

## CHAPTER 4

### MODES OF ACTION INVOLVED IN ANTAGONISM OF *BACILLUS SUBTILIS* AGAINST FUNGAL POSTHARVEST PATHOGENS OF AVOCADO

#### 1. ABSTRACT

Postharvest diseases are of economic importance to the avocado industry. The most effective control of postharvest fungal pathogens causing these diseases is through the use of fungicides. However, due to the global movement away from the excessive use of chemicals, research into alternative control measures, such as biocontrol have become important. In biocontrol, two basic strategies can be followed, one a preventative and the other a curative approach. *Bacillus subtilis*, previously described as an effective antagonist against postharvest pathogens of avocado, was further evaluated *in vitro* and *in vivo* to determine its mode of action. Scanning electron microscopy studies aimed at elucidating the interactions between *B. subtilis* and *Colletotrichum gloeosporioides* indicated that the antagonist can prevent conidia of *C. gloeosporioides* from germinating. The antagonist also attached to the hyphae of *C. gloeosporioides* and caused extensive lysis. Inhibitory substances were produced *in vitro* by *B. subtilis* that may represent antibiotics, enzymes or siderophores. *In vitro* tests showed that *B. subtilis* expressed chitinase, extracellular amylase, lipase and proteinase activity. Siderophores are also produced by *B. subtilis*. Volatiles produced by *B. subtilis* inhibited *Phomopsis perseae*, *Dothiorella aromatica* and *Lasiodiplodia theobromae* *in vitro*. However, *C. gloeosporioides* showed no sensitivity towards these volatiles.

#### 2. INTRODUCTION

All subtropical fruit are prone to fungal attack either during fruit development or after harvesting. Postharvest diseases, such as stem-end rot (SE) and anthracnose, cause major economic losses in avocado production. Several fungal pathogens have been associated with SE, and include *Dothiorella aromatica* (Sacc.) Petrak & Sydow (Darvas & Kotzé, 1987; Korsten *et al.*, 1995), *Thyronectria pseudotrichia* (Schw.) Seeler, *Lasiodiplodia theobromae* (Pat.) Griffon & Maubl., *Colletotrichum gloeosporioides* Penzig., *Phomopsis perseae* Zerova, *Pestalotiopsis versicolor* (Speg.) Steyaert, and *Fusarium* species. However, infection patterns of the pathogens differ and therefore require different disease control strategies. Most SE pathogens may enter fruit through freshly cut stem-end lesions during harvesting, or postharvestly due to handling and bruising (Darvas *et al.*, 1987; Koomen & Jeffries, 1993). The postharvest pathogen, *C.*

*gloeosporioides*, typically infect preharvestly and either cause lesions or remain latent until fruit ripen, resulting in postharvest infections (Koomen & Jeffries, 1993).

The wide spectrum of fungi associated with SE and their infection patterns make control of this disease difficult. Biological control strategies can either focus on curative or preventative approaches depending on the target pathogen. Keeping the diversity in fungal pathogen infection mechanisms in mind, it could be important to select both approaches in order to achieve total control of avocado diseases. Latent infections, employed by *C. gloeosporioides*, are especially problematic since the pathogen has already attached itself to the fruit surface and developed appressoria. Infection is only seen when fruit soften and ripen, decreasing the amount of inhibitory substances in the avocado skin (Prusky & Plumbley, 1992).

To develop a successful control strategy that will target all types of infection patterns, the specific mode of action of an antagonist has to be taken into consideration (Andrews, 1992; Droby & Chalutz, 1994; Guetsky *et al.*, 2002). Knowledge of the mode of action may also help in optimising the method and timing of application, as well as enhancing the activity of antagonists by modifying its formulation (Wilson & Wisniewski, 1989). In addition, information regarding the antagonist and its mode of action is necessary for product registration.

Several different modes of action can be involved in antagonism between pathogens and antagonists. These include antibiosis (Baker *et al.*, 1983; Fravel, 1988; Asaka & Shoda, 1996; Bull *et al.*, 1998), competition for space and nutrients (Wisniewski *et al.*, 1989; Dik, 1991; Calvente *et al.*, 1999), induced resistance in the host (Wilson *et al.*, 1994; Arras, 1996; Elad, 2000) and direct interactions between the pathogen and antagonist (Droby & Chalutz, 1994; Nielsen & Sørensen, 1997). The modes of action involved in *Bacillus subtilis* antagonism against *C. gloeosporioides* were previously investigated (Korsten & de Jager, 1995; Havenga *et al.*, 1999). Three modes of action were found to be involved, namely competitive colonization, competition for nutrients and antibiosis.

In this study the modes of action of *B. subtilis* against a spectrum of SE pathogens were investigated. In all assays, *C. gloeosporioides* were included for comparative purposes. Interactions between *B. subtilis* and *C. gloeosporioides* were viewed using Scanning Electron Microscopy (SEM). The possibility that antibiotic substances, enzymes and volatiles are involved was also evaluated.



### 3. MATERIALS AND METHODS

#### 3.1. Isolates

Subculture MI-14 of *B. subtilis* (Chapter 3) was used throughout this study. Stock cultures of antagonist, *B. subtilis* were stored in 30 % glycerol with Ringer's solution (Merck, Johannesburg) at  $-70^{\circ}\text{C}$ . Cultures were maintained on standard 1 nutrient agar (STD1) (Biolab, Merck) and plates were incubated at  $28^{\circ}\text{C}$  for 24 h before use.

Fungal pathogens isolated and identified in Chapter 3 were used throughout this study (*C. gloeosporioides*, *P. perseae*, *D. aromatica* and *L. theobromae*). All fungi were maintained on both potato dextrose agar (PDA) (Biolab) slants and mycelium-containing agar plugs in sterile water, kept at room temperature. An agar disk (5 mm) containing the fungus was placed on PDA and incubated at  $25 - 28^{\circ}\text{C}$  for three days prior to use.

#### 3.2. Direct interaction between *Bacillus subtilis* and *Colletotrichum gloeosporioides* on avocado fruit surfaces using scanning electron microscopy

Ripe Fuerte cultivar avocado fruit were obtained from Westfalia Estate for use in *in vivo* antagonism studies. The *B. subtilis* isolate was streaked onto STD1 and incubated for 24 h at  $25^{\circ}\text{C}$ . Cells were suspended in sterile quarter strength Ringer's using a sterile glass rod. Cell counts were determined with a Petroff-Hausser counting chamber and adjusted to  $10^7$  cells/ml. Fungal spores of *C. gloeosporioides* were harvested from seven-day-old cultures and spore counts were made using a Haemocytometer. Spore suspensions were adjusted to contain  $10^4$  spores/ml.

