

Mode of Action of *Bacillus subtilis* for Control of Avocado Post-harvest Pathogens

L. Korsten and E.E. De Jager

Department of Microbiology and Plant Pathology, University of Pretoria, Pretoria, 0002, RSA

ABSTRACT

Bacillus subtilis (isolate B246), *Bacillus cereus* (isolate B247 and B249) and *Bacillus licheniformis* (isolate B248) were inhibitory to avocado post-harvest pathogens *Colletotrichum gloeosporioides*, *Phomopsis perseae*, *Drechslera setariae*, *Pestalotiopsis versicolor* and *Fusarium solani*; when tested with the dual culture technique. *In vitro* growth of *Thyronectria pseudotrichia* was not suppressed by any of the antagonists, while *B. cereus* isolate B247 did not inhibit growth of *Dothiorella aromática*. *B. Cereus* isolate B249 actually stimulated development of *Lasiodiplodia theobromae*. Diffusable metabolites from the four antagonists impeded growth of *C. gloeosporioides*, *D. aromática*, *P. perseae*, *L. theobromae*, but not of *T. pseudotrichia*. *B. subtilis* (B246) mode of action was determined using an *in vitro* spore-germination assay. *In vitro* the antagonist effectively reduced percentage spore germination of *C. gloeosporioides*, *D. aromática*, *P. perseae*, *L. theobromae*, *F. solani*, *P. versicolor* and *D. setariae*. In addition, bulb formation and lysis of hyphae were observed, as well as bacterial movement towards germinating spores and subsequent attachment to the spores. The possible mode of action of *B. subtilis* is discussed.

INTRODUCTION

Avocado (*Persea Americana* Mill.) is susceptible to various fruit diseases, including black spot, anthracnose, *Dothiorella/Colletotrichum* fruit rot complex (DCC), stem-end rot (SE), and, to a lesser extent, sooty blotch and sooty mould (Darvas, 1982; Blakeman, 1985; Smith *et al*, 1985). At present, limited control of these diseases in South Africa is primarily achieved by means of preharvest sprays with fungicides like copper oxychloride and benomyl (Lonsdale & Kotzé, 1989; Lonsdale, 1991). However, fungicide sprays tend to produce visible residues on fruit surfaces, which have to be removed manually in the packinghouse (Dermer & Kotzé, 1986). Furthermore, prolonged use of fungicides can lead to a build-up of resistance in pathogens (Kotzé *et al.*, 1982). Add to this the growing public awareness around pesticide pollution, and the need for alternative control measures such as biological control becomes obvious.

Considering the exceptional performance of *Bacillus subtilis* (Ehrenberg) Cohn (isolate B246) against avocado fruit diseases (Korsten, 1993), it is inevitable that interest in commercialisation of this product will follow. However, in order to commercialise and register a biocontrol agent, detailed information is needed on its mode of action

(McLaughlin *et al.*, 1990). During the initial *in vitro* screening of bacterial isolates against avocado post-harvest pathogens (Korsten, 1993), antibiosis was indicated as a possible mechanism of action in view of the formation of prominent inhibition zones. Thus far, a broad spectrum of antifungal compounds produced by *Bacillus* spp. have been identified and described (Pusey, 1990) and mode of action of the well known antagonist *B. subtilis* has been attributed to antibiosis (Nandi & Sen, 1985; Vaseduva & Chakravarti, 1954; Swinburne *et al.*, 1975; McKeen *et al.*, 1986; Gueldner *et al.*, 1988). However, in nature more than one type of interaction can occur between microbial populations, depending on microbial density (Atlas & Bartha, 1987).

This paper provides evidence on *in vitro* inhibition of pathogen growth and spore germination by *B. subtilis* (isolate B246), *Bacillus cereus* Frankland and Frankland (isolates B247 and B249) and *Bacillus licheniformis* (isolate B248) using the dual culture technique, and on production of diffusible metabolites by the antagonists.

MATERIALS AND METHODS

Antagonists

B. subtilis (B246), *B. cereus* (B247 and B249) and *B. licheniformis* (B248) previously isolated from avocado leaf and fruit surfaces, and with *in vitro* inhibitory action against avocado post-harvest pathogens (Korsten, 1993), were cultured and maintained on nutrient agar (NA) (Biolab) slants for 24 h at 28°C.

