Developing biopesticides for control of citrus fruit pathogens of importance in global trade

By

Joseph Obagwu

Submitted in partial fulfillment of the requirements for the degree of Ph.D. (Plant Pathology)
in the Faculty of Natural and Agricultural Sciences
University of Pretoria

2003

DECLARATION

I, the undersigned hereby declare that the work reported herein is the result of my original research findings.

Name: Joseph Obagwu **Signature & Date**: 2003

ACKNOWLEDGEMENTS

The author would like to thank the following:

- My supervisor Prof. Lise Korsten, and co-supervisor Dr Thierry Regnier for academic guidance and support. They were more like colleagues than supervisors, and I thank them for their simplicity.

- Staff and students' of Plant Pathology Laboratories, Department of Microbiology and Plant Pathology, University of Pretoria for technical support.

- My parents Mr and Mrs Michael Obagwu, and all members of the Obagwu Okpeh and Ella Agbama families for their love.

- Ahmadu Bello University, Zaria, Nigeria for granting me a study fellowship.

- The financial and/or material assistance received from the following bodies or organizations is gratefully acknowledged: German Academic Exchange Service (DAAD), the South African National Research Foundation (NRF), the South African Technology and Human Resources for Industry Programme (THRIP) South African Citrus Growers' Association (SACGA) and Letaba Estates, South Africa.

- Above all, I thank God Almighty for answered prayers.

DEDICATION

This thesis is dedicated to my wife Mrs Joy Ene Obagwu. Words cannot express the way I feel about you. Thank you for your love.

CONTENT

CHAPTER ONE

GENERAL INTRODUCTION

Citrus belongs to the genus *Citrus* L., sub tribe Citrinae, subfamily *Aurantioideae*, family *Rutaceae*, order *Sapindales*, superorder *Rossidae* and subclass *Ddityledoneae*. Citrus fruit is rich in vitamin C and has been used for the treatment of scurvy since the 17th century (FAO, 1998). The sweet orange (*Citrus sinensis* Osbeck) is one of the world's most popular fruit crops (Hui, 1999). The exact center of origin of citrus is unknown. It is however, generally believed that all commercially important citrus varieties originated in Southeast Asia (Janick *et al.*, 1981; Whiteside *et al.*, 1988; Zohary and Hopf, 1993; Davies and Albrigo, 1994), where there is greater diversity in varieties than anywhere else in the world (McPhee, 1967). Citrus is currently grown throughout the world. It grows particularly well in areas where there are sufficient rainfall or irrigation to sustain growth and where freezing conditions are not severe enough to kill the tree (Whiteside *et al.*, 1988). Brazil is currently the largest citrus producer followed by the United States of America (USA) and China (FAO, 2002). South Africa is the 12th largest producer; however, over 60% of its produce is exported, making it the third largest exporter in the world following Spain, and the USA (FAO, 2001). The first citrus tree was said to have reached South Africa (the Cape) in 1654 from the Island of St. Helena (Oberholzer, 1969). Citrus is a major foreign exchange earner for exporting countries and provides employment for many people throughout the world.

The citrus tree can be attacked by many pathogens that can affect the roots, leaves and fruits. Of the most important pathogens affecting the fruit are *Guignardia citricarpa* Kiely. the cause of citrus black spot (CBS), and *Penicillium digitatum* Sacc. and *P. italicum* Wehmer the cause of citrus green- and blue mold respectively. These pathogens can cause huge economic losses annually, particularly in fruits destined for export. *Guignardia citricarpa* is primarily a pre-harvest pathogen and the black spot it causes is mainly a cosmetic disease on fruits, which affects only the rind and not the internal quality of the fruit. However, fruits with CBS symptoms are unacceptable for export due to the phytosanitary risks for the importing country in the case of it being CBS free. Citrus black spot lesions on fruits can therefore lead to the rejection of whole export consignments in international trade (Kotzé, 1981). Although, pre-harvest fruit symptoms gets identified and

removed prior to packing; latent infections (red spots) can still develop on black spot infected fruit postharvestly, while in transit. In order to gain access to certain lucrative markets such as the European Union (EU) or United States of America (USA), citrus groves must be CBS free.

Of the postharvest diseases, the so-called wound pathogens, *P. digitatum* and *P. italicum* are common in all citrus producing regions of the world. Global figures of postharvest losses due to these diseases are difficult to obtain because of poor record keeping, but are known to be huge. Green mold alone for instance is reported to cause annual losses of up to $50 million in California (Eckert and Eaks, 1989).

Citrus fruit diseases are currently being managed with synthetic fungicides applied either pre- or postharvestly. However, there is a growing global concern over the use of synthetic chemicals on food crops because of the continuous exposure of man to low levels of pesticides through his diet (Anonymous, 1987; Norman, 1988). The impact these chemicals have on the environment is an additional concern that affects the well-being of mankind indirectly. These aspects have lead to the implementation of more restrictive legislations regarding the maximum residue levels (MRL) of chemical residues on fruits exported to particularly European markets. Currently, all pesticides must be re-registered in the USA and EU. In addition to this is the growing concern over reported cases of reduced efficacy of certain fungicides particularly the ones used in the postharvest arena including thiabendazole (TBZ) (MacDonald *et al.*, 1979; Eckert, 1988) and imazalil (Eckert, 1987; Dave *et al.*, 1987; Brown, 1989). This is mainly due to a build up of pathogen resistance, particularly with *P. digitatum*. Due to these recent developments, it is becoming critically important to identify alternative, environmentally safer, and where possible, cheaper alternative control measures. One such option is biological control with the use of microbial antagonists (Droby *et al.*, 1991).

Plant surfaces harbor a large population of microorganisms that are well adapted to colonizing that particular niche (Janisiewicz and Korsten, 2000). 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 on fruits. One such example is *Bacillus subtilis* (Avogreen) registered in South Africa by the University of Pretoria according to

the Fertilizer, Farm Feeds, Agricultural and Stock Remedies Act of 1947 (Act 36 of 1947). Other biocontrol agents for control of fruit diseases have been registered in other countries such as the *Pseudomonas syringae* (BioSave 110) and *Pichia guilliermondii* (Aspire) marketed by Village Farms LLC and Ecogen Inc. respectively. However, there are obviously untapped pools 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.

Biological control agents, unfortunately, are often not as effective and consistent in their activity as synthetic chemicals. This is mainly because biological control agents are living entities, rapidly responding to environmental changes which in turn affect their activity (Conway *et al.*, 1999). The combination of more than one antagonist with different modes of action, and/or more than one control strategy has been advocated as a better approach to provide a wider spectrum of activity, more consistent and/or better control of postharvest diseases (Baker and Cook, 1982; Korsten, 1993; Moline, 1994; Pusey, 1994).

The potential of plant extracts for control of plant diseases have long been identified (Ark and Thompson, 1959). The actual use of these products in plant disease control is however, still limited. The antifungal properties of garlic (*Allium sativum* L.) have been reported (Bisht and Kamal, 1994; Obagwu *et al.*, 1997; Sinha and Saxena, 1999), as has other extracts of indigenous plants (Louw, 2002). There are however, no references in the literature on the use of such extracts for control of CBS and citrus green - and blue molds. Information on the horticultural value of *Coprosma repens* Hook. F. abound, but no reference is made of its antimicrobial activity.

Fruits and vegetables grown in natural environments harbor a wide variety of both beneficial and harmful microorganisms. Reported outbreaks of food-borne illnesses involving fruits and vegetables have recently increased (Schlundt, 2002; Tauxe, 2002), and have become a major concern in export of fresh fruits. In the United States of America, it has been estimated that 76 million cases of food-borne illnesses occur each year resulting in 325 000 hospitalizations (Schlundt, 2002). Although only a small percentage of these causes are associated with the consumption of fresh fruits, it has however, become a major issue in international trade. Due to these concerns, the European community requires that

all export fruit be certified within a food safety framework using systems such as Good Agricultural Practice (GAP), and Hazard Analysis Critical Control Point (HACCP).

The prime cause of food-borne illnesses includes bacteria mainly *Escherichia coli* 0157-H7, *Salmonella*, *Staphylococcus*, *Campylobacter*, *Vibrio,* and *Shigella* species. Currently, there are limited methods available for the control of food-borne pathogens on fresh fruits and vegetable surfaces. A number of "generally regarded as safe" chemicals have been reported to possess bactericidal activity against *E. coli* 0157:H7, *Listeria monocytogenes*, and *Salmonella enteritidis* (Friedman *et a*l., 2002). However, none of these chemicals can significantly reduce populations of food-borne pathogens. Although biological control is becoming an effective alternative to fungicides, only an integrated approach is most likely to provide the consistent and effective control obtainable with synthetic fungicides. In order to address the most important concerns of export citrus namely CBS that represents a postharvest concern and technical barrier to trade, and other postharvest diseases that develop during long transit periods of export (particularly *Penicillium* rots), and also to prevent food-borne pathogens from establishing on the fruit surface, the following aspects were investigated. The overall objective of this investigation is to identify biocontrol system(s), which could be used globally to address the problems stated earlier:

1. Commercial and natural microorganisms were evaluated either on their own, or in combination with other non-chemical products for control of *G. citricarpa, P. digitatum* and *P. italicum.*

2. Combinations of antagonists were evaluated to exploit synergistic relationships between them.

3. Natural plant extracts from garlic and *C. repens* were evaluated either alone, or in combination with other non-chemical products for control of *G. citricarpa, P. digitatum* and *P. italicum,* and food-borne bacterial pathogens (*E. coli* 0157-H7, *S. typhimurium*, *S. aureus* and *V. cholerae*) that may occur on citrus fruit surfaces destined for export.

Literature Cited

Anonymous. 1987. Regulating Pesticide in Food-The Delaney Paradox. US National Academy of Sciences, Washington, DC.

Ark, P.A. and Thompson, J.P. 1959. Control of certain diseases of plants with antibiotics from garlic (*Allium sativum* L.). Plant Disease Reporter 43: 276.

Baker, K. and Cook, R.J. 1982. Biological control of plant pathogens. The American Phytopathological Society. St. Paul MN .

Bisht, S.S. and Kamal, G.B. 1994. Garlic extract - an effective antifungal treatment for the control of storage rot of apple. Proceeding of the National Academy of Sciences India 64: 233-234.

Brown, G.E. 1989. Baseline sensitivity of Florida isolates of *Penicillium digitatum* to imazalil. Plant Disease 73: 773-774.

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.

Dave, B., Sales, M.C. and Walia, M. 1987. Resistance of different strains of *Penicillium digitatum* to imazalil treatment in California citrus packhouses. Proceeding of the Florida state Horticultural Science Society 102: 178-179.

Davies, F.S. and Albrigo, L.G. 1994. Citrus. CAB International, Great Britain.

Droby, S., Chaltutz, E., Weiss, B. and Wilson, C.L. 1991. Biological control of postharvest diseases of citrus fruits. Pages 60-70 in: Wilson.C.L.and Chaltutz, E. (Eds) Proceeding of the Biological Control of Postharvest Diseases of Citrus Fruits and Vegetables Workshop, West Virginia, 1990.

Eckert, J.W. 1987. Biotypes with reduced sensitivity to imazalil. Phytopathology 72: 1728.

Eckert, J.W. 1988. Dynamics of benzimidazole resistant *Penicillium* in the development of postharvest decays of citrus and pome fruits. Pages 31-35 in: Fungicide resistance in North America. Delp, C.J. (Ed) American Phytopathological Society St. Paul MN.

Eckert, J.W. and Eaks, J.L. 1989. Postharvest disorders and diseases of citrus fruit. Pages 179-260 in: The citrus industry. Vol. 5. Reuther, W. Calavan, E.E. and Carman, G.E. (Eds). University of California, Division of Agriculture and Natural Resources, Ock.

Food and Agricultural Organization (FAO) 1998. Oranges and lemons on the agenda at international meeting (http/www.fao.org/NEWS/1998/981005-e.htm).

Food and Agriculture Organization (FAO) 2001. Citrus fruits annual statistics.

Food and Agriculture Organization (FAO) 2002. Citrus fruits annual statistics.

Friedman, M., Henika, P.R. and Mandrell, R.E. 2002. Bactericidal activities of plant essential oils and some of their isolated constituents against *Campylobacter jejuni*, *Escherichia coli*, *Listeria monocytogenes*, and *Salmonella enterica*. Journal of Food Protection 65: 1545-1560.

Hui, S. 1999. Sweet oranges: The biogeography of *Citrus sinensis* http:/www.sfu.ca/-shui/resources/orange.

Janick, J.S.R.W., Woods, F.W. and Rutan, V.W. 1981. Plant Science: An Introduction to World Crops. 3rd edition, Freeman & Co. California.

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. Marel Dakker, New York.

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

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

Louw, C.A.M. 2002. Antimicrobial activity of indigenous bulbous plant extracts to control selected pathogens. MSc Thesis, University of Pretoria, Pretoria.

MacDonald, R.E., Risse, L.A. and Hilerbrand, R.M. 1979. Resistance to thiabendazole and benomyl in *Penicillium digitatum* and *P. italicum* isolated from citrus fruit from several countries. Journal of the American Society of Horticultural Science 104: 333-335.

McPhee, J. 1967. Oranges. Farrar, Straus and Giroux, New York.

Moline, H.E. 1994. Pre-harvest management for postharvest biological control. Pages 57-61 in: Wilson, C.L. and Wisniewski, M.E. Biological control of postharvest diseases, CRC press, Roca Raton.

Norman, C. 1988. EPA sets new policy on pesticide cancer risks. Science 242: 366-367.

Obagwu, J., Emechebe, A.M. and Adeoti, A.A. 1997. Effect of extracts of garlic (*Allium sativum* L.) bulb and neem (*Azadirachta indica* Juss) seed on the mycelial growth and sporulation of *Colletotrichum capsici*. Journal of Agricultural Technology 5: 51-55.

Oberholzer, P.C.J. 1969. Citrus culture in Africa south of the Sahara. Proceeding of the International Citrus Symposium 1: 111-119.

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.

Schlundt, I. 2002. New directions in food-borne disease prevention. International Journal of Food Microbiology 78: 1-17.

Sinha, P. and Saxena, S.K. 1999. Inhibition of fruit rot fungus and fruit fly by leaf extracts of onion and garlic. Indian Journal of Agricultural Sciences 69: 651-653.

Tauxe, R. 2002. Emerging food-borne pathogens. International Journal of Food Microbiology 78: 31-41.

Whiteside, J.O., Garnsey, S.M. and Timmer, L.W. 1988. Compendium of citrus diseases. The American Phytopathological Society Press, St. Paul, MN.

Zohary, D. and Hopf, M. 1993. Domestication of plants in the Old World: The origin and spread of cultivated plants in West Asia, Europe and the Nile valley. 2nd edition, Oxford University Press, England.

CHAPTER TWO

LITERATURE REVIEW

2.1. Introduction

The global fruit trade is dominated by citrus, which is exported mainly as a fresh fruit, or as juice. Globally, about 91 million tons of citrus are produced annually and about 10 million tons are traded in the international market generating over $1.5 billion (FAO, 2001). Brazil is the largest producer followed by the United States of America (USA), China, and Mexico (Table 2.1). South Africa is the 12th largest producer, but exports about 60% of its fresh produce, making it the third largest exporter after Spain and the USA (Table 2.2) (FAO, 2002).

Most of the citrus that is traded in the international market is produced in Asia and Africa far away from the export markets, which are mainly the European Union (EU) and the USA. Export fruits therefore, have to be transported over long distances, which often takes several days, if not weeks, to reach their final destination. Although fruits destined for export are treated postharvestly with fungicides to prevent postharvest losses, postharvest development of latent infections of citrus black spot (CBS) caused by *Guignardia citricarpa* Kiely and *Penicillium* rots, for instance can still occur in transit. The development of latent infections (red spot) of *G. citricarpa* on export consignments can lead to an outright rejection of an entire consignment due to a phytosanitary risk for the importing country if it is CBS free, thus making it an important factor in international trade. Postharvest *Penicillium* rot may not necessarily lead to total rejection of a consignments but the cost of re-packaging might be enormous and undesirable.

Citrus fruit diseases are currently being managed with synthetic fungicides applied pre- or postharvestly. However, concerns over the real or perceived negative effects of synthetic pesticides on both man and his environment (Norman, 1988), has lead to the implementation of more restrictive legislations regarding the Maximum residue levels (MRL) of pesticides, particularly to European markets. In addition to the growing concern over the use of pesticides, is the reported increase in the outbreaks of food-borne illnesses.

Although only a small percentage of these causes are associated with the consumption of fresh fruits, it has however, become a major concern in international trade, prompting the European community to request that all exports of fresh fruits be certified through some kind of food safety systems such as Good Agricultural Practice (GAP) and Hazard Analysis Critical Control Point (HACCP). Unlike postharvest fungal diseases, no compounds are known to provide effective control of food-borne bacterial pathogens.

Table 2.1 Major citrus producing countries in the world in 2000 and 2001 (FAO, 2002)

Country	Production (thousand tons)	
	2000	2001
Brazil	19 716.0	19 100.1
United States of America	14 810.7	14 049.3
China	10 787.0	8 783..2
Mexico	5 529.9	5 680.0
Spain	5 624.5	5 400.7
Italy	3 217.2	3 009.8
India	4 466.0	3 000.0
Argentina	2 580.0	2 706.1
Iran	2 811.2	2 593.0
Egypt	2 231.6	2 508.2
Turkey	1 826.2	1 531.5
South Africa	1 473.1	1 525.8
Japan	1 817.0	1 487.0
Greece	1 354.2	1 229.2
Pakistan	1 838.5	1 109.0

Due to these recent developments, it is becoming critically important to identify alternative, environmentally safer, and where possible, cheaper alternative control measures. The use of one microorganism to control another (biological control), natural fungicides (plant extracts), and hot water treatments are amongst several non-chemical control options that are being intensively researched as alternatives to synthetic pesticides. This chapter briefly reviews fruit postharvest diseases generally, with particular emphasis

on those that are important in international trade. This is in addition to control measures (particularly non-chemical measures) that have been studied, or are currently being used in the postharvest arena. Factors responsible for and/or that encourage the development of citrus postharvest diseases are also reviewed. The importance of food-borne pathogens, particularly in the light of new regulations in international trade is also discussed.

Table 2.2 Major citrus export countries in the world in 2000 and 2001 (FAO, 2002)

Country	Export volume (thousand tons)	
	2000	2001
Spain	3 221.4	2 858.7
United States of America	1 046.0	1 086.0
South Africa	782.5	813.2
Greece	323.4	423.3
Turkey	489.5	499.4
Argentina	289.0	408.0
Morocco	596.8	393.3
Mexico	281.7	267.0
Egypt	226.0	225.5
Israel	220.0	194.0
China	157.9	157.2
Cyprus	118.5	98.2
Australia	170.9	94.3
Brazil	114.0	90.0
Uruguay	120.5	73.6

2.2. Postharvest Diseases

Citrus fruits are susceptible to attack by several pathogens both pre- and postharvestly. However, three of these diseases: CBS and green- and blue mold caused by *Penicillium digitatum* Sacc. and *P. italicum* Wehmer respectively are particularly important because they are perceived barriers to international trade and can result in huge economic losses.

Citrus black spot is primarily a pre-harvest disease, but can cause significant economic losses postharvestly as a result of latent infections that develop into typical red spots on export fruits in transit. Although CBS is a cosmetic disease and the red spot that develops in the rind has no effect on the quality of the fruit, these spots are unacceptable for the export market due to its phytosanitary status and could result in the rejection of a whole export consignment (Kotzé, 1981). This makes the disease both politically and economically important. *Penicillium digitatum* and *P. italicum* are found in all citrus growing regions of the world and the rots they cause can result in huge economic losses (Eckert and Ogawa, 1985). These three diseases will therefore be discussed in greater detail. The causal agent, symptoms, epidemiology and control of the other important postharvest diseases of citrus are briefly reviewed in Table 2.3. Most of these diseases unlike the previous three have narrow geographical distributions and in some cases are sporadic in occurrence and not as economically important. Sour rot caused by *Geotrichum candidum* Link for instance is an important disease in South Africa but may not be economically important in other citrus growing regions. Sour rot, like most of the other diseases is however, easily managed with fungicides and unlike CBS does not constitute a major barrier to international trade.

2.2.1. Citrus Black Spot

Citrus black spot is primarily a pre-harvest disease. Unlike green- and blue mold, CBS is more or less a localized disease. The disease is reported to occur in South Africa, Argentina, Australia and Brazil, but has not been reported on citrus in Europe or the USA (Kotzé, 1996). It was first reported in South Africa in 1929 by Doidge (Doidge, 1929), reaching epidemic proportions in parts of the country between 1956 and 1959 (Kotzé,1996).

Symptoms

Guignardia citricarpa produces a spectrum of symptoms both on leaves and fruits but it is the later that are normally important as far as international trade is concerned. On fruit, the nature of lesions produced are determined by the stage of maturity of fruit and the ambient temperature at the time of infection. Fruit symptoms have been classified into three categories by Kiely (1948):

Table 2.3 Description of some major citrus fruit diseases that may cause significant losses postharvestly

Disease	Description of causal agent	Fruit symptom	Disease cycle and epidemiology Disease cycle and epidemiology	Control	Reference
Alternaria Rot	*Alternaria citri* Elli &Pierce. • Mycelium on Potato dextrose agar(PDA) yellowish or oliveaceaus and hyaline. • Conidia vary in size and shape and are dark brown in color. • Conidia 4 to 6 septate and slightly contracted at the septa. The conidia is also divided by one or more septa.	• Premature ripening • Light brown to blackish discoloration of the rind at the stylar end. • A black rot that is only visible when the fruit is cut i.e. no external symptom is presented. • In lemon, typically affected fruit develop stem-end rot, browning as well as center rot	• The pathogen grows saprophytically on dead citrus tissue where it produces air-borne conidia. • Pathogen establishes a quiescent infection in the button or stylar end of the fruit. • The fungus grows from the button or stylar end into the fruit only after the senescence of the button. • Stress and physiological disorder helps in the promotion of the disease. • The optimum temperature for infection is about 23°C	• Delay harvesting to allow infected fruits to drop. • Harvest fruits at optimum maturity. • Post harvest chemical testament with 2-4-D and or imazalil.	Whiteside *et al.*, 1988; Nel *et al.*, 1999.
Anthracnose	*Colletotrichum gloesporioides*.Penz. • Acervuli erumpent and superficial, 90-200 µm in diameter. • Conidia-oval or oblong, 10-16x5-7 µm. • Colony color vary from white to gray to black. • Degree of sporulation varies with type of isolate.	Symptom normally appears on fruits injured by other agents. • Brown or black spots about 1.5cm in diameter appear on fruits. • The decay may be dry and firm or soft. • Spores on lesion may be pink, salmon-colored or brown to black depending on humidity. • As the decay progresses, the rind becomes brown to grayish black, and eventually a soft rot occurs.	• Conidia are produced on dead twigs and are spread by rain or irrigation water. • The conidia so produced enters the fruit, and germinates but remain dormant until the tissue is weakened by other factors. • Ethylene treatment is believed to trigger the growth of the pathogen. A dose of ethylene above optimum is said to increase the occurrence of the disease.	• Careful handling of fruits to avoid injury. • Postharvest application of fungicide e.g. thiabendazole (TBZ).	Whiteside *et al.*, 1988. Davies and Albrigo, 1994.

Table 2.3 Continued

Disease	Description of causal agent	**Fruit symptom**	Disease cycle and epidemiology Disease cycle and epidemiology	Control	Reference
Aspergillus Rot	*Aspergillus niiger* Van Tiegh. • Spores are produced in chains. • Conidia-2.5 to 4.0 µm. in diameter and rough walled.	• Symptoms include a light-colored, very soft decay, which punctures easily. • Lesions on orange eventually become sunken and wrinkled. • The rotten surface is normally covered with black powdery layer of spores.	• The fungus survives as a saprophyte. • Spores are carried by wind to fruit surfaces. • Infection occurs through injuries. • Infection can spread in packed containers. • Optimum growth temperature is near 32°C • The disease spreads rapidly at about 25° C but the fungus can grow at below 10 °C.	• Storage at low temperature (i.e. 10 C or below). • Use of fungicide such as TBZ or imazalil.	Whiteside, *et al.*, 1988; Agrios, 1997
Brown Rot	*Phytophthora citriphthora* (Sm & Sm) Leonian and *P. parasitica*. Dart. • *P. parasitica* is the most common and most widespread cause of brown rot. • Sporangia-papillate, and pear shaped to spherical with average dimension of 38-50 by 30-40 µm in *P.* *parasitica* and 45-90 by 27-60 µm in *P. citriphthora.*	• The decay first appears as light brown discoloration of the rind. • The affected area remains firm and leathery. • Delicate white mycelium forms on the rind surface under humid conditions. • Effected fruits have a characteristic pungent, rancid odor, which distinguishes the disease from stem-end rots.	• Under wet conditions, zoospores are spread from the soil onto low hanging fruits. • Spores produced on fruits are then splashed higher into the canopy. • Fruits infected before harvest may not show symptom until in storage.	• Cultural practices including proper irrigation and drainage. • Pruning to remove low hanging branches. • Avoid harvesting from poorly drained grooves and during rainy periods. • Avoid harvesting fruits lying low close to the ground to minimize picking infected fruits.	Whiteside, *et al.*, 1988; Agrios, 1997

Table 2.3 Continued

Disease	Description of causal agent	**Fruit symptom**	Disease cycle and epidemiology Disease cycle and epidemiology	Control	Reference
Diplodia stem-end rot	*Diplodia natalensis* Evans. • Pycnidia-subglobose to globose, 300-700 µm in diameter. • Spores are17-30 µm by 10-18 µm . • Young spores are hyaline , non- septate, while the mature spores are unseptate	• Lesions appear in 7-10 days of harvest as dark discoloration of the rind in the stem-end of the fruit. • Typical decay is formed at both ends of the fruit before involving the entire fruit. • There is usually a sour fermented odor and sometimes the fruit will become quite black. • The disease does not spread from diseased to healthy fruits in packed containers.	• The fungus grows on dead wood on the tree where it produces spores. • Spores are carried in rain water or irrigation water to immature fruits. • The fungus becomes established in dead tissue of the button surface where it stays dormant until harvest. • Optimum temperature (about 25° C) and humidity in the degreening room encourage the growth of the pathogen. • Ethylene treatment causes senescence and abscission of the button that allow entry of the pathogen into the base of the fruit.	• Cultural practices including removal of dead wood from trees. • Harvest at optimum maturity to reduce time required for degreening. • Pre-harvest treatment with benlate or drenching with TBZ before degreening and application of TBZ on the packline. • Immediate cooling of fruits after harvest.	Brown, 1994; Nel *et al., 1999.*
Gray mold	*Botrytis cinerea* Pers. ex Fr. • The fungal colony is greenish gray, or dark olive . • Conidia colorless to dark brown, elliptical to oblong.	• At very high humidity, distinctive patches of gray brown to olive spore masses appear on the fruit surface. • A brown leathery decay develops on the fruit. • Infection spreads from healthy to infected fruits in packed containers.	• The pathogen inoculum is produced on organic debris in orchards and dispersed by wind or rain splash. • Dispersed inoculum infects flowers. • The fungus forms a quiescent infection at the stem-end of the fruit. • The fungus becomes active after harvest and causes postharvest decay.	• Avoid harvesting fruits on or close to the soil surface. • Minimize injuries to fruits. • Packhouse treatment applied for the control of *Penicillum* diseases is also effective against gray mold.	Whiteside,*et al.*, 1988; Agrios, 1997.

Table 2.3 Continued

Disease	Description of causal agent	**Fruit symptom**	Disease cycle and epidemiology Disease cycle and epidemiology	Control	Reference
Sour rot	*Geotrichum candidum* Link ex Pers. • The mycelium is hyaline and septate. • The fungus grows rapidly on PDA producing a dull gray-white colony.	• Initial symptom similar to blue and green molds. • Lesion first appears as water soaked, light to dark yellow slightly raised spots. • The cuticle is however more easily removed from the epidermis than in lesions formed by blue and green molds. • Cell degrading enzyme produced by the fungus causes the fruit to disintegrate into a slimy watery mass. • Following exposure to high RH the lesion may be covered with a yeasty sometimes wrinkled layer of white or cream colored mycelium.	• The fungus occurs commonly on soil from where it is dispersed to fruit surfaces. • The fungus invades the rind through injury made by insects or mechanical means. • Susceptibility to infection increases with maturity of fruits. • The amount of moisture on the rind greatly influences the susceptibility of the fruit. • Spore laden watery debris from infected fruits spread the decay to healthy fruits.	• Minimize injury to fruits. Immediate storage of packed fruits at 10° C to delay the onset of the disease. • Proper hygiene at packhouses. • Pre-harvest fungicide treatment e.g. with guazatine gives some measure of control.	Howard, 1936; Whiteside *et al.*, 1988.
Trichoderma Rot	*Trichoderma viride* Pos ex Gray. • The fungus is a ubiquitous soil saprophyte but grows readily on wood products. • The mycelia are white and the conidia are almost globose and rough	• Diseased fruit becomes cocoa brown and the infected peel remains leathery and pliable. • Rotted fruits have a characteristic coconut - odor, which distinguishes it from other rots. • The pathogen cannot penetrate sound fruit directly	• The spores may be disseminated with soil particles or the fungus may infect fruits in contact wood of infested storage boxes Infection may be initiated at any location on the fruit, but decay normally start at the stem-end or stylar end of the fruit.	• Good cultural practices i.e. removal of dead wood to reduce inoculum source • Minimize injury to fruits • Rapid cooling of fruits after harvest because the fungus does not spread fast at 10° C. • Prompt removal of infected fruits,	Whiteside *et al.*, 1988; Nel *et al.*, 1999.

- **Hard spot:** usually appears at the beginning of fruit maturity. It is characterized by a brown circle with a depressed light brown to grey-white centre and surrounded by a green halo.
- **Freckle spot**: being similar to hard spot but it usually appears after the fruit has changed from green to orange colour.
- **Virulent spots**: normally develop late in the season and are enhanced by increase in temperature. The sunken necrotic lesions are brown to brick red at the periphery.

