Evaluation of biological control systems for control of mango post-harvest diseases

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DECLARATION

I the undersigned declare that the dissertation, which I hereby submit for the degree of Master of Science at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at another university.

Name: _____ Signature & Date:_____

DEDICATION

In memory of a wonderful person and loving brother-in-law

Reginald Denis Vengtas

14/09/1958 – 06/06/2004

You blessed my life with your presence and your character will forever remain an inspiration to me.

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CHAPTER 1

General introduction

1.1 Introduction

The mango, *Mangifera indica* L., is well known for its excellent flavour and versatility. This fruit is native to the Asian continent, but is cultivated globally over an estimated area of 2.2 million acres (Salunkhe and Desai, 1984). Although India is the epicenter of mango production, South Africa, followed by Mexico are the major exporters of the fresh fruit (Arauz, 2000; FAO, 2001). Mango was first introduced into South Africa in 1920, but commercial plantings only started in 1962 (Finnemore, 1999). Of the crops cultivated in South Africa, mango is the fifth most important in terms of export volume following citrus, banana, avocado and pineapple (Subtropical fruits, http://www.nda.agric.za). Due to the limited shelf life of the fruit, the mango is susceptible to various pre- and post-harvest diseases (Kruger *et al*., 1995). Diseases of major concern in South Africa are anthracnose caused by *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc. in Penz. and stem-end rot (SR) caused by *Botryosphaeria* spp. (Johnson *et al.*, 1990; Donkin and Oosthuyse, 1996).

Limited post-harvest disease control can be achieved with pre-harvest applications of copper-based fungicides (Lonsdale, 1993; Ippolito and Nigro, 2000). Similarly, hot water dips incorporating products such as prochloraz (Omega) (Johnson *et al.*, 1997; Cooke and Jacobi, 2000; Orme and Kegley, 2002) can be used at a post-harvest level for control of anthracnose and SR. However, reported build-up of pathogen resistance against fungicides, environmental pollution due to excessive or incorrect use of pesticides and harmful effects on human health have resulted in negative public perception and new requirements by the United States and European Union that all pesticides must be re-registered. Availability of fungicides to be used on export fruit has been effectively reduced particularly for smaller niche crops such as mango. In light of the above, there is increased scientific interest in biological control of plant pathogens as an alternative form of disease control (Cook, 1993).

Recently, biological control has emerged as an effective strategy to combat major post-harvest diseases of fruit (Janisiewicz, 1994). This approach exploits the activities

of one organism, namely the antagonist, to inhibit the development of a second organism, the pathogen (Janisiewicz and Korsten, 2000). The antagonist is reported to function by at least four modes of activity. These modes of action include antibiosis, competition for nutrients and space, induction of resistance in the host and direct interaction with pathogens (Baker and Cook, 1974; Droby and Chalutz, 1994).

Several biocontrol agents have been commercialised globally and include products such as BIOSAVE-110 (*Pseudomonas syringae*) for the control of *Penicillium* spp. (Fravel, 2000; El Ghaouth *et al*., 2002). In South Africa, the biocontrol products that are registered for the control of fruit diseases include Avogreen (*Bacillus subtilis*) used to control cercospora spot on avocado (Korsten *et al.*, 1997) and YieldPlus (*Cryptococcus albidus*) used in the post-harvest control of *Botrytis cinerea* (Pers.), *Penicillium expansum* (Link.) and *Mucor* spp. on apples, pears and other stone fruit (Graham Reid, Anchor Bio-technologies, personal communication).

Biocontrol programs on mango were initiated in South Africa in 1987 when natural mango epiphytes were screened for antagonism against bacterial black spot (Burger and Korsten, 1988). The potential antagonist was subsequently identified as *Bacillus licheniformis* and was tested in pre- and post-harvest trials to control anthracnose and SR (Korsten *et al*., 1991; Korsten *et al*., 1992; Korsten *et al*., 1993; De Villiers and Korsten, 1994). However, variable results were obtained in these trials and the product was never registered for commercial use. Despite its appeal and potential, biological control has not effectively made the transition from small-scale research to large commercial trials (Emmert and Handelsman, 1999).

In order to fully understand the effectiveness of certain biological control programs, we need to first understand the dynamics of host-pathogen-antagonist interactions and the impact of the environment on these systems. The next step to successful implementation of biocontrol is the knowledge of the different modes of action of the antagonist. This information can be applied in such a way as to optimise the activity of the antagonist for more efficient control. The aim of this study was therefore to use *B. licheniformis* as a model system to obtain a better understanding of biological control systems in the post-harvest arena.

This aim will be achieved through the following objectives:

1. Elucidating the mode of action of *B. licheniformis*;
2. Evaluating attachment of *B. licheniformis* on fruit surfaces and;
3. Further assess the antagonists performance in terms of consistency and effectiveness in a semi-commercial environment evaluated alone or in combination with other products.

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CHAPTER 2

Literature review

2.1 Introduction

Mango, *Mangifera indica* L., belongs to the dicotyledonous family Anacardiaceae. The genus *Mangifera* consists of 62 invariably arborescent species, which includes mango and other economically important trees. The mango forms an erect, branched, evergreen tree with a wide crown (Singh, 1960; Samson, 1986). The fruit is a large fleshy drupe, embedding a laterally compressed fibrous and woolly stone (Singh, 1960; Ploetz *et al*., 1994).

Although the mango originated from India, it is currently grown throughout the tropic and subtropical regions of the world (Salunkhe and Desai, 1984). It is rated as the world's third most important crop in the tropics proceeded by citrus and banana (Nakasone and Paull, 1998). The popularity of the fruit in international markets is due to its excellent flavour, attractive fragrance, beautiful color, delicious taste and health giving properties (Arauz, 2000; Salunkhe and Desai, 1984). Importance of mango production is currently reflected in the following production volumes: India producing 12 0000 tons, followed by Pakistan with 937 705 and Philippines with 931 500, while South Africa only produces 115 152 tons (Fig 2.1) (FAOSTAT, 2001). Even though India produces 70% of the world's mangoes, only 0.3% is exported compared to South Africa that exports 27.9% of fresh fruit (FAO, 2001).

2.2. Local production and export potential

Although commercial mango plantings dates back to 1962 in South Africa, the industry only recently started exporting large volumes of fruit (Finnemore, 1999; http://www.mango.co.za). The most important cultivars currently grown in South Africa are Tommy Atkins (26%), Sensation (13%), Kent (12%), Heidi (9%), Keitt (8%) and Zill (8%) (http://www.mango.co.za). Mangoes are mainly grown in the northern and eastern provinces of South Africa and the major centers of production are Tzaneen (36%), Hoedspruit (28%), Malelane (20%) and Komatipoort (20%) (Finnemore, 1999). The average annual rainfall in the major mango growing regions

varies from 300 to 1000mm and temperatures from 3°C (winter night) to 40°C (summer day).

Mango fruit is harvested, packed and exported from late December till mid March. Difference in time of harvest for a specific cultivar may be as long as three to six weeks (http://www.mango.co.za). Tommy Atkins is an early seasonal cultivar while Keitt and Sensation are late seasonal fruit. During January, South African mango volumes on the export markets compete with fruits from South American countries (Finnemore, 1999). The harvest to consumption fruit chain in South Africa is as follows: fruit are picked by hand when they are physiologically mature (hard and green). At the packhouses, fruit are handled in either a wet or dry receival followed by a disinfectant wash. Fruit are then handsorted according to size and directly packed for either local or export, or alternatively for processing (juice, dried, canned etc). Mangoes that are sold as dried-fruit are normally processed in a separate facility that is in the vicinity of the packhouse. Fruit are stored in cold storage (10°C) prior to being cool transported (7°C) to the export ports for direct shipping overseas. Export fruit takes on average 27 days until arrival at its destined overseas markets (Appendix A). The largest proportion of exports (90%) goes directly to European markets, while the rest is diverted to Middle and Far Eastern countries as well as to Canada (Finnemore, 1999).

In terms of fresh produce, approximately 30-50% of the total tonage produced in South Africa is sold on local markets, which are mainly Cape Town, Durban, Johannesburg and Pretoria (Sevenoaks, 1998; Finnemore, 1999). As of 1996, the export figures showed that Tommy Atkins (58%) was the most favoured, cultivar followed by Kent (29%), Sensation (8%) and Keitt (8%) (Finnemore, 1999; http://www.mango.co.za). Quality criteria of mangoes inspected for export includes: 1) physiologically mature, commencing ripening with 30 to 50% coloration; 2) relative firmness; 3) minimum sugar content of 10%; 4) uniform shape; 5) free from disease, decay, sun-scald, cracks, bruises, latex stains, insect or mechanical damage and 6) conformity to the weight and size specifications set by the European Union (http://www.fintrac.com/indoag/phguides/fintrac/mango.htm).

2.3. Mango fruit diseases and disorders

Mangoes are climacteric fruit, which means that the fruit shows a large increase in carbon dioxide and ethylene production rates during ripening. Like most fresh produce, mangoes are susceptible to many pre- and post-harvest diseases, which can result in significant losses. Pathogens frequently infect unripe fruits, causing relatively minor damage until ripening, when extensive decay sets in (Prusky and Keen, 1993). Mango fruit are also prone to damage and injury during post-harvest handling (lenticel spotting, sapburn), temperature injury (chilling and heat injury) as well as mechanical damage, which occur in the packhouses (brushing, abrasion, pressure and impact damage) (Johnson *et al.*, 1995). The most severe of diseases affecting mangoes in South Africa are anthracnose (pre- and post-harvest), casual organism *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc. in Penz and stem-end rot (SR) (post-harvest), casual organism *Botryosphaeria* spp. (Lonsdale, 1993a).

2.4 Post-harvest diseases of mangoes

2.4.1 Anthracnose

2.4.1.1. Description of *Colletotrichum gloeosporioides*

Colletotrichum gloeosporioides is a facultative parasite and belongs to the order *Melanconiales* (Dickman *et al.*, 1983). The colour of the fungal mycelium on potato dextrose agar (PDA) can vary between white to grey and orange with slimy conidial masses (Fig. 2.2A), which are formed as the acervuli mature. Conidia are hyaline, unicellular and cylindrical to ellipsoidal, 7-20µm long and 2.5–5µm wide formed on slightly pigmented conidiophores in irregularly shaped acervuli. Masses of conidia appear pink or salmon coloured. Conidia germinate and form germ tubes within 3-8 hours at temperatures between 25 and 30°C (Dodd *et al*., 1997).

2.4.1.2 Symptoms

Colletotrichum gloeosporioides is the casual agent of anthracnose, which is the most important fungal disease of mangoes. Major losses have been reported to occur from flowering till fruit set and again after harvest. Anthracnose is highly prevalent in areas with high rainfall and humidity. It causes pre-harvest lesions on fruit, which is characteristically black and tear shaped (Fig. 2.2B) running down the fruit from the stem-end (Lonsdale, 1993a; Ploetz *et al*., 1994; Johnson *et al.*, 1995).

Post-harvest anthracnose appears as rounded black to brown lesions with a rounded border on the fruit surface. Lesions are normally restricted to the peel, but in severe cases, the fungus can invade the pulp (Johnson *et al*., 1995; Arauz, 2000). In advanced stages of the disease, the fungus produces acervuli and abundant orange or pink masses of conidia in the lesions (Freeman *et al*., 1998; Arauz, 2000).

2.4.1.3. Disease cycle of anthracnose

The disease cycle of anthracnose is outlined in Appendix B. The pathogen initially infects intact, non-wounded immature green fruit in the field. The pathogen produces conidia from lesions on leaves, twigs, panicles and mummified fruit (Gantotti and Davies, 1993; Koomen and Jeffries, 1993). Spores germinate and form appressoria on the fruit surface. The fungus using its appressorium penetrates the cuticle and then remain sub-cuticular until the post-harvest stage (Dickman *et al.*, 1983; Jeffries and Dodd, 1990). Once the climacteric period of the fruit starts, lesions begin to develop. There is no fruit-to-fruit infection. However, post-harvest anthracnose is a monocyclic disease (Arauz, 2000).

2.4.2. Stem-end rot

2.4.2.1 Description of *Botryosphaeria parva*

Botryosphaeria spp. occurs as endophytes or sapropytes in several hosts and as a pathogen of many woody trees (Johnson *et al.*, 1990; Roux, 1993). Stem-end rot (SR) caused by *Botryosphaeria parva,* (Pennycook and Samuels) previously known as *Dothiorella dominicana* (Petrak and Cif) (Jacobs, 2002) manifests itself either pre- and or post-harvestly and is the teleomorph of the fungus. *Botryosphaeria* anamorphs affecting mango produces abundant, dark grey-black, fluffy mycelium on either oatmeal or PDA (Fig. 2.2C) (Johnson *et al.*, 1992; Dodd *et al.*, 1997).

2.4.2.2 Symptoms

The pathogen colonizes the blossom often resulting in blossom blight. If environmental conditions are favourable for the pathogen, it moves down the main axis and colonizes stem tissue, causing twig die-back and extensive cankers of stems and trunks. The fungus remains latent in or on fruit until onset of ripening (Johnson *et al.*, 1992; Lonsdale, 1993b). The decay manifests itself at the peduncle and pedicel tissues and in extreme cases covers the entire body of the fruit (Fig. 2.2D). At the

stem end of the mango fruit a brown soft decay starts and rapidly spreads through the whole fruit. A straw-coloured fluid drains from the stem-end and steel-grey mycelium may cover the surface of the fruit.

In extreme cases the decay manifests itself in the same way except that it does not start at the peduncle and pedicel tissues but rather covers the entire body of the fruit. The fungus also infects the fruit in the orchard, however, it remains latent until the fruit begins to ripen after harvest when it resumes activity and colonizes the fruit, giving rise to SR (Lonsdale, 1993b). Affected fruit may be split open as they collapse and a straw-coloured fluid drains from splits in the side of the fruit. *Botryosphaeria* spp. can quickly spread from affected to healthy adjacent fruit causing significant losses to the industry due to decay at the end of the export chain (Ploetz *et al*., 1994). In certain cases *C. gloeosporioides* may be found associated with SR.

2.4.2.3. Proposed disease cycle of *Botryosphaeria* spp.

The proposed disease cycle of *Botryosphaeria* spp. is outlined in Appendix C. *Botryosphaeria* survives the winter as black pycnidia and perithecia in wart-like stromata on living and dead cankered limbs of trees or "mummified" fruit. Wounds or breaks in the cuticle are necessary for infection. The perithecia forcibly discharges ascospores during spring rains, while the conidia produced within the pycnidia ooze out and are washed and rain-splashed to other parts of the trees. The spread of the conidia may continue throughout the rainy season. Fruit become infected fairly early but the rotting does not develop much until the fruit is almost mature (http://www.ipm.uiuc.edu/diseases/series800/rpd813#cycles).

Post-harvest symptom development on fruit obtained in this way is generally known as SR or soft-brown rot, which is discussed together under the term of stem-end rot (SR), in this study. It has been found that both these symptoms are caused by the same pathogen (Jacobs, 2002). The inflorescence can also develop symptoms known as blossom blight (Johnson *et al.*, 1992; Lonsdale, 1993b). *Botryosphaeria* and fruit rot infection is favored by any condition that reduces tree vigour, drought, high temperatures, winter injury, sunscald, poor pruning practices as well as low or unbalanced nutrition
(http://www.ipm.uiuc.edu/diseases/series800/rpd813#cycles).

2.5. Control measures

In the control of mango diseases, pre-harvest spray programs are routinely used to control post-harvest infections. Pre-harvest control is important in providing some degree of control to previously established infections, such as quiescent and incipient ones (Ippolito and Nigro, 2000). Previously, pre-harvest control relied mainly on copper fungicidal field sprays, while post-harvest treatments included hot water dips (55°C for five mins) with fungicides such as prochloraz (Omega) often incorporated in the water (Pelser and Lesar, 1989; Korsten *et al*., 1992).

Current pre-harvest control measures include the use of the copper-based sprays, benomyl (Benlate) and propiconazole (Tilt) (Harry Grove, Bavaria Estates, Hoedspruit, personal communication). A hot dip in a suspension of benomyl (100g/100L) or a hot dip in prochloraz (180ml/1L) emulsion is registered as a post-harvest treatment of mangoes in South Africa (Oosthuyse, 2000; Swart *et al.*, 2002). In a study by Bosquez *et al*. (2000), chemical and organoleptic quality of fruit were not altered by the chemical products, such as those used in this study.

Various forms of alternative disease control methods have been applied to mangoes. Growth regulators have been used for instance to hasten or delay maturity of mangoes to extend the harvesting season (Salunkhe and Desai, 1984). In addition other forms of control included 24 hours exposure of post-harvest mangoes to high concentrations of CO_2 which enhanced concentrations of pre-formed antifungal compounds. Incorporating CO_2 (gaseous form) into pallets filled with mangoes during simulated sea shipments proved to be costly and not suited for commercial sea or air transportation (Salunkhe and Desai, 1984; Lonsdale, 1993c).

Successful reduction in post-harvest decay in grapefruit and papayas, have been achieved by exposure to shortwave infra-red (IR) and ultra-violet (UV) radiation (Moline *et al.*, 1999). The reason for IR or UV not being implemented with success on mango fruit was the impact on quality factors such as scalding of fruit or alteration of taste (Lonsdale, 1993c; Moline *et al.*, 1999). In a recent study, mineral oils (Wenfinex, Citrole, Wenspray and Orchex) were sprayed onto fruit when the mango scale insects were at the crawling stage. All oils proved to be efficient (Daneel *et al.*,

2002). This study is important in a sense that insects cause wounds that provide entry points for most plant pathogens (Daneel *et al.*, 2002).

Research on avocados and other crops has shown that internal fruit disorders are directly related to low calcium levels. Calcium is known to play a vital role in increasing membrane stability and cell wall strength (Conway *et al.*, 1999; Penter and Stassen, 2000). In mangoes, elevated levels of cell calcium have been reported to decrease chilling injury and extend shelf-life (Hetherington *et al*., 2000). Post-harvest treatment of mangoes with calcium chloride combined with a surfactant (Tween 80) improved the uptake of calcium in the skin and flesh of mango. Treatments with calcium significantly delayed fruit ripening and increased fruit firmness and reduced fruit rot as compared to the untreated fruits (Singh *et al.*, 2000).

Current research into control measures, specifically at the post-harvest phase, used a combination of methods such as heat treatments, use of calcium or bicarbonate salts and biological control agents. This has been more effective and the different methods complement one another to help minimise the shortcomings of each (Palmer *et al*., 1997; Conway *et al*., 1999). The ultimate goal of using alternative treatments either alone or in combination is to reduce our dependency on fungicides (Conway *et al*., 1999). Implementation of biological control as an alternative disease control method is based on our current knowledge and awareness of natural processes of the antagonist-pathogen interaction (Janisiewicz and Korsten, 2002).

2.6. Biological control

Biological control is an approach using microorganisms to suppress plant diseases. It offers a powerful alternative to the use of synthetic chemicals (Campbell, 1989; Emmert and Handelsman, 1999; El Ghaouth *et al.,* 2002). In addition, biological control attempts to increase crop production within existing resources and avoids development of pathogens resistant to chemicals. This approach has been used in one way or another since the beginning of organized arable agriculture (Campbell, 1989). In the 1920's, there was a sudden increase in the number of publications reporting the control of diseases by antagonistic fungi, actinomycetes or general soil, fruit and vegetable populations (Campbell, 1989). Recently, some of the microbial taxa that have proved to be successful antagonists included bacteria belonging to the genera

Agrobacterium, *Bacillus*, *Pseudomonas* and *Streptomyces* and fungi belonging to the genera *Ampelomyces*, *Candida* and *Trichoderma* (McLaughlin *et al*., 1990; Karabulut and Baykal, 2003; Batta, 2004). *Bacillus* spp. has been tested on a wide variety of plant species for their ability to control plant diseases. With antibiosis as their postulated mode of action (Wilson and Wisniewski, 1994), they are appealing candidates for biocontrol due to their production of endospores that are tolerant to heat and desiccation (Weller, 1988; Emmert and Handelsman, 1999).

Multiple findings of pesticide residues in different commodities, as well as toxins produced by fruit pathogens have stimulated public demand for fruits and vegetables free of hazardous chemical residues (Janisiewicz, 1994; Larkin *et al*., 1998; Janisiewicz and Korsten, 2002). This has paved the way for the implementation of biological control specifically in the post-harvest arena. The post-harvest environment is an artificial ecological ecosystem and thus appears to present a more suitable milieu for biological control than if implemented under field conditions (Korsten *et al*., 1993a; Spurr, 1994). It is generally regarded that there are four strategies in control of post-harvest diseases namely: 1) reduction in inoculum; 2) prevention or eradication of field infection; 3) inactivation of wound infection and; 4) suppression of disease development and spread (Korsten *et al*., 1993b; De Villiers and Korsten, 1994). Currently, there are several published examples of biological control being evaluated with success. Table 1 provides a summary of progress in biological control research over the past ten years.

The fruit surface is an excellent source of naturally occurring antagonists. Searching for antagonists on healthy fruit in the orchard or during storage where disease may develop seems an obvious strategy. This approach has resulted in the isolation of many ecologically fit bacterial and yeast antagonists effective against post-harvest pathogens of various fruits (Table 1) (Janisiewicz and Korsten, 2002). In addition, the bacterium used in this study was initially isolated from the mango fructoplane during initial biological control studies in South Africa. It was subsequently identified as *Bacillus licheniformis* and showed potential as an antagonist *in vitro* (Burger and Korsten, 1988).

