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Determining appropriate parameters for an integrated disease control system for biological predictive modelling

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ABSTRACT

Increased economic and environmental pressure has led to the need for more accurate and safe crop protection measures. Although existing cercospora spot forecasting models currently contribute to targeted disease control programs for avocados, integration of such a model with a biocontrol predictive system may provide growers with a disease control program that adheres to the requirements of organic programmes and EurepGap. This model will also focus on eliminating variable parameters that may influence product performance. These parameters include climatic variables and agricultural practices. During this preliminary study, the effect of certain agricultural practices on the survival of Bacillus subtilis *(Avogreen) were evaluated. It was found that mist blower and hand gun application methods were equally effective in delivering the antagonist to the phylloplane, but that spray volumes could affect performance. Repeated pre-harvest applications resulted in a higher build-up of bacterial numbers on the leaves and increased the success of postharvest disease control. Results showed that spreading and sticking agents should be avoided to ensure survival of the bacteria. The compilation of a predictive system for* B. subtilis *will consist of a combination of climatological information (to be assessed during 2004) and antagonist behaviour as influenced by the above-mentioned agricultural practices.*

INTRODUCTION

Recently, there has been an increased demand for healthier, safer food without compromising quality. Although current disease control programs have proven relatively effective in maintaining lower pathogen infection levels, the need to reduce chemical applications due to consumer preferences and regulatory issues, has led to intensified research efforts to develop alternative control strategies (Ippolito & Nigro, 2000; Emmert & Handelsman, 1999; Weaver *et al.*, 1990). Biological control offers an environmentally friendly alternative to the use of pesticides for the control of plant diseases. The complexity of interactions between microbes, the involvement of different suppression mechanisms and the ability of these organisms to adapt to the environment, contribute to the belief that biocontrol may also be more sustainable than chemical disease control (Emmert & Handelsman, 1999).

Avocados (*Persea americana* Mill.) are susceptible to various fruit diseases, including cercospora spot, anthracnose, *Dothiorella/ Colletotrichum* fruit rot complex and stem-end rot (Korsten *et al.*, 1994). The integrated pre-harvest disease control program for these diseases involves the use of biological and chemical agents (benomyl and copper-based fungicides) (Towsen, *et al.*, 1995). Biocontrol of avocado fruit diseases has been investigated extensively at the University of Pretoria for the past 16 years and led to the development and registration of Avogreen (*Bacillus subtilis*) as South Africa's first pre-harvest biological control agent on avocado. Sporulation, production of antibiotics, glucanalytic activity and competition are known to contribute to survival on the phylloplane and control of especially cercospora leaf spot (Collins & Jacobsen, 2003; Collins *et al.*, 1993; Korsten & De Jager, 1995). Avogreen can be used in organic production systems and is fully EurepGap compatible. Yet, few growers have opted for this approach, mainly due to uncertainty of product performance under variable environmental conditions.

Antagonist performance is based on the ability of the organism to survive in the environment. If parameters affecting survival can be correlated with disease control, it may be possible to manipulate or promote these parameters in order to improve control of the disease (Collins & Jacobsen, 2003). Epidemiological parameters such as temperature and humidity have been known to affect antagonist survival and performance. Agricultural practices such as timing of application and application method may also influence the antagonist (Sutton & Peng, 1993). Incorporation of these parameters into an integrated disease system may provide a more targeted window of opportunity to establish the antagonist.

Cercospora spot forecasting models have been developed on a regional basis and focus on pathogen inoculum levels, spore release patterns and environmental factors (Darvas, 1982). Integration of these models with an antagonist predictive system could provide growers with disease control programs that reduce the number of copper sprays which is

Table 1. Treatment regimes for pre-harvest application of Benlate, Copper Oxychloride and Avogreen (Bacillus subtilis).

	Application			Month of Application			
Treatment program	Rate / ℓ	Method	Volume	Oct	Nov	Dec	Jan
Mist blower 15 ℓ							
Benlate	0.5 g	Hand gun	70 ℓ/ tree	+	-	-	-
Copper Oxychloride	3 g	Hand gun	70 ℓ/ tree	-	+	+	+
Avogreen	2.5 ml	Mist blower	15 ℓ/ tree	-	+	+	-
Mist blower 30 ℓ							
Benlate	0.5 g	Hand gun	70 ℓ/ tree	+	-	-	-
Copper Oxychloride	3 g	Hand gun	70 ℓ/ tree	-	+	+	+
Avogreen	2.5 ml	Mist blower	13 ℓ/ tree	-	+	+	-
Hand gun 70 ℓ							
Benlate	0.5 g	Hand gun	70 ℓ/ tree	+	-	-	-
Copper Oxychloride	3 g	Hand gun	70 ℓ/ tree	-	+	+	+
Avogreen	2.5 ml	Mist blower	70 ℓ/ tree	-	+	+	-
Copper 70 ℓ							
Benlate	0.5 g	Hand gun	70 ℓ/ tree	+	-	-	-
Copper Oxychloride	3 g	Hand gun	70 ℓ/ tree	-	+	+	+

in line with EurepGap requirements.

