ALTERNATIVE APPLICATION METHODS OF ANTAGONISTS TO AVOCADO FLOWERS TO CONTROL STEM-END ROT PATHOGENS

BY

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Submitted in partial fulfilment of the requirements for the degree of

MASTER OF SCIENCE

Faculty of Natural and Agricultural Sciences
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South Africa

2005

DECLARATION

I, the undersigned, hereby declare that the thesis submitted herewith for the degree Master of Science to the University of Pretoria contains my own independent work and has not been submitted for any degree at any other university.

Besrat Tesfagiorgis Demoz

June 2005

DEDICATION

This thesis is dedicated to my father Tesfagiorgis Demoz, my mother Elen Gebregziabher, my sisters Berikti and Asmeret and my brothers Semere and Kibreab whom I haven't had a chance to see in all these years.

ACKNOWLEDGEMENT

I would like to thank the following, without whom this project would not have been a success:

My Heavenly Father, for all answered prayers

Prof. Lise Korsten, my supervisor, for your invaluable guidance and support throughout the project. Thank you for being my anchor and compass, and for being a 'mother' to me through all trying times

Dr. Gina Swart, my co-supervisor, for great guidance during the laboratory work

Amanda Redmond, Mashudu Silimela, Shadrack Phophi and Itani Tshivhandekano, for great help in the fieldwork. I wouldn't have done it without you

René Jacobs, for your invaluable inputs and editorial assistance

Allan Hall, for unlimited help in microscopy

Trish Beart, for your smiles and care

All staff members and students of Plant Pathology, for your support and making me feel at home

Mr. Bill Dearing, for allowing me to use your farm for my experiments

Habtom Berhe, for your love and encouragement

Most of all, my parents, sisters and brothers, for all your love, support and prayers from across the miles. I cannot thank you enough

SUMMARY

ALTERNATIVE APPLICATION METHODS OF ANTAGONISTS TO AVOCADO FLOWERS TO CONTROL STEM-END ROT PATHOGENS

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Biological pre- and postharvest disease control strategies depend on successful colonisation and survival of the introduced antagonists on the infection court. Effective and economical applications involve targeting the antagonists where they are critically needed i.e. the infection court. Honeybee dispersal is one method of antagonist application to such specific sites. In view of this, an *in vitro* experiment was conducted to investigate attachment, colonisation and survival of *Bacillus subtilis* on avocado flowers. Scanning electron microscopy studies showed that the bacterium could attach and colonise avocado flower surfaces. It can also survive on the flowers for longer periods of time. *In vivo* mode of action of the antagonist against stem-end rot (SER) pathogens was also studied where results showed lysis and degradation of hyphae and conidia. However, no viable colonies were retrieved from bee antagonist dispersal under field conditions. Bee antagonist dissemination was compared with antagonist and fungicide spray applications in terms of SER control and the added effect on other diseases such as Cercospora spot and anthracnose. Spray applications of the antagonist were more effective in reducing the incidence of SER than bee dissemination. Integrated sprays of the antagonist and fungicides significantly reduced the incidence of both pre- and postharvest diseases. The identity of *Dothiorella aromatica*, one of the most important SER pathogens, was investigated at a molecular level. RAPD techniques using the discriminatory OPC02 primer successfully separated isolates into three groups based on banding profiles. A further study using RFLP identified the pathogen as a *Botryosphaeria* spp. The most dominant specie was *B. parva* followed by *B. rhodina*. Further studies should focus on assessing the distribution of these pathogens within avocado-growing regions of South Africa.

TABLE OF CONTENTS

CHAPTER 1

GENERAL INTRODUCTION

Avocado (*Persea americna* Mill.) is a member of the Lauraceae family, which is indigenous to the warm subtropical Pacific regions (Bergh, 1992). All taxa that are close relatives of the avocado originated from the same epicentres i.e. Central Mexico through Guatemala into adjoining Central America. Several hybrids have originated from the native West Indian, Guatemalan and Mexican races (Bergh, 1992). Today, avocados are produced commercially in many parts of the world including South Africa.

Since its introduction into South Africa in 1882, the avocado industry has grown, with the bulk of fruit being exported. Production increased from 48 150 metric tons (mt) in 1994/95 to 100 000 mt in 2003/04 while exports steadily increased from 28 400 mt to 43 000 mt during the same period (http://www.fas.usda.gov/psd/complete_tables/HTP-table6-101.htm). The bulk of the fruit exported are directed towards the European markets (59%) (Van Zyl and Ferreira, 1995). An important aspect affecting fruit quality is the distance to export markets (Darvas, 1982). Since the bulk of the fruit (97%) is exported by sea, long transit periods of up to three weeks often result in significant postharvest losses at the retail end.

In South Africa, postharvest diseases remain the most important threat. The most important postharvest diseases include anthracnose, caused by *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc. in Penz., stem-end rot (SER), most commonly caused by *Thyronectria pseudotrichia* (Berk. & M. A. Curtis) Seeler., *C. gloeosporioides, Dothiorella aromatica* (Sacc.) Petr. & Syd., *Lasiodiplodia theobromae* (Pat.) Griffon and Maubl. and *Phomopsis perseae* Zerova and the Dothiorella/Colletotrichum fruit rot complex (DCC) (Le Roux *et al*., 1985; Darvas and Kotzé, 1987). According to Sanders and Korsten (1997), Bezuidenhout and Kuschke reported losses of up to 36% due to anthracnose and 13% due to SER for South African avocados exported in 1983. Similar losses have also been reported for export consignments from other countries. For instance, 23% of New Zealand Hass avocados exported to Sydney were affected with SER (Ledger, *et al*., 1993 in Everett, 1996).

The pathogens associated with SER vary from country to country. Johnson and Kotzé (1994) indicated that *L. theobromae* predominates as a SER pathogen in Israel while in South Africa the dominant pathogens have been described as *D. aromatica* and *T. pseudotrichia*. In Australia, New Zealand and the United States *Botryosphaeria* species and their anamorphs are the major causal agents of SER. Of these pathogens, the taxonomy of the anamorphs of *Botryosphaeria* species has been a major point of controversy. These anamorphs are commonly placed under *Dothiorella* but could more correctly belong to *Fusicoccum* Corda (Johnson and Kotzé, 1994). On mango, for example, *B. ribis*, has been described as *D. dominicana* but has now been correctly identified as a *Fusicoccum* anamorph of *B. ribis* in South Africa (Jacobs, 2002).

The SER pathogens infect fruit from endophytically colonised inflorescences through the stem-end tissues i.e. the pedicel and adjacent peduncle (Johnson and Kotzé, 1994). Once infected, fruit remain symptomless until they start to ripen postharvest. This is mainly due to the inhibitory effect of the high levels of antifungal dienes in unripe fruit (Prusky *et al*., 1998). This makes early detection and control of the disease difficult leading to successful establishment of the pathogens in the neck of the fruit with resultant postharvest losses.

Limited control of SER can be achieved with preharvest sprays using copper oxychloride or benomyl (Darvas and Kotzé, 1987) or postharvest applications using prochloraz (Darvas, 1985) or thiabendazole (Nel *et al*., 2003). However, continuous use of chemicals can result in build-up of resistance in target pathogens (Darvas and Kotzé, 1987; Eckert, 1990; Ippolito and Nigro, 2000), as has been reported for benomyl (Darvas *et al*., 1987). Copper oxychloride also leaves visible residues that have to be removed manually in pack houses adding to labour costs (Korsten, 1993). In addition to these, prochloraz has still not been given product clearance for use on fruit exported to France. Currently, only prochloraz and thiabendazole are registered in South Africa for postharvest treatments against avocado postharvest diseases including SER (Nel *et al.,* 2003).

Due to increased public concern over the build up of chemical residues in the food chain and environmental pollution (Wisniewski and Wilson, 1992; Piano *et al*., 1997; Ippolito and Nigro, 2000), a need for alternative methods of disease control arose (Janisiewicz and Bors, 1995; Vinas *et al*., 1998).

Biological control represents one such alternative (Piano *et al*., 1997; Vinas *et al*., 1998) that can provide an environmentally safer product with potentially reduced risk to human health (Shtienberg and Elad, 1997). Effective biological control has been reported for postharvest diseases of several fruits including pome (Janisiewicz 1987; Janisiewicz and March, 1992; Benbow and Sugar, 1999), stone (Pusey and Wilson, 1984) and several tropical and subtropical fruits (Korsten *et al*., 1994; Lima *et al*., 1997; Schena *et al*., 1999).

Natural resident microbial populations on fruit surfaces have been investigated at the University of Pretoria for use in biological control programs of postharvest diseases since 1985. Some of the investigations involved postharvest strategies while others utilised preharvest applications of biocontrol agents. *Bacillus subtilis* B246 has subsequently been developed and registered as Avogreen for pre- and postharvest treatments against Cercospora spot on avocado (Korsten *et al*., 1997). Moreover, Korsten (1993) and Korsten *et al*. (1994) reported effective control of postharvest decay for anthracnose, SER and DCC by using preharvest field sprays of this antagonist.

However, the level of effective SER control varied over time (Korsten *et al.*, 1998). This was attributed to variable environmental conditions and the product formulation used. Another important aspect that impacted on biocontrol efficacy was the method of application. The ideal biological control system is one where the antagonists are introduced only when and where they are required or where they are most effective, i.e. infection courts to minimise wasteful application (Sutton and Peng, 1993).

Biological methods and strategies involve timely manipulation of antagonistic populations to suppress pathogens in various inoculum sources or on host plants (Sutton and Peng, 1993). The latency of SER pathogens poses specific challenges in terms of disease control. With postharvest biological control strategies, it is difficult to achieve a significant level of control against quiescent infections. The most feasible option remains a preharvest strategy of reducing initial inoculum thereby minimising the level of infection in the orchard. Application of antagonists at flowering or at early fruit set proved to be effective in controlling latent infections on strawberries (Lima *et al.*, 1997).

Today, plant disease control is focused more on integration of best strategies that will reduce the risk to the consumer and the environment. In integrated chemical and biological control, antagonists are applied when and where they are likely to be effective to reduce the number of chemical sprays while achieving the same level of disease control (Shtienberg and Elad, 1997). Honeybee dissemination of biocontrol agents to flowers enables delivery of antagonists to the infection court at timely intervals as flowers open. The potential of using bees as vectors of biocontrol agents was considered long after they were found to be vectors of plant pathogens (Thomson *et al.*, 1992; Johnson *et al.*, 1993a). Bees have been tested successfully for the dissemination of antagonist for the control of *Erwinia amylovora* in pear blossoms (Johnson *et al.*, 1993b).

Because of the phenology of avocado flowers, farmers usually keep bees in the orchards to aid pollination. This opportunity can be utilised to disseminate biocontrol agents to avocado flowers so that they can colonise the infection court prior to the arrival of the pathogen thereby preventing infection. To test the hypothesis that biocontrol agents applied during flowering directly targeting the infection can potentially ensure more effective control of SER, the following objectives were set:

- To evaluate attachment, colonisation and survival of honeybee disseminated antagonists on avocado flowers
- To evaluate antagonist-pathogen interactions on avocado flowers
- To evaluate SER control by honeybee disseminated antagonist as compared with field spraying and chemical control
- To identify, compare and determine the status of pathogens associated in SER in avocado

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CHAPTER 2

LITERATURE REVIEW

The avocado (*Persea americana* Mill.) is a native crop to the highlands of Mexico and Latin America. It has been cultivated globally in a wide range of habitats including tropical and subtropical regions (Zentmyer, 1994). The avocado is a shallow rooted tree that varies in canopy shape, from tall, upright to having widely spreading branches (Nakasone and Paull, 1998). It is regarded as an evergreen tree although some cultivars shed leaves gradually or during flowering. The fruit is a one seeded berry and mainly pear shaped (Scora *et al*., 2002).

Bergh and Ellstrand (1986) classified the commercial avocado, ***P. americana***, into three subspecies: ***americana***, ***guatemalensis***, and ***drymifolia***. These three types are also known as the West Indian, Guatemalan and Mexican horticultural races, respectively. The West Indian variety is a tropical one and bears large fruits with low oil content. The Guatemalan variety grows in the subtropical zone. Fruit size is intermediate and has the thickest and roughest skin, while fruit is rounder. The third variety, the Mexican, thrives best in the subtropics and has the smallest fruit with the highest oil content and thinnest skin.

WORLD AVOCADO PRODUCTION

Avocados are widely distributed throughout the tropical and subtropical world and are amongst the top six contributors of food to the New World. Although production varies from year to year, Mexico remains the leading avocado producer in the world followed by California, Dominican Republic and Brazil (Bergh, 1992). In Africa, the main producer is South Africa followed by Madagascar and other west central countries. Only Israel and South Africa are major avocado exporters (Bergh, 1992).

AVOCADO PRODUCTION IN SOUTH AFRICA

The avocado has been known in South Africa since the arrival of the first Dutch settlers (Durand, 1990). There is no exact date when the first avocado was planted in South Africa but the first tree was found in Natal in 1882 (http://www.knet.co.za/avocado). Today, the main production areas are concentrated in the Mpumalanga and Limpopo provinces,

namely Tzaneen with 38% of the trees, followed by Nelspruit and Hazyview with 33%, Levubu with 21% and KwaZulu-Natal with 8% (Sippel, 2001). According to the last tree census by the South African Avocado Growers' Association (SAAGA) (1998), the area covered by avocado plantation expanded from approximately 2 000 ha in 1970 to 12 500 ha in 1997. The predominant cultivars are Fuerte and Hass, which comprise 42% and 33%, respectively, of the area planted. However, due to preferences in the European markets the local industry has shifted with new plantings being Hass. This is also reflected in older Fuerte orchards that are in the process of being phased out. The other commercial cultivers Ryan and Pinkerton cover 11% and 8.5%, respectively, of the area planted (Vorster, 2001).

South African avocado production and export figures have dramatically increased over the past ten years (Table 1). Approximately 50% of total South African exports are destined for France while 25% goes to the United Kingdom and 20% to other European countries (Van Zyl and Ferreira, 1995). Other markets include Germany, Scandinavia, and more recently, Middle- and Far Eastern destinations.

REPRODUCTIVE BIOLOGY

Based on flowering phenology, avocados can be classified into two types (Robbertse, 2001):

1. Type A consists of cultivars such as Hass, Hayes and Reed, with flowers that open as female in the morning, then close to reopen as male flowers in the afternoon of the following day.
2. Type B consists of cultivars such as Fuerte, Sharwil and Zutano, which have female flowers that open in the afternoon, then close overnight and reopen in the male stage the following morning.

Thus, the avocado is known as a typical cross-pollinated species due to its synchronously protogynous flowering rhythm. Therefore, pollen transfer occurs when Type A pollen is available to Type B female flowers in the afternoon and vice versa. Pollen tends to clump in a sticky mass and is usually transported by bees or other large flying insects. For this reason, farmers keep beehives in their avocado orchards.

Table 1: Production, supply and exports of fresh avocados in South Africa from 1994 to 2004* (in 1000 tonnes)

Year	Production	Imports	Total supply/distribution	Exports	Fresh domestic consumption	Processed
1994/1995	48 150	5	48 155	28 400	19 755	0
1995/1996	55 782	211	55 993	27 417	28 576	0
1996/1997	54 000	312	54 312	22 704	31 608	0
1997/1998	100 000	0	100 000	52 000	38 000	10 000
1998/1999	65 000	0	65 000	33 000	24 000	8 000
1999/2000	104 000	0	104 000	54 000	38 000	12 000
2000/2001	83 000	0	83 000	34 000	36 000	13 000
2001/2002	110 000	576	110 576	47 741	40 000	22 835
2002/2003	82 000	600	85 600	38 000	23 000	24 600
2003/2004	100 000	600	100 600	43 000	30 000	27 600

* Source: http://www.fas.usda.gov/psd/complete_tables/HTP-table6-101.htm

DISEASES

Avocados are attacked by a range of insects and microbial pathogens that affect the yield and quality of fruit. Both pre- and postharvest fruit diseases have become a major threat to successful exports. In warm production areas, preharvest diseases such as Cercospora spot can cause losses of up to 70% in unsprayed orchards. Postharvest diseases such as anthracnose and stem-end rot (SER) remain a major problem in terms of losses in export revenue (Manicom, 2001). Since the focus of this project is on SER this review will further focus on this topic.

STEM-END ROT

The incidence of SER in South Africa is lower than that recorded for anthracnose but can be as high as 25% of locally marketable fruit (Sanders and Korsten, 1997). Pathogens involved in SER are mainly *Dothiorella* species, *Lasiodiplodia theobromae* (Pat.) Grifd.

and Maubl., *Thyronectria pseudotrichia* (Schw.) Seeler, *Colletotrichum gloeosporioides* (Penz.) Penz. and Sacc. in Penz., *Phomopsis perseae* Zerova, and *Fusarium decemcellulare* Brick (Darvas and Kotzé, 1987; Johnson and Kotzé, 1994). Other pathogens such as *Bipolaris setariae* (Sawada) Shoemaker, *Fusarium sambucinum* Fuckel, *Fusarium solani* (Mart.) Sacc., *Pestalotiopsis versicolor* (Speg.) Steyart, and *Rhizopus stolonifer* (Ehrenb.:Fr.) Vuill (Darvas and Kotzé, 1987) are occasionally associated with the disease.

Johnson and Kotzé (1994) indicated that *L. theobromae* predominates in Israel while in South Africa the dominant pathogens have been described as *D. aromatica* and *T. pseudotrichia*. In Australia, New Zealand and the United States *Botryosphaeria* species and their anamorphs are the major SER pathogens.

SYMPTOMS

Stem-end rot fungi may infect avocado fruit endophytically growing in tissue without showing symptoms. The fungi penetrate the fruit via the stem-end and symptoms develop as fruit ripen. Skin and flesh decay starts at the pedicel-end and advances through the pulp via the vascular bundles to the blossom-end. Externally, the skin becomes dark at the affected area with a well-defined margin surrounding the lesion. Mycelial growth is occasionally seen on the surface of lesions, particularly during advanced infection stages and under humid storage conditions. Except for SER caused by *C. gloeosporioides*, the vascular tissues become discoloured in advance of flesh decay (Johnson and Kotzé, 1994). Stem-end rot caused by *C. gloeosporioides* is characterised by production of salmon-coloured spore masses on the surface of lesions. Stem-end rots can be detected earlier by slight shrivelling of the stem-end and presence of mycelium on the abscission scar when the stem button is removed.

Infections remain quiescent until harvest, when antifungal dienes that are found in fungitoxic amounts (Prusky *et al*., 1998) in the skin of the fruit break down due to degradation by lipoxygenase activity (Karni, 1989). The most active of these dienes is 1-acetoxy-2-hydroxy-4-oxo-hereicosa-12, 15-diene (Prusky *et al*., 1998). The pathogens resume growth and invade the fruit to cause postharvest rots (Prusky *et al*., 1990; Prusky *et al*., 1991; Adikaram *et al*., 1992).

THE PATHOGENS

In this section, some of the most important SER pathogens will be discussed.

Dothiorella spp.

Various species of *Dothiorella*, such as, *Dothiorella aromatica* (Sacc.) Petrak and Sydow, *Dothiorella gregaria* Sacc., *Dothiorella dominicana* Pet. et Cif., and *Dothiorella mangiferae* H. et P. Syd. et But. (Snowdon, 1990; Johnson and Kotzé, 1994) have been reported as causal agents of SER on avocado. The taxonomy of this genus and associated teleomorphs (*Botryosphaeria* spp.) has been controversial. It has been suggested that the fungi usually classified under *Dothiorella* should be placed in the genus *Fusicoccum* (Pennycook and Samuels, 1985). Pegg *et al*. (2002) also mentioned that *D. aromatica*, one of the most important SER pathogens on avocado, may be correctly classified as *Fusicoccum luteum*. On mango, *Botryosphaeria ribis*, which was previously described as *D. dominicana* has now been correctly identified as the *Fusicoccum* anamorph of *B. ribis* in South Africa (Jacobs, 2002).

Dothiorella aromatica produces sparse, aerial mycelia on potato dextrose agar (PDA) with dark, dendritic undersides and radially aligned, immersed conidiomata (Johnson and Kotzé, 1994). Conidia are narrowly fusiform to clavate, hyaline and granular. Once invaded by the fungus, the flesh becomes discoloured and develops an offensive odour. The fungus usually grows on dead leaves, dead leaf margins, and dead branches.

