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Honey bee dissemination of *Bacillus subtilis* to citrus flowers for control of *Alternaria*

BY

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Submitted to the Faculty of Natural and Agricultural Sciences
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In partial fulfillment of the requirements for the degree of
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Summary

Honey bee dissemination of *Bacillus subtilis* to citrus flowers for control of *Alternaria*

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The initial phase in the development of a biological control strategy is screening of biological control agents. Secondary to this phase is the establishment of accurate, effective application techniques. However, successful control requires a thorough understanding of all factors affecting the relationship between host plant, pathogen and other microbes. The purpose of this study was to screen and identify potential bacterial antagonists against *Alternaria*, a fungal citrus pathogen, attachment of the antagonists to bees, and bee dissemination of the antagonist to citrus flowers. A total of 568 bacterial epiphytes were screened on agar plates for antagonism against *Alternaria*. Only eight of these isolates, which were identified as *Bacillus subtilis*, *B licheniformis, B. melcerons, B. polymyxa, B. thermoglycodasius, B. sphaericus, B. amiloliquefaciens,* and *B. coagulans*, showed inhibitory effects on the growth of *Alternaria.* The most effective isolates were *B. subtilis* and *B. licheniformis.* Further screening was done with *B. subtilis* and *B. subtilis* commercial powder (Avogreen). These bacteria were sprayed on citrus flowers for colonisation studies. Mean populations of *B. subtilis* and the commercial powder recovered from the flowers were 10^4 and 10^3 cfu/stamen respectively. The organisms colonised the styler end and ovary of the flowers when observed under scanning electron microscope (SEM). Avogreen was placed in an inoculum dispenser, which was attached to the entrance of the hive. Honeybees emerging from the beehive acquired 10^4 cfu/bee. The powder attached to the thorax and thoracic appendages, as revealed by SEM. One active beehive was placed in an enclosure with fifteen flowering citrus nursery trees in pots for dissemination trials. Mean populations of commercial *B. subtilis* recovered from the flowers visited by bees were 10^4 cfu/stamen. Electron microscope studies revealed that the antagonist was colonising the styler end and ovary of the flowers. Field dissemination studies were unsuccessful due to low yields.

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TABLE OF CONTENTS

CHAPTER 1 GENERAL INTRODUCTION **1**

1.1 References **4**

CHAPTER 2 LITERATURE REVIEW **7**

2.1 Introduction **7**

2.1.1 Geographical distribution and host range 8

2.1.2 Economic importance of the pathogen 8

2.2 Disease epidemiology **9**

2.2.1 The pathogen 9

2.2.2 Life cycle 9

2.3 Mode of entry of the pathogen **10**

2.4 Symptomology **10**

2.5 Transmission and survival **12**

2.6 Control **13**

2.6.1 Chemical control 15

2.6.2 Cultural control 16

2.6.3 Integrated control 17

2.6.4 Biological control 17

2.7 Discussion **20**

2.8 References **21**

CHAPTER 3 *IN VITRO* SCREENING OF BACTERIAL EPIPHYTES AND COMMERCIAL *BACILLUS SUBTILIS* FOR ANTAGONISM AGAINST *ALTERNARIA ALTERNATA* **27**

3.1 Introduction **27**

3.2 Materials and Methods **29**

3.2.1 *In vitro* screening of potential antagonists 29

3.2.2 Identification of potential antagonists 30

3.2.3 Colonisation of *Bacillus* antagonists and *Alternaria alternata* on citrus flowers 30

3.2.4 Interactions between commercial *Bacillus subtilis* and *Alternaria alternata* on citrus flowers 31

3.3 Results **32**

3.3.1 *In vitro* screening of potential antagonists 32

3.3.2 Identification of potential antagonists 32

3.3.3 Colonisation of *Bacillus* antagonists and *Alternaria alternata* on citrus flowers 32

3.3.4 Interactions between commercial *Bacillus subtilis* and *Alternaria alternata* on citrus flowers 36

3.4 Discussion **37**

3.5 References **39**

CHAPTER 4 HONEY BEE DISSEMINATION OF COMMERCIAL *B. SUBTILIS* TO CITRUS FLOWERS **42**

4.1 Introduction **42**

4.2 Materials and methods **43**

4.2.1 Acquisition of antagonistic bacteria by honeybees 43

4.2.2 Inoculum dispersal by honeybees in an enclosure 44

4.2.3 Field dissemination of commercial *Bacillus subtilis* to citrus flowers by honeybees 45

4.3 Results **46**

4.3.1 Acquisition of antagonistic bacteria by honeybees 46

4.3.2 Inoculum dispersal by honeybees in an enclosure 46

4.3.3 Field dissemination of commercial *Bacillus subtilis* to citrus flowers by honeybees 48

4.4 Discussion **49**

4.5 References **50**

CHAPTER 5 GENERAL CONCLUSIONS **52**

5.1 References **55**

APPENDIX A **58**

APPENDIX B **59**

CHAPTER 1

GENERAL INTRODUCTION

Citrus, like any other crop, is affected by various diseases and insect pests. *Alternaria alternata* (Ell & Pierce) is amongst the most important pathogens causing pre- and postharvest diseases. These diseases include black rot, navel-end rot of navels (Wager, 1939), brown spot and black rot of mandarins (Whiteside, 1976), Alternaria rot in Valencias and rough lemons (Brown, 1988), as well as leaf spot of rough lemons (Brown, 1988). Losses caused by this pathogen have reached alarming proportions in many South African orchards (Schutte *et al.*, 1994). In South Africa, losses can be as high as 30% due to fruit drop as recorded in Riverside, and 27% fruit drop as recorded in Baddaford and Millbank in the Kat River Valley (Wager, 1939). Similarly, navel-end splitting of about 34 fruits per tree was reported by Wager (1941). Depending on the citrus species infected, many fruits lose sales appeal due to unsightly spots on the rind. In addition, a considerable amount of rot occurs inside the fruit before the first symptoms appear on the rind. These internal infections can lead to inferior quality juice due to rancid flavours of the affected fruits (Schutte *et al.,* 1994).

Control of *A. alternata* in citrus is a major challenge. Disease symptoms are not easily detected on young fruit or the harvested crop, since *Alternaria* is a weak parasite. Infection remains quiescent and only progresses when the fruit is reaching maturity, usually postharvest (Coit & Hodgson, 1918). The infection process is triggered by rupturing of juice vesicles during fruit maturation. Rupturing may also be caused by protrusions of rudimentary fruitlets (Schutte *et al.*, 1994). This phenomenon is prevalent in navel oranges. The flowering period in citrus is another factor which complicates control of *Alternaria*. The bloom period can extend over two months, and every flower that opens is prone to infection. This makes conventional chemical sprays uneconomical and impractical on many farms. Furthermore, the use of contact chemicals such as benzimidazole fungicides was found to be inefficient due to a lack of penetration into infection sites, especially at the navel-end of fruit (Schutte *et al.*, 1994).

Another important aspect in the etiology of the disease is the pathogen's mode of entry. Conidia are airborne and can be brought into contact with the flowers by wind (Whiteside, 1988). According to Wager (1941), entry can only occur in one of two ways: when conditions are favourable, the style breaks off and fruitlets and young fruit will form a closed navel. However, if conditions are harsh, the style persists on the fruitlet, which causes formation of cracks in the navel-end. It is through these cracks that the pathogen gains entry to the plants and navel-ends. During harsh weather, the primary style turns brown and dries out, and the secondary style continues to grow and enlarges, causing longitudinal cracks in the outer stylar tissue. Once again infection can occur through these cracks. In some instances, the style does not break off cleanly, thereby creating a space or opening to the exterior in which the fungus can grow down into the fruitlet without penetrating any tissue (Wager, 1939). In tangerines, the rind remains susceptible to infection 16 weeks after petal fall (Whiteside, 1976). These aspects are important in designing a control strategy that targets the infection court.

The use of alternative control strategies can be explored regardless of the complexities of the pathogen infection process. One such strategy is biological control. This term was first used in relation to plant pathogens by Von Tubeauf in 1914 (Baker, 1987). Biological control is the decrease of inoculum or the disease producing activity of a pathogen accomplished through one or more organisms, including the host plant, but excluding man (Baker, 1987). According to Cook (1983), proponents of biological control advance certain motivations to justify its use as an alternative to chemical control, i.e.: increasing production within existing resources; avoiding development of pathogen resistance to chemicals; maintaining relatively pollution and risk free control and adopting practices compatible with sustainable agriculture. Environmental pollution and the presence of chemical residues in food are two issues currently receiving much attention from the general public and retailers. It is for these reasons that the United States Environmental Protection Agency has withdrawn chemicals such as Captan and Benomyl (Wisniewski & Wilson, 1992). Similarly, the European Parliament has voted in favour of a total ban on postharvest pesticide treatments of fruits and vegetables as soon as the practice becomes feasible (Wisniewski & Wilson, 1992). It is clear that there is a need for change in disease control strategies. Successes with this control strategy have been achieved in several cases, including anthracnose on mango (Korsten *et al.*, 1991), Cercospora spot and anthracnose on avocado

(Korsten & Kotzé, 1992), postharvest fruit rot of litchi (Korsten *et al.,* 1993), stem end rot, anthracnose, and Dothiorella / Colletotrichum fruit rot complex of avocado (Korsten *et al*., 1995), avocado black spot (Korsten *et al*., 1997), peach brown rot (Pusey & Hotchkiss, 1988), blue mould of apples (Janisiewicz, 1987) citrus blue mould (Chalutz & Wilson, 1990) brown rot of cherry (Utkhede & Sholberg, 1986), Rhizopus rot of peach (Wilson *et al*., 1987), stem end rot of citrus, (Singh & Deverall, 1984), Botrytis rot of pears (Benbow & Sugar, 1999), fire blight of apples (Johnson & Stockwell, 2000) and grey mould of pears (Mao & Capellini, 1989). Despite these studies, relatively few biocontrol products have been registered and successfully commercialised. One of the success stories is "Avogreen", a preharvest *Bacillus subtilis* biocontrol product that has been registered for control of Cercospora spot on avocado (Janisiewicz & Korsten, 2002).

Selection of biological control agents can be done by manipulating resident microorganisms on the fruit or leaf surfaces, or by using introduced microorganisms (Wilson & Wisniewski, 1989). However, a thorough understanding of the nature of the relationship between host, pathogen and associated microflora, as well as the character of the plant surface and the environment, is essential (Blakeman, 1985; Romantchuk, 1992). Any control strategy would be efficient on condition that it is economical, consistent and easy to implement and evaluate. Given the infection process of *Alternaria*, it would be uneconomical to use conventional field spraying techniques due to the extended flowering period in citrus. Bees (*Apis mellifera*) have been tested successfully for the dissemination of antagonists for control of *Erwinia amylovora* in pears (Johnson *et al*., 1993; Vanneste, 1997). Therefore, the use of foraging bees was evaluated for the dissemination of antagonists to citrus flowers for the control of *A.* a*lternata* in this study. The use of bees was chosen as an efficient, more targeted approach, as bees could visit the actual infection court directly. Since bees visit every flower as it opens, the antagonist can be deposited effectively on all flowers throughout the flowering period.

The main objectives of this mini-dissertation were therefore to screen and identify potential antagonists; determine colonisation and attachment of potential antagonist on blossoms; determine the attachment of potential antagonist on bees; and test the efficiency of bee dissemination of the antagonists to the flowers.

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CHAPTER 2

LITERATURE REVIEW

2.1 INTRODUCTION

The genus *Citrus* is a member of the subtribe Citranae in the family Rutaceae. Within the subtribe, citrus is known to be one of the major crops produced worldwide in more than 19 countries. Most citrus is produced in the Mediterranean Region (18.2 million metric tonnes) and the USA (14.8 million tonnes) (FAO, 2000). Other important producers are Spain, China and Mexico. The Mediterranean Region is the largest exporter (5.5 million metric tonnes), followed by Spain (3.2 million metric tonnes). Globally, South Africa is the fourth largest exporter of citrus (FAO, 2000). The citrus industry is operating on 47 422 ha of land with a total of 1 339 farmers in the country (Outspan Annual Report, 1996). The industry produces 1.1 million metric tonnes per annum, making it the third largest producer in the Southern Hemisphere (FAO, 2000).

