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Evaluation of an Ethanolic Extract of Propolis as a Potential Pre- and Post-Harvest Fungicide for 'Fuerte' Avocado (*Persea americana* Mill.) Fruits and Orchards

Lorenzo Corrado Giovanelli

A dissertation submitted to the Faculty of Science, University of the Witwatersrand, Johannesburg, in fulfillment of the requirements for the degree of Master of Science.

Johannesburg, March 2008

Declaration

I declare that this dissertation is my own work. It is being submitted for the Degree of Master of Science to the University of the Witwatersrand, Johannesburg. It has not been submitted to any other university for any other purpose or examination.

_____

Lorenzo Corrado Giovanelli

_____ day of _____ 2008

Abstract

Propolis has been used by man for millennia for its antimicrobial and pharmaceutical properties. However, its use as an agricultural antimicrobial agent has only recently been assessed. This study assessed the use of an ethanolic extract of propolis (EEP) for the control of avocado fruit fungal pathogens. Qualitative analyses of EEP indicated flavonoids as the main antimicrobial constituents. Quantitative analyses detected 16.35 mg ml^{-1} total flavonoids and 3.28 mg ml^{-1} total phenolics. The Minimum Inhibitory Concentration (MIC) of EEP was determined as 5 mg ml^{-1} against *Colletotrichum gloeosporioides*, *Pestalotiopsis guipinii*, a complex of *Colletotrichum gloeosporioides* and *Pseudocercospora* sp. (CgP complex), *Verticillium* sp., *Fusarium* sp. and *Monilia* sp., isolated from avocado fruits, using the agar dilution method, at a concentration gradient from 1 to 10 mg ml^{-1}. Electron micrographs of *Pestalotiopsis guipinii*, *Colletotrichum* sp. and *Colletotrichum gloeosporioides*/*Pseudocercospora* sp. (CgP complex) incubated on agar media containing EEP clearly indicated signs of cell wall damage with large pores within the hyphae. Conidial germination of *Colletortichum* sp. and *P. guipinii* was inhibited by 98.95 % and 40.41 % respectively by EEP. Trees infected with *Colletotrichum* sp., *P. guipinii* or CgP complex conidia were incubated within greenhouse conditions and treated with 5 mg ml^{-1} EEP at weekly intervals from once every week to once every six weeks. Disease indices from experimental and control trees were similar but noticeable control of CgP disease symptoms was observed from treatment with EEP. 'Fuerte' avocado trees were treated with copper hydroxide, bore-hole water or 5 mg ml^{-1} EEP during the 2006-2007 growing season. All fruit were similar after harvesting with respect to pre-harvest disease. The occurrence of post-harvest diseases was analysed after simulations of import and export markets. EEP treated fruit were similar to bore-hole treated fruits (control). Similar results were observed after trials to assess the use of EEP as a post-harvest dip. The stem-end of prematurely harvested 'Fuerte' fruit were dipped into 5 mg ml^{-1} EEP, and incubated until ripe, to assess control of stem-end rot (SER). EEP reduced the occurrence of SER by 30 %. EEP was further assessed to inhibit infection or disease spread by *Colletotrichum* conidia. Fruits were inoculated with *Colletotrichum* conidia and either treated with 5 mg ml^{-1} or 10 mg ml^{-1} EEP either after or before infection. The fruits were incubated until ripe. Both treatments reduced the occurrence of disease ($P < 0.001$). In conclusion, EEP did not efficiently

control disease in the field, but showed high potential as a future fungicide for avocado fruit. Optimisation of EEP includes higher concentrations, the addition of stickers, and/or more frequent spraying of trees.

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Preface

The majority of economically important diseases of avocado fruits are post-harvest diseases. Post-harvest disease pathogens of avocado fruit usually infect the fruit epidermal layers during the growing season, after which they enter a dormant state. The post-harvest pathogens re-enter a vegetative state once the fruits are harvested and begin to ripen. They spread into the surrounding epidermal tissues and degrade or liquefy the host cells. This causes depressions on the fruits surface which take on a darker colour resulting in noticeable rotting of the fruit. Mature lesions spread into the flesh of the fruit resulting in unpalatable fruit. Examples of such diseases are: anthracnose caused by *Colletotrichum gloeosporioides*; Dothiorella rot caused by *Dothiorella aromatica*; DCC fruit rot caused by a complex of *D. aromatica* and *C. gloeosporioides*. A disease called stem-end rot (SER) has a dormant state similar to the above mentioned diseases. The fungi initially infect the ovaries of the flowers and enter a dormant state as the fruit develops and situate themselves within the stem of the fruit. When the fruit is harvested and ripens, the fungi infect the fruit through the vascular system, rotting the flesh. Only mature infections express external and visual symptoms. Due to the dormant state of the post-harvest pathogens the majority of infected fruits are usually sold to fruit markets as they seem visually healthy. Once the fruits ripen the disease causes much customer dissatisfaction and economic loss.

To control the diseases, pre-harvest treatments, post-harvest treatments or both treatments are applied to the fruits. Pre-harvest treatments are applied to the trees, usually as a copper-based spray, several times during the growing season. The fruits are dipped into the fungicide for times of between 30 seconds to 5 minutes, depending on the fungicide used.

The most economically important pre-harvest disease of avocado fruits is caused by the fungus *Pseudocercospora purpurea*, expressing a disease known as Cercospora spot. Cercospora spot is defined as a scab-like lesion on the external epidermal layer. Only very mature lesions penetrate the flesh and cause rotting of the flesh thereof. Consumers reject the fruit due to the unsatisfactory superficial look, thereby resulting in economic

loss to markets. Cercospora spot is conventionally treated by pre-harvest spraying with copper-based fungicides.

South Africa is an internationally important exporter of avocado fruit to the United Kingdom (U.K.) and Europe. The quality standards allowed for sale of the South African grown fruits is, therefore, depicted by the U.K standards and European Union (E.U.). However, with ever increasing awareness of the impact of agricultural practices on the environment, the U.K. and E.U. have been increasing their pressure on farmers to implement more environmentally safe agricultural methods. An example of such a pressure has been the call for all companies manufacturing pesticides to re-register their products. This has resulted in many products not being re-registered and therefore a decrease in the variety of chemical pesticides. Other examples of pressures have been the lowering of fungicide concentrations and implementing organic farming practice. This has resulted in copper-based fungicides as the only approved pre-harvest fungicides to control diseases of avocado fruit. However, concern over future build up of copper within the soil, underground water table and surrounding environments has resulted in the E.U. requesting all farmers to reduce their concentration of copper-based fungicides.

There are no known alternate fungicides that are environmentally friendly, and with no adverse health risks, on the market to date. Besides biological control, there has been very little recent research in the development of alternate fungicides for the avocado industry. This study therefore looked at the possibility of using an ethanolic extract of propolis as a fungicide for the avocado industry which could be applied as either a pre- or post-harvest fungicide.

Propolis is a sticky substance produce by honey bees by collecting residues and extracts of different plant species. They masticate and regurgitate the residues and mix bees-wax to form propolis. The bees use propolis to surround their hive walls and entrances as a protection from wind, to aid in climate control, and to maintain sterile conditions especially near the larvae. The antimicrobial activity of propolis has been known and used by man for pharmaceutical purposes for as much as 2000 years. However, not much interest in the use of propolis for agricultural methods has been persisted.

The main objectives of this study were:

- To efficiently extract the antimicrobial compounds from a propolis sample.
- To determine the efficacy of the antifungal activities in *in vitro* trials against fungal pathogens of avocado fruit and other commonly found fungi.
- To determine, during greenhouse trials, how often the propolis extract is needed to spray on avocado trees during pre-harvest treatments.
- To determine the efficacy of the propolis extract as a pre- and post-harvest fungicide to control both pre- and post-harvest diseases of avocado fruit.

The study is structured in six chapters. The first chapter is a review of the literature on alternative fungicides, the diseases of avocado fruits, and the antimicrobial activity of propolis. The extraction of the antimicrobial compounds from propolis and their chemical analysis is described in chapter two. Chapter three describes studies to determine the efficacy, *in vitro,* of the propolis extract. Chapter four describes the *in vivo* trials, within greenhouse conditions and is followed by the assessment of the extract as a pre- and post-harvest fungicide for avocado fruits during *in situ* trials in chapter five. Final conclusions of the entire study are given in chapter six.

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Chapter 1

Literature Review

1.1 Introduction

Organic farming practices are intensifying due to an ever increasing awareness and demands by consumers for healthier and environmentally risk free produce. However, there are no efficient alternative fungicides as compared to synthetic fungicides registered to date (Karasuda *et al..,* 2003; Gamagae *et al..*, 2004; Spadaro and Gullino, 2004; Tripathy and Dubey, 2004). Therefore, further developments of alternative fungicidal methods, which are non-toxic, environmentally friendly and pose no threat to health, are urgently required.

Over the past few years, several non-hazardous, environmentally friendly alternative strategies for the control of pre- and post-harvest fungal pathogens have been developed. The most common of these strategies has been (i) the use of microorganisms to antagonize plant pathogens (known as biological control) (Spadaro and Gullino, 2004); (ii) the application of natural products produced by plants and/or animals with antifungal properties or to induce plant defence mechanisms (Tripathi and Dubey, 2004); and (iii) the utilization of non-selective fungicides that are non-toxic and biodegradable, (Gamagae *et* al., 2004, Tripathi and Dubey, 2004).

1.2 Biological Control

Several biofungicides have been commercialized. The two most common international biocontrol products are BioSave 110, which is primarily the bacterium *Pseudomonas syringae*, active against *Botrytis*, *Penicillium*, *Mucor*, and *Geotrichum* sp.; and the yeast *Candida oleophila* active against *Botrytis* and *Penicillium* sp. (Spadaro and Gullino, 2004).

An example of a successful study using BioSave was that of Errampalli (2003). They observed an 89 % reduction of blue mold infecting 'Empire' apples by treatment with 5 x 10^{10} CFU ml^{-1} BioSafe, and stored in cold storage. Errampalli and Brubacher (2006) integrated BioSave with cyprodinil, a reduced-risk fungicide, as an attempt to enhance BioSave efficiency. They demonstrated that 3 x 10^{7} CFU ml^{-1} of *P. syringae* with 20 μg ml^{-1} of cyprodinol gave complete control of blue mold in 'Empire' and 'McIntosh' apples under cold storage. In the subsequent shelf-life storage of the apples, the same combination of BioSave and cyprodinil reduced blue mold in 'Empire' and 'McIntosh' apples by 97 % and 86 % respectively (Errampalli and Brubacher, 2006).

Arras *et al.* (2007) treated Tangelo Mapo fruit with *C. oloephila* Montrocher 13L to control postharvest decay by the fungal pathogen *Penicillium digitatum*. The yeast biocontrol agent inhibited up to 92 % of decay. However, in combination with oxalic acid only 86 % of fruit decay was obtained. Similarly, Gamagae *et al.*. (2004) integrated *C. oleophila* with sodium bicarbonate mixed with a paraffin-based wax as an integrated biological control agent against anthracnose of papaya fruit, a disease commonly caused by *Colleotrichum gloeosporioides*. The treatment reduced the incidence of anthracnose by over 40 % (Gamagae *et al.*., 2004).

Biological control has its disadvantages. In long term usage, biological antagonists have been reported to be inconsistent and/or to have decreasing efficiency (Tripathy and Dubey, 2004). The disease control level required for biological control is alarmingly high at 95-98 % reduction in diseased fruit, especially in post-harvest diseases. A major impact to the use of biological control is the relatively small number of markets accepting biological control treated produce (Spadaro and Gullino, 2004).

1.3 Use of Natural Compounds Derived from Organisms

Kim and Chung (2004) isolated an unknown protein from *Bacillus amyloliquefaciens* MET0908, which showed antifungal activity against the watermelon anthracnose causing fungus, *Colletotrichum lagenarium*. The molecular weight of the protein was estimated at

40 kDa and was found to be stable at 80 °C for 20 min. After applying the protein to *C. lagenarium*, which was then examined examined under the electron microscope, it was found that the protein acted on the cell wall of the fungus. Further examination of the protein's activity showed it to have β-1,3-glucanase activity. The protein was also shown to be novel from any other known proteins to date, and showed high potential for commercialization. Similarly, Karasuda *et al.*. (2003) isolated and purified plant chitinases (chitinase E, family 19, class IV) from yam (*Dioscorea opposite*) tubers. They proceeded to test the application of the chitinases with β-1,3-glucanase in a solution called Zymolyase 20T, against the strawberry powdery mildew (*Sphaerotheca humula*) infecting 3 month old strawberry plants (in greenhouse conditions). A week after spraying the diseased plants, with a mixture of 3 μM chitinase E and 0.6 % Zymolyase 20T, the powdery mildew disappeared completely and did not appear again for at least an additional two weeks. Once the disease re-established, it did not spread and the infected spots gradually turned from white to brown. Using scanning electron microscopy (SEM), it was illustrated that the hyphae and conidia of *S. humula* were extensively damaged. The hyphae contained visible holes through their surface and the conidia were not complete. SEM of *S. humula* treated with chitinase E only showed damage to the hyphae, but was not as extensive as with the Zymolyase 20T. Karasuda and associates (2003) therefore demonstrated that the enzymatic treatment of powdery mildew on strawberry plants can be more efficient than fungicides as the fungi were affected at a faster rate and lasted longer than synthetic fungicides.

1.4 Exploitation of Natural Products and/or Biodegradable Chemicals

Exploitation of natural products and/or low-hazardous, biodegradable chemicals as fungicides is a more promising strategy for the control of fungal pathogens. Lima *et al.*. (1998) determined the use of natural products, including organic and inorganic salts, organic acids, gums, chitosan, and propolis for antifungal activity against *Botrytis cinerea* and *Penicillium expansum*. They obtained 100 % inhibition of B. *cinerea* and *P. expansum* using $NaHCO_3$, sodium silicate, NH_4HCO_3, K_2CO_3, $KNaCO_3$ (1 % w/v), propionic acid (0.5 % w/v), or chitosan (50 mg ml^{-1}). They also attained a high inhibition

using lactic acid (1 % w/v), calcium silicate (1 % w/v), potassium sorbate (0.5 % w/v) and propolis (0.5 % w/v) (Lima *et al.*., 1998).

Chitosan is a soluble form of chitin, a compound of fungal cell walls. Applied to plants as a control agent, chitosan induces the plants immune systems to attack the pathogens (Karasuda *et al.*., 2003, Tripathi and Dubey, 2004). The use of chitosan has been successfully used in the control of blue mold in apples (Tripathi and Dubey, 2004). Vivekananthan *et al.*. (2004) integrated the use of biological control with chitin (similar to chitosan) by combining with *Pseudomonas fluorescens*, a plant growth promoting rhizobacterium (PGPR). The chitin-*P. fluorescens* mixture caused a 60 % reduction in anthracnose on mangoes. This was a more efficient result than with the commonly used fungicide, carbendazim (Vivekananthan *et al.*., 2004). Additional, naturally occurring compounds that have been used to induce plant responses to fungal pathogenesis are jasmonates (jasmonic acid and methyl jasmonates). They are naturally occurring plant growth regulators and play a significant role as signalling molecules in plant defence responses. It has been shown that spraying of jasmonates can significantly reduce grey mold rot of strawberries caused by *B. cinerea* (Tripathy and Dubey, 2004).

In Tripathy and Dubeys' (2004) review article, they described many additional examples of other naturally occurring compounds that have been used as alternative fungal control strategies. The most interesting are flavour compounds, acetic acid, and essential oils. Flavour compounds are volatile, secondary metabolites, which are easily absorbed by fruits and plants. An example commonly used is acetaldehyde, effective against *B. cinerea*, *Rhizopus stolonifer*, *Erwinia carotovora*, *Pseudomonas fluorescens*, *Monilinia fructicola*, and *Penicillium* sp. Acetic acid is a metabolic intermediate produced by most fruit and penetrates microbial cells inducing toxic activities (specific to microorganisms at low concentrations). It is commonly used as a fumigant to control post-harvest diseases in stored fruit and is very effective against *B. cinerea* conidia. Essential oils have been well documented on their antifungal activities, especially in a vapour state. Essential oils have been proposed as a possible fumigant on stored fruit, to

suppress post-harvest diseases. It has been effective against the control of *Aspergillus versicolor*, *A. flavus*, and *B. cinerea* (Tripathy and Dubey, 2004).

Propolis, however, is a natural product produced by bees, which has not had a great deal of attention, but which has plenty of potential.

1.5 Propolis: a Possible Control Agent of Plant Pathogenic Fungi

1.5.1 What is Propolis?

Propolis is a resinous substance collected by worker honeybees from buds and exudates of a variety of plants (Pieta *et al.*., 2002; Silici and Kutluca, 2005; Gómez-Caravaca, *et al.*. 2006; Quiroga *et al.*., 2006). Honeybees produce propolis to glue their hive onto the beehive wall, cover the wells of the hive to maintain aseptic conditions, smooth out the internal walls of the hive, and to avoid entry of wind into the hive by sealing holes (Matsushige *et al.*., 1997; Pieta *et al.*, 2002; Gómez-Caravaca, *et al.*, 2006; Quiroga *et al.*, 2006). The main function of propolis is therefore to protect the hive, hence the meaning of propolis - 'defence of the city'- derived from the Greek words *pro*- meaning 'in defence' and -*polis* meaning 'city' (Burdock, 1998).

Hatoum (1997) confirmed that the honeybees are trained in the collection of the constituents of propolis from selected plants. The worker bees (usually 41 to 63 days old) collect the constituents using their mandibles and front legs, later transferring it to their corbicula before returning to the hive. The propolis-collecting worker bees sometimes display a dance in order to recruit 8 to 16 bees to a potential 'hot-spot' of propolis materials. The constituents are collected during the hottest times of the day when the plants secrete exudates the most. Younger workers (15 to 39 days old) receive the propolis constituents from the propolis-collecting workers inside the hive. They then manipulate the propolis by masticating it, thereby adding salivary enzymes and partially digesting it. Finally the propolis is supplemented with beeswax and is placed in the vital areas of the hive (Marcucci *et al.*, 1997; Santos and Message, 1997; Burdock, 1998).

1.5.2 What are the Constituents of Propolis?

The constituents of raw propolis can be divided into three parts: 1) resin; 2) wax; and 3) residue (Cunha *et al.*, 1997). The wax comprises of beeswax, whereas the residue consists primarily of pollen grains (5 % weight), heavy metals, and insoluble materials (Barth *et al.*, 1997; Buenos *et al.*, 1997). The resin constitutes the active compounds which give rise to the aseptic properties of propolis, and it is the resin that is usually extracted for experimentations and/or pharmaceutical purposes.

The antimicrobial constituents of propolis resins and extracts are commonly phenols, flavonoids, aromatic acids and diterpenic acids (Silici and Kutluca, 2005; Uzel *et al.*, 2005). Kumazawa *et al.* (2004) quantitatively analyses several samples of propolis from 14 countries around the world. They determined the concentrations of total phenolics and total flavonoids by means of spectrophotometry. All samples had higher concentrations of total phenolics than total flavonoids. The sample with the highest total phenolics was from China (Hubei) with 299.0 mg g^{-1} whereas the sample with the lowest had 31.2 mg g^{-1} from Thailand. The highest and lowest concentrations of total flavonoids were 176.0 mg g^{-1} and 2.5 mg g^{-1} from Hungary and Thailand respectively. Additionally, by use of high performance liquid chromatography (HPLC) coupled with photo-diode array (PDA) and mass spectrometry (MS) Kamazawa *et al.* (2004) identified the major constituents of the propolis samples. Identified chemicals were caffeic acid, coumaric acid, cinnamic acid derivatives, pinobanksin, quercetin, apigenin, kaempferol, pinobanskin, chrysin, pinocembrin, galangin, caffeate acids, tectochrysin, and artepillin. A comprehensive study on all the constituents of extracted propolis resin was performed by Uzel *et al.* (2005) on Anatolian propolis samples by use of high-temperature high resolution gas chromatography (GC) coupled to MS. Several aromatic alcohols, aromatic acids, the aromatic aldehydes benzaldehyde, cinnamic acid and its ester, fatty acids, linear hydrocarbons and their acids, flavanones and flavonones were detected. Similarly, Silici and Kutluca (2005) identified flavonoids, aliphatic acids, aromatic acids esters, alcohols, terpenes, and quinones.

The above mentioned studies had clearly showed difference in the composition of propolis samples, and so have the comparisons of most studies of propolis samples (Bankova, 2005a). The reason is that the plants from which honeybees collect the propolis, vary greatly from region to region resulting in a large variety of possible flora producing propolis constituents (Buenos *et al.*, 1997; Marcucci *et al.*, 1997). In Europe it is commonly known that propolis is collected mainly from popular (*Populus*) trees, and to a lesser extent from beech, horsechestnut, birch and/or conifer trees in the Mediterranean regions (Pieta *et al.*, 2002). Furthermore, the race of the bee community is a determinant of the composition as indicated by Silici and Kutluca (2005). Propolis samples from *Apis mellifera caucasica*, *A. mellifera carnica* and *A. mellifera anatilica* were collected from the same region and analysed using GC-MS. All three propolis samples contained the general compounds mentioned earlier, however the specific compounds in each compound family differed. For example, *A. mellifera anatolica* and *carnica* samples both contained the flavonoid naringenin whereas *A. mellifera caucasica* samples only contained acacetin. All three samples contained the aliphatic acids 9-ocatdecoanoic acid and hexadecanoic acid but *carnica* samples additionally contained decanoic acids (Silici and Kutluca, 2005).

However, raw propolis from all regions of the world, on a general note, contains 50 % vegetable balsam and resin, 30 % wax, 10 % essential and aromatic oils, 5 % pollen, and 5 % an assortment of additional substances such as organic debris (Uzel *et al.*, 2005; Pieta *et al.*, 2002; Gómez-Caravaca *et al.*, 2006). Importantly, all the compounds comprising propolis are common compounds found in food products and their additives, and are recognised as GRAS safe substances.

1.5.3 Antimicrobial Activity of Propolis

Propolis has been used by humans as a natural remedy against infections and wounds since ancient times. However, only recently (the past 30 years) have pharmaceutical companies shown a keen interest in the antimicrobial activities of propolis, and extensive research has been undertaken (Bankova, 2005b).

Much of the antimicrobial assays of propolis have been against pathogenic bacteria and yeasts such as *Klebsiella pneumonia*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Candida albicans* to name but a few. However, each publication describes a novel propolis sample from individual regions so as to compare with previously published activities. Such an example of a study is from Katircioğlu and Mercan (2006). They determined the antimicrobial activity of ethanolic extracts of propolis samples, at concentrations of 50 and 200 mg ml^{-1}, from three regions of Turkey against *E. coli*, *K. pneumonia*, *P. aeruginosa*, *Morganella morganii*, *S. aureus*, *Bacillus subtilis*, *Proteus vulgaris*, and *C. albicans*. The three samples inhibited the gowth of all the organisms assayed, except for *K. pneumonia* and *M. morganii*, which were only inhibited by one of the propolis samples. Similarly Uzel *et al.* (2005) determined the MIC values of four Anatolian propolis samples (ethanolic extracts of the propolis samples) against two *Streptococcus* strains, two *Staphylococcus* strains, *Enterococcus faecalis*, *Micrococcus luteus*, *E. coli*, *Enterobacter aeroginosa*, *Salmonella typhimurium*, *P. aeruginosa*, and three *Candida* strains. All organisms were susceptible to all three propolis samples with the least susceptible being *E. coli* with a Minimum Inhibitory Concentration (MIC) range between 16 and 128 µg ml^{-1}, whereas the most susceptible was *S. sorbrinus* with an MIC range of between 2 and 8 µg ml^{-1}.

Stepanović *et al.* (2003) demonstrated the antimicrobial activity of ethanolic extracts of propolis (EEP) samples from 13 regions in Serbia against 39 microorganisms, of which 14 were resistant to antibiotic drugs. The antimicrobial properties were determined by the agar diffusion and agar dilution method. Stepanović and colleagues showed that the antimicrobial activities against Gram-positive bacteria and yeasts were high (MIC of 0.078-1.25 % and 0.16-1.25 % respectively). However activities against Gram-negative bacteria were low with a MIC of between 1.25 to 5 %. The most important discovery made by the Stepanović group was that the antimicrobial effects of the propolis samples were species specific, in that the MIC differed for each microbial genus and species types. The most resistant Gram positive and Gram negative bacteria were *Enterococcus faecalis* (MIC of 0.31-1.25 %) and *Salmonella* spp. (MIC of 2.5-5 %) respectively. The most resistant yeast was *Candida albicans* (MIC of 0.31-2.5 %). They also indicated that

the microbial strains which were resistant to antimicrobial drugs were susceptible to the EEP. The findings of the different MIC required for different microbial species was confirmed by Garedew *et al.* (2004). The Garedew group determined the antimicrobial activities and MIC of several propolis samples from Colombia, Ethiopia, Germany, Italy, Kazakhstan, Poland, Russia, and South Africa, all extracted either by water or ethanol, or the volatiles were extracted. The antibacterial activities (against *Bacillus subtilis*, *B. megaterium*, *B. brevis*, *M. luteus*, *E. coli*, and *P. syringae*) were determined by the use of the Petri dish bioassay method or flow microcalorimetry. Antifungal activity against filamentous fungi (*Aspergillus niger*, *Penicillium chrysogenum*, and *Trichoderma viride*) and the yeast *Saccharmycete cerevisiae* was determined only by the Petri dish bioassay method. Filamentous fungi were shown to be less susceptible to propolis than bacteria. The MIC values for the bacterial cultures were between 0.005-0.5 %. The MIC values for the filamentous fungi were between 0.5-2.5 %. *S. cerevisiae* was susceptible to the propolis samples at an MIC of 0.1-5.0 %. The propolis extracted with water was significantly less efficient ($P<0.05$, *t*-test) than the propolis extracted with ethanol. The same occurred for propolis volatiles, only that some bacteria and filamentous fungi were almost completely unaffected by the propolis volatiles. This was demonstrated again in the calorimetric investigations, whereby the EEP required for inhibition was much lower than for propolis extracted with water and propolis volatiles (Garedew *et al.*, 2004).

Flavonoids are powerful antioxidants, effective free radical scavengers, chelate metal ions, inhibit enzyme activity and have antimicrobial activities. Physiologically, they inhibit lipid peroxidation, have antihypertensive, anti-arthritic, oestorgenic, antiallergenic, vascular, and cyrotoxic antitumour activities (Prytzyk *et al.*, 2003; Cushnie and Lamb, 2005). Flavonoids are commonly produced by plants and function in providing colours to flowers as to attract pollinators; promote physiological survival in leaves, such as protection from fungal pathogens and UV-B radiation; and are involved in photosensitization, energy transfers, actions of plant growth hormones and growth regulators, controlling photosynthesis and respiration, morphogenises and sex determination (Cushnie and Lamb, 2005; Treutter, 2006). Prytzyk *et al.* (2003) determined the antibacterial, antifungal, and trypanocidal activities of flavonoids

extracted from Bulgarian propolis samples. They investigated the trypanocidal activities against the parasite *Trypanosoma cruzi*, the antibacterial activities against *S. aureus* and *E. coli* and the antifungal activities against *C. albicans*. Activity against *T. cruzi* was shown to be as effective as the general treatment, crystal violet (187.4 ± 10.4 ED_{50}/24h in μg ml^{-1}). The propolis flavonoids were effective against *S. aureus* and the fungus *C. albicans*, but no antibacterial activity was noticed against *E. coli* (Prytzyk *et al.*, 2003). Flavonoids are also known to inhibit the germination of plant pathogenic fungal spores, however most studies are aimed at human fungal pathogens (Cushnie and Lamb, 2005). Such examples are 7-hydroxy-3,4-(methylenedioxy)flavan and 5,7,4-trihydroxy-8-methyl-6-(3-methyl-[2-butenyl])-(2S)-flavone with activity against *C. albicans*; 6,7,4-trihydoxy-dimethoxyflavone, 5,5-dihydroxy-8,2,4-trimethoxyflavone, and 5,7,4-trihydroxy-3,5-dimethoxyflavone against *Aspergillus flavus*; and galangin, a common flavonoid found in propolis, with high activity against *A. tamarii*, *A. flavus*, *Cladosporium sphaerospermum*, *Penicillium digitatum*, and *P. italicum*. It so happens that most propolis antimicrobial activity seems to be attributed to the flavonoid galangin and a second flavonoid, pinocembrin (Cushnie and Lamb, 2005).

Basim *et al.* (2006), to our knowledge, were the first and seemingly only group, to extensively determine the efficacy of propolis against plant pathogenic bacteria. They determined the antibacterial activity of Turkish propolis against 13 plant pathogenic bacteria, namely: *Agrobacterium tumefaciens*, *A. vitis*, *Clavibacter michiganensis*, *Erwinia amylovora*, *E. carotovora*, *Pseudomonas corrugata*, *P. savastanoi*, *P. syringae* (4 strains), *Ralstonia solanaceraum*, *Xanthomonas campestris*, and *X. axonopodis*. *P. syringae* pv. *phaseolicoli* was the most sensitive to propolis at a MIC of 1\10 concentration. The least active concentration toward the bacteria was 1/1000 of the propolis extract. Basim and associates (2006) had therefore demonstrated that propolis could be a candidate for the control of plant diseases caused by bacterial infections.