Fig. 2.1 Symptoms of citrus black spot caused by *Guignardia citricarpa*

Disease cycle and epidemiology

The citrus black spot fungus has two sexual stages. *Guignardia citricarpa* is the teleomorph (sexual) stage and produces ascospores, while *Phyllostica citricarpa* (asexual) stage produces pycnidiospores. Both spore types are dispersed with the aid of moisture (rain) and air currents (Kotzé, 1962). Most fruit infections appear to originate from pycnidiospores spread by rain splash after liberation from infected, late hanging or out-of-eason fruits and dead twigs (Fig. 2.2). Infection by ascospores takes place in the presence of moisture when spores germinate and produce apppresoria.

A thin infection peg penetrates the cuticle and expands forming a small mass of mycelium between the cuticle and the epidermal wall (Mc Onie, 1967). This constitutes the so-called latent infection that months later produce the typical black spot symptoms (Kotzé, 1981).

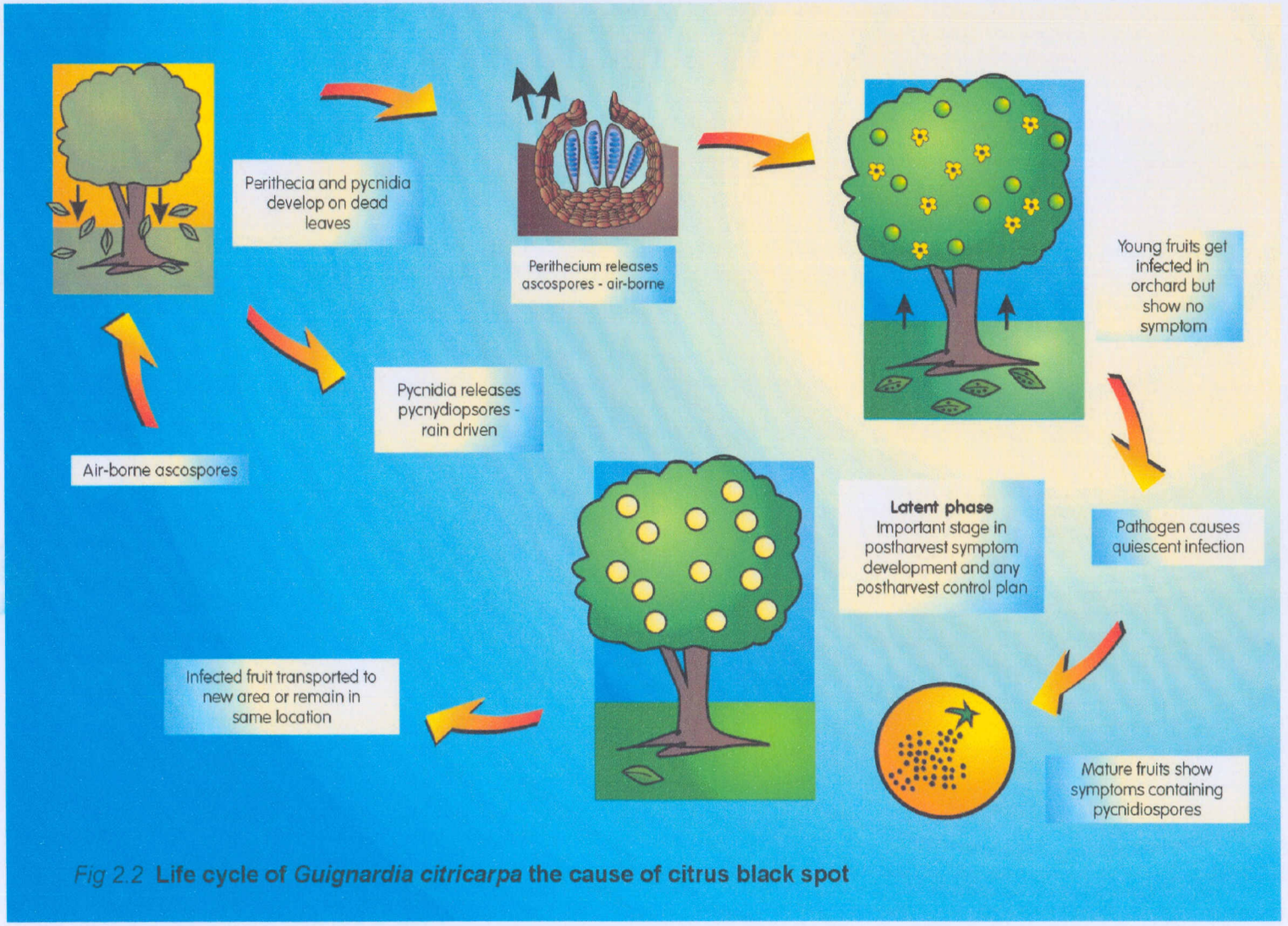


Fig 2.2 **Life cycle of *Guignardia citricarpa* the cause of citrus black spot**

Diagram courtesy of Prof. J.M. Kotzé

The success of any control measure is said to hinge on the latent period. Pycnidia are solitary, sometimes aggregated, globose, dark brown and between 115-190µm in diameter. Conidia are obovate to elliptical and 8-10.5 x 5.5-7.0µm (CMI 1966).

2.2.2. The penicillia

The name *Penicillium* comes from 'Penicillus', which means brush (Howard, 1936). The genus *Penicillium* was created by Link in 1809 for mold producing brush like sporulating structures (Fig. 2.3a). Over 99 species of *Penicillium* have been described (Carlos, 1982). Two species, *P. digitatum* and *P. italicum* are of great economic importance on citrus because they cause two important postharvest diseases of citrus fruits namely green - and blue mold respectively.

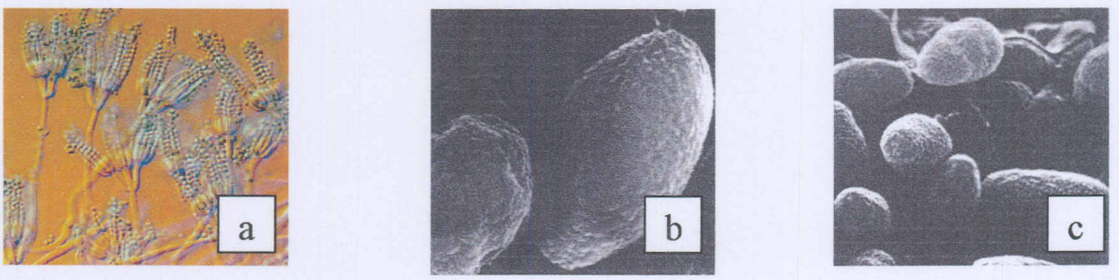


Fig.2.3 Reproductive structures of *Penicillium*, a) Conidiophores bearing spore producing phialides, b) *Penicillium digitatum* spores, c) *Penicillium italicum* spores (Carlos, 1982; Anonymous, 1999).

2.2.2.1. *Penicillium digitatum* (green mold)

Penicillium digitatum produces spores that are at first cylindrical, becoming elliptical, smooth and thick walled (Fig. 2.3b). Conidia vary in shape and dimensions, from 3.5-5.0 µm x 3.0-3.5µm at first, then 6-8µm by 4.0-6.0µm (Howard, 1936; Carlos, 1982).

Symptoms

Initial fruit symptoms include a water-soaked, soft area on the rind, which is easily punctured on impact. This enlarges rapidly, and eventually a white mycelium appears on the surface, followed by the development of olive green powdery spore masses, which forms a cloud when disturbed. The main diagnostic characteristic of the disease is the wide, white margin ahead of the green area while the soft central core is enlarging (Fig.

2.4). The colour of the spore masses varies somewhat with age, and could be pea green, greenish olive, or olive green (Howard, 1936; Agrios, 1997). Both *P. digitatum* and *P. italicum* require similar growth conditions. The optimum growth temperature is near 24° C. The pathogen grows slowly above 30° C and below 10° C. The rot is almost completely inhibited at 1° C (Whiteside *et al.*, 1988).

Fig. 2.4. Symptoms of citrus green mold caused by *Penicillium digitatum*.

Disease cycle and epidemiology

The disease develops rapidly at temperatures near 24° C and much more slowly above 30° C and below 10° C. The rot is almost completely inhibited at about 1° C (Whiteside et *al.*, 1988). The pathogen also grows over a wide range of pH but growth is best around neutral pH. In terms of disease epidemiology, the following conditions apply:

- The pathogen survives from season to season in orchards primarily as conidia
- Infection is initiated by airborne spores which enter the rind through injuries
- Conidia can germinate and form new spores within four days
- The infection and sporulation cycle can be repeated several times during a season in a packhouse, in transit or in storage (Whiteside *et al.*, 1988).

The following factors are reported to enhance the development of both green- and blue mold:

- The oil liberated from injured glands cause surface cells to break down and allow entrance of mold fungi.

- Juice leaking from injured fruits creates an ideal medium for germination and entrance of mold fungi.
- Acids leaking from decayed fruits are believed to break down the resistance of the rind especially in the case of blue mold.
- The more matured the fruit, the faster the decay. Overripe fruits result in more decay than normal fruits and fruits left in the sun after harvest decay faster than those left in the shade. (Howard, 1936).

2.2.2.2. *Penicillium italicum* (blue mold)

Penicillium italicum, produces spores that are typically cylindrical at first, becoming elliptical, or even sub-globose at maturity (Fig 2.3c). The conidia are extremely variable in size but are generally between 5-6µm x 3.0-3.5µm (Carlos, 1982).

Symptoms

Symptoms of blue mold are very similar to green mold, except for the blue spore masses (Fig. 2.5). The fungus however, has a greater tendency to spread from one fruit to the next apparently through uninjured skin (Howard, 1936).

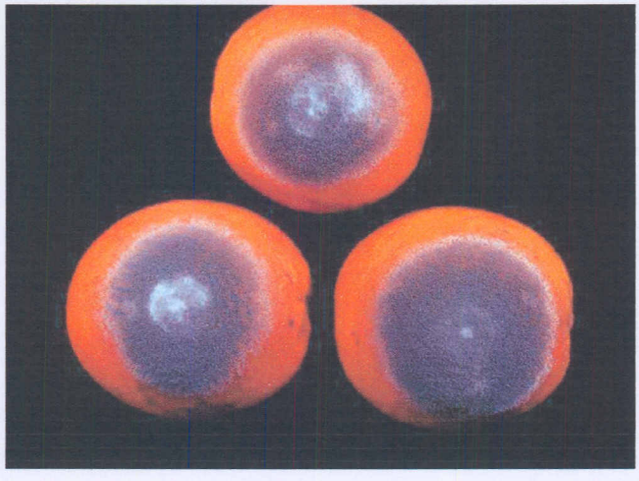


Fig. 2.5 Symptoms of citrus blue mold caused by *Penicillium italicum*

Disease cycle and epidemiology

Disease cycle and epidemiology is similar to that described for green mold.

2.3. Control of Postharvest Diseases

The development of modern fungicides and the continuous improvement in cold storage facilities and cold chain management systems since the 1960's have greatly improved the shelf life of perishable fruits and vegetables after harvest. Not withstanding, losses of up to 20% could still be recorded even in countries with advanced cold storage facilities (Cappellini and Ceponis, 1984). In developing countries, postharvest losses of up to 50% have been reported which is mainly due to poor or a lack of cold chain management systems (Eckert and Ogawa, 1985). Postharvest losses have hitherto been reduced mainly through the application of fungicides (Eckert and Ogawa, 1985) and to a lesser extent through postharvest management practices. Such practices include care during harvesting and processing to minimize injury to fruits, heat treatment, biological control with antagonistic microorganisms, and good sanitation practices. Synthetic fungicides are effective even in the control of latent infections and generally provide a longer protection of the commodity than most, if not all non-chemical measures. Chemical and particularly non-chemical measures that have been evaluated for control of postharvest diseases of citrus and other fruits are briefly reviewed in the following section.

2.3.1. Chemical control

Market losses have traditionally been prevented through the use of effective fungicides applied postharvestly. However, postharvest use of fungicides has increasingly been curtailed by more restrictive legislation driven by the perception that pesticides are harmful to man and his environment (Norman, 1988). These concerns have lead to the implementation of more restrictive regulations regarding MRL's of chemicals used on fruits exported to the USA or EU. The indiscriminate use of pesticides has lead to the proliferation of resistant strains of pathogens to many fungicides including thiabendazole (TBZ) (MacDonald *et al.*, 1979; Eckert, 1988; Timmer and Duncan, 1999) and imazalil (Eckert, 1987; Timmer and Duncan, 1999). Some of these fungicides like the benzimidazoles, which were previously used to control a broad spectrum of fungi, have been withdrawn from the market. The indiscriminate use of synthetic chemicals have also resulted in an ecological shift or imbalance in microbial populations, often leading to a reduction in natural antagonistic populations, which naturally helps to keep diseases in check.

Other problems affecting the continuous use of synthetic fungicides include difficulty in registering new products due to increased toxicological testing and information required. Despite these concerns, Jeffries and Jeger (1990) believed that agrochemicals would remain the preferred choice for farmers until alternative control options can provide the same level of consistent control achieved with synthetic fungicides. Such products, especially those with systemic activity are particularly effective in control of incipient infections, which represent a major deficiency in most biological control systems.

Fungicidal action can be expressed in one of two physically visible ways i.e. inhibition of spore germination and/or inhibition of mycelial growth. The end result of these products is normally a disruption in physiological processes such as electron, enzyme or nucleic acid transport within the cells (Matheron, 2001). The imidazoles for example are believed to inhibit the synthesis of ergosterol, which is essential for membrane structure and function in many fungi. The benzimidazoles on the other hand inhibit DNA synthesis (nuclear development), while the organophosphates are believed to disrupt amino acid metabolism (Tomlin, 1995; Matheron, 2001; Anonymous, 2002).

Despite the high efficacy of synthetic fungicides, the time lag between infection and treatment application is still critical for effective control to be achieved. The maximum time lag between harvest of citrus fruit and treatment varies, but as a general rule should not exceed 24 hours, otherwise, infection could be initiated which would be difficult to control (Anonymous, 1999). For economically important crops, several postharvest fungicides are still available that can be used with great success to effectively control postharvest diseases. The fungicides registered for postharvest control of citrus diseases in South Africa are listed in Table 2.4. Only guazatine has been reported to provide effective control of sour rot caused by *Geotrichum candidum* Link. It is therefore normally used in combination with imazalil for a more comprehensive control of *Penicillium* and sour rots.

Table 2.4 A summary of registered fungicides currently available for commercial use to control postharvest diseases in South Africa and their mode of action and range of activity.

Fungicide	Active ingredient	Mode of action	Remarks	Reference
Imazalil	Imazalil	A systemic fungicide with both protective and curative action	Control a wide range of diseases on fruits and vegetables. It is not effective against *Geotrichum* sp	Nel *et al.*, 1999; Anomymous, 2002.
Guazatine (Decitine)	Guazatine	A broad fungicide with broad spectrum activity	Provides good control of *Penicillim* spp. and *Geotrichum*. It does not inhibit sporulation of *Penicillium* on diseased fruits	Nel *et al.*, 1999; Anomymous, 2002.
Sportak	Prochloraz	A fungicide with both protective and eradicant action	Effective against *Penicillium* spp but only moderately effective against *Alternaria*, *Diplodia* and *Phomopsis* stem-end rots. It is not effective against sour rot.	Nel *et al.*, 1999; Anomymous, 2002.
Tecto	Thiabendazole	A systemic fungicide with both protective and curative action	Effective against *Penicillium* spp. and the stem-end rot fungi especially *Diplodia* and *Phomopsis*.	Nel *et al.*, 1999; Anomymous, 2002.

2.3.2. Biological control with microbial antagonists

At harvest, the fruit microflora is complex in composition, and numerically variable. Natural antagonists selected for biological control systems belong to different taxonomic groups including, bacteria, yeast, and filamentous fungi. The inhibiting activity of the fruit microflora plays an important role in disease control. Biological control, which involves the use of one microorganism to control another, is increasingly becoming an effective alternative to synthetic fungicides for control of pathogens in the postharvest arena (Janisiewicz and Korsten, 2000). The progress in biological control of postharvest diseases using microbial antagonists may be attributed to the uniqueness and relative simplicity of the postharvest system. For example, environmental conditions such as temperature and relative humidity can be managed to favor antagonist survival, and biotic interference is minimal, so the antagonist encounters minimal competition from indigenous microorganisms (Janisiewicz and Korsten, 2002). Biological control of postharvest diseases of citrus has made great advances in the recent past and is reviewed in Table 2.5. The review shows that *B. subtilis* is effective in the control of *Penicillium* rots but mostly ineffective in the control of sour rot. More promising control of sour rot has been reported with yeast.

Other reports where biocontrol systems have been evaluated in the postharvest arena for control of other fruit diseases include amongst others; Korsten *et al.* (1991), Korsten and Kotzé (1992) and Korsten *et al.* (1995; 1997) with avocado, Koomen and Jeffries (1993); Govender and Korsten (2001); Silimela and Korsten (2001), with mango, Leibinger *et al.* (1997); Chand-Goyal and Spotts (1997); Janisiewicz *et al.* (1999); El-Ghaouth *et al.* (2000 a; b; c) with apple, Conway *et al.* (1999) and Williamson and Groenewald (2001), with nectarines, and Benbow and Sugar (1999), with pear. Despite these positive reports, many attempts at transferring potentially effective biocontrol systems from the laboratory to the field have remained largely unsuccessful. The failure in most cases is attributed to a lack of knowledge of the biocontrol agent and/or the environment under which it is applied (Janisiewicz and Korsten, 2002).

Table 2.5. Examples of citrus postharvest biocontrol research programs focusing on different diseases.

Antagonist	Disease controlled				Reference
	Green mold	Blue mold	Sour rot	*Alternaria* rot	
Bacillus subtilis	+	-	-	+	Singh and Deverall, 1984.
B. subtilis	+	+	-	-	Korsten *et al.*, 2000.
B. subtilis	+	+	-	-	Obagwu *et al.*, 2000.
B. pumulis	+	-	-	-	Huang *et al.*, 1992.
Pseudomonas species	+	-	-	-	Smilanick and Dennis-Armie, 1992.
Pichia guilliermondii	+	-	+	-	Droby *et al.*, 1991; 1997.
P. guilliermondii	+	-	-	-	Hofstein *et al.*, 1991.
P. guilliermondii	-	+	-	-	Arras *et al.*, 1998.
Candida famata	+	-	-	-	Arras, 1996.
C. guilliermondii	+	-	-	-	McGuire. 1994.
C. saitoana	+	+	+	-	El-Ghaouth *et al.*, 2000 a, b, c.
Trichoderma viride	+	-	-	-	Boras and Ahuilar, 1990.

"+" means disease(s) against which the control was targeted

Table 2.6 Biocontrol products registered for use in the postharvest arena for control of fruit diseases in South Africa and the United States of America

Country	South Africa		United States of America		
Product Name	Avogreen	Yieldplus	Aspire	Biosave 10 LP, 100	Serenade
Biocontrol agent	*Bacillus subtilis*	*Cryptococcus albidus*	*Candida oleophila* 1-182	*Pseudomonas syringae*	*B. subtilis* QST716
Target pathogen/Disease	Pre- and postharvest diseases of avocado fruit	Postharvest diseases of apple and pear	*Botrytis* and *Penicillium* on citrus and pome fruit	*Botrytis*, *Penicillium*. *Geotrichum candidum* on pome and citrus	Mildews, brown rot, *Cercospora* leaf spot etc on grapes, stone fruit and others
Application method	Spray and dip	Dip	Drench, dip or spray	Drench, dip or spray	Spray
Manufacturer	Stimuplant cc.	Anchor Yeast	Ecopen Inc.	Village Farms LLC	AgraQuest Inc.
Distributor	Ocean Agriculture	Anchor Yeast	Ecopen Inc.	Village Farms LLC	AgraQuest Inc.
Registration held by	University of Pretoria	Anchor Yeast	-	-	-
Registered at	National Department of Agriculture		Environmental Protection Agency (EPA)		

The mechanism of biological control of postharvest diseases is poorly understood. Relatively few attempts have been made to study this field, probably due to the absence of appropriate methods to study microbial interactions in wounds of fruits (Janisiewicz and Korsten, 2002). Information on the mode of action of a biocontrol agent is necessary, not only for the purpose of optimizing the performance of the organism, but also for commercialization. Various mechanisms have been recorded in plant-microbial environments including: site exclusion (Janisiewicz, 1988), competition for nutrient and space (Lim and Rohrbach, 1980; Chalutz and Wilson, 1990; Korsten, 1993; Korsten *et al.*, 1997; Filonow, 1998; Arras *et al.*, 1999; Castoria *et al.*, 2001; Janisiewicz *et al.*, 2000), antibiosis (Wilson and Chalutz, 1989; Korsten, 1993; Korsten *et al.*, 1995; Arras, 1996), induction of host defense mechanisms (Janisiewicz, 1987; Chalutz and Wilson, 1992; Arras, 1996), and direct interaction (Dubos, 1984; Arras, 1996). Some yeast antagonists e.g *Candida* species are capable of colonizing the fungal mycelium where they out-compete the pathogen for nutrients (Arras, 1996). Information presented in Table 2.6 shows that commercial biocontrol products are applied in ways similar to fungicides i.e. as a dip application, spray application or drench. This method of application is compatible with current packhouse practices of treatment application meaning that no alterations might be necessary before a packhouse can begin to use a biocontrol product.

Antibiosis, generally defined as antagonism mediated by specific or non-specific metabolites of microbial origin, by lytic agents, enzymes, volatile compounds, or other toxic substances (Jackson, 1965), is a common phenomenon responsible for the biocontrol activity of many organisms developed as biocontrol agents such as *Pseudomonas*, *Bacillus, Trichoderma* spp (Alabouvette and Lamanceau, 1999). Many phenotypically identical microorganisms are capable of producing vastly different kinds of secondary metabolites, each of which could be highly target specific (Cutler, 1986). Such metabolic compounds may include 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 so give them a competitive advantage over organisms with no such capabilities.

Many *Bacillus* species including *B. subtilis* produce, as major products of glucose fermentation, alcohol (especially low molecular weight ethanol) enzymes, and polypeptide antibiotics all of which may be lytic (Buchanan and Gibbons, 1974). Secondary

metabolites of *B. subtilis* have proven to be inhibitory to several plant pathogenic fungi (Babad *et al.*, 1952; Asante and Neal, 1964). This dilemma can be illustrated by the use of *B. subtilis* and its diverse metabolites. Depending on the source, and mostly because of environmental pressure, *B. subtilis* produces different types of metabolites including antibiotics (Cutler and Hill, 1994). The most commonly produced antibiotic is reported to be iturin A. Even within the iturin producing strains of *B. subtilis* the congeners produced and consequently the ability to control certain phytopathogens may vary (Cutler and Hill, 1994). One of the most successful applications of iturin A in agriculture has been the use of a strain of *B. subtilis* (B-3) for postharvest control of brown rot, *Monilinia fructicola* (Wint) Honey in peaches nectarines, apricots and plums (Cutler and Hill, 1994).

In addition to these secondary metabolites, other compounds such as siderophores (Neilands, 1981; Leong, 1988) are reported to also play a role in the biocontrol of some bacteria including *Pseudomonas* species (Simeoni *et al.*, 1987; Alabouvette and Lemanceua, 1999), and *Enterobacter cloacae* (Fravel 1088). Siderophores, defined as "low-iron-induced virtually ferric-specific ligands" (Neilands, 1993), are produced by most aerobic and facultative anaerobic microorganisms in response to low iron stress. The main function of siderophores are the supply of iron to the cell. Apart from their role in transport of iron (III), siderophores may act as growth factors and some are said to be potent antibiotics (Neilands, 1981). Siderophores from *Rhodotorula glutinis* was reported to reduce apple decay caused by *P. expansum* Link (Calvente *et al.*, 1999). Volatiles are also reported to play a role in the biocontrol of some bacteria. Ammonium isolated from the volatile cultures of *E. cloacae* (Fravel 1088) were reported to inhibit fungal growth when added to fresh media.

Often, more than one mechanism is implicated, and in no one case has a sole mechanism been found responsible for biological control of an antagonist. As with fungicides, the time lag between infection and treatment with biocontrol products is also critical for the success of the product. The maximum time lag will depend on the efficacy of the biocontrol agent in question, which will largely be determined by its mechanisms of action. In any case, delaying treatment for up to 24 hours means that the pathogen would develop beyond the point of control.

The requirements for registration of a microorganism as a biocontrol product as well as the length of time to obtain registration are less than for a chemical pesticide (Powell *et al.*, 1991). These requirements may differ slightly between nations but basically the following information/data are required before an approval is given:

1. Identification data – information on taxonomy of the biological pesticide
2. Toxicological data – information on the infectivity of the living agent, multiplication *in vivo*, and its allergenic potential.
3. Residue data – information on identification and measurement of residues on edible crops at harvest and on non-target organisms is needed.
4. Environmental data – information on expected effects on the environment and its biota is necessary.
5. Efficacy data – quantitative trial demonstrating the efficacy of the product over a period of at least two years.
6. Biological properties – information on spectrum of activity, specificity etc under different environmental and geographical situations.

2.3.3. Plant extracts

The potential of plant extracts for control of plant diseases have long been recognized (Ark and Thompson, 1959). The actual use of these products for control of plant diseases generally, and postharvest pathogens of citrus in particular, is however, still limited. Plant extracts are one of several alternative control options that are currently being intensively researched. Oils from *Artemesia ofra* Jacq ex Willd, *Lavandula angustifolia* Mill., *Eriocephalus punctulatus*, and *Mentha piperia* L. were found effective against *Alternaria citri* Ell. & Pierce, the citrus navel-end rot pathogen (Poswal, 1996). Alcohol and water extracts of *Piper betle* L., *Ocimum sanctum* L., and *Citus limon* (L.) Burm, were effective in inhibiting the growth of *Colletotrichum lindemuthianum* (Sacc. & Magnus) both in culture and in field trials (Amadioha, 1999). Literature on the medicinal values of garlic abound (Gabe Mirkin, 2001). However, only a few references are available describing the potential of garlic extracts for control of plant pathogens, including *Colletotrichum gloeosporoides* (Penz) Sacc. (Pordesimo and Ilag, 1976), *Colletotrichum capsici* (Syd.) Butler & Bisby (Obagwu *et al.*, 1997), *Fusarium oxysporum* f.sp *phaseoli* (Russell and Musa, 1977), *Aspergillus* spp. (Garcia and Garcia, 1990; Bisht and Kamal, 1994; Sinha and

Saxena, 1999) and *Alternaria alternata* (Fries:Fries) Von Keissler (Bisht and Kamal, 1994).

The antifungal activity of garlic is attributed to allicin (diallyl thiosulfinate), the biologically active component of garlic extracts (Focke *et al.*, 1990; Miron *et al.*, 2000). Allicin is produced when the garlic clove is crushed or cut and thought to protect garlic from soil parasites and fungi (Anon., www/3mistral.co.uk/garlic/allicin). Allicin is a very reactive compound, slightly soluble in water and soluble in alcohol (North and Quadrini, 2000). Allicin extracted normally breaks down quickly because of its reaction with other chemicals in garlic (Anon., www/3mistral.co.uk/garlic/allicin). The mode of action is believed to be interference with enzymes required by pathogens to initiate infection in the host plant (Miron *et al.*, 2000). Literature on the ornamental values of *Coprosma repens* Hook. F. also abounds. As far as could be determined, there is no reference in the literature on research done to characterize extracts of this plant and/or evaluate them for any antimicrobial activity.

Plant secondary metabolites

The term phenolics have been used to describe a group of structurally diverse plant secondary metabolites (Wong, 1973). The group includes metabolites derived from the concentration of acetate units (e.g. terpenoids), those produced by the modification of aromatic amino acids (e.g. phenylpropanoids, cinamic acids, lignin procusors, coumarins etc), flavanoids, and many others. Phenolics are almost universally present in higher plants (Harborne, 1980). Woody plants can synthesize and accumulate in their cells a great variety of compounds including low molecular weight phenolics (hydroxybenzoic and hydroxycinnamic acids, acetophenones, flavonoids, stilbenes and lignans) and oligo- and polymeric forms (hydrolysable and condensed tannins and lignins) (Harborne, 1980).

The function of most phenolics is not well described. They are however, traditionally believed to play an important role in plant herbivore interactions (tannins), and in disease resistance of plants (phytoalexins). Polyphenolics are thought to constitute one of the most important groups of higher plants' defensive secondary metabolites (Haslam and Lilley, 1985). It has been observed that most plants that exhibit some antimicrobial activity contain phenols, alkaloids, glycosides and saponins (Samy *et al.*, 1998; Ahmad and Beg, 2001). Citral, a secondary metabolite found in citrus peel is believed to influence the

resistance of the fruit to disease attack (Rodov *et al.*, 1995) Secondary metabolic compounds in plant extracts could alter such systems like the Salicyclic acid pathway (SAP) and /or Jasmonic acid pathway (JAP). The SAP is believed to promote the production of enzymes such as peroxidase, which has been associated with fungal cell wall degradation and pathogen defence signaling (Matheron, 2001). The JAP on the other hand, is believed to promote the accumulation of pathogenesis related protein such as chitinase, which has been implicated in the break down of fungal cell wall. The phenolic content of a plant may therefore serve as an indicator, but is not necessarily a measure of its antimicrobial potential (Moure *et al.*, 2001).

Extraction techniques

Alcohol is the most frequently used solvent for the extraction of plant compounds. Of the alcohols, methanol (Kelmanson *et al.*, 2000) and ethanol (Campbell *et al.*, 2000) are the most frequently used solvents. These solvents are efficient and have little or no negative effects on plant phenolics (Sauvesty *et al.*, 1991). Water is less frequently used because of its poor extraction quality (Jager *et al.*, 1996). Other frequently used solvents include acetone, chloroform, dichloromethane and hexane. These solvents are either used singly or in mixtures. Excessive heat encourages oxidation and/or hydrolysis in phenolic compounds, which could cause structural/chemical changes that impact negatively on such compounds (Sauvesty *et al.*, 1991; Moure, *et al.*, 2000).

