INTEGRATED USE OF YEAST, HOT WATER AND POTASSIUM SILICATE TREATMENTS FOR THE CONTROL OF POSTHARVEST GREEN MOULD OF CITRUS AND LITCHI

By

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ABSTRACT

There is a growing recognition globally that many agrochemicals are hazardous to humans, animals and the environment. Therefore, there is a need to substitute these chemical products with biological and physical treatments, and to change agronomic practices in order to control pests and diseases in agriculture.

The primary objective of this thesis was to develop and test in laboratory, field and commercial packhouses trials as alternative control measures against green mould of citrus (caused by *Penicillium digitatum* Pers: Fr. Sacc) and *Penicillium* molds of litchi (caused by several *Penicillium*).

A South African isolate of *P. digitatum,* isolated from an infected orange fruit, was found to be resistant to imazalil (the standard postharvest fungicide used in South Africa). Sixty yeast and 92 *Bacillus* strains were screened for their antagonistic activity against this isolate of *P. digitatum*. None of the yeasts or *Bacillus* isolates produced a curative action against *P. digitatum* on oranges. However, yeast Isolate B13 provided excellent preventative control of *P. digitatum,* superior to all the *Bacillus* isolates, when it was applied to citrus fruit prior to artificial inoculation with *P. digitatum.* Electron microscopy showed that yeast Isolate B13 inhibited conidial germination of *P. digitatum*. For the control of *P. digitatum* pre-harvest, trees were sprayed with a yeast, Isolate B13, a few months or a few days before harvest. However, this treatment alone proved to be ineffective in providing preventative control of green mould on Valencia oranges.

For the control of *P. digitatum* preharvest, trees were treated with potassium silicate for a full season. Regular potassium silicate treatments resulted in a significant preventative control of *P. digitatum* infection on both navel and Valencia oranges. Treatment of Eureka lemons with potassium silicate as a postharvest treatment for the control of *P. digitatum* resulted in reduced disease lesion diameters when applied preventatively or curatively. Electron

microscopy showed that potassium silicate inhibited germination of *P. digitatum* conidia and growth of its mycelium.

Hot-water dip treatment at 50-58°C for 60-180 seconds (in increments of 15 seconds), significantly reduced infection development in inoculated wounds of Valencia oranges compared with control fruit treated with tap water, without causing any rind damage.

The integration of the yeast, a hot water dip and potassium silicate pre-and postharvest applications provided control of *P. digitatum* control in multiple packhouse trials. The control achieved by the yeast Isolate B13 or hot-water, and potassium silicate in the packhouse alone was superior or equivalent to that provided by imazalil.

A similar study was also carried out to determine possible control measures for *Penicillium* sp. on litchis. In this study, a total of 23 yeast and 13 *Bacillus* isolates were obtained from litchi fruit surfaces. Ten yeast and 10 *Bacillus* isolates that had shown good efficacy against *P. digitatum* of citrus were added to these for screening against *Penicillium* sp. of litchis. None of the yeasts or *Bacillus* isolates produced a curative action against *Penicillium* sp. infection on litchis. However, several yeast isolates (YL4, YL10, YLH and B13) resulted in reduced severity of the pathogen, when applied preventatively, compared with an untreated control. The yeast isolates were superior to all the *Bacillus* isolates, when applied to litchis prior to artificial inoculation by *Penicillium* infection on litchis. Potassium silicate as a postharvest treatment for the control of the pathogen caused reduced lesion diameters when applied preventatively or curatively to naturally infected litchis.

The results presented in this thesis highlight the use of biological, physical and agronomic practices singly or in combination as an alternative control strategy against citrus postharvest green mould. This thesis also provides an insight into expanding these strategies, partly or fully, for the control of other postharvest *Penicillium* infections using litchi as an example.

DECLARATION

I, ABRAHA OKBASILLASIE ABRAHAM, declare that

(i) The research reported in this thesis, except where otherwise indicated, is my original work.

(ii) This thesis has not been submitted for any degree or examination at any other university.

(iii) This thesis does not contain other persons' data, pictures, graphs or other information, unless specifically acknowledged as being sourced from other persons.

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a) their words have been re-written but the general information attributed to them has been referenced;

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INTRODUCTION

Postharvest diseases caused by pathogenic fungi result in great losses to the food industry, and especially to fruit industry (Sommer, 1985). Postharvest losses are not easy to estimate reliably (Tian, 2002). However, citrus losses can reach to 50% (Janisiewicz and Korsten, 2002). *Penicillium* spp. causes severe postharvest storage problems wherever citrus are grown, with significant annual losses up to $50 million in California alone (Eckert and Eaks, 1989). *Penicillium* sp. is also the major postharvest fungal pathogens of litchis (Jiang *et al*., 2001). Losses of litchis are estimated to be 20-30% of the harvested fruit (Jiang *et al*., 2001). In severe cases losses of litchis can also reach 50% before consumption (Jiang *et al*., 2001). Global losses of all fruit probably run into hundreds of millions of dollars. This is a loss incurred at the end of all the farming activities, from land preparation to maturity management, harvesting, packing, and transport and storage management. Thus, there is a great demand for effective measures to reduce the loss.

Current control measures largely revolve around the application of synthetic fungicides. Increasingly, public concern over food safety and the development of fungicide resistance by strains of *Penicillium* has increased the search for alternative means which are less harmful to human health and the environment (Holmes and Eckert, 1999). The search for microbial antagonists to control postharvest decay of fresh fruit has been widely pursued and has emerged as a promising approach for managing postharvest fruit diseases (Ippolito and Nigro, 2000; Palou *et al.,* 2008). Use of materials that are generally regarded as safe (GRAS) (Larrigaudiere *et al*., 2002; Palou *et al.,* 2008), physical control measures and changes in agronomic practices (Lurie, 1998; Palou *et al.,* 2008) also appear to show promise as alternatives for postharvest control of citrus fruit infection.

The overall objective of this study was to investigate the application of a multi-component approach, combining biocontrol agents (yeast and *Bacillus* spp.) with potassium silicate (preharvest and postharvest) and a hot water dip treatment to control of the green mould (caused by *Penicillium digitatum* Pers: Fr. Sacc) of citrus. A preliminary investigation was also conducted on the

control of *Penicillium* sp. of litchi fruit using biocontrol agents (yeast and *Bacillus* spp.) and postharvest application of potassium silicate.

Specific objectives of the thesis were:

1. To investigate the effect of the number of conidia of *P. digitatum* on its pathogenicity on citrus fruit.

2. To evaluate the efficacy of the fungicide imazalil to control *P. digitatum.*

3. To identify yeast and *Bacillus* isolates antagonistic to *P. digitatum* and further investigate their efficacy in controlling infection by the pathogen under *in vivo* conditions.

4. To investigate possible modes of action of effective antagonists in the control of *P. digitatum* by observing interactions in citrus fruit wounds, using environmental scanning electron microscopy (ESEM).

5. To assess the ability of yeast Isolate B13 to colonize citrus fruit surfaces and to investigate its efficacy in controlling postharvest infection by *P. digitatum,* when applied prior to harvest.

6. To investigate the effects of preharvest applications of potassium silicate for the control of *P. digitatum* infection of navel and Valencia oranges, and to determine the silicon content of leaves and fruit of navel and Valencia oranges after harvest.

7. To evaluate the effect of potassium silicate as a postharvest application in order to manage *P. digitatum* and to identify possible modes of action using ESEM.

8. To evaluate the use of a hot water dip as a stand-alone treatment under laboratory conditions.

9. To investigate the individual or combined effect of a hot water dip, yeast Isolate B13 and potassium silicate treatments in three commercial packhouses for the control of *P. digitatum* of Valencia oranges.

10. To isolate yeast and *Bacillus* antagonistic to *Penicillium* spp. of litchis and investigate their efficacy in controlling infection by *Penicillium* spp. on litchis.

11. Use of potassium silicate for the control of *Penicillium* spp. on litchis.

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CHAPTER 1
INTEGRATED CONTROL OF POSTHARVEST GREEN AND BLUE MOULDS OF CITRUS FRESH FRUIT

LITERATURE REVIEW

1.1. INTRODUCTION

Fruit are important food crops (Chakraverty and Singh, 2001) and provide important nutritional values for humans (Kays, 1997). There is a need to increase food production in order to provide nutritional requirements to the world population (Chakraverty and Singh, 2001).

The supply of food can be increased by increasing production and by reducing postharvest losses. The use of high yielding cultivars, fertilizers, water and modern crop management has been attributed to the significant increase of food production during the last few decades (Chakravery and Singh, 2001). However, these researchers noted that hunger and malnutrition can still exist, despite adequate food production, due to uneven distribution of production, biotic and abiotic crop losses and the deterioration of food resources during postharvest operations.

Losses of fresh fruits after harvest may be significant and are particularly high in under-developed countries, accounting for losses up to 50%, and most of them are due to postharvest pathogen attacks (Wilson and Wisniewski, 1989). Fungal diseases are the most common sources of postharvest fruit decay and losses. Harvested produce has a higher value than the same produce in the field. A harvested crop carries the collective cost of the whole farming operation, including soil preparation, planting, fertilization, watering, disease, pest and weed control, harvesting, storage, distribution, and sales. Hence, a 20% loss of a high value produce has substantial impact on the total food production budget (Wilson and Pusey, 1985).

Citrus is the world’s leading fruit crop in terms of volume (quantity) of production. Further, it is grown on six continents in over 100 countries worldwide, in the tropics and sub-tropics (Saunt, 1999). Citrus fruits are

susceptible to a number of postharvest diseases that cause significant losses during the marketing of fresh citrus. Green mould (caused by *Penicillium digitatum* Pers: Fr. Sacc) and blue mould (caused by *Penicillium italicum* Wehmer.) are major sources of citrus fruit decay (Brown and Miller, 1999). For example, *P. digitatum* causes an annual loss up to $50 million in California alone (Eckert and Eaks, 1989). Global losses may run into hundreds of millions of dollars. Other most common postharvest fungal diseases of citrus fruit include stem-end rot (caused by *Physalospora rhodina* Berk. and Curt.) or *Diaporthe citri* H.S. Fawc.), sour rot (caused by *Endomyces geotrichum* E.E. Butler and L.J. Petersen) and brown rot (caused by *Phytophthora citrophthora* R.E. Sm. and E.H. Sm. Leonian), (Brown and Miller, 1999). This review will focus on green and blue moulds.

Maintaining the health of the fresh citrus fruit during all the postharvest processes such as storage, marketing, and consumption is crucial to the achievement of the producer and consumer satisfaction (Brown and Miller, 1999). Crop losses during the postharvest period results not only in a loss of investment or income, but also a loss of consumer confidence, wholesaler contracts and market share (Brown and Miller, 1999), undermining the complex citrus industry as a whole.

1.2. *PENICILLIUM* MOULDS

The *Penicillium* species are ubiquitous, and produce prolific numbers of asexual conidia. Their inoculum is found in soil, on plant surfaces, in dump tank water, (Spotts *et al*., 1988; Spotts and Cervantes, 1993), in contaminated wooden bins (Sanderson and Cervantes, 1995) as well as in the atmosphere (Eckert and Eaks, 1989; Brown, 2006). They are prevalent during the citrus harvest season and often infect citrus fruit through wounds incurred during harvesting and handling (Eckert and Eaks, 1989).

1.2.1. Green mould (caused by *Penicillium digitatum*)

Green mould, caused by *P*. *digitatum,* is a widespread postharvest disease of citrus. It produced chains of varying size and shape conidia (4-7 × 6-8 µm)" (Brown and Eckert, 2000). It is readily recognized by the mass of green conidia produced on infected fruit. Infection takes place through wounds and fruit decay

begins at the infected injury spots. The initial infection area shows a soft watery spot. As the lesion develops, a white mycelium develops and this produces the green conidia (Ismail and Zhang, 2004).

After establishing itself in a wound as the primary point of infection, mycelium of *Penicillium* spp. produces cell wall-degrading enzymes that cause a breakdown of the fruit epidermal cell walls, allowing for a rapid spreadof the fungus over the surface of the infected fruit. The vegetative mycelium then starts to sporulate, with the result that the entire fruit surface may be covered with green conidia (Ismail and Zhang, 2004; Smilanick *et al*., 2006).

1.2.2. Blue mould (*Penicillium italicum*)

Blue mould, caused by *Penicillium italicum*, also develops on citrus fruit through injuries as in the case of green mould. “It produced chains of cyliderical to elliptical or slightly ovate conidia (2-3 × 3-5 µm)” (Brown and Eckert, 2000). It is widespread in the citrus growing world. Blue mould is easily recognized by the mass of blue conidia produced on infected fruit. Initial lesions are similar to the lesions of green mould, but the conidia are blue in colour and are surrounded by white mycelium (Ismail and Zhang, 2004).

1.2.3 *Penicillium* spp. of litchi

Litchis are susceptible to postharvest infection by microorganisms such as bacteria and filamentous fungi (Lonsdale, 1988). *Penicillium* spp. symptoms appear as white foci which later become green to blue (Lichter *et al*., 2004). *Penicillium* spp. is one of the major postharvest fungal pathogens of litchis and the disease is made worse by the fact that it can not be controlled by sulphur fumigation (Kremer-Kohne & Lonsdale, 1990; Jacobs & Korsten, 2004).

1.2.4. *Penicillium* disease epidemiology

Understanding the epidemiology of the disease organisms is essential in order to control postharvest disease (Anonymous, 2005). In particular, understanding the different ways by which the pathogens come into contact, and infects fruit is important in formulating post-harvest control strategies.

Penicillium lives on in the field, on soil debris and produces conidia that infect wounded fruit on the tree and on the ground. The conidia of *Penicillium* are

adaptated to survive unfavourable environmental conditions. Under favourable conditions of temperature and moisture, *Penicillium* develops prolific numbers of conidia. Wind currents disseminate conidia to fruit in the tree canopy and exposed surfaces of the fruit (Brown, 2006). Even tiny injuries provide points of infection for these pathogens (Brown, 2006; Smilanick *et al*., 2006).

The fungus does not usually spread directly as mycelium from decayed fruit to healthy fruit in packed boxes. However, a situation labelled as “soilage” occurs when conidia produced on infected fruit are disseminated to healthy fruit through contact (Brown, 2006). This ability of *P. digitatum* to produce conidia prolifically enables it to develop strains with resistance to fungicide treatments. A fresh infection can cover a whole fruit in only 2-3 days (Brown, 2006). Once active growth has occurred and the *Penicillium* pathogen enters below the epidermal layer of the fruit, control becomes difficult (Anonymous, 2005).

1.3 CONTROL OF *PENICILLIUM* MOULD OF CITRUS FRUIT

1.3.1 Past and current chemical control

Control of postharvest pathogens has been accomplished mainly with the use of chemicals. *Penicillium* postharvest pathogens developed resistance to the fungicide thiabendazole (TBZ) in the California citrus industry in 1981 (Holmes and Eckert, 1999). In a subsequent study, isolates of *Penicillium digitatum* were systematically collected from packhouses in California and screened for fungicide resistance. Some isolates were resistant to the fungicides TBZ, sodium ortho-phenylphenate (SOPP) and imazalil 1-[2-(2,4-dichlorophenyl)-2-(2-prophenyloxy)ethyl]-1H-imidazole *(*Kinay *et al*., 2007*)*. South Africa has also experienced fungicide resistance problems in the citrus industry for the last 30 years (Lesar, 2008). The fungicides sodium ortho-phenylphenate and prochloraz have not been used in citrus packhouses for the last 20 years because of resistance problems (Lesar, 2008). The fungicide imazalil has been the standard fungicide for the treatment of citrus fruit against *Penicillium* postharvest pathogens in South African packhouses for the last 20 years but resistant isolates are now widespread in South Africa (Lesar, 2008).