Colletotrichum gloeosporioides

Colletotrichum gloeosporioides is an important pathogen on a very wide range of tropical and subtropical crops. On avocado, it gets into the fruit through the stem-end, wounds, or Cercospora spot or scab lesions. After arriving on the fruit surface, the spores germinate within seven hours (Parbery, 1981 *In* Pegg *et al*., 2002), forming appressoria and infection pegs that penetrate the fruit wax, causing small brown to black spots surrounding the lenticels. Once the fruit is ripe, the fungus resumes growth, producing typical visible anthracnose symptoms. When the fruit is cut into halves through the stem-end, rot extending into the flesh in a hemispherical pattern can be seen.

Phomopsis perseae* and *Pestalotiopsis versicolor

Pegg *et al*. (2002) described *P. perseae* as producing felted, white to buff mycelia with scattered, discrete pycnidia or multilocular stromatic structures. Pycnidia are produced under the epidermis, later becoming erumpent with an associated colour change to black. Pycnidia consist of one, seldom two or three cavities, the upper wall is thick, while the lower wall is thin, first yellow or indistinct, tapering with a round pore on top. Conidiophores are indistinct and conidia are fusiform with two lipid fragments at the two ends. Darvas (1982), according to Zerova (1940), described *P. versicolor* as producing thick, cottony white mycelia on PDA that appear yellowish when wet. Spores exude from scattered acervuli in glistening, greenish black drops.

Thyronectria pseudotrichia

Thyronectria pseudotrichia is generally seen in its conidial stage as *Stilbella cinnabarina* (Mont.) Wollenw. The name *T. pseudotrichia* is designated as the sexual stage of the fungus. It produces immersed mycelia with sparse aerial hyphae and phialides that produce conidia in balls (Johnson and Kotzé, 1994). Perithecia usually develop in caespitose clusters of 3 to 20 or more and they measure 200-590µm in diameter. Their colour is bright orange-red, weathering to dark brown and finally almost black. Asci are clavate when young, tapering towards the apex, later broad, closely following contours of spores, finally avenescent and measure 50-100 x 10-25µm. There are approximately eight ascospores in an ascus. Ascospores are muriform, broadly ellipsoid, sometimes curved and slightly tapered terminally. Their colour is hyaline to pale yellow or light brown. The basically three-septate ascospores are constricted with many other, often conspicuous transverse and longitudinal septae (Pegg *et al*., 2002).

DISEASE CYCLE AND EPIDEMIOLOGY

Stem-end rot pathogens occur as endophytes in avocado stems. Infections occur from endophytically colonised inflorescence and stem-end tissues (Johnson and Kotzé, 1994). These remain quiescent until fruit ripen after which infections resume, growing into the flesh. The spectrum of pathogens that cause SER is predetermined by environmental conditions (Johnson and Kotzé, 1994). Hot conditions promote infection by *L. theobromae*, whereas wet conditions promote infection by *C. gloeosporioides* and *T. pseudotrichia*. Water stress promotes endophytic infection. Subsequent storage conditions

determine which pathogens predominate. Cool storage prompts *C. gloeosporioides* and *P. perseae* over *T. pseudotrichia*, whereas *L. theobromae* will predominate over *Dothiorella* spp. at 30ºC. On mango, symptomless infections by *Dothiorella* spp. and *Phomopsis mangiferae* were reported occurring endophytically in stem tissue prior to inflorescence emergence (Johnson *et al*., 1993). These infections can later move down into the fruit and cause SER.

Dothiorella species, *L. theobromae, P. perseae, C. gloeosporioides*, and *T. pseudotrichia* produce spores in tree litter and on dead leaves, twigs and branches in the canopy. Spores are spread by water and air movement. Infection can also occur through wounds or by direct penetration of the fruit surface. Generally, SER pathogens produce inoculum in abundance on many aboveground parts of the tree and mummified infected fruits and leaves become sources of inoculum for primary infection (Darvas, 1982).

CONTROL

Because of long transit periods, postharvest losses due to diseases are often greater in export than domestic consignments. Postharvest losses also occur in the market, at the retail end and at the consumer level. Because harvested commodities carry the cumulative cost of harvesting, storage, distribution, and sale, they require effective control programs to ensure quality.

Cultural control

Stem-end rot is promoted by water stress. Johnson and Kotzé (1994) indicated that endophytic infections might be reduced by avoiding water stress, defoliation and optimising nutrition. Mulching under trees promotes tree litter decomposition and thereby reduces available inoculum. Saline conditions, which cause necrotic spots to develop on leaves, have to be avoided because the fungus will live in the dead areas. Tree pruning and removal of dead leaves and twigs within the canopy can help reduce pathogen inoculum levels. Stem-end rot arising from endophytic colonisation can be reduced by maintaining good tree vigour (Pegg *et al*., 2002).

Chemical control

Successful control of avocado fruit diseases requires that all susceptible plant parts be thoroughly coated with fungicides to prevent establishment of the pathogen. Sprays applied after pathogen establishment and infection often are less effective on disease control.

Recent research showed the efficacy of azoxystrobin (a strobilurin-analogue fungicide) field sprays for control of SER (Coats *et al*., 2001 *In* Pegg *et al*., 2002). Limited control can also be achieved by means of preharvest spays using copper oxychloride or benomyl or postharvest application of prochloraz (Muirhead *et al*., 1982; Darvas *et al*., 1987; Lonsdale and Kotzé, 1989). However, copper oxychloride leaves unsightly residues, which must be removed manually in the pack house (Korsten, 1993) while prolonged use of benomyl can lead to build up of pathogen resistance (Darvas and Kotzé, 1987). Although prochloraz is at present registered for use on avocado in South Africa (Nel *et al*., 2003), product clearance for use on fruit exported to France has still not been given (Boshoff *et al*., 1995). Furthermore, none of these chemicals is registered for control of SER in South Africa.

Because flowers are the infection court for SER pathogens, spraying trees during flowering could be another option. However, field sprays targeting flowers is difficult since flowers do not open in a synchronised way. Multiple sprays are therefore required which is neither practical nor cost effective. In addition, spraying may not deliver the antagonist to the exact infection court on the flowers.

Biological Control

Fungicides are the primary means of controlling postharvest diseases of fruits and vegetables (Ekert and Ogawa, 1985; Lima *et al*., 1997; Ippolito and Nigro, 2000). However, as harvested fruits are treated with fungicides to retard postharvest diseases, there is a greater likelihood of direct, human exposure to them than to other pesticides applied solely to protect foliage (Wisniewski and Wilson, 1992). This together with the increasing international concern over environmental pollution has forced fruit industries to look at alternative measures (Janisiewicz *et al*., 1994).

The use of alternative control strategies can be explored regardless of the complexities of the pathogen infection process. Biological control is one such alternative (Pusey, 1989), which either on its own or as part of an integrated control strategy can result in reduced pesticide usage (Teixido *et al*., 1998). Biological control has been utilised by man since the early days to control plant pests and diseases (Campbell, 1989). Although the discovery of powerful pesticides changed the focus away from natural control, its detrimental effect on the environment and human health resulted in eventual swing back to biological means of controlling diseases (Gunasekaran and Weber, 1996).

The term biological control was first used in relation to plant pathogens in 1914 by C. F. von Tubeuf (Baker, 1987). Biological control of plant diseases is defined as the reduction of inoculum density or disease-producing activities of a pathogen in its active or dormant state, by one or more organisms, accomplished naturally or through manipulation of the environment, the host, antagonist, or by mass introduction of one or more antagonists (Baker and Cook, 1974).

The history of biocontrol research on fruit crops has largely been successful in the laboratory, but often a failure in the field (Wilson *et al.,* 1991) mainly due to the numerous variable uncontrollable environmental conditions. Although several biocontrol agents are in commercial use today, some have been marketed with insufficient efficacy data and have tended to reduce public faith in this approach. Despite the challenges involved in developing biological control systems, several antagonistic microorganisms capable of controlling a large number of fruit pathogens are known.

Biological control of postharvest diseases has been more successful (Janisiewicz *et al*., 1994) than field applications, primarily due to the more stable environment in storage and packhouses. However, one of the major obstacles to the development of postharvest biocontrol agents is its inability to control previously established infections (Ippolito and Nigro, 2000). Therefore, it would be advantageous to apply antagonists before harvest (Leibinger *et al*., 1997), which could reduce initial infections.

However, relatively few studies have been carried out to control postharvest diseases by means of preharvest applications of microbial antagonists (Benbow and Sugar, 1999;

Ippolito and Nigro, 2000). Pusey (1989) indicated that there are three reasons for failure of preharvest biological control: the inability to control environmental conditions in the field, the difficulty to target biocontrol agents to effective sites in the field and economical feasibility of control procedures for harvested commodities. Despite these obstacles there are some examples of successful preharvest biocontrol of fruit diseases (Korsten *et al*., 1994; Leibinger *et al*., 1997; Lima *et al*., 1997; Teixidó *et al*., 1998; Benbow and Sugar, 1999; Schena *et al*., 1999).

The application of biological control agents prior to harvest is advantageous since it enables early colonisation of fruit surfaces thereby potentially preventing establishment of quiescent infections. Preharvest sprays can also help in arresting infections that occur during harvesting and postharvest handling. After achieving poor control of natural infections of brown rot of nectarine and peach by postharvest applications, Smilanick *et al*. (1993) concluded that early application of biocontrol agents in the field might make early colonisation possible with subsequent protection against infections.

In South Africa, extensive research has been done on biological control of pre- and postharvest diseases of avocado. Preharvest applications of *B. subtilis* to avocado trees resulted in sustained control of Cercospora spot (Korsten *et al*., 1997). Korsten *et al*. (1992) also reported that preharvest biological and integrated treatments gave as effective control as the fungicide on soft brown rot of mangoes. Korsten *et al*. (1989) applied both pre- and postharvest treatments of *B. subtilis* and *Bacillus licheniformis* on avocado and mango fruits, respectively, and achieved reasonable levels of control of postharvest diseases. Besides, preharvest biocontrol was as effective as postharvest treatments and more effective that the standard commercial copper oxychloride spray programmes in reducing SER. Korsten *et al*. (1994) also reported effective control of postharvest decay from anthracnose, SER and Dothiorella/Colletotrichum fruit rot complex by using preharvest *B. subtilis* field sprays.

However, to be successful in preharvest applications, biocontrol agents must be able to tolerate adverse factors such as low-nutrient availability and fluctuating temperatures (Leibinger *et al*., 1997; Ippolito and Nigro, 2000). Wilson and Wisniewski (1989) described an ideal antagonist as one that is genetically stable, effective at low

concentrations, able to survive well under adverse environmental conditions and efficacious against a wide range of pathogens on a variety of crops. It also has to be amenable to growth on an inexpensive medium in fermenters and preparable in a form that can be effectively stored and dispensed, not produce secondary metabolites that may be deleterious to humans, compatible with other chemical and physical treatments and non-pathogenic to the host.

Integrated control

Emphasis on the chemical control of postharvest diseases of fruits and vegetables is evident and many trials proved successful. One sustainable way of controlling diseases is the usage of multiple control strategies called integrated disease management (IDM) (Cooley, 1996). Concepts underlying IDM include: optimisation of disease control in an ecologically and economically sound manner, emphasis on co-ordinated use of multiple tactics to enhance stable crop production and maintenance of disease damage below injurious levels while minimising hazards to humans, animals, plants and the environment (Cooley, 1996). Multiple control procedures are usually necessary to achieve consistent success by utilizing the cumulative effect of combined measures (Baker and Cook, 1974). Other motivations are to develop cost saving production techniques and the prevention of disease resistance to chemicals. Korsten *et al*. (1997) showed that integrated control is consistently more effective over time and location compared with sole applications of biocontrol agents or commercial fungicides and therefore has the greatest potential for acceptance by growers.

In order to minimise postharvest diseases of avocado, an integrated suite of strategies should be implemented. Both pre- and postharvest protocols and procedures are important in the ultimate control of plant pathogens responsible for postharvest diseases (Everett, 1996). Furthermore, a basic understanding of the infection processes and the period which represents the highest risk for infection are required in order to more effectively target control measures (Everett, 1996). Generally biocontrol agents have a relatively narrow spectrum of activity compared to fungicides. Therefore in reality, chemical control has not been replaced on a large scale by biological control agents. However, the use of biological control can be justified on its own merits, without elevating its perceived importance at the expense of chemical controls (Cook, 1993).

DISPERSAL OF BIOCONTROL AGENTS

Stem-end rot pathogens infect through flowers and inflorescences (Johnson and Kotzé, 1994). Smith and Korsten (1996) isolated *C. gloeosporioides* from avocado flowers. Other investigations showed that this fungus is associated with poor fruit set in citrus (Agostini *et al*., 1993). Thomas *et al.* (1994) also indicated that some fungi present on the pistils of avocado flowers could play a role in flower abscission. Therefore, it is important to control fungi that colonise flowers during the preharvest period of flowering.

Pusey (1989) indicated that it is difficult to target biocontrol agents to effective sites in the field especially in cases where infection takes place through flowers. Moreover, in some crops, flowers do not open simultaneously during the flowering period (Thomson *et al*., 1992). This requires multiple applications, which are not feasible, are inefficient and uneconomical. Therefore, there is a need to develop and evaluate alternative application strategies that provide more effective control.

Flower-visiting insects, like honeybees, seeking nectar and pollen are known to be inadvertent vectors of pathogens as well as beneficial vectors of antagonists. Honeybees can be used to disseminate antagonistic inoculum formulated as dust of freeze-dried bacterial cells (Johnson *et al*., 1993a), pollen that has been soaked in a suspension of the antagonist and then dried (Thomson *et al*., 1992) or fungal spores formulated as a powder (Sutton and Peng, 1993). To use bees for this purpose a ‘pollen insert’, which contains antagonist inoculum, is attached to the entry platform of a beehive. Bees become contaminated with the preparation of the antagonists as they exit the hive through the pollen insert. The antagonist powder will then be deposited in the infection court as bees forage on flowers (Johnson and Stockwell, 2000).

Honeybees can be used to deliver biocontrol agents to the precise site where the pathogen and the antagonist interact (Hattingh *et al.,* 1986; Rundle and Beer, 1987). Field applications of biocontrol agents during flowering by means of bees proved to be effective in suppressing the incidence of *Botrytis cinerea* on strawberry (Peng *et al*., 1992). Wittig *et al*. (1997) reported a reduction in the number of latent infections of brown rot in green fruit by the application of *Aureobasidium pullulans* (de Bary) Arnaud and *Epicoccum*

purpurascens Ehrenburg to sweet cherry blossoms. Thomson *et al*. (1992) found that bees could disperse antagonistic bacteria to flowers in commercial apple and pear orchards.

Johnson *et al.* (1993a, b) also confirmed that bees could disperse *Erwinia amylovora* and its antagonistic bacteria *Pseudomonas fluorescens* in apple and pear blossoms. However, they concluded that the efficacy of honeybees as vectors of antagonists were probably not as efficient as orchard sprayers for primary establishment of bacterial antagonists in blossoms. The efficacy of using bees for primary establishment of antagonists depends on seasonal variation in the rate in which blossoms open and become colonised, dependence of bee-foraging activity on weather conditions, presence of other flowering plants, which may draw inoculated bees away from the orchard, and the need for frequent monitoring of the pollen inserts to ensure that exiting bees are contaminated sufficiently with bacterial antagonists (Johnson *et al*., 1993a).

While honeybees may not be the best method to introduce antagonists into orchards, there is evidence that bees play an important role in the secondary movement of bacterial antagonists from colonised to non-colonised blossoms (Nuclo *et al*., 1998).

CONCLUSION

Postharvest diseases pose a major threat to the avocado industry. Stem-end rot is one of the important postharvest diseases, which infects preharvest and remains quiescent until fruit ripen. Combating SER can be a major challenge due to its latency, which makes early detection of infection difficult. Fungicides have only provided limited control. In addition, the possibility of build up chemical fungicides in the environment and the food chain has posed a need for safer alternative control measures. Biological control is considered as one such method. Given the fact that flowers are the infection court of SER pathogens, targeting antagonists to flowers can be an effective strategy. Honeybees, which are used for pollination in avocado orchards, can serve as agents that can disperse antagonists to the flowers. This strategy, however, may not be used as a sole control measure but rather as part of an integrated disease control system.

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CHAPTER 3

Bacillus subtilis ATTACHMENT, COLONISATION AND SURVIVAL ON AVOCADO FLOWERS AND ITS MODE OF ACTION ON STEM-END ROT PATHOGENS

ABSTRACT

Stem-end rot (SER) is an economically important postharvest disease of avocado. It is caused by several fungi, which infect fruits through inflorescences. *Bacillus subtilis* has been registered and commercially used as a biocontrol agent against avocado fruit diseases both pre- and postharvest. Targeting the flowering stage in the disease cycle for dispersal of antagonists is believed to be an alternative application strategy for controlling SER. The aim of this study was therefore to determine the ability of *B. subtilis* to attach, colonise and survive on avocado flowers and to study the interaction of the SER pathogens and the antagonist on avocado flowers. Avocado flowers inoculated with a liquid formulation of the antagonist were observed over time under the Scanning Electron Microscope (SEM). Population dynamics of the antagonist on the flowers was determined. Flowers were also inoculated with antagonist pathogen (*Dothiorella aromatica* and *Phomopsis perseae*) combinations to determine the *in vivo* interaction. The SEM observations and population dynamics study confirmed that the antagonist could attach, colonise and survive on avocado flowers. It could also attach to conidia and hyphae of the pathogens and cause cell degradation.

INTRODUCTION

The avocado (*Persea americana* Mill.), like any other tropical or subtropical fruit, is susceptible to various postharvest diseases, of which anthracnose, stem-end rot (SER), and Dothiorella/Colletotrichum fruit rot complex (DCC) (Darvas and Kotzé, 1987) are the most important. Losses of 36% due to anthracnose and 13% due to SER have been reported for South African avocados on overseas market (Sanders and Korsten, 1997). One of these diseases, SER, is caused by a number of fungal pathogens of which the most important are *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc. In Penz., *Lasiodiplodia theobromae* (Pat.) Griffon and Maubl., *Dothiorella aromatica*. (Sacc.) Petr. & Syd., *Thyronectria pseudotrichia* (Schw.) Seeler, and *Phomopsis perseae* Zerova

(Darvas and Kotzé, 1987; Sanders and Korsten, 1997). These pathogens infect fruit at an early growth stage through the stem-end and remain quiescent until fruit ripens. Stem-end rot symptoms appear when the fruit start to ripen postharvest.

Smith and Korsten (1996) investigated fungi that inhabit the stem and flowers of avocado and reported the presence of C. *gloeosporioides*, one of the causal agents of SER. The study indicated that flowers might be important infection route of this fungus. On citrus, *Alternaria alternata* (Ell & Pierce) is also known as an endophyte that infects through the flowers. Similarly with strawberries, *Botrytis cinerea* Pers.:Fr. infects through flowers and remains latent (Kovach *et al*., 2000) until fruit ripens.

Limited control of avocado SER can be achieved with preharvest fungicide sprays such as copper oxychloride (Korsten and Cook, 1996). However, at present there are no preharvest fungicides registered for control of SER although prochloraz and thiabendazole are registered as postharvest treatments (Nel *et al*., 2003). Postharvest fungicide treatments against latent infections cannot ensure complete protection unless the fungicide can penetrate through the fruit cuticle and reach the infection site. In addition, public pressure against the use of fungicides on harvested commodities consumed fresh has increased (Roberts, 1990; Wisniewski and Wilson, 1992; Piano *et al*., 1997; Ippolito and Nigro, 2000). This, together with the increasing prevalence of pesticide resistance in plant pathogens, is shifting the focus of plant disease control towards alternative methods with reduced risk to the consumer and the environment.

One such alternative is biological control (Piano *et al*., 1997; Vinas *et al*., 1998). Although various researchers have reported successful control of postharvest fruit diseases, little has been reported on preharvest approaches to control postharvest fruit diseases. One of the difficult aspects in preharvest biocontrol is the successful delivery of the antagonist to the infection court at the critical stage of infection.

Combating SER pathogens at the flowering stage can prove to be effective in suppressing the incidence of the disease. Because SER pathogens infect through flowers, targeting antagonist application to flowers could provide an alternative approach. The biocontrol agent must however be able to attach, colonise and survive on the flowers so that the

pathogens will be prevented from attaching and colonising the flowers. Avogreen, *Bacillus subtilis* B246, has been successfully tested as a preharvest application against SER pathogens (Korsten *et al*., 1989). Currently, Avogreen has been registered for commercial use against Cercospora spot and anthracnose. Though variable results have been obtained from season to season, good control has been achieved with preharvest applications of the antagonist to control anthracnose. The aim of this experiment was therefore to determine the potential of *B. subtilis* to attach, survive and colonise avocado flowers with the objective of *in situ* inhibition of the pathogens.