The aim of this study was to examine the effect of agricultural practices normally employed during commercial spray programs on the survival of *B. subtilis* in the field. This included an investigation into the effect of application method, spray frequency and spreading / sticking agents on the survival of *B. subtilis* on the phylloplane. This information is necessary to predict the behaviour of the antagonist for incorporation into a cercospora spot forecasting model.

MATERIALS AND METHODS

Population fluctuations and the percentage of disease free fruit was monitored in order to examine the effect of application method, application frequency and spreaders / stickers on the survival and efficacy of *B. subtilis*.

1. The effect of application method on the establishment / survival of *Bacillus subtilis* on the phylloplane.

Twenty-year-old Fuerte trees on the farm Amana in the Limpopo Province were used for this trial. Ten replicates were selected for each treatment described in Table 1.

Survival of Bacillus subtilis *on the phylloplane*

For the enumeration of *B. subtilis* from the phylloplane, ten leaves were collected randomly from each of five trees and pooled to form a sample. For each treatment, two samples were collected and bacterial counts made based on a modified method (De Jager, 1999). Three disks were punched from every leaf with a sterile cork borer and mixed. One hundred disks were randomly selected and vortexed for five minutes in ten ml sterile ¼ strength Ringer's solution (Merck). The spread plate method was used to enumerate *Bacillus* on Standard 1 Nutrient agar (Biolab) at 24°C.

Incidence of pre- and postharvest diseases on fruit

A minimum of 70 fruit were randomly harvested (March 2002) from six trees in each treatment and transported to Amondel packhouse (Levubu) where pre- and postharvest disease assessment took place. Fruit were rated for cercospora infection on a 0 – 6 scale, where 0 represented uninfected fruit and 6 severe cercospora infection. Fruit from each treatment were subjected to the normal packhouse treatment, which employs a Biocoat (1 ℓ/ 29 t fruit) and Avoshine (1 ℓ/ 55 lugs fruit) bath. One half of the fruit from each treatment were stored at 5°C to simulate export conditions. To induce ripening, the temperature was increased to 18°C after three weeks. Avocados destined for the local market were stored at 18°C immediately after the packhouse treatment. Ripe fruit were assessed for anthracnose and stem-end rot on a 0 – 10 scale (0 = uninfected fruit; 10 = severely infected fruit).

2. The effect of spray frequency on the establishment / survival of *Bacillus subtilis* on the phylloplane.

Seven-year-old Fuerte trees on the farm Delaja in the Levubu area were selected for this investigation. Treatment one followed an integrated disease control program (Table 2). Both copper oxychloride and Avogreen was applied during November 2001, December 2001 and January 2002. In treatment two, the monthly copper sprays were combined with bi-weekly Avogreen sprays. Treatment three served as the chemical control and received only the three copper oxychloride sprays. Twenty four replicates were used for each treatment. A mist blower calibrated at 15 ℓ/ tree was used during all applications.

Survival of Bacillus subtilis *on the phylloplane*

The survival of *B. subtilis* on the phylloplane was investigated using the modified method of De Jager (1999). Four samples were collected for each treatment as described before.

Incidence of pre- and postharvest diseases on fruit

Pre- and postharvest disease assessments were conducted as described previously. In

addition to the normal packhouse treatment that included Biocoat (1 ℓ/ 29 ton fruit) and Avoshine (1 ℓ/ 55 lugs fruit) , fruit was also subjected to an Avogreen bath containing 2.5 ml / ℓ Avogreen.

3. The effect of spreading and sticking agents on the establishment / survival of *Bacillus subtilis* on the phylloplane.

Table 2. Treatments employed for pre-harvest disease control on Fuerte during 2001/02 season.

Treatment Program	Rate / ℓ	Week of Application 1	2	3	4	5	6
Avogreen							
Copper Oxychloride	3 g	+	-	+	-	+	-
Avogreen	2.5 ml	+	-	+	-	+	-
Avogreen x 2							
Copper Oxychloride	3 g	+	-	+	-	+	-
Avogreen	2.5 ml	+	+	+	+	+	+
Copper							
Copper Oxychloride	3 g	+	-	+	-	+	-

Laboratory investigation

Antagonist survival in the presence of spreaders and stickers was studied in a liquid medium using a Spectronic 20 spectrophotometer (Milton Ray Company). A standard curve was prepared with known concentrations of the antagonist in 25 ml test tubes containing 18 ml Nutrient broth (Biolab). Optical density was measured as percentage transmission at 520 nm and a standard curve was plotted to determine cell concentrations.