Recently, a new dual component product for postharvest disease control, combining imazalil and pyrimethanil, has been formulated into a single

formulation known as Philabuster® by Janssen Phamaceutica (Belgium) and has been registered in South Africa (Wever, 2008). This fungicide has resulted in high levels of control of imazalil resistant strains of *P*. *digitatum* (Lesar, 2008). However, this applies strong selection pressure for *Penicillium* to develop resistance against pyrimethanil because the dual fungicide is effectively acting as a single fungicide since the imazalil is ineffective.

1.3.2 Future regulations and issues related to chemical control

Although imazalil is still in use in the citrus industry in South Africa, Lesar (2008) reported that 20 isolates of *Penicillium* were found to be resistant to imazalil, during the period of 2001-2005 as a result of *in vivo* screening of several hundreds *Penicillium* isolates. The existence of biotypes of *Penicillium* resistant to imazalil has also been documented in all major citrus production areas (Eckert *et al*., 1994; Wild, 1994) and terminal markets (Bus *et al*., 1991). Resistance to imazalil by *Penicillium expansum* on apples developed both in the field and in packhouses (Kinay *et al*., 2007). Given the underlying biology of fungicide resistance, it is unlikely that any systemic fungicide will provide a long-term solution to the problem of postharvest *Penicillium* diseases of citrus.

Over time, chemical control has proved to have other negative side effects such as high costs of registrations, negative effects on beneficial microorganisms (Utkhede, 1992) and on the environment, causing soil and water pollution (Akhtar, 1998). It is widely accepted that there is a need to substitute polluting chemicals with less dangerous chemicals or, preferably, cultural practices and biological control measures (Walker and Morey, 1999).

1.4 SANITATION PRACTICES FOR THE CONTROL OF *PENICILLIUM* POSTHARVEST MOULDS

1.4.1 Soil and field conditions

Infected plant material in the field can support large numbers of conidia of *Penicillium* postharvest pathogens, which can be disseminated and distributed by wind onto fruit trees (Anonymous, 2005).

1.4.2 Contaminated water

Pond and stream water may be contaminated by runoff from fields and packhouses that may have large numbers of *Penicillium* postharvest propagules. Therefore, using water from these sources for irrigation may contaminate produce in the field (Anonymous, 2005).

1.4.3 The harvesting and packing pathway

Injury of the flavedo (peel) of citrus fruit typically occurs during harvesting, and subsequent packhouse processes of washing and packing. These wounds provide infection points for *Penicillium* conidia to enter the epidermal layer of the fruit and cause decay. However, careful management of the harvesting and packing of fruit can reduce losses significantly (Anonymous, 2004; Smilanick *et al*., 2006) [For more detailed information, refer to Smilanick *et al*. (2006)].

Penicillium propagules carried with the fruit into the packhouse will contaminate all working surfaces. The propagules may remain viable for months on surfaces such as dump tank, grading lines, and brushes (Anonymous, 2005). All fruit handling tools need to be sterilized daily to remove dirt, condia and infected fruit. It is important to keep the packhouse and its vicinity clear of any infected produce, including culls piles (Anonymous, 2005).

1.4.4 Airborne Conidia

Sanitation may not completely stop contamination of fresh fruit by *Penicillium* conidia because of their presence in the air as suspended particles. The best prevention against such propagules would be sanitation with chlorine (Anonymous, 2005), UV or or ozone.

1.5 CONTROL OF POSTHARVEST *PENICILLIUM* MOULDS OF CITRUS BY SODIUM CARBONATE, SODIUM BICARBONATE AND OTHER FOOD ADDITIVE PRODUCTS

Several additives have potential for disease control, such as silicates (Belanger *et al*., 1995), essential oils (Arras and Usai, 2001), carbonates and bicarbonates (Smilanick *et al*., 1999; Conway *et al*., 2007; Janisiewicz *et al*., 2008; Smilanick *et al*., 2008), chitosan (El-Ghaouth *et al*., 1992) and various acids (Sholberg,

1998). Food additives that control post-harvest diseases would be ideal because they may be applied to the food system without restriction (Maga and Tu, 1994).

Carbonic acid salts, such as sodium carbonate (soda ash) and sodium bicarbonate (baking soda) have been shown to control plant diseases (Palou *et al*., 2001). Treatments of citrus in solutions of sodium carbonate and sodium bicarbonate reduce the incidence of postharvest green moulds (Palou *et al*., 2001). Good control of green mould of oranges has also been achieved in South Africa using both salts (Lesar, 2007). Sodium carbonate has shown the potential to control green mould when applied long after pathogen inoculation (Smilanick *et al*., 1995) and are being used in the California citrus industry (Smilanick *et al*., 1999). Sodium silicate and calcium chloride have been shown to control green and blue moulds of Clementine mandarins (Ligorio *et al*., 2007).

Carbonic acids have been shown to provide pathogen control on other crops, for example, applications of potassium bicarbonate has reduced postharvest infection of sweet pepper (Fallik *et al*., 1997). This product is inexpensive and can be used with minimal danger of injury to fruit (Palou *et al*., 2001).

Other compounds such as sulphur dioxide and ethanol have also been used to control green moulds of lemons without injury to fruit (Smilanick *et al*., 1995). Ethanol occurs in many additives; however, elevated concentrations could be a safety concern because of flammability and negative effects on the air quality of packhouses (Mlikota *et al*., 2004).

Soluble silicon applications as a pre- and postharvest treatment have provided control of fungal pathogens of many crops (Menzies and Belanger, 1996). Some successful examples are given in the Tables 1.1 and 1.2 below.

Table 1.1 Some examples of successful preharvest application of soluble silicon for the control of fungal pathogens

Crop	Pathogen/Disease	Reference
Peas	*Mycosphaerella pinodes*	Dann and Muir (2002)
Rice	Blast	Seebold *et al*. (2001)
Cucumber	Pythium root rot	Cherif *et al*. (1994)
Cucumber	Powdery mildew	Menzies *et al.* (1991)

Table 1.2 Some examples of successful postharvest application of soluble silicon for the control of fungal pathogens of fruit

Crop	Pathogen/Disease	Reference
Chinese Cantaloupe	*Trichothecium roseum*	Guo *et al*. (2007)
Hami melons	*Alternaria alternata*	Bi *et al*. (2006)
	Fusarium spp.	
	Trichothecium roseum	
Sweet Cherry	*Penicillium expansum*	Qin and Tian (2005)
	Monilinia fructicola	
Peach	*Monilinia fructicola*	Biggs *et al*. (1997)

Mechanisms by which silicon provides protection against plant diseases are:

1. Accumulation of the silicon in cell walls around infection sites providing a physical wall to pathogen penetration (Heath and Stumpf, 1986; Carver *et al*., 1987; Datnoff *et al*., 1997).
2. Triggering biochemical defense activities such as phenolic compounds in infected plants (Cherif *et al*. 1994; Epstein, 1999).

1.6 HOT WATER TREATMENT FOR THE CONTROL OF *PENICILLIUM* MOULDS OF CITRUS

Heat treatment is a promising method for reducing postharvest disease infection (Terry, 2007). This treatment can be used on fresh produce after harvest as

brief hot water dips, hot dry air, hot vapour or brief water rinsing and brushing (Terry and Joyce, 2004). Postharvest hot water treatments have been studied for the control of citrus fruit infections and recommendations are provided by numerous authors (Barkai-Golan and Phillips, 1991; Lurie, 1999; Palou *et al*., 2001) and some examples are given in Table 1.3. A detailed review on hot water treatments for the control of postharvest pathogen on a number of crops was provided by Fallik (2004).

Ben-Yehoshua *et al*. (1998) and Schirra *et al*. (2000) reported that the mode of action of heat treatment on citrus fruit is by inducing resistance through increased lignin formation, heat shock related proteins and phytoalexins production against green mould caused by *Penicillium digitatum*.

Table 1.3 Some examples of successful hot water treatments for the control of *Penicillium* disease of citrus fruit

Crop	**Optimal Temperature (**°C)	**Time (seconds)**	**Reference**
Clementine	45	150	Larrigaudiere *et al.* (2002)
Lemon	52-53	120	Nafussi *et al*. (2001)
Minneola tangerines, Shamouti oranges and 'Star Ruby' red grapefruit	56	20	Porat *et al*. (2000)
Mandarin	50-54	180	Schirra and D'hallewin (1997)
Oranges	53	180	Schirra *et al*. (1997)
Grapefruit and lemon	53	120-180	Rodov *et al*. (1995)

1.7 CONTROL OF *PENICILLIUM* MOULDS OF CITRUS USING BIOCONTROL AGENTS

Biocontrol of plant diseases has been defined by Baker (1987) as, “the decrease of inoculum or the disease producing activity of a pathogen accomplished through one or more organisms”. With inundative biocontrol, antagonistic microbes are produced in a laboratory or factory, and are then released to compete with disease causing organisms for nutrient resources, space, or by attacking them directly with secretions of antibiotics, enzymes, and other substances (Baker, 1987).

Biocontrol agents have been widely investigated and promising results have been achieved (Droby and Chalutz, 1998; Janisiewicz and Korsten, 2002). Biological control agents such as bacteria, yeast, and filamentous fungi have been used effectively to protect a number of fresh fruit and vegetables against pathogens (Chalutz and Wilson, 1990). They have been selected because of their antagonistic activity, suitable mode of action and their harmlessness to human beings and to the environment (Arras *et al*., 1999). Many successful laboratory trials have been reported on apples, stone fruit, citrus, grapes, and other fruit. Some semi-commercial scale trials in packhouses have been carried out with success using biological control agents (El-Ghaouth *et al*., 2000). However, the large-scale production and sale of commercial biocontrol agents against postharvest diseases has been limited to two products, Aspire® and YieldPlus® (Droby *et al*., 2002 b).

1.7.1 Use of *Bacillus* spp. for the biological control of *Penicillium* moulds of citrus

The genus *Bacillus* belongs to the family Bacillaceae. *Bacillus* spp. are rod-shaped and generally motile bacteria. The motility is an advantage since it enables the bacteria to scavenge more efficiently for limited nutrients (Brock and Madigan, 1991). Members of the genus *Bacillus* are common residents of the soil and rhizosphere environment (Holl and Chanway, 1992; Mazzola, 1999).

Advantages of *Bacillus* spp. for use as biological control agents include:

1. The ability to form resistant endospores. This inherently improves shelf life (Emmert and Handelsman, 1999).
2. Their ability to produce a multitude of broad-spectrum antibiotic compounds (Rytter *et al*., 1989; Mavingui and Heulin, 1994).
3. Rapid growth and ability to use a wide range of substances as either carbon or nitrogen sources (Glick, 1995).
4. Ease to grow and their spores can be stored as a dried powder (Singh and Deverall, 1984).

Bacillus spp. have been used for many years as biocontrol agents for the control of plant pathogens and to increase plant growth (Turner and Backman, 1991; Holl and Chanway, 1992; Manero *et al*., 1996; Kim *et al*., 1997). Tables 1.4, 1.5 and 1.6 below summarize some of the *Bacillus* spp. that have been investigated for their biological control potential against citrus postharvest diseases, postharvest diseases of other crops, and the use of preharvest applications for the control of postharvest of other crops respectively. In terms of human safety, most *Bacillus* spp. are considered to be safe and of little clinical significance, with the exception of *Bacillus anthracis* and *B. cereus* that pose a risk of infection to humans (Drobniewski, 1993).

Suggested mechanisms of disease control by *Bacillus* spp. include:

1- Competitive antagonism with invading pathogens through competition for nutrients and suitable niches (Larkin and Fravel, 1998; O'Sullivan and O'Gara, 1992).
2- Antibiosis by broad-spectrum antibiotics able to suppress more than one pathogen (Fiddaman and Rosal, 1994; Emmert and Handelsman, 1999).
3- Synthesis of antifungal enzymes such as chitinase and ß-1,3-gluconases (Mauch *et al*., 1988).
4- Induction of systemically acquired resistance (Liu *et al*., 1995).

Table 1.4 Examples of successful biological control by *Bacillus* spp. of *Penicillium* postharvest disease of citrus fruit

Antagonist	Disease/Pathogen	Crop	Authors
Bacillus subtilis	*Penicillium digitatum*	Mandarin	Leelasuphakul *et al*. (2008)
Bacillus subtilis	*Penicillium digitatum and P. italicum*	Valencia orange and 'Shamouti'	Obagwu and Korsten (2003)
Bacillus-pumilus	*Penicillium digitatum*	Valencia orange	Huang *et al*. (1992)
Bacillus pumilus	*Penicillium digitatum*	Lemon	Huang *et al*. (1992)
Bacillus subtilis	*Penicillium digitatum*	Valencia orange	Singh and Deverall (1984)

Table 1.5 Examples of successful biological control by *Bacillus* spp. of postharvest plant pathogenic fungal diseases of fruit crop other than citrus

Antagonist	Disease/Pathogen	Crop	Authors
Bacillus subtilis	*Penicillium* sp.	Litchi	Sivakumar *et al*. (2008)
Bacillus mycoides	*Botrytis cinerea*	Strawberry	Guetsky *et al*. (2002)
Bacillus subtilis	*Peronophythora litchi*	Litchi	Jiang *et al.* (2001)
Bacillus subtilis	*Penicillium expansum* and *Botrytis cinerea*	Apple	Leibinger *et al*. (1997)
Bacillus subtilis	*Monilinia fructicola*	Peach Nectarine Apricot Plum	Pusey and Wilson (1984)

Table 1.6 Examples of successful biological control of plant pathogenic fungal diseases on other crops by preharvest application of *Bacillus* spp.

Crop	**Disease/Pathogen**	**Antagonist**	**Authors**
Loblolly pine	Fusiform rust	*Bacillus pumilus*	Enebak and Carey (2000)
Tomato	*Phytophthora infestans*	*Bacillus subtilis* MB1600 and MB 1205	Knox *et al*. (2000)
Wheat	Take-all and *Rhizoctonia* root rot	*Bacillus cereus* A47 and *Bacillus subtilis* M908	Ryder *et al*. (1999)
Cucumber	*Pythium aphanidermatum*	*Bacillus subtilis* BACT-O	Utkhede *et al*. (1999)
Cucumber plants	Anthracnose Angular leaf spot Cucurbit wilt	*Bacillus pumilus* INR7 *and B. subtilis* GB03	Raupach and Kloepper (1998)
Wheat	*Rhizoctonia solani* AG8 and *Pythium* root rot	*Bacillus subtilis* L324-92	Kim *et al*. (1997)
Avocado	*Pseudo-cercospora purpurea*	*Bacillus subtilis*	Korsten *et al*. (1997)
Cotton	*Fusarium* wilt	*Bacillus subtilis*	Zhang *et al*. (1996)
Beans	*Rhizoctonia solani* AG-4	*Bacillus subtilis*	Turner and Backman (1991)
Geranium leaf	Geranium rust	*Bacillus subtilis*	Rytter *et al*. (1989)
Apple seedlings	*Phytophthora cactorum*	*Bacillus subtilis*	Utkhede (1984)

1.7.2 Biocontrol of citrus green or blue moulds by bacteria other than *Bacillus* sp.

Penicillium disease of citrus can be inhibited by biocontrol bacteria other than *Bacillus* (Wilson and Chalutz, 1989). Two bacterial isolates, *Pseudomonas cepacia* Palleroni and *P. syringae* Van Hall were effective at providing protection of citrus against green or blue mould (Smilanick *et al*., 1996). Smilanick *et al*. (1996) discovered an isolate of *Pseudomonas cepacia* that

controlled green mould very effectively. However, it also caused severe black pit of citrus.

Table 1.7 summarizes bacterial species that have been studied for their biological control of postharvest diseases.

