CHAPTER FIVE

TESTING POTENTIAL BIOCONTROL PRODUCTS FOR CONTROL OF ITRUS GREEN MOLD UNDER SIMULATED EXPORT CONDITIONS

5.1. Abstract

Three *Bacillus subtilis* isolates (F1, L2 and L2-5) were evaluated along with other commercial biocontrol products *Bacillus subtilis* (Avogreen powder and Avogreen liquid), and *Candida saitoana* (Biocure and Biocoat) for their antifungal activity against *Penicillium digitatum,* the cause of citrus green mold, under simulated export conditions in 2000, 2001 and 2002. The *B. subtilis* isolates were evaluated either alone or in combination with sodium bicarbonate (SB) at 1% (w/v). The efficacy of treatment was negatively affected by time of treatment application. Treatments were generally more effective when applied at the beginning of the season than when used later in the season when fruits have started 'ageing'. Neither the *B. subtilis* isolates on their own, nor the formulated products were as effective as the commercial fungicide treatment, which gave complete control of the disease throughout the season. Combining *B. subtilis* isolates with SB resulted in a remarkable improvement in the biocontrol activities of all isolates. Isolate F1 combined with SB was as effective as the fungicide treatment in some instances.

5.2. Introduction

As indicated earlier (Chapter Two, Table 2.3), the citrus fruit is susceptible to attack from several diseases both pre- and postharvestly. Some of these diseases particularly citrus black spot (CBS) caused by *Guignardia citricarpa* Kiely and green- and blue mold caused by *Penicillium digitatum* Sacc. and *P. italicum* Wehmer respectively are particularly important because of the huge economic losses normally associated with their infections, and/or because they are barriers to international trade. The increasing negative perception regarding the safety of synthetic chemicals for man and his environment, has in the recent past, resulted in several research studies aimed at identifying alternative control measures for plant diseases. Of particular interest have been the increasing numbers of

microorganisms evaluated for their antagonistic properties (biological control) (Janisiewicz and Korsten, 2002).

The increased interest in the use of microorganisms for disease control however, has been accompanied by many unsuccessful attempts at transferring potentially effective biological control systems from the laboratory into commercially viable products. Despite these failures, some successful control in *in vivo* evaluations has been reported (Arras, 1996; Arras *et al.,* 1999; Auret, 2000; El-Ghaouth *et al.,* 2000a, b, c; Korsten *et al.,* 2000; Northover and Thou, 2002). Several microorganisms with antagonistic properties have also been patented (see Chapter Two, Table 2.5) and are being used for control of several plant diseases in different countries. Testing the performance of a potential antagonist under simulated export conditions is a prerequisite for commercialization of a biological control agent.

The aim of this study was therefore to screen three *Bacillus subtilis* isolates (F1, L2, and L2-5) that gave promising results in previous *in vitro* and *in vivo* trials for control of green mold caused by *Penicillium digitatum* Sacc. The isolates were evaluated on their own or with SB and formulated biocontrol products which included Avogreen, Biocure, and Biocoat.

5.3. Materials and Methods

Penicillium digitatum was chosen as the test pathogen because it occurs more readily and is more damaging i.e. economically more important than *P. italicum* the causal agent of blue mold. This trial was conducted at Letaba Estates, a commercial citrus farm in Limpopo Province of South Africa in 2000 and 2001. In 2002, the trials were repeated as before but using the postharvest facilities at Plant Pathology Laboratories, University of Pretoria. Not all products (treatments) were evaluated in all the years and months. Some treatments had either not been identified at the time of such trials, not available, or had to be withdrawn at later a stage because of poor performance. *Bacillus* isolates for example were only introduced in September 2000. Cold storage of fruit was only done in July 2000 due to logistical problems. Treatments were done three times in a season; May-June, July-August, and September-October. Refer to Appendix 8 for details of treatments and methodology used. Treated fruits were stored at two temperature regimes: 25° C for two

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weeks, and 6° C for four weeks. The rationale behind storing fruits at the former temperature regime was to test product performance under extreme conditions such as under local marketing conditions where refrigeration may not be available, while the later temperature regime was selected to simulate export conditions.