For each treatment, a set of three test tubes

Table 3. Spray program for pre-harvest application of Benlate, Copper oxychloride and Avogreen (Bacillus subtilis) during the 2001/02 season.

Treatment program	Rate/L	Application method	Volume	Month of application Oct	Nov	Dec	Jan
1) Unsprayed Control							
2) Avogreen				-	-	-	-
Knowin	0,5 ml	Hand gun	70 ℓ/ tree	+	-	-	-
Copper oxychloride	3 g	Hand gun	70 ℓ/ tree	-	+	+	+
Avogreen	2,5 ml	Mistblower	13 ℓ/ tree	-	+	+	+
3) Avogreen + Superfilm							
Knowin	0,5 ml	Hand gun	70 ℓ/ tree	+	-	-	-
Copper oxychloride	3 g	Hand gun	70 ℓ/ tree	-	+	+	+
Avogreen	2,5 ml	Mistblower	13 ℓ/ tree	-	+	+	+
Biofilm	0,25 ml			-	+	+	+
4) Avogreen + Biofilm							
Knowin	0.5 ml	Hand gun	70 ℓ/ tree	+	-	-	-
Copper oxychloride	3 g	Hand gun	70 ℓ/ tree	-	+	+	+
Avogreen	2.5 ml	Mistblower	13 ℓ/ tree	-	+	+	+
Superfilm	0.25 ml			-	+	+	+
6) Copper							
Knowin	0.5 ml	Hand gun	70 ℓ/ tree	+	-	-	-
Copper oxychloride	3 g	Hand gun	70 ℓ/ tree	-	+	+	+

was prepared containing 18 ml nutrient broth and the recommended concentration test compound. These included 0.25 ml / ℓ Superfilm (Plaaskem), 0.25 ml / ℓ Biofilm (Plaaskem), 0.18 ml / ℓ Allgral (Hyper Agrochem) and 0.26 ml / ℓ Nufilm (Plaaskem). To the control 0.5 ml / ℓ Jik was added. One ml of a 29 x 10^6 *B. subtilis* cell suspension was inoculated into each of the tubes. A separate control was also prepared that contained no test compound. All treatments were shake incubated (160 rpm) for 24 hours at room temperature (23/25 °C). Optical density was measured as percentage transmission and the standard curve was used to calculate the *B. subtilis* concentration. The experiment was designed as a completely randomised design. The six compounds were tested for an analysis of variance (ANOVA, Table 6). Data was acceptably normal with homogeneous least significant differences (LSD) at the 1% test level of significance (Snedecor & Cochran, 1980), if the F-probability of the ANOVA was significant at 1%.

Table 4. Effect of application method on the survival of B. subtilis on the leaf surface.

Treatment	Spray date	*Bacillus* count one day after spray (cfu/cm²)	*Bacillus* count one week after spray (cfu/cm²)
Mist blower 30 L	21/11/01	70.00	0
Mist blower 15 L		30.16	0
Handgun 70 L		61.73	0
Control		0	0
Mist blower 30 L	03/01/02	*	2.55
Mist blower 15 L		*	0
Handgun 70 L		*	1.91
Control		*	0

* No sampling due to rain

Table 5. Effect of spray frequency on the survival of B. subtilis on the leaf surface.

Treatment	Spray date	*Bacillus* count one day after spray (cfu/cm²)	*Bacillus* count one week after spray (cfu/cm²)
Control	No	0	0
Avogreen x 2	21/11/01	33.98	0
Avogreen	21/11/01	18.71	0
Control	No	0	0
Avogreen x 2	3/12/01	36.27	0.64
Avogreen	No	0	0
Control	No	0	0
Avogreen x 2	14/12/01	311.82	11.45
Avogreen	14/12/01	114.55	0
Control	No	*	0
Avogreen x 2	10/01/02	*	5.09
Avogreen	No	*	0
Control	No	0	0
Avogreen x 2	23/01/02	2757.54	26.47
Avogreen	23/01/02	21.13	0

* No sampling due to rain

Field investigation

Thirty trees per treatment were selected from Orchard 74 (five-year-old Fuerte trees) on

the farm Springfield in the Limpopo Province. Copper oxychloride was applied to treatments two, three, four and five at 3 g / ℓ with spray dates corresponding with commercial spray dates for the area (Table 3). Avogreen was applied to treatment two, three and four. Superfilm was added to the Avogreen spray tank in treatment four and Biofilm to the Avogreen spray tank in treatment five. Treatment one was left unsprayed and served as the control, with treatment five serving as the chemical control. All applications were made using a high volume applicator fitted with hand lances at 50 ℓ/ tree.

