infection as well as sustaining quality are also important in postharvest disease management (Palou *et al.,* 2008). Therefore, the incorporation of essential oil in wax or coating should be investigated to maintain essential oil in contact with the fruit peel to exert a continuous inhibitory effect on pathogens. Furthermore, the aroma of essential oil may improve the sensory quality of the fruit. In future studies, it is recommended that naturally infected fruit should be used in order to provide more information on the suitability of the application methods investigated in this study in preventing decay of apples caused by *B. cinerea*, *P. expansum* and *N. alba*. This would allow assessment not only of the effects of essential oil application on disease incidence but also of sensory evaluation for visual and organoleptic attributes of fruit.

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APPENDIX 1

Conference proceeding produced during PhD candidature

- Mbili, N.C., Vries, F. and **Lennox, C.** (2013) *In vitro* effects of essential oils and their combinations against *Botrytis cinerea*, *Penicillium expansum* and *Neofabraea alba*. 2nd International Symposium on Discovery and Management of Innovative Strategies for Postharvest Disease Management, Fantasia Hotel De Luxe, Kusadasi, Turkey, 29 April - 2 May 2013 (Poster).
- **Mbili, N.C.**, Vries, F., Opara, U.L. and Lennox, C. (2014) Chemical composition and antifungal activity of citrus and lemongrass essential oils in combination with cold storage regimes against *Botrytis cinerea*, *Penicillium expansum* and *Neofabraea alba*. 1st ARC-Professional Development Programme Conference: ARC-Infruitec, Stellenbosch, South Africa, June 2014 (Poster).
- **Mbili, N.C.**, Vries, F., Opara, U.L. and Lennox, C. (2014) Chemical composition and antifungal activity of citrus and lemongrass essential oils in combination with cold storage regimes against *Botrytis cinerea*. 10 International IOBC Conference: STIAS, Stellenbosch, South Africa, 24-28 November 2014 (Oral).
- **Mbili, N.C.**, Vries, F., Opara, U.L. and Lennox, C. (2014) Volatile composition and antifungal activity of citrus and lemongrass essential oils in combination with cold storage regimes against *Botrytis cinerea*. 49th Southern African Society for Plant Pathology: Bains Lodge, Bloemfontein, South Africa, 18-21 January 2015 (Poster).
- **Mbili, N.C.**, Vries, F., Opara, U.L. and Lennox, C. (2015) The use of essential oils in combination with controlled atmosphere to control postharvest decay caused by *Botrytis cinerea* and *Penicillium expansum* on apples. Henry Ford Building, Berlin, Germany, 24-27 August 2015 (Poster).
- **Mbili, N.C.**, Vries, F., Opara, U.L. and Lennox, C. (2015) The mode of antifungal action of lemongrass (*Cymbopogon citratus* (DC.) Strapf) essential oil on *Botrytis cinerea*. Henry Ford Building, Berlin, Germany, 24-27 August 2015 (Poster).