MATERIALS AND METHODS

Collection sites of avocado flowers

Flowers were collected during September 2001 from four avocado trees at the experimental farm, University of Pretoria. One branch was taken at four points around each tree representing north, south, east and west. Samples were placed in paper bags and transported to the laboratory in cooler boxes for processing.

Antagonist inoculation

Bacillus subtilis B246 (Avogreen) (Stimuplant CC, Pretoria), originally isolated from avocado leaf surfaces and effective in controlling pre- and postharvest diseases of avocado (Korsten, 1993) was used in this study. Three replicates of ten flowers were used for scanning electron microscopy (SEM) observation. Flowers were detached from the panicles and put on a surface disinfected tray covered with tissue paper moistened with sterile distilled water to avoid desiccation. Each flower was inoculated with 1µℓ of Avogreen liquid (1x 10^9 viable cells of *B. subtilis* per ml) twice at 15 min intervals. Control flowers were treated with sterile distilled water. The tray was covered with a lid to avoid contamination and water loss.

Scanning electron microscopy sample preparation

Flowers were prepared for SEM immediately after inoculation and 1, 2, 4, 6, 12, 24, and 48 hrs later. One flower from each replicate was taken for each time interval. All floral parts except the pistils were excised under a stereo microscope and discarded. The pistils were fixed overnight in 2.5% glutaraldehyde in 0.075 M phosphate buffer (Coetzee and van der Merwe, 1994). Samples were rinsed three times after 24 hrs in 0.075 M phosphate

buffer for 15 min each, followed by successive 15 min dehydrations in 50, 70, and 90% ethanol, and finally three times for 15 min each in 100% ethanol. Samples were dried in a Biorad drier (Biorad Polaron Division, England) under CO_2. Mounted specimens were coated for 2.5 min with 10 mÅ of gold-palladium (Polaron Equipment Ltd., England) and examined under a JEOL (JSM-840) SEM operating at 5 kV.

Population dynamics of antagonists on avocado flowers

Flowers were prepared and inoculated as mentioned before (section 2.3) during October 2002. Dilution series were made immediately and 1, 2, 4, 8, 48, and 72 hrs after inoculation. For each replicate 1 g of flowers was used. Flowers were put in test tubes containing 9 ml of 25% sterile Ringer's solution (Oxoid, Hampshire) supplemented with 0.001% (v/v) Tween 80 (Unilab, Krugersdorp). Test tubes were vortexed for 30 sec at 7000 Hz and a dilution series were made from each washing up to 10^{-7}. Dilutions were plated on Standard-1 nutrient agar (Biolab, Midrand) plates and incubated at 37ºC. After 24 hrs, *B. subtilis* type colony forming units (cfu) were counted and recorded for each washing.

Interactions between stem-end rot pathogens and *Bacillus subtilis* on avocado flowers

In this experiment, two SER pathogens were selected, namely *D. aromatica* and *P. perseae*, previously isolated from avocado fruits as these pathogens were dominant during the 2001 season. Identity of the pathogens was confirmed by Prof. F. C. Wehner, Department of Microbiology and Plant Pathology, University of Pretoria. Spores from actively sporulating cultures were removed and diluted in 25% sterile Ringer's solution. The concentration of all pathogens were standardised to 10^3 spores/ml using a Haemacytometer counting chamber. The concentration of *B. subtilis* (Avogreen) was standardised at 10^5 cells/ml using the Petroff Hauser counting chamber. Avocado flowers were collected as described before. Moisture chambers were prepared in Petri dishes using sterile distilled water. A 5 µℓ droplet of Avogreen was applied 24 hrs before, simultaneously, and 24 hrs after a 5 µℓ spore suspension of the pathogen was inoculated onto flowers in three replicates. Control flowers were inoculated with pathogens only. Negative control inoculations of water only were also included to assess background populations. Moisture chambers were sealed with Parafilm to avoid moisture loss and

contamination. After 24, 48, and 72 hrs flowers were removed from moisture chambers and prepared for SEM as mentioned before.

RESULTS

Scanning electron microscopy observations

It was noted that spore forms of the bacteria were evident from the formulated product. After *B. subtilis* inoculum was applied with a microdroplet technique, fibril like strands could be observed similar to glycocalyx formation (Fig. 1A,B). Bacteria were generally confined around papilla and in the depressions between epidermal cells. Two hours after application, bacterial cell aggregates were noted on the surfaces of papillae (Fig. 1A and B). Four hours after application, some bacteria were observed attached to the surface polarly and others were lying down. Bacterial attachment to papillae was observed on samples prepared four hours after application (Fig. 2A and B) and multiplication and colonisation were evident (Fig. 2 C, D). Depressions were colonised and bacteria were observed multiplying 24 hrs (Fig. 3A and B) after application. Bacteria multiplied and colonised papillae and depressions in large numbers forming bigger colonies 48 hrs after application (Fig. 4A, B). Bacteria could not be seen on samples taken immediately and one and six hours after inoculation. This might be because the samples were mounted on the side with the site where bacteria colonised facing down. No bacteria were observed on control flowers treated with distilled water.

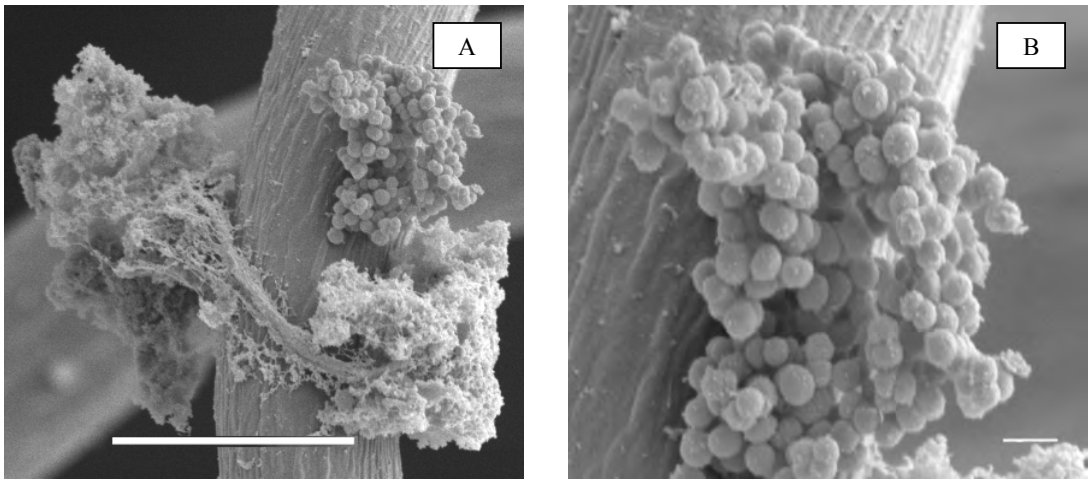

Figure 1. Scanning electron micrographs of avocado flowers two hours after applying *Bacillus subtilis* showing: (A) Bacteria embedded in fibrillar material on the surface of papillae; (B) Aggregates of bacteria attached to papillae (bar: A= 10 µm; B=1 µm).

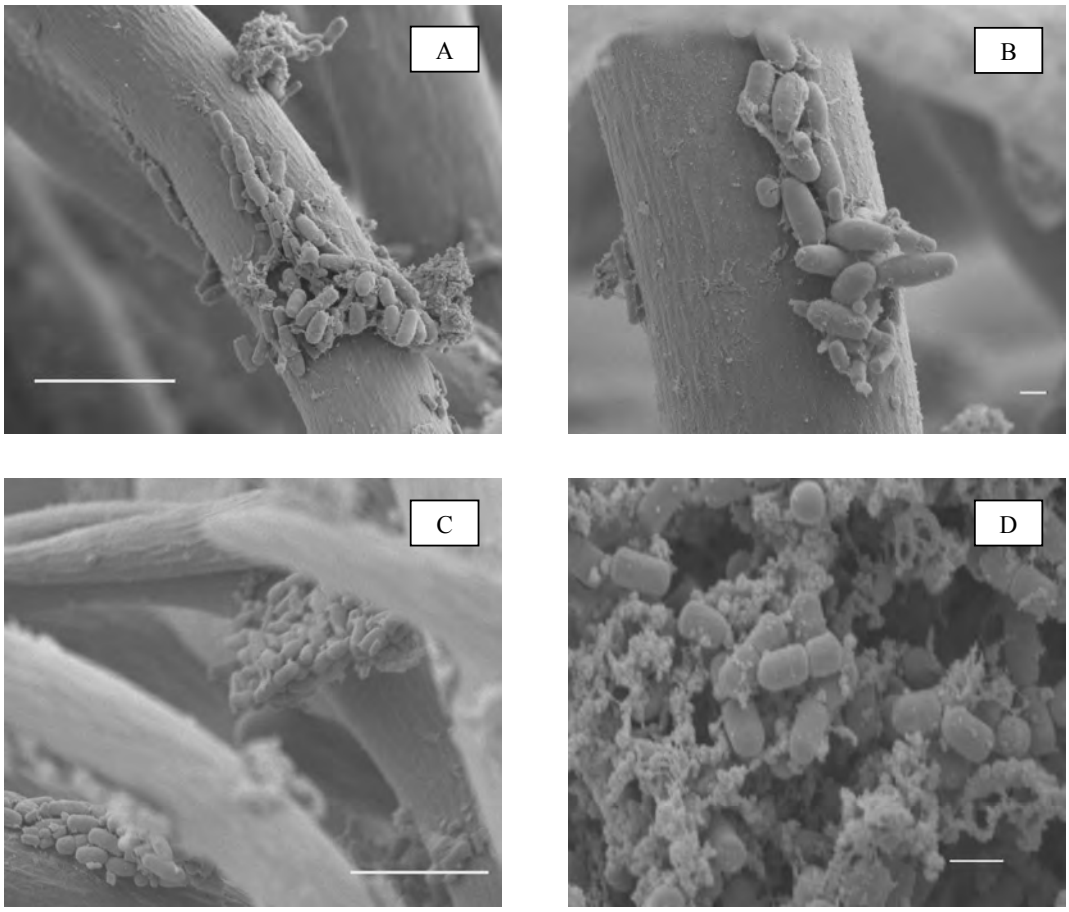

Figure 2. Scanning electron micrographs of avocado flowers four hours after application of ***Bacillus subtilis*** showing: (A) Bacteria attached to papillae (note polar attachment with some bacteria; (B) Bacteria colonising papillae from point of attachment; (C) Bacteria forming colonies on papillae surfaces; (D) Bacterial cells embedded in fibril-like material on papillae and multiplying (bar: A= 10 µm; B= 1 µm; C= 10 µm; D= 1 µm).

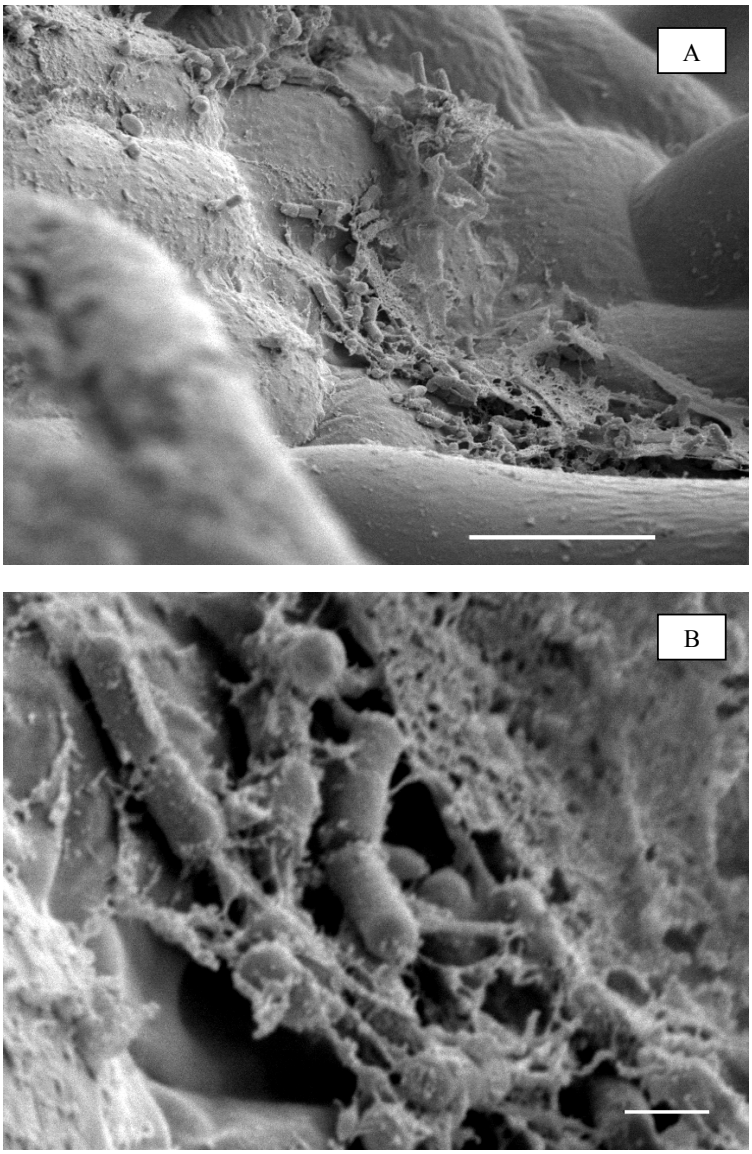

Figure 3. Scanning electron micrograph of avocado flowers 24 hours after application of *Bacillus subtilis* showing (A) and (B) Bacteria colonising and multiplying in depressions on pistil surface (note the presence of fibril-like material) (bar: A = 10 µm; B = 1 µm).

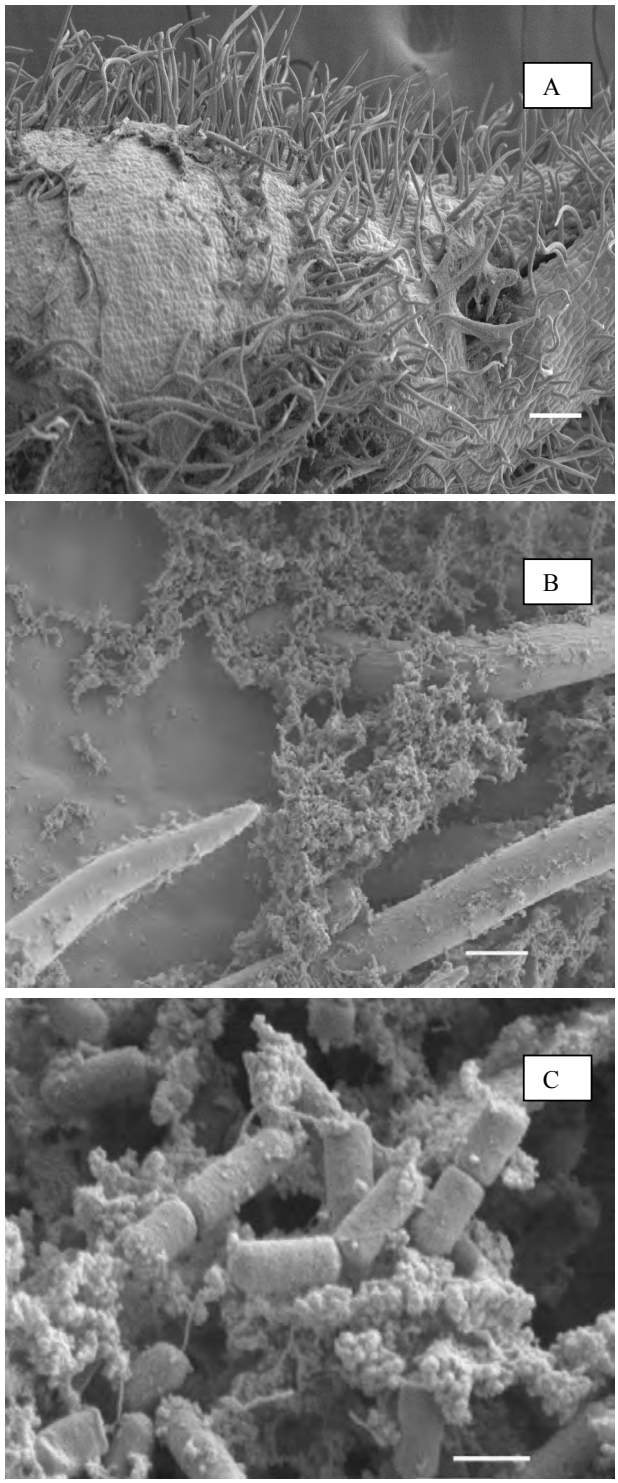

Figure 4. Scanning electron micrographs of avocado flowers 48 hours after application of ***Bacillus subtilis*** showing: (A) Region on the pistil where colony formation and further multiplication were observed; (B) Extensive bacterial colonies formed on surfaces of papillae and in depressions on pistil surfaces; (C) Multiplication of bacteria (bars: 1 μm).

Population dynamics on avocado flowers

Bacterial colony counts were observed to increase over time which indicates that *B. subtilis* can colonise and survive on avocado flowers. The bacterial count was observed to increase for the first 48 hrs and then decline (Fig. 6).

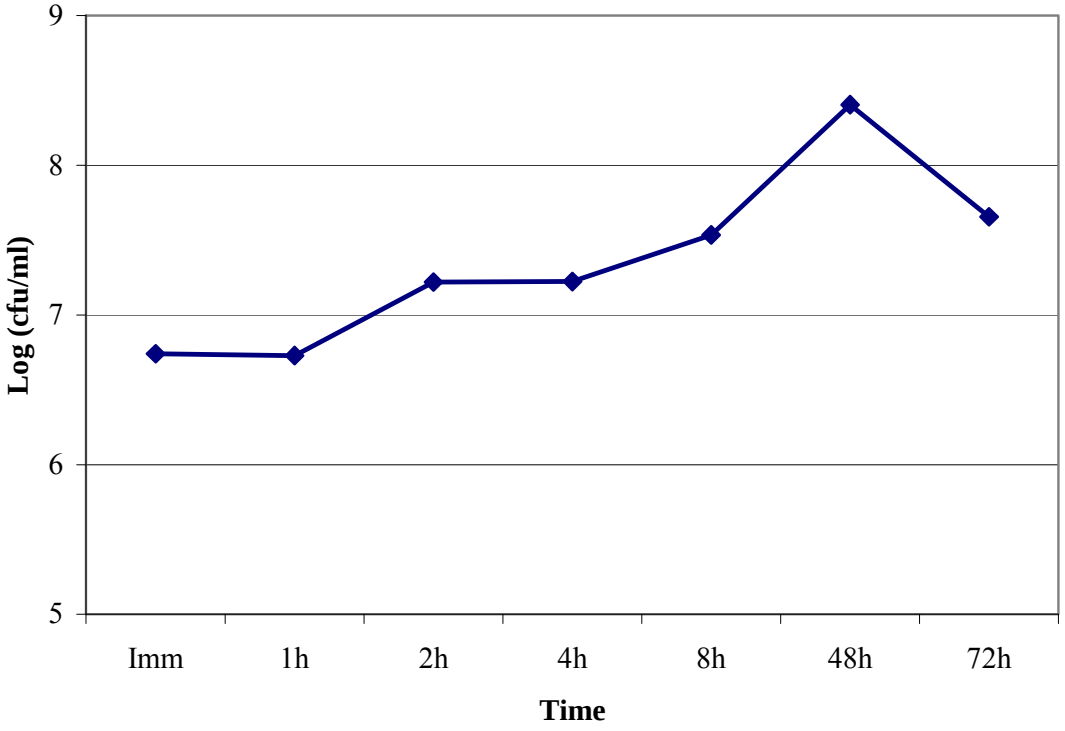

Figure 6. *Bacillus subtilis* viable counts on inoculated avocado flowers.

Pathogen - antagonist interaction on avocado flowers

The positive control treatment inoculated only with the pathogen showed no bacterial colonisation (Fig. 7, 10). Bacteria attached end-on to hyphae and extensively colonised the surfaces of both pathogens including their conidia. Fibrillar material was seen connecting bacterial cells to each other and to the surfaces of the hyphae or conidia (Fig. 8). Shadows around many attached cells suggest the formation of depressions in the hyphal cell wall (Fig. 8A). Evidence of partial or total degradation of hyphae and conidia were evident indicated by an extensive collapse of cell walls and breakage of conidia (Fig. 9, 11B, 13).