Table 1.7 Examples of successful biological control of postharvest plant pathogenic fungal diseases on other fruit crops by application of bacterial antagonists other than *Bacillus* sp.

Antagonist	Disease/Pathogen	Crop	Authors
Pseudomonas cepacia	*Monilinia fructicola*	Nectarine/peach	Smilanick *et al*. (1993)
Pseudomonas syringae	*Botrytis cinerea* / *Penicillium expansum*	Pear	Janisiewicz and Marchi (1992)
Pseudomonas cepacia	*Penicillium digitatum*	Lemon	Smilanick and Denis-Arrue (1992)
Pseudomonas cepacia	*Botrytis cinerea* / *Penicillium expansum*	Apple/pear	Janisiewicz *et al.* (1991)
Pseudomonas cepacia	*Botrytis cinerea* / *Penicillium expansum*	Apple/pear	Janisiewicz and Roitman (1988)
Pseudomonas fluorescens	*Monilinia fructicola*	Peach/nectarine, apricot and plum	Pusey and Wilson (1984)

1.7.3 Use of yeasts for the biological control of green and blue moulds of citrus

Several yeast biocontrol agents have been reported to effectively control postharvest infection on a variety of fruit (Chalutz and Wilson, 1990; Gholamnejad *et al*., 2009; 2010). Tables 1.8, 1.9 and 1.10 summarize some of the successful biocontrol agents that have been studied for their biological control potentials for citrus postharvest *Penicillium* control, other postharvest diseases of other crops and preharvest applications for the control of postharvest diseases respectively. The mode of action of yeast biocontrol agents include:

1. Competing for space and nutrients (Arras *et al*., 1998; Lima *et al*., 1999),

2. Colonizing the fungal hyhae and a direct effect of live yeast cells on germination of pathogen conidia (Janisiewicz and Korsten, 2002),
3. Extracellular lytic enzymes, such as glucanase and chitinase (Janisiewicz and Korsten, 2002), and
4. Eliciting host resistance (Arras, 1996; Arras *et al*., 1998).

Table 1.8 Examples of postharvest application of yeasts for the control of *Penicillium* diseases of citrus fruit

Antagonist	**Crop**	**Pathogen/Disease**	**Reference**
Various yeasts	Mandarins and tangelo	*Penicillium digitatum*	Arras *et al*. (2007)
Candida spp.	Lemon	*Penicillium italicum*	El-Neshawy (2007)
Various yeasts	Orange	*Penicillium digitatum*	Bouzerda *et al*. (2003)
Candida oleophila	Grapefruit	*Penicillium digitatum*	Droby *et al*. (2002 a)
Candida saitoana	Orange, lemon	*Penicillium digitatum*	El-Ghaouth *et al*. (2000)
Pichia guilliermondii	Grapefruit	*Penicillium digitatum*	Droby *et al*. (1997)
Pichia guilliermondii, Aureobasidium pullulans, Debaryomyces hanseii	Various citrus fruit	*Penicillium digitatum/ Penicillium italicum*	Chalutz and Wilson (1990)
Various yeasts	Lemon	*Penicillium digitatum*	Cheah and Tran (1995)

Table 1.9 Examples of postharvest application of yeasts for the control of fungal pathogens of crops other than citrus

Antagonist	**Crop**	**Pathogen/Disease**	**Reference**
Cryptococcus laurentii	Sweet cherry	*Penicillium expansum* *Monilinia fructicola*	Qin and Tian (2005)
Kloeckera apiculata and *Candida guilliermondii*	Grape/ Peach/ Apple	*Rhizopus stolonifer*	McLaughlin *et al.* (1992)
Sporobolomyces roseus	Apple	*Penicillium expansum*	Janisiewicz *et al*. (1994)
Various yeasts	Chilli	*Colletotrichum capsici*	Chanchaicha-ovivat *et al*. (2007)
Candida guilliermondii/ Pichia membranefaciens	Peach/ nectarine	*Botrytis cinerea*	Tian *et al*. (2002)
Candida spp.	Apple	*Botrytis cinerea/ Penicillium expansum*	McLaughlin *et al.* (1990)
Candida spp.	Peach/ nectarine	*Botrytis cinerea/ Penicillium expansum*	Karabulut *et al*. (2002)
Candida saitoana	Apple	*Botrytis cinerea/ Penicillium expansum*	El-Ghaouth *et al*. (2000)
Candida sake	Pear	*Botrytis cinerea/ Penicillium expansum*	Nunes *et al.* (2002)

Table 1.10 Examples of successful preharvest application of yeasts for the control of fungal pathogens of crops other than citrus

Antagonist	Crop	Pathogen/Disease	Reference
Candida sake	Apple	*Penicillium expansum*	Teixido *et al*. (1998)
Aureobasidium pullulans and *Rhodotorula glutinis*	Apple	*Penicillium expansum*	Leibinger *et al*. (1997)
Cryptococcus infirmo-miniatus, *C. laurentii* and *Rhodotorula glutinis*	Pear	*Penicillium expansum*	Benbow and Sugar (1999)

1.8 INTEGRATION OF BIOCONTROL AGENTS WITH OTHER CONTROL PRACTICES IN THE PROCESSING CYCLE OF CITRUS

Innovative research techniques suggest that biocontrol agents may be important alternatives to chemicals (Lima *et al*., 2007). Bacteria and yeast isolated from fruit surfaces possess properties considered useful for postharvest biocontrol of fruit and vegetables (Lima *et al*., 2007). However, biocontrol agents often fail to consistently control postharvest infections. For this reason, optimizing or improving the performance of biocontrol agents by combining them with other control methods is essential (Karabulut *et al*., 2002).

Several studies have shown that biocontrol efficacy of different selected antagonists can be improved by combining them with: (1) other antagonists, (2) adjuvants (see Table 1.11) on citrus fruit [For more detailed information refer Lima *et al*. (2007)], (3) low dosages of fungicides, (4) physical treatments such as a hot water treatment, (5) preharvest applications of antagonists, or (6) a multi-component approach, as proposed by Lima *et al*. (2007).

Preharvest application of biocontrol agents in the field prior to harvest may enable early colonization of fruit and better protection of wounds from pathogen

infection (Ippolito *et al*., 2004). Hot water, sodium carbonate and sodium bicarbonate have shown poor protection against conidia. Thus, additional treatments would be essential to provide protection for the fruit from reinfection with biocontrol agents that can persist for longer periods, which may achieve this objective as suggested by Smilanick *et al.* (1999). The same author found that the effectiveness of sodium carbonate and sodium bicarbonate against green mould was significantly enhanced when these treatments were followed by the application of *P. syringae* Strain ESC10. Obagwu and Korsten (2003) also found that combining *Bacillus* strain (F1) with sodium bicarbonate was as effective as the recommended fungicide (guazatine and imazalil) treatment, which provided complete control of both green and blue molds of Valencia and Shamouti orange cultivars.

Research by Karabulut *et al.* (2002) has shown that a postharvest treatment combination of hot water brushing at 60°C followed by a yeast antagonist (*Candida* spp.) as a postharvest treatment was very successful in controlling natural infections of *Monilinia fructicola* (G. Winter) Honey. and *Penicillium expansum* (Link) Thom. of peach and nectarine, compared with the hot water treatment alone.

Research by Tian *et al*. (2007) has shown that a postharvest treatment combination of sodium silicate with yeast antagonists (*Cryptococcus laurentii* Kufferath C.E. Skinner. and *Rhodotorula glutinis* Harrison.*)* provided synergistic effects in controlling postharvest diseases caused by *P. expansum*, *M. fructicola* and *A. alternata* in sweet cherry, peach and jujube fruit. It also increased the population density of the antagonistic yeasts.

The application of a multi-component approach that combines biocontrol agents with other control measures would optimize antagonistic efficacy. This could include combining biocontrol agents with more than one of the common field and/or postharvest methods (Lima *et al*., 2007). According to Lima *et al*. (2007), a number of studies have shown that a multi-component approach can provide enhanced or synergistic effects to biocontrol and noted that it should be able to totally control the development of postharvest infections. The multi-component approach has been successfully applied to apples in order to control *Penicillium expansum* (Link) Thom optimum control of postharvest fungal infection has

been achieved by combining heat treatment, calcium chloride and the antagonist *P. syringae* (Conway *et al.,* 1999) or a biocontrol mixture, applied with a heat treatment and/or sodium bicarbonate (Conway *et al.,* 2005).

Table 1.11 Examples of preharvest application of yeasts in combination with adjuvant to enhance efficacy against *Penicillium* disease of citrus

Antagonist	**Adjuvant(s)**	**Pathogen/ Disease**	**Fruit**	**Reference**
Candida saitoana	Glycolchitosan	*Penicillium digitatum*	Oranges	El-Ghaouth *et al*. (2000)
Pichia guillermondii	Ca-chloride	*Penicillium digitatum*	Grapefruit	Droby *et al*. (1997)

In conclusion, citrus is susceptible to a number of postharvest diseases. The green and blue moulds are the most prevalent postharvest diseases affecting citrus fruit. Implementation of an integrated disease control program that includes sanitation, physical treatments, inorganic salts, biocontrol agents as both preharvest and postharvest measures, as discussed in this review, would minimize postharvest losses of citrus, enhance consumer confidence and increase economic returns to the producer.

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CHAPTER 2

THE PATHOGENICITY OF *PENICILLIUM DIGITATUM* AND ITS SENSITIVITY TO THE FUNGICIDE IMAZALIL, IN SOUTH AFRICA

ABSTRACT

Wounded navel oranges were inoculated with one ml of *Penicillium digitatum* suspension at concentrations of 1, 10, 20, 50, 100, 500, 1×10^3, 1×10^4, and 1×10^5 conidia ml^{-1} in the laboratory. There was no significant effect in the wound lesion diameters as the result of the different conidial concentrations. A one conidia ml^{-1} suspension caused damage equivalent to the higher concentrations of conidial inoculum. Other samples of wounded navel oranges inoculated with *P. digitatum* were treated with 100 µl of imazalil suspension at the recommended concentration. *Penicillium digitatum* infection was not significantly reduced by the fungicide treatment compared to the untreated control. This indicates that the pathogen used is resistant to imazalil.

2.1 INTRODUCTION

Green mould caused by *Penicillium digitatum* (Pers: Fr. Sacc.) is the most important postharvest infection of citrus fruit worldwide (Bancroft *et al*., 1984), including South Africa (Lesar, 2007). This wound-dependent pathogen has a relatively short disease cycle (3-5 days) at 25°C and, on a single fruit, can produce 1-2 billion conidia with relative ease (Holmes and Eckert, 1995). Potentially, *P. digitatum* arising from a single conidium can produce about 100 million conidia on an infected fruit in seven days under optimum environmental conditions (Brown and Miller, 1999).

Varying conidial concentrations of *P. digitatum* in green mould disease evaluations have been used by different researchers, e.g. Droby *et al*. (1997) on grapefruit and Porat *et al*. (2000) on citrus fruit inoculated 10^4 conidia ml^{-1}, while Palou *et al.* (2001) and Eckert *et al*. (1994) used 10^6 conidia ml^{-1} on oranges, Holmes and Eckert (1999) on Eureka lemons inoculated with 10^7 or 10^8 conidia ml^{-1}. It demonstrates that a wide range of conidial concentrations of

the pathogen have been recorded to induce infection. However, the minimum dose of pathogenic conidia has not been investigated.

In most South African packhouses, citrus fruit are treated with imazalil to control the incidence of *P. digitatum* infection and sporulation (Holmes and Eckert, 1999). Imazalil (1-[2-(2,4-dichlorophenyl)-2-(2-prophenyloxy)ethyl]-1H-imidazole) was first adopted by the Californian citrus industry in 1981 as an additive to fruit coating formulations to replace thiabendazole, which was rendered ineffective due to the development of pathogen resistance (Holmes and Eckert, 1999). Kinay *et al.* (2007) reported that isolates of *Penicillium digitatum* in Californian citrus packhouses were found to be resistant against the fungicide imazalil. Boubaker *et al*. (2009) also reported that 19% (55/290) isolates were found to be resistantant against imazalil. This fungicide is used in a manner that is highly conducive to the selection and proliferation of resistant biotypes of *P. digitatum.* Continuous selection pressure for resistance to the fungicide is applied in the farm and packhouse environments because the fungicide is continuously used for a six months harvest period in a multitude of packhouses, dump tanks, on conveyor belts and inside timber lug boxes. The fungicide is also applied to all citrus fruit surfaces. Furthermore, in the case of exported fruit, the fungicide is exposed to a six week period in refrigerated containers, followed 2-3 weeks at room temperature in the market place. This provides for additional selection pressure on the pathogen population to develop resistance against the fungicide (Holmes and Eckert, 1999).

Imazalil-resistant biotypes of *P. digitatum* were reported just five years after its introduction as a commercial treatment in California packhouses (Holmes and Eckert, 1999). The existence of resistant biotypes of *P. digitatum* to imazalil has been documented in all major citrus production areas (Eckert *et al*., 1994; Wild, 1994) and terminal markets (Bus *et al*., 1991).

This investigation was undertaken: (1) to evaluate the influence of conidial concentration on *P. digitatum* pathogenicity and (2) to determine the imazalil sensitivity of the strain of *P. digitatum* used in subsequent trials.

2.2 MATERIALS AND METHODS

2.2.1 Isolation of *P. digitatum* from navel oranges

A single spore isolate of *P. digitatum* was cultured from conidia of infected navel oranges obtained from Gateway packhouse (29.53 S 30.17 E), Thornville, Pietermaritzburg, South Africa by directly plating the conidia onto PDA (Merck Laboratory, South Africa) amended with 0.15 g ℓ^{-1} of Rose Bengal (BDN Laboratory, England). Plates were incubated at 25°C for 10 days. Pure cultures of *P. digitatum* isolate were established by sub-culturing on malt extract agar (MEA) (Merck Laboratory, South Africa) plates after identification and verification of *P. digitatum* conidia under a compound microscope. For long term storage the conidia were maintained in double autoclaved, distilled water storage at 25°C. Regular transfers onto fresh citrus fruit were performed to maintain the aggressiveness of the pathogen. A conidial suspension was prepared by washing a 10 days old culture of *P. digitatum* onto MEA medium, with sterile distilled water containing 0.01% (wt/vol) Tween 80 (Uni Laboratory, South Africa) for inoculation purposes.

2.2.2 Pathogenicity of an isolate of *P. digitatum* on navel oranges

Penicillium digitatum conidia isolated from navel oranges as described in Section 2.2.1 was used. Navel fruit were collected from citrus trees at Ukulinga Research Farm, University of KwaZulu-Natal, Pietermaritzburg, South Africa (29.36 S 30.24 E). They were surface disinfected with 70% alcohol for one minute, dried, and were wounded with a dissecting needle (25 mm in length × 3 mm in depth) at one site at the equator of the fruit. The wounded fruit were inoculated with one ml (applied as two deposits) of the conidia suspension of *P. digitatum* at concentrations of 1, 10, 20, 50, 100, 500, 1×10^3, 1×10^4, or 1×10^5 conidia mℓ^{-1}, adjusted with a haemocytometer and applied as two deposits. Distilled water (one mℓ) was pipetted into wounds to serve as controls. Inoculated fruit were kept at room temperature (24±1°C). Two boxes, with five fruit per box (each fruit was placed in a discrete compartment to avoid fruit touching other fruit) were used per treatment and placed on a bench in a complete randomized block design (CRBD). Lesion diameters (mm) of each infected wound were determined 10 days after inoculation. Lesion diameter was measured taking the mean of horizontal and vertical diameter of the lesion.