The surfaces of ten avocado fruit were wiped with 70 % ethanol. Fruit were left for five min to air dry before three  $25\text{ mm}^2$  areas were marked with a water insoluble marker. The protocol described in Table 4.1 was followed. For each treatment, three samples of inoculated avocado areas were aseptically removed 6, 24 and 72 h after application and prepared for viewing using SEM. Sterile distilled water, as well as *B. subtilis* and *C. gloeosporioides* alone served as controls.

Excised avocado skin samples were placed in 0.075 M Phosphate buffer ( $0.15\text{ M Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$  (Saarchem, Merck);  $0.15\text{ M NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$  (Saarchem) containing 2.5 % Gluteraldehyde (Dawson *et al.*, 1969) for 24 h. Samples were washed three times in 0.075 M Phosphate buffer. Samples were dehydrated through a series of ethanol concentrations (1 x 50 %, 1 x 70 %, 1 x 90 % and 3 x 100 %) for 15 min each.

Samples were dried in a Hitachi HCP-2 critical point dryer and mounted on aluminium stubs. Specimen stubs were coated with gold palladium in an Eiko IB-3 ion coater. Stubs were viewed in a Hitachi 840 JEOL SEM operating at 5 kV. All samples were observed and photos were taken of possible interactions between *C. gloeosporioides* and *B. subtilis*, as well as the control treatments.

Table 4.1: Treatment of avocado fruit surfaces prior to scanning electron microscopy viewing

Treatment number	Treatment	Time interval between applications			
		0 h	4 h	24 h	48 h
1	Sterile distilled water (100 µl)	+	-	-	-
2	<i>B. subtilis</i> alone (50 µl of $10^7$ cells/ml + 50 µl sterile water)	+	-	-	-
3	Fungal suspension alone (50 µl of $10^4$ spores/ml + 50 µl sterile water)	+	-	-	-
4	<i>B. subtilis</i> (50 µl of $10^7$ cells/ml). Application of fungal test culture (50 µl of $10^4$ spores/ml) after time intervals	+	+	+	+
5	Fungal test culture (50 µl of $10^4$ spores/ml). Application of <i>B. subtilis</i> (50 µl of $10^7$ cells/ml) after time intervals	-	+	+	+

+ indicates application of treatment

- indicates no application

### 3.3. Diffusible inhibitory metabolites produced by *Bacillus subtilis* in vitro active against *Colletotrichum gloeosporioides*

Antibiotic production medium (Chapter 6) was inoculated with *B. subtilis*. The inoculated liquid culture medium was shake incubated at 25 °C for seven days and two ml of the suspension was daily filtered through a 0.22 µm pore sized acetate filter (Millipore, Separation Scientific, Johannesburg). Spore suspensions of *C. gloeosporioides* were made by pipetting sterile water onto PDA plates and removing mycelia and spores with a sterile streaking rod. Spore suspensions were spread plated over PDA. Wells were made in the agar with a sterile five mm diameter cork borer and 0.25 ml of the bacterial liquid culture filtrate was pipetted into the wells. The absence or presence of inhibition zones were noted and were measured when formed. A filtrate of sterile antibiotic production medium was used as a negative control. The averages and standard deviation between formed inhibition zone diameters were calculated.

### 3.4. Enzyme activity of *Bacillus subtilis* and fungal postharvest avocado pathogens in vitro

The production of chitinase, amylase, lipase and proteinase were investigated *in vitro* on selective media. Plates were either streak inoculated with *B. subtilis* or inoculated with a four mm diameter agar plug



containing *C. gloeosporioides*, *P. perseae*, *D. aromatica*, or *L. theobromae*. Three plates were streak inoculated on four equidistant spots with *B. subtilis* culture. For each test fungal culture, three plates were inoculated with a five mm diameter agar plug taken from the actively growing periphery of the fungus on PDA. Petridishes were inoculated with four plugs equidistant from each other. Plates were incubated at 25 °C for up to seven days and monitored daily.

#### 3.4.1. Chitinase activity

The medium of Frändberg & Schnürer (1994) was modified to contain 4 % chitin instead of 1.5 % w/v. The medium contained the following: 4 % w/v colloidal chitin from crab shell; 8.6 mM  $K_2HPO_4$ ; 11.0 mM  $KH_2PO_4$ ; 2.8 mM  $MgSO_4 \cdot 7H_2O$ ; 8.6 mM NaCl; 6.7 mM KCl (all from Saarchem); 0.9 mM  $CaCl_2 \cdot 2H_2O$  (Fluka, Sigma-Aldrich); 0.05 % w/v yeast extract (Biolab); and 2 % w/v bacteriological agar (Biolab). The pH was adjusted to 6.6. The medium was autoclaved for 15 minutes at 121 °C. The plates were inoculated as previously described, incubated at 25 °C and the presence or absence of growth on the minimal medium was noted after 7 days. Results were compared qualitatively in relation to the presence or absence of growth.

#### 3.4.2. Extracellular amylases

To test for extracellular amylase activity, Petridishes containing starch medium (Skinner & Lovelock, 1979) was prepared. The medium contained: 50 ml Czapek solution A (94.1 mM  $NaNO_3$ ; 26.8 mM KCl; 0.2 mM  $MgSO_4 \cdot 7H_2O$ ); 50 ml Czapek solution C (23 mM  $K_2HPO_4$  (all from Saarchem)); 1 ml zinc solution (3.5 mM  $ZnSO_4 \cdot 7H_2O$  (AnalaR, British Drug Houses (BDH)); 1 ml copper solution (2 mM  $CuSO_4 \cdot 5H_2O$  (Pro Analysis, Merck)); 50 ml starch solution (20 % w/v starch (Biolab) in distilled water; heated slowly to 70 – 80 °C and slowly added to the rest of the media); 1.2 % w/v bacteriological agar (Biolab); and 850 ml distilled water. The medium was sterilized for 30 min at 121 °C. Plates were inoculated as previously described, incubated at 25 °C for three days and then covered with Gram's iodine (Sigma). The presence or absence of clear zones in the agar surrounding bacterial and fungal growth was noted. Results were compared qualitatively in relation to the presence or absence of clear zones.

