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Effect of additives and copper fungicide on *Bacillus subtilis* to control avocado (*Persea Americana* Mill.) fruit diseases

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ABSTRACT

Effective biological control programmes depend on successful establishment of the antagonist on the plant surface. Colonising strategies of these organisms are the key to effective biocontrol systems. In order to ensure more consistent levels of disease control, it is important to understand what influence additives might have on in situ *performance of the product. By applying the antagonist earlier (winter spray) it might contribute to more effective colonisation by the antagonist. During this study, it was confirmed that the use of spreading and sticking agents do not enhance attachment and colonisation of* B. subtilis *and that the use of a winter spray did not improve biocontrol effectiveness. The combination of* B. subtilis *and copper oxychloride had a negative effect on the survival of the organism, while Carbendazim had no effect.*

INTRODUCTION

Amongst the first successful biopesticides in agriculture were members of the genus *Bacillus.* Because of the safety and effectiveness of these products, there has been a steady increase across the world in their use. By 1994, Kodiak (*Bacillus subtilis*) was already used on two million hectares of crops in the US (Emmert & Handelsman, 1999). *Bacillus subtilis* has been registered worldwide as a biocontrol agent and as a plant stimulant on various crops. These include general registrations such as Epic, Kodiak and System 3 (USA); MBI 600 and Stimulex (United Kingdom); Rhizo-Plus (Germany); Rotor (Thailand); *Bacillus subtilis* GBO3 (USA) registered against *Rhizoctonia, Fusarium, Alternaria* and *Aspergillus* on cotton, peas, and beans; and Serenade® registered against bacterial spot on tomatoes and grapes (Davis, 2003; Agrobiologicals, 2000; US EPA, 1999). The success of these products is contributed to their ease of formulation and storage. Because of the ability of *B. subtilis* to form spores, these products are characterised by a consistent shelf life (Emmert & Handelsman, 1999; US EPA, 1999; Korsten & Cook, 1996). Another advantage of this biocontrol agent is the absence of visible residues on fruit (Korsten & Cook, 1996). Since *B. subtilis* functions as a preventative fungicide, it can be applied up to the day of harvest (Buyot, 2003). Pre-harvest application for postharvest disease control has also proved to be affective due to its protective colonisation attributes (Yiang *et al*., 2001).

In South Africa, uncertainty over the ability of the biocontrol agent Avogreen (a.i. B. subtilis B426) to perform under variable environmental conditions has restricted the use of this organism in disease control programs. Performance is mainly influenced by the ability of the organism to colonise and survive in the environment (Ippolito & Nigro, 2000). Timing of application and the use of additives included in biocontrol applications may optimize colonisation and improve survival strategies. Knowledge of these interactions may result in prediction of antagonist performances. In this study, the effect of additives such as spreading and sticking agents and fungicides on the antagonist's ability to effectively colonise and control disease was investigated, as was the feasibility of a winter spray application.

MATERIALS AND METHODS

Effect of fungicides on the survival of *Bacillus subtilis*

Antagonist survival in the presence of chemical fungicides was studied in a liquid medium. For each treatment a test tube was prepared that contained 18 ml sterile nutrient broth (Biolab) and one millilitre of a 29 x 10^5 *B. subtilis* (Stimuplant, Pretoria) culture. Coprox Super

(Copper Oxychloride, Saadchem) (powder formulation) was added to a total concentration of 3 g/L in treatment 1, Knowin 500SC (Carbendazim, Plaaschem) (systemic fungicide) to a total concentration of 0.5 ml/L in treatment 2 and Jik (Sodium hypochlorite: 3.5% m/v, Reckitt Benckiser™) to a total concentration of 0.5 ml/L in treatment 3 (negative control). Treatment 4 (positive control) contained only the *B. subtilis* culture without any test compound. All treatments were shake incubated (160 rpm) for 24 hours at room temperature. *Bacillus subtilis* was enumerated from each of the flasks at time intervals 0, 1, 2, 4, 8, 12 and 24 hours on Standard 1 Nutrient agar and incubated at 24ºC for 48 hours. The experiment was repeated three times. Data from the treatments was evaluated using analysis of variance (ANOVA). Data was acceptably normal with homogeneous significant differences (LSD) at the 1% test level of significance (Snedecor & Cochran, 1980), if the F-probability of the ANOVA was significant at 1%.