***In vitro* screening of bacterial epiphytes for antagonism**

All bacteria isolated from the avocado phyllo- and fructoplane were initially screened *in vitro* for antagonism against one of the most important avocado postharvest pathogens, viz. *Dothiorella aromática* (Sacc.) Petrak & Sydow (Korsten, 1993), using the dual culture technique described by Fokkema (1987). The three most promising antagonists (B246, B248 and B249) were evaluated further in this study as well as isolate B247 for comparative purposes. Avocado post-harvest pathogens *Colletotrichum gloeosporioides* Penzig, *D. aromática*, *Thyronectria pseudotrichia* (Schw.) Seeler, *Lasiodiplodia theobromae* (Pat.) Griffon & Maubl., *Phomopsis perseae* Zerova, *Drechslera setariae* (Sawada) Subram. & Jain., *Pestalotiopsis versicolor* (Speg.) Steyart and *Fusarium solani* (Mart.) Appel & Wr. emend. Snyd. & Hans. (Darvas & Kotzé, 1987) were freshly isolated, identified, and their pathogenicity confirmed (Darvas *et al.*, 1987) before being used in the *in vitro* antagonism test. Fungal isolates described above were cultivated on potato-dextrose agar (PDA) (Biolab). Discs (5 mm diameter) were aseptically punched with sterile corkborer (No. 5) from the periphery of actively-growing fungal cultures and placed off-centre on nutrient agar (NA) (Biolab) plates. After three days incubation at 25°C, plates were streak-inoculated with a bacterial isolate 45 mm from the fungal disc. Plates not inoculated with bacteria served as controls. Triplicates were used throughout for each pathogen-antagonist combination. Percentage growth inhibition was determined after 7 and 14 days incubation at 25 °C by the formula of Skidmore (1976):

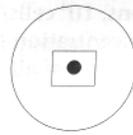
$$\frac{K_r - r_1}{K_r} \times 100 = \% \text{ GI}$$

Where K_r represents the radius of the control pathogens growth, and r_1 the radius of the pathogen's growth towards the bacterial antagonist.

Effect of diffusable metabolites from *Bacillus* spp.

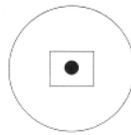
An indirect agar plate technique essentially similar to the one described by Skidmore and Dickinson (1976) was used to detect the production of diffusible metabolites by the antagonist inhibiting growth of *C. gloeosporioides*, *D. aromática*, *T. pseudotruchia*, *P. perseas* and *L. theobromae*. Agar wells (5 mm diam.) extending to the bottom of the plate were made in the centre of 25 ml NA plates using a sterile corkborer. Bacterial antagonists B246, B247, B248 and B249 were grown individually in test tubes containing 10 ml standard 1 nutrient broth (STD) (Biolab) for 24 h at 30°C. One hundred microlitres of each antagonist culture suspension were pipetted into each of 15 agar wells. After 24 h incubation at 25°C, each well was covered with a 5 cm square sterile dialysis membrane (MW 12000-14000). Discs (5 mm diam.) were aseptically punched from the periphery of actively-growing fungal cultures, and three discs from each species were placed on top of a membrane in each of three plates. Control plates received 100 µl sterile STD per well instead of antagonist suspensions. Plates were incubated for a further 14 days at 25°C, and growth patterns (GP) were scored after 7 and 14 days as described in Figure 1. Triplicates were used throughout. Percentage fungal growth (G) was determined using the formula $r_1/K_1 \times 100 = G$, where K_r represents the control fungal growth radius on dialysis membrane, over antagonist in agar well and r_1 represents the fungal growth radius, as determined after 14 days incubation.

A. NO GROWTH ON DIALYSIS MEMBRANE



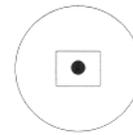
Score = 0

No mycelial growth or sporulation



Score = 1

Mycelial growth



Score = 2

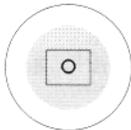
Sporulation

B. GROWTH ON DIALYSIS MEMBRANE



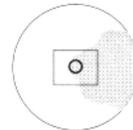
Score = 3

Mycelial growth only on dialysis membrane



Score = 4

Mycelial growth covering entire dialysis membrane



Score = 5

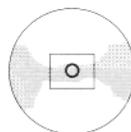
Mycelial growth on a section of the dialysis membrane and medium

C. GROWTH ON MEDIUM ONLY



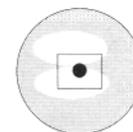
Score = 6

Mycelial growth on edge of medium



Score = 7

Mycelial growth in two directions



Score = 8

No mycelial growth on dialysis membrane, but on surrounding medium

Score = 9
Fungi overgrow media

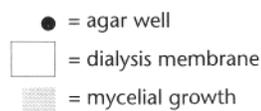


Figure 1

Nine possible growth patterns (GP) on media exposed to metabolites of antagonists using an indirect technique essentially similar to Skidmore & Dickinson (1976)

Data was subjected to analysis of variance and differences in antagonism compared using Duncan's multiple range test. Statistical analysis of only the 14 day data is presented. Marginal means for combined antagonists or pathogens are included.

