CHAPTER 7

GENERAL DISCUSSION

Avocado, *Persea americana* Mill., is an economically important crop in South Africa (Keevy, 1999). Major losses occur due to pre- and postharvest diseases. Postharvest diseases include stem-end rot (SE), caused by *Dothiorella aromatica* (Sacc.) Petrak & Sydow (Darvas & Kotzé, 1987; Korsten *et al.,* 1995), *Thyronectria pseudotrichia* (Schw.) Seeler, *Lasiodiplodia theobromae* (Pat.) Griffon & Maubl., *Colletotrichum gloeosporioides* Penzig., *Phomopsis perseae* Zerova, *Pestalotiopsis versicolor* (Speg.) Steyaert, and *Fusarium* species. Anthracnose is caused by *C. gloeosporioides*. The pathogens causing avocado diseases are mainly controlled using chemical pesticides. With the worldwide movement away from excessive use of chemicals, a need for alternative control strategies evolved in the avocado industry. Biocontrol has been investigated as an alternative approach, especially when combined within an integrated pest management system (Roberts, 1994). In 1987, a *Bacillus subtilis* isolate was found on avocado leaves that showed promise as a biocontrol agent for control of avocado fungal diseases (Korsten, 1993). The isolate was evaluated and found effective in laboratory and semi-commercial trials, both in the field and in the packhouse (Korsten *et al.*, 1989; Korsten *et al.*, 1993; Korsten *et al.*, 1994; van Dyk *et al.*, 1997). Disease control using the biocontrol agent was often comparable to that achieved with commercially used chemicals, but occasionally results were found to be variable.

The main aim of this thesis was to investigate the modes of action involved in the antagonism of *B. subtilis* against postharvest pathogens of avocado. Previous studies showed that various modes of action might be involved (Korsten & de Jager, 1995; Havenga *et al.*, 1999). Since there is a wide range of pathogens involved in pre- and postharvest diseases of avocado, various infection patterns can be used, making effective control difficult. The biocontrol agent needs to act in both a preventative and curative way. The modes of action of *B. subtilis* against fungal postharvest avocado pathogens found in this study may play a role in both.

For biocontrol to be most effective, the biocontrol agent needs to be present before the pathogen arrives, when using the preventative approach. The antagonist needs to out compete the pathogen for space and nutrients. *In vivo* studies on avocado fruit using scanning electron microscopy (SEM) showed that where *B. subtilis* was present before the pathogen, conidia of *C. gloeosporioides* were unable to germinate.

Competition for nutrients was proposed as one of the main modes of action. However, *C. gloeosporioides* is a specialized necrotrophic pathogen and does not require any additional nutrients for germination (Blakeman, 1985). In *in vitro* studies, it was found that *B. subtilis* produces inhibitory substances. These substances may prevent conidia of *C. gloeosporioides* from germinating. It was also found that *B. subtilis* produces antifungal volatiles that may play a further role in the inhibitory activity. This finding is in accordance with Fiddaman & Rossall (1993) who evaluated volatile substances produced by *B. subtilis* that inhibits the growth of *Rhizoctonia solani* (J. G. Kühn) and *Pythium ultimum* (Trow). However, if the pathogen has already established an infection, the curative approach is the only alternative to ensure control.

Enzyme activity of *B. subtilis* was investigated in this study and chitinase, amylase, protease and lipase activity was found. Various lytic enzymes are reported to be produced by *B. subtilis* and are implicated in biocontrol, including chitinase (Frändberg & Schnürer, 1998; Helistö *et al.*, 2001), chitosanase, laminarinase, lipase and protease (Helistö *et al.*, 2001), as well as glucanolytic and proteolytic enzymes (Nielsen & Sørensen, 1997). These enzymes may cause the damage to *C. gloeosporioides* hyphae where *B. subtilis* attached itself directly onto the fungal cell wall which was observed during direct interaction studies on avocado fruit observed using SEM. In this study, cell-free inhibitory metabolites as well as inhibitory volatile substances were found to be produced by *B. subtilis* and may also act directly on a cellular level against the pathogen.

Since *B. subtilis* is a well-known producer of antibiotic substances, antibiotic production by the biocontrol agent was investigated. Most antibiotic substances produced by *B. subtilis* are peptides and lipopeptides (Katz & Demain, 1977; Shoji, 1978; Peypoux *et al.*, 1984; Loeffler *et al.*, 1986; Sakajoh *et al.*, 1987; Chen *et al.*, 1995; Tsuge *et al.*, 1996). Few studies focused on phenolic compounds utilised by biocontrol agents (Pinchuk *et al.*, 2002). In this study, a combination of thin layer chromatography (TLC) and high performance liquid chromatography combined with UV illumination was used to study free acid phenolic compounds produced by *B. subtilis*. Four fluorescent spots were observed on silica coated glass plates using acetic acid:methanol:water (8:1:1, v/v/v) as solvent. Two of these spots were inhibitory against *Cladosporium cladosporioides* (Fresen.) G.A. de Vries (a standard test organism for antifungal metabolites on TLC plates). The free acid phenolic compounds produced by *B. subtilis* belong to the hydroxycinamic acids group. Further characterisation of these spots is of importance and should be considered in future studies.