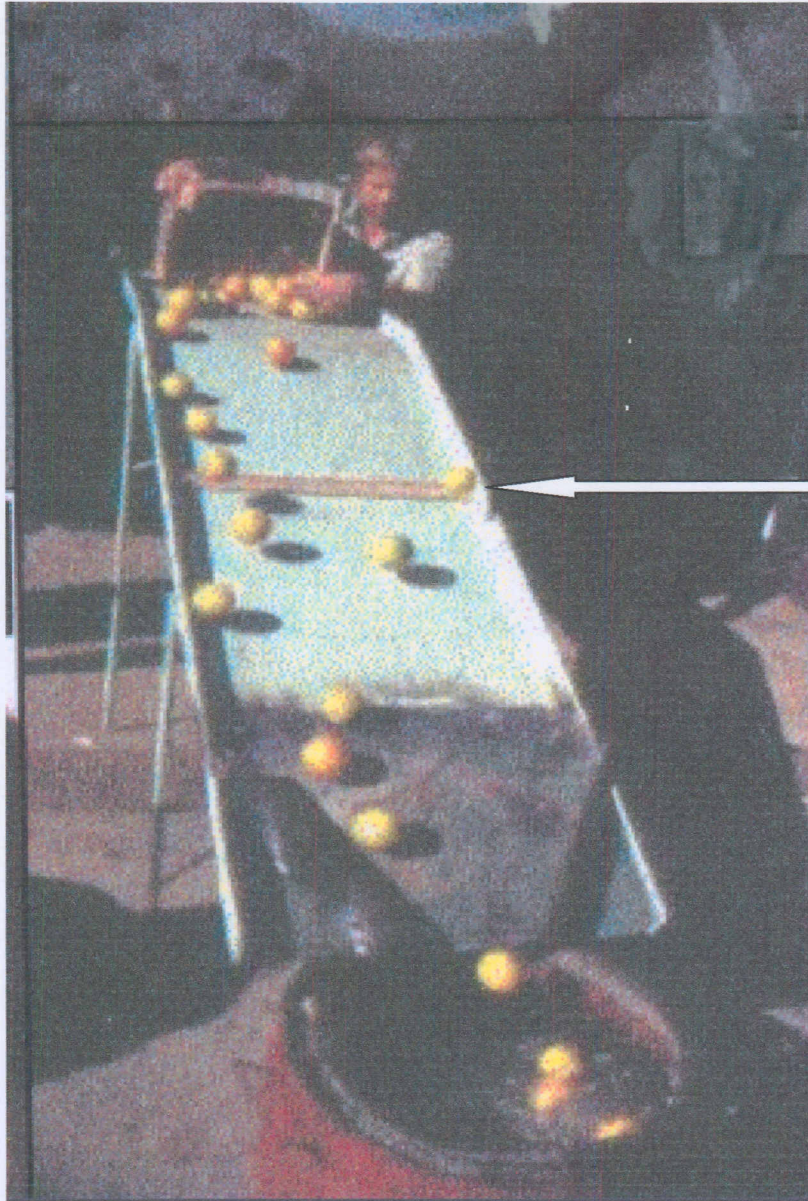### 5.3.1. Preparation of antagonist suspension

A cell suspension of *Bacillus subtilis* (isolates F1, L2 and L2-5), was prepared as described in Chapter Three (3.3.3.2) with an initial concentration (cell count) of 10^8 cell ml^{-1}. The choice of this concentration was based on results obtained in Chapter Four. Commercial products were used according to the registered rates. Avogreen powder was used at 75g/100 L of water, Avogreen liquid at 200ml/100 L of water. Biocoat and Biocure were used at 406g/ formulated product/15L of water.

5.3.2. Pathogen

For pathogen inoculum preparation, refer to Chapter Three (3.3.2 and 3.3.3.1).

5.3.3. Fruit Inoculation and Treatment

Refer to Appendix 8 for detail treatments and the methodology used. Freshly harvested Valencia oranges were artificially wounded using a fruit wounder (see Fig. 5.1). The wounder was made from a slanting flat steel board, about 1.5 m long and 75 cm wide with size-staples placed directly down the slant. Staples stick out when placed upright thereby ensuring even wounding of fruits as they roll down the slant. An average of five wounds were made per fruit which were immersed for two minutes in a pathogen suspension (10^6 spores ml^{-1}). Inoculated fruits were left overnight (18 hours) before treatment was applied. Fruits were immersed for two minutes in the product suspension and stored at either 6° C for four weeks or 25° C for two weeks, after which fruits were assessed for decay. Fruits immersed in tap water or fungicides (Appendix 8) served as negative and positive controls respectively. Disease assessment was based on a scale of 0 and 1; where 0 = healthy fruits and 1 = diseased fruits. A fruit was considered diseased if there were any visible signs of decay at the inoculation point. This is because the entire fruit is usually damaged within a

