CHAPTER THREE

SCREENING *BACILLUS* SPECIES FOR ANTAGONISTIC ACTIVITY AGAINST *PENICILLIUM DIGITATUM* AND *PENICILLIUM ITALICUM*

3.1. Abstract

Several *Bacillus* species originally isolated from citrus fruit surfaces were evaluated *in vitro* for possible antagonistic activity against *Penicillium digitatum* and *P. italicum*, the cause of citrus green- and blue molds respectively. Fifty percentage of the isolates screened possessed some degree of antagonistic properties, and were effective in checking the growth of both pathogens. Three isolates, F1, L2-5 and L2 were particularly effective in this respect. Several possible modes of action were investigated including antibiosis, competitive colonization, production of siderophores and production of volatile compounds. No one isolate exhibited all of these characteristics. Some of the characteristics were either completely absent in some isolates or weakly present.

3.2. Introduction

Fungicides have for many years been the most effective method of control of postharvest diseases of fruits. Citrus is no exception and growers have for years used postharvest fungicides such as imazalil and quazatine effectively to control the major postharvest diseases. This technology enabled growers to export their fruits to distant markets due to the protective activity of these fungicides. The South African citrus growers have therefore built a highly successful industry with the bulk of their fruit being exported to distant markets in mainly European countries.

However, the use of fungicides has increasingly been curtailed by the development of pathogen resistance to many key fungicides and the negative public perception regarding their safety (Janisiewicz and Korsten, 2002). These negative perceptions regarding the effects of pesticides on human health have compelled the United States of America (USA) and European community to introduce more restrictive legislation regarding the tolerance level of pesticides in the food chain. These developments have necessitated a need to

identify more acceptable methods of control in order for growers to remain relevant at an international level.

Biological control of postharvest diseases is strongly emerging as an effective alternative to the use of synthetic fungicides. Few areas of research within plant pathology have attracted more interest during the last 20-25 years than has the use of introduced microorganisms for biological control of plant pathogens (Cook, 1993). The fruit microflora at harvest is a rich resource of antagonists (Smilanick, 1994). The inhibitory activities of some of these microorganisms play an important role in the natural control of numerous plant diseases. Many microorganisms with antagonistic properties have been identified, evaluated and registered for commercial use such as *Bacillus subtilis* "Avogreen", registered in South Africa by the University of Pretoria, for the control of avocado fruit diseases, and the yeast, "Aspire" registered for control of citrus mold and marketed by Ecogen Inc.in the USA. However, there is obviously an untapped pool of microorganisms of which many more beneficial microorganisms are yet to be discovered. The search for new microorganisms with antagonistic properties is therefore a continuous process.

Bacillus species produce spores that are resistant to desiccation, heat, UV irradiation, and organic solvents (Roberts and Hitchins, 1969). These qualities make them more resistant to adverse weather conditions. The antagonistic activity of *Bacillus* species against many postharvest pathogens (particularly citrus) has been demonstrated (Singh and Deverall, 1984; Huang *et al.*, 1992; Auret, 2000; Korsten *et al.*, 2000). Although several *Bacillus* species have been successfully isolated and screened *in vitro* and *in vivo* for control of postharvest pathogens, concerns over the possibility of antibiotic production have been raised particularly when used directly on fruit surfaces. However, it has not been shown that antibiotics are produced on fruit surfaces or that it will pose a major threat to human health if introduced into the food chain through the application of biocontrol agents. Many phenotypically identical microorganisms are reported to be capable of producing vastly different kinds of secondary metabolites, each of which might be highly target specific (Cutler, 1986). This is particularly true for *Bacillus* species.

Information on the mode of action of a biocontrol agent is necessary, not only for the purpose of optimizing the performance of such an organism, but also for registration

purposes. The main objective of this study was therefore to screen *Bacillus* species originally isolated from citrus fruit surfaces for possible antagonistic activity against two important citrus postharvest pathogens, *Penicillium digitatum* Sacc. and *P. italicum* Wehmer, the cause of citrus green- and blue mold respectively. In addition to identifying isolates with high antagonistic activities, the possible mode(s) of action of these bacterial isolates were also investigated.

3.3 Materials and Methods

3.3.1 Pathogen

Isolates used in this study were selected from the culture bank of the Plant Pathology Laboratories, University of Pretoria. *Penicillium digitatum,* isolate Q103 and *P. italicum,* isolate JO/1/01 were originally isolated from diseased fruits and were selected for this study based on their high repeated virulence on citrus fruits. Professor F.C. Wehner of the Department of Microbiology and Plant Pathology, University of Pretoria originally confirmed their identity. Isolates were maintained on potato dextrose agar (PDA) (Biolab), in MacCartney bottles at 7° C until use. Stock cultures of the test cultures were prepared for use throughout this study and were maintained in the culture collection of Plant Pathology Laboratories, University of Pretoria.

3.3.1.1. Preparation of pathogen spore suspension

Before each trial, cultures were grown on PDA at 25° C for seven days. Spores were harvested by gently swabbing the culture surface with a sterile swab and shaking the spore-laden swab in sterile distilled water to dislodge the spores. A conidial suspension was prepared in Tween 80 [Fluka, (0.05% wt/vol)] and the inoculum concentration (10^6 spores ml^{-1}) determined with the aid of a haemocytometer. Although this inoculum concentration is generally regarded as being too high, for *Penicillium* evaluation, it is commonly used in citrus experiments (Eckert and Ogawa, 1985).