Enumeration of Bacillus subtilis

Four leaf samples were used to enumerate *B. subtilis* on the phylloplane with the modified method of De Jager (1999).

Incidence of pre- and postharvest diseases on fruit

Pre- and postharvest disease assessment took place at Springfield packhouse. Fruit were rated for cercospora infection and subjected to a Gezogerm (25 ml / ℓ) bath. Export and local market conditions were simulated after which fruit was ripened and assessed for anthracnose and stem-end rot. Data from all experiments was analysed using the GenStat statistical program (2000).

Table 6. Split plot of ANOVA of 6 compounds with 3 replicates.

Source of variation	Degrees of freedom
Compound	5
Residual	12
Total	17

RESULTS AND DISCUSSION

When the observations made on each experimental unit are counts, as in the case of this study – the number of clean fruit, the Chi-square test is usually applied. The Chi-square test for the R x C table is useful when interest lies with the number of objects falling into various classes and when the differences between these frequencies must be determined. This test has certain limitations (Siegel, 1965), e.g. no more than 20% of the cells may have an expected frequency of less than five and no cell may have an expected frequency of less than one. If these limitations are not met, combination of frequencies is suggested.

1. The effect of application method on the establishment / survival of *Bacillus subtilis* on the phylloplane.

While a great deal of emphasis is placed on the development of biocontrol agents, little effort has been made to evaluate application methods for these agents that will improve disease control (Ozkan *et al.*, 2001; Bateman, 1993). In deciding which application method

Table 7. Growth curves for* B. subtilis *in the presence of spreading / sticking agents.

Time	Compound							
(hours)	Superfilm	Biofilm	Allgral	Nufilm	*B. subtilis*	Jik	F pro.	LSD
0	2.48 a	0.93 b	1.94 a	2.29 a	0.46 b	0.46 b	P> 0.001	0.944
1	1.39 ab	0.62 bc	1.85 a	1.72 a	0.54 c	0.46 c	P> 0.001	0.783
2	0.82 a	0.66 a	1.51 a	0.91 a	0.46 a	0.46 a	P> 0.001	0.882
4	0.46 a	0.46 b	0.15 a	1.20 a	0.46 b	0.46 b	P> 0.001	0.286
8	0.46 b	0.87 b	0.74 b	4.98 a	9.80 a	0.46 b	P> 0.001	0.690
10	0.46 b	4.57 a	2.15 b	8.27 a	15.51 a	0.46 b	P> 0.001	1.837
12	0.46 b	0.66 b	5.01 a	9.81 a	15.35 a	0.46 b	P> 0.001	0.856
24	13.1 b	11.8 b	20.4 a	11.1 b	22.50 a	0.46 c	P> 0.001	2.148

Values in the same row followed by the same letter do not differ at the 1% level of significance

to use, a good starting point is to consider the delivery systems already in place for fungicide application during avocado disease management (Pusey, 1994). In order to evaluate the effect of application methods currently employed during avocado disease control on the survival of *B. subtilis*, population fluctuations were monitored. Table 4 illustrates results obtained for *B. subtilis* enumeration from the avocado phylloplane following different application methods. Samples could not be collected one day after the second spray due to rain.

Within one week of the first application, the antagonist decreased to undetectable levels in all treatments. This phenomenon continued throughout the trial in the 15 ℓ/ tree mist blower treatment. The effect of spray volumes was reflected when 30.16 cfu / ml *Bacillus subtilis* survived after spraying with a mist blower at 15 ℓ/ tree and almost double this amount (70 cfu/ml) after spraying at 30 ℓ / tree. Spray volumes therefore seemed to play a considerable role in the number of detectable bacteria surviving on the leaf surface. Application of Avogreen at 30 ℓ/ tree with a mist blower and at 70 ℓ/ tree with hand guns proved to be equally favourable for the establishment and survival of the antagonist on the phylloplane. Employment of these two methods resulted in detection of the antagonist on the leaf surface for up to one week after the second spray. A significant Chi-square test indicated that frequencies for preharvest and postharvest disease rating differed significantly ($P < 0.001$) at the 0.01% test level. No significant differences between treatments could be established for cercospora spot rated fruit or stem-end rot and anthracnose rated on local market fruit. Data is therefore not shown. Although not significant, the 30 ℓ mist blower treatment ($P = 0.111$) and 70 ℓ hand gun treatment ($P = 2.452$) showed a higher level of stem-end rot control than the 15 ℓ mist blower treatment in fruit simulated for export conditions (Figure 1). The hand gun application was probably more effective in reaching stem ends and resulted in slightly higher control efficiency than the mist blower. It could also be due to higher volumes used.