The antagonistic efficacy of *B. subtilis* was evaluated *in vitro* using the dual culture technique, initially used to select the isolate (Korsten *et al.*, 1989). A high variability was found in the antagonistic activity with the subcultures evaluated. However, this effect can be ascribed to phenotypic variability in the culture (Reinheimer *et al.*, 1995) or even consecutive subculturing. Genetic comparisons between the different subcultures were done using DNA fingerprinting with RISA PCR. All of the representative subcultures showed identical banding patterns and differed from other *B. subtilis* reference strains. Even though phenotypic variability was found, environmental conditions may also play a role in the consistent performance of the biocontrol agent.

By providing optimal conditions for antagonist performance, more consistent disease control results can be ensured. The commercial formulation of the biocontrol agent can play a role in product performance and efficacy (Korsten *et al.*, 1998). Various nutrients were tested *in vitro* to determine their effect on antagonism against four postharvest fungal pathogens. The effect of these nutrients on the pathogens was also investigated. It was recommend that only nutrients that enhance antagonism, while not supporting the growth of the pathogen should be incorporated into the commercial formulation. The nutrients identified were L-glutamic acid, L-glutamine and L-(+)-asparagine as nitrogen source as well as D-arabinose and D-(+)-mannitol as carbon source. The efficacy of the new formulation is currently being tested in the field and in packhouse trials under commercial conditions.

In this study it was found that the environmental conditions also affect the biocontrol agent's effectiveness. In previous packhouse trials, *B. subtilis* was applied in the wax after the fruit were washed and was followed by storage in cold chambers (Korsten *et al.*, 1998). However, significant control was not achieved (Korsten *et al.*, 1998). The effect of various temperatures on *in vitro* antagonism of *B. subtilis* against postharvest fungal pathogens indicated that *B. subtilis* did not grow well at 10 ºC, which is the commercially used temperature for export. In a study by Huang *et al.* (1995), *Pseudomonas glathei* was applied to oranges. After application the fruit were stored at 30 ºC for 24 h prior to cold storage and an increase in efficacy of the biocontrol agent was found. A similar approach, taking into consideration the conditions necessary for fruit quality and shelf life, should perhaps be considered in future using *B. subtilis*. Temperature also affects the mode of action utilised by a biocontrol agent and can be used to enhance the efficacy.

In conclusion, *B. subtilis* is an effective biocontrol agent of avocado fungal pathogens. Results from this study provide some evidence of the mode of action employed by *B. subtilis*. This information can be used to

improve future biocontrol product formulation and application. By understanding the factors affecting the antagonist's efficacy a more stringent application program can be devised to support more consistent performance. This study confirms the involvement of antibiosis in the form of cell-free secondary metabolites, siderophores and volatiles, as well as direct interaction through lytic enzymes as modes of action used by *B. subtilis* against fungal postharvest avocado pathogens. Since *B. subtilis* employs such a wide range of modes of action, working directly and indirectly, few if any fungal pathogens will be able to overcome its control.

REFERENCES

Blakeman, J.P. 1985. Ecological succession of leaf surface micro organisms in relation to biological control. Pages 6 – 30 In: Biological Control on the Phylloplane. Windels, C.E. & Lindow, S.E. (Eds). The American Phytopathological Society, St. Paul, Minnesota, USA.

Chen, C.L., Chang, L.K., Chang, Y.S., Liu, S.T. & Tschen, J.S.M. 1995. Transposon mutagenesis and cloning of the genes encoding the enzymes of fengycin biosynthesis in *Bacillus subtilis*. Molecular and General Genetics 248: 121 – 125.

Darvas, J.M. & Kotzé, J.M. 1987. Fungi associated with pre-and post-harvest diseases of avocado fruit at Westfalia Estate, S.A. Phytophylactica 19: 83 – 85.

Fiddaman, P.J. & Rossall, S. 1993. The production of antifungal volatiles by *Bacillus subtilis*. Journal of Applied Bacteriology 74: 119 – 126.

Frändberg, E. & Schnürer, J. 1994. Chitinolytic properties of *Bacillus pabuli* K1. Journal of Applied Bacteriology 76: 361 – 367.

