South African Avocado Growers' Association Yearbook 1999. 22:12-20

Factors affecting biocontrol efficacy of *Bacillus subtilis* against *Colletotrichum gloeosporioides*

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ABSTRACT

Biological control programmes aimed at controlling anthracnose caused by *Colletotrichum gloeosporioides* depend on either establishing the antagonist preharvestly to prevent infection or post-harvestly to inhibit symptom development. *Bacillus subtilis*, effective in controlling avocado pre- and post-harvest anthracnose, was formulated for commercial use and evaluated in commercial packinghouses. The formulated product resulted in insignificant control of anthracnose. The impact selective nutrition has on antagonistic activity and the subsequent direct pathogen-antagonist interactions on fruit surfaces were therefore studied. Antagonistic activity was significantly influenced by nutrition. Citric acid, D (+)galactose, pyruvate and benzoate as sole carbon sources and L(+)asparagine and L-aspartic acid as sole nitrogen sources were crucial in sustaining antagonism of *B. subtilis*. Through scanning electron microscopy studies it was observed that *B. subtilis* may operate by a combination of different modes of action, as previously speculated. These interactions include preventative action by using either competitive exclusion or pre-emptive colonization and its curative action by the production of anti-fungal substances. These interactions are also directly influenced by selective nutrition. Results may facilitate methods in improving the existing formulation for effective biological control of post-harvest avocado diseases.

INTRODUCTION

Avocado (*Persea americana* Mill.) is susceptible to various fruit diseases with anthracnose regarded as one of the most important in South Africa (Darvas and Kotzé, 1987). *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc., the causal agent of anthracnose in avocado, is a semi-biotrophic pathogen (Bailey *et al.,* 1992) capable of epiphytic growth phases on aerial surfaces (Korsten, 1993), often before latent fruit infections are established. Control is therefore mainly focused on reducing the pre-harvest inoculum potential with chemical sprays and delaying post-harvest anthracnose development on marketed fruit using post-harvest chemicals (Lonsdale and Kotzé, 1989). Recently, the build-up of resistance in pathogens due to prolonged use of fungicides (Kotzé et al, 1982; Darvas and Kotzé, 1987) and the international concern over fruit safety and the continued use of fungicides (Lichtenberg and Zilberman, 1987),

drives the need for alternative disease control strategies such as the use of beneficiary microorganisms.

The potential of microbial antagonism to control avocado post-harvest diseases was initially demonstrated by an avocado phylloplane isolate of *Bacillus subtilis* (ATCC55466/B246) (Korsten etf al, 1988; Korsten, 1993). Its exceptional performance as preharvest treatment (Korsten, 1993) to control post-harvest anthracnose, and its efficacy as post-harvest antagonist was further confirmed on semi commercial scale (Korsten et al., 1989; 1991; 1993; 1995; Van Dyk et al., 1997). For commercial use, the antagonist was formulated in powder form (Avogreen® [Stimuplant, Pretoria, South Africa]) to ensure recommended viable cell counts with extended shelf life (Towsen et al., 1998). However, commercial trials at six avocado packinghouses resulted in insufficient control, with more severe external anthracnose observed on Avogreen® treated fruit than control fruit (Towsen et al., 1 998). This could be ascribed to the product formulation's direct effect on the pathogen-antagonist interactions as it either stimulated the pathogen by serving as a nutrient base or inactivated the antagonist's mode of action (Towsen *et a*l., 1998).

It was suggested by Korsten and De Jager (1995) that several modes of action may be involved in the biocontrol activity of *B. subtilis;* including antibiosis, competitive exclusion and nutrient competition. This paper reports on nutritional factors influencing the antagonistic performance of *B. subtilis* to control C. *gloeosporioides,* the pathogens nutrient requirements and provide evidence on the direct curative and preventative action of the antagonist.

MATERIALS AND METHODS

Antagonist: *B. subtilis* (ATCC55466/ B246), previously shown to be effective against avocado post-harvest pathogens was used throughout this study (Korsten, 1 993; Korsten *et al.,* 1988; 1989; 1991; 1993; 1995; Van Dyk et al., 1997).

