CHAPTER 3

COMPARISON OF *BACILLUS SUBTILIS* SUBCULTURES' EFFICACY IN CONTROLLING FUNGAL AVOCADO PATHOGENS *IN VITRO*

1. ABSTRACT

With the global move away from pesticides, biocontrol has become a viable alternative. Postharvest diseases, anthracnose and stem-end rot of avocado are difficult to control. Previously, *Bacillus subtilis* was isolated and proved effective as biocontrol agent of avocado pre- and postharvest diseases. However, during semi-commercial trials levels of control were not constant. The *in vitro* efficacy of the subcultures used in these studies was therefore compared to determine possible reasons for product performance variability. The dual culture technique was used and all subcultures and reference isolates were tested against four important fungal pathogens (*Phomopsis perseae, Colletotrichum gloeosporioides, Dothiorella aromatica* and *Lasiodiplodia theobromae*) causing postharvest diseases of avocado. The efficacy of the subcultures differed significantly. Subculture MI-14 was the most effective. RISA PCR was used to ascertain if the subcultures were still closely related, even though their efficacies differed. All subcultures showed the same DNA fingerprint, differing from that of other *B. subtilis* isolates included as reference strains.

2. INTRODUCTION

Pre- and postharvest fruit diseases are of major concern for avocado growers. Postharvest diseases are difficult to control since there is a wide spectrum associated fungal pathogens involved in disease development, especially in the case of stem-end rot (SE) (Darvas & Kotzé, 1987; Korsten *et al.*, 1995). Control of anthracnose caused by *Colletotrichum gloeosporioides* Penzig. is also challenging since the pathogen makes use of latent infections. As alternative to chemical control, biocontrol has received increasing attention and have been used either on its own or as part of an integrated disease management system (Roberts, 1994).

In previous studies, *Bacillus subtilis* was isolated and proved effective against various postharvest diseases of avocado (Korsten, 1993). The antagonist was evaluated successfully on a semi-commercial scale in field and packhouse trials (Korsten *et al.*, 1989; 1991; 1992; 1993; 1994; 1997; van Dyk *et al.*, 1997). However, control was not always consistent when evaluated over 15 years. Initially, stock cultures were made of the

original isolate. Subcultures were used in subsequent studies. The subcultures of *B. subtilis* used in most of these studies were incorporated over time into the bacterial culture collection of the Department of Microbiology and Plant Pathology, University of Pretoria, South Africa.

The main objective of this study was to compare the *in vitro* efficacy of the abovementioned subcultures. The dual culture technique (Skidmore, 1976), initially used to select *B. subtilis* amongst other avocado epiphytes, was used to determine if all subcultures are still efficacious. DNA fingerprinting, using the RISA (rDNA internal spacer analysis) PCR (Polymerase Chain Reaction), was employed to ascertain if the subcultures originated from the same isolate are still closely related. The RISA PCR targets the spacer regions between the 23S and 16S ribosomal genes (Campbell *et al.*, 1993; Jensen *et al.*, 1993; Farber, 1996). The spacer region is highly conserved and the length as well as the sequence of these regions can be used to compare related bacteria, even though the RISA PCR is usually employed in the analysis of bacterial communities (García-Martínez *et al.*, 1999; Ranjard *et al.*, 2000).

3. MATERIALS AND METHODS

3.1. Strains

Bacillus subtilis (B246), isolated in 1984 by Korsten (1993) from avocado (*Persea americana* Mill.) leaves in Tzaneen, South Africa, was used throughout this study. Subcultures for use were stored in 30 % glycerol with quarter strength Ringer's (Merck, Johannesburg, S.A.) solution at –70 ºC, and grown on standard 1 nutrient agar (STD1) (Biolab, Merck) for use. Freshly streaked cultures on STD1 were incubated at 28 ºC for 24 h prior to use. Consecutive subculturing of isolate was recorded over time and summarized in Table 3.1. The identity of all strains was verified using the API system (API 50 CH for Gram-positive bacteria (BioMérieux)).