Bacterial cells were observed dividing on the surface of hyphae or conidia. Several conidia were seen completely covered by fibril-like structures produced by the attached bacterial cells and no germination was observed from such conidia (Fig. 12). This is in contrast to the positive controls where conidia were observed germinating (Fig. 10). Bacterial attachment and colonisation was observed in all three treatments, often completely covering the hyphae (Fig. 11A). However, where the antagonist was inoculated first, more extensive attachment and colonisation was observed.

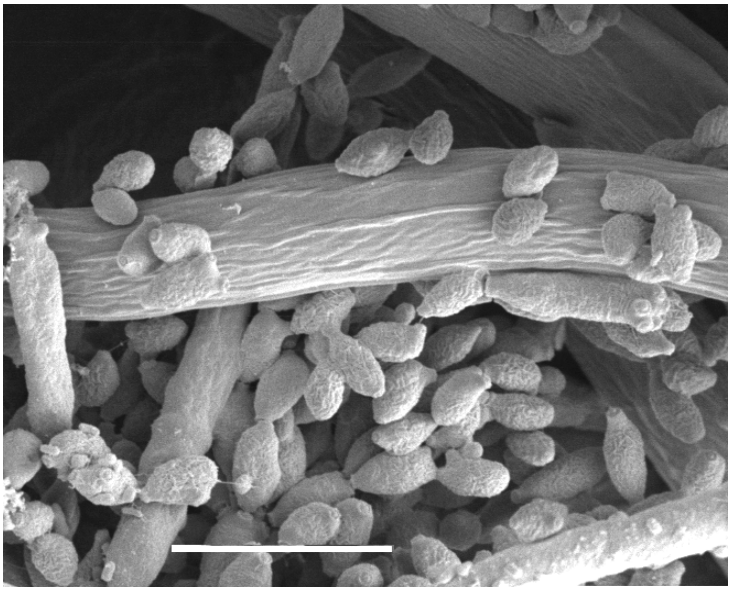

Figure 7. Scanning electron micrograph of conidia of *Dothiorella aromatica* on papillae of control avocado flowers inoculated with the pathogen only (bar: 10 µm).

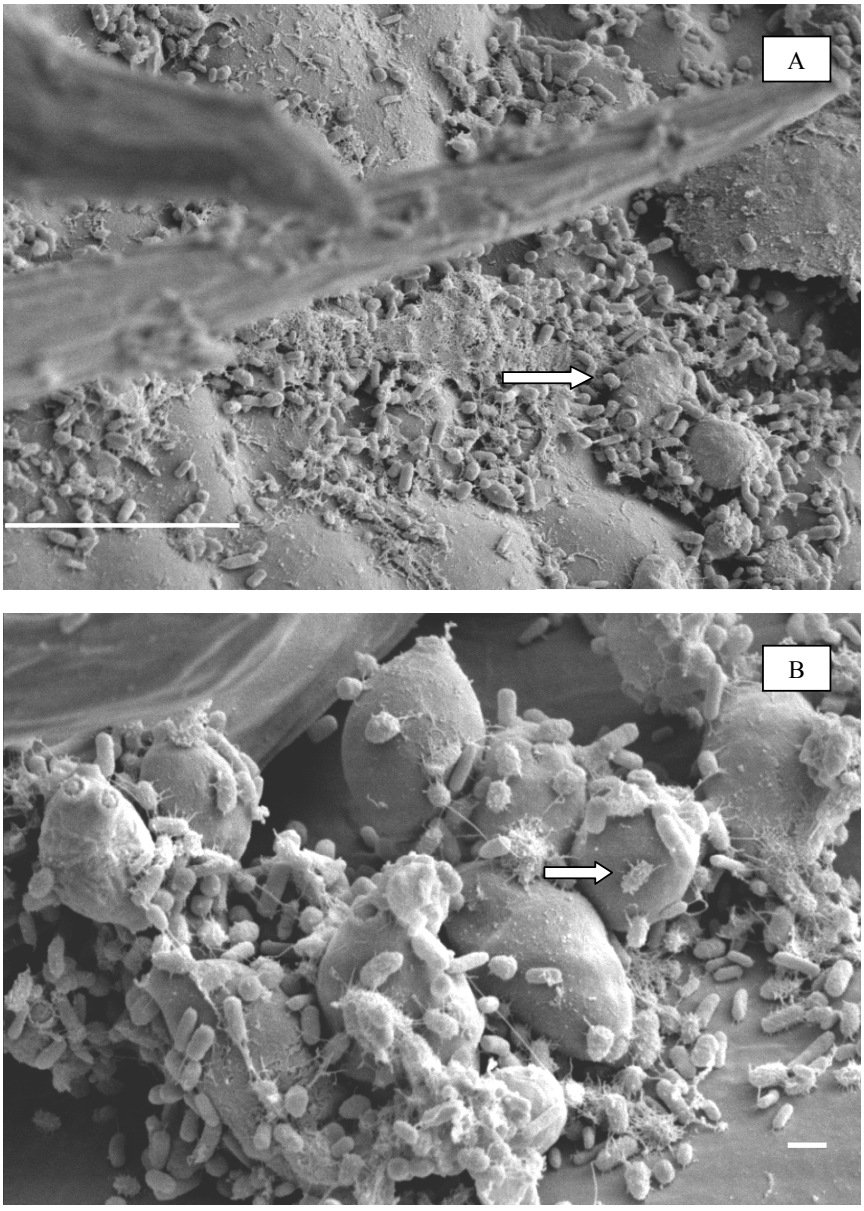

Figure 8. Scanning electron micrographs of ***Bacillus subtilis*** attached to conidia of ***Dothiorella aromatica*** 48 hrs after application of the pathogen to antagonist-inoculated avocado flowers. Note (A) extensive bacterial colonisation on conidia in depressions of pistil surface and (B) the fibrillar structures of bacteria and the shadowy area on conidia (bar: A= 10 µm; B= 1 µm).

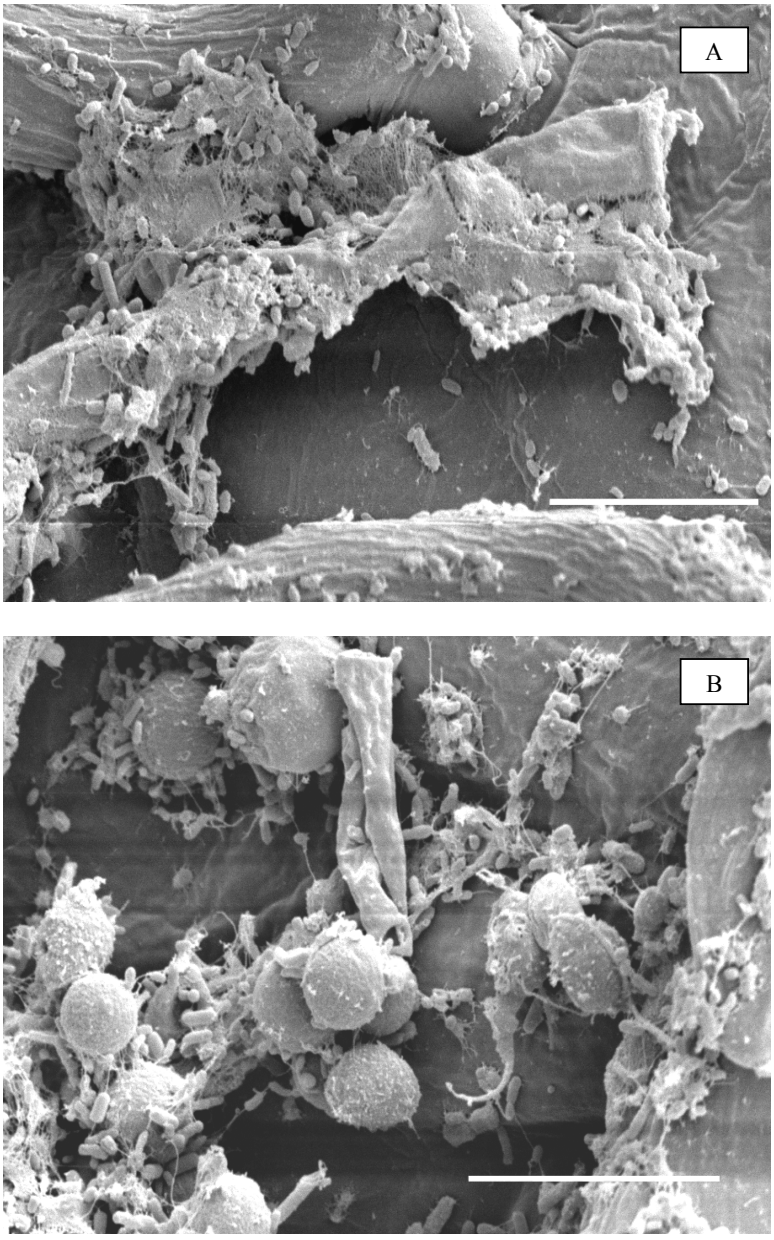

Figure 9. Scanning electron micrograph of hyphae of *Dothiorella aromatica* (A) colonised and completely degraded by *Bacillus subtilis* 48 hrs after application of antagonist to pathogen-inoculated avocado flowers and (B) degraded by *Bacillus subtilis* 48 hrs after simultaneous application of both pathogen and antagonist (bars: 10 µm).

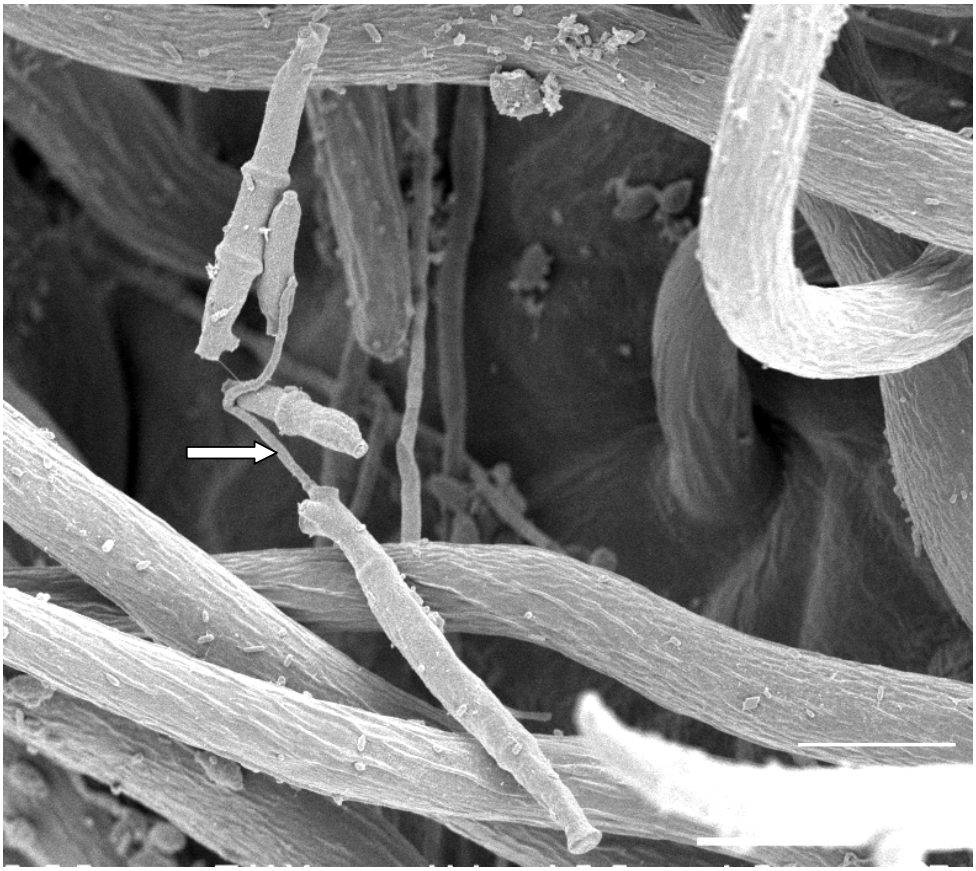

Figure 10. Scanning electron micrograph of conidia of *Phomopsis perseae* on control avocado flowers inoculated with the pathogen only. The arrow indicates a germinating conidium (bar: 10 µm).

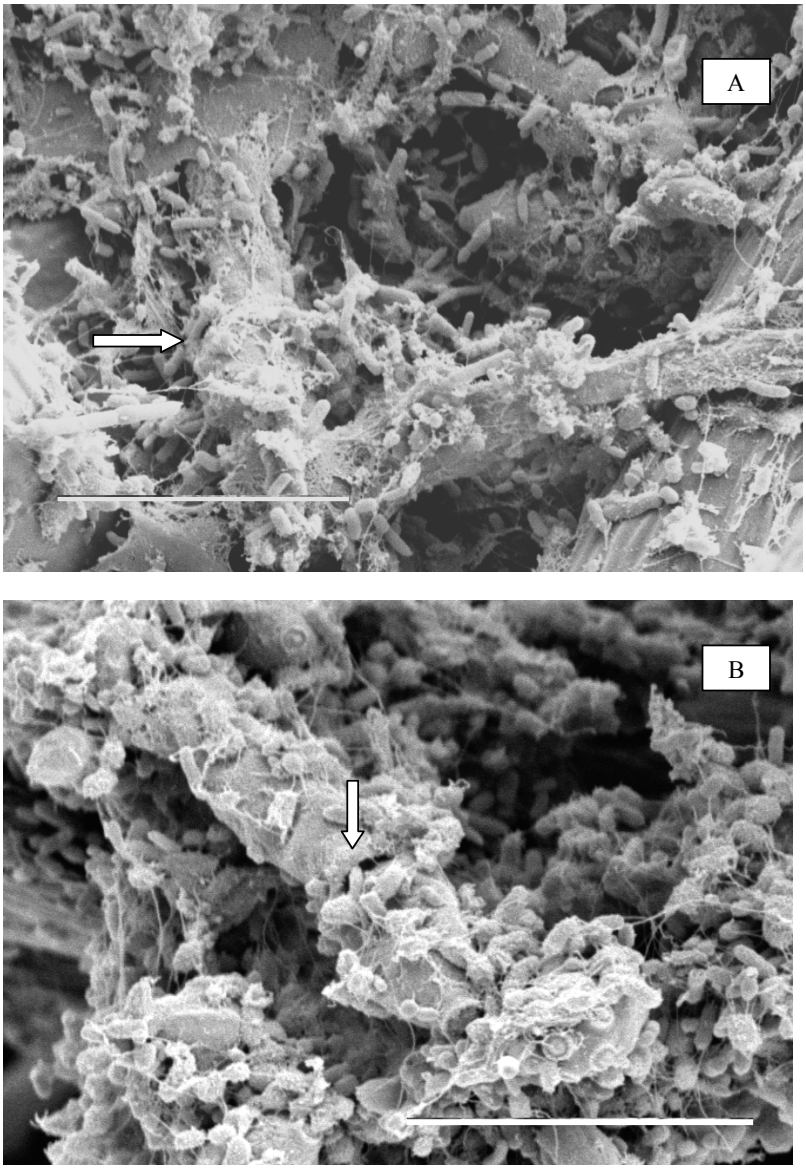

Figure 11. Scanning electron micrographs of *Phomopsis perseae* (A) hyphae colonised and (B) conidia colonised and degraded by *Bacillus subtilis* 48 hrs after application of pathogen to antagonist-inoculated avocado flowers. The arrow shows pit formation by the bacteria on the conidia (bars: 10 µm).

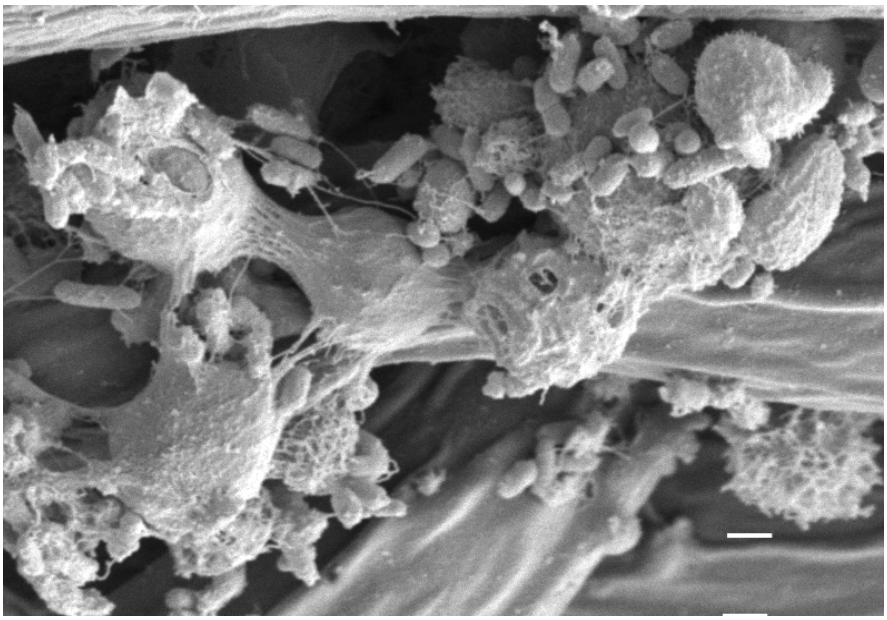

Figure 12. Scanning electron micrograph of conidia of *Phomopsis perseae* completely covered by fibrillar material produced by *Bacillus subtilis* 24 hrs after simultaneous application of pathogen and antagonist to avocado flowers (bar: 1 µm).

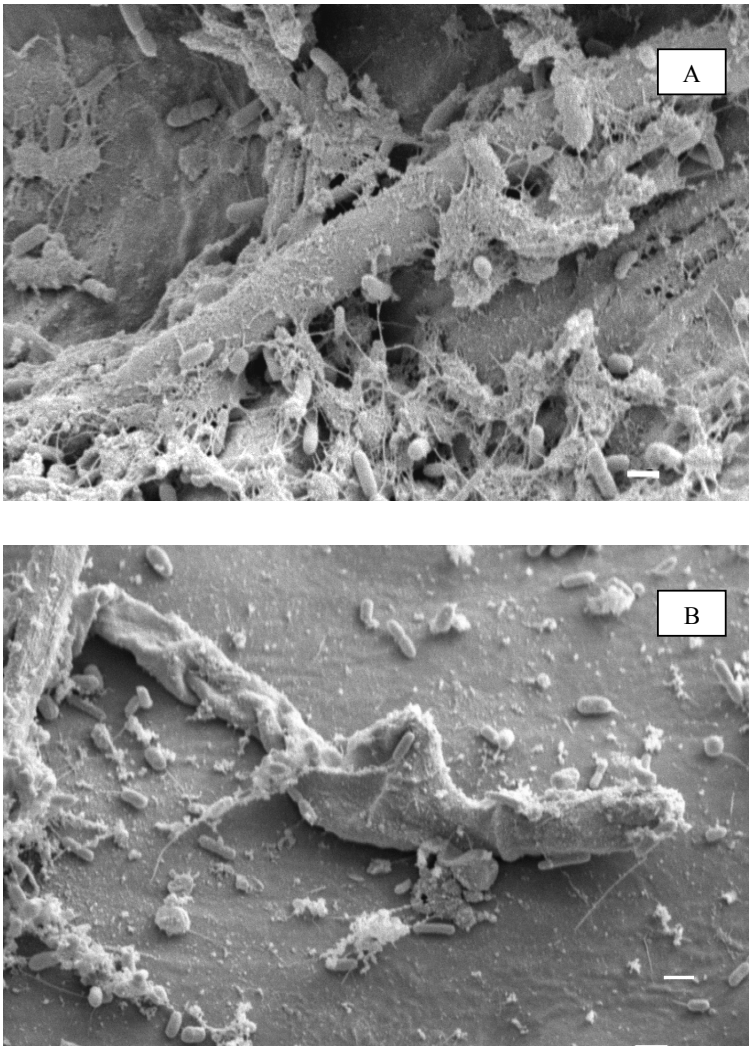

Figure 13. Scanning electron micrographs of *Phomopsis perseae* hyphae (A) colonised and (B) degraded by *Bacillus subtilis* 48 hrs after application of antagonist to pathogen-inoculated avocado flowers (bars: 1 µm).