The control of phytopathogenic fungi by propolis has been proposed by Hegazi and El-Hady (2002) and Quiroga *et al.* (2006). Hegazi and El-Hady (2002) determined the use of Egyptian propolis to control nine post-harvest harvest and aflotoxin producing fungi on

fruits and vegetables. They successfully inhibited the fungi with MIC values ranging between 1.2 – 3.6 mg ml^{-1}. Quiroga *et al.* (2006) assayed the use of Argentinean propolis against several species of xylophagous and phytopathogenic fungi. Similarly to Hegazi and El-Hady (2002), they successfully demonstrated the inhibition of the fungal pathogens by the propolis samples. MIC values to inhibit germination of all the fungi were between 77 and 349 μg ml^{-1} and to inhibit hyphal radial growth was as high as 81.7 % at the low concentration of 1.16 mg ml^{-1}.

Alternatively, Ngeope and Straker (2004) investigated the use of propolis against the rose bush (*Rosa*) pathogens *Nigrospora* and *Alternaria*. They determined the effective concentration range of the propolis antifungal activity by varying the concentration from 1 to 50 mg ml^{-1} (long range concentration gradient) and 0 to 15 mg ml^{-1} (short range concentration) by using the agar dilution method. Results indicated that the effective concentration range was between 5 and 50 mg ml^{-1}. They further evaluated the propolis against *Alternaria* sp. infections on rose bushes (*Rosa* hybrid). They treated the rose bushes with 5 mg mg^{-1} propolis followed by inoculating with *Alternaria* sp. Controls were untreated inoculated trees and uninoculated trees treated with Tween 1 %. Complete inhibition of the *Alternaria* sp. infections was observed from bushes treated with the propolis.

1.6 Economically Problematic Diseases of Avocado Orchards

Important diseases of avocado fruit in South Africa are the pre-harvest disease Cercospora spot and the post-harvest diseases anthracnose, stem-end rot (SER), Dothiorella rot and Dothiorella/Colletotrichum complex (DCC) fruit rot (Muirhead *et al.*, 1982; Darvis and Kotzé, 1987; Darvis *et al.*, 1987; Willis and Duvenhage, 2003).

Darvis (1977) identified, via Koch's postulates, fungi that are the cause of the post-harvest disease anthracnose and stem-end rot of South African avocados. Six fungi were found to be capable of causing stem-end rot of avocado plants, namely *Pestalotia* sp., *Dothiorella* sp., *C. gloeosporioides*, *Fusarium solani* and *Phomopsis* sp. (Darvas, 1977).

Darvas (1977) determined that *Colletotrichum gloeosporioides* (its perfect stage is *Glomerella cingulata*) was accountable for the disease anthracnose. Similarly, Darvas and Kotzè (1987) determined the fungi associated with pre- and post-harvest diseases of avocado fruits within the Westfalia estate, South Africa. They confirmed that *C. gloeosporioides* was the cause of anthracnose. They also confirmed the causal species of SER isolated by Darvas (1997), as well as *Thyronectria pseudotrichia Botryodiplodia theobromae, Drechslera setaria*, and *Rhizopus stolonifer*. Additionally, they identified *Pseudocercospora purpurea* as the causal agent of Cercospora spot; *Dothiorella aromatica* as the causal agent of Dothiorella rot; and *C. gloeosporioides* and *D. aromatica* as the causal agent of DCC fruit rot.

The symptoms of the post-harvest diseases usually only occur after ripening of the fruit, usually during storage. This makes them impossible to detect and diagnose after harvest, causing much loss to export markets. Both anthracnose and DCC rot symptoms are very similar, at first. Early symptoms of anthracnose are circular depressions of the fruit skin with darkening of the fruit skin. As the lesions mature, pink slime develops near the centre of the depression, which are conidia of *C. gloeosporioides*. Mature lesions penetrate the underlying flesh and liquefy it (Darvas and Kotzé, 1987; Darvas *et al.*, 1987). Early symptoms of DCC rot differ to anthracnose in that the fruit skin develops a reddish brown colour. Mature lesions penetrate the fruits flesh and causes watery rot (Darvas and Kotzé, 1987). Dothiorella rot is characterised by the superficial discolouration of the fruit skin. Mature disease results in darkening of the skin and the underlying flesh becomes dark-brown in colour (Darvas *et al.*, 1987). Stem-end rot is usually the hardest disease to diagnose externally. The fungi migrate from the pedicel end of the fruit, infecting the vascular system of the fruit, towards the base of the fruit as it matures. The infected vascular bundle darkens in colour to a dark-brown often resulting in softening and rotting of the mesoscarp. Rotting is usually accompanied by a foul odour (Darvas and Kotzé, 1987).

Kotzè (1978) described how *C. gloeosporioides* infects the fruit and remains dormant until the fruit ripens. The conidia of *C. gloeosporioides* germinate on the avocado fruit,

producing germination tubes. The germination tubes penetrate the thick waxy layer, which surrounds the fruit above the cuticle, and forms dark appressoria. The appressoria results in the latent phase of infection as they become inactive. Once the avocado fruits are picked, however, the appressoria become active again and form infection pegs, penetrating the cuticle and epidermal layers. As the fruit soften in storage, *C. gloeosporioides* mycelia invade both the peel and the pulp, rendering the fruit unfit for consumption (Kotzè, 1978). The life cycle of *C. gloeosporioides* is illustrated in Figure 1.1.

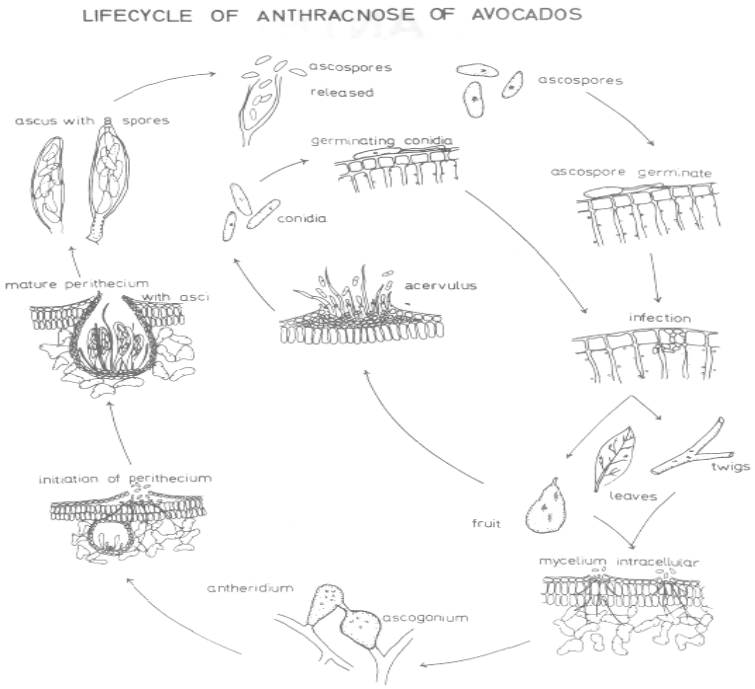


Figure 1.1: Schematic presentation of the life cycle of *Colletotrichum gleosporioides* on avocados (Kotzè 1978)

The pre-harvest disease Cercospora is easily diagnosed well before harvesting. It is characterised by a shiny, raised, black lesion on the surface of the fruit skin. Young lesions are generally 1-3 mm in diameter. Mature lesions can reach sizes of 6 mm and usually has cracks and shows corking. The oldest lesions often coalesce and become sunken with soporiferous areas in the centre (Darvas and Kotzé, 1987). This disease is the most problematic of diseases in South Africa attributing to many losses of fruit to the markets.

Darvas and Kotzè (1979) carried out experiments on Fuerte avocados to determine the critical infection period and suitable chemical control of *P. purpurea* in the Westfalia Estate in the Limpopo Province, RSA. They enclosed the avocado fruits with paper bags on the trees, and the fruits were allowed to be exposed to natural infection throughout the 1978/1979 growing season. Conidia were collected using a Hirsch type spore trap, as a means to determine the growth amplitude of *P. purpurea*. They determined that the critical period for *P. purpurea* was the early rainy season and that the fungi had a latent phase in the disease cycle of at least 3 months. They demonstrated that infection occurs as early as October, with a mean spore count (as collected by the Hirsch type spore trap) of 23 for October 1978. The spores count drastically increased to 66 for the month of November (1978), 74 for December (1978), and 109 for January (1979). The increase in the spore count correlated with the increase in rainfall for each month. This indicated that the sporulation of *P. purpurea* is directly related to the amount of rainfall. They also determined the most effective fungicide to be used in conjunction with the most efficient sticker. Best results were obtained using Benlate (benomile) 0.025% fungicide with or without Tecto 0.05% fungicide, both with the sticker Nu film 17 0.02%, resulting in 1.2 and 1.3 Cercospora spots per fruit. Other fungicides were significantly less efficient, such as CGA 64251 0.025% a.i. and Nu Film 17 (8.1 Cercospora spots $fruit^{-1}$) and CGA 64250 0.025% a.i. and Nu Film 17 (7.0 Cercospora spots $fruit^{-1}$) (Darvas and Kotzè, 1979). This study demonstrated the importance of a properly managed spray programme in which fungicide applications should be in conjunction with rainfalls.

The only registered fungicides to control avocado diseases today are copper-based. However, chemical fungicides such as prochloraz and benomyl have been successfully used in the past, but have been taken off the markets due to adverse environmental and/or health effects or to pathogen resistance build up (Willis and Duvenhage, 2003).

Darvas (1981) determined several products for the control of both anthracnose and stem-end rot of Fuerte avocados grown in Westfalia Estate, South Africa. Each fungicide tested was sprayed on 8 trees each, twice, once during mid-November 1979 and the other during mid-January 1980. After 23 May 1980, 100 fruits were picked from each tree and

placed in cold storage (6 °C) for 28 days. After storage, the fruits were exposed to room temperatures to ripen and evaluated for the respective diseases. They determined the efficiency of Benlate (50 % Benomyl), Aliette (80% fosetyl-AI), Cupravit (85% copper oxychloride), Difolatan (80% captafol), Kocide 101 (77% copper hydroxide), Glyodin experimental material, Plyac experimental material, Solvaid experimental material, B77 experimental material, Baycor experimental material and PP 296 experimental material. Darvas showed that the best control for anthracnose was obtained by Difolatan, but was closely followed by copper oxychloride (CuOC*l*) and Baycor. Baycor was the most efficient spray for the control of stem-end rot. The B77 and PP296, which were experimental at the time, showed unsatisfactory results (0.62 and 0.59 rating, respectively, of anthracnose and 0.94 and 0.40 rating respectively for stem-end rot compared to 0.11 and 0.07 by Difolatan and Baycor for anthracnose and stem-end rot respectively) (Darvas 1981). Kotzè *et al.* (1982) determined the efficacy of seven fungicides (CGA 64250, CGA 64251, pro-cymidone, Benomyl, procymidone / Benomyl, benomyl / captab and CuOC*l*) on South African avocados to combat anthracnose, sooty blotch, and Cercospora spots during the 1980-1981 growing season (in much the same methodology as Darvas 1981). Copper oxychloride was the most efficient reducing anthracnose, sooty blotch and Cercospora spots by 84 %, 100%, and 100 % respectively. CGA64250, pro-cymidone/benomyl, benomyl/captab and Benomyl were also significantly effective against *C. gloeosporioides* (Kotzè *et al.*, 1981).

Benomile was the most common pre-harvest fungicide used in South African avocado farms until 1982. It was largely replaced by CuOC*l*, due to the occurrence of benomile-resistant fungal strains. Copper oxychloride sprays were found to contain an efficacy over a broad spectrum of fungal pathogens on numerous plant hosts, as well as adhering well to plant surfaces, resulting in its protective fungicidal properties (Boshoff *et al.*, 1996). Boshoff *et al.* (1996) determined the most effective intervals and periods to apply CuOCl to avocado trees, in order to control Cercospora spots and post-harvest diseases. Copper oxychloride was sprayed monthly from September (1994) to March (1995) on both Fuerte and Hass cultivars at the Everdon Estate, KwaZulu-Natal, South Africa. The fruits were harvested in June 1995 for Fuerte and August 1995 for Hass. The fruits were stored

at 5.5 °C for 28 days to simulate export conditions. Boshoff and colleagues showed that the critical infection period for *P. purpurea* was February to March (which correlated with the study of Darvas and Kotzè, 1979). They concluded that the severity of Cercospora spots were determined by both the high-risk infection period, or availability of conidia, along with weather conditions favourable for infection; and the time or latent phase which must elapse between infection and symptom development. Also shown was that the most efficient periods for CuOCl sprays were from September to March, October to March, or November to March, all achieving successful control over pre- and post-harvest infections such as anthracnose and stem-end rot (Boshoff *et al.*, 1996). According to Willis and Duvenhage (2003), CuOCl can be applied twice to five times sometimes with the addition of Benomyl once during the raining season, for effective control of fungal infections of both the avocado fruit and trees (Willis and Duvenhage, 2003).

However, due to an increasing copper build-up in soils, as well as increasing export market demands, alternative products were sought out for the control of Cercospora spots by Willis and Duvenhage (2003) at the Westfalia Estate, RSA, during the 2001/2002 season. Among the products tested were Bravo 500SC (chlorothalonil), Ortiva (azoxystrobin), and Thiovit Jet (sulphur). Willis and Duvenhage (2003) also tested several additives to CuOCl (Demildex), as to reduce the amount of CuOCl addition to crops, namely: ferric chloride ($FeCl_3 \cdot 6H_2O$); Tecsaclor (Chlorine dioxide); and Prasin Agri (QAC combination product). Analysis were based upon 140 randomly picked fruit and assessed for signs of Cercospora spots, sooty blotch and visible spray residues. The best treatment remained CuOCl, applied twice or three times during the season (no significant difference between the two applications). The only other controls that had any significant control of Cercospora spots and sooty blotch were azoxystrobin (4 ml/10 l) and CuOCl combined with $FeCl_3 \cdot 6H_2O$, however, these were not significantly efficient (Willis and Duvenhage, 2003).

1.7 Propolis as a Potential Control Agent for Fungal Avocado Diseases

Vallabh and Straker (2005) determined the MIC of EEP against the avocado pathogens *C. gloeosporioides* and *P. guepinii* using the agar dilution method as well as testing EEP spray on avocado trees infected with *C. gloeosporioides*. The MIC values were determined to be 10 and 7 mg ml^{-1} for *C. gloeosporioides* and *P. guepinii* respectively. After analyses of twenty leaves from three trees each for each treatment (untreated trees, treated with ethanol, or treated with EEP, prior to inoculation of *C. gloeosporioides*) no lesions were observed for leaves treated with EEP, whilst 18.3 % of untreated leaves showed disease symptoms. Vallabh and Straker (2005) also demonstrated that below the MIC values the antimicrobial activities were masked by the ethanol comprising EEP. Above the MIC for EEP, the antifungal activities were only attributed to the EEP and not the ethanol.

1.8 Aim

The aim of this study was to evaluate the potential of an ethanolic extract of propolis (EEP) to control pre- and post-harvest diseases of avocado fruit, either as a post-harvest dip or pre-harvest spray, by evaluating during *in vitro*, *in vivo*, and *in situ* trials. The mode of action of EEP on the pathogenic fungi was also evaluated by electron microscopy and germination assays. To further understand the chemical nature of the antimicrobial activity of EEP, chemical analyses of the EEP was assessed.

The specific objectives of this study were:

- To optimize the ethanolic extraction of antimicrobial compounds of the propolis as described by Ngoepe and Straker (2004) and Vallabh and Straker (2005).
- To qualitatively analyse the antimicrobial constituents of the ethanolic extract of propolis (EEP).
- To quantitatively analyse the antimicrobial constituents of the EEP.

- To determine the Minimum Inhibitory Concentration (MIC) of the EEP against avocado fungal pathogens by use of the agar dilution method.
- To determine, using the electron microscope, morphological changes of the avocado fungal pathogens as a result of the antifungal activity of the EEP.
- To assess the germination inhibition activity of EEP against avocado fungal pathogens.
- To assess the control of fungal infections on avocado trees by treatment with EEP within greenhouse conditions.
- To assess, *in vivo*, if EEP could prevent infections by pathogenic fungal conidia.
- To assess, *in vivo*, if EEP could prevent disease symptoms of infected avocado fruit by fungal pathogens.
- To assess the use of EEP to control pre-harvest avocado fruit diseases by treatment of avocado trees, within the field, during the fruit growing season.
- To assess the use of EEP to control post-harvest avocado fruit diseases by treatment of avocado trees, within the field, during the fruit growing season
    - To assess results after local market simulations.
    - To assess results after export market simulations.
- To assess the control of stem-end rot (SER) disease of harvested avocado fruits.
- To assess the use of EEP as a post-harvest dip to control post-harvest diseases of avocado fruits
    - To assess results after local market simulations.
    - To assess results after export market simulations.

Chapter 2

Chemical Analysis of Potential Antimicrobial Compounds Comprising South African (Nelspruit) Propolis

2.1 Introduction

Honey bees collect specific compounds from buds and exudates of leaves from a diversity of plants to produce propolis (Silici and Kutluca, 2005; Gómez-Caravaca, *et al.* 2006; Quiroga *et al.*, 2006). Propolis is a resinous substance that is usually dark in colour, and is placed around the bee hive to maintain aseptic conditions, to block out wind, and to mummify their carcases (Pieta *et al.*, 2002; Gómez-Caravaca *et al.*, 2006; Quiroga *et al.*, 2006).

The most common antimicrobial compounds in propolis are polyphenols (flavonoids and phenolic acids and their esters), aromatic acids, and diterpenic acids (Kujumgiev *et al.*, 1999; Pietta *et al.*, 2002; Kumazawa *et al.*, 2004; Bankova, 2005b; Silici and Kutluca, 2005; Uzel *et al.*, 2005; Gómez-Caravaca *et al.*, 2006; Mani *et al.*, 2006). The success of the use of propolis as an antimicrobial agent has been well documented for pharmaceutical purposes against *Staphylococcus auereus*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Candida albicans, C. parapsilosis*, *C. krusei, Streptococcus* sp., *Enterococcus* sp., *Micrococcus* sp., *Enterobacter* sp., *Salmonella typhimutium*, *Morganella morganii*, *Klebsiella pneumoniae*, *Porphyromonas gingivalis*, *Prevotella intermedia*, *Campylobacter rectus*, and *Fusobacterium nucleatum* (Kujumgiev *et al.*, 1999; Stepanović *et al.*, 2003; Popova *et al.*, 2005; Sahinler and Kaftanoglu, 2005; Silici and Kutluca, 2005; Sonmez *et al.*, 2005; Uzel *et al.*, 2005; Katircioğlu and Mercan, 2006; Mani *et al.*, 2006).

It is well known that the chemical composition of propolis can vary greatly between different geographic regions as a result of the different flora in which the constituents are collected (Bankova 2005a). The chemical profile and characteristics of a propolis

Table 2.1: Total Flavonoids and total phenolics determined in propolis samples from different regions of the world

Reference	Total Flavonoids	Total Phenolics	Propolis origin	Proportions of Flavonoids to Phenolics
Popova *et al.* (2004)	11.00-21.10 mg ml^{-1}	22.40-26.00 mg ml^{-1}	Bulgaria (2 types)	0.49-0.81
	18.00-22.00 mg ml^{-1}	23.80-24.40 mg ml^{-1}	Italy (2 types)	0.76-0.90
	13.90-17.20 mg ml^{-1}	19.00-23.90 mg ml^{-1}	Switzerland (2 types)	0.72-0.73
Kosalec *et al.* (2003)	0.68-10.05 mg ml^{-1}	2.66-16.03 mg ml^{-1}	Croatia (contental region)	0.26-0.63
	0.11-10.74 mg ml^{-1}	0.11-16.79 mg ml^{-1}	Croatia (Adriatic region)	0.64-1.00
Quiroga *et al.* (2006)	5.00 mg g^{-1}	47.00 mg g^{-1}	Argentina	0.11
Buratti *et al.* 2007)	ND	105.00 mg 100 g^{-1} caffeic acid	Germany	
	ND	114.00 mg 100 g^{-1} caffeic acid	Hungary	
	ND	108.00-181.00 mg 100 g^{-1} caffeic acid	Italy (2 types)	
	ND	29.00-99.00 mg 100 g^{-1} caffeic acid	China (2 types)	
	ND	2.00-170.00 mg 100 g^{-1} caffeic acid	Unknown (6 types)	
Kumazawa *et al.* (2004)	130.00 mg g^{-1}	212.00 mg g^{-1}	Argentina	0.61
	145.00 mg g^{-1}	269.00 mg g^{-1}	Australia	0.54
	51.90 mg g^{-1}	120.00 mg g^{-1}	Brazil	0.43
	157.00 mg g^{-1}	220.00 mg g^{-1}	Bulgaria	0.71
	116.00 mg g^{-1}	210.00 mg g^{-1}	Chile	0.55
	136.00-147.00 mg g^{-1}	262.00-298.00 mg g^{-1}	China (3 types)	0.49-0.52
	176.00 mg g^{-1}	242.00 mg g^{-1}	Hungary	0.73
	152.00 mg g^{-1}	237.00 mg g^{-1}	New Zealand	0.64
	50.80 mg g^{-1}	99.50 mg g^{-1}	South Africa	0.51
	2.50 mg g^{-1}	32.20 mg g^{-1}	Thailand	0.08
	63.70 mg g^{-1}	255.00 mg g^{-1}	Ukraine	0.25
	168.00 mg g^{-1}	187.00 mg g^{-1}	Uruguay	0.90
	122.00 mg g^{-1}	256.00 mg g^{-1}	United States	0.48
	94.20 mg g^{-1}	174.00 mg g^{-1}	Uzbekistan	0.54
de Funari et *al* (2007)	2.64 w/w	7.39 w/w	Brazil	0.36
Mohammadzadeh *et al.* (2007)	1.22-7.79 g 100 g^{-1}	3.08-8.46 g 100 g^{-1}	Iran	0.40-0.92

A range is a result from more than one propolis type analysed from a country or region.
ND = No Data

sample is usually determined as an attempt to understand the antimicrobial and/or biological activities of propolis. The chemical characterization of propolis is also a means to standardize propolis due to its inconsistent chemical composition from different geographic positions and climatic zones (Bankova 2005a). According to Gómez-Caravaca *et al.* (2006) a complete chemical analysis of propolis involves both qualitative and quantitative chemical assessments.

The conventional qualitative methods to analyse propolis samples is the use of chromatography such as high performance liquid chromatography (HPLC) and gas chromatography (GC), usually coupled to mass spectrometry (MS) to obtain molecular weight, structural information and identification (Bankova 2005a, 2005b; Gómez-Caravaca *et al.* 2006). Even though these techniques provide a sufficient profile and identification of the compounds analysed (Gómez-Caravaca *et al.* 2006), they are usually difficult to perform and are inefficient in identifying all the components within propolis (Popova *et al.* 2004). An added disadvantage to GC-MS is that the propolis has to be derivatised in order to increase the concentration of volatile compound for detection. However, not all compounds comprising propolis are able to be derivatised or become volatile after derivatisation (Gómez-Caravaca *et al.* 2006). HPLC does offer a solution for the non-volatile compounds and therefore its popularity is increasing with levels of higher efficiency (Pietta *et al.*, 2002; Kosalec *et al.*, 2003; Kumazawa *et al.*, 2004; Popova *et al.*, 2004; Gómez-Caravaca *et al.*, 2006; Quiroga *et al.* 2006; Volpi and Bergonzini, 2006; de Funari *et al.*, 2007).

This study aimed at using methodology that was inexpensive and simpler than chromatography. We therefore looked at ionisation techniques utilizing MS. FAB ionisation has been gaining popularity for the identification of flavonoids (Volpi and Bergonzini, 2006) and, to our knowledge in published data, has not yet been used to analyse propolis.

Bankova (2005a) proposed three parameters for quantitative analysis of propolis, namely total flavone and flavonol, total flavanone and dihydroflavonol, and total

phenolics. These parameters only analyse the antimicrobial compounds within propolis but give important data on which class of compounds are the most or least concentrated. In so doing, the overall antimicrobial activity of the propolis samples is further comprehended. Table 2.1 is a summary of a few selected published data on the quantitative analysis of propolis from different international regions, with total flavone and flavonol and total flavanone and dihydroflavonol combined to indicate total flavonoids.

In this chapter we extracted the active ingredients from South African (Nelspruit) propolis, using ethanol, for the use as a fungicide on avocado trees. The methods were designed for efficacy and ease of production. A qualitative and quantitative chemical analysis on the ethanolic extract of propolis (EEP) was performed using Fast Atomic Bombardment (FAB) ionisation and spectrophotometery respectively. The quantitative analysis determined the concentrations of the three parameters proposed by Bankova (2005a). The quantitative and qualitative analysis of EEP was to demonstrate, at a chemical level, the antimicrobial activities of the propolis samples.

2.2 Material and Methods

2.2.1 Preparation and Purification of Ethanol Extract of Propolis (EEP)

Several balls of propolis (approximately 500 g) from Roodewal farm (Nelspruit, South Africa) donated. The propolis was collected during the experimental period from honeybee hives within the avocado orchards.

The propolis was extracted based on methods described by Ngoepe (2004), with modification. The propolis was solubilised in 100 % laboratory grade ethanol (Merck) (30 g propolis in 100 ml ethanol) at 70 °C for 5 hours. Solubilisation was under a double surface condenser (Quickfit) to prevent ethanol evaporation loss. The process was repeated three times for optimal extraction of the active compounds. Between each repeat, the propolis-ethanol solution was stored at 4 °C overnight.

To optimize purification, centrifugation at high speeds was proposed. Three speeds were tested, 1 700 *g*, 6 700 *g*, and 14 500 *g*, in a Sorval centrifuge RC-5C with a SS-34 rotor at 25 °C. All Samples were centrifuged for 25 minutes in Oak Ridge centrifuge tubes, PPCO, 50 ml (Nalgene). The supernatant was stored overnight at ambient temperatures. The supernatant was further filtered through filter paper (Whatman no. 1) and stored at ambient temperatures in a Schott bottle. This was compared to purification used by Ngoepe (2004) which did not involve a centrifugation step. The final solution was termed 'ethanol extract of propolis' (EEP). EEP solutions were combined to produce a final solution from various propolis samples.

Production of EEP was repeated numerous times throughout the experimental period.

To determine the mass of dissolved propolis constituents in EEP, 10 ml was aliquoted onto an evaporation dish, allowed to dry, and the mass of the remaining compound was measured and calculated to g ml^{-1}. This was repeated three times.

2.2.2 Fast Atomic Bombardment Spectrometric Analysis of EEP

The ethanol from EEP was evaporated using a rotary evaporator at 40 °C for approximately 1 h followed by further drying under vacuum. The resulting mixture was thick viscous oil with a dark red-brown colour and strong odour. This was a pure form of the propolis extract (PE). The final mixture was analysed using Fast Atomic Bombardment (FAB) ionisation MS at the School of Chemistry, University of the Witwatersrand.

The solvent for FAB ionisation was nitrobenzyl alcohol. The resolution was 1000 with positive polarity and mass range of 4000 amu (6 Kv). The scan rate was 3 sec $decade^{-1}$ (external). Compounds were identified using the chemical databases ChemFinder.com, CambridgeSoft Corporation (last viewed 31 July 2007).

2.2.3 Spectrophotometric Quantification of Antimicrobial Compounds Comprising EEP

Quantification of antimicrobial active compounds in South African propolis was based on methods described by Popova *et al.* (2004) with modifications.

An aliquot of 3 ml and an aliquot of 1.5 ml of EEP were each added to 50 ml 100 % methanol (Merck) to produce two stock solutions. This was used for all quantification studies and termed EEP-methanol solution.

Quantification of the Flavone and Flavonol Content:

The spectrophotometric assay is based on the carbonyl and hydroxyl groups of the flavones and flavonols which form a complex with the aluminium (III) ion, which absorbs light at a wavelength of 425 nm (Popova *et al.*, 2004).

A standard curve was constructed using galangin (Fluka) at a concentration of 4-32 μg ml^{-1} in methanol, and absorbance was measured at 425 nm absorbance (Fig. 2.1). The blank was methanol. The EEP-methanol solution (2 ml) was further diluted in methanol to a final volume of 20 ml. A 1 ml aliquot of aluminium chloride (Merck) solution (5 %, w/v) was added to the diluted EEP-methanol solution and made up to a final volume of 50 ml with methanol. The solution was left at room temperature for 30 min. The absorbance was read at 425 nm, with the blank as aluminium chloride solution without EEP.

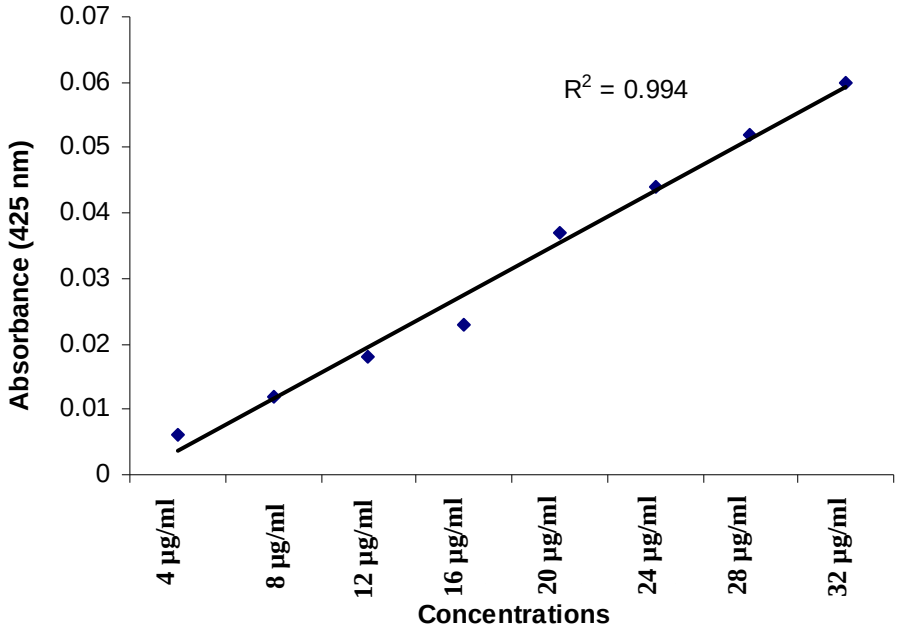


Figure 2.1: Standard curve for galangin, measured using spectrophotometry, for the quantification of flavones and flavonols comprising EEP. $R^2 = 0.994$. All data are from an average of three replicates.