Pathogen: *C. gloeosporioides,* isolated from symptomatic avocado fruit, was supplied by G.M. Sanders, and identity positively confirmed by F.C. Wehner (Department of Microbiology and Plant Pathology, University of Pretoria, Pretoria). Isolates were maintained on potato-dextrose agar (PDA) (Biolab) slants and in water.

Determining nutrient requirements for effective antagonistic interactions*:*

The dual culture technique (Skidmore, 1976) was used to determine the ability of *B.* subtilis to inhibit mycelia growth of *C. gloeosporioides* as affected by different carbon and nitrogen sources.

A minimum salt-based medium with the following composition: 3.49g $Na_2HPO_4 \cdot 2H_20$; 2.77g KH_2PO_4; 20ml of Hutner's vitamin-free mineral base supplemented with 0.01 mg D(+) biotin; 1.0 mg nicotinic acid; and 0.5 mg of thiamine hydrochloride (Janisiewicz *et al.*, 1992); 15g Bacto agar (Biolab); and 1000ml double distilled water was used for each test. The pH of the mediums was adjusted with NaOH or HCI to 6.8 before autoclaving.

For the carbon source assay the minimal salt-based medium was supplemented with one of the following filter sterilized carbon sources at final concentration of 1 %w/v: D(-) arabinose; L(-) arabinose; acetate; benzoate; cellulose; D(+)cellobiose; citric acid; citrate; D(-)fructose; D(+)galactose; D-gluconic acid; D(+)glucose; glycerol; myo-inositol; lactose; D(-) lyxose; L(-)lyxose; L(-) malic acid; D(+)maltose; D(-) mannitol; D(+) mannose; pectin; peptone; pyruvate; D(-)rhamnose; L(-)rhamnose; D(-)ribose; D(-)sorbitol; L(-)sorbitol; starch; sucrose; D(+) trehalose; D(+)xylose and L(+)xylose. NH_4Cl was added at 0.1 % w/v as nitrogen source.

To determine the effect of amino acids, the minimal salt-based medium was supplemented with 2g/ℓ of the following filter sterilized amino acids: L-alanine; L-arginine; L(+) asparagine; Laspartic acid; L-cystein; L-glutamine; L-glutamic acid; glycine; L-histidine; L-isoleucine; L-leucine; L-lysine; L-methionine; L-phenylalanine; L-proline; L-serine; L-threonine; DL-thryptophan; L-thyrosine and L-valine. D(+)glucose was added at 1% w/v as carbon source. Merck supplied all chemicals.

B. subtilis was respectively streak inoculated at fixed positions 35mm from the center of a 90mm petri dish containing different media. Inoculated plates were incubated at 25°C for 24h. A 5mm-diameter agar disc was cut from the edge of an actively growing culture of *C. gloeosporioides* using a sterile cork borer. The discs were placed in the center of the petri dish containing the different media with inoculated antagonistic cultures. Control plates were inoculated with *C. gloeosporioides* only. Growth of *C. gloeosporioides* was measured after seven days.

The following formula was used to calculate *inhibition zone: R2-R1. Where RI is the farthest radial distance growth by* C. gloeosporioides in the direction of *B. subtilis* and R2 is the farthest radial distance growth of the antagonist. To determine the nutrient requirement of the *C. gloeosporioides*, diameters of fungal colonies were measured after seven days.

There were three replicates per treatment and data was statistically analyzed by varians of analysis using Duncan's multiple range test (p=0.05) to determine significant differences between inhibition zones and fungal colonies on growth media.

Examination of direct interaction between *B. subtilis* and *C. gloeosporioides* on avocado fruit.

Direct curative and preventative interactions between *B. subtilis and C. gloeosporioides* was investigated using scanning electron microscopy (SEM).

The antagonist, *B. subtilis,* was cultured in 100-mℓ Erlenmeyer flasks containing 50 *mℓ* of nutrient broth (NB) (Biolab). After 24-h shake incubation (rotary shaker, 70rpm) at 28°C, cells were harvested by centrifugation for 60 sec at 12500 g. The resultant pellet was dissolved in 1 mℓ of sterile quarter-strength Ringer's solution (commercial isotonic diluent, Merck), cell concentrations were determined with a Petroff Hausser counting chamber, and a dilution series was made.