3.3.2. Isolation of potential antagonist

Valencia and Shamouti oranges are susceptible to both green- and blue mold. The potential antagonist were isolated from the surface of these two cultivars from fruit lots

that had received no fungicide treatment. The fruits were collected from Letaba Estates, a commercial farm in the Limpopo Province of South Africa. Isolations were made from 90 freshly harvested fruits of each cultivar at different time intervals of the harvesting season (June, July and August) in 2000 to capture the microbial spectrum on the fruit surfaces over the season. Individual, visually 'clean' fruits were placed in 1L-glass beakers containing 250 ml sterile distilled water. Beakers were placed on a rotary shaker (67 rpm) for 30 minutes. One hundred microlitres of the wash water was plated out onto standard 1 nutrient agar (STD 1) (Biolab). Plates were incubated at 27 °C for 24 hours. Isolates were selected based on their typical *Bacillus* colony growth and morphological characteristics. The identities of isolates selected were confirmed using the API 50 CH system. Forty-one isolates were selected from the initial fruit isolation. Purified cultures were stored in glycerol at -70° C until use.

3.3.3. Screening

3.3.3.1. Preparation of bacteria cell suspension

Isolates were grown on STD 1 at 27° C for 24 hours. A loop of each culture was transferred to a 250 ml conical flask containing 50 ml sterile nutrient broth (NB) (Biolab), and incubated on a rotary shaker (67 rpm) for 48 hours at 25±2° C. Cultures were centrifuged for 15 minutes at 7500-x g using Labofuge ^{GL} (Haraeus-Christ GMBH Osterode), Premier Technologies. The resulting pellet was resuspended in sterile distilled water and centrifuged a second time. Washed cells (pellets) were suspended in quarter strength Ringer's solution (Merck). Bacterial concentrations were determined with a Spectrophotometer (LKB 4050), Separations Scientific (Pty) Ltd, at 420 nm. The cell concentration was determined from a calibration curve that equates absorbance with number of colony forming units (CFU) per ml determined from a dilution plating series on STD 1.

3.3.3.2. Minimal inhibitory concentration

To determine the minimum inhibitory concentration, 250 µl of 10^6, 10^7 and 10^8 cell ml^{-1}, of bacterial cells prepared as described in 3.3.3.1 was dispensed separately in microtiter (Nunc; AEC,-Amersham (Pty) Ltd), plate wells and inoculated with *P. digitatum* and *P. italicum* (10^6 spores ml^{-1}) respectively prepared as described in 3.3.1.1. Each microtiter well represented a replicate and each treatment was replicated four times in a completely

randomized design (CRD). Plates were incubated for one to three hours at 25° C following which 50 µl of cell-spore suspension was removed from each well and pipetted onto PDA plates amended with chloramphenicol (250 ppm L^{-1}), to prevent bacterial growth. Inoculated PDA plates were incubated at 25° C for 10 days following which fungal colony diameter was measured. This involved measuring the radial growth of the pathogen along two perpendicular lines drawn on the underside of each Petridish with a meter rule and the mean calculated. Five plates were used per treatment, and the experiment was repeated once. The control plate was inoculated with pathogen spores suspended in sterile distilled water only. Only treatments that allowed an average growth of 50 mm or less relative to the control plate for both pathogens were recorded. All further tests were evaluated with three potential antagonists (F1, L2 and L2-5). These isolates were selected based on their *in vitro* performance and on their ease of cultivation i.e. growth in culture media. On the basis of these criteria other potential antagonist such as isolate 143 was not included in further *in vivo* evaluations.

3.3.4. Mode of Action

Many microorganisms are reported to produce secondary metabolites. When grown *in vitro*, some of these organisms produce metabolites within a narrow time frame of two to three days (Cutler and Hill, 1994). Experiments were designed to give an insight into the possible mode(s) of action of isolates which includes production of secondary metabolites, volatile compounds, and production of siderophores, all of which have previously been reported as possible modes of action of microbial antagonists. The ability of isolates to attach to fruit surface, and establish at the wound site was also investigated.

3.3.4.1. Production of secondary metabolites

Antibiosis

Three vertical lines, 25 mm apart, were drawn on the underside of 90 mm Petridish containing 25 ml aliquots of PDA. A loop full of cell suspension (10^8 cell ml^{-1}) of each test bacterial isolate was streaked out on the two outer lines. Inoculated plates were incubated at 27° C for 24 hours to allow bacterial growth, following which the middle lines in each plate was streaked with either *P. digitatum* or *P. italicum* (10^6 spores ml^{-1}) using the same procedure. Control plates were streaked on the borderlines with sterile distilled water instead of bacteria. Plates were further incubated at 27° C for 10 days and fungal

growth determined thereafter. This involved measuring the colony width at two points (4-5 cm apart), along the vertical line using a meter rule, and determining the mean. Pathogen growth on these media was compared with the control. Five plates were used per treatment, and each experiment was repeated once. Data obtained were statistically analysed.

The antibiotic activity of isolates was evaluated using the method described by Mc Keen *et al.* (1986). The fungicidal properties of the crude "antibiotic" extract obtained from 5-day old "potential antagonist" cultures grown up in nutrient broth at 25° C, were tested at three concentrations (100, 500, and 1, 000 μg ml^{-1}) using a slightly modified version of the direct soak method described by Mc Keen *et al. (*1986). Spores of *P. digitatum* and *P. italicum* (10^6 spores ml^{-1}) prepared in 0.1 M sodium phosphate buffer (pH 7.5) were suspended in 2 ml of buffered crude inhibitory extract in centrifuge tubes. Two controls were included, 1) spores suspended in buffer alone, and 2) spores suspended in a solution of commercial fungicide prepared as described for imazalil (Janssen), 1 000 ppm and quazatine (Aventis) 1 000 ppm. After 12 and 24 hours, the spore suspensions were centrifuged at 7, 500 x g for 10 minutes. The pellet was washed twice in sterile distilled water. Fifty microlitres of the resultant spore suspension was pipetted onto PDA plates and incubated at 25° C for 10 days following which colony diameter was measured as described earlier (3.3.3.2). Five plates were used per treatment, and each experiment was repeated once. Data obtained were statistically analysed.