The following fungal pathogens were used in this study: *Colletotrichum gloeosporioides* (Dr. G. Swart, Department of Microbiology and Plant Pathology, University of Pretoria, S.A.), *Dothiorella aromatica* (Sacc.) Petrak & Sydow and *Lasiodiplodia theobromae* (Pat.) Griffon & Maubl. (both isolated by M. Schoeman, Department of Microbiology and Plant Pathology). *Phomopsis perseae* Zerova was isolated from SE lesions on avocado fruit as described in the following section. All fungi were maintained on potato dextrose agar (PDA) (Biolab, Merck) slants as well as in sterile water at 25 ºC. An agar disk containing fungus was placed on PDA and incubated at room temperature for three days at near UV light, the purity thereof checked and subsequently used throughout this study.

3.2. Isolation and pathogenicity of fungal postharvest avocado pathogens

Fuerte cultivar avocados were collected from the Pretoria fresh produce markets and were ripened at room temperature. All fruit showing typical SE symptoms were used for isolation. Fruit were surface sterilized by wiping with 70 % ethanol. Small sections of skin and pulp surrounding the diseased lesions (2 x 2 mm) were aseptically cut and placed on PDA plates. The plates were incubated at 25 ºC for three to six days. Fungi growing from the samples were isolated, purity ensured and identified. Prof. F.C. Wehner confirmed the isolates' identity (Department of Microbiology and Plant Pathology). Pathogenicity was confirmed using the fruit inoculation method described below.

Unripe Fuerte avocados were surface sterilized as described and three holes were made with a five mm diameter cork borer on the equatorial region of the fruit. Three fruit were used per isolate. Agar disks were punched from the periphery of the actively growing fungal pathogen cultures using a five mm diameter cork borer and were placed in the holes with the side containing fungal growth inserted first. Holes were sealed with the removed avocado plugs and covered with Parafilm. The negative control consisted of agar disks containing no fungal growth. Fruit were incubated at 25 ºC to ripen and observed daily for the development of decay symptoms. As soon as typical black necrotic lesions developed, fungi were re-isolated and identity confirmed (as described previously).

3.3. In vitro *evaluation of antagonistic efficacy of* Bacillus subtilis *subcultures*

The dual culture method of Skidmore (1976) was modified and used in this study. Fungal isolates were cultured on PDA for seven days at 25 ºC and used in this assay. Disks were aseptically punched from the periphery of the actively growing fungal culture using a cork borer with a four mm diameter. Disks were placed in the center of a round 90 mm Petridish containing PDA, with the fungal growth in contact with the PDA plate. Plates were incubated at 25 ºC until the fungal colony reached a five mm diameter. Bacterial cultures were streaked on STD1 and incubated for 24 h at 25 ºC. Bacteria were subsequently streak inoculated (two cm long streaks) 30 to 35 mm from the center of the fungal disk on three equidistant places on the Petridish. Plates containing only the test fungal culture, without any bacterial streaks, served as controls. Figure 3.1 depicts what the test plates looked like.

Table 3.1: *Bacillus subtilis* subcultures and other reference bacteria used for comparison purposes

