CHAPTER FOUR

EVALUATING THE POTENTIAL OF INTEGRATED STRATEGIES FOR POSTHARVEST CONTROL OF CITRUS GREEN- AND BLUE MOLD AND BLACK SPOT

4.1. Abstract

Bacillus subtilis isolates F1, L2, and L2-5, isolated from citrus fruit surfaces were evaluated along with *Candida saitoana* (Biocoat and Biocure) for control of citrus green- and blue mold, and citrus black spot caused *by Penicillium digitatum, P. italicum,* and *Guignardia citricarpa* respectively. Isolates were evaluated either on their own, or in combination with sodium bicarbonate or hot (45° C) water treatment on artificially inoculated Valencia and Shamouti orange fruits in the case of *P. digitatum* and *P. italicum* respectively. Treated fruits were stored at 9° C and 90 to 95% relative humidity for four weeks. When used on their own, all isolates were more effective than the control (water treatment) in checking the incidence of both green- and blue mold, but were not as effective as the fungicide (quazatine plus imazalil) treatment. Isolate F1 of *B. subtilis* was the most effective in this respect. A significant increase in biocontrol activity of all isolates was recorded when isolates were combined with sodium bicarbonate (1% w/v), or when applied following hot (45° C) water treatment. The treatments comprising F1 combined with sodium bicarbonate or F1 applied following hot water treatment were as effective as the fungicide treatment, which gave total control of both green- and blue mold on both cultivars. Isolates were effective in control of *G. citricarpa* compared to the control treatment, in terms of controlling the development of new black spot lesions on fruit. However, none of these isolates completely stopped the development of new lesions.

4. 2. Introduction

Biological control is increasingly becoming an effective alternative to synthetic fungicides in plant disease control (Conway *et al.*, 1999; El-Ghaouth *et al.*, 2000; Korsten *et al.*, 2000). Biocontrol agents are however, often inconsistent, and in most cases less effective than many of the conventional fungicides currently in use. For acceptance by growers, biological

control efficacy has to be comparable to the level of control provided by conventional fungicides. Achieving such high levels of control and consistency is difficult with biological control systems and an integrated approach rather than the use of a single biocontrol product is more likely to provide the answer (Pusey, 1994). Janisiewicz (1988), for example, achieved a more effective control of apple rot with a mixture of *Acremonium breve* and a *Pseudononas* sp. compared to either antagonist used on its own. Research into the use of antagonist mixtures for control of citrus postharvest pathogens is still limited, and in most cases, poorly understood.

There are reports of improvements in biocontrol activity of yeast antagonists when combined with calcium salts (Wisniewski *et al.*, 1995; Droby *et al.*, 1997; El-Ghaouth *et al.*, 2000; Tian *et al.*, 2002). The inhibitory activity of sodium bicarbonate (SB) on fungal pathogens (Barger, 1928; Palou, *et al.*, 2001), and the antifungal activity of *Bacillus* species (Singh and Deverall, 1984; Huang *et al.*, 1992; Korsten *et al.*, 2000), amongst others, has been reported. There is however no reference in the literature on research conducted to determine the compatibility of *Bacillus subtilis* isolates with (SB), or possible improvements in biocontrol activity resulting from such integration, particularly in post-inoculation control of citrus mold. Hot water dip treatments are commercially used for fungal pathogen control on both fruits and vegetables (Lurie, 1999). Temperatures that are not injurious to the rind however, are unlikely to provide complete control of most citrus postharvest pathogens such as *P. digitatum* and *P. italicum* on citrus. Such a temperature could however retard pathogen development (Huang *et al.*, 1992; Conway *et al.*, 1999), and give a biocontrol agent a competitive advantage over a pathogen and thereby prevent disease development. Auret (2000) successfully evaluated the effects of integrated hot water treatments with *Bacillus* species on control of *Penicillium* mold on citrus. Similarly, El-Ghaouth *et al.* (2000) effectively controlled citrus postharvest diseases with a postharvest application of *Candida saitoana* supplemented with glycolchitosan.

Fruits with citrus black spot (CBS) caused by *Guignardia citricarpa* Kiely are unacceptable for export due to the phytosanitary risks for the importing country that is CBS free. Citrus black spot lesions on fruits can therefore lead to the rejection of entire export consignments in international trade (Kotzé, 1981). The main objective of this study was to screen three *B. subtilis* isolates (F1, L2, and L2-5), that gave promising results *in vitro*, in the preceding chapter (Chapter Three) *in vivo* i.e. on fruit under laboratory conditions. The possibility of

using these isolates to provide additional control of latent infections of *G. citricarpa* on citrus fruit was also investigated. The compatibility of *B.* subtilis isolates with SB, and its ability to enhance its biocontrol activity for control of *Penicillium* rot, was also evaluated. Finally, additional benefits of applying isolate following hot water treatments, or combining them with other biocontrol products was evaluated *in vivo*.

4.3. Materials and Methods

4.3.1. Fruit

Two orange (*Citrus sinensis* (L.) Osbeck) cultivars, Valencia and Shamouti collected from a commercial orchard at Letaba Estates in the Limpopo Province were used in this investigation. No postharvest treatment was applied and fruits were either used immediately after harvest, or stored at 9° C until used, usually no longer than two weeks.

4.3.2. Pathogen

For the *Penicillium* isolates used in this investigation and inoculum preparation refer to Chapter Three (Section 3.3.3.1). In addition, *G. citricarpa* was included in this study. The isolate (7HS1-2), which is a pathogenic isolate originally isolated from citrus fruit was received from Dr. Linda Meyer of the Department of Microbiology and Plant Pathology, University of Pretoria. The isolate was maintained on potato dextrose agar (PDA) (Biolab), in MacCartney bottles at 7° C until use. Stock cultures of this isolate were prepared for use throughout this study.