Isolation of pure pharmacologically active constituents from plants is a long, and mostly tedious process. Chemical screening is normally performed to allow localization and targeted isolation of new or useful types of constituents with potential activity (Hostettmann, 1997). Numerous techniques are available, and employed in the characterization of plant organic compounds. Thin layer chromatography (TLC) is the simplest and cheapest method of detecting plant constituents. The method is easy to run, reproducible and requires little equipment. However, for efficient separation of metabolites, good selectivity and sensitivity of detection, high performance liquid chromatography (HPLC) techniques are preferred (Hostettmann, 1997). The combination of HPLC with different detection methods gives detailed analysis of plant extracts. For example, HPLC coupled to ultra-violet (UV) photodiode array detector gives useful information on the type of constituents, and in the case of certain classes of compounds such as the polyphenols, indications of oxidation patterns. High Performance Liquid

Chromatography coupled to mass spectrometry (LC/MS) is one of the most sensitive methods of molecular analysis and gives information on the molecular weight as well as on the structure of the analytes (Hostettmann, 1997).

Detection of compounds with the desired activity in complex plant extracts depends on the reliability and sensibility of the test systems used. The system should be sensitive enough to detect active principles that are generally present in small concentrations in plant extracts. Their selectivity should be such that the number of false positives are reasonably small (Hostettmann, 1997). Bioautography is a very convenient and simple way of testing plant extracts for their effects on plant pathogenic microorganisms and other pathogens, and can be employed in the target-directed isolation of active constituents. Three bioautographic methods have been described. This includes, agar diffusion, direct TLC bioautographic detection and agar overlay (Rios *et al.*, 1988). Direct bioautography is applicable to microorganisms that can grow directly on TLC plates. Agar-overlay is a hybrid of the other two methods, and is applicable to a broad spectrum of microorganisms. It produces well-defined zones of inhibition and is not sensitive to contamination. Active compounds are transferred from the stationary phase to the agar layer by a diffusion process. After incubation, the plate is sprayed with a tetrazolium salt e.g. MIT, which is converted to a formazan dye by the microorganism in the agar. Inhibition zones are then observed as clear spots against a purple background (Hostettmann, 1997).

2.3.4. The use of inorganic salts in postharvest disease control

The beneficial effects of sodium and calcium salts in postharvest disease control has been widely reported (Barger, 1928; Sharples and Johnson, 1977; Conway, 1982; Conway *et al.*, 1988; 1991; 1992; Droby *et al.*, 1997; Smilanick *et al.*, 1999; El Ghaouth *et al.*, 2000; Palou *et al.*, 2001; Tian *et al.*, 2002). Sodium bicarbonate (SB) is classified under products 'generally regarded as safe' (GRAS) by the United States Food and Drug Administration, and has been used as a disinfectant for citrus fruit since the 1920's (Barger, 1928). Recently, Palou *et al.* (2001) reported a reduction of up to 90% in the incidence of both citrus green- and blue molds following treatment of artificially inoculated fruits with different concentrations of SB. Application of 68 to 136 nM $CaCl_2$ to grapefruit surface wounds reduced the incidence of green mold (Droby *et al.*, 1997). Calcium salts applied to fruit tissues played an important role in reducing physiological disorders, and delaying

senescence (Sharples and Johnson, 1977; Conway, *et al.*, 1988; 1991; 1992; Droby *et al.*, 1997). The mode of action of both salts are believed to be inhibition of spore germination and germ tube elongation (Marloth, 1931; Conway *et al.*, 1988; Droby *et al.*, 1997), as well as the formation of calcium cross linkages in the cell wall which neutralizes the effects of cell wall macerating enzymes secreted by the pathogen (Conway *et al.*, 1988; Droby *et al.*, 1997). However, there are no reports of these salts being used on their own to provide control of postharvest diseases, probably because they do not have a residual effect (Marloth, 1931).

2.3.5. Heat treatment

Hot water dips are commonly utilized for fungal pathogen control on fruits and vegetables (Lurie, 1999; Auret, 2000). Some fruits and vegetables can tolerate temperatures of up to 75° C (Palou *et al.*, 2001). Most plant pathogens on the other hand can hardly survive temperatures above 40° C. *Penicillium digitatum* and *P. italicum* for example, grow slowly at 30° C, and cannot survive above 35° C (Carlos, 1982). Treating apple fruits with hot air (38 to 46° C) for 12 to 16 hours reduced decay caused by *B. cinerea* and *P. expansum* (Fallik *et al.*, 1993). Heat treatment creates stress in tissue, resulting in the production (in some plants), of compounds such as phytoalexins, or the synthesis of fungistatic aromatic aldehydes and lignin (Eckert *et al,.* 1996). Heated citrus fruit was found to contain high concentrations of the phytoalexin scoparone, which is believed to have antifungal properties (Kim *et al.,* 1991). However, improper application of heat could encourage, rather than retard pathogen development. In grapefruit for example, fruit rot caused by *Penicillium* spp. increased as a result of hot water treatments when used to control Caribbean fruit fly (Muller *et al.*, 1988). Improperly applied heat could cause both external and internal damages such as browning, yellowing and pitting in some fruits (Klein and Lurie, 1992; Wolf and Laing, 1996; Jacobi *et al.*, 1996). The use of heat treatment alone for control of green - and blue mold is accompanied by a high risk of rind injury (Palou *et al.*, 2001).

2.3.5. Integrated control

Most biological control systems are less effective, and less consistent in their activities than most of the conventional fungicides currently used in the postharvest arena. For

acceptance of biological control systems by growers, efficacy and consistency has to be comparable to that provided by conventional fungicides. Achieving such high levels of control is difficult with biological control systems, and the use of an integrated approach, rather than a single antagonist is advocated to provide this required level of control (Pusey 1994). A mixture of antagonists should theoretically mean a wider spectrum of complimentary modes of action, and thus improved activity (Pusey, 1994). Unfortunately, selecting antagonist mixtures that are compatible with each other is difficult. Most frequently, antagonistic behaviour rather than synergistic interaction predominate in antagonist mixtures. The compatibility of biocontrol agents with current commercial practices is of paramount importance in planning an integrated control approach. Compatibility between microbial antagonists and synthetic fungicides for example may allow the use of such an antagonist with a reduced dosage of the fungicide to achieve complete control. Such an integrated approach should be more acceptable from a safety point of view (Droby *et al.*, 1991). Certain groups however, feel that such an approach might encourage the build up of chemical resistance due to the continuous use of low levels of chemicals.

So far, Korsten *et al.* (1991) found that *Bacillus* spp. was compatible with quarter-strength benomyl, prochloraz and chlorine (Korsten, 1993). Better control of anthracnose on mango was achieved with a combination of hot water dip treatments using antagonist *Bacillus licheniformis* than either treatment on its own (Korsten *et al.* 1991). Sodium bicarbonate (SB) combined with hot (45° C) water was more effective than either treatment on its own for the control of citrus blue mold (Palou *et al.*, 2001).

Different salts and other compounds are reported to enhance the biocontrol activity of antagonists. The activity of *Candida oleophila* isolate 182 for example was enchanced by the addition of 90-100 nM $CaCl_2$ (Wisniewski *et al.*, 1995). The increased activity is believed to have resulted from both the direct inhibitory effects of Ca on spore germination and metabolism, and indirectly due to the ability of the isolate to maintain normal metabolism in the presence of toxic levels of Ca (Droby, *et al.*, 1997). Combining $CaCl_2$ applied through pressure infiltration with *Pseudomonas syringae* (isolate ESC-11) used in Biosave 110) resulted in greater control than either treatment on its own (Janisiewicz *et al.*, 1998). A combination of heat treatment with Ca infiltration followed by treatment with

Pseudomonas syringae (ESC-11) was more effective than the individual treatments on their own (Laverentz *et al.*, 2001).

Chitosan and its derivatives, including glycolchitosan, were reported to inhibit fungal growth and induce host defense responses in plants, and harvested commodities (Allan and Hadwiger, 1979; Wilson *et al.*, 1994). Combining 0.2% glycolchitosan with the antagonist *Candida saitoana* was more effective than either treatment on its own in the control of green mold of oranges and lemon caused by *P. digitatum* (El-Ghaouth *et al.*, 2000a). Combining 10% ethanol with heat (50° C) treatment for two minutes was better than either method for control of decay in peaches and nectarines (Dennis *et al.*, 1997).

2.4. Food-Borne Pathogens

Raw fruits and vegetables grow in natural environments and therefore can be expected to carry a wide variety of microorganisms both beneficial and harmful to mankind. Food-borne pathogens are a major cause of food poisoning. The prime cause of food-borne illness associated with fruits and vegetables includes bacteria such as *Escherichia, Salmonella*, *Staphylococcus*, *Campylobacter* and *Shigella* spp. (Evers, 1998). Intrinsic agents such as allergens and toxic compounds present in food as contaminants may also cause food poisoning. In the case of food poisoning caused by microorganisms, the food may serve either as an active vehicle in which multiplication occurs, or as a passive one in which no growth takes place (Varman and Evans, 1991).

Reported outbreaks of food-borne illness involving fresh fruits and vegetables have increased during the last decade (Drapeau and Solomon, 1998). There has been some reports of food-borne illnesses linked to the consumption of unpasteurized orange juice (Anon, htp/www.stop-usa.org/news/priocom/11999com.htm). The main organisms implicated in these outbreaks were *Escherichia coli* 0157-H7 and *Salmonella* spp., and the source of the contamination is believed to be the internalization of pathogens on the fruit peel. Accurate figures on financial losses as a result of food-borne illness and related consequences are difficult to find because of poor record keeping (Schlundt, 2002). In the United States of America, it has been estimated that 76 million cases of food-borne diseases may occur each year resulting in 325 000 hospitalizations (Schlundt, 2002). It is anticipated that the problem is more severe in developing countries (Kaferstein and

Abdussalam, 1999). Although only a small percentage of these causes are associated with the consumption of fresh fruits, it has however, become a major issue in international trade, prompting the European community to require that all fresh fruits imported be certified through some kind of food safety system such as Good Agricultural Practice (GAP), and Hazard Analysis Critical Control Point (HACCP).

Fungicides are ineffective in the control of food-borne pathogens (Janisiewicz and Korsten, 2002), and non-chemical measures have to date, also proved ineffective. Although a number of "generally regarded as safe" chemicals have been reported to possess anti-microbial activity on *Escherichia coli* 0157:H7, *Listeria monocytogenes*, and *Salmonella enteritidis* for example (Friedman *et al.*, 2002), none of these substances could singly reduce significantly populations of bacterial pathogens on fruits and vegetables. Essential oil of cloves, dispersed (0.4% v/v) in a concentrated sugar solution, had a marked germicidal effect against various bacteria including *S. aureus, Klebsiella pneumoniae, Pseudomonas aeruginosa, Clostridium perfringens*, and *E. coli*. (Briozzo *et al.*, 1998).

Conclusion

From this review, it is obvious that great advances has been made in the recent past in testing alternative control measures, especially the use of microorganisms for control of postharvest pathogens. The issue then is not if or when these alternative methods will be used, but how broad their applications will be. Alternative control measures have their limitations under some circumstances, but many of these limitations can be improved by manipulating the environment under which they are applied to their advantage. It would be inappropriate however to equate alternative control, particularly biological control with chemical treatments without considering the advantages and limitations of both methods, which often differ (Janisiewicz and Korsten, 2002).

Literature Cited

Agrios, G.N. 1997. Plant Pathology. Academic press, New York.

Ahmad, I. and Beg, A. 2000. Antimicrobial and photochemical studies on 45 Indian medicinal plants against multi-drug resistant human pathogens. Journal of

Ethnopharmacology 74; 113-123.

Allabouvette, C. and Lemanceau, P. 1999. Joint action of microbials for disease control, Chapter 8 in: Hall, F.R and Menn, J.J. (Eds). Methods in Biotechnology, vol. 5: Biopesticides: Use and Delivery. Humana Press Inc.New Jersey.

Allan, C.R. and Hadwiger, L.A. 1979. The fungicidal effect of chitosan on fungi of varying cell wall composition. Experimental Mycology 3: 285-287.

Amadioha, A.C. 1999. Evaluation of some plant extracts against *Colletotrichum lindemuthianum* in cowpea. Archives of Phytopathology and Plant Protection 32: 141-149.

Anonymous, 1999. Mold control. South Australian Research and Development Institute. Citrus handling guide, 1999.

Anonymous, 2002. Fungicide Resistance Action Group (FRAC)-Fungicide group names and codes. htp/www.frac.info/publication/fraccode-sept2002.

Ark, P.A. and Thompson, J.P. 1959. Control of certain diseases of plants with antibiotics from garlic (*Allium sativum* L.). Plant Disease Reporter 43: 276.

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

Arras,G., De Cicco, V., Arru, S. and Lima, G. 1998. Biocontrol by yeast of blue mould of citrus fruits and the mode of action of an isolate of *Pichia guilliermondii*. Journal of Horticultural Science and Biotechnology 73: 413-418.

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.

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.

Babad, J., Punsky, A., Turner-Graff, R. and Sharon, N. 1952. An antifungal polypeptide produced by *Bacillus subtilis*. Nature 170: 618-619.

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

Benbow, J.M. and Sugar, D. 1999. Fruit surface colonization of and biological control of postharvest diseases of pear by pre-harvest yeast applications. Plant Disease 83: 839-844.

Bisht, S.S. and Kamal, G.B. 1994. Garlic extract-an effective antifungal treatment for the control of storage rot of apple. Proceeding o the National Academy of Sciences India 64: 233-234.

Boras, A.D. and Aguilar, R.V. 1990. Biological control of *Penicillium digitatum* by *Trichoderma viride* on postharvest citrus fruits. International Journal of Food Mibrobiology 11:179-184.

Briozzo, J., Nunez, L., Chirife, J., Herszage, L. and D'Aquino, M. 1998. Antimicrobial activity of clove oil dispersed in a concentrated sugar solution. http:/www.bth.co.uk/EssentialOils.htm.

Brown, G.E. 1994. *Diplodia* Stem-end Rot. University of Florida Cooperative Extension Fact sheet. pp 136.

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

Calvente, V., Benuzzi, D. and de Tosetti, M.I.S. 1999. Antagonistic action of siderophores from *Rhodotorula glutinis* upon the postharvest pathogen *Penicillium expansum*. International Biodetector. Biodegrad 43: 167-172.

Campbell, W.E., Nair, J.J., Gammon, D.W., Codina, C., Bastida, J., Viladomat, F., Smith, J.P. and Albrecht, C.F. 2000. Bioactive alkaloids from *Brunsvigia radulosa*. Phytochemistry 53: 587-591.

Cappellini, R.A. and Ceponis, M.J. 1984. Postharvest losses in fresh fruits and vegetables. Pages 24-30 in: Moline, H.E. (Ed) 1984. Postharvest pathology of fruits and vegetables: Postharvest losses in perishable crops. UC, Berkeley: Agric. Experimental Station.

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

Castoria, R., De Curtis, F., Lima, G., Caputo, L., Pacifico, S. and De Cicco, V. 2001. *Aureobasidium pullulans* (LS-30) an antagonist of postharvest pathogens of fruits: study on its modes of action. Postharvest Biology and Technology 22: 7-17.

Chalutz, E. and Wilson, C. L. 1990. Biocontrol of green and blue mold, and sour rot of citrus fruit by *Debaryomyces hansenii*. Plant Disease 74: 134-137.

Chalutz, E. and Wilson, C.L. 1992. Biological control of postharvest diseases of fruits and vegetables through manipulation of epiphytic plant microflora. Pages 259-266 in: Bills, D.D. and King, S.D (Eds). Proceeding of the second International symposium of Biotechnology and Food safety 259-266.

Chand-Goyal, T. and Spotts, R.A. 1997. Biological control of postharvest diseases of apple and pear under semi-commercial and commercial conditions using three saprophytic yeast. Biological Control 10: 199-206.

Commonwealth Mycological Institute (CMI) 1966. Description of Pathogenic Fungi and Bacteria no. 85. CAB, Great Britain.

Conway, W.S. 1982. Effect of postharvest calcium treatment on decay of delicious apples. Plant Disease 66: 402-403.

Conway, W.S., Gross, K.C., Boyer, C.D. and Sams, C.E. 1988. Inhibition of *Penicillium expansum* polygalacturonase activity by increased apple cell wall calcium. Phytopathology 78: 1052-1055.

Conway, W.S., Sams, C.E., Abbott, J.E. and Bruton R.D. 1991. Postharvest treatment of apple fruits provide broad-spectrum protection against postharvest pathogens. Plant Disease 75: 620-622.

Conway, W.S., Sams, C.E., McGuire, R.G. and Kaman, S. 1992. Calcium treatment of apples and potatoes to reduce postharvest decay. Plant Disease 76: 329-334.

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.

Cutler, H.G. 1986. The science of allelopathy. John Willey & Sons, New York.

Cutler, H.G. and Hill, R.A. 1994. Natural fungicides and their delivery systems as alternatives to synthetics. Chapter 12 in: Wilson, C. L. and Wisniewski, M.E. (Eds) Biological control of post harvest diseases. CRC Press, Roca Raton.

Davies, F.S. and Albrigo, L.G. 1994. Citrus. CAB International, Great Britain.

Dennis, A.M., Joseph, L.M., Gilbert, P.S. and Delmer, J.H. 1997. Combination of hot water and ethanol to control postharvest decay of peaches and nectaries. Plant Disease 81: 1405-1409.

Doidge, E.M. 1929. Some diseases of citrus prevalent in South Africa. South African Journal of Science 26: 320-325.

Drapeau, C. and Solomon, N. 1998. Blue-Green Algae: A powerful immune system enchancer.htp/www.galaxymall.com/virtual/shgalgae/research.htm.

Droby, S., Chalutz, E., Weiss, B. and Wilson, C.L. 1991. Biological control of postharvest diseases of citrus fruits. Pages 60-70 in:Wilson, C.L. and Chaltutz, E. (Eds) Proceeding of the Biological control of postharvest diseases of citrus fruits and vegetables Workshop, West Virginia, 1990.

Droby, S., Wisnieswski, M.E., Cohen, l., 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 guilliermondi*i. Phytopathology 87: 310-315.

Dubos, B. 1984. Fungal antagonism in aerial agrobiocenoses. Pages 107-135 in: Chet, J. (Ed). Innovative approaches to plant disease control. John Willey and Sons, New York.

Eckert, J.W. 1987. Biotypes with reduced sensitivity to imazalil. Phytopathology 72: 1728.

Eckert, J.W. 1988. Dynamics of benzimidazole resistant *Penicillium* in the development of postharvest decays of citrus and pume fruits. Pages 31-35 in: Delp, C.J (Ed), Fungicide resistance in North America. The American Phytopathology Society, St. Paul, MN.

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

Eckert, J.W. and Brown, G.E. 1986. Postharvest citrus diseases and their control, in: Wardowski, W.F., Nagy, S and Grierson, W. (Eds) Fresh citrus fruits, AVI Publishers Co. Inc.

Eckert, J.W., Rotmayake, M., Sievert, J.R. and Strange, R.R. 1996. Curing citrus fruits to control postharvest diseases. Proceeding of the International society of Citriculture, 46.

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.

Evers, W.D. 1998. Electronic Food RAP.
Volume 8 (35). htp/www.cfu.purdue.edu/extension/efr/efrframe.htm

Fallik, E., Klein, J.D., Grinberg, S., Lomansier, E., Lurie, S. and Lalazar, A. 1993. Effect of postharvest heat treatment of tomatoes on fruit ripening and decay caused by *Botrytis cinerea*. Plant Disease 77: 985-988.

Filonow, A.B. 1998. Role of competition for sugars by yeast in the biocontrol of gray mold of apple. Biocontrol Science and Technology 8: 243-256.

Food and Agriculture Organization (FAO). 2001. Citrus fruit annual statistics.

Food and Agriculture Organization (FAO). 2002. Citrus fruit annual statistics.

Focke, M., Field, A. and Lichtenthalar, 1990. Allicin a naturally occurring antibiotic from garlic. FEBS Letters 206: 106-108.

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

Friedman, M., Henika, P.R. and Mandrell, R.E. 2002. Bacterial activities of plant essential oils and some of their isolated constituents against *Campylobacter jejuni*, *Escherichia coli*, *Listeria monocytogenes*, and *Salmonella enterica*. Journal of Food Protection 65: 1545-1560.

Gabe Mirkin, M.D. 2001. Allicin in garlic. htp/www/3mistral.co.uk/garlic/allicin.

Garcia , R.P. and Garcia, M.I. 1990. Laboratory evaluation of plant extracts for the control of *Aspergilus* growth and aflatoxin production. Proceeding of the Japanese Association of Mycotoxicology. In: Horticultural Abstracts 60: 6543.

Govender, V. and Korsten, L. 2001. Evaluating biological control systems for mango postharvest disease control. South African Mango Growers Association Yeakbook 21: 6-11.

Harborne, J.B. 1980. Plant phenolics. Pages 329-402 in: Bell, F.A. and Chartwood, B.V. (Eds), Encyclopedia of plant Physiology. New series. Vol. 8. Springer Verlag, New York.

Haslam, E. and Lilley, T.H. 1985. New phenolics for old tannins. Annual Proceedings of the Phytochemical Society of Europe 25: 237-256.

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

Hofstein, R., Droby, S., Chalutz, E., Wilson, C.L. and Friedlender, B. 1991. Scaling-up the production of an antagonist – from basic research to R and D. Pages 188-201 in: Wilson, C.L. and Chalutz, E. (Eds). Proceedings of the Biological control of Postharvest diseases of fruits and vegetables workshop.

Hostettmann, K. 1997. Strategy For the biological and chemical evaluation of plant extracts. Invited lecture presented at the International Conference on Biodiversity and Resources: Conservation and Utilization, 22-27 November, 1997, Phuket, Thailand.

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.

Jackson, R.M. 1965. Antibiosis and fungistasis of soil microorganisms. In: Baker, K.F. and Synder, W.C. (Eds), Ecology of soil-borne plant pathogens. University of California Press Barkeley.

Jacobi, K.K., Wong, L.S. and Giles, J.E. 1996. Postharvest quality of zucchini following high humidity hot air disinfestation treatments and cold storage. Postharvest Biology and Technology 7: 309-316.

Jager, A.K., Hutchings, A. and Van Staden, J. 1996. Screening of Zulu medicinal plants for prostaglandin-synthesis inhibitors. Journal of Ethnopharmacology 52: 95-100.

Janisiewicz, W.J. 1987. Postharvest biological control of blue mold on apple. Phytopathology: 77, 481-485.

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. Marel Dakker, New York.

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

Janisiewicz, W.J., Conway, W.S., Glenn, D.M. and Sams, C.E. 1998. Integrating biological control and calcium treatment for controlling postharvest decay of apples. HortScience 33: 105-109.

Janisiewicz, W.J., Conway, W.S. and Leverentz, B. 1999. Biological control of postharvest decays of apple can prevent growth of *Escherichia coli* 0157:H7 in apple wounds. Journal of Food Protection 62: 1372-1375.

Janisiewicz, W.J., Tworkoski, T.J. and Sharer, C. 2000. Characterizing the mechanism of biological control of postharvest diseases on fruits with a simple method to study competition for nutrients. Phytopathology 90: 1196-1200.

Jeffries, P. and Jeger, M.J. 1990. The biological control of postharvest diseases of fruits. Postharvest News and Information 1: 364-368.

Käferstein, F. and Abdussalam, M. 1999. Food safety in the 21st century. Bulletin of the World Health Organization 77; 347-351.

Kelmanson, J.E., Jager, A.K. and Van Staden, J. 2000. Zulu medicinal plants with antimicrobial activity. Journal of Ethnopharmacology 69: 241-246.

Kiely, T.B. 1948. Preliminary studies on *Guignardia citricarpa* species; the ascigerous stage of *Phoma citricarpa* McAlp and its relation to black spot of citrus. Proceeding of the Linnean Society, New South Wales 93: 249-292.

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.

Klein, J.D. and Lurie, S. 1992. Heat treatment for improved postharvest quality of horticultural crops. HortTechnology 2: 316-320.

Koomen, J. and Jeffries, P. 1993. Effects of antagonistic microorganisms on the postharvest development of *Colletotrichum gloesporoides* on mango. Plant Pathology 42: 230-237.

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

Korsten, L. and Kotzé, J.M. 1992. Postharvest biological control of avocado postharvest diseases. Proceeding of the Second World Avocado Congress, California, 1991, 473-477.

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., Kotzé, 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 Villers, E.E., Wehner, F.C. and Kotzé, J.M. 1997. Field sprays of *Bacillus subtilis* and fungicides for control of postharvest fruit diseases of avocado in South Africa. Plant Disease 81: 455-459.

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

Kotzé, J.M. 1962. Studies on the black spot disease of citrus caused by *Giugnardia citricarpa* with particular reference to its epidemiology and control at Letaba. DSc (Agric) Thesis. University of Pretoria, Pretoria.

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

Kotzé, J.M. 1996. History and epidemiology of citrus black spot in South Africa. Proceeding of the International Society of Citriculture 2: 1296-1299.

Laverentz, B., Janisiewicz, W.J., Conway, W.S. and Saftner, R.A. 2001. Effect of combining biocontrol, heat treatment, and MCP treatment on the reduction of postharvest decay of 'Delicious' apples. Phytopathology 91: 555.

Leibinger, N., Breuker, B., Hahn, M. and Mendgen, K. 1997. Control of postharvest pathogens and colonization of the apple surface by antagonistic microorganisms in the field. Phytopathology 67: 1103-1110.

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

Lim, T.K. and Robrbach, K.G. 1990. Role of *Penicillium funiculosum* strains in the development of pineapple fruit diseases. Phytopathology: 70, 663-665.

Louw, C.A.M. 2002. Antimicrobial activity of indigenous bulbous plant extracts to control selected pathogens. MSc Thesis, University of Pretoria, Pretoria.

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

MacDonald, R.E., Risse, L.A. and Hilerbrand, M.R. 1979. Resistance to thiabendazole and benomyl in *Penicillium digitatum* and *Penicillium italicum* isolated from citrus from several countries. Journal of the American Society of Horticultural Science 104: 333-335.

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

Matheron, M. 2001. Mode of action for plant disease management chemistry. Paper Presented at the Annual Desert Vegetable Crop Workshop, Yuma, AZ., December 6, 2001.

McGuire, R.C. 1994. Application of *Candida guuilliermondi*i in commercial citrus coating s for biocontrol of *Penicillium digitatum* on grapefruits. Biological Control 4: 1-7.

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*. Phytopathology 76: 136-139.

Mc Onie, K.C. 1967. Germination and infection of citrus by ascospores of *Guignardia citricarpa* in relation to control of black spot. Phytopathology 57: 743-746.

Miron, T., Rabinkov, A., Mirelman, D., Wilchek, M. andWeiner, l. 2000. Mode of action of allicin. Biochim Biophysta Acta 1463, 30-30.

Moure, A., Cruz, J.M., Franco, D., Dominguez, J.M., Sineiro, J., Dominguez, H., Nunez, M.J. and Jarajo, J.C. 2001. Natural antioxidants from residual sources. Food Chemistry 72: 145-171.

Muller, W.R., McDonald, R.E., Hatton, T.T. and Ismail, M. 1988. Phytotoxicity to grapefruit exposed to hot water immersion treatment. Proceeding of the Florida State Horticultural Society 101: 192-195.

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.

Nel., A., Krause, M. and Van Zyl, K. 1999. A guide for the control of plant diseases. National Department of Agriculture, Republic of South Africa.

Norman, C. 1988. EPA sets new policy on pesticide cancer risks. Science 242: 366-367.

North, C. and Quadrini, F. 2000. Allicin-the smell of health.
htp/www.chem.ok.ac.uk/moom/allicin.htm.

Obagwu, J., Emechebe, A.M. and Adeoti, A.A. 1997. Effect of extracts of garlic (*Allium sativum* L.) bulb and neem (*Azadirachta indica* Juss) seed on the mycelial growth and sporulation of *Colletotrichum capsici*. Journal of Agricultural Technology 5: 51-55.

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.

Pordesimo, A.N. and Ilag, I.I. 1976. Toxicity of garlic juice to plant pathogenic organisms. Philippines Journal of Biology 5: 251-258.

Poswal, M.A.T. 1996. Antifungal activity of natural plant oils against *Alternaria citri*, the Navel-end rot pathogen. Proceeding of the International Society of Citriculture. 478-481.

Powell, K.A., Faull, J.L. and Remwick, A. 1991. The commercial and regulatory challenge. Pages 445-463 in: Biological control of plant pathogens. Oxon, UK: CAB Int.

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, Roca Raton.

Rios, J.L., Recio, M.C. and Villar, A. 1988. Journal of Ethnopharmacology 23: 127-149.

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

Russell, P.E. and Mussa, A.E. 1977. The use of garlic to control foot rot of *Phaseolus vulgaris* caused by *Fusarium solani* f.sp *phaseoli*. Annals of Applied Biology 86: 369-372.

Samy, R.P., Ignacimuthu, S. and Raja, D.P. 1998. Preliminary screening of ethno medicinal plants from India. Journal of Ethnopharmacology 66: 235-240.

Sauvesty, A., Page, F. and Eliot, J.A. 1991. A simple method for extracting plant phenolic compounds. Canadian Journal for Research 22: 654-659.

Schlundt, I. 2002. New directions in food-borne disease prevention. International Journal of Food Microbiology 78: 1-17.

Sharples, R.O. and Johnson, D.S. 1977. The influence of calcium on senescence changes in apple. Annals of Applied Biology 85: 450-453.

Silimela, M. and Korsten, L. 2001. Alternative methods for preventing pre- and postharvest diseases and sunburn on mango fruits. South African Mango Growers Association Yeakbook 21: 59-43.

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.