Some microorganisms are better adapted than others to withstand fluctuations in temperature and other environmental conditions (Campbell, 1989; Agrios, 1997). The lack of knowledge in terms of mode of action may be attributed to our limited understanding of interactions between host, pathogen and antagonist (Campbell, 1989; Droby and Chalutz, 1994; Janisiewicz *et al*., 2000). Many useful bacterial biocontrol agents have been found by observing zones of inhibition on Petri plates, resulting from their *in vitro* interaction with pathogens (antibiosis) (Freeman *et al*., 2000). This screening method does not necessarily imply antibiotic production and may identify biocontrol agents with other modes of action such as parasitism, competition for space and nutrients and excretion of different metabolites such as fatty acids, ethanol etc (Gardener and Fravel, 2002).

2.6.1 Mode of Action

Several modes of action exist in nature and are density dependent. Nature maintains population dynamics by regulating the level of positive and negative interactions as a function of species diversity and richness (Campbell, 1989; Agrios, 1997). Competition is defined as niche overlap, resulting from a situation where there is simultaneous demand on the same resource by two or more microbial populations (Campbell, 1989; Droby and Chalutz, 1994; Buysens *et al.*, 1996). Competition is also possible for oxygen, space and in the case of autotrophs, light (Campbell, 1989, Elad *et al.*, 1994). In the past, competition for nutrients and space has often been implied, but direct proof of the importance of this mechanism in the fruit system is lacking (Janisiewicz *et al*., 2000). Currently, methods used to detect competition include evaluating microbes that rapidly colonize habitats and have the ability to exclude other organisms that attempt to invade the space (Gardener and Fravel, 2002; Spadaro and Gullino, 2003).

Competition can be an effective biocontrol mechanism when the antagonist is present in sufficient quantities at the correct time and location and if it can utilize limited nutrients or resources more efficiently than the pathogen (Larkin *et al*., 1998). In terms of competition for space, certain microorganisms (yeasts and bacteria) have the added advantage of the formation of an extracellular polysaccharide capsule that can promote adhesion to the fruit surface (Spadaro and Gullino, 2003). The effects of competition show that there are many different possibilities for biological control

such as 1) reducing inoculum potential by nutrient competition, 2) increasing saprotrophic competition for initial resources in substrate colonization and 3) reducing the actual amount of the pathogen in either the dormant survival or pathogenic growth phases (Campbell, 1989; Spurr, 1994).

Parasitism or predation occurs when the antagonist feeds on or within the pathogen, resulting in a direct destruction or lysis of propagules and structures (Campbell, 1989; Larkin *et al*., 1998). Direct parasitism by the antagonist on the pathogen propagules has been reported to play a role in biological control of foliar plant diseases (Droby and Chalutz, 1994). Methods to prove parasitism include burying and retrieving propugules of the pathogen to isolate the antagonist (Gardener and Fravel, 2002). A strong adhesion was observed *in vitro* between the antagonist *Pichia guillermondii* and *Botrytis cinerea* Pers. Ex Nocca Balb mycelium, which was through the formation of a pectin link. The yeast showed a high ß-1.3-glucanase enzyme activity that resulted in the degradation of the fungal cell wall (Spadaro and Gullino, 2003).

Antibiosis refers to the inhibition or destruction of the pathogen by a metabolic product of the antagonist, such as the production of a specific toxin, antibiotics or enzymes (Larkin *et al*., 1998; Nakayama *et al.*, 1999; Heungens and Parke, 2001). To be effective, antibiotics must be produced *in situ* in sufficient quantities at the precise time of interaction with the pathogen (Larkin *et al*., 1998; Emmert and Handelsman, 1999; El Ghaouth *et al*., 2002). It was discovered that bacteriocins, which are antibacterial proteins, produced by bacteria, kill or inhibit the growth of other bacteria (Agrios, 1997; Cleveland *et al*., 2001). Bacteriocins function by forming pores in the membrane of target cells and depleting the trans-membrane potential. This results in the leakage of cellular materials (Cleveland *et al*., 2001). One well-known example is Pyrrolnitrin, a natural product produced by some *Pseudomonas* spp. This compound provided the chemical model for development of Fludioxonil, a broad-spectrum fungicide used as seed treatment, foliar sprays or soil drenches (Gardener and Fravel, 2002). Another well-known example of bacteria that produce toxic compounds is *Bacillus thuringiensis,* which produces a BT toxin (Gerhardson, 2002). In a study conducted by Jiang and co-workers (2001), *Bacillus subtilis* was found to be the most effective antagonist against the main pathogens of litchi fruit (*Pernophythora litchi*). The activity of the antifungal compound produced by *B. subtilis* reached a maximum

after 48 hours (Jiang *et al*., 2001). In addition, *Bacillus subtilis* produces iturin, which is a powerful antifungal peptide as well as gramicidin S (Jiang *et al*., 2001; Cho *et al*., 2003).

Gliocladium, *Penicillium* and *Trichoderma* spp. were screened for possible antagonism against *Fusarium* spp. The antagonists were able to produce cell wall degrading enzymes in the presence of the pathogen and they showed an ability to colonize the rhizosphere (Roberti *et al.*, 2000). Possible modes of action were antibiosis and hyperparasitism. For the control of *B. cinerea* by *Brevibacillus brevis*, it was found that the antagonist had two modes of action. Firstly, the production of Gramicidin S that inhibits conidial germination and secondly, the production of a biosurfactant, which reduces periods of leaf wetness that encourage infection (McHugh and Seddon, 2000).

Plant defence mechanisms include the hypersensitive response, synthesis of phytoalexins, lignification of plant cell walls, synthesis of lytic enzymes as well as expression of a wide range of pathogenesis related proteins (Glazebrook, 1996; Hannusch and Boland, 1996; Ippolito and Nigro, 2000). Several *Candida* strains, applied to the fruit surface, are able to cause chemical and osmotic changes in apple tissues, favouring antagonist settlement (Spadaro and Gullino, 2003). Fungal pathogens must overcome several barriers before they are able to initiate disease in plants. The pathogen must locate and adhere to susceptible host tissue and initiate infection. Contact with underlying plant tissues present a different set of barriers, most notably, preformed antibiotic compounds, morphological barriers and phytoalexins induced by the host (Prusky and Keen, 1993; Villajuan-Abgona *et al*., 1996). Evidence suggests that preformed antifungal compounds might be involved in the resistance of mango fruits to fungal development and many plants may have the ability to accumulate phenolic compounds, especially tannins, in response to stress (Sauvesty *et al*., 1991; Prusky and Keen, 1993). A phytoalexin accumulation (scoparon and scopoletin) was noted in citrus fruits treated with yeast cells (Spadaro and Gullino, 2003). Research into the mechanisms by which plants resist bacterial pathogens has led to the discovery of harpin. This is a protein that is now being used to activate crop defences prior to pathogen attack. A variety of pathogenic and non-pathogenic microorganisms can induce plant defences and may be useful as

biocontrol agents (Gardener and Fravel, 2002). In a study by Tian and Wan (2003), salicyclic acid was used to enhance biocontrol efficacy in cherries. The Salicylic acid treatment induced a significant increase in polyphenoloxidae, phenylalanine ammonia-lyase and ß-1,3-glucanase activity in cherry fruit thereby increasing the biochemical defense response.

2.6.2 Integrated application methods

Schmitt and co-workers (2000) studied the effects of combinations of different biological disease control methods on grey mold disease development caused by *B. cinerea* in grapes. The main objective was to enhance plant health by reducing the use of chemical fungicides, induction of resistance in the plant and integration of bacterial and fungal antagonists. The use of plant extracts were combined with the use of antagonists and it was clear that when used in combination, several control strategies targeted at different stages of disease development of the pathogens occurred. Having a system that targets more than one site in the life cycle of the pathogen can result in higher reliability and efficacy (Schmitt *et al.*, 2000). In the control of powdery mildew (*Oidium mangiferae*) on mango, sodium bicarbonate has been used in combination with fungicide sprays (http://www.crfg.org/pubs/ff/mango.html). In a study by Fan and Tian (2001), *Cryptococcus albidus*, isolated from peach fruit was found to be effective against post-harvest rot in apples. In addition, calcium chloride facilitated control of *B. cinerea* and *Penicillium expansum* (Link.) by *C. albidus* (Fan and Tian, 2001). An integrated approach was studied for the control of post-harvest diseases of peaches. This included application of Candida oleophila and hot water treatments and storage in modified atmosphere. The highest efficacy was achieved by the combination of all three strategies (Karabulut and Baykal, 2003). Elevated calcium levels have been reported to reduce chilling injury and extend fruit storage life (Hetherington, 2000). This has lead to the investigation of the influence of many food additives (calcium salts and sodium bicarbonate) on the efficacy of biocontrol agents (Fan and Tian, 2001; Obagwu and Korsten, 2002). The effect of calcium proprionate, sodium bicarbonate and sodium ethylene-diamine-tetra-acetic acid (EDTA) on 1) post-harvest pathogens of apples and 2) the efficacy of the biocontrol product Aspire were tested. Sodium bicarbonate exhibited a consistent ability to enhance the biocontrol performance (curative and protective effect) against *Botrytis* and

Penicillium rot of apples (El Ghaouth *et al*., 2000; Droby *et al.*, 2003; Wszelaki and Mitcham, 2003).

2.7. Conclusion

A successful biocontrol agent has several modes of action, which often act in a synchronised way, which is crucial for the end result (Gerhardson, 2002, Leverentz *et al*., 2003). Multiple interactions between the antagonist and pathogen, as well as other microbes can interfere with the elucidation of the biological control agent's mode of action (Jijakli *et al*., 2000). Biological control has enormous potential to improve or replace existing disease control strategies. However, biological control systems require proper implementation and integration with current production strategies (Larkin *et al*., 1998). The most effective approach to overcome variability of product performance is by formulating the product for effective storage attributes and for commercial triats such as shelf life and efficacy (Spadora and Gullino, 2003). In addition, the application of adjuvants to protect and stimulate establishment of antagonists on host surfaces may enhance product performance (Spadaro and Gullino, 2003). The use of calcium salts that have previously been shown to enhance the activity of several biocontrol agents can easily be integrated on a commercial scale (Hetherington, 2000; Droby *et al.*, 2003; Leverentz *et al*., 2003). Significant progress has been made towards biological and integrated control strategies in the post-harvest environment. Therefore biological control should be viewed as an important if not essential component of an integrated disease management approach resulting in a significant reduction in the use of pesticides (Spadaro and Gullino, 2003).

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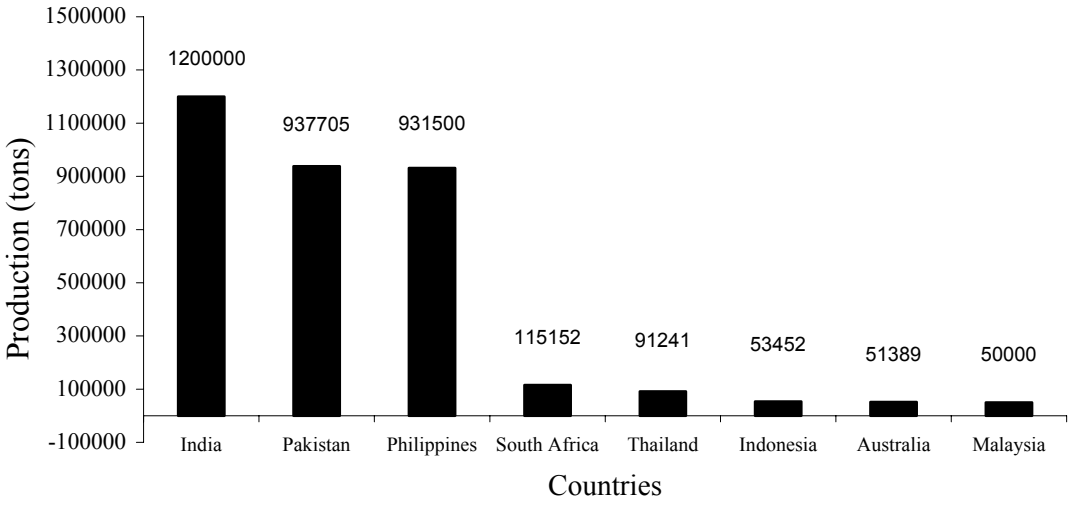

Fig. 2.1 Global mango production volumes in tons (FAOSTAT, 2001).

Table 1: Summary of recent publications regarding biological control (post-harvest) for fruit crops

Commodity	Pathogen	Antagonist	Mode of action[a]	Status of research[b]	Reference
Apple (*Malus dosmetica Borkh.*)	*Botrytis cinerea* (Pers. Ex Nocca and Balb)	*Candida oleophila* (Berkhout)	HR	*In vivo*	Mercier and Wilson, 1995
Apple	*B. cinerea* and *Penicillium.expansum* (Link)	*Cryptococcus albidus* (Kütz)	NM	*In vitro*	Fan and Tian, 2001
Apple	*P.expansum*	*Cryptococcus laurentii* and *candida*	Competition	*In vitro*	Vero *et al.*, 2002
Apple	*P. expansum*	*Metchnikowia pulcherrima*	NM	*In vivo*	Janisiewicz *et al.*, 2003
Apple	*P. expansum* (Link.) and *Monilinia fructicola* (G. Wint.)	*Heat tolerant yeast*	Competition	*In vitro* and *combination*	Leverentz *et al.*, 2003
Apple	*B. cinerea*	*Trichoderma harzianum* (Pers.)	NM	*In vitro*	Batta, 2004
Apples and **Peaches** *(Pyrus communs, L)*	*Colletotrichum sp* (Penz.) and *Penicillium digitatum* (Pers.:Fr)	*Muscodor albus* (Worapong. Strobel)	Production of volatiles	*In vitro*	Mercier and Jimencz, 2004
Banana *(Musa acuminata Colla)*	*Fusarium* sp (Link.)	*Pseudomonas syringae*	NM	*In vitro*	Willamson *et al.*, 1998

Table 1: Continued

Commodity	Pathogen	Antagonist	Mode of action[a]	Status of research[b]	Reference
Cherry *(Prunus salicifolia HBK)*	*Rhizopus stolonifer* (Erhenb.: Fr Vuill)	*Cryptococcus infirmo-miniatus*	Competition	*In vitro*	Spotts *et al.*, 1998
Cherry	*P. expansum and Alternaria. Alternata* (Fr.) Keissler	*Rhodotorula glutinis and Cryptococcus laurentii* and Salicyclic acid	HR	*In vitro*	Qin *et al.*, 2003
Cherry	*A. alternata* and *P. expansum*	*Trichosporan pullulans, C. laurentii*	NM	*In vitro*	Qin *et al.*, 2004
Cherries and **Grapes** *(Vitis rotundifolia Michx.)*	*B. cinerea and Monilinia laxa*	*Aureobasidium pullulans* (Viala and Boyer)	NM	*In vitro*	Schena *et al.*, 2003
Citrus *(Citrus sinensis)*	*Penicillium digitatum* (Pers.:Fr)	*Saccharomyces* sp	Competition	*In vitro*	Cheah and Tran, 1995
Citrus	*P. digitatum*	*Candida famata*	Direct interaction	*In vitro* and *in vivo*	Krihak *et al.*, 1996
Citrus	*P. italicum* (Wehmer)	*P. syringae*	Competition	*In vivo*	Smilanick *et al.*, 1996
Citrus	*P. digitatum*	*Pantoea agglomerans*	Direct interaction	*In vivo*	Plaza *et al.*, 2000

Table 1: Continued

Commodity	Pathogen	Antagonist	Mode of action[a]	Status of research[b]	Reference
Citrus	*P. italicum* and *P. digitatum*	*B. subtilis*	Production of volatiles	*In vitro* and *combination*	Obagwu and Korsten, 2003
Grapes	*Ulocladium atrum*	*Botrytis spp*	Competition	*In vivo*	Kohl *et al.*, 2000
Lychee *(Litchi chinensis Sonn*)	*Peronophythora*	*B. subtilis*	Bioactive compound	*In vitro*	Jiang *et al.*, 2001
Papaya *(Carica papaya L)*	*Colletotrichum*	*C. oleophila*		*In vivo*	Gamagae *et al.*, 2003
Pear *(Pyrus communis)*	*B. cinerea*	*C. albidus*	NM	*In vitro* and *In vivo*	Chand-Goyal and Spotts, 1996
Pear	*P. expansum*	*Rhodotorula glutinis*	Competition	*In vivo,* Packhouse trials	Sugar and Spotts, 1999
Pears	*P.expansum*, *B. cinerea* and *R.stolonifer*	*P. agglomerans*	Parasitism	*In vitro*	Nunes *et al.*, 2001
Peaches	*B. cinerea* and *P. expansum*	*C. oleophila*	NM	*In vitro*	Karabulut and Baykal, 2003
Plums *(Prunus salicina Lindl)*	*M. fructicola*	*Trichoderma viride* (Pers. Ex S.F. Gray)	NM	*In vitro*	Hong *et al*., 1998
Nectarine (Prunus perica var nucipersica) and **apricot** *(Prunus mandishurica)*	*M. laxa and Rhizopus stolonifer*	*Panteoa agglomerans*	Direct interaction	*In vivo*	Bonaterra *et al.*, 2003
Pome and Citrus	*P. digitatum and B. cinerea*	*Candida saitoana*	Competition	*In vitro* and semi-commercial	El Ghaouth *et al.*, 2000

Table 1: Continued

Commodity	Pathogen	Antagonist	Mode of action[a]	Status of research[b]	Reference
Strawberry	*B. cinerea and C. acutatum*	*Trichoderma spp*	Antibiosis	*In vitro* and GH	Freeman *et al.*, 1998
Strawberry	*B. cinerea*	*P. guilliermondii*	NM	*In vivo*	Wszelaki and Mitcham, 2003

[a] NM= Not mentioned, HR = Induction of Host resistance
[b] GH = Green house trials

*Botanical names obtained from: http://www.thefruitpages.com
** Authorities for fungal names obtained from: http://www.indexfungorum.org/Names/fundic.asp

CHAPTER 3

Putative mechanisms of biological control of *Bacillus licheniformis*

Abstract

Bacillus licheniformis, initially isolated from the mango phylloplane, was assessed for its ability to inhibit *Colletotrichum gloeosporioides* and *Botryosphaeria parva in vitro*. Radial growth of the pathogens were inhibited *in vitro* when evaluated with the dual culture technique. Several modes of action were investigated, including antibiosis, production of volatiles, competition for nutrients and bioactive compounds. The isolate used in this study showed antibiosis as one of its modes of action. Production of a volatile compound was confirmed by an *in vitro* assay and the separation of the bioactive compound was achieved using high performance liquid chromatography. Siderophore production was evaluated as possible competitive advantage the antagonist could have by removing iron from the iron-rich media before the pathogens can. In addition, different concentrations of the antagonist were evaluated *in vitro* for inhibition of the pathogens growth. The optimum concentration of the antagonist was found to be 10^7 cells/ml. Evaluation of the effect of temperature on antagonist-pathogen interaction showed that a lower temperature (10ºC) was more effective for antagonism compared to 25ºC. The addition of calcium salts (calcium carbonate and calcium chloride) was instrumental in enhancing the *in vitro* activity of the antagonist. This study showed that more than one mode of action of *B. licheniformis* were found against mango post-harvest fruit pathogens.

3.1 Introduction

General lack of knowledge of the mode of action of the antagonists is attributed to our limited understanding of complex interactions between the host, pathogen and the antagonist at the site of infection. This information is important to optimise application methods and for product registration of biocontrol agents (Campbell, 1989; Droby and Chalutz, 1994).

Traditional screening procedures for antagonist favoured selection of isolates that could inhibit the pathogen by direct interactions or by secretion of antibiotics (Droby and Chalutz, 1994; Cho *et al*., 2003; Spadaro and Gullino, 2003). Microbial interactions are based on population densities and comprise more than one type of interaction over time (Atlas and Bartha, 1998). Negative microbial interactions reported in biocontrol systems can represent any of the following: competition, site exclusion and direct interaction between the antagonist and the pathogen (Campbell, 1989; Droby and Chalutz, 1994; Agrios, 1997).

The term competition has been defined as niche overlap, where there is simultaneous demand on the same resources by two or more microbial populations (Campbell, 1989; Droby and Chalutz, 1994; Gardener and Fravel, 2002). Competition can be an effective biocontrol mechanism when the antagonist is present in sufficient quantities at the correct time and location and if it can utilize limited nutrients or resources more efficiently than the pathogen (Larkin *et al*., 1998). In terms of competition for space, certain microorganisms (yeasts and bacteria) have the added advantage of the formation of an extracellular polysaccharide capsule that can promote adhesion to the fruit surface (Spadaro and Gullino, 2003).

Induced resistance has been recognised as an important biocontrol mechanism (Qin *et al.*, 2003). Some reports have shown that certain post-harvest biocontrol agents may affect host responses, in particular at wound sites, leading to enhanced wound-healing processes (Wisniewski *et al.,* 1991; Droby and Chalutz, 1994). Direct parasitism by the antagonist on the pathogen propagules has been reported to play an important role in biological control systems particularly in soil-borne and to a lesser extent foliar diseases (Bonaterra *et al.*, 2003). Secretion of substances by one organism inhibitory to another is a common phenomenon in nature (Droby and Chalutz, 1994; Gardener

and Fravel, 2002). Antibiosis has often been described as the most important mode of action in biocontrol systems of post-harvest diseases (Campbell, 1989; Freeman *et al.*, 2000).

Biocontrol programs on mango were initiated in South Africa in 1987 when natural mango epiphytes were screened for antagonism against bacterial black spot (Burger and Korsten, 1988). The isolate was identified as *Bacillus licheniformis* and was evaluated successfully *in vitro* as well as in field and packhouse trials. The focus of this chapter is to verify the *in vitro* mode of action of *B. licheniformis,* against *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc. in Penz. and to evaluate the effect on *Botryosphaeria parva,* previously known as *Dothiorella dominicana* (Petrak and Cif).