#### 3.4.3. Lipase activity

To test for lipase activity, Petridishes containing Tween-80 medium (Skinner & Lovelock, 1979) was prepared. The medium contained: 1 % w/v peptone (Biolab); 8.6 mM NaCl (Saarchem); 0.7 mM  $CaCl_2 \cdot 2H_2O$  (Fluka); 0.05 mM bromocresol purple (Pro Analysis); and 1.5 % w/v biological agar (Biolab). The pH was adjusted to 5.4. A 10 % v/v tween-80 stock solution was made by adding tween-80 (Sigma) to distilled water

that was heated to  $65 \pm 5$  °C. Both medium and tween-80 stock solution was autoclaved for 10 minutes at 121 °C. The final medium contained 10 ml of the tween-80 stock solution and 90 ml of the medium before plates were poured. Plates were inoculated as previously described, incubated at 25 °C for four days and monitored for change in colour of the medium from yellow to purple-blue. Results were compared qualitatively in relation to whether the medium colour changed or not.

#### 3.4.4. Proteinase activity

To test for proteinase activity, Petridishes containing casein hydrolysis medium (Skinner & Lovelock, 1979) was prepared. The medium contained: 7.3 mM  $\text{KH}_2\text{PO}_4$ ; 6.7 mM KCl; 0.8 mM  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  (all from Saarchem); 0.7 mM  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$  (Fluka); 1 % w/v glucose (Sigma); 2.5 % v/v skim milk; and 1.2 % w/v bacteriological agar (Biolab). The pH was adjusted to 5.4 and the medium was autoclaved for 30 min at 121 °C. The plates were inoculated as previously described, incubated at 25 °C and monitored for four days for the formation of clear zones surrounding growth. The presence or absence of clear zones in the agar was noted. Results were compared qualitatively in relation to the presence or absence of clear zones.

#### 3.5. Siderophore production by *Bacillus subtilis* and fungal postharvest avocado pathogens in vitro

The methods of Schwyn & Neilands (1987), Buyer *et al.* (1989), and Alexander & Zuberer (1991) were combined to evaluate the production of siderophores. In brief, 2 mM chrome azurol S (CAS) (Sigma) was slowly added to an iron solution (1 mM  $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$  (AnalaR) in 10 mM HCl (Sigma)) in a 5:1 v/v ratio to make solution A. Solution B consisted of 5 mM hexadecyltrimethylammonium bromide (HDTMA) (Fluka) dissolved over low heat distilled water. Solution A was slowly added to solution B in a ratio of 3:2 v/v and autoclaved (CAS stock solution). The following was autoclaved for 15 min at 121 °C and cooled to 50 °C to form the RSM stock solution: 6.35 mM  $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$  (Saarchem); 2 mM  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  (Saarchem); 0.2 M N-(2-Acetamido)-2-aminoethanesulfonic acid (ACES) (Fluka); 0.1 M NaOH (Merck); 30 % w/v bacteriological agar (Biolab). The following were each autoclaved before adding to the RSM stock solution: 1 M  $\text{KH}_2\text{PO}_4$  (Saarchem) at 1:0.002 v/v; 10 % casaminoacid solution (Difco) at 1:0.17 v/v; and 30 % sucrose (Saarchem) at 1:0.07 v/v. The following was filter sterilized before adding to RSM stock solution: 0.007 M  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$  (AnalaR) at 1:0.002 v/v; 0.009 M  $\text{MnSO}_4 \cdot \text{H}_2\text{O}$  (Saarchem) at 1:0.002 v/v; 0.02 % w/v thiamine HCl (Sigma); and 0.001 % w/v biotin (Fluka). The CAS stock solution was slowly added to RSM stock solution, to keep foam from forming, at 1:9 v/v and dispensed into Petridishes. Plates were either streak inoculated with *B. subtilis* or inoculated with a four mm diameter agar plug containing *C. gloeosporioides*, *P. perseae*, *D. aromatica* or *L. theobromae*. Three replicate plates were inoculated for each organism and the experiment



was repeated four times. Plates were incubated at 25 °C for four days and monitored for the formation of yellow to orange zones surrounding the growth on the blue plates. The presence or absence of yellow or orange zones was noted. Results were compared qualitatively in relation to whether medium colour changed or not.

### 3.6. *Antifungal volatiles produced by Bacillus subtilis active against fungal postharvest avocado pathogens*

The sealed plate (3.6.1) and the aerated plate (3.6.2) methods of Fiddaman & Rossall (1993) were followed. The radial growth of the fungal pathogens were measured and compared to the untreated control by determining the percentage inhibition (Chapter 3). The means of percentage inhibition for each fungus were calculated. Data was analysed using the statistical program GenStat (2000). Weighted analysis of variance was used to test for differences between the main effects for both pathogens and mediums, as well as the pathogen-by-medium interaction. Fisher's protected t-test least significant difference was used to test for differences between means per day (Snedecor & Cochran, 1980).

#### 3.6.1. *Sealed plate method*

The method of Fiddaman & Rossall (1993) was followed. The antagonist was shake incubated at 25 °C overnight in Nutrient Broth (NB) (Biolab) and 100 µl was spread plated over Nutrient Agar (NA) (Biolab) or STD1. Plates were incubated at 25 °C for 24 h. Fungal pathogens were grown on PDA for four to seven days. Five mm diameter plugs, obtained from the periphery of actively growing cultures, were used to centrally inoculate PDA plates. The treatment consisted of fungal pathogen inoculated plates inverted and placed over the antagonistic bacterial cultures, so that the antagonist could not contaminate the fungal cultures. The plates were sealed together with Parafilm and incubated at 25 °C. Control treatment consisted of two identical pathogen-containing plates sealed together. Three replicates were made from each fungus and the experiment was repeated four times. The experiment was designed to determine if the antagonist could produce antifungal volatiles. Measurements of fungal radial growth were taken over a three-day period incubated at 25 °C. Data was statistically analysed as previously described in 3.6.

#### 3.6.2. *Aerated plate method*

As in the method of Fiddaman & Rossall (1993), an agar strip, 5 mm wide, was removed from the centre of PDA containing plates, creating a division. The antagonist was shake incubated at 25 °C overnight in NB

and 50 µl was spread plated over one of the PDA containing sides. Petridishes were incubated at 25 °C for 24 h. The other PDA side was inoculated with a 5 mm diameter plug obtained from the periphery of actively growing fungal cultures on PDA. Plates were incubated at 25 °C for four days. Plates inoculated with the fungus alone served as control. Three replicates were made from each of the fungi and the experiment was repeated four times. The pathogen's colony diameter was measured daily for four days. Data was statistically analysed as previously described in 3.6.