Culture	*MPUP Code	ºReference Code	Known history	Reference
Bacillus cereus	2	DSMZ 9	Reference strain	None
Bacillus licheniformis	214	5A1	Reference strain	None
Bacillus licheniformis	494	B40	Reference strain	None
Bacillus licheniformis	-	PS-Mango	Commercial isolate	None
Bacillus licheniformis	-	MAL	Trial mango antagonist	None
Bacillus licheniformis	-	MAH	Trial mango antagonist	None
Bacillus spp.	19	Natto	Reference strain	None
Bacillus subtilis	16	Avo 13	Isolated by L. Korsten	Korsten *et al.*, 1989; Korsten, 1993
Bacillus subtilis	17	Avo 32	Isolated by L. Korsten	Korsten *et al.*, 1989; Korsten, 1993
Bacillus subtilis	18	Avo 66	Isolated by L. Korsten	Korsten *et al.*, 1989; Korsten, 1993
Bacillus subtilis	22	Avo 225	Isolated by L. Korsten	Korsten *et al.*, 1989; Korsten, 1993
Bacillus subtilis	23	ATCC 663	Reference strain	None
Bacillus subtilis	24	ATCC 6051	Reference strain	None
Bacillus subtilis	25	ATCC 11774	Reference strain	None
Bacillus subtilis	27	A6	Unknown	Korsten *et al.*, 1988; Korsten *et al.*, 1989
Bacillus polymyxa	32	DSMZ 36	Reference strain	None
Bacillus subtilis	35	A6 (Daleen)	Unknown	Korsten *et al.*, 1989; Korsten, 1993
Bacillus subtilis	44	A6 (87)	Unknown	Korsten *et al.*, 1989; Korsten, 1993
Bacillus subtilis	45	A6 (91)	Unknown	Korsten *et al.*, 1989; Korsten, 1993
Bacillus subtilis	46	A6 (Lise)	Unknown	Korsten *et al.*, 1989; Korsten, 1993
Bacillus subtilis	47	A6 (Lise - 1)	Unknown	Korsten *et al.*, 1989; Korsten, 1993
Bacillus subtilis	48	A6 (89 - 1)	Unknown	Korsten *et al.*, 1989; Korsten, 1993
Bacillus subtilis	49	A6 (Lise - lig)	Unknown	Korsten *et al.*, 1989; Korsten, 1993
Bacillus subtilis	50	A6 (Lise - wit)	Unknown	Korsten *et al.*, 1989; Korsten, 1993
Bacillus subtilis	51	A6 (89 - lig)	Unknown	Korsten *et al.*, 1989; Korsten, 1993
Bacillus subtilis	52	A6 (89 - wit)	Unknown	Korsten *et al.*, 1989; Korsten, 1993
Bacillus subtilis	79	A6 (88)	Unknown	Korsten *et al.*, 1989; Korsten, 1993
Bacillus subtilis	80	ATCC 55466	Deposited at ATCC	None
Bacillus subtilis	-	A6 (Estelle)	Unknown	Towsen *et al.*, 1995, Korsten *et al.*, 1998
Bacillus subtilis	-	A6 (Erika)	Unknown	Korsten *et al.*, 1991; Korsten *et al.*, 1992; Korsten *et al.*, 1993; Korsten *et al.*, 1994; Van Dyk *et al.*, 1997
Bacillus subtilis	-	PS-S	Commercial isolate	None
Bacillus subtilis	-	PS-B	Commercial isolate	None
Bacillus subtilis	-	MI-14	Subculture used 1999	Havenga *et al.*, 1999; Korsten & de Jager, 1995

* MPUP code as assigned to isolates in the culture collection at the University of Pretoria
ºReference code: DSMZ (German Collection of Microorganisms and Cell Cultures), ATCC (American Type Culture Collection), A6 (original biocontrol bacterium), other codes are the researcher's own codes
No MPUP code assigned

The antagonists were also streak inoculated on PDA plates without fungal cultures as an additional control. Duplicate plates were used and the experiment was repeated three times. Plates were maintained at 25 °C. Growth of the fungus, measured from the centre of the fungal disk to the periphery of the culture (in the direction of the bacterium), as well as the distance to the nearest edge of the bacterium's growth were measured 3, 6, 10 and 14 days after inoculation with the antagonist. The percentage growth inhibition was determined by the following formula: $(K_r - r1) / K_r \times 100 = \%$ Growth Inhibition. K_r represents the radius of the control fungal pathogen's growth and r1 represents the radius of the pathogen's growth towards the bacterial antagonist as measured from the centre of inoculation. The means of the percentage inhibition for each bacterium were calculated for each fungus. The most resistant or most susceptible groups of treatments, with a probability of 95% for the correct decision, were selected according to the Multiple t-distribution test procedure of Gupta & Panchapakesan (1979). Data were analysed using the statistical program GenStat (2000).