2.2.3 Sensitivity of an isolate of *P. digitatum* to imazalil on lemons

To assess the effects of imazalil (500 mg ℓ$^{-}$1) for the control of *P. digitatum* on lemon, fruit were treated similarly to that described in Section 2.2.2. Wounded fruit were separately treated with a 100 µℓ of imazalil. After the wound site had dried for three hours, each wound was inoculated with 100 µl of conidial suspension of *P. digitatum* (1 ×10^{4} conidia mℓ$^{-1}$). Wounds inoculated with 100 µℓ of conidial suspension of *P. digitatum* only served as the control. Fruit were kept at room temperature (24±1°C). Two boxes, with five fruit per box, were used per treatment and placed on a laboratory bench in a CRBD. The lesion diameters (mm) of infected wounds were determined 10 days after inoculation. Lesion diameter was measured by taking the mean of the horizontal and vertical diameters of each lesion.

2.2.4 Statistical analysis

Data were subjected to an analysis of variance (ANOVA) using Genstat® Executable Release 9.1 Statistical Analysis Software (Anonymous, 2006). To determine differences between treatments, Fisher's Least Significant Difference Test was used ($P<0.05$).

2.3 RESULTS

2.3.1 Pathogenicity of an isolate of *P. digitatum* on navel oranges

Lesion diameter of navel oranges wounds inoculated with *P. digitatum* at 10 conidia per ml was significantly lower than those navel fruit wounds inoculated with 500, 10,000 or 100, 000 conidia per ml. However, it was not significantly lower than the rest of the fruit inoculated at different concentrations of *P. digitatum* (a detailed statistical analysis of the data in Figure.2.1 is provided in Appendix 2A).

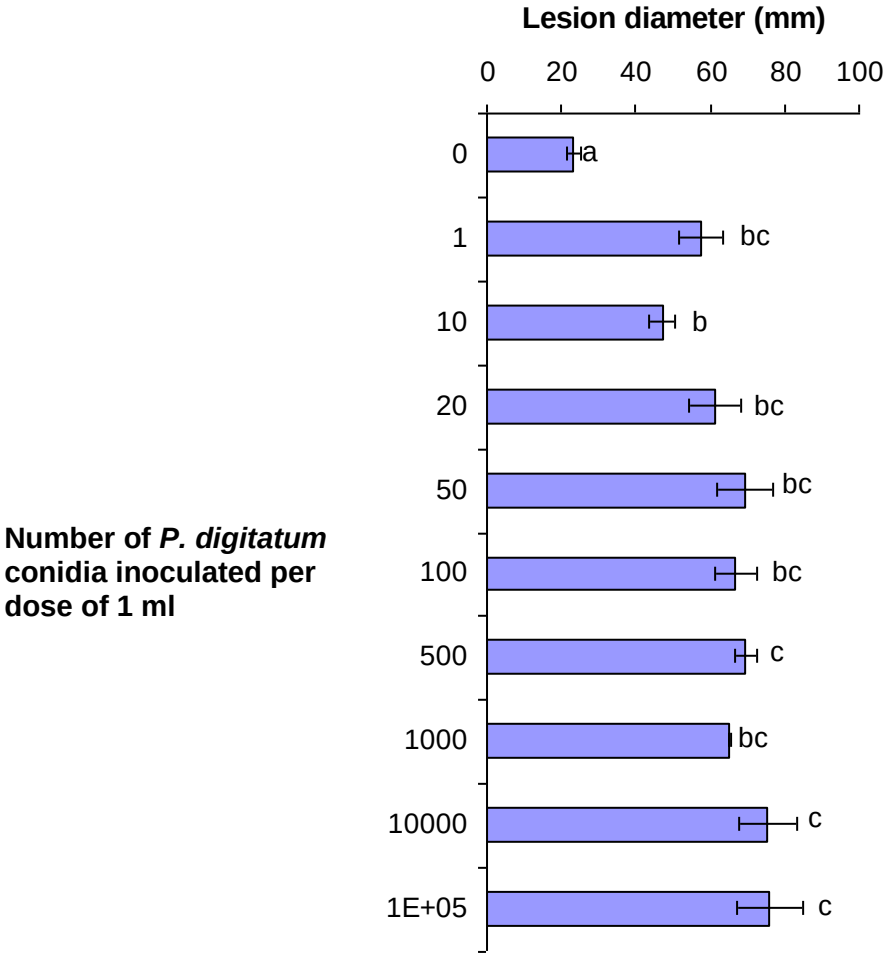

Figure 2.1 Lesion diameter as a result of different inoculum doses applied, as conidia $m\ell^{-1}$, of *P. digitatum* ($p \leq 0.05$), 10 days after inoculaion.

2.3.2 Sensitivity of *P. digitatum* isolate to imazalil on lemons

There was no significant difference in lesion diameter between the inoculated control and treated with imazalil suspension on lemons (a detailed statistical analysis of the data in Figure 2.2).

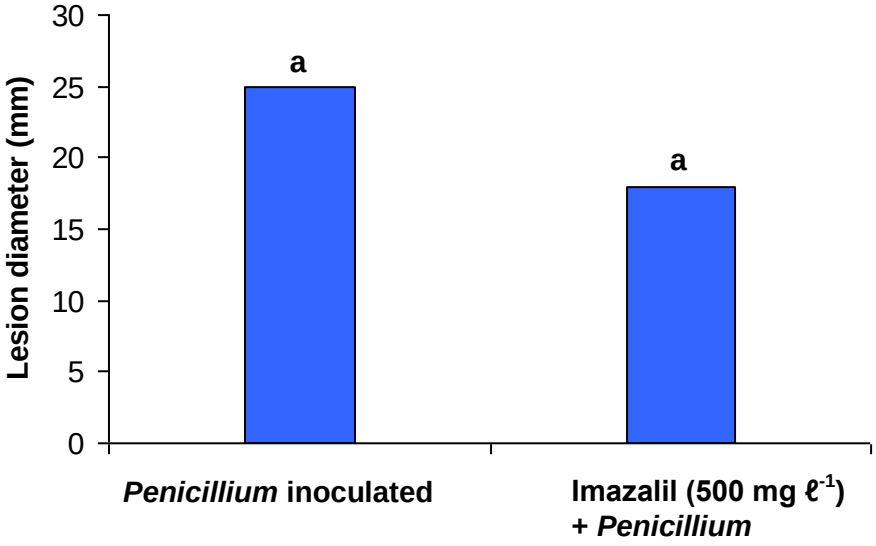

Figure 2.2 Sensitivity of of *P. digitatum* isolate to imazalil treatment on artificially inoculated lemons 10 days after inoculation.

2.4 DISCUSSION

All the conidial concentrations of *P. digitatum* caused similar lesion sizes. This may have been due to competition between conidia. Alternatively, disease development could be limited by the availability of nutrients, limiting pathogen development to close to that of a conidial concentration of 1 conidia $m\ell^{-1}$. A lesion diameter of 20 mm was recorded on uninoculated navel oranges. This could have been caused by pre-existing infection of the pathogen on the fruit, which may have occurred during preharvest activities or transport or contamination due to interplot interference.

A single conidium of *P. digitatum* can infect a citrus fruit. Brown and Miller (1999) recorded that a single conidium of *P. digitatum* produced up to 100 million conidia on an infected fruit within one week. Therefore, it is not necessary to use high concentrations of conidial suspensions of *P. digitatum* for inoculations for disease evaluations.

The isolate of *P. digitatum* used was not effectively controlled by imazalil, indicating that the *P. digitatum* isolate used in this research was resistant to imazalil. This isolate was also found to be resistant in an agar plate test (Moon, 2008). Similar findings have been reported by Eckert (1987); Bus *et al*. (1991);

Eckert *et al*. (1994) and Wild (1994). Additional reports suggest that resistance problems with imazalil have also occurred on soft skin citrus fruit in the Western Cape Province of South Africa (Beghin, pers. comm. 2006). Further incidences of resistance have occurred in Richmond, KwaZulu-Natal, South Africa (Herrer, pers. comm. 2006). Subsquent research by Moon (2008) showed that 10 out of 10 isolates of *P*. *digitatum* that were collected in packhouses in northern KwaZulu-Natal were resistant to imazalil. Boubaker *et al*. (2009) found that 55 out of 290 isolates of *P*. *digitatum* developed resiatnce against imazalil. To the best of our knowledge, this is the first formal report of an imazalil resistant *P. digitatum* isolate derived from citrus fruit grown in KwaZulu-Natal, South Africa.

2.5 CONCLUSION

Clearly, postharvest disease of citrus fruit can result from exposure to very low levels of inoculum. This coupled with the failure of imazalil to control of some isolates of green mould (Boubaker *et al*., 2009), poses a serious threat to the citrus industry in South Africa. These results suggest that there is a need for alternative methods for control of *P. digitatum* in the citrus industry in South Africa, and globally.

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CHAPTER 3

ISOLATION AND *IN VIVO* SCREENING OF YEAST AND *BACILLUS* ANTAGONISTS TO INHIBIT *PENICILLIUM DIGITATUM* AND CONTROL GREEN MOULD OF CITRUS FRUIT

ABSTRACT

A total of 60 yeast and 92 *Bacillus* isolates were isolated from the fruit surface of papaya and several varieties of citrus from various orchards in South Africa, and screened for antagonism to *Penicillium digitatum*. Ten yeast and 10 *Bacillus* isolates reduced the surface area of visible *P. digitatum* growth ≥50%, when applied three hours before inoculation with the pathogen. Two yeast isolates (B13 and Grape), when applied 48 hours prior to inoculation with *P*. *digitatum,* prevented decay of navel oranges and lemons, and ≤5% incidence on Valencia oranges, compared with an untreated control that had ≥50% incidence of infection. The application of isolates to lemons and Valencia oranges did not produce a curative action against *P*. *digitatum* when applied three hours post infection. The yeast isolates B13 and Grape were superior to all the *Bacillus* isolates, and provided excellent control of *P. digitatum,* when applied to citrus fruit prior to artificial inoculation by *P. digitatum*.

3.1 INTRODUCTION

Green mould of citrus, caused by *Penicillium digitatum* (Pers.: Fr) Sacc. is a major cause of postharvest disease of citrus fruit worldwide (Bancroft *et al*., 1984). Control of the disease has depended upon the use of synthetic fungicides. However, the development of strains of *P. digitatum* and other fungi that are resistant to fungicides (El-Goorani *et al*., 1984; Eckert *et al*., 1994) and increasing public concern over food safety, and the environment (Holmes and Eckert, 1999) are driving a search for alternative postharvest disease control methods. Biological control has been proposed as a key alternative control method, and some effective antagonistic microorganisms have already been used in the global fruit industry (McLaughlin *et al.*, 1992).

Successful control of infections caused by a number of postharvest pathogens using biological control agents have been reported on citrus fruit (Wilson and Chalutz, 1989; Chalutz and Wilson, 1990; Cheah and Tran, 1995; El-Ghaouth *et al*., 2000; Bouzerda *et al*., 2003). Numerous yeast antagonists have been reported to successfully control postharvest infection on a variety of fruit (Chalutz and Wilson, 1990; Leelasuphakul *et al*., 2008; Gholamnejad et al., 2009; 2010). Yeasts are particularly suitable as antagonistic agents because they grow rapidly, colonizing fruit surfaces and limiting nutrient availability to pathogens through competition, and are tolerant of most agrochemicals (Richard and Prusky, 2002). Two yeast products, Aspire® (*Candida oleophila* Montrocher) and Yield-Plus® (*Cryptococcus albidus* (Saito) Skinner) are commercially available (Vero *et al*., 2002).

Bacillus spp. are also used as biological control agents (Leifert *et al*., 1995; Kim *et al*., 1997; Enebak and Carey, 2000). *Bacillus subtilis* Ehrenberg Cohn strains typically have the ability to survive on citrus fruit surfaces and some are antagonistic to pathogens (Gutter and Littauer, 1953).

The objectives of the study were: (1) to identify yeast and *Bacillus* isolates antagonistic to *P. digitatum*; and (2) to investigate their efficacy in controlling infection by *P. digitatum in vivo.*

3.2 MATERIALS AND METHODS

3.2.1 Fruit used for isolation of potential antagonists

Fruit of papaya (*Carica papaya* L.), granadilla (*Passiflora quadrangularis* L.) and a range of citrus [i.e., navel orange (*Citrus sinensis* [L] Osbeck), Valencia orange (*Citrus sinensis* [L] Osbeck), lemon (*Citrus limon* Burmann), grapefruit (*Citrus paradisi* Macf) and mandarin (*Citrus reticulata* Blanco)] were harvested from commercial orchards and home guardens in KwaZulu-Natal and Mpumalanga, South Africa. Undamaged fruit were processed either immediately, after storage for 2-3 days at room temperature or after storage in a cold room at 8±1°C for 5-7 days.

3.2.2 Preliminary investigation of microorganisms on the fruit surface

Navel and Valencia oranges that had not been sprayed with fungicides were collected from citrus orchards located at Ukulinga Research Farm, University of KwaZulu-Natal, Pietermartizburg (29.36 S 30.24 E), South Africa, and stored at 25°C for two weeks. Whole oranges were rinsed with sterile water in order to remove any potential antagonists. The rinsing water was then serially diluted (10 fold dilution series was made up to 10^{-4}) and plated onto potato dextrose agar (PDA) (Merck Laboratory, South Africa). The plates were incubated at 25°C for four days. Yeasts or/and *Bacillus* colonies were selected and identified visually by their typical colony morphologies.

3.2.3 Isolation of antagonistic yeasts and *Bacillus*

Bacillus and yeast isolates were recovered from the peel of 3-5 mature fruit from a range of the fruit samples, as described in Section 3.2.2. The fruit peel was cut into 10-15 small pieces, weighing 50 grams and placed in separate 250 mℓ Erlenmeyer flasks containing 100 mℓ sterile distilled water plus quarter strength Ringer's Solution and shaken in a water bath (G.F.L. 1083, Labortechnik, Germany) at 120 rotations per minute (rpm) for one hour at 30°C. Fruit peel pieces were removed and the liquid suspension was used to make a serial dilution of 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4}. An aliquot of 0.2 mℓ of each dilution was plated onto PDA amended with 0.15 g ℓ^{-1} of Rose Bengal (BDN Laboratory, England) for recovery of yeast isolates and incubated at 25°C for three days. Pure cultures of yeast were made by sub-culturing from discrete colonies on the plates. For isolation of *Bacillus*, the same serial dilution prepared for the yeast isolates was used, after heat treatment at 80°C for 15 minutes in a water bath. Each aliquot of 0.2 mℓ was poured onto a tryptone soy agar (TSA) (Merck Laboratory, South Africa) plate. Plates were incubated for 3 days at 28°C, after which representative colonies were arbitrarily selected and streaked onto fresh TSA plates to obtain single colonies. Isolates were stored in sterile distilled water and -80°C freeze in 20% glycerol.

3.2.4 Screening of yeasts and *Bacillus* isolates against *P. digitatum*

3.2.4.1 Preliminary Screening

A total of 60 yeast and 92 *Bacillus* isolates (see Section 3.2.1) were tested on navel oranges. Each fruit was surface disinfected with 70% alcohol for one minute, dried, and then wounded (2 mm in width and 1 mm in depth) with a

disinfected needle 10 times at both ends of the fruit. The wound was dipped into a suspension of yeast cells or *Bacillus* (1×10^8 cells $m\ell^{-1}$) for one minute. Three hours after the wound site had dried, each wounded fruit was dipped into a suspension of conidia of *P. digitatum* at 1×10^4 conidia $m\ell^{-1}$, isolated from infected navel oranges, collected at Gateway Packhouse (29.53 S 30.17 E), Thornville, Pietermaritzburg, South Africa. The conidial suspension was quantified using a haemocytometer and then adjusted to the final concentrations by dilution. Control fruit were treated with sterile distilled water. Fruit were kept at room temperature (24±1°C) for 10 days. One box with three fruit was used per treatment. Treatments were placed on a bench. Fruit were examined for percentage fruit surface area covered by *P. digitatum* using visual estimations for the initial screening. The criterion used to select the antagonists was the ability to reduce growth or development of *P. digitatum* to ≤50%.