Effect of volatile compounds on pathogen growth

Fifty microlitres of a suspension of each isolate (10^8 cell ml^{-1}) prepared as described earlier (3.3.3.1) was spread out on 90-mm Petridish containing 25 ml aliquots of STD 1. Another set of plates containing the same quantity of PDA was inoculated with either *P. digitatum* or *P. italicum* (10^6 spores ml^{-1}) by centrally placing 5μl of spore suspension in the Petridish. Once the surface dried (usually after one hour), the lids were removed and the bacterial plates were placed open ended on the fungal plates and sealed with parafilm. Plates were incubated at 27° C for between seven and 14 days following which fungal colony diameter was measured as described earlier (3.3.3.3). The control consisted of STD 1 plates streaked with sterile distilled water instead of bacteria. Each treatment was replicated five times, and the experiment was repeated once. Data obtained were statistically analysed.

Production of siderophores

The "CAS" assay (Schwyn and Neilands, 1987) was adopted. This assay is based on the removal of ferric iron from the deep blue Chrome Azurol sulfonate ferric complex to yield a bright orange-colored ligand. Isolates were inoculated on a 90-mm Petridish containing 25 ml aliquots of siderophore indicator medium. The chemical composition of this medium is indicated in Appendix 3. Plates were incubated at 25° C for 10 days, after which the diameter of the golden-yellow halo formed on the siderophores media, was measured using the procedure described earlier (3.3.3.3). The diameter of the halo was assumed to be proportional to the amount of siderophores produced by each isolate. Data obtained were statistically analysed.

3.3.4.2. Fruit attachment

Scanning electron microscopy (SEM) was performed to observe the attachment of test isolates on the fruit surface and in wound sites. Valencia oranges were wound inoculated with each test isolate with sterile dissecting needles dipped in the bacterial cell suspension (10^8 cell ml^{-1}). Inoculated spots were marked with a waterproof pen. Twelve and 24 hours after inoculation, three pieces of peel tissue (5 x 5 mm) were removed from the inoculation point with a sterile scalpel. Samples were fixed in 3.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.0) for 24 hours at 9° C and washed in three changes (15 minutes each) of sodium phosphate buffer (pH 7.2). Washed samples were suspended in 2% osmium tetraoxide (OsO_4) for two hours, and further rinsed in buffer as described above. Samples were dehydrated in an ethanol series 50, 70, 90, and 100% (15 minutes each) and further dried (critical point drying) for SEM viewing. Dried samples were mounted on aluminum stubs, and coated with gold-palladium, and viewed with a JEOL 540 SEM at 5 Kv.

3.3.5. Data analysis

All data was statistically analysed using the GenStat statistical program. One-way analysis of variance (ANOVA) was used to test for differences in average means between treatments. Treatment means were separated using Fishers' protected t-test at a 5% level of significance.

3.4. Results

3.4.1. Minimal inhibitory concentration

Forty-one *Bacillus* species were identified from the isolations made from the fruit surface (Appendix 1), representing approximately 30% of the microflora. Of this number, none could completely inhibit the growth of both *P. digitatum* and *P. italicum* at all cell concentrations tested. The level of growth inhibition was not significant for 21 isolates tested when compared with the control even at the highest cell concentration evaluated (10^8 cell ml^{-1}). These results were therefore not included in the data presented. Results presented in Table 3.1 shows a general increase in the biocontrol activity of most isolates with increase in cell concentration. A growth of 50 mm or less was recorded in 20 of the 41 isolates at the highest concentration (10^8 cell ml^{-1}) for both pathogens (Table 3.1). The growth of both fungi in treatments tested against isolates F1, L2-5, and L2 was lower than 27 mm and did not differ significantly.

3.4.3. Production of secondary metabolites

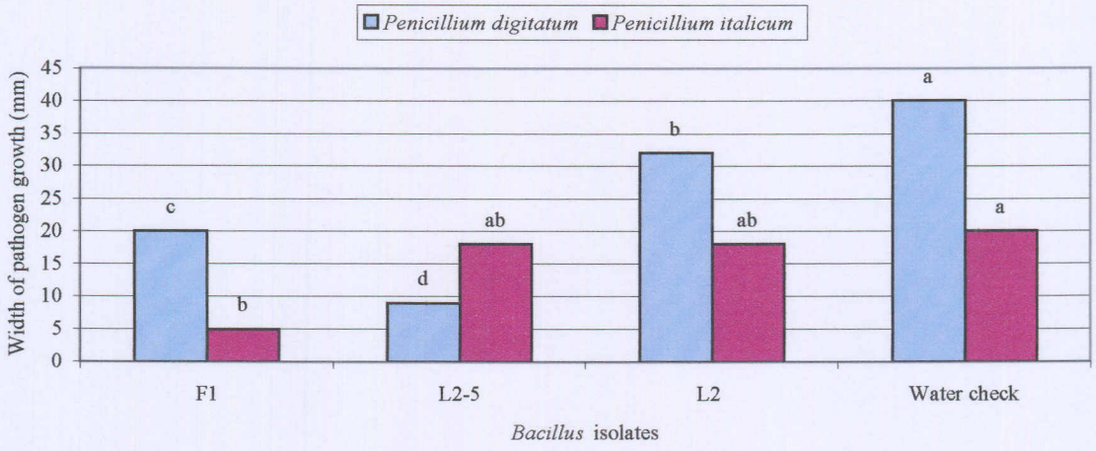
Antibiosis - All isolates tested did produce secondary metabolites with inhibitory properties. However, the degree of production and efficacy of such products as measured by the inhibition of pathogen growth on PDA varied between pathogens and between isolates (Fig. 3.1 and 3.2). The effect of antibiosis was generally more visible on *P. digitatum* than *P. italicum*. The metabolic products produced by isolates L2-5 and L2 for example were not effective in inhibiting the growth of *P. italicum* when compared with the control treatment. The crude antibiotic extract method used resulted in none of the extracts from the test isolates inhibiting the growth of the pathogens tested (data not presented).