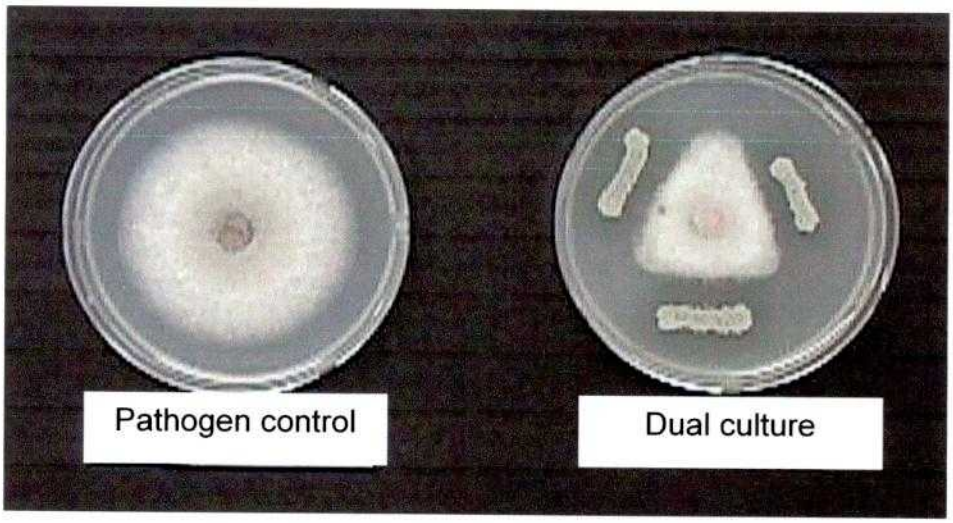

Figure 3.1: Dual culture technique: *Bacillus subtilis* inhibiting *Colletotrichum gloeosporioides in vitro* on potato dextrose agar plates.

3.4. *Genomic fingerprinting of* Bacillus subtilis *subcultures using RISA primers for polymerase chain reaction*

3.4.1. DNA extraction

Template DNA was extracted from bacterial isolates (2, 23, 25, 35, 79, 80, A6 (Estelle), A6 (Erika), PS-S, MI-14) using a modification of the rapid lysis method (Maniatis *et al.*, 1982). Single colonies were transferred to Luria Bertani broth (LB) (Biolab, Merck) and shake incubated (70 rpm) for 24 h at 37 °C. One ml of the

suspension was centrifuged (14 000 x g) for 10 min. The supernatant was removed and the pellet was washed twice by resuspending the pellet in one ml quarter strength Ringer's solution and centrifuged (14 000 x g) for 10 min. The pellet was resuspended in 100 µl sterile milli Q water and heated for 10 min at 95 °C. Cell lysate was immediately placed on ice.

3.4.2. Amplification of DNA

RISA primers (MWG Biotech Germany), 16S rDNA (5'-TTG TAC ACA CCG CCC GTC A-3') and 23S rDNA (5'-GGT ACC TTA GAT GTT TCA GTT C-3') were used for the amplification of DNA (McManus & Jones, 1996). A reaction mixture containing 1 x reaction buffer (100 mM NaCl; 50 mM Tris-HCl, pH 8.0; 0.1 mM DDT; 50 % glycerol and 1 % Triton X-100) (Promega, Johannesburg), 50 pmoles of each of the oligonucleotide primers, 0.625 mM of each of the dNTPs, 2.5 mM $MgCl_2$, milli Q water and 0.2 U of *Taq* DNA polymerase (Promega) were set up to which 1.5 µl of cell lysate was added. A Perkin Elmer 2400 was used for thermal cycling. The following cycle was selected: initial denaturation for seven min AT 95 °C followed by thirty cycles of denaturation for one min at 94 °C, annealing for one min at 52 °C and extension for three min at 72 °C. The reaction was completed with a final 10 min extension at 72 °C. As positive control, a pure *Pseudomonas aeruginosa* DNA sample (B. Steyn, Department of Microbiology and Plant Pathology, University of Pretoria) was included. Water alone was used as negative control.

3.4.3. Separation of DNA bands

Ten µl of the amplification products were separated on a 1.2 % agarose (Sigma) gel in Tris-Borate buffer (TBE) (45 mM Tris-borate, 1 mM $Na_2EDTA.2H_2O$) and visualized by ethidium bromide staining (Maniatis *et al.*, 1982) and transillumination. The Vilber Lourmat camera system was used to photograph the DNA gels. A 1 kbp standard ladder was included for band size reference.

4. RESULTS

4.1. Isolation and pathogenicity of fungal postharvest avocado pathogens

Pathogenic efficacy of the isolated fungi, *P. perseae*, *C. gloeosporioides*, *D. aromatica* and *L. theobromae* is summarised in Figure 3.2. Of the four fungi evaluated for pathogenicity, *D. aromatica* and *L. theobromae* caused the greatest lesion formation followed by *C. gloeosporioides* and *P. perseae*. All test fungi were re-isolated from lesions produced and Koch's postulate could therefore be confirmed.