3.2.4.2 Secondry Screening

Further tests were conducted on the most promising yeast (10) and *Bacillus* (10) isolates (a detailed description of locations from where fruit has been obtained and isolates of yeast and *Bacillus* recovered is presented in Appendix 3A) following the procedures described in Section 3.2.4.1. However, in this instance Valencia oranges were used for the evaluation, and fruit was wounded (3 mm in length × 3 mm in depth) at one site on the fruit equator with a dissecting needle. The wound was then treated with a 100 µℓ cell suspension of the test organism (yeast or *Bacillus* at 1×10^8 cells $m\ell^{-1}$). After the wound site had dried for three hours, 100 µℓ of the conidial suspension of *P. digitatum* (1×10^4 conidia $m\ell^{-1}$) was inoculated into the wound. Wounds inoculated with the same amount of *P. digitatum* isolate, but no biocontrol pretreatment, served as the control. Fruit were kept at room temperature (24±1°C). Two boxes, with five fruit per box, were used per treatment and placed on a bench in a complete randomized block design (CRBD). The criterion used to select the antagonist was the reduction of lesion diameters (mm) caused by *P. digitatum* 10 days after inoculation. Lesion diameter was measured by taking the mean of the horizontal and vertical diameters of each lesion.

3.2.5 A selected yeast and *Bacillus* isolates antagonistic against *P. digitatum*

3.2.5.1 Preventative Action

Ten yeasts and 10 *Bacillus* isolates were tested for their preventative action against *P. digitatum*. This was achieved by treating fruit with an antagonist 48 hours before inoculation with the pathogen. The procedures described in Section 3.2.4.2 were followed for the preparation of the antagonist, *P. digitatum*, and for the treatment application. Navel and Valencia oranges, as wellas lemons were used in this trial. The fruit wound was extended to 25 mm in length × 3 mm in depth. The wound was treated with 100 µℓ of cell suspension of the test organism (yeast or *Bacillus* at 1×10^8 cells $m\ell^{-1}$). After the wound site had dried for 48 hours, each wound was inoculated with 100 µℓ of the suspension of conidia of the *P. digitatum* isolate (1×10^4 conidia $m\ell^{-1}$). Wounds inoculated with the same amount of *P. digitatum* isolate served as the control. Fruit were kept at room temperature (24±1°C). Two boxes, with five fruit per box, were used per treatment and placed on a bench in a CRBD. Lesion diameter (mm) of each infected wound was determined 10 days after inoculation. Lesion diameter was measured by taking the mean of the horizontal and vertical diameters of each lesion.

3.2.5.2 Curative Action

Similar procedures as described in Section 3.2.5.1 were followed, with the difference that navel oranges were not included because they were out of season. Valencia oranges or lemons were wounded, and then inoculated with 100 µℓ of the suspension of *P. digitatum* conidia (1×10^4 conidia $m\ell^{-1}$). After the wound site had dried for three hours, the wound was treated with a 100 µl of the test organism (yeast or *Bacillus* at 1×10^8 cells $m\ell^{1}$). Wounds inoculated with *P. digitatum* conidial suspension served as a control. Fruit were kept at room temperature (24±1°C). Two boxes, with five fruit per box, were used per treatment and placed on a bench in a CRBD. Lesion diameter was measured by taking the mean of the horizontal and vertical diameters of each lesion.

3.2.6 Dose effect of two yeast isolates, B13 and Grape, applied preventatively on lemons for the control of *P. digitatum*

The effect of various concentrations of two yeast isolates, namely, B13 and Grape (identified as strains of *Candida fermentati* (Saito) Bai. by Botes[1], were studied for their preventative action on lemons against *P. digitatum*. Lemons were surface disinfected with 70% alcohol for 1 minute, dried, and then wounded. Fruit were wounded (25 mm in length × 3 mm in depth) at one site on the equator with a dissecting needle as described in Section 3.2.5.1. Cell suspensions (100 µℓ) of both yeasts of 1×10^5, 1×10^6, 2.5×10^6, 1×10^7 and 1×10^8 cells mℓ$^{-1}$ were inoculated into each wound site. The technique used was based on that reported by Tian *et al*. (2002). Wounds treated with 100 µℓ of distilled water served as a control. After 48 hours, all yeast-treated wounds and control wounds were inoculated with a 100 µℓ conidial suspension of *P. digitatum* (1×10^4 conidia mℓ$^{-1}$). Fruit were kept at room temperature (24±1°C). Two boxes, with five fruit per box, were used per treatment and placed on a bench in a CRBD. Lesion diameter was measured by taking the mean of the horizontal and vertical diameters of each lesion.

3.2.7 Statistical analysis

With one exception, all data sets were analysed using a REML (REsidual Maximum Likelyhood) Variance Component Analysis using Genstat® Executable Release 9.1 Statistical Analysis Software (Anonymous, 2006). Where the F test was significant, differences between treatment means were determined using Duncan's Multiple Range Test ($P \leqslant 0.05$). Detailed analyses are presented in Appendixes 3B-3E.

The exception was in the case of measuring dose effects of two yeast isolates, B13 and Grape, for the control of *P. digitatum*, when applied preventatively on lemons. In this case, the data was subjected to an analysis of variance (ANOVA) using Genstat® Executable Release 9.1 Statistical Analysis Software (Anonymous, 2006). To determine differences between treatments, Fisher's Least Significant Difference Test was used ($P<0.05$).

[1] Dr Botes, A. University of Stellenbosch, South Africa.

3.3 RESULTS

3.3.1 Preliminary investigation of microorganisms on the fruit surface

The potato dextrose agar plates yielded dense bacterial and yeast populations with little growth of filamentous fungi being apparent (Figure 3.1).

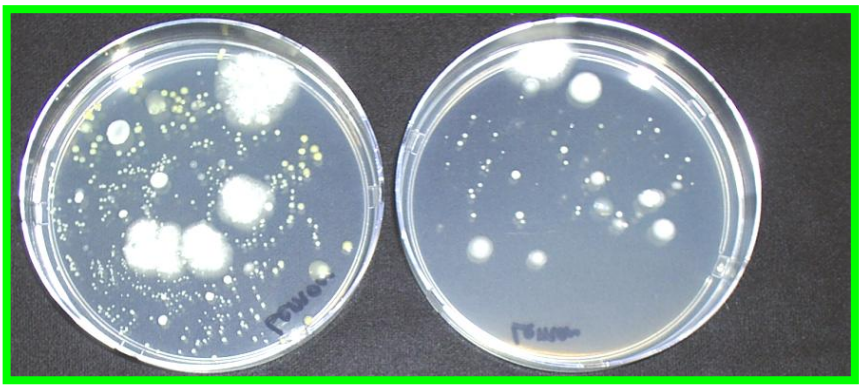

Figure 3.1 Cultures of microorganisms isolated from the surface of navel and Valencia oranges, after four days growth on potato dextrose agar: direct plating of the rinsing water (left), plating of rinsing water after a tenfold dilution with sterile water (right).

3.3.2 Preliminary screening of antagonistic yeast and *Bacillus* isolates against *P. digitatum* on navel oranges

All 60 yeasts and 92 *Bacillus* isolates recovered from the surface of numerous different fruit reduced *P. digitatum* development compared to the untreated controls, which developed 90% infection by *P. digitatum* (Table 3.1). Only 20 isolates (10 yeast and 10 *Bacillus* isolates) (for the source of isolates see Appendix 3A) reduced the fruit surface area infected by *P. digitatum* on navel oranges by ≥50% (Table 3.1). Among the 10 yeast isolates, four isolates (Grape, ON3, EP and RG2) reduced the area of the fruit surface to *P. digitatum* ≤30%. The best *Bacillus* isolate (Si-1) reduced *P. digitatum* deases development by 53% (Table 3.1). Yeast and *Bacillus* isolates that reduced the percentage of fruit surface area infected to ≤50% were chosen for further screening.

Table 3.1 Preventative treatment of navel oranges with yeast and *Bacillus* isolates to control *Penicillium digitatum*

Isolate and Control	**Isolate Type**	**% Surface Area of Fruit Infected by *P. digitatum***	**% Reduction of *P. digitatum by* Antagonists**
S1-1	*Bacillus*	42	53
B7	*Bacillus*	43	52
B9 (1)	*Bacillus*	45	50
B9 (2)	*Bacillus*	48	47
Papaya	*Bacillus*	49	46
B3	*Bacillus*	50	44
B6	*Bacillus*	50	44
B8	*Bacillus*	50	44
GW1	*Bacillus*	50	44
S1-2	*Bacillus*	50	44
Grape	Yeast	19	79
ON3	Yeast	21	77
EP	Yeast	24	73
GR2	Yeast	26	71
Lemon P	Yeast	30	67
UL3	Yeast	37	59
SPL	Yeast	38	58
GR1	Yeast	40	56
B13	Yeast	48	47
B-a	Yeast	50	44
Others (132 isolates)	*Bacillus* and yeasts	>50	44
Water (control)	*Penicillium digitatum* only	90	0

3.3.3 Screening of selected yeast and *Bacillus* isolates antagonistic to *P. digitatum* on Valencia oranges

Yeast isolates, Grape, EP, ON3, RG2, Lemon P, GR1, B13, and B-a, and the *Bacillus* isolate B3 (for the original source of the isolate see Appendix 3A) significantly ($p \leq 0.05$) reduced lesion diameter on Valencia oranges compared to the other treatments and the untreated control in reducing lesion diameter (a detailed statistical analysis of the data in Table 3.2 is provided in Appendix 3B).

Table 3.2 Preventative treatment of Valencia oranges with 10 yeast and 10 *Bacillus* isolates to control *Penicillium digitatum*

Isolate and Control	**Isolate type**	**% surface area of fruit covered by *P. digitatum***
B3	*Bacillus*	28.6 ab
B7	*Bacillus*	35.8 bcdf
B9 (2)	*Bacillus*	37.0 bcdef
GW1	*Bacillus*	39.8 cdefg
Papaya	*Bacillus*	40.3 cdefg
S1-1	*Bacillus*	41.8 defg
S1-2	*Bacillus*	42.2 efg
B6	*Bacillus*	44.1 fg
B8	*Bacillus*	48.6 g
B9 (1)	*Bacillus*	48.9 g
Grape	Yeast	24.2 a
EP	Yeast	28.4 ab
GR1	Yeast	30.8 abc
GR2	Yeast	30.9 abc
B13	Yeast	31.4 abc
ON3	Yeast	32.1 abc
B-a	Yeast	32.5 abcd
Lemon P	Yeast	33.4 abcd
SPL	Yeast	36.9 bcdef
UL3	Yeast	39.2 cdef
Water (control)	*Penicillium digitatum* only	45.2 fg
P Value		<0.001***

*= 0.05 significant, **=0.01 highly significant, ****=0.001 very highly significant

3.3.4 Preventative action of selected yeasts and *Bacillus* isolates antagonistic to *P. digitatum* on navel and Valencia oranges and lemons

On navel oranges, yeast isolates B13, Grape, SPL, B-a, and RG2 provided significant control ($p \leq 0.05$) of *P. digitatum*, relative to the untreated control. The *Bacillus* isolate from papaya did not reduce the level of green mould relative to the untreated control. The other yeast and *Bacillus* isolates tested developed significantly less green mould than the untreated control (a detailed statistical analysis of the data in Table 3.3 is presented in Appendix 3C).

On Valencia oranges, *Bacillus* isolates B3, B6 and B9 (2) did not control green mould. The other yeasts and *Bacillus* isolates reduced the levels of green mould, relative to the untreated control. Yeast isolates Grape and EP provided the best control of *P. digitatum.* (A detailed statistical analysis of the data in Table 3.3 is presented in Appendix 3D).

On lemons, yeast isolates B13, Grape, EP, ON3, SPL, B-a, RG2 and GR1 (for the source of isolates, see Appendix 3A) gave significant protection against *P. digitatum*, when compared with the control. Yeast isolate UL3 and *Bacillus* isolates B3, B9 (2), B6, B8, and S1.1 did not control green mould effectively (a detailed statistical analysis of the data in Table 3.3 is provided in Appendix 3E).

The two yeast isolates, B13 and Grape, provided complete protection against *P. digitatum* infection on navel oranges and lemons, but did not provide complete protection on Valencia oranges (Table 3.3 and Figure 3.3). Three other yeast isolates, SPL, B-a, and RG2, also provided excellent protection on navel oranges and lemons, but less protection on Valencia oranges. The *Bacillus* isolate Papaya did not perform significantly better than the control on navels and lemons; however, it reduced disease development relative to the control on Valencia oranges. The best yeast isolates were more effective than the *Bacillus* isolates on navel oranges (Table 3.3). Two yeast isolates, Grape and EP, provided the best protection on Valencia oranges (Table 3.3). The *Bacillus* isolates B3, B6 and B9 (2), did not protect Valencia oranges and lemons from green mould. *Bacillus* isolates B8 and S1-1, and yeast isolate UL3 did not lemon fruit, but provided significant protection of navels and Valencia oranges (Table 3.3).

Table 3.3 Preventative treatment of navel and Valencia oranges and lemons with 10 yeast and 10 *Bacillus* isolates to control *Penicillium digitatum*

Isolate and control	Biocontrol Agent	Green Mould Lesion Diameter (mm) Navels	Valencia	Lemon
B3	*Bacillus*	18.9 b	63.0 jk	59.1 e
B9 (2)	*Bacillus*	18.9 b	68.0 k	54.0 de
GW1	*Bacillus*	19.4 b	46.5 fg	16.9 b
B7	*Bacillus*	22.1 bc	54.1 h	39.9 c
B6	*Bacillus*	29.9 d	64.5 jk	56.6 de
B8	*Bacillus*	30.7 d	55.7 hi	57.0 de
S1-1	*Bacillus*	34.8 de	53.5 h	53.8 de
B9 (1)	*Bacillus*	38.8 ef	53.4 h	10.9 b
S1-2	*Bacillus*	46.9 g	51.6 gh	44.3 c
Papaya	*Bacillus*	62.0 h	49.8 gh	50.7 d
Grape	Yeast	0.0 a	2.8 ab	0.0 a
B13	Yeast	0.0 a	25.3 c	0.0 a
SPL	Yeast	1.7 a	41.2 ef	1.37 a
B-a	Yeast	2.9 a	33.2 d	3.6 a
GR2	Yeast	6.1 a	22.7 c	3.9 a
GR1	Yeast	18.2 b	24.3 c	3.4 a
Lemon P	Yeast	21.1 b	19.3 c	11.6 b
EP	Yeast	28.1 cd	6.2 b	0.0 a
ON3	Yeast	28.5 cd	38.0 de	0.0 a
UL3	Yeast	44.5 fg	40.4 e	58.0 e
Water (control)	None (*Penicillium digitatum* only)	68.4 h	60.4 ij	58.2 e
P Value	-	<0.001***	<0.001***	<0.001***

*= 0.05 significant, **=0.01 highly significant, ****=0.001 very highly significant.