Avocado pathogen, *C. gloeosporioides*, was cultured on PDA for 14 days at 25°C under a near UV-light source. Spores were harvested in Ringer's, counted with a hemacytometer, and diluted in 10 fold increments.

Mature Fuerte fruit, at ready-to-eat stage, were used to examine direct pathogen-antagonist interactions. Fruit were sprayed with 70% ethanol until run-off and wiped with sterile paper cloth to dry. Squares ($1cm^{-2}$) were then drawn with a black waterproof pen on one side of each fruit to give three squares per fruit.

The following procedure was followed to investigate the antagonist's curative interactions: Squares were inoculated with *50*µℓ of the *C. gloeosporioides* suspensions at 10^4 conidia $m\ell^{-1}$. Fruit was left to air dry at room temperature before being challenged with the antagonist. At different time intervals, viz. immediately after, 4, 12 and 48h after fruit has dried, squares were inoculated with *50*µℓ of the antagonist suspension at 10^7cells $m\ell^{-1}$. Fruit inoculated with *50*µℓ Ringer's represented pathogen controls.

Inoculated fruit were randomly packed in cardboard boxes lined with absorbent paper and moistened cotton wool to obtain humidity. Boxes were stored at 25°C and samples taken after 6, 24 and 72 h of antagonist-pathogen incubation.

Inoculated fruit surfaces were prepared according to the method of Dawson *et a*l. (1969) for SEM. Each prepared fruit treatment was cut into $0.5cm^2$ sections before drying in a Hitachi HCP-2 critical point drier (Hitachi Koki Co, Ltd., Tokyo, Japan). Each section was fixed on a specimen stub and coated with gold-palladium in an Eiko IB-3 ion coater (Eiko Engineering Co, Ltd., Japan) and viewed with a 840 JEOL SEM operating at 5 kV

RESULTS AND DISCUSSION

The post-harvest biocontrol potential of *B subtilis* has been described on several commodities against a number of pathogens (Pusey and Wilson, 1984; Singh and Deverall, 1984; Utkhede and Shollberg, 1986; Pusey *et a*l., 1988 Pruvost and Luisetti, 1991; Korsten *et al*., 1995). This however is the first report dealing with the influence nutrition has on the antagonistic activity of *B*. *subtilis* to control mycelia growth of *C. gloeosporioides* and describing the direct pathogen-antagonist interactions on avocado fruit.

Determining nutrient requirement for effective antagonistic interactions:

Antagonism of *B subtilis* was greatly influenced by 34 carbon sources (Table 1) and 20 amino acids (Table 2). Citric acid, D(+) galactose, pyruvate and benzoate were carbon sources most effective in sustaining inhibition zones, whereas D(+)xylose, D(+)mannose, starch and cellulose were least effective (Table 1). Carbon sources most important for antagonism were also among those least required for *C. gloeosporioides* growth (Table 1). Upadhyay *et al*. (1991) found D(+) xylose as carbon source most effective in enhancing the antagonistic activity of *Pseudomonos cepaceae* against *Trichoderma viridae*, a soilborne pathogen. De Cal *et al.* (1990) reduced infection of *Monilinia laxa* by 38-80% with pre-harvest applications of the antagonist *Penicillium frequentants* suspended in malt and yeast extract.

Although Blakeman and Brodie (1977) suggested that bacteria are scavengers for amino acids, only L (+) asparagine and L-aspartic acid were utilized by the antagonist to sustain antagonism in this study (Table 2). These amino acids were also significantly

less important as nitrogen sources than for instance lysine which was most important in C. gloeosporioides' metabolism. Janisiewicz ef *al.* (1 992), enhanced biocontrol of blue mold on apples by increasing the population densities of the antagonist Pseudomonas syringae on fruit with L-asparagine and L-proline. The addition of amino acids to snap bean leaves reduced populations of *P.* syringae and reduced bacterial brown spot (Morris and Rouse, 1 985). In addition, amino-acid applications have also been reported to stimulate host resistance (Van Andel, 1966).