The 30 ℓ mist blower treatment differed significantly from the 15 ℓ mist blower treatment and the chemical control in export fruit

Table 8. Effect of spreading and sticking agents on the survival of* B. subtilis *on the leaf surface.

Treatment	Spray date	*Bacillus* count one day after spray (cfu/cm²)	*Bacillus* count one week after spray (cfu/cm²)
Control	01/12/01	0	0
Avogreen		29.27	14.64
Avogreen + Biofilm		30.80	17.18
Avogreen + Superfilm		29.53	0.64
Control	02/01/02	0	0
Avogreen		115.82	23.80
Avogreen + Biofilm		138.73	9.29
Avogreen + Superfilm		83.37	1.27
Control	15/02/02	0*	0
Avogreen		48.75*	26.73
Avogreen + Biofilm		53.46*	7.64
Avogreen + Superfilm		59.31*	2.93

* Sampled four days late due to rain

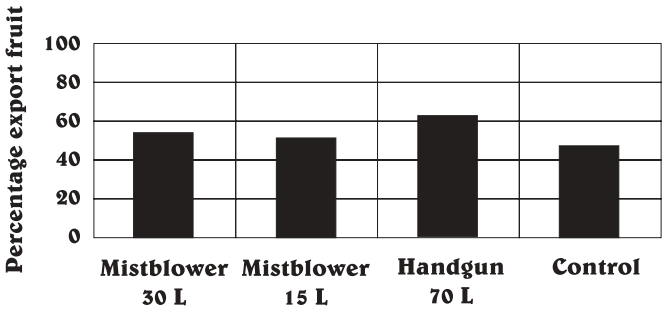

Figure 1. Effect of application method on biocontrol efficacy on export market simulated fruit rated for stem-end rot.

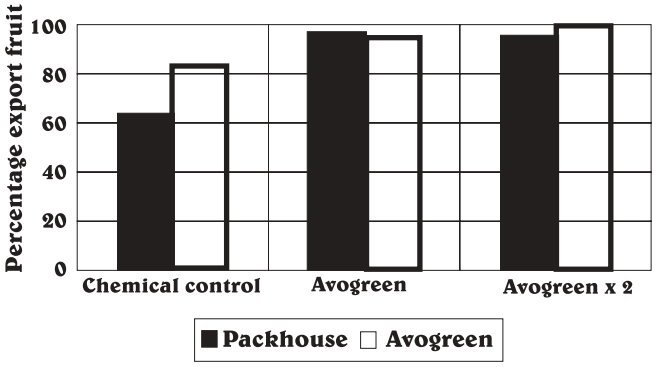

Figure 3. Effect of spray frequency on biocontrol efficacy on export market simulated fruit rated for stem-end rot.

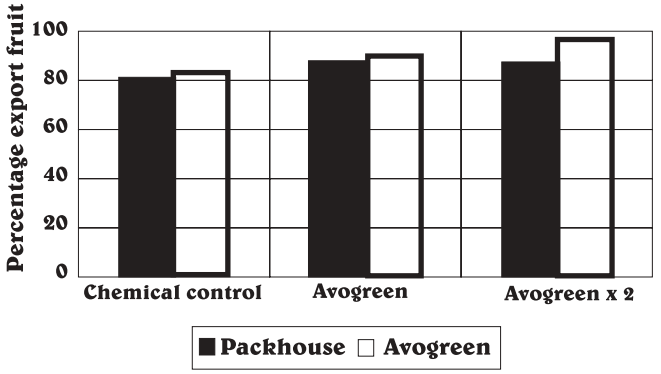

Figure 5. Effect of spray frequency on biocontrol efficacy on fruit simulated for the export market and rated for anthracnose.

rated for anthracnose (Figure 2). Although *Bacillus subtilis* population densities proved to be slightly higher in the 30 ℓ mist blower treatment (Table 4), the number of clean fruit did not differ significantly from the 70 ℓ hand

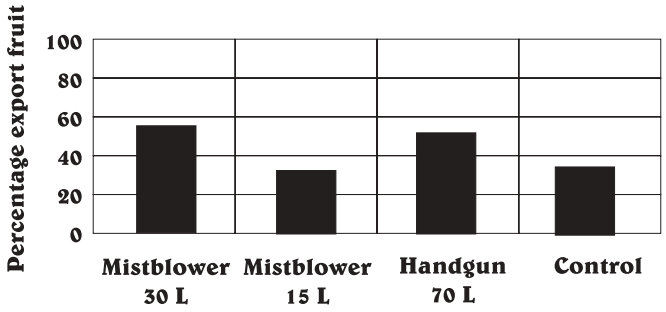

Figure 2. Effect of application method on biocontrol efficacy on export market simulated fruit rated for anthracnose.