DISCUSSION

The present study confirmed that *B. subtilis* could attach, survive and colonise avocado flowers. Towsen (1996) showed similar effective attachment, survival and colonisation of the same antagonist on avocado leaves. The antagonist *B. subtilis* was originally isolated from avocado leaf surfaces and was subsequently effectively screened *in vitro* and *in vivo* for antagonism against the most important postharvest pathogens (Korsten, 1993). This study therefore provides a new insight into the ability of the same antagonist to colonise another niche. Within two hours after *B. subtilis* were applied to avocado flowers, fibril-like strands were observed. Similar fibril-like strands have been reported in other similar studies. Towsen (1996) reported observation of attachment strands within 20 sec after *B. subtilis* application to avocado leaves; Latham *et al*. (1978) described 30 min with *Ruminococcus flavefaciens*; Matthyse (1983), two hrs for *Agrobacterium tumefaciens*; and Mariano and McCarter (1993), 72hrs with *Pseudomonas viridiflava*.

Different attachment times were reported for different surfaces and bacterial combinations. Leben and Whitmoyer (1979) found *Pseudomonas lachrymans* adhering to rye grass after 30 min. The differences in contact time could be attributed to various attachment and subsequent colonisation phases as described by Laurence *et al*. (1987) that included motile attachment, reversible attachment, irreversible attachment, growth and colonisation. These phases however, occur in water systems where bacteria need to attach effectively to surfaces to prevent removal by water. Romantschuk (1992) described an initial binding of bacteria to plant surfaces as an early reversible and occasionally rather unspecific phase, followed by irreversible attachment. Romantschuk (1992) stated that the binding capacity of bacteria might be beneficial or sometimes essential for survival and colonisation. This would improve the overall efficacy of the biocontrol agent. No conclusion could be made whether the fibril-like strands observed in this study are part of the irreversible attachment phase.

An important consideration in preharvest applications of biocontrol agents is the ability of microorganisms to survive at sufficient populations on the plant or fruit surfaces after application to ensure effective prevention of pathogen establishment or colonisation (Benbow and Sugar, 1999). Since *B. subtilis* was originally isolated from the avocado phylloplane it indicates that it should be able to tolerate these conditions and build up a

sustainable population, which can prevent pathogen spores from germinating as they arrive. In this study, *B. subtilis* was observed surviving on avocado flowers in high numbers. Towsen (1996) also reported survival of the same antagonist on avocado leaves. Similar observations have also been reported by Stockwell *et al*. (1992, 1998) whereby *Pseudomonas syringae* P*f*A506 was established in large numbers on pear and apple blossoms (10^5 - 10^6 cfu/blossom) under field conditions. The populations established by another antagonist, *Erwinia herbicola* E*h*C9-1, on blossoms averaged between 10^4 and 10^6 cfu/blossom. Biocontrol populations can be adversely affected by weather, pesticide sprays or washed off the plant or fruit surface during rain or spraying.

Knowledge about the mode of action of an antagonist is of importance in improving biocontrol strategies. A biocontrol agent can have a combination of modes of action (Havenga *et al*., 1999). Korsten and de Jager (1995) described *in vitro* interactions between *B. subtilis* and *C. gloeosporiodes*. It was reported that production of lytic enzymes was evident by 'bulb formation' of germinating spores followed by the release of cell contents. Havenga *et al*. (1999) also demonstrated both preventive and curative action of *B. subtilis*. It was shown that antagonist cells applied prior to the pathogen resulted in total inhibition of spore germination, which reflected competitive exclusion or pre-emptive colonisation. The curative effect was also shown with the collapse of germinating fungal hyphae if the pathogen was applied prior to the antagonist. This study revealed the ability of the antagonist to cause lysis of hyphae of the pathogens and degradation of conidia. This could be one of the modes of action of the antagonist on the pathogens.

The associations of bacteria with plant tissue or other microorganisms involve complicated and dynamic interactions. Bacteria exhibit both loose (Roberts *et al*., 1994) and intimate associations (Hood *et al*., 1998) with plant tissues. The latter is mostly mediated by fimbriae, pilli, or cellulose fibrils (Romantschuk, 1992). The presence of fibrillar structures by bacteria attached to pathogen hyphae or conidia as was found in this study shows the ability of the bacteria to remain attached. This could be an initial mechanism of mode of action.

While investigating potential biocontrol agents, Toyota and Kimura (1993) discovered that *Pseudomonas stutzeri* and *Pimelobacter* spp. could colonise chlamydospores of *Fusarium*

oxysporum f. sp. *raphani* and penetrate the outer cell wall. *Pseudomonas stutzeri* was also observed causing a marked reduction in germination of chlamydospores. Nelson *et al*. (1986) reported strong agglutination of cell wall fragments of *Pythium ultimum* by living cells of *Enterobacter cloacae* and random attachment of the bacteria on mycelial surfaces. The positive controls in this study showed germination of pathogen conidia while no conidial germinations were observed from the pathogen-antagonist combinations. This could be an indication to the ability of the antagonist to prevent germination of pathogen conidia. Cook *et al*. (1997) also reported formation of depressions in hyphal cell walls of *Botrytis cinerea* and partial or total degradation of hyphae by *Candida sake*. The presence of depressions and degradation of hyphae and conidia in this study could also depict the possible mode of action of the bacteria on the pathogens. Hence it is suggested that microbial attachment is a means by which antagonism can be delivered to specific pathogen sights and represents an addition to the contemporary biocontrol mode of action model.

Korsten and de Jager (1995) reported high performance of *B. subtilis* against avocado postharvest pathogens like *Dothiorella aromatica* and *Fusarium solani in vitro* on dual culture. Diffusible metabolites from *B. subtilis* were also highly effective in inhibiting these pathogens (Korsten and de Jager, 1995). Thus it is assumed that more than one mode of action is involved in the inhibition of avocado postharvest pathogens by *B. subtilis* as previously postulated by Korsten and de Jager (1995). Further study in understanding all modes of action is recommended.

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CHAPTER 4

EVALUATION OF DIFFERENT *Bacillus subtilis* APPLICATION METHODS TO CONTROL AVOCADO FRUIT DISEASES

ABSTRACT

Several pathogens are known to cause avocado postharvest diseases i.e. anthracnose and stem-end rot (SER). Fungicides have traditionally provided effective control but not for SER. Public pressure has also increased on the agricultural industry to practice organic farming where alternative, safer products can be used. An alternative strategy to use natural antagonistic microorganisms to control plant pathogens has been developed and in some cases commercialised. One such example is Avogreen (*Bacillus subtilis*) that has been registered for use on avocado to control Cercospora spot anthracnose. Extending its application to control other avocado fruit diseases, SER in particular, has been the focus of this study. Dispersing the biocontrol agent to avocado flowers using honeybees can provide an effective means to establish the antagonist population at the infection court of SER pathogens at an early stage. In this study, honeybee dispersal of *Bacillus subtilis* was compared to preharvest sprays using the same antagonist to control avocado fruit diseases such as Cercospora spot, anthracnose and SER. Honeybee antagonist dispersal integrated with field sprays using lower concentrations of copper oxychloride was effective in controlling Cercospora spot and anthracnose. Commercial copper oxychloride sprays were the most effective in controlling both pre- and postharvest diseases. Antagonist sprays alternated with lower concentrations of copper oxychloride were as effective as commercial copper oxychloride sprays. Spray treatments gave higher percentages of healthy fruits compared to honeybee dispersal for control of SER. For Cercospora spot, honeybee antagonist dispersal gave higher percentages of healthy fruit.

INTRODUCTION

The avocado (*Persea americana* Mill.) is a highly prized tropical/subtropical fruit for which demand has increased in world markets making it a more common fresh product in the

consumers' food basket. However, like all other tropical and subtropical fruits, avocado is affected by several preharvest diseases like Cercospora spot, caused by *Pseudocercospora purpurea* (Cke) Deighton, and postharvest diseases including anthracnose caused by *Colletotrichum gloeosporioides* (Penz.) Penz. and Sacc. in Penz., and stem-end rot (SER) caused by a complex of fungi, which include: *D. aromatica*, *C. gloeosporioides*, *Thyronectria psedotrichia* (Schw.) Seeler and *Phomopsis perseae* Zerova (Darvas and Kotzé, 1987). Cercospora spot is the most serious preharvest disease of avocado in South Africa. Stem-end rot, the primary focus of this study, is one of the most important postharvest diseases for the South African avocado industry with losses of up to 13% being reported on overseas markets (Bezuidenhout and Kuschke, 1983 *in* Sanders and Korsten, 1997). Fungal infections via the stem end through endophytic colonisation occur at a preharvest stage and remain quiescent until fruit ripen. This quiescence makes early detection and control difficult.

Currently, growers use extensive copper fungicide sprays to control both pre- and postharvest diseases of avocado. However, these sprays provide only limited control (Pegg *et al*., 2002) and leave visible residues on fruits, which have to be removed manually in packhouses (Korsten, 1993). In addition, build up of copper levels in soils has reached unacceptable levels resulting in certain retailers specifying maximum allowable levels (L. Korsten, personal communication). Growers are thus required to reduce the number of sprays and monitor copper levels in soils. Due to these reasons it has become important to explore alternative disease control options.

The use of microbial antagonists for biological control is one of the most promising alternatives to chemical control as part of an integrated system to reduce pesticide inputs (Archer, 2002). For avocado, successful biological control of pre- and postharvest diseases has been achieved with preharvest *Bacillus subtilis* spray applications or postharvest dip or wax treatments (Korsten *et al*., 1991; 1994). Korsten *et al*. (1997) found that integrated programs of fungicide and *B. subtilis* preharvest sprays give consistently effective control of Cercospora spot. Korsten *et al*. (1989; 1994) also reported that preharvest biocontrol with *B. subtilis* was more effective in reducing SER than standard copper sprays. Similarly,

postharvest applications of *B. subtilis* controlled SER on Fuerte avocado as effectively as and more effectively than prochloraz. However, consistency in product performance could not be shown over time (Korsten *et al*., 1998). One of the reasons for inconsistency in product performance has been attributed to ineffective targeting of the antagonist to the infection court. Alternative delivery strategies like honeybee dissemination of antagonists have been shown to provide effective control of strawberry diseases (Peng *et al*., 1992). Honeybees are believed to deliver the antagonist to the exact infection court on flowers before the arrival of the pathogen.

Because biocontrol does not stand alone as a complete control strategy, integration with reduced chemical applications has been providing more consistent results (Korsten *et al*., 1997). Hence, the aim of this study was to investigate alternative and potentially more effective control strategies primarily for SER pathogens and to determine additional effect on Cercospora spot and anthracnose by comparing preharvest *B. subtilis* on its own or integrated sprays with honeybee dispersal of antagonists.

MATERIALS AND METHODS

Antagonists and fungicides

A commercial liquid formulation of *B. subtilis* isolate B246 (Avogreen) (Stimuplant CC, Pretoria) was used for this trial. The concentration used was $1x10^9$ viable cells/ml for the 2002 season and 5×10^8 viable cells/ml in 2003. The product was diluted in water at a rate of 1ℓ/500ℓ in 2002 and 1ℓ/200ℓ in 2003. Copper oxychloride (Universal Crop Protection Pty. Ltd., Kempton Park) with 85% active ingredient was used for this experiment at the recommended rate. The statistical design of all experiments was done in consultation with the statistician, Ms. Marie Smith, Agricultural Research Council, Pretoria.

Honeybee dispersal of Avogreen to avocado flowers

The bee antagonist dispersal experiment was initially performed at the experimental farm of the University of Pretoria, Pretoria, on 15-year-old Fuerte avocado trees. One beehive with an active colony of bees was used in this experiment. The beehive was placed three meters from

a row of avocado trees. A dispenser was fitted three days prior to dispersal of antagonists to allow bees to get used to the device. The Avogreen powder formulation was mixed with a fluorescent powder (Croda Chemicals, Johannesburg) and placed into the dispenser at 10:00 AM when bee activity was high. Dispenser refilling was done at 11:00 AM and again at 1:00 PM. This was done for three consecutive days. Monitoring was done during the evening of each day using a portable UV light torch. Flower samples were collected for total viable counts as described in Chapter 3.

To determine the amount of antagonist carried by individual bees, ten control bees were collected early in the morning as they crawled out of the dispenser before the antagonist powder was placed into the device. The bees were placed individually into test tubes containing 9 ml of 25% sterile Ringer's solution (Oxoid, Hampshire) and kept in a cool box until all bee samples were collected. Two hours after the antagonist powder was placed into the dispenser, another ten bees exiting from the hive were collected, kept in the same manner as described for control bees and transported back to the laboratory for processing. Test tubes were vortexed for 30 sec and the suspension was dilution-plated on Standard-1 nutrient agar (STD-1) (Biolab, Midrand) to estimate the number of colony forming units (cfu) per bee. Plates were incubated at 37℃ for 24 hrs before counting. This experiment was repeated to check consistency.

A similar experiment was done on Avondshoek Estate, Tzaneen, South Africa, on 15-year-old Fuerte avocado trees in block B in 2003. Five dispensers were fitted on five beehives in the block. Dispensers were filled three times a day, viz. at 08:00 and 10:00 AM and 1:00 PM. The experiment was carried out for 14 days. Nine trees around the beehives were selected and marked. Three treatments were incorporated in this experiment after bee antagonist dispersal. Treatments included: Avogreen liquid sprays (1ℓ/200ℓ) and copper oxychloride sprays (100g/100ℓ; one third of commercially recommended concentration). All sprays were done in three-week intervals form November 2002 until January 2003. Three trees were used for each treatment using a completely randomised design. Due to the practical difficulty of selecting control trees, an additional unsprayed and unvectored control block was included for

comparative purposes. Trees were randomly selected from these blocks. Fruits were harvested in March 2003, evaluated for Cercospora spot and kept in cold storage (6°C) for 21 days and then ripened at room temperature (14-17°C) before evaluations for anthracnose and SER.

Field spray trials

The field spray experiment was conducted in 2002 and 2003 on 15-year-old Ryan avocado trees in block M on Avondshoek Estate. A completely randomised design was used involving four treatments in four replicates. Four trees of similar size and maintained under uniform cultural practices were used per treatment. Treatments were unsprayed control, Avogreen liquid sprays at a rate of 1ℓ/500ℓ (2002) and 1ℓ/200ℓ (2003), commercial copper oxychloride sprays at the recommended rate of 300g/100ℓ, and integrated sprays of copper oxychloride at a rate of 100g/100ℓ alternated with Avogreen at a rate of 1ℓ/500ℓ (2002) and 1ℓ/200ℓ (2003). Biofilm (Plaaschem, Witfield), a sticking and spreading agent, was added to each spray mix at a rate of 25 ml/100ℓ to enhance product attachment to the fruit and leaf surfaces. Trees were sprayed from early November to January in each season at three-week intervals. One tree was left unsprayed between consecutive treatments to avoid spray drift. Spraying was done with a high volume sprayer using hand lances. Trees were sprayed until run-off to obtain full coverage of leaves, branches, stems and fruits.

Fruits were commercially harvested in June 2002 and 2003. Evaluation for Cercospora spot was done immediately after harvest. One crate of fruit (~70 fruits/crate) from each of the four trees in each treatment and for each replicate were paired and fruits were mixed and then divided into two boxes to simulate local and export marketing conditions. Fruits for the local market simulations were ripened under room temperature (14-17°C) while those for export market simulations were kept in cold storage (6°C) for 21 days and then ripened at room temperature (14-17°C) before evaluation for anthracnose and SER.

Evaluation of fruit and data analysis

For Cercospora spot, fruits were evaluated externally using a 0-3 scale where 0 = clean fruit, 1 = one to three spots, 2 = four to seven spots, 3 = more than eight spots (Appendix 1). Fruits

with ratings 0 and 1 were considered as marketable fruit (National Department of Agriculture, South Africa, http://www.nda.agric.za) and analysis was done accordingly. For anthracnose and SER fruits were evaluated when they were at the ready-to-eat ripeness level. Each fruit was cut open longitudinally to evaluate SER internally and peeled to rate anthracnose infections. For both diseases a 0 - 5 scale was used where 0 = clean fruit, 1 =1- 10% infection, 2 = 11 - 20% infection, 3 = 21 - 40% infection, 4 = 41 - 70% infection, and 5 = > 71% infection (Appendix 2).

Data analysis was done using the statistical program GenStat (2000). One-way analysis of variance (ANOVA) was done to differentiate percentage healthy fruits among treatments within each trial according to Fisher's Protected Least Significant Difference test at a 5% level of significance. A Chi-square test was done to compare percentage healthy fruits between the spray trial of 2003 and the bee dispersal experiment. Paired treatments were: Avogreen sprays vs. bee dispersal, Avogreen sprays vs. the integration of bee dispersal and Avogreen sprays, and the integration of Avogreen and copper oxychloride sprays vs. the integration of bee dispersal and copper oxychloride sprays.

RESULTS

Honeybee dispersal of Avogreen to avocado flowers

The fluorescent indicator powder was found all over the flowers monitored. The number of flowers with fluorescent powder increased from four flowers/panicle on day one to 10 flowers/panicle on day three. However, no viable *B. subtilis* colonies were recovered from these fluorescent flowers. The bacterial population recovered from bees that exited through the dispenser containing the antagonist powder had an average of 4 x 10^4 cfu per bee. No *B. subtilis* colonies were observed on plates from control bees.

In the field trial, the integration of bee antagonist dispersal and copper oxychloride sprays gave the most effective control for Cercospora spot followed by bee antagonist dispersal on its own (Fig. 1; Appendix 3). The integration of bee antagonist dispersal and copper

oxychloride sprays was the most effective treatment in controlling anthracnose. No significant difference amongst treatments was observed for SER.

Field spray trial of chemical and antagonist to avocado trees

In both seasons, the highest percentage marketable fruit for Cercospora spot was obtained from commercial copper oxychloride sprays followed by the integrated sprays (Fig. 2). No significant difference was observed between Avogreen sprays and the unsprayed control.

Under local market simulated conditions, copper oxychloride gave the highest level of control for anthracnose in 2002 and 2003 (Fig. 3). The other treatments were not effective in 2002. However, in 2003 the integrated treatment gave the second best control of anthracnose. There was no significant difference between treatments in both years in controlling SER (Fig. 3) (see also Appendix 4). Under export market simulated conditions, copper oxychloride gave the best results in controlling anthracnose in 2002 (Fig. 4) with no significant difference between the other treatments. In 2003, copper oxychloride and the integrated treatment gave the same level of anthracnose control. Stem-end rot was most effectively controlled by copper oxychloride in 2002 but there was no significant difference between the treatments in 2003 (Fig. 5) (see also Appendix 5).

The Chi-square test showed significantly higher percentage healthy fruit from bee antagonist dispersal on its own for Cercospora spot compared to Avogreen sprays (Appendix 6). The integration of bee antagonist dispersal and copper oxychloride sprays gave a higher percentage healthy fruit compared to the integration of Avogreen and copper oxychloride sprays. There was no significant difference between Avogreen sprays and the integration of bee antagonist dispersal followed by Avogreen sprays. No significant difference in percentage healthy fruit was observed between the paired treatments for anthracnose. For SER, Avogreen sprays gave significantly higher percentage healthy fruit compared to the bee antagonist dispersal on its own and the integration of bee antagonist dispersal and Avogreen sprays. There was no significant difference between the integration of Avogreen and copper

oxychloride sprays and the integration of bee antagonist dispersal and copper oxychloride sprays.