4.3.3. Preparation of aqueous antagonist suspension

For preparation of aqueous cell suspension of *B. subtilis* isolates *F1*, L2, and L2-5, refer to Chapter Three, (Section 3.3.3.2). Two yeast (*Candida saitoana*) biocontrol products commercially produced as Biocoat and Biocure respectively (Anchor Yeast, Cape Town) were evaluated in combination with the *Bacillus* isolates. The yeast products were prepared according to the registered dosage (406g of formulated product dissolved in 15 L of water) and were applied as a dip treatment.

4.3.4. Effect of sodium bicarbonate on growth of *Bacillus subtilis* isolates

Two hundred and fifty microliter of 1, 3, and 5% (w/v) SB was dispensed separately in microtiter (Nunc; AEC-Amersham (Pty) Ltd) plate wells and inoculated with an antagonist cell suspension (10^8 cell ml^{-1}) prepared as described in Chapter Three (3.3.3.2). The microtiter plate was incubated at 25° C for 1, 12, and 24 hours. The choice of this antagonist concentration was based on results obtained in Chapter Three (3.3.3). At each time interval, 50μl of antagonist-salt suspension was pipetted onto fresh standard 1 nutrient agar (STD 1) (Biolab), plates containing 25ml aliquots of medium. Plates were incubated at 25° C for 24 hours and colony diameter measured as described earlier in Chapter Three (3.3.3.3). Plates inoculated with cells suspended in sterile distilled water were used as positive control. A serial dilution was prepared at the end of each time interval and the number of viable cells (colony forming units) in each salt concentration determined using the spread plate technique. Each treatment was replicated five times and the experiment repeated once.

4.3.5. Effect of sodium bicarbonate on citrus peel

This trial was conducted to determine if SB had any detrimental effect on Valencia and Shamouti orange fruit. Fruits were immersed in 1, 3, and 5% (w/v) SB for two minutes, and air-dried. Treated fruits were stored at 9° C and 90 to 95% relative humidity (RH) for four weeks, and observed thereafter for any sign of scorching or external damage. Fruits treated with distilled water acted as control. Ten fruits were used per replicate, and each treatment replicated three times. The experiment was repeated once.

4.3.6. Effect of combining antagonists and sodium bicarbonate treatments on *in vivo* control of *Penicillium digitatum* and *Penicillium italicum*

Fresh, visually healthy fruits were surface sterilized in 70% ethanol and wound inoculated with either *P. digitatum* or *P. italicum* (10^6 spore ml^{-1}) by pricking using sterile dissecting needles. Four wounds each approximately 1 mm wide and 5 mm deep were made per fruit. Inoculation points were marked with a waterproof pen. Six hours after inoculation fruits were immersed for two minutes in antagonist-SB (1% w/v) suspension prepared as

described previously (Chapter Three, section 3.3.3.2). The choice of this SB concentration was based on results obtained in 4.3.5. Fruits immersed in 1% SB solution, distilled water or a mixture of fungicides that included decotine (guazatine 1 000 ppm) (Aventis), and Fungazil (imazalil 1000 ppm) (Janssen), were included as controls. Fruits were stored at 9° C and 90 to 95% RH for four weeks and assessed thereafter for decay symptoms. Disease assessment was based on a scale of 0 and 1; where 0 = healthy fruits and 1 = diseased fruits. A fruit was considered diseased as long as there was a visible sign of decay at the inoculation point irrespective of the diameter of the symptom. This is because the entire fruit is usually damaged within a few days following infection, especially if fruits are kept at temperatures around 25° C. Thirty fruits were used per replicate each treatment replicated three times. The experiment was repeated twice.

4.3.7. Effect of combining hot water treatment with antagonist on *in vivo* control of *Penicillium digitatum* and *Penicillium italicum*

Fruits were inoculated as described earlier (4.3.6). Six hours after inoculation, fruits were immersed in hot (45° C) distilled water in a water bath for two minutes and air-dried. They were further immersed for one to two minutes in the antagonist suspension (10^8 cell ml^{-1}). Fruits immersed in hot water alone, distilled water, or fungicides (see 4.3.6) acted as controls. Thirty fruits were used per treatment replicated three times. Fruits were stored and assessed as described before (4.3.6). The experiment was repeated twice.

4.3.8. Effect of *Bacillus subtilis* on its own or in combination with *Candida saitoana* on *in vivo* control of *Penicillium digitatum* and *Penicillium italicum*

Fruits inoculated as described above (4.3.6) were immersed six hours after inoculation, for two minutes in aqueous suspension of antagonists comprising *Bacillus* isolates on their own (10^8 cell ml^{-1}), or the yeasts on their own (see 4.3.3), or a combination of *Bacillus* and yeast. Treated fruits were stored in cardboard boxes at 9° C and 90 to 95 % RH for four weeks. Treatments were assessed thereafter for decay symptom development as described previously (4.3.6). Thirty fruits were used per replicate, and each treatment was replicated three times. Fruits immersed in distilled water or treated with fungicides (see 4.3.6) were used as controls. The experiment was repeated twice.

4.3.9. Effect of antagonists on *in vivo* development of new black spot lesions

Valencia oranges used in this study were collected from a block with a known history of CBS at Letaba Estates. As with the *Penicillium* experiments, the fruits also received no postharvest fungicide treatment. Fruits with some characteristic black spot lesions (hard spots) were selected, and marked with a waterproof pen. Marked fruits were first immersed in hot (45° C) distilled water for two minutes, air-dried, and further immersed for another two minutes in the test antagonist suspension prepared as described previously (3.3.3.2). Treated fruits were stored at 9° C and 90 to 95 % RH for three weeks and then 25° C for seven days. Fruits were assessed thereafter for development of new black spot lesions (red spots). Fruits immersed in distilled water acted as control. Thirty fruits were used per replicate and each treatment replicated three times. The experiment was repeated once.