***In vitro* spore germination evaluation**

B. subtilis (B246), the most promising antagonist, was cultured in a 250 ml Erlenmeyer flask containing 200 ml STD. After 48 h shake-incubation (Rotary shaker 67 r.p.m.) at 28°C, cells were harvested by centrifugation for 20 min at 16 081 G in a Sorval

superspeed refrigerated centrifuge with a GSA rotor. The pellet was dissolved in 20 ml sterile quarter-strength Ringer's (Merck) and cell concentration was determined with a Petroff-Hausser counting chamber before a dilution series was made in Ringer's to obtain a concentration range of 10^8 , 10^7 , 10^6 and 10^5 cells/ml. Sterile ELISA 96 well (Nunc) plates were used in this checkerboardtype assay. Fifty microlitres of each *B. subtilis* cell concentration solution was placed in each well in a vertical row, starting with the highest concentration in the left row, and the lowest concentration in the fourth row. The last row on the right was used as control, receiving only Ringer's.

Post-harvest pathogens *C. gloeosporioides*, *D. aromatica*, *T. pseudotruchia*, *P. perseae*, *F. solani* and *P. versicolor* were incubated on PDA plates for 14 days at room temperature (22-28°C) under near-UV light. Spores were harvested in sterile quarter-strength Ringer's solution, counted in a haemocytometer and diluted to obtain a concentration range of 10^6 , 10^5 , 10^4 and 10^3 spores/ml. Each fungal spore suspension was placed in each well in a horizontal row, starting with the highest concentration at top, and the lowest concentration in the fourth row. Three replicate plates for each pathogen-antagonist combination was used. Plates were incubated at 28°C for 12 h. Percentage spore germination was determined within 6 h by dividing the number of germinating spores by total number of spores present per well as observed under a Nikon inverted microscope (IM). Three counts per microscopic field (40 x magnification) were made per well. In addition, effect of antagonists on germination and growth was also recorded under the IM. Variance of analysis was used to separate mean percentages and Duncan's multiple range test was applied to test for variables. Least significant (LS) means were compared to determine disease threshold levels at a 50 % germination cut-off point.

RESULTS

***In vitro* screening of bacterial epiphytes for antagonism.**

All four antagonists (B246, B247, B248 and B249) inhibited growth of *C. gloeosporioides*, *P. perseae*, *D. setariae*, *P. versicolor* and *F. solani* when tested with the dual culture technique (Table 1). *In vitro* growth of *T. pseudotruchia* was not suppressed by any of the antagonists, while *B. cereus* isolate B247 was ineffective against *D. aromatica* (Table 1). *B. cereus* isolate B249 had a stimulatory effect on *L. theobromae*. The four antagonists were equally effective in inhibiting *C. gloeosporioides* and *P. versicolor* (Table 1). However, isolate B246 performed best against *D. aromatica* and *F. solani*.

Table 1
Effect of selected bacterial antagonists on *in vitro* growth inhibition of avocado fruit pathogens

<i>Pathogen</i>	Bacillus subtilis B246	Bacillus cereus B247	Bacillus licheniformis B248	Bacillus cereus B249	<i>F value</i> <i>Pr > F</i>
<i>Colletotrichum gloeosporioides</i>	26.9 a	30.8 a	6.4 a	12.8 a	0.4558
<i>Dothiorella aromatica</i>	42.2 a	0.0 c	9.5 b	09.5 b	0.0001
<i>Thyronectria pseudotrichia</i>	0.0 a	0.0 a	0.0 a	0.0 a	0
<i>Lasiodiplodia theobromae</i>	3.6 ab	3.6 ab	11.9 a	-14.3 b	0.1359
<i>Phomopsis perseae</i>	31.3 a	18.1 b	15.7 b	24.1 ab	0.0677
<i>Drechslera setariae</i>	48.1 a	39.7 b	44.2 ab	39.1 b	0.0001
<i>Pestalotiopsis versicolor</i>	23.9 a	29.6 a	25.4 a	21.1 a	0.0011
<i>Fusarium solani</i>	67.7 a	38.3 b	39.4 b	37.1 b	0.0001

Values represent percentage growth inhibited (GI) using the modified formula of Skidmore (1976): $K_r - r_i / K_r \times 100 = \% \text{ GI}$, where K_r represent the radius of the control pathogen's growth, and r_i , the radius of the pathogens growth towards the test bacterial antagonist, after 14 days incubation.