3.2 Materials and methods

3.2.1 Antagonist

The antagonist, *B. licheniformis* (isolate Mal) previously isolated from the mango phylloplane, was obtained from the bacterial culture collection of Plant Pathology Laboratories, University of Pretoria. The isolate was maintained on nutrient agar (Merck, Johannesburg), supplemented with 0.1% cycloheximide (Sigma, Johannesburg) throughout the duration of the study. Subcultures were made as required and plated on fresh media and incubated at 25°C for 48 hrs prior to use in the respective experiments. A cell suspension was made using sterile ¼ strength Ringer's solution (Oxoid, Johannesburg). This was done to wash cells aseptically from the surface of the plate. A standard dilution series was made and the concentration determined using the Petroff-hauser counting chamber. A dilution series was made to obtain a final concentration of 10^7 cells/ml for all tests. Experiments were performed using a 24 hr old culture at a standard concentration of 10^7 cells/ml, unless otherwise mentioned.

3.2.2 Pathogen

A virulent culture of *C. gloeosporioides* was obtained from the fungal culture collection of Plant Pathology Laboratories, University of Pretoria. This pathogen was originally isolated, by Dr. G.M. Swart, from mango fruit with symptoms of

anthracnose. An isolate of *Botryosphaeria parva,* culture originally isolated as endophytes from twigs of mango trees in Hoedspruit. This pathogen was isolated and identified by Ms. Leylani Grobler (Plant Pathology Laboratories, University of Pretoria). This fungus was subsequently tested for its pathogenicity on mango fruit and both the pathogens were maintained on potato dextrose agar (PDA) supplemented with chloramphenicol (Rolab, Johannesburg), unless otherwise mentioned. During experimentation, *C. gloeosporioides* was maintained on oats agar (Bacteriological agar (Merck), oats and distilled water) and left in the ultra-violet chamber for 72 hrs to induce sporulation. *Botryosphaeria parva* was maintained on Bacteriological agar containing a piece of sterile mango twig. Plates were incubated under ultra-violet in order to induce sporulation. Sterile ¼ strength Ringer's was added to the plate and spores were disrupted using a sterile glass rod. A total spore count was done using a haemocytometer and a final cell concentration of 10^5spores/ml was made using a standard dilution series.

3.2.3 Dual culture technique

A 5mm plug was taken from the edge of an actively growing fungal colony (*C. gloeosporioides* and *B. parva*) with a cork borer. The plugs were inoculated separately onto the centre of a 90mm PDA plate. Plates were incubated at 25°C for 24 hr before the antagonist was streaked 35mm from the centre of the plate (Fig. 3.1). Colony growth and radii were measured, using digital callipers, after 48 hrs incubation at 25°C. Controls consisted of plates inoculated with either a culture of *C. gloeosporioides* or *B. parva* on their own. This experiment was done in triplicate and repeated three times. Data were analysed using the statistical program GenStat (2000). Analysis of variance (ANOVA) was used to test for differences between treatments. The angular transformation for percentages was used to stabilise treatment variances. Treatment means were separated using Fishers' protected t-test least significant difference (LSD) at the 5% level of significance.

3.2.4 Antibiotic Production

Antibiotic production medium (McKeen *et al.*, 1986) was used for this experiment. The pH of the medium was adjusted to 6.0 and the media autoclaved at 121ºC for 15 min. The prepared liquid media was inoculated with 1ml of 10^7 cells/ml *B. licheniformis*. The flasks were shake incubated at 30ºC for seven days before two ml

aliquots of the actively growing suspension were filtered through a 0.22µm pore sized acetate filter. Serial dilutions (6.25, 12.5, 25, 50 and 100%) were made of the filtered media. Spore suspensions of the pathogens were prepared as described previously (3.2.2) and spread plated on PDA. In each plate four holes were made in the agar of each with a sterile 5mm diameter cork borer and 0.25ml of each of the bacterial dilutions were pipetted into a hole per plate. A filtrate of sterile antibiotic production medium was used as a negative control. Plates were incubated at 25ºC and monitored daily for development of inhibition zones. Control plates consisted of plates inoculated with *C. gloeosporioides* or *B. parva*. alone. The presence or absence of inhibition zones was noted and measured at seven days. This experiment was done in triplicate and repeated three times. Data was analysed as described previously (3.2.3).

3.2.5 Production of Volatiles

In order to determine production of and inhibitory effect from volatiles on the pathogens, nutrient agar plates were spread plated with freshly prepared *B. licheniformis* at a concentration of 10^7 cells/ml. Five mm fungal plugs were obtained from the edge of actively growing pathogen cultures, using a sterile cork borer. These plugs were placed onto the centre of PDA plates. The lids of the inoculated Petridishes were removed and the bottoms containing either *C. gloeosporioides* or *B. parva* were placed on top of the antagonist plates and sealed together with parafilm. The fungal bottom plate faced downward. All taped plates were incubated at 25ºC and were monitored daily for fungal inhibition for a period of up to two weeks. Mycelial diameter was measured using digital callipers. Control plates consisted of the fungal pathogens on their own without the antagonist. This experiment was done in triplicate and repeated three times. Data were analysed as described previously (3.2.3).

3.2.6 Optimum concentration of *Bacillus licheniformis* effective against the pathogens

In order to determine the lowest concentration at which the antagonist is inhibitory to the pathogen, a checker-board type titration assay was used (Korsten, 1993). Antagonists prepared for analysis as described in section 3.2.1 was used at a concentration range of 10^5, 10^6 and 10^7 cells/ml. The test pathogen was similarly prepared as described in section 3.2.2 for use in a concentration gradient of 10^3, 10^4 and 10^5 spores/ml. Fifty microlitres of each test concentration was placed aseptically

into a Elisa plate (Sterilin, Johannesburg). For each antagonist concentration tested, two rows of the Elisa plate were used and for each pathogen concentration, three columns were used (Fig. 3.2). For each combination tested six replicate wells were used. Controls were wells inoculated with either the pathogen or antagonist on its own, as indicated by the shaded wells of Fig. 3.2. Plates were incubated at 25°C for 16 hrs, after which the pathogen was inoculated into the appropriate wells. Plates were read immediately using the Anthos Reader 2000 (Anthos Labtec instruments) at a wavelength of 492nm. This value was used as the zero reading. Plates were incubated at 25°C and optical density readings were taken at one, three, five and seven day intervals. A control consisting of Ringer's only, was included. This experiment was done in triplicate and repeated three times. Data were analysed as described previously (3.2.3).

3.2.7 Role of hydrolytic enzymes in mode of action of *Bacillus licheniformis*

This experiment was a qualitative analysis designed to test if either the antagonist or pathogen hydrolysed complex compounds. The usage of these complex compounds demonstrated competition between antagonist and the pathogen. Starch, casein, chitin and cellulose media (Skinner and Lovelock, 1979; Claus, 1989) were made (Appendix D). Media were streak inoculated according to the template shown in Fig. 3.1. Plates were monitored daily for zones of inhibition and colorimetric reaction noted and growth was compared to the control.

3.2.8 Role of siderophore production in antagonism

Method of Buyer *et al*. (1989) were used to evaluate the production of siderophores. The RSM-CAS medium (Appendix E) was prepared and inoculated according to the template shown in Fig. 3.1. Plates were incubated at 25°C and monitored daily for the formation of yellow to orange zones surrounding the growth of either pathogen or antagonist on the blue agar plates. Each combination evaluated consisted of five plates and this experiment was repeated three times. Results consisted of colorimetric reactions where either the antagonist or pathogen was able to take up iron from the media. The bright yellow around the organisms were noted accordingly.

3.2.9 Separation of bioactive compounds from *Bacillus licheniformis*

3.2.9.1 Preparation of antagonist for extraction

The antagonist was maintained and prepared as described previously (3.2.1). Aliquots of 10^7 cells/ml were inoculated into flasks containing nutrient broth. Flasks were shake incubated at 6000rpm (25°C) for a period of 20 days. Controls consisted of flasks containing only nutrient broth. Flasks were removed at 0, 1, 4, 7, 10 and 20-day intervals and samples were filtered using a 0.22µm pore size syringe driven filter (Millipore). The filtered samples were divided into five flasks in order to determine: 1) the total phenolic compounds, 2) the amount of non-conjugated phenolic compounds, 3) the amount of soluble glycoside-bound phenolic content and 4) the soluble ester-bound phenolic acids, according to methods of de Ascensao and Dubery (2003). Filtered samples were either used immediately or kept at 4°C for further use.

3.2.9.2 Extraction of phenolics

Extraction of Free acids: The first sample was acidified with 50µl of 1M Trifluoro-acetic acid and the solution was extracted three times with equal volumes of diethylether (Saarchem, Johannesburg) (Cvikrová *et al.*, 1993). The ether extract was reduced to dryness and the resulting precipitate was re-suspended in 100µl of methanol. This was used to determine the free phenolic content with the Folin-Ciocalteu reagent (Swain and Hillis, 1959).

Extraction of soluble esters-bound phenolics: The second sample was used to extract the soluble ester-bound phenolic acid which were extracted after alkaline hydrolysis. In addition 0.125ml 2M NaOH was added to the supernatant and the tubes were sealed and allowed to stand at room temperature in a dark cupboard. After hydrolysis, the tubes were cooled to -4ºC for 30min before addition of 60µl of 1M HCl. The phenolics were extracted three times as described above.

Determination of glycoside-bound phenolic content: The third sample was hydrolysed in 50µl concentrated HCl for 1 hr at 96° C, in a water bath, and thereafter extracted three times with equal volumes of anhydrous diethylether as described previously.

3.2.9.3 Quantification of total soluble phenolic acids from *Bacillus licheniformis* extracts

The concentration of total phenolic compounds in the first sample was determined using the Folin-Ciocalteau reagent (Swain and Hillis, 1959). The volumes were modified for the wells in the microtiter plates. Four replicates of the extract (5µl) was diluted to 175µl, with sterile distilled water, and added to 25µl of Folin-Ciocalteau reagent (Sigma) and mixed. After three min, 50µl of aqueous sodium carbonate (Saarchem) (20% w/v) was added, mixed thoroughly and incubated at 40°C for 30 min. Distilled water was used as a blank. The absorbance was read at 690 nm with an Elisa plate reader (Multiskan Ascent VI. 24 354-00973, Version 1.3.1). Gallic acid (Sigma) was used as a phenolic standard to construct a standard curve ranging from 0-400µg, r^2 = 0.9989. The concentration of phenols in the various extracts was calculated from the standard curve and expressed as mg gallic acid equivalent g^{-1} dry weight.

3.2.9.4 High Performance Liquid Chromatography

Bacillus licheniformis extracts were prepared as described earlier. These samples were analysed on a Varian HPLC Prostar 9010 equipped with a 20µl loop injection valve. A Varian Chrompak C18 reverse-phase column (250 x 4.6 mm, 5ul particle size) was used. Data was analysed by STAR chromatography system software. The following conditions were used: flow rate of 1ml/min, linear gradient of 10-45% acetonitrile (Saarchem) within 30 min, followed by 70% (v/v) acetonitrile-water (0.1% phosphoric acid, Saarchem) within 90 min. Elution was monitored at 280 and 320 nm with a diode array UV monitor.

3.2.10 Factors effecting mode of action of *Bacillus licheniformis*

3.2.10.1 Temperature

The antagonist and pathogens were maintained and prepared as described previously. In order to determine the effect of temperature on the activity of the antagonist, PDA plates were inoculated with the pathogen and antagonist according to the pattern illustrated in Fig. 3.1. Plates were incubated at the following temperature regimes: 6, 10, 25, 37 and 40ºC. Radii of the mycelium growth was measured daily, up to seven days, using digital callipers and expressed in mm inhibition values. Each set consisted

of five plates and this experiment was repeated three times. Data was analysed as described previously (3.2.3).

3.2.10.2 Additives

The effect of calcium carbonate and -chloride (Fluka, Johannesburg) were tested on the growth of the pathogens. Calcium carbonate and -chloride at a concentration of 0.5% and 1.0% (w/v) respectively were mixed in sterile distilled water (0.5g/500ml media) and added to PDA plates. Plates were inoculated as shown in Fig. 3.1 and incubated at 25°C and 10°C. Plates without calcium carbonate or -chloride served as controls. Plates were assessed after 3, 5 and 7 days for changes in growth patterns and rate compared to the control. The radii of mycelial growth were measured in mm using digital callipers. Each set consisted of five plates and this experiment was repeated three times. Data were analysed as described previously (3.2.3).

3.3 Results

All data was acceptably normal, but with heterogenous treatment variances.

3.3.1 Dual Culture technique

By challenging the pathogens (*C. gloeosporioides* and *B. parva*) with *B. licheniformis,* a significant decrease in radial growth was observed (Fig. 3.3). Of the two pathogens screened, the former was more effectively inhibited.

3.3.2 Antibiotic Production

Inhibition zones were noted after seven days for both pathogens. Only the 25% dilution of the antagonist extract was effective in inhibiting the growth of both pathogens compared to the controls (Table 3.1). In the case of *B. parva,* 50% was also an effective dilution point.

3.3.3 Production of Volatiles

The radial growth of both pathogens was equally effectively inhibited by *B. licheniformis* volatiles (Fig. 3.4).

3.3.4 Optimum concentration of *Bacillus licheniformis* effective against the pathogens

A concentration of 10^7 cells/ml was identified as the optimal concentration of the antagonist that was effective against both the pathogens. Results not illustrated, as there were no significant differences between the other dilutions (Pr = 0.001).

3.3.5 Role of hydrolytic enzymes in mode of action of *Bacillus licheniformis*

In the presence of the antagonist, the pathogen was unable to hydrolyse complex compounds such as chitin and cellulose as compared to the control (Fig. 3.5).

3.3.6 Role of siderophore production in antagonism

The extraction of iron from the RSM-CAS medium by *C. gloeosporioides* and *B. parva* were restricted when the pathogen was grown together with the antagonist. (Fig. 3.6).

3.3.7 Separation of bioactive compounds from *Bacillus licheniformis*

Results from the HPLC showed that a peak was present on the chromatogram for the ten-day sample when compared to the control. The elution of the peak at 22 min is an indication of the presence of a compound produced by the antagonist that is not present in the media (Fig. 3.7).

3.3.8 Factors affecting mode of action of *Bacillus licheniformis*

3.3.8.1 Temperature

Of the temperature ranges that were evaluated, only the plates kept at 10°C showed a significant decrease in the growth of the pathogens compared to the control. At this temperature the antagonist inhibited the growth of *C. gloeosporioides* totally compared to the control (Fig. 3.8).

3.3.8.2 Additives

For plates stored at 10°C there was no significant reduction in growth rate. Results therefore not presented (Pr = 0.107). Calcium carbonate was found most effective in enhancing the effect of the antagonist by inhibiting the growth of *C. gloeosporioides,*

when compared to the control at room temperature (Pr = 0.001). In contrast, *B. parva*'s growth was not significantly affected (Fig. 3.11).

3.4 Discussion

The antagonistic potential of *B. licheniformis* was confirmed in this study. This was in agreement with the initial screening assay done *in vitro* by Burger and Korsten (1988). Both the radial growth of *C. gloeosporioides* and *B. parva* was significantly restricted *in vitro* in comparison with their respective controls when using the dual culture assay. Previous studies described a similar *in vitro* dual culture activity for *Bacillus* spp. (Pusey and Wilson, 1984; Singh and Deverall, 1984; Utkhede and Scholberg, 1989). Although this technique has previously been described as inadequate for initial biocontrol screening (Jijakli *et al.*, 2000), it can still rapidly provide insight into possible modes of action that include antibiosis, production of volatiles by the antagonist and production of siderophores in the process of iron uptake.

In the antibiosis assay, the inhibition zones were only noted around the pathogen after seven days at a twenty-five percent dilution of the antagonist. This is important in terms of application time, concentration and method. The interaction of the antagonist and pathogen *in situ* does not always lead to immediate inhibition of the pathogen due to possible higher, less effective concentrations of secondary metabolites being produced. Microorganisms are capable of producing different types of secondary metabolites (Fravel, 1988; Atlas and Bartha, 1998) and antibiotics (Mc Keen *et al.*, 1986; Emmert *et al*., 2004) that are inhibitory to other microorganisms (Cho *et al*., 2003). The concentration of antagonists that was most inhibitory to the pathogens was found to be 10^7 cells/ml. It was therefore shown in this study that *B. licheniformis* had a competitive *in vitro* growth advantage against the pathogens.

The production of volatile compounds by bacteria and fungi is also reported to play an important role in biocontrol (Jiang *et al*., 2001; Mercier and Jimenez, 2004). The same bacillus species as was used in this study, has previously been reported to produce a volatile, toxic to various other bacterial species (Tomas-Barberan and Robbins, 1997; Mikkola *et al.*, 2000). From the results we can deduce that after seven days of incubation, the antagonist did produce a volatile compound that was

inhibitory to both pathogens, when compared to the control. For *B. parva*, which is a fast growing pathogen, twenty percent decrease in growth compared to the control was significant.

Studies of competition as a mode of action has been lacking in terms of fruit diseases (Chalutz *et al.*, 1998; Wilson and Chalutz, 1989, McLaughlin *et al.*, 1990). Competition was evaluated *in vitro* by assessing the ability of the pathogen and antagonist to hydrolyse complex compounds (starch, chitin and cellulose) and more importantly, to test for the ability of the pathogen to hydrolyse these products in the presence of the antagonist. The growth of the pathogens was restricted when cultured together with the antagonist, implying competition for essential nutrients from the medium. The ability of *B. licheniformis* to produce extracellular amylases and proteinases was confirmed in this study. Previous studies by Edwards (2001) reported that selection of *B. licheniformis* and other Bacillus spp. was ideal to compete with *Vibrio* spp. since it was able to utilize certain micronutrients required by this organism.

In addition, iron uptake from the medium is important for growth of certain microorganisms (Crosa and Walsh, 2002). Competition for iron is reported to be one of the modes of action by which fluorescent *Pseudomonas* limits the growth of pathogenic fungi and reduce disease incidence and severity (Alabouvette and Lemanceua, 1999). Siderophores are excreted into the medium to remove iron (Neilands, 1981; Schwyn and Neilands, 1987). In this study, the antagonist and pathogen were cultured together on an iron-rich medium. The antagonist produced siderophores within 24 hours thereby removing iron from the medium and restricting the growth of the pathogens.

From the preliminary HPLC results we observed the presence of a phenolic compound produced by the antagonist after ten days incubation at room temperature. This compound has a maximum absorbance at 320nm and is present only in the sample extracted with diethylether. This result showed that the bacillus is able to produce a compound from the hydroxycinnamic family. The separation of this compound using HPLC confirms results obtained with both the antibiotic and volatile assays.

Temperature studies are important when considering the interaction between the antagonist and pathogen *in situ* (Hetherington, 2000). Total inhibition of *C. gloeosporioides* was observed when *in vitro* dual culture plates were incubated at 10ºC. This is of major importance since mango fruit destined for the export markets are handled in the cold chain at temperatures of 10-11ºC.

In this study, two calcium salts (calcium chloride and calcium carbonate) was tested for their ability to enhance the activity of the antagonist. A one percent solution of calcium carbonate was effective in reducing the growth of *C. gloeosporioides*, when combined with the antagonist and compared with the control. Calcium salts are known to play a vital role in increasing membrane stability and cell wall strength as well as enhancing the activity of the antagonist (Conway *et al.*, 1999, Hetherington, 2000; Fan and Tian, 2001; Droby *et al*., 2003; Wszelaki and Mitcham, 2003).

The results presented in this study offer a better understanding of *B. licheniformis* modes of action. These observations confirm earlier reports that antagonists may exhibit more than one mode of action such as antibiosis together with competition for micronutrient (Korsten *et al.*, 2000; Leverentz *et al.*, 2003). In nature not only one mode of action function and these are independent of the other Further in depth studies should focus on characterising the inhibitory products and evaluating different modes of action at lower temperatures to fully understand *in vitro* activity.

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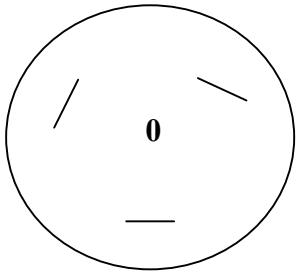
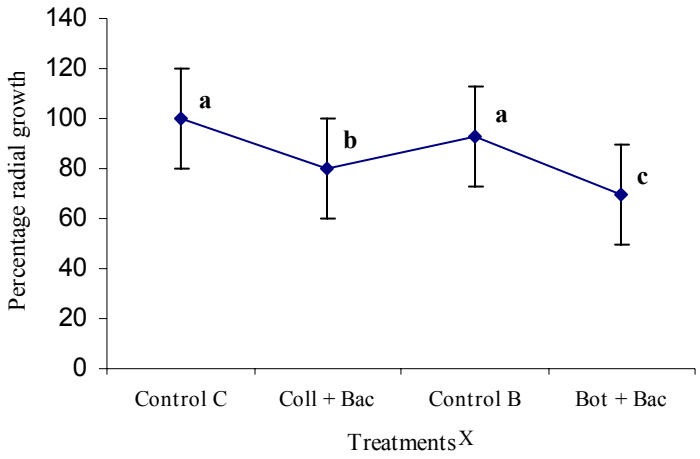


Fig. 3.1 Diagrammatic illustration of *in vitro* screening assays. The lines indicate three replicates of *Bacillus licheniformis* (20mm streaks), 35mm from the central fungal plug (**0**, used as the test isolate).

Pathogen

		1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
		10^0			10^3				10^4			10^5	
Control	10^0												
		B											
Antagonist	10^5	**C**											
		D											
	10^6	**E**											
		F											
	10^7	**G**											
		H											

Fig. 3.2 Schematic representation of inoculation pattern on Elisa plates. The shaded areas are controls for the various concentrations.