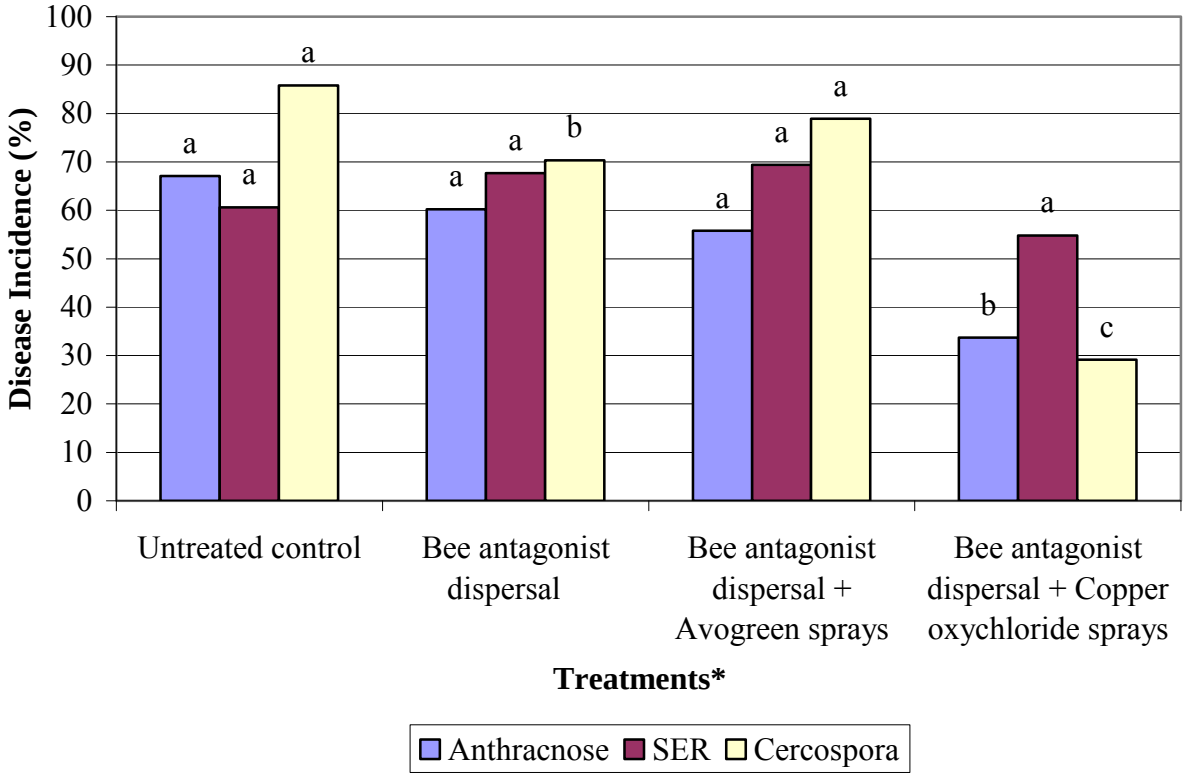

Figure 1. Effect of honeybee dispersal of Avogreen powder to avocado flowers and incorporation of Avogreen liquid and copper oxychloride sprays in controlling pre- and postharvest diseases of avocado during the 2003 season.

* Treatments: Untreated control; Bee antagonist dispersal to avocado flowers on its own; Bee antagonist dispersal to avocado flowers followed by Avogreen liquid sprays at a rate of 1ℓ/200ℓ of water; Bee antagonist dispersal to avocado flowers followed by copper oxychloride sprays at a rate of 100g/100ℓ of water;. Letters on bars indicate significant differences among treatments according to Fisher's Protected Least Significant Difference test at 5 % level of significance.

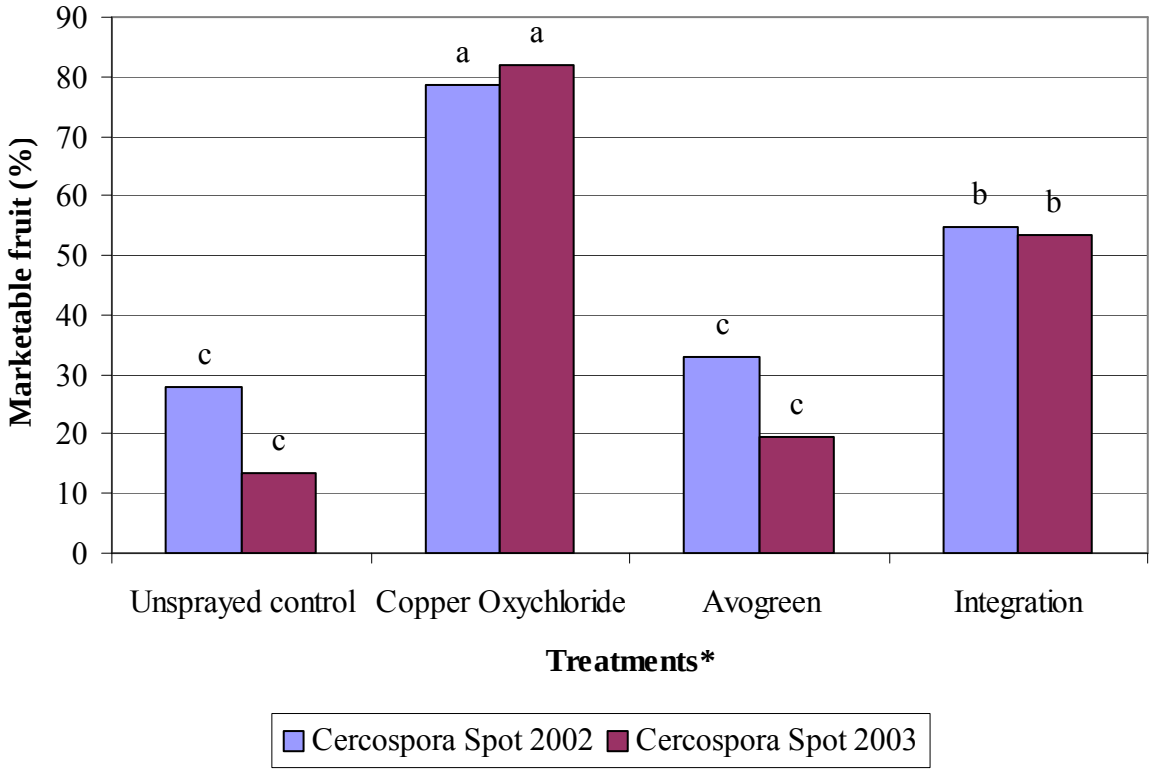

Figure 2. Effect of Avogreen and copper oxychloride sprays on control of Cercospora spot on avocado during the 2002 and 2003 seasons.

* Treatments: Unsprayed control; Commercial copper oxychloride sprays at a rate of 300g/100ℓ of water; Avogreen liquid sprays at a rate of 1ℓ/500ℓ (2002) and 1ℓ/200ℓ (2003) of water; Integration of Avogreen at rate of 1ℓ/500ℓ (2002) and 1ℓ/200ℓ (2003) of water and copper oxychloride at a rate of 100g/100ℓ of water. Letters on bars indicate significant differences among treatments according to Fisher's Protected Least Significant Difference test at 5 % level of significance.

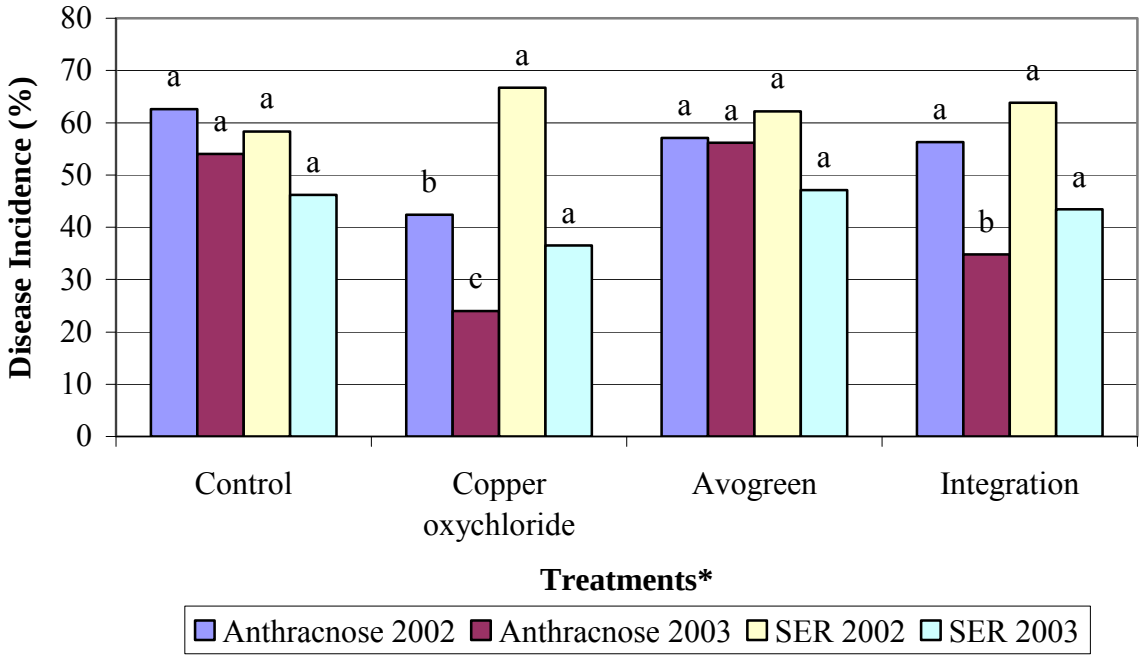

Figure 3. Effect of Avogreen and copper oxychloride sprays on anthracnose and stem-end rot of avocado during the 2002 and 2003 seasons for fruit evaluated at local market simulation conditions.

* Treatments: Unsprayed control; Commercial copper oxychloride sprays at a rate of 300g/100ℓ of water; Avogreen liquid sprays at a rate of 1ℓ/500ℓ (2002) and 1ℓ/200ℓ (2003) of water; Integration of Avogreen at rate of 1ℓ/500ℓ (2002) and 1ℓ/200ℓ (2003) of water and copper oxychloride at a rate of 100g/100ℓ of water. Letters on bars indicate significant differences among treatments according to Fisher's Protected Least Significant Difference test at 5 % level of significance.

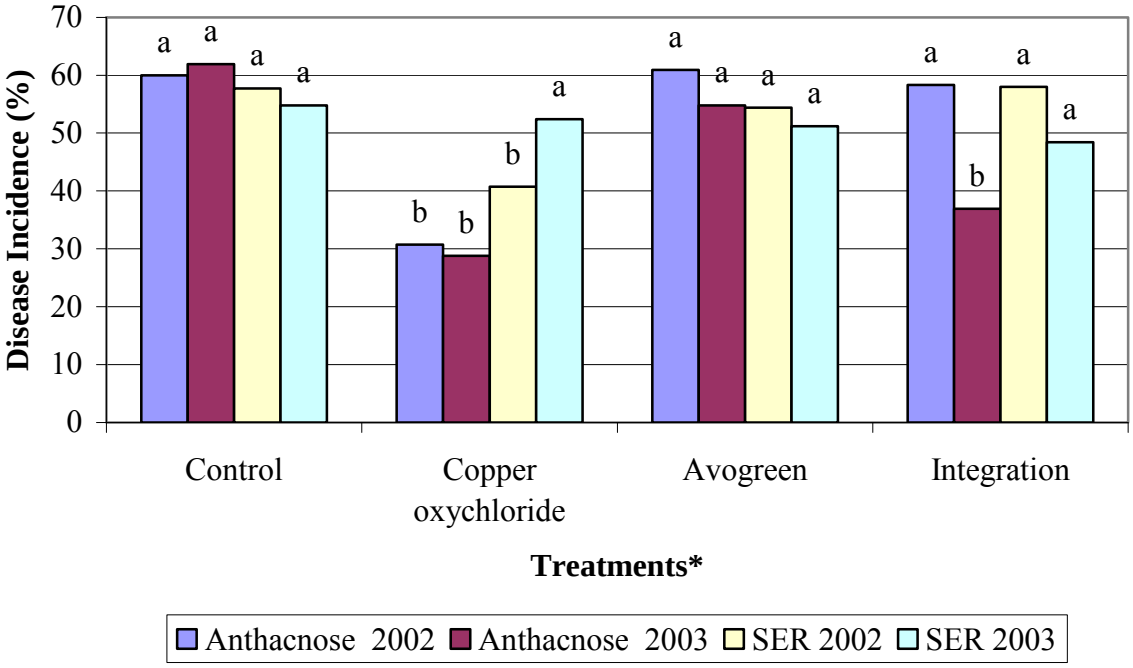

Figure 4. Effect of Avogreen and copper oxychloride sprays on control of anthracnose and stem-end rot of avocado during the 2002 and 2003 seasons for fruit evaluated at export market simulation conditions.

* Treatments: Unsprayed control; Commercial copper oxychloride sprays at a rate of 300g/100ℓ of water; Avogreen liquid sprays at a rate of 1ℓ/500ℓ (2002) and 1ℓ/200ℓ (2003) of water; Integration of Avogreen at rate of 1ℓ/500ℓ (2002) and 1ℓ/200ℓ (2003) of water and copper oxychloride at a rate of 100g/100ℓ of water. Letters on bars indicate significant differences among treatments according to Fisher's Protected Least Significant Difference test at 5 % level of significance.

DISCUSSION

Stem-end rot pathogens infect through flowers at the early stage of fruit development. One of the major aims of this experiment was, therefore, to investigate alternative and more effective control strategies to control SER pathogens. Bee antagonist dispersal to avocado flowers was therefore compared with spray applications of antagonist and fungicides. Although previous *in vivo* results (Chapter 3) showed that the antagonist could attach, colonise and survive on avocado flowers, no viable counts of *B. subtilis* were retrieved from flowers visited by bees carrying antagonist powder despite high bee activity during the experiment. Studies conducted on apples showed good recovery of antagonistic bacteria from blossoms visited by inoculated bees (Johnson *et al.*, 1993). However, Archer (2002) reported a considerable variation in the level of fungal biocontrol agent recovered from apple blossoms inoculated in a similar way and attributed these variations to a number of factors including bee foraging behaviour being temperature dependant and viability of fungal propagules over time. These factors could be some of the reasons for the failed recovery of the antagonist from the avocado flowers. The survival of antagonists is enhanced by the ability to colonise certain sites, which provide protection against UV-light and dry adverse conditions on the surface (Andrews, 1992). The pistilate surface of avocado flowers is covered with papillae, which may interfere with deposition and may not protect antagonists from adverse environmental parameters. It is also possible that very little amount of the antagonist formulation was deposited on the flowers by the bees. This also applies for the insignificant level of SER control achieved by bee antagonist dispersal to avocado flowers. The integration of bee antagonist dispersal and lower concentration of copper oxychloride sprays also did not give any control. This confirms that fungicide sprays cannot reach infection sites of SER pathogens and hence cannot offer protection against them.

Compared to honeybee dispersal, Avogreen sprays during flowering resulted in a higher percentage healthy fruit without SER. However the fact that the spray trial was done for two consecutive seasons should be taken into consideration. During the season there could have been a build up of antagonist population on the trees, which could have resulted in an improved level of disease control as was previously reported by Korsten *et al.* (1997). The

bee antagonist dispersal on the other hand was done only for one season. In addition, spray application covers the whole tree thereby reducing inoculum within the tree canopy for the following season while bee vectoring is very specific targeting flowers of that season. Although a reduction in SER incidence was observed with copper oxychloride sprays in 2002, no significant level of disease control was observed the following year. This indicates once again that fungicides are ineffective in providing protection against SER pathogens.

The second aim of this study was to investigate the potential of these alternative strategies in controlling other avocado fruit diseases like Cercospora spot and anthracnose. Preharvest and latent infections are difficult to control with postharvest biocontrol strategies (Ippolito and Nigro, 2000). Field application of the biocontrol agents may enable early colonisation of the fruit surfaces, protecting them from these infections. Although these strategies are difficult to use to reduce diseases (Peng and Sutton, 1991), significant reduction of natural infections of Cercospora spot on avocado has been reported with preharvest sprays of *B. subtilis* (Korsten *et al*., 1992) and integrated treatments of *B. subtilis* and copper fungicides (Korsten *et al*., 1997). In this experiment, copper oxychloride sprays were the most effective in reducing both pre- and postharvest diseases of avocado. The second best treatment was the integrated Avogreen and copper oxychloride sprays, which is in agreement with Korsten *et al*. (1997). Korsten *et al*. (1989) and Korsten (1993) also reported effective control of postharvest decay by using preharvest *B. subtilis* sprays. Contrary to previous work (Korsten *et al*., 1992), Avogreen sprays on its own gave no significant control in both pre- and postharvest diseases. It should however be noted that Korsten *et al*. (1992) used laboratory prepared formulation of the antagonist while in this experiment a commercial product was used.

Korsten *et al*. (1997) reported less effective control of Cercospora spot with biological and integrated treatments during the first spray season, which improved over subsequent seasons. Acceptable control of plant diseases through biological or integrated treatments is not always evident in the first season of the trial (Korsten *et al*., 1997). Therefore, it needs repetition over consecutive seasons. This enables the biocontrol agent to gradually build up its populations on the plants and adapt to different environmental parameters. In addition, Van Eeden and

Korsten (2003) reported a pronounced increase in the establishment and survival of antagonist on avocado trees with higher spray frequencies. The improved levels of control of anthracnose obtained with integrated treatments in 2003 in both local and export market simulated conditions may indicate build up of antagonist populations on the trees over time. Although previous research showed the usefulness of sticking and spreading agents (Korsten *et al*., 1994), Van Eeden and Korsten (2003) recommended the exclusion of sticking and spreading agents like Biofilm during field applications of *B. subtilis* for biological control on avocado as they reduce the efficacy of the biocontrol agent. This could be one reason for the low level of disease control achieved with the antagonist in this study.

Copper oxychloride sprays gave the highest percentage marketable fruit for control of Cercospora spot in both seasons compared to the rest of the treatments. A decrease in percentage marketable fruit was observed for Avogreen sprays in 2003 as compared to 2002. This could probably be due to higher inoculum pressure in the later season.

In this study, the biocontrol only approaches were not successful in controlling most pre- and postharvest diseases of avocado except anthracnose, while the integrated approach with reduced copper oxychloride concentrations provided control for most avocado diseases. A biocontrol only approach may therefore not be a long-term solution for replacing copper sprays and an integrated approach should rather be followed.