Means within columns followed by the same letter do not differ significantly ($p \leq 0,05$) according to Duncan's multiple range test.

Effect of diffusable metabolites from *Bacillus* spp.

The four isolates were equally effective in inhibiting *in vitro* growth of *C.gloeosporioides* (Table 2). *B. subtilis* was the most effective antagonist against *D. aromatica*, whereas *B. cereus*. (B247) had no inhibitory effect on this pathogen. Diffusable metabolites from *B. cereus* (B249) resulted in a stimulation of *L. theobromae* (Table 2). *P. perseae* was most effectively inhibited by *B. subtilis*, although not significantly better than by *B. cereus* (B249). *C. gloeosporioides*, *D. aromatica* and *P. perseae* were the most sensitive to inhibition when marginal means of the pathogens were compared (Table 2). Antagonist marginal means indicate that *B. subtilis* was the most effective in inhibiting growth of avocado post-harvest pathogens by means of a diffusable metabolite, albeit not significantly better than *B. licheniformis* and *B. cereus* (B247). *T. pseudotrichia* was not affected by any of the antagonists. *B. cereus* (B247 and B249) and *B. licheniformis* did not sustain their inhibitory effect on *D. aromatica* by day 14, while *B. subtilis* limited fungal growth to the dialysis membrane.

Table 2

Evaluation of the inhibitory effect of diffusible metabolites from selected bacterial antagonists on the growth of the four most important avocado post-harvest pathogens

Bacterial antagonist	Isolate	Growth pattern (GP) ^w and percentage pathogens growth (G) ^x										Marginal mean ^y
		Colletotrichum gloeosporioides		Dothiorela aromatica		Thyronectria pseudotrichia		Phomopsis perseae		Lasiodiplodia theobromae		
		GP ^w	G ^x	GP ^w	G ^x	GP ^w	G ^x	GP ^w	G ^x	GP ^w	G ^x	
<i>Bacillus subtilis</i>	B246	3	73 a	3	55 c	9	100 a	3	69 b	4	96 ab	79 b
<i>Bacillus cereus</i>	B247	5	69 a	9	100 a	9	100 a	4	82 a	5	96 ab	84 ab
<i>Bacillus licheniformis</i>	B248	4	94 a	9	91 b	9	100 a	4	84 a	4	88 b	87 ab
<i>Bacillus subtilis</i>	B249	5	87 a	9	91 b	9	100 a	3	76 ab	4	114 a	93 a
Pr > F			0,4558		0,0001		1,000		0,0677		0,1359	0,0618
Marginal mean ^z			77 c		80 c		100 a		77 c		91 b	

^w Growth pattern represent type of inhibition as indicated in Fig. 1: 0–2 = no inhibition; 3–5 = growth on dialysis membrane; 6–8 = growth of medium only; 9 = fungal growth overgrows media as determined after 14 days incubation.

^x Represents percentage pathogen growth (G) calculated from the formula: $r_i/K_i \times 100$, where K_i represents fungal growth radius on dialysis membrane over antagonist in agar well and r_i represents the control fungal growth radius, as determined after 14 days incubation. Method essentially similar to Skidmore & Dickinson (1976).

^y Mean percentage growth of all pathogens as affected by each antagonist (horizontal rows).

^z Mean percentage growth of all pathogens as affected by each antagonist (vertical columns).

Means within columns followed by the same letter do not differ significantly ($p \leq 0,05$) according to Duncan's multiple range test.

***In vitro* spore germination evaluation**

At all concentrations of *C. gloeosporioides*, *D. aromatica* and *P. versicolor*, spore germination was significantly less than in the control when challenged by any of the different antagonist concentrations (Table 3). The 10^3 spore concentration of *P. perseae* and *F. solani*, as well as the 10^4 concentration of the latter pathogen and of *T. pseudotrichia*, were inhibited only by the 10^8 antagonist concentration. Spore germination of *T. pseudotrichia* at 10^3 was not reduced by antagonist concentrations 10^7 and 10^6 (Table 3). Comparing the marginal means of the various antagonist concentrations, 10^8 cells/ml was the most effective concentration for inhibiting spore germination of all pathogens tested, except for *C. gloeosporioides* and *D. aromática*, where the 10^7 cells/ml was equally effective. At high spore concentrations self-inhibition of germination was evident for all fungi tested (Table 3).