Table 3.1 Effect of *Bacillus* species cell suspension on the growth of *Penicillium digitatum* and *P. italicum* on potato dextrose agar following incubation at 25° C for 10 days

Bacillus isolate [a]	*Penicillium digitatum* [b]			*Penicillium italicum* [b]		
	Bacterial cell concentration (cell ml^{-1})					
	10^6	10^7	10^8	10^6	10^7	10^8
268	38	38	35	33	33	30
F1	34	31	25	23	21	17
L2-5	42	41	26	25	23	19
OPF1	40	40	41	44	43	37
OP2-5	44	39	40	43	38	38
L3	47	44	38	32	30	27
719C	44	39	39	40	33	27
OPL2A	52	48	42	45	38	34
143	30	20	22	34	31	28
T1	61	47	40	40	33	32
L2	41	37	25	30	23	18
565	41	35	28	31	31	26
T2	47	40	32	31	31	26
L2-2	45	41	41	47	40	31
LIA	48	37	35	35	30	30
80	55	49	46	52	45	41
2	52	44	44	52	43	40
814	47	40	33	28	24	21
642	41	41	36	31	31	27
341	40	37	38	33	34	36
Water control	61	-	-	52	-	-

[a] Refer to Appendix 1 for identity of isolates.

[b] Colony diameter of pathogen (mm) representing mean of five replicates and two repetitions.



Treatments having same letter are not significantly different according to Fishers' protected t-test (P = 0.05).

Fig. 3.1 Inhibition ability of secondary metabolites produced by different isolates of *Bacillus* against the growth of *Penicillium digitatum* and *P. italicum* potato dextrose agar plates incubated for 10 days at 25° C.

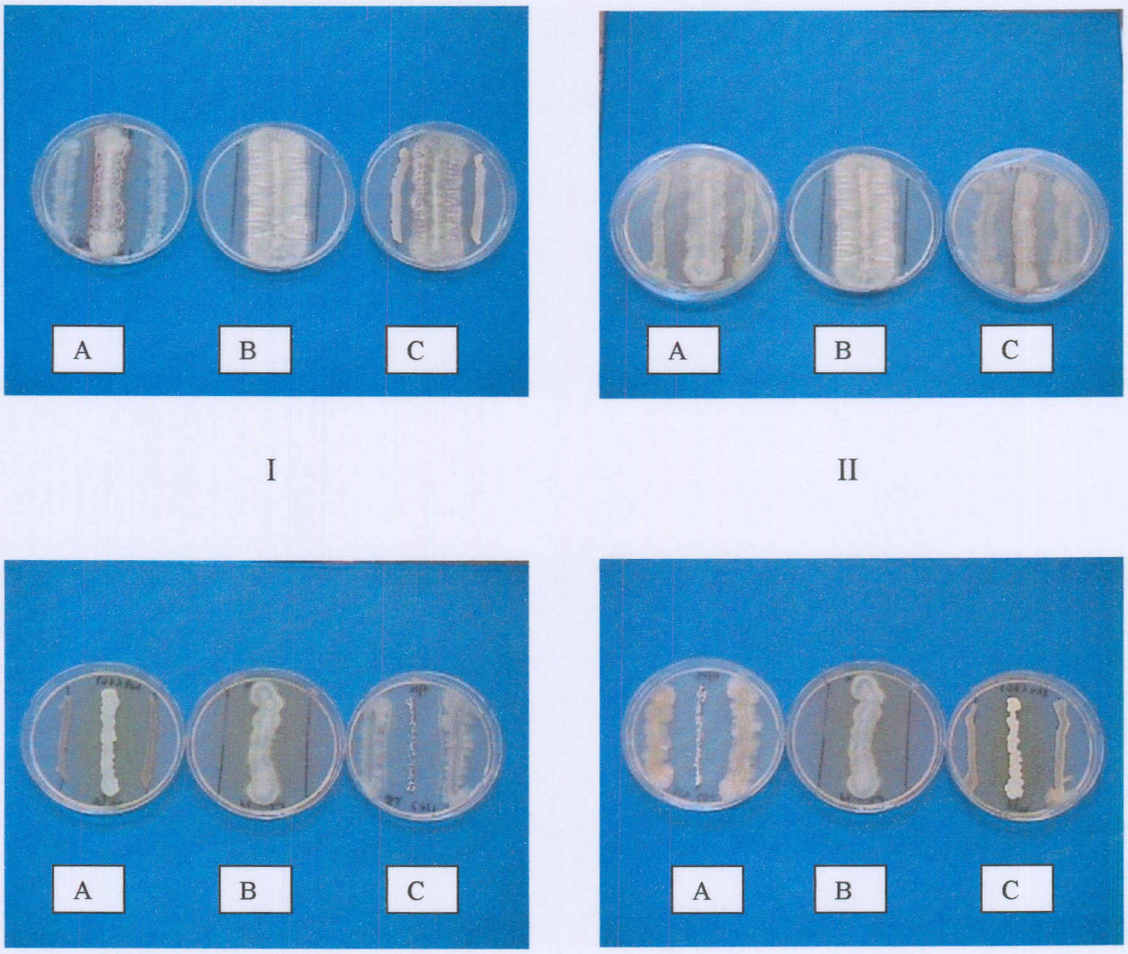


Photo I

A = Isolate F1/*Penicillium digitatum*

B = *P. digitatum* alone (check)

C = Isolate L2/*P. digitatum*

Photo II

A = Isolate L2-5/*P. digitatum*

B= *P. digitatum* alone (check)

C = Isolate 143/*P. digitatum*

Photo III

A = F1/*P. italicum*

B = *P. italicum* alone (check)

C = L2/*P. italicum*

Photo IV

A = L2-5/*P. italicum*

B = *P. italicum* alone (check)