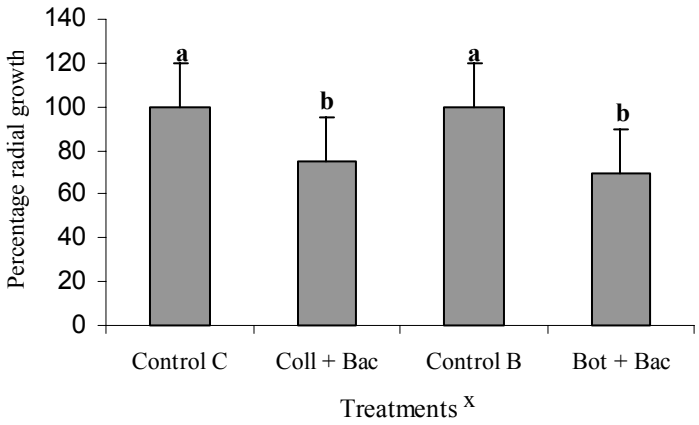
Percentage radial growth of *Colletotrichum* or *Botryosphaeria* when grown in dual culture with *Bacillus licheniformis* as compared to growth of the control. [x] Coll = *Colletotrichum*, Bot =*Botryopshaeria* and Bac = *B. licheniformis*. Different letters above data points indicate significant differences between treatments [Pr = 0.100]

Fig. 3.3 *In vitro* effect of *Bacillus licheniformis* on the growth of *Colletotrichum gloeosporioides* and *Botryosphaeria parva* when grown on potato dextrose agar.

Table 3.1 The effect of various concentrations of *Bacillus licheniformis* filtrate on the growth of *Colletotrichum gloeosporioides* and *Botryosphaeria parva*

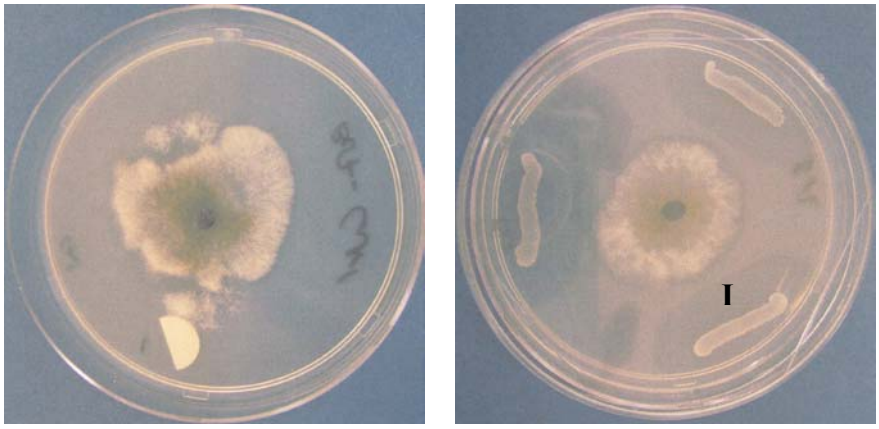
Treatments	**Inhibition zones noted after seven days[x]**	
	Colletotrichum gloeosporioides	*Botryosphaeria parva.*
Positive Control	-	-
Negative Control	-	-
100 % dilution	-	-
50 % dilution	-	+
25 % dilution	+	+
12.5 % dilution	-	-
6.25 % dilution	-	-

[x] The "-" indicates no inhibition zones and "+" indicates the presence of an inhibition zone.



Percentage radial growth of *Colletotrichum* and *Botryosphaeria* inhibited by *Bacillus licheniformis*.[x] Control C = *Colletotrichum* control, Coll = *Colletotrichum*, Bac = *Bacillus licheniformis*, Control B = *Botryosphaeria* control and Bot = *Botryosphaeria*. Data points containing the same letters did not differ significantly [Pr =0.348]

Fig. 3.4 Effect of *Bacillus licheniformis* volatiles on the *in vitro* growth of *Colletotrichum gloeosporioides* and *Botryosphaeria parva*.



A **B**

Fig. 3.5 Effect of *Bacillus licheniformis* on the ability of *Colletotrichum gloeosprioides* to hydrolyse chitin medium (**A**) compared to the control. Zones of inhibition (**I**) around the antagonist indicate the ability of the antagonist to produce chitinase (**B**).

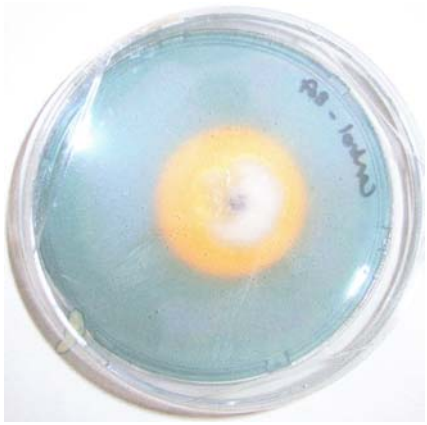


Fig. 3.6 Siderophore production by *Colletotrichum gloeosprioides* forty-eight hours after inoculation.

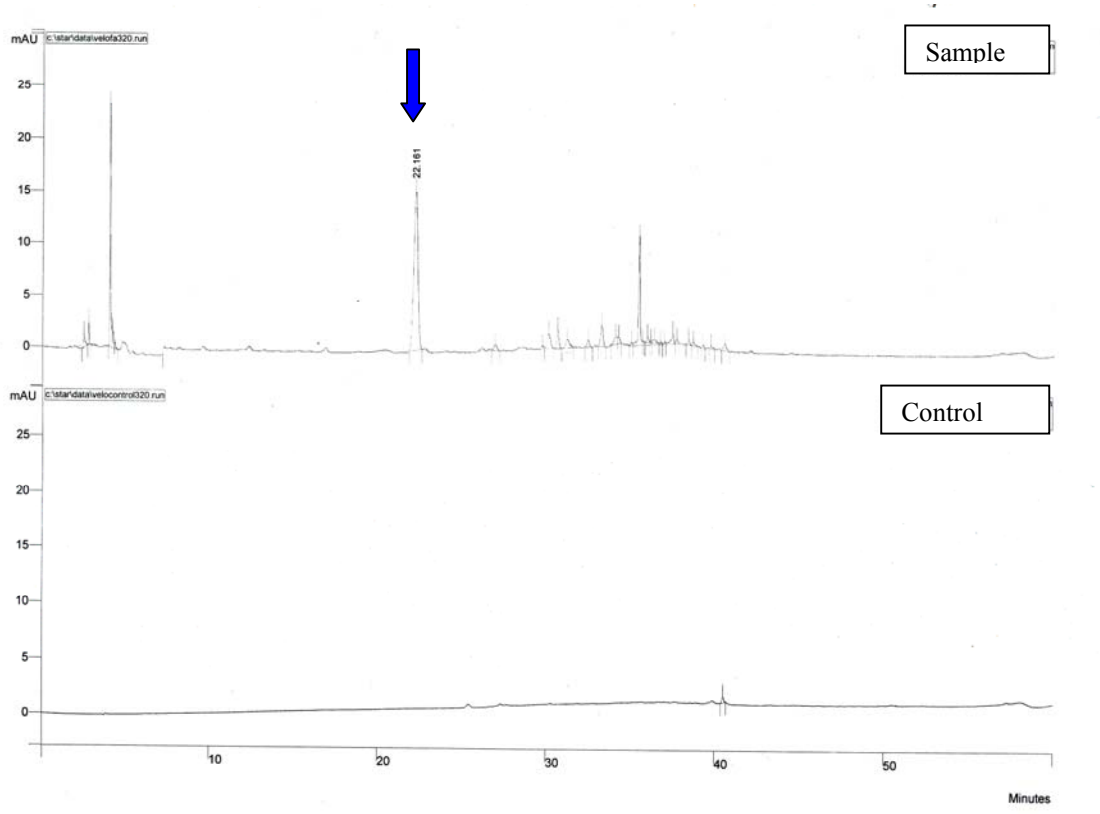
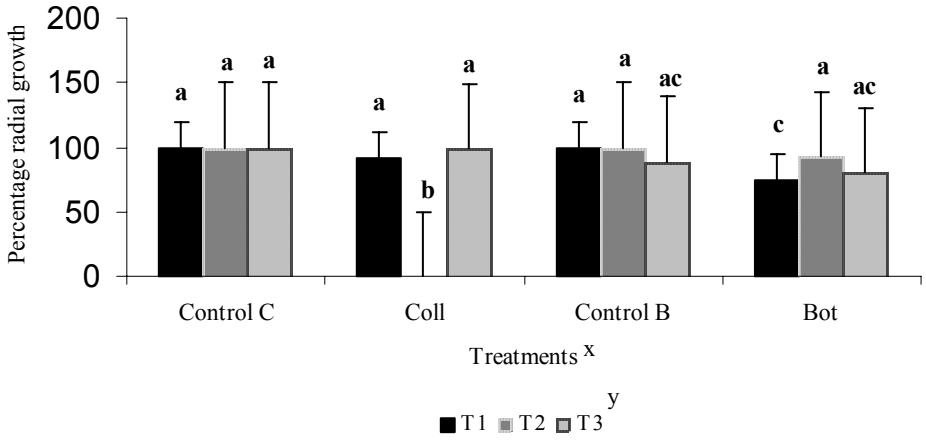
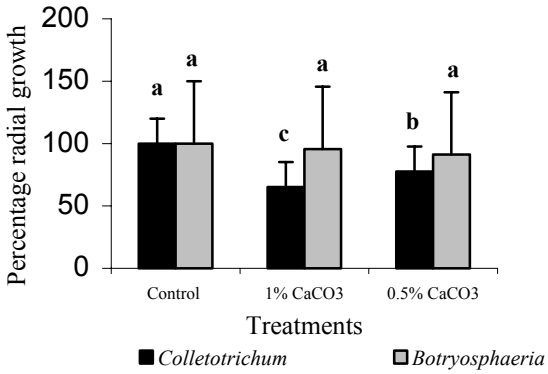


Fig 3.7 Chromatograms showing elution of bioactive compound from *Bacillus licheniformis* extract at 22 min when compared to control where there was no elution of a compound.
Separation was achieved through High Performance Liquid Chromatography.



Comparison of the growth of *Colletotrichum* and *Botryosphearia* spp when grown under different temperature conditions. [x]Control C = *Colletotrichum* control, Coll = *Colletotrichum* + Bacillus, Control B = *Botryosphaeria* control and Bot = *Botryosphaeria* + Bacillus. [y]T1 = 6ºC (Pr = 0.105), T2 = 10ºC (Pr <0.001) and T3 = 25ºC (Pr = 0.005)

Fig. 3.8 Influence of different temperatures on *Bacillus licheniformis* inhibition of *Colletotrichum gloeosporioides* and *Botryosphaeria parva*.



Comparison of the growth of both pathogens (*Colletotrichum* and *Botrysphaeria*) on Potato dextrose agar when calcium salts was incorporated into PDA. Control plates showed 100% growth. Control = PDA plates without Calcium carbonate and CaCO3= Calcium Carbonate. Bars containing the same letters do not differ significantly [Pr <0.001].

Fig. 3.9 Effect of adding calcium carbonate to potato dextrose agar, when *Bacillus licheniformis* and *Colletotrichum gloeosporioides* and *Botrysphaeria parva* are grown together.

CHAPTER 4

Evaluation of *Bacillus licheniformis* biocontrol potential *in vivo*

Abstract

Bacillus licheniformis isolated from the mango phylloplane, in South Africa, was successfully evaluated *in vitro,* for its ability to inhibit *Colletotrichum gloeosporioides* and *Botryosphaeria parva.* Different concentrations of the antagonist and pathogens were cross-checked *in situ* to determine an optimum dosage for biocontrol evaluation. The minimum concentration where the antagonist could still effectively inhibit both pathogens *in vivo* was 10^7 cells/ml. The microbial ecology on green mango, after fruit were treated in a dip solution of the antagonist, was compared to dip-treated ripened fruit. The yeasts, fungal and total microbial counts increased as fruit ripened. Scanning electron microscopy studies revealed that wax application provided an effective means of trapping fungal spores and confirmed that the antagonist attaches to the surface of the mango fruit. The antagonist could effectively be recovered from the mango fruit at 5 log units decreasing to 3 logs at the onset of fruit ripening. Temperature studies confirmed that even though the antagonist is effective at ambient temperature it was most effective in inhibiting the pathogens at cold storage conditions. Additives in combination with bacillus were evaluated for providing an enhanced activity of the bacterium. Mango fruit treated with a combination of calcium carbonate and the antagonist gave a lower disease incidence when compared to the untreated control. This study confirmed that *B. licheniformis* is antagonistic *in vivo* towards the mango post-harvest fruit pathogens.

4.1 Introduction

Over the last decade, the usage of beneficial bacteria and yeasts to control post-harvest fruit diseases has increased (Roberts, 1990; Chand-Goyal and Spotts, 1995; Janisiewicz, 1998; Droby *et al.,* 1999). The dominant bacterial species on the fructoplane was reported to belong to the genus Bacillus (80%) (Arrias, 1999; De Jager, 1999). Further the consistent recovery of *Bacillus spp* from the fructoplane during fruit growth and development indicated that this species was a permanent and dominant coloniser of the fructo-microflora making it an ideal microorganism for biocontrol programs (McGuire, 1998; Arias, 1999).

The knowledge of the mode of action of these naturally occurring biocontrol agents is however often limited. This is mainly due to the complexity of the mechanisms involved, lack of follow-up research and assumption that successful antagonist/pathogen interactions *in vitro* will occur *in vivo* (Smilanick *et al*., 1993; Janisiewicz, 1998). Most assays are performed *in vitro* and essential screening was generally not conducted *in vivo* (Janisiewicz, 1998; Emmert and Handelsman, 1997).

Competition for nutrients has been suggested to play an important role in *in situ* antagonism. Bacteria and yeasts that occur naturally on the fructoplane can be used or managed to compete against pathogens for nutrients in the exudates (McGuire, 1998, Pingsheng and Wilson, 2002). Even *in vivo,* biological control agents must be effective under a wide range of temperatures and storage atmospheres (Spotts and Sanderson, 1994). One method of ensuring that the activity of the biocontrol agent on the fructoplane is enhanced is to add these antagonists to coatings that are often applied to fruits and vegetables. The coatings must be formulated in such a manner to ensure the growth of these biocontrol agents (McGuire, 1998; Wilson and El Ghaouth, 2000).

Screening of naturally occurring mango epiphytes was initiated in South Africa in 1987 as part of a biological control program on mangoes. Amongst the potential antagonists isolated, *Bacillus licheniformis* were the most effective *in vitro* and was further successfully tested in pre- and post-harvest trials to control anthracnose and stem-end rot (SR) (Korsten *et al*., 1991; Korsten *et al*., 1992; Korsten *et al*., 1993a; De Villiers and Korsten, 1994; Govender and Korsten, 2001).

It has been suggested by Cladera-Olivera and co-workers (2003) that *B. licheniformis* acts against fungi by producing both an antibiotic agent and an anti-fungal enzyme, *in vitro* and *in vivo*. A bacteriocin-like substance produced by *B. licheniformis* was characterized presenting a broad spectrum of activity against pathogenic and spoilage organisms (http://www.epa.gov/pesticides/biopesticides.htm). In Chapter three *in vitro* studies indicated that *B. licheniformis* may act by means of antibiosis and production of a volatile compound. The aim of this chapter was to confirm if the antagonist was effective *in vivo* against the pathogens *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc. in Penz. and *Botryosphaeria parva,* previously known as *Dothiorella dominicana* (Petrak and Cif).

4.2 Materials and methods

4.2.1 Antagonist

The antagonist, *B. licheniformis* (isolate Mal) was previously isolated from the mango phylloplane was obtained from the bacterial culture collection of Plant Pathology Laboratories, University of Pretoria and was maintained on nutrient agar (Merck, Johannesburg), which was supplemented with 0.1 % cycloheximide (Sigma, Johannesburg). Preparation of the antagonist was done as described in the previous chapter.

4.2.2 Pathogen

A virulent culture of *C. gloeosporioides* was obtained from the fungal culture collection of Plant Pathology Laboratories, University of Pretoria. This pathogen was originally isolated from mango fruit with symptoms of anthracnose. An isolate of *Botryosphaeria parva,* culture originally isolated from twigs of mango trees in Hoedspruit. This pathogen was isolated and identified by Ms. Leylani Grobler (Plant Pathology Laboratories, University of Pretoria). This fungus was subsequently tested for its pathogenicity on mango fruit and both the pathogens were maintained on potato dextrose agar (PDA) supplemented with chloramphenicol (Rolab, Johannesburg), unless otherwise mentioned. Pathogens were prepared as described for the previous chapter.

4.2.3 Minimum inhibitory concentration of the antagonist effective against the pathogens

The antagonist and pathogens were prepared for this test as described in sections 4.2.1 and 4.2.2 respectively. This experiment was done using freshly harvested mango fruit, cultivar Tommy Atkins following a checker-board titration type method (Fig. 4.1) (Korsten, 1993b). Fruit were surface sterilized for 1 min using 70% ethanol. Blocks of 10mm x 10mm were made on the fruit surface, using a scalpel. Fruit were inoculated by first wounding it (5mm into fruit), in the middle of the block using a sterile microfine needle (Becton and Dickinson). Aliquots (100µl) of the appropriate dilution were added into each block. The antagonist was inoculated first and 16 hours later the pathogen. Controls were blocks inoculated with either the pathogen or antagonist on its own. A control, consisting of distilled water only was included. Fruit were stored either at ambient (25ºC) or at cold storage (10ºC) temperatures. Fruit were monitored daily for lesion development which was noted as presence or absence of lesions. The blocks on each fruit were done in triplicate and fruit packed into boxes. This entire was done in triplicate and repeated three times. Data were analysed using the statistical program GenStat (2000). Analysis of variance (ANOVA) was used to test for differences between treatments. The angular transformation for percentages was used to stabilise treatment variances. Treatment means were separated using Fishers' protected t-test least significant difference (LSD) at the 5% level of significance.

4.2.4 Population dynamics on mango fruit surface

Two sub-experiments were conducted: 1) to confirm the presence of the different epiphytes on the fructoplane and 2) to assess the recovery of the antagonist from the fruit surface. Week old cultivar Keitt fruit was used for this assay. Ten fruit were dipped in a solution of the antagonist at 10^7 cells/ml. The microbial ecology of green fruit, that was dip treated was compared to that of fruit with no dip treatment. In addition some fruit that had been dipped were left to ripen, at ambient temperature, so that the microbial ecology of both green and fruit that had ripened could be compared. Five fruit were washed when still green in colour, five were left for two weeks, to ripen prior to washing. Fruit were placed in a beaker containing ¼ strength Ringer's supplemented with 0.001% Tween (Fluka chemicals, Johannesburg). The beaker with fruit was vortexed for four min on Heidolph Reax vortex. The beaker, containing the

fruit, was placed in an ultrasonic bath (UMC5, Ultrasonic manufacturing company, Krugersdorp) and sonicated for 30sec. The washing solution was serially diluted and plated onto NA supplemented with cycloheximide (0.1%) (Sigma) and PDA, supplemented with Rifampicin (Rolab). This experiment was done in triplicate and repeated three times. Plates were incubated at 25ºC. Total bacterial, yeasts and fungal colonies were counted and species diversity noted and data was analysed as described previously. In the second part of the experiment the recovery of the antagonist from fruit after dip was compared to that of fruit that had ripened, prior to washing. This was done using the above described standard dilution series. This aspect was conducted concurrently with preliminary packhouse trials, described in the next chapter. Therefore the epiphytes on fruit treated with the standard commercial packhouse treatments (Table 4.1) were compared in green fruit to ones that had ripened. Bacterial, yeasts and fungal counts were done and expressed as colony-forming units/ml. Data was analysed as previously described (4.2.3).

4.2.5 Attachment of *Bacillus licheniformis* on fruit surface

In order to study the attachment of the antagonist to the fructoplane fruit that had been treated with the standard commercial packhouse treatments were used for this experiment. Excised mango peel pieces (cultivar Tommy Atkins) were dehydrated. Five (5mm X 5mm) samples were dried in a Hitachi HCP-2 critical point dryer and mounted on aluminium stubs. Specimen stubs were coated with gold palladium in an Eiko IB-3 ion coater. Stubs were viewed in a Hitachi 840 JEOL SEM operating at 5kV. In addition mangoes that had been dip treated as described above were prepared for SEM as described. Ripened fruit were also prepared for SEM and in addition to evaluating whether there was attachment of the antagonist to the fruit surface. In addition fruit treated with wax (Citrashine) used at the packhouse was prepared for SEM as described.

4.2.6 Factors affecting mode of action of *Bacillus licheniformis*

4.2.6.1 Temperature

Antagonist and pathogens were prepared as described in 4.2.1 and 4.2.2 respectively. Fruit were inoculated with as described previously in 3.2.10.1 and stored at ambient (25ºC) and cold storage (10ºC) temperatures. Fruit were monitored daily for lesion

development and the presence or absence of lesions was noted. This experiment was done in triplicate and data was analysed as described for previously (4.2.3).

4.2.6.2 Additives

Untreated mango fruit (cultivar Tommy Atkins) were used to assess the effect of calcium carbonate on the *in vivo* efficacy of the antagonist. Fruit were dipped in a Bacillus (10^7 cells/ml) suspension for three min and left to air dry before being dipped into a solution of calcium carbonate (1%) (w/v). Fruit were air-dried and then packed into boxes (six per box). Half the boxes were left at room temperature and the other half in cold storage (10º C). Fruit were monitored daily for lesion development and were evaluated for the onset of anthracnose and SR. Disease rating for anthracnose and SR were manually done using a 0-5 scale with zero indicating healthy fruit and 5 indicating total decay of fruit. Data of this experiment was analysed as described previously (4.2.3).