### 4.3.10. Statistical analysis

All data obtained were statistically analysed using the SAS statistical program. One-way analysis of variance (ANOVA), was used to test for differences in average means between treatments. Treatment means were separated using Duncan's multiple range test (DMRT) at 5% level of significance.

4.4. Results

4.4.1. Effect of sodium bicarbonate on growth of *Bacillus subtilis* isolates

All *Bacillus subtilis* isolates evaluated did grow when suspended in 1, 3 or 5% SB solution. The rate of growth was however influenced by both concentration and period of cell suspension in salt solution (Fig. 4.1). All isolates grew normally after suspension in 1% concentration for 24 hours. Normal or retarded growth was observed and refers to the radial growth of the isolate on STD 1 following suspension in salt solution relative to cells suspended in sterile distilled water (control). Isolates L2 and L2-5 grew normally only up to 12 hours of suspension in 3% solution, while isolate F1 on the other hand maintained a normal growth even after 24 hours of suspension in that concentration. None of the isolates grew normally following suspension in 5% solution after 24 hours. Viable cell counts, as determined by the number of colony forming units at each time interval, showed a marked

81

decrease in the number of viable cells in treatments, where retarded growth was observed (Fig.4.1)
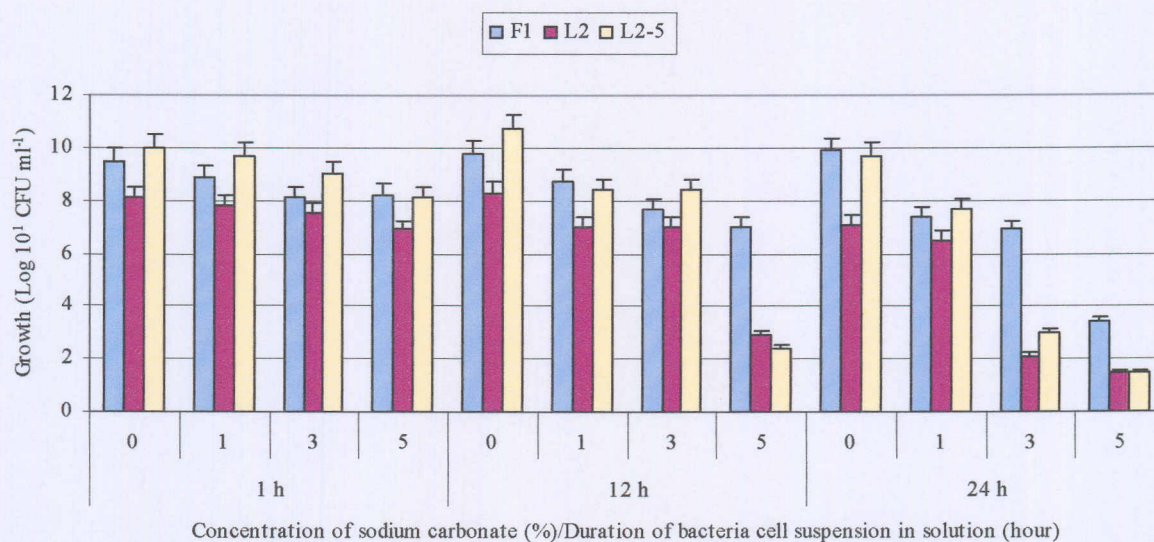
Treatments having same error bars are not significantly different at 3% level

Fig. 4.1 Growth of *Bacillus subtilis* isolates as affected by different concentrations of sodium bicarbonate and duration of cell suspension exposure in salt solutions before plating on nutrient agar and incubation at 25° C for 1, 12 and 24 hours. .

## 4.4.2. Effect of sodium bicarbonate on citrus peel

After two weeks of storage, no visible/external symptoms of damage were observed on fruits treated with 1, 3, or 5% (w/v) SB solution. By four weeks however, blotching type symptoms were visible on both Valencia and Shamouti fruits immersed in 5% SB. Symptoms were more severe on Shamouti than Valencia. Fruits immersed in 1 and 3% solution showed no visible signs of damage.

4.4.3. Effect of combining antagonists and sodium bicarbonate treatments on *in vivo* control of *Penicillium digitatum* and *Penicillium italicum*

The influence of *B. subtilis* isolates on their own, or in combination with SB on control of citrus green - and blue mold are presented in Fig. 4.2. There were variations in percentage

disease incidence between experiments and between cultivars. The performance of isolates did not however vary much between repetitions and results presented are the average values. All isolates evaluated on their own were effective when compared to the water control in checking the incidence of both green - and blue mold. They were however, not as effective as the fungicide treatment which gave total control of both diseases (Fig. 4.2). The incidence of both green - and blue mold on F1 treated fruits was lower than 21%. This level of control was significantly better than that achieved with isolates L2 and L2-5, which was around 30%. The latter isolates did not differ significantly. Treatment F1 on its own was not better than SB as evaluated under the conditions used during the assay.

Addition of SB to isolate suspensions resulted in a remarkable improvement in the biocontrol activity of all isolates. The addition of SB to isolate F1 suspension for example resulted in complete control of both green - and blue mold similar to that achieved with the fungicide treatment (Fig. 4.2). The addition of SB to isolates L2 and L2-5 did not result in the same level of control. The percentage control achieved was however significantly higher than either treatment on its own. No damage to the citrus peel was observed after treatment. Isolations made from the fruit surface at the end of the storage period (data not presented) indicated that all tests antagonist could be successfully re-isolated.