In a second experiment the effect of additives on the growth of the antagonist was investigated in a disk inhibition experiment. Sterile Standard 1 Nutrient Agar (Biolab) (20 ml) was poured into Petri dishes and cooled down to approximately 30ºC before one milliliter of a 29 x 10^7 *B. subtilis* was added. After gently rotating the dishes and allowing the medium to solidify, six holes were made in the agar using a sterile cork borer (10 mm diameter). Different concentrations of the test compound (200 µl) were inoculated into each of the holes. Test compounds included Coprox Super at 100% (dampened with sterile distilled water), 6 g/L; 3 g/L and 1.5 g/L and Knowin 500SC at 100%, 1 ml/L; 0.5 ml/L and 0.25 ml/L. Jik (undiluted) and sterile distilled water was inoculated into holes four and five as the positive and negative control. Twelve replicates were prepared for each compound. Plates were incubated for 24 hours at 24ºC after which the formation of clear zones around holes were measured as an indication of growth inhibition. The experiment was repeated and data were analysed as described before.

Effect of additives and a winter spray to improve Avogreen product performance

Percentage disease free fruit was monitored in a commercially sprayed orchard in order to determine the enhanced effect of spreading and sticking agents and agricultural oil on the survival and efficacy of *B. subtilis*.

Thirty trees per treatment were randomly selected from Orchard 74 (5-year-old Fuerte trees) on the farm Springfield in the Limpopo Province. Avogreen (10^8 cells/ml Stimuplant, Pretoria) was applied to treatments 1, 2, 3 and 5 at the time intervals indicated in Table 1. Superfilm (Plaaschem) (sticker / spreader) was added to the Avogreen spray tank in treatment three and Citrex (Plaaschem) (oil / spreader) to the Avogreen spray tank in treatment two. Coprox Super was applied to treatment four (chemical control) at 3 g/L. All spray schedules corresponded with the dates for the commercial programme for the area (Table 1). An additional Avogreen application was included in treatment 5 during August 2002 (3 months before the trail commenced). Treatment 6 was left unsprayed and served as the control. All applications were made using a mistblower at 13 L/tree. Fruit was harvested during the commercial harvest season.

Pre- and postharvest disease assessment took place at Springfield packhouse. Fruit was rated

Table 1. Spray program for pre-harvest applications of copper oxychloride and Avogreen (*Bacillus subtilis*) during the 2002/03 season

Treatment no	Treatment program	Rate	Month of application				
			Aug	Oct	Nov	Dec	Jan
1	Avogreen	5 ml /L	-	-	+	+	+
2	Avogreen and Citrex	5 ml/L 0.25 ml/L	-	-	+	+	+
3	Avogreen and Superfilm	5 ml/L 0.25 ml/L	-	-	+	+	+
4	Chemical control Copper Super Knowin 500SC	 3 g/L 0.5 ml/L	 - -	 - +	 + -	 + -	 + -
5	Avogreen winter	0.5 ml/L	+	-	+	+	+
6	Control*		-	-	-	-	-

(* not employed for post-harvest evaluation).

for Cercospora infection before subjecting it to a Desogerm™ (Quarternary ammonium compund) (25 ml / L) bath. Fruit from each treatment were commercially packed before the treatments were split into two batches. One batch were kept at room temperature for immediate ripening to simulate local marketing conditions and the other were first commercially stored at 5ºC at the packhouse for 28 days prior to ripening at room temperature to simulate export conditions. Once ready to eat all fruit was assessed for anthracnose and stem-end rot.