## 4. RESULTS

### 4.1. In vivo studies

Scanning electron micrographs (Figure 4.1) show the avocado fruit surface being characteristically corrugated providing multiple microbial sites to colonise. The avocado surface has distinct protruding wax platelets.

Germinating conidium of *C. gloeosporioides* was observed attaching to the fruit surface, forming an attachment sheath (Figure 4.2). Cells of *B. subtilis* were typically found to be at least five times shorter than *C. gloeosporioides* spores (Figure 4.2). Inoculation of the avocado fruit surface with *B. subtilis* followed by fungal conidia, showed inhibition of germination of *C. gloeosporioides* spores (Figure 4.2).

In Figure 4.3 *B. subtilis* cells were observed actively dividing. Extracellular slime and micro colonies were observed (Figure 4.3). In addition to attaching to the avocado fruit surface, *B. subtilis* also colonized *C. gloeosporioides* hyphae (Figure 4.3).

Conidia of *C. gloeosporioides* inoculated onto avocado fruit germinated, forming germ tubes and hyphae. When *B. subtilis* was inoculated 24 hours later, it colonized the hyphal surface. In certain cases hyphal lysis was observed (Figure 4.4). Colonization of *C. gloeosporioides* by *B. subtilis* did not always coincide with damage to hyphae (Figure 4.4).





Figure 4.1: Scanning electron micrographs of the surface of avocado fruit showing characteristic wax platelets (Photo 1: 4300 x, 2: 3500 x, and 3: 10000 x magnification).

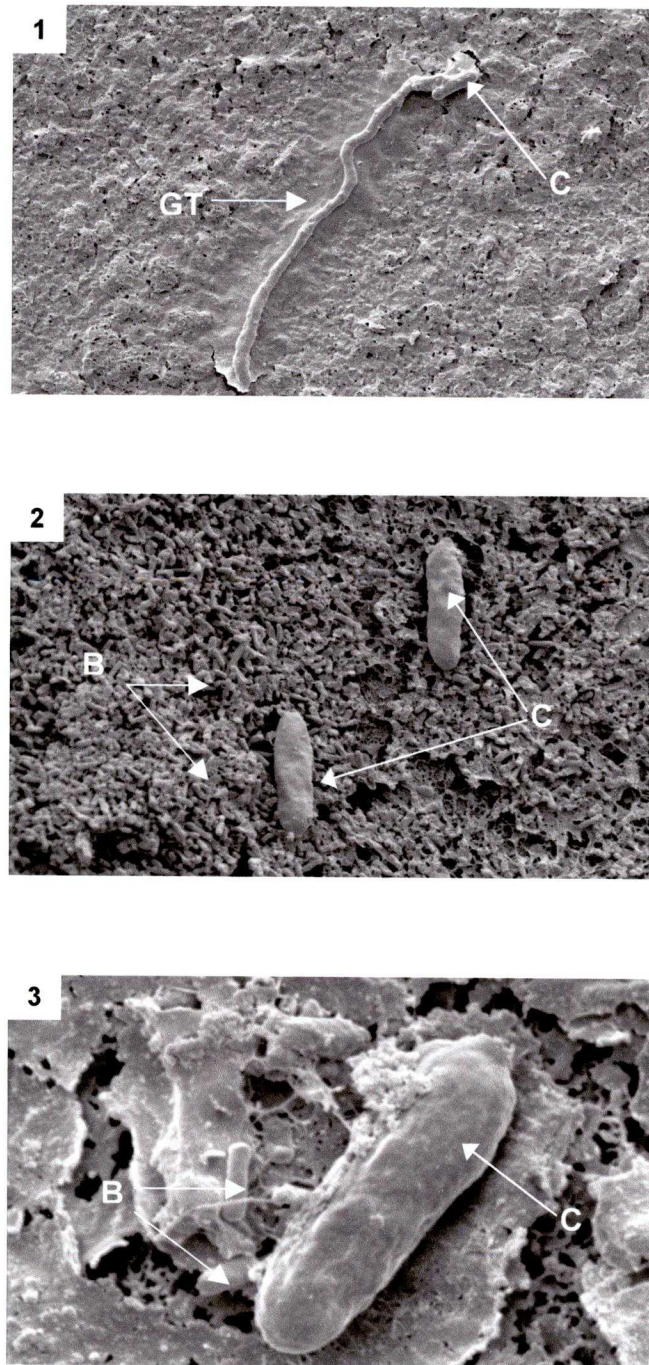


Figure 4.2: Scanning electron micrographs of avocado fruit inoculated: 1) with conidia of *Colletotrichum gloeosporioides* alone, 2) and 3) with conidia of *C. gloeosporioides* followed by *Bacillus subtilis* (Photo 1: 1200 x, 2: 7500 x, and 3: 2300 x magnification).

Abbreviations: C = conidium of *C. gloeosporioides*, B = *B. subtilis* cells, GT = germ tube of *C. gloeosporioides*