A

B
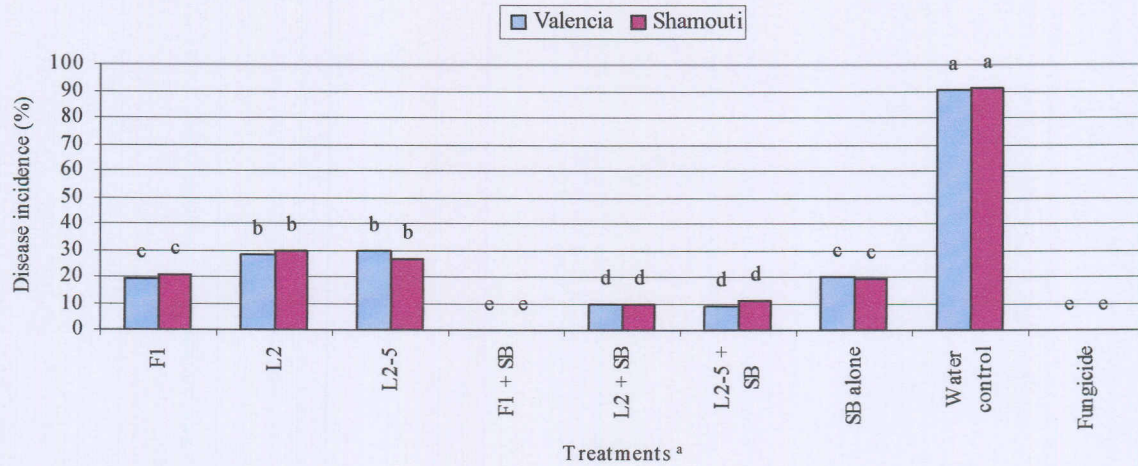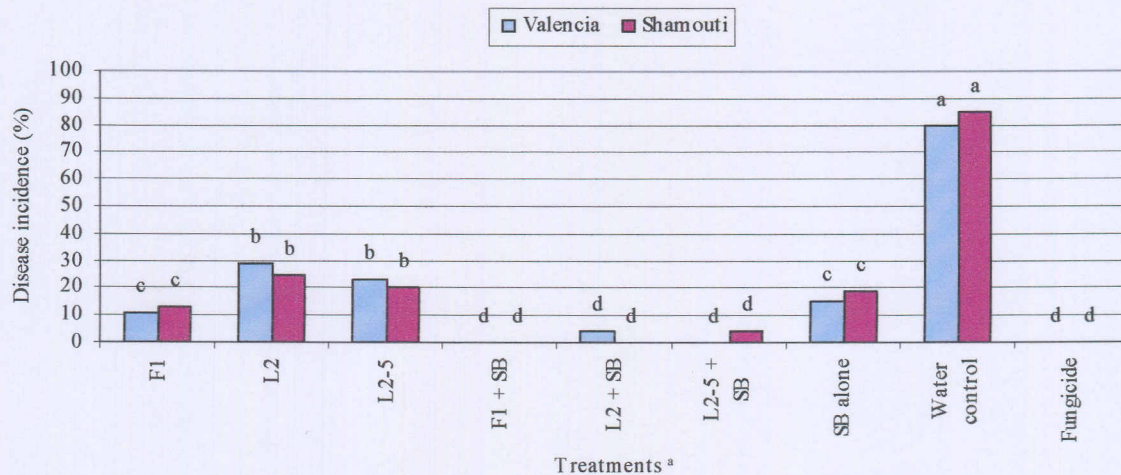
[a] F1, L2 and L2-5 represent *Bacillus subtilis* isolates (10^8 cells ml^{-1}); SB represent sodium bicarbonate (1% w/v); Fungicide represent Fungazil (imazalil 75% a.i. at 1g L^{-1}) plus Decotine (quazatine 20% a.i. at 1 ml L^{-1}). Data represent mean of three repetitions. Treatments with same letter are not significantly different according to Duncan's multiple range test (P = 0.05).
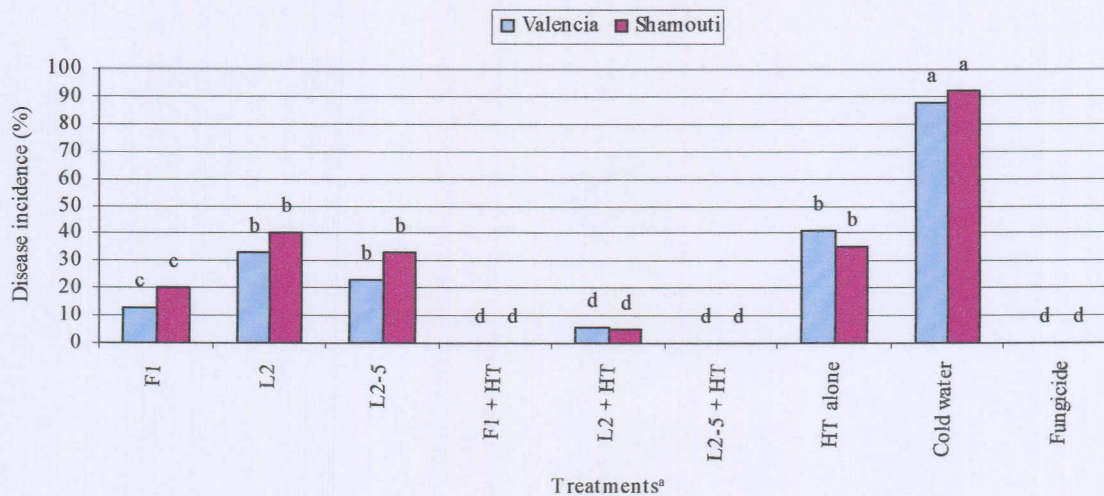
Fig. 4.2 Performance of *Bacillus subtilis* isolates on their own or in combination with sodium bicarbonate to control citrus green (*Penicillium digitatum*) (A) and blue (*P. italicum*) (B) mold on artificially inoculated fruits stored at 9° C and 90 to 95% relative humidity for four weeks.