Mercer *et al*. (1970) demonstrated that excess nutrients encourage *Colletotrichum lindemuthianum* to develop saprophytically with an apparent suppression of appresoria formation and thus normal pathogenic behavior. The application of nutrients on leaves has been found not only to enhance the competitive ability of natural antagonists of pathogens, but also to neutralize the effect agricultural chemicals had on the natural microbial inhabitants (Dik, 1991)

Although it was suggested by other authors that *B*. *subtilis* operates solely by the production of anti-microbial substances (Lenné and Parbery 1976; Pusey and Wilson, 1984; Singh and Deverall, 1984; McKeen *et al.,* 1986; Utkhede and Shollberg, 1986; Pusey *et al.,* 1988; Pruvost and Luisetti, 1991), this study showed however that the production of antimicrobial substances are dependent on the availability of selective nutrients. Therefore competition for nutrients might indirectly be a part of the antagonist's mode of action.

Conceptually, any attempt to alter environmental factors such as nutrition to favor the antagonist must consider the effects these changes might have on the pathogen.

Examination of direct interaction between *B. su*btilis and *C. gloeosporioides* on avocado fruit:

Understanding the mode of action of an antagonist is vital in improving biocontrol, as a combination of different modes of action may be involved, *in vitro* interactions between *B subtilis* and *C. gloeosporioides* were first described by Korsten and De Jager, 1995. The production of lytic substances was demonstrated by "bulb formation" of germinated spores followed by the release of cell contents. This study however demonstrates the curative and preventative action of *B*. *subtilis.*

Antagonist cells applied prior to the pathogen resulted in total inhibition of spore germination (Fig. 1), irrespective of application times. Therefore the antagonist's preventative action might be an indication of competitive exclusion or pre-emptive colonization. However, non-germinated spores collapsed in the presence of the antagonist, which may indicate the production of lytic substances as suggested by Lenné and Parbery (1976).

Demonstrating the curative effect when the pathogen was applied prior to the antagonist, germinating fungal hyphae collapsed in direct contact with viable bacterial cells (Fig. 2), also irrespective of application times. This further confirmed the production of anti-fungal metabolites by the antagonist. Antagonist cells were able to stick to the fungal surface, especially on sites of leaching such as growing germ tube (Fig. 3c) and damaged hyphal regions.

Tabel 1. ***Effect of different carbon sources on growth[a] of Colletotrichum gloeosporiodes and inhibition zone[b] between Bacillus subtilis and C. gloeosporioides.***

Carbon sources	*C. gloeosporioides* growth (mm)[a]	Inhibition zone (mm)[b]
D(-)Arabinose	74.11 abcdef	-07.78 abcde
L(-)Arabinose	74.11 abcdef	-18.00 efghi
Acetate	66.00 g	-10.78 bcdef
Benzoate	27.00 k	+01.22 ab
D(+)Cellobiose	74.58 abcdef	-28.78 hijkl
Cellulose	76.00 abcde	-32.11 l
Citrate	49.89 l	-0.211 abc
Citrid acid	46.67 l	+05.11 a
D(+)Fructose	76.89 abcd	-10.00 bcdef
D(+)Galactose	40.56 j	+02.33 ab
D-Gluconic acid	76.22 abcde	00.00 abc
D(+)Glucose	72.78 bcdef	-22.44 fghijkl
Glycerol	72.22 bcdef	-07.44 abcde
Myo-Inositol	78.67 ab	-19.22 efghijkl
Lactose	70.11 efg	-28.00 hijkl
D(-)Lyxose	75.33 abcdef	-27.78 hijk
L(-)Lyxose	36.89 j	-00.78 abc
L(- Malic acid	73.79 abcdef	-25.44 ghijkl
D(+)Maltose	*79.56 a	-29.11 hijkl
D(-)Mannitol	70.78 defg	-19.00 efghijkl
D(+)Mannose	75.00 abcdef	-31.33 jkl
Pectin	*80.00 a	-30.89 ijkl
Peptone	*79.56 a	-03.56 abc
Pyrovate	70.00 efg	+02.11 ab
D(-)Rhamnose	77.78 abc	-18.33 efghij
L(-)Rhamnose	75.11 abcdef	-18.67 efghijk
D(-)Ribose	77.89 abc	-26.89 ijkl
D(-)Sorbitol	72.44 bcdef	-13.00 cdefg
L(-)Sorbitol	60.22 h	-11.89 cdef
Starch	75.00 abcdef	-31.89 kl
Sucrose	71.67 cdefg	-04.44 abcd
D(+)Trehalose	69.22 fg	-04.44 abcd
D(+)Xylose	77.33 abc	-31.22 ijkl
L(+)Xylose	75.56 abcdef	-16.56 defgh
N	306	306
p >	0.0001	0.0001