Fig.5.1. Fruit wounder used for fruit inoculation

few days following infection, especially if fruits are kept at temperatures around 25° C. There were three replicates of 400-450 fruits per treatment.
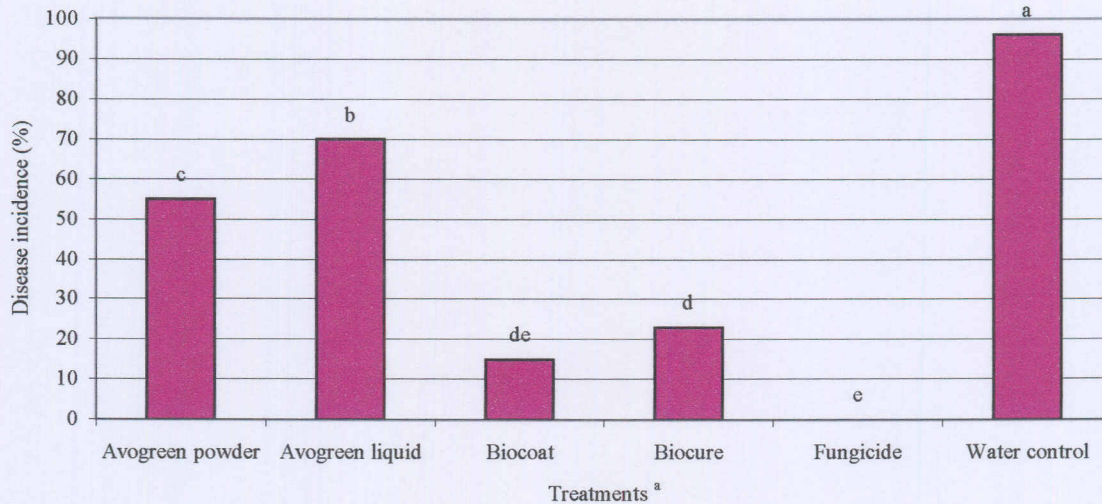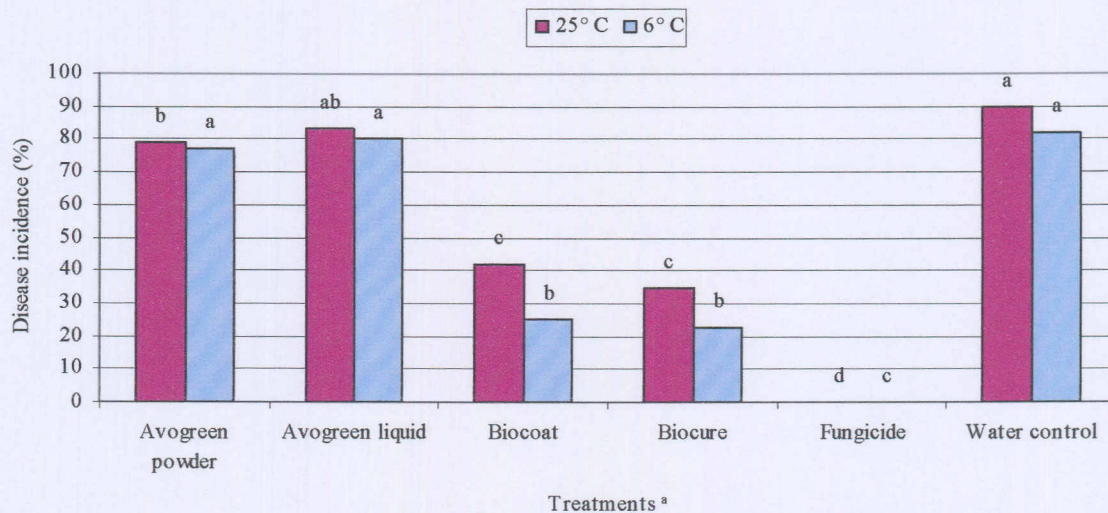
5.4. Results

2000 season

The results presented in Fig.5.2A show that all products were effective in the control of *Penicillium* rot compared to the water treatment in June. They were however, less effective than the fungicide treatment, which gave complete control of the disease. The percentage disease incidence in treated fruits varied between 15 and 70%. Biocoat was more effective than all other treatments with a percentage disease incidence of 15%, followed by Biocure with 23%. Both Avogreen powder and liquid formulation were not as effective with percentage disease incidence ranging between 55 and 70% respectively.

In August, fruits kept under cold storage (6° C) had a lower disease incidence than those stored at 25° C (Fig. 5.2 B). All treatments were less effective than the fungicide treatment, which gave complete control of the disease. The percentage disease incidence in Biocoat and Biocure treated fruits was 23 and 25%, and 36 and 41% respectively in fruits stored at 6° C and 25° C and did not differ significantly. This performance was however lower than that obtained in the first trial in June. Neither Avogreen powder nor liquid were effective.

In September, treatment performance (percentage disease incidence) varied between 5 and 80% under cold storage and was more effective than for fruits kept at 25° C. Results presented in Fig. 5.3 shows that all treatments were effective relative to the water control, but less effective than the fungicide treatment. The percentage disease incidence in Biocoat, F1, and Biocure treated fruits kept under cold storage (6° C) was lower than 30% and did not differ significantly. The percentage disease incidence in F1 treated fruits stored at 25° C was 40% and was more effective than either Biocoat or Biocure both of which were 53%. Avogreen liquid was not more effective than the water control at 25° C. A remarkable increase in biocontrol activity of *Bacillus subtilis* isolates was observed when isolates were combined with sodium bicarbonate (SB) under both storage conditions.
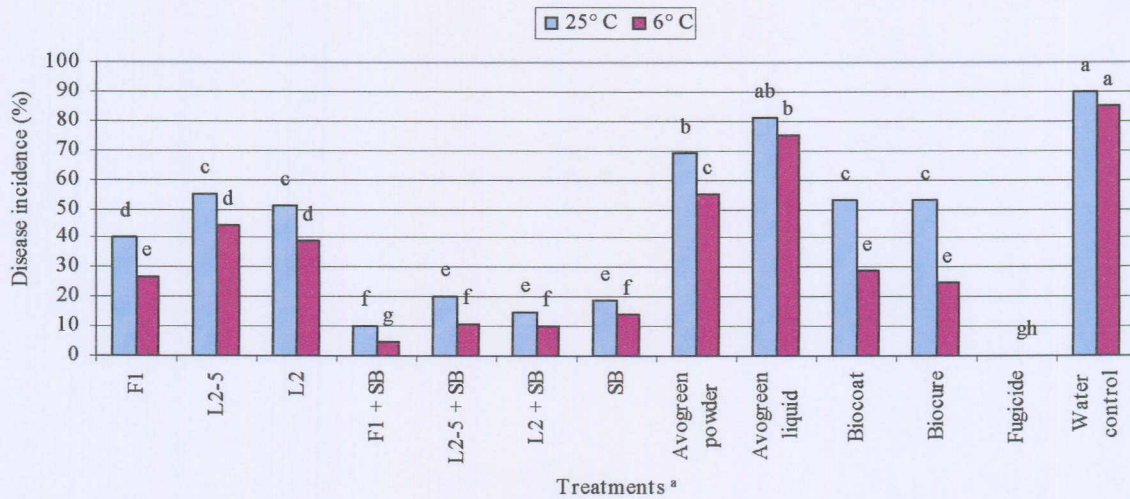
A