Havenga, W., De Jager, E.S. & Korsten, L. 1999. Factors affecting biocontrol efficacy of *Bacillus subtilis* against *Colletotrichum gloeosporioides*. South African Avocado Growers' Association Yearbook 22: 12 – 20.

Helistö, P., Aktuganov, G., Galimzianova, N., Melentjev, A. & Korpela, T. 2001. Lytic enzyme complex of an antagonistic *Bacillus* sp. X-b: isolation and purification of components. Journal of Chromatography B 758: 197 – 205.

Huang, Y., Deverall, B.J. & Morris, S.C. 1995. Postharvest control of green mould on oranges by a strain of *Pseudomonas glathei* and enhancement of its biocontrol by heat treatment. Postharvest Biology and Technology 5: 129 – 137.

Katz, E. & Demain, A.L. 1977. The peptide antibiotics of *Bacillus*: Chemistry, biogenesis, and possible functions. Bacteriological Reviews 41: 449 – 474.

Keevy, C. 1999. Foreword. South African Avocado Growers' Association Yearbook 22: v.

Korsten, L. 1993. Biological Control of Avocado Fruit Diseases. Ph.D. thesis, University of Pretoria, Pretoria.

Korsten, L. & de Jager, E.S. 1995. Mode of action of *Bacillus subtilis* for control of avocado post-harvest pathogens. South African Avocado Growers' Association Yearbook 18: 124 – 130.

Korsten, L., Bezuidenhout, J.J. & Kotzé, J.M. 1989. Biocontrol of avocado postharvest diseases. South African Avocado Growers' Association Yearbook 12: 10 – 12.

Korsten, L., de Villiers, E.E., Rowell, A. & Kotzé, J.M. 1993. Postharvest biological control of avocado fruit diseases. South African Avocado Growers' Association Yearbook 16: 65 – 69.

Korsten, L., de Villiers, E.E., Duvenhage, J.A. & Kotzé, J.M. 1994. Control of avocado pre-harvest diseases with *Bacillus subtilis* and fungicide sprays. 17: 32 – 34.

Korsten, L, Towsen, E. & Claasens, V. 1998. Evaluation of Avogreen as post-harvest treatment for controlling anthracnose and stem-end rot on avocado fruit. South African Avocado Growers' Association Yearbook 21: 83 – 87.

Loeffler, W., Tschen, S. -M., Vanittanakom, N., Kugler, M., Knorpp, E., Hsieh, T. –F. & Wu, T. –G. 1986. Antifungal effects of bacilysin and fengymycin from *Bacillus subtilis* F-29-3: A comparison with activities of other *Bacillus* antibiotics. Journal of Phytopathology 115: 204 – 213.

Nielsen, P. & Sørensen, J. 1997. Multi-target and medium-independent fungal antagonism by hydrolytic enzymes in *Paenibacillus polymyxa* and *Bacillus pumilus* strains from barley rhizosphere. FEMS Microbiology Letters 22: 183 – 192.

Peypoux, F., Pommier, M.T., Das, B.C., Besson, F., Delcambe, L. & Michel, G. 1984. Structures of bacillomycin D and bacillomycin L peptidolipid antibiotics from *Bacillus subtilis*. The Journal of Antibiotics XXXVII: 1600 – 1604.

Pinchuk, I.V., Bressollier, P., Sorokulova, I.B., Verneuil, B. & Urdaci, M.C. 2002. Amicoumacin antibiotic production and genetic diversity of *Bacillus subtilis* strains isolated from different habitats. Research in Microbiology 153: 269 – 276.

Reinheimer, J.A., Morelli, L., Bottazzi, V. & Suárez, V. 1995. Phenotypic variability among cells of *Lactobacillus helveticus* ATCC 15807. International Diary Journal 5: 97 – 103.

Roberts, R.G. 1994. Integrating biological control into postharvest disease management strategies. HortScience 29: 758 – 762.

Sakajoh, M., Solomon, N.A. & Demain, A.L. 1987. Cell-free synthesis of the dipeptide antibiotic bacilysin. Journal of Industrial Microbiology 2: 201 – 208.

Shoji, J. 1978. Recent chemical studies on peptide antibiotics from the genus *Bacillus*. Advances in Applied Microbiology 24: 187 – 214.

Tsuge, K., Ano, T. & Shoda, M. 1996. Isolation of a gene essential for biosynthesis of the Lipopeptide antibiotics plipastatin B1 and surfactin in *Bacillus subtilis* YB8. Archives of Microbiology 165: 243 – 251.

van Dyk, K., de Villiers, E.E. & Korsten, L. 1997. Alternative control of avocado post-harvest diseases. South African Avocado Growers' Association Yearbook 20: 109 – 112.