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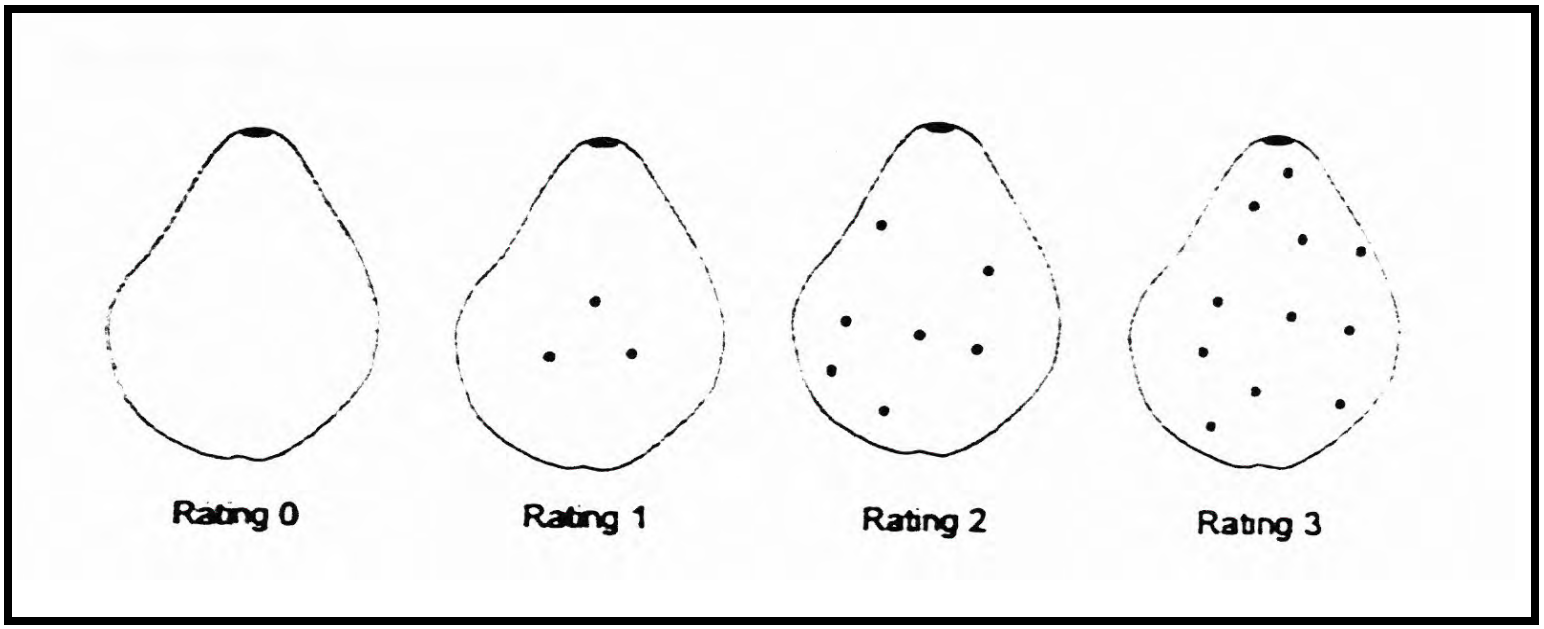
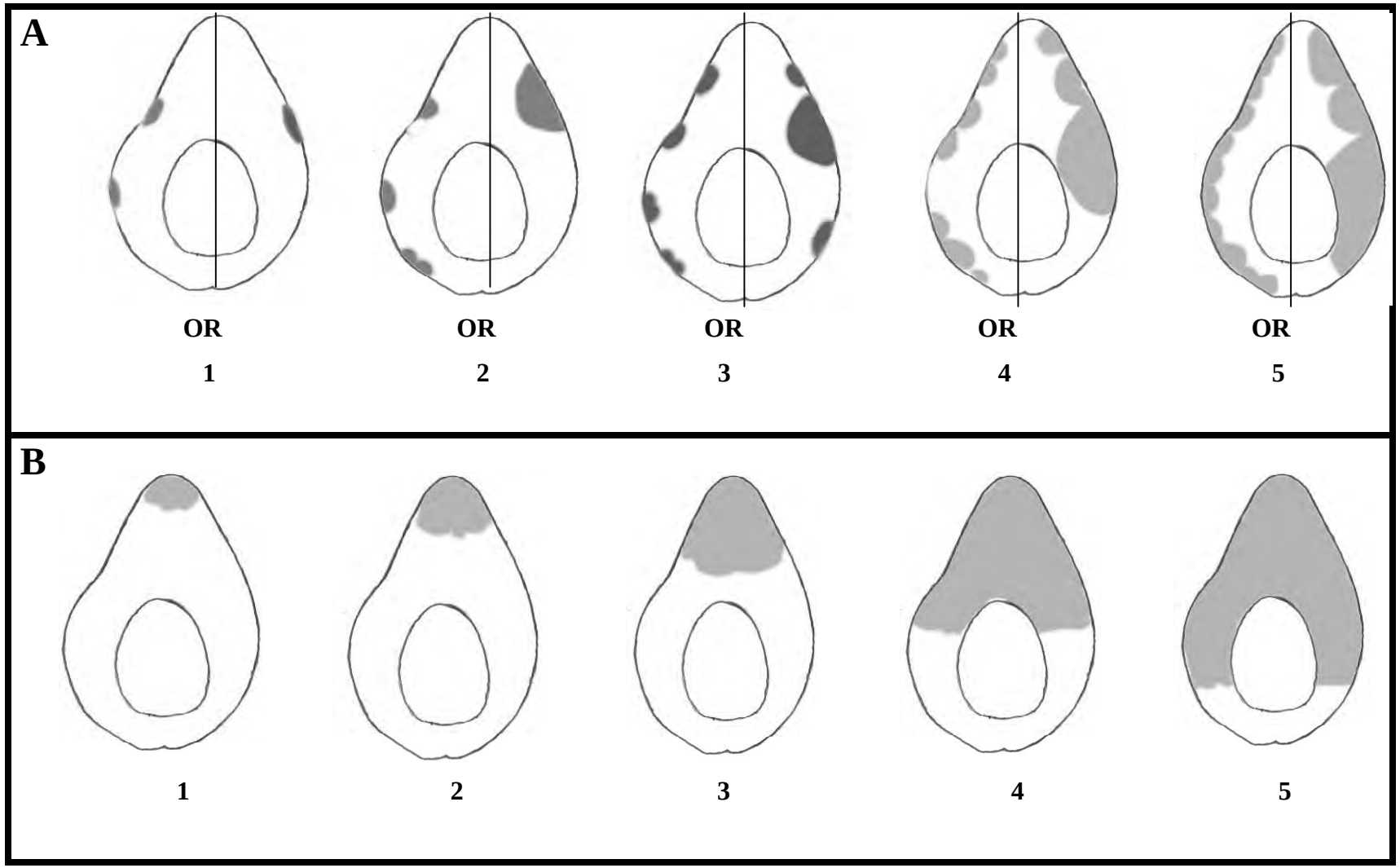
Appendix 1. Rating criteria for Cercospora spot where 0 represents clean fruit, 1 = 1 to 5 spots, 2 = 6 to 10 spots, 3 = >10 spots



Appendix 2. Rating criteria for evaluation of internal (A) anthracnose and (B) stem-end rot where 0 = clean fruit, 1 =1-10% infection, 2 = 11 - 20% infection, 3 = 21 - 40% infection, 4 = 41 - 70% infection, and 5 = > 71% infection



Appendix 3: Percent healthy fruit for export market simulations from honeybee dissemination of antagonist powder to avocado flowers in 2003

Treatments [1]	Anthracnose	SER	Cercospora spot
1	32.93 a	39.4 a	14.26 c
2	39.77 a	32.3 a	36.83 b
3	44.22 a	30.6 a	36.02 b
4	66.28 b	45.2 a	84.59 a
SEM[2]	9.85	6.12	3.72
Fpr.[3]	0.016	0.065	<0.001
LSD (5%)[4]	18.55	-	7.0

[1]Treatments: 1 = Unsprayed control, 2 = Bee antagonist dispersal on its own, 3 = Bee antagonist dispersal + Avogreen sprays (1ℓ/200ℓ of water), 4 = Bee antagonist dispersal + Copper oxychloride sprays (100g/100ℓ of water); [2]SEM = Standard error of means; [3]Fpr. = Calculated F value; [4]LSD (5%) = Least Significant Difference at 5 % level of significance.

Appendix 4. Percent marketable fruit for Cercospora spot from spray trials in 2002 and 2003

Treatments[1]	2002	2003
Untreated control	28.01 c	13.44 c
Commercial copper oxychloride sprays	78.58 a	81.84 a
Avogreen sprays	33.03 c	19.54 c
Avogreen + Copper oxychloride sprays	54.64 b	53.27 b
SEM[2]	7.40	4.08
Fpr.[3]	<0.001	<0.001
LSD (5%)[4]	7.74	13.42

[1]Treatments: 1 = Unsprayed control, 2 = Commercial copper oxychloride (300g/100ℓ of water), 3 = Avogreen (1ℓ/500ℓ of water in 2002 and 1ℓ/200ℓ of water in 2003), 4 = Integrated treatment [Avogreen1ℓ/500ℓ of water (2002) and 1ℓ/200ℓ of water (2003) and copper oxychloride 100g/100ℓ of water]; [2]SEM = Standard error of means; [3]Fpr. = Calculated F value; [4]LSD (5%) = Least Significant Difference at a 5% level of significance.

Appendix 5. Percent healthy fruit for local and export market simulations from spray trial in 2002 and 2003

Treatments[1]	Local				Export			
	2002		2003		2002		2003	
	Anth.[2]	SER[3]	Anth.	SER	Anth.	SER	Anth.	SER
1	37.4 a	41.7 a	46.0 a	47.9 a	40.0 a	42.3 a	38.1 a	45.2 a
2	57.6 b	33.3 a	76.0 c	64.3 a	69.3 b	59.3 b	71.2 b	47.6 a
3	42.9 a	37.8 a	44.0 a	46.4 a	39.1 a	45.6 a	45.2 a	48.8 a
4	43.7 a	36.2 a	65.2 b	51.6 a	41.7 a	42.0 a	63.1 b	51.6 a
SEM[5]	7.91	8.84	5.10	9.42	4.72	6.68	8.48	12.55
Fpr.[6]	0.021	0.610	<0.001	0.410	<0.001	0.01	<0.001	0.907
LSD (5%)[7]	12.81	13.62	7.86	-	7.27	10.29	13.06	-

[1]Treatments: 1 = Unsprayed control, 2 = Commercial copper oxychloride (300g/100ℓ of water), 3 = Avogreen (1ℓ/500ℓ of water in 2002 and 1ℓ/200ℓ of water in 2003), 4 = Integrated treatment [Avogreen1ℓ/500ℓ of water (2002) and 1ℓ/200ℓ of water (2003) and copper oxychloride 100g/100ℓ of water]; [2]Anth. = Anthracnose; [3]SER = Stem-end rot; [4]CS = Cercospora spot; [5]SEM = Standard error; [6]Fpr. = Calculated F value; [7]LSD (5%) = Least Significant Difference at a 5 % level of significance.

Appendix 6. Chi-square (χ^2) comparisons between spray trials and honeybee antagonist dispersal in 2003

Treatments compared[1]	Anthracnose		SER		Cercospora spot	
	Clean fruit %	χ^2 value [2]	Clean fruit %	χ^2 value	Marketable fruit %	χ^2 value
Spray 3 vs Bee 1	31.3 vs 26.3	1.115 NS	36.7 vs 15.8	22.060 S	11.9 vs 36.2	56.95 S
Spray 3 vs Bee 2	31.1 vs 31.5	0.00 NS	36.7 vs 12.7	43.645 S	11.9 vs 34.0	66.13 S
Spray 4 vs Bee 3	62 vs 69.2	2.142 NS	36 vs 33.3	0.22. NS	73.7 vs 98.7	45.26 S

Tabled chi-square value = 9.801

Test level = 0.00179

Degree of freedom = 1

[1] Treatments compared: Spray 3 = Avogreen sprays; Spray 4 = Integrated sprays of Avogreen and copper oxychloride; Bee 1 = Bee antagonist dispersal on its own; Bee 2 = Bee antagonist dispersal + Avogreen sprays; Bee 3 = Bee antagonist dispersal + copper oxychloride sprays; [2] χ^2 = Chi-square value; NS = Non-significant; S = Significant.

CHAPTER 5

MOLECULAR COMPARISON AND IDENTITY CONFIRMATION OF *Botryosphaeria* SPECIES FROM AVOCADO IN SOUTH AFRICA

ABSTRACT

Knowledge of pathogen identity is crucial to develop effective control strategies. Taxonomic confusion regarding *Dothiorella* spp. has recently been resolved by combining molecular and morphological identification techniques. The pathogen is now placed under the genus *Botryosphaeria*. In this study, molecular comparisons were performed to identify differences amongst isolates previously described as *Dothiorella* from avocado. Twenty-six *Dothiorella* isolates obtained from avocado fruits, sixteen isolates from mango leaves were used in this study. Two isolates of *Botryosphaeria parva* and *Botryosphaeria dothidea* isolated from mango in Australia were included in this experiment for comparative purposes. A 10-mer primer OPC02, randomly amplified the template DNA. The primer gave discriminatory banding patterns among the isolates separating them into three groups. The ITS regions of 12 avocado and 7 mango isolates from South Africa were further included in the RFLP identification system for *Botryosphaeria*. The ITS-PCR amplicons were digested with *Cfo*I restriction enzyme that resulted in polymorphic banding patterns. Three *Botryosphaeria* species were distinguished with this enzyme. *Botryosphaeria parva* was the most frequent species among all isolates. This can be used as base information for further studies on the fungus from avocado.

INTRODUCTION

Stem-end rot of avocados is caused by a number of fungal pathogens, one of the major species being *Dothiorella aromatica* (Sacc.) Petr. and Syd (Darvas and Kotzé, 1987). The taxonomy of this pathogen spp. has been controversial. The species of *Dothiorella*, *Lasiodiplodia* Ellis and Everh., *Fusicoccum* Corda and *Diplodia* Fr. are the anamorph states of Botryosphaeriaceous fungi (Jacobs and Rehner, 1998). The type species of *Dothiorella* was recently syonymised under *Diplodia*, raising questions about the correct genetic affinities of all species presently placed in *Dothiorella* (Crous and Palm, 1999). Researchers have reported *Fusicoccum* species from mango and avocado. Hartill (1991) examined Botryosphaeriaceous fungi from avocado, which have previously been

described as *Dothiorella* spp. and concluded that these fungi should reside in the fungus *Fusicoccum*.

The taxonomy of *Botryosphaeria* spp. had mostly been based on morphological characteristics of the associated anamorphs. However, these characters overlap among anamorphs and teleomorphs making differentiation difficult. Recently, molecular diagnosis together with morphological characterisation has been used in identifying *Botryosphaeria* anamorphs (Jacobs, 2002). Various DNA-based techniques have been applied in identifying fungi. These techniques include the Random Amplified Polymorphic DNA (RAPD) and Restriction Fragment Length Polymorphism (RFLP).

In RAPD-PCR, a single primer, generally a 10'mer binds at low annealing temperatures to DNA. The primer finds homology in the DNA added to the PCR reaction and initiates extension. It generates random amplified polymorphic DNA, which can be analysed reproducibly from differences in the sizes of bands, indicating real sequence differences in the DNA of different strains or species (Foster *et al*., 1993). This technique can amplify many different sequences spread randomly throughout the genome by using a series of different primers of random sequence.

RAPD-PCR has been successfully used for comparison between and within species (William *et al*., 1990 in Duncan *et al*., 1993). The technique has been successful in differentiating and identifying several fungal pathogens, for example, the apple powdery mildew fungus *Podosphaera leucotricha* (Ell. Et Ev.) Salm (Urbanietz and Dunmann, 2000), the grapevine dieback fungus *Eutypa lata* (Pers:Fr.) Tul and C.Tul. (Péros *et al*., 1997), *Serpula lacrymans* (Wulfen: Fr.) J. Schröt. apud Cohn that causes dry rot and decay on timber (Theodore *et al*., 1995) and *Colletotrichum* spp. causing anthracnose diseases of various fruits and *Stylosanthes* spp. (Freeman *et al*., 1998; Munaut *et al*., 1998). RAPD-PCR is useful mainly because it is simple and quick and no prior sequence data is needed (Duncan *et al*., 1993).

A further extension of the PCR procedure for differentiation between closely related fungal species is Restriction Fragment Length Polymorphisms (RFLP) (Tan *et al*., 1996). This technique has been successfully used to identify fungi up to species level (Buscot *et*

al., 1996). Jacobs (2002) also developed a PCR-RFLP identification protocol that could distinguish between four *Botryosphaeria* species from mango in South Africa.

Proper identification and classification of fungal pathogens is important in developing effective disease control strategies. It is also vital in understanding the prevalence and economic importance of the pathogenic species. The aim of this experiment is therefore to confirm the taxonomy of *Botryosphaeria* isolates from avocado and identify the prevalent species.

MATERIAL AND METHODS

Isolation

Isolates were obtained from Ryan avocados harvested from Avondshoek Estate, Tzaneen, South Africa in 2002 from trees used for the spray trial (Chapter 4). Symptomatic fruits were randomly selected from each treatment and isolations were made. Fruits were first sprayed with 70% ethanol to avoid epiphytic contamination and left to air dry. Isolations were subsequently made by aseptically cutting fruit into halves and cutting 0.5 mm^2 sections from the pulp of the fruit at the stem-end. Sections were plated on Potato Dextrose Agar (PDA) (Biolab, Midrand) and plates were incubated at 25°C for four to five days before subcultures were made. Morphological identity of pure cultures was confirmed by Prof. F. C. Wehner, Department of Microbiology and Plant Pathology, University of Pretoria. Cultures were maintained on agar slants and in sterile distilled water. Sixteen isolates of the same fungus from mango were also included for comparison (Table 1).

Isolates were grown on Water Agar (WA) (Agar Bacteriological) (Biolab, Midrand) together with double sterilised avocado twigs or carnation leaves and placed under near UV light to induce sporulation. Subsequent spores were then transferred to WA plates and incubated at 25ºC for two to three hours to induce germination. These plates were then studied under the stereo microscope (WILD M7-A) and single germinating spores were transferred to PDA (Biolab, Midrand) plates and incubated at 25ºC for five days. The single spore cultures were preserved in sterile distilled water and used for molecular identification throughout this study.

Fungal mycelium preparation

To produce mycelium for DNA extraction, the method of Sreenivasaprasad *et al*. (1993) was used. All isolates were cultured on PDA (Biolab, Midrand) for five days at 25℃. For fungal mycelium preparation, eight 5 mm diameter discs were cut from the edge of actively growing cultures on PDA (Biolab, Midrand) and placed in a 50 ml volume of liquid medium (10 g glucose; 1g $NH_4H_2PO_4$; 0.2 g KCl; 0.2 g $MgSO_4.7H_2O$; 5 g yeast extract (Difco); 1 ml of 0.5% w/v $CuSO_4.5H_2O$; 1 ml of 1% w/v $ZnSO_4.7H_2O$ in 1ℓ of sterile distilled water). Flasks were incubated at 25℃ for six days. Mycelium was harvested by filtration through Whatman No. 3 filter paper, immediately frozen in liquid nitrogen, pulverized, lyophilised and stored at – 70℃ until further use.

DNA extraction

To extract template DNA a DNeasy plant mini kit (Qiagen) was used and the extraction was done according to manufacturer's specifications. Extracted DNA was stored at -35℃ until further use. Two DNA samples were obtained from Mr. B. Slippers, Forestry and Agricultural Biotechnology Institute, University of Pretoria. The identity of these isolates is *Botryosphaeria parva* (CMW7026) and *Botryosphaeria dothidea* (CMW7020) isolated from mango in Australia.

RAPD PCR amplification

Reactions were performed in 50 µl volumes with 1 µl template DNA, 0.4 µl of the 100pmol OPC02 primer (5'-GTG AGG CGT C-3') (Operon Technologies), 0.5 µl *Taq* polymerase (Promega), 5 µl magnesium-free buffer (supplied with *Taq* polymerase), 1 µl dNTP mix (Promega), 4 µl $MgCl_2$, and 38.1 µl SABAX water. PCR was performed as follows: one initial denaturation cycle at 94℃ for 2 min followed by 35 cycles of denaturation at 94℃ for 30 sec, annealing at 35℃ for 45 sec and extension at 72℃ for 90 sec, followed by a single final extension cycle at 72℃ for 10 min. Analysis was done by adding 3 µl loading buffer to 10 µl PCR product which was loaded onto a 1.2 % horizontal agarose gel stained with 10% v/v ethidium bromide (Merk) using a Tris-Borate buffer (TBE) system. A 100bp molecular mass marker (Promega) was also loaded onto each gel to estimate the size of products. Electrophoresis was performed at 100 V for 2 hours and visualised under UV illumination. This was repeated twice.

PCR-RFLP amplification

PCR-RFLP reactions were performed in 50 µl volumes with 1µl template DNA, 0.2 µl each of a 100pmol ITS1 primer (5'-TTT CCG TAG GTG AAC CTG C-3') and ITS4 primer (5'-TCC TCC GCT TAT TGA TAT GC-3'), 5 µl buffer with $MgCl_2$, 4 µl dNTP mix (Promega), 0.2 µl *Taq* polymerase (Promega) and 40.1 µl SABAX water. PCR was performed as follows: one initial denaturation cycle at 95℃ for 2 min followed by 40 cycles of denaturation at 93℃ for 30sec, annealing at 55℃ for 45 sec and extension at 72℃ for 90 sec, followed by a single final extension cycle for 7 min at 72℃. The PCR products, 20 µl of each sample, were digested with 0.5 µl restriction enzyme C*fo*I in 2.5 µl buffer and visualised on a 3% horizontal agarose gel using a TBE buffer electrophoresis system. A 100bp molecular mass marker (Promega) was used to estimate the size of products. Isolates were then identified according to the protocol developed by Jacobs (2002) for *Botryosphaeria* spp. from mango.

RESULTS

DNA was successfully extracted from the 39 isolates studied in this experiment. The OPC02 primer, known for its ability to produce discriminatory banding patterns for *Botryosphaeria* (previously known as *Dothiorella*) populations (Schoeman, 2002) gave banding patterns for most isolates (Fig. 1). Genetic variation was observed in the populations tested in this study. According to the dendogram constructed from these data three distinct groups could be generated based on the number of bands (Fig. 2). This is in agreement with Schoeman (2002). Group I consisted of 14 isolates (nine from avocado and five from mango). These isolates had RAPD profiles of two to seven bands. Group II had nine isolates (five from avocado and four from mango) with bands ranging from three to eight. Group III consisted of sixteen isolates (eleven from avocado and five from mango) with bands ranging from one to four. The first two groups are closely related and group III was the most dissimilar of all three.