B

[a] Biocure and Biocoat represent *Candida saitoana*; Avogreen liquid and Avogreen powder represent *Bacillus subtilis*; Fungicide represent Fungazil (imazalil 75% a.i. at 1g L^{-1}) plus Decotine (quazatine 20% a.i. at 1 ml L^{-1}); SB represent sodium bicarbonate (1% wt/vol). Values Are mean of three replicates. Treatment having same letter are not significantly different according to Duncan's multiple range test (P =0.05).

Fig 5.2   Evaluation of alternative postharvest disease control options for control of citrus green mold caused by *Penicillium digitatum* on Valencia orange after two weeks of storage at 25° C or four weeks at 6° C in June 2000 (A) and August 2000 (B).

^{a} Biocure and Biocoat represent *Candida saitoana*; Avogreen liquid, Avogreen powder, F1, L2 and L2-5 represent *Bacillus subtilis* isolates; Fungicide represent Fungazil (imazali75% a.i. at 1g L^{-1}) plus Decotine (quazatine 20% a.i. at 1 ml L^{-1}); SB represent sodium bicarbonate (1% wt/vol). Values are mean of three replicates. Treatments having same letter are not significantly different according Duncan's multiple range test (P = 0.05)

Fig 5.3   Evaluation of alternative postharvest disease control options for control of citrus green mold caused by *Penicillium digitatum* on Valencia orange after two weeks of storage at 25° C or four weeks at 6° C in September 2000.

Disease incidence in the treatment comprising F1 combined with SB for example was 5%, and was better than either treatment on its own. The combination of isolates L2, and L2-5 was not as effective (Fig. 5.3). The treatments comprising L22 + SB and L2-5 + SB at 25° C were not significantly better than SB on its own.
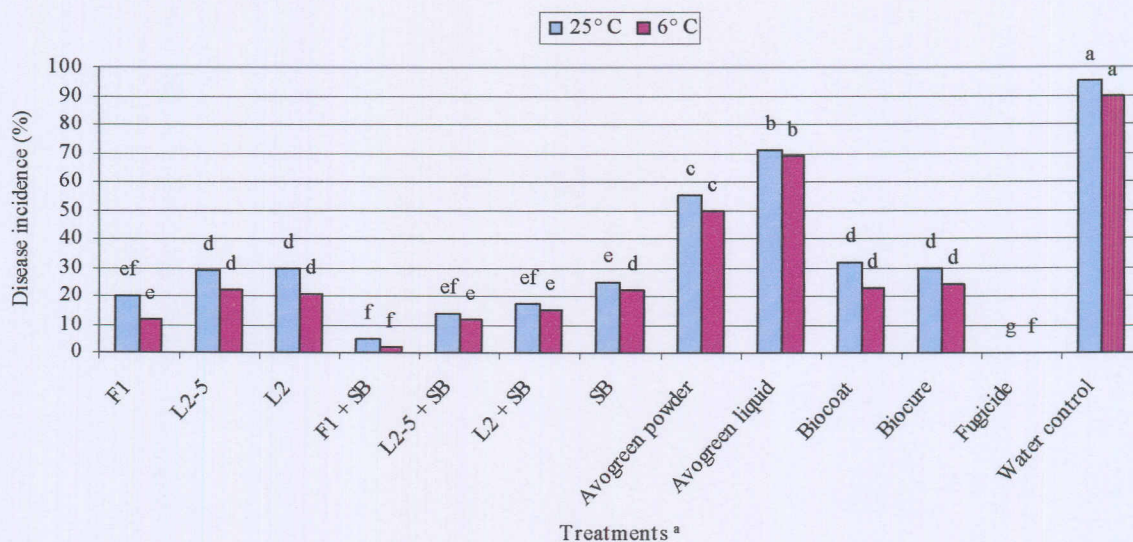
2001 Season