Sinha, P. and Saxena, S.K. 1999. Inhibition of fruit rot fungus and fruit fly by leaf extracts of onion and garlic. Indian Journal of Agricultural Sciences 69: 651-653.

Smilanick, J.L. and Denis-Arrue, R. 1992. Control of green mold of lemons with *Pseudomonas* species. Plant Disease 76: 481-485.

Smilanick, J.L., Margosan, D.A., Mlikota, F., Usall, J. and Michael, F. 1999. Control of citrus green mold by carbonate and bicarbonate salts and the influence of commercial post harvest practices on their efficacy. Plant Disease 83: 139-145.

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.

Timmer, L.W. and Duncan, L.W. (Eds). 1999. Citrus Health Management, American Phytopathological Society Press, St. Paul, MN.

Tomlim, C. (Ed). 1995. The pesticide manual 10th edition. Crop Protection Publications.

Varnam, A.H and Evans, M.G. 1991. Foodborne pathogens: An illustrated text. Mosby Yearbook Inc., New York.

Whiteside, J.O., Garnsey, S.M. and Timmer, L.W. 1988. Compendium of citrus diseases. The American Phytopathological Society Press, St. Paul, MN.

Williamson, L. and Groenewald, M. 2001. Successful biocontrol of postharvest decay on plums and nectarines. Paper presented at the 39th Annual South African Society for Plant Pathology Congress held at Greenway Woods, Nelspruit, South Africa 21-24 January, 2001.

Wilson, C.L. and Chaltutz, E. 1989. Postharvest biological of *Penicillium* rots with antagonist yeast and bacteria. Scientific Horticulture 40: 105-112.

Wilson, C.L., El-Ghaouth, A., Chalutz, E., Droby, S. and Stevens, C. 1994. Potential of induced resistance to control postharvest diseases of fruits and vegetables. Plant Disease 78: 837-843.

Wisniewski, M., Droby, S., Chaltutz, E. and Eilam,Y. 1995. Effect of Ca and Mg on *Botrytis cinerea* and *Penicillium expansum in vitro* and on the biocontrol activity of *Candida ailurophile*. Plant Pathology 44: 1016-1024.

Wolf, A.F. and Laing, W.A. 1996. Avocado fruit skin fluorescence following hot water treatment and pretreatments. Journal of American Society of Horticultural Science 121: 147-151.

Wong, E. 1973. Plant phenolics, in: Butler, G. W. and Bailey, R.W (Eds). Chemistry and Biochemistry of herbage, vol. 1. Academic Press, London.

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

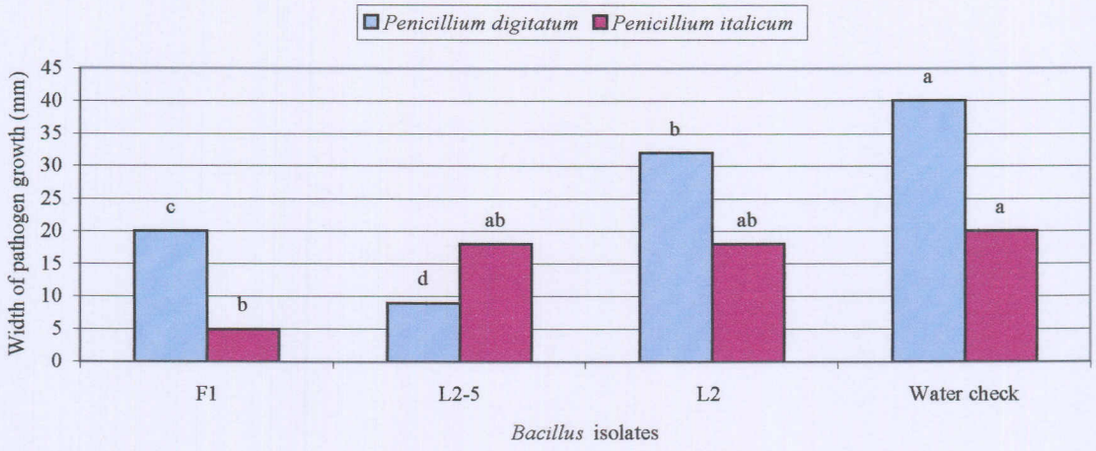
Antibiosis - All isolates tested did produce secondary metabolites with inhibitory properties. However, the degree of production and efficacy of such products as measured by the inhibition of pathogen growth on PDA varied between pathogens and between isolates (Fig. 3.1 and 3.2). The effect of antibiosis 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.

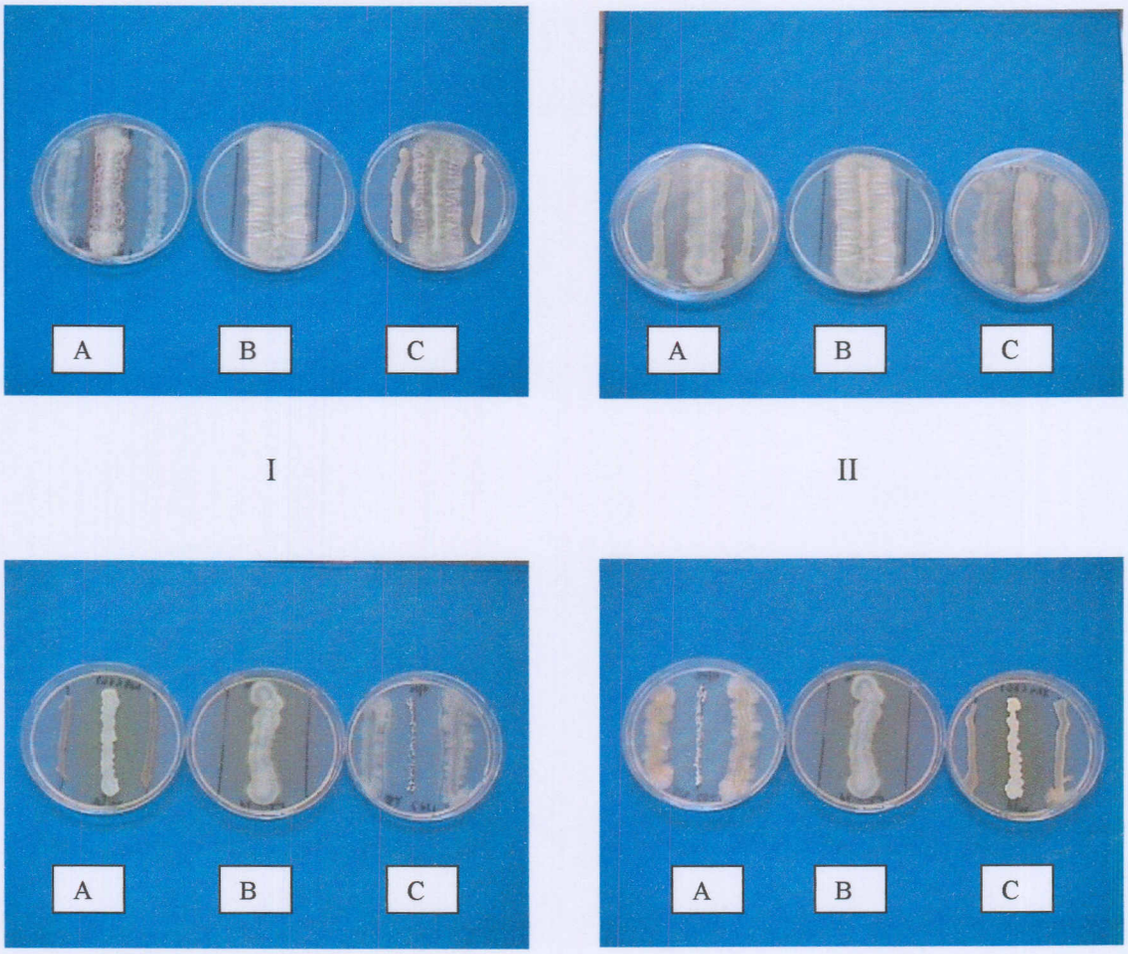


Photo I

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

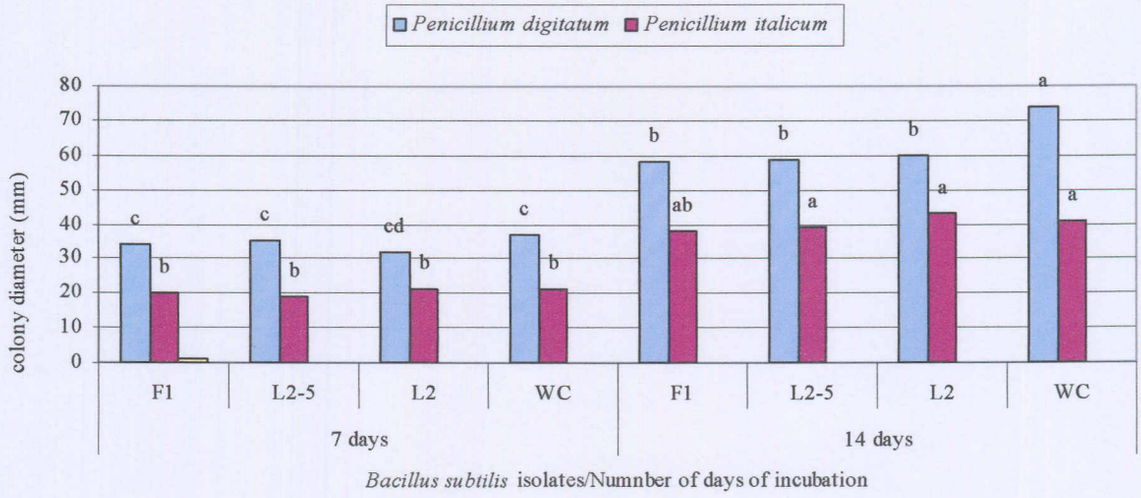
None of the isolates produced volatile compounds, which completely inhibited the growth of both *P. digitatum* and *P. italicum* (Fig. 3.3). Also, none of the isolates' volatiles significantly inhibited the growth of both pathogens when compared to the control after seven days incubation. At 14 days 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.

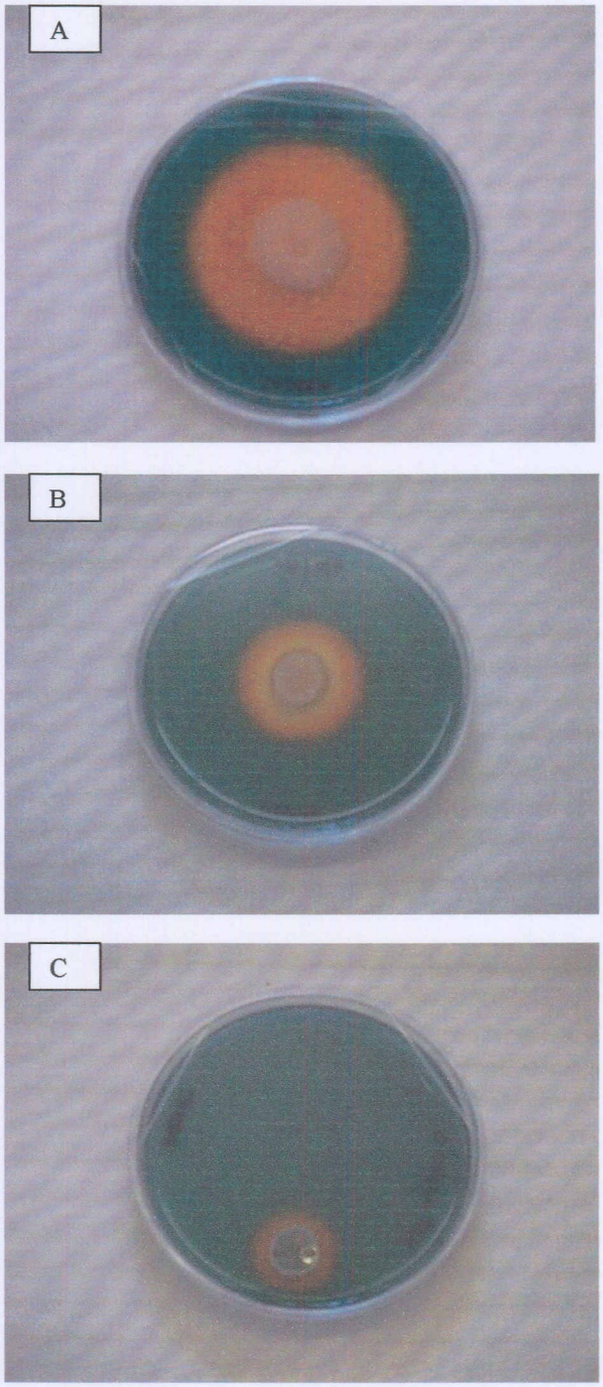


Fig. 3.4 Siderophore production ability (in yellow halo) of *Bacillus subtilis* antagonist after 10 days of incubation at 25° C, with photo **A** representing isolate L2-5; **B** isolate L2; and **C** the pathogen, *Penicillium digitatum.*

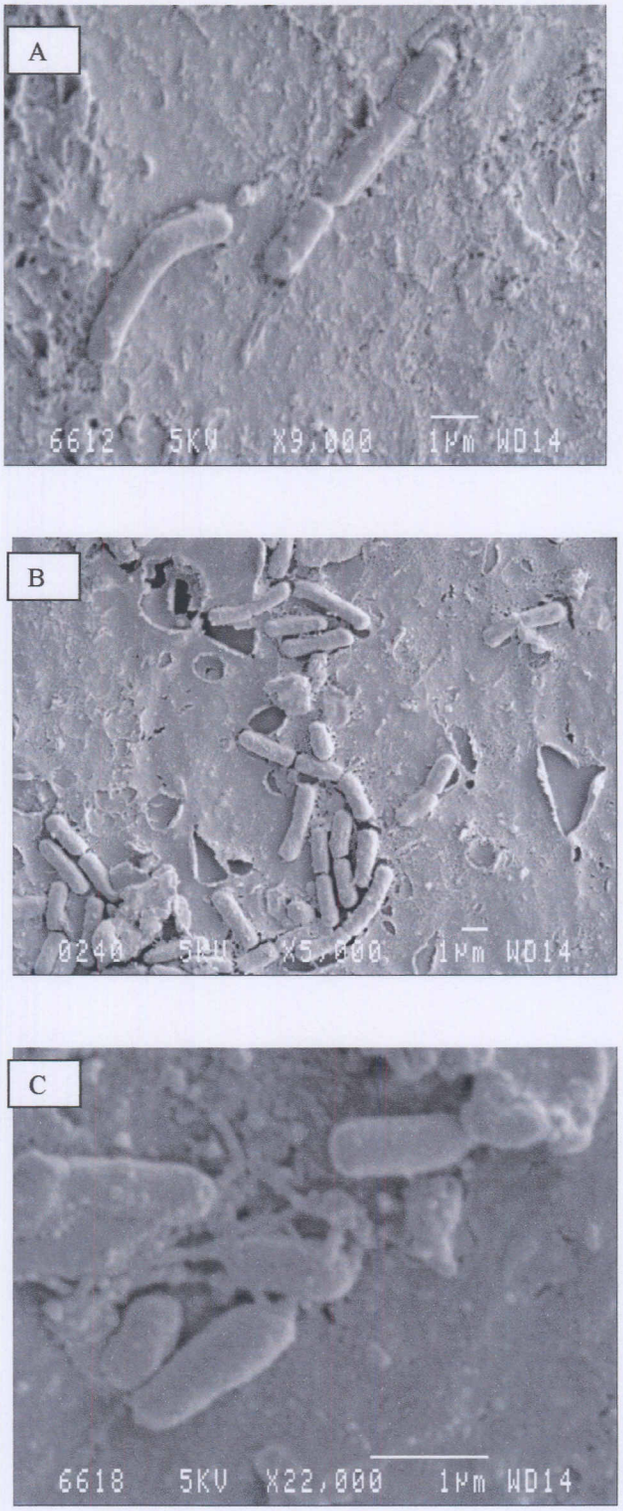


Fig. 3.7 Colonization and attachment of *Bacillus subtilis* on Valencia orange, Photo **A** and **B** represent colonization of the fruit surface by isolate F1 after 12 and 24 hours of application, while **C** shows attachment of isolate L2 at the wound site after 12 hours of application

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.

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

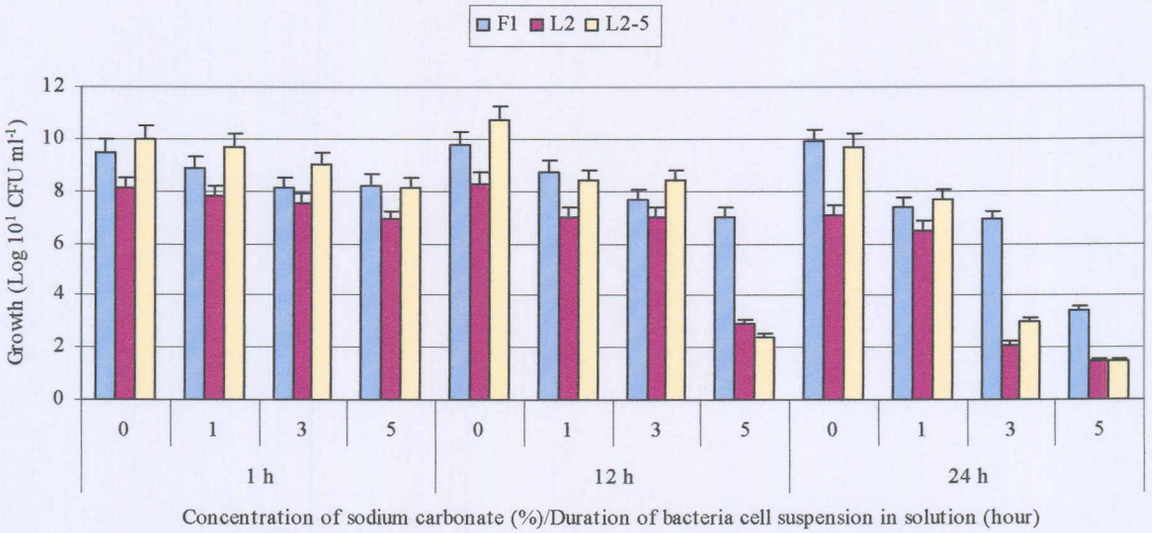
All data obtained were statistically analysed using the SAS statistical program. One-way analysis of variance (ANOVA), was used to test for differences in average means between treatments. Treatment means were separated using Duncan's multiple range test (DMRT) at 5% level of significance.

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

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

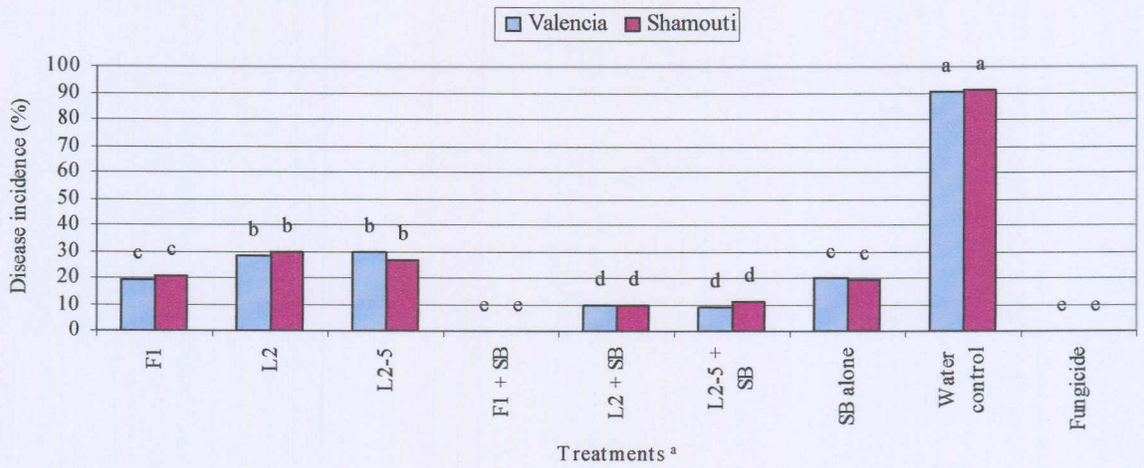
After two weeks of storage, no visible/external symptoms of damage were observed on fruits treated with 1, 3, or 5% (w/v) SB solution. By four weeks however, blotching type symptoms were visible on both Valencia and Shamouti fruits immersed in 5% SB. Symptoms were more severe on Shamouti than Valencia.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

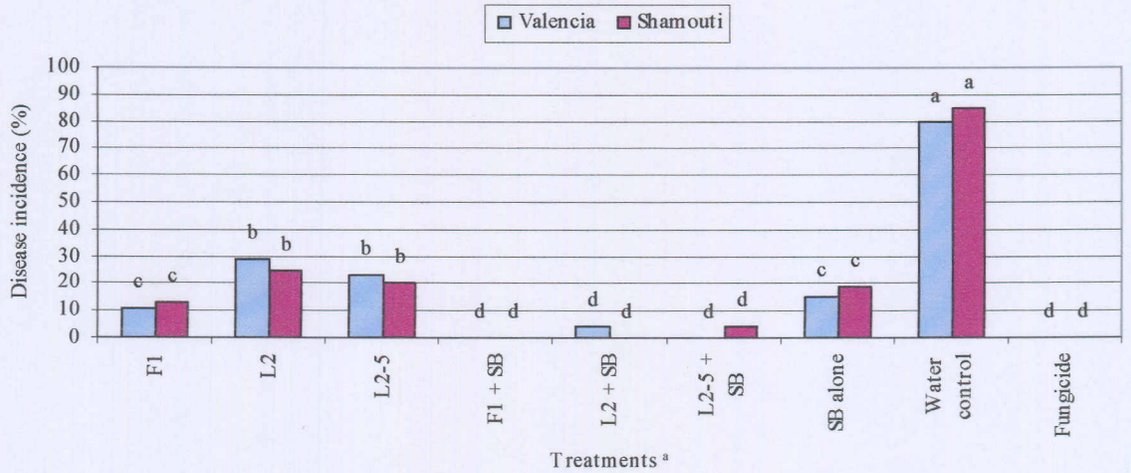
The influence of *B. subtilis* isolates on their own, or in combination with SB on control of citrus green - and blue mold are presented in Fig. 4.2. There were variations in percentage

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).

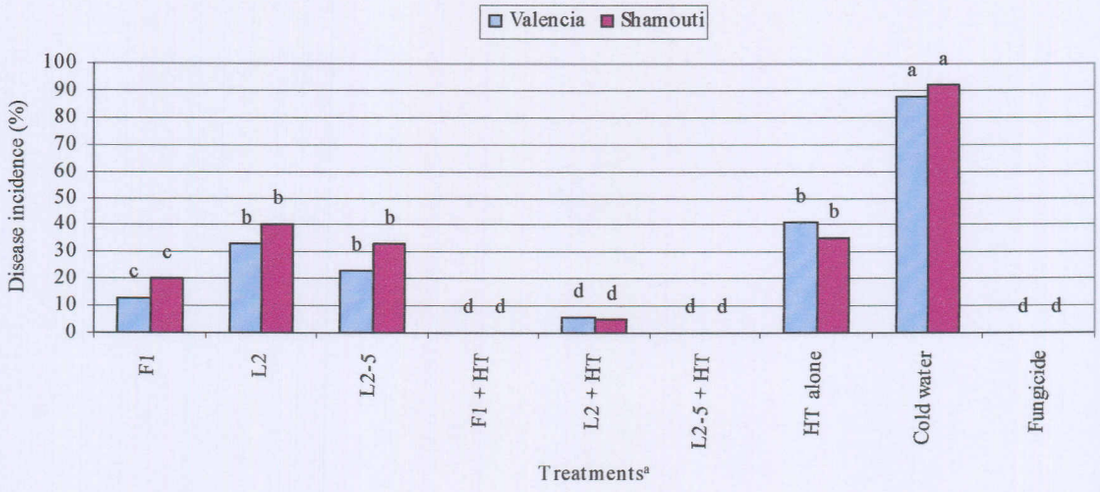
Fig. 4.2 Performance of *Bacillus subtilis* isolates on their own or in combination with sodium bicarbonate to control citrus green (*Penicillium digitatum*) (A) and blue (*P. italicum*) (B) mold on artificially inoculated fruits stored at 9° C and 90 to 95% relative humidity for four weeks.

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

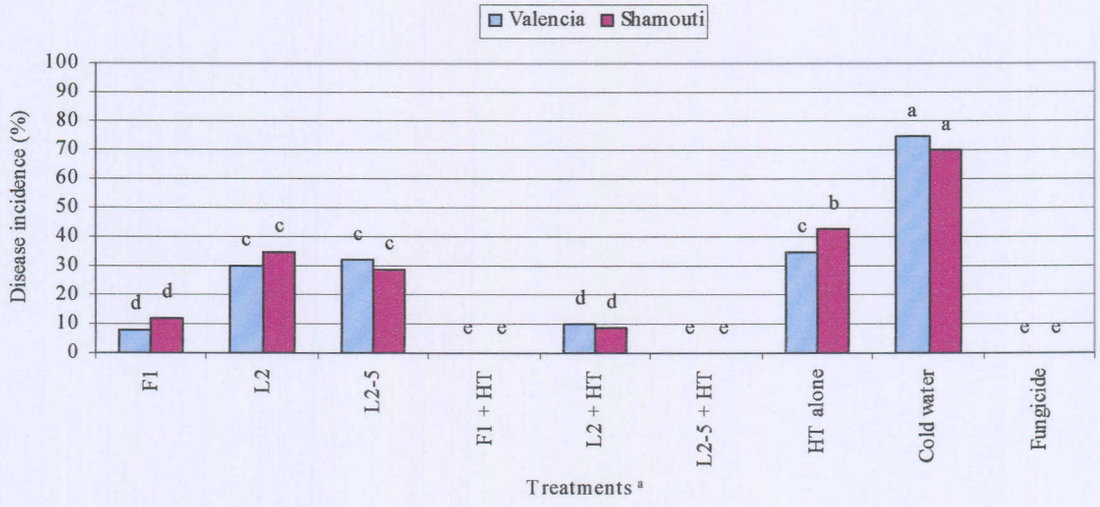
Results presented in Figs 4.3 indicate a significant difference between the control (cold water) and other treatments. Isolate F1 on its own was more effective than L2 and L2-5, in checking the incidence of both green - and blue mold on both cultivars. The percentage incidence in L2 and L2-5 treated 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.



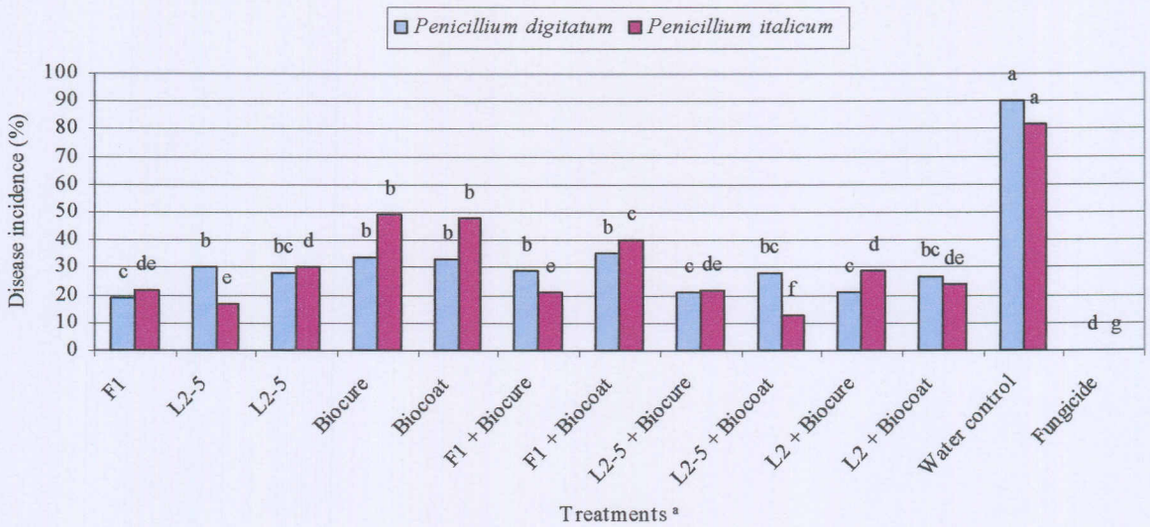
A



B

[a] F1, L2 and L2-5 represent *Bacillus subtilis* isolates (1 x 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.

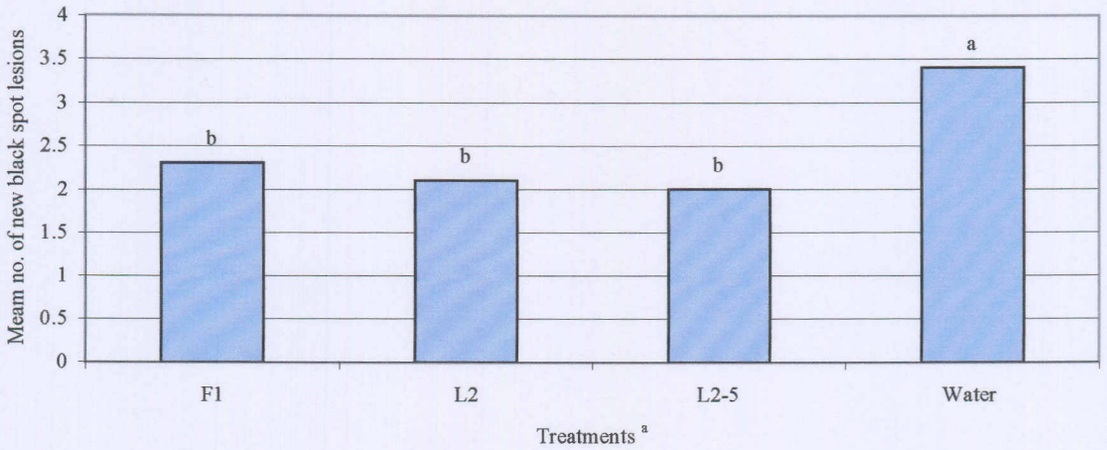


[a] F1, L2 and L2-5 represent *B. subtilis* isolates (1 x 10^8 cells ml^{-1}); Biocure and Biocoat represent *Candida saitoana*; Fungicide represent Fungazil (imazalil 75% a.i. at 1g L^{-1}) plus Decotine quazatine 20% a.i. at 1 ml L^{-1}). Data represent mean of three repetitions. Treatments having 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, l,, 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.