Citrus production is affected worldwide by a range of fungal, bacterial and viral diseases. Of these, *Alternaria* spp. cause significant economic losses, with both pre- and postharvest diseases including black rot of navel oranges (Wager, 1939), brown rot of mandarins and black rot of Satsuma (Whiteside, 1986), Alternaria rot and leaf spot of rough lemons (Brown, 1988).

This chapter explores the epidemiology and control of *Alternaria* in citrus. Traditional control measures in different countries will be discussed together with methods that have potential for control of the pathogen, but which are not widely used in the industry. The control methods to be discussed include chemical, biological and cultural control, as well as integrated disease management.

2.1.1 GEOGRAPHICAL DISTRIBUTION AND HOST RANGE

Alternaria spp are widespread and occur in practically every citrus producing country in the world. The pathogen is important, especially in areas where mandarins, lemons and navels are grown. Mandarin hybrids, e.g. minneolas, are also susceptible to *Alternaria* infections (Schutte *et al.,* 1994).

In the interior valleys of California and in Arizona, *Alternari*a was originally reported as the causal agent of fruit drop in Washington navels (Coit & Hodgson, 1918). In 1976, brown spot of tangerines was attributed to *Alternaria* spp in Florida (Whiteside, 1976). Many citrus producing countries of the world e.g. Spain, Brazil, Zimbabwe, Cuba, Japan and Australia have reported high incidences of *Alternaria* spp, particularly as a causal agent of storage disease (Schiffman-Nadel *et al*., 1981). In Israel, it was isolated from valencias, where it was reported to cause stem end rot and internal black rot (Schiffman-Nadel *et al*., 1981). In the Kat River Valley of South Africa, the pathogen was described to cause early fruit drop (November drop) and navel end rot on Washington navels (Coit & Hodgson, 1918).

Alternaria is a ubiquitous parasite with a wide host range. *Alternaria* diseases are prevalent in many vegetable, ornamental and fruit crops (Agrios, 1988). In many of the hosts, it attacks the stem, leaves, flowers and fruit.

2.1.2 ECONOMIC IMPORTANCE OF THE PATHOGEN

Losses caused by *Alternaria* spp in citrus have serious economic impacts on the industry in many countries. Depending on the citrus cultivar affected, many infected fruits can lose sales appeal due to spots on the rind. However, at this point, a considerable amount of rot would have occurred inside the fruit prior to appearance of the first symptoms on the rind (Schutte *et al*., 1994). This interior infection is also a problem in consignments destined for processing, since low levels of infection can result in off-flavoured juice. Losses in monetary value were estimated at $1 225 000 to $1 750 000 in the valleys of California and Arizona (Coit & Hodgson, 1918).

Split fruits were recorded as high as 34 per tree on navel oranges (Wager, 1939), and yield losses of up to 30% were reported in Israel (Solel, 1991). The total percentage of fruits that dropped in Riverside, Baddaford and Millbank were 30%, 27% and 47% respectively (Wager, 1939). However it is difficult to accurately interpret yield losses due to factors such as other fungal diseases, insect damage and natural fruit drop, which can result in considerable yield losses (Wager, 1939).

2.2 DISEASE EPIDEMIOLOGY

2.2.1 The pathogen

Alternaria spp have been reported as causal agents of citrus diseases in Arizona (Pierce, 1902), Florida (Whiteside, 1976), Australia (Kielly, 1964; Pegg, 1966), Israel (Solel, 1991) and South Africa (Doidge, 1929; Kellerman *et al.*, 1979). At species level researchers differ in opinion regarding the identity of *Alternaria* pathogens. Ninety percent of organisms referred to in publications did not resemble the type of pathogen described by Pierce (Simons, 1990). Since there is little difference in conidium morphology between *Alternaria* species, it cannot be used as an identification characteristic (Doidge, 1929). Conidiophore length and width are also not useful for identification purposes (Rotem, 1994). *Alternaria* strains should therefore be identified at molecular and genetic level (Huang *et al.*, 1987; Petrunak & Christ, 1992). Although *A. citri* was reported to be the causal agent of Alternaria rot of citrus in South Africa, later findings confirmed *A. alternata* as the pathogen causing the disease (Swart *et, al.,* 1998).

2.2.2 Life cycle

Alternaria alternata grows saprophytically on dead citrus tissue and produces airborne conidia (Whiteside, 1988). The spores are disseminated mainly by air currents, though splashing rain may contribute to spread of the inoculum. The fungus grows into the fruit after senescence of the button (Brown, 1988). According to Whiteside (1988), overwintering and survival takes place on infected leaves, stems and out of season fruits in citrus orchards. Infected leaves drop and infected fruits are harvested, therefore the mycelia and conidia may overwinter on infected shoots and serve as primary source of inoculum. In navels, infection takes place through longitudinal cracks in the primary style

or space between the primary and secondary ovary (Fig.2 1). The infection remains quiescent until fungal growth is stimulated by rupturing juice vesicles, resulting in fruit decay (Schutte *et al.*, 1994). Post harvest decay is triggered by overmaturing injuries to juice vesicles and large navel-ends.

2.3 MODE OF ENTRY OF THE PATHOGEN

Alternaria alternata causes latent infections, i.e. infection takes place preharvest through flowers, but the symptoms appear postharvest. Conidia are airborne and can be brought into contact with flowers by wind dispersal. Once conidia have landed on a flower, entry can occur in two ways.

a) When conditions are favourable, the style breaks cleanly from the fruitlets and young fruit will form on a closed navel. If conditions are harsh, the style persists on the fruitlet, which results in the formation of cracks in the navel-end. Infection takes place through these cracks (Wager, 1939).

b) The pathogen can gain entry through the flowers. During harsh weather, the primary style turns brown and dries out. The secondary style continues to grow, causing longitudinal cracks in the outer stylar tissue through which the pathogen gains entry (Schutte *et al.*, 1994). In some instances, the style does not break off clearly, creating a space or opening to the exterior in which the fungus can grow down the young fruit without penetrating any tissue (Wager, 1939). In tangerines, which become susceptible even 3-4 months after bloom, the fungus can penetrate through the rind since fruit only become resistant to infection 16 weeks after petal fall (Whiteside, 1988).

2.4 SYMPTOMOLOGY

Different names given to *Alternaria* diseases in citrus refer to the development of typical symptoms after infection has occurred. In oranges, infected tissue turns brown, giving rise to common names like black rot and black center rot. In mandarins and mandarin hybrids, lesions develop on the side of the fruit and the infected peel appears brown, hence the name brown spot of mandarin. Depending on the citrus species attacked, the symptoms vary greatly on leaves (Fig. 2.2) and fruits (Table 2.1).

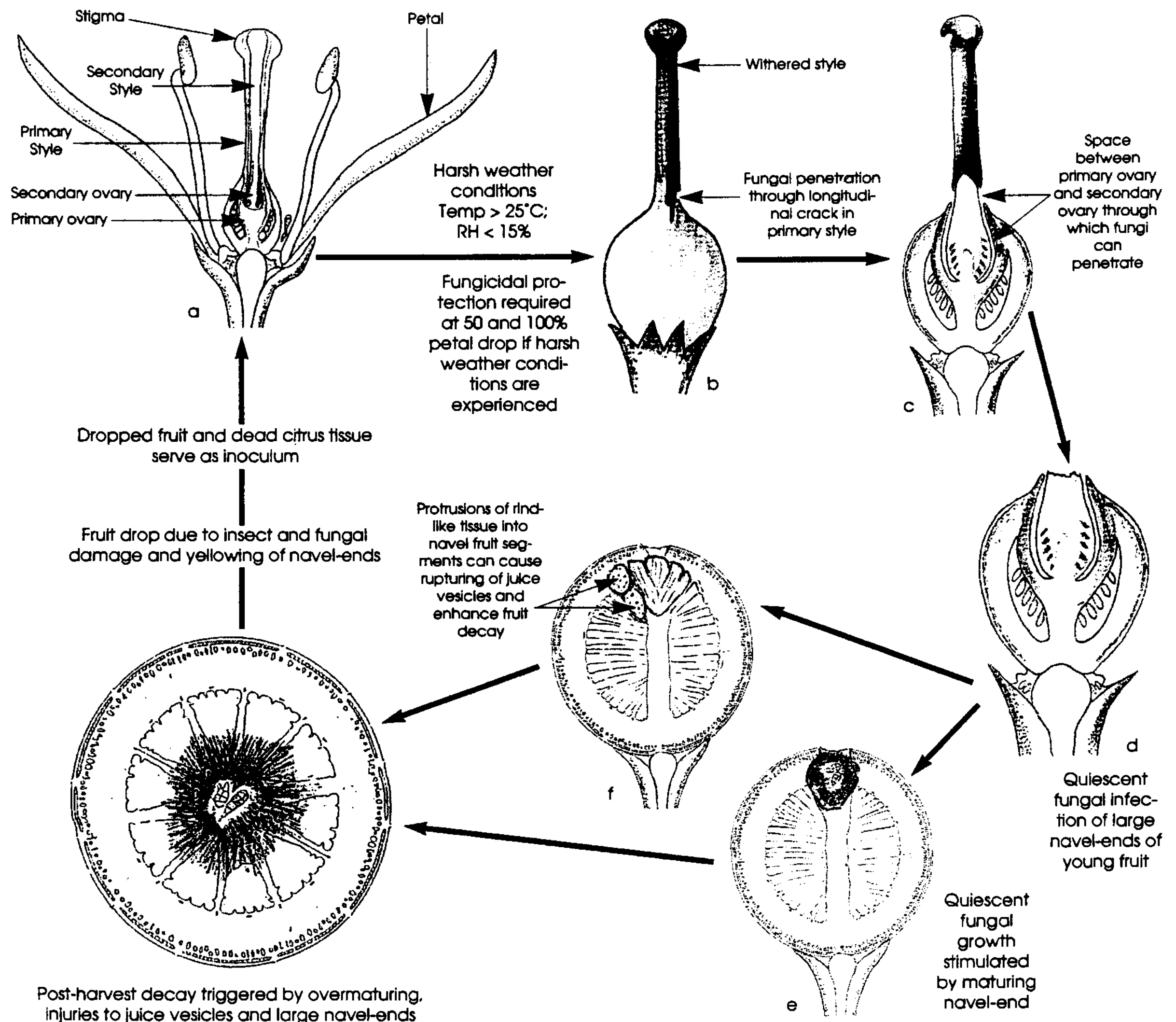

Figure 2.1 **Life cycle of *Alternaria* (Schutte *et al.*, 1994).**

Table 2.1 **Diseases caused by *Alternaria* on different citrus species**

Species	Disease	Reference
Navels	Black rot, navel-end rot, navel-end splitting	Wager, 1939
Mandarins and mandarin hybrids	Brown spot of mandarin, Black rot of Satsuma	Whiteside, 1988
Valencia	*Alternaria* rot of citrus	Brown, 1988
Lemons	*Alternaria* rot, *Alternaria* leaf spot of rough lemon	Brown, 1988

Whiteside (1988) described specific symptoms associated with different citrus species as follows:

a) **Symptoms on mandarins and mandarin hybrids**

- large, necrotic, blighted areas to circular spots
- slowly expanding necrotic brown spots
- fruit infected after petal fall are usually dropped
- corky eruptions on the rind of older fruit (Fig. 2.3)

b) **Symptoms on navels**

- infected fruit colour prematurely
- light brown to blackish discoloration of the rind at stylar end
- internal decay observed when fruit is cut open (black or centre rot)
- navel end rot and split
- excessive fruit drop

c) **Rough lemons**

- pulp becomes greyish-brown, soft and slimy
- stem end browning
- centre rot
- small black spots on the rind
- short internodes
- lesions extend outwards along the veins (Fig. 2.2)
- large, necrotic, blighted areas to small, circular spots
- necrotic areas surrounded by chlorotic halo
- die back of shoot apices due to stem infection and defoliation

2.5 TRANSMISSION AND SURVIVAL

Spores are disseminated mainly by air currents and splashing rain. *Alternaria* conidia are mainly released during the day. Dry leaf surfaces are important for spore liberation and dispersal by wind and wind velocity therefore plays an important role in spore release and transmission (Rotem, 1994).