4.3 Results

The data was acceptably normal, but with heterogenous treatment variances.

4.3.1 Minimum inhibitory concentration of the antagonist effective against the pathogens:

For fruit stored at cold storage the optimum concentration at which the antagonist was effective was 10^7 cells/ml (Pr = 0.101). For fruit kept at room temperature there was lesion development on the checker-board titration that was prominent on the fruit and therefore the results could not be presented due to no significant differences between the concentrations (Pr = 0.405).

4.3.2 Population dynamics on mango fruit surface

The yeast and fungal counts increased as fruit ripened (Fig. 4.2). Although the total microbial count was higher on the surface of green fruit than ripened fruit (Fig. 4.2). In addition recovery of the antagonist was confirmed by bacterial counts done on fruit after treatment and on fruit that had ripened. Recovery of the antagonist was higher on green fruit than fruit that had ripened but was still higher than control (Fig. 4.3).

4.3.3 Attachment of *Bacillus licheniformis* on fruit surface

The attachment of the antagonist to the fruit surface was confirmed by SEM studies (Fig. 4.4A). This study also confirmed that the wax used on the fruit provides an ideal area for the fungal spores to attach and germinate (Fig. 4.4B).

4.3.4 Factors affecting mode of action of *Bacillus licheniformis*

4.3.4.1 Temperature

Storing fruit at different temperatures revealed that lesion development was more prominent on fruit stored at room temperature than those stored first under cold storage conditions (Table 4.2). Results are presented as presence or absence of lesion development (Pr = 0.407).

4.3.4.2 Additives

Calcium carbonate was effective in enhancing the activity of the antagonist *in vitro* and *in vivo*. The calcium solution did enhance the activity of the antagonist and was more effective than the treatment consisting of the antagonist on its own (Fig. 4.5).

4.4 Discussion

In this chapter the effectiveness of *B. licheniformis* was tested *in vivo*. Previous studies have been focused on preliminary *in vitro* testing as well as pre- and post-harvest trials using this isolate of *B. licheniformis* (Korsten *et al*., 1993a; De Villiers and Korsten, 1994). The experiments reported in this chapter were designed to assess effectiveness of *B. licheniformis* on the fructoplane. When different concentrations of the antagonist were evaluated for their efficacy against different concentrations of the pathogens, the minimum inhibitory concentration was found to be 10^7 cells/ml. This optimum concentration has previously been confirmed for *B. subtilis* in studies conducted by Korsten and co-workers (1993c).

The evaluation of the epiphytes on the fructoplane showed that the yeast and fungal epiphytic populations increased on the fruit surface that had ripened when compared to green fruit. Apart from dominant bacterial species (*Bacillus* and *Pseudomonas*), the phylloplane epiphytes consisted of mainly yeasts i.e. *Aureubasidium*, *Candida* and *Cryptococcus* and fungi *Alternaria alternata*, *Penicillium* spp. and *Trichoderma* spp. Population studies confirmed results obtained previously (De Jager, 1999), in which

the organisms on the leaf and fruit surface were compared. It was reported that the bacterial population was higher on the fruit than leaf surface. Yeast and filamentous fungi made up a smaller percentage of the total microbial population but increased as fruit ripened as confirmed in this study. The importance of all the epiphytes present on the fruit surface was illustrated in a study conducted by Fallik and co-workers (2000) where it was discovered that hot water brushing of mangoes improved general appearance but also caused a 3 to 4 log reduction of the ephiphytic pathogen population. This is significant since recovery of the antagonist from the fructoplane after fruit had been treated could be confirmed in this study. Comparison of treated green versus ripe fruit showed a 2 log unit reduction of the antagonist concentration. Apart from inhibitory effects the antagonist has on the pathogens, it should also establish on the fruit surface and be able to have a competitive advantage over the rest of the fruit population.

Scanning electron microscopy studies revealed that the antagonist is able to attach to the fructoplane, displaying some biofilm formation. This was previously reported for *B. licheniformis* by studies conducted by Silimela (2003). According to Marques and co-workers (2002), biofilm formation is a major virulence factor in diseases. It is known that differences in attachment times of *Bacillus subtilis* were explained by various attachment and subsequent colonization phases, which included reversible and irreversible attachment (Towsen, 1996). This study also revealed that the wax applied to fruit in the packhouse provided an effective medium to trap fungal spores. This could explain why the antagonist is sometimes ineffective in ensuring effective control. In this study, successful recovery of the antagonist was found after treatment compared to the untreated control. This was similarly found in previous studies, using *B. subtilis* (Towsen, 1996; Guetsky *et al.,* 2001).

In this study, the results obtained in the previous *in vitro* study was confirmed where the antagonist was most effective in inhibiting the pathogens at 10ºC. Treating fruit with the antagonist and calcium carbonate enhanced the activity of *B. licheniformis.* This was evident when comparing the integrated treatment with the untreated control and the antagonist on its own. However, the calcium-bacillus treatment was most effective for fruit kept at room temperature. In a study by Bernal and co-workers (2002) calcium ions at a level of 0.5% had a significant effect on antibiotic production

of *B. subtilis* by promoting the production of antibiotics. As this study confirmed that the antagonist is effective on the fruit surface, further studies should focus on establishment of the antagonist on the fructoplane during the different phases of fruit ripening. Other aspects could include the effect the antagonist could have on induced resistance in the mango fruit. It is imperative that *B. licheniformis* is able to function in both environments. The next step of this study is to evaluate the feasibility of incorporating the antagonist in a commercial packhouse line, integrated with the other packhouse treatments.

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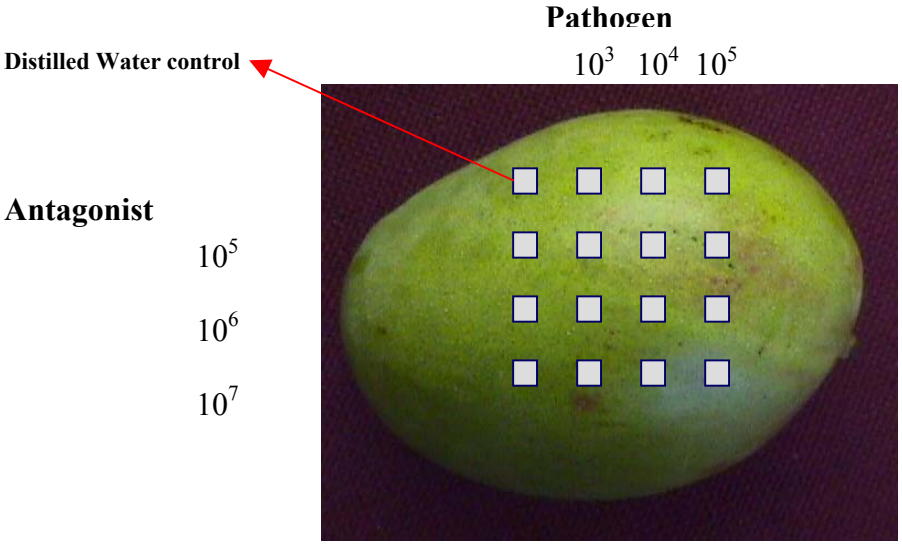


Fig 4.1 Diagrammatic illustration of the checker-board titration inoculation method used on mango fruit.

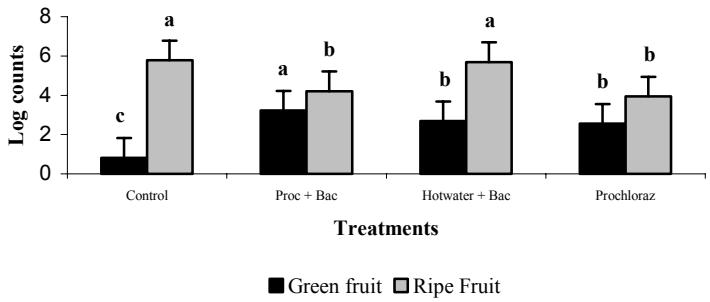
Table 4.1: Post-harvest treatments that mango (cultivar Tommy Atkins) fruit was subjected to prior to population dynamic studies

Treatments[a]	**Hot water bath**	**Prochloraz**	**Wax**	***Bac* (powder)**
Control	-	-	-	-
Prochloraz	+	+	+	-
Bacillus	+	-	-	+
Bac + Prochloraz	+	+	+	+

[a] Bac = *Bacillus licheniformis*

All chemicals were used at the manufactures recommended rates as described in Chapter five.

X



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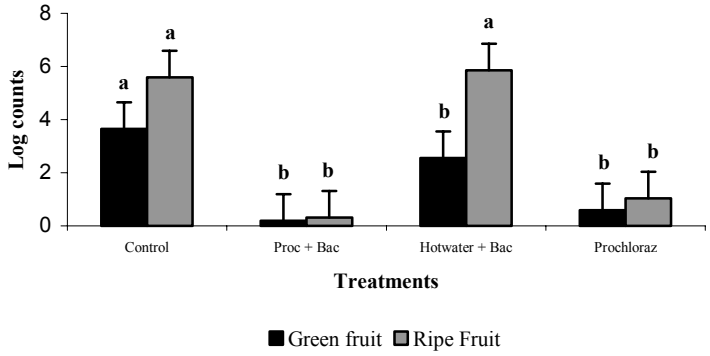

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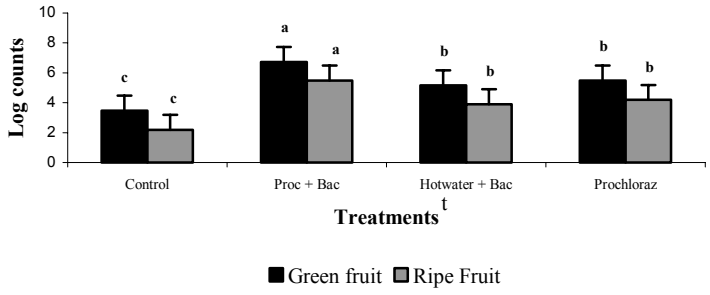
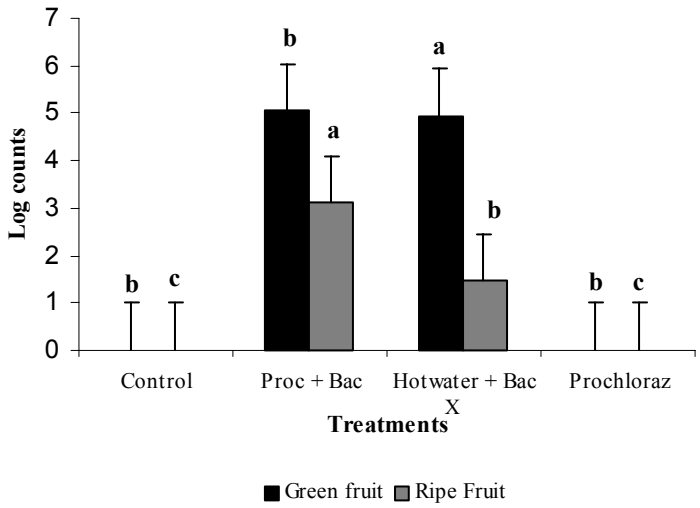


Comparison of the changes in the number of microbial epiphytes on the surface of green mangoes and those that have ripened. X) Yeasts counts; Y) Fungal counts and Z) Total microbial count. [t]Proc = Prochloraz; Bac = *B. licheniformis*. Bars with same letters did not differ significantly (Pr < 0.001).

Fig. 4.2 Comparison of microbial epiphytes present on the surface of green and ripe mango fruit after dip treatment in *Bacillus licheniformis and* Prochloraz.



Comparison of the different treatments and the bacterial counts after treatment and after fruit had ripened. [X] Proc + Bac = Prochloraz + *Bacillus licheniformis*; Hotwater + Bac = Hotwater (55°C) with *B. licheniformis*. Bars with same letters did not differ significantly (Pr <0.001).

Fig. 4.3 *Bacillus licheniformis* recovery rate for the different treatments, compared to the untreated control.

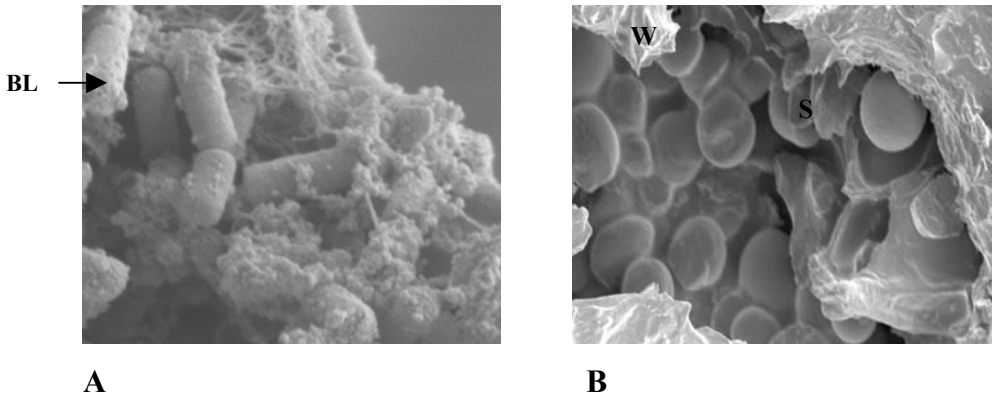
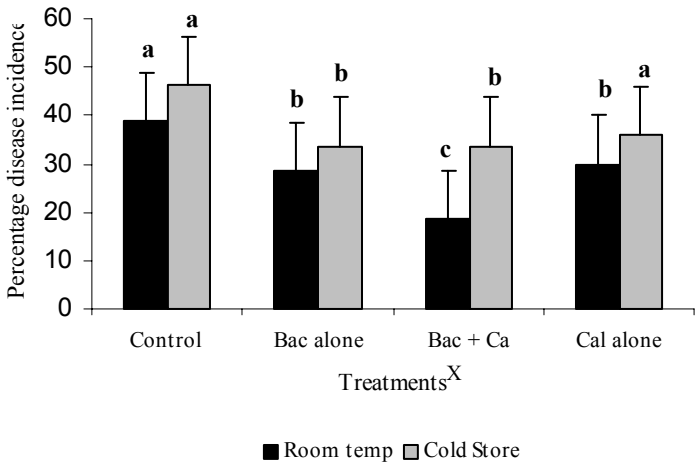


Fig. 4.4 Attachment of *Bacillus licheniformis* (**BL**) on the surface of mango fruit showing a matrix of bacteria (**A**) and Fungal spores (**S**) trapped inside the wax covering (**W**) on the surface of mango fruit after fruit has been waxed in the packhouse. (A= 12 000 and B = 1600 X magnification)

Table 4.2: The *in vivo* effect of various concentrations of *Bacillus licheniformis* on pathogen development on mango fruit (cv Keitt)

Concentrations comparisons	**Presence or absence of lesion development[x]**			
	Colletotrichum gloeosporioides		***Botryosphaeria parva***	
	Room Temp	Cold Store	Room Temp	Cold Store
Pathogen control	+	+	+	+
Antagonist control	-	-	-	-
Distilled water control	-	-	-	-
$P10^3A10^5$	+	+	+	+
$P10^3A10^6$	+	+	+	-
$P10^3A10^7$	-	-	-	-
$P10^4A10^5$	+	+	+	+
$P10^4A10^6$	+	+	+	-
$P10^4A10^7$	-	+	+	+
$P10^5A10^5$	+	+	+	+
$P10^5A10^6$	+	+	+	-
$P10^5A10^7$	-	+	-	+

[x] P = Pathogen, A = Antagonist, Positive (+) = indicates lesion development and negative (-) indicates no lesion development.



Comparison of treatments using calcium carbonate with those with the antagonist alone. [X] Control = untreated fruit; Bac = *Bacillus licheniformis,* Ca = calcium carbonate and Cal = calcium alone. Bars containing the same letters did not differ significantly (Pr < 0. 0001).

Fig. 4.5 Effect of calcium carbonate on the efficacy of *Bacillus licheniformis in vivo.*

CHAPTER 5

Semi-commercial evaluation of *Bacillus licheniformis* for control of mango post-harvest diseases

Abstract

Different formulations of *Bacillus licheniformis* were evaluated on their own and in combination with prochloraz and stroburilin for their ability to reduce mango post-harvest fruit diseases when applied as dips under semi-commercial packhouse conditions. Untreated fruit and fruit treated with either prochloraz or stroburilin alone served as controls. In preliminary trials treatments integrating chemical pesticides with *B. licheniformis* controlled anthracnose and stem-end rot as effectively as the chemical control. The antagonist was more effective especially in the control of post-harvest diseases when fruit were kept in cold storage to simulate export conditions. In most of the trials, results obtained when fruit was treated with the antagonist in combination with the commercial chemical was comparable to that obtained with the commercial chemical control. A final commercial trial conducted during the 2004 season confirmed results from semi-commercial trials obtained previously (2001, 2002 and 2003). In the case of the commercial trial all factors such as color development, marketability and the effect of post-harvest treatments on fruit firmness was included as evaluation criteria. In fruit that were treated with the antagonist color development were less than other treatments making the fruit visually more acceptable. The marketability and firmness of the fruit increased when fruit were treated with the antagonist integrated with prochloraz and wax in the packhouse. In this study it was found that the antagonist used in a packhouse commercial trial could provide an effective alternative to fungicides and can be incorporated into normal packhouse operations.

5.1 Introduction

Mangoes are highly perishable and maintain an active metabolism post-harvestly. The major factors causing the early termination of storage life of fresh produce are fungal infections, senescence and transpiration. Factors that accelerate senescence and favor microbial growth, such as mechanical injuries, physiological status of the host and exposure to undesirable storage conditions, can promote post-harvest decay (Arul, 1994; Janisiewicz and Korsten, 2002). Post-harvest losses have been significant in decreasing the export potential of mangoes, thus resulting in a negative economic impact for the industry (Korsten *et al.*, 1991; Korsten *et al*., 1993; Lonsdale, 1993; Sanders and Korsten, 1996; Rong *et al*., 1999).

Major strategies for disease management have included pre- and post-harvest fungicide applications and agricultural practices such as crop rotation and orchard hygiene (Finnemore, 1999; Wellington, 2003). Environmental concerns, development of pathogen resistance to pesticides, new restrictive regulations governing the use of fungicide and its allowable residue levels on crops is now resulting in an increased need to find alternative disease control strategies (Wellington, 2003). This together with the new regulation that all pesticides need to be re-registered in the European Union (http://europa.eu.int/pol/agr/index_en.htm) has resulted in increased interest in alternative disease control strategies. Due to the pressure on agricultural industries, development of new fungicides has decreased and those that have been re-registered are mainly for major crops, leaving smaller ones in dire need of protective pesticides (Wilson and Wisniewski, 1994).

Developing post-harvest disease management strategies has become an economic imperative rather than an option (Roberts, 1994). Elimination of post-harvest chemicals necessitates an alternative approach to disease control and special emphasis has been placed on biological control as a viable alternative to synthetic fungicides (Korsten *et al*., 1991; Koomen and Jeffries, 1993; Wilson and Wisniewski, 1994). The post-harvest cycle of the fruit (from harvest to final packing and storage for either local or export markets) is unique and relatively simple, yet the tremendous diversity of crop, pathogen and environment combinations presents a challenge to the

commercialization of biological control products (Spotts and Sanderson, 1994; Janisiewicz and Korsten, 2002).

This chapter focuses on the use of an alternative disease management option. *Bacillus licheniformis* has previously shown potential as an effective antagonist for the control of *Xanthomonas campestris pv. mangiferaeindicae* and post harvest diseases anthracnose and stem-end rot (SR) (Burger and Korsten, 1988; Korsten *et al.*, 1991; Korsten *et al*., 1993; De Villiers and Korsten, 1994). The mode of action of this antagonist was assessed *in vitro* (Chapter three) and *in vivo* (Chapter four). The aim of this chapter is therefore to assess the potential of a commercially prepared biological control product in a commercial environment to determine efficacy over time. In addition the product was tested in combination with other products.

5.2 Materials and methods

5.2.1 Description of antagonist

Bacillus licheniformis (isolate Mal) was previously isolated from the mango phylloplane. For this study, the isolate was prepared and maintained as described in chapter three (3.2.1).

5.2.2 Preparation of the antagonist *in vitro*

Flasks containing Nutrient broth (Merck) were inoculated with a 24hr culture of *B. licheniformis* and incubated on a mechanical shaker at 7 000 rpm for 24 hrs. Cells were harvested by centrifugation at 10 000 rpm for 10 mins. The supernatant was discarded and the resultant cell mass re-suspended in quarter strength Ringer's solution (Oxoid, Johannesburg). The cell suspension was made up to a final concentration of 10^7cells/ml for experimental purposes.

5.2.3 Description of commercial formulations of *Bacillus licheniformis*

Commercial liquid and powder formulations of the biocontrol product (Mangogreen) were obtained from Stimuplant cc (Pretoria). The commercial formulations were kept at room temperature (25°C) prior to being used in the trials. The formulations were prepared for the trials as follows: the liquid product was made up to the required volume of 40L and used at a final concentration of 10^7 cells/ml and the powder

formulation was added to a hot-water bath (temp: 50º C, Capacity: 4000L) and made up to a final concentration of 10^7 cells/ml as per manufacturers recommendation.

5.3 Preliminary packhouse experiments: 2001 and 2002 mango seasons

Two packhouse trials were conducted on a semi-commercial scale, during March 2001, at Letaba Pakkers in Tzaneen (Limpopo Province). Cultivars and number of fruit were used based on seasonal availability. In the first trial, cultivar Sensation was used. Fruit were randomly selected from the same freshly harvested consignment and packed into crates. For trial one a total of 180 fruit were used per treatment. All chemicals were used according to manufacturers recommended rates outlined in Table 1. The products and treatments that were used in the packhouse trial are set out in Table 2. The treatments in trial one included an untreated control and a commercial packhouse treatment that was included as a reference standard. Different biological and integrated treatments were compared to these controls (Table 2).