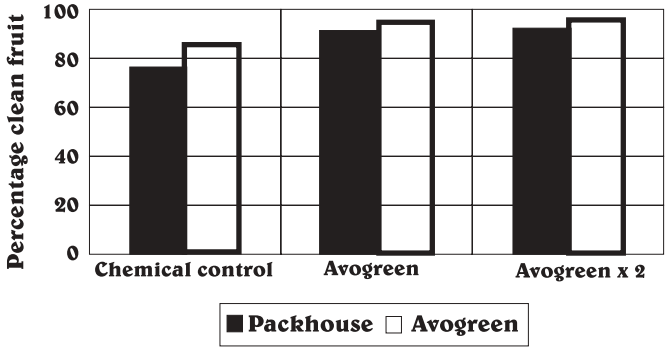

Figure 4. Effect of spray frequency on biocontrol efficacy on local market simulated fruit rated for anthracnose.

gun treatment (P = 0.157) (Figure 2). Results indicated that the 30 ℓ mist blower application and the 70 ℓ hand gun application methods may be equally sufficient in ensuring antagonist survival on the leaf surface. Small droplets produced during mist blower application may however lack sufficient energy to deposit deep into a canopy and may result in uneven distribution of the antagonist. These droplets are also more susceptible to wind movement (Ozkan *et al.*, 2001, Dent, 1993). To ensure effective pest control, optimal coverage of the target area must be accomplished (Dent, 1993). Practical considerations such as cost effectiveness, spray volume, concentration of the antagonist and penetration efficiency would therefore determine the preferable method of application.

2. The effect of spray frequency on the establishment / survival of *B. subtilis* on the phylloplane.

When Avogreen was sprayed according to the normal spray program for the area (monthly), results obtained for *Bacillus subtilis* survival on the phylloplane (Table 5) corresponded with results obtained during the mist blower and handgun trial (Table 4). The antagonist decreased to undetectable levels within one week after Avogreen was applied at 15 ℓ / tree with a mist blower. Increased spray frequency however had a pronounced effect on establishment of the antagonist. The number of viable cells on the phylloplane increased after each spray and frequently resulted in a higher survival level one week later. Counts decreased following the unsprayed period in December (farm was closed). When spraying commenced again, a large number of bacterial cells were able to establish on the phylloplane (2757 cfu/ml). A significant Chi-square test indicated that frequencies for pre- and postharvest disease rating differed significantly ($P = 0.05$) at the 5% test level. No significant differences could be established between treatments for cercospora spot rated fruit or local market fruit rated for stem-end rot and data is therefore not shown.

Van Dyk, *et al.* (1997) found that *Bacillus subtilis* was able to control anthracnose and stem-end rot as effectively or better than chemical packhouse treatments. During this study, the only significant difference between the packhouse bath and Avogreen bath was obtained for the bi-weekly Avogreen treatment in export simulated fruit rated for anthracnose (Figure 5). The tendency was, nevertheless, observed for most of the treatments. Avogreen may therefore provide a more environmentally friendly disease control procedure for growers / packers. Market acceptability requirements must however still be met.

Field application of biocontrol agents enables early colonisation of the antagonist, resulting in early protection of the fruit surface. This may prove to be an appropriate strategy for fruits subjected to damage or spoilage during postharvest handling (Jiang *et al.*, 2001; Ippolito & Nigro, 2000). Pre-harvest application of Avogreen, although not significant for all treatments, appeared to reduce the incidence of stem-end rot and anthracnose on postharvest fruit (Figures 3–5). Observations made after the packhouse bath, proved that pre-harvest application of *B. Subtillus* significantly reduced the incidence of stem-end rot in export fruit (Figure 3) and anthracnose (Figure 4) in local market fruit, when compared to the chemical control. Fruit subjected to an increased pre-harvest application rate resulted in an even higher percentage of disease free fruit. Following the Avogreen bath, fruit from the bi-weekly Avogreen application were significantly cleaner than the control in export fruit rated for stem-end rot (Figure 3) and local market fruit rated for anthracnose (Figure 4).

Increased pre-harvest application in all instances resulted in a higher percentage disease free fruit, but no significance could be established between treatments. This implied that repeated pre-harvest application of the antagonist might contribute to a faster build-up of numbers on the phylloplane (Table 5) and therefore improved disease control. If establishment occurred and survival is high, *B. subtilis* may be able to protect the fruit against disease, from field treatment, through to storage and during the entire shelf life when integrated with a pre-harvest disease control program.