Quantification of the Flavanone and Dihydroflavonol Content:

The principles of the reaction is based on the interaction of the flavanones and dihydroflavonols with acidified 2,4-dinitrophenylhydrazine (DNP). The resulting phenylhydrazone is absorbent at 486 nm (Popova *et al.*, 2004).

A measure of 1 g of DNP (dinitrophenylhydrazine) (Sigma) was added to 2 ml of 98 % sulphuric acid (Saarchem) and diluted to a final volume of 100 ml with methanol. An aliquot of 1 ml of the EEP-methanol solution was added to 2 ml of the DNP-sulphuric acid solution and the mixture was heated to 50 °C for 50 min. After allowing cooling to room temperature, the solutions were diluted to a final volume of 10 ml with 10 % potassium hydroxide (Merck) in methanol (w/v). A 1 ml aliquot of the solution was further diluted to a final volume of 50 ml in methanol. Samples of the reactions were centrifuged at 2000 *g* for 5 min to sediment the precipitate. The absorbance of the supernatant was read at 486 nm. The standard curve was constructed using pinocembrin (Fluka) at a concentration of 0.25-2.00 mg ml^{-1} after the same reactions and treatments

(Fig. 2.2). The blank was diluted DNP-sulphuric and potassium hydroxide solution without pinocembrin or EEP.

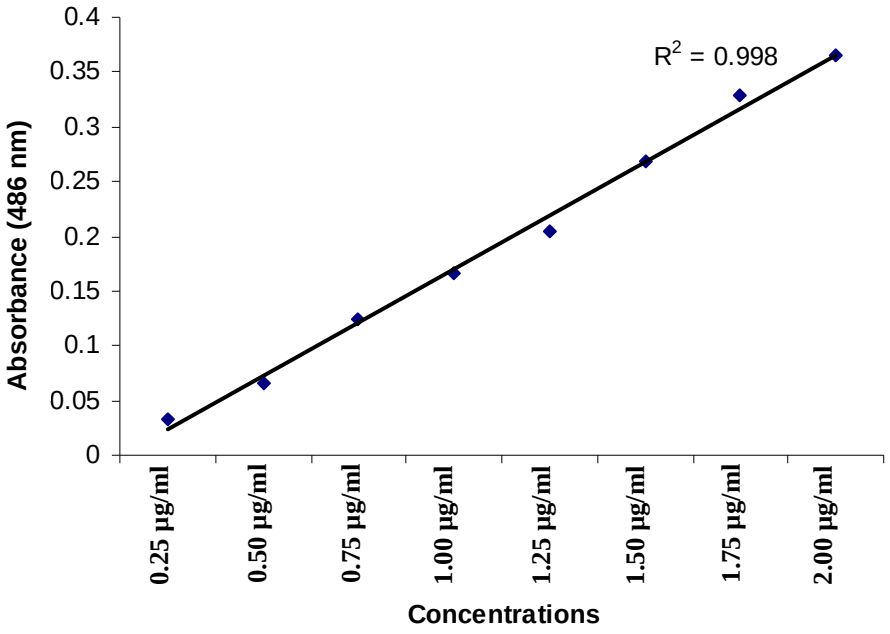


Figure 2.2: Standard curve for pinocembrin, measured using spectrophotometry, for the quantification of flavanones and dihydroflavonol comprising EEP. R^2 = 0.998. All data are from an average of three replicates.

Quantification of Total Phenolics:

This is based on the standard Folin-Ciocalteu method whereby the phenols reduce the phosphomolybdic-phosphotungstic reagent resulting in a coloured reaction which is detectable at 760 nm (Woisky and Salatino, 1998).

A 4 ml aliquot of Folin-Ciocalteu (Fluka) was added to 15 ml of distilled water followed by 1 ml EEP-methanol. An aliquot of 6 ml 20 % sodium carbonate (Merck) solution (w/v) was added to the solution. The solution was diluted to a final volume of 50 ml with distilled water and allowed to stand at room temperature for 2 hours. The absorbance was read at 760 nm. According to Popova *et al.* (2004), gallic acid is commonly found in

tropical samples of propolis therefore it was presumed safe to determine the presence of gallic acid in South African propolis and used as a standard to construct a standard curve (Fig. 2.4). Caffeic acid was also used as a standard (Fig. 2.4) along with pinocembrin:galangin (2:1) (Fig. 2.3) proposed by Popova *et al.* (2004). The standards were diluted to concentrations of 0.037-0.296 mg ml^{-1} for both caffeic (Fluka) and gallic acid (Sigma) and 40.75-244.50 µg ml^{-1} for pinocembrin:galangin (2:1). They were treated with Folin-Ciocalteu and sodium carbonate and absorbance read at 760 nm. The blank was Folin-Ciocalteu reagent and sodium carbonate solution without EEP or standards.

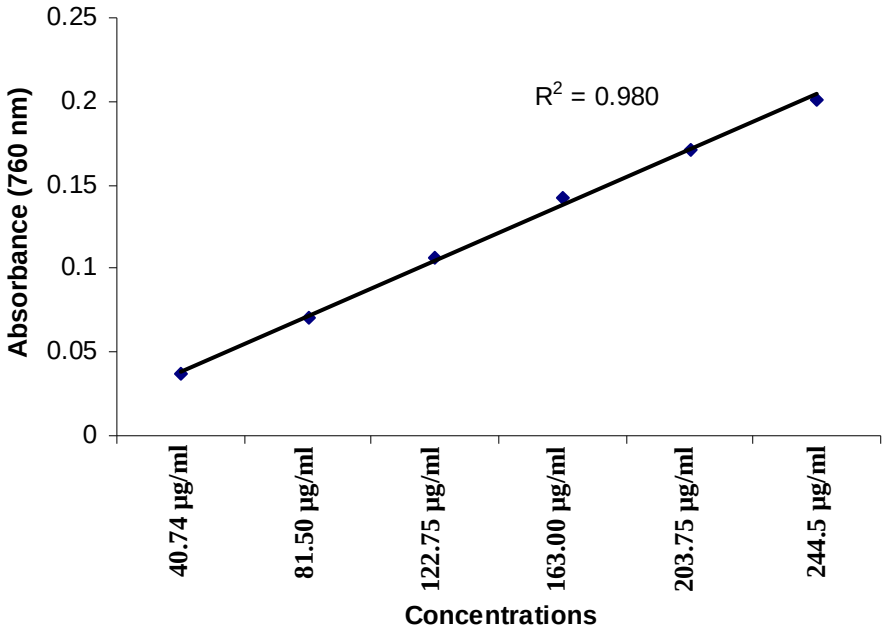


Figure 2.3: Standard curve for pinocembrin:galangin (2:1), measured using spectrophotometry, for the quantification of total phenolics comprising EEP. R^2 = 0.980. All data are from an average of three replicates.

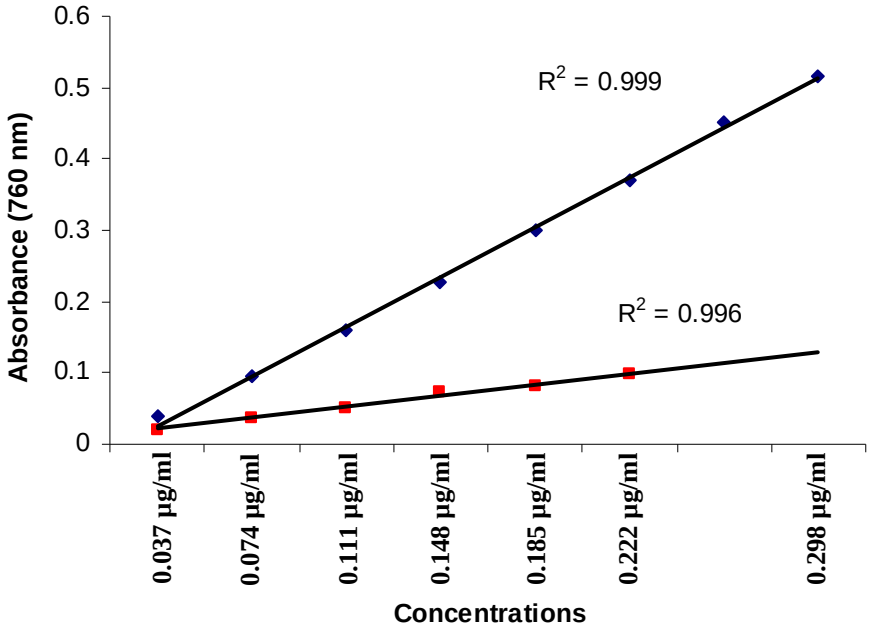


Figure 2.4: Standard curve for caffeic acid and gallic acid, measured using spectrophotometry, for the quantification of total phenolics comprising EEP. R^2 caffeic acid = 0.999. R^2 gallic acid = 0.996 All data are from an average of three replicates.

2.3 Results

2.3.1 Characteristics of EEP

Solubilization of the propolis extract in absolute ethanol under a double surface condenser resulted in no ethanol lost to evaporation (data not shown), and therefore the concentration of the EEP within the final solution to be calculated.

Purification of the propolis-ethanol solution via centrifugation was optimal at 14 500 g for 25 minutes. The resulting supernatant was a clear dark-brown solution with no visible suspensions of wax. However, after storing at ambient temperatures overnight, some wax precipitated and was suspended in the solution. A final purification step was added to filter out any precipitated wax through filter paper. This produced a high quality ethanolic extract of propolis with little wax (EEP).

The final EEP product was dark brown with a strong, sweet, leather-like odour. The EEP was transparent but upon dilution with water, the EEP formed an oil-in-water emulsion. Adherence to most surfaces (metal, wood, and glass) was common, especially to human skin (with no hazardous effects). The concentration of propolis compounds that were dissolved in ethanol to produce EEP was 0.12 g ml^{-1}.

2.3.2 Chemical Composition of EEP

FAB ionisation detected at least 36 compounds from the propolis extract (Table 2.2 and 2.3). The molecular weights (MW) ranged from 54.1 to 618.8 (Table 2.3). However, only 11 compounds were identifiable (Table 2.2). Compounds to note were cinnamic acid, pinocembrin, pinostrobin, quercetin, and a derivative of flavanone.

To determine the relative concentration of the potential antimicrobial compounds within the propolis extract, specrophotometric analysis was achieved. Flavones and flavonols were quantified as the highest concentration at 15.168 mg ml^{-1} as represented by galangin (Figure 2.1 and Table 2.4). Flavanone and dihydroflavonol were the lowest concentration at 1.186 mg ml^{-1} as represented by pinocembrin (Figure 2.2 and Table 2.4). Flavonoids were therefore, a total concentration of 16.354 mg ml^{-1}. Total phenolics were a concentration of 3.28 mg ml^{-1} as determined from caffeic acid (highest concentration of the three reference compounds [Figure 2.3 and 2.4]) (Table 2.4).

Table 2.2: Compounds identified from the analysis of South African propolis (Nelspruit) extract using FAB ionisation.

Molecular Weights	Identified Compounds
108.1	Benzyl alcohol
122.2	Benzoic acid
148.2	Cinnamic acid
158.2	2-Naphthalenemethanol
202.3	Hydrocarbon
228.3	Myristic acid
256.3	Pinocembrin
270.3	Pinostrobin
282.3	Quercetin
296.3	Derivatives of flavanone
310.4	Oleic acid

The resolution was 1000 with positive polarity and mass range of 4000 amu (6 Kv). The scan rate was 3 sec decade^{-1} (external). The solvent was nitrobenzyl alcohol.

Table 2.3: Molecular weights of compounds that could not be identified from the analysis of South African propolis (Nelspruit) extract using FAB ionisation.

Unidentified Molecular Weights	
54.1	186.3
64.1	172.3
68.1	188.3
76	217.3
80.1	240.2
90.1	324.4
94.1	338.4
618.8	392.5
118.2	408.6
130.2	424.5
134.2	452.5
144.2	466.5
	618.8

The resolution was 1000 with positive polarity and mass range of 4000 amu (6 Kv). The scan rate was 3 sec decade^{-1} (external). The solvent was nitrobenzyl alcohol.

Table 2.4: Antimicrobial active compounds found in South African propolis (Nelspruit) extract via spectrophotometry.

Contents/ Concentration (mg ml^{-1})	Flavone and flavonol	Flavanone and dihydroflavonol	Total phenolic
Concentration (mg ml^{-1})	15.168 (± 0.057)	1.186 (± 0.001)	$3.28^{\#}$ (± 0.001)

= calculated from three standard curves: pinocembrin:galangin, (Figure 2.3) gallic acid (Figure 2.4), and caffeic acid (Figure 2.4) whereby caffeic acid indicated the highest phenolic concentration. Flavone and flavonols were calculated from standards curves for galangin (Figure 2.1). Flavanone and dihydroflavonols were calculated from standard curves fro pinocembrin (Figure 2.2). Values represent means of three replicates (± SEM).

2.4 Discussion

Methodology described by Ngoepe (2004) to extract ethanol required replenishing of ethanol regularly during the three day extraction due to extensive evaporation loss. The use of the double surface condenser within this study durng the extraction period resulted in no loss of ethanol and therefore reduced the required amount of ethanol substantially.

Ngoepe's (2004) method of straining the propolis-ethanol solution through filter paper after solubilization proved very difficult, especially when the propolis samples were high in wax content. As soon as the solution cooled to room temperature in the filter paper, the remaining wax precipitated and the entire solution solidified. Solidification rendered the sample impossible to filter. However, centrifugation at 14 500 *g* for 25 minutes proved efficient, even when the solution solidified due to high wax content. The supernatant was a clear dark-brown solution with no visible precipitants.

Ethanol is conventionally used as a solvent for antimicrobial compounds found in propolis (Gómez-Caravaca *et al.* 2006). According to Gómez-Caravaca *et al.* (2006) most of the waxes found in propolis are insoluble in ethanol, allowing for a higher purity of the active compounds. However, a small percentage of the waxes were still soluble in the EEP after centrifugation. Only by cooling the EEP to ambient temperature overnight

(more effective by placing the EEP in 4 °C - data not shown), were the waxes precipitated out.

Our initial thought was to have a small amount of wax present in the EEP to allow for a sticker property, i.e. to aid in the EEP to adhere to leaves and fruit of the avocado trees. Absolute ethanol allows for a small percentage of wax, and according to Gómez-Caravaca *et al.* (2006) aqueous ethanol (70 %, 80 %, or 95 % conventionally used) allows for a solution free of wax. The presence of wax in our EEP, however, posed problems when using it in spray bottles and guns to treat avocado trees. Upon dilution in water, more of the wax precipitated out. This blocked the nozzles of the spray bottles and guns. The addition of the filtration step through filter paper proved effective in removing the precipitated waxes and provided with a solution with enough soluble waxes to result in the required sticker property.

During FAB ionisation neutral atoms, usually xenon or argon, are charged through a charge-cell and beamed at the sample. The charged atoms ionise the molecules comprising the sample and causes desorption (the ionised molecules enter the gaseous phase). Once desorbed, the compounds are able to be detected by the mass spectrometer (MS) (Gruber, 2000). The nitrobenzyl alcohol was used as the matrix, which served to aid in the ionisation and desorption of the sample, as well as to serve some form of protection from damage to the sample during the ionisation process.

FAB ionisation proved effective in identifying 36 compounds within the propolis extract (Table 2.2 and Table 2.3). This is similar to chemical identifications of propolis by Volpi and Begonzini, 2006 (26 compounds) using online-HPLC-electrospray MS; Uzel *et al.*, 2005 (45 compounds) and Silici and Kutluca, 2005 (34 compounds at most) using GC-MS; and Prytzyk *et al.*, 2003 (27 compounds) using HT-HRGC-MS. However, the MS was only accurate to one decimal point and this proved difficult in identification of compounds based on molecular weight, so identified compounds are therefore presumptive and not confirmed.

Cinnamic acid, pinostrobin, pinocembrin, quercetin, and a derivative of flavanone were all identified in our propolis extract (Table 2.2). Gómez-Caravaca *et al.* (2006) reviewed the chemical compounds identified in propolis by recent published articles using GC-MS, HPLC, or capillary electrophoresis (CE). Most of the reviewed propolis samples were similar in composition to the PE tested in this study with cinnamic acid, pinostrobin, pinocembrin, and/or quercetin commonly occurring. In contrast, South African propolis analysed by Kumazawa *et al.* (2004), using HPLC coupled with photo-diode array (PDA) and MS, did not detect quercetin, pinostrobin, or any cinnamic acids. Pinocembrin was detected in fairly high concentrations (69.8 mg g^{-1} EEP) in the South African propolis compared to the other 15 samples collected with the lowest pinocembrin concentration at 9.2 mg g^{-1} from Ukraine and the highest at 99.7 mg g^{-1} from New Zealand. Most of the samples tested by Kumazawa and associates (2004) did contain the compounds identified from our PE. Interestingly, propolis from Thailand did not contain any of the common identifiable compounds (Kumazawa *et al.* 2004).

It is important to note that pinostrobin, pinocembrin, quercetin, and the derivative of flavanone are all flavonoids. It is common belief that polyphenols (flavonoids and phenolic acids and their esters), aromatic acids, and diterpenic acids are the main antimicrobial active compounds of propolis (Kujumgiev *et al.*, 1999; Pietta *et al.*, 2002; Kumazawa *et al.*, 2004; Bankova, 2005b; Silici and Kutluca, 2005; Uzel *et al.*, 2005; Gómez-Caravaca *et al.*, 2006; Mani *et al.*, 2006; Mohammadzadeh *et al.* 2007). Therefore, based on the chemical composition of the Nelspruit (South African) propolis and comparison to the propolis constituents in other studies, it has been shown that the propolis samples had potential antimicrobial properties.

Bankova (2005a) proposed that instead of the conventional chemical identification of propolis to determine the main antimicrobial compounds, the chemical profile can be determined by three parameters: 1) total flavone and flavonol, 2) total flavanone and dihydroflavonol, and 3) total phenolics. This allows for an overview of which class of compounds known for their antimicrobial activity in propolis are the most concentrated. In other words, Bankova's method allows for an overall understanding of the

antimicrobial activities of the propolis instead of trying to construe which specific chemicals are responsible.

Since the FAB analysis did indicate possible antimicrobial compounds in our propolis sample but only few, Bankova's approach seemed more realistic. The methodology of Popova *et al.* (2004), was used as it detected the concentrations of all three parameters discussed above.

The chemical composition of propolis originating from Nelspruit is not known. Therefore, the preferred reference compounds (pinocembrin and galangin) determined by Popova *et al.* (2004) to quantitatively analyse poplar-type propolis were used. These are the two compounds found in most propolis and are presumed to be among the most antimicrobial active compounds of propolis (Uzel *et al.* 2005; Katircioğlu and Mercan 2006; Burdock 1998). Gallic acid and caffeic acid are commonly found in tropical-type propolis (Popova *et al.* 2004) and were thus used as additional reference compounds to assay the Nelspruit propolis, which is subtropical. This proved correct as caffeic acid showed the highest detection of 3.28 mg ml^{-1} total phenolics (Table 2.4).

Total flavonoids (16.354 mg ml^{-1}) were almost 5 x higher than total phenolics (3.28 mg ml^{-1}) with a flavonoid – phenolic ratio of 4.99. Table 2.1 indicates a summary of some recent data from quantitative research on propolis. All the flavonoid – phenolic ratios were below 1.00, except propolis from the Adriatic region of Croatia with a maximum ration of 1.00 (Kosalec *et al.*, 2003). The lowest proportion was from Thailand of value 0.08, which is a 12.5 x higher concentration of phenolics than flavonoids (Kumazawa *et al.*, 2004). However, the propolis samples analysed by Kumazawa *et al.* (2004) from South Africa had a ration of 0.51, i.e. flavonoids were approximately half the concentration of the phenolics. The region from where the propolis samples were collected is not known, and could be an important reason for the different compositions.

In contrast, Uzel *et al.* (2005) analysed propolis samples from four different Anatolian regions using GC-MS, and concluded that the majority of compounds were flavonoids.

Some of these flavonoids were the flavanones pinocembrin (7.01-16.26 % of total ion current), pinostrobin (4.46-13.06 % of total ion current), and chrysin (1.45-9.86 % of total ion current) and the flavonones pinobanksin (4.3-11.5 % of total ion current) and quercetin (1.1-5.1 % of total ion current). Conversely, Silici and Kutluca (2005) indicated that propolis samples from Turkey (East Anatolia) of three different races of honey bees (*Apis mellifera anatolica*, *A. mellifera carnica* and *A. mellifera caucasica*) showed exceptionally low content of flavanones (data from GS-MS). They all had high contents of aromatic acids and amino acids. All three honeybees produced similar propolis, but Silici and Kutluca (2005) demonstrated that they did differ in content, even though they were from the same region. Examples of such dissimilarities were the presence of naringenin and vanillin only in *A. mellifera anatolica* and *carnica* propolis, benzyl cinnamate in *anatolica* and *caucasica* propolis, chrysin in only *carnica* propolis, and ferulic acid in *anatolica* propolis. Therefore, Silici and Kutluca (2005) demonstrated that the composition of the propolis not only depends on the local flora surrounding the hive, but the race of the honeybees as well.

However, the EEP will be expected to change slightly in composition throughout the year (propolis can be harvested anytime of the year). This is due to the change in seasons and therefore a change in flora in which the constituents are collected. Systematic analyses will therefore be required on seasonal bases, but only for a short period. After a year or two of analyses, good estimation of the chemical composition will be obtained, if the same honeybee race is maintained and in the same location, after which periodic evaluation using the three parameters of Bankova (2005a) will suffice for monitoring the antimicrobial content of the product.

The high flavonoid concentration of the EEP in this study could therefore be attributed to both the local flora surrounding the honeybees and the race of the honeybees. However, sufficient floral and faunal data is required for verification.

Due to the high flavonoid concentrations, the flavonoids could be safely presumed to be the major antimicrobial compounds of the EEP.

2.5 Conclusion

- In terms of its chemical composition EEP showed its probability as an antimicrobial agent and therefore it's potential as a phyto-fungicide.

Chapter 3

In vitro Evaluation of South African Propolis (Nelspruit) as a Possible Fungicide against Fungi found associated with *Persea americana* (Avocado)

3.1 Introduction

It is apparent from published literature on the antimicrobial activity of propolis samples that the majority of these studies are aimed at medical and/or pharmaceutical intentions. The fungi in these studies are usually species of the yeast *Candida* (Strepanović *et al.*, 2003; Silici and Kutluca, 2005; Katircioğlu and Mercan, 2006; Uzel *et al.*, 2005).

However, the first report of antimicrobial action of propolis against fungal pathogens of plants, were that of Ghaly *et al.* (1998). They determined the efficacy of an ethanolic extract of propolis (termed PEE) against the ascomycete and mold fungus *Aspergillus flavus* in hopes to reduce aflotoxin production. At concentrations between 1-4 g l^{-1} the PEE effectively reduced the dry mycelial mass by 11-80 % and reduced aflotoxin B1 production by 34-100 %. In contrast, the chemical, ultragriseofulvin (UG), decreased the dry mycelial mass by 16-88 % and aflotoxin production by 48-98 % at four times lower concentrations (0.25-1 g l^{-1}) than PPE. However, to the best of our knowledge, no further studies have been published on the activity of propolis to prevent *Aspergillus* mold or aflotoxin production. *Aspergillus* is a problematic fungus causing much economic loss due to postharvest diseases (mold rots) in grains and legumes (Agrios, 2005). Further research into the use of propolis to control this pathogen could have been beneficial to industries today.

Similarly, Hegazi and El-Hady (2002) determined the antifungal activities of Egyptian propolis, in terms of Minimum Inhibitory Concentrations (MICs), against nine fungal genera namely *Cladosporium*, *Mucor*, *Scopulariopis*, *Penicillium*, *Rhizopus*, *Fusarium*, *Aspergillus*, *Alternaria*, and *Rhodotorula*. These fungi are known to cause postharvest rot of fruits and vegetables and the MIC values were in the range of 1.2 – 3.6 mg ml^{-1} . The

efficacy of propolis as an anti-phytofungal agent was also demonstrated by Quiroga *et al.* (2006) against several xylophagous (*Ganoderma applanatum*, *Lenzites elegans*, *Pycnoporus sanguineus*, and *Schizophyllum commune*) and phytopathogenic fungi (*Aspergillus niger*, *Fusarium* sp., *Macrophomina* sp., *Penicillium notatum*, *Phomopsis* sp., *Thrichoderma* sp.). MIC values to inhibit germination of all the fungi were between 77 and 349 μg ml^{-1}. Hyphal radial growth was inhibited by 81.7 % at the low concentration of 1.16 mg ml^{-1} (Quiroga *et al.*, 2006).

To further establish the efficacy of propolis as an agricultural antifungal agent, the ability of South African propolis (Nelspruit) against six fungal pathogens isolated from avocado fruit and foliage was determined. The MIC was used to determine an efficient concentration for the use of the propolis as a foliage preharvest spray for the avocado trees. Germination inhibition of *Pestalotiopsis guipinii* and *Colletotrichum* sp. was assayed at the determined MIC. To further understand the mechanisms of antimicrobial activities of EEP, scanning electron microscopy of *P. guipinii*, CgP complex (*Colletotrichum gloeosporioides* and *Pseudocercospora* sp.) and *Colletotrichum* sp. was performed to visually see if any external damage had occurred.

3.2 Materials and Methods

3.2.1 Determination of Minimum Inhibitory Concentrations for Six Fungal Pathogens isolated from Avocado Fruit and Foliage

Culture Collection and Isolation:

Pestalotiopsis guipinii (Cooke) Steyaert PPRI 7860 and a fungal complex comprising *Colletotrichum gloeosporioides* Penz. and a *Pseudocercospora* sp. PPRI 6008 were obtained from the Plant Protection Research Institute (PPRI) culture collection. Despite numerous attempts the two fungi of the complex could not be separated from each other. The complex hereafter is referred to as CgP complex.

Twelve avocado fruits (Fuerte and Pinkerton), showing symptoms of anthracnose and Cercospora spot, were purchased from local markets within the east rand of Johannesburg, South Africa. Fruits were stored overnight at ambient temperatures. The fruits were surface sterilized with 70 % ethanol (Merck). Fruits epidermal layers showing symptoms of disease were excised and surface sterilized in 70 % ethanol and 10 % household sodium hypochlorite, each for 1 min, with rinsing in sterile distilled water between each step. One hundred lesions were isolated. The tissue pieces were placed in the centre of Malt Extract Agar (MEA) (Merck) plates. The plates were incubated at 25 °C untilthe fisrt fungal growth was visible. Using a dissecting microscope (Zeiss) and a sterile inoculation needle, single hypha were transferred to fresh MEA and incubated at 25 °C until sporulation occurred. If purity was not achieved, spores were collected by the spreading of sterile Tween 80 (0.5 %) over the culture and collected. An aliquote of 0.1 ml of the Tween 80-spore solution was spread on fresh MEA and incubated at 25 °C until growth from single spores were observed. Each germinated spore was transferred to fresh MEA using a sterile cork borer and incubated at 25 °C until sporulation was visible. The fungi were only identified to genus level based on hyphal and spore morphology (Barnett and Hunter, 1987; Baxter *et al*., 1994). The most predominant fungi isolated from the fruit were *Colletotrichum* sp., *Fusarium* sp., *Monilia* sp., and *Verticillium* sp. and these were additionally used for the assays as a means to assay the broad range of antifungal activities of EEP. All the cultures were maintained on MEA at 25 °C or 4 °C.

Agar Dilution Method to Determine the Minimum Inhibitory Concentration (MIC) of EEP:

The EEP was diluted in MEA to construct a dilution series of 1, 2, 5, 7, and 10 mg ml^{-1} of EEP (five replicates) based on methodology of Ngoepe and Straker (2004). Based on the mass of the propolis determined in chapter 3, the EEP was diluted with sterile distilled water to the required dilutions. The EEP was filter-sterilized (Zetapor, 2 µm pore size) into molten MEA after autoclaving. The control was MEA with no EEP. Preliminary results (Vallabh and Straker, 2005, unpublished) had successfully shown no inhibitory action of ethanol solvent alone at these concentrations and therefore an ethanol

control was omitted. Each of the fungi was placed onto the centre of the MEA plates using a sterile cork borer of 10 mm diameter. The plates were incubated at 25 °C in a lighted chamber for 30 days. Growth was assessed by daily measurements (mm) of diameter growth.

The average mean growth was calculated for the 30 day period and was compared statistically between each concentration of EEP tested for all fungi using one-way ANOVA (software GraphPad Instat version 3.00).