In June, all treatments were effective in controlling the incidence of the disease relative to the control, but were less effective than the fungicide treatment, which gave complete control of the disease. The percentage disease incidence in F1 treated fruits stored at 25° C was 20% and this performance was lower than all the other antagonists treatments used on their own. The percentage disease incidence in Biocoat, Biocure, L2, L2-5 varied between 29 and 32%, and did not differ significantly. Avogreen powder was more effective than
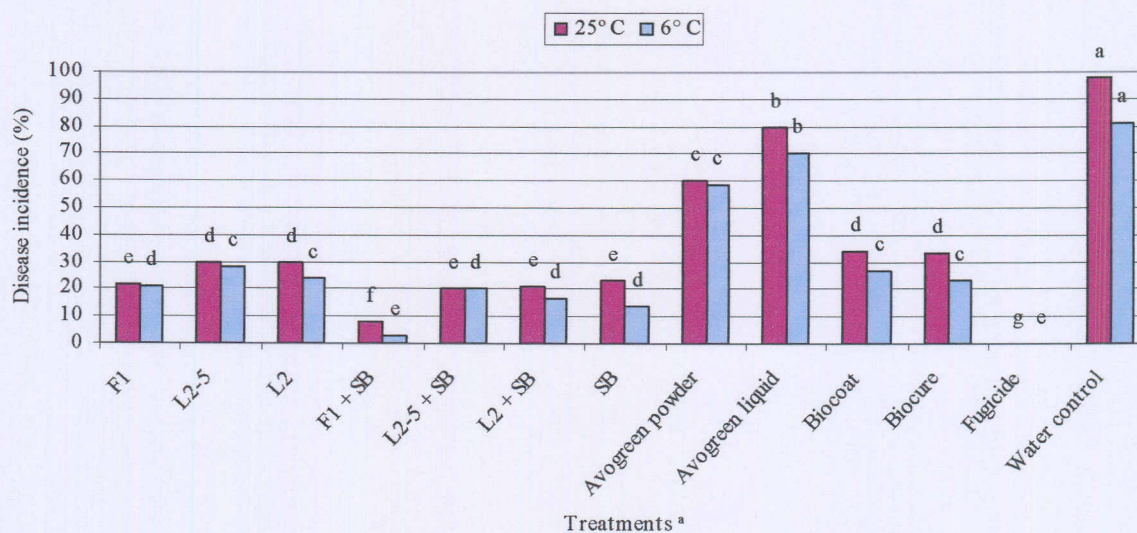
Avogreen liquid. The observed increase in biocontrol activity of *Bacillus subtilis* products following addition of SB was again evident. When integrated, the treatment comprising F1 combined with SB was more effective than all treatments evaluated with a percentage disease incidence of only 2% under cold storage. This performance was not significantly different from the fungicide treatment.

In August, the biocontrol agents used on their own were once again not as effective as the fungicide treatment. The percentage disease incidence when Biocure, Biocoat, L2, and L2-5 were evaluated at 25° C were lower than 35%, and did not differ significantly. The percentage disease incidence in the treatment comprising F1 plus SB at 6° C was lower than 4% (Fig. 5.4B). The combination of L2 + SB and L2-5 + SB was not as effective as SB on its own at 25° C. Avogreen treatments were not as effective as the other treatments evaluated.

During the third trial in September, a higher level of disease incidence in all treatments and at both temperature regimes was recorded relative to the two previous trials (Fig. 5.5). The percentage disease incidence in treatments F1, L2-5, Biocoat, and Biocure at 25° C was lower than 45%, and did not differ significantly. The percentage incidence in these treatments under cold storage varied between 30 and 33% and again did not differ significantly. As previously observed, an improvement in biocontrol activity was recorded with addition of SB and treatment F1 = SB was more effective than all the other antagonists with an incidence of lower than 7% under cold storage. The treatments comprising L2 + SB and L2-5 + SB were not significantly better than SB on its own (Fig. 5.5).
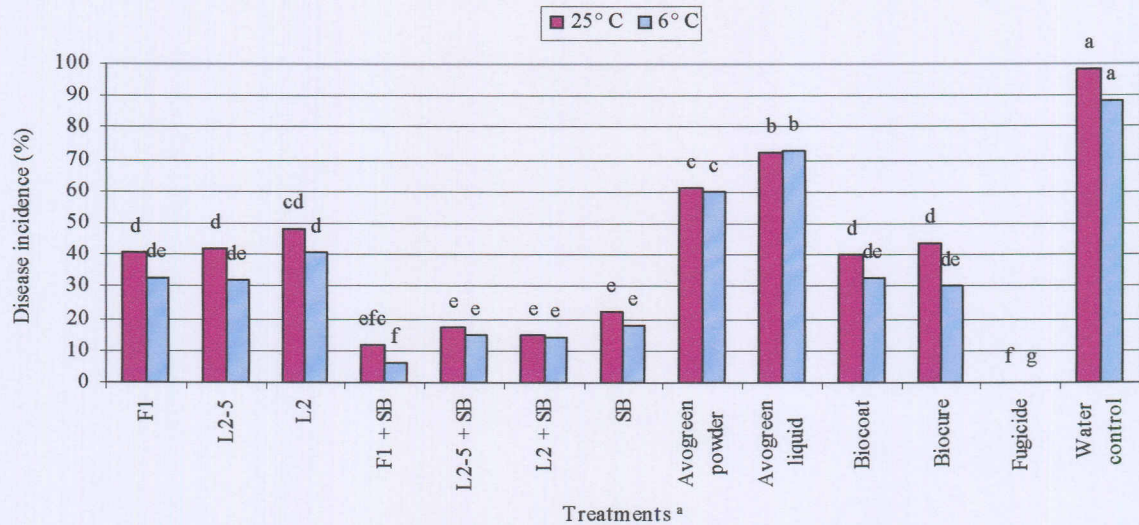
A