The pathogen survives in stem lesions and infected leaves which remain on the tree canopy. Infected leaves and stem lesions may serve as primary inoculum for the subsequent seasons (Whiteside, 1988). It can also survive saprophytically on dead citrus tissues and other substrates. Postharvest, arthrospores washed from the fruit can also accumulate on conveyer belts, brushes and elevators, causing infection of injured fruits (Wild, 1977). Mandarins may be more prone to infection than oranges if stored above 8°C for several weeks (Hall, 1973). Sweet oranges and grape fruit stored at 1°C for 4-12 weeks were found to have low incidences of Alternaria rot (Smoot, 1969). Grapefruit develop high incidences of stem end rot if subjected to freezing temperatures (Shiffman-Nadel *et al.*, 1975). Light plays an important role in conidium production because conidia are mainly produced in the night and dispersed by day (Rotem, 1994). Temperatures between 12 and 28°C, with a day length of twelve hours, are ideal for conidium production (Rotem *et al.*, 1989).

2.6 CONTROL

Control of diseases caused by *Alternaria* is a major challenge for the citrus industry. It is not easy to detect the symptoms on young infected fruit or fruit that are being packed since *Alternaria* is a weak parasite and symptoms remain internal. Infection remains quiescent and only progresses when the fruit reaches full maturity, usually after harvest. The infection is triggered by the rupturing of juice vesicles as the fruit matures, which may be the result of pressure from the large navel-ends, causing splitting and rupturing of juice vesicles (Schutte *et al.*, 1994). Rupturing may be caused by protrusions made by other rudimentary fruitlets. These protrusions could result in the rupturing of juice vesicles, a phenomenon which is prevalent in navel oranges. Another challenging aspect in the control of *Alternaria* spp is its point of entry. The bloom period in citrus can last for up to two and a half months depending on the climatic conditions and cultivar type. The rind of Dancy tangerines remains susceptible to infection 16 weeks after petal fall, as reported by Whiteside (1988).

Figure 2.2 ***Alternaria* leaf spot lesions on Dancy tangerine (Whiteside, 1976).**

Figure 2.3 ***Alternaria* lesions on Dancy tangerine (Whiteside, 1976).**

Given this scenario, it is clear that conventional spray methods used on many farms may not be efficient or economical. Every single flower that opens in the orchard is prone to infection. There will thus be many flowers opening per day that may not be sprayed and the challenge is to get complete protection agaisnt *Alternaria* on a daily basis. To ensure effective control of this disease, sound, economic and sustainable strategies are required. It may be uneconomical for many farmers to control one pathogen on a continuous basis for more than two months. However, control measures have been used in several citrus producing countries, with the emphasis on chemical control, which will be discussed below, together with other strategies such as biological, cultural and integrated control (Schutte *et al.,* 1994).

2.6.1 Chemical control

A list of chemicals generally used both internationally and in South Africa for control of *Alternaria* is given in Table 2.2. The growth regulator 2,4-D delays senescence of the fruit button, thereby restricting movement of the pathogen into the fruit. The use of benomyl and CGA-64251 proved successful in Florida. This trial was done on fruit stored for 17 weeks at 1°C and one week at 21°C (Table 2.2). At the Volcani Centre in Israel, use of metalaxyl along with CGA 64251 produced efficient control (Schiffman-Nadel *et al.*, 1981). The use of triazoles, namely difenconazole and tebuconazole in the Eastern Cape in South Africa, were used successfully (Schutte *et al.*, 1994). Mancozeb, cupric hydroxide and procymidone are registered for control of *Alternaria* in practically all citrus producing areas in South Africa, and are sprayed from October to December in two to three applications (Krause *et al.*, 1999). Chemical control has been achieved by the use of either imazalil or 2,4-D, or both, on harvested fruit (Eckert & Bretschneider, 1981). Effective control of black rot was achieved with the active ingredient AECL-69, an antibiotic derived from *Bacillus subtilis*. This antibiotic was applied at a two cm radius around the stem-end, which prevented decay in Valencia and Kinow mandarins (Farooqi, 1981). Fruits were stored for a period of 3-4 months and the antibiotic was used as a pre-storage treatment.

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Table 2.2 Postharvest fungicides used in *Alternaria* control on citrus

Chemicals	Source
Imazalil, 2,4-D	Eckert & Bretschneider, 1981
Difenoconazole	Eckert & Bretschneider, 1981
Tebuconazole	Schutte *et al.*, 1994
Prochloraz	Tuset, 1981
C.G.A.-64251	Brown, 1981
Mancozeb	Krause *et al*, 1999
Cupric hydroxide	Krause *et al*, 1999
Procymidone	Krause *et al*, 1999

2.6.2 Cultural control

Abiotic stresses can result in plants being susceptible to less virulent pathogens. In preventing such conditions, methods that reduce disease-producing activity, e.g. cultural practices that delay or prevent stresses on the host, are most relevant (Cook, 1983). Cultural control has been used successfully in the control of citrus postharvest diseases (Porat *et al.,* 2000; Palou *et al.*, 2001; Auret, 2001).

Cultural methods applicable in the control of *Alternaria* include:

- avoid late harvesting of fruit, since the fungus does not seem to cause infection in healthy, vigorous fruit;
- judicious irrigation, scheduling and fertilisation to avoid the development of inferior fruits, which are prone to infection;
- the establishment of windbreaks to restrict transmission of airborne conidia.

Reduction of wind velocity may also help to reduce cracks that form on the style through which spores can enter the plant. Windbreaks also minimise premature dropping of infected fruits.

2.6.3 Integrated control

Emphasis on the chemical control of *Alternaria* is evident and many trials proved successful even though large-scale applications have not been commercially adopted (Ippolito & Nigro, 2000; Harman, 2000). One sustainable way of controlling diseases is the use of multiple control strategies, also called integrated disease management (IDM). According to Cooley (1996) concepts underlying IDM include: optimisation of disease control in an ecologically and economically sound manner, emphasis on coordinated 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. Other motivations are to develop cost saving production techniques and the prevention of disease resistance to chemicals. Today, IDM systems put more emphasis on food safety and environmental sustainability. Intergrated control offers the opportunities to reduce the level of chemical residues on harvested produce (Ippolito & Nigro, 2000). In designing IDM for *Alternaria*, the first step would be to move from insurance sprayings, that are calendar based, to control that considers the pathogen's life cycle, mode of infection and the totality of environmental predisposing factors. However, there is little information available regarding the control of *A. alternata* on citrus with IDM programmes.

2.6.4 Biological control

Von Tubeuf first used the concept biological control in relation to plant pathogens in 1914 (Baker, 1987). The first attempt at direct application of biological control of plant diseases was made in 1921 for the control of damping off caused by *Pythium debaryanum* (Baker, 1987). New strategies include the use of plant-growth promoting rhizobacteria, seed inoculants, biotic systems that exclude the pathogen from the host and induced resistance (Jacobson & Backman, 1993). Biological control is an alternative to synthetic chemical pesticides and can play an important role in integrated pest management systems (Conway *et al.,* 1999; El-Ghaouth *et al.*, 2000; Korsten *et al.*, 2000). Diseases of several crops have been controlled successfully, e.g. anthracnose of mango (Korsten *et al.*, 1991); Cercospora spot and anthracnose of avocado (Korsten & Kotzé, 1992); postharvest fruit rot of litchi (Korsten *et al.*, 1993); peach brown rot (Pusey & Hotchkiss, 1988); blue mould of apples (Janisiewicz, 1988); brown rot of cherry (Ukhede & Sholberg, 1986) and grey mould of pears (Mao

& Capellini, 1989). Similarly, diseases of citrus have been controlled successfully in recent years (Table 2.3).

Table 2.3 Biocontrol agents evaluated for control of citrus diseases

Disease	Pathogen	Biocontrol agent	Reference
Green mould	*Penicillium digitatum*	*Bacillus subtilis*	Chalutz & Wilson, 1990
Green mould	*P. digitatum*	*Pichia guilliermondi*	Wisniewski *et al.*, 1988
Blue mould	*P. italicum*	*P. guilliermondi*	Chalutz *et al.*, 1988
Blue mould	*P. digitatum*	*Candida oleophila*	Droby *et al.*, 1998
Green mould	*P. digitatum*	*C. oleophila*	Brown *et al.*, 2000
Green mould	*P. digitatum*	*B. pumilus*	Huang *et al.*, 1987
Green mould	*P. digitatum*	*C. saitoana*	El-Ghaouth *et al.*, 2000

The selection of biocontrol candidates is an important factor in the development of biological control programmes (Andrews, 1985). Approaches often selected include the use of resident microorganisms or the use of introduced biocontrol agents (Wilson & Wisniewski, 1989). However a thorough understanding of the pathogen and host relationship is essential in developing biological control strategies (Blakeman, 1985). Microorganisms should also possess certain characteristics to be effective in the control of diseases. A successful antagonist on the aerial plant parts must have a high reproductive capacity and be able to survive unfavourable weather conditions (Wood & Tveit, 1955). These qualities are often found in spore-forming members of the family Bacillaceae.

The mode of action of biocontrol agents must be fully understood in order to make optimum use of a particular biological control agent. Modes of action of many antagonists are competition for nutrients, space, and antibiosis (Janisiewicz, 1988). Antibiosis and competition for space have been reported as the modes of action of *B. subtilis* (Pusey, 1989; Utkhede & Rahe, 1980).

Biological control agents should be compatible with normal cultural practices as part of a holistic disease management strategy (Harman, 2000; Johnson & Stockwell, 2000). Compatibility with commonly used agrochemicals is also important for the acceptance of biocontrol agents (Obagwu

& Korsten, 2003). This is important, since both agents are often applied simultaneously. Consistency in product performance is essential if the antagonist is used to replace chemicals or mixed with chemicals at low concentrations.

Another important aspect of biological control strategy is the accurate application of the product (Mukertji & Garge, 1988). The recent observation of bee vectoring of biocontrol agents (Johnson *et al.*, 1993) made a century after bees were first found to vector plant pathogens, has opened new possibilities for the efficient control of flower infecting pathogens. Foraging bees (*Apis mellifera*) were tested successfully for their ability to vector bacterial antagonists to flowers for control of *Erwinia amylovora* (Thompson *et al.*, 1992; Vanneste, 1996; Johnson & Stockwell, 2000). Vectoring involves acquisition, transport and deposition of inoculum and is influenced by many factors including inoculum formulations, bee activity on crop plants and weather variables (Sutton & Peng, 1993). Vectoring also requires considerations of quantitative, spatial and temporal relationships of bee populations with flowers of crops and many competing food sources for bees. Bees can vector fungal spores, formulated as powders (Peng *et al.*, 1992) or bacterial antagonists that are prepared as freeze dried products or absorbed onto apple or cattail pollen (Johnson *et al.,* 1993). The powders adhere to setae and other external surfaces as bees crawl through formulations in dispensers (Peng *et al* 1992). Acquisition of vectored microorganisms has been reported in the range of 10^5 colony forming units (cfu) per bee (Thompson *et al.*, 1992), 10^4 cfu/bee (Peng *et al.*, 1992) and 10^6 cfu/bee (Johnson *et al.*, 1993). In citrus, bees move in rotational patterns and the repeated contact of legs and bodies with stamens and other flower parts are key factors in influencing the deposition of the antagonist and on the flowers (Sutton & Peng, 1993). Careful consideration should be given when harmful chemical sprays are applied at any time while bees are foraging in the orchard.