Relative inhibition (RI) values were also calculated according to the following formula:

$$RI = \frac{(\text{Hyphal extension of control [mm]} - \text{Hyphal extension of experiment [mm]})}{\text{Hyphal extension of control [mm]}} \times 100$$

and compared statistically using one-way ANOVA (software GraphPad Instat version 3.00).

The lowest concentration of EEP to produce a stiatisitical difference (i.e. inhibition) growth as compared to the control was determined the MIC.

3.2.2 Scanning Electron Microscopy

Preparation was based upon methodology of Karasuda *et al.* (2003) with modifications. *Colletotrichum* sp., *P. guipinii*, and the CgP complex were incubated on MEA media containing 5 mg ml^{-1} EEP; MEA containing 100 % ethanol equivalent to amount in 5 mg ml^{-1} EEP (as a control) and MEA (as a control). Fungi were incubated at 25 °C until growth on EEP reached approximately 10 mm in diameter. Approximately 3-5 mm of the leading edge of the fungal growth, with the media, was excised using a sterile scalpel. The fungal samples on agar were fixed in 2.5 % gluteraldehyde for 2 h, followed by a series of dilutions in ethanol (50, 70, 80, 90, 99, and 100 %) twice at each concentration for 20 min each. The samples were further treated, twice, with 100 % butan-1-ol (Saarchem) for 20 minutes. The samples were lyophilized for 1.5 h. The samples were

coated with gold (Ag) and were examined using a scanning electron microscope (SEM), JSM-840 (Jeol Ltd., Tokyo, Japan) at 15 keV. Three samples of each fungus were prepared and viewed.

3.2.3 Inhibition of Fungal Conidial Germination at 5 mg ml^{-1} EEP

Germination viability of conidia treated with 5 mg ml^{-1} EEP was determined based on methodology of Dimbi *et al.* (2004) with modifications. A 0.25 µl aliquot of a conidial suspension of *Colletotrichum* sp. (5.6 x 10^4 conidia ml^{-1}) or *P. guipinii* (1.144 x 10^6 conidia ml^{-1}) in 0.5 % Tween 80 (Sigma) was spread plated on to MEA (Merck) as a control. A spread plate of the conidia on MEA containing a concentration of 5 mg ml^{-1} EEP was used to determine if the conidia could germinate in the presence of EEP. A second assay was the addition of EEP to the spore suspension to a final concentration of 5 mg ml^{-1}, followed by the spread plating onto MEA. This was to determine if conidia could germinate with a coating of EEP. Three surface sterilized cover slips (flame sterilized with 70 % ethanol) were placed on each plate, separately from each other. The plates were incubated for 24 h at 25 °C. Germination was observed under a light microscope (Carl Zeiss) at 400 x magnification. Germination tubes longer than the conidial body indicated germination (Dimbi *et al.* 2004). One hundred conidia were counted per slide and evaluated for germination. The experiment was repeated three times. Statistical analysis used was a two-tailed Student,s t-test (software GraphPad Instat version 3.00).

3.3 Results

3.3.1 Minimum Inhibitory Concentrations of EEP for Six Fungal Pathogens isolated from Avocado Fruit and Foliage

Figure 3.1 depicts the growth curves for *P. guipinii*, CgP complex, *Colletotrichum* sp., *Verticillum* sp., *Fusarium* sp., and *Monilia* sp. grown in the presence of EEP at

concentrations of 1, 2, 5, 7, 10 mg ml^{-1}, with growth on MEA without EEP (0 mg. ml^{-1}) as the control.

P. guipinii was completely inhibited by 10 mg ml^{-1} EEP, whereas all the other fungi were inhibited the most by 10 mg ml^{-1} EEP, but not completely. Significant inhibition was achieved at EEP concentrations of 5 and 7 mg ml^{-1} for all fungi ($P<0.001$). EEP concentrations of 5 mg ml^{-1} showed greater inhibition than 7 mg ml^{-1} against *P. guipinii*, CgP complex, *Fusarium* sp., and *Monilia* sp. No significant difference between 5 and 7 mg ml^{-1} were found ($P > 0.05$) against *Colletotrichum* sp., *Fusarium* sp. and *Monilia* sp. Concentrations of 1 and 2 mg ml^{-1} EEP were significantly less inhibitory than 5, 7 and 10 mg ml^{-1} EEP ($P<0.001$), except for the CgP complex in which 7 mg ml^{-1} was statistically similar to 1 and 2 mg ml^{-1} EEP ($P>0.05$). Both concentrations (1 and 2 mg ml^{-1}) were similar ($P>0.05$) for all the fungi tested, except *Monilia* sp., which showed a significantly higher inhibition at 2 mg ml^{-1} ($P < 0.01$). No difference ($P>0.05$) was observed between 10 and 7 mg ml^{-1} against *Colletotrichum* sp. and between 10 and 5 mg ml^{-1} against *Fusarium* sp. All fungal growth tended towards sigmoidal shaped growth curves.

Final RI values (Figure 3.2) were recorded up to the tenth day of growth when the control plates reached their maximum hyphal extension. EEP at concentrations of 1 and 2 mg ml^{-1} stimulated growth in both *P. guipinii* and *Fusarium* on the first day. *P. guipinii* was stimulated by 11.11 % and 10.68 % at 1 and 2 mg ml^{-1} EEP respectively, whereas *Fusarium* was stimulated by 88.33 % and 40 % respectively. However, on the second day, growth of both *P. guipinii* (23.33 % and 8.33 % at 1 and 2 mg ml^{-1} respectively) and *Fusarium* (14.40 % and 36.36 % at 1 and 2 mg ml^{-1} respectively) was inhibited. *P. guipinii*, *Colletotrichum* and *Monilia* demonstrated no growth for the first 10 days at 1 and 2 mg ml^{-1}. *Verticillium* demonstrated the fastest growth on 1 and 2 mg ml^{-1} achieving full growth on day 6.

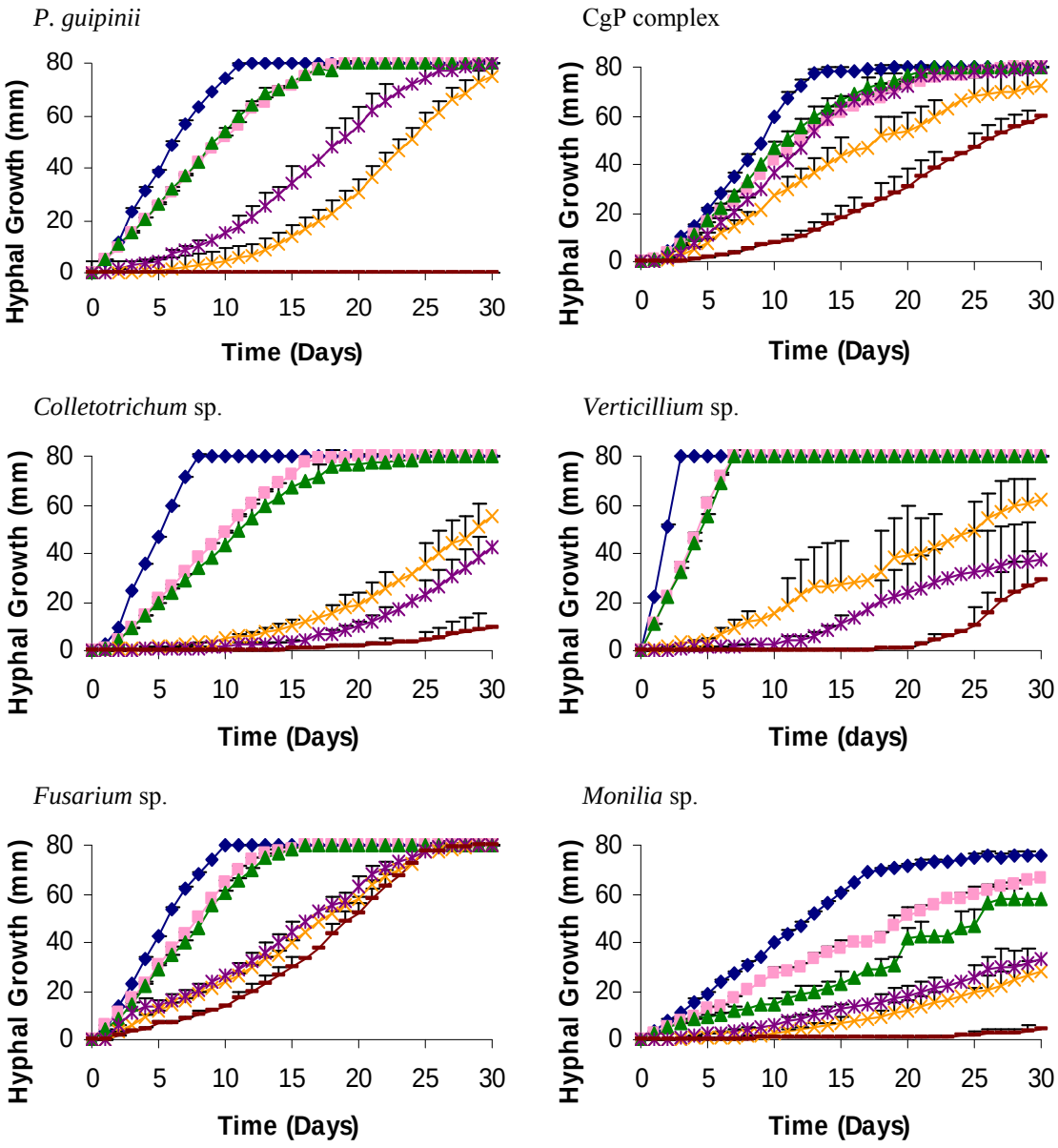


Figure 3.1: Growth inhibition of six phytopathogens of avocado trees and fruit by an ethanolic extract of propolis (EEP) within Malt Extract Agar at different concentrations (1-10 mg ml^{-1}), over a 30 day period at 25 °C. ♦ = 0 mg ml^{-1}; ■ = 1 mg ml^{-1}; ▲ = 2 mg ml^{-1}; X = 5 mg ml^{-1}; ∗ = 7 mg ml^{-1}; - = 10 mg ml^{-1}. Standard error bars indicated.

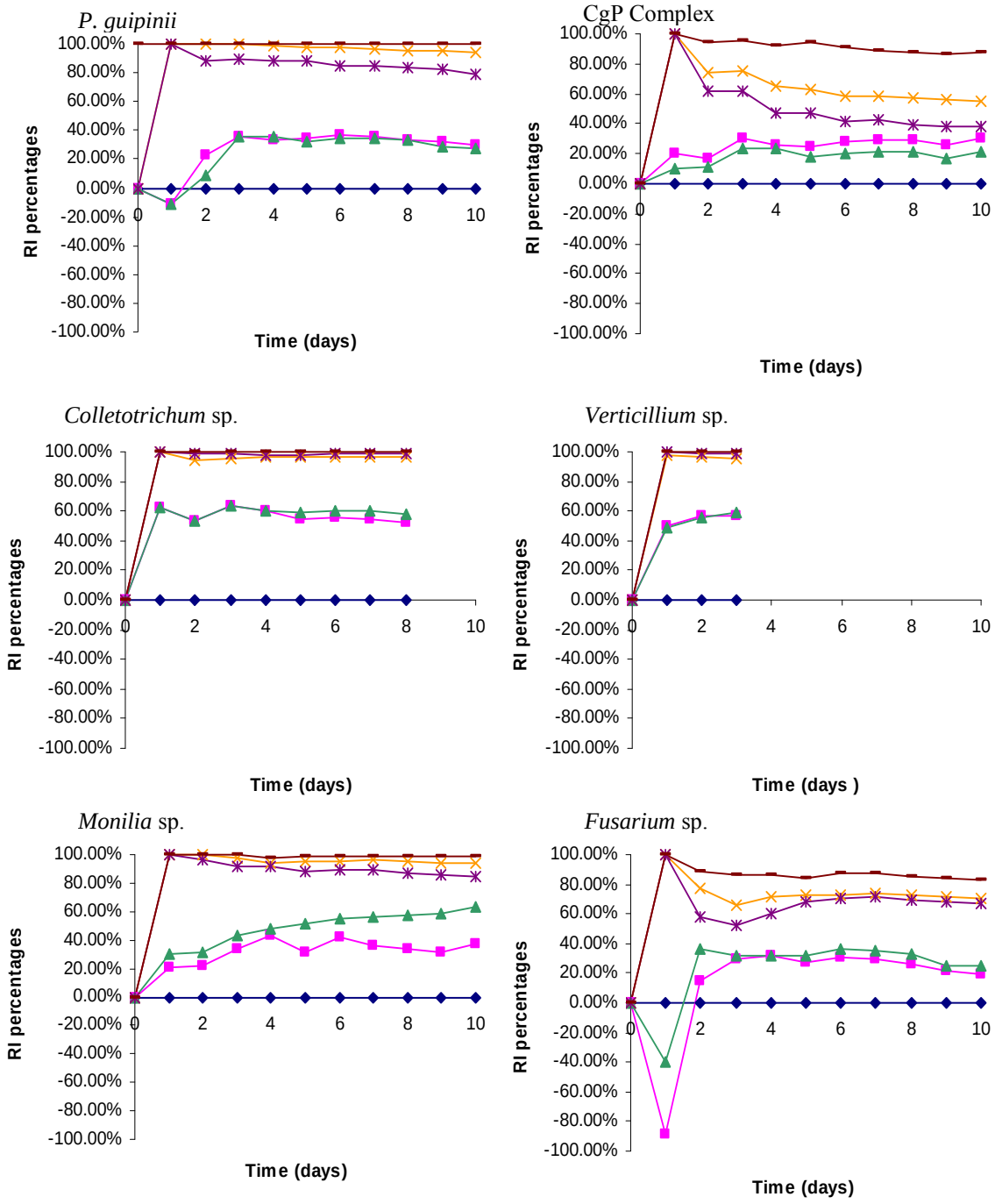


Figure 3.2: The RI percentage of fungal phytopathogens in the presence of EEP at different concentrations over a period of 10 days. Cultures were incubated at 25 °C. ♦ = 0 mg.ml^{-1}; ■ = 1 mg.ml^{-1}; ▲= 2 mg.ml^{-1}; X = 5 mg.ml^{-1}; ∗= 7 mg.ml^{-1}; - = 10 mg.ml^{-1}. Data capturing ceased after control plates reached maximum hyphal extension.

All fungi for the first 10 days of growth, at concentrations of 1 and 2 mg ml^{-1} demonstrated a slight increase or stabilized in inhibition activity. In contrast, concentrations of 5 and 7 mg ml^{-1} showed a decrease in inhibitory activity for all fungi except *Colletotrichum*, with the CgP complex demonstrating the highest decrease (74.69 % to 54.79 % inhibition and 62.03 % to 38.15 % inhibition respectively for 5 and 7 mg ml^{-1}). *Colletotrichum* showed a stable inhibition rate against concentrations of 5 and 7 mg ml^{-1} EEP of between of 94.68 % and 97.07 % at 5 mg ml^{-1} EEP and between 98.02 % and 98.94 % at 7 mg ml^{-1}.

3.3.2 Use of Scanning Electron Microscopy to Visually Determine External Damage to Fungal Hyphae due to Antifungal Activity of EEP

All the fungi tested showed signs of pores within the cell walls of the hyphae when grown on EEP containing media.

Colletorichum sp.:

Severe damage of the *Colletotrichum* sp. hyphae was observed in the form of large pores when incubated on 5 mg ml^{-1} EEP (Figure 3.3B). The pores were clearly within the hyphal matrix with raised outer edges. The pores were more severe and numerous in hyphae which were in direct contact with the agar. Most of the hyphae which were not in contact with the agar, i.e. growing over other hyphae, were undamaged and resembled that of the control (grown on standard MEA-Figure 3.3A).

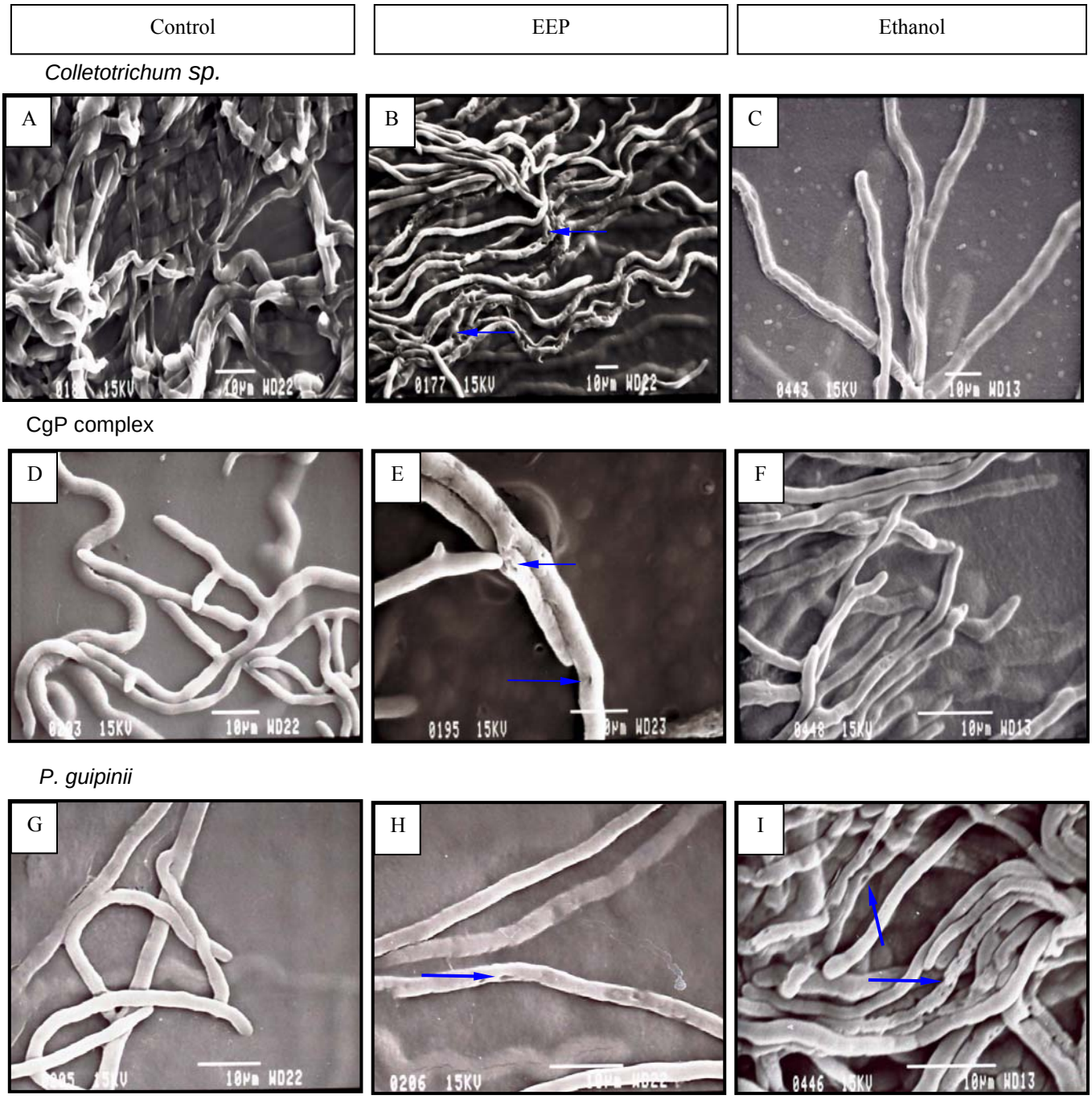


Figure 3.3: Scanning Electron Micrographs of *Colletotrichum* sp., CgP complex, and *P. guipinii* incubated on Malt Extract Agar (control), Malt Extract Agar with 5 mg ml^{-1} EEP, and Malt Extract Agar with ethanol equivalent to concentration found in 5 mg ml^{-1} EEP (negative control). Blue arrows indicate pores present in the fungal hyphae.

The hyphal apex and Spitzenkörper region seemed to be intact compared to the control. The *Colletotrichum* sp. incubated on the ethanol showed no signs of damage with smooth textured cell walls, similar to the control (Figure 3.3C). The hyphal apex and Spitzenkörper region were intact.

CgP complex:

Pores were observed in the CgP hyphae grown in EEP (Figure 3.3B), but smaller than that found in *Colletotrichum* hyphae and fewer. Some hyphal walls were irregular in texture. A few hyphae were extensively damaged as though the cell wall structure had been degraded. Overall, however, the hyphal grown in EEP looked far healthier than that of *Colletotrichum* in EEP. The hyphae grown in ethanol showed no signs of damage and resembled that of the control hyphae (Figure 3.3C). The hyphal apex and Spitzenkörper region for both treatments were intact

P. guipinii:

The hyphae of *P. guipinii* showed far fewer pores than *Colletotrichum* and CgP and were much healthier (Figure 3.3B). The hyphal apex and Spitzenkörper region were intact. In contrast to *Colletotrichum* and CgP, the hyphae grown in ethanol contained pores similar to the EEP treated hyphae of all the fungi (Figure 3.3C). The pores were also numerous but smaller than for the EEP treated hyphae. In dissimilarity to the EEP treated *Colletotrichum* and CgP, the hyphae of *P. guipinii* were affected both in contact and grown above the agar. Some hyphae had numerous pores as compared to the other two fungi.

3.3.3 Inhibition of Fungal Conidial Germination at 5 mg ml^{-1} EEP

Colletotrichum sp. conidial germination was inhibited by 98.95 % when incubated on MEA containing 5 mg ml^{-1} EEP, as opposed to 14.33 % inhibition when incubated with a coating of 5 mg ml^{-1} EEP (Figure 3.4). *P. guipinii* conidial germination was inhibited by

40.41 % when incubated on MEA containing 5 mg ml^{-1} EEP. In contrast, *P. guipinii* conidia germination was inhibited by 0.84 % with a coating of 5 mg ml^{-1} EEP (Figure 3.4). Inhibition and germination of *Colletotrichum* sp. and *P. guipini* spores were similar ($P > 0.05$) when incubated with a coating of EEP. However, the two fungi were significantly different ($P < 0.01$) when incubated on MEA with EEP.

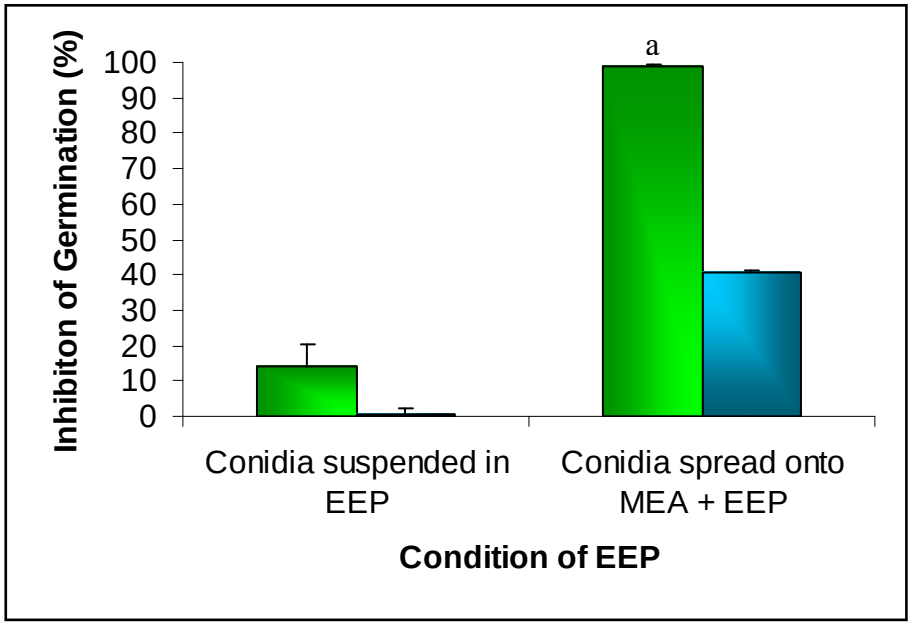


Figure 3.4: Inhibition of conidial germination of *Colletotrichum* and *P. guipinii* conidia in the presence or within 5 mg ml^{-1} EEP. All conidia were spread onto MEA and incubated for 24 h at 25 °C. EEP was added to either the spore suspension or to the MEA. = *Colletotrichum*. = *P. guipinii*. Inhibition was calculated as a percentage relative to the controls, therefore the control is omitted from the figure. Significant difference within each treatment is represented by different letters.

3.4 Discussion

The review by Treutter (2006) discussed the significance of flavonoids in the protection and resistance of plant to phyto-pathogens, especially fungi. A natural product that is well known for its high flavonoids content and used for medicinal properties for millennia is propolis (Cushnie and Lamb, 2005). However, the development of a propolis extract for the use as an agricultural fungicide has not been given much attention (Hegazy and El-Hady, 2002; Quiroga *et al.*, 2006).

This study aimed at developing an ethanolic extract of propolis (EEP), in *vivo*, as a fungicide intended for *in situ* trial on avocado (*Persea americana* Mill.) orchards. For *in situ* trials (chapter 5) the avocado orchards were treated with propolis at the MIC determined in this study. It was also determined in this chapter if EEP was able to inhibit germination of the fungal pathogens' conidia. This would mean that the EEP possibly could result in an additional barrier for the fruits exterior in inhibiting infections.

The majority of fungi used in this study were isolated from avocado epidermal layers. Species such as *Monilia* sp. and *Verticillium* sp. were commonly isolated from the lesions; however they have not been reported as common microbiota of avocado fruit They were included in this study to determine the antifungal properties of EEP on a more diverse range of fungal species other than only fungal pathogens of avocado fruit.

All the assessed fungi within this study were sensitive to all the concentrations of EEP tested, with high inhibition occurring at 5, 7, and 10 mg ml^{-1} (Figure 3.1). These results were similar to those of previous studies by Ngoepe and Straker (2004) and Vallabh and Straker (2005). Ngoepe and Straker (2004) determined the optimal concentrations for antifungal activities of EEP using both a long range concentration gradient (0, 1, 5, 10, 25, and 50 mg ml^{-1}) and a short range concentration gradient (0, 1, 5, 7, 9, 11, 13, and 15 mg ml^{-1}) using the agar dilution method. They assayed against *Nigrospora* and *Alternaria*. Results showed that the most effective concentrations were between 5 and 50 mg ml^{-1}. This corresponded to the results within this study, whereby concentrations less than 5 mg ml^{-1} (i.e. 1 and 2 mg ml^{-1}) had very little inhibitory effect on the assessed fungi. Vallabh and Straker (2005) used a concentration gradient of 1, 2, 5, 7, and 10 mg ml^{-1} against *P. guipinii* and *Glomerella cingulata* (asexual stage is *C. gloeosporioides*) with the agar dilution method. These results proved effective in showing inhibition at 5 mg ml^{-1} and higher, and the growth curves and inhibition behaviour of the fungi were almost identical to this study's data. The MIC within this study was therefore concluded to be 5 mg ml^{-1} for all the fungi as this was the lowest concentration resulting in a significant inhibition (with respect to significant differences from statistical analyses).

Interestingly, the MIC values of Hegazi and El-Hady (2002) were much lower than the MIC values in this study. They determined the efficacy of propolis as an antifungal agent against *Alternaria*, *Aspergillus*, *Cladosporium*, *Fusarium*, *Mucor*, *Penicillium Rhodotorula*, *Rhizopus*, and *Scopulariopsis*. MIC values were determined by the agar dilution assay at different concentrations. The MIC values obtained were between 1.20 and 3.60 mg ml^{-1} with the former for *Alternaria* and *Fusarium* and latter for *Aspergillus* and *Penicillium*. A concentration of 1 and 2 mg ml^{-1} showed very little inhibition on the *Fusarium* tested in this study, with the lowest effective concentration as 5 mg ml^{-1}. Additionally, Hegazi and El-Hady (2002) MIC values were 1.4 - 4.2 times lower than the MIC value obtained in this study.

Similarly, Quiroga *et al.* (2006) obtained lower MIC values to the presented data. They determined the antifungal properties of propolis against xylophagous fungi (*Ganoderma applanatum*, *Lenzites elegans*, *Pycnoporus sanguineus*, and *Schizophyllim commune*) phytopathogenic fungi (*Aspergillus niger*, *Fusarium* sp., *Macrophominia* sp., *Penicillium notatum*, *Phomopsis* sp., and *Trichoderma* sp.) and yeasts (*Saccharomyces carlsbergensis* and *Rhodotorula* sp.). MIC values were calculated from germination assays of the fungal spores (1×10^4 conidia ml^{-1}) at a concentration gradient of 0 to 349 μg ml^{-1} EEP after 2 – 3 days. The lowest MIC value was obtained from *S. carlsbergensis* at a concentration of 77 μg ml^{-1}. The highest MIC value was obtained from *Trichoderma*, *P. notatum* and *Fusarium* at a concentration of 349 μg ml^{-1}. These values are 65 times and 14 times lower respectively than the MIC value obtained from the present study.

To further understand the properties of the propolis sample, Quiroga and associates (2006) assayed the inhibition of hyphal extent after 4 -5 days at a concentration gradient of 0 – 1.164 mg ml^{-1}. *Macrophomina* sp. was the most resistant fungus with an inhibition of 8 % on 1.16 mg ml^{-1} EEP. The most sensitive fungus was *Phomopsis* sp. with an inhibition of 81.7 % on 1.16 mg ml^{-1}. The most sensitive fungus in this study was *Colletotrichum* with an inhibition of 55.8 and 60.5 % at 1 and 2 mg ml^{-1} respectively after the fifth day. The most resistant was CgP with an inhibition of 28.4 and 20.6 % at 1 and 2 mg ml^{-1} respectively. Most of the fungi tested were inhibited between 11.1 % and 60.8 %

with the majority below 40 %. This showed a generally lower inhibitory activity compared to Quiroga *et al.* (2006) (general inhibition range between 39.4 % and 81.7 %). Comparisons of concentrations in published results are incomparable as most authors do not explain the methods for concentration determination.