4.4.4. Effect of combining hot water treatment with antagonist on *in vivo* control of *Penicillium digitatum* and *Penicillium italicum*

Results presented in Figs 4.3 indicate a significant difference between the control (cold water) and other treatments. Isolate F1 on its own was more effective than L2 and L2-5, in checking the incidence of both green - and blue mold on both cultivars. The percentage incidence in L2 and L2-5 treated fruits varied between 24 and 40%. A remarkable improvement in biocontrol activity of all isolates was recorded when they were applied following a hot water treatment, against both diseases and both cultivars tested. Application of isolates F1 and L2-5 following hot water treatment for example resulted in total control of both diseases, which was similar to the levels of control achieved with the fungicide treatment (Figs. 4.3 A and B). Of the combinations, L2 was the least effective. However, the level of control achieved was significantly higher than either treatment on its own. No damage to the citrus peel was observed after treatment.

4.4.5. Effect of *Bacillus subtilis* antagonists on its own or in combination with *Candida saitoana* on *in vivo* control of *Penicillium digitatum* and *Penicillium italicum*

Neither *Bacillus* nor yeast on its own, or their combinations gave total control of both green- and blue mold (Fig. 4.4). Both antagonists were however, more effective than the water control in checking the incidence of both diseases. The percentage disease incidence in fruits treated with isolates F1, L2, and L2-5 was lower than 31%. Combining bacteria and yeast antagonists produced mixed results. The combination appears to result in an antagonistic rather than a synergistic interaction with respect to the activity of the bacterial isolates. Combining F1 with biocoat for example resulted in a reduction in the percentage control of *Penicillium digitatum* from 70 to 64% and *P. italicum* from 77 to 60%. On the other hand, results presented in Fig 4.4 shows that the performance of biocure in the control of *P. italicum* increased from about 50% when used on its own to about 75% when combined with all bacterial isolates.
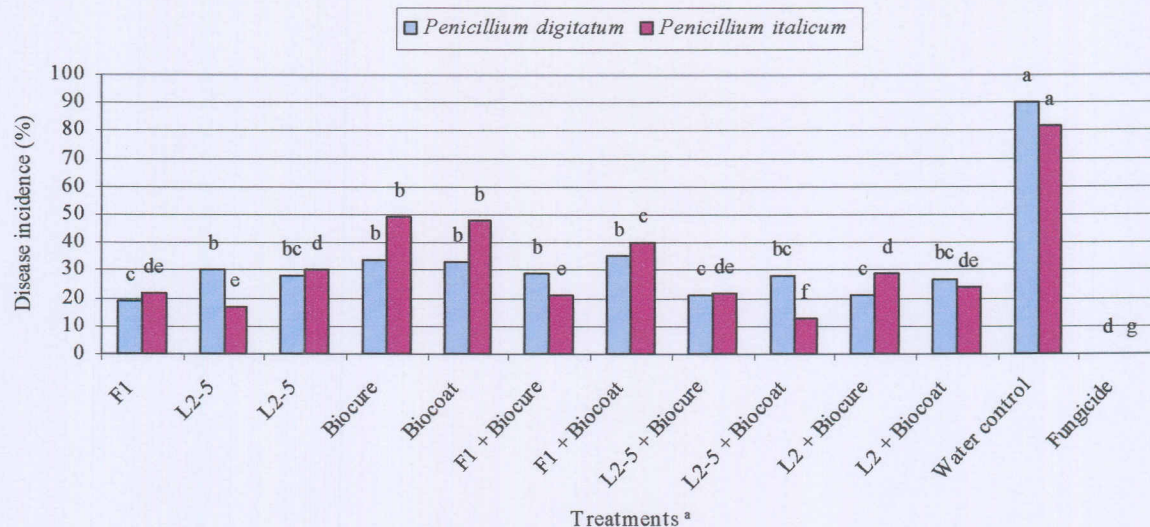
A

B
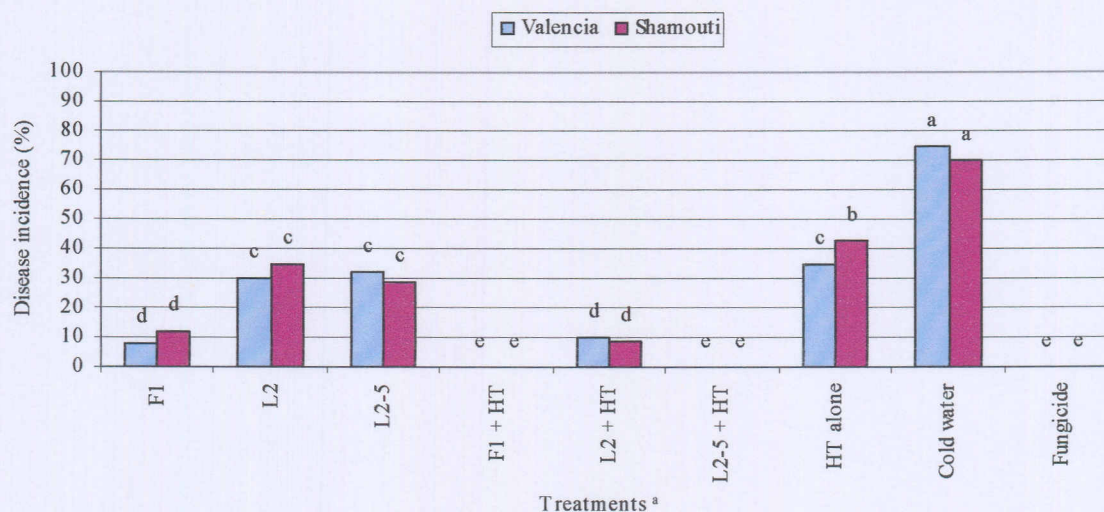
[a] F1, L2 and L2-5 represent *Bacillus subtilis* isolates (1×10^8 cells ml^{-1}); HT represent heat treatment ($45°$ C for two minutes); Fungicide represent Fungazil (imazalil 75% a.i. at 1g L^{-1}) plus Decotine (quazatine 20% a.i. at 1 ml L^{-1}). Data represent mean of three repetitions. Treatments having dame letter are not significantly different according to Duncan's multiple range test (P = 0.05).