The efficiency of bees in the dissemination of antagonists is due to the fact that bees disseminate the antagonist straight to the target site, i.e. flowers, where the pathogen enters the host. By depositing the antagonist to every flower as it opens, complete coverage throughout flowering can be assured. Given the infection process and duration of susceptibility in citrus, the control of *Alternaria* using this approach seems a viable option. Spraying the antagonist during bloom may be another option, but may result in a very small percentage of the antagonist being deposited on

all the flowers. Multiple applications may be necessary, as flowers do not open at the same time. It is against this background that dissemination by bees seems to make more economical and viable sense.

2.7 DISCUSSION

Sound control measures of *Alternaria* are a necessity in the sustainable growth of the citrus industry. It is also a known fact that losses due to *Alternaria* infections are a limiting factor in profitable citrus production, especially on susceptible citrus species, e.g. minneola, tangelo, navels and rough lemons. It is of paramount importance to implement control strategies which would produce efficient, economical and sustainable control. Controlling an omnipresent pathogen that causes several diseases is extremely difficult and requires more than one control strategy. Limited successes have been achieved with chemical control. However, the ideal way of controlling *Alternaria* is to prevent preharvest infections. The complexity of *Alternaria*'s life cycle, mode of infection and selected infection sites, complicates its control, especially in large commercial scale farming systems. Since the pathogen is a classical opportunist, cultural control practices, including adequate fertilisation and irrigation to maintain plant and fruit vigour, are also important. Injuries to fruits and late harvesting should be avoided because infection occurs mainly in over-matured and injured fruits. Integrated control is one of the strategies that can be employed in the control of *Alternaria* and in the development of such a programme, the following can be suggested: amelioration of the environment or conditions that predispose the host to the pathogen. In the case of *Alternaria*, this system could include the growing of windbreaks and the use of parasites and antagonistic mixtures, depending on their compatibility. This strategy works on the principle that the pesticide weakens the pathogen or suppresses its population while giving the antagonist a competitive edge to colonise and multiply on the host. This could lead to the use of a lower concentration of chemicals in disease control, a desirable factor in terms of environmental pollution and pesticide poisoning of humans. Biological control is practised in many situations as part of the strategies employed to control plant diseases. In many instances, biological control takes place in the form of destruction of existing inoculum, suppression of the pathogen after infection and exclusion of the pathogen from the host (Cook, 1983). One of the key factors contributing to a successful biological control strategy is the accurate application of the biocontrol agents to the

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infection court. In the case of Alternaria rot of citrus, this can be achieved economically and efficiently by the use of bees as disseminating agents of the antagonist, as bees will visit every flower that opens, ensuring efficient coverage of the infection court. Global awareness of environmental pollution and its detrimental effects on human health necessitates a change in attitude towards disease control strategies. Biological control can therefore be part of the answer to this problem.

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CHAPTER 3

IN VITRO SCREENING OF BACTERIAL EPIPHYTES AND COMMERCIAL *BACILLUS SUBTILIS* FOR ANTAGONISM AGAINST *ALTERNARIA ALTERNATA*

ABSTRACT

A total of 568 bacterial epiphytes isolated from citrus flowers and leaves were screened on agar plates for antagonism against *Alternaria*. Only eight of the isolates tested showed *in vitro* antagonistic activity against the pathogen's growth. The isolates were identified as *Bacillus subtilis*, *B. licheniformis*, *B. melcerons*, *B. polymyxa*, *B. thermoglycodasius*, *B. sphaericus*, *B. amyloliquefaciens*, and *B. coagulans*. The eight isolates and the commercial *B. subtilis* powder (Avogreen, Stimuplant cc, Pretoria) were sprayed on citrus flowers to test their ability to colonise and survive on citrus flowers. Bacterial populations of *B. subtilis* (citrus isolate and Avogreen) were recovered from the flowers at concentrations of 10^4 and 10^3 cfu/stamen respectively. Based on electron microscopy, these two organisms multiplied preferentially on the stylar end and ovary of the flowers.

3.1 INTRODUCTION

Citrus is an economically important crop that is produced in most regions of the world. Currently it is one of the major fruit crops in global retail and is mainly exported from developing to developed countries. South Africa annually exports 50% of its total citrus crop (FAO, 2000). The strong focus on export in the South African citrus industry indicates that it is well developed with a strong emphasis on research, extension and quality control through end point inspections. Postharvest rot at the retail end causes substantial economic losses to the citrus industry. Alternaria rot of citrus is one of the most important postharvest diseases (Schutte *et al.*, 1994). Control of *Alternaria* starts during flowering, since the initial infection takes place through this unique infection court.

Control of many pre- and postharvest fruit diseases is mainly achieved by the use of agrochemicals (Eckert & Ogawa, 1985; Ipollito & Nigro, 2000). However, contact pesticides are ineffective in controlling *Alternaria* diseases because the products cannot penetrate the infection site, i.e. the stylar end of the flowers. In certain cases, it has been reported that application of benzimidazole fungicides can increase disease incidence (Schutte *et al.*, 1994). Environmental drawbacks often associated with chemical control renders this control strategy undesirable from the public point of view. Increasing restrictions on the use of particularly postharvest fungicides and the growing organic market requires an urgent re-evaluation of alternative environmentally friendly disease control options (El Ghaouth *et al.*, 1995).

Biological control can be used as an alternative to conventional agrochemicals used extensively in many crop production systems (Jacobsen & Backman, 1993; El-Ghaouth *et al.*, 2000). The selection of biocontrol agents is pivotal in ensuring successful biological control strategies (Andrews, 1985). Microorganisms are known to colonise plant surfaces and some have the ability to suppress disease development on plants (Fokkema, 1993; Baker & Cook, 1974). Interaction between microorganisms is, however, influenced by environmental, chemical and nutritional variables (Blakeman, 1985). By understanding the nature of and relationship between host, pathogen and associated microflora, effective biological control strategies can be established (Blakeman, 1985; Romantchuk, 1992).

A successful antagonist on the aerial plant surfaces should have high reproductive capacity and the ability to survive unfavorable environmental conditions (Wood & Tveit, 1955). These qualities are more often found in spore forming members of the family Bacillaceae (Wood & Tveit, 1955). The use of *Bacillus* spp. and other antagonists as biocontrol agents have shown great promise in recent years (Korsten *et al.*, 1993; Chalutz & Wilson, 1990; Qing & Shiping, 2000). The purpose of this study was to evaluate natural epiphytes and a commercial antagonist formulation for antagonism against *A. alternata* and to determine its colonising potential on citrus flowers.

3.2 MATERIALS AND METHODS

3.2.1 *In vitro* screening of potential antagonists

A total of 568 bacterial epiphytes previously isolated from citrus leaves and flowers (Juckers, unpublished data), were screened for antagonism against *A. alternata*. Pure cultures of all isolates were prepared for analysis and stocks were stored in cryotubes with glass beads in 60% glycerol at -70°C. Commercial *B. subtilis* (Avogreen, Stimuplant cc, Pretoria) was obtained in powder formulation for *in vitro* screening purposes. *Alternaria alternata* was isolated from symptomatic minneola fruits. Fruits were surface sterilised with 70% ethanol and left to air dry. Edges from the advancing minneola fruit lesions were cut with a sterile scalpel and placed on the center of potato dextrose agar plates (PDA) (Biolab). Plates were incubated at 25°C for 14 days. Cultures were further purified using the block and ring method (Kirsop & Snell, 1984). Three bacterial replicates were prepared on each medium for each isolate. Pathogenicity of *Alternaria* was confirmed by spraying a spore suspension of the pathogen onto new opening flowers, and measuring flower drop. Preliminary identification was done based on colony growth and morphological features. Identity of the fungus was confirmed by Prof. F.C. Wehner (Department of Microbiology and Plant Pathology, University of Pretoria). Discs of pure cultures were maintained in sterile distilled water in McCartney bottles and stored at room temperature.

Initial screening was done using the dual culture technique described by Fokkema (1978). Discs of *A. alternata* ($6mm^3$) were cut from the edge of actively growing two-week old cultures on a PDA plate, and placed off-center on Corn meal agar (CMA) (Biolab) and PDA plates. After 24 h incubation at 25°C, the plates were each streak-inoculated with either one of the epiphytes, 40mm from the fungal disc. Control plates were not inoculated with the different bacterial isolates. Measurement of pathogen growth inhibition was determined after 21 days of incubation at 25°C. Percentage inhibition was determined using Skidmore's method (1976): **Kr-r1/ Kr*100=G1**

Where **Kr** = radius from the point of inoculation to the edge of fungal growth.
r1 = radius from the point of inoculation to the edge of bacterial streak.
G1 = percentage growth inhibition.

Data from this experiment was analysed statistically using the SAS program, Analysis of Variance (ANOVA) and Duncan's multiple range test was done to determine significant differences between isolates.

3.2.2 Identification of potential antagonists

Preliminary identification tests were conducted to identify isolates which showed *in vitro* inhibition using the Gram stain, morphological comparison, catalase and cytochrome oxidase tests (Schaad, 1988). Further identification of potential antagonists was done using the BIOLOG system (Biolog Inc.) according to the manufacturer's instructions.

3.2.3 Colonisation of *Bacillus* antagonists and *Alternaria alternata* on citrus flowers

Selected potential antagonists were further evaluated for their colonisation potential of flowers. Bacterial cultures were grown on Standard One Nutrient agar (STD 1) (Biolab) and incubated at 30°C for 24 hours. Cells were harvested by pouring sterile quarter-strength Ringer's solution (Merck) into Petri dishes and harvesting the cultures with sterile spreaders. Potato carrot agar was used to culture *A. alternata*. The medium was prepared by submerging 20g unpeeled potatoes and 20g finely cut carrots in 250ml water, in a one-litre Erlenmeyer flask. This medium was subsequently autoclaved for 15 minutes. Water extract from the autoclaved carrot-potato pieces (1:1 ratio) was mixed with PDA and autoclaved again. The same procedure used to harvest bacterial cultures was used to harvest spores of *A. alternata*. Concentrations of both the pathogen and antagonists were standardised at 10^5 cells/ml using a Haemacytometer and Petroff-Hauser counting chamber respectively. Moisture chambers were prepared in glass Petri dishes, which were first autoclaved for 15 minutes. Citrus flowers were collected from 15 Palmer navel trees grown in pots in the greenhouse at the experimental farm, University of Pretoria. Flowers (three per Petri dish) were sprayed until run-off, with a small plastic hand spray with aqueous suspensions of either *Bacillus subtilis, B. licheniformis, B. amyloliqueaciens, B. sphaericus, B. coagulans, B. polymyxa, B. thermoglucosidasius, B. melcerons* and 24 h later with *A. alternata.* Petri dishes were sealed with parafilm to prevent moisture loss. This process was repeated by inoculating the pathogen first. Control flowers were sprayed with the pathogen only. An

unsprayed control was included, in the trial by spraying flowers with sterile distilled water. The experiment was repeated using a concentration of 10^3 spores/ml for the pathogen. After 24, 48, and 72 h, flowers were removed from the moisture chambers and prepared for scanning electron microscopy (SEM). Five stamens were excised from the inoculated flowers, fixed with 5% osmium tetracycline (Biorad) and allowed to dry for three days in a critical point dryer. The specimens were divided into stigma, style and ovary, and coated with gold palladium in an Eiko I.B. ion coater. The three specimen divisions were mounted on stubs and each viewed in a Hitachi SEM operated at 5 KV.

Another set of 30 blossoms was collected from the same Palmer Navel trees for antagonist survival studies. The flowers were placed in sterile moisture chambers (three flowers per Petri dish) and sprayed separately with the 10^5cfu/ml aqueous solution of the eight bacterial antagonists. After 24h, the flowers were removed from the moisture chambers and placed in sterile test tubes (three per test tube), containing quarter-strength Ringer's solution. The test tubes were vortexed for 30 seconds and 1ml of the wash solution was plated on Mundt and Hinckle (MH) agar media, selective for growth of *Bacillus* spp (Schaad, 1988). Agar plates were incubated at 30ºC and characteristic colonies similar to those of *B. subtilis* were counted after 24h. Data was analysed statistically as described before.