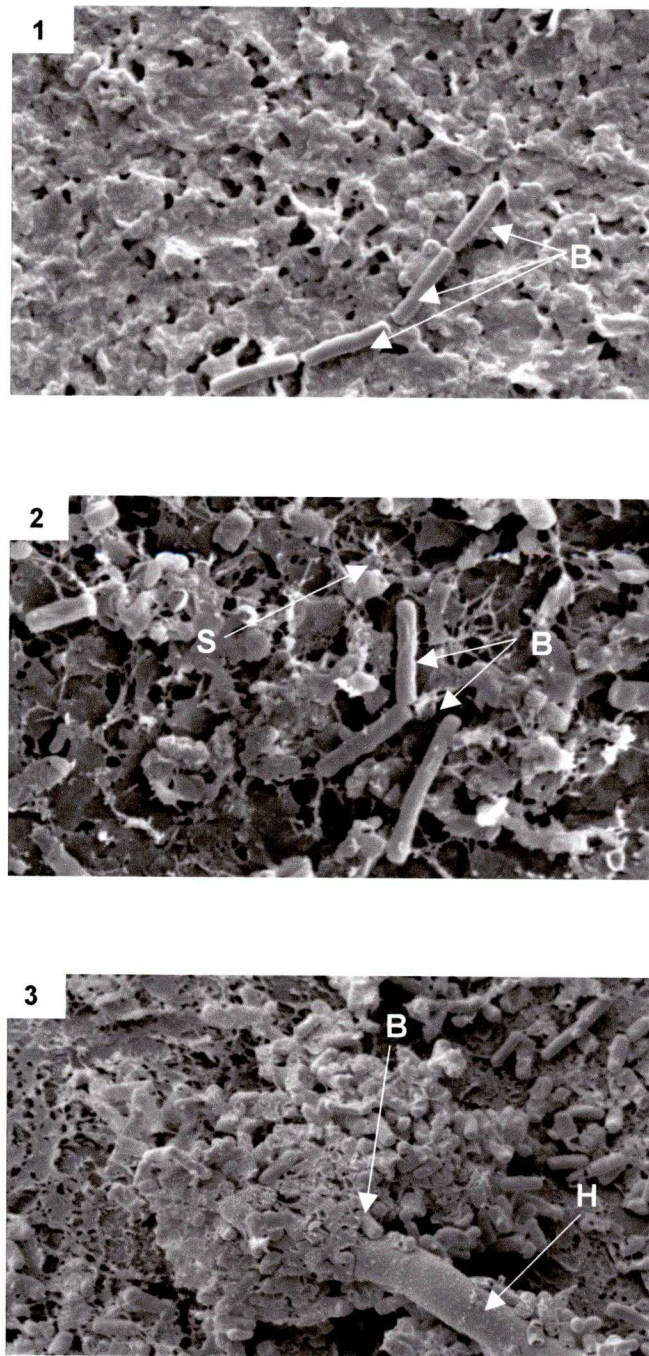


Figure 4.3: Scanning electron micrographs of avocado fruit surfaces inoculated with: 1) *Bacillus subtilis*, 2) *B. subtilis* showing attachment structures, and 3) both *B. subtilis* cells and *Colletotrichum gloeosporioides* hyphae (Photo 1: 6000 x, 2: 8500 x, and 3: 5000 x magnification).  
Abbreviations: B = *B. subtilis* cells, H = *C. gloeosporioides* hyphae, S = slime formed by *B. subtilis*

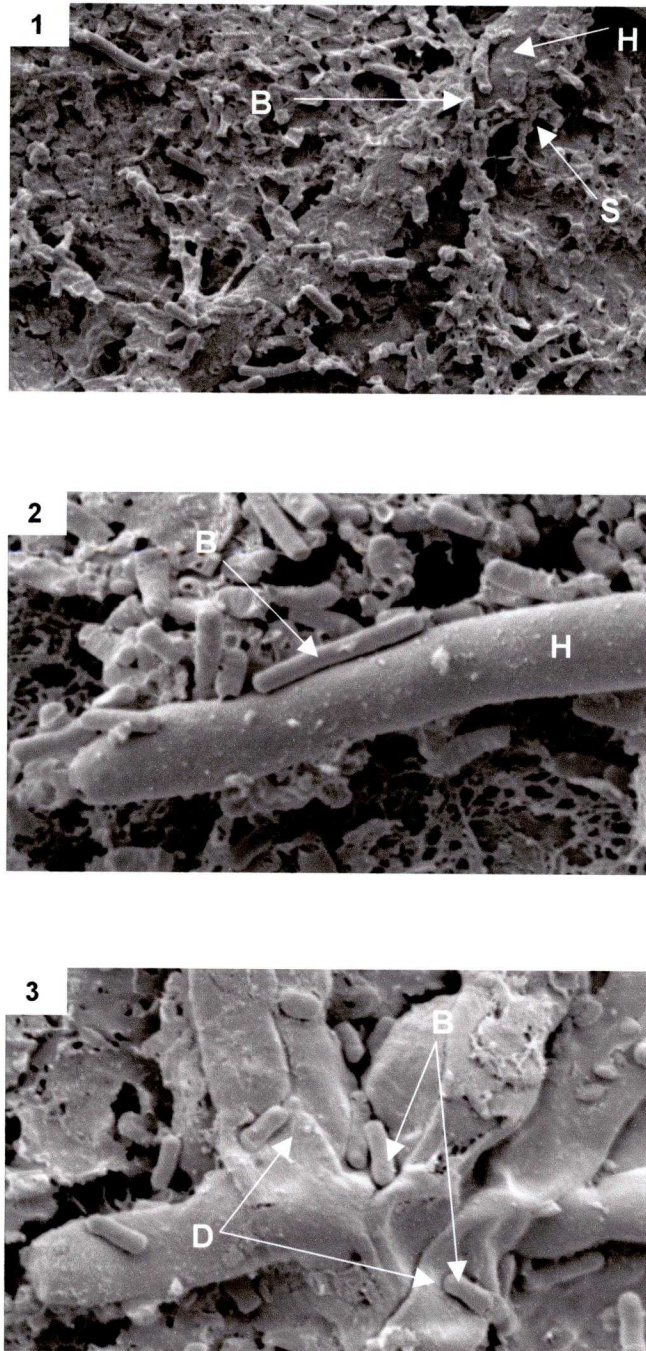


Figure 4.4: Scanning electron micrographs showing avocado fruit surfaces inoculated with *Colletotrichum gloeosporioides* and *Bacillus subtilis* (Photo 1: 4300 x, 2: 8500 x, and 3: 7000 x magnification). Abbreviations: B = *B. subtilis* cells, D = Damage caused by *B. subtilis* to fungal hypha, H = *C. gloeosporioides* hyphae, S = slime formed by *B. subtilis*



#### 4.2. Diffusible inhibitory metabolites produced by *Bacillus subtilis* in vitro active against

##### *Colletotrichum gloeosporioides*

Extracellular inhibitory substances were produced by *B. subtilis* from day two and consistently increased over time (Figure 4.5).