C = 143/*P. italicum*

Fig.3.2. Inhibition ability of secondary metabolites produced by different isolates of *Bacillus* against the growth of *Penicillium digitatum* and *Penicillium italicum* grown on potato dextrose agar and incubated for 10 days at 25° C

3.4.3.3. Effect of volatile compounds on pathogen growth

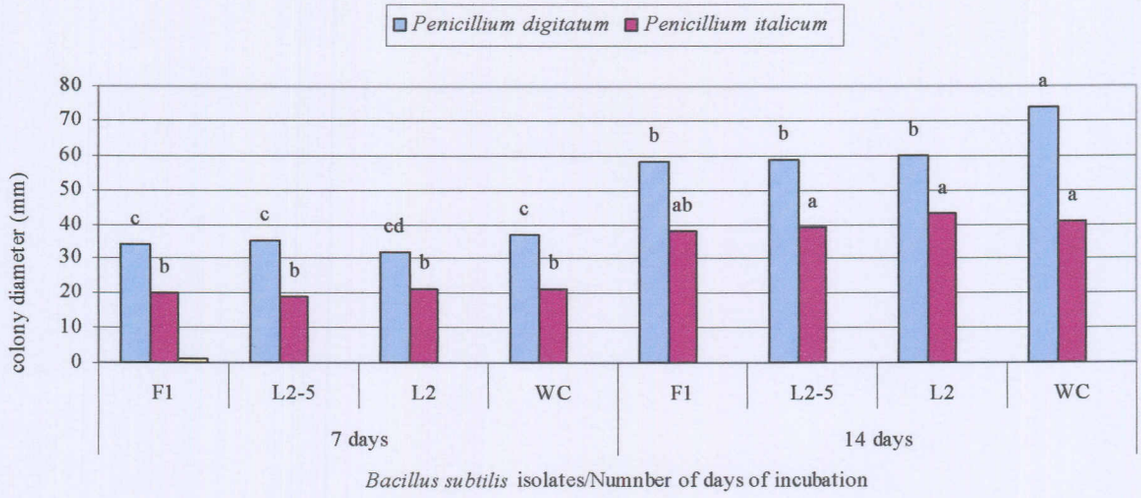
None of the isolates produced volatile compounds, which completely inhibited the growth of both *P. digitatum* and *P. italicum* (Fig. 3.3). Also, none of the isolates' volatiles significantly inhibited the growth of both pathogens when compared to the control after seven days incubation. At 14 days however, a significant difference was recorded between the growth on the control and the other treatments.

3.4.3.4. Production of siderophores

Not all the isolates tested were capable of producing siderophores, and even within the siderophore producers, the degree of production varied between isolates (Fig.3.4). Isolate L2-5 for example-produced siderophores readily as indicated by the bright orange coloured zone. Similarly, isolate L2 produced siderophores but to a lesser extent than isolate L2-5. Isolate F1 failed to produce siderophores. Siderophore production as indicated by the orange coloured zone, was visible in isolate L2-5 from as early as 24 hours following incubation. No visible colour change was observed in L2 plates until 48 hours after incubation. Results presented in Fig.3.4 shows that the pathogen (*P. digitatum*) also produced siderophores but to a limited extent. Unlike the bacterial isolates however, it took an average of seven days for the orange coloured zone to become visible, and the quantity of siderophores produced was far less than that of isolates L2-5 and L2. When both antagonists and pathogens were seeded on the same plate, the pathogen failed to grow probably because the inoculation point was over run by the yellow halo produced by the antagonists (data not presented).

3.4.3.5. Fruit colonization

Results presented in Fig 3.5 indicate that the isolates were capable of attaching and colonizing fruit surfaces and the wound site.



Data represent means of two repetitions. Means having same letter are not significantly different according to Fishers' protected t -test (P = 0.05).

Fig. 3.3 Effects of volatile compounds produced by *Bacillus subtilis* isolates on the growth of *Penicillium digitatum* and *P. italicum* on potato dextrose agar after seven and 14 days of incubation at 25° C.