The lower inhibition activity and higher MIC values of the propolis sample tested can be explained by the constituents of the propolis, which can vary greatly from different geographical regions, as well as between different bee colonies (Bankova, 2005a; Silici and Kutluca, 2005). As indicated in Chapter 2, the flavonoid concentration in the propolis sample was almost five times higher than the phenolic concentration (flavonoids were 16.35 mg ml^{-1} compared to phenols at 3.28 mg ml^{-1}). This is a unique concentration ration of flavonoids and phenolics as such a ration has not been reported to date. This can be attributed to the location of the propolis samples which were collected form bee hives within Roodewal farm avocado orchards in Nelspruit, South Africa. Chemical analysis on neither propolis from an avocado orchard nor from Nelspruit, South Africa has been reported to date. The low activity is evidence that phenolics might play an important role in the antimicrobial activity of propolis samples. A higher concentration of phenolics could result in a higher antifungal activity. Other sources of variation could be the specific flavonoids, and even phenolics, comprising the propolis sample Identification of the specific compounds comprising the propolis sample is therefore necessary. The compounds with antifungal activities could be compared to published identified compounds, which will determine if the compounds have a lower efficacy in antifungal activities.

Interestingly, 5 mg ml^{-1} and 1 mg ml^{-1} were more efficient than 7 mg ml^{-1} and 2 mg ml^{-1} respectively in inhibiting *P. guipinii*, *CgP complex*, (1 and 2 mg ml^{-1}) and *P. Guipinii*, CgP complex, *Monilia* sp. and *Fusarium* sp. (5 mg ml^{-1} and 7 mg ml^{-1}) (Figure 3.1). This phenomenon in which an inhibiting agent results in a stimulation effect on the target organisms at specific concentrations is termed hormesis (Stebbing, 1982; Barreto *et al.*, 2002). According to the four types of hormetic effects (α, β, γ and δ) proposed by Stebbing (1982), this type of data exemplifies δ-hormesis in which an increase in the

toxins' (antimicrobial agent in this case) concentration results in periodic stimulation and inhibition of the target organism, but tending toward greater inhibition. Similar results were obtained by Barreto *et al.* (2002) against the fungi *C. gloeosporioides* and *Rhizoctonia solani* using ethanolic extracts of the seaweeds *Osmundaria serrata* and *Stypopdium zonale*. *C. gloeosporioides* and *R. solani* demonstrated hormetic growth on low concentrations of *O. serrata*. However, *C. gloeosporioides*, never showed hormetic growth on extracts of *S. zonale*. This study and Barreto *et al.* (2002) indicate that *C. gloeosporioides* can demonstrate hormesis when in contact with antifungal agents and consequently the concentration at which antifungal agents should be applied to plants should be carefully determined.

An EEP concentration of 10 mg ml^{-1} proved to be the most inhibitory concentration to all the fungi tested within this study. However, the MIC was determined as 5 mg ml^{-1}. The most suitable concentration for use of a fungicide should be the lowest, as a lower expense fungicide will result in a more affordable product. Hence 5 mg ml^{-1} was chosen for further testing as a possible concentration for the development of a fungicide for avocado trees.

The apparent stimulation of growth on both 1 and 2 mg ml^{-1} EEP for *P. guipinii* and *Fusarium* sp. on day 1 (Figure 3.2) could have been a result of obtaining nutrients mostly or entirely from the agar plug and not the MEA with EEP. It was observed that these two fungi were ridged in initial extension and thereby had no contact with the plate agar for the first day. The other fungi extended horizontal onto the plate agar within the first day of growth. This possibly indicated that antifungal activity of EEP was only effective in direct contact with the fungal hyphae, and if any volatile compound were emitted by EEP they were either ineffective or inefficient. As a possible fungicide, propolis being efficient only in direct contact with the target fungi, i.e. not emitting efficient volatile compounds, could result in fungal infection to occur on untreated areas of the avocado tree and fruit. Therefore, when applying the propolis to the avocado trees, complete coverage of the trees would need to be meticulous to ensure maximum antifungal protection.

The presence of flavonoids is common in the plant kingdom (Medina, 2004; Treutter, 2006). Flavonoids are secondary metabolites within the plants and have various functions such as UV-B protection, signalling, protection from environmental stress, to serve as allelochemicals, and most importantly antimicrobial protection, particularly towards fungal phytopathogens (Treutter, 2006). Examples of the mode of action of flavonoids against fungi are: the crosslinking of enzymes; inhibition of fungal cellulases, xylanases, and pectinases; the formation of chelates around essential metal ions; and the formation of physical barriers in the form of crystals (Treutter, 2006). Flavonoids are therefore, an additional barrier which fungi have to overcome for successful infection. Fungi, consequently, have developed counter-defence mechanisms to the antimicrobial flavonoids in the form of extracellular enzymes (Medina *et al.*, 2004; Pedras and Ahiahonu, 2005; Treutter, 2006).

There are two types of defence against flavonoids by extracellular enzymes of fungi, namely (i) detoxification of the flavonoids (Pedras and Ahiahonu, 2005) and (ii) metabolizing of the flavonoids (Medina, 2004; Pedras and Ahiahonu, 2005). Most detoxifications of flavonoids involve the addition of one or more hydroxyl groups. An example of detoxification of flavonoids is that of *Aspergillus saitai* in which it detoxifies the flavonoid phytoalexin daidzein to 8-hydroxydaidzein by the addition of a single hydroxyl group to the 8^{th} carbon. The extracellular enzyme is, to date, unknown (Pedras and Ahiahonu, 2005). However, the metabolism of the flavonoid rutin is fairly well understood (Medina, 2004). Rutin (which is a 3-*O*-glycoside of quercetin) is hydrolyzed to quercetin, rhamnose and glucose by the enzymes rutinase, β-glucosidase and α-rhamnosidase. Quercetin is then oxidatively cleaved to carbon monoxide and the depside 2-protocatechuoylphloroglucinoal carboxylic acid by the enzyme quercetinase (flavonol 2,4-dioxygenase). Phenol carboxylic acid acyl esterase finally hydrolyses the depside to protocatechuic acid and phloroglucinol carboxylic acid (Medina, 2004). Such a defence may have been what was observed by the steady decrease in RI percentages in the fungi within this study.

Further research is therefore required for the detection, isolation and identification of any enzymes that might be involved in the detoxification or metabolising of the flavonoids within the propolis study. Such information would aid in the estimation of the time taken to neutralize the EEP and in the estimation of the application rate and spray intervals of EEP to the avocado trees.

On a microscopic level, distinctive pores were visible within the fungal cell walls when incubated on MEA containing 5 mg ml^{-1} EEP (Figure 3.3). However, *P. guipinii* incubated on the ethanol control had similar pores within its cell walls but they were smaller and fewer. This sensitivity to ethanol by *P. guipinii* could indicate that the *Colletotrichum* sp. and CgP complex were able to overcome the effects of the concentration of ethanol within the ethanol control. However, upon the presence of propolis with the ethanol, the propolis either (i) amplified the effects of the ethanol to a point that the fungi were unable to inhibit the formation of the pores; or (ii) the propolis itself had a similar effect as ethanol on the fungi, but on a greater scale. The latter is the more likely as flavonoids are chemically defined as a 2-phenyl-benzo[α]pyrane ring, which is two benzene rings linked together through a heterocyclic pyrane ring (Cushnie and Lamb, 2005). The functional group are hydroxide ions and have the same reactivity properties of alcohols. However, due to the stability of the benzene ring and its resonance structure, reactivity is directed towards it functional groups, resulting in a higher reactivity by the functional groups (McMurry 1996). This would result in a higher and more rapid sensitivity to the flavonoids by the fungi.

The exact reactivity site of the EEP in the fungal hyphae cannot be deduced from the micrographs (Figure 3.3). However, McNally *et al.* (2003) demonstrated the uptake of C-glycosyl flavonoid phytoalexins by the powdery mildew fungus *Podosphaera xanthii* (syn. *Sphaerotheca fuliginea*, Castagne; Braun and Shishkoff) in infected cucumber leaves (*Cucumis sativus*) by use of fluorescence microscopy (488 nm). Their micrographs clearly indicated that the entire haustorial complex, as well as the extrahaustorial matrix, contained high concentrations of the phytoalexin. Soon afterwards, surface conidial chains collapsed followed by death of the fungi. McNally and associates (2003)

concluded that the glycosyl group aided in the uptake of the flavonoids by the fungus and that the antifungal activities of the flavonoids were a result of the inhibition of fungal enzymes, such as peroxidases, lipoxygenases, reverse transcriptase, pectinases, and β-glucosidase. The presence of pores in the hyphae of the fungi in this study could have been as a result of enzyme inhibition of important enzymes involved in the development of cell wall structures, ion channels, or receptors, or the inhibition of enzymes required for translation or transcription of any of the cell wall structures. A further molecular understanding of the inhibition activity of flavonoids within fungi, especially phytopathogenic fungi, is required. This will aid in the further development of propolis as a phyto-fungicide, and most likely novel alternative phyto-fungicides.

The rationale behind the germination assays was to simulate, *in vitro*, the spraying of avocado fruit within the field to see (i) if the conidia were able to germinate on previously treated/sprayed fruit and (ii) if the conidia adhered to fruit, followed by the fruit being treated/sprayed, were still able to germinate.

Significant inhibition ($P < 0.001$) 98.85 % and 40.41 % respectively for *Colletotrichum* and *P. guipinii* was achieved when incubated on MEA containing 5 mg ml^{-1} EEP (Figure 3.4). Similarly, Ghaly *et al.* (1998) demonstrated the germination inhibition properties of an ethanol extract of propolis (termed PEE) against conidia of *Aspergillus flavus* at a concentration gradient of 1 to 4 mg ml^{-1}. The resultant mycelial dry mass was weighed to determine percentage of inhibition. At 1 g l^{-1} germination was inhibited by 11 % as compared to 80 % at 4 g l^{-1}. This corresponds to the data of this study in which more efficient inhibition resulted from a concentration of 5 mg ml^{-1} EEP to 10 mg ml^{-1}, and 1 mg ml^{-1} EEP results in very low inhibition (Figure 3.1), as well as to the high germination inhibition (Figure 3.4).

However, inhibition activities were not significant ($P > 0.05$) when the conidia were coated with EEP. The EEP probably diffused into the agar once the spore-EEP mixture was spread onto the MEA which would dilute the concentration of EEP. Quiroga *et al.* (2006) described methodology whereby dilutions of the EEP are aliquoted into sterile

wells to which the conidia are added, followed by incubation to allow germination. This would ensure that the conidia are continually coated with a given concentration of EEP for the whole incubation period.

The CgP complex was used as a candidate for this study as it was commonly isolated from Cercospora-like lesions on the avocado fruit. This is the first report of the isolation of the possible CgP complex. Fungal complexes infecting avocado foliage and/or fruit are not uncommon. The *Dothiorella*/*Colletotrichum* fruit rot complex is well known and documented in the South African avocado industry (Anderson, 1986; Darvas and Kotzé, 1987, Darvas *et al.*, 1987, Boshoff *et al.*, 1996). The *Dothiorella/Colletotrichum* complex results in similar external symptoms to anthracnose (caused by *C. gloeosporioides*) on avocado fruit. It is defined as resulting in a reddish brown colouration to the avocado fruit skin around the infected area. As the disease matures it darkens gradually and the disease infects the flesh of the fruit producing a watery rot. Sporulation of the fungi usually only occurs at later stages of disease development (Darvas and Kotzé, 1987). The CgP complex was isolated from lesions very similar to Cercospora spots (caused by *Pseudocercospora purpurea*) but not similar to anthracnose. Stem-end rot (SER) diseases on avocado fruit are also known to be a result of fungal complexes. The most common fungi to result in SER are *C. gloeosporioides*, *Lasiodiplodia theobromae*, *Dothiorella aromatica*, *Tyronectria pseudotichia* and *Phomopsis perseae*. However, no reports have been made of these fungal complexes having any resistance to fungicides. The CgP complex, as compared to *Colletotrichum* sp., did show higher resistance to EEP at all concentrations (Figure 3.1 and 3.2).

Further research could answer if a possible synergistic relationship between the fungi of the CgP complex is occurring. If so, are the resistant properties of the CgP complex fungi a result of an abundance of secondary metabolites and/or extracellular enzymes to degrade or detoxify the EEP? Or, is there a molecular relationship between the fungi, activating more defence genes creating an overall resistance of the complex to EEP? Studies were not performed on *Pseudocercospora* sp. as the fungus was impossible to separate from the *Colletotrichum gloeosporioides*. Further studies would be to compare

the resistance of *Pseudocercospora* sp. to that of the CgP complex in the presence of EEP. This would assess the *Pseudocercospora* sp. contribution to resistant to EEP, resulting in the complex to be resistant.

3.5 Conclusion

- This study showed that EEP at a final concentration of 5 mg ml^{-1} would be feasibile as a potential phyto-fungicide against fungal pathogens.

Chapter 4

In Vivo Assay to Determine Spray Intervals of EEP to Control Diseases of *Persea Americana* Mill. (Avocado) Trees

4.1 Introduction

Despite increasing awareness of the use of propolis as an agricultural fungicide, very few studies have been conducted in *in vivo*.

Ngoepe and Straker (2004) determined the efficacy of an ethanolic extract of propolis (EEP) against the rose bush (*Rosa* hybrid) pathogen, *Alternaria* sp. The rose bushes were inoculated with *Alternaria* sp. or left untreated (negative control) or treated with Tween 1 % (positive control); or the trees were initially treated with 5 mg ml^{-1} EEP followed by the inoculations with *Alternaria* sp. Complete inhibition of infections from *Alternaria* sp. were observed from bushes treated with propolis. Similarly, Vallabh and Straker (2005) determined the usage of 10 mg ml^{-1} EEP against *Glomerella cingulata* (asexual *Colletotrichum gloeosporioides*) infections on *Persea americana* (avocado) trees. Complete inhibition was obtained from treatments with EEP.

Alternatively, Stompor-Chrzan (2004) demonstrated the efficacy of EEP on the prevention of fungal root pathogen infections of bean plants. They compared 4 % and 10 % EEP as a seed coat before planting the bean seeds in pots. Best results were obtained at 10 % EEP, whereby pathogenic fungi were inhibited completely and the sprouting bean plants were noticeably healthier than the control plants.

However, the success of an agricultural fungicide is not only determined by its antifungal efficacy but by the seasonal period of application to the trees and/or crops and the time intervals between each spray (Sanders and Korsten, 1999). It is common practice to spray trees after the first rains of the seasons, as well as any periods of high

atmospheric moisture when high concentrations of pathogen inocula are usually dispersed. A well timed spray programme during the fruiting season is mandatory to maintain as it sustains low infection from the pathogens throughout fruit development (Sanders and Korsten, 1999).

It is, therefore, important to not only to determine if EEP is efficient in inhibiting infection from fungal pathogens on avocado trees but to also determine the optimal time interval between sprays to maintain low infections during fruit development. This study determined the efficacy of 5 mg ml^{-1} EEP on root stock avocado trees, in greenhouse conditions, to inhibit C. *gloeosporioides*, CgP complex, and *Pestalotiopsis guipinii* foliage infections, as well as to determine the optimal time interval between sprays for use *in situ*.

4.2 Materials and Methods

4.2.1 Conidial Inocula

Agar plugs of *P. guipinii*, *Colletotrichum* sp. (wild type), and *Colletotrichum gloeosporioides-Pseudocercospora* sp. (CgP) complex (10 mm) were cultured on Malt Extract Agar (MEA) (Merck); a modified version of the Modified Merlin Norkrans (MMN) medium with cellulose as the carbon source (cellulose 7.5 g, malt extract 3.0 g, glucose 20.0 mg, ammonium hydrogen phosphate [$(NH_4)_2HPO_4$] 250.0 mg, potassium dihydrogen phosphate [KH_2PO_4] 500.0 mg, hydrated magnesium sulphate [$MgSO_4.7H_2O$] 150.0 mg, hydrated calcium chloride [$CaCl.2H_2O$] 65.0 mg, ferrous chloride (1 %) [$FeCl_2$] 1.2 ml, sodium chloride [NaCl] 25 mg, thiamine HCl 0.1 g, peptone 750 mg, agar 15.0 g), and oat meal agar (OA) (oatmeal [Jungle Oats] 60.0 g, agar 12.5 g) respectively. The selected media were as a result from a pilot study to assess media for optimal sporulation of each fungus. The plates were incubated for two weeks at 25 °C. Spores were isolated by the spreading of Tween 80 (0.5 %) with a glass spreader and pouring off the Tween-spore solution into sterile glass Schott bottles. The conidial inocula were strained through cheese cloth to remove hyphae. The concentrations of the

conidia were calculated using a haemocytometer (Neubauer). Conidial inocula were stored overnight at 4 °C in the Tween solution. Final conidial concentrations were *P. guipinii* = 3.56 x 10^5 conidia ml^{-1}, *Colletotrichum* sp. = 7.24 x 10^5 conidia ml^{-1}, and CgP complex = 1.30 x 10^5 conidia ml^{-1} *C. gloeosporioides* and 9.25 x 10^4 conidia ml^{-1} *Pseudocercospora*.

4.2.2 Greenhouse Conditions

A tunnel greenhouse with wet wall cooling system was used. Trees (23) were watered every second day via a dripper irrigation system (*ca* 800 ml per tree). The greenhouse had an average day:night temperature of 25 °C:14 °C.



Figure 4.1: External view of the tunnel greenhouse. Day:night temperatures of the greenhouse were 25 °C:14 °C.

4.2.3 Inoculation of Avocado Trees

Persea americana (avocado) root stock trees were kindly donated by Westfalia Nursery, Tzaneen, South Africa. As instructed by Westfalia Nursery, root stock avocado trees were used as to insure successful infections. Randomly chosen leaves (10 per tree) were injured using sterile carborundum. Conidial inocula were sprayed on to the injured leaves using an artists airbrush (Aztek A4308 Airbrush set) connected to a compressor ("mini compressor" CP101 220v/50 Hz, Colored Drawing Enterprise Co., LTD.) from an

average distance of 10 cm. The trees were incubated in the greenhouse for three days to allow for infection to occur. A set of trees was left uninoculated as positive controls. Seven trees for each fungal conidial inoculum were inoculated.

4.2.4 Treatment of Inoculated Root Stock *Persea americana* Trees with 5 mg ml^{-1} EEP

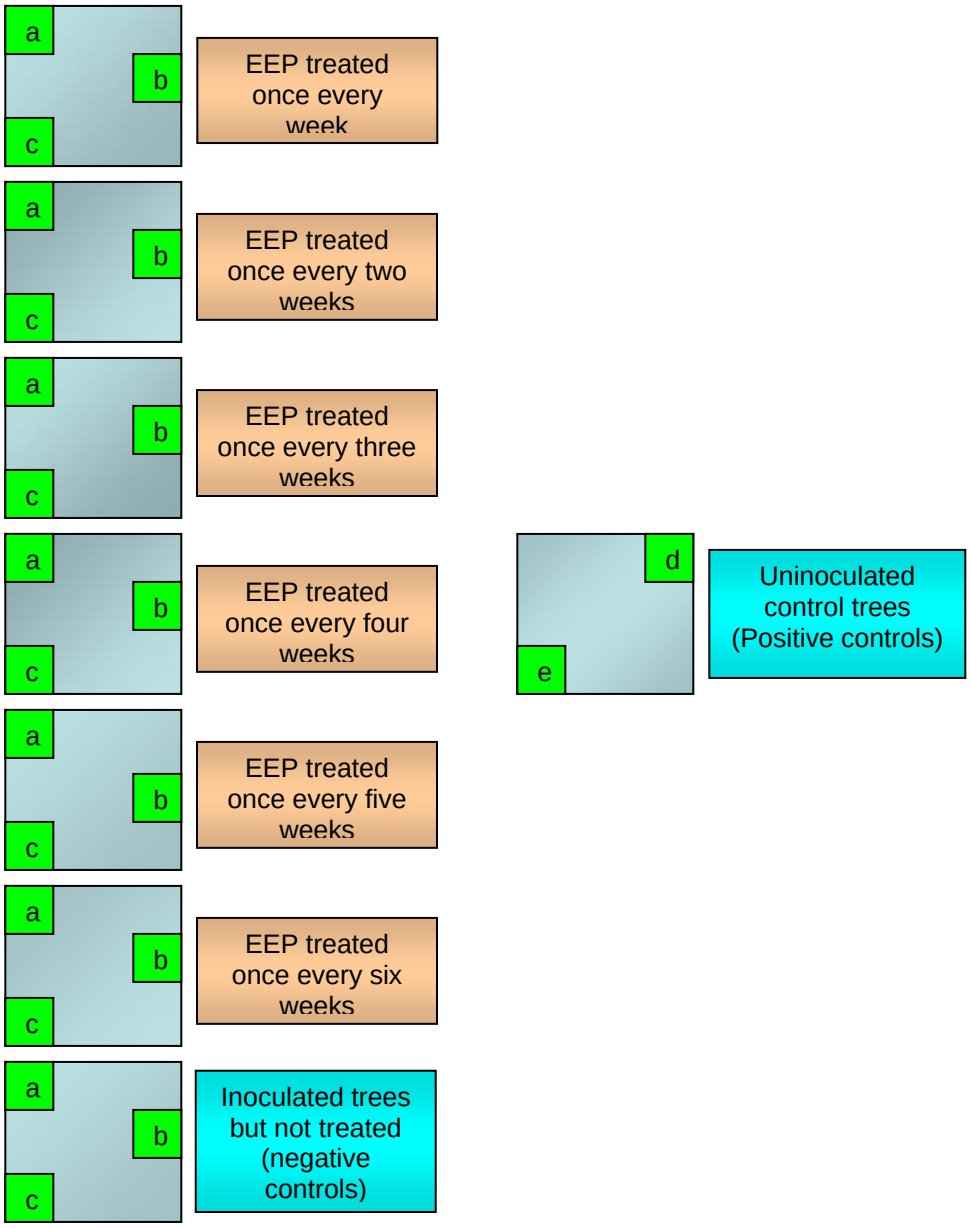


Figure 4.2: Layout and design of greenhouse trials with root stock *Persea amerciana* trees (green blocks). The trees were inoculated with (a) = *P. guipinii*, (b) = *Colletotrichum* sp. and (c) = CgP complex. Trees were left for 24 hours before treatments started. Each group of three trees were treated in intervals of once every week to once every sixth week with 5 mg ml^{-1} EEP. Negative control trees were inoculated but treated with sterile water. Positive control trees were uninoculated but treated with sterile water = d or EEP = e.

Ethanol Extract of Propolis (EEP) was diluted to 5 mg ml^{-1}, as determined in Chapter 3. Positive control trees were treated with either sterile water or EEP on weekly time intervals using a standard garden hand-held spray bottle (Canyon). Inoculated trees were grouped in threes of each fungal inoculum (Figure 4.2). The trees were treated with 5 mg ml^{-1} EEP with incrementing weekly time periods of once every week for the first group to once every six weeks for the penultimate group. The final group was treated with water as a negative control. The greenhouse experiment occurred over a six week period.

4.2.5 Analysis of Results

A disease evaluation was recorded after the sixth week, based on the disease index for anthracnose by Guyot *et al.* (2005), from foliage disease symptoms: 0 = no lesion; 1 = small brown necrotic lesions; 2 = few lesions of diameter less than 5 mm and/or only slight necrotic lesions with no to slight distortion of the leaf; 3 = numerous small lesions of diameter < 5 mm with distortion of the leaf and/or two or more large (> 5 mm) lesions with slight distortion; 4 = < 75 % necrosis or large lesions with much distortion; 5 = > 75 % necrotic or leaf fallen off.

The average disease index for each treatment was calculated and analysed using one-way ANOVA (software SAS Enterprise 3.0).

4.3 Results and Discussion

Disease symptoms and lesions on all the infected trees were a spreading brown necrotic lesion with a lighter brown margin. Symptoms on the CgP inoculated trees increased in severity with increasing spray intervals up to once every fourth week treatments (Figure 4.3). The CgP inoculated tree which was treated every fourth week showed severe symptoms similar to the negative control. However, treatment intervals of every fifth week and every sixth week with EEP resulted in few lesions.

The majority of the infected trees tended towards the lower category disease indices (≤ disease index 3) (Figure 4.3). According to Guyot *et al.* (2005) category one represents aborted infection, i.e. the spores were able to infect the plant tissue but disease development was inhibited. Disease index 2 and 3 represent successful infections but disease spread was inhibited, possibly to a point where the fungi were inhibited completely. The exceptions were CgP infected trees treated once every third and fourth week and *Colletotrichum* sp. infected tree treated once every two weeks, with a general disease index trend towards higher category infections (> disease index 3).

However, comparisons between (1) each treatment; and (2) between treatments and controls for *P. guipinii* and *Colletotrichum* sp. inoculated trees showed no significant differences ($P > 0.05$). A significant difference between the CgP complex inoculated trees and the positive control trees was observed ($P < 0.05$). Additionally, every fifth and sixth week treatments of CgP inoculated trees were similar ($P > 0.05$) to every week and second week treatments of CgP inoculated trees. These treatments showed lower disease indices than treatments of every third and fourth weeks. Similarly, *P. guipinii* and *Colletotrichum* sp. inoculated trees treated every fifth and sixth weeks showed generally lower disease indices than more frequent treatment times.

All the inoculated trees treated every fifth and every sixth week was visually healthier than inoculated trees treated less than every fifth week. Treated trees of every fifth and sixth weeks had numerous new shoots and the leaves were further developed. Treatment intervals of every fifth and sixth weeks were also visually efficient in the suppression of the symptoms on the CgP inoculated trees.

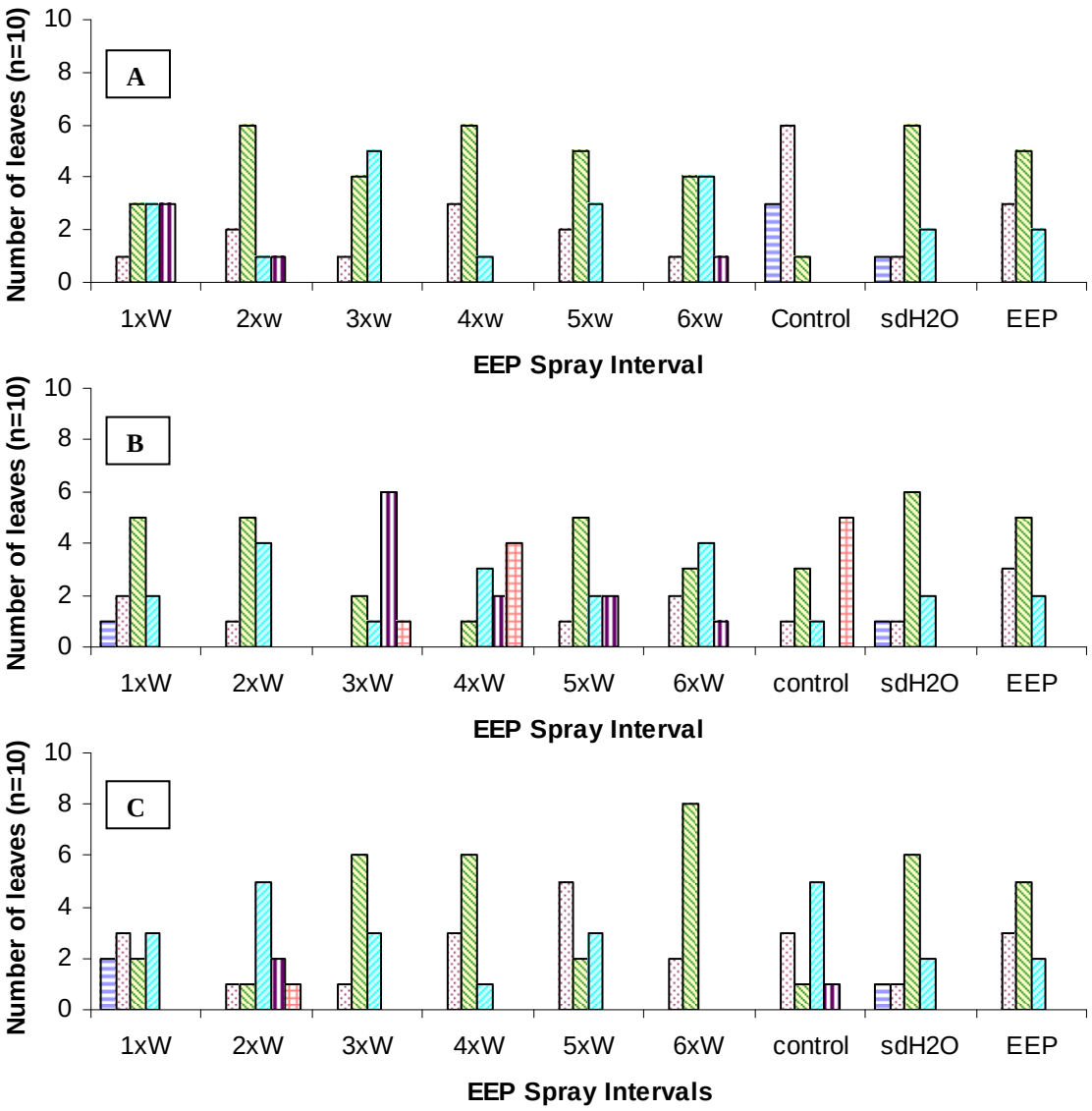


Figure 4.3: Occurrence of disease index after six weeks of 10 leaves from each avocado plant, incubated in greenhouse conditions, infected with **A** = *P. guipinii*, **B** = CgP complex, and **C** = *Colletotrichum* sp, and treated with EEP at weekly intervals (1xW = once a week spray to 6xW = every sixth week spray). Disease index range from 0 (no symptoms) to 5 (> 75 % necrotic or leaf fallen off). Control trees were inoculated but treated with sd H_2O. Uninoculated trees treated with sdH2O and EEP served as positive controls. = disease index 0; = disease index 1; = disease index 2; = disease index 3; = disease index 4; = disease index 5.