Values followed by the same letter do not differ significantly according to Duncan's multiple range test (p=0.05)

a Represents *C gloeosporioides* diameter growth measured after 7 days incubation.

b Represents inhibition zones calculated from the formula: R2-R1, where R1 is the farthest radial distance growth of *C. gloeosporioides* on different minimal salt-based media supplemented with different carbon sources and R2 is the farthest radial growth distance of *B. subtillis* (Skidmore, 1976).
Negative values represents *C. gloeosporioides* over grow *B. subtillis*
Positive values indicate inhibition zones between *C. gloesporioides* and *B. subtillis*

Table 2. Effect of different amino acids on growth[a] of Colletotrichum gloeosporioides and inhibition zone[b] between Bacillus subtilis and C. gloeosporioides.

Amino acids	*C. gloeosporioides* Growth (mm)[a]	Inhibition zone (mm)[b]
L-Alanine	71.67 abcde	-02.22 ab
L-Arginine	73.22 abcd	-04.11 ab
L(+)Asparagine	71.00 abcde	+01.11 a
L-Aspartic acid	70.22 cde	+04.00 a
L-Cystein	69.78 de	-20.89 defg
L-Glutamine	68.11 e	-31.56 g
L-Glutamic acid	72.22 abcde	-02.56 ab
Glycine	70.22 cde	-25.56 defg
L-Histidine	72.22 abcde	-02.79 ab
L-Isoleucine	74.89 abc	-25.11 defg
L-Leucine	71.11 bcde	-28.79 defg
L-Lysine	76.11 a	-30.00 fg
L-Methionine	69.33 de	-19.67 defg
L-Phenylalanine	71.33 abcde	-29.00 efg
L-Proline	73.00 abcde	-17.67 cd
L-Serine	75.78 ab	-30.44 fg
L-Threonine	71.56 abcde	-27.44 defg
DL-Thryptophan	46.22 f	-09.67 bc
L-Tyrosine	74.00 abcd	-28.11 defg
L-Valine	70.22 cde	-18.00 cde
N	180	180
p >	0.0001	0.0001

Values followed by the same letter do not differ significantly according to Duncan's multiple *range test (p=0.05)*

a Represents *C gloeosporioides* diameter growth measured after 7 days incubation

b Represents inhibition zones calcualted from the formula: R2-R1, where R1 is the farthest radial distance growth of *C. gloeospoioides* on different minimal salt-based media supplemented with different amino acids and R2 is the farthest radial growth distance of *B. subtillis* (Skidmore, 1976).
Negative values represents *C. gloeosporioides* over grow *B. subtillis*
Positive values indicate inhibition zones between *C. gloeosporioides* and *B. subtillis*

B. subtilis multiplied rapidly four hours after inoculation onto fruit surfaces (Fig. 3a). The antagonist accumulates in fruit depressions and around germinating conidia (Fig. 3b) as water and nutrients are limited on fruit surfaces. Fruit were kept under high humidity and two types of cells were observed; elongated rods and groups of shorter cells (Fig. 1-3).