Table 3
Effect of *B. subtilis* on spore germination of avocado post-harvest pathogens

Pathogen	Pathogen concentration (spores/ml)	Percentage spore germination ^x					Pr > F	Marginal mean ^y
		B. subtilis concentration (cells/ml)						
		10 ⁸	10 ⁷	10 ⁶	10 ⁵	0 (control)		
<i>Colletotrichum gloeosporioides</i>	10 ⁶	0,03 cd B	0,07 b B	0,10 c B	0,14 b B	38,2 b A	0,0001	9,6 c
	10 ⁵	0,07 bc C	0,10 b C	0,13 c C	20,0 b B	90,7 a A	0,0001	22,8 bc
	10 ⁴	0,18 ab D	0,21 b D	27,0 b C	63,3 a B	96,4 a A	0,0001	31,0 ab
	10 ³	0,29 a C	13,2 a C	78,7 a AB	73,4 a B	91,5 a A	0,0001	45,9 a
	Pr > F	0,0005	0,0043	0,0001	0,0001	0,0001		0,0009
	Marginal mean ^y	0,14C	3,4C	26,5B	39,2B	79,2A	0,0001	
<i>Thyronectria pseudotrichia</i>	10 ⁶	0,1 b B	0,1 c B	0,2 c B	0,2 c B	32,7 b A	0,0001	8,3 c
	10 ⁵	0,3 b D	16,1 b CD	51,9 b B	24,2 b C	83,6 a A	0,0001	88,0 b
	10 ⁴	38,6 a B	83,7 a A	73,1 a A	70,5 a A	77,9 a A	0,0001	68,4 a
	10 ³	39,8 a B	83,3 a A	74,3 a A	53,1 a B	85,0 a A	0,0001	70,6 a
	Pr > F	0,0001	0,0001	0,0001	0,0001	0,0001		0,0001
	Marginal mean ^y	19,7C	45,8B	49,9B	37,0B	69,8A	0,0001	
<i>Dothiorella aromatica</i>	10 ⁶	0,4 c B	0,4 c B	0,1 b B	0,3 b B	26,4 c A	0,0001	6,8 b
	10 ⁵	3,0 ab B	6,2 c B	5,9 b B	3,0 b B	83,6 b A	0,0001	24,7 a
	10 ⁴	3,1 a D	20,9 a C	27,9 a BC	37,6 a B	82,0 ab A	0,0001	33,2 a
	10 ³	1,6 bc D	12,1 b CD	27,7 a BC	35,3 a B	91,5 a A	0,0001	33,5 a
	Pr > F	0,0001	0,0001	0,0001	0,0001	0,0001		0,0007
	Marginal mean ^y	2,0 C	9,9 BC	15,4 B	19,1B	70,9 A	9,0001	
<i>Pestalotiopsis versicolor</i>	10 ⁶	0,6 b B	0,7 c B	0,4 c B	0,9 c B	24,5 c A	0,0001	6,5 d
	10 ⁵	1,4 b C	7,9 c BC	2,4 c C	14,4 c B	80,4 b A	0,0001	23,0 c
	10 ⁴	6,3 b C	37,6 b B	46,0 b B	41,6 b B	90,8 a A	0,0001	45,2 b
	10 ³	23,8 a C	65,8 a B	73,5 a AB	63,5 a B	89,2 ab A	0,0001	63,1 a
	Pr > F	0,0001	0,0001	0,0001	0,0001	0,0001		0,0001
	Marginal mean ^y	8,0 C	28,0 B	30,6 B	30,1 B	71,2 A	0,0001	
<i>Fusarium solani</i>	10 ⁶	0,09 b B	0,14 c B	0,13 b B	0,20 c B	14,1 c A	0,0001	3,6 c
	10 ⁵	0,14 b C	54,5 b B	78,9 a A	55,6 b B	79,2 b A	0,0001	53,2 b
	10 ⁴	12,8 a B	82,3 a A	86,0 a A	93,3 a A	95,8 a A	0,0001	69,2 a
	10 ³	18,9 a B	81,2 a A	83,3 a A	85,1 a A	88,7 a A	0,0001	68,0 a
	Pr > F	0,0013	0,0001	0,0001	0,0001	0,0001		0,0001
	Marginal mean ^y	8,0 B	54,6 A	62,1 A	58,5 A	69,5 A		
<i>Phomopsis perseae</i>	10 ⁶	0,0 b B	0,03 d B	0,03 d B	0,03 c B	20,0 c A	0,0001	5,0 b
	10 ⁵	0,03 ab E	15,0 c D	22,4 c C	57,0 b B	76,4 b A	0,0001	28,5 b
	10 ⁴	0,03 ab C	76,6 b B	78,3 b B	83,1 a B	92,6 a A	0,0001	61,9 a
	10 ³	0,1 a B	91,0 a A	89,4 a A	83,1 a A	92,6 a A	0,0001	68,3 a
	Pr > F	0,0855	0,0001	0,0001	0,0001	0,0001		0,0001
	Marginal mean ^y	0,40 B	45,7 A	47,6 A	55,8 A	70,4 A	0,0001	

^xMean percentage spore germination at each pathogen concentration as affected by the mean antagonist concentration.