B

[a] Biocure and Biocoat represent yeast (*Candida saitoana*) products; Avogreen liquid, Avogreen powder, F1, L2 and L2-5 represent *Bacillus subtilis*; Fungicide represent Fungazil (imazalil 75% a.i. at 1g L^{-1}) plus Decotine (quazatine 20% a.i. at 1 ml L^{-1}); SB represent sodium bicarbonate (1% wt/vol). Values are mean of three replicates. Treatments having same letter are not significantly different according to Duncan's multiple range test (P = 0.05)

Fig 5.4 Evaluation of alternative postharvest disease control options for control of citrus green mold caused by *Penicillium digitatum* on Valencia orange after two weeks of storage at 25° C or four weeks at 6° C in June 2001 (A) and August 2001 (B).
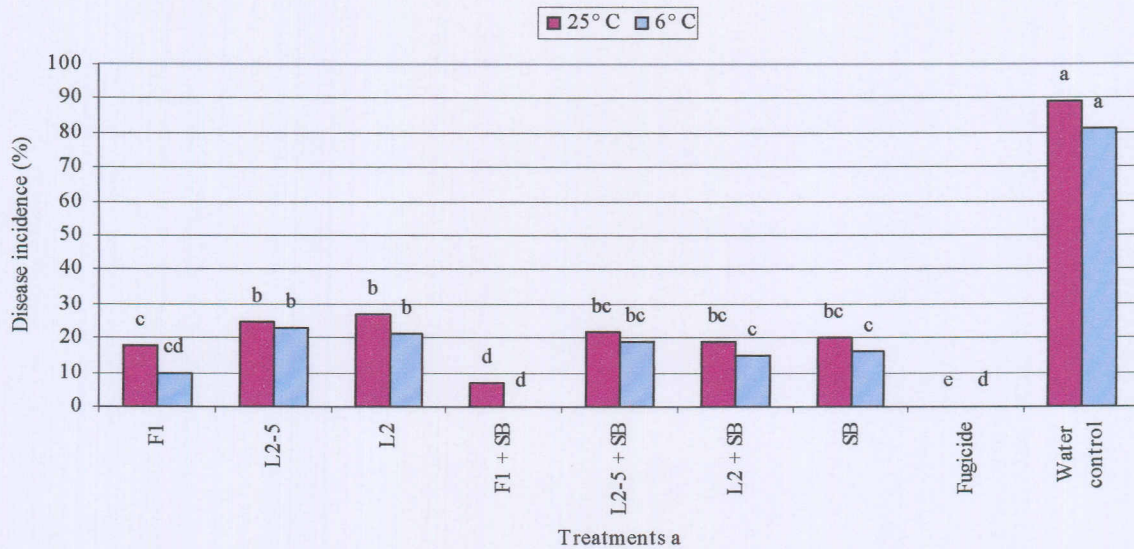
[a] Biocure and Biocoat represent yeast (*Candida saitoana*) products; Avogreen liquid, Avogreen powder, F1, L2 and L2-5 represent *Bacillus subtilis*; Fungicide represent Fungazil (imazalil 75% a.i. at 1g L^{-1}) plus Decotine (quazatine 20% a.i. at 1 ml L^{-1}); SB represent sodium bicarbonate (1% wt/vol). Values are mean of three replicates. Treatments having the same letter are not significantly different according to Duncan's multiple range test (P = 0.05).
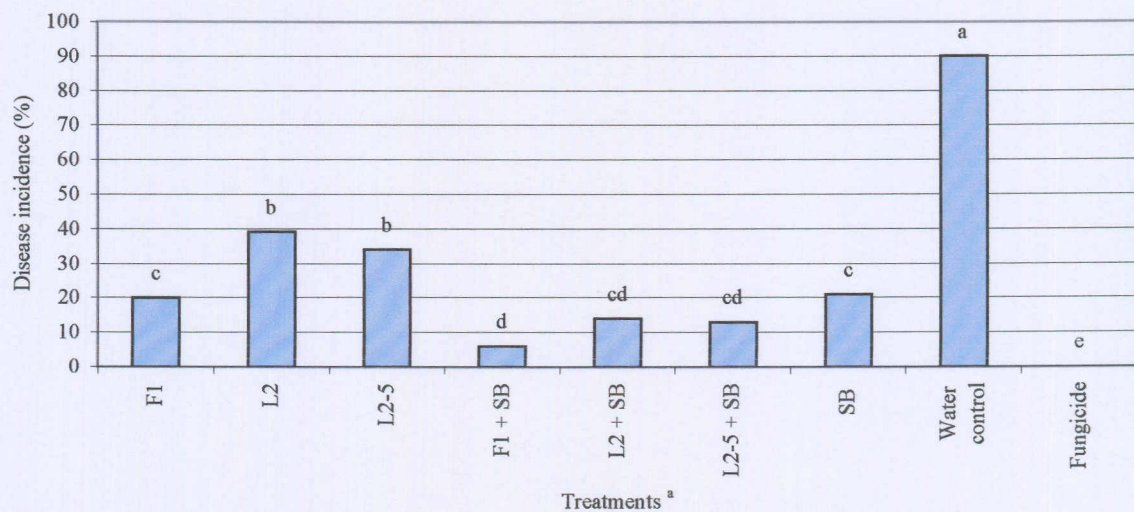
Fig 5.5   Evaluation of alternative postharvest disease control options for control of citrus green mold caused by *Penicillium digitatum* on Valencia orange after two weeks of storage at 25° C or four weeks at 6° C in September 2001.