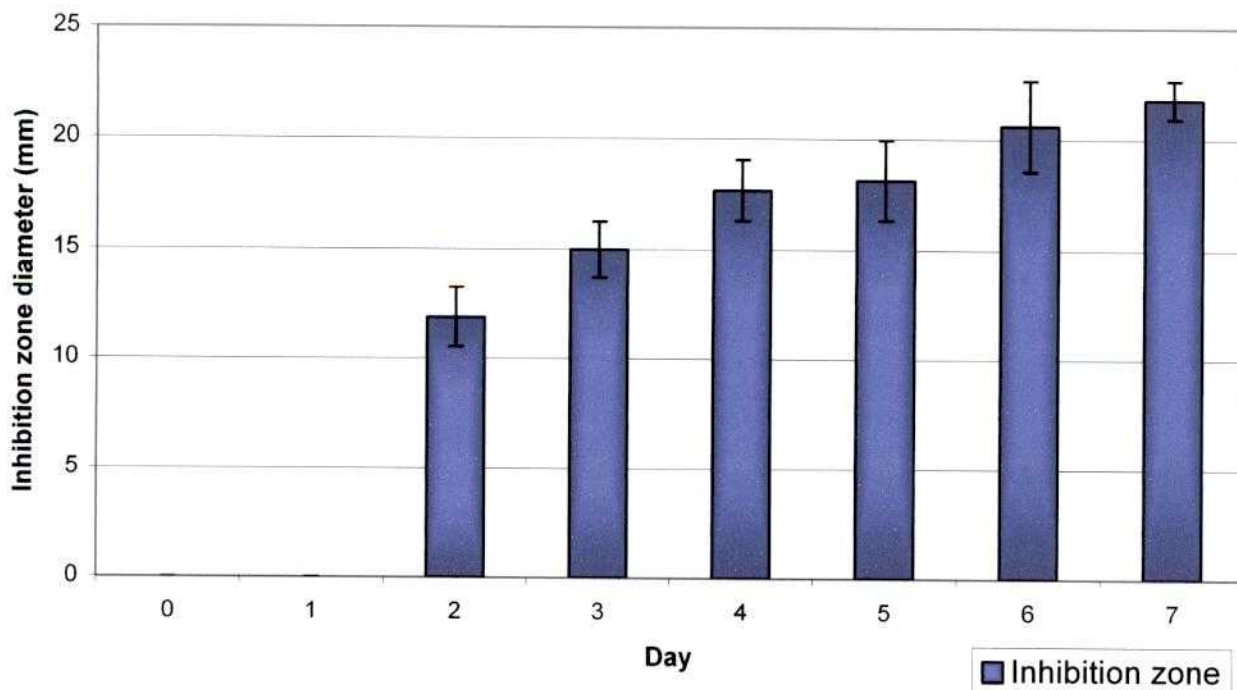


Figure 4.5: Diameter of inhibition zones formed by *Bacillus subtilis* cell-free filtrates on spread plates of *Colletotrichum gloeosporioides*.

#### 4.3. Enzyme activity of *Bacillus subtilis* and fungal postharvest avocado pathogens in vitro

Qualitatively, all fungal pathogens tested, as well as *B. subtilis*, were observed to break down chitin, starch, lipids and casein.

#### 4.4. Siderophore production by *Bacillus subtilis* and fungal postharvest avocado pathogens in vitro

Siderophore production was observed on all tested organisms, except *P. perseae*.

#### 4.5. Antifungal volatile production in vitro by *Bacillus subtilis* active against fungal postharvest avocado pathogens

For both the sealed and aerated plate method, the data was acceptably normally distributed, but the treatment variances were excessively heterogeneous and significance was obtained at the 5 % level of significance.

#### 4.5.1. Sealed plate method

Significant radial mycelial growth inhibition was observed for *P. perseae* (Figure 4.6), *D. aromatica* (Figure 4.8) and *L. theobromae* (Figure 4.9). A volatile substance was produced by *B. subtilis* that is able to decrease the growth rate of the fungi, when compared to the control. Although the volatile substance inhibited radial growth of *P. perseae* and *L. theobromae*, it affected *C. gloeosporioides* (Figure 4.7) less. The highest percentage fungal pathogen inhibition was higher on STD1 when compared to NA.

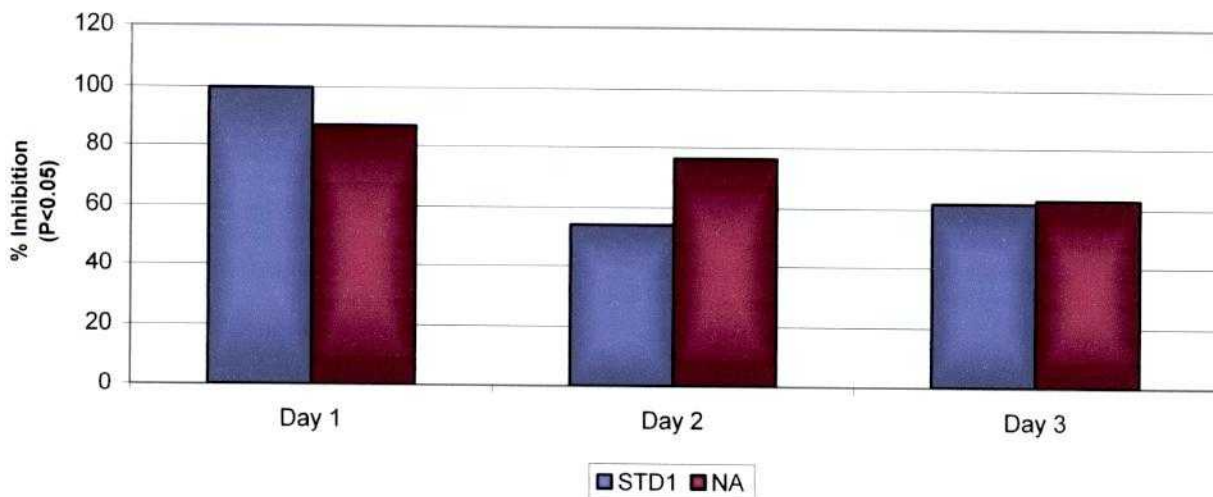


Figure 4.6: Comparison between the mean percentage inhibition of *Phomopsis perseae* by *Bacillus subtilis* measured on days 1 to 3 after inoculation.

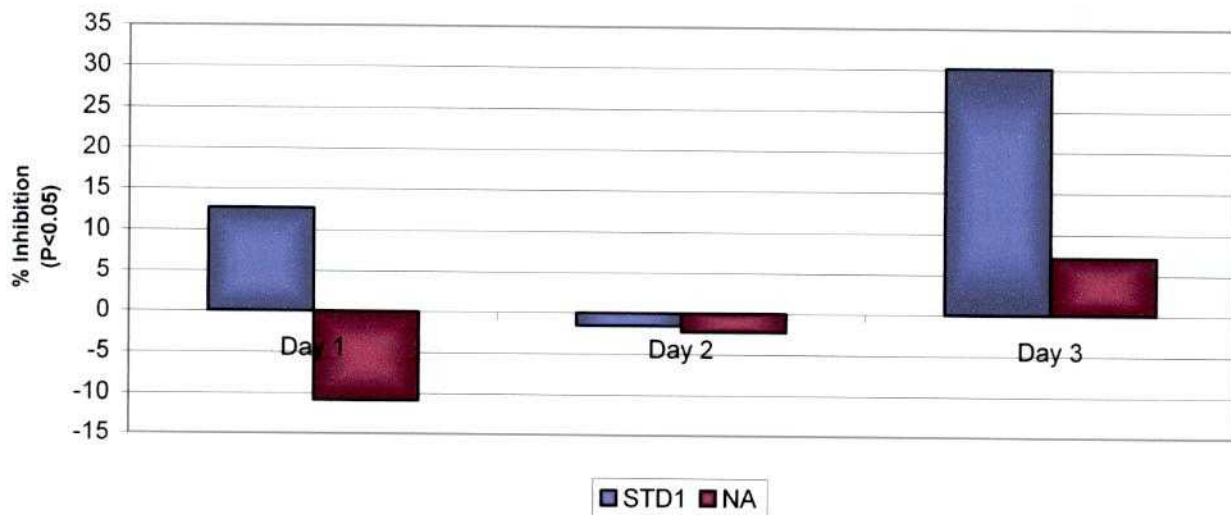


Figure 4.7: Comparison between the mean percentage inhibition of *Colletotrichum gloeosporioides* by *Bacillus subtilis* measured on days 1 to 3 after inoculation.