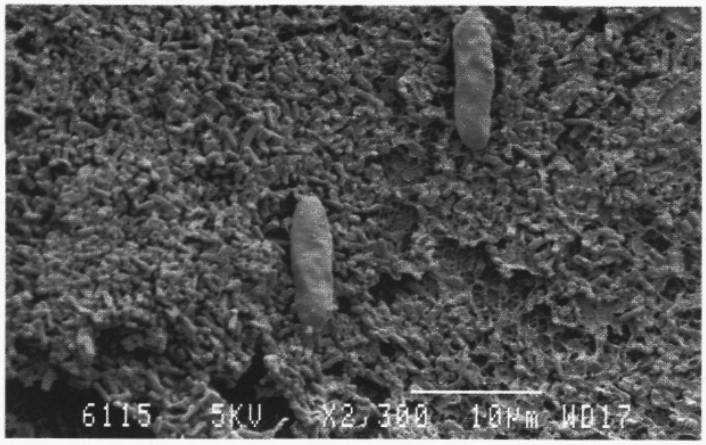

Figure 1: In vivo preventative interaction between Bacillus subtilis *(ATCC 55466/B246) and* Colletotrichum gloeosporioides *on avocado fruit surfaces showing non-germinated spores collapsed in the presence of the antagonist (2300 x magnification).*

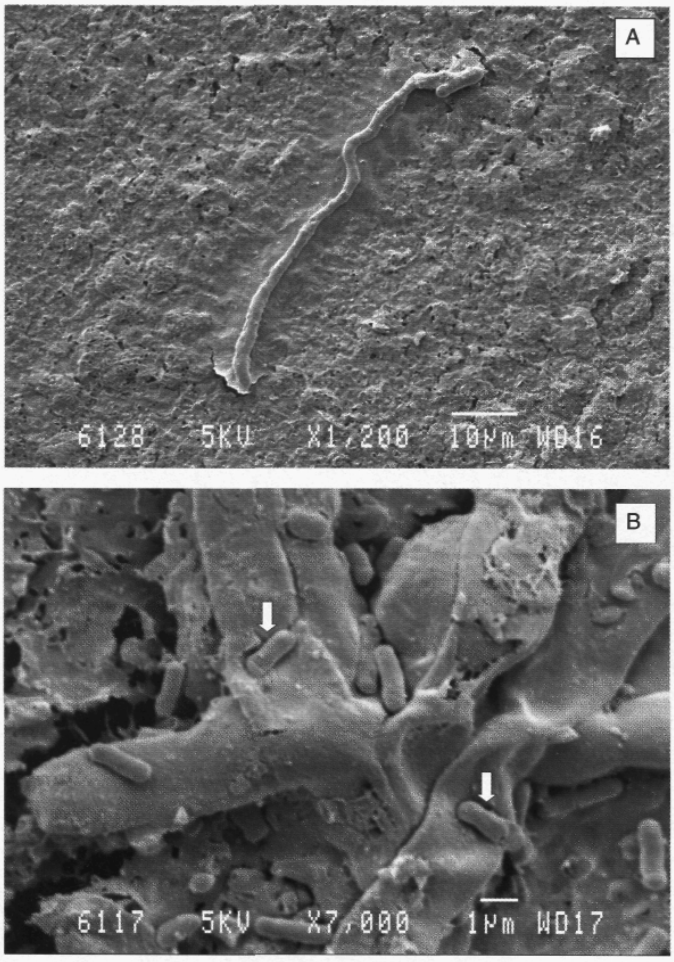

Figure 2: In vivo curative interaction between Bacillus subtilis *(ATCC 55466/B246) and* Colletotrichum gloeosporioides *on avocado fruit surfaces showing (A) control germination of* C. gloeosporioides *(1200 x magnification) and (B) germinating fungal hyphae collapsed in direct contact with viable antagonist cells (7000 x magnification).*