3.2.4 Interactions between commercial *Bacillus subtilis* and *Alternaria alternata* on citrus flowers

Fifteen flowering Palmer navel budlings kept in the greenhouse were assigned to this experiment. Fungal cultures were prepared as described in 3.2.3, except for the concentration of *A. alternata*, which was used at 10^3 spores/ml. Commercial *B. subtilis* (Avogreen, Stimuplant cc, Pretoria) was used in powder form in this experiment at a concentration of 10^7 spores/gram. The antagonist powder was dusted on flowers using a paint brush at 10^7 spores/gram, 24h before applying the pathogen. The experiment was repeated but the pathogen was applied first, as described in 3.2.3.

3.3 RESULTS

3.3.1 *In vitro* screening of potential antagonists

Of the 568 isolates screened, only eight species showed *in vitro* inhibitory activity against *A. alternata* (Table 3.1). Of these isolates, code number 245 was found to be the most inhibitory although not significantly more than isolate 244.

3.3.2 Identification of potential antagonists

All isolates that had inhibitory effects against *A. alternata* were identified as *Bacillus* spp (Table 3.1). *Bacillus amyloliquefaciens* and *B. coagulans* were the most dominant amongst the group of potential antagonists (four isolates each), *B. polymyxa* (three) and *B. licheniformis* (two). In addition *B. sphaericus, B. thermoglucadasius, B. melceron*s were also effective. All isolates which inhibited *A. alternata* by 60% or more were selected for further screening, i.e: (244, 523, 545, 572, 223, 221 and 506).

3.3.3 Colonisation of *Bacillus* antagonists and *Alternaria alternata* on citrus flowers

The stylar end and ovary of uninoculated blossoms are shown in Fig. 3.1a and 3.1b. After 48 h, spores of *A. alternata* were observed germinating on the specimen inoculated with the pathogen only (Fig. 3.1c). On some specimens single hyphae threads were visible (Fig. 3.1d). *Bacillus subtilis* cells were observed in clusters 72 h after inoculation (Fig. 3.2). No bacterial cells were observed on specimens sprayed with *B. amyloliquefaciens*, *B. polymyxa*, and *B. melcerons* (Table 3.2). No bacterial cells were observed on uninoculated control flowers. Dense mycelial mats were observed 48 h after inoculation on all specimens sprayed with *A. alternata* only (Table 3.2). Bacterial populations recovered from stamen wash solutions were 10^4 cfu/stamen for isolate number 245 and 10^3 cfu/stamen for isolate 244.

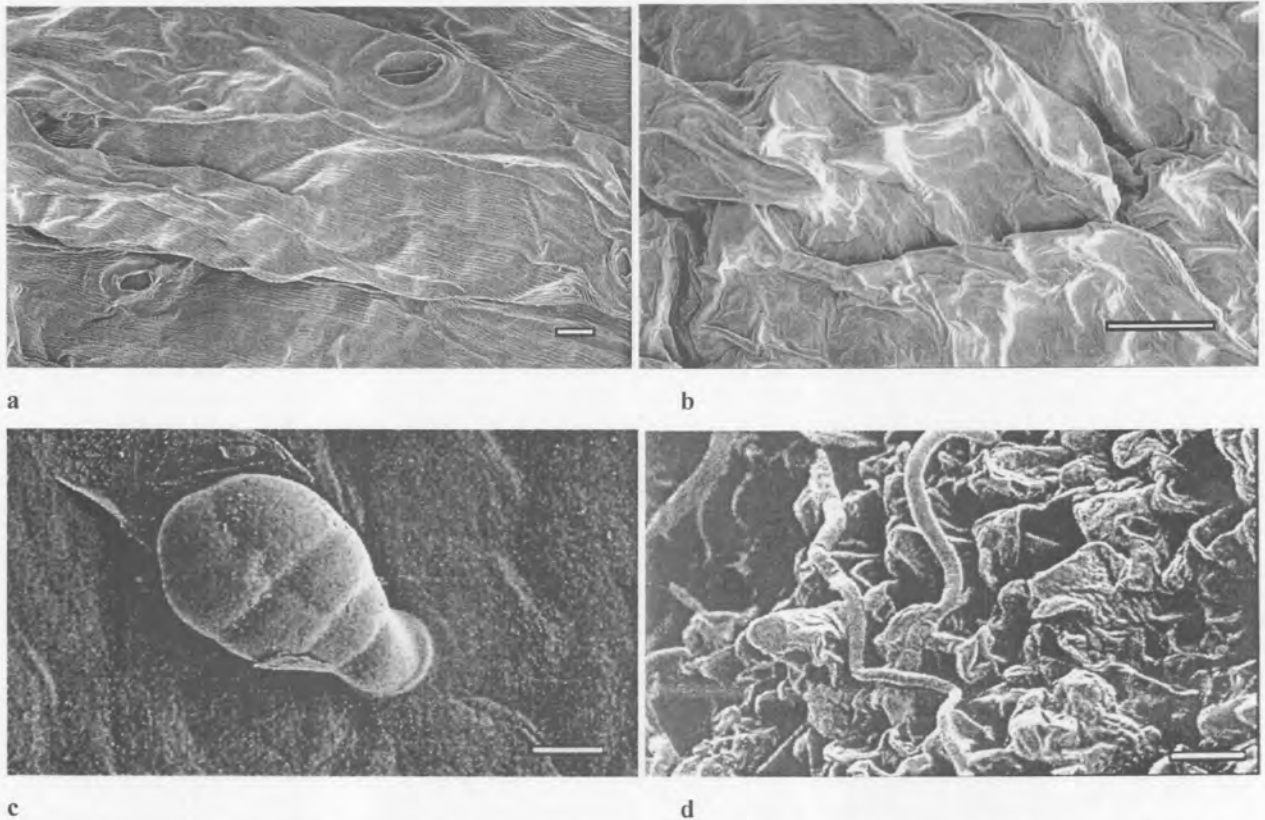

Figure 3.1 Scanning electron micrographs (5kV) of the surface of an uninoculated stylar end of citrus flowers: (a) micrograph of the surface of an uninoculated citrus flower ovary, (b) 48h after inoculation, (c) an *Alternaria* spore onto the stylar end of citrus flowers and (d) inoculated *Alternaria* showing mycelium growing over the surface of the style of citrus flowers [Bar = 10μm].

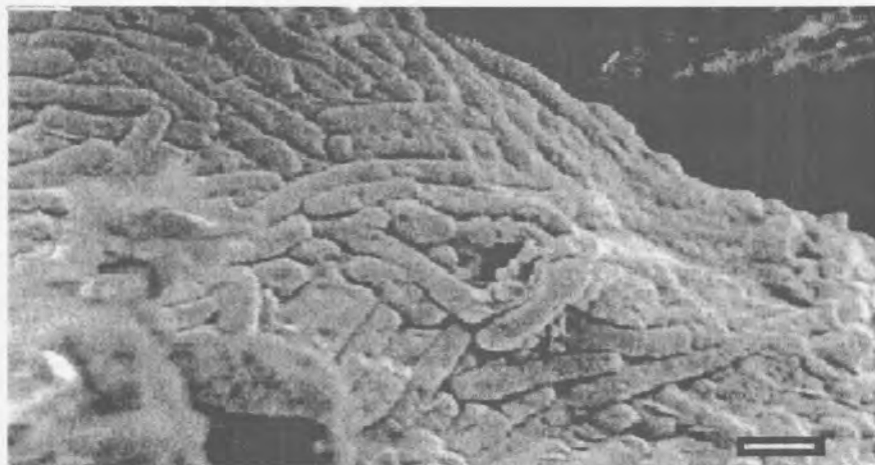

Figure 3.2 Scanning electron micrograph (5kV) of *Bacillus subtilis* showing complete colonisation of the stylar end of citrus flowers 72h after dual inoculation with *Alternaria* [Bar = 1μm].

Table 3.1 Percentage *in vitro* inhibition of *Alternaria alternata* by *Bacillus* spp

Antagonist code	Percentage inhibition	Identification results
221	62.821 bc	*Bacillus melcerons*
222	57.692 bc	*B. coagulans*
223	65.385 bc	*B. polymyxa*
231	60.684 bc	*B. amyloliquefaciens*
244	71.795 ba	*B. licheniformis*
245	87.179 a	*B. subtilis*
283	55.983 bc	*B. coagulans*
388	52.991 bc	*B. sphaericus*
506	64.104 bc	*B. thermoglucodasius*
513	52.765 bc	*B. melcerons*
523	68.803 bc	*B. sphaericus*
543	62.393 bc	*B. licheniformis*
545	66.667 bc	*B. amyloliquefaciens*
568	61.111 bc	*B. amyloliquefaciens*
572	65.812 bc	*B. coagulans*
576	53.418 bc	*B. thermoglucodasius*
578	53.419 bc	*B. polymyxa*
587	58.974 bc	*B. coagulans*
600	55.556 bc	*B. polymyxa*
611	61.966 bc	*B. amyloliquefaciens*

Values followed by the same letter in the same column are not significantly different according to Duncan's multiple range test (P= 0.05).

Table 3.2 **Rating of colonisation of *Bacillus* antagonists and *Alternaria alternata* on citrus flowers**

	Electron microscope observations (hours after application)		
Specimens	**24**	**48**	**72**
Bacillus melcerons	0[a]	0	3
B. polymyxa	0	3	3
B. licheniformis	0	1	1
B. subtilis	0	1	2
B. thermoglucodasius	0	1	1
B. sphaericus	0	1	1
B. amyloliquefaciens	0	0	0
B. coagulans	0	1	1
Uninoculated control	0	3	0
A. alternata	0	5	5

[a] Indication: 0 = no cells observed 1 = single bacterial cells, 2 = clusters of bacteria, 3 = foreign bodies, 4 = single mycelial threads, 5 = dense mycelial threads.

Table 3.3 **Rating of interactions of *Bacillus* spp with *Alternaria alternata* at 10^3 spores/ml on citrus flowers when the pathogen was inoculated first**

	Electron microscope observations (hours after application)			
Antagonist	**0**	**24**	**48**	**72**
Bacillus melcerons	0[a]	5	4	5
B. polymyxa	0	3	5	5
B. licheniformis	0	5	4	5
B. subtilis	0	1	2	2
B. thermoglucodasius	0	5	4	5
B. sphaericus	0	5	4	5
B. amyloliquefaciens	0	5	5	5
B. coagulans	0	4	5	5
Uninoculated control	3	0	3	3

[a] Indication: 0 = no cells observed, 1 = single bacterial cells, 2 = clusters of bacteria, 3 = foreign bodies, 4 = single mycelial threads, 5 = dense mycelial threads.

3.3.4 Interactions between commercial *Bacillus subtilis* and *Alternaria alternata* on citrus flowers

No signs of microbial growth were observed on uninoculated specimens (Table 3.3). Traces of bacterial cells were observed on specimens sprayed with *B. subtilis* only, twenty-four hours after inoculation (Table 3.4). During the same period dense mycelia of *A. alternata* were visible on specimens sprayed with the pathogen only. However, when the antagonist and the pathogen were interacting, clusters of bacterial cells were observed on the stylar end of the blossom, 72 h after inoculation, as ilustrated in Table 3.5. The colonisation pattern was not affected by the application of the pathogen first or *vice versa.*

Table 3.4 Rating of interactions of *Bacillus* antagonists with *Alternaria alternata* at 10^3 spores/ml on citrus flowers when the antagonist was applied first

	Electron microscope observations (hours after application)			
Antagonist	**0**	**24**	**48**	**72**
Bacillus melcerons	0[a]	5	4	5
B. polymyxa	0	3	5	5
B. licheniformis	0	5	4	5
B. subtilis	0	1	2	2
B. thermoglucodasius	3	5	5	5
B. sphaericus	0	5	3	5
B. amyloliquefaciens	0	5	5	5
B. coagulans	0	4	5	5
Uninoculated control	3	0	3	3

[a] Indication: 0 = no cells observed 1 = single bacterial cells, 2 = clusters of bacteria, 3 = foreign bodies, 4 = single mycelial threads, 5 = dense mycelial threads.