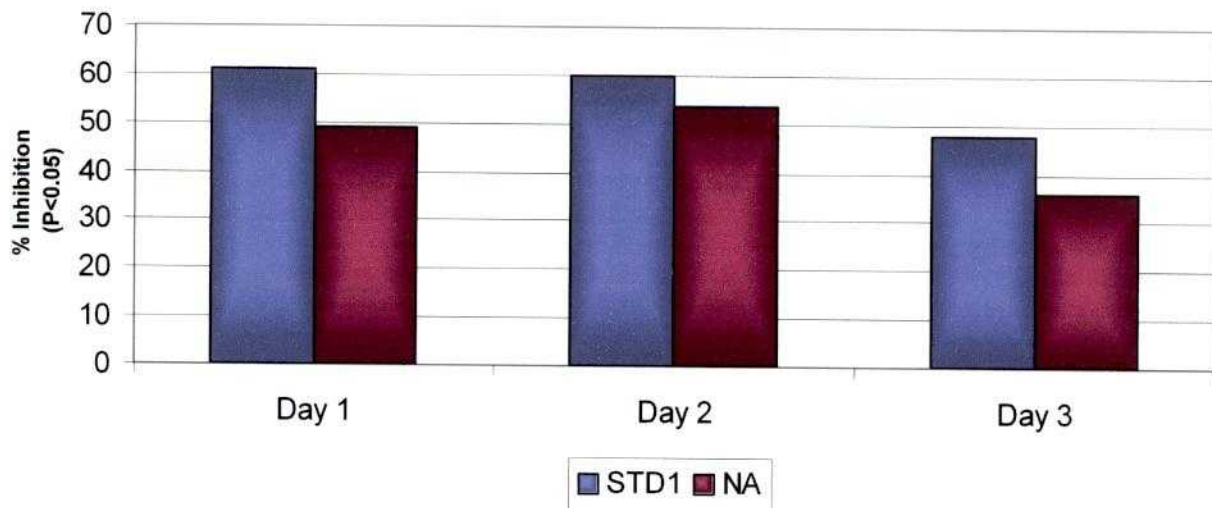


Figure 4.8: Comparison between the mean percentage inhibition of *Dothiorella aromatica* by *Bacillus subtilis* measured on days 1 to 3 after inoculation.

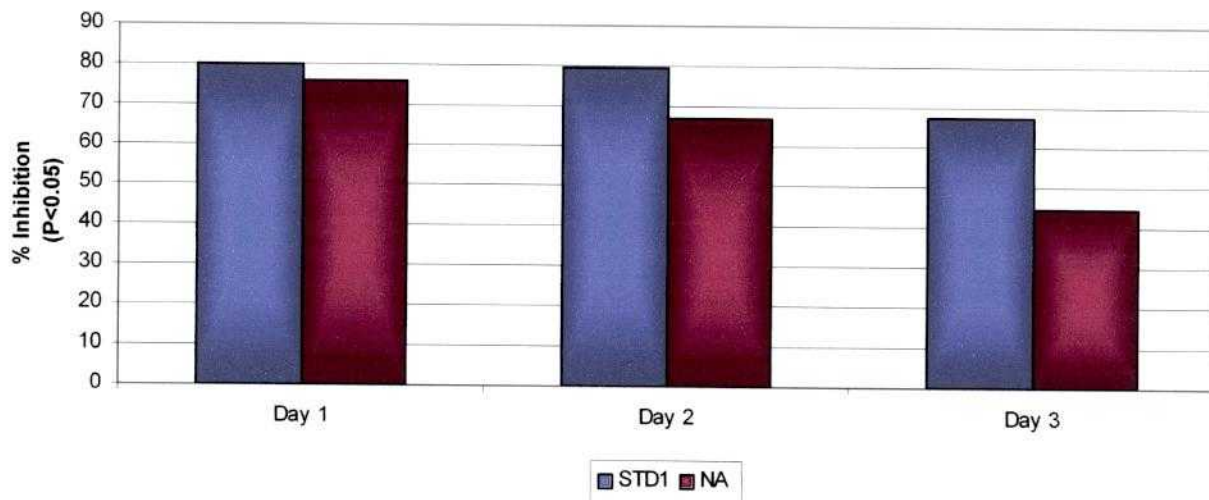


Figure 4.9: Comparison between the mean percentage inhibition of *Lasiodiplodia theobromae* by *Bacillus subtilis* measured on days 1 to 3 after inoculation.

#### 4.5.2. Aerated plate method

The radial mycelial growth of all fungi was inhibited to some extent, as seen in Table 4.2. This implied that *B. subtilis* produced some volatile substance that was able to inhibit the growth of these pathogens. However, the extent to which the pathogens were inhibited was much lower than that observed when using the sealed plate method. There were no significant differences when the percentage inhibition of the four pathogens was compared (Table 4.2). After two days, the small area available for growth of *L. theobromae* was overgrown and no further measurements could be taken.

Table 4.2: Mean percentage inhibition of fungal pathogens caused by volatile substances produced by *Bacillus subtilis* using the aerated plate method

Pathogen	Day 1		Day 2		Day 3		Day 4	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
<i>Phomopsis perseae</i>	-5.94	32.8	-8.905	12.103	2.647	5.344	1.496	3.841
<i>Colletotrichum gloeosporioides</i>	13.50	2.3	-0.689	0.961	-0.700	0.676	3.760	0.256
<i>Dothiorella aromatica</i>	12.02	9.5	-16.847	7.093	-15.942	13.401	-8.225	4.777
<i>Lasiodiplodia theobromae</i>	0.57	10.6	2.820	5.379	-	-	-	-
Fprob	P = 0.633		P = 0.154		P = 0.454		P = 0.072	
% CV	50.5		5.45		9.72		78.38	

SEM = Standard error of means

- Indicates no measurements were taken on these days

Data analysed using weighted ANOVA.