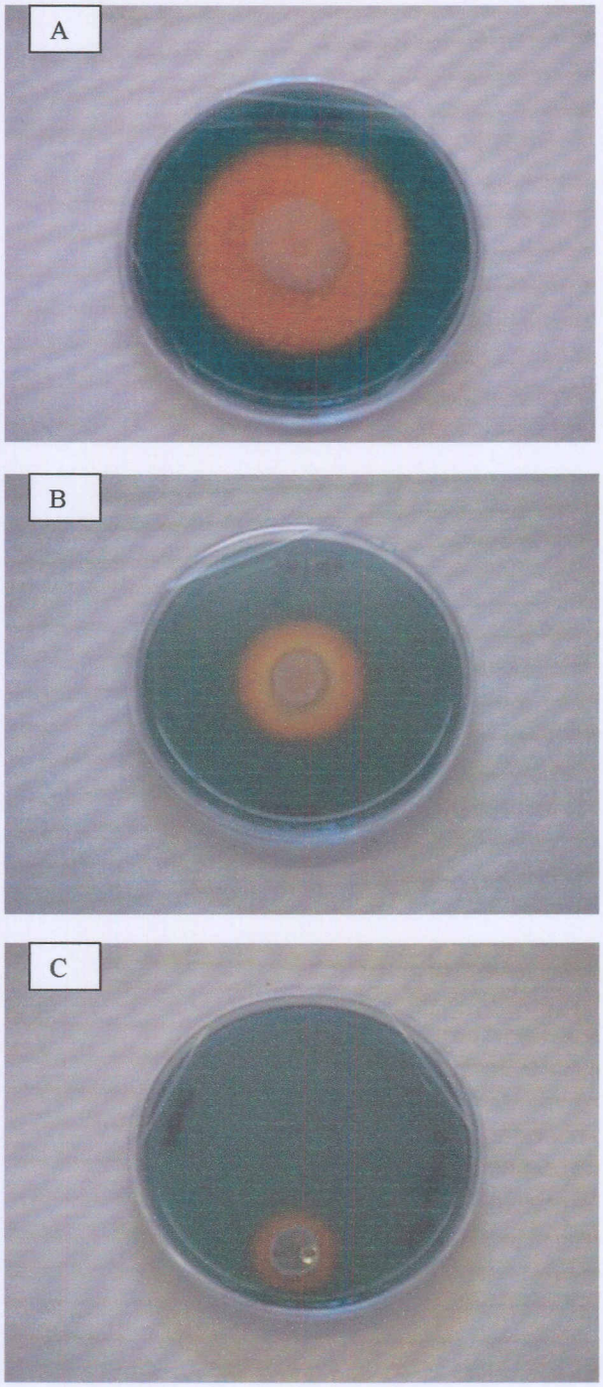


Fig. 3.4 Siderophore production ability (in yellow halo) of *Bacillus subtilis* antagonist after 10 days of incubation at 25° C, with photo **A** representing isolate L2-5; **B** isolate L2; and **C** the pathogen, *Penicillium digitatum.*

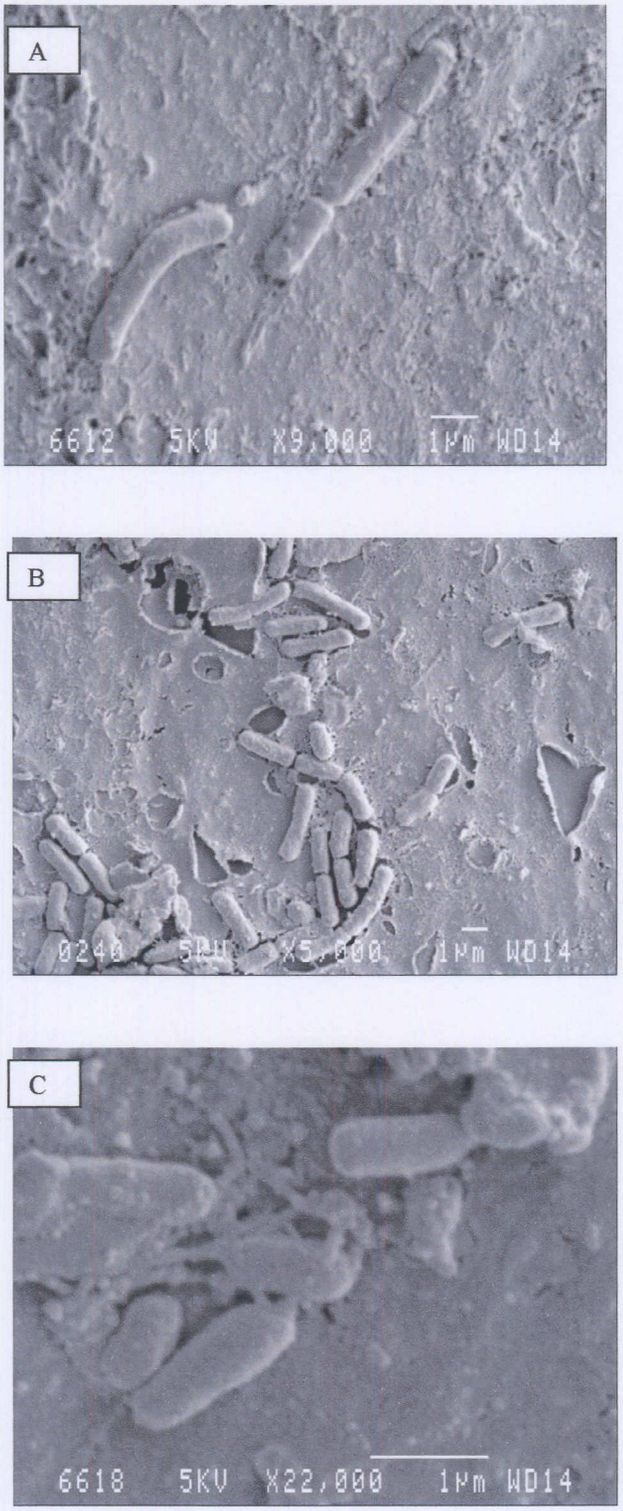


Fig. 3.7 Colonization and attachment of *Bacillus subtilis* on Valencia orange, Photo **A** and **B** represent colonization of the fruit surface by isolate F1 after 12 and 24 hours of application, while **C** shows attachment of isolate L2 at the wound site after 12 hours of application

3.5. Discussion

Thirty percent of the microorganisms isolated from the surfaces of Valencia and Shamouthi orange cultivars were *Bacillus* species. This observation compares favourably with other research findings (Singh and Daverall, 1984; Arras, 1996) where several *Bacillus* species were isolated from similar environments. *Bacillus* species has also been isolated from other types of fruit surfaces such as avocado (Korsten, 1093, Korsten *et al.*, 1995) and mango (Korsten *et al.*, 1991). Fifty percent of the *Bacillus* species screened *in vitro* for antagonism against both *P. digitatum* and *P. italicum*, showed some potential activity against both pathogens. This observation agrees with earlier reports i.e. Singh and Deverall (1984), Huang *et al.* (1992), Auret (2000), Korsten *et al.* (2000) on the antifungal properties of *Bacillus* species. Four of the isolates (F1, L2, L2-5 and 143) were particularly effective in inhibiting the growth of both pathogens. Of these, F1 proved most effective against both pathogens.

Based on the result obtained in the present study a few possible modes of action could be postulated which include antibiosis, production of siderophores, and colonization of wound sites and fruit surfaces. Two of the isolates (L2-5 and L2) possessed all of the above characteristics. Some characteristics especially the ability to produce siderophore was however, either strongly observed (L2-5), weakly present (L2), or absent (F1). These observations confirm earlier reports (Pusey, 1994) that most antagonists exhibit more than one mode of action and in nature, no one mode of action is actually exclusive of the other.