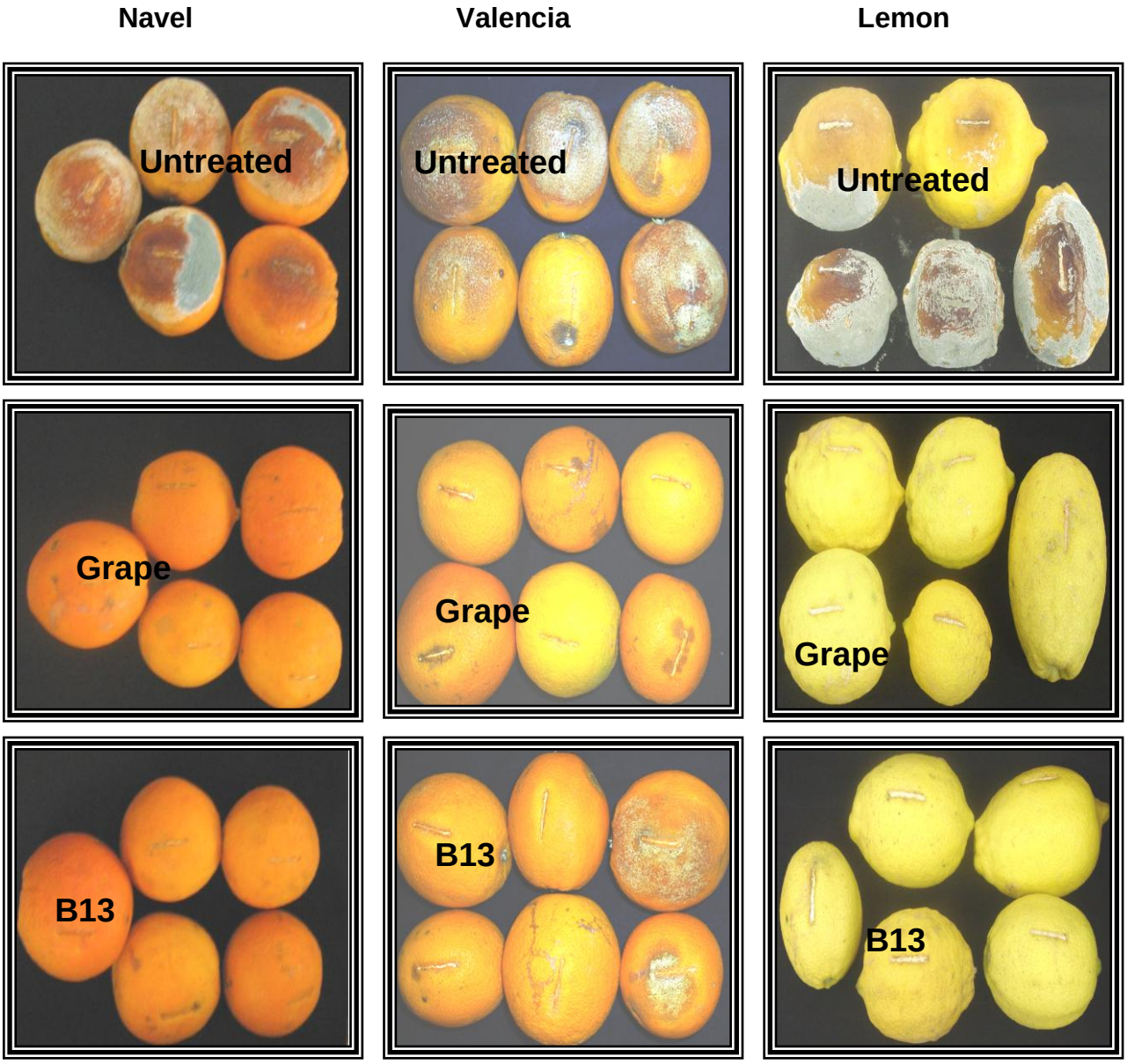

Figure 3.2 Preventative control of navel and Valencia oranges as well as lemons with three yeast isolates to control *Penicillium digitatum*. Untreated (inoculated with *P. digitatum* only), on top row navel oranges (left), Valencia oranges (center), and lemons (right). Center row shows fruit treated with yeast isolate Grape, (1×10^8 cells $m\ell^{-1}$) and inoculated with *P. digitatum* (1×10^4 conidia $m\ell^{-1}$), navel oranges (left), Valencia oranges (center) and lemons (right). The bottom row shows fruit treated with yeast Isolate B13 (1×10^8 cells ml^{-1}) and inoculated with *P. digitatum* (1×10^4 conidia $m\ell^{-1}$), navel oranges (left), Valencia oranges (center) and lemons (right).

3.3.5 Curative action of selected yeast and *Bacillus* isolates antagonistic to *P. digitatum* on Valencia oranges and lemons

When tested on Valencia oranges and lemons by application to wounds three hours after inoculation with the pathogen, none of the yeasts or *Bacillus* isolates produced a significant curative action against *P*. *digitatum* (Table 3.4).

Table 3.4 Curative treatment of Valencia oranges and lemons with 10 yeast and 10 *Bacillus* isolates to control *Penicillium digitatum*

		Green Mould Lesion diameters (mm)	
Treatment	**Isolate types**	**Valencia**	**Lemon**
B7	*Bacillus*	48.1	59.8
B6	*Bacillus*	49.5	60.5
B8	*Bacillus*	52.2	60.7
B9 (2)	*Bacillus*	55.3	51.7
GW1	*Bacillus*	55.9	58.5
B3	*Bacillus*	56.5	58.1
S1-2	*Bacillus*	59.5	61.3
B9 (1)	*Bacillus*	61.0	59.2
S1-1	*Bacillus*	60.1	57.1
Papaya	*Bacillus*	62.0	59.6
Lemon P	Yeast	48.5	58.6
B-a	Yeast	48.9	58.9
Water	*P. digitatum* only	49.3	57.9
GR2	Yeast	49.5	60.3
EP	Yeast	49.5	57.9
ON3	Yeast	51.2	58.6
Grape	Yeast	52.2	58.1
B13	Yeast	52.3	60.2
SPL	Yeast	54.9	61.0
GR1	Yeast	55.5	60.3
UL3	Yeast	60.2	53.9
P Value		0.08 NS	0.074 NS

NS= Not significant (>0.05), *= 0.05 significant, **=0.01 highly significant, ****=0.001 very highly significant.

3.3.6 Dose effect of two yeast isolates, B13 and Grape, on the control of *P. digitatum*, when applied preventatively on lemons

The results of the different concentrations of the two yeast isolates against *P. digitatum* showed that both yeast isolates B13 and Grape provided significantly smaller lesion diameters compared to the control (0 cells ml^{-1}) (a detailed statistical analysis of the data in Figure 3.3 is provided in Appendix 3F).

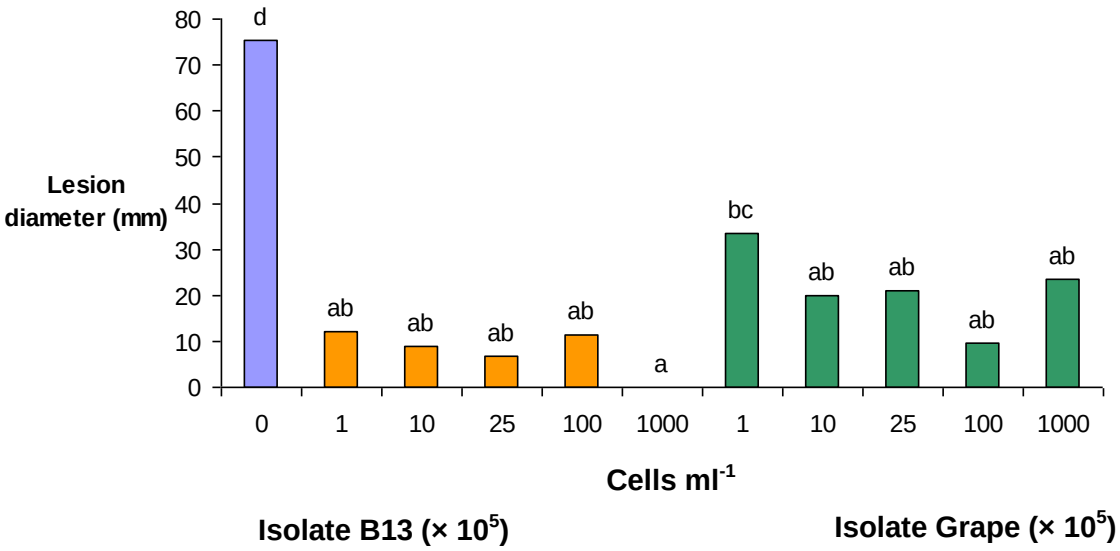

Figure 3.3 Dose effect of two yeasts (isolates B13 and Grape) on the the lesion diameter of *Penicillium digitatum* on lemon, when applied preventatively.

3.4 DISCUSSION

The major objective of this study was to isolate yeasts and *Bacillus* spp. antagonistic to *P*. *digitatum* and to assess their potential ability for biological control of green mould of citrus. This approach has been reported by others (Wilson and Chalutz 1989; Chalutz and Wilson, 1990; Smilanick *et al.,* 1993; Bouzerda *et al.,* 2003). However, it is the first reported study in which yeasts and *Bacillus* have been isolated in South Africa and their potential antagonistic ability assessed against *P*. *digitatum*. Isolation of yeast and *Bacillus* isolates from a particular geographical location may be more effective and specific against the pathogen isolates present in that region (Vero *et al*., 2002; Bouzerda *et al.,* 2003).

A pilot study on the microorganisms present on the surfaces of mature citrus was conducted. Dense bacterial and yeast populations were found, with little growth of filamentous fungi. This indicates that microorganisms present on fruit in orchards may be used as a rich source of yeast and *Bacillus* isolates (Figure 3.1). The presence of microorganisms on mature fruit reflect the microorganisms' abilities to tolerate the hostile conditions present on the surface of fruit, namely: low nutrient availability, UV radiation, rapid climatic changes (Leibinger *et al*., 1997), the presence of agrochemicals (Köhl and

Fokkema, 1998); and reflect their ability to colonize and survive on the target host tissue.

All 60 yeasts and 92 *Bacillus* isolates recovered from the surface of the fruit reduced disease development by *P. digitatum* on navel oranges in the initial screening. However, only 20 isolates (10 yeast and 10 *Bacillus* isolates) reduced the fruit surface area infected by *P. digitatum* on navel oranges by ≤50% (Table 3.1). Isolates of yeast and *Bacillus* which failed to reduce infection levels by *P. digitatum* to ≤50 on the fruit surface were considered inadequate to warrant further screening (Table 3.1).

In subsequent screenings, yeast isolates provided superior control of *P. digitatum* compared with the best *Bacillus* isolates (Table 3.3). In general, yeast isolates effectively reduced disease development by *P. digitatum* on navel and Valencia oranges, and lemons. In a preventative trial with 20 yeast and Bacillus isolates, yeast isolates B13 and Grape, originally isolated from the skin of a Valencia orange and a grapefruit, respectively, exhibited the highest antagonistic activity against *P. digitatum* infection on navel oranges and lemons (Table 3.3). On Valencia oranges, Isolate Grape was more effective than Isolate B13. However, in the dosage trial, which compared the performance of these two isolates at a range of cell concentration, Isolate B13 consistently, provided better control of green mould on lemons than Isolate Grape (Figure 3.3). Isolate B13 was therefore selected for further studies.

In these trials, yeast antagonists were effective when applied preventatively but not when applied curatively. This is in agreement with the results obtained by Qing and Shiping (2000), who showed that the yeast *Pichia membranefaciens* Hansen was only effective when applied to nectarine fruit 24-48 hours before inoculation with *Rhizopus*. Similarly, de Capdeville *et al*. (2002) showed that yeasts reduced the progress of *Penicillium expansum* (Link) Thom. on apple fruit more effectively when applied to the fruit 24 hours or 96 hours before inoculation with the pathogen than when applied 24 hours after inoculation. Chalutz and Wilson (1990) also found that the efficacy of a yeast isolate of *Debaryomyces hansenii* (Zopf) Lodder and Kreger-van Rij against green and blue moulds of grapefruit was reduced if application was delayed by three hours or more after inoculation of the pathogen.

The lack of curative disease control by yeast antagonists could be due to the pathogen penetrating into the fruit tissues and therefore being out of reach of the antagonist, as suggested by Mercier and Smilanick (2005). An alternative hypothesis is that biocontrol yeasts act by competitively colonizing wounds faster than *Penicillium*. Once established in a fruit wound, the yeasts utilize all the nutrients released from the wound. In this scenario, no nutrients are left to stimulate *Penicillium* spores to germinate. However, if the *Penicillium* spores have access to wound nutrients for three hours before the yeasts are applied, then the stimulation of *Penicillium* spores to germinate will have already occurred before the yeasts are applied. Hence, yeasts isolates would not be effective in a curative role.

The study of the biocontrol activities of different concentrations of the two yeast isolates B13 and Grape against *P. digitatum* demonstrated that both yeasts reduced the lesion diameters of *P. digitatum* on lemon, irrespective of the yeast concentrations. Isolate B13 demonstrated greater biocontrol efficacy and completely controlled *P. digitatum* development at 1×10^8 cells $m\ell^{-1}$. Similar findings were reported by Tian *et al*. (2002) with *Candida guilliermondii* (Castellani) Langeron and Guerra and *P. membranefaciens* at a concentration of 10^8 cells $m\ell^{-1}$, which completely controlled infection by *Rhizopus stolonifer* on peaches and nectarines. Chanchaichaovivat *et al*. (2007) were able to control anthracnose on chilli (6.5% disease incidence) caused by *Colletotrichum capsici*, with a yeast *P. guilliermondii* Strain R13, applied at 10^8 cells $m\ell^{-1}$. Zheng *et al.* (2005) were also able to control green mould of oranges with yeast *Rhodotorula glutinis* (Harrison), applied at a concentration of 1×10^9 cells $m\ell^{-1}$. Reports by Janisiewicz (1988) and Hong *et al*. (1998) demonstrated that a direct relationship exists between the population density of the biocontrol agents and the effectiveness of the postharvest biological control treatment.

Isolate B13 is an isolate of *Candida fermentati*, which was previously thought to be the anamorph of *Pichia guilliermondii*, cosmopolitan yeast associated with numerous habitats (Kurtzman and Fell, 2000). However, low DNA base sequence relatedness, different electrophoretic karotypes and nucleotide divergence of the D1/D2 region have all lead to the reinstatement of *Candida fermentati* as a separate species (Vaughan-Martini *et al*., 2005). To the best of our knowledge, this yeast has not been reported as a biocontrol agent, possibly

because its activities as an effective biocontrol agent have been credited to *P. guilliermondii*.

Candida fermentati has been isolated from the gut of beetles (Suh and Blackwell, 2004). It has not been reported to pose a risk to human health. Therefore, rigorous toxicological tests would be needed before its use as a biocontrol agent could be commercialized.

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CHAPTER 4

SCANNING ELECTRON MICROSCOPY STUDIES OF THE INTERACTIONS OF YEAST ISOLATE B13 AND *PENICILLIUM DIGITATUM* IN LEMON WOUNDS

ABSTRACT

Environmental scanning electron microscopy (ESEM) studies showed effective colonization of lemon wounds by yeast Isolate B13. Based on ESEM observations, when applied preventatively to control *Penicillium digitatum,* yeast Isolate B13 (*Candida fermentati* (Saito) Bai.) inhibited or restricted conidial germination of *P. digitatum*. The yeast Isolate B13 demonstrated good colonization of lemon wounds and provided protection against *P. digitatum.*

4.1 INTRODUCTION

Yeasts that occur naturally on fruit have been targeted by many researchers as potential antagonists of postharvest pathogens because they exhibit a number of traits that enhance their potential for colonizing fruit surfaces (Droby *et al*., 2000). Yeasts protect a variety of harvested commodities against a number of postharvest pathogens (Janisiewicz *et al*., 1994; Chand-Goyal and Spotts, 1996; Leibinger *et al*., 1997). Successful control of *Penicillium digitatum* (Pers: Fr. Sacc.) of citrus has been reported by a number of workers. Chalutz and Wilson (1990) found that the yeast strain *Pichia guilliermondii* Wickerham and *Debaryomyces hansenii* (Zopf) Lodder and Kreger-van Rij inhibits the incidence of *P. digitatum*; El-Ghaouth *et al*. (2000) controlled *P. digitatum* with *Candida saitoana;* and Bouzerda *et al*. (2003) found that yeast isolates L13 and L22 limited the level of infection of citrus by *P. digitatum*.