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

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

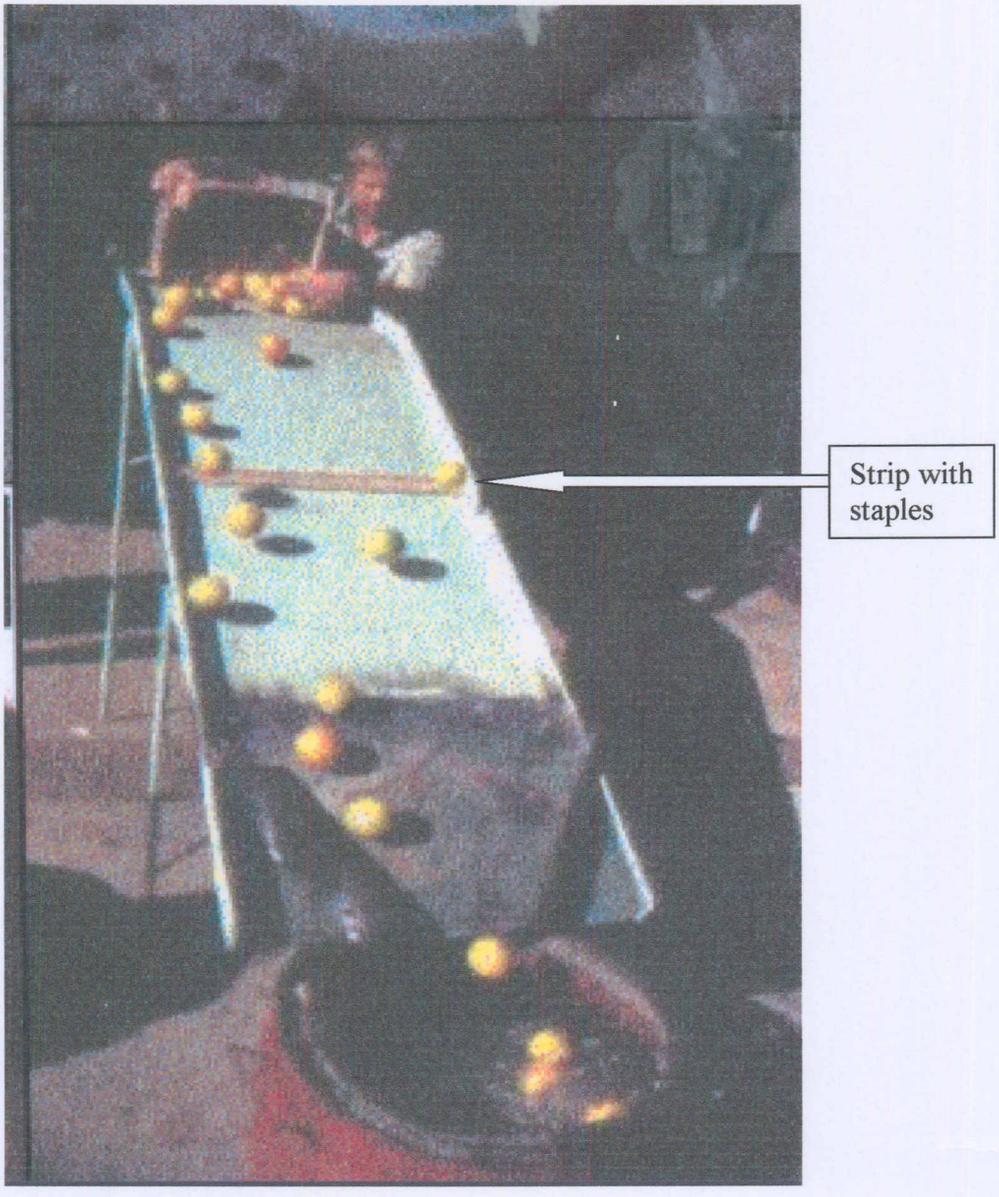


Fig.5.1. Fruit wounder used for fruit inoculation

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

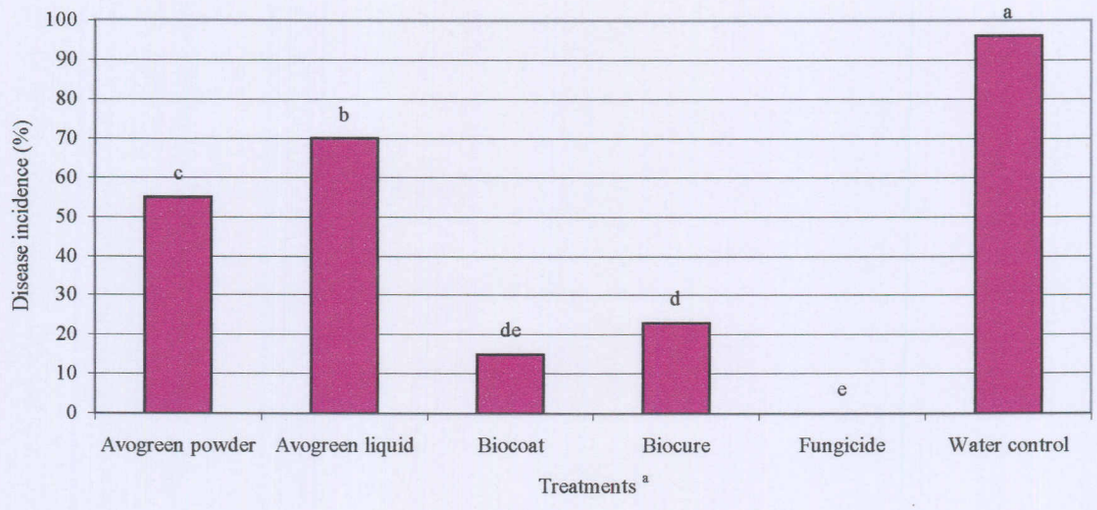
5.4. Results

2000 season

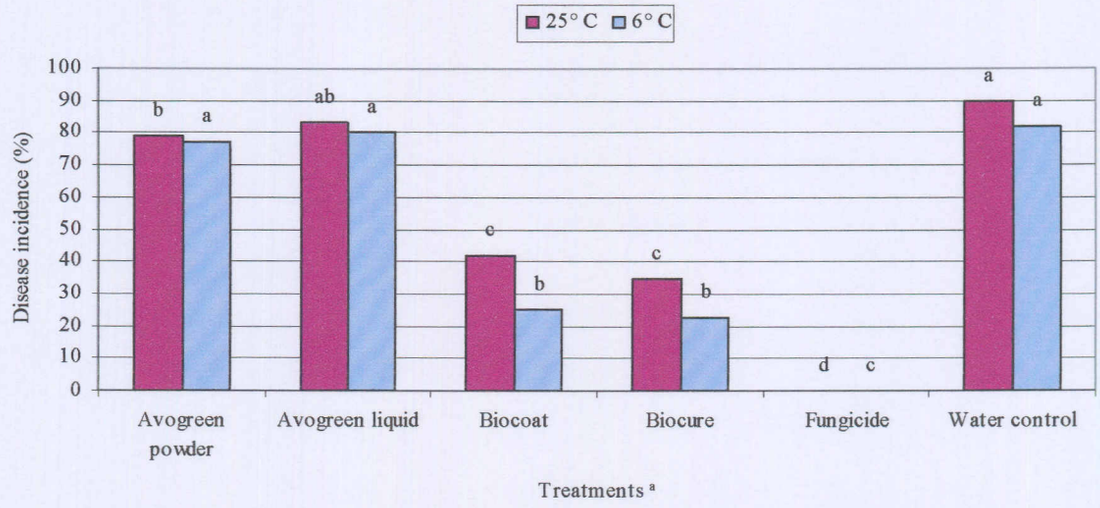
The results presented in Fig.5.2A show that all products were effective in the control of *Penicillium* rot compared to the water treatment in June. They were however, less effective than the fungicide treatment, which gave complete control of the disease. The percentage disease incidence in treated 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.



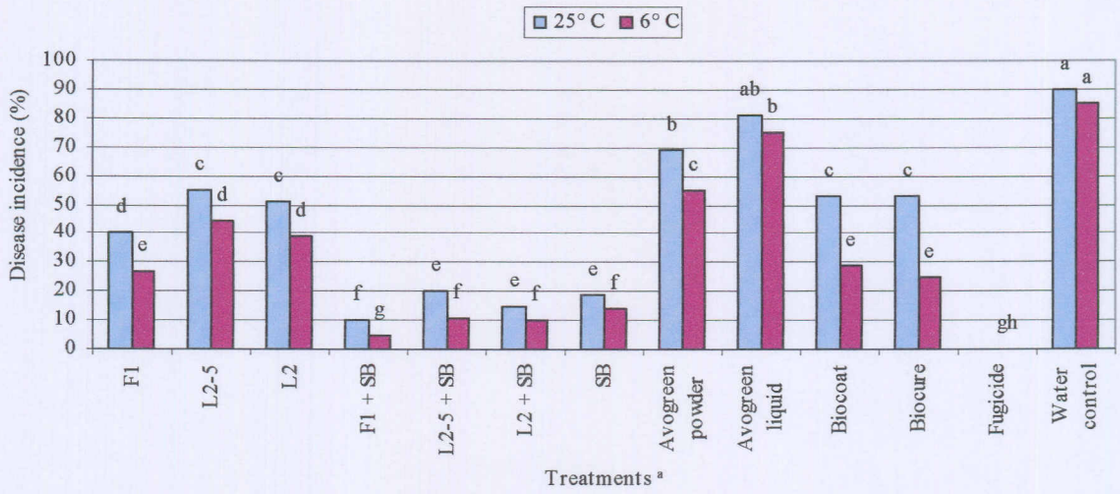
A



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.

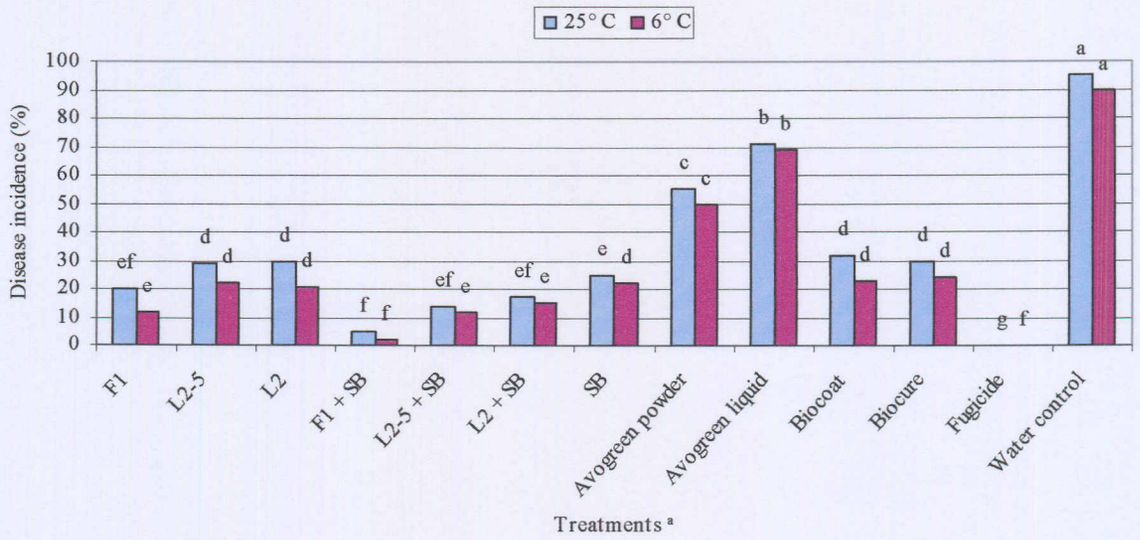
2001 Season

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

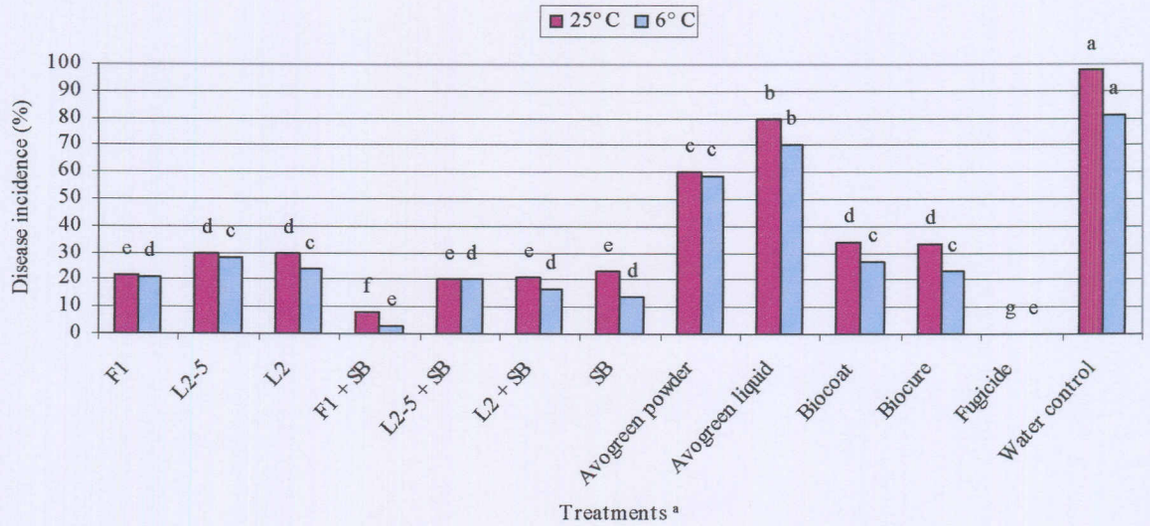
Avogreen liquid. The observed increase in biocontrol activity of *Bacillus subtilis* products following addition of SB was again evident. When integrated, the treatment comprising F1 combined with SB was more effective than all treatments evaluated with a percentage disease incidence of only 2% under 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).



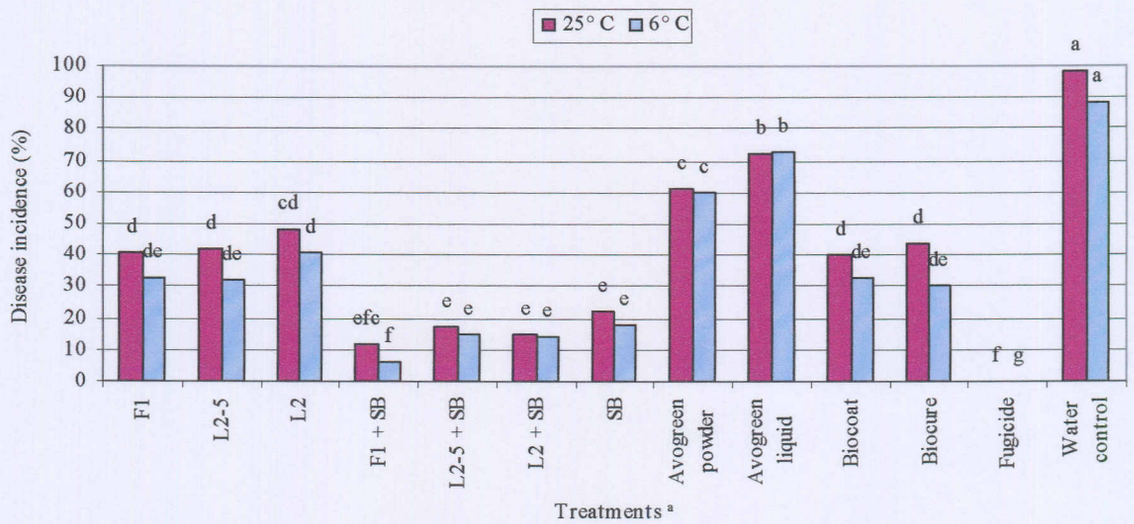
A



B

[a] Biocure and Biocoat represent yeast (*Candida saitoana*) products; Avogreen liquid, Avogreen powder, F1, L2and 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).

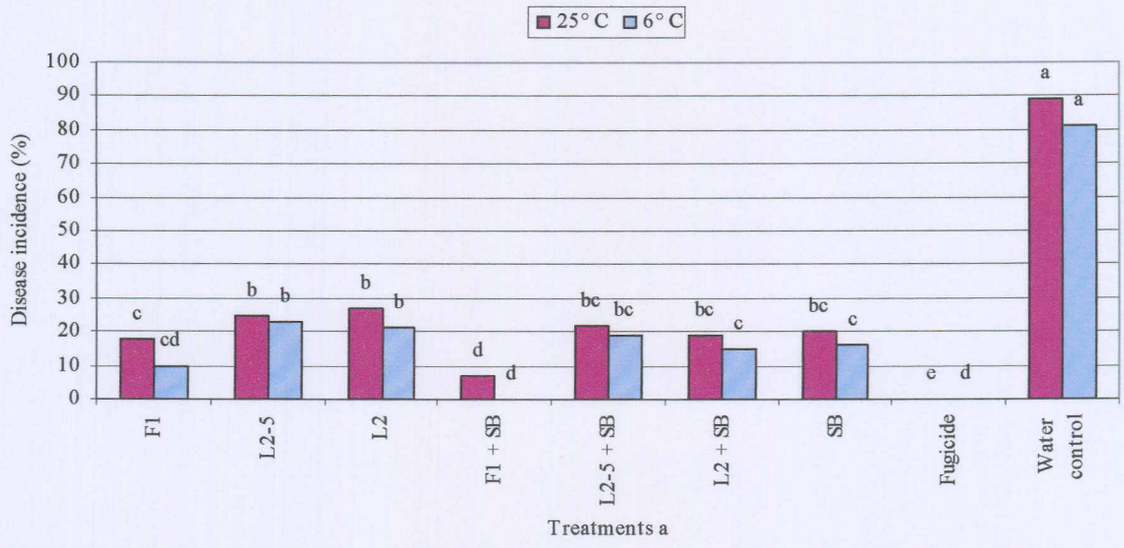


[a] Biocure and Biocoat represent yeast (*Candida saitoana*) products; Avogreen liquid, Avogreen powder, F1, L2 and L2-5 represent *Bacillus subtilis*; Fungicide represent Fungazil (imazalil 75% a.i. at 1g L^{-1}) plus Decotine (quazatine 20% a.i. at 1 ml L^{-1}); SB represent sodium bicarbonate (1% 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).

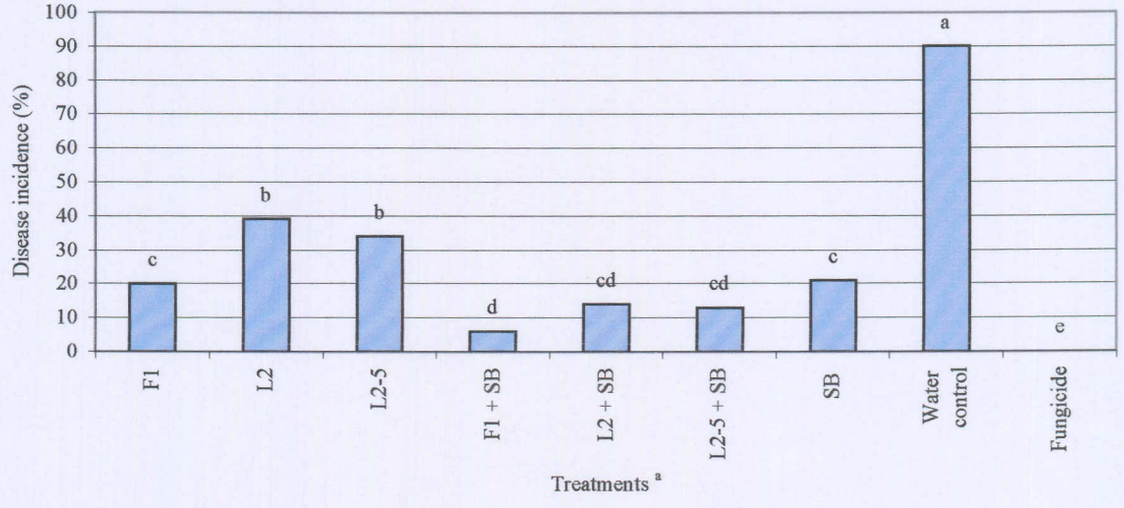
Fig 5.5 Evaluation of alternative postharvest disease control options for control of citrus green mold caused by *Penicillium digitatum* on Valencia orange after two weeks of storage at 25° C or four weeks at 6° C in September 2001.

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.

CHAPTER SIX

SCREENING PLANT EXTRACTS FOR BIOLOGICAL ACTIVITY AGAINST *PENICILLIUM DIGITATUM*, *PENICILLIUM ITALICUM* AND *GUIGNARDIA CITRICARPA*

6.1. Abstract

Garlic (*Allium sativum*) clove and *Coprosma repens* extracts were evaluated for their biological activity against *Penicillium digitatum*, *P. italicum*, and *Guignardia citricarpa* both *in vitro* and on artificially inoculated (in the case of *P. digitatum* and *P. italicum*) and naturally infected (in respect of *G. citricarpa)* Valencia oranges stored at 8±1° C and 90-95% relative humidity (RH) for four weeks. Garlic was evaluated alone or in a mixture with vegetable cooking oil (0.1% v/v). Both garlic and *Coprosma* exhibited varying degrees of antifungal activity against all pathogens, and all concentrations of extracts were significantly effective when compared with the control in checking disease incidence, but were not as effective as the commercial fungicide which gave complete control of both *P. digitatum* and *P. italicum*. Mixing garlic extracts with oil remarkably improved its activity. As a result, the treatment comprising garlic extracts (1 000 ppm) mixed with oil was as effective (100% control) as the fungicide treatment in the control of both *P. digitatum* and *P. italicum*. *Coprosma repens* extract on its own was not as effective and the percentage control achieved varied between 67 and 81%. *In vitro* studies indicate that the mode of action of extracts is inhibition of spore germination and germ-tube development.

6.2. Introduction

Plants contain thousands of constituents and are a valuable source of new and biologically active molecules. Although the potential of these products has long been recognized (Ark and Thompson, 1959), their actual use in this respect is however, still limited. This is particularly true for control of postharvest diseases. Of the several hundred thousand plant species around the globe, only a small proportion has been investigated both phytochemically and pharmacologically (Hostettmann, 1997). Plant extracts are therefore

one of several non-chemical control measures currently being intensively researched for control of plant diseases. Alcohol and water extracts of *Piper betle* L., *Ocimum sanctum* L., and *Citrus limon* (L.) Burm were effective against *Colletotrichum lindemuthianum* (Sacc. & Magnis) in culture, and in checking the incidence and spread of the disease in the field (Amadioha, 1999). Most research with garlic (*Allium sativum* L.) to date has focussed on its medicinal values (Gabe Mirkin, 2001), with few (Pordesimo and Ilag, 1976; Russell and Mussa, 1977; Shashikanth *et al.*, 1981; Garcia and Garcia, 1990; Obagwu *et al.*, 1997; Sinha and Saxena, 1999) reports of its other possible applications such as plant disease control. To date there are no references in the literature on the use of extracts of garlic clove for control of *Penicillium digitatum* Sacc., *P. italicum* Wehmer., and *Guignardia citricarpa* Kiely. Literature abounds on the horticultural values of *Coprosma repens* Hook F. There are however, no references on the use of its extracts in plant disease control.

The aim of this study was therefore to evaluate the efficacy of water and ethanol extracts of garlic clove and *C. repens* for control of *P. digitatum*, *P. italicum*, and *G. citricarpa* and characterize extracts of *C. repens*. The active component of garlic, allicin, is reported to break down easily (Anon., hyp/www/3mistral.co.uk/garlic/allicin). Extracts will therefore be evaluated together with vegetable oil to exploit possible delay of the breakdown and improvement of its biological activity following such a combination. Garlic extracts will be evaluated as a spray application because a dip application might not be economical. Attempts will be made to characterize *C. repens* extracts with a view to identifying the active compound(s).

6.3. Materials and Methods

Twenty-nine plants were screened for their antifungal properties *in vitro* i.e. in microtiter plates (Nunc; AEC-Amersham (Pty) Ltd) at the beginning of this study (unpublished data). Only those that gave promising results in the preliminary studies were evaluated further. In these preliminary studies, garlic and *C. repens* were most promising and were therefore included in further screening.

6.3.1. Fruit

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

6.3.2. Pathogen

For the source and identity of pathogens used in this study, refer to section 3.3.1 (Chapter Three).

6.3.3. Preparation of Extracts

6.3.3.1. Garlic

Preliminary *in vitro* trials in microtiter plate (result not presented) showed that a higher quantity of powder was required compared to fresh samples to achieve the same level of pathogen inhibition. Only extracts obtained from fresh samples were therefore evaluated further. Similarly, no significant difference in activity was observed between two garlic cultivars (Nootka Rose and Rose du Var). Only Rose du Var was evaluated further.

Preparation of extracts from fresh cloves

Samples were surface disinfested for two minutes in 70% ethanol and washed in three changes of sterile distilled water. One, 3, 5, 7 and 10g sample were separately pulverized in sterile porcelain mortar with a pestle. The resulting pulp was suspended in half the required quantity of solvent (50ml water or 20% ethanol) in 250ml Erlenmeyer flask to form a paste. The paste was agitated for one minute and filtered through sterile cotton wool into a 100ml Erlenmeyer flask and the volume of filtrate made up to 50ml with sterile distilled water or 20% ethanol respectively.

6.3.3.2. *Coprosma repens*

Preparation of extracts from fresh leaves

Leaf samples were washed under tap water for two minutes. Different quantities of samples were separately crushed in solvent (water or 20% ethanol) using a small porcelain mortar and pestle to obtain different concentration of extracts, which was sieved through two layers of cheesecloth to remove uncrushed leaf particles. Samples were sterilized using a 0.22µm pore size syringe driven filter (Millipore). Extracts were either used immediately or kept at 7° C for future use.

Preparation of extracts from dry samples

Leaf samples were washed in tap water for two minutes and blotted dry with tissue paper. Samples were further dried in an oven at 40±1° C for seven days. Dry samples were ground into powder using a small porcelain mortar and pestle. Different quantities (5 000, 10 000 and 20 000 ppm) of powder were suspended in either water or 20% ethanol to obtain different concentrations of extracts, which were sterilized and treated as described in 6.3.3.2.

6.3.4. *In vitro* Screening

6.3.4.1. Characterization of *Coprosma repens* Extracts

The Thin layer chromatography (TLC) and High Performance Liquid Chromatography (HPLC) studies reported below were conducted as preliminary trials to basically identify the family of compounds possibly responsible for the activity of *C. repens* extracts. Detailed analysis to identify the exact compound responsible for extract activity will not be undertaken in this study.

6.3.4.1.1. Chemical analysis of *Coprosma repens* extracts

Determination of total soluble phenolics- (Folin-Ciocalteu's Reaction)

One hundred and seventy microlitres of distilled water was dispensed separately in microtiter plate wells. Next, five microlitre of extract prepared as described in (6.3.4.2) was added to each well, followed by 50 µl of 20% (w/v) sodium carbonate solution. Lastly, 25 µl of Folin and Ciocalteu's Phenol Reagent (Sigma) was added as a colorimetric

indicator to each well. A blank, consisting of identical composition but replacing the sample with water, served as a control. The mixture was properly mixed with a pipette, and the plate incubated at 40° C for 30 minutes. Five wells were used per treatment and the experiment was repeated once. Absorbance was read at 690nm with a Multiskan Ascent V1.24 354-00973 (Version 1.3.1). Data was calculated as equivalent ferulic acid in mg ml^{-1} extract from the standard curve using an equation: $y = 0.9886x + 0.0108$ ($R^2 = 0.997$).

Extraction of free acids

Tetrafluoacetic acid (TFA) 20% was added to extracts prepared as described previously (6.3.4.2) at the rate of 25µl per 1.25 ml extract in an Eppendorf tube in order to obtain a pH of 2.6. An equal volume of diethylether was added and the mixture shaken and allowed to stand briefly to allow separation of fractions, after which the upper phase, was removed with a pipette and placed in a new Eppendorf tube. This procedure was repeated four times. The separated upper phase layers were combined together and diethylether evaporated. One hundred and fifty microlitres of methanol was added and the extracts stored at 5° C until further use.

Extraction of esters

Ammonium sulphate was added to the remaining part of the aqueous phase (6.3.5.1.2) at the rate of 2% w/v (i.e. 0.2 g/1.25 ml extract) and the same volume of ethylacetate was added. The mixture was shaken and the supernatant removed as described above (6.3.5.1.2). The procedure was repeated four times. At the fourth time, 100 µl of methanol was added before the supernant was removed. The supernatants were bulked together and evaporated until dry, after which 150 µl of methanol was added. Extracts were stored at 5° C until further use.

Thin Layer Chromatography

Thin layer chromatography is the simplest and cheapest method of detecting plant constituents. It was therefore used to determine the chemical constituent of *C. repens* in this study. A preliminary trial was conducted to determine the best solvent for use. The solvent systems tested included Hexane/Acetic acid (5:95), Toluene/Acetic acid (4:1), Benzene/Acetic acid/Water (6:7:5) and water alone. Toluene/Acetic acid gave the best separation and was used in subsequent trials. Also both aluminium pre-coated TLC plates

(SIL G-100 UV $_{254}$) Macherey-Nagel and pre-coated glass plates (Silica Gel 60 F-254) Merck were tested to determine which resulted in the best separation. Aluminium was found to be better than glass and was therefore used in subsequent trials. Extracts prepared as described earlier (6.3.3.2) was analyzed on aluminium TLC silica gel plates (10 x10 cm) using the Toluene/ Acetic acid (4:1) solvent system. All assays were run in duplicate. Spots and bands were visualized with a CAMAT 50Hz UV lamp (254 and 366nm). The visibility of compounds on plates was amplified where necessary by flooding developed plates with liquid nitrogen. Three fluorescent bands (1, 2, and 3, Fig. 6.2B) were visible. For identification purpose, band 1 (RF 0.32) was eluted from the TLC plate with methanol (50 µl) for 20 hours in an Eppendorf tube. The mixture was centrifuged for one minute in a microcentrifuge (Helttich Micro Rapid/K) at 7500 x g. The resultant supernant was collected in a new Eppendorf tube and analyzed by HPLC to identify the class of compound present. The second set of TLC plate was used for bioautographic tests.