3. The effect of spreading and sticking agents on the establishment / survival of *B. subtilis* on the phylloplane.

Spreading and sticking agents are frequently used to enhance the effectiveness of agrochemicals during chemical disease control (Kucharek, 1997). This is a simple and effective method that is also often used to increase the efficacy of biocontrol agents (Ippolito & Nigro, 2001). Spreaders and stickers generally prevent wash off after rain (Kucharek, 1997) and can therefore reduce fruit losses (Mathews, 1979). The desired re-

sult is increased survival rate and larger population sizes of the antagonist.

The standard curve obtained for *B. subtilis* proved to be linear (r = 0.9956) between bacterial concentrations of 0.6 x 10^{-6} and 26 x 10^{-6} cfu / ml. Growth curves compiled for *B. subtilis* in a liquid medium containing different spreading and sticking agents are illustrated in Figure 6 and Table 7. After inoculation of a bacterial culture into fresh medium, growth only begins after an initial lag phase. During this time period bacteria usually synthesise new enzymes and repair damaged cells (Brock *et al.*, 1994). When inoculated into fresh medium without any spreaders / stickers, the lag phase for *B. subtilis* lasted approximately four hours (Table 7, Fig. 6). During the lag phase of *B. subtilis*, cultures containing Superfilm, Allgral and Nufilm showed significantly higher bacterial counts than the control. This lasted for two hours, after which a negative growth rate was followed for the following two hours. The *B. subtilis* culture then entered an exponential (growth) phase. The phase was characterised by two distinct stages: a stage of rapid growth (growth rate of 2.3 cfu/ml/h) and a second slower stage with a growth rate of 0.8 cfu/ml/h. The exponential phase generally results from repeated cell division within the culture and is a function of environmental influences and the availability of nutrients (Brock *et al.*, 1994). The only other culture that entered the exponential phase at this time period, and didn't differ significantly from the *B. subtilis* culture, was the Nufilm culture with a growth rate of 0.9 cfu/ml/h. After 12 hours the Nufilm culture reached the stationary phase (no net increase or decrease in cell numbers) that proceeded for the remaining of the experiment and displayed a bacterial count significantly lower than in the *B. subtilis* culture. Growth in the Allgral culture commenced into an exponen-

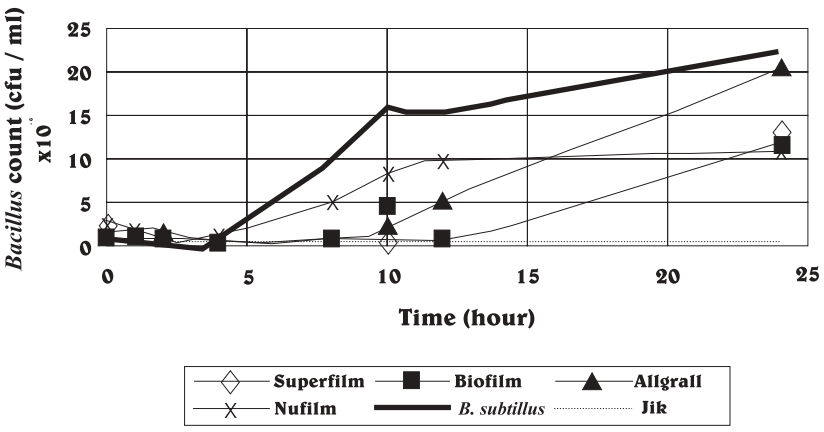

Figure 6. Growth curves plotted for* B. subtilis *in the presence of spreading / sticking agents.

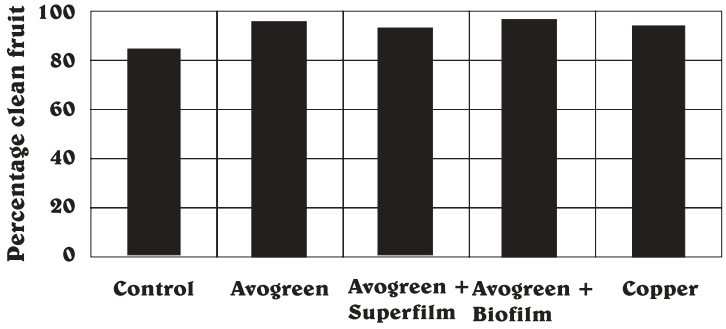

Figure 7. Effect of spreading and sticking agents on biocontrol efficacy on fruit simulated for the local market and rated for stem-end rot.