The antagonist (liquid formulation) was added to the commercial water bath and was applied on the existing packing line (Appendix F). After each treatment, a crate of fruit was run through the packing line to reduce contamination of one treatment by another. After treatment, fruit were packed (12 fruit per box) into fifteen boxes per treatment. The boxes were transported back to the University of Pretoria. Seven boxes were stored for two weeks at room temperature (23-25ºC) to simulate local market conditions and eight were placed in cold storage (10ºC) for three weeks, to simulate export conditions during trial one. Fruit were then stored at room temperature for ripening. Fruit were monitored regularly for symptom development, which was determined by change in fruit colour (from green to yellow). Disease rating for anthracnose and stem-end rot (SR) were manually done using a 0-5 scale with zero indicating healthy fruit and five indicating total decay of fruit (Appendix G).

Data of trial one were analysed using the statistical program GenStat (2000). Analysis of variance (ANOVA) was used to test for differences between treatments. Data was acceptably normal, but with heterogeneous treatment variances. The angular transformation for percentages was used to stabilise treatment variances. Treatment means were separated using Fishers' protected t-test least significant difference (LSD)

at the 5% level of significance. In the subsequent mango season only treatments that showed promise were repeated.

For the second trial, cultivar Keitt was used and the trial consisted of five treatments (Table 3). Untreated and commercial packhouse controls were included as reference standard as described for trial one. The commercial powder formulation (Mangogreen) was evaluated in this trial and was compared to laboratory prepared *B. licheniformis*. Fruit were packed (six fruit per box) with 15 boxes per treatment, transported and stored at room temperature as described for the first trial. However, cold storage was done at Letaba packhouse (10ºC) for three weeks prior to being transported to the University of Pretoria, where it was left at room temperature for ripening. Fruit evaluation and analysis was done as described in trial one.

5.4 Semi-commercial packhouse trial: 2003 mango season

Two trials were conducted at Letaba Pakkers (Limpopo Province). The mango cultivars that were available at this packhouse at the time of experimentation were Kent and Keitt. In each trial, three crates of fruit were randomly selected from the same harvested consignment. The various products used in these trials are set out in Table 1. All chemicals were used according to the manufacturers recommended rates. Details of treatments for the trials were the same as described for the previous season using cultivar Sensation (Table 2 and Table 3).

After treatment, fruit were packed as previously described. Due to the size of the mangoes, six fruit was packed per box. There were fourteen boxes per treatment. The boxes were transported back to the University of Pretoria where the trial was split, with half the fruit going into cold storage (10ºC), and half left at room temperature (23-25ºC). After three weeks, the fruit kept in cold storage were removed and placed at room temperature for ripening. Both sets of fruit were monitored regularly for symptom development and evaluated at a ready to eat stage. This was determined by colour change as described previously. Evaluation of fruit was done manually. Disease rating for anthracnose and SR were done as described in Appendix G. Data were analysed as described before (5.3).

5.5 Commercial packhouse trial: 2004 mango season

A commercial trial was conducted at Bavaria Estates (Hoedspruit) during March 2004, to evaluate the best treatment and confirm results obtained in previous mango trials. Cultivars and number of fruit were chosen based on seasonal availability. Cultivar Keitt was used and 180 fruit was used per treatment. Fruit were randomly selected from the same freshly harvested consignment and packed into crates. The products (Table 4) and treatments that were used in the packhouse are set out in Table 5. All chemicals were used at the manufacturers recommended rates and fruit were treated on the commercial line.

After treatment, fruit were packed in boxes as previously described. Fruit that were to be stored at room temperature (23-25ºC) were transported back to the University of Pretoria. The remainder of the trial was placed into cold storage (10ºC) at Bavaria Estates. After three weeks, the fruit kept in cold storage were removed and transported back to University of Pretoria. Both sets of fruit were evaluated at a ready to eat stage and monitored regularly for colour and symptom development as described before. In addition fruit that were kept at cold storage to stimulate export market conditions were also evaluated for marketability and firmness. The color rating was done according to a 1-5 visual rating (Appendix H); marketability according to a 1-7 visual rating and firmness of fruit was done using a hand held firmness tester (Bareiss puufgratebau GmbH, Germany). Disease rating for anthracnose and SR were done as described previously. Data were analysed as described previously (5.3).

5.6 Results

5.6.1 Preliminary packhouse experiments: 2001 and 2002 mango season

In all cases disease incidence was lower in the cold stored fruit and all treatments could effectively reduce anthracnose incidence. Of these treatments, the antagonist combined with the chemical control or Stroburilin was the most effective, although not more effective than the chemical control. Surprisingly, the antagonist applied on its own in the water bath increased anthracnose above that of the control for fruit kept at room temperature (Fig. 5.1). For control of SR only the chemical treatment gave effective control, for both fruit stored at room temperature and cold storage. For fruit

placed in cold storage the antagonist combined with the chemical and the one applied in wax improved product efficacy (Fig. 5.2).

Commercial powder was more effective than the antagonist prepared in the laboratory and could provide equal effective control of anthracnose when compared with the commercial chemical (Fig. 5.3). The commercial powder on its own and integrated with the chemical control could control SR as effective as the chemical when fruit were placed at cold storage. Chemical control was effective for fruit kept at room temperature and cold storage (Fig. 5.4).

5.6.2 Semi-commercial packhouse trial: 2003 mango season

For fruit kept at cold storage, antagonist incorporated into wax was found to be as effective as the chemical control. The integrated treatment and chemical control gave lower disease incidences but did not differ significantly from other treatments incorporating the antagonist only. Results not presented graphically [Pr = 0.045]. In the control of SR on cultivar Keitt, the chemical control was the best treatment. Of significance is that the antagonist combined with the chemical control increased the incidence of SR. Results not presented graphically [Pr <0.862)].

In terms of anthracnose control, the chemical treatment was found to be the most effective treatment for fruit kept in cold storage and at room temperature (Fig. 5.5). For fruit kept at room temperature, the antagonist combined with chemicals gave total control as with the commercial chemical treatment (Fig. 5.5). The chemical control was the most effective treatment for fruit kept at room temperature giving total control of SR. The antagonist on its own or combined with the chemical or incorporated in the wax was also effective in preventing SR. This was predominant in cold storage fruit while the antagonist applied in the wax gave total control of SR under cold storage conditions (Fig. 5.6).

5.6.3 Commercial packhouse trial: 2004 mango season

The antagonist combined with the standard commercial treatment once again was effective for the treatment of anthracnose (Fig. 5.7) and SR (Fig. 5.8). The color development after three weeks was slower with the integrated treatment, this meant fruit were visually appealing for a longer time period and the marketability index

increased (Fig. 5.9). Fruit that were treated with the commercial antagonist (powder) combined with the standard packhouse treatment were also firmer after three weeks in cold storage (Fig. 5.10).

5.7 Discussion

Biocontrol on its own or integrated with chemical products were effective in inhibiting the development of post-harvest diseases and was either as or more effective compared to the commercial chemical treatment. The effectiveness of *B. licheniformis* applied post-harvestly to control post-harvest diseases of mango has previously been shown (Korsten *et al*., 1991; Lonsdale, 1993; De Villiers and Korsten, 1994). However, in this study trials were run over several seasons and were done in several packhouses and on different cultivars and focused more on consistency of product performance. Previous studies focused on using laboratory preparation of the bacteria and variable results were found (De Jager and Korsten, 1995; Korsten *et al*., 1992; Korsten *et al*., 1993). Of special interest is that the powder formulation of the product was more effective than both the liquid formulation and the laboratory preparation of the antagonist. In addition, by combining the commercial product with prochloraz, increased control could be achieved. Integrated control has been studied and used with similar success in the control of apple (Leverentz *et al.*, 2003; Krihak *et al*., 1996) and citrus post-harvest diseases (El Ghaouth *et al.*, 2000; Obagwu and Korsten, 2003). Integrated control has always been studied as a viable option to bring about a more complete disease control strategy (Korsten *et al.,* 1991; Korsten *et al*., 1992; Korsten *et al.,* 1993; De Villiers and Korsten, 1994; Serfontein *et al.*, 2002) this was also tested in this study and antagonist combined with chemicals brought about control that was comparable to the standard treatment used in the packhouse.

The efficacy of the biological control product gave variable results between the beginning and end of the season. When the powder formulation was used on Keitt at the beginning of the season it was more effective than the liquid formulation and gave control comparable to the chemical control. When used on the same cultivar later in the next season, treatments incorporating the powder did not differ significantly. A factor that needs to be taken into consideration when implementing biocontrol at the post-harvest level is maturity and physiological fitness of the fruit, which changes

depending on the season and time delays between harvest and packing. Furthermore, McLaughlin *et al*. (1990) showed that maturity markedly affects biocontrol efficacy. Results from the previous chapter showed that the yeasts population increased as fruit ripened. Establishment of the antagonist on the fruit surface is imperative for effective control and its ability to compete effectively with the existing epiphytes should be assessed as fruit matures and ripens.

Janisiewicz *et al*. (1991) reported that as storage temperatures decreases, the efficacy of the biological agent used in disease control increases. It was found in this study that disease incidence were in some instances generally lower for fruit kept at cold storage. Furthermore all the treatments incorporating the antagonist gave more effective control or was equal to the chemical treatment. Since low temperatures are imperative for prolonged storage, it is important that the effectiveness of antagonists be tested under prevalent storage conditions rather than at ambient temperature (Arul, 1994; Spotts and Sanderson, 1994). Similar results were obtained in the *in vitro* and *in vivo* temperature studies reported in Chapter three and four where the antagonist most effectively inhibited the growth of *C. gloeosporioides* at 10°C. This correlates well with the more effective anthracnose control at simulated export conditions.

Due to the loss of fruit from storage during these trials, only two cultivars could be compared. Keitt and Sensation are harvested in the middle and end of the mango season respectively. Sensation is known to be a fairly susceptible cultivar (http://www.mango.co.za), and all treatments effectively controlled anthracnose. Results obtained in these trials were comparable with those obtained by Korsten and co-workers (1993), where treatments were generally more effective for cultivar Keitt than cultivar Sensation. Further studies should focus on the use of the antagonist formulations in commercial trials on various cultivars, throughout the mango season and from different geographical locations.

In all the preliminary trials, the chemical control provided effective control. This is comparable with results obtained previously by Roux and Boshoff (1999). In their studies it was found that with semi-commercial trials, anthracnose was most effectively controlled when Prochloraz was included in the treatment. There were no significant differences between treatments when combining the antagonist with either

prochloraz and the results of treatments were mostly comparable with the chemical control. The antagonist combined with both the disinfectant and the Prochloraz used in the packhouse generally indicated a lower disease incidence than the *Bacillus* on its own as was previously found (Korsten *et al.,* 1993; De Villiers and Korsten, 1994).

Being able to incorporate the antagonist into the hot water bath in the packing line indicated that the biocontrol agent was compatible with current commercial packhouse systems (Spotts and Sanderson, 1994). Despite commercial practices, it is not feasible to change the water daily at most packhouses and build-up of inoculum is therefore eminent. Evaluation of a water sample from the water bath (unpublished data) revealed the presence of *Pseudomonas* spp., several species of *Enterobacteria* and Protozoa (Wilma du Plooy, ISO 17025 Test method: TR 02/01/01, Plant Pathology Laboratories, University of Pretoria; personal communication). Competition from other organisms found in the water bath could be a possible explanation as to why in some trials, the antagonist did not provide any protective action.

One of the keys to the successful commercialisation of biological control of post-harvest diseases lies in understanding and controlling the post-harvest environment (Spotts and Sanderson, 1994). In addition, other aspects of export such as colour development and marketability after fruit were in cold storage, were taken into consideration. There was a direct correlation between the effectiveness of the integrated treatment, marketability, color development and firmness of fruit. These factors are important for the assessment of shelf-life and consumer appeal of export mangoes. Since it was established that the antagonist is comparable to chemical control and was effective under cold storage conditions, other studies should focus on testing the antagonist efficacy throughout the cold chain. It was also shown that the formulation of the antagonist is extremely important, as the antagonist can be incorporated into the standard packing line. Future studies should focus on testing the effectiveness of the antagonist against quarter- and half-strength concentrations of the chemical use.

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Table 1: Products and application methods used in post-harvest treatments to control mango fruit diseases on cultivars Sensation and Keitt

Products	**Supplier**	**Concentration**	**Application method**
Hot water bath	-	-	55°C, for 5 min.
Prochloraz	Saarchem	180ml/100L	Dip for 20 sec.
Wax	Citrashine	1L/ton fruit	Brush application.
Stroburilin	BASF	10ml/1L	Incorporated with wax.
B. licheniformis (liquid formulation)	Stimuplant	10^7 cells/ml	Incorporated into water bath (10^7 cells/ml)
B. licheniformis (powder formulation)	Stimuplant	10^7 cells/ml	Incorporated into water bath (10^7 cells/ml)

Table 2: Treatments conducted, during the 2001/2002 trials at Letaba Pakkers using the cultivar Sensation

Treatments[a]	**Laurolatex**	**Hot water bath**	**Prochloraz**	**Wax**	**Strob**	***Bac*. (Liquid)**
Control	-	-	-	-	-	-
Chem cntrl	+	+	+	+	-	-
Bacillus	+	+	-	-	-	+
Bac + Strob	+	+	-	+	+	+
Bac + Chem cntrl	+	+	+	+	-	+
Bac + Wax	+	+	-	+	-	+

[a] Chem cntrl = Chemical control; Bac = *Bacillus licheniformis*; Strob = Stroburilin;
All chemicals were used at the standard registered rate outlined in Table 1.

Table 3: Treatments conducted during the 2003 trials at Letaba Pakkers on cultivar Kent and Keitt

Treatments[a]	Hot water bath	Prochloraz	Wax	*Bac* (powder)	*Bac* (cells)
Control	-	-	-	-	-
Chem cntrl	+	+	+	-	-
Bacillus	-	-	-	-	+
Bac + Chem cntrl	+	+	+	+	-
Bac powder	+	-	-	+	-

[a] Chem cntrl = Chemical control; Bac = *Bacillus licheniformis*

All chemicals were used at the standard registered rate outlined in Table 1.

Table 4: Products and application methods used in post-harvest treatments to control mango fruit diseases on cultivar Keitt at Bavaria Estate

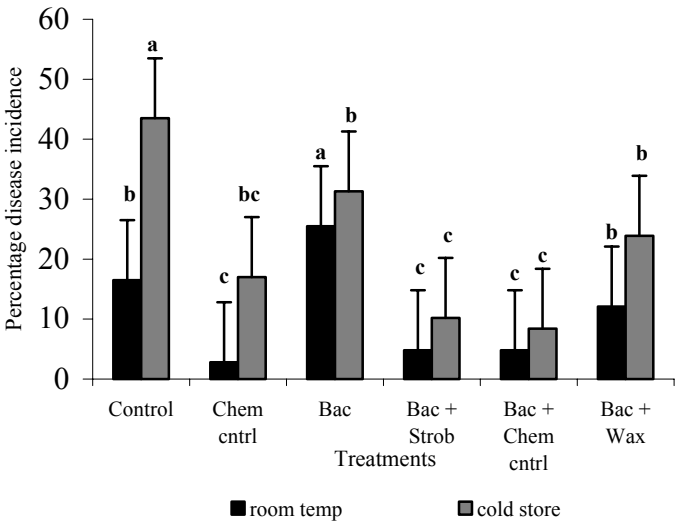
Products	Supplier	Concentration	Application method
Wet receival	-	-	Dip for 30 sec
Hot water bath	-	-	50°C, for 5 min.
Prochloraz	Saarchem	180ml/100L	Dip for 20 sec.
Wax	Citrashine	1L/ton fruit	Brush application.
B. licheniformis (powder formulation)	Stimuplant	10^7 cells/ml	Incorporated into water bath (10^7 cells/ml)

Table 5: Treatments conducted during the commercial trial at Bavaria Estates on cultivar Keitt

Treatments[a]	Hot water bath	Prochloraz	Wax	*Bac* (powder)
Control	-	-	-	-
Chem cntrl	+	+	+	-
Bacillus	+	-	-	+
Bac + Chem cntrl	+	+	+	+

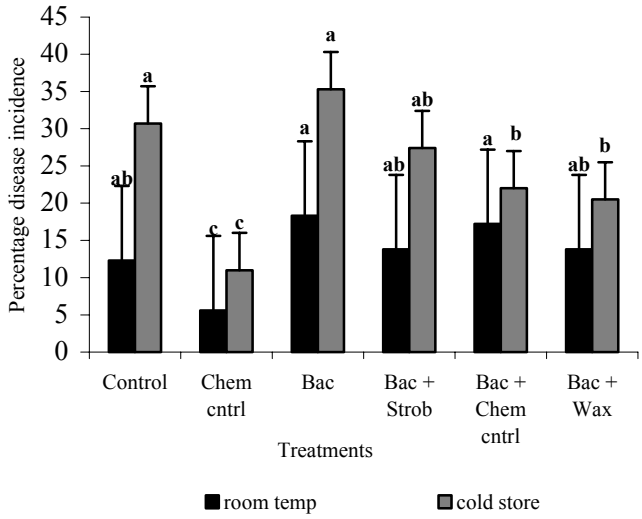
[a] Chem cntrl = Chemical control; Bac = *Bacillus licheniformis*

All chemicals were used at the standard required rate outlined in Table 4.



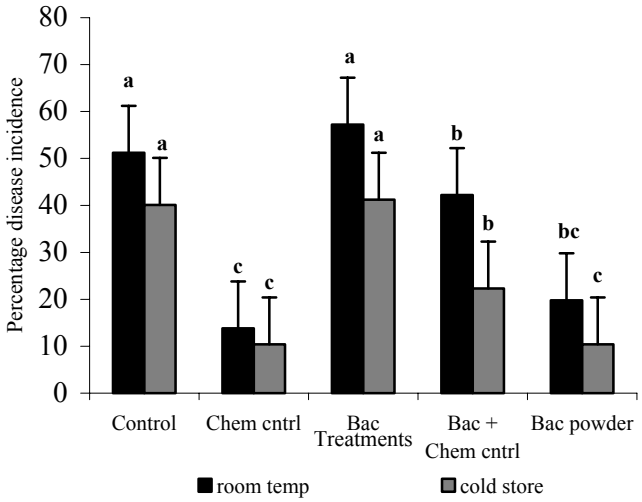
Comparison of post-harvest dip treatments to control anthracnose in trial one for mango fruit, cv Sensation kept at room temperature and in cold storage. Chem cntrl = Chemical control; Strob = Stroburilin; Bac = *Bacillus licheniformis*. Bars containing the same letters do not differ significantly [Pr (room temp, cold storage) <0.001].

Fig. 5.1 Effectiveness of post-harvest treatments for control of anthracnose on cultivar Sensation in the preliminary trials 2001: mango season.



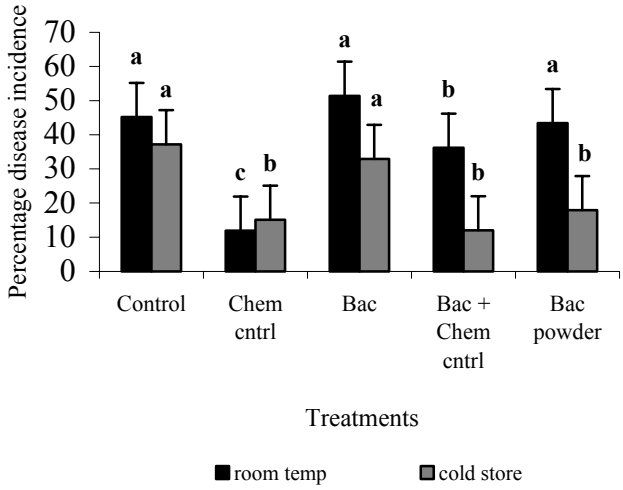
Comparison of post-harvest dip treatments on SR in trial one for mango fruits at room temperature and cold storage. Chem cntrl = Chemical control; Strob = Stroburilin; Bac = Bacteria/antagonist. Bars containing the same letters did not differ significantly [Pr (room temp = 0.407, cold storage <0.001)].

Fig. 5.2 Effectiveness of post-harvest treatments for control of SR for trial one on cv Sensation in preliminary trial: 2001 mango season.



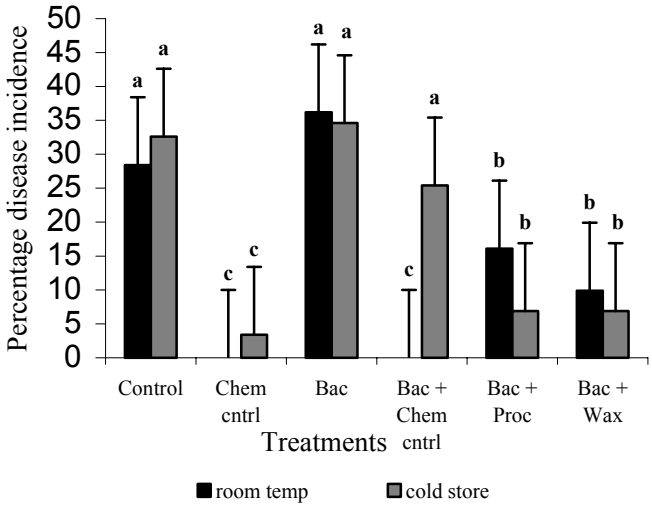
Comparison of post-harvest dip treatments on anthracnose in trial two for mango fruits at room temperature and cold storage. Chem cntrl = Chemical control; Bac = Bacteria/antagonist. Bars containing the same letters did not differ significantly [Pr (room temp<0.001, cold storage= 0.003)].