Bioautography

Chromatogram developed as described above was sprayed with a fungal spore suspension prepared in a nutrient base medium which composed of glucose (30%), (Solution A) with KH_2P0_4 (7g L^{-1}), Na_2HP0^4 (3g L^{-1}), KNO_3 (4g L^{-1}), $MgSO_4.7H_20$ (1g L^{-1}) and NaCl (1g L^{-1}) (Solution B). Solution A and B were mixed in a ratio of 1:6. *Penicillium digitatum*, *P. italicum* and *G. citricarpa* were tested, at a concentration of 10^6 spores ml^{-1}. The TLC plate was incubated for between 48 and 96 hours at 25° C in humidity chambers. Plates were monitored afterwards for germination of pathogen spores. Inhibition zones indicated the presence of active compounds. Where no visible growth was recorded, plates were sprayed with tetrazolium salt (8%). A red colouration indicated respiratory activity taking place indicating that pathogen spores germinated and therefore no active compound was present. An inhibition zone on the other hand, indicated the presence of active compounds.

High Performance Liquid Chromatography (HPLC)

Ten microlitres of *C. repens* extracts prepared as described earlier (6.3.4.2) was injected into the HPLC machine. For qualitative analysis, a gradient elution schedule consisted of an initial one minute run of 10% acetonitrile in ultra distilled water followed by a linear gradient to 50% acetonitrile over 15 minutes at a flow rate of 2 ml per minute. The chromatographic system consisted of Varian 9012 high-pressure pumps (3 phases), a manual injector, an integrated system controller, a MALsil C18 5 micron reverse-phase

analytical column (250 x 4.6 mm, 5µm particle size), and a System Spectra 6000 LP UV diode array detector with an attached analysis computer and data storage system (OS/2 WARP, Thermo Separation Products). Ferrulic and p-coumaric acids were also injected for comparative purposes.

6.3.5. Effect of Extracts on Pathogen Growth *In vitro*

6.3.5.1. Garlic

Two hundred and fifty microlitres of different concentrations (1 000, 3 000, 5 000, 7 000, and 10 000 ppm) of extracts prepared as described earlier (6.3.3.1) was dispensed in microtiter plate wells and inoculated with either *P. digitatum*, *P. italicum*, or *G. citricarpa* spore (1 x 10^6 spore ml^{-1}). The plate was incubated at 25° C for 1, 6 and 12 hours. At each time interval, 50µl extract-spore suspension was pipetted onto 90-mm Petri-plate containing 25ml aliquot of potato dextrose agar (PDA) (Biolab). Inoculated plates were incubated at 25° C for 10 days in the case of *P. digitatum* and *P. italicum*, and 14 days for *G. citricarpa*. Pathogen growth was determined as described earlier (3.3.2.3). Plates inoculated with spores suspended in water served as control. Five plates were used for every concentration tested. The experiment was repeated twice. To determine the effect of treatment on spore development, 100 randomly selected spores were viewed under an inverted microscope (Nikon-TMS, Japan) at 40x magnification and the percentage of spores germinated following 72 hours of incubation at 25° C, recorded.

6.3.5.2. *Coprosma repens*

Extracts prepared as described previously (6.3.4.2) were evaluated for their antifungal activity as described in 6.3.6.1.1. Percentage spore germination was also determined as described in 6.3.6.1.1.

6.3.6. *In vivo* Screening

6.3.6.1. Effect of Extracts on Disease Control

6.3.6.1.1. Garlic

Treatments included; extracts alone, extracts combined with sunflower cooking oil (Black Cat), extracts combined with fruit wax (Polyorange at 0.1% vol/vol), oil alone and wax alone. The choice of "Black Cat" was based on results of a preliminary *in vitro* trial (data

not presented) to test the effects of different sources of vegetable oil on germination of *P. digitatum* spores. Although all oils were effective in inhibiting spore germination, "Black Cat" was cheaper and more readily available. *Guignardia citricarpa* was not included as a treatment because of logistic problems. *Penicillium digitatum*, and *P. italicum* inoculated fruits were sprayed about six hours after inoculation with one of five treatments prepared as described earlier using a spraying bottle (Efekto). The control consisted of fruits sprayed with sterile distilled water or commercial fungicides (see section 4.3.6). Treated fruits were stored in cardboard boxes at 9 °C, and 90 to 95% RH for four weeks, and assessed for decay symptoms as described previously (4.3.6).

Effect of treatment on pathogen development on fruit

To determine the effect of treatments on the development of *Penicillium,* peel pieces were taken from the inoculation point at 36-48 hours after inoculation with a no.1 cork borer. Six peel pieces; namely two per fruit were taken and bulked. The peel macerate prepared in 10 ml sterile distilled water was sieved through two layers of sterile cheesecloth. One hundred spores were observed under the inverted microscope (Nikon TMS) for germination

6.3.6.1.2. *Coprosma repens*

Unlike garlic, *C. repens* was evaluated as a dip treatment. This is because the plant is readily available and large volumes of extracts could easily be prepared. Fruits artificially inoculated with *Penicillium* as described in 4.3.6 were immersed for one to two minutes in different concentrations of extracts prepared as described previously (6.3.4.2). Treated fruits were dried and then waxed. Storage and disease assessment was done as described in 6.3.7.1.1 and 4.3.6 respectively. In the case of *G. citricarpa*, old lesions on naturally infected fruits were marked as described earlier (4.3.9) and fruits sprayed as described above. Efficacy of treatment was based on the number of new black spot lesions appearing following treatment. Thirty fruits were used per replicate and each treatment was replicated three times. Treated fruits were stored at 9 °C for three weeks, and 23±1° C for one week. The experiment was repeated twice.

Effect of storage duration and storage temperature on efficacy of *Coprosma repens* extracts

Extracts prepared as described in 6.3.4.2 were stored at 23±1° C and 9 °C for 30, 60, and 90 days. Extracts were evaluated at each time interval (i.e. 30, 60 and 90 days) as described earlier (6.3.7.1.2) for its activity against *P. digitatum*, *P, italicum*, and *G. citricarpa*.

6.3.7. Statistical analysis

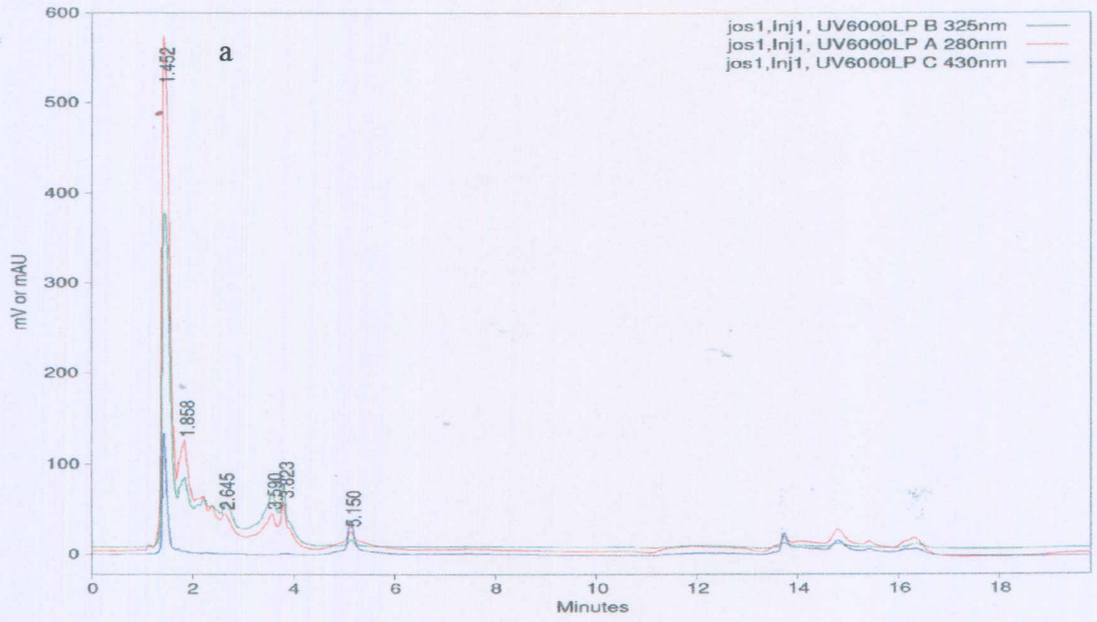
Data was statistically analysed using the GenStat statistical program. Two-way analysis of variance (ANOVA) was used to test for differences in average means between treatments. Treatment means were separated using Fishers' protected t-test at a 5% level of significance.

6.4. Results

6.4.1. *In vitro* Screening

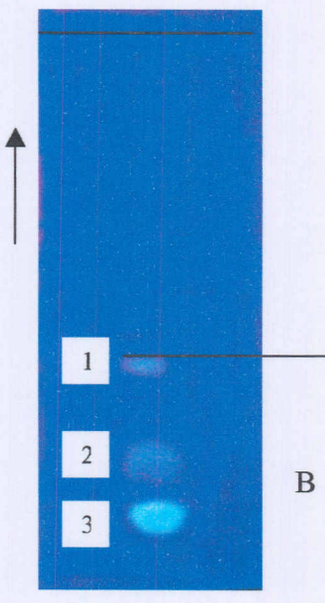
6.4.1.1. Characterization of *Coprosma repens* Extracts

Results of the chemical analysis of extract constituents and the subsequent anti-microbial activity are summarized in Figs. 6.1-6.3. The HPLC run of crude extract indicated one major peak (a0 and five minor peaks in the chromatogram (Fig.6.1A). The extract chromatograph on silica gel using Toluene/ Acetic acid (4:1) as solvent, showed two prominent blue fluorescent spots at 350 nm wavelength (spot 1 and 2, Fig. 6.1B), with Rf values of 0.32 and 0.11 respectively. This solvent only allows the migration of the free acids. The third spot indicated as 3 in Fig 6.1B represents the loading spot of the crude extract and showed a milk-coloured fluorescent. When spot 1 (Rf 0.32) was extracted in methanol and further characterized by HPLC, results obtained (Fig 6.1C) indicated one peak in the chromatogram which corresponded to the peak "a" in Fig. 6.1A. The spectrum (Fig.6.2) and the fluorescence and Rf value of the band 1 and therefore peak "a" are similar to those obtained for the hydroxycinamic acids and so confirm the allocation of the compound (peak A) to this family. Results obtained from the bioautographic test indicated that free acid extracts were effective in inhibiting spore germination at all concentration tested (Fig.6.3). Spot 2 also has a similar Rf to those recorded for p-coumaric acid and is

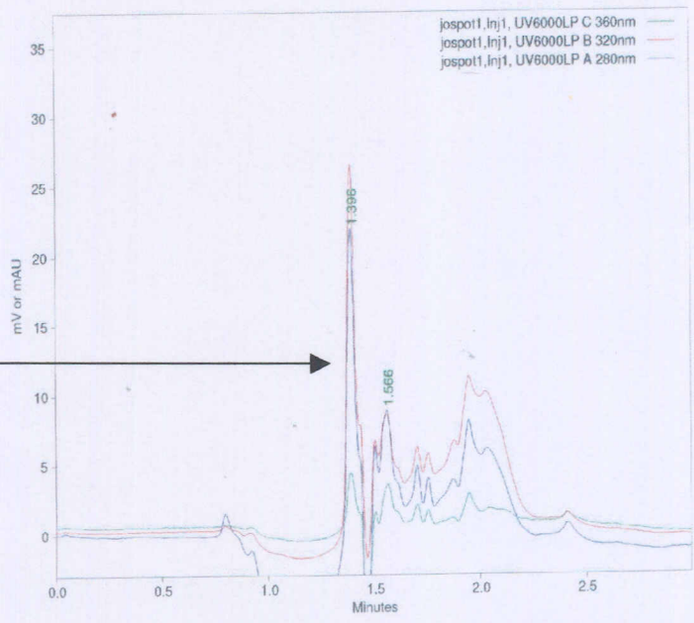


A

Toluene/AA



B



C

(A) HPLC chromatographic run of crude extract (B) TLC chromatographic run of crude extract (C) HPLC chromatographic run of fraction 1 ran at 320nm (see arrow). 1 and 2 represent different fractions (bands), from extract, fraction 3 represent injection point of crude extract.

Fig. 6.1 Chemical screening of *Coprosma repens* leaf extracts using High Performance Liquid Chromatography and Thin Layer Chromatography

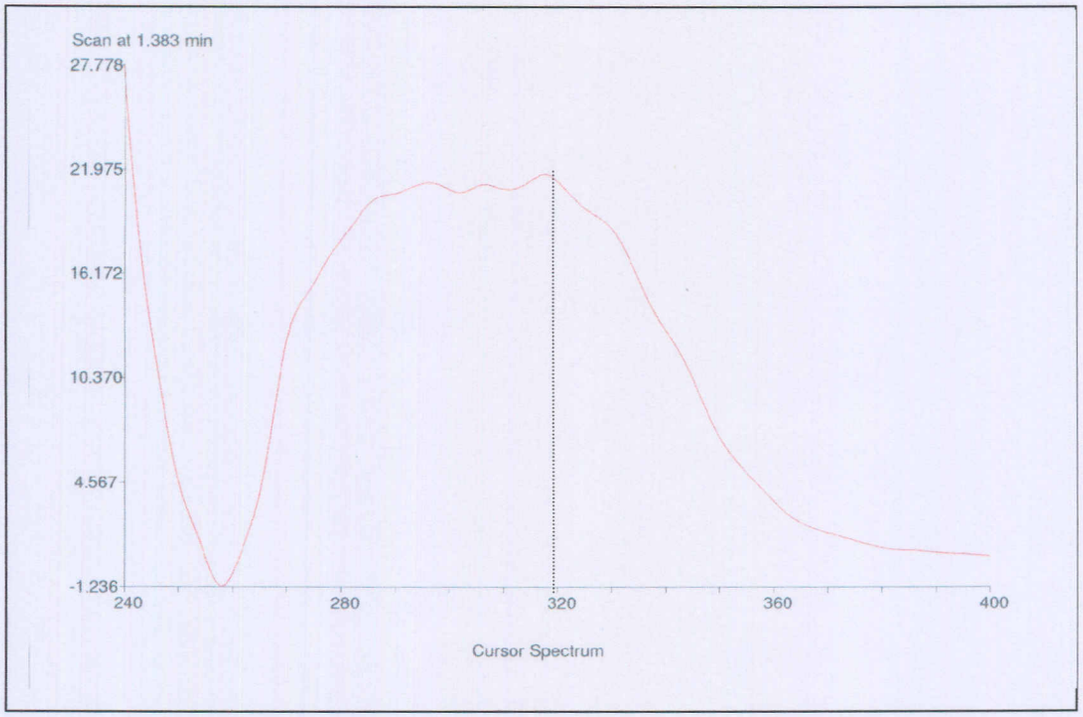
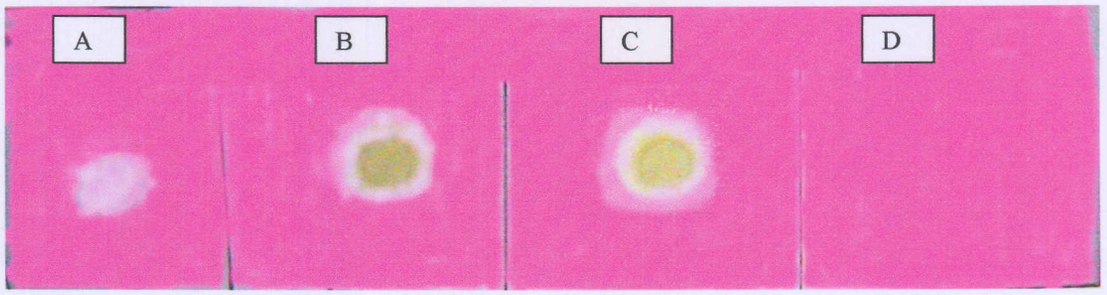


Fig. 6.2 Chromatographic spectrum of "band 1" corresponding to compound "A" (see Fig. 6.2A & B).



A, B, C, and D represent 25, 50, 100 and 0% (control) concentration of free acids White zones indicate inhibitory activity of compound.

Fig. 6.3 TLC bioautographic assay showing effects of free acids extracted from *Coprosma repens* leaf on germination of *Penicillium digitatum* spores after 72 hours of incubation at 25° C.

possibly a compound from the hydroxycinamics. The pool of soluble esters was not effective and results are therefore not presented.

6.4.1.2. Effect of Extracts on Pathogen Growth *In vitro*

6.4.1.2.1. Garlic

Garlic clove extracts had a significant effect on the growth of all three pathogens screened. The concentration of extracts had a significant effect on its efficacy, and all concentrations were effective when compared with the check in the control of pathogen growth. Results presented in Table 6.1 shows a proportional decline in fungal colony diameter with increasing extract concentration and duration of spore suspension. Extracts were more effective in the control of *G. citricarpa* than *P. digitatum* and *P. italicum*. As a result, all concentrations above 1, 000 ppm completely inhibited the growth of the pathogen *in vitro*. Extracts gave better control of *P. italicum* compared to *P. digitatum*. When spores were inoculated on PDA within one hour of suspension in extracts, none of the concentrations completely inhibited the growth of both *P. digitatum* and *P. italicum*. After three hours of suspension, however, complete inhibition of growth was observed for both pathogens at 7 000 and 10 000 ppm respectively.

Extracts had a negative effect on spore germination. Results presented in Table 6.2 show a progressive decline in percentage spores germinated with increasing concentration of extracts. No visible growth of both *P. digitatum* and *P. italicum* was observed in treatments comprising 7 000 ppm and above, and in all concentrations in the case of *G. citricarpa*. Results presented in Table 6.2 however, indicated that some spores did germinate at these concentrations.

6.4.1.2.2. *Coprosma repens*

Data presented in Table 6.3 shows that the *C. repens* extract contains a valuable amount of phenolic compounds. It was observed that over 80% of phenolic compounds were extracted when the suspension was allowed to stand for six hours or more.

Table 6.1 Effects of aqueous garlic extracts on the growth of *Penicillium digitatum, Penicillium italicum*, and *Guignardia citricarpa* on potato dextrose agar incubated for eight days at 25±1° C.

Concentration of garlic extracts ('000 ppm)	Time of spores suspension in extract (h)/Fungal colony diameter (mm) *					
	1 h			3 h		
	Pd [x]	*Pi* [y]	Gc [z]	*Pd* [x]	*Pi* [y]	Gc [z]
0	80.1 a	69.5 a	15. 0 a	77.2 a	51.1 a	16.5 a
1	61.1 b	52.2 b	0.0 b	42.2 b	31.3 b	0.0 b
3	53.0 c	49.4 c	0.0 b	30.1 c	22.1 c	0.0 b
5	35.3 d	34.7 d	0.0 b	10.0 d	5.2 d	0.0 b
7	30.9 d	30.1 d	0.0 b	0.0 e	0.0 e	0.0 b
10	24.4 e	16.5 e	0.0 b	0.0 e	0.0 e	0.0 b

[x] represent *P. digitatum*; [y] represent *P. italicum*; [z] represent *G. citricarpa*

* Mean of five replicates. Means having the same letter in the same column are not significantly different according to Fishers' protected t-test (P = 0.05).

Table 6.2 Effects of aqueous extracts of garlic and *Coprosma repens* on germination of *Penicillium digitatum, Penicillium italicum*, and *Guignardia citricarpa* spores.

Treatment	Pathogen/Percentage spore germination [a]		
	Pd[x]	Pi[y]	*Gc*[z]
1 000 ppm garlic	43	38	6
3 000 ppm garlic	20	30	0
5 000 ppm garlic	12	30	0
7 000 ppm garlic	10	10	0
10 000 ppm garlic	0	10	0
2 5 00 ppm *C. repens*	30	21	8
5 000 ppm *C. repens*	5	20	0
Water control	68	48	25

[x] represent. *P. digitatum*; [y] represent *P. italicum*; [z] represent *G. citricarpa*

[a] Mean of five replicates

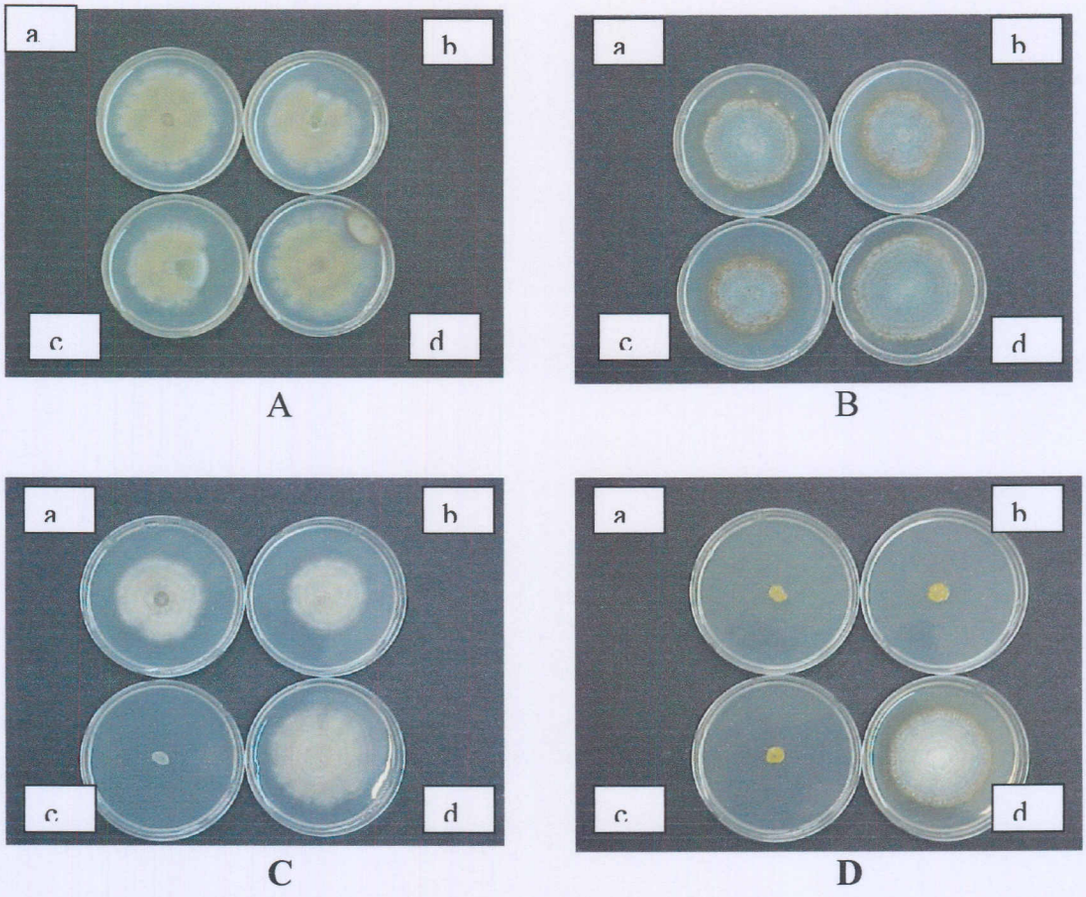


Fig. 6.2 Growth of *Penicillium digitatum* (A and C) and *Penicillium italicum* (B and D) on potato dextrose agar after spore suspension in 3 000 (a), 5 000 (b), 7 000 (c), and 10 000 ppm (d) aqueous garlic extracts for one hour (A and B), and three hours (C and D), and 10 days of incubation at 25° C.

Table 6.3 Quantity of soluble phenolics extracted from *Coprosma repens* leaf powder at each extraction using 20% ethanol as solvent.

Number of extractions	Total soluble phenolics (equivalent ferulic acid mg ml^{-1})	Standard error
1	2.855	0.384
2	2.115	0.304
3	0.775	0.244
4	0.446	0.076

Coprosma repens extracts have great potential for control of all three pathogens screened in the present study. From the results presented on Table 6.4 it is clear that extracts were effective when compared with the water check in inhibiting pathogen growth. A progressive decline in fungal colony diameter was recorded with increasing extract concentration. Extracts were more effective in the control of *G. citricarpa* compared to *P. digitatum* and *P. italicum*. When assayed against G. *citricarpa*, complete inhibition of growth was observed at concentrations above 5 000 ppm, unlike *P. digitatum* and *P. italicum* where none of the concentration tested completely inhibited pathogen growth. The growth of both pathogens at 20 000 and 10 000 ppm did not differ significantly.

6.4.2. *In* vivo Screening

6.4.2.1. Effects of Extracts on Disease Control

6.4.2.1.1. Garlic

All concentrations of extracts were effective compared with the water check in controlling the incidence of both green- and blue mold. They were however, less effective than the fungicide treatment, which gave complete control of both diseases (Table 6.5). The results also show that extracts were more effective on Valencia than Shamouti. A significant increase in the biological activity of extracts was observed when treatments were combined with oil. Consequently, the treatments comprising 1 000, 3 000, and 5 000 ppm combined

Table 6.4 Effect of ethanol extracts of *Coprosma repens* on the growth of *Penicillium digitatum* and *Penicillium italicum on* potato dextrose agar after eight days of incubation at 25±1° C or 14 days in the case of *Guignardia citricarpa*.

Treatment	Fungal colony diameter (mm) *		
	Pd [x]	Pi [y]	Gc [z]
CP 20 000 ppm	5 a	10 a	0 a
CP 10 000 ppm	6 a	10 a	0 a
CP 5 000 ppm	10 b	20 b	8 b
20% Ethanol	21 c	28 c	21 c
Water control	57 d	69 d	30 d

[x] represent *P. digitatum*'; [y] represent *P. italicum*; [z] represent *G. citricarpa*.

* Mean of five replicates. Means having the same letters in the same column are not significantly different according to Fishers' protected t-test (P = 0.05).

with oil (0.1%), for example were as effective (100% control), as fungicide in the control of green mold. The increased activity was more obvious on Valencia and the same level of control was not achieved when extracts were combined with wax. A comparatively higher level of disease incidence was recorded on treatments involving ethanol extracts relative to water extracts (Table 6.5).

Results presented in Table 6.6 shows that treatments were more effective in controlling blue than green mold. As a result, the treatments comprising 3 000 and 5 000 ppm plus oil were as effective as the fungicide treatment which gave complete control of blue mold on both Valencia and Shamouti. This was in contrast to green mold where complete control with the above treatments was achieved only on Valencia.

Table 6.5 Effects of garlic extracts on their own, or in combination with vegetable oil, or fruit wax, on control of citrus green mold on artificially inoculated fruits stored at 9° C and 90 to 95% relative humidity for four weeks.

Treatment	Percentage disease control *			
	Valencia		Shamouti	
	WE [x]	EE [x]	WE [x]	EE [x]
1 000 ppm garlic	83 b	50 d	70 c	44 c
3 000 ppm garlic	83 b	50 d	60 d	44 c
5 000 ppm garlic	83 b	65 c	75 c	26 e
1 000 ppm garlic + oil	100 a	93 a	85 b	44 c
3 000 ppm garlic + oil	100 a	85 b	60 d	50 c
5 000 ppm garlic + oil	100 a	65 c	50 d	39 d
Oil alone (0.1% v/v)	67 c	65 c	23 e	22 e
1 000 ppm garlic + wax	67 c	61 c	65 c	60 b
3 000 ppm garlic + wax	55 d	56 d	55 d	58 b
5 000 ppm garlic + wax	83 b	80 b	60 d	57 b
Wax alone	55 d	55 d	60 d	62 b
20% ethanol	-	25 e	-	37 d
Water control	17 e	-	15 e	-
Fungicide [y]	100 a	-	100 a	-

[x] WE represent water extracts; EE represent ethanol extracts; [y] Fungicide represent Fungazil (imazalil 75% a.i. at 1g L^{-1}) plus Decotine (quazatine 20% a.i. at 1 ml L^{-1}).

* Mean of three replicates. Means followed by the same letter in the same column are not significantly different according to Fishers' protected t-test (P = 0.05).

Table 6.6 Effects of garlic extracts on their own, or in combination with vegetable oil, or fruit wax, on control of citrus blue mold on artificially inoculated fruits stored at 9° C and 90 to 95% relative humidity for four weeks.

Treatment	Percentage disease control *			
	Valencia		Shamouti	
	WE [x]	EE [x]	WE [x]	EE [x]
1 000 ppm garlic	80 c	72 c	70 c	76 c
3 000 ppm garlic	91 b	87b	84 b	81 b
5 000 ppm garlic	92 b	83 b	80 b	82 b
1 000 ppm garlic + oil	91 b	77 c	89 b	80 b
3 000 ppm garlic + oil	100 a	85 b	100 a	82 b
5 000 ppm garlic + oil	100 a	85 b	100 a	80 b
Oil alone	52 e	46 e	44 e	52 e
1 000 ppm garlic + wax	71 d	65 d	69 c	60 d
3 000 ppm garlic + wax	88 b	82 b	88 b	70 c
5 000 ppm garlic + wax	92 b	84 b	85 b	81 b
Wax alone	50 e	47 e	52 d	31 f
20% ethanol	-	42 e	-	37 f
Water control	31 e	-	27 f	-
Fungicide	100 a	-	100 a	-

[x] WE represent water extracts; EE represent ethanol extracts; [y] Fungicide represent Fungazil (imazalil 75% a.i. at 1g L^{-1}) plus Decotine (quazatine 20% a.i. at 1 ml L^{-1}

* Mean of three replicates. Means followed by the same letter in the same column are not significantly different according to Fishers' protected t-test (P = 0.05).