Table 3.5 Interactions between commercial *Bacillus subtilis* and *Alternaria alternata* at 10^3 spores/ml pathogen concentration

Pathogen first	**Electron microscope observations (hours after application)**			
	0	**24**	**48**	**72**
B subtilis (control only)	0[a]	0	1	2
A. alternata (control only)	0	3	4	5
Uninoculated control	3	0	5	5
B. subtilis & *A. alternata*	0	3	2	2
Antagonists first				
B subtilis (control only)	0	3	2	2
A. alternata (control only)	0	3	4	5
Uninoculated control	3	0	0	3
B. subtilis & *A. alternata*	0	3	2	2

[a] Indication: 0 = no cells observed, 1 = single bacterial cells, 2 = clusters of bacteria, 3 = foreign bodies, 4 = single mycelial threads, 5 = dense mycelial threads.

3.4 DISCUSSION

In this study only 1% of the 568 isolates screened *in vit*ro showed inhibitory action against *A. alternata.* These isolates all belonged to the genus *Bacillus.* Low ratios of natural antagonists as part of the microflora on plants has been reported extensively (Leben, 1964; Chakravati *et al.*, 1972; Korsten, 1993; Towsen, 1996). Gram-positive spore forming members of the family Bacillaceae are common inhabitants of aerial plant parts and have typical antagonistic characteristics (Wood & Tveit, 1955). *In vitro* screening of natural epiphytes has been described by numerous authors (Cubeta *et al.*, 1985; Janisiewicz, 1988; Korsten *et al.*, 1997; Utkhede & Sholberg, 1986). *Bacillus subtilis* and *B. licheniformis* were the most inhibitory of all the isolates evaluated in this study. The antagonistic activities of *B. subtilis, B. licheniformis and B. amyloliquefaciens* are also well documented (Korsten *et al.,* 1991; Pruvost & Luisetti, 1991; Korsten *et al.*, 1992). *Bacillus subtilis* has been used successfully in the control of many plant diseases, e.g. anthracnose of avocado (Korsten *et al.*, 1991), Alternaria brown rot of cherries (Utkhede & Sholberg, 1986), green mould, sour rot and Alternaria rot of citrus (Singh & Deveral,

1984), brown rot of nectarines, Rhizopus rot of peaches (Pusey & Wilson, 1984) and black spot of mangoes (Pruvost & Luisetti, 1991). The control of black rot was achieved successfully on Valencias and Kinov mandarins using AECL-69, an antibiotic derived from *B. subtilis* (Farooqi, 1981). In this study, the antagonist was challenged with the pathogen on the stamen region of citrus flowers. Studies of Morris *et al.* (2002) showed the formation of biofilms on the plant surface which is in agreement with the observations of this study in which only *B. subtilis* formed clusters of cells indicating biofilm formation on the stamen surface. This colonisation strategy by bacteria was previously thought to be only possible within water ecosystems. Of interest was that biofilm formation was observed after 72h in the first colonisation experiment but formed after 48h in the second study when the antagonist was applied first. The effective *in vitro* inhibitory action of *B. subtilis* and the other *Bacillus* spp in this study is indicative that antibiosis could be one of the modes of action, because dual culture screening assays typically indicate this type of interaction (Baker & Cook, 1974). The mode of action of *B. subtilis* is reported to be mainly antibiosis (Utkhede & Rahe, 1980). In this trial, an effective colonisation test was selected for second tier screening. The eight antagonists, which produced over 60% inhibition against *A. alternata in vitro*, were further tested *in vivo* on citrus flowers, to determine if pre-emptive colonisation could be an alternative mode of action. Infection patterns of *A. alternata* during flowering require both antibiosis and competitive colonisation for effective control.

Of interest was the fact that only *B. subtilis* could effectively colonise the infection court and prevent growth of *A. alternata*, indicating that competitive colonisation is an additional mode of action (Appendix B, Fig. B1). This is the first report where such activities of *B. subtilis* have been shown on flowers. The remaining seven antagonists failed to colonise the surface and were overgrown by the pathogen's mycelia. When applied on citrus flowers in a moisture chamber, *B. subtilis* colonised preferentially on stylar ends of flowers. Preferred colonisation sites of *B. subtilis* were previously reported on avocado leaves between depressions of epidermal cells and areas in close proximity to stomata (Towsen, 1996). Andrews (1992), potential higher concentrations of nutrient and water retention are generally preferred for colonisation. Colonisation sites of many bacteria are areas protected from direct exposure to UV light and environmental factors (Andrews, 1992). A similar pattern of colonisation was observed with the commercial *B. subtilis* powder formulation, also used in this study for comparative purposes.

In vitro screening is not an end in itself but a means to an end. More often, good performance on the agar plate does not confirm efficient results in the field. *In vivo* screening should therefore be conducted to substantiate these *in vitro* results. This study clearly shows superior performance and potential of *B. subtilis.*

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CHAPTER 4

HONEY BEE DISSEMINATION OF COMMERCIAL *BACILLUS SUBTILIS* TO CITRUS FLOWERS

ABSTRACT

Foraging bees were tested for their ability to disseminate commercial *Bacillus subtilis* to citrus flowers for control of *Alternaria* spp. Commercial *B. subtilis* powder (Avogreen, Stimuplant cc, Pretoria) was placed in pollen inserts in the entrance of the beehive. Honeybees emerging from the hive acquired 10^4 cfu/bee. Observations under the scanning electron microscope (SEM) revealed that the powder attaches to the abdomen and legs of these bees. The mean population size of *B. subtilis* recovered from flowers visited by bees was 10^4 cfu/stamen. When observed under the (SEM), *B. subtilis* attached and colonised the ovary and stylar end of the flowers. The field trial experiment was abandoned because selected flowers from commercial trees did not produce enough fruits for evaluation.

4.1 INTRODUCTION

Accurate application techniques aimed at the target site are necessary for effective establishment of biocontrol agents and the subsequent effective control of the disease (Mukertji & Garge, 1988; Johnson & Stockwell, 1998). Most biocontrol agents are currently sprayed mechanically and some manually for the control of plant diseases (Stockwell *et al.*, 1998). Mukertji and Garge (1988) reported different application methods for biocontrol of belly rot caused by *Rhizoctonia solani*. These methods include using a brush, modified pruning shears and nail guns to apply commercial inoculum of *Peniophora gigantea* spores. However, some of these methods are inaccurate in that only a small percentage of the biocontrol agent is deposited on the actual infection court. Due to flowers opening in an unsynchronised manner over an extended period of time (two months in the case of citrus), multiple applications are required to ensure full coverage of all flowers and fruits. The recent observation of bee vectoring of biocontrol agents made a century after bees were first found to vector a plant pathogen, have opened up new possibilities

for efficient application of agents to control flower and fruit diseases (Johnson *et al.*, 1993; Stockwell *et al.*, 1998; Vanneste, 1997). Vectoring involves acquisition, transport and deposition of biocontrol inoculum and is influenced by many factors including inoculum formulations, bee-loading activity, crop plants and weather variables (Sutton & Peng, 1993). Vectoring also requires consideration of quantitative, spatial and temporal relationships of bee populations with the flowers of crops and with other competing food sources for bees. Because of their foraging habits, bees can be used effectively to disseminate the antagonist to the flowers. Bees can vector fungal spores formulated as powders (Peng *et al.*, 1992), or bacterial antagonists that are freeze-dried or absorbed onto apple or cattail pollen (Johnson *et al.*, 1993; Thompson *et al.*, 1992). When using bees to disseminate antagonist formulations to flowers, pollen inserts are attached to the entrance of hives. The pollen inserts are designed in such a manner that bees leaving the hive must crawl through the antagonist powder, and returning bees normally avoid re-inoculation by re-entering through the sides of the dispenser (Johnson *et al.*, 1993). Given the infection process of the pathogen and duration of the susceptible phase in citrus flowers, control of *Alternaria* using this approach seems to be a viable option.

The aim of this study was therefore to investigate the efficiency of honeybee dissemination of a natural antagonist to citrus flowers for the control of *A. alternata* the causal agent of Alternaria rot of citrus.

4.2 MATERIALS AND METHODS

4.2.1 Acquisition of antagonistic bacteria by honeybees

One active beehive maintained at the experimental farm at the University of Pretoria was used to determine bacterial acquisition by bees when leaving the hive. A pollen insert (Antles Pollen Inc, U.S.A.) was placed onto the hive three days before the addition of antagonist powder (Avogreen, Stimuplant cc, Pretoria). This was done to allow bees to get used to the device before the powder was added to the dispenser. Ten control bees were collected early in the morning as they crawled out of the pollen insert. Bees were placed individually in test tubes containing 9 ml quarter-strength Ringer's solution (Merck) and kept in a cool box until all bee samples were collected.

Ten grams of the antagonist powder was placed inside the pollen dispenser at 10 a.m., when the temperature was between 15 and 21°C. The dissemination process ran for six continuous hours. Bees were collected 30 minutes after the addition of the powder. Ten bees crawling through the pollen insert were collected individually in sterile test tubes containing 9 ml quarter-strength Ringer's solution. All bee samples were taken to the laboratory for processing. Each test tube containing one bee was vortexed for 30 seconds. A 0.1-ml aliquot of the wash solution was spread plated on selective agar media (Mundt & Hinckle (MH)) for *Bacillus* (Schaad, 1988). Plates were incubated at 30°C for 24 hours and examined for the presence of colonies similar to that of *B. subtilis*.

Five bees emerging from the pollen trap were collected after addition of Avogreen, but using empty sterile test tubes, for scanning electron microscopy (SEM) processing. Bees were vapour dried in 5% osmium tetracycline for three days. Dry specimens were divided into head, thorax and abdomen with a sterile scalpel. The specimens were coated with gold palladium in an Eiko IB.3 ion coater for five minutes and mounted on stubs for SEM viewing at 5KV. Ten control bees collected earlier on were prepared in the same manner described above for SEM processing.

4.2.2 Inoculum dispersal by honeybees in an enclosure

A total of 15 Palmer navel flowering budlings in pots were assigned to the experiment. Trees were put in a shade cloth enclosure with the following dimensions: 12 m length, 6m width and 4m height. The enclosure was constructed using the following materials: aluminum pipes and 30% polypropylene shade cloth (2.2 mm mesh). Spacing between trees was 1 m between rows and 2 m within rows. A single beehive was placed in the centre of the enclosure when the trees were 10-15% in bloom. A pollen insert was attached to the hive and left for 24 h to enable bees to become used to the dispenser. A total of 75 control flowers were collected (five from each tree) before antagonist powder was placed into the insert. Collected flowers were placed in sterile McCartney bottles, placed in a cooler box and taken to the laboratory for plate counts and SEM processing. Sixty stamens were excised from these flowers with a sterile scalpel, placed in three test tubes containing 9ml quarter-strength Ringer's solution and processed in the same manner described for viable counts. Each test tube therefore contained 20 stamens. Stamens for

SEM viewing were processed as described in 4.2.1. The dried specimens were divided into stigma, style and ovary and after gold palladium coating mounted on stubs for SEM viewing at five to ten kV. Ten grams of the antagonist powder was placed into the insert before the dissemination trials started. The dissemination process lasted six hours for three consecutive days. Refilling of the pollen trap was performed half-hourly. The rate of bee inoculation was defined as the number of bees exiting the hive through the insert every 30s. The number of bees foraging in the trees was established by closely examining the tree canopy and counting the number of bees per 30s interval. Blossoms were sampled randomly after two hours, after which a total of 75 blossoms were taken to the laboratory and processed as described 4.2.1 for viable counts. This was repeated three times per day in the morning, midday and afternoon during the dissemination process. In each sampling period, five stamens were collected and processed in the same manner as for SEM viewing.