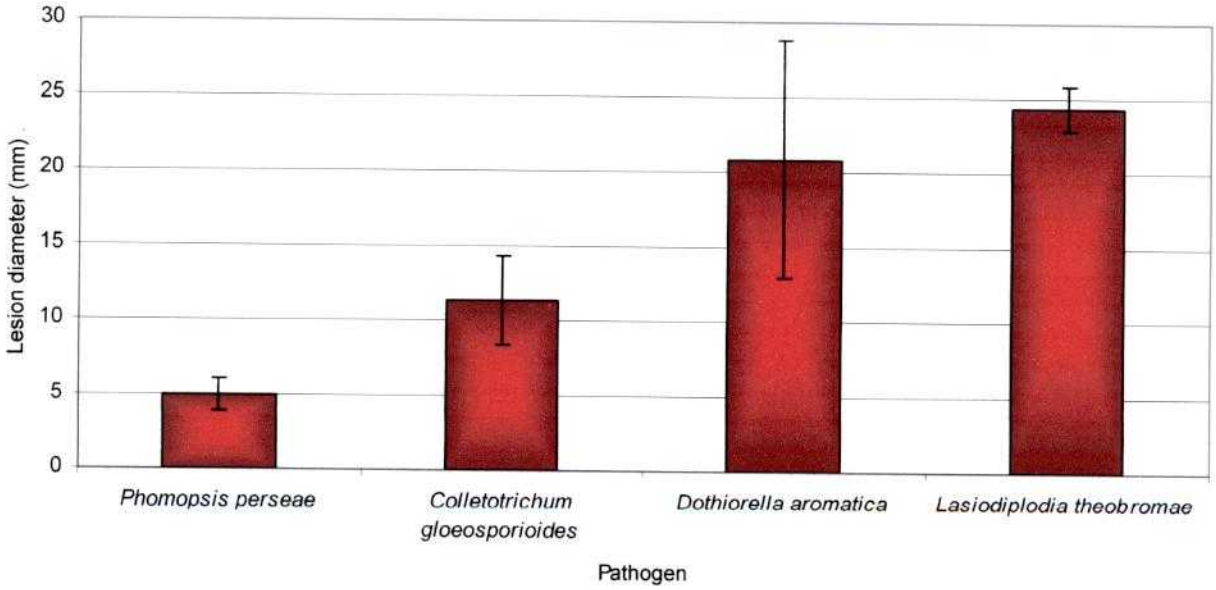

Figure 3.2: Diameter of lesions formed on avocado fruit by fungal postharvest pathogens.

4.2. Evaluation of in vitro *antagonism of* Bacillus subtilis *subcultures and reference strains*

The inhibitory efficacy of subcultures and reference strains against fungal avocado pathogens is summarised in Table 3.2. The antagonists, listed from the best (indicated in clear blocks) to the least effective antagonist (grey blocks) and the two blocks significantly less effective by a probability of $P < 0.05$ according to the Multiple t-distribution test. The most effective antagonists against all four pathogens *in vitro* are 16, 22, MI-14, MAH, 214, MAL, PS-M and A6 Erika, of which only MI-14 and A6 Erika are subcultures of the initial biocontrol organism. Antagonists MAH, MAL, PS-M and 214 are all *B. licheniformis* isolates. The commercial isolates (PS-S and PS-B), however, is still efficacious, but not to the same extent as subculture MI-14. Antagonist A6 Erika is the most effective and A6 Estelle the least effective compared to the rest of the subisolates.

4.3. Genomic fingerprinting of Bacillus subtilis *subcultures using the rDNA internal spacer analysis polymerase chain reaction*

Figure 3.3 depicts the fingerprint of the antagonists. Isolates MI-14, PS-S, A6 (Erika), A6 (Estelle), 80, 79 and 35 all have the same characteristic set of DNA bands. Isolate 80 contained less DNA and the band is not as bright as the rest. The band pattern of isolates 2, 23 and 25 are unique and are not subcultures of the initial biocontrol organism.