Effect of treatment on pathogen development on fruit

Results obtained were similar to the *in vitro* trials and data is therefore not presented. Pathogen growth on plates seeded with spores harvested from treated fruits was much slower compared to the control i.e. fruits treated with only water. Growth rate was not significantly dependent on concentration of extract, and there was no significant difference between spores harvested after 6 and 12 hours of treatment. There was also no difference in growth between spores harvested from Valencia or Shamouti. None of the treatments completely inhibited pathogen growth after six days of incubation at 25°C. The growth of spores harvested from treatment combinations with oil was however, much smaller (not more than 7 mm), and unlike the control, no sporulation of the fungus was observed on these treatments.

6.4.2.1.2. *Coprosma repens*

All concentrations of *C. repens* were effective when compared with the water check in the control of all three pathogens screened. Extracts were more effective in controlling green mold than blue mold. The percentage disease control varied between 80 to 82%, and 64 to 71% in terms of green- and blue mold respectively (Table 6.7). Unlike the *in vitro* study, results of the *in vivo* evaluation showed no significant difference in the percentage disease control obtained at 5 000, 10 000 and 20 000 ppm.

Effect of storage duration and storage temperature on efficacy of extracts

Both storage temperature and storage time (up to 90 days) had little effect on efficacy of extracts. Results presented in Table 6.8 indicated that a lower incidence of blue mold was recorded at all time intervals and temperatures relative to green mold. No remarkable difference in percentage disease control was recorded at 30, 60, and 90 days following storage at both temperature regimes. The percentage control under cold storage varied between 85 and 64% in *Penicillium* and 91 and 87% for *G. citricarpa.*

Table 6.7 Efficacy of ethanol extracts of *Coprosma repens* for the control of *Penicillium digitatum*, *Penicillium italicum*, and *Guignardia citricarpa* on fruits stored at 9° C and 90 to 95% relative humidity for four weeks (in respect of *P. digitatum* and *P. italicum*), and 7° C for three weeks followed by 23° C for one week (in the case of *G. citricarpa*).

Concentration of extract ('000 ppm)	Percentage disease control *		
	Pd [x]	Pi [y]	Pc [z]
20	82 a	71 a	95 a
10	80 a	67 ab	88 ab
5	80 a	64b	83 b
0 (control)	5 b	10 c	13c

[x] represent *P. digitatum*; [y] represent *P. italicum*; [z] represent *G. citricarpa*

* Mean of three replicates and three repetitions. Means followed by same letter in the same column are not statistically significant according to Fishers' protected t-test (P = 0.05).

Table 6.8 Effects of storage duration and temperature on the *in vivo* efficacy of *Coprosma repens* extracts for control of *Penicillium digitatum*, *Penicillium italicum*, and *Guignardia citricarpa*

Concentration of extract ('000 ppm)	Storage temperature/Percentage disease control *					
	23±1° C			9° C		
	Pd [x]	Pi [y]	Gc [z]	Pd [x]	Pi [y]	Gc [z]
After 30 days						
20	72 a	68 a	-	82 a	71 a	90 a
10	70 a	69 a	-	80 a	67 ab	88 a
5	70 a	67 a	-	80 a	64 b	88 a
0 (control)	1 a	3 b	-	5 b	10 c	10 b
After 60 days						
20	73 a	70 a	-	85 a	75 a	91 a
10	71 a	70 a	-	82 a	74 a	87 a
5	71 a	68 a	-	83 a	71 ab	88 a
0 (control)	3 b	5 b	-	3 b	6 c	15 c
After 90 days						
20	75 a	69 a	-	80 a	77 a	89 a
10	71 ab	70 a	-	78 a	70 a	89 a
5	70 ab	68 a	-	80 a	69 a	88 a
0 (water control)	8 b	5 b	-	8 b	3 b	11 c

[x] represent *P. digitatum*; [y] represent *P. italicum*; [z] represent *G. citricarpa*.

* Mean of three replicates and three repetitions. Means followed by same letter in the same column are not statistically significant according to Fishers' protected t-test (P = 0.05).

6.5. Discussion

The increasing reports of pathogen resistance to many conventional fungicides is partly the result of a continuous and often indiscriminate use of the same compound(s) on the same organisms for long periods of time. Part of the solution to this problem is the use of an integrated approach, using different methods/means of control either together or in sequence (Janisiewicz and Korsten, 2002). It is in the light of this new approach to disease management that the results obtained in this study are useful as an alternative means of control of these three citrus pathogens.

Results obtained in the present study indicated that garlic extracts are effective in the control of *P. digitatum, P. italicum,* and *G. citricarpa*. This finding is in agreement with earlier reports (Russell and Mussa, 1977; Garcia and Garcia, 1990; Obagwu *et al.*, 1997) amongst others, on the antifungal properties of garlic clove extracts. This is, however, the first report where garlic clove extracts were evaluated in combination with vegetable oil and plain fruit wax to assess possible improvement in biological activity. The efficacy of extracts, especially in the inhibition of *P. digitatum* and *P. italicum* was positively related to both extract concentration and duration of spore suspension in extracts. Extracts were more effective in inhibition of G. *citricarpa* than *Penicillium* species. This observation is probably due to the fact that *G. citricarpa* is a much slower growing fungus than the *Penicillium* species. Results obtained in this study indicate that garlic extracts had a negative effect on pathogen spore germination, with percentage inhibition positively related to extract concentration.

No growth of *P. digitatum* or *P. italicum* was recorded on treatments comprising 7 000 ppm garlic and above. Results obtained however, indicate that some spores did germinate at these concentrations. This shows that in addition to inhibiting spore germination, extracts also have negative influences on mycelial development; hence, although some spores did germinate at these concentrations, the residual activity of extracts inhibited further mycelial development and so no visible growth was recorded on media. *In vivo* bioassays indicated that extracts were more effective in inhibiting *P. italicum* than *P. digitatum*. This was contrary to observations made in *in vitro* studies. Both pathogens require similar growth conditions. However, under the same environmental conditions, *P. digitatum* grows faster than *P. italicum* (Carlos, 1982). This slower growth rate under

similar conditions could explain the lower growth and/or disease incidence observed in most instances.

A remarkable improvement in biological activity was observed when garlic extracts were mixed with oil. As a result, the treatment comprising 1 000 ppm combined with oil was as effective as the fungicide treatment in the control of both green- and blue mold on Valencia oranges. Our observation also showed that, unlike spores recovered from fruits treated with extracts alone, less than 14% of spores recovered from fruits treated with extracts mixed with oil germinated. None of these spores was capable of further growth when plated on PDA, thus indicating that the treatment affected both spore germination and further pathogen development. Allicin, the main biologically active component of garlic clove is reported to lose its beneficial properties quickly as it breaks down and evaporates (Anon, htp/www/3mistral.co.uk/garlic/allicin). Addition of oil to extracts could therefore have provided a light coating that helped to slow down the evaporation process, prevent oxidation and increased the retention time of allicin on the fruit and thus the contact period with spores.

Results obtained shows that a more effective inhibition was recorded when spores were suspended in extracts for a longer period i.e. three-hour was better than one. Allicin is soluble in alcohol and only slightly soluble in water (North and Quadrini, 2000). Our study however, showed that ethanol extracts were not as effective as water extracts. Because ethanol is a volatile compound, it could enhance the volatilization and break down of allicin in ethanol extracts faster than would occur in water extracts. Consequently, though the percentage allicin in ethanol extracts may have been higher than in aqueous extracts immediately following extraction, the percentage lost was equally higher and thus the efficacy and subsequent control much lower. Also, the main antimicrobial effect of allicin is due to its chemical reaction (blockage) of the thio groups of various enzymes, including alcohol dehydrogenase (ADH), thioredoxin reductase, and RNA polymerase required for the pathogenesis of many microorganisms (Ankri and Miralman, 1999). A study by Massantini *et al.* (2000) indicated that ethanol greatly stimulated ADH activity. This means that the activity of ADH in garlic extracted in ethanol would be equally stimulated, resulting in an abundance of ADH in the extract. This development may result in the blockage or impairment of allicin activity in ethanol extracts thus resulting in reduced efficacy of allicin. All these are possible factors that could have contributed to the

reduced efficacy of ethanol extracts relative to the water extracts of garlic recorded in this study.

Results obtained in the present study shows that *C. repens* possesses antifungal properties, and has great potential for control of the three pathogens screened in this study. The possible role of phenolic compounds in plant resistance to diseases has been reported (Harborne, 1980; Ahmed and Beg, 2001, Moure *et al.*, 2001). This is however, the first report where extracts from *C. repens* was evaluated for control of these pathogens in the post harvest arena. HPLC results indicate that hydroxycinamic acid derivatives are the main component of the phenolic compound pool. The involvement of the hydroxycinamic derivatives in plant pathogen resistance/disease control has been reported (Lattanzio *et al.*, 1994; Agioni *et al.*, 1998; Ejechi *et al.*, 1999; Uzi *et al.*, 1999). They were reported to have antifungal activity against several plant pathogens including *P. digitatum* (Lattanzio *et al.*, 1994). Since this family of compounds is the most abundant, it is most likely that they are largely responsible for the activity of the extract. The TLC bioautographic results showed that all concentrations of free acid extracts were inhibitory and prevented germination of all three pathogens. Both ferrulic and *p*-coumaric acids which were found to be the main phenolics in the extracts are believed to be important components of the cell wall where they play an important role in cell wall lignification and ultimately plant resistance to pathogens (McDougall, 1993; Regnier, 1994).

From the results obtained, we can deduce that that extracts of *C. repens* were effective in the control of all three pathogens screened, but a more effective control of *G. citricarpa* was achieved relative to *P. digitatum* and *P. italicum*. Unlike the results obtained for *Penicillium* control, efficacy of extracts did not appear to depend on extract concentration and duration of spore suspension in extracts. The compound identified as the active constituent of *C. repens* i.e. phenolics is more stable than allicin and may have contributed to the effective control recorded. Phenolics have been implicated in the activity of many plant extracts (Matheron, 2001), mainly by the inhibition of spore germination and possibly mycelial development.

Perhaps the most interesting observation with *C. repens* extracts in this study was its activity against *G. citricarpa*. Infection by this pathogen takes place at the immature stages of fruit development and post harvest control has thus far proved difficult. The level

of control in this study was high (over 90% for CBS) and consistent. These results are very promising and the use of this extract in pre-harvest applications, might give total control of *G. citricarpa*. Temperature did not seem to affect extract activity. This characteristic is of importance especially if extracts are used under export conditions where fruits are stored under cold storage for extended periods of time. We advocate that further studies be undertaken to concentrate the extracts so that it could be used in smaller quantities, and as a spray treatment, preferably as an emulsion to enhance the retention of the active compound on the fruit surface. Before the use of these extracts can be recommended however, it has to be registered, in which case it has to pass toxicology tests and other requirements that accompany product registration.

A detailed cost-benefit analysis was not carried out in this study to determine the economic benefits of garlic-oil treatments. Results obtained however showED that over 500 fruits can be treated with a litre of garlic-oil mixture. The average cost of 400g fresh garlic in South Africa at the time of this report was less than 1 US dollar and the cost of 750 ml vegetable oil about 70 cents. This treatment should therefore be economical. Garlic is edible and has not been reported to have any harmful effects. The preparation of extracts is also simple and straightforward. *Coprosma repens* is abundant and the preparation of the extracts is practicable. These technologies should therefore be easy to adopt. Some of the suggestions made with respect to *C.* repens however need further studies. Also, the possibility of using pure allicin rather than aqueous extracts should be exploited. The identification of an acceptable fragrance capable of suppressing the so-called "bad smell" of garlic without interfering with its biological activity needs further research. Observation from the present investigation showed that this smell reduces with time, probably as the allicin concentration drops.

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.

Ahmad, I. and Beg, A. 2001. Antimicrobial and photochemical studies on 45 Indian medicinal plants against multi-drug resistant human pathogens. Journal of Ethnopharmacology 7: 113-123.

Amadioha, A.C. 1999. Evaluation of some plant extracts against *Colletotrichum lindemuthianum* in cowpea. Archives of Phytopathology and Plant Protection 32: 141-149.

Ankri, S, and Miralman, D. 1999. Antifungal properties of allicin from garlic. Miros Infection 1: 125-129.

Ark, P.A. and Thompson, J.P. 1959. Control of certain diseases of plants with antibiotics from garlic (A*llium sativum* L.). Plant Disease Reporter 43: 276.

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

Ejechi, B.O., Nwafor, O.E. and Okoko, F.J. 1999. Growth inhibition of tomato rot fungi by phenolic acids and essential oils extracts of pepperfruit. Food Research International 32: 395-399.

Gabe Mirkin, M.D. 2001. Allicin in garlic. htp/www/3mistral.co.uk/garlic/allicin

Garcia, R.P. and Garcia, M.I. 1990. Laboratory evaluation of plant extracts for the control of *Aspergillus* growth and aflatoxin production. Proceeding of the Japanese Association of Mycotoxicology. In: Horticultural Abstracts 60: 65-43.

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

Harborne, J.B. 1980. Plant phenolics, in: Encyclopaedia of plant Physiology. New series. Bell, F.A. and Chartwood, B.V. (Eds) Vol. 8: pp 329-402. Springer Verlag.

Hostettmann, K. 1997. Strategy For the biological and chemical evaluation of plant extracts. Invited lecture presented at the International Conference on Biodiversity and Resources: Conservation and Utilization, 22-27 November, 1997, Phuket, Thailand.

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

Lattanzio, V., De-Cicco, V., Di-Venere, D., Lima, G. and Salerno, M. 1994. Actifungal activity of phenolics against fungi commonly encountered during storage. Italian Journal of Food Science 6: 23-30.

Massantini, R., Gobattoni, E., Botondi, R. and Mencarelli, F. 2000. Role of alcohol dehydrogenase on ripening of ethanol-treated tomato and kiwifruit. Acta Horticulturae 379: 297-304.

Matheron, M. 2001. Modes of action for plant disease management chemistry. Paper presented at the 11th Annual Desert Vegetable Crop Workshop, December 6 2001, Yuma AZ.

McDougall, 1993. Phenolic cross-links in growth and development of plants. Pages 119-136 in: Scalbent, A (Ed). Polyphenolic phenomena. INNA Eds,, Paris.

Moure, A., Cruz, J.M., Franco, D., Dominguez, J.M., Sineiro, J., Dominguez, H., Nunez, M.J. and Jarajo, J.C. 2001. Natural antioxidants from residual sources. Food Chemistry 72: 145-171.

North, C. and Quadrini, F. 2000. Allicin-the smell of health. htpwww.chem.ok.ac.uk/moom/allicin.htm

Obagwu, J., Emechebe, A.M. and Adeoti, A.A. 1997. Effect of extracts of garlic (*Allium sativum* L.) bulb and neem (*Azadirachta indica* Juss) seed on the mycelial growth and sporulation of *Colletotrichum capsici*. Journal of Agricultural Technology 5: 51-55.

Pordesimo, A.N. and Ilag, I.I. 1976. Toxicity of garlic juice to plant pathogenic organisms. Philippines Journal of Biology 5: 251-258.

Regnier, T. 1994. Les composes phenoliques blé dur: variations au cours du developpement et de la maturation du grain, relations avec e' apparition du la moucheture. Ph.D. Thesis, Universite' Montpellier II, France.

Russell, P.E. and Mussa, A.E. 1977. The use of garlic to control foot rot of *Phaseolus vulgaris* caused by *Fusarium solani f.sp phaseoli.* Annals of Applied Biology 86: 369-372.

Shashikanth, K.N., Basappa, S.C. and Murthy, V.S. 1981. Studies on antimicrobial and stimulatory factors of garlic. Journal of Food Science and Technology 18: 147-148.

Sinha, P. and Saxena, S.K. 1999. Inhibition of fruit rot fungus and fruit fly by leaf extracts of onion and garlic. Indian Journal of Agricultural Sciences 69: 651-653.

Uzi, A., Jameta, O., Shmuel, C., Victor, R. and Moshe, B.J. 1999. Umbelliferone, a phytoalexin associated with resistance of immature Marsh grapefruit to *Penicillium digitatum*. Phytochemistry 50: 1129-1132.

CHAPTER SEVEN

CONTROLLING THE ESTABLISHMENT OF FOOD-BORNE PATHOGENS WITH *COPROSMA REPENS* EXTRACTS

7.1. Abstract

Ethanol extracts of *Coprosma repens* were evaluated *in vitro* for their biological activity against four food-borne bacterial pathogens; *Salmonella typhimurium*, *Staphylococcus aureus*, *Escherichia coli* 0157-H7, and *Vibrio cholerae*, and *in vivo* against the establishment of these pathogens on orange fruit surfaces. Extracts were evaluated both as a protective and curative treatment. All concentrations of extracts were effective in checking the establishment of the pathogens both *in vitro* and *in vivo* when compared with the control. Efficacy of extracts was dependent on concentration and varied between pathogens. Extracts were more effective when used as a protective treatment, and gave more effective control of *S. typhimurium* and *V. cholerae* than *S. aureus* and *E. coli*. All concentrations above 5000 ppm completely stopped the establishment of *S. typhimurium* and *V. cholerae* both *in vitro* and *in vivo*.

7.2. Introduction

Reported outbreaks of food-borne illnesses involving fruits and vegetables have increased during the last decade (Drapeau and Solomon, 1998; Schlundt, 2002; Tauxe, 2002). A broad spectrum of microbial pathogens can contaminate human food and water supplies, and cause illness after the organism or their toxins are consumed (Tauxe, 2002). The primary cause of food-borne illnesses include mainly bacteria such as *Escherichia coli* 0157-H7, *Salmonella typhimurium*, *Staphylococcus aureus*, and *Vibrio cholerae,* and other organisms which includes *Clostridium*, *Cyclospora* and *Cryptosporidia* (Evers, 1998). Raw fruits and vegetables grow in a natural environment and therefore carry a wide variety of both beneficial and harmful microorganisms (Evers, 1998). In the case of food poisoning due to microorganisms, the food may serve either as an active vehicle in which multiplication occurs, or as a passive vehicle in which no growth takes place (Varman and Evans, 1991). Accurate records of food-borne illnesses are difficult to come by. In the

United States of America, it has been estimated that 76 million cases of food-borne diseases may occur each year resulting in 325, 000 hospitalizations (Schlundt, 2002). The problem is more severe in developing countries (Kaferstein and Abdussalam, 1999).

Since 1977, new or newly characterized food-borne pathogens have been recognized at the rate of approximately one every two years (Tauxe, 2002). This includes microbial contaminants on uncooked fruits and vegetables, and the identification of microorganisms not previously known to be food-borne pathogens. There is also an increase in the emergence of bacterial strains that are resistant to antibiotics (Conko, 1998). This development has been attributed partly to the widespread use of antimicrobials in hospitals and the community (Schlundt, 2002).

The increasing reports of food-borne illnesses originating from the consumption of fruits and vegetables have resulted in a strict legislation being placed on import of fresh fruits and vegetables into most European countries and the United States. This legislation requires that foods imported into these countries be certified within a food safety framework using systems such as Good Agricultural Practice (GAP) and Hazard Analysis Critical Control Point (HACCP). Another area where more restrictive legislation has been put in place pertains to the maximum residue levels (MRL) allowed on foods including fresh fruits and vegetables. Currently, there are no effective methods available to kill food-borne bacteria on fruits and vegetables. A number of "generally regarded as safe" chemicals have been reported to possess bactericidal activity against food-borne pathogens including *Escherichia coli* 0157:H7, *Listeria monocytogenes*, and *Salmonella enteritidis* for example (Friedman *et al.*, 2002). However, none of these chemicals can singly significantly reduce populations of bacterial pathogens. Food-borne pathogens have been managed mainly with disinfectants such as chlorine. Unfortunately, these products have no residual activity and the possibility of re-contamination in treated consignments destined for export is high. Ideally, any product that will provide effective control of food-borne pathogens should therefore possess some degree of residual properties.

Plants contain thousands of constituents and are a valuable source of new and biologically active molecules. Plant extracts including garlic have been reported to be effective in checking the establishment of food-borne pathogens (Briozzo *et al.*, 1998). However, there is still a lack of knowledge on the potential of other plant extracts in this respect. In a

previous trial (Chapter Six), extracts of *Coprosma repens* Hook F. were effective in controlling *Penicillium digitatum* Sacc., *P. italicum* Wehmer, and *Guignardia citricarpa* Kiely. The aim of this study was therefore to screen *C. repens extracts* for any bactericidal activity against *S. typhimurium*, *S. aureus*, *E. coli* 0157-H7, and *V. cholerae.* This is with a view to using the extracts to provide additional control of these food-borne pathogens on citrus fruit surfaces, in addition to controlling the fungal pathogens listed above. These pathogens were chosen based on their frequency of occurrence and the severity of the illnesses that often result from consuming products contaminated by them.

7.3. Materials and Methods

7.3.1. Food-borne-pathogens

Pathogenic isolates of *E. coli* 015-H7, *S. typhimurium*, *S. aureus* and *V. cholerae* were used in this study. The cultures were received from Wilma du Plooy of the Food Safety Laboratory, Plant Pathology Laboratories, University of Pretoria, and maintained at 7±1° C in the media listed in Appendix 4-7.

7.3.1.1. Preparation of pathogen inoculum

In nature, the population i.e. colony forming units (CFU) of food-borne pathogens on fruits and vegetables vary considerably depending on the type of plant and environmental conditions under which the plant is grown or found (Thunberg *et al.*, 2002). The initial inoculum load i.e. CFU used in this investigation varied between 100 to 150. This concentration is rather high but was chosen to test the efficacy of the extracts under extreme inoculum pressure. To obtain this concentration, a serial dilution was prepared from fresh (24-hour) old cultures of pathogens and plated on *Vibrio* Diagnostic Agar (in the case of *V. cholerae*), Violet-Red-Bile-Mug Agar (in the case of *E. coli*), MacConkey agar (in the case of *S. typhimurium)* and Standard 1 Nutrient Agar (in the case of *S. aureus*). See Appendix 4-7 for composition of media. Colony forming units were counted after 24 hours of incubation at 27±1° C. The dilution yielding a population of between 100-150 CFU was used in further evaluations.

7.3.2. Extracts

The extract used in this investigation was prepared as described previously (Chapter Six, 6.3.3.2).

7.3.3. *In vitro* Screening

Two hundred and fifty microlitres of different concentrations of extracts prepared as described earlier (6.3.3.2) was dispensed separately in microtiter plate (Nunc; AEC-Amersham (Pty) Ltd), wells and inoculated with pathogen cells prepared as described in 7.3.1.1. Sufficient cell suspension was added to give a final concentration of 100-150 CFU ml^{-1}. Such a volume was always predetermined in preliminary trials before the actual inoculation in the microtiter plate. The plate was incubated for between 45 minutes to one hour following which 50 µl of extract-cell suspension was pipetted onto 90-mm Petridishes containing 25 ml aliquots of the different media listed previously depending on the bacteria in question. The suspension was evenly spread using a sterile, plastic Beijerinck rod. Plates were incubated at 27±1° C for 24 hours and the CFU on each plate counted. There were five Petridish/replicates per treatment and each treatment was repeated twice. The control consisted of cell suspended in quarter-strength Ringers solution (Merck). Where growth was observed following treatment, the colonies were compared with the original isolate to confirm their identity. Treatments/concentrations that gave promising results in *in vitro* trials were further evaluated *in vivo* (on fruits).

7.3.4. *In vivo* Screening

The biological activity of extracts was evaluated on Valencia orange. Fresh, healthy fruits were surfaced sterilized with 90% ethanol for one minute and air-dried. Squares, approximately 2 cm^2 were drawn on the fruit surface with a waterproof pen to make a checkerboard pattern. Each square represented a replicate. Extracts were evaluated for both protective and curative activity. In screening extracts for protective activities, different concentrations of extracts prepared as described previously (6.3.3.2) were applied on the marked squares using sterile swabs. The extracts were allowed to air dry. Thirty minutes to one hour following application of extracts, treated squares were challenged with food-borne pathogens. This involved dipping a sterile swab in an inoculum suspension

prepared as described in 7.3.1.1, and gently swabbing the squares treated with extracts with the suspension. Three to six hours after challenge-inoculation, swabs were taken from the treated squares using sterile, moist, swabs and streaked out on different media as in 7.3.1.1. Plates were incubated and assessed as described earlier (7.3.3). Controls included squares treated with sterile distilled water only. To ensure that experimental materials were not sources of contamination, plates streaked with swabs only or swabs taken from sterilized but untreated squares were included as controls. To assess the curative properties of extracts, the same procedure described above was followed except that the pathogens were first applied before treatment with extract. Colonies recovered from test trials were always compared with the original cultures to confirm their identity. Where any contamination was observed the experiment was discarded. There were three replicates (squares) per concentration and the experiment was repeated three times.

7.3.4.1. Residual activity of extracts

To assess the residual activity of extracts, fruits treated as described above were stored at 8±1° C for four weeks following which they were evaluated for survival of pathogens as described earlier (7.3.3.1).

7.3.5. Statistical analysis

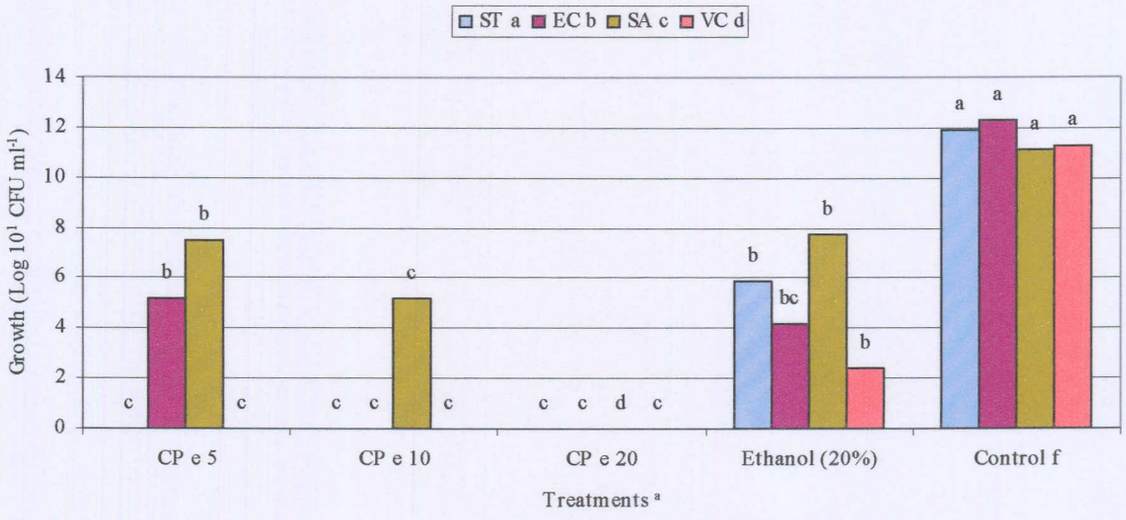
Data was statistically analysed using the GenStat statistical program. One-way analysis of variance (ANOVA) was used to test for differences in average means between treatments. Treatment means were separated using Fishers' protected t-test at a 5% level of significance.

7.4. Results

7.4.1. *In vitro* Screening

Results obtained from the present study indicate that ethanol extracts of *C. repens* possess bactericidal properties. From the results presented in Fig.7.1 it is clear that extracts were effective in checking the growth of *E. coli* 0157-H7, *S. typhimurium*, *S. aureus* and *V. cholerae in vitro* when compared with both the water control, and 20% ethanol on its own. Efficacy of extracts improved with increase in concentration but not in all cases. The

minimum inhibitory concentration (MIC) that completely stopped the growth of *S. typhimurium* and *V. cholerae* for example was 5 000 ppm powder. On the other hand, only concentrations of 10 000 ppm and above completely stopped the growth of *E. coli* 0157-H7 and complete inhibition of *S. aureus*, was achieved only at 20 000 ppm.

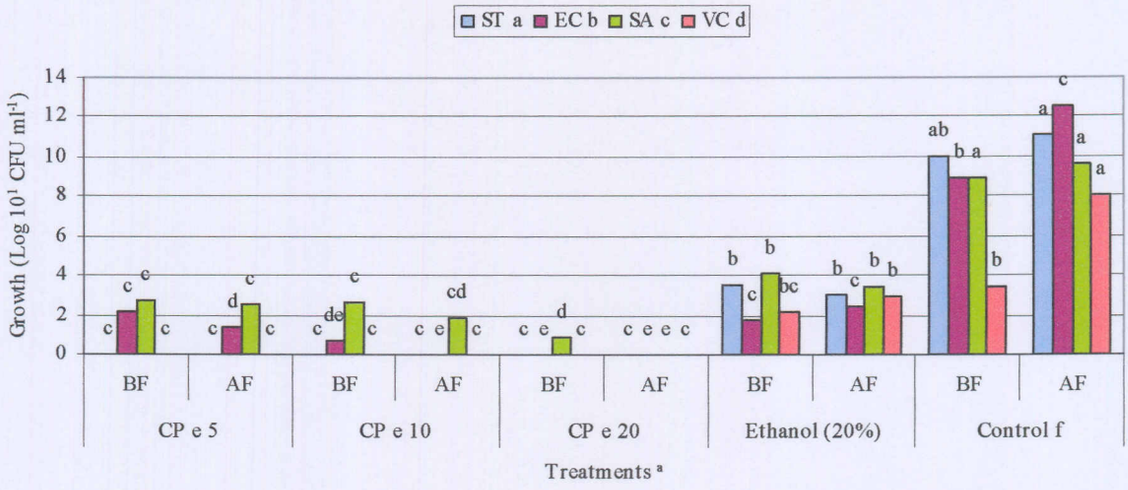


[a] CP 5, 10 and 20 represent concentration of *Coprosma repens* extracts (in 1 000 ppm); Control represents quarter strength Ringer's solution; ST represent *Salmonella typhimurium*; EC represent *Escherichia coli* 0157-H7; SA represents *Staphylococcus aureus*; VC represents *Vibrio cholerae*. Treatments having same letter are not significantly different according to Fishers' protected t-test (P = 0.05).