With the antibiosis assay, it was evident that all three isolates could inhibit the growth of both *Penicillium* species by means of a secondary metabolite. Many phenotypically identical microorganisms are capable of producing vastly different kinds of secondary metabolites including alcohols and acetic acids (Atlas and Bartha, 1998), ammonium (Fravel, 1988), and antibiotics (Mc Keen *et al.*, 1986; Atlas and Bartha, 1998), which may be inhibitory to other micoorganisms and thereby giving them a competitive advantage over other competing organisms. Many *Bacillus* species including *B. subtilis* produce, as a major product of glucose fermentation, alcohol (especially low molecular weight ethanol), a range of enzymes and polypeptide antibiotics all of which may be lytic (Buchanan and Gibbons,(1974). Secondary metabolites of *B. subtilis* were inhibitory to several plant pathogenic fungi including *Ceratocystis ulmi* Bruisman (Asante and Neal, 1964),

Monilinia fructicola Wint. (Mckeen *et al.*, 1986) and *Colletotrichum gloesporoides* Penz. (Korsten *et al.*, 1991). In this study the *B. subtilis* isolates proved effective against *P. digitatum* and *P. italicum*, similar to studies by Asante and Neal, (1964), and Singh and Deverall (1984).

None of the tested isolates produced antibiotics when evaluated according to the technique described by Mc keen *et al.*, (1986). Depending on the source, and mostly because of environmental pressure, organisms may produce different types of metabolites. In the case of *B. subtilis*, the most commonly produced antibiotic is iturin (Cutler and Hill, 1994). However, even within the iturin producing strains of *B. subtilis* the congeners produced and consequently the efficacy to control certain phytopathogens may vary (Cutler and Hill, 1994). The fact that none of the tested isolates produced antibiotics using this assay could indicate that it is not a mode of action viz antibiotic production or it may be that the technique used was inadequate to detect all antibiotics.

Antibiosis (as measured by the inhibition of pathogen growth) was better expressed by isolate L2-5, relative to F1 and L2 in the assays selected. However, a single metabolite generally does not account for all the antagonistic activity of a biocontrol agent (Loper and Lindow, 1993), and even when antibiosis plays an important role in the biocontrol of plant diseases, it is generally not an exclusive role (Fravel, 1988). Since a given strain of an organism often produces several types of metabolites. The most practical and convincing way to prove the involvement of a given metabolite in the antagonistic activity of a biocontrol agent is to produce mutants unable to synthesis such metabolite(s), and demonstrate that they no longer possess inhibitory activities against the pathogen or disease in question (Weller and Thomashow, 1993).

In addition to antibiosis (secondary metabolites), other coumpounds such as siderophores (Neilands, 1981; Leong, 1986) are also reported to play a role in the biocontrol of some bacteria including *Pseudomonas* species (Simeoni *et al.*, 1987), and *Enterobacter cloacae* (Fravel 1988). In the study, isolate L2-5 produced siderophores within 24 hours. The role of siderophores in the biocontrol activity of *B. subtilis* is not well documented. Competition for iron (through production of siderophores) is reported to be one of the modes of action by which fluorescent *Pseudomonas* limit the growth of pathogenic fungi and reduce disease incidence and severity (Alabouvette and Lemanceua, 1999). Of interest

in this study is the limited production of siderophores by *P. digitatum* when grown on its own, and particularly the non growth of the pathogen when inoculated together with the antagonist. This indicates that the antagonist in combination with *P. digitatum* could effectively remove the iron from the environment giving it a competitive advantage over the pathogen during competition in an iron deficient environment. It is therefore possible that competition for iron is one of the modes of action of particularly isolate L2-5. Competition for one element is however not exclusive of other minerals necessary for growth by the pathogen. Siderophores in some instances could serve as potent antibiotics (Neilands, 1981). Unlike isolates L2-5 and L2 however, isolate F1 was incapable of producing siderophores, which obviously means that competition for iron is most likely not a possible mode of action of this isolate. Siderophores are commonly produced by aerobic and facultative anaerobic bacteria and by fungi (Neilands, 1993). There is however no reference in the literature on the production of siderophores by *Penicillium* species. Most of the research on the role of siderophores in pathogen control has also been on the control of soil-borne pathogens (Leong, 1986). Results obtained in this study therefore give an additional insight into the modes of action of *Bacillus* species generally and in particular the isolates tested in this study.

The production of volatile compounds is also reported to a play role in the biocontrol of some bacteria. Ammonium isolated from the volatiles produced by *Enterobacter cloacae* (Fravel 1088) was reported to inhibit fungal growth when added to fresh media. From the results obtained in the present study we can deduce that after seven days of incubation, none of the potential antagonists tested produced volatile compounds with visible inhibitory activity against both *P. digitatum* and *P. italicum*. Some observable differences were however, recorded after 14 days incubation indicating that all four test isolates produced some volatiles active against *P. digitatum*. However, since the inhibitory activity of such volatiles against the pathogens was not evident after seven days; the contribution of such volatiles to the *in vivo* control of a fast growing pathogen like *Penicillium* is therefore questionable.

Scanning electron micrograph shows that the antagonists can attach, multiply and colonize the fruit surface and wound site. These observations are similar to those of Korsten *et al.*, (1995) on avocado. These characteristics should enhance the ability of the potential antagonists to compete for both nutrients and space.