4.2.3 Field dissemination of commercial *Bacillus subtilis* to citrus flowers by honeybees

Field trials were conducted on flowering Palmer navel trees in full production on the Crocodile Valley Citrus Estate in Nelspruit, Mpumalanga Province, South Africa. A total of 18 trees in the block were randomly chosen, marked with chevron tape and used in this experiment. Six flowering trees were selected at random from the 18 trees used as control. All open flowers from the selected trees were plugged off before dissemination started. Only flower buds that were not open were left to bear fruit, which would be used as control during disease evaluation. A total of sixty flowers were collected as control samples from the remaining 12 trees and taken to the laboratory for processing as described in Chapter 4 (4.2.1) for viable counts. Four full colony active beehives were moved to the orchard at night before the dissemination process started. Pollen inserts were placed in the hive entrances as described previously. The hives were spaced at two-meter intervals in a row of trees that were used in the experiment. All trees were within a 40 m radius from the hives. Ten grams of the antagonist powder was placed in each of the four pollen inserts before the dissemination process started, which lasted for 72 hours. The number of bees foraging on trees was determined as described in 4.2.2, except that in the morning only the northern and eastern sides of the trees were monitored as they were warmer, and in the afternoon the cooler western and southern sides were monitored. Blossom sampling was done in the

morning and afternoon of each dissemination day on all trees. Bees from the hives, identified by the presence of white powder covering their bodies, were followed until they landed on the flowers of the selected trees. A total of 10 flowers per tree were collected and placed in sterile McCartney bottles in a cooler box and taken to the laboratory for immediate processing. The rest of the flowers visited by bees were left on the trees and were covered with insect proof polyethylene bags (30 flowers per cluster covered). Fruits from these clusters were to be used later for disease evaluation once fruits matured. Four clusters of flowers were treated in this way on the northern, southern, eastern and western side of the twelve trees.

4.3 RESULTS

4.3.1 Acquisition of the antagonistic bacteria by honeybees

In contrast to control bees, all ten bees collected after the addition of the powder to the pollen insert were visually covered with antagonist powder. The bacteria recovered from bees which exited through the insert containing the antagonist powder, had an average population of 10^4 cfu per bee. No *Bacillus* type colonies were observed from plates made from control bee samples. When observed under the SEM, it was found that the powder formulation attached to the lower part of the abdomen and thoracic appendages (Fig. 4.1).

4.3.2 Inoculum dispersal by honeybees in an enclosure

Control flowers were found to be free of any colonies resembling those of *B. subtilis*. However, *B. subtilis* colonies were recovered in washes of blossoms that were sampled after the addition of the antagonist powder to the pollen insert. The mean population size was 10^4 cfu per stamen. The total number of bees exiting through the insert was 50 per 30s interval (Table 4.1). A total of 15 bees were counted on individual tree canopies per 30s (Table 4.1). Twenty-four hours after the commencement of the dissemination process, traces of bacterial cells were observed on the stylar end of the inoculated stamen (Fig. 4.2). Dense masses of bacteria were observed on the stylar end of the stamen 72 hours later (Fig. 4.3).

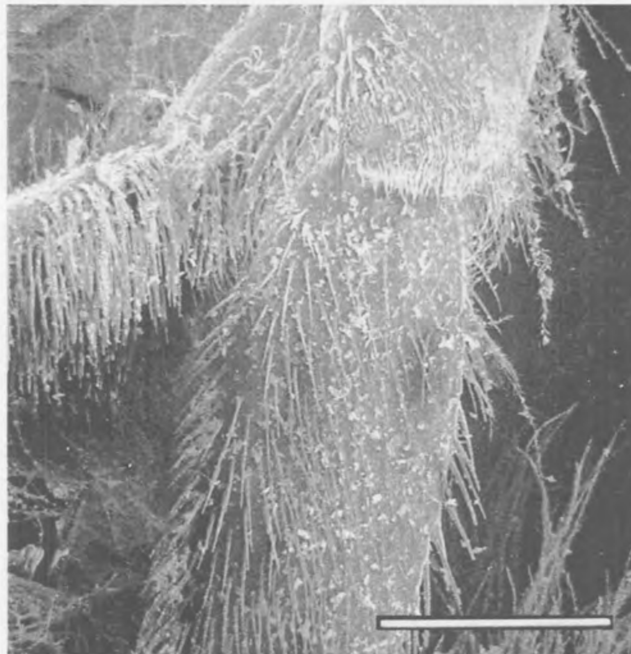

Figure 4.1 Scanning electron micrograph (10kV) of antagonistic powder of ***Bacillus subtilis*** adhering to body hair of bees [Bar = 10μm].

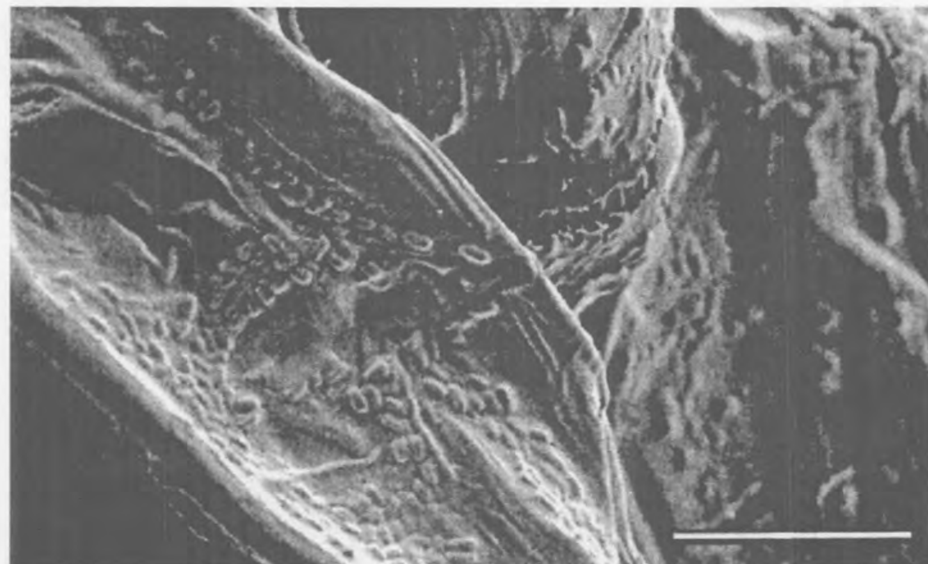

Figure 4.2 Scanning electron micrograph (5kV) showing traces of ***Bacillus subtilis*** on the stylar end of citrus flowers 24 h after bee dissemination [Bar = 10μm].

Table 4.1 Honeybee dispersal of commercial *Bacillus subtilis* powder to citrus flowers in an enclosure

Experimental conditions	Dissemination observations (days)		
	1	2	3
Hours/day bees were inoculated	6	6	6
Amount of inoculum dispensed/day (g)	120	120	120
Bee inoculation rate [a] (bees/30 s)	48	53	49
Average number of bees/tree/30 s [b]	17	16	13

[a] Average number of bees per second that exited an inoculum dispenser containing bacterial antagonist.

[b] Mean of individual number of bees foraging in each tree with counting done at hourly intervals.

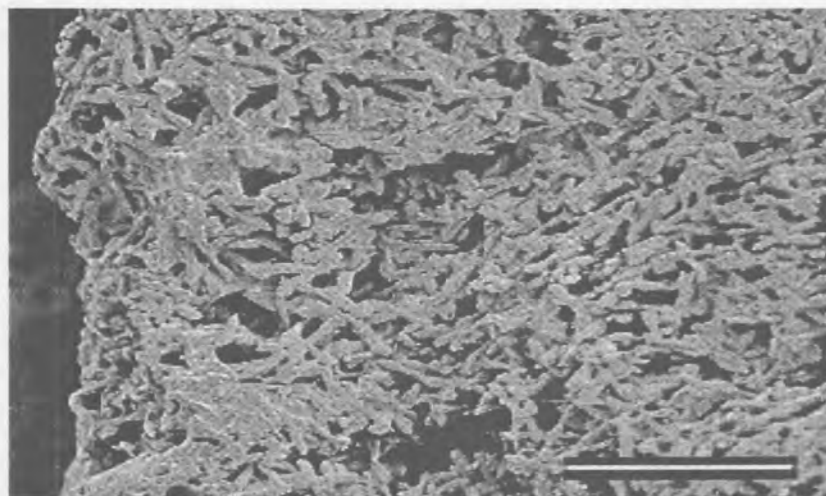

Figure 4.3 Scanning electron micrograph (5kV) of *Bacillus subtilis* showing complete colonisation of the stylar end of citrus flowers, 72 h after bee dissemination [Bar = 10µm].

4.3.3 Field dissemination of commercial *Bacillus subtilis* to citrus flowers by honeybees

Control flowers were found to be free of any bacteria resembling *B. subtilis* on agar plates. The number of bees foraging on trees was found to be five to six bees per minute. No colonies resembling *B. subtilis* were recovered from flowers visited by bees from the four hives. Fruiting on all flowers covered with mesh polyethylene bags was very poor; therefore, no representative sample could be taken to investigate inhibitory activity of *B. subtilis* on *A. alternata* infections.

4.4 DISCUSSION

Honeybees were used successfully to disperse *B. subtilis* from the hive to citrus flowers. This is the first report were biological control agents were dispersed to citrus flowers to control *A. alternata*. In this experiment, individual bees carried 10^4 cfu/bee *B. subtilis*. Populations of 10^4-10^6 cfu/bee were previously recorded by other authors (Thompson *et al.*, 1992; Johnson *et al.*, 1993; Peng *et al.*, 1992). In addition, the carrier material used to grow *B. subtilis* in this study was an inert material whereas Thompson *et al.* (1992) used pollen of cattail (*Typha latifolia*). Johnson *et al.* (1993) also used cultures of *Pseudomonas* which were harvested on nutrient agar, and their suspensions lyophilised for 48h. Seventy-two hours after the dissemination process started, colonies of up to 10^4 cfu/stamen were recovered from stamens, whereas Johnson *et al.* (1993) and Thompson *et al.* (1992) reported populations ranging from 10^2 to 10^4 cfu/stamen. However, their studies were conducted using *Pseudomonas* antagonists on apple and pear blossoms for control of *Erwinia*. Antagonist powder was observed on the lower side of the abdomen and the thoracic appendages of the bees. Peng *et al.* (1992) reported that formulations adhered to setae and other external surfaces as bees crawled through the dispensers.

When bees visited flowers, they moved in rotational patterns, repeatedly touching flowers with their legs and body. These are key factors in determining the effective deposition of antagonists and the success of biocontrol (Sutton, & Peng, 1993). In this study it was found that the antagonist attached to the abdomen and the legs of the bees. The effective deposition of the antagonist through these organs is therefore possible when flowers are visited. It is important to bear in mind that when bees are kept in an enclosure, the microclimate around them is modified and foraging patterns differ from those in the orchard where bees forage freely. The results from field experiments may therefore differ from those obtained under a controlled environment.

When stamens were observed under the SEM, dense masses of bacterial cells were visible on the stylar end of the stamen and also on the ovary. This suggests that *B. subtilis* successfully colonised and multiplied preferentially on these parts of the stamen. This is in agreement with the studies by (Singh & Deverall, 1984; Huang *et al.*, 1992; Korsten *et al.*, 2000). *Alternaria* spores gain entry into citrus flowers through growth cracks forming at the stylar end when windy

conditions or thunderstorms prevail Schutte *et al.* (1994). They can also enter through spaces between the primary and secondary ovaries, which form in navel oranges. The dense masses of bacteria, which were observed on the stylar end and ovary, can therefore serve as a physical barrier that prevents pathogen entry and thus indicate competitive colonisation as mode of action. Failure to detect bacteria disseminated by bees under extensive field conditions was also reported (Thompson *et al.* 1992; Stockwell *et al.*, 1998). They however ascribed this failure to the total area to be covered by bees during dissemination process, and the amount of innoculum needed to ensure viable population and survival of antagonists on flowers. Thompson *et al.* (1992) detected no bacteria on flowers when dissemination was performed on 20 hectare unit using two bee hives, but was able to detect 10^2 cfu per flower after two days when the area to be covered during dissemination was reduced to 1.5 hectares.