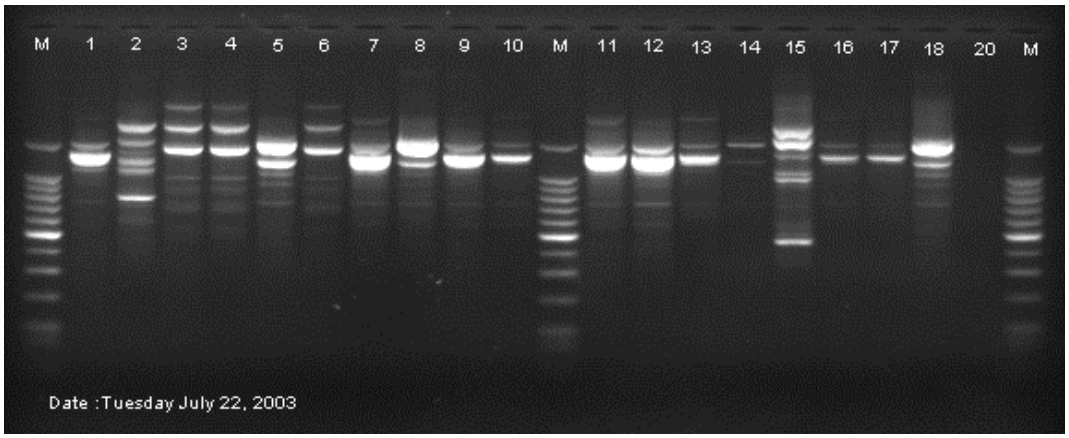
Cleavage of the ITS amplicons with restriction enzyme (RE) C*fo*I and visualisation of polymorphic banding patterns differentiated between the different *Botryosphaeria* spp. (Fig. 3). *Botryosphaeria parva* Pennycook and Samuels (*Fusicoccum parvum*) was the most dominant spp. with *Botryosphaeria rhodina* (Berk. and M.A. Curtis) Arx (*Lasiodiplodia theobromae*) being the second most dominant.

DISCUSSION

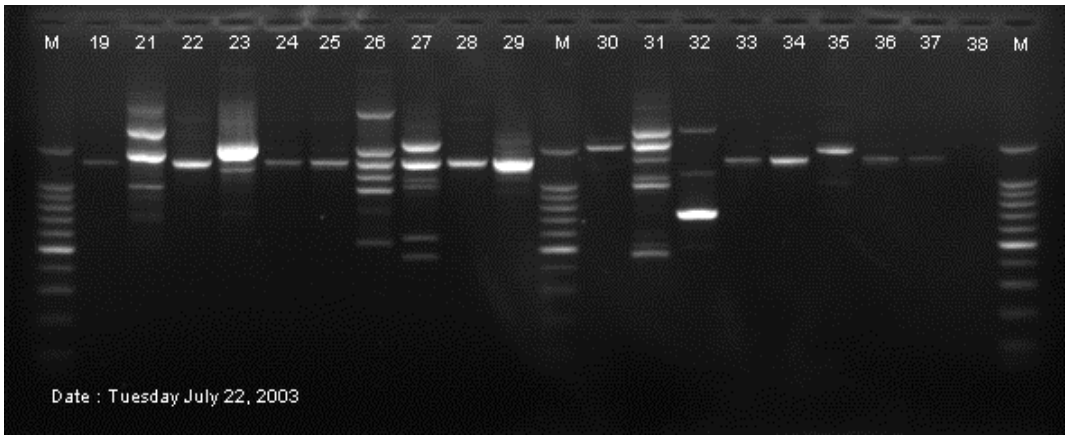
Analysing *Botryosphaeria* spp. isolates previously described as *Dothiorella* using RAPD profiles has proven to be an efficient and rapid method in this experiment. The OPC02 primer gave discriminatory banding patterns because of the repeated presence of the primer sequences in the isolates tested. This data shows that there are three distinct populations of the pathogen in the Tzaneen area and isolates that belong to these groups can be identified by their unique RAPD patterns using the OPC02 primer (Schoeman, 2002).

Table 1: *Botryosphaeria* isolates used for identity confirmation

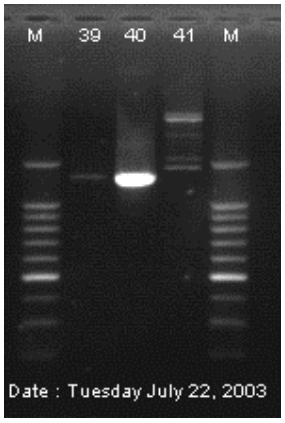
CODE	ISOLATE	SOURCE	CODE	ISOLATE	SOURCE
1	SR1I13S1	Avocado, SA	22	SR3W3	Avocado, SA
2	SR1I19S1	Avocado, SA	23	SR2B4	Avocado, SA
3	SR3W17	Avocado, SA	24	SR2B25	Avocado, SA
4	SR2Y20	Avocado, SA	25	WK9T9	Mango, SA
5	SR2B26	Avocado, SA	26	1A14T5	Mango, SA
6	SR3Y39	Avocado, SA	27	1R10B3W8 L2.2	Mango, SA
7	SR2B12	Avocado, SA	28	SR3B9	Avocado, SA
8	SR1I17S2	Avocado, SA	29	KEB9L7	Mango, SA
9	SR3Y17	Avocado, SA	30	JA8L5	Mango, SA
10	SR2B1	Avocado, SA	31	1R7B6W8 L2.1	Mango, SA
11	SR2W52	Avocado, SA	32	RVK10C5	Mango, SA
12	SR1I9	Avocado, SA	33	KW9T7	Mango, SA
13	SR3R10	Avocado, SA	34	SSB5T10	Mango, SA
14	SR2W28	Avocado, SA	35	1R7B6W8 L2.2	Mango, SA
15	1R10B8W2 L1.2	Mango, SA	36	WK9T7	Mango, SA
16	SR3W1	Avocado, SA	37	SR2W55	Avocado, SA
17	SR2W72	Avocado, SA	38	SR3W15	Avocado, SA
18	SR2Y18	Avocado, SA	39	KEB9T1	Mango, SA
19	SR2B34	Avocado, SA	40	CMW7026	Mango, Aus.
20	SR2R19	Avocado, SA	41	CMW7020	Mango, Aus.
21	SR1I3	Avocado, SA			



A



B



C

Figure 1. Agarose gel electrophoresis of RAPD fragments from DNA of Botryosphaeria isolates generated by primer OPC02.

Electrophoresis was done on 1.2% agarose gel run at 100V for 2 hrs. Lanes designated M are the 100 bp ladders. (A) Lanes 1-14, 16-18, 20 represent avocado isolates and lane 15 is mango isolate. (B) Lanes 19, 21-24, 28, 37, 38 represent avocado isolates and lanes 25-27, 29-36 are mango isolates. (C) Lane 39 is avocado isolate and lanes 40 and 41 are mango isolates

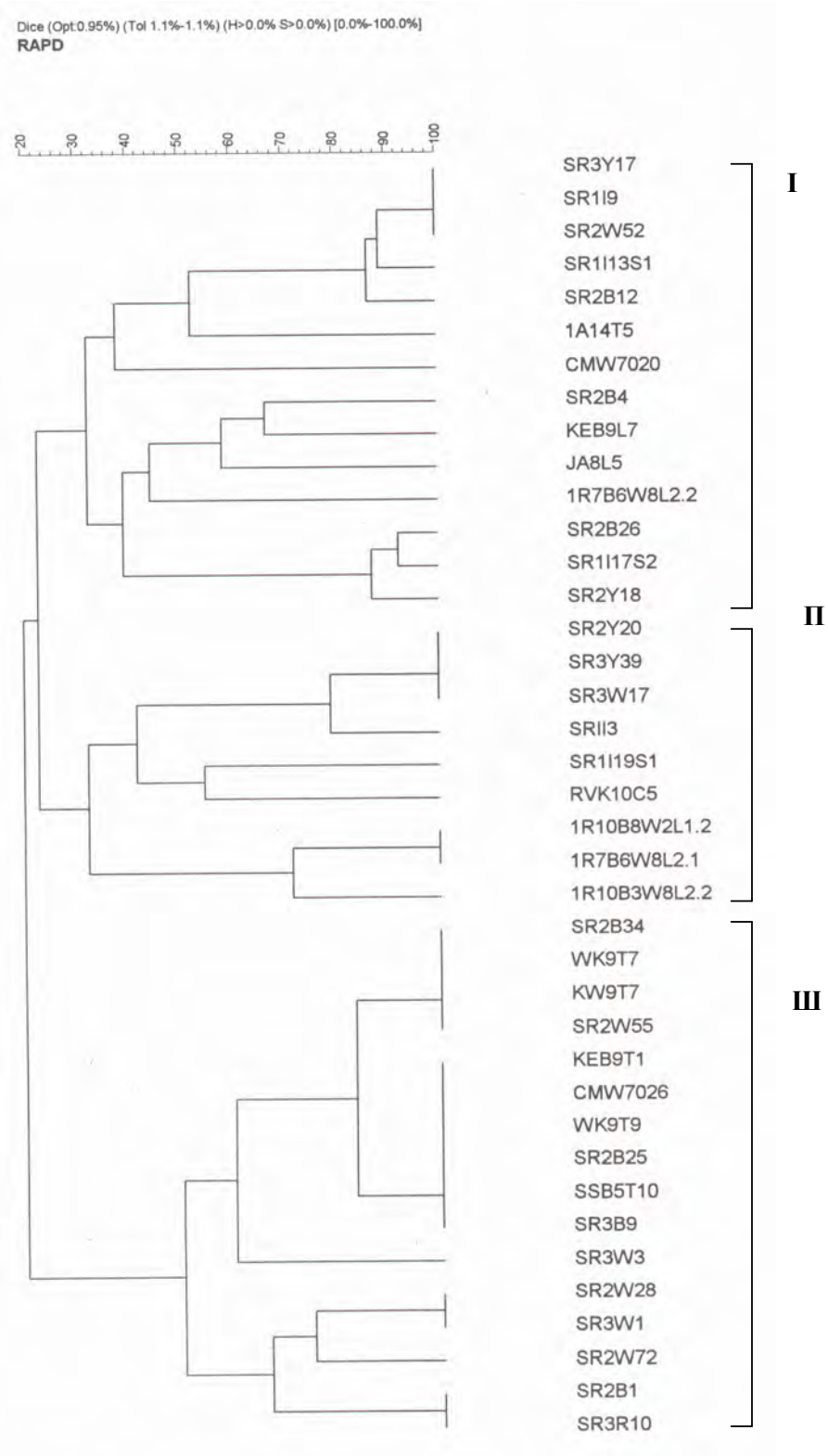


Figure 2. Dendogram showing the grouping of representative RAPD band profiles of *Botryosphaeria* isolates (I, II and III represent the three groups identified).

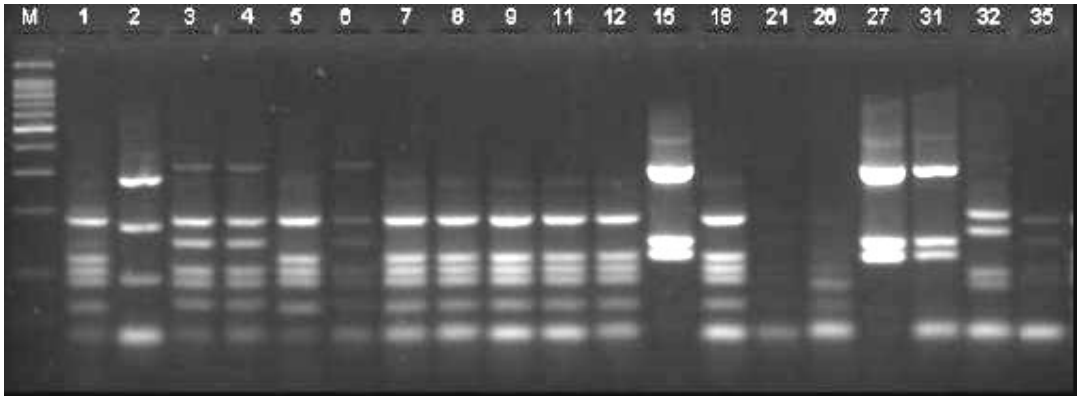


Figure 3. A fingerprint banding pattern of PCR-RFLP fragments from DNA of *Botryosphaeria* spp. isolates generated by restriction digest with restriction enzyme C*fo*I.

Electrophoresis was done on 3% agarose gel run at 100V for 2 hrs. Lanes with mark M are the 100 bp ladders. Lanes 1-12, 18, 20, and 21 represent avocado isolates and lanes 15, 27, 31, 32 and 35 represent mango isolates.

The taxonomy of *Dothiorella* was mostly based on morphological characteristics such as properties of the colonies (Johnson and Kotzé, 1994) and structure of fruiting bodies and conidial shapes (Darvas, 1982). In addition, it is a slow sporulating fungus and fruiting bodies are difficult to see (Darvas *et al*., 1987) even when cultures are grown under UV light, making quick identification difficult. Morphological characteristics do overlap among species and cannot always distinguish between species resulting in placement of different species under the same name or vice versa. Hence, it is important to integrate morphological characterisation with molecular methods to get conclusive identification.

In this study, *Dothiorella* isolates were compared at a molecular level and identified to species level. The PCR-RFLP identification system developed by Jacobs (2002) was successfully used to identify these isolates as *Botryosphaeria* up to species level. Some isolates that were morphologically identified as *Dothiorella* (isolates 1R10B8W2 L1.2, 1R10B3W8 L2.2 and 1R7B6W8 L2.1) were identified using RFLP as non-Botryosphaeriaceous species. This indicates that RFLP is a more accurate method than RAPD in overcoming difficulties of morphological characterisation. It is also more repeatable and consistent in comparison to RAPD techniques.

The PCR-RFLP can further be utilised in assessing the distribution of *Botryosphaeria* species in all avocado-growing regions of South Africa. This information is also useful in preventing the introduction of *Botryosphaeria* species that are not yet found in South Africa. The speed at which results can be obtained would aid in the confirmation of the identity of the fungus as the main cause of SER. This can further help in tracing the origin of newly introduced species and consequently assist in developing control strategies.

Botryosphaeria parva was the dominant species found in this study. Jacobs (2002) reported the dominance of this species on mango. It can therefore be speculated that *B. parva* is the main cause of economic losses among all the *Botryosphaeria* species and control practices should focus on this species. However, further investigation is needed to assess its distribution over all avocado-growing regions of South Africa.

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CHAPTER 6

GENERAL DISCUSSION

Avocado (*Persea americana* Mill.) is among the most important tropical and subtropical fruit crops in South Africa. Annual production of 110 000 metric tonnes (mt) and export volumes amounting to 42 000 mt have been recorded during the 2001/2002 season (http://www.fas.usda.gov/psd/complete_tables/HTP-table6-101.htm). Avocado production in South Africa is mostly export-orientated with 59% of total volume destined for mainly European markets (Van Zyl and Ferreira, 1995). Since the bulk of the fruit (97%) is exported by sea, long transit periods of up to three weeks often result in significant losses at the retail end due to postharvest diseases.

One of the most important diseases of avocado is stem-end rot (SER) (Darvas, 1992; Manicom, 2001) caused by a complex group of pathogens. The taxonomic placement of one of the SER pathogens, particularly *Dothiorella aromatica* (Sacc.) Petr. and Syd., has been controversial. Knowledge of the correct classification of the pathogens involved in the SER disease complex is vital in developing effective control strategies. Stem-end rot pathogens infect through flowers and remain latent until fruit is ripe thereby making early detection difficult. Losses of up to 25% of locally marketable fruit and 13% of export fruit have been recorded (Sanders and Korsten, 1997) due to SER. Due to the number of pathogens associated with SER disease control is primarily dependent on the use of fungicides (Lonsdale and Kotzé, 1989). However, fungicide sprays are unable to reach the infection court of these pathogens i.e. flowers thereby making effective disease control difficult. Alternative control strategies have been investigated (Korsten, 1993; Korsten *et al*., 1994) and successful results have been reported.

In view of this, a study was initiated to look into alternative disease control strategies for SER. the concept of utilizing honeybee dissemination of antagonists to flowers to colonise the infection court was compared to the use of standard spray applications of antagonists and

fungicides. To explore the possible extension of these strategies further studies focused on confirming the identity of *D. aromatica* using molecular techniques.

For successful bee antagonist dispersal the antagonist must first attach, colonise and survive on avocado flowers. The antagonist must then be able to antagonise the pathogens within the infection court. Results obtained from this study show that *B. subtilis* can attach, colonise and survive on avocado flowers. Within two hours after *B. subtilis* were applied to avocado flowers, fibril-like strands were observed. These strands represent attachment structures for the bacterium. Similar fibril-like strands have been reported in other similar studies (Latham *et al*., 1978; Matthyse, 1983; Mariano and McCarter, 1993; Towsen, 1996). Population dynamic studies indicated that the antagonist could survive on avocado flowers for extended periods of time. Interactions between the antagonist and pathogens on the flowers showed bacterial degradation and lysis of fungal hyphae and formation of pits on fungal spores. These results confirm the antagonist when applied to flowers can build up population densities and protect infection court from fungal pathogens.

Based on the positive colonisation and *in situ* inhibition studies, field evaluations of bee antagonist dispersal and spray applications were carried out to evaluate antagonism under field conditions. Under field dissemination conditions the antagonist could not be detected on the flowers, which can be attributed to insufficient product deposition by bees or poor survival of the antagonist under field conditions. In addition, the bee dissemination trials did not result in effective SER control. None of the other avocado diseases could effectively be reduced with this approach. Several factors could be identified in this study that could have contributed to ineffective control including the dependence of bee foraging behaviour on temperature, viability of the antagonists under field conditions and protection provided by the inoculation site against harsh environmental parameters. Similar studies also reported insufficient deposition of antagonists on apple flowers for similar reasons (Archer, 2002).

Acceptable control of plant diseases through biological or integrated treatments is not always evident in the first season of the trial (Korsten *et al*., 1997). Improvements in levels of disease

control of postharvest disease such as anthracnose with biocontrol and integrated spray applications were observed during the second season of the experiment. This was attributed to the antagonist first having to build up sufficient population densities over time to exert any significant pressure on the pathogens. Repeated sprays over consecutive seasons enabled the biocontrol agent to gradually build up its population numbers on the plants and to adapt to different environmental parameters. This was also confirmed by van Eeden and Korsten (2003) who reported a pronounced increase in the establishment and survival of antagonist on avocado trees with higher spray frequencies.

Fungicide sprays were the most effective treatment for control of Cercospora spot and anthracnose but not SER. This indicates that the fungicide sprays could also not provide protection of the infection court. Using the biocontrol on its own also proved to be ineffective in controlling most pre- and postharvest diseases of avocado except for anthracnose. The integrated approach using reduced copper oxychloride sprays alternated with antagonist applications provided control of diseases such as Cercospora spot and anthracnose. A biocontrol only approach may therefore not provide the desired long-term solution for replacing copper sprays. In this study, it was therefore found that the integrated approach can provide a longer-term solution to disease control. This finding is in agreement with previous studies (Korsten *et al*., 1997) where most effective control was reported with integrated treatments.

Genetic heterogeneity among the previous *D. aromatica* isolates was confirmed by molecular analysis using random amplified polymorphic DNA (RAPD). This method has been used to characterise fungal strains and individual isolates with in a species (Guthrie *et al*., 1992; Tan *et al*., 1996; Péros *et al*., 1997; Freeman *et al*., 1998; Munaut *et al*., 1998; Urbanietz and Dunemann, 2000). In this study, RAPD analysis indicated that three groups of *D. aromatica* isolates could be identified with profiles ranging from one to eight bands. This is in agreement with Schoeman (2002), who also found three groups with banding profiles ranging from three to eight. The presence of one hundred percent similarities between some of the tested isolates may indicate a possibility of true clonality, which imply interchange within a population

(Satyaprasad *et al*., 2000). This may be due to the fact that the sexual stage of this fungus is rarely seen (Darvas, 1982).

The PCR-RFLP identification system developed by Jacobs (2002) was successfully used in this study to identify isolates of *Botryosphaeria* up to species level. With this approach difficulties with morphological characterisation can be avoided. This approach can be further utilised in assessing the distribution of *Botryosphaeria* species in all avocado-growing regions of South Africa. This information could be useful in assessing the homogeneity of populations in a region and could be used to determine if a new specie has been introduced into that region. This technique also distinctively segregated isolates that have been morphologically identified as *D. aromatica* (now *Botryosphaeria* spp.) (Jacobs, 2002). The technique is useful in identifying disease causing *Botryosphaeria* spp. and ultimately implementing more appropriate targeted control strategies.

Botryosphaeria parva Pennycook and Samuels was the dominant species found in this study. Jacobs (2002) reported the dominance of this specie in mango tree die back situations. Data from this study therefore indicates that *B. parva* is one of the major pathogens of SER. However, further investigation is required to assess its importance and distribution over all avocado-growing regions in South Africa.

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