RESULTS AND DISCUSSION

Effect of fungicides on the survival of *Bacillus subtilis*

The commercial biocontrol agent followed a characteristic growth curve (Figure 1) when inoculated into a liquid medium. This experiment represents a closed biological system since no new nutrients are added and no metabolic waste products are removed (Pelczar *et al*., 1993). The four phases of growth in such a system are collectively called the growth curve (Pelczar *et al*., 1993; Brock *et al*., 1994). The first phase (known as the lag phase) is characterised by a period of no growth (bacteria synthesize new enzymes and repair damaged cells), followed by the exponential phase, a period of rapid growth. During the stationary phase, nutrients are depleted from the environment up to the point where growth decreases until all the micro-organisms die (death phase) (Pelczar *et al*., 1993; Brock *et al*., 1994). In this study it was found that the lag phase lasted approximately seven hours, followed by a very rapid exponential growth phase of three hours. The stationary phase was found to be very short (approximately 1 hour) and the death phase was characterised by a steady decrease in microbial numbers between time intervals 12 and 24 hours. When *B. subtilis* was combined with copper and Knowin 500SC to simulate field applications, the test culture containing Knowin 500SC, followed the same standard growth curve for *B. subtilis*, but with a longer exponential phase. It can therefore be concluded that a combination of Knowin 500SC and *B. subtilis* mixed in the same spray tank would not negatively affect the biocontrol product. Coprox Super proved to have a negative effect on the growth of *B. subtilis*. These results were supported by the disk inhibition experiment (Table 2) where Knowin 500SC had no effect on the growth of *B. subtilis* at any of the test concentrations. However, the addition of Coprox Super showed an inhibition zone of 3.25 mm at 100% concentration, although no visible effect was observed at lower concentrations.

Current avocado disease control programs recommend the integration of copper oxychloride and Avogreen (Korsten *et al*., 1997). These products are applied in alternate sprays but have in certain cases been applied mixed into one spray tank. Previous studies by Korsten *et al*. (1992) indicated that pre-harvest application of Avogreen on its own or integrated with fungicide sprays (copper oxychloride or Benomyl) effectively reduced Cercospora severity. Results

Table 2. Inhibition zones for* Bacillus subtilis *in the presence of Coprox Super, Knowin 500SC and Jik

Treatment No	Compound	Rate	Zone / Growth
1	Coprox Super	100%	3.25 mm inhibition zone, normal growth
2	Coprox Super	6 g / L	Normal growth
3	Coprox Super	3 g / L	Normal growth
4	Coprox Super	1.5 g / L	Normal growth
5	Knowin 500SC	100%	Normal growth
6	Knowin 500SC	1 ml / L	Normal growth
7	Knowin 500SC	0.5 ml / L	Normal growth
8	Knowin 500SC	0.25 ml / L	Normal growth
9	Jik	100%	3 mm inhibition zone, normal growth
10	Distilled water		Normal growth

Table 3. Split plot of ANOVA of 4 compounds with 3 replicates

Source of variation	Degrees of freedom
Compound	3
Residual	8
Total	11

Table 4. Growth curves for* Bacillus subtilis *in the presence of Coprox Super, Knowin 500SC and Jik

Time (hours)	Compound				F pro.	LSD
	Knowin 500SC	Coprox Super	*B. subtilis*	Jik		
0	0.5 a	0.5 a	0.24 b	0.0 b	P < 0.001	0.2565
1	4.67 a	5 a	6.67 a	0.0 b	P = 0.012	5.046
2	0.04 b	0.05 b	0.3 a	0.0 c	P < 0.001	0.0316
4	0.63 a	0.01 b	1.07 a	0.0 a	P = 0.001	0.492
8	2.87 a	0.0 b	2.07 a	0.0 b	P = 0.008	2.399
12	646.7 a	0.0 b	516.7 a	0.0 b	P = 0.002	464.9
24	233.3 a	0.0 b	186.7 a	0.0 b	P = 0.002	165.9

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

obtained in this study indicate that the direct combination of copper formulations and the antagonist in one spray tank can have an inhibitory effect on the antagonist growth. Combination of the two products in the same spray tank should therefore be avoided. However, alternate spraying of copper and then Avogreen is still the most effective program ensuring effective control and reduced copper sprays. Future research will focus on the effect of different copper compounds on the organism and the effect of timing intervals between applications in an integrated program.