^yMean percentage spore germination of each pathogen as affected by each antagonist concentration.

Means within rows followed by the same capital letter, as well as means in horizontal columns followed by the same small letter do not differ significantly according to Duncan's multiple range test ($p \leq 0,05$).

In the presence of the antagonist, spores of *P. versicolor* swelled abnormally before germination, with subsequent bulb formation. Bulb formation was also observed in all pathogen-antagonist combinations evaluated. This appeared to be associated with release of cell contents, as was the case with *F. solani* (Figure 2). Abnormal mycelial growth with swellings after germination occurred in *D. aromatica* (Figure 3) and *C. gloeosporioides* (Figure 4). Bacteria were often observed moving rapidly towards germinating spores and attaching polarly to the surface (Figure 5).

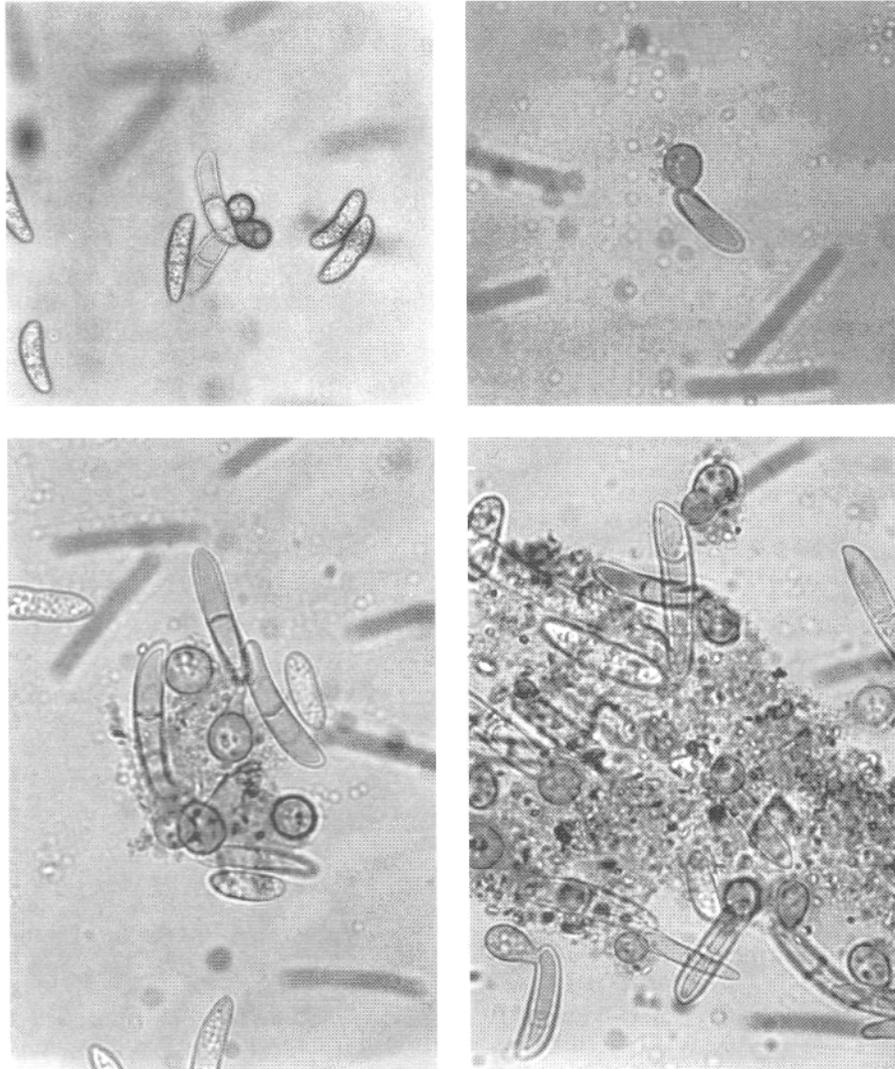


Figure 2
In vitro interaction between *Bacillus subtilis* (B246) and *Fusarium solani* showing bulb formation and release of cell contents (400 × mag.)