Failure to detect *B. subtilis* in the Palmer navel orchard indicated that in future a more extensive method and a longer time exposure was required to disseminate the powder to establish viable bacterial populations on flowers. To enhance viable counts and colonisation on the flowers the antagonists can be sprayed on the trees using a machine before dissemination by bees starts, preferably during early bloom (Stockwell *et al.*, 1998). This is because the presence of biocontrol agents at the initial phases of the disease cycle is crucial in the effective suppression of diseases by antagonists (Ippolito & Nigro, 2000)

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CHAPTER 5

GENERAL CONCLUSIONS

Control of *Alternaria alternata* the causal agent of Alternaria rot of citrus is done mainly by the use of fungicides (Schutte *et al*., 1994). Several systemic fungicides are used to control postharvest diseases of many fruits (Schutte *et al*., 1994; Dahmen & Staub, 1992a & 1992b; Tuset *et al.,* 1981; Swart *et al.,* 1998). However, Hutton (1989) reported resistance to iprodione by *A. alternata* sprayed for four consecutive years. Contact fungicides, are often not effective because they cannot penetrate the infection court, i.e. within the navel-end and stylar end of the flower (Schutte *et al*., 1994). In addition, there is a constant withdrawal of key fungicides due to growing international concern over pesticide pollution in the environment and their detrimental effects on human health (Ippolito & Nigro, 2000). This resulted in an upsurge in research into alternative disease control options. One such alternative is biological control (Droby *et al*., 1989; Conway *et al*., 1999; El Ghaouth, 2000; Janisiewicz & Korsten, 2002). Biological control has been evaluated successfully on control of fruit diseases (Korsten *et al*., 1991; El Ghaouth *et al*., 1995). For instance, *B. subtilis* was for instance evaluated extensively as a pre- and postharvest biocontrol agent (Pusey & Wilson, 1984; Pusey *et al.,* 1986; Korsten *et al*., 1989), and was used successfully in the control of *Alternaria* rot of citrus (Singh & Deverall, 1984). One of the most important aspects of successful establishment of biocontrol agents is the correct application method aimed at the infection court. Due to the nature of *Alternaria* infections during flowering, the ultimate way to control the disease would be by applying the antagonist directly to the flowers preferably prior to arrival of the pathogen. This is critical for the suppression of disases by antagonists (Ippolito & Nigro, 2000). Previous studies with fire blight caused by *Erwinia amylovora* which also infects through flowers was successfully achieved through bee vectoring (Thompson *et al*., 1992; Stockwell *et al.,* 1998). The objectives of this study were therefore to: screen potential antagonists, study their colonisation patterns of citrus flowers and evaluate honeybee dissemination of commercial *B. subtilis* to citrus flowers for the control of Alternaria rot of citrus.

Of a total number of 568 bacterial isolates screened in this study, only 1% showed *in vitro* inhibitory action against *A. alternata*. The low presence of natural antagonists in the phylloplane corroborates with reports by Leben (1964) and Chakravarti *et al.* (1972). Given the mode of infection of *Alternaria* in citrus, the dual culture screening technique was chosen as a preliminary assay since this method selects mainly for antibiosis (Pusey, 1989). However, agar assays do not always correlate with the results in the field (Baker and Cook 1974). When evaluated *in vitro*, *B. subtilis* and *B. licheniformis* showed the best inhibitory effect of all the isolates identified. After confirmation of identity, *B. subtilis* was chosen for further studies because it effectively colonised the flowers and was readily available in commercial powder form, which could be used in bee dissemination trials. Furthermore, the application of *Bacillus* spp. to flowers by bees surely presents a safer and more effective alternative than direct application to the fruits to control Alternaria rot of citrus. When observed under the scanning electron microscope (SEM), bacterial cells resembling those of *B. subtilis* were found largely on the stylar end of the flower specimens. This area is more shaded than the stigma or upper style and is a site that can provide protection against UV light and dry adverse conditions. These shaded sites favour colonisation and the survival of microorganisms (Andrews, 1992). The stylar end in particular is immersed in nectar, which can be a source of nutrients for the microbes. Such sites are more colonised than others (Andrews, 1992). No bacterial cells were observed on the stigma and other more exposed parts of the flowers. This is in line with reports that bacterial populations tend to decrease upon exposure to UV radiation (Leben & Whitmoyer, 1979; Ippolito & Nigro, 2000). It is therefore important to identify niches that biocontrol agents favour to colonise. Timely application of the antagonist to these sites may prevent pathogen infection (Hatting *et al.*, 1986; Qing & Shiping, 2000). *Alternaria alternata* infects through cracks at the stylar end of the flower. In this study, it was found that these sites were effectively colonised with dense masses of *B. subtilis* as observed under the SEM. Morris *et al.* (2002) reported the formation of these assemblages, known as biofilms in the phylloplane. These biofilms can serve as a barrier for the pathogen and therefore function on the principle of competitive exclusion.

Another challenging aspect of biocontrol is the development of appropriate application techniques of biological control agents (Makerji & Garge, 1988; Sutton & Peng, 1993; Stockwell *et al.*, 1998). In this study, honeybees were used successfully in the dissemination of *B. subtilis.*

The use of a pollen insert attached to the entrance of a beehive proved to be effective in ensuring acquisition of biocontrol products and dissemination to citrus flowers. The mean population of *B. subtilis* acquired by bees was 10^4 cfu/bee. This compares favourably with studies done by Thompson *et al.* (1992) on biological control of *E. amylovora* on apple and pears, using *Gliocladium roseum.* Peng *et al.* (1992) also reported successful biological control of *Botrytis cinerea* on strawberries by *Pseudomonas fluorescens* and *E. herbicola.* In this study, the transmission and successful establishment of the antagonist to the blossoms was 10^4 cfu/stamen. Johnson *et al.* (1993), Thompson *et al.* (1992) and Andrews (1992) also reported a population range of 10^2-10^4 cfu/stamen. The ability of honeybees to disseminate *B. subtilis* was remarkable in this study. Scanning electron microscopy studies revealed that the preferential colonisation site was the stylar end of the flowers. The stylar end is the point of entry for *A. alternata.* Effective colonisation of this specific site can serve therefore as an exclusion barrier for pathogens. Alexander (1971) reported that pioneering species normally exclude late-comers and this is regarded as a general principle of community ecology. Field dissemination of commercial *B. subtilis* did not however generate positive results. Viable *B. subtilis* could not be detected in the dilution plate studies from flowers visited by bees. Thomson *et al.* (1992) reported a similar findings and was also unable to to detect *E. herbicola* after one week of dissemination in a 20 ha experimental unit. However he was able to detect *E. herbicola* at an average of 10^2 cfu/flower within one day of dissemination when the experimental unit was reduced to 1.5 ha. However, failure to detect viable bacteria on agar plate does not mean the complete disappearance of the organism. The observation of aggregates of *B. subtilis* on the flowers visited by bees during the dissemination process further substantiate this. Supplementary detection methods that are more sensitive such as Enzyme Linked Immunosorbent Assays should be used in future together with viable counts to optimise field recovery of introduced organisms.

Biocontrol of plant pathogenic microorganisms will grow and expand. However, to be accepted as a dependable strategy, effective formulations and application methods should be developed. A microbial ecology study focused on the colonising ability and competitive interactions of the antagonists needs to be undertaken prior to commercialisation of biocontrol programmes. Future studies should focus on labelling the biocontrol product with fluorescent markers and modernisation of the pollen insert to fit with commercial farming operations. Thus far we have

designed an automatic feeder system to ensure continued supply of biocontrol product to the pollen trap.

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Appendix A

In this study, pollen inserts were found to be an effective means of inoculating honeybees with the antagonist. However, the antagonist powder was found to be emptied out after 30 min. In practice, it would be uneconomical to keep on refilling the antagonist powder. Subsequently, an automatic feeder system was developed to ensure continued feeding of the powder into the pollen insert (Appendix A, Fig. A1). The automatic feeder was tested under field conditions on a full colony active beehive. The number of bees exiting through the automated insert was 30 per 30 s. The hissing noise made by the rotating conveyer belt and the electric motors, driving the equipment, does not seem to affect the bee trafficking patterns. Normally, the centre of the hive is used when bees exit the hive and they enter through the corners of the hive on re-entry. The automated feeder developed in this study is unique in terms of providing an effective continuous feeding system of powder products.

This apparatus supplies the compound to be disseminated by bees to flowers. A conveyor belt, carrying the substance from a container, is driven electrically or mechanically, as shown on the side view of the device.

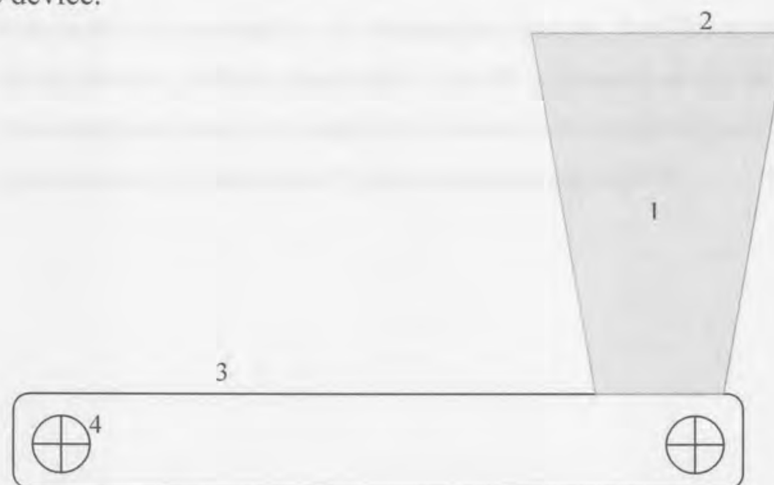

Figure A1. Schematic presentation of a compound distributor, consisting of a container (1), providing the substance (2) to a conveyor belt (3), driven by a power source (4).

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Appendix B

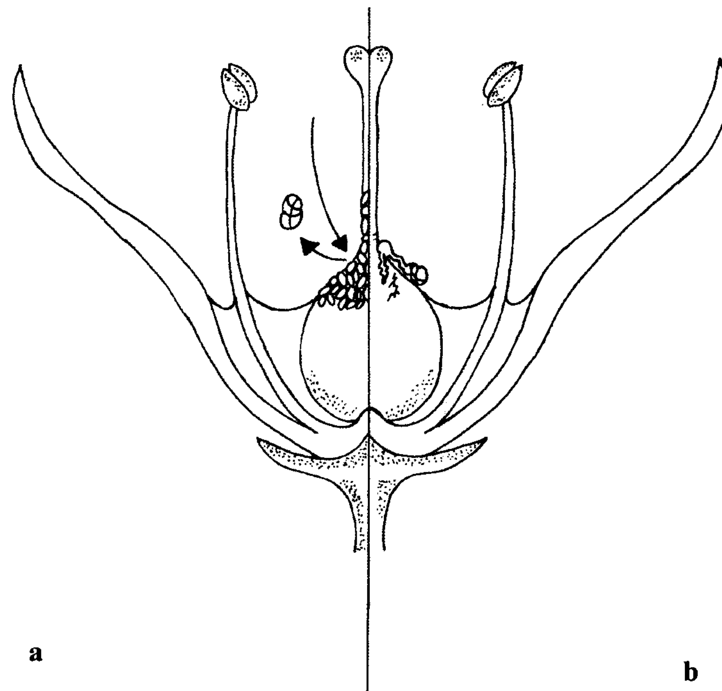

Figure B1 Schematic representative of colonisation sites by ***Bacillus subtilis*** on the stylar end of citrus flowers. When a layer of ***B. subtilis*** is present on the citrus flower, spores of ***Alternaria alternata*** are unable to colonise (a), whereas spores are able to attach and germinate in the absence of the bacterial antagonist (b). **Picture: B. Porter, 2002.**