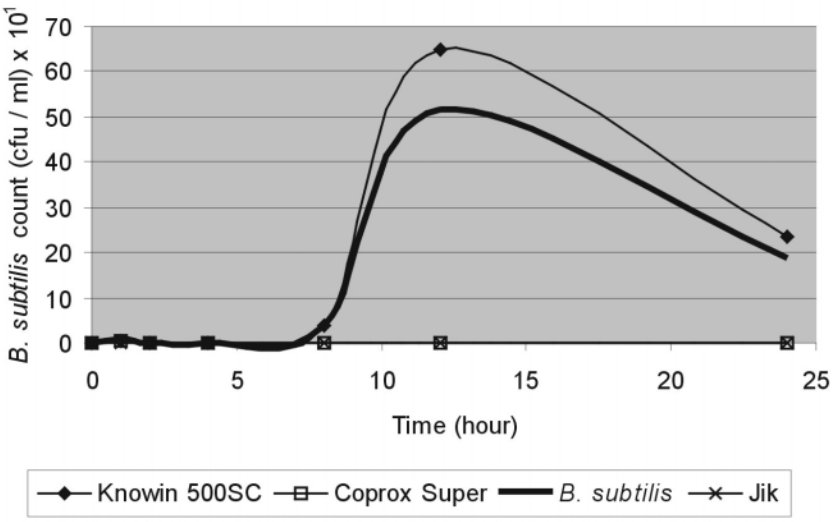

Figure 1. Growth curves plotted for* B. subtilis *in the presence of Knowin 500SC, Coprox Super and Jik

Effect of additives and a winter spray to improve Avogreen product performance

All treatments significantly reduced Cercospora spot (Figure 2). No significant difference could be found between the various Avogreen and copper sprays with all giving 100% or 99% control compared to the 12% for the untreated treatment. This data is in agreement with previous reports on the effective control of avocado fruit diseases using *B subtilis* pre-harvest field sprays *(*Korsten *et al*., 1997). When a sample of individuals is classified according to two attributes results are presented in a two-way frequency table known as an r x c contingency (Snedecor & Cochran, 1980). In this study, individual values were classified according to each treatment and to percentage clean or export fruit. This information was required to determine if the distribution of clean fruit is the same for each treatment. The Chi-squire row-by column test is useful to determine if there are significant differences between the two independent attributes. This test has certain limitations (Siegel, 1956), namely, no category may have an expected frequency of less than three.

Control of anthracnose and stem-end rot proved to be more effective under simulated export compared to local simulated marketing conditions (Figure 2 & 3). This was probably due to lower storage temperatures employed during simulated export conditions, which previously

have shown to benefit disease control Denner *et al.* (1986). At low temperatures, micro-organisms present on fruit surfaces become metabolically inactive (Atlas & Bartha, 1993), resulting in growth inhibition or death. Spore germination of *Colletotrichum gloeosporioides* (anthracnose) and *Dothiorella aromatica* (stem-end rot complex) are severely affected by temperature. The optimum growth temperature for these pathogens is approximately 28ºC. Temperatures above 30ºC and below 10ºC inhibit growth and no growth will occur due to delay of spore germination at 5ºC (Denner *et al.,* 1986). The optimal growth temperature for *B. subtilis* is 30ºC to 37ºC, with a minimal temperature of 18ºC and a maximum temperature of 43ºC (Korsten & Cook, 1996). Sporulation takes place at temperatures above optimal growth temperatures and under the optimal growth temperatures. The spores of *B. subtilis* are known for their resistance and survival potential at extreme temperatures (Atlas & Bartha, 1993), which may become viable at more favourable environmental conditions, favouring early colonisation (before other micro-organisms) on the plant surface (Atlas & Bartha, 1993; Agrobiologicals, 2000).

Results found in this study indicate that the use of spreaders and stickers (Superfilm and Citrex) might reduce disease control of *B. subtilis* (Figure 3 & 4). The chi-square test was significant (χ^2 = 17.8; degrees of freedom = 4; P = 0.001) for local market fruit rated for stem-end rot. The use of Superfilm reduced the ability of *B. subtilis* to control stem-end rot and anthracnose. Although the use of Citrex increased the antagonist's ability to control stem-end rot in fruit simulated for export conditions, the percentage clean fruit was lower than the Avogreen treatment in the other disease assessments. These findings support previous results (Van Eeden & Korsten, 2003) that the use of these compounds could have an inhibitory effect on the survival of *B. subtilis*.