## 5. DISCUSSION

This study was the first to show the activity of volatile substances produced by *B. subtilis* that is inhibitory against fungal pathogens of avocado fruit. The production of antifungal volatile substances by *B. subtilis* is not unusual. Knox *et al.* (2000) observed *B. subtilis* producing antifungal volatiles active against *Fusarium oxysporum* (Schltdl.: Fr.). The fungal pathogens differed in their sensitivity to the volatile substance, with *P. perseae*, *L. theobromae* and *D. aromatica* being more sensitive. However, *C. gloeosporioides* was not affected by the volatile substance, indicating that the volatile might not play a role in *in vivo* antagonism against *C. gloeosporioides*. Therefore, other modes of action must be involved in the interaction between *C. gloeosporioides* and *B. subtilis*. This observation corresponds to that reported by Fiddaman & Rossall (1993). They found that various fungi react differently to volatiles produced by *B. subtilis* NCIMB 12376. They also stated that this variation in activity might reflect differences in sites of action of the volatiles, or even differences in the ability of the fungi to detoxify these metabolites. The volatiles produced by *B. subtilis* NCIMB 12376 were tentatively identified as alcohols, aldehydes, ketones and esters. In the current study, two different agar media were used to test for the production of antifungal volatiles, STD1 and NA. A greater inhibitory activity was found with STD1 medium, especially against *L. theobromae* and *D. aromatica*. The carbon sources and peptone levels in STD1 are known to be higher than that of NA. Fiddaman & Rossall (1993; 1994) found that by increasing the D-glucose levels in the medium, a significant increase in the volatile activity could be observed. Furthermore, volatile production increased with the addition of complex carbon sources, like starch, even though the same inhibitory levels yielded by D-glucose was not reached. Knox *et al.* (2000) found that with the interaction between *B. subtilis* and *F. oxysporum*, volatile production



increased with higher nitrate levels in the media. Similarly, peptone was able to increase volatile production in *Streptomyces* (Fiddaman & Rossall, 1994). However, Fiddaman & Rossall (1994) found that there was not necessarily a correlation between the ability of a medium to support growth and its ability to inhibit the fungal pathogens. They state that some nutrients may yield higher volatile production, without increasing bacterial growth and that a range of different volatiles may act synergistically. They also found the inhibitory effect of the sealed plate being more effective than the aerated plate method (Fiddaman & Rossall, 1993).

*In vivo* inhibition of *C. gloeosporioides* germination by *B. subtilis* was observed when conidia were applied to avocado fruit after *B. subtilis*. This indicates competitive colonisation or competition for nutrients, as suggested by Korsten & de Jager (1995). When bacteria colonise fruit surfaces they utilise nutrients from the surrounding area (Brodie & Blakeman, 1976). This could imply that only limited nutrients would be available for other microbes. However, *C. gloeosporioides* is a specialised necrotrophic pathogen that does not require exogenous nutrients for germination and appressoria formation (Blakeman, 1985). The involvement of nutrient competition can thus be excluded, leaving only direct inhibitory interaction by enzymes or antibiotics produced by the biocontrol agent. Upadhyay & Jayaswal (1992) also observed this phenomenon in the interactions between *Pseudomonas cepacia* and phytopathogenic fungi.

The antagonist, *B. subtilis*, produced a substance effective against *C. gloeosporioides in vitro*. The cell-free filtrate contained diffusible substances, which may be enzymes or antibiotics. In this study, the amount of inhibitory substance/s produced by *B. subtilis* in liquid culture increased over time. The stationary phase is usually the signal for *B. subtilis* to switch on its survival genes (Msadek, 1999). In this study, single cells that colonised the hyphal surface of the pathogen did not cause damage to the fungal mycelium. However, when *B. subtilis* was present in high numbers, hyphal lysis was observed. In this case cell density was related to hyphae damage. This concept is known as quorum sensing or cell density-dependent regulation, which is a type of gene regulatory system. It has been found to play a role in antibiotic production (Dunny & Leonard, 1997; Msadek, 1999). With *B. subtilis*, a small regulator molecule (usually a small peptide or modified peptide) is produced at the basal level (Dunny & Leonard, 1997). When the bacterial cell concentration reaches a certain level, these molecules attain a certain concentration, which result in a signal to switch on selected genes (Dunny & Leonard, 1997). Quorum sensing may thus impact on the efficiency of *B. subtilis* as a biocontrol agent.

The production of chitinase, amylase, lipase and proteinase was observed in this study. Enzymes reported to be produced by *B. subtilis* and implicated in biocontrol include chitinase (Frändberg & Schnürer, 1998; Helistö *et al.*, 2001), chitosanase, laminarinase, lipase and protease (Helistö *et al.*, 2001), as well as glucanolytic and proteolytic enzymes (Nielsen & Sørensen, 1997). These enzymes may cause damage to *C. gloeosporioides* hyphae where *B. subtilis* attached itself directly onto the fungal cell wall. Keeping in mind that effective biocontrol is not necessarily ensured by the potential of an organism to produce cell wall degrading enzymes (Elad, 2000), further studies should be done to determine their role in *in vivo* biocontrol of postharvest pathogens by *B. subtilis*.

In this study it was found that *B. subtilis* produces siderophores. *Bacillus* species are known to produce siderophores of which at least two have been identified, namely 2,3-dihydroxybenzoate (Rowland & Taber, 1996; Bsat & Helmann, 1999) and itoic acid (Ito, 1993). Lindow & Wilson (1999) described siderophores that may play a dual role in antagonism, both competing for nutrients and inducing resistance in the host. However, the involvement of siderophores in *in vivo* antagonism against fungal postharvest avocado pathogens has yet to be proven.

This study shed some light on the different types of modes of action that may be involved in the control of fungal avocado postharvest pathogens by *B. subtilis*. Results indicate that antibiosis is involved through the production of inhibitory volatile substances and soluble cell-free filtrates, which may contain antibiotics, enzymes or siderophores. The direct interaction studies using SEM also indicates that competitive colonization is a potential mode of action. Further studies should focus on identifying the siderophore, volatile substance or antifungal component involved in antagonism since there is evidence that more than one of these substances may be involved. Determining which nutrients and temperatures could enhance antagonism will also be useful when designing better formulations and applications for the commercial biocontrol product.

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