Fig. 5.3 Effectiveness of post-harvest treatments for the control of anthracnose for trial two on cv Keitt during the preliminary trial: 2002 mango season.



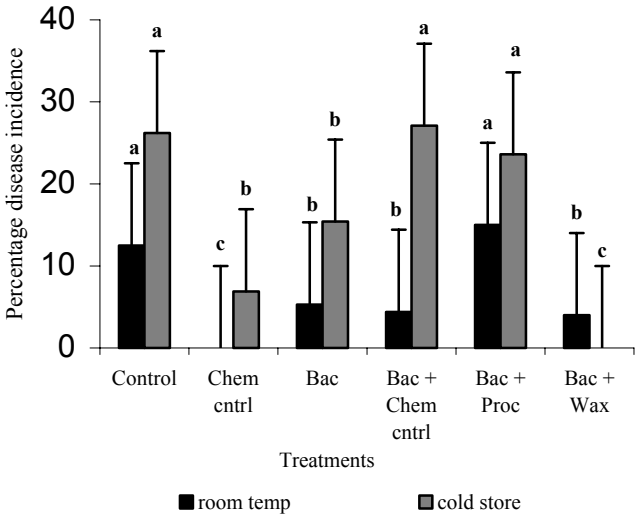
Comparison of post-harvest dip treatments on SR in trial two for mango fruits at room temperature and cold storage. Chem cntrl = Chemical control; Bac = Bacteria/antagonist. Bars containing the same letters did not differ significantly [Pr (room temp<0.001, cold storage= 0.003)].

Fig. 5.4 Effectiveness of post-harvest treatments for the control of SR for trial two on cv Keitt during the preliminary trial: 2002 mango season.



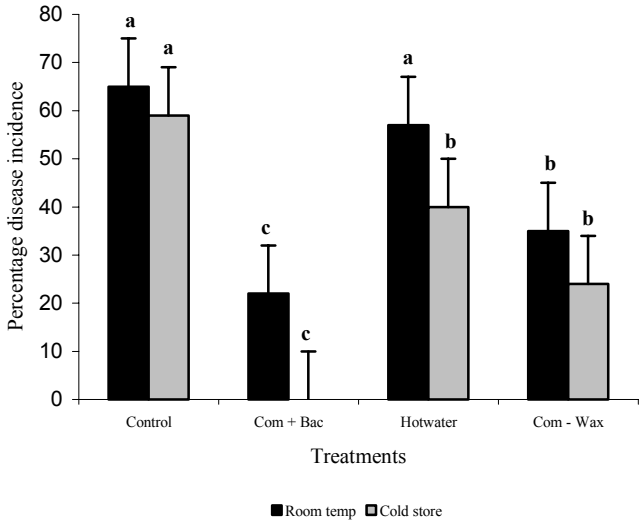
Comparison of post-harvest dip treatments on anthracnose in trial six for mango fruits at room temperature and cold storage. Chem cntrl = Chemical control; Bac = Laboratory preparation of Bacteria/antagonist; Proc = Prochloraz. Bars containing the same letters did not differ significantly [Pr (room temp = 0.164; cold storage <0.001)].

Fig. 5.5 Effectiveness of post-harvest treatments for the control of anthracnose on cv Keitt during semi-commercial trials: 2003 mango season.



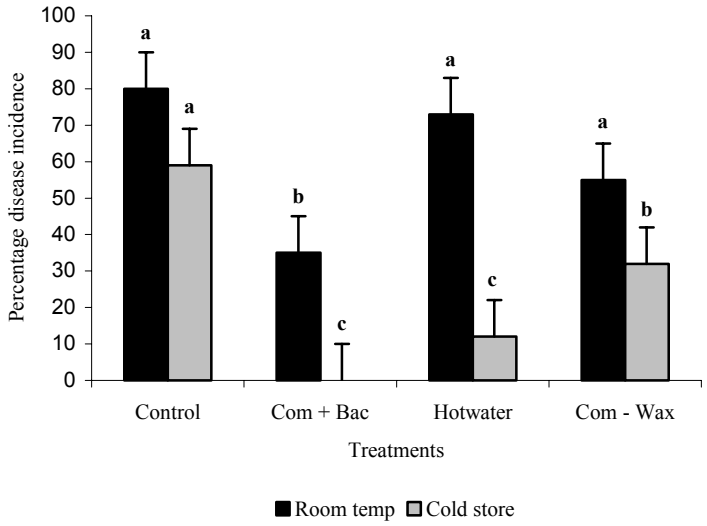
Comparison of post-harvest dip treatments on SR in trial six for mango fruits at room temperature and cold storage. Chem cntrl = Chemical control; Bac = Bacteria/antagonist; Proc = Prochloraz. Bars containing the same letters did not differ significantly [Pr (room temp, cold storage) <0.001].

Fig. 5.6 Effectiveness of post-harvest treatments for the control of SR on cv Keitt during the semi-commercial trial: 2003 mango season.



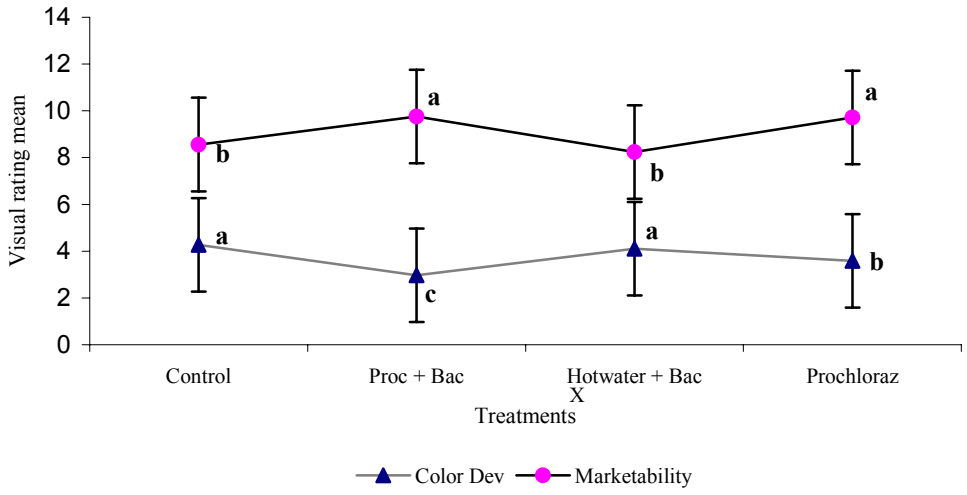
Comparison of post-harvest dip treatments on anthracnose in semi-commercial trial for mango fruits at room temperature and cold storage. Com = standard packhouse treatment; Bac = Bacteria/antagonist; Hotwater = Hotwater bath with bacillus added. Bars containing the same letters did not differ significantly [Pr (room temp, cold storage) <0.001].

Fig. 5.7 Effectiveness of post-harvest treatments for the control of anthracnose on cv Keitt during commercial trial: 2004 mango season.



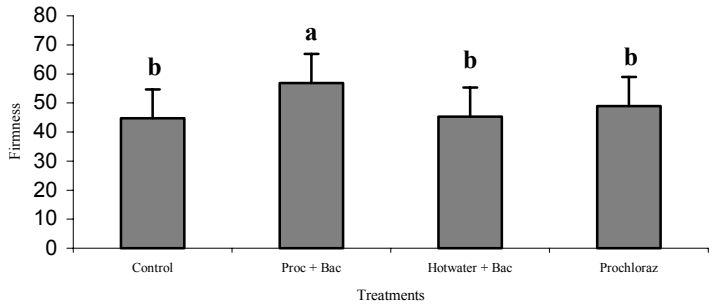
Comparison of post-harvest dip treatments on SR in trial six for mango fruits at room temperature and cold storage. Com = standard packhouse treatment; Bac = Bacteria/antagonist; Hotwater = Hotwater bath with Bacillus powder. Bars containing the same letters did not differ significantly [Pr (room temp, cold storage) <0.001].

Fig. 5.8 Effectiveness of post-harvest treatments for the control of SR on cultivar Keitt during commercial trial: 2004 mango season.



Comparison of the effect that post-harvest treatments had on colour development and marketability of fruits. [X] Proc = Prochloraz; Bac = *Bacillus licheniformis*; Hotwater = Hotwater bath with Bacillus powder added. Bars containing the same letters did not differ significantly [Pr (color, marketability) <0.001].

Fig. 5.9 Effect of post-harvest treatments on colour development and marketability for commercial trial on cultivar Keitt.



Comparison of the effect that post-harvest treatments had on firmness of mango fruits. Com = standard packhouse treatment; Proc = Prochloraz; Bac = *B. licheniformis*; Hotwater = Hotwater bath with Bacillus powder added. Bars containing the same letters did not differ significantly [Pr (firmness) <0.001].

Fig. 5.10 Effect of post-harvest treatments on firmness of fruits for commercial trial on cultivar Keitt.

CHAPTER 6
Final discussion

6.1 Discussion

Control of post-harvest diseases has challenged mankind particularly since the beginning of extensive global trade in fresh produce. Traditionally, fungicides were extensively used and provided the required protection of fruit to ensure quality produce on the export markets. However, reported pathogen resistance, negative impact on the environment and on human health has resulted in a move away from the extensive use of particularly post-harvest fungicides. It was not until the second half of this century that the use of beneficial microorganisms in crop protection was extensively exploited as an alternative to pesticides (Baker and Cook, 1974; Korsten *et al.*, 1994). Biological control has since been successfully evaluated for several fruit commodities including apples (Mercier and Wilson, 1995; Fan and Tian, 2001; Vero *et al.*, 2002; Leverentz *et al.*, 2003), cherries (Spotts *et al.*, 1998; Qin *et al.*, 2003), citrus (Krihak *et al.*, 1996; Smilanick *et al.*, 1996; Obagwu and Korsten, 2003) and mangoes (Korsten *et al.*, 1992; Korsten *et al.*, 1993; De Villiers and Korsten, 1994).

In South Africa, the major post-harvest diseases affecting mango fruit are anthracnose caused by *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc. in Penz. and stem-end rot (SR) caused by *Botryosphaeria* spp. (Johnson *et al.*, 1990; Donkin and Oosthuyse, 1996). Thus far, limited post-harvest disease control has been achieved with pre-harvest applications of copper-based fungicides (Lonsdale, 1993; Ippolito and Nigro, 2000). At a post-harvest level, hot water dips incorporating products such as prochloraz (Omega) (Johnson *et al.*, 1997; Cooke and Jacobi, 2000; Orme and Kegley, 2002) are currently still in use and provide effective control.

The main focus in research within the mango industry of South Africa has shifted to finding alternative control methods for pre-harvest diseases and sapburn (Silimela and Korsten, 2001; Duvenhage, 2002), insects (Daneel *et al.*, 2002, Willis and Duvenhage, 2002; Lagadec and Bruwer, 2002) and post-harvest diseases (Kruger *et al.*, 2001). In this

study, the antagonistic potential of *Bacillus licheniformis* was confirmed *in vitro* and *in vivo*. This antagonist was originally isolated from the mango phylloplane and successfully screened *in vitro* and *in vivo* against bacterial black spot, anthracnose and stem-end rot diseases (Burger and Korsten, 1988). In this study, its inhibitory activity against mango post-harvest pathogens were confirmed which included *C. gloeosporioides* and *Botryosphaeria parva* previously known as *Dothiorella dominicana* (Petrak and Cif).

Although, the dual culture technique has been described as inadequate for initial biocontrol screening (Jijakli *et al*., 2000), this technique does give an indication as to the degree of antagonist inhibition on pathogen mycelial growth. Antibiosis in the genus Bacillus has previously been shown (Mc Keen *et al.*, 1986; Cho *et al*., 2003; Emmert *et al*., 2004). Several secondary products may be produced and play a role in antibiosis. Although it is known that several antibiotics are produced by *B. licheniformis,* including subtilism A, Proticin and Bacitracin (Johnson *et al.*, 1945; Bernal *et al.*, 2002; Cladera-olivera *et al.*, 2004), inhibitory substances in this study were not further characterised. In order to further elucidate the mode of action the production of volatiles by *B. licheniformis* were studied *in vitro.* In liquid culture, the antagonist produces an inhibitory compound in the medium after ten days incubation at ambient temperature. Chromatograms from high performance liquid chromatography revealed the presence of this compound, which must still be characterised. Further studies should also focus on identifying the volatile compounds and testing if the compound is still effective or produced at 10ºC. From this study it could be shown that *B. licheniformis* has a wide range of *in vitro* activity against mango post-harvest pathogens. This was similar to reports by Silimela (2003), in which mango pre- and post-harvest pathogens were effectively inhibited in *in vitro* assays.

Competition as a mode of action *in vitro* and *in vivo* has only recently been proven (McLaughlin *et al.*, 1990). In this study, the ability of the antagonist to produce extracellular enzymes, e.g. chitinase, were evaluated and it was noted that the ability of both pathogens to utilize starch, chitin and cellulose were restricted when grown in culture with *B. licheniformis.* Many bacillus spp, including, *B. licheniformis* has

previously been chosen for its ability to compete, *in vitro* for micronutrients with *Vibrio* spp. (Edwards, 2001). In addition to the enzymes production by *B. licheniformis,* the ability of the pathogens to take up iron from an iron rich medium was restricted, in the presence of the antagonist. Siderophore production is an important aspect of survival for most microorganisms (Crosa and Walsh, 2002), but the role it plays in biological control has not been well documented for *B. licheniformis*. Further studies should focus on quantifying the amount of iron taken up from the media by both the antagonist and the pathogens.

As promising results were obtained for *in vitro* assays, the ability of the antagonist to be effective *in vivo* was evaluated. Since this was the first time the antagonist was tested on the mango fructoplane, the study focused more on population dynamic studies and attachment of the antagonist to the mango fruit surface rather than induced resistance on the host. Population dynamic studies are important to ensure survival of the antagonist on the fruit surface, if an antagonist is unable to colonize the fruit surface then it is ineffective as a biological control agent. In this study, the yeast and fungal epiphytic populations increased on the fructoplane during ripening when compared to green fruit. Dominant phylloplane epiphytes, apart from bacterial species (*Bacillus* and *Pseudomonas)* consisted of yeasts (*Aureubasidium*, *Candida* and *Cryptococcus*) and fungi (*Alternaria alternata*, *Penicillium* spp. and *Trichoderma* spp.). This confirmed previous microbial ecological studies done by De Jager (1999). In a study by Fallik *et al.* (2000), it was proven that treatment and handling of mangoes may improve general appearance but also cause reduction in the number of naturally occurring epiphytes. It is important therefore that the antagonist be able to attach to the mango surface and effectively compete with the epiphytes and give protection against the pathogens.

Scanning electron microscopy studies confirmed that the antagonist was able to attach to the fructoplane. In addition, the recovery rate of the antagonist after fruit were dipped in a solution of the antagonist was a 2 log unit decrease after fruit had ripened. In recent studies done by Silimela (2003), it was shown that *B. licheniformis* had the ability to effectively attach and colonise the mango leaf surface. The minimum time required for

the antagonist to attach to the leaf surface was found to be 15 min, similar studies were conducted by Towsen (1996), using *B. subtilis*.

Laboratory antagonist preparations of have previously been tested in pre- and post-harvest trials (Korsten *et al*., 1991; Korsten *et al*., 1992; Korsten *et al*., 1993; De Villiers and Korsten, 1994). Although it was previously shown to have potential in packhouse trials, performance was not evaluated under commercial conditions. This study focused on efficacy of performance of the commercial formulation of *B. licheniformis* (Mangogreen). Mangogreen was tested alone or in combination with chemicals. These trials were designed to fit in with the current commercial system used in South African mango packhouses. Integrated treatments gave effective control specifically for anthracnose. This was in line with results obtained previously (Korsten *et al*., 1993). Another aspect that linked *in vitro*, *in vivo* and packhouse trial results was temperature studies. The antagonist was most effective under cold storage (10ºC) conditions, similar to actual export conditions. This correlated well with results obtained for control of *C. gloeosporioides,* where the antagonist is able to maintain appressorium dormancy at such low temperatures thereby preventing infection.

Knowledge obtained from results in the *in vitro* and *in vivo* studies assisted with the design of packhouse trials. An important aspect was found to be the production of the bioactive compound, which still needs to be quantified. As the number of the antagonists decreased as fruit ripened, effective control was not found to always be possible after harvest, specifically for faster growing pathogens like *B. parva.* Further studies should focus on effective establishment of the antagonist on the fruit surface at the onset of ripening. This study was different from previous studies as the commercial formulations of the antagonist were tested against laboratory preparations of *B. licheniformis*. The *in vivo* testing was structured so as to provide evidence for the effectiveness of this antagonist on the fructoplane and not just *in vitro.* The experiments were simply designed and were structured depending on availability of mango fruit. Results from the packhouse trial showed consistency over three mango seasons. This consistency showed that the

powder formulation of the antagonist was more effective. Results were also dependant on fruit age where there was variability in control, within a cultivar.

Further studies could focus on the efficacy of the biocontrol agent over thee entire mango seasons. It was shown that the antagonist could successfully be integrated into the normal packing line operation. Future work should focus on testing the antagonist against half- and quarter strength concentrations of the chemicals used in the packhouse to reduce cost. An ideal biological control agent must be compatible with other systems used. It should have combined action against the full spectrum of pathogens. There should be consistent levels of efficacy and consistency under different inoculum pressures. The essence of this study was that it showed potential to commercialize and register the Mangogreen product.

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Summary

Evaluation of biological control systems for the control of mango post-harvest diseases

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Mangifera indica L has become an important export crop. Two of the most important diseases on mangoes of major concern in South Africa are anthracnose (*Colletotrichum gloeosporioides* (Penz.) Penz. and Sacc. in Penz) and stem-end rot (SR) [*Botryosphaeria parva*, previously known as *Dothiorella dominicana* (Petrak and Cif)]. Limited post-harvest control may be achieved with pre-harvest applications of copper-based fungicides. However, reduction in the number of fungicides re-registered due to stricter requirements, pathogen resistance, negative effects on the environment and on human health has left most of the smaller industries, such as the mango producers, in search for alternative control measures. In light of the above, there was increased scientific interest in biological control. The antagonist (*Bacillus licheniformis*) used in this study was previously isolated from the mango phylloplane and shown to have some potential as a biological control agent. The aim of this study was therefore to use *B. licheniformis* as a model system to obtain a better understanding of biological control systems in the post-harvest arena. The objectives of this study were; 1) to elucidate the mode of action of *B. licheniformis,* 2) to evaluate attachment, colonisation and survival of *B. licheniformis* on the fructoplane and 3) to further assess the antagonist's performance in terms of consistency of efficacy in a commercial environment, alone or in combination with other products. Results from this study confirmed the *in vitro* antagonism of *B. licheniformis*. In addition the antagonist inhibited *in vitro* growth of *C. gloeosporioides* and *B. parva* by means of antibiosis, production of volatiles and competition. A bioactive compound was produced after ten days incubation. Competition was confirmed when the antagonist was able to produce extracellular enzymes, such as chitinase, to hydrolyse complex compounds, while restricting the pathogens ability to hydrolyse

these compounds. Iron-uptake from an iron-rich medium was achieved by the antagonist and gave *B. licheniformis* an *in vitro* growth advantage. *In vivo,* the minimum concentration of the antagonist that was effective at inhibiting the pathogens was 10^7 cells/ml. Antagonist applications integrated with chemicals gave most effective post-harvest control that was equal to or more effective than the commercial chemical used. Scanning electron microscopy studies revealed that the antagonist attaches to the fruit surface. The recovery of the antagonist from treated fruit was high proving increased survival on the fructoplane. Temperature studies *in vitro* and *in vivo* revealed that the antagonist was most effective in inhibiting growth of the pathogens under cold storage conditions (10ºC). This was significant as results from commercial trials confirmed those obtained in the laboratory. Calcium carbonate added *in vitro* and *in vivo* enhanced the activity of the antagonist. In this study, *B. licheniformis* was effective against the pathogens with *in vitro* assays, on the fructoplane and lastly in a commercial set up. As it is feasible to incorporate this commercial formulation into the standard packing line, further work should focus on testing this antagonist efficiency throughout the mango season and on all cultivars.

An ideal biological control agent must be compatible with other systems used.
It should have combined action, either not be affected by the other organisms on the fructoplane and also be able to function in an integrated system. There should be high efficacy as well as consistency of efficacy. A biological control agent should function over a broad spectrum of organisms. If a biological control agent if efficient over a long period of time, then large scale production should be tested.