2002 Season

In August, all treatments were more effective than the water control. Results presented in Fig. 5.6A shows that the percentage disease incidence in treatment F1 under cold storage was 10%, and it was more effective than the other isolates, The percentage disease incidence in SB treated fruits was lower than 16%, and it was more effective than isolates L2, and L2-5 on their own. The combination of these isolates with SB was not significantly better than SB on its own. The treatment comprising F1 combined with SB was as effective as the fungicide treatment, giving complete control of the disease under cold storage.

A

B

[a] F1, L2 and L2-5 represent *Bacillus subtilis* species; Fungicide represent Fungazil (imazalil 75% a.i. at 1g L^{-1}) plus Decotine (quazatine 20% a.i. at 1 ml L^{-1}); SB represent sodium bicarbonate (1% wt/vol). Values are mean of three replicates. Treatments having same letter are not significantly different according to Duncan's multiple range test (P = 0.05).

Fig 5.6   Evaluation of alternative postharvest disease control options for control of citrus green mold caused by *Penicillium digitatum* on Valencia orange after two weeks of storage at 25° C or four weeks at 6° C in August 2002 (A) and September 2002 (B).

The same treatment combination at 25°C was not as effective, but was better than either treatment on its own.

In September-October, all treatments were again less effective than the fungicide treatment (Fig.5.6B). The percentage disease incidence on F1 treated fruit was 20%, and the treatment was again more effective than isolates L2 and L2-5 with a percentage incidence of 39 and 34% respectively. It was however, not more effective than SB (1%) on its own. The percentage incidence in treatments L2 + SB and L2-5 + SB was 13 and 15, and did not differ significantly.

5.5. Discussion

Results obtained in this study further confirm earlier observations on the potential of isolate F1 for biocontrol of *Penicillium* decay. The treatment comprising F1 integrated with SB was consistent in its activity in controlling green mold and further supports the benefits of integrated control as a more consistent alternative disease control option. None of the isolates/products on their own were as effective as the fungicide treatment. Biocontrol agents, as living entities, respond to environmental changes, which may in turn affect their survival and activity (Conway *et al.*, 1999). They may also react to changes within their host tissue, including changes in pH, all of which may interfere with their establishment and activity. The biocontrol agent has to first establish itself at the wound sites before it will produce the secondary, inhibitory metabolites against the pathogen. Fungicides on the other hand are more stable and is immediately active i.e. its activity is less affected by environmental factors. The better performance of fungicides compared to biocontrol agents is therefore understandable.

A higher level of disease incidence was recorded on fruits stored at 25° C relative to those kept under cold storage (6° C). The optimum growth temperature for *P. digitatum* is around 25° C (Carlos, 1982). At this temperature the pathogen grows fast and is difficult to control. At temperatures lower than 10° C however, the pathogen grows slowly, and in the present study, it took more than 72 hours for any growth to be observed on potato dextrose agar (PDA) - data not included. On the other hand, visible growth of the *Bacillus subtilis* isolates evaluated in this study was evident within 48 hours at the same temperature. The better performance of isolates under cold storage may therefore have

resulted from a combination of negative effects of temperature on pathogen growth and a better competitive colonization of the wound site by the faster and better growing *Bacillus* isolates under these conditions. Earlier results on possible modes of action of these isolates (Chapter Three) indicated that isolate F1 for example could colonize both fruit surfaces and flavedo tissue easily.

We observed a general and progressive decline in product efficacy as the season advanced with fruits maintained at both storage conditions. This was more obvious when isolates were used on their own compared to when they were integrated with SB. The physiological state of the fruit is believed to directly influence the efficacy of control measures in the postharvest arena (Howard, 1936). As the season advances, the fruit tissue becomes weaker as it begins to loose its integrity, thus becoming less resistant, and more vulnerable to attack by pathogens. This probably explains the decline in product efficacy. As the season advances, there might also be a reduction in the content of certain chemical compounds (Rodov *et al.*, 1995) and minerals such as calcium within the fruit, which play a crucial role in cell wall integrity and ultimately fruit resistance. Citral for example, is one preformed chemical compound that has been positively linked to disease resistance in citrus fruit (Ben-Yehosshua *et al.*, 1995, Rodov *et al.*, 1995). The concentration of citral was found to decline with fruit age (Ben-Yehosshua *et al.*, 1995). Rodov *et al.* (1995) also reported that the flavedo of green lemon contains 1.5-2.0 times more citral as compared to yellow fruit. They concluded that the level of citral in the flavedo was related to disease resistance in lemon. A compound, 7-geranoxycoumarin, found to be occurring naturally in the flavedo tissue of "Star Ruby" grapefruit was found to be toxic antifungal activity against *P. digitatum* (Agioni *et al.*, 1998). In addition to a higher pathogen activity at 25° C, tissue firmness and integrity is lost faster at this temperature compared to cold storage. This development may also have contributed to the higher disease incidence observed at this temperature.