Although the biocontrol activity of antagonistic yeasts has been demonstrated on a variety of commodities, evaluation of control of *P. digitatum* has been largely based on percentage of infected fruit (El-Ghaouth *et al*., 2000; Chalutz and Wilson, 1990) or lesion diameter (Droby *et al*., 2002). Similarly, assessment of the antagonist on the fruit wound has been based on cell counts on plates, as demonstrated by Bouzerda *et al*. (2003). This technique does not provide

information on the colonization and establishment ability of biocontrol agents, nor does it provide information on the mode of action of specific biocontrol agents on a pathogen. Suggested mechanisms of biocontrol include: antibiosis, lysis, competition and mycoparasitism (Chet and Baker, 1980; Papavizas, 1985; Sid Ahmed *et al*., 1999). The purpose of this study was therefore to investigate the ability of the yeast Isolate B13 (*Candida fermentati* (Saito) Bai.) to colonize fruit wounds and to determine the modes of action of Isolate B13 by observing surface colonization and pathogen germination and infection processes, using environmetal scanning electron microscopy.

4.2 MATERIALS AND METHODS

4.2.1 *In vitro* reaction of yeast Isolate B13 against *Penicillium digitatum*

Yeast Isolate B13, grown on nutrient agar (NA) (Merck Laboratory, South Africa) was streaked on potato dextrose agar (PDA) (Merck Laboratory, South Africa) on both sides at a distance of about 10 mm from edge of the petri dish with agar with a 4 mm plug of *Penicillium digitatum* (grown on PDA) occupying the center of the plate and incubated (incubator: Babotec, South Africa) at 25°C for seven days. The treatments were evaluated for evidence of inhibition of *P. digitatum* by Isolate B13 by measuring the width of the inhibition zone between the two fungi.

4.2.2 Preparation of the yeast Isolate B13 on agar, and investigation of its colonization ability in lemon wounds, using scanning electron microscopy

A quarter of a loop filled with culture of the yeast Isolate B13 was taken from a discrete colony grown on nutrient agar (NA) (Merck Laboratory, South Africa). This was then streaked onto nutrient agar plates. The plates were incubated (Incubator: Labotec, South Africa) at 25°C for four days. Blocks of 10 mm × 10 mm agar with a colony of the yeast on the surface were prepared for electron microscopy studies.

To assess colonization by the yeast Isolate B13 in wounds of lemons, lemon fruit was surface disinfected with 70% alcohol for one minute, dried, and then wounded (25 mm in length × 3 mm in depth) at one site at the equator of the

fruit using a dissecting needle. A 100 µl aliquot of cell suspension of Isolate B13 (1×10^8 cells $m\ell^{-1}$) was pipetted into each wound site. This concentration was used because it provided a complete inhibition of *P. digitatum* in a previous trial (Chapter 3.3.6). Fruit were kept on a laboratory bench at room temperature (24±1°C). After three days, colonized wounds on the fruit were excised and prepared for observation by scanning electron microscopy.

4.2.3 Preparation of the yeast Isolate B13 and *P. digitatum* to examine their interactions in lemon wounds, using scanning electron microscopy

Lemons were surface disinfected (with 70% alcohol for one minute, dried) and wounded as described avove in Section 4.2.2. A cell suspension of 100 µℓ of the yeast Isolate B13, at 1×10^8 cells $m\ell^{-1}$, was pipetted into each wound site. Wounds treated with the same amount of distilled water served as a control. After 48 hours, all wounds were inoculated with a conidial suspension of 100 µℓ of *P. digitatum* (1×10^4 conidia $m\ell^{-1}$). Fruit were kept at room temperature (24±1°C). Two boxes (disinfected with 70% alcohol), with five fruit per box, were used per treatment and placed on a bench. Ten days after inoculation, wounded tissue from the treated and the control fruit were excised and used for scanning electron microscopy studies.

4.2.4 Scanning electron microscopy studies of the growth of the yeast Isolate B13 on nutrient agar and its colonization ability in wounds of lemon

Samples were taken of yeast Isolate B13 grown on nutrient agar, together with excised wounds of lemons inoculated with combinations of Isolate B13 and *P. digitatum*, plus a control treatment. The samples were fixed for eight hours in 2% buffered osmium tetroxide (10 ml 4% OsO_4, 5ml 0.2M sodium cacodylate buffer and 5 ml distilled water) the samples were dehydrated in an ethanol series (10 minutes each in 30%, 50%, 70%, 80%, 90%, and 3 × 10 minutes in 100% ethanol) in a fume cupboard. The specimens were then transferred into critical point drier baskets under 100% ethanol and placed in a pre-cooled Hitachi HCP-2 critical point drier. Following critical point drying (CPD) and gold-palladium sputter coating (Polaron Equipment Limited ESEM, coating unit E5100), the samples were viewed in an ESEM (Philips, FEI XL 30, Holland) at

an accelerating voltage of 15 keV. Samples from five fruit from each treatment, and a sample of yeast Isolate B13 on the surface of nutrient agar were viewed.

4.2.5 Scanning electron microscopy studies of the interactions of yeast Isolate B13 and *P. digitatum* in wounds of lemon fruit

Sample tissue from the untreated wounded lemon was excised (Figure 4.1A) for ESEM observations. Similarly, tissue sample were obtained from wounded lemon fruit that were treated with the yeast Isolate B13 and subsequently inoculated with *P. digitatum* (Figure 4.1B), after which tissue samples were excised for ESEM observations (Figure 4.2C). The tissue samples were held overnight in a fixative of 3% (v v^{-1}) glutaraldehyde in 0.05 M sodium cocodylate buffer (pH 7.2) and dehydrated in an ethanol series (as reported in Section 4.2.4), transferred into critical point drier baskets and gold-palladium sputter coated. The ESEM equipment used was as described above in Section 4.2.4. Samples from five fruit from each treatment were viewed.

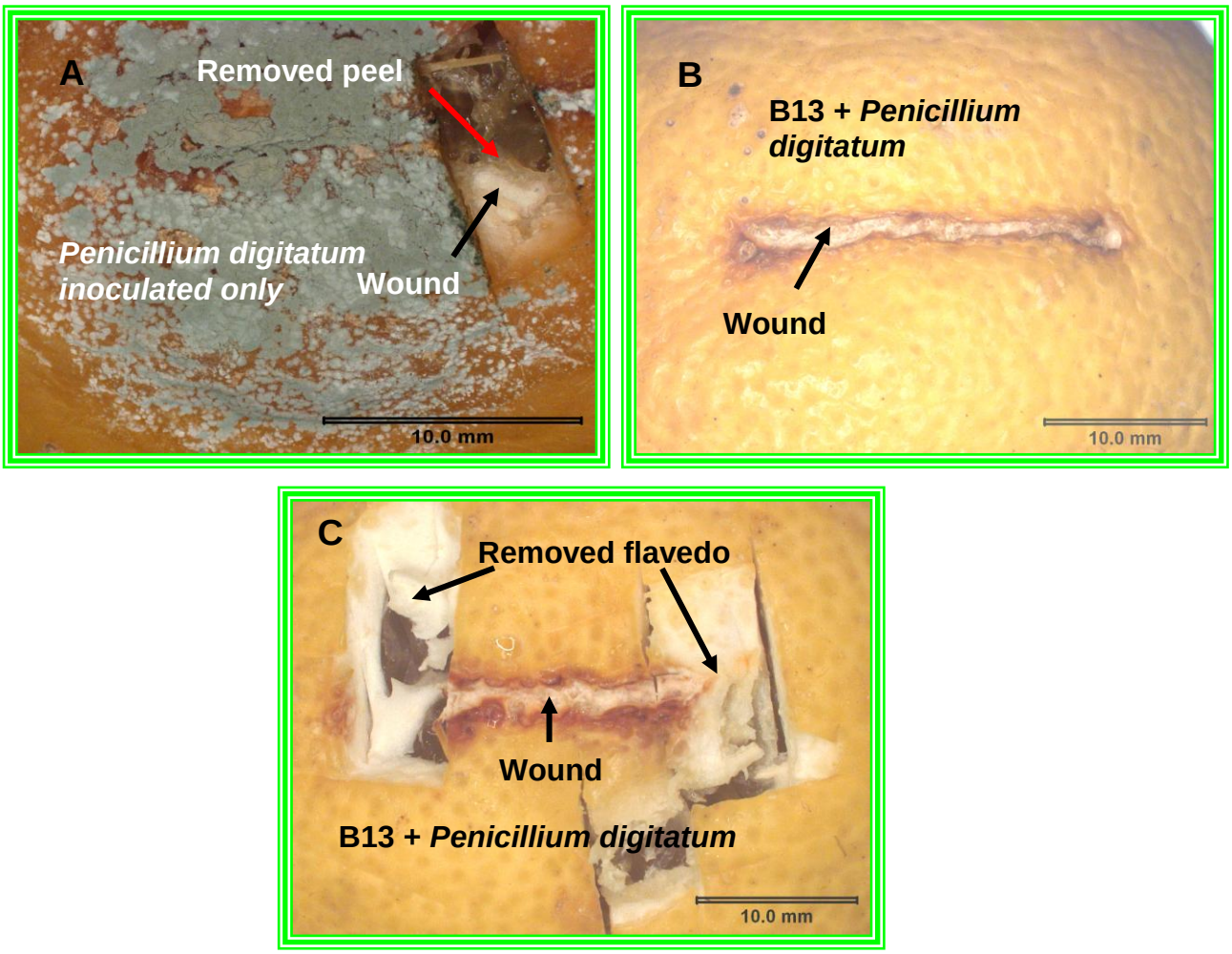

Figure 4.1 (A) *Penicillium digitatum* inoculated into a lemon wound, showing infection and how sample tissue was taken from the wounded fruit for ESEM; (B) *In vivo* interaction of the yeast Isolate B13 and *P. digitatum* on lemon, with no infection developing; (C) the same fruit surface used for electron microscope studies by excising surface samples from the wounded fruit.

4.3 RESULTS

4.3.1 *In vitro* reaction of yeast isolate B13 against *Penicillium digitatum*

Yeast isolate B13 showed no inhitory activity against *P. digitatum*. There was no gap between colonies of B13 and the pathogen. Furthermore, there was no visual or microscopic evidence of hyperparasitism by Isolate B13 on the pathogen.

4.3.2 Scanning electron microscopy observations of the yeast Isolate B13 on nutrient agar and its colonization ability in wounds of lemons

Environmental scanning electron microscopy observation showed growth of the yeast Isolate B13 on agar (Figure 4.2A). Environmental scanning electron microscopy observations showed good colonization of lemon wounds by the yeast Isolate B13, where it densely colonized the fruit surface after 3 days of treatment as in Figure 4.2B making the wound invisible.

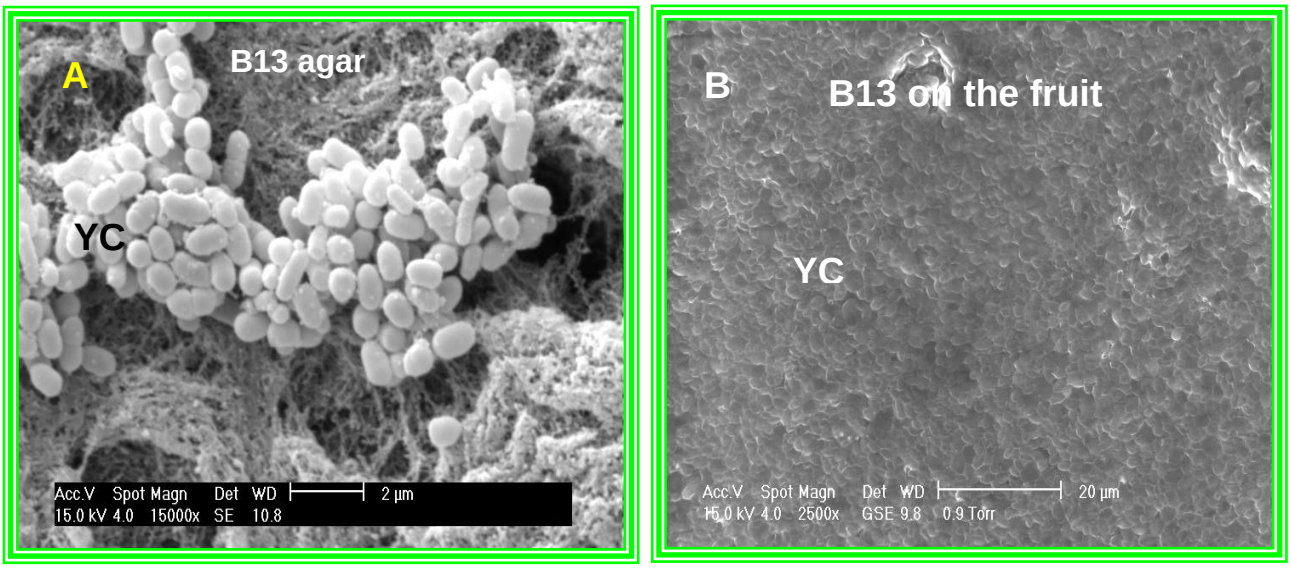

Figure 4.2 (A) Scanning electron micrographs of the yeast Isolate B13 cells on nutrient agar; (B) ESEM of the yeast Isolate B13 cells growing in a lemon wound. YC= yeast cells.

4.3.3 Visual observation of wounds of lemons inoculated with *P. digitatum* only

Observation on lemon wounds inoculated with *P. digitatum* showed visible green conidia of *P. digitatum* and/or softening on the fruit surface 10 days after inoculation (Figure 4.3).

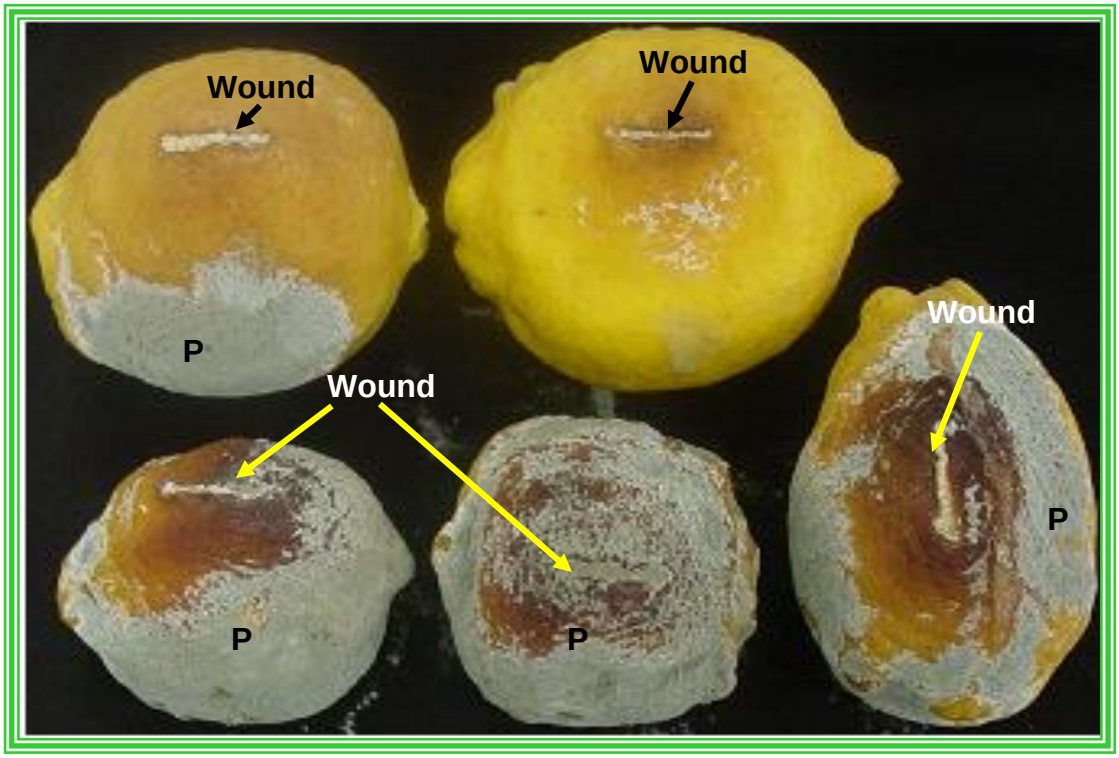

Figure 4.3 Lemons infected with *Penicillium digitatum* (P) 10 days after inoculation.