Table 3.2: *In vitro* inhibition of avocado fungal pathogens, *Phomopsis perseae*, *Colletotrichum gloeosporioides*, *Dothiorella aromatica* and *Lasiodiplodia theobromae* by *Bacillus isolates*

Phomopsis perseae		*Colletotrichum gloeosporioides*		*Dothiorella aromatica*		*Lasiodiplodia theobromae*	
Antagonist	Mean % Inhibition	Antagonist	Mean % Inhibition	Antagonist	Mean % Inhibition	Antagonist	Mean % Inhibition
16	61.1	16	56.57	PS-M	54.45	16	41.38
MAH	61.1	MAH	56.2	MAH	49.78	MAH	40.8
214	60.92	PS-M	54.22	22	48.07	214	38.2
PS-M	60.67	214	54.15	16	40.72	PS-M	37.02
18	58.57	18	51.72	18	39.73	18	31.25
MI-14	57.32	22	49.82	214	38.95	22	30.7
24	56.22	MI-14	48.82	MI-14	35.92	48	24.67
22	55.83	MAL	46.98	24	34.3	45	24.05
80	53.47	24	44.52	MAL	34.1	A6 Erika	22.43
MAL	53.12	A6 Erika	42.02	PS-B	32.63	MAL	22.22
A6 Erika	52.82	45	40	PS-S	32.3	MI-14	20.92
PS-S	52.53	48	38.93	23	32.15	17	19.95
PS-B	51.95	23	37.76	A6 Erika	32.15	24	19.8
17	51.92	80	37.57	48	31.95	79	18.18
45	50.88	35	35.58	35	30.92	PS-B	17.23
44	49.7	79	35.53	79	30.62	80	16.73
35	49.23	51	35.28	17	30.5	PS-S	16.47
79	48.68	PS-S	35.1	45	30.35	23	16.1
52	48.57	44	34.55	80	28.6	35	15.97
48	48.05	52	33.93	44	28.4	44	15.77
51	45.43	PS-B	33.82	19	16.65	51	15.22
23	44.2	17	32.85	51	14.17	52	14.93
19	29.83	19	26.65	52	12.22	19	0
49	28.25	25	22.92	25	1.73	25	0
27	27.35	A6 Estelle	22.38	A6 Estelle	1.67	27	0
47	25.33	49	21.3	47	0.97	46	0
46	23.63	27	20.4	46	0.42	47	0
A6 Estelle	22.6	46	17.65	27	0	49	0
50	19.32	50	16.77	49	0	494	0
25	13.48	47	16.32	494	0	50	0
494	10.17	494	10.87	50	0	A6 Estelle	0
P < 0.001		P < 0.001		P < 0.001		P < 0.001	
SEM = 4.06		SEM = 2.73		SEM = 5.53		SEM = 5.11	