As earlier observed, the integration of *B. subtilis* with SB resulted in a remarkable improvement in their biological activity. This was particularly true for isolate F1, where in some instances; the treatment combination was as effective as the fungicide treatment, which gave complete control of the disease. This observation is of interest in our search for alternative control measure for *P. digitatum*. As reported previously (Barger, 1928; Palou, 2001) and confirmed by us in Chapter Three, SB impacts negatively on spore germination

and subsequent development of the pathogen. This development creates a vacuum, which is then exploited by the antagonist to its advantage. Sodium bicarbonate is a non-living entity, and so its activity is less dependent on environmental conditions as does biocontrol agents. This probably explains the more consistent performance recorded with SB treatments. However, since SB might not cause complete lysis of spores, and the integrity of the tissue on which it is applied weakens with time, its efficacy is also likely to be affected, and this probably explains the slight decline observed in its performance as the season advanced.

From the present study, it is evident that none of the products evaluated on their own were as effective as the conventional fungicide treatment in the control of *P. digitatum*. An integration of F1 with SB was the most promising treatment. This treatment combination was consistent in its performance under laboratory conditions (Chapter Three and Four), and in semi-commercial evaluation for three seasons. It is possible that the biocontrol activity of the other products evaluated along with the *B. subtilis* isolates might also improve when integrated with SB. In the present study, fruits were artificially inoculated with the pathogen, and the inoculum concentration used (10^6 spores ml^{-1}) were not a true reflection of natural conditions. The probability that the products evaluated in this study will perform better under natural conditions is therefore very high. The use of these products, especially the combination of F1 with SB under export conditions is therefore advocated. We observe however that these products will not give desirable control when used to treat fruits stored at 25° C for up to two weeks.

The findings reported within this chapter are only the first step in the commercialization of any biocontrol agent. These biocontrol agents still have to go through all the protocols and toxicological tests necessary for product registration. The tedious task of optimising product formulation also has to be completed. These aspects however, fall outside the scope of this study.

Literature cited

Agioni, A., Cabras, P.D., Hallewin, G., Pirini, F.M., Remiero, F. and Schirra, M. 1998. Synthesis and inhibitory activity of 7-geranoxycoumarin against *Penicillium* species in citrus fruit. Photochemistry 47: 1521-1525.

Arras, G. 1996. Inhibitory action of microorganisms isolated from citrus fruit against *Penicillium digitatum*. Proceeding of the International Society of Citriculture, 1996, 456-460.

Arras, G., Dessi, R., Sanna, P. and Aru, S. 1999. Inhibitory activity of yeast isolated from fig fruits against *Penicillium digitatum*. Acta Horticultural 485: 37-42.

Auret, E.L. 2000. Control strategies for citrus post harvest diseases. MSc Thesis, University of Pretoria, Pretoria.

Ben- Yehosshua, S., Rodov, V., Fang, D.Q. and Kim, J.J. 1995. Preformed antifungal compounds of citrus fruit; Effect of postmarked treatments with heat and growth regulators. Journal of Agricultural Food Chemistry 43: 1062-1066.

Barger, W.R. 1928. Sodium bicarbonate as a citrus disinfectant. California Citrograph 12: 164-174.

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.

El-Ghaouth, A., Smilanick, J.L. and Wilson, C.L. 2000a. 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.

El-Ghaouth, A., Smilanick, K.J., Brown, J.E., Ippolito, A., Wisniewski, M. and Wilson, C.L. 2000b. Application of *Candida saitoana* and glycolchitosan for the control of postharvest diseases of apple and citrus fruit under semi-commercial conditions. Plant Disease 84: 243-248.

El-Ghaouth, A., Smilanick, K.J., Brown, J.E. , Ippolito, A., Wisniewski, M. and Wilson, C.L. 2000c. Improved control of apple and citrus fruit decay with a combination of *Candida saitoana* and 2-4 Deoxy-D- glucose. Plant Disease 84: 249-253.

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

Janisiewicz, W.J. and Korsten, L. 2002. Biological control of postharvest diseases of fruits. Annual Review of Phytopathology 40: 411-441.

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, Orlando, Florida, USA, 2000.

Northover, J. and Thou, T. 2002. Control of Rhizopus rot of peaches with postmarked treatments of tecuconazole, fludioxonil, and *Pseudomonas syringae*. Canadian Journal of Plant Pathology 24: 144-153.

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.

Rodov, V., Ben-Yehoshua, S., Fang, D.Q. and Kim, J.J. 1995. Preformed antifungal compounds on lemon fruit; Citral and its relation to disease resistance. Journal of Agricultural and Food Chemistry 43: 1057-1061.