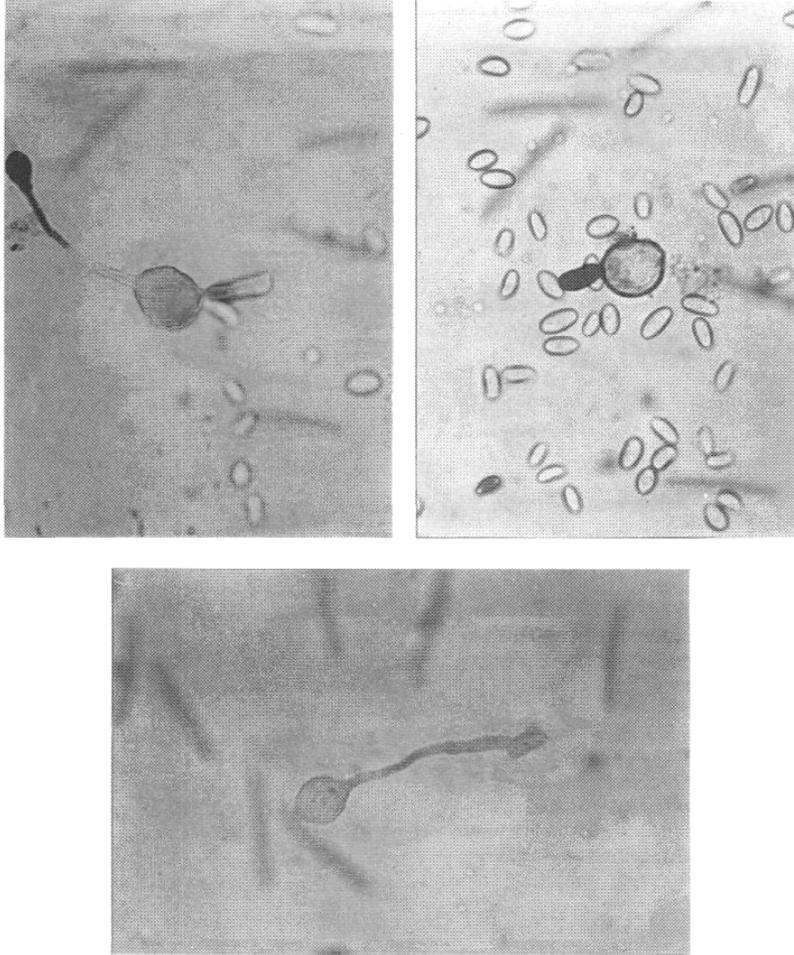
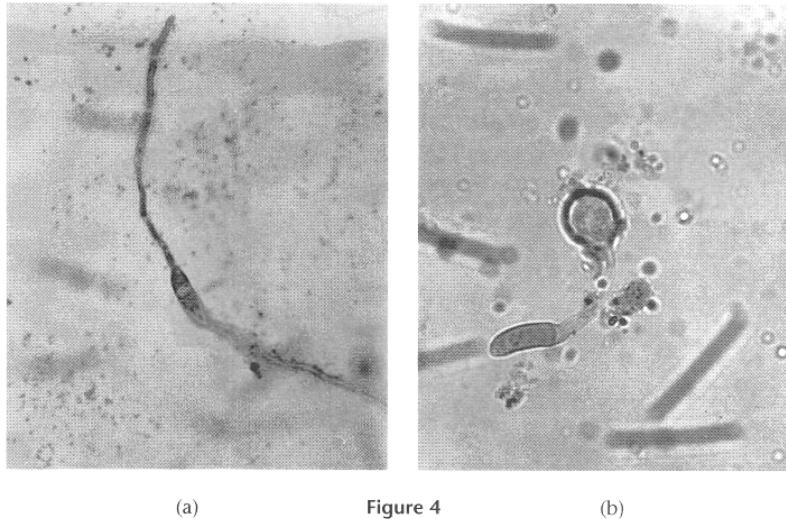


Figure 3

In vitro interaction between *Bacillus subtilis* (B246) and *Dothiorella aromatica* showing germination in the presence of the antagonist with subsequent "bulb" formation (400 × mag.)



(a) **Figure 4** (b)
In vitro interaction between *Bacillus subtilis* (B246) and *Colletotrichum gloeosporioides* showing (a) control germination of *C. gloeosporioides* in ELISA plates and (b) germination in the presence of the antagonist (400 × mag.)

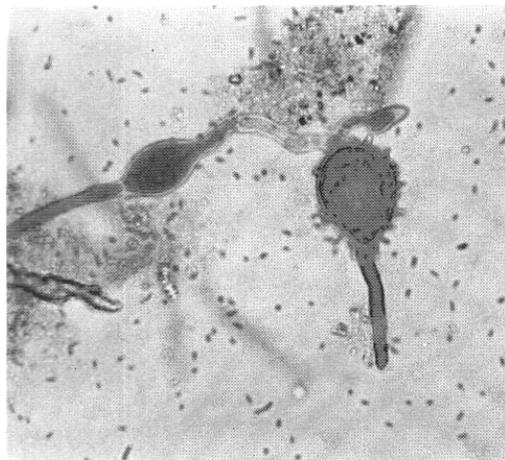


Figure 5
In vitro interaction showing *Bacillus subtilis* (B246) being attracted to the germinating fungal spore and subsequent polar attachment of the bacteria to the fungal surface (400 × mag.)