Fig. 4.3 Performance of *Bacillus subtilis* isolates either on their own or in combination with hot ($45°$ C) water treatment in the control of citrus green (*Penicillium digitatum*) (A) and blue (*P. italicum*) (B) mold on artificially inoculated fruits stored at $9°$ C and 90 to 95% relative humidity for four weeks.

□ *Penicillium digitatum* ■ *Penicillium italicum*

[a] F1, L2 and L2-5 represent *B. subtilis* isolates (1×10^8 cells ml^{-1}); Biocure and Biocoat represent *Candida saitoana*; Fungicide represent Fungazil (imazalil 75% a.i. at 1g L^{-1}) plus Decotine quazatine 20% a.i. at 1 ml L^{-1}). Data represent mean of three repetitions. Treatments having same letter are not significantly different according to Duncan's multiple range test (P = 0.05).

Fig. 4.4   Performance of *Bacillus subtilis* isolates either on their own or in combination with *Candida saitoana* on the control of citrus green-and blue mold on artificially inoculated fruits Valencia orange fruits stored at 9° C and 90 to 95% relative humidity for four weeks.).

4.4.6. Effects of antagonist on *in vivo* development of new black spot lesions

The incidence of new black spot lesions on treated fruits was low.   From the results presented (Fig 4.6) we can observe that all isolates were effective in preventing the development of new spots when compared with the water control. However, none of the isolates completely stopped the development of new spots. The performance of the isolates did not differ significantly.
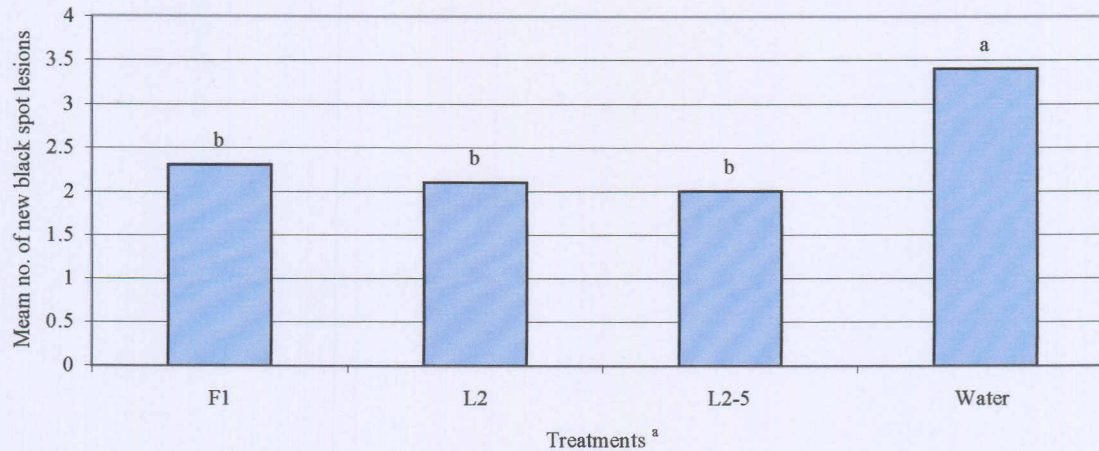
[a] F1, L2 and L2-5 represent *Bacillus subtilis* isolates   Data represent mean of three repetitions. Treatments having same letter are not significantly different according to Duncan's multiple range test (P = 0.05).

Fig. 4.5   Effect of *Bacillus subtilis* isolates on the development of new black spot lesions on naturally infected Valencia oranges after storage at 9° C and 90 to 95 % relative humidity for three weeks, and 25° C for one week.

4.5. Discussion

The increasing negative perception over the continuous use of synthetic fungicides in the food chain has resulted in many attempts in the recent past to develop non-chemical methods to control postharvest decays on various commodities including citrus.   These attempts included the use of microbial antagonists (Huang *et al*., 1992; Auret, 2000; El-Ghaouth *et al*., 2000; Korsten *et al*., 2000; Janisiewicz *et al*., 2001; Tian *et al*., 2002), application of substances generally regarded as safe (GRAS) ((El-Ghaouth *et al*., 2000; Palou, *et al*., 2001) and treatment with hot air or water (Eckert *et al*., 1996; Schirra *et al*., 1998; Auret, 2000; Palou *et al*., 2001) amongst others. Although some of these methods provided satisfactory levels of control when used alone, most appeared to give high and consistent levels of control only when used in an integrated program as a result of additive or synergistic activity.

From the results obtained in the present study all antagonists were effective in the controlling both green - and blue mold. When used on their own, the antagonists were not as effective as the fungicide treatment, which gave total control of both diseases. Isolate F1

of *B. subtilis* controlled both diseases more effectively than any of the other isolates and was also the most effective on both cultivars. These results are therefore in agreement with earlier reports on the use of microbial antagonists for control of postharvest diseases including the use of *Bacillus* isolates (Auret, 2000; Korsten *et al.*, 2000). The addition of SB (1% w/v) to *B. subtilis* isolate suspensions resulted in a remarkable improvement in its activity against both diseases. The potential of *Bacillus* species as biological control agents has been reported previously. The activity of SB against phytopathogens including *Penicillium* has also been reported (Barger, 1928; Palou *et al.*, 2001). This is however, the first report of the evaluation of *B. subtilis* isolates in combination with SB to improve its biocontrol activity.