The data demonstrated the efficacy of every fifth and sixth week treatments of avocado trees to potentially reduce and control diseases *in situ*. Frequent treatments of the inoculated trees resulted in higher disease indices. Therefore a treatment interval of every fifth or sixth week was concluded as feasible for the application of 5 mg ml^{-1} EEP on avocado trees.

Timing and intervals of fungicidal applications to crops and trees are of utmost importance for the control of pre- and postharvest diseases. Fungicide application should coincide with periods of high rainfall as the dispersal of pathogen conidia is high (Sanders and Korsten, 1999). Therefore, spray programmes, in terms of regular spray intervals, generally occur throughout the rainfall seasons. Roodewal farm (in which field trials were commenced – see Chapter 5) has an average spray interval, using copper hydroxide (CopStar), of every fifth week from late October to early February the following year. However, if high rainfall over a period of a few days occurs, the spraying of the avocado trees are commenced a week or two earlier to prevent a high incidence of infections.

Therefore, a spray interval of every fifth week at Roodewal farm coincides with the data of this study. The EEP, however, should be applied after periods of high rainfall, even if it is a week or two earlier, which is a similar strategy used for conventional fungicides. This is to ensure maximum protection of the trees and fruit from phytopathogen germinations and infections. It is common practice on Roodewal farm, as well as other avocado farms, to spray fungicides only when the trees are dry (usually late morning to early afternoon), as should EEP. This is to ensure that the fungicides are applied evenly and are not diluted or washed off before they dry.

The occurrence of disease symptoms on the positive control trees (sdH_2O and EEP) were low disease index categories and were possibly as a result from injury during transportation of the trees to the greenhouse.

The use of 5 mg ml^{-1} in this study correlated with that of Ngoepe and Straker (2004) whereby a treatment of 5 mg ml^{-1} EEP onto leaves of *Rosa* hybrids before inoculation with *Alternaria* sp. resulted in 100 % inhibition of infection. Correspondingly, Vallabh and Straker (2005) sprayed 10 mg ml^{-1} EEP onto avocado trees, *in vivo*, infected with *Colletotrichum gloeosporioides* with complete inhibition of the disease symptom.

The negative control trees infected with the fungi indicated predominantly low category infection (Figure 4.3). Low category infections indicated unfavourable conditions for infection to occur. Fungal infection of avocado trees and fruit are known to be most active during the hot and rainy seasons (Darvas and Kotzé 1979; Boshoff *et al.* 1996; Willis and Duvenhage 2003). Furthermore, Guyot *et al.* (2005) determined the impact moisture and moisture development has on infection by *Colletotrichum* on leaves. They indicated that the disease index (0 - 4) increased with an increasing duration of moisture on rubber tree leaves (6 - 20 h). In this study, leaves were usually dry as a dripper irrigation system was used adding water directly into the soil. In addition, the moisture from the spraying of the spores dried rapidly in the greenhouse. To obtain successful infections of the spores, the inoculated leaves should be covered, with for example a polyethylene bag as described by Guyot *et al.* (2005), for a period of 20 h. The high moisture maintained by the bags would induce higher concentrations of conidia germinating and resulting in high infections of the avocado leaves. It would also maintain a low respiration rate of the leaves inducing unfavourable conditions for the leaves, increasing the chance for successful pathogen infections. The trees should then be incubated in the greenhouse with the bags removed and at a relative humidity range of 80-95 % (Guyot *et al.*, 2005) to allow for the natural disease cycle to progress.

Temperature was, nevertheless, ideal for infection with an average day temperature of 25 °C, according to Denner *et al.* (1986). Denner *et al.* (1986) demonstrated the germination of *C. gloeosporioides* and *Dothiorella aromatica* conidia at a temperature range of 5-40 °C. The optimum germination temperatures were between 10 - 35 °C and 25 - 30 °C for *C. gloeosporioides* and *D. aromatica* respectively. Germination for both the fungi was inhibited at 5 and 40 °C. Most importantly, the appresoria of *C.*

gloeosporioides was temperature sensitive and only formed between 10 and 30 °C. With an average day:night temperature of 25 °C:14 °C of the greenhouse within this study, the temperatures were ideal for the germination and appresoria formation for the fungal spores.

The success of EEP as a seed coat for beans was successfully determined by Stompor-Chrzan (2004) in greenhouse studies. They evaluated the potential of EEP at 4 and 10 % as a seed coat agent to inhibit damping-off disease by pathogenic fungi in experimental and pot trials with been seeds. It was shown that 10 % EEP was the most efficient in inhibiting fungal infections and resulted in visually healthy plants. However, sprouting seedlings were low in the treated pots. These results, in conjunction with this study, demonstrated the high potential of EEP to be used as a fungicide, both in mature plants and seedlings, broadening the application and uses of EEP.

4.4 Conclusion

- This study demonstrated the feasibility of an EEP spray schedule of every fifth week in avocado orchards using hand spray guns.

Chapter 5

In Situ Evaluations of EEP as a Pre- and Post-Harvest Treatment for the Control of Pre- and Post-Harvest Fungal Diseases of Avocado Fruit

5.1 Introduction

Avocado farming practices (both organic and conventional) use copper-based pre-harvest fungicides (occasionally with one or two treatments of benomyl per season) to control both pre- and post-harvest diseases of avocado fruit (Duvenhage, 2002). Copper (Cu) fungicides are broad spectrum and are able to control a wide range of fungal pathogens. An added advantage to Cu fungicides is their ability to adhere to the surfaces of fruits and leaves (Boshoff *et al.*, 1996).

The most common diseases of avocado fruit are the post-harvest diseases anthracnose, Dothiorella rot, Dothiorella/Colletotrichum complex (DCC) fruit rot and stem-end rot (SER) and the pre-harvest disease Cercospora spot (black spot) (Muirhead *et al.*, 1982; Darvis and Kotzé, 1987; Darvis *et al.*, 1987; Willis and Duvenhage, 2003). The pathogen causing avocado anthracnose is *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc. Dothiorella fruit rot is caused by the fungus *Dothiorella aromatica* (Sacc.) Petr. & Syd., whereas DCC fruit rot is caused by both *D. aromatica* and *C. gloeosporioides*. Cercospora spot is caused by the pathogen *Pseudocercospora purpurea* (Cooke) Deighton. The causal agents of SER are fungi and can vary between species, often involving more than one from different genera. The most common fungi causing SER in South African avocado fruit are *Thyronectria pseudotrichia* (Berk. M.A. Curtis) Seeler, *Dothiorella aromatica*, *Lasiodiplodia theobromae* (Pat.) Griffon & Maubl., *Phomopsis persea* Zerova, *Colletotrichum gloeosporioides* and/or several *Fusarium* spp. (Darvas and Kotzé 1987, Demoz and Korsten 2006).

Anthracnose is defined as a circular pink slime of *C. gloeosporioides* conidia. Mature lesions result in penetration and the liquefying of the underlying flesh (Darvas and Kotzé, 1987; Darvas *et al.*, 1987). DCC rot is similar to anthracnose at first but results in the fruit skin to discolour to a reddish brown at the infected areas. Discolouration is followed by a watery rot of the underlying flesh (Darvas and Kotzé, 1987). Dothiorella rot is a superficial discolouration of the fruit which darkens as the disease matures. The underlying flesh becomes dark-brown in colour (Darvas *et al.*, 1987). Stem-end rot (SER) migrates from the pedicel end of the fruit towards the base of the fruit internally. The vascular bundle is always affected as the fungal hyphae extend within the vascular systems resulting in discolouration, softening and rotting of the mesoscarp, usually accompanied by a foul odour (Darvas and Kotzé 1987). The symptoms of these four diseases (anthracnose, DCC, Dothiorella rot and SER) usually only appear upon ripening of the fruit. This makes it almost impossible to diagnose before shipment (export and local) to retail stores and results in much economic loss once ripened. depression of the fruit skin and later stages have a

Cercospora is diagnosed as a shiny, raised black lesion on the surface of fruit skin approximately 1-3 mm in diameter. As it matures it grows in size to approximately 6 mm, often with signs of cracking and corking. Very mature lesions often coalesce and become sunken with sporiferous areas in the centre (Darvas and Kotzé, 1987). Cercospora is the most problematic of avocado diseases in South Africa attributing to major loss of fruit to markets (D. Taylor, personal communication), but is easily diagnosed.

With the sub-tropical climates of several regions of South Africa and the high quality of fruit, South Africa has a large export market to the European Union (EU). However, concerns over the build-up of copper in orchard soils and the surrounding environments are increasing as a high concentration could potentially lead to unfit soil for production, adverse health risks, and adverse environmental risks. Therefore the European Union (EU) has new strict regulations on the maximum residues of copper fungicides which regulates the concentrations at which the fungicides are applied (Duvenhage, 2002; Willis and Duvenhage, 2003; van Eeden and Korsten, 2003; Silimela and Korsten, 2007). The

EU has also reduced the variety of additional chemical fungicides that are registered for use due to their potential or confirmed adverse health and environmental effects. These concerns and regulations have resulted in an urgent need for alternative and biologically safe fungicides.

This study determined the potential of an ethanolic extract of propolis (EEP) at a concentration of 5 mg ml^{-1}, to control Cercospora spots, anthracnose and SER diseases of avocado fruit (most economical important diseases on Roodewal farm). The EEP was assessed as both a post-harvest and pre-harvest fungicide on 'Fuerte' avocado fruit. The potential of EEP to control SER was also evaluated. Additionally, *in vitro* studies determined if EEP could prevent infection or inhibit established infections by *C. gloeosporioides* conidia.

5.2 Materials and Methods

5.2.1 Conditions and Dimensions of Orchard 7 on Roodewal Farm

The experimental site was at Roodewal farm in Nelspruit, South Africa (Figure 5.1) on orchard 7, which contained Fuerte avocado trees. Orchard 7 (Figure 5.2) was approximately 2.5 hectares and the trees were young and small (approximately 2-3 metres in both height and width) with approximately 2-3 metres between each tree. The size and distance of the trees allowed some ease in controlling the amount of each treatment per tree.

5.2.2 Partition, Experimental Design, and Experimental Procedure in Orchard 7

Three experimental treatments were implemented: 1) treated with bore-hole water (control); 2) treated with CopStar 120 SC (150 g/ 100 l copper hydroxide, Ag-Chem Africa) + a non-ionic extender sticker spreader Nu-Film 17 (Di-p-Menthene, Hygrotech) and 3) treated with 5 mg ml^{-1} EEP (see Chapter 3). This was to compare the efficacy of

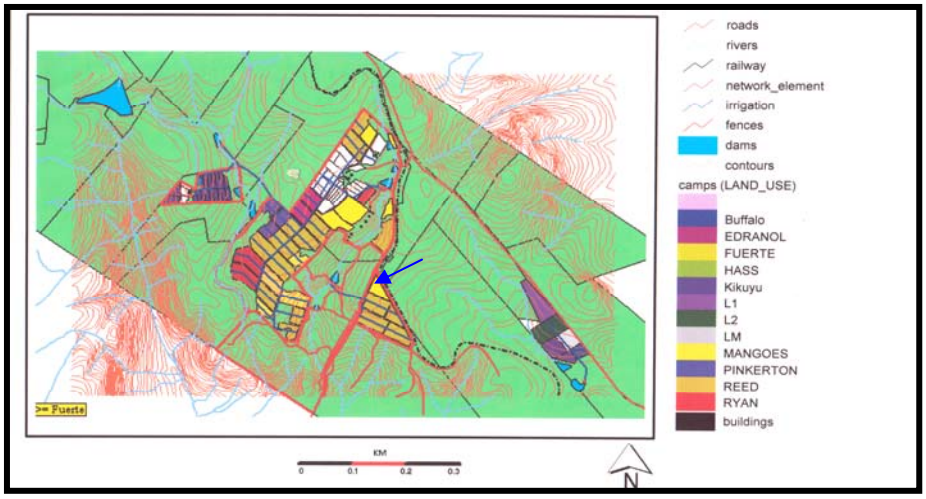


Figure 5.1: Geographic map of Roodewal farm, Nelspruit, South Africa, generated on ESRI ArcExplorer 1.1. Orchard 7 indicated by arrow.

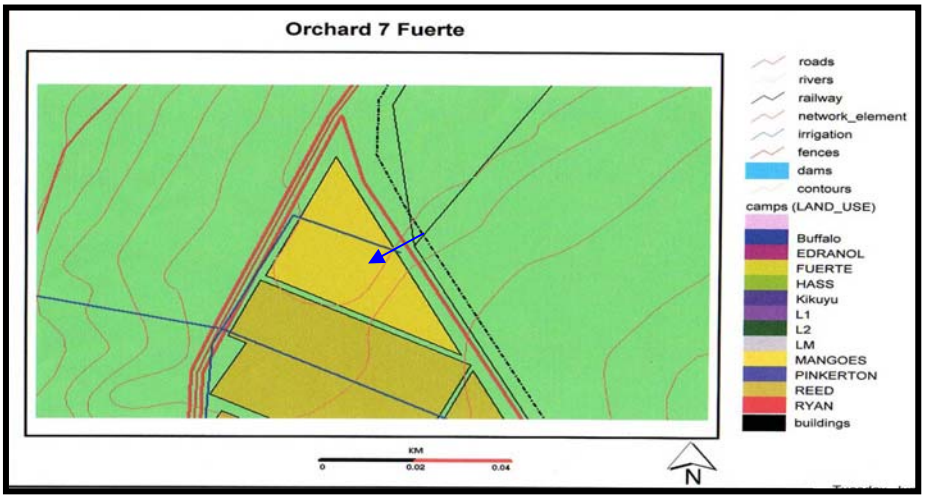


Figure 5.2: Geographic map of Orchard 7 of Roodewal farm, Nelspruit, South Africa, generated on ESRI ArcExplorer 1.1. Orchard 7 indicated by arrow. Size of Orchard 7 was 247.135 m X 199. 150 m X 338.774 m.

the antifungal properties of EEP with the efficacy of CopStar 120 SC. CopStar 120 SC is conventionally used at Roodewal farm.

Bore hole water and CopStar 120 SC were sprayed with a tractor pulled tank and pump connected to a hose with an adjustable nozzle at a pressure of 3000 kPa (30 bar). The EEP was sprayed with a backpack pump action hand gun.

Partitioning of the trees was set out to minimize contamination of the sprays by cross wind (Figure 5.3). The orchard was set out in grids of 6 rows of trees. A row of trees in a centre grid of the orchard was chosen as the experimental row (20 trees). The two rows on either side of the experimental row were sprayed with bore-hole water to serve as negative control as well as barriers against cross wind contamination of CopStar 120 SC. Within the experimental row, five randomly picked trees were sprayed with EEP until dripping (*ca* 4 l per tree). The trees partitioning each experimental tree sprayed with EEP were sprayed with bore-hole water as a control. The remaining trees in the orchard were sprayed with CopStar 120 SC.

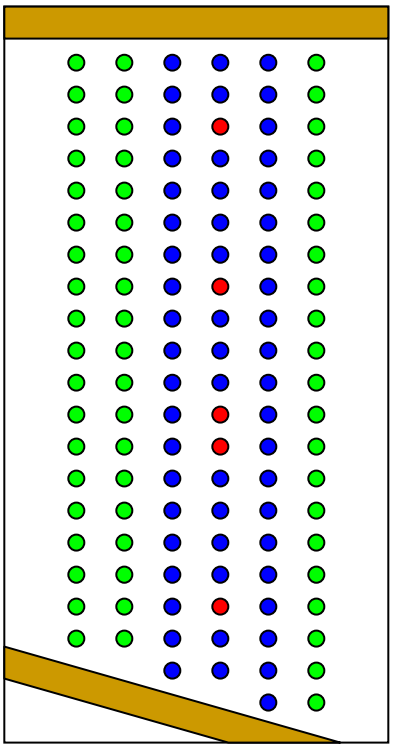


Figure 5.3: A representation of the grid of six rows of avocado trees used for field trials on Orchard 7, Roodewal farm. Circles represent the avocado trees. Red = treated with 5 mg ml^{-1} EEP. Blue = Treated with bore hole water. Green = Treated with CopStar 120 SC. Brown = boundary roads.

Within the CopStar 120 SC treated trees and bore-hole treated trees, 5 random trees as well as the five experimental trees sprayed with EEP were chosen for evaluations.

Sprayings occurred on the 24 October 2006, 28 November 2006, 3 January 2007, and the 6 February 2007.

5.2.3 Rainfall for the 2006-2007 Growing Season

High rainfall was obtained on Roodewal farm during August (32 mm), November (165 mm) and December (163 mm) 2006 and April (149 mm) 2007 compared to the fifteen year averages (11 mm, 114 mm, 143 mm and 51 mm respectively). Lower rainfall was obtained during September (8 mm), October (67 mm) 2006 and January (47 mm), February (91 mm) and March (43 mm) 2007 as compared to the fifteen year averages (32 mm, 73 mm, 123 mm, 124 mm and 115 mm respectively). No rainfall was obtained during May 2007. The fifteen year average rainfall for May was 14 mm.

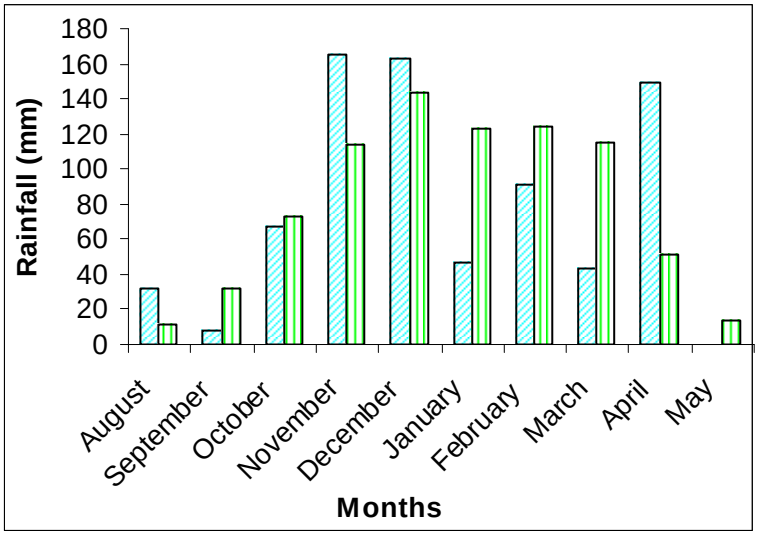


Figure 5.4: Monthly volumes of rainfall on Roodewal farm during the 2006-2007 growing season as compared to a 15 year average rainfall volumes for each month. ▨ = 2006-2007 rainfall season. ▯ = 15 Year average rainfall.

5.2.4 Evaluation of Fruits after Harvesting

All fruits from orchard 7 were harvested during early June 2007, standard commercial harvesting season. The five trees treated with 5 mg ml^{-1} EEP as well as five randomly picked trees each from the bore-hole water treatments and CopStar 120 SC treatments

trees were harvested and separated. Harvesting was achieved by hand cutting. Randomly chosen fruits (n = 120 for each treatment) were separated and placed in cardboard cartons of 20 fruits each (six cartons per treatment). The avocado fruit were passed through the packing line to be brushed off of all dirt, residue and sooty mould, as per conventional methods by Roodewal farm for both export and local markets.

All the sampled fruit were evaluated for disease based on Boshoff *et al.* (1996) disease index with modification. Fruits were evaluated for both Cercospora spots (Black spots) and anthracnose at a scale of 0-3, whereby 0 = no visual symptoms, 1 = less than 5 black spots/anthracnose, 2 = 5-10 black spots/anthracnose, 3 = more than 10 black spots/anthracnose. The percentage of fruits in each disease index were analysed statistically using ANOVA with Tukey-Kramer multiple comparison test (software GraphPad Instat version 3.0).

5.2.5 Simulations of Local and Export Markets

The cartons from the harvesting trials were divided into two groups of three cartons each, whereby one group was simulated for local markets, and the other for export markets. For local markets, the fruit were ripened at ambient temperatures (*ca* 20 °C ± 1 °C) for two weeks in the laboratory. To simulate shipment of fruit for export markets, cartons were stored at 7 °C for 28 days in the packhouse cold rooms. The fruits were ripened at ambient temperatures (*ca* 20 °C ± 1 °C) in the laboratory.

After ripening, fruits in both export and local market simulations were evaluated for diseases symptoms with the modified Boshoff *et al.* (1996) disease index. The percent of the fruit surface area infected was analysed based on Bautista-Baños *et al.* (2003) with modifications: 1 = 0 %, 2 = 1-20 %, 3 = 21-40 %, 4 = 41-60 %, 5 = 61-80 %, 80-100 %. This index was termed percentage disease fruit cover index (PDFC index). Both indices were each analysed using one-way ANOVA with Tukey-Kramer multiple comparison test (software GraphPad Instat version 3.0).

5.2.6 The Potential of EEP as a Dipper to Inhibit Post-Harvest Diseases of Avocado Fruit

'Fuerte' avocado fruit, treated with CopStar 120 SC during the growing season at Roodewal farm (Nelspruit, South Africa), were harvested on the 6 June 2007. Randomly selected fruit (n = 240) were packed into 12 cardboard cartons with 20 fruits in each. The diseases index was analysed on all the fruit based on the modified Boshoff *et al.* (2006) disease index. All fruit were passed through the packing line brushers to remove all dirt and sooty mould. Six cartons were dipped in 5 mg ml^{-1} EEP for 30 seconds (as instructed by Roodewal farm) and dried in ambient conditions. The remaining six cartons were untreated. Three cartons from each group were ripened at 20 °C (± 1 °C) to simulate local markets. The remaining three were stored at 7 °C for 28 days followed by ripening at 20 °C (± 1 °C) to simulate export conditions. After ripening, the fruits were evaluated by the disease index and the PDFC index. The experiment was not repeated.

The indices were each analysed using one-way ANOVA with Tukey-Kramer multiple comparison test (software GraphPad Instat version 3.0).

5.2.7 The Potential Use of EEP as a Post-Harvest Control Dip for Stem-End Rot

'Fuerte' avocado fruits were picked during the early months of February 2007. The pickings of the fruits were at most two months premature, in order to induce stem-end rot (SER). Symptoms of SER usually develp more frequently from immature fruits picked from Roodewal farm. Ten fruits were dipped at the stem-end in 5 mg ml^{-1} EEP for 15 s. Ten fruits were left untreated. Both sets of fruits were incubated at ambient temperatures for 14 days, until ripe. The fruits were cut in half and SER was assessed visually. Data was collected as a percentage of fruit with SER.

5.2.8 The Potential of EEP to Prevent Infection of *Colletotrichum*

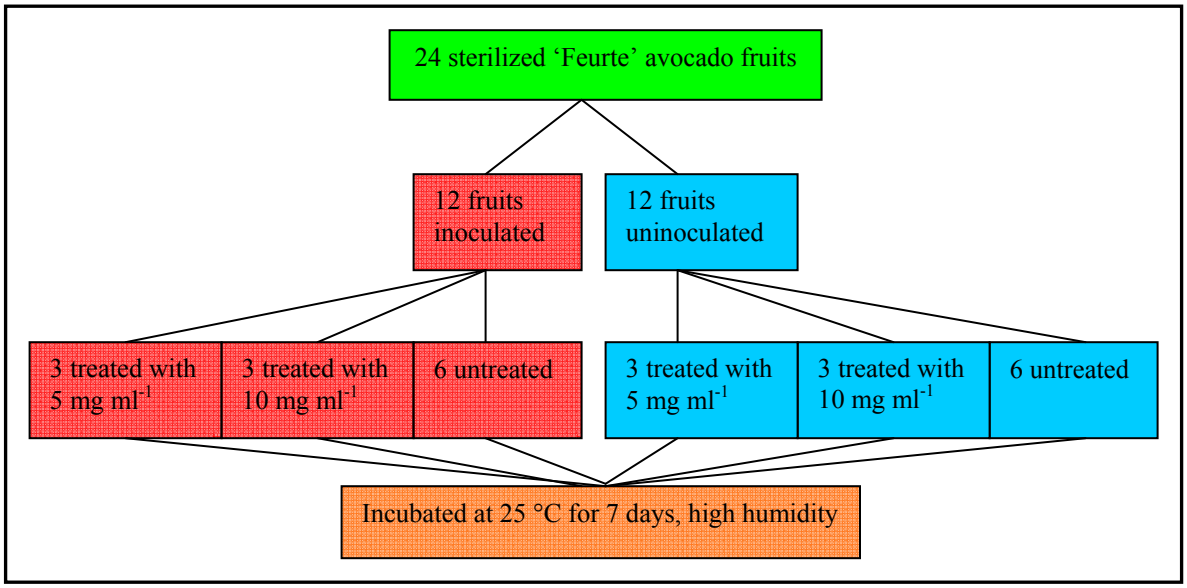


Figure 5.5: Diagrammatic representation of the experimental design for determining the potential of EEP to prevent infection of *Colletotrichum* to avocado fruit.

Ripe 'Fuerte' avocado fruit were surface sterilized by immersion in 1 % sodium hypochlorite (JIK) for 10 min followed by rinsing in water three times. The fruits were left to air dry. Fruits were further wiped with 70 % ethanol (Merck) and allowed to air dry. Three circular markings with permanent marker pens on the surface of each fruit were made as a guide for infection sites. A flamed sterilized needle of 2 mm length and 1 mm thick was used to pierce the fruit once within each marked circle. Twelve of the fruits were inoculated with 15 µl of *Colletotrichum* sp. conidia (1 x 10^5 conidia ml^{-1}) and air dried (Figure 5.5). Three of the inoculated fruits were treated with 5 mg ml^{-1} EEP, three treated with 10 mg ml^{-1} EEP and the remaining six were untreated. All treated fruits were treated by spraying EEP with a standard spray bottle and air dried. Uninoculated fruits were treated similarly to inoculated fruits. The fruits in each treatment were placed in aluminium trays with three Petri dishes with sterile distilled water and the lids cracked to allow increased evaporation. The water increased the humidity within the boxes during incubation to allow infection to occur. The boxes were loosely covered with cling-wrap

plastic to allow sufficient aeration. The fruits were incubated in 25 ºC for seven days until disease symptoms occurred.

5.2.9 The Potential of EEP to Inhibit Disease Symptoms of Infected Fruit by *Colletotrichum* Conidia.

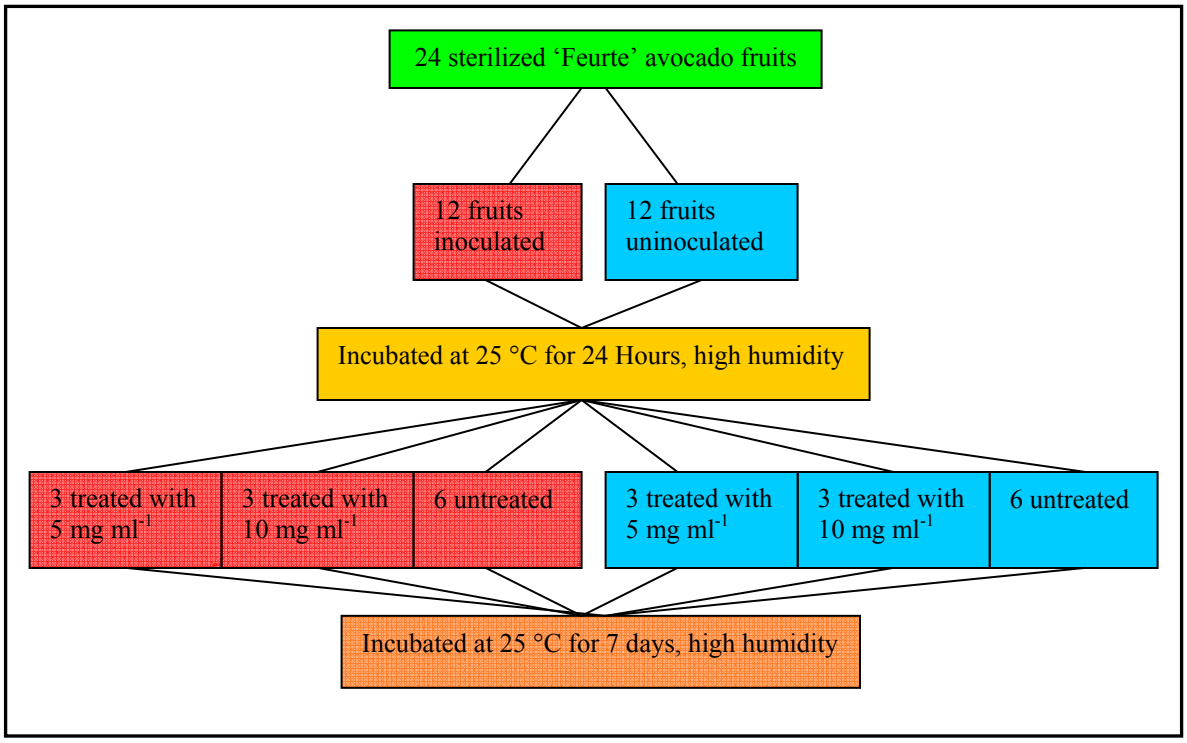


Figure 5.6: Diagrammatic representation of the experimental design for determining the potential of EEP to inhibit disease symptoms of infected fruit by *Colletotrichum* conidia.