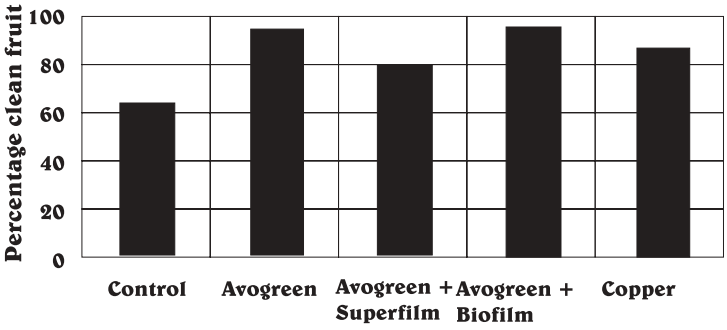

Figure 8. Effect of spreading and sticking on biocontrol efficiency on fruit simulated for the local market and rated for anthracnose.

tial fashion after 10 h, with a growth rate of 0.7 cfu/ml/h. The Biofilm culture initially fluctuated at low levels and entered the exponential phase after 10 h, displaying a growth rate of 0.8 cfu/ml/h. The exponential phase for Superfilm also started after 10 h, with the same growth rate as the Biofilm culture. If incubation continued for these cultures, the death phase would follow on the stationary phase. This is the phase during which toxic products build up, nutrients becomes depleted and growth ceases (Brock *et al.*, 1994). It was therefore concluded that the use of spreading and sticking agents didn't promote the growth of the antagonist, especially during the first four hours. This is the critical time period during which field spray would occur.

Table 8 shows the survival of *B. subtilis* during in the phylloplane during the field spray. Higher numbers of bacteria were observed after each succeeding application of Avogreen. The addition of Superfilm to the spray tank resulted in a clear decrease in numbers of the antagonist over time. The same tendency was shown by the treatment that contained Biofilm, but to a lesser extent.

A significant Chi-square test indicated that frequencies for pre- and postharvest disease rating differed significantly ($P < 0.001$) at the 0.01% test level. No significant differences between treatments could be established for cercospora spot rated fruit or fruit simulated for export conditions and rated for stem-end rot and anthracnose. Data is therefore not shown.

Although not significant, local fruit rated for stem-end rot exhibited a lower percentage of clean fruit when Superfilm was used in the spray program (Figure 7) than when it was used without spreading / sticking agents. This observation was more significant in local market fruit rated for anthracnose (Figure 8). The behaviour of the antagonist on the leaf surface in the presence of Superfilm therefore correlated with the growth curve obtained for *B. subtilis* in liquid cultures containing Superfilm (Figure 6). Addition of Biofilm to the spray tank did not seem to have a pronounced effect on the antagonist. Similar results were obtained in a separate field trial on guava's, where the use of Superfilm and Nufilm inhibited antagonist survival on the phylloplane (unpublished data). It is therefore strongly recommended to exclude the use of spreading and sticking agents during the application of *B. subtilis* as biological control agent on avocado. The mechanism of this phenomenon is unknown. According to Matthews (1979), the use of spreaders and stickers may lead to the protection of the deposit to such an extent that the availability of it to a pest is reduced. There is therefore a possibility that *B. subtilis* is isolated to such an extent from its immediate environment, nutrients and even the pathogen by the sticker / spreader, that it is difficult for the organism to survive or act as biocontrol agent.

CONCLUSION

The phyllosphere is a very harsh environment with limited nutrients and enormous fluctuations in environmental conditions (Collins & Jacobsen, 2003). A trait of a good biological control agent is the ability to colonise and survive in this environment. Poor commercial success is mostly due to insufficient colonisation, but can be enhanced through management of the agricultural practices employed (Ippolito & Nigro, 2000). This study proved that agricultural practices (parameters) having an effect on the ability of *B. subtilis* to colonise the phyllosphere include application method, spray frequency and the use of additives. Manipulating the behaviour of a *B. subtilis* to enhance its disease controlling ability in the agro-ecosystem should include increased spray frequency and the exclusion of spreading and sticking agents during the spray program. The preferable application method should be optimised regarding application volume.

During this study the leaves supported bacterial counts lower than 100 cfu / cm^2 after the first week. Collins *et al.*, (2003) made the

same observation for *B. subtilis* on sugar beet. They postulated that disease control might be the result of a resistance mechanism that is not necessarily dependent on complete leaf coverage. They also found that high population numbers were more likely to occur in the presence of a nutrient source.

Future research will focus on the relationship between leaf coverage and biocontrol. Application volumes, recommended concentration, the effect of high-pressure application on the bacterial cell, the ability of mist blower spray to reach the inside canopy of a tree and the effect of weather conditions on antagonist behaviour will also receive attention.

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