Any microorganism with antagonistic properties and ability to disrupt pathogen growth and development is of great interest in biocontrol systems. Three isolates of *Bacillus subtilis* screened in this study (F1, L2-5 and L2) exhibited high antagonistic activity against *P. digitatum* and *P. italicum* and therefore seems to be potential antagonists for citrus postharvest diseases caused by *Penicillium*. Results obtained in this study indicated that the isolates tested did not produce antibiotics or that the inhibitory activity of such substance(s) was negligible. One of the arguments against the use of *Bacillus* species as antagonist is the production of antibiotics because of the real or perceived side effects in the food chain. Although *Bacillus* species are believed to produce antibiotics, they are also reported to produce several other non-antibiotic secondary metabolites (Buchanan and Gibbons, 1974) all of which are reported to have inhibitory properties. Equally important is the observation that iturin, the most frequently produced antibiotic by most *B. subtilis* strains is not only easily degraded, but also has a low toxicity. Also, under laboratory conditions, some microorganisms may produce antibiotics which can be demonstrated to be potent inhibitors of other microbial populations; however, the role of antibiotics in nature is subject to debate as conditions that favour the production of these compounds are not normally found in natural habitats (Atlas, and Bartha, 1998). So, the fact that a microorganism produces antibiotics *in vitro* does not necessarily mean it will do so *in vivo*, and that such compounds will play any significant role in the mode of action of the organism. Isolates F1, L2-5 and L2 will therefore be evaluated further on fruits to determine if the biological activity recorded in this trial could be repeated under laboratory conditions.

The debate surrounding the mode of action of microorganisms in general and *Bacillus* species in particular is one that will continue and may never fully be understood in natural ecosystems. The results presented in this chapter are therefore by no means a detailed study of the mode of action of this group of organisms, but only a brief insight into the possible mode(s) of action of the three *B. subtilis* isolates that gave promising results in *in vitro* assays based on what is already known or reported.

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. 5: Biopesticides: Use and Delivery. Humana Press Inc. NJ.

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

Asante, G.S. and Neal, A.L. 1964. Characterization of fungistatic substances produced by a *Bacilus* antagonistic to *Ceratocystis ulmi*. Phytopathology 54: 819-822.

Atlas, R.M. and Bartha, R. 1998. Microbial Ecology: Fundamentals and Applications, 4th Edition. Addison Wesley Longman, California.

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

Buchanan, R.E. and Gibbons, N.E. (Eds) 1974. Bergey's Manual of Determinative Bacteriology. William & Wilkins Co., Baltimore.

Cook, R.J. 1993. Making greater use of introduced microorganisms for biologically control of plant pathogens. Annual Review of Phytopathology 31: 53-80.

Cutler, H.G. 1986. The science of allelopathy. John Wiley & Sons, London.

Cutler, H.G. and Hill, R.A. 1994. Natural fungicides and their delivery systems as alternatives to synthetics. Pages 135-152 in Wilson, C. L. and Wisniewski, M.E. Biological control of post harvest diseases. CRC Press, Boca Raton.

Eckert, J.W. and Ogawa, J.M. 1985. The chemical control of postharvest diseases: subtropical and tropical fruits. Annual Review of Phytopathology 23: 421-454.

Fravel, D.R. 1988. Role of antibiosis in the control of plant diseases. Annual Review of Phytopathology 26: 75-91.

GenStat for Windows 2000. Release 4.2, Fifth Edition, VSN International Ltd, Oxford, UK.

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

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

Korsten, L. 1993. Biological control of avocado fruit diseases. PhD Thesis, University of Pretoria, Pretoria.

Korsten, L., Van Harmelen, M.W.S., Heitmann, A., De Villers, E.E and De Jager, E.S. 1991. Biological control of postharvest mango diseases. South African Mango Growers Association Yearbook 11, 65-67.

Korsten, L., De Jager, E.S., De Villers, E.E., Lourens,A., Kotze, J.M. and Wehner, F.C. 1995. Evaluation of bacterial epiphytes isolated from avocado leaf and fruit surfaces for biocontrol of avocado postharvest diseases. Plant Disease 79: 1149-1156.

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.

Leong, J. 1986. Siderophores: Their production and possible role in the biocontrol of plant pathogens. Annual Review of Phytopathology 24: 187-209.

Loper, J.E. and Lindow, S.E. 1993. Role of competition and antibiosis in suppression of plant diseases by bacterial biological control agents, in Pest Management: Biologically Based Techniques, Lumsden, R.D. and Vaughn, J.L. (Eds). American Chemical Society, Washington, DC.

Mc Keen, C.D., Relly, C.C. and Pusey, P.L. 1986. Production and partial characterization of antifungal substances antagonistic to *Monilinia fructicola* from *Bacillus subtilis*. Phytopathololology. 76: 136-139.

Neilands, J.B. 1981. Microbial iron compounds. Annual Review of Biochemistry 50, 715-731.

Neilands, J.B. 1993. Perspectives in Biochemistry and Biophysics - Siderophores. Archives of Biochemistry and Biophysics 302: 1-3.

Pusey, P.L. 1994. Enhancement of biocontrol agents for postharvest diseases and their integration with other control strategies. Pages 77-88 in: Wilson, C.L. and Wisniewski, M.E (Eds). Biological control of postharvest diseases. CRC press, Boca Raton.

Roberts, T.A. and Hitchins, A.D. 1969. Resistance of spores. In: Gould, G.W. and Hurst, A. (Eds). The bacteria spore. Academic Press, London.

Schwyn, B. and Neilands, J.B. 1987. Universal chemical assay for the detection and determination of siderophores. Analytical Biochemistry 160: 47-56.

Simeoni, L.A., Lindsay, W.L. and Baker, R. 1987. Critical ion levels associated with biological control of *Fusarium* wilt. Phytopathology 77: 1057-1061.

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.

Smilanick, J.L. 1994. Strategies for the isolation and testing of biocontrol agents. Pages 25-41 in: Wilson, C.L.and Wisniewski, M.E. (Eds). Biological control of postharvest diseases. CRC Press, Boca Raton.

Weller, D.M. and Thomashow, L.S. 1993. Microbial metabolites with microbial activity against plant pathogens, in: Pest Management: Biologically Based Techniques, Lumsden, R.D. and Vaughn, J.L. (Eds). American Chemical Society, Washington, DC.