Ripe 'Fuerte' avocado fruit were surface sterilized by immersion in 1 % sodium hypochlorite (JIK) for 10 min followed by rinsing in water three times. The fruits were left to air dry. Fruits were further wiped with 70 % ethanol (Merck) and allowed to air dry. Three circular markings with permanent marker pens on the surface of each fruit were made as a guide for infection sites. A flamed sterilized needle of 2 mm length and 1 mm thick was used to pierce the fruit once within each marked circle. Twelve of the fruits were inoculated with 15 µl of *Colletotrichum* sp. conidia (1 x 10^5 conidia ml^{-1}) (Figure 5.6). All fruits were placed in aluminium trays with three Petri dishes each with sterile distilled water and the lids cracked to allow increased evaporation. The boxes were loosely covered with cling-rap plastic to allow sufficient aeration. The fruits were

incubated for 24 hours to allow sufficient infection of the fruits. After incubation, three of the infected fruits were treated with 5 mg ml^{-1} EEP, three treated with 10 mg ml^{-1} EEP and the remaining six were untreated. Uninoculated fruits were treated similarly to inoculated fruits. The fruits were placed back into the aluminium trays with the Petri dishes and incubated in 25 ºC for seven days until disease symptoms occurred.

5.3 Results

5.3.1 EEP as a Pre-Harvest Treatment for 'Fuerte' Avocado Fruit

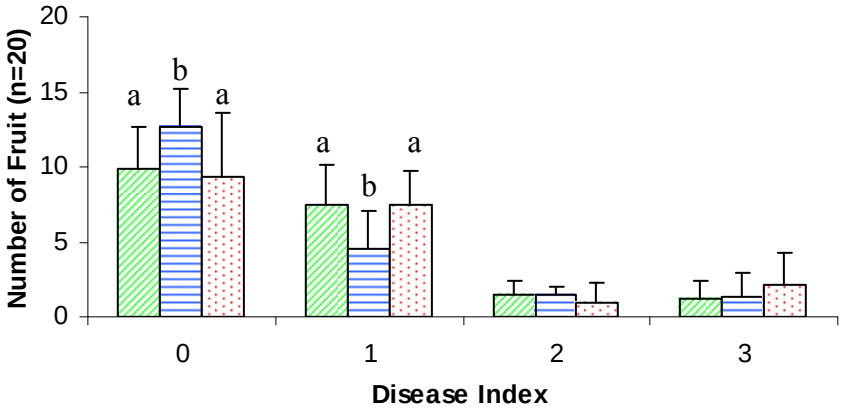


Figure 5.7: A comparison of the number of fruit (n=20), after harvesting, within each disease index from trees treated with 4 pre-harvest sprays of 5 mg ml^{-1} EEP ( ), CopStar 120 SC (copper hydroxide) ( ) and bore-hole water (control) ( ). Disease index 0 = no visual symptoms, 1 = less than 5 black spots/anthracnose, 2 = 5-10 black spots/anthracnose, 3 = more than 10 black spots/ anthracnose. Spraying occurred on 24 October 2006, 28 November 2006, 3 January 2007, and 6 February 2007. Standard deviation represented as Y-error bars. Significant differences within disease index indicated by different letters.

Figure 5.7 indicates the disease index for harvested fruit treated with 5 mg ml^{-1} EEP, CopStar 120 SC or bore-hole water (control) during the growing season. The CopStar 120 SC treated fruit had a significant higher average number of fruit (63.4 %) with no symptoms of disease (disease index 0) after harvesting compared to the EEP treated (49.2 %) ($P < 0.01$) and control (bore-hole water) fruit (46.7 %) ($P < 0.001$). However, CopStar 120 SC treated fruit (22.5 %) were significantly lower ($P < 0.01$) within disease index 1 than both EEP (38 %) and control fruit (38 %). EEP treated and control fruit were similar within all disease indices.

CopStar 120 SC treated fruits had the lowest percentage of 36.7 % overall fruits diseased compared to 50.8 % for EEP treated and 53.3 % for bore-hole water treated fruits.

Local Market Simulation:

Figure 5.8 indicates the disease index before and after ripening of fruit treated with 5 mg ml^{-1} EEP, CopStar 120 SC or bore-hole water (control) in a local market simulation. No significant differences ($P > 0.05$) were observed prior to ripening and after ripening in either the EEP treated fruit or the bore-hole water treated fruit within any of the disease indices. In contrast, a significant decrease in disease index 0 (33.3 % decrease) was observed in CopStar 120 SC treated fruit ($P < 0.05$). The only significant difference between any of the treatments after ripening was within disease index 1 where CopStar 120SC (25.0 %) was lower than the bore-hole water (58.4 %) treatments ($P < 0.05$). The highest decrease in disease index 0 (disease free fruit) after harvest was 67.9 % from the bore-hole water treated fruit. The lowest decrease within disease index 0 after ripening was 34.2 % from EEP treated fruit. CopStar 120 SC treated fruit expressed a 47.6 % decrease in disease index 0 after ripening. CopStar 120 SC treated fruits had the highest number of fruits before and after ripening within disease index 0. Disease index 2 and 3 were similar between all treatments, before and after ripening.

After ripening, the overall percentages of fruits diseased were 61.67 %, 53.33 % and 30 % for EEP, bore-hole water and CopStar 120 SC treatments respectively.

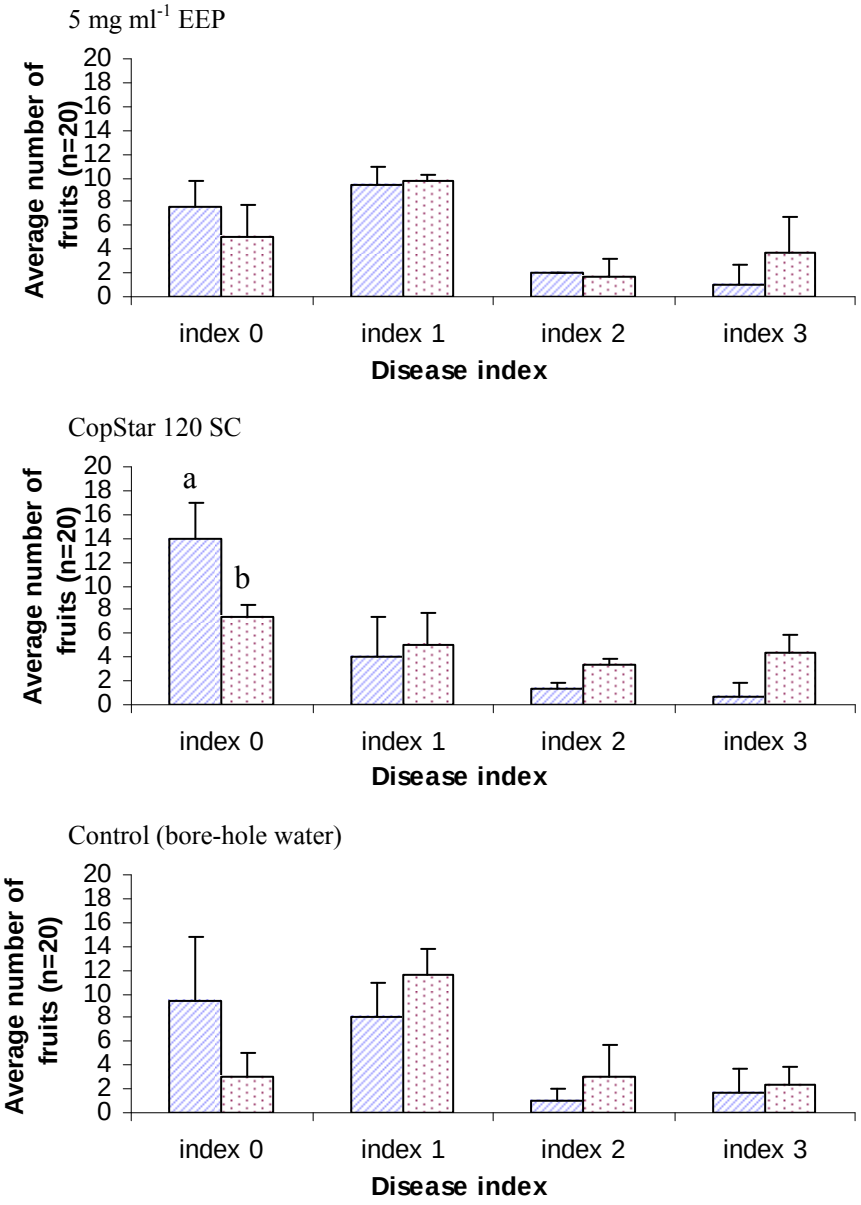


Figure 5.8: A disease index comparison of before ripening (▨) and after ripening (⁝) of avocado fruit treated with 5 mg ml^{-1} EEP, CopStar 120 SC, or bore-hole water (control) as a pre-harvest spray to control fruit fungal diseases. Fruit were ripened at 20 °C (± 1 °C) in a local market simulation. Disease index 0 = no visual symptoms, 1 = less than 5 black spots/anthracnose, 2 = 5-10 black spots/anthracnose, 3 = more than 10 black spots/ anthracnose. Spraying occurred on 24 October 2006, 28 November 2006, 3 January 2007, and 6 February 2007. Standard deviation represented as Y-error bars. Significant differences within disease index indicated by different letters.

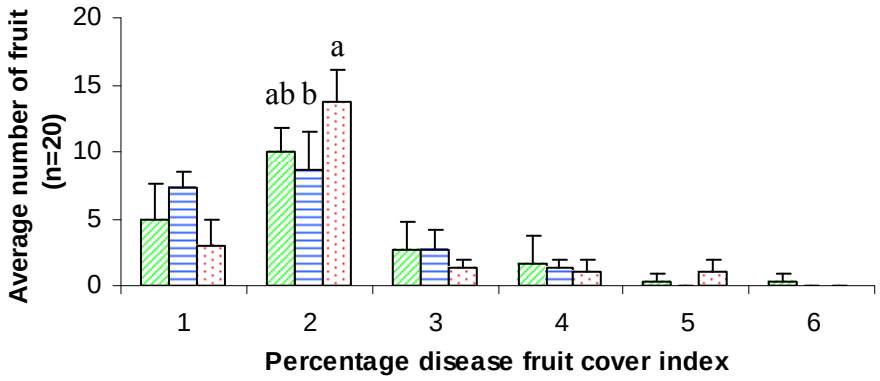


Figure 5.9: The percentage of disease covering the surface of ripe 'Fuerte' avocado fruits harvested from trees which had 4 pre-harvest sprays of 5 mg ml^{-1} EEP ( ), CopStar 120 SC (copper hydroxide) ( ), and bore-hole water (control) ( ). Fruit were ripened at 20 °C (± 1 °C) to simulate local markets. 1 = 0 % disease, 2 = 1-20 % disease cover, 3 = 21-40 % disease cover, 4 = 41-60 % disease cover, 5 = 61-80 % disease cover, 6 = 81-100 % disease cover. Standard deviation represented as Y-error bars. Different letters indicate significant difference within disease index.

The majority of fruits expressing disease symptoms in all treatments had 1- 20 % of the surface diseased (disease index 2) with the highest in bore-hole water treatment (68.4 %) and the lowest in CopStar 120 SC treated (43.4 %) (Figure 5.9). The EEP and CopStar 120 SC treated fruits within percentage disease fruit cover (PDFC) index 2 were similar, whereas, the bore-hole water treated fruits were significantly higher than the CopStar 120 SC treated fruit ($P < 0.05$). All treatments were similar within PDFC index 0, 1 and 3-6; however EEP treated fruits was the only treatment within PDFC index 6 (100 % of the fruit surface covered with disease).

Export Market Simulation:

Figure 5.10 indicates the disease index before and after ripening of fruit treated with 5 mg ml^{-1} EEP, CopStar 120 SC or bore-hole water (control) in an export market simulation. The number of fruits expressing no symptoms of disease (disease index 0) was significantly reduced after ripening in export simulations for all treatments ($P < 0.01$ for bore-hole water treatments and $P < 0.001$ for CopStar 120 SC and EEP treatments). CopStar 120 SC treated fruit decreased by 79 % within disease index 0. EEP had a 91.67 % decrease within disease index 0 after ripening, whereas bore-hole water treated

obtained a 100 % decrease. The only other significant difference in the treatments between before and after ripening was an increase by 30 % within disease index 2 of EEP treated fruits ($P < 0.05$).

Within each disease index the patterns were similar for all treatments. The overall percentage of diseased fruits after harvest was 88.33 %, 95 % and 100 % for EEP treatment, CopStar 120 SC treatment and bore-hole water treatment respectively.

All treatments were also similar within all PDFC indices for the export simulation (Figure 5.11). The highest amount of diseased fruit in each treatment indices were index 4 for EEP treatment (35.0 %), index 2 for CopStar 120 SC treatment (33.4 %) and index 3 for bore-hole water (41.7 %). The lowest amount of fruit in each treatment within the PDFC indices were index 6 for EEP treatment (1.7 %) and CopStar 120 SC treatment (6.7 %) and index 0 for bore-hole water treatment (0 %). Bore-hole water treatment index 3 was significantly higher than index 1, 5 and 6 ($P < 0.01$). EEP treatment index 4 was significantly higher than index 1 and 6 ($P < 0.01$). CopStar 120 SC treatment indices were not significantly different ($P > 0.05$) although highest values occurred in index 2 and 4.

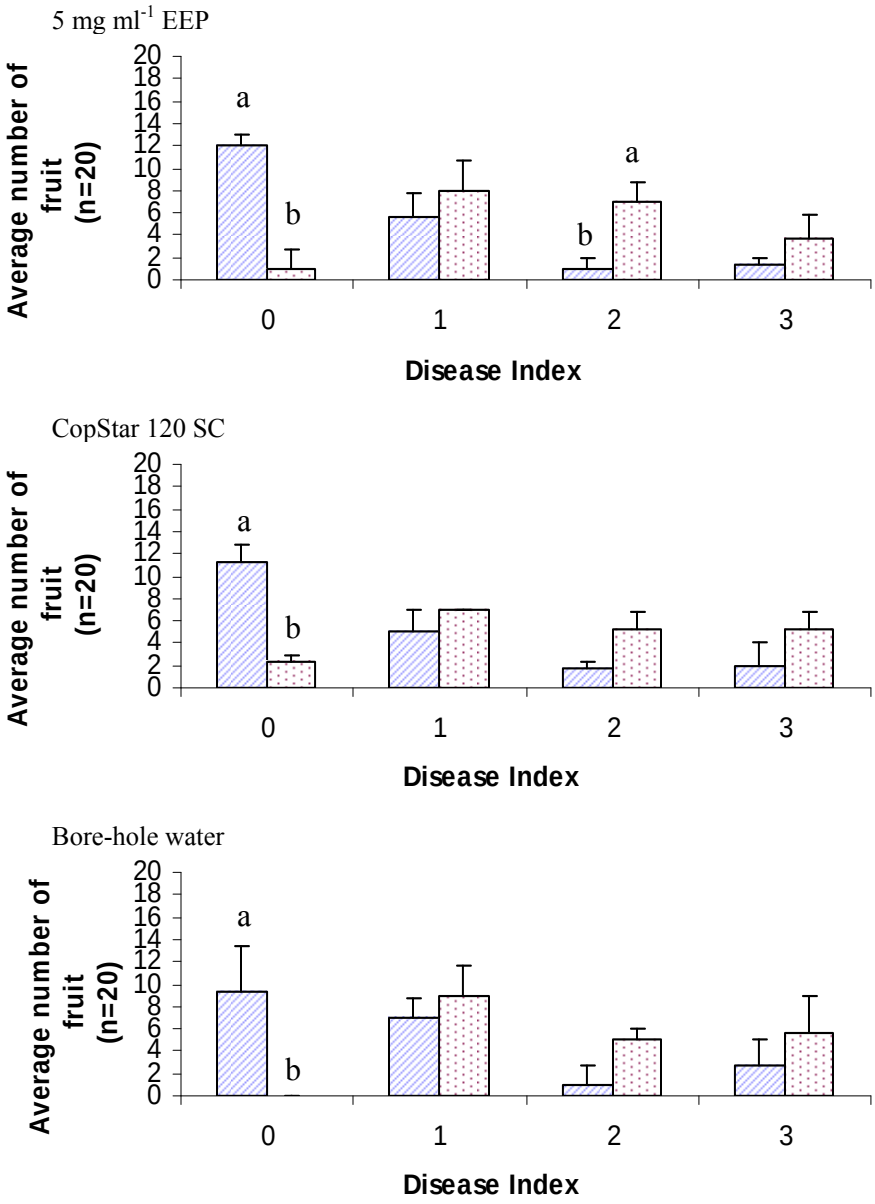


Figure 5.10: A disease index comparison of before ripening (▨) and after ripening (▩) of avocado fruit treated with 5 mg ml^{-1} EEP, CopStar 120 SC, or bore-hole water (control) as a pre-harvest spray to control fruit fungal diseases. Fruit were stored at 7 °C for 28 days and ripened at 20 °C (± 1 °C) as an export market simulation. Disease index 0 = no visual symptoms, 1 = less than 5 black spots/anthracnose, 2 = 5-10 black spots/anthracnose, 3 = more than 10 black spots/ anthracnose. Spraying occurred on 24 October 2006, 28 November 2006, 3 January 2007, and 6 February 2007. Standard deviation represented as Y-error bars. Significant differences within disease index indicated by different letters.

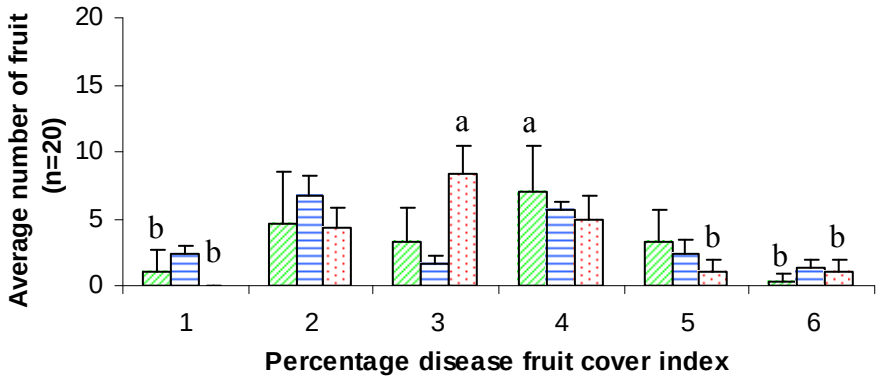


Figure 5.11: The percentage of disease fruit covering the surface of ripe 'Fuerte' avocado fruits harvested from trees which had 4 pre-harvest sprays of 5 mg ml^{-1} EEP (▨), CopStar 120 SC (copper hydroxide) (▤), and bore-hole water (control) (⁝). Fruit were stored at 7 °C for 28 days and ripened at 20 °C (± 1 °C) to simulate export markets. 1 = 0 % disease, 2 = 1-20 % disease cover, 3 = 21-40 % disease cover, 4 = 41-60 % disease cover, 5 = 61-80 % disease cover, 6 = 81-100 % disease cover. Standard deviation represented as Y-error bars. For each treatment, different letters indicate significant differences between disease indices ($P < 0.05$).

5.3.2 EEP as a Post-Harvest Treatment for 'Fuerte' Avocado Fruit

Local Market Simulation:

Figure 5.12 indicates the disease index before and after ripening of 'Fuerte' avocado fruit dipped in EEP (5 mg ml^{-1}) for 30 s, in a local market simulation. The control fruit were untreated. Dipped fruit and the control fruit before and after ripening were similar within all disease indices. The percentages of fruit diseased before ripening were 30.00 % and 46.67 % for the control group and experimental group of fruit respectively. After ripening, the percentages of fruits diseased were 63.33 % and 77.97 % for the control and dipped fruit respectively.

For the control treatments the only significant difference between before (70.0 %) and after (36.7 %) ripening was in index 0 ($P < 0.05$). Before ripening, the number of fruits in the control disease index 0 was significantly higher than the disease indices 1-3 ($P < 0.001$). However, after ripening the disease index 0 for the control was statistically similar to the disease indices 1-3 ($P > 0.05$).

In the EEP treatment before and after ripening, values were similar within all disease indices. The disease index 0 decreased from 51.7 % fruits before ripening to (21.7 %) fruits after ripening.

Disease index 3 increased from 3.35 % fruit to 21.7 % fruit and 1.7 % fruit to 21.7 % fruit for both the control and dipped fruit respectively. Disease index 2 increased from 6.7 % fruit to 16.7 % fruit and from 6.7 % fruit to 28.4 % fruit for the control and dipped fruit respectively.

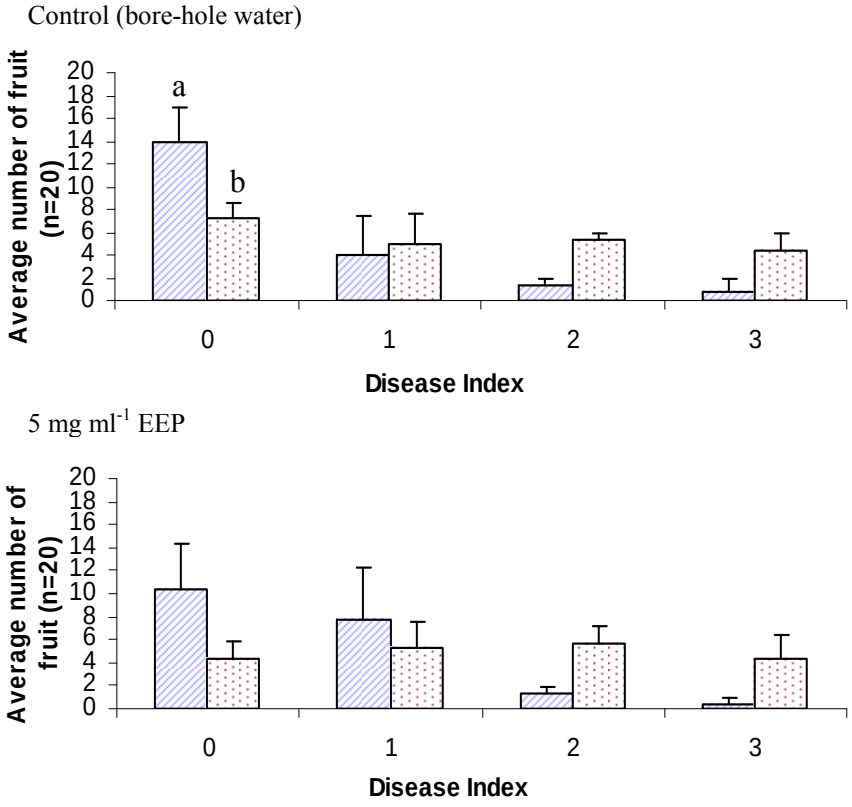


Figure 5.12: A comparison of before ripening (▨) and after ripening (▩) of avocado fruit treated with 5 mg ml^{-1} EEP as a post-harvest dipper to control post-harvest diseases, in a local market simulation. Disease index 0 = no visual symptoms, 1 = less than 5 black spots/anthracnose, 2 = 5-10 black spots/anthracnose, 3 = more than 10 black spots/ anthracnose. Fruit were ripened at 20 °C (± 1 °C). Standard deviation represented as Y-error bars. Significant differences within disease index indicated by different letters.

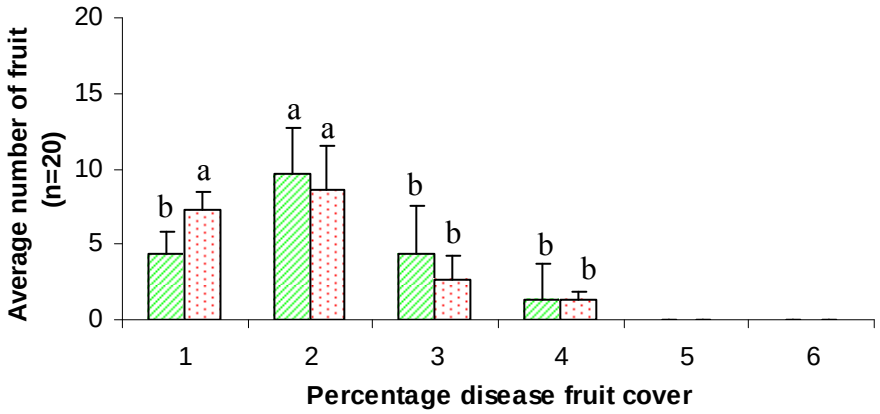


Figure 5.13: The percentage of disease covering the surface of ripe 'Fuerte' avocado fruits dipped in 5 mg ml^{-1} EEP for 30 s (▨) or control with no treatment (▨). Fruit were ripened at 20 °C (± 1 °C) to simulate local markets. 1 = 0 % disease, 2 = 1-20 % disease cover, 3 = 21-40 % disease cover, 4 = 41-60 % disease cover, 5 = 61-80 % disease cover, 6 = 81-100 % disease cover. Standard deviation represented as Y-error bars. For each treatment, different letters indicate significant differences between disease indices ($P \leq 0.05$).

No fruits expressed surface disease within PDFC index 5 or 6 from the control or dipped treatments in the local simulation (Figure 5.13). Both treatments had the highest number of fruits in index 2 with 48.4 % and 43.4 % fruits for EEP dip treated and control fruits respectively. Index 2 was significantly higher than index 4, 5 and 6 for EEP dip treatments ($P < 0.001$). Control treatment index 2 was significantly higher that index 3, 4, 5, and 6. Control treatment index 1 was significantly higher than index 4, 5, and 6. Both treatments were similar within all PDFC indices.

Export Market Simulation:

Figure 5.14 indicates the disease index before and after ripening of 'Fuerte' avocado fruit dipped in EEP (5 mg ml^{-1}) for 30 s, in an export market simulation. The control fruits were untreated. The total percentage of fruits diseased before ripening was 30.00 % and 43.33 % for the control group and experimental group of fruits respectively (not significant, $P = 0.140$). However, after ripening the dipped fruit had significantly higher percentage ($P = 0.013$) of diseased fruit (98.33 %) compared to the control fruit (88.33 %).

The disease index 0 for both the control and dipped treated fruit before ripening showed a significant decrease after ripening with a 45 % decrease and 68.35 % ($P < 0.001$) respectively. However, a significant increase ($P < 0.05$) of 26.7 % within disease index 1 occurred in the EEP treatment. EEP treatments disease index 0 was significantly lower than disease indices 1 ($P < 0.01$) and 2 ($P < 0.05$) after ripening, whereas disease index 1 was significantly higher than disease index 3 ($P < 0.05$) after ripening. Control treatment disease index 0 was significantly lower than disease index 1 ($P < 0.05$) after ripening.

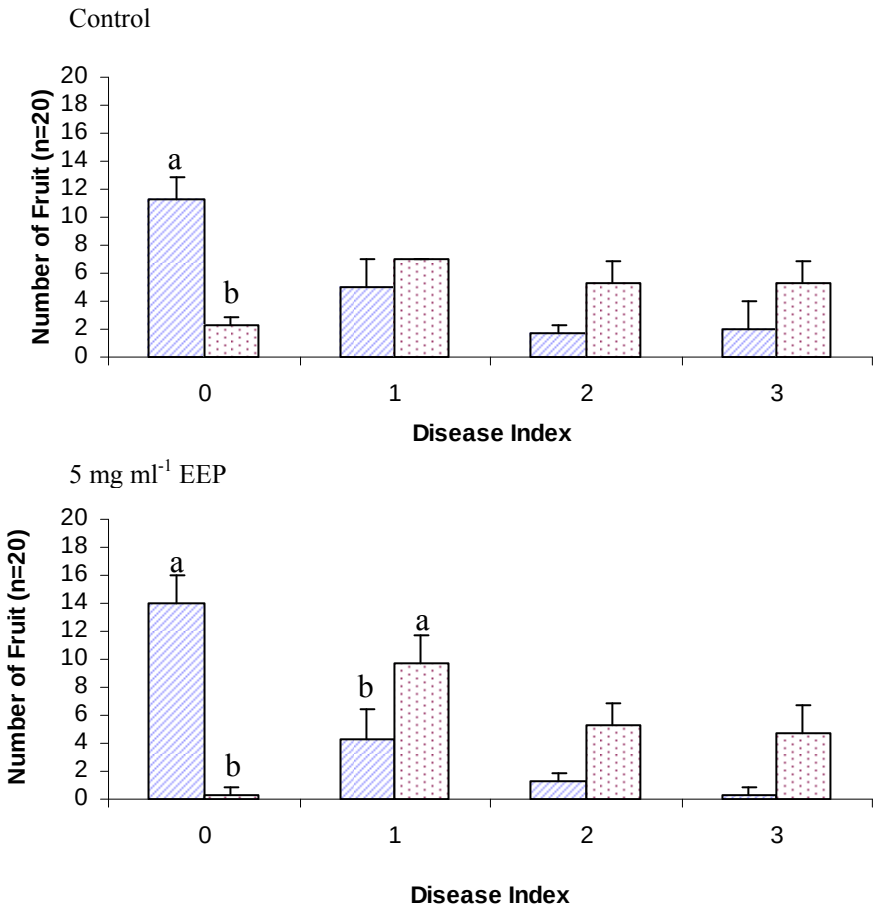


Figure 5.14: A comparison of before ripening (▨) and after ripening (⁚) of avocado fruit treated with 5 mg ml^{-1} EEP as a post-harvest dip to control post-harvest diseases, in an export market simulation. Disease index 0 = no visual symptoms, 1 = less than 5 black spots/anthracnose, 2 = 5-10 black spots/anthracnose, 3 = more than 10 black spots/ anthracnose. Fruit were stored at 7 °C for 28 days before ripening at 20 °C (± 1 °C). Standard deviation represented as Y-error bars. Significant differences between 'before' and 'after' ripening indicated by different letters.