DISCUSSION

Knowledge of the mechanism of antagonism may be used to improve biocontrol (Spurr, 1981). In addition, an antagonist may operate according to more than one mode of action. Exploiting all modes of action will increase efficacy of the biocontrol agent. Five basic mechanisms were postulated by Blakeman & Brodie (1976). These include: direct parasitism, the production of extracellular antibiotics or other substances, competition on the host and stimulation of host defences.

The production of antibiotics or other inhibitory substances by *B. cereus*, *B. licheniformis* and *B. subtilis* against several avocado post-harvest pathogens was demonstrated in

our study using the dual culture technique as well as using the indirect agar-plate method. However, *T. pseudotrichia* was not inhibited as effectively by diffusible metabolites of the antagonists as were the other pathogens. Nevertheless, *B. subtilis* inhibited spore germination of all pathogens tested. Upadhyay and Jayaswal (1992) reported a similar inhibitory effect on spore germination due to the presence of *Pseudomonas espacia*. Effective inhibition of germination in minimal medium indicates competition for nutrients (Droby *et al.*, 1990). Furthermore, since antibiotics are mainly produced in the presence of excess substrate concentrations (Atlas & Bartha, 1987), the inhibitory effect in the minimal medium used in our study suggests nutrient competition as mode of action.

Stimulation of appressorium formation by *C. gloeosporioides* and *D. aromática* in response to antagonists is in agreement with various workers who described promotion of appressorium development in the presence of high populations of bacteria (Lenné & Parbery, 1976; Blakeman & Brodie, 1977; Blakeman & Parbery, 1977; Parbery, 1985). It has been suggested that the main interaction in such situations is competition for nutrients, which accelerates the starvation effect and therefore causes nutrient stress in the germinating fungus (Blakeman & Parbery, 1977). However, according to Lenné and Parbery (1976) there is a possibility that lytic enzymes from bacteria may influence the process. Lysis of fungal protoplasts as observed *in vitro*, will contribute significantly to the inactivation of the pathogen (Austin *et al.*, 1977). Under such circumstances lysis may reflect permeability changes in the protoplasmic membrane caused by the action of antibiotics, enzymes or metabolites (Austin *et al.*, 1977). Plasmolysis and alternating areas of high and low staining intensities (Barring) within the protoplast described by Austin *et al.* (1977), was also evident.

In this study it was shown that *in vitro* results were complementary to *in vivo* effects described by Korsten (1993). It thus seems reasonable to assume that more than one mode of action is involved in the inhibition of post-harvest pathogens by *B. subtilis*. This most probably include production of inhibitory substances and competition for nutrients. In a previous study, Korsten (1993) showed that pre-emptive colonisation could also play a role. Smaller lesions developed on avocado fruit in *in vivo* studies when the antagonist was applied several days before the pathogen was introduced, compared to when it was introduced simultaneously. Korsten (1993) also reported more effective control of cercospora spot on avocado with early pre-harvest field sprays than with later seasonal sprays. A hypothetical model was subsequently developed to set out the possible modes of action which could play a role in the control of disease when antagonists are applied pre- or post-harvestly (Figure 6). Current research is aimed at providing efficient evidence for verifying the hypothetical model and to identify and characterise the inhibitory substances associated with the antagonists.

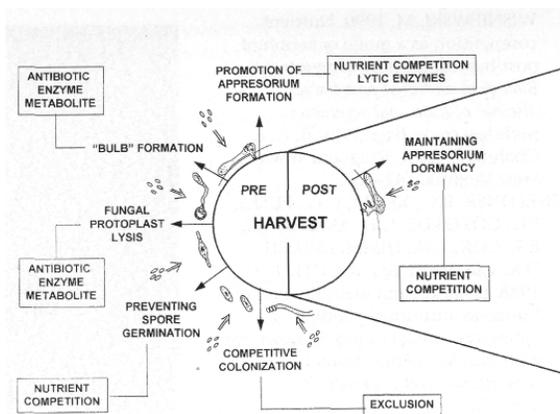


Figure 6
Hypothetical model showing possible modes of action of *Bacillus subtilis* for control of avocado post-harvest pathogen *Colletotrichum gloeosporioides*.

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