The increased effectivity following combinations of antagonists with SB could be due to several factors. Our observation showed that suspending spores of *P. digitatum* and *P. italicum* in SB solution resulted in reduced germination and retarded mycelial growth. This observation agrees with an earlier study (Marloth, 1931), which reported that SB and sodium carbonate caused spore mortality in both *P. digitatum* and *P. italicum*. The hydrogen ion concentration (pH) of sodium is believed to play an important role in the observed activity of sodium compounds against many plant pathogens (DePasqualle and Montville, 1990). Sodium bicarbonate alone does not provide long term protection of fruits against reinfection after treatment. On the other hand, viable antagonist cells were isolated from fruit surfaces and wound sites after storage, thus indicating that the long-term protection was provided by the antagonist. The integration therefore complimented the shortcomings of either treatment used on its own.

Although this is the first report where *B. subtilis* isolates were integrated with SB, similar improvement in biocontrol activity of yeast antagonists following addition of calcium salts (Mclaughlin *et al.*, 1990; Wisniewski *et al.*, 1995; Droby *et al.*, 1997; Conway et *al.*, 1999) have been reported. In these reports, the reasons postulated for the observed increase in biocontrol activity of the antagonists included amongst others; osmotic tolerance of the biocontrol agent (yeast) to the calcium salt, inhibition of pathogen spore germination, inhibition of germ tube elongation, and the pectinolytic activity of the pathogen (*P. expansum*). In the present study, isolate F1 which gave total control of both green - and blue molds when combined with SB was also observed to be the most tolerant when exposed to different concentrations of SB. This characteristic means that the isolate can benefit from

the disruption in pathogen development caused by SB. Because of the isolate's high tolerance level to SB, its inhibitory activity will be improved as it now encounters a 'less aggressive' pathogen and hence the biocontrol performance should be more effective.

In the present study, we recorded a significant improvement in the activity of the antagonists when they were applied following hot (45° C) water treatment. The benefits of hot water treatment for control of fruit pathogens have been reported (Eckert *et al.*, 1996; Schirra *et al.*, 1998; Auret, 2000; Palou *et al.*, 2001). Heat treatment is reported to retard pathogen development (Huang *et al.*, 1992; Conway *et al.*, 1999). Temperature plays an important role in the development of decay caused by *Penicillium* species (Howard, 1936; Schirra *et al.*, 1998). The effect of heat on microbes will ultimately result in the creation of a vacuum that could be filled afterwards by an antagonist. In the case of *Penicillium*, the pathogen is reported to grow slowly at 30° C, and can hardly survive above 35° C (Carlos, 1982). Judging from this report, the temperature evaluated in this study (45° C) should have been high enough to kill the pathogens. It is important however to note that this temperature was only the temperature of the water in the tank and not the peel temperature or that underneath the peel, where the pathogen had been placed by artificial inoculation. Due to the short exposure time of the fruits used in this study the temperature underneath the peel could have been lower than the surrounding water. It was obvious however, that although the temperature regime did not give complete control of both pathogens, it may have retarded pathogen development as stated previously and thus created a vacuum that was occupied by the antagonists. This development might have contributed to the observed increase in the activity of isolates when applied following hot water treatment. Heat is also reported to promote the formation of compounds such as phytoalexins (Kim *et al.*, 1991; Eckert *et al.*, 1996), which increased the resistance of the host tissue to infection.

In nature, the microbial population on the fruit surfaces is made up of a diversity of microorganisms including bacteria and yeast. (Alabouvette and Lemanceau, 1999), and the inhibitory activities of these microbes are believed to play a significant role in the natural protection of plants against pathogen attack (Janisiewicz and Korsten, 2000). Results obtained in the present study however, indicated that the combination of *Bacillus* and yeast antagonists did not result in any remarkable improvement in bioactivity. In many instances, antagonistic rather than synergistic activity was recorded. This observation confirmed earlier reports of the problems associated with the use of antagonist mixtures in biological

control systems. It is known that microorganisms can switch between different modes of interaction depending on availability of nutrient resources; with positive interactions dominating when there is excess nutrients and available space, while negative interactions dominate when space and nutrients become limited (Atlas and Bartha, 1998).

Although the results obtained in the present study indicated that the *B. subtilis* isolates evaluated had great potential for use in control of citrus green- and blue mold in the postharvest arena, they were however, ineffective in the control of citrus black spot. Unlike the wound pathogens (*Penicillium* species), CBS is an incipient disease, and only active compounds with systemic activity is most likely to reach inside the tissue where the pathogen is embedded, for any control to be effected.

The impact of external factors on product efficacy is greater on biocontrol agents than fungicides. As a result, biological control systems are generally less effective than many conventional fungicides (Conway *et al.*, 1999). The importance of external factors becomes more relevant when a biocontrol agent is challenged against an opportunistic and fast growing fungus like *Penicillium*. Any measure therefore, that reduces the pathogenic ability of such a fungus, and increases the activity of a biocontrol agent is a welcome development in the search for alternative control measures to synthetic fungicides.

In the present study, isolate F1 exhibited a high degree of tolerance to SB. Sodium bicarbonate is classified as a "GRAS" product (Palou *et al.*, 2001). Hot water treatment is also a normal practice in many packhouses. The present technology of combining *B. subtilis* isolates (especially isolate F1) with SB or hot water treatment is therefore in line with already existing citrus packhouse practices and should be easy to adopt. Isolate F1 therefore holds great promise for use in the postharvest arena for control of citrus green- and blue mold. From the results obtained in Chapter Three it is obvious that antibiotic production is not the main mode of action of this isolate. This property makes the chances of acceptance of this potential biocontrol agent greater, although first tier toxicological tests will still have to be successfully completed to ensure product registration. The technology tested in the present study however, need to be evaluated further on a semi-commercial scale to confirm the observed activities before their commercial adoption can be recommended.