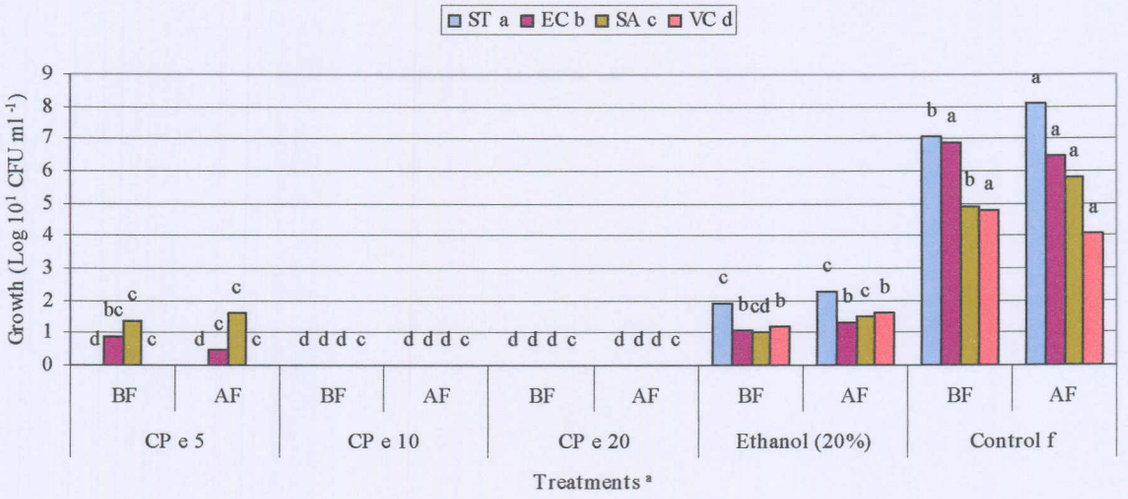
Fig. 7.1 Effect of ethanol extracts of *Coprosma repens* on the population (number of colony forming units) of food-borne bacterial pathogens *in vitro* following incubation at 30° C for 24 hours.

7.4.2. *In vivo* Screening

Results of the *in vivo* study were variable, but extracts were generally more effective in *in vivo* than *in vitro* trials, perhaps because the pathogen population (as determined by CFU) was lower. In general, extracts were more effective when used as a protective rather than a curative treatment. Results presented in Fig. 7.2A shows that the MIC required to completely stop the establishment of both *S. typhimurium* and *V. cholerae* was 5 000 ppm, irrespective of whether it was used as a protective or curative treatment.



A



B

[a] CP 5, 10 and 20 represent concentration of *Coprosma repens* extracts (in 1000 ppm); Control represents quarter strength Ringers solution; ST represent *Salmonella typhimurium*; EC represent *Escherichia coli* 0157-H7; SA represents *Staphylococcus aureus*; VC represents *Vibrio cholerae*; **BF** means fruit is treated with extract before inoculation with pathogen, **AF** means the opposite. Treatments having same letter are not significantly different according to Fishers' protected t-test (P = 0.05)

Fig. 7.2 Effect of ethanol extracts of *Coprosma repens* on the establishment of food-borne bacteria pathogens on Valencia orange fruits surface (A), and the residual effects of extracts on the establishment of pathogens after four weeks of storage at 8±1° C. (B).

Complete control of *E. coli* on the other hand was achieved only at 10 000 ppm, (Fig. 7.2A). Extracts were less effective in the control of *S. aureus*. As a result, only a MIC of 20 000 ppm could completely stop the establishment of the pathogen, and only when the extract was used as a protective treatment.

7.4.3. Residual Activity of Extracts

From the results presented in Fig. 7.2B it is clear that pathogen survival as determined by the CFU was lower after four weeks of cold storage compared with the trials where the extract activity was determined within a few hours after application (7.4.2). As a result, extract performance was higher than the *in vitro* and *in vivo* trials. Unlike the preceding trials however, all concentrations above 10 000 ppm completely inhibited the establishment of all pathogens, irrespective of whether they were used as a protective or curative treatment (Fig. 7.2B).

7.5. Discussion

Results obtained in the present study shows that *C. repens* possess bactericidal properties. All concentrations of extracts were effective when compared with the control (quarter strength Ringer's solution) in checking the establishment of all four food-borne pathogens screened including *S. typhimurium*, *S. aureus*, *E. coli* 0157-H7, and *V. cholerae*. The fungicidal activity of *C. repens* was demonstrated earlier (Chapter Six). In this study, it was found that the compound responsible for extract activity belonged to the hydroxycinamic acids group. Although the antifungal activity of these compounds (including *Penicillium* species.) has been reported (Agioni *et al.*, 1998; Uzi *et al.*, 199), there are no available records of their role as anti-bacterial agents. This is the first report where the anti-bacterial activity of extract of this plant against the four food-borne pathogens screened in this study has been demonstrated.

The efficacy of extracts varied with concentration and between pathogens. Extracts were more effective in checking the establishment of *S. typhimurium* and *V. cholerae* compared to *E. coli* 0157-H7 and *S. aureus*, resulting in complete control of the former pathogens at 5 000 ppm and above. All concentrations of extracts (except 5 000 ppm, and only in the case of *E. coli* 0157-H7) were more effective than the ethanol treatment thus indicating

that ethanol was not solely responsible for extract activity. The exact reason for the lower pathogen population recorded in *in vivo* trials relative to the *in vitro* is not completely understood. The "dry" surface of fruits in cold storage is however not an ideal environment for bacterial growth, most of which require warm and moist environments for optimum growth. Suspending cells of the above pathogens in rind extracts (prepared by homogenizing citrus peel in sterile, distilled water), prior to plating did not affect pathogen growth significantly (data not presented), thus indicating that the lower colony count (population) may not have resulted from any negative effect from the rind on growth.

From the results obtained in the present study, we can deduce that extracts possess some residual activity. After four weeks of storage at 8±1° C, no growth was recorded in all extract concentrations above 5 000 ppm. The same level of control was not recorded with the ethanol treatment and the water control. Although ethanol does have antimicrobial properties, it has a poor residual activity because of its tendency to evaporate quickly. This characteristic reduces its contact period with the pathogen and probably its activity. Results obtained in this study indicated that the activity of extracts was independent of temperature. This property is an advantage over the conventional disinfectant such as chlorine whose activity is easily influenced by changes in temperature. The combined effects of the bactericidal activities of extracts, and low temperature may therefore have resulted in higher cell lysis on fruits kept under cold storage. This observation is particularly interesting as it does indicate that extracts will remain effective even under export conditions where fruits are kept under cold storage conditions for up to four weeks or more.

Results obtained in the present study are also of interest in our effort to control food-borne pathogens especially in the fruit industry where the water used to wash fruit may harbour one or more of the bacteria screened in this study. The effective control achieved with *V. cholerae* is of particular interest since it is the causal agent of cholera disease, an illness that causes many deaths in developing countries. These extracts therefore hold great promise as a potential natural product that could be used as a disinfectant in our quest to reduce food-borne illnesses. There is however, a need for further studies to concentrate the active compound in this extract so that it can be used in smaller volumes as a spray treatment rather than in large volume treatments. Although *C. repens* is not listed as a poisonous plant (Appendix 2), which theoretically makes it safe, there is still the need to

investigate possible side effects of the active component of this extract on man to be sure of its safety when consumed. This will also facilitate its use on other food products. This study however, falls outside the scope of the present investigation.

Literature Cited

Agioni, A., Cabras, C.D., Hallevin, G., Pirini, F.M., Remiero, F. and Schirra, M. 1998. Synthesis and inhibitory activity of 7-gernoxycuramicrin against *Penicillium* species in citrus fruit. Phytochemistry 47: 1521-1525.

Briozzo, J., Nunez, L., Chirife, J., Herszage, L. and D'Aquino, M.. 1998. Antimicrobial activity of clove oil dispersed in a concentrated sugar solution. http//www.bth.co.uk/EssentialOils.htm.

Conko, 1998. On the plate. http:/www.consumeralert.org/pubs/OnthePlate/may1998.htm.

Drapeau, C. and Solomon, N. 1998. Blue-Green Algae: A powerful immune system enchancer.TheExperts'Optimal Health Report.htp/www.galaxymall.com/virtual/shgalgae/research.htm.

Evers, W.D. 1998. Electronic Food RAP. Volume 8 (35). htp/www.cfu.purdue.edu/extension/efr/efrframe.htm.

Friedman, M., Henika, P.R. and Mandrell, R.E. 2002. Bacterial activities of plant essential oils and some of their isolated constituents against *Campylobacter jejuni*, *Escherichia coli*, *Listeria monocytogenes*, and *Salmonella enterica*. Journal of Food Protection 65: 1545-1560.

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

Käferstein, F. and Abdussalam, M. 1992. Food safety in the 21st century. Bulletin of the World Health Organization 77: 347-351.

Schlundt, I. 2002. New directions in food-borne disease prevention. International Journal of Food Microbiology 78: 1-17.

Tauxe, R. 2002. Emerging food-borne pathogens. International Journal of Food Microbiology 78: 31-41.

Thunberh, R.L., Tran, T.T., Bennett, R.W., Matthews, R.R. and Belay, N. 2002. Microbial evaluation of selected fresh produce obtained at retail markets. Journal of Food Protection 65: 677-682.

Uzi, A., Jameta, O., Shmuel, C., Victtor, R. and Moshe, B.J. 1999. Umbelliferone, a phytoalexin associated with resistance of immature grapefruit to *Penicillium digitatum*. Phytochemistry 50: 1129-1132.

Varnam, A.H. and Evans, M.G. 1991. Foodborne pathogens: An illustrated text. Mosby Yearbook Inc. New York.

CHAPTER EIGHT

GENERAL DISCUSSION AND CONCLUSION

The citrus plant is susceptible to attack by many diseases both pre- and postharvestly. Citrus black spot (CBS) caused by *Guignardia citricarpa* Kiely is responsible for high economic losses annually resulting from the rejection of export consignments to countries regarded as CBS free. The molds caused by *Penicillium* species, particularly *Penicillium digitatum* Sacc. and *P. italicum* Wehmer the cause of citrus green- and blue mold respectively, are of economic importance in all citrus producing regions of the world (Eckert and Eaks, 1989). These diseases are currently managed with pre-harvest fungicides (CBS) or postharvestly, in the case of green- and blue mold. However, growing concern over the residual effects of chemicals in the food-chain, and the subsequent danger posed to human health (Norman, 1988), have resulted in a renewed search for alternative disease control measures. Reports of food-borne illnesses have increased in the last decade (Drapeau and Solomon, 1998; Schlundt, 2002; Tauxe, 2002). Thus far, no control measure has proved effective against food-borne pathogens on fruits. All these aspects are of importance in terms of international trade and therefore a study was initiated focusing on developing new postharvest control options for CBS, green- and blue mold. The study was therefore conducted to evaluate *Bacillus* species, either on their own or in combination with other non-chemical products for their biological activity against the most important trade pathogens. The choice of *Bacillus* was based on some desirable morphological characteristics such as endospore formation, which makes them resistant to adverse environmental conditions including long term cold storage conditions and because of previous successful reports in controlling citrus diseases (Huang *et al.*, 1992; Auret, 2000; Korsten *et al.*, 2000), and reports of previous inhibitory activity. Plant extracts were also screened for possible activity against these pathogens due to renewed interest in this untapped resource. The possibility of using plant extracts to control the establishment of food-borne pathogens on citrus fruits in addition to fungal pathogens was also investigated.

Results obtained in the present study showed that some of the *Bacillus* species screened have excellent antagonistic properties and therefore high potential for use in control of *P. digitatum* and *P. italicum* in the postharvest arena. The isolates on their own were not as

effective as the commercial fungicide treatment (imazalil 1 000 ppm + quazatine 1 000 ppm) that gave total control of both pathogens in all experiments. Three of the *Bacillus subtilis* isolates screened; F1, L2 and L2-5, were very effective in controlling *P. digitatum* and *P. italicum* both *in vitro* and *in vivo* (i.e. on fruit). Of these three, F1 was particularly outstanding in its performance and consistency. A remarkable improvement in biocontrol activity of isolates was observed when they were combined with sodium bicarbonate (SB). When combined with SB, isolate F1 was as effective as the fungicide treatment in the control of both *P. digitatum* and *P. italicum*. The antifungal activity of *Bacillus* species (Singh and Deverall, 1984; Huang *et al.*, 1992; Korsten *et al.*, 2000; Obagwu *et al.*, 2001) and the inhibitory activity of SB (Barger, 1928; Smilanick *et al.*, 1999; Palou *et al.*, 2001) have been reported. This is however, the first report where *B. subtilis* isolates were evaluated together with SB to exploit the potential benefits of such integration. These results are of interest for the postharvest arena in the search for alternative control options. It is generally thought that the main mode of action of *Bacillus* species is antibiotic production. Results obtained in this study however showed that antibiotics were not the main mode of action of the three *B. subtilis* isolates tested. This property should make these isolates more acceptable for use in postharvest treatments. The use of F1 combined with SB for control of *P. digitatum* and *P. italicum* is therefore recommended. However, observations reported herein are only the first step in the commercialization process of a biocontrol product. These isolates therefore need to be further screened for non-target activities, toxicological properties and consistency under commercial conditions.

In the present study, we observed that extracts of garlic (*Allium sativum* L.), clove and *Coprosma repens* Hook F. were effective in the control of *P. digitatum*, *P. italicum* and *G. citricarpa*. Extracts on their own were not as effective as the commercial fungicide treatment. A remarkable improvement in the biocontrol activity of garlic extracts was observed when the extract was combined with vegetable oil. A combination of extracts (1 000 ppm) with vegetable oil (0.1%) for example was as effective as the fungicide treatment which gave complete control of both *P. digitatum* and *P. italicum* on fruits. The antifungal activity of garlic has been reported (Bisht and Kamal, 1994; Obagwu *et al.*, 1997; Sinha and Saxena, 1999). This is however the first report where garlic clove extracts were evaluated together with vegetable oil to improve its biological activity. Ethanol extracts of C. *repens* was effective in both *in vitro* and *in vivo* evaluation in controlling the growth of the three pathogens listed. Of particular interest was the observed activity

against *G. citricarpa* as measured by the inhibition of the development of new CBS lesions. The postharvest control of CBS has proved to be very difficult, and so far, no fungicide is known to provide total control of postharvest symptom development. *Coprosma repens* extracts therefore hold great promise as an alternative control measure for citrus postharvest pathogens and CBS in particular. Since CBS infection is initiated at the immature stages of fruit development, we postulate that a combination of pre-harvest applications, supplemented with postharvest treatments as was done in this study might provide more effective control of this pathogen. The possibility of build up of pathogen resistance should however, be investigated if a pre- and postharvest approach is to be followed. This approach should therefore be evaluated in further studies. The possibility of concentrating the active component of this extract and using it in smaller quantities, as an emulsion spray to improve the retention of the active component on the fruit surface is also advocated, and should be investigated. Another interesting observation with respect to *C. repens* extract was its activity against the establishment of the four food-borne pathogens tested in this investigation i.e. *Salmonella typhimurium*, *Staphylococcus aureus*, *Escherichia coli* 0157-H7, and *Vibrio cholerae*. The effective control of these pathogens with this extract is interesting not only because of the importance of these pathogens, but also because to date, there are still no effective means of controlling these pathogens on fruits.

In conclusion, this study has shown that the combination of isolate F1 of *B. subtilis* with SB was effective and consistent in its performance and therefore has great potential for commercial use in the postharvest arena for control of both *P. digitatum* and *P. italicum* on citrus. The use of this technology for the export market is therefore recommended. It is important to note however, that a commercial formulation of F1 was not tested and no commercial trials were carried out. Equally important is the fact that preliminary results showed that these biocontrol systems were not effective in the control of sour rot. No further testes were therefore carried out or reported. Also recommended is the use of a mixture of garlic clove extracts (1 000 ppm) with vegetable oil (0.1%) for control of *P. digitatum* and *P. italicum* on citrus. Based on the results obtained in this study, ethanol extracts of *C. repens* have great potential for the management of CBS, postharvest *Penicillium* rots, and the establishment of the four important food-borne pathogens tested in this study. We strongly advise that this extract be evaluated further.

Suggestions for future studies:

1. Commercial evaluation of treatment F1 + SB under exact export conditions, monitoring fruits overseas.
2. Evaluate the effects of a combined pre- and postharvest application using extracts of *C. repens* for control of CBS.
3. Evaluate the effects of integrating *C. repens* extracts with other non-chemical control measures (such as SB).
4. Determine the non-target effects of *C. repens*.
5. Evaluate product consistency by repeating commercial trials in different regions, on several cultivars and throughout the season.
6. Conduct further studies to determine factors that affect genetic stability of isolates.

Literature Cited

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

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

Bisht, S.S. and Kamal, G.B. 1994. Garlic extract-an effective antifungal treatment for the control of storage rot of apple. Proceeding of the National Academy of Sciences India 64: 233-234.

Drapeau, C. and Solomon, N. 1998. Blue-Green Algae: A powerful immune system enchancer.The Experts'Optimal Health Report.htp/www.galaxymall.com/virtual/shgalgae/research.htm

Eckert, J.W. and Eaks, J.L. 1989. Postharvest disorders and diseases of citrus fruit. Pages 179-260 in: The citrus industry. Vol. 5. Reuther, W. Calavan, E.E. and Carman, G.E. (Eds). University of California, Division of Agriculture and Natural Resources, Ock.

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.

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.

Norman, C. 1988. EPA sets new policy on pesticide cancer risks. Science 242: 366-367.

Obagwu, J., Emechebe, A.M. and Adeoti, A.A. 1997. Effect of extracts of garlic (*Allium sativum* L.) bulb and neem (*Azadirachta indica* Juss) seed on the mycelial growth and sporulation of *Colletotrichum capsici*. Journal of Agricultural Technology 5: 51-55.

Obagwu, J., Korsten, L., Wehner, F.C. and Paul, I. 2001. Inhibitory action of natural epiphytes isolated from citrus fruit surface against *Penicillium digitatum* Sacc., the cause of citrus green mold. Paper presented at the 39th South African Society for Plant Pathology Congress. Greenway Woods, Nelspruit, South Africa, 21-24 January 2001.

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.

Schlundt, I. 2002. New directions in food-borne disease prevention. International Journal of Food Microbiology 78: 1-17.

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.

Sinha, P. and Saxena, S.K. 1999. Inhibition of fruit rot fungus and fruit fly by leaf extracts of onion and garlic. Indian Journal of Agricultural Sciences 69: 651-653.

Smilanick, J.L., Margosan, D.A., Mlikota, F., Usall, J. and Michael, F. 1999. Control of citrus green mold by carbonate and bicarbonate salts and the influence of commercial post harvest practices on their efficacy. Plant Disease 83: 139-145.

Tauxe, R. 2002. Emerging food-borne pathogens. International Journal of Food Microbiology 78: 31-41.

Developing biopesticides for control of citrus fruit pathogens of importance in global trade

By

Joseph Obagwu

Promoter: Prof. Lise Korsten
Co-promoter: Dr. Thierry Regnier
Department: Microbiology and Plant Pathology
Faculty: Natural and Agricultural Sciences
Degree: Ph.D. (Plant Pathology)

RESUMÈ

Bacillus isolates originally isolated from Valencia and Shawmut oranges were screened for inhibitory activity against citrus pathogens; *Guignardia citricarpa* cause of citrus black spot, *Penicillium digitatum* and *P. italicum* the cause of green- and blue mold respectively. The potential antagonists were tested both *in vitro* and *in vivo*, and isolates were tested either on their own or in combination with sodium bicarbonate (SB). Plant extracts, which included aqueous garlic clove and *Coprosma repens* were also evaluated for antifungal activity against the fungal pathogens. In addition, *C. repens* extracts were also evaluated for their antibacterial properties against *Escherichia coli* 0157-H7 *Salmonella typhimurium*, *Staphylococcus aureus* and *Vibrio cholerae,* known food-borne pathogens of importance in food safety of fresh fruits. The study revealed the following:

1. Fifty percent of the *Bacillus* isolates showed *in vitro* inhibitory activities against *P. digitatum* and *P. italicum.* However, only three isolates: F1, L2 and L2-5 provided effective and consistent control of both pathogens in both *in vitro* and *in vivo* tests. Isolates were not effective in controlling latent infections of *G. citricarpa*.

2. The combination of isolates with SB (1% w/v) resulted in a synergistic reaction. Subsequently, a remarkable improvement in the biocontrol activity of the three

isolates was recorded particularly with F1. The combination of F1 with SB was as effective as the commercial fungicide treatment giving complete control of both diseases. Consistency of product performance was shown throughout the season.

3. Effective *in vivo* control of both *P. digitatum* and *P. italicum* was achieved with aqueous garlic clove extracts. Extracts alone (1 000 - 10 000 ppm) was not as effective as the commercial fungicide treatment. Combining extracts with vegetable oil improved biological activity. A combination of extracts (1 000 ppm) with oil (0.1% v/v) was as effective as the fungicide treatment which gave complete control of both diseases.

4. Ethanol extracts of *C. repens* were effective in preventing the development of black spot lesions, and in controlling both green- and blue mold. More effective control of CBS was achieved compared to green- and blue mold. The active compound in the plant extracts falls within the family of the hydroxycinamics.

5. In addition to its antifungal activities, extracts of *C. repens* possessed antibacterial activities and were effective in controlling both the *in vitro* and *in vivo* establishment of *E. coli* 0157-H7, *S. typhimurium*, *S. aureus* and *V. cholerae*.

Appendix 1. Identity of *Bacillus* species screened and status of evaluation

Code	Name	Status [a]	
		In vitro	*In vivo*
268	*Bacillus subtilis*	x	-
F1	*Bacillus subtilis*	x	x
L2-5	*Bacillus subtilis*	x	x
OPF1	*Bacillus subtilis*	x	-
OP2-5	*Bacillus subtilis*	x	-
L3	*Bacillus subtilis*	x	-
719 C	*Bacillus subtilis*	x	-
OPL2A	*Bacillus subtilis*	x	-
143	*Bacillus amyloliquefasciens*	x	-
T1	*Bacillus subtilis*	x	-
L2	*Bacillus subtilis*	x	x
565	*Bacillus subtilis*	x	-
T2	*Bacillus subtilis*	x	-
L2-2	*Bacillus subtilis*	x	-
LIA	*Bacillus subtilis*	x	-
80	*Bacillus subtilis*	x	-
2	*Bacillus subtilis*	x	-
814	*Bacillus licheniformis*	x	-
642	*Bacillus subtilis*	x	-
341	*Bacillus subtilis*	x	-

[a] "x" means applicable, "-" means not applicable

Appendix 2. Common poisonous plants growing in gardens and in the wild

American Ivy
Apple
Apricot
Azalea
Bird-of-Paradise
Bittersweet
Black locust
Bunch Berry
Caladium
Calla Lily
Castor Bean
Cherry
Choke Cherry
Colocasia
Convallaria Majalis
Cowbane
Crowfoot
Daffodil
Delphinium
Daphne
Dieffenbachia
Digitalis
English Ivy
Foxglove
Hedera Helix
Hemlock
Hens and Chickens
Holly
Hyscinth
Hydrangea
Iris
Jack-in-the-Pulpit
Jessamine
Jimsonweed
Jonquil
Lantana
Larkspur
Ligustrum
Lily-of-the-Valley
Locoweed
Locust
Mayapple
Mistletoe
Monstera
Morning Glory
Narcissus
Nightshade
Oleander
Philodendron
Plum
Poinciana
Poinsettia
Poison Oak
Pokeweed
Prickly Poppy
Privet
Rhubarb
Rhododendron
Ricinus Communis
Skunk Cabbage
Strawberry Bush
Sweet Pea
Thorn Apple
Wisteria
Yew

Reference: www.webhome.idirect.com/bom2luv/plants.htm.

Appendix 3. Chemical composition of the RSM-CAS medium (Leong, 1986)

Treatment	Molecular weight	Weight of chemical	Volume of water	Concentration
$FeCl_3.6H_2O$	27.0	23.0	27.0	1M
$KH_2.PO_4$	126.09	13.61	0.1 L	1 M
Sucrose	302.31	30.81	0.3 L	30%
$ZnSO_4.7H_2O$	287.56	0.2013	1 L	0.0007 M
$MnSO_4.4H_2O$	169.01	0.1521	1 L	0.0009 M
Thiamine. HCl	337.27	0.02	1 L	20mg L^{-1}
Casamino acids	-	30	0.3 L	10%
Biotin	233.31	0.001	1 L	1mg L^{-1}
HCl	-	1ml	1 L	10nM

Appendix 4. Vibrio Diagnostic Agar, a medium used for the isolation of *Vibrio cholerae*

Ingredient	Quantity (g)
Sodium Chloride	10.0
Sucrose	20.0
Sodium Citrate	10.0
Sodium Thiosulphate	10.0
Special Peptone	10.0
Ox Bile	5.0
Yeast Extract	5.0
Sodium Taurocholate	3.0
Ferric Citrate	1.0
Bromothymol Blue	0.04
Thymol Blue	0.04
Agar	15.0

Appendix 5. Standard 1 Nutrient Agar, a medium used for the enumeration, isolation and enrichment of bacteria

Ingredient	Quantity (g)
Special Peptone	15.6
Yeast Extract	2.8
Sodium Chloride	5.6
D (+) Glucose	1.0
Agar	12.0

Appendix 6. Violet-Red-Bile-MUG Agar, a selective medium for the simultaneous detection and enumeration of *Escherichia coli*

Ingredient	Quantity (g)
Brain Heart Infusion	7.0
Peptone	4.0
Lactose	9.0
Bile Salt No.3	1.5
Neutral Red	0.03
Crystal Violet	0.002
MUG	0.1
Sodium Chloride	4.5
di-Sodium Phosphate	1.0
Agar	13.0

Appendix 7. MacConkey Agar, a selective agar for the isolation of *Salmonella*

Ingredient	Quantity (g)
Peptone	20.0
Lactose	10.0
Bile Salt No.3	1.5
Sodium Chloride	5.0
Neutral Red	0.003
Crystal Violet	0.001
Agar	13.5

Appendix 8. Identity, methodology and time of dip applications of different treatments evaluated on a semi-commercial scale

Treatment	Product	Concentration	Date
Experiments done in 2000 (A)			
Control	Water	-	June & August
Avogreen powder	*Bacillus subtilis*	75g/100 L water	June & August
Avogreen liquid	*Bacillus subtilis*	250 ml/100 L water	June & August
Biocoat	*Candida saitoana*	406g product/ 15 L water	June & August
Biocure	*Candida saitoana*	406g product/ 15 L water	June & August
Fungicide	Imazalil + guazatine	1g +1 ml product/ L water	June & August
Experiments done in 2000 (B)			
Control	Water	-	September
Avogreen powder	*Bacillus subtilis*	75g/100 L water	September
Avogreen liquid	*Bacillus subtilis*	250 ml/100 L water	September
Biocoat	*Candida saitoana*	406g product/ 15 L water	September
Biocure	*Candida saitoana*	406g product/ 15 L water	September
Fungicide	Imazalil + guazatine	1g +1 ml product/ L water	September
F1	*Bacillus subtilis*	10^8 cell ml^{-1}	September
L2-2	*Bacillus subtilis*	10^8 cell ml^{-1}	September
L2	*Bacillus subtilis*	10^8 cell ml^{-1}	September
F1 + SB	-	-	September

Appendix 8 continued

Treatment	Product	Concentration	Date
L2-5 + SB	-	-	September
L2 + SB	-	-	September
SB	Sodium bicarbonate	1% w/v	September

Experiments done in 2001 (A)

Control	Water	-	June & August
Avogreen powder	*Bacillus subtilis*	75g/100 L water	June & August
Avogreen liquid	*Bacillus subtilis*	250 ml/100 L water	June & August
Biocoat	*Candida saitoana*	406g product/ 15 L water	June & August
Biocure	*Candida saitoana*	406g product/ 15 L water	June & August
Fungicide	Imazalil + guazatine	1g +1 ml product/ L water	June & August
F1	*Bacillus subtilis*	10^8 cell ml^{-1}	June & August
L2-2	*Bacillus subtilis*	10^8 cell ml^{-1}	June & August
L2	*Bacillus subtilis*	10^8 cell ml^{-1}	June & August
F1 + SB	-	-	June & August
L2-5 + SB	-	-	June & August
L2 + SB	-	-	June & August
SB	Sodium bicarbonate	1% w/v	June & August

Experiments done in 2001 (B)

Control	Water	-	September
Avogreen powder	*Bacillus subtilis*	75g/100 L water	September
Avogreen liquid	*Bacillus subtilis*	250 ml/100 L water	September

Appendix 8 continued

Treatment	Product	Concentration	Date
Biocoat	*Candida saitoana*	406g product/ 15 L water	September
Biocure	*Candida saitoana*	406g product/ 15 L water	September
Fungicide	Imazalil + guazatine	1g +1 ml product/ L water	September
F1	*Bacillus subtilis*	10^8 cell ml^{-1}	September
L2-2	*Bacillus subtilis*	10^8 cell ml^{-1}	September
L2	*Bacillus subtilis*	10^8 cell ml^{-1}	September
F1 + SB	-	-	September
L2-5 + SB	-	-	September
L2 + SB	-	-	September
SB	Sodium bicarbonate	1% w/v	September
Experiments done in 2002			
Control	Water	-	August & September
Fungicide	Imazalil + guazatine	1g +1 ml product/ L water	August & September
F1	*Bacillus subtilis*	10^8 cell ml^{-1}	August & September
L2-2	*Bacillus subtilis*	10^8 cell ml^{-1}	August & September
L2	*Bacillus subtilis*	10^8 cell ml^{-1}	August & September
F1 + SB	-	-	August & September

Appendix 8 continued

Treatment	Product	Concentration	Date
L2-5 + SB	-	-	August & September
L2 + SB	-	-	August & Sept