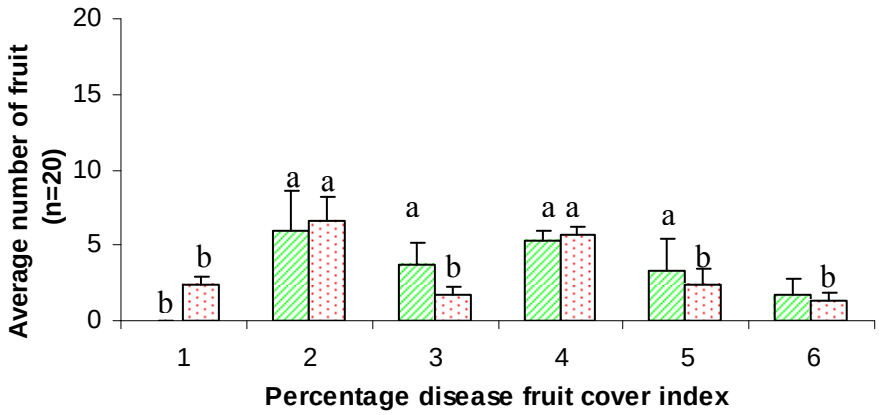


Figure 5.15: The percentage of disease covering the surface of ripe 'Fuerte' avocado fruits dipped in 5 mg ml^{-1} EEP as a post-harvest dipper to control post-harvest diseases. Fruit were stored at 7 °C for 28 days and ripened at 20 °C (± 1 °C) to simulate export markets. 1 = 0 % disease, 2 = 1-20 % disease cover, 3 = 21-40 % disease cover, 4 = 41-60 % disease cover, 5 = 61-80 % disease cover, 6 = 81-100 % disease cover. ▨ = fruits dipped in 5 mg ml^{-1} EEP for 30 s. □ = control fruit with no post-harvest treatments. Standard deviation represented as Y-error bars. For each treatment, different letters indicate significant differences between disease indices ($P \leq 0.05$).

EEP and control treatments were similar within all the PDFC indices (Figure 5.15). Both treatments had the highest number of fruits within PDFC index 2 (1-20 % of the fruit surface expressing disease) at an average of 30 % and 33.35 % respectively. The control treatment PDFC index 2 was significantly higher than index 1 ($P < 0.05$), 3 ($P < 0.01$), 5 ($P < 0.05$) and 6 ($P < 0.01$). The EEP dip treatment PDFC index 2 was significantly higher than index 1 ($P < 0.001$) and 6 ($P < 0.05$). No fruits from the EEP treatment were within PDFC index 1 (fruits expressing no disease symptoms).

5.3.3 The Potential of EEP as a Post-Harvest Dip to Control SER

Sample number for the control and EEP treated fruits were only 10. Nevertheless, 40 % of the untreated fruit showed SER symptoms and in contrast 10 % of the fruit dipped stem-end in EEP (5 mg ml^{-1}) showed symptoms of SER (Figure 5.16). This related to a 30 % reduction of the occurrence of SER.

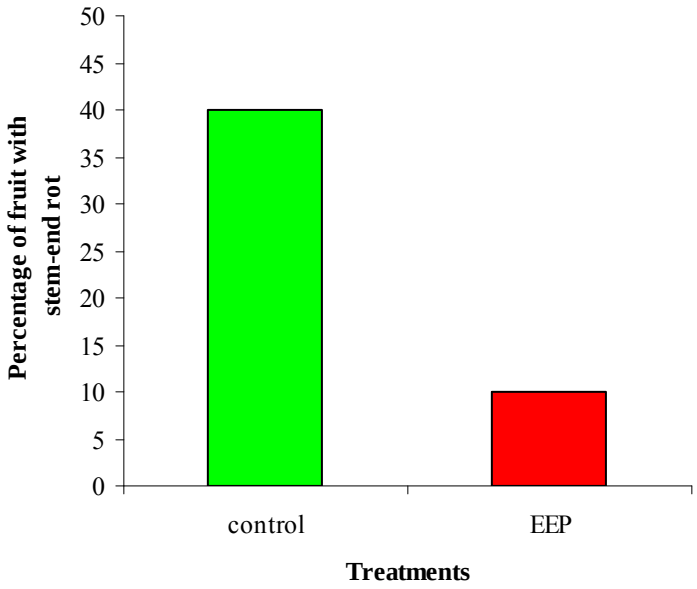


Figure 5.16: The percentage of Stem-end rot occurrence within 'Fuerte' avocado fruit (n=10) treated with either EEP or no treatment (control) and ripened at ambient temperature for 14 days. Control had 40 % occurrence of SER. EEP treated fruit had 10 % occurrence of SER.

5.3.4 The Potential of EEP to Prevent Infection of *Colletotrichum* Conidia

Figure 5.17 demonstrates the assay that determined the possibility of EEP to prevent infection of avocado fruit by *Colletotrichum* sp. conidia. No differences were observed between 5 and 10 mg ml^{-1} treatments. Both uninoculated treatments expressed no signs of disease. Both inoculated treatments were similar. With a treatment of 5 mg ml^{-1} an average of 66.7 % infections occurred and an average of 77.7 % infections occurred without treatment. With 10 mg ml^{-1} EEP treatment 55.7 % infections occurred and without treatment 100 % infections occurred.

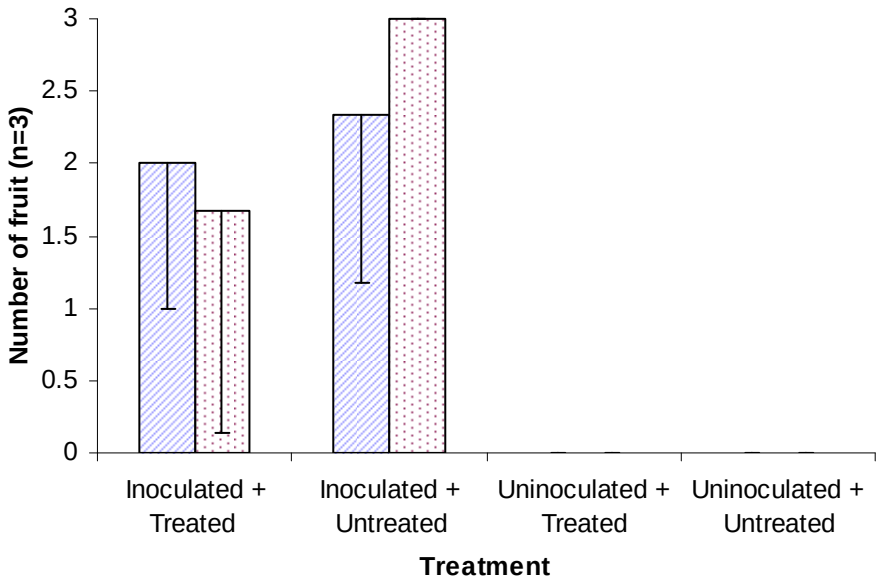


Figure 5.17: The potential of EEP to prevent infection of avocado fruit by *Colletotrichum* sp. conidia. 'Fuerte' avocado fruit were infected with *Colletotrichum* spores and treated with either 5 mg ml^{-1} ( ) or 10 mg ml^{-1} ( ) EEP. Standard deviation represented by Y-error bars.

5.3.5 The Potential of EEP to Inhibit Disease Symptoms of Infected Fruit by *Colletotrichum* Conidia.

Figure 5.18 demonstrates the assay that determined the potential of EEP to inhibit disease symptoms of infected avocado fruit by *Colletotrichum* sp. conidia. Both 5 and 10 mg ml^{-1} EEP treatments were similar with respect to decreasing the levels of disease symptoms. The uninfected fruits expressed no symptoms of disease. The infected untreated fruits expressed noticably higher levels of disease than infected fruits treated with 5 and 10 mg ml^{-1} EEP.

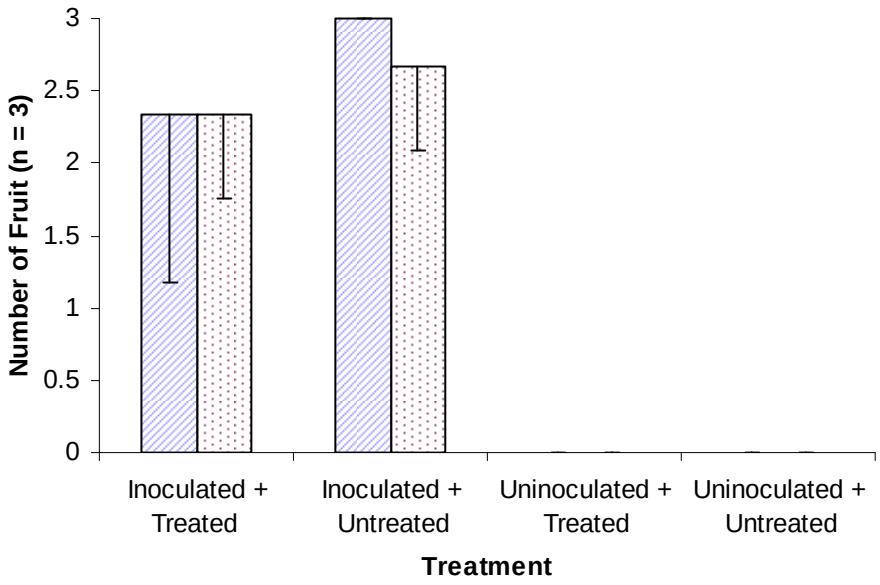


Figure 5.18: The potential of EEP to inhibit disease symptoms of infected avocado fruit by *Colletotrichum* sp. conidia. 'Fuerte' avocado fruit were infected with *Colletotrichum* spores and treated with either 5 mg ml^{-1} ( ) or 10 mg ml^{-1} ( ) EEP after 24 hour incubation. Standard deviation represented by Y-error bars.

5.4 Discussion

When deciding upon the applications of a fungicide, one should assess what application methods are to be used (van Eeden and Korsten, 2003). An efficient and optimal method of fungicide application would decrease the inoculum levels of the pathogens in the field and decrease further infections, thereby optimizing the function of the fungicide (Sanders and Korsten, 1999).

The standard method of fungicide application is to spray the fungicides under ultra high volume with an average of 10 000 *l* of the fungicide per ha per application (Duvenhage and Köhne, 1999). To reduce the amount of volume sprayed onto avocado orchards, Duvenhage and Köhne (1999) evaluated and compared the use of thermal fogging to ultra-high volume sprays to control the pre-harvest disease Cercospora spot. They evaluated and compared two copper based fungicides (Cu oxychloride and Cu ammonium carbonate) and three systemic triazole fungicides (flusilazole, benomyl, and carbendazim). A glycol carrier fogging enhancer, VK II 2, was added to the fungicides

for thermal fogging. The systemic fungicides and Cu oxychloride under ultra-high volume sprays resulted in high control of Cercospora spot, whereas Cu ammonium carbonate was not as effective. The best control was from Cu oxychloride resulting in 93 % of fruit expressing no disease symptoms. Both copper fungicides resulted in 97 % of disease free fruit under thermal fogging application, however only in low disease pressure orchards. The best thermal fogging control of Cercospora spot was from the systemic fungicides. However, the thermal fogging application was unable to penetrate deep within the foliage of large trees. This resulted in high control of outside fruit but no control of fruit within the foliage of the trees. Duvenhage and Köhne (1999) concluded that the most efficient method was standard fungicide Cu oxychloride applied at ultra-high volumes.

Similarly, Rowell (1986) compared mist blowers to hand guns (high volume spray) for the application of fungicides. Rowell (1986) showed that the mist blowers were unable to penetrate the foliage and resulted in high diseased fruit as compared to the hand gun.

The use of a hand held pump-action sprayer to apply the EEP was sufficient in penetrating the foliage and covered the surface of fruits and leaves evenly. The droplets of the EEP were small and easily managed. Surprisingly, the volumes of EEP needed to efficiently cover the trees were an average of 4 l compared to the average 13-16 l of the ultra-high volume spray for CopStar 120 SC. The EEP could therefore possibly be efficiently applied to the avocado trees with low-volume, high pressured hand guns.

After harvesting from the pre-harvest field trials, no control of the pre-harvest disease Cercospora spot was observed from the EEP pre-harvest treatment. The number of disease-free fruit and the overall percentage of diseased fruit from EEP pre-harvest treatments were similar to bore-hole treated fruit (control). Similarly, upon evaluation of local and export markets, no effective control was observed by EEP on post-harvest diseases. Interestingly, CopStar 120 SC did not result in effective control of highly diseased fruit (disease index 2 and 3) but only resulted in changes between disease index 1 and 0 before and after harvest. The PDFC indices also showed no effective control on

high spreading of diseases (PDFC indices 4-6) by CopStar 120 SC. However, very few fruit expressed 100 % of the surface diseased by both pre- and postharvest diseases from all pre-harvest treatments.

In a similar study, over two growing seasons, Duvenhage (2002) compared several alternative fungicides to standard Cu oxychloride for the control of avocado diseases. The fungicides tested were lime sulphur, salicylic acid, Westfalia Biocoat (organic acids), flusilazole, Avogreen (*Bacillus subtilis*), three strobulorins (Stroby, Flint and Ortiva), and Solanicure (*Bacillus* sp.). Two sprays of Cu oxychloride and an alternation of Cu oxychloride and salicylic acid gave the best control of Cercospora spot with over 90 % of the fruits expressing no symptoms. High levels of Cercospora control were also obtained by the alternation of Cu oxychloride and lime sulphur, Ortiva, Avogreen, or Solanicure. However, no control of Cercospora spot was achieved by use of Westfalia Biocoat, Stroby or flusilazole. Anthracnose was controlled by Cu oxychloride, Flint, Westfalia Biocoat and alternations of copper oxychloride and Flint or Ortiva. Duvenhage (2002) successfully demonstrated the effectiveness of alternative fungicides, mixture of reduced copper fungicides and alternative fungicides and the alternation of standard copper and the alternative fungicides. The alternations of fungicides gave a higher control than the fungicides alone or mixtures of Cu and the alternative fungicides.

In comparison, Willis and Duvenhage (2003) compared the efficacy of chlorothalonil, Ortiva, Thiovit Jet (sulphur based), Cu oxychloride and mixtures of Cu oxychloride and ferric chloride, chlorine dioxide or a QAC combination product (Prasin Agri) to control post-harvest diseases of avocado. Each treatment was applied by ultra high volume to 7 trees for each treatment. Fruits were ripened at 23 °C. The best control of Cercospora spot was from two to three applications of Cu oxychloride during the growing season. Ortiva or Cu oxychloride with Ferric chloride gave little control, however no other fungicide resulted in control of Cercospora spot. However, anthracnose was control by Cu oxychloride (9.77 % occurrence of anthracnose), and a lowered concentration of Cu oxychloride with added chlorine dioxide (5.96 % occurrence of anthracnose) or Ortiva

(3.58 % occurrence of anthracnose). Willis and Duvenhage (2003) concluded Cu oxychloride as the most efficient fungicide.

Both the Duvenhage (2002) and Willis and Duvenhage (2003) study demonstrated the effectiveness of alternative fungicides, mixture of reduced copper fungicides and alternative fungicides or the alternation of standard copper and the alternative fungicides. The alternations of fungicides gave a higher control than the fungicides alone or mixtures of Cu and the alternative fungicides in the Duvenhage (2002) study. Reducing the concentrations of copper fungicides will meet the EU regulations, and therefore the mixing of an alternative fungicide with Cu-based fungicides seems a promising solution. The mixing of EEP and Cu hydroxide could be a potential solution to maintaining efficacy of disease control and should be evaluated.

Boshoff *et al.* (1996) sprayed Cu oxychloride on 'Hass' and 'Fuerte' avocado trees at monthly intervals to assess critical times for treatments during the growing season (trials ran from September to March). Fruit were stored at 5.5 °C to simulate export condition before ripening. They assessed fruits for both pre- and post-harvest diseases. One spray in March was similar to untreated fruit with a high level of Cercospora spot. However all the treatments significantly reduced SER and DCC on Fuerte fruit with the most effective treatment from September to March. However, no significant control of anthracnose was observed. Anthracnose was controlled in the Hass avocados with effective control from September to March, November to March, December to March, and March treatments. Boshoff *et al.* (1996) showed that the incidence of disease correlated with the rainfall season and the critical times to spray the trees were during the months of high rainfall. Boshoff *et al.* (1996) indicated that fungicides should be applied on avocado trees when humidity, rain and temperatures are high.

Similarly, Darvas and Kotzé (1979) showed that Cercospora spot of avocado increased during months of high rainfall and warm temperature. Fruit on the trees were covered with paper bags and exposed to natural infections. The concentration of *P. purpurea* conidia were assessed by using a Hirsch type spore trap. Interestingly, the conidial

concentrations of *P. purpurea* significantly decreased when temperatures were low even with high rainfall. Most conidia were trapped during the early hours of the morning from 01h00 to 06h00. Most importantly, Darvas and Kotzé (1979) indicated that the latent period of Cercospora was longer than 3 months.

The Boshoff *et al.* (1996) and Darvas and Kotzé (1979) studies demonstrated how high rainfall, high humidity and high temperatures increase the disease pressure within avocado orchards. For this reason, spraying of fungicides commence from October to February. The season for high rainfall is usually during the summer months (October to February) in which temperatures can average 30 °C at Roodewal farm. Roodewal farm had high rainfall during the months of November 2006, December 2006 and April 2007 (Figure 5.4). These three months were higher than the 15 year average with 165, 163 and 149 mm rain respectively compared to the respective 114, 143 and 51 mm from the 15 year average.

Early infections of fruit within the growing season would result in larger, more mature lesions of Cercospora spot after harvesting during June/July. Few of the fruit usually express symptoms of post-harvest diseases after harvesting. These fruit usually result in a greater percentage of fruit with higher disease and PDFC indices once ripened. However, the majority of fruits from the pre-harvest trials expressed low disease indices (0-1) and low PDFC indices (1-3). Evidently, the majority of infections were later within the growing season. April was the only month late in the season with high rainfall and no fungicidal applications. Therefore, the majority of infections possibly could have been within the month of April. EEP had no sticker and possibly could have been washed off during the late seasonal rains. This would have resulted in a lack of fruit protection and the resulting high infections of the fruit. The EEP should have been applied to the trees at least once more later within the season to provide sufficient protection. This study had therefore confirmed the assertions of Sander and Korsten (1999) and Boshoff *et al.* (2003) that an adequate spray programme for the application of fungicides is vital to the control of avocado diseases.

An alternative solution to improve EEP efficacy could be the addition of a sticker-spreader to the EEP-water mix. The sticker increases the adhesion of the fungicide to plant surfaces, thereby reducing the amount of fungicides washed of by rain or irrigation (Silimela and Korsten, 2007; D.Taylor, personal communication). Denner and Kotzé (1986) determined the efficacy of three stickers (Nu-Film, Triton-1956-B, and Agral 90) to improve the distribution and adherence of Cu oxylchloride and benomyl on avocado leaves. All the stickers effectively distributed both fungicides. However, Agral 90 lowered the surface tension spreading the fungicides more evenly. Surprisingly, Nu-film increased the antifungal effect of benomyl *in vitro*. Conversely, Denner and Kotzé (1986) concluded Nu-Film as the most efficient sticker as it maintained a high concentration of the fungicides on the leaves. Today, Nu-film is conventionally used on many avocado farms, especially Roodewal farm (D. Taylor, personal communication). The addition of Nu-Film to EEP should be evaluated further.

No effective control from EEP as a post-harvest dip was observed within the local and export simulation. Surprisingly, EEP significantly increased the occurrence of post-harvest diseases within the export simulation. However, the PDFC indices indicate no dissimilarity between the dipped and untreated fruits.

Surprisingly, EEP as a post-harvest dip reduced SER by 30 %. However, due to low replication numbers, statistics could not be performed. This study, however has demonstrated a high potential for EEP to inhibit SER and further studies with high replication numbers should be evaluated. There is no effective post-harvest product on the market for organic farmers to reduce SER (D. Taylor, personal communication). SER is a problematic disease in that fruits with SER can not be diagnosed before ripening. Therefore SER poses great economic loss to avocado industries. A product that would effectively control SER alone could therefore increase marketable fruit and hence the economics of the avocado industry.

In a similar study, Muirhead *et al.* (1982) compared four post-harvest fungicides (prochloraz, carbendazim, CGA 64251 and guazatine) for the control of post-harvest

diseases of avocado fruit. Fruit were dipped into the fungicides for 5 minutes. The treated fruits were either dipped or sprayed with ethylene (150-300 ppm) to induce even ripening. Fruit were ripened at 25 °C. Prochloraz was the only fungicide to control anthracnose with 0.02-0.2 % of fruits infected, however all the fungicides controlled mild SER. Prochloraz, CGA 64251 and carbendazim controlled severe SER with 0-4 % of fruits infected. Prochloraz was further assayed at different concentrations (0.125, 0.25, 0.5 and 0.1 g acl^{-1}) and at different dipping times of 0.5, 1.0, 2.0 and 4 min to control post-harvest diseases. Concentrations of 0.25-1.0 g acl^{-1} and at all times resulted in high control of anthracnose (ratings were 0.6 or below). However, prochloraz did not control SER. Muirhead *et al.* (1982) concluded that the most efficient fungicide was prochloraz resulting in 80 % of disease free fruit and 100 % acceptable fruit.

Similar to Muirhead (1982), the EEP should be evaluated at different time intervals. As instructed by Roodewal farm the fruits were only dipped for a short period as it would be economically feasible. A longer dipping period could result in higher efficacy of EEP. The EEP concentration of 5 mg ml^{-1} was determined by *in vitro* assays (chapter 3). However, higher concentrations of EEP, such as 10 mg ml^{-1} (see chapter 3) should be evaluated for higher efficacy.

For control of SER, fruits were dipped for a very short time as conventional farming practises press the stem-ends of the fruits into a sponge soaked with the fungicide, usually prochloraz (D. Taylor, personal communication). A higher concentration of EEP could improve the efficiacy of SER control. Alternatively, longer dipping times could be assessed for inmproved SER control.

Infection studies of avocado fruits to determine if EEP could either prevent or inhibit *C. gloeosporioides* infection and disease spread was low in replication number and thus data showed high variation. However, inhibition of the pathogen's conidia to infect the fruit as well as to extend the latent period of the disease was observed at both 5 and 10 mg ml^{-1}. The higher concentration of EEP showed a slight higher inhibition of infection than the 5 mg ml^{-1}. This study has confirmed that EEP had the potential to inhibit pathogens

infecting fruit within the field thereby potentially reducing the amount of disease. The EEP had the potential to delay the latent phase of the post-harvest diseases by inhibiting the fungi after infection. This potentially could result in slightly prolonged storage, which would benefit retailers.

5.5 Conclusions:

- A spraying programme of every fifth week with 5 mg ml^{-1} EEP on avocado orchards proved ineffective to control pre- and post-harvest diseases of avocado fruit
- EEP at a concentration of 5 mg ml^{-1} proved a possible post-harvest dipper to control Stem-End Rot of avocado fruit.

Chapter 6

Final Conclusions

South Africa has a large export market in avocado fruit to Europe. According to the Fruit Produce Export Forum of South Africa (fpef-sa), during the 27th, 29th and 30th shipping weeks of the 2006 export season more than 500 000 cartons, 4 kg each, of avocado fruits were exported to the EU (http://www.fpef.co.za/exstatisticssubtropical.htm). With South Africa playing a major role in avocado export, strict regulations are placed on the avocado farming practices to ensure high quality fruit. This also ensures that South Africa maintains good export status to the receiving countries. The regulations are usually based on EU regulations and set in place by the South African Avocados Growers Association (SAAGA) and fpef-sa.

However, an ever increasing awareness by consumers of the health and environmental effect of chemical sprays on fruit to control disease results in more demand for healthier and safer produce, but still maintaining quality (van Eeden and Korsten, 2003). This has resulted in fewer chemicals allowed for application to control diseases as well as an increasing demand to reduce the number of applications of chemicals (van Eeden and Korsten, 2003).

The most economically important diseases of avocado fruits are anthracnose, Cercospora spot, Dothiorella rot, and Dothiorella/Colletotrichum fruit rot (DCC rot) (Darvis and Kotzè, 1987, Willis and Duvenhage, 2003, van Eeden and Korsten, 2003). These diseases are mainly controlled by copper based fungicides, such as copper hydroxide and copper oxychloride, by both organic and conventional farmers. Depending on the cultivar, two to five sprays of ultra-high volume copper fungicides (± 10 000 l/ha per application) per growing season is sufficient to control most of the diseases (Duvenhage and Köhne, 1999; Willis and Duvenhage, 2003). However, export markets are aware of copper build up in soils and therefore require fewer applications to prevent potential adverse environmental and/or health effects (Willis and Duvenhage, 2003).

However, chemical fungicides, such as prochloraz and benomyl, have either been disallowed for use due to adverse health and environmental effects or pathogens have developed resistance. Benomyl is still used on some farms once a season to reduce application of copper sprays (Willis and Duvenhage, 2003; D. Taylor, personal communication). Rowell (1986) showed that hand-gun sprays overspray as much as 30 % of chemicals. This method is still the most popular but attributes to high build up of chemicals in the environment.

Therefore, to meet consumer demands and to stay in the forefront of avocado export, South Africa is in urgent need of new alternative fungicides. This research has assayed the potential of an ethanolic extract of propolis (EEP) as a fungicide to control diseases of avocado fruit. Since 'Fuerte' avocado fruit are the most susceptible to diseases on Roodewal farm, it was used for all assays.

In *in vitro* experimentations, EEP demonstrated a strong potential as a fungicide by inhibiting growth of avocado pathogens as well as potential opportunistic pathogens. EEP was also efficient in inhibiting germination of *P. guipinii* and *Colletotrichum* sp. Similarly, in *in vivo* assays, EEP at 5 and 10 mg ml^{-1} concentrations were effective at inhibiting infection of the fruit by *Colletotrichum* sp. as well as the prevention of disease development within the infected fruit.

However, no significant control of diseases was observed after pre-harvest and post-harvest *in situ* trials. Possibly the EEP was washed off the leaves and fruit during the rains. Further studies are required to determine if a sticker could increase the endurance of EEP on the fruit. Alternatively, an extra spray during the high rainfall of April could have resulted in more effective control as it was noticed that the majority of the disease symptoms were within low disease indices and PDFC indices indicated late infections during the growing season (possibly during the month of April). A possible solution to the use of EEP as a post-harvest dip is to increase the dipping time to as long as five minutes. The concentration of EEP could also be increased to 10 mg ml^{-1} for both uses as

a pre- and post-harvest applicantion. Field trials could also be increased to more than 20 trees to allow for a greater replication number. This could reduce variation around the mean. However, one has to balance these experimental design considerations against the loss of produce to the farmer.

In contrast, EEP demonstrated high potential to decrease SER disease. However, the replication number of the experiment was low and therefore it should be repeated on a bigger scale. Storage of avocado fruits is limited by SER which can cause more than 13 % of fruit loss (Muirhead *et al.*, 1982; Demoz and Korsten, 2006). Unfortunately, there are no registered fungicides for the control of SER in South Africa (Demoz and Korsten, 2006). Therefore, the potential for EEP as a product for control of SER is high as any reduction in the occurrence of the disease can result in much economic advantage.

The chemical composition of the EEP indicated high antimicrobial properties with high flavonoid concentrations as well as phenolic concentrations. These compounds are known to demonstrate high antimicrobial activities (Cushnie and Lamb, 2005; Gómez-Caravaca, 2006) and are the most common constituents of propolis (Gómez-Caravaca, 2006). The constituents of propolis could be increased, however, during extraction by lowering the percentage of alcohol to between 70 and 80 %. This would lower the concentration of wax and increase the concentrations of the polyphenolics (Gómez-Caravaca, 2006), thereby increasing the antimicrobial activities.

The actual mode of action of propolis on fungi has received little attention. Therefore, this study attempted the first step in understanding the site of activity by observing any structural damage to the fungal hyphae. It was discovered that the propolis was creating large pores through the hyphal cells. This could indicate a possible disruption of cell wall constituents either directly or at a molecular level and this hypothesis should be explored further.

Overall, the use of EEP as a fungicide for avocado fruit is still promising. The lack of efficacy in the field could be linked to environmental factors, but the EEP was shown to

be effective with all respects to antifungal activity. This study was the first step into EEP fungicide development and much research is still required for optimization, which include increased concentration; the addition of a sticker; more frequent sprays or sprays later in the growing season; and longer dipping times.

Chapter 7

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