Literature Cited

Alabouvette, C. and Lemanceau, P. 1999. Joint action of microbials for disease control. Pages 117-135, in Hall, F.R. and Menn, J.J. (Eds). Methods in Biotechnology, vol. 6: Use and delivery. Humana Press Inc., Totowa, NJ.

Atlas, R.M. and Bartha, R. 1998. Microbial Ecology: Fundamentals and Application. Addison Wesley Longman Inc. New York.

Auret, E.E. 2000. Control strategies for citrus postharvest diseases. M.Sc Thesis, University of Pretoria, Pretoria.

Barger, W.R. 1928. Sodium bicarbonate as a citrus fruit disinfectant. California Citrograph 12: 64-174.

Carlos, R. 1982. Manual and atlas of the Penicillia. Elsevier Biomedical Press, Netherlands.

Conway, W.S., Janisiewicz, W.J., Klein, J.D. and Sams, C.E. 1999. Strategy for combining heat treatment, calcium infiltration, and biological control to reduce postharvest decay of gala apple. HortScience 34: 700-704.

DePasqualle, D.A. and Montville, T.J. 1990. Mechanism by which ammonium bicarbonate and ammonium sulfate inhibit mycotoxigenic fungi. Applied Environmental. Microbiology 56: 3711-3717.

Droby, S., Wisniewski, M.E., Cohen, 1,, Weiss, B., Tomiton, D., Eilan, Y. and Chalutz, E. 1997. Influence of calcium chloride on *Penicillium digitatum*, grapefruit peel tissue and biocontrol activity of *Pichia guilliermondii*. Phytopathology: 87: 310-315.

Eckert, J.W., Rotmayake, M., Sievert, J.R. and Strange, R.R. 1996. Curing citrus fruits to control postharvest diseases. Congress of the International Society of. Citriculture, Sun City, South Africa, 48.

El-Ghaouth, A., Smilanick, J.L. and Wilson, C.L., 2000. Enhancement of the performance of *Candida saitoana* by the addition of glycolchitosan for the control of postharvest decay of apple and citrus fruit. Postharvest Biology and Technology: 19, 103-110.

Howard, F.S. 1936. Citrus diseases and their control. McGraw-Hill Company Inc., New York.

Huang, Y., Wild, B.L. and Morris, S.C. 1992. Postharvest biological control of *Penicillium digitatum* decay on citrus fruit by *Bacillus pumilus*. Annals of Applied Biology 130: 367-372.

Janisiewicz, W.J. 1988. Biocontrol of diseases of apple with antagonist mixtures. Phytopathology 78: 194-198.

Janisiewicz, W.J. and Korsten, L. 2000. Control of postharvest diseases and microbial spoilage of vegetables by application of microbes, Chapter 23, in: Bartz, J (Ed). The physiology and microbiology of vegetables after harvest. Marcel Dekker, New York

Janisiewicz, W.J., Tworkoski, T.J. and Kurtzman, C.P. 2001. Biocontrol potential of *Metchnikonvia pulcherrima* strains against blue mold of apple. Phytopathology 91: 1098-1108.

Kim, J.J., Yehoshua, S., Shapiro, B., Henis, Y. and Carmeli, S. 1991. Accumulation of scoparone in heat- treated lemon fruit inoculated with *Penicillium digitatum* Sacc. Plant Physiology: 97: 880-885.

Korsten, L, de Jager, E.S., Paul, I., Obagwu J. and El-Ghaouth, A., 2000. Alternative control of citrus postharvest diseases. Poster presented at the International Society of Citriculture at Orlando, Florida, USA, 3-7, December 2000.

Kotzé, J.M. 1981. Epidemiology of and control of citrus black spot in South Africa. Plant Disease 65: 945-950.

Lurie, S. 1999. Postharvest heat treatments of horticultural crops. Horticultural Review 22: 91-121.

Marloth, R.H. 1931. The influence of hydrogen-ion concentration and of sodium bicarbonates and related substances on *Penicillium italicum* and *P. italicum*. Phytopathology 21: 169-198.

Mclaughlin, R.J., Wisniewski., M.E., Wilson, C.L. and Chalutz. E. 1990. Effect of inoculum concentration and salt solution on biological control of postharvest diseases of apple with *Candida* species. Phytopathology 80: 456-461.

Palou, L., Smilanick, J.L., Usall, J. and Virias, I. 2001. Control of postharvest blue and green molds of oranges by hot water, sodium carbonate and sodium bicarbonate. Plant Disease 85: 371-376.

Pusey, P.L. 1994. Enhancement of biocontrol agents for postharvest diseases and their integration with other control strategies, in: Wilson, C.L. and Wisniewski, M.E. (Eds) Biological control of postharvest diseases. CRC Press, Boca Raton.

Schirra, M., D'hallewin, G., Cabras, P., Anioni, A. and Garau, V.L. 1998. Seasonal susceptibility of Tarocco oranges to chilling injuries affected by hot water treatment and thiabendazole postharvest dip treatments. Journal of Agricultural Food Chemistry 46: 1177-1180.

Singh, V. and Daverall, S.J. 1984. *Bacillus subtilis* as a control agent against fungal pathogens of citrus fruit. Transaction of the British Mycological Society 83: 487-490.

Tian, S.R., Fan, Q., Xu, Y. and Jaing, A.L. 2002. Effects of calcium on biocontrol activity of yeast antagonists against the postharvest fungal pathogen *Rhizopus stolonifer*. Plant Pathology 51: 352-358.

Wisniewski, M., Droby, S., Chalutz, E. and Eilam, Y. 1995. Effect of Ca^{2+} and Mg^{2+} on *Botrytis cinerea* and *Penicillium expansum in vitro* and on the biocontrol activity of *Candida oleophila*. Plant Pathology 44: 1016-1024.