Next step is toxicology studies, patenting etc
Application
Study of marketability

APPENDICES

Appendix A: Summary of the harvest to export mango fruit chain in South Africa

HARVEST

Hand picked when physiologically mature [a]

Put into picking crates / bins and later loaded onto trucks

Transport to packhouse

PACKHOUSE

Wet receival

20 sec

Disinfectant sprays

20 sec

Pre-sorting table

Lower quality fruit are sorted and redirected for processing, drying etc

Hot water bath [b]

Hot air drying

2 min

Chemical bath [c]

20 sec

Drying and Waxing[d]

55° C

Start of the cold chain 10° C

Local Chain

Fresh Produce Markets

Jan Feb Mar Apr

Tommy Atkins

Kent

Keitt

Sensation

25° C

Consumers

Retailer

Repacked

Break in the cold chain

Distributer

Export market

Refrigerated shipping 7° C for 27 days

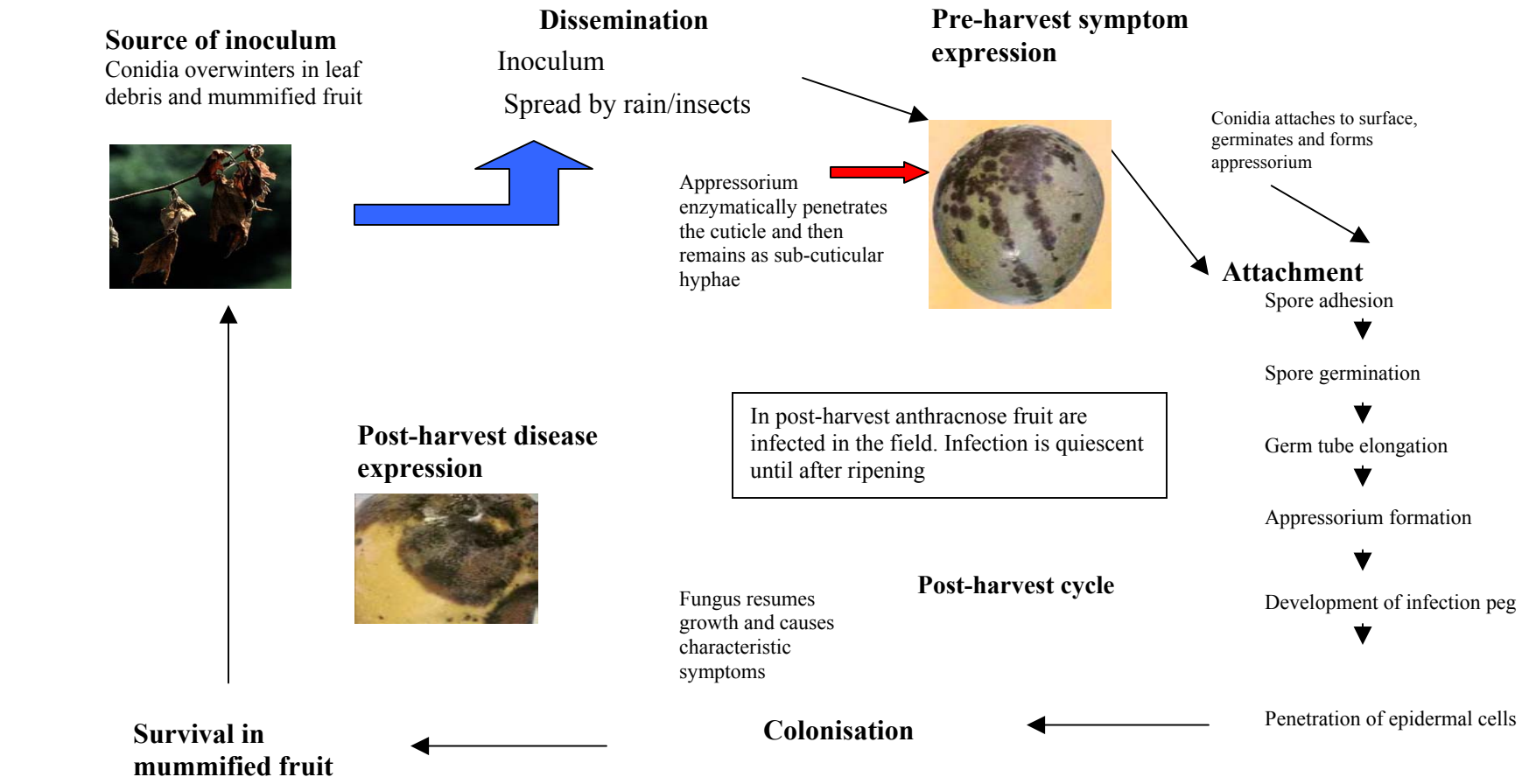
Holding facility at the ports 7° C

Refrigerated trucks

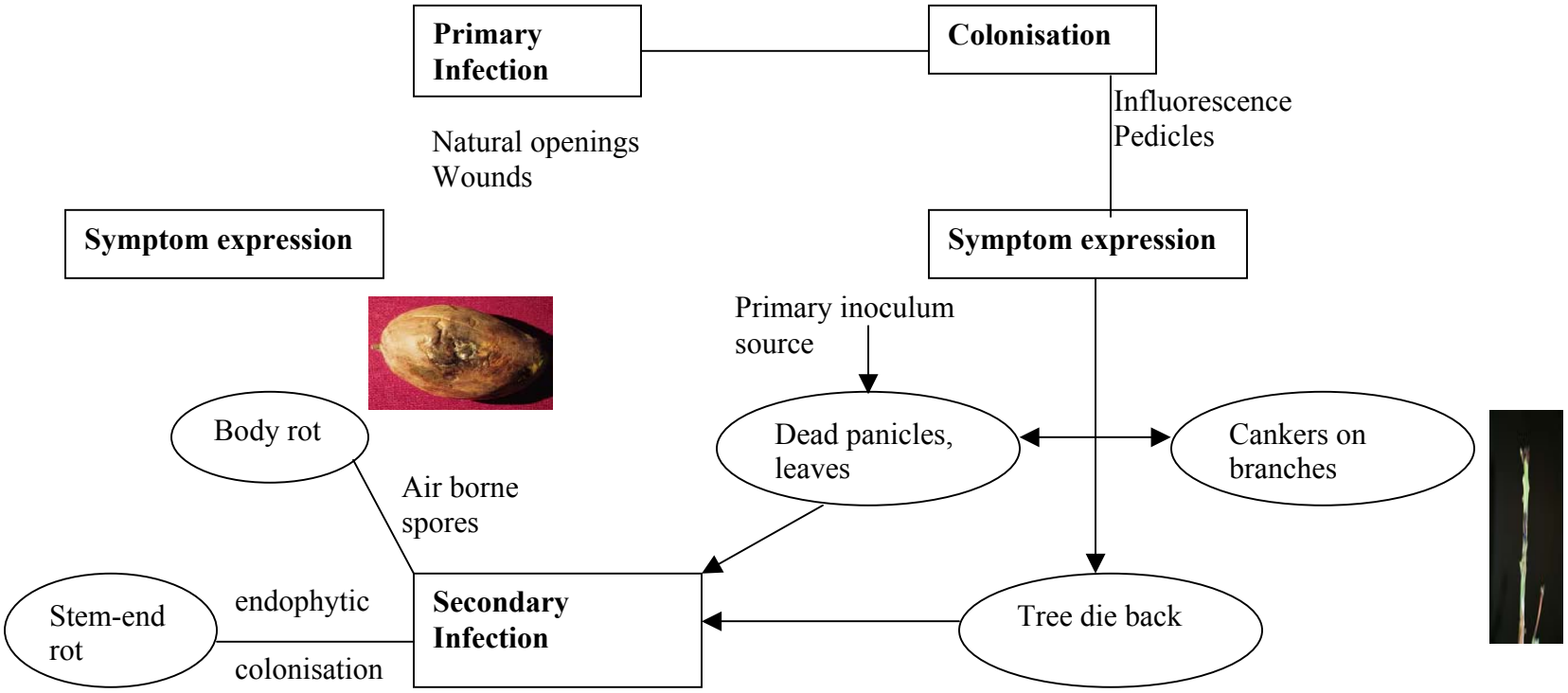
= End of packing line

[a] Physiologically mature index based on size of fruit.
[b] Hot water bath at 50° C for 3 min. replenishment rate varies from twice weekly to weekly depending on packhouse. Volumes differ between 4000L to 5000L.
[c] Chemical bath consist of prochloraz (180ml/L) in most packing houses. Time in chemical bath 20 sec.
[d] Citrashine as spray application (1L/Ton)

Appendix B: Summary of the disease cycle of *Colletotrichum gloeosporioides*



Appendix C: Summary of the disease cycle of *Botryosphaeria* spp (Leylani Grobler, Plant Pathology Laboratories, unpublished data)



Appendix D: Media prepared for evaluation of antagonist and pathogen's ability to produce extracellular enzymes (adapted from Skinner and Lovelock, 1979; Claus, 1989)

Casein Hydrolysis medium

KH_2PO_4 (1.0g); KCl (0.5g); $MgSO_4.7H_2O$ (0.2g); $CaCl_2.H_2O$ (0.1g); 15% Milk powder solution (25ml); Glucose (10g); Agar (12g); distilled water (1L).

A 15% milk powder solution was prepared by dissolving 3.75g milk powder in 25ml distilled H_2O until a creamy, smooth texture is achieved. The solution was added to the medium. The final pH was adjusted to 5.4 and the medium was autoclaved for 30min at 121º C.

Cellulose medium

$NH_4H_2PO_4$ (1.0g); KCl (0.2g); $MgSO_4$(0.2g); $CaCl_2$ (0.2g); 4% Cellulose(25ml); Agar (12g); distilled water (750ml).

Starch medium

Czapek solution A ($NaNO_3$; KCl; $MgSO_4.7H_2O$; distilled water);
Czapek solution B ($NH_4H_2PO_4$; KCl; $MgSO_4.7H_2O$; distilled water);
Zinc solution
Copper solution
Starch solution
Agar
Distilled water

The starch solution was prepared by dissolving 10g soluble starch in 50ml water while slowly heating at 70-80 º C for 1-2 min. Stirring until formation of a smooth paste. The starch was then added to the medium and sterilized for 30 min at 121º C.

Appendix E: RSM-CAS medium for siderophore screening (modified from Buyer *et al.*, 1989)

1. Preparation of CAS stock solution

Solution A

Dissolve 60.5mg CAS (chrome azurol S) [1.21 mg ml-1]

50 ml distilled water

Mix with 10ml iron (III) solution (solution = 1mM FeCl3.6H2O in 10mM HCl)

} Dissolve over low heat while stirring constantly

72.9 mg HDTMA (hexadecyltrimethylammonium bromide) [1.82 mg ml^{-1}]

40 ml distilled water

} Solution B

Slowly add solution A to B (=dark blue dye stock solution) and autoclave (+/- 100ml stock solution)

2. Preparation of 1 liter of RSM agar medium

Dissolve 0.75g $Ca(NO_3)_2.4H_2O$)

0.246g $MgSO_4.7H_2O$

18.22g ACES (2-[(2-amino-2-oxoethyl)-amino]ethanesulfonic acid

2g NaOH

15g Agar

853ml deionized water

} Autoclave and cool to 50º C

Add sterile stock solutions of the following to # 2

1ml 1M KH2PO4 *

100ml 10% casamino acids

33.3ml 30% sucrose*

* Autoclave separately and cool before adding to agar medium

1ml of $7x10^{-4}$ M $ZnSO_4.7H_2O$

1ml of $9x10^{-4}$ M $MnSO_4.4H_2O$

1ml of 20mg/l thiamine HCl

1ml of 1mg/l biotin

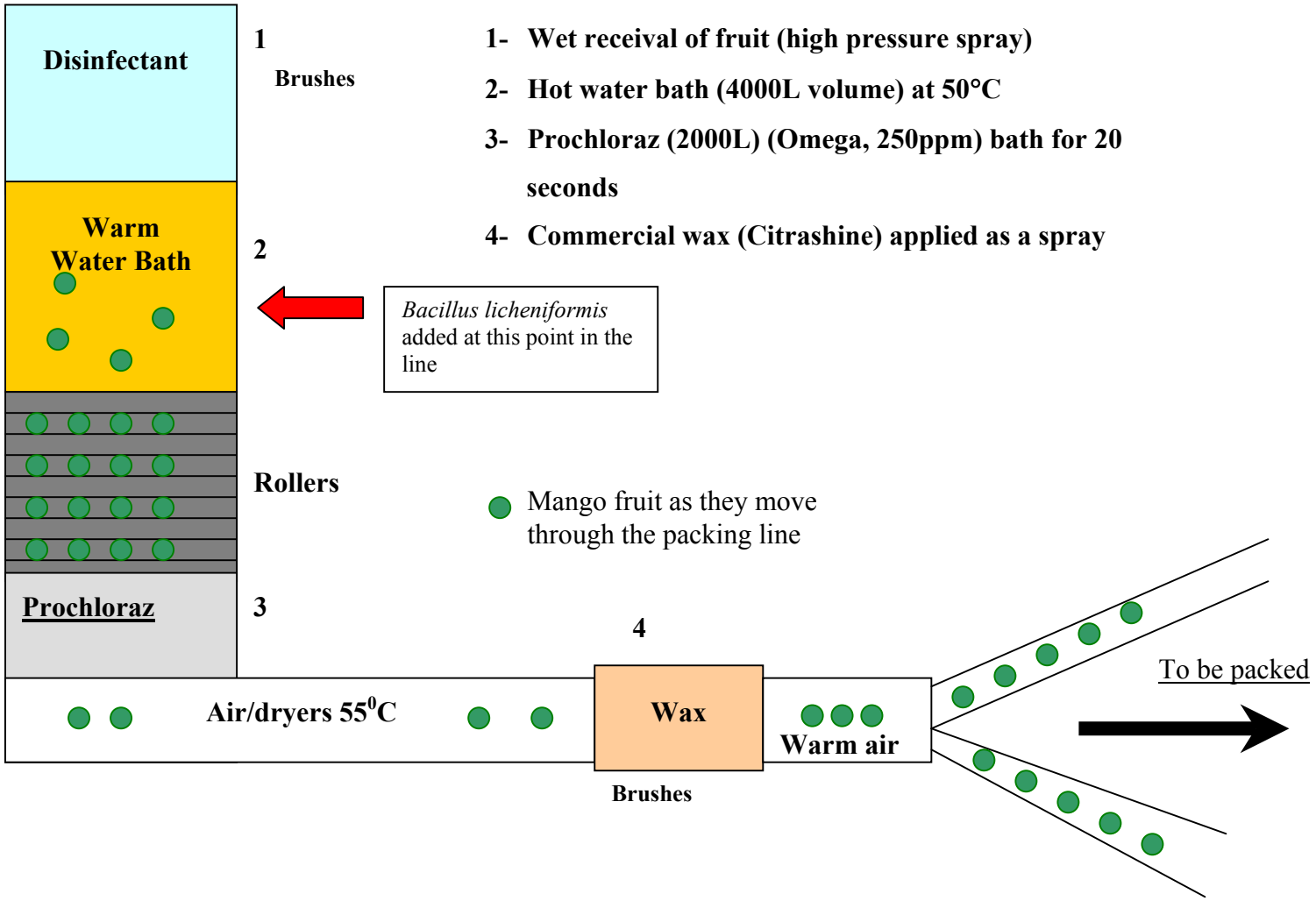
} Filter-sterilize before adding to agar medium (to prevent precipation of insoluble salts and avoid degradation of vitamins)

3. Preparation of RSM-CAS plates

100ml autoclaved CAS-stock solution
900ml autoclaved RSM agar medium

} Add stock solution along glass wall to RSM with enough agitation to achieve mixing without generation of foam

Appendix F: Schematic layout of a commercial packing line used for the preliminary, semi-commercial and commercial trials



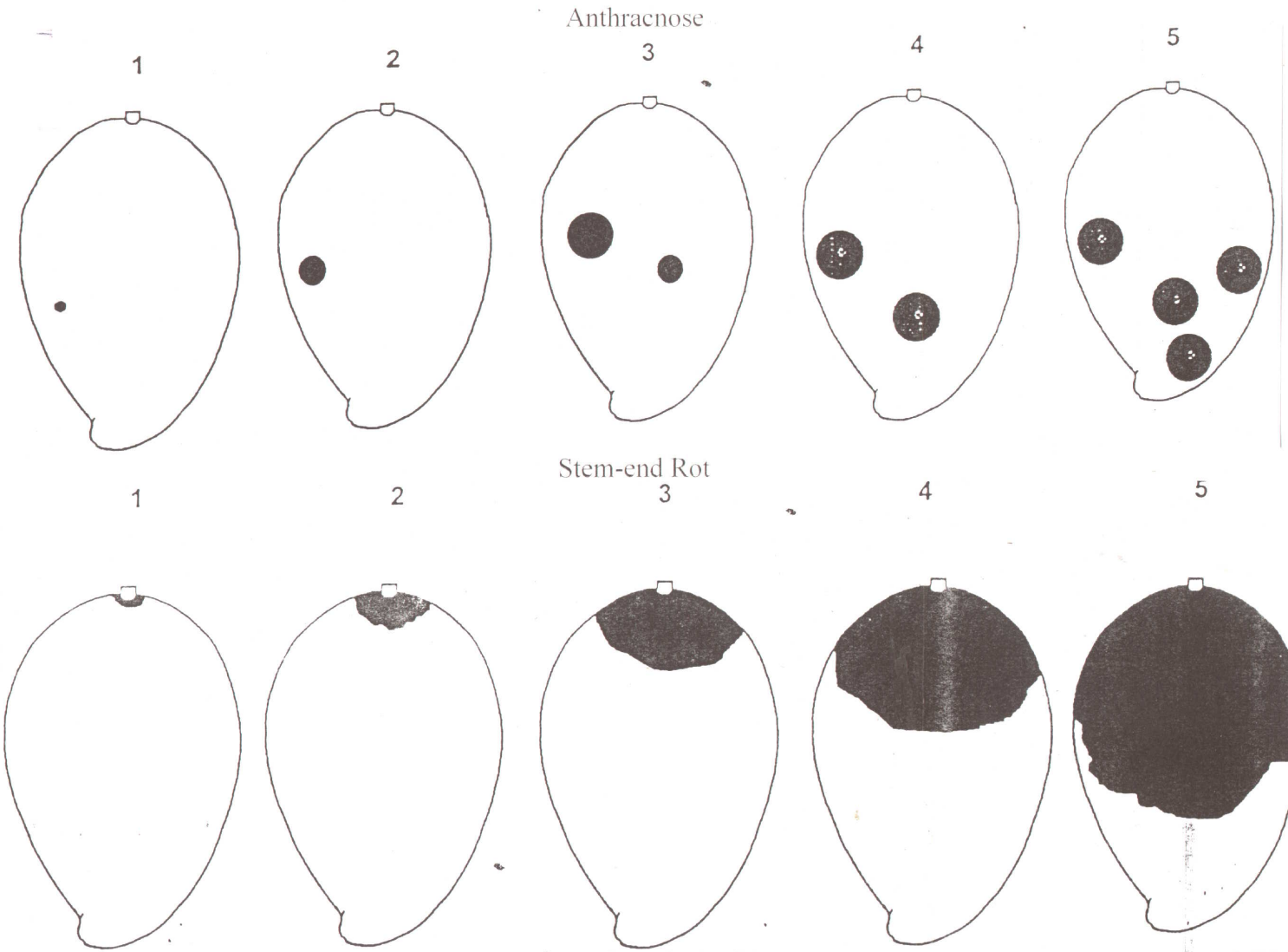
1- Wet receival of fruit (high pressure spray)

2- Hot water bath (4000L volume) at 50°C

3- Prochloraz (2000L) (Omega, 250ppm) bath for 20 seconds

4- Commercial wax (Citrashine) applied as a spray

Appendix G: Evaluation for anthracnose and Stem-end rot (SR) was done according to a 1-5 disease rating as shown below



Appendix H: Mango fruit colour development was evaluated on a 1-5 visual rating scale

A firm fruit, mature mango right after harvest is mostly green with some red blush and a

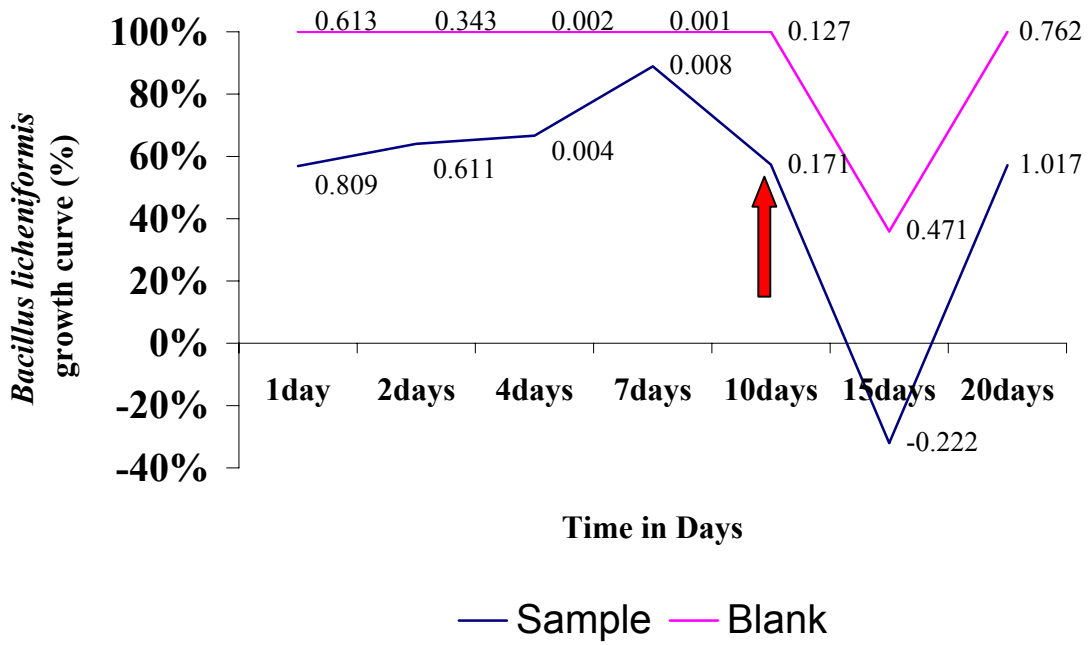
As the mango fruit begins to ripen, it turns a lighter green to yellow colour, with a light red blush

The ripening mango fruit is now half green and half yellow. Any red blush will continue to brighten.

When a mango fruit has reached full ripening it is completely yellow with some full bright red blush (depending on the variety).

As the fruit ripening process continues, the mango turns more yellow and begins to soften slightly. Depending on the variety, there will be varying degree of red blush. A fragrant aroma will emanate from the stem end.

Appendix I: Growth curve of *Bacillus licheniformis* during incubation for evaluation of the production of a bioactive compound



Red arrow indicates Day 10 at this point in the bacterial growth there was production of a volatile compound.

Blank consisted of the growth medium only.