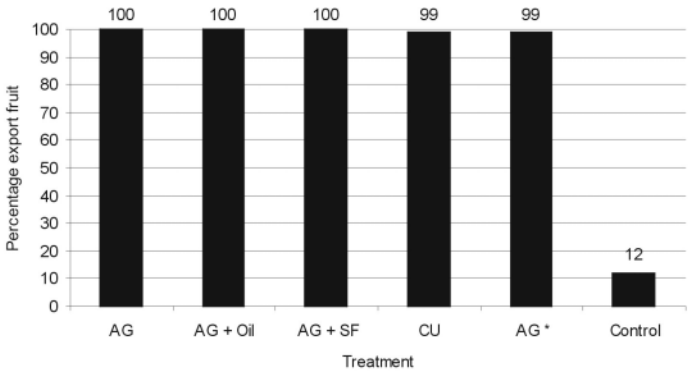

Figure 2. Effect of Superfilm, Citrex and a winter spray on biocontrol efficacy on fruit rated for Cercospera spot (AG = Avogreen, AG + OIL = Avogreen + Citrex, AG + SF = Avogreen + Superfilm, CU = Coprox Super, AG*= Avogreen with winter spray)

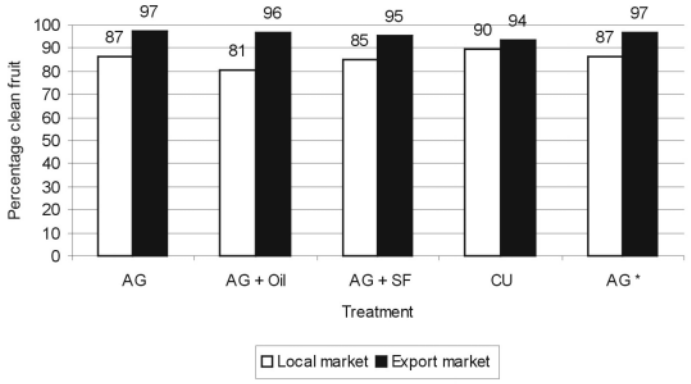

Figure 3. Effect of Superfilm, Citrex and a winter spray on biocontrol efficacy for anthracnose (AG = Avogreen, AG + Oil = Avogreen + Citrex, AG + SF = Avogreen + Superfilm, CU = Coprox Super, AG* = Avogreen with winter spray)

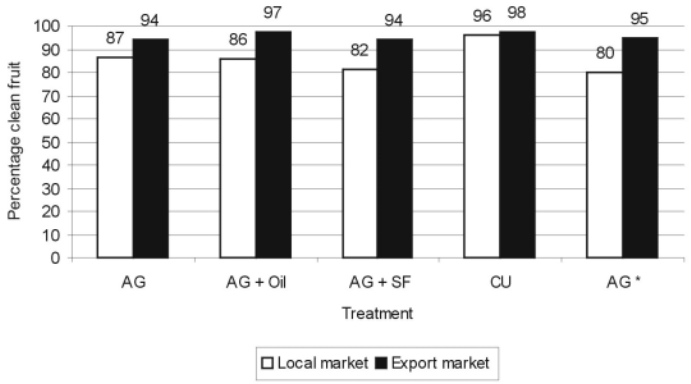

Figure 4. Effect of Superfilm, Citrex and a winter spray on biocontrol efficacy for stem-end rot (AG = Avogreen, AG + Oil = Avogreen + Citrex, AG + SF = Avogreen + Superfilm, CU = Coprox Super, AG* = Avogreen with winter spray)

It is generally assumed that early application allows early colonisation of the antagonist, resulting in early protection of the fruit surface and therefore enhancing protective biological control (Jiang *et al*., 2001; Ippolito & Nigro, 2000). Timing of application is also of crucial importance during biological control (Bhatt & Vaughan, 1962). The winter spray application did however not result in increased biocontrol efficiency for anthracnose or stem-end rot (Figures 3 & 4). In the present study application dates were mainly based on spray dates for the commercial spray programme for the area. The significant reduction of Cercospora infection in all treatments (Figure 2) may indicate that Cercospora spore release did not occur between the winter application and the start of the first seasonal sprays, but only thereafter. It also suggests that the normal biological and chemical spray programmes are sufficient in addressing infection at later stages. The winter spray had no enhanced effect on post-harvest disease control and although stem-end rot was lower for local market fruit, this was not significant. Optimised application will in the future be aimed at spore release patterns for Cercospora in this area.

CONCLUSION

The ability of *B. subtilis* to serve as a biocontrol agent against anthracnose, stem-end rot and Cercospora, was clearly demonstrated in this study. Coprox Super and spreading / sticking agents did not enhance Avogreen performance, and neither did an additional winter spray.

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