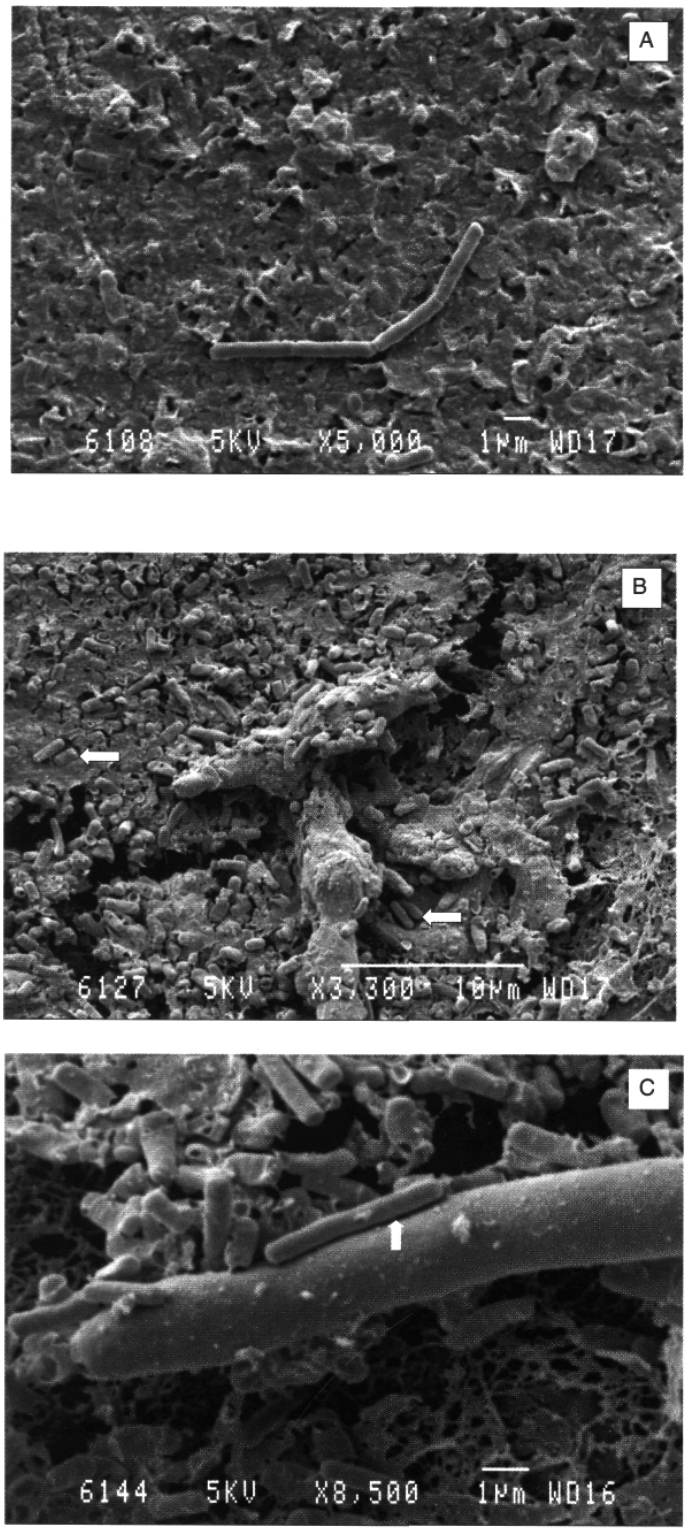

Figure 3: Bacillus subtilis *(ATCC 55466/B246) on avocado fruit surfaces showing (A) multiplication of* B. subtilis cells *into elongated rods (5000 x magnification), (B) accumulation in fruit depressions and on hyphae of* Colletotrichum gloeosporioides *(3300 x magnification), and (C) attachment to growing germ tube of* C. gloeosporioides *(8500 x magnification).*

Cells were not coated with mucilaginous matrix as was previously reported (Korsten 1993). Whether or not the production of mucilaginous matrix was influence by the preparation process should be determined.

Current research is focused on improving the biocontrol potential of *B. subtilis* by means of a liquid formulation incorporating selective nutrients, and to characterize the inhibitory substance produced by the antagonist.

CONCLUSION

B. subtilis and *C. gloeosporioides* are able to use various carbohydrates and amino acids as sole carbon and nitrogen sources respectively. This study showed that the availability of specific carbohydrates (citric acid, D(+)galactose, pyruvate and benzoate) and amino acids (L(+)asparagine and L-aspartic acid) is crucial to *B. subtilis'* effective performance as antagonist. Nutrition therefore has a significant impact on pathogen-antagonist interactions. Direct pathogen-antagonist interactions showed that the antagonists' mode of action might be a combination among competitive exclusion, preemptive colonization and the production of anti-fungal substances. These interactions are directly influenced by selective nutrition. Previous antagonist formulations were not suitable for effective disease control.

ACKNOWLEDGMENT

The authors wish to thank SAAGA for financial support, Westfalia Estates for providing the avocado fruit, Alan Hall for SEM assistance and Amanda Lourens for statistical analysis.

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