SEM = Standard error of means

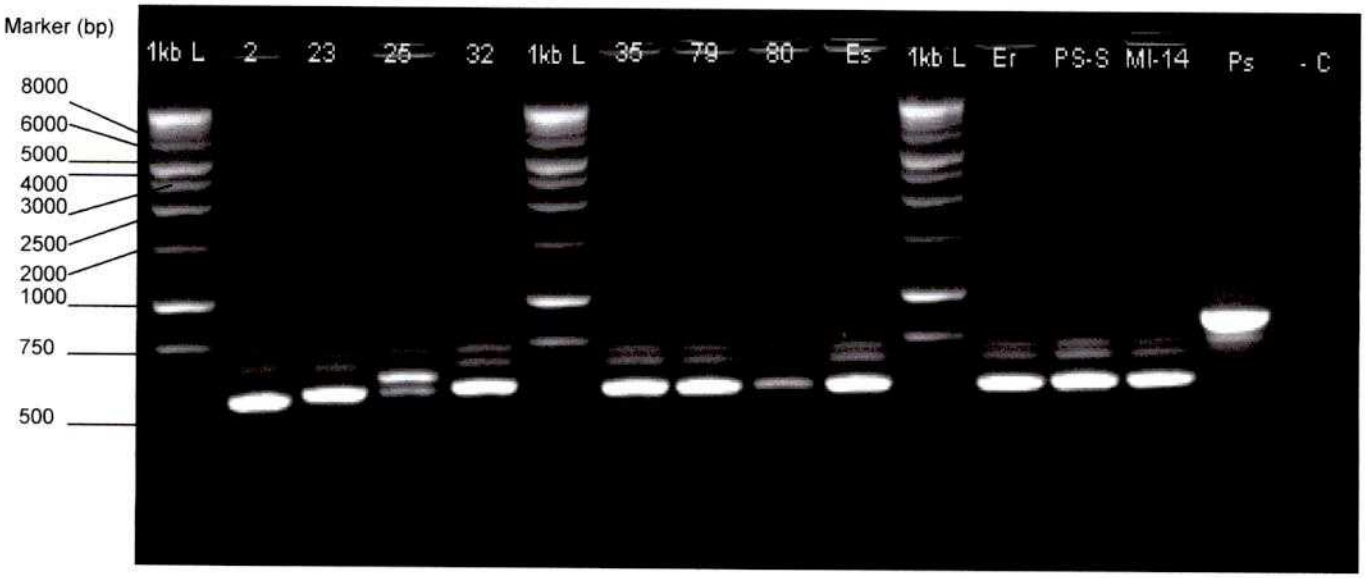

Figure 3.3: Banding patterns of DNA products after rDNA internal spacer analysis polymerase chain reaction on selected isolates of *Bacillus subtilis* separated using sodium dodecyl sulphate polyacrylamide gel electrophoresis. Isolates 35, 79, 80, Es, Er, PS-S and MI-14 are all subcultures of *B. subtilis* biocontrol agent of avocado postharvest diseases. Isolate 2 (*Bacillus cereus*), 23 (*Bacillus subtilis* ATCC 663) and 25 (*B. subtilis* ATCC 6051) are reference cultures used. Isolate Ps is pure DNA from *Pseudomonas aeruginosa* as positive control and –C is water used as a negative control.

5. DISCUSSION

In this study significant differences were found in the *in vitro* activity of *B. subtilis* subcultures against postharvest fungal avocado pathogens. The subcultures used in various studies over a 15 year period differed in terms of their biocontrol effectivity *in vitro*. Theoretically, the activity of the subcultures should not differ, but some were found to be less effective. Isolate A6 Erika performed well against *P. perseae* and *L. theobromae*. Korsten *et al.* (1997) found this subculture to be effective against preharvest diseases of avocado. Black spot, caused by *Pseudocercospora purpurea* Cooke, and sooty blotch, caused by a specie of *Akaropeltopsis* that was controlled effectively for three consecutive years with and without combining it with the commercial fungicide, copper oxychloride. The commercial isolates (PS-S and PS-B), however, is efficacious, but not to the same extent as subculture MI-14, used in the mode of action studies (Korsten & de Jager, 1995; Havenga *et al.*, 1999).

The variability in the effectiveness of *B. subtilis* subcultures to inhibit fungal pathogens of avocado may be ascribed to phenotypic variation. Variation in efficacy under commercial and semi-commercial conditions was observed (Korsten *et al.*, 1993; Korsten *et al.*, 1998) and subculturing in order to obtain pure cultures may contribute thereto. Reinheimer *et al.* (1995) found variants in a community of *Lactobacillus helveticus*

ATCC 15807 that are able to ferment mannose and fructose. However, these variants make up a very small percentage of the total population. This implies that during subculturing, selection might take place if care is not taken to preserve stock cultures from the initial isolate. Rainey & Rainey (2003) found that individuals of *P. fluorescens* making up a population work together for the benefit of the group, even though it may be costly to the individual itself. They state that some phenotypic characteristics are sacrificed for the benefit of the population. Specific traits may be lost while others are selected for during continuous subculturing.

Since the subcultures differed significantly in their *in vitro* efficacy, contamination was suspected. All subcultures showed identical DNA banding patterns or fingerprints using RISA primers in PCR. The observed banding pattern of the subcultures differed from those of the reference strains *B. subtilis* ATCC 663 and *B. subtilis* ATCC 11774. Thus, the possibility of contamination is ruled out as a possible reason for the observed variability in product performance.

This study highlights the importance of managing stock cultures, subcultures and culture purity to ensure consistent results. The subculture MI-14 was used further throughout this study. Of all the subcultures, it showed the greatest antagonistic activity against all fungal pathogens tested. No differences were observed between the subcultures using RISA PCR. Sequencing of the subcultures may further support these findings and should be considered for future studies.

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