4.3.4 Scanning electron microscopy observations of wounds of lemon inoculated with *P. digitatum* only

Environmental scanning electron microscopy showed hyphal growth of the *P. digitatum* infection on the wounded lemon fruit (Figure 4.4A). Dense hyphal growth and conidiophore formation was evident under 1200× magnification (Figure 4.4B). Conidia were densely packed on the fruit inoculated with *P. digitatum* only (Figure 4.4C).

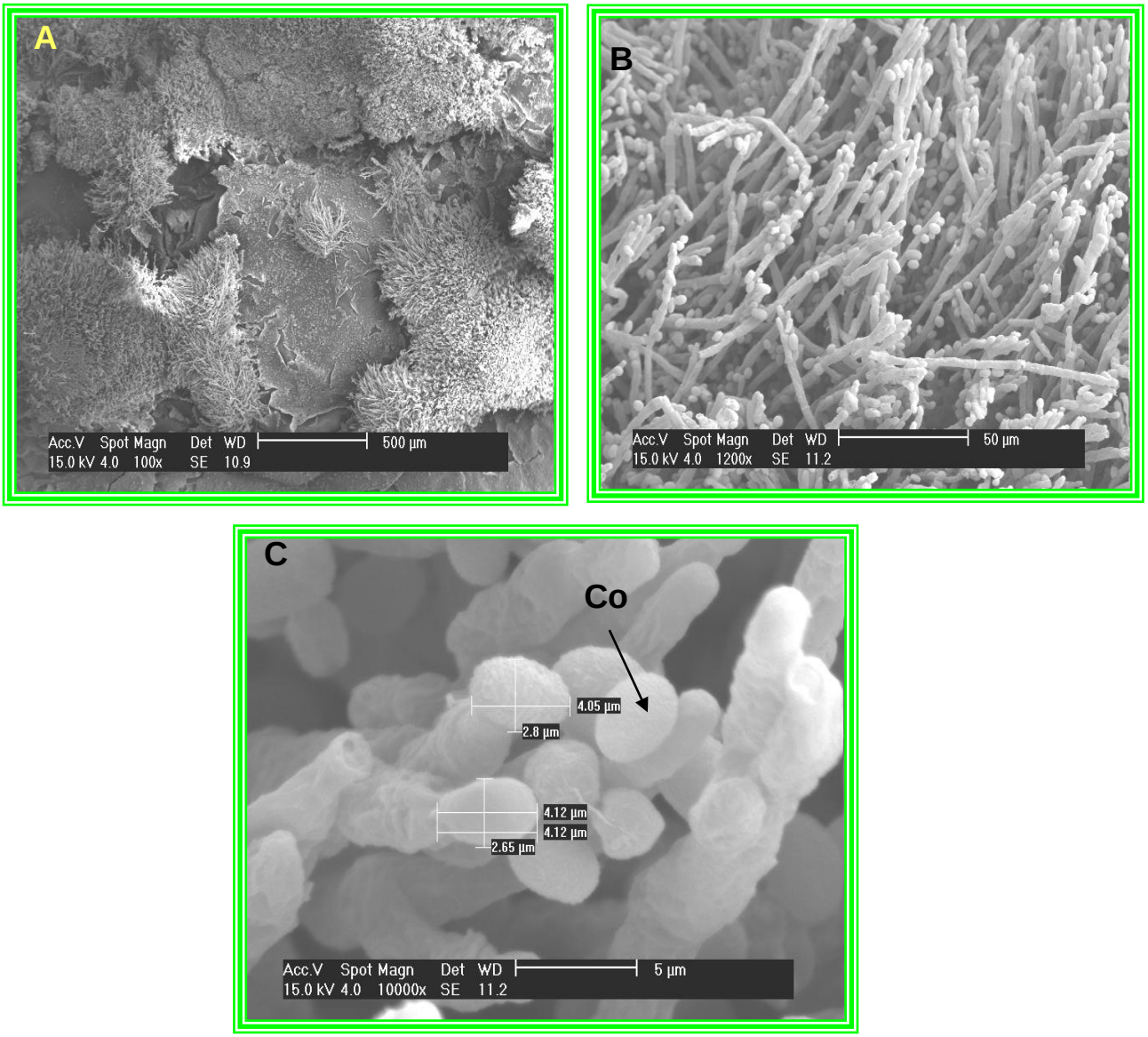

Figure 4.4 Scanning electron micrographs of the untreated control (inoculated with *Penicillium digitatum* only*)*: (A) mycelial growth of *P. digitatum* in the lemon wound surface; (B) hyphal growth of *P. digitatum* in the wound surface; (C) hyphae and conidial structures of *P. digitatum*. Co = conidium.

4.3.5 Visual observation of wounds of lemons treated with the yeast Isolate B13 and subsequently inoculated with *P. digitatum*

In vivo interactions on wounded lemons treated with the yeast Isolate B13, then 48 hours later inoculated with *P. digitatum,* showed no visible *P. digitatum* mycelium or softening of the wounded fruit surface (Figure 4.5) 10 days after inoculation.

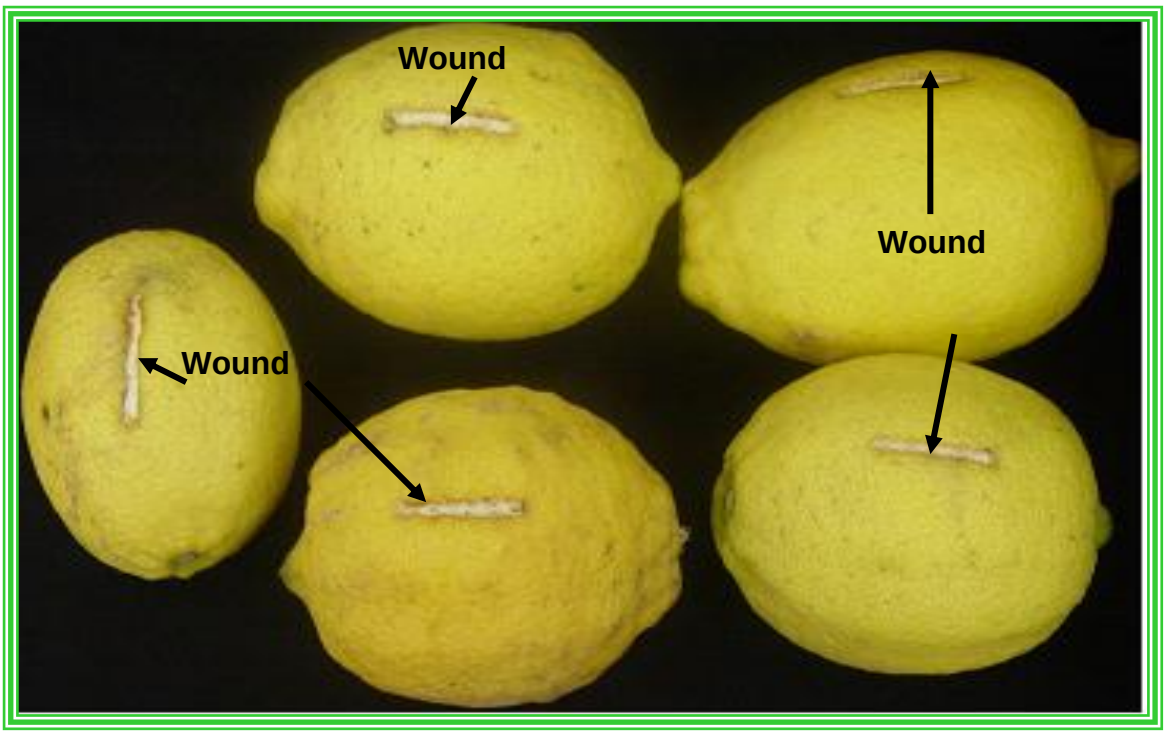

Figure 4.5 *In vivo* interactions of the yeast Isolate B13, applied preventatively on lemon wounds, and *Penicillium digitatum*, inoculated 48 hours later.

4.3.6 Scanning electron microscopy observations of interaction of the yeast Isolate B13 and *P. digitatum* in lemon wounds

Inhibition of conidial germination of *P. digitatum* was observed as a result of the prior inoculation of the yeast Isolate B13 onto wounds of lemons, using ESEM (Figures 4.6A and B). Restricted conidial germination of *P. digitatum* was observed in some cases (Figure 4.6C), compared with the untreated control (Figures 4.4B and C). In addition, no mycelial growth was observed on wounded lemons treated with the Isolate B13 and then inoculated with *P. digitatum* (Figure 4.6), compared to the dense mycelia that grew when the pathogen was applied alone (Figure 4.4).

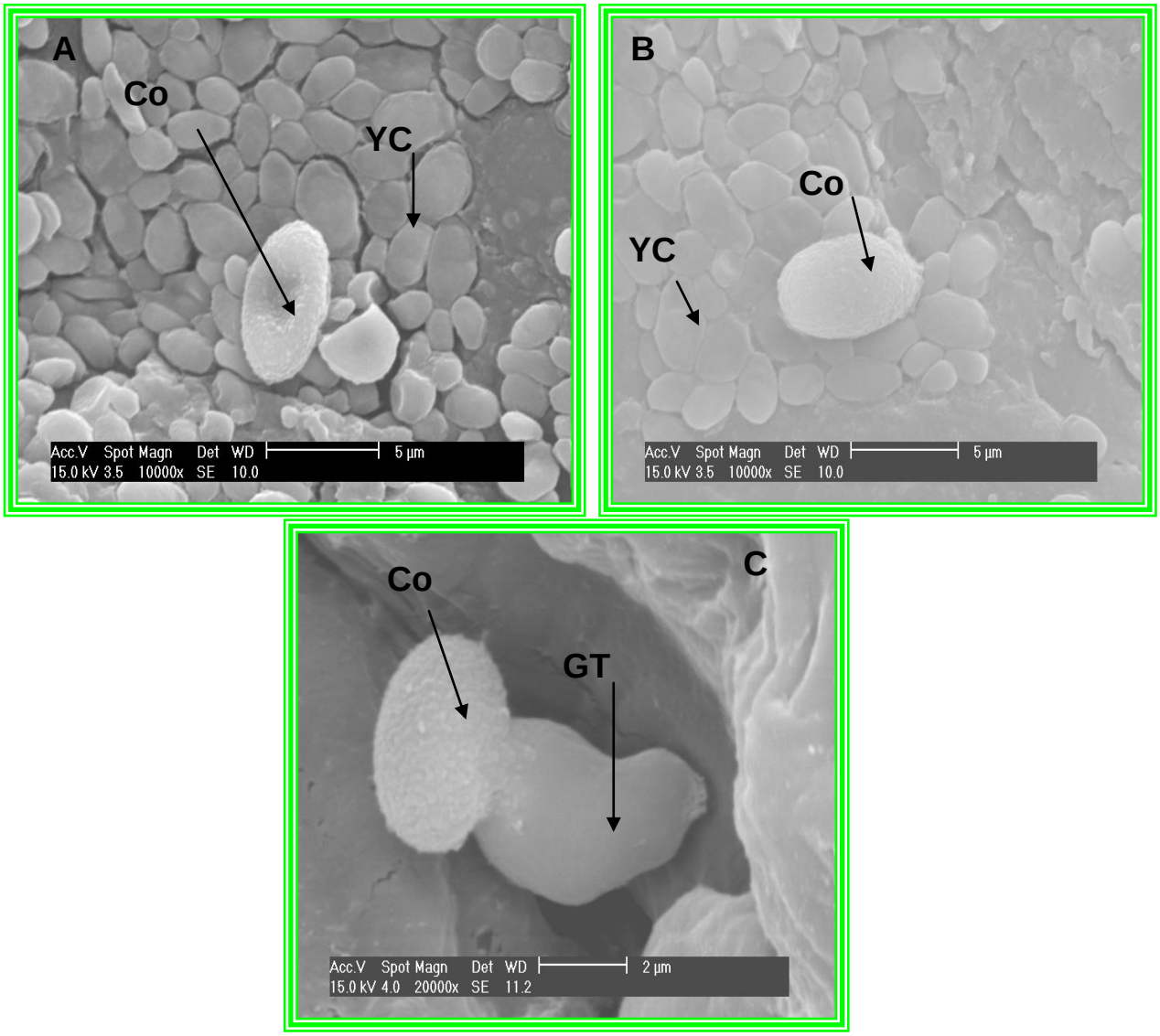

Figure 4.6 Scanning electron micrographs of the yeast Isolate B13 and *Penicillium digitatum* interacting in wounded lemons: (A and B) germination of conidia of *P. digitatum* was inhibited; yeast cells dominated the wound surface; (C) germinating *P. digitatum* conidia in a lemon wound. Co= conidium; YC= yeast cells; GT= germ tube.

4.4 DISCUSSION

Germination and growth of *P. digitatum* in lemon wounds, treated preventatively with the yeast Isolate B13, were observed using ESEM (Figure 4.6). Observations showed that complete inhibition of conidial germination (Figure 4.6A and B) or restricted germination of conidia (Figure 4.6C), compared with the water-treated control where normal conidial germination, germ tube growth, hyphal growth and sporulation were evident (Figure 4.4).

Yeast antagonists often exert antagonistic activity through competition for nutrients or space (Roberts, 1990). For effective biocontrol activity, antagonists must multiply and colonize wounds rapidly (Wilson and Wisiniewski, 1989; Wilson *et al*., 1993). The yeast Isolate B13 was able to multiply, colonize and survive in lemon wounds (Figure 4.2B). It also densely colonized wounds in the presence of *P. digitatum* conidia (Figure 4.6A and B). Although no population counts of the Isolate B13 were done in these studies, but research on other yeasts have shown good adaptation, colonization and multiplication in citrus fruit wounds (Tian *et al*., 2002; Bouzerda *et al*., 2003). Rapid and comprehensive colonization of the wound environment by the yeast Isolate B13 was observed (Figures 4.2B and 4.6A and B). We hypothesize that the prolific colonies of Isolate B13 used up all available nutrients and that this resulted in the observed control of *P. digitatum* by inhibiting its conidia from germinating, a theory proposed earlier by Wilson and Wisiniewski (1989).

This hypothesis of nutrient competition was supported by *in vitro* tests, in which Isolate B13 failed to produce any antagonistic activity by antibiosis, or to display any evidence of hyperparasitic activity against *P. digitatum* (see Section 4.3.1). Similar findings were reported by Zheng *et al*. (2005) that tests on agar plates with yeast isolate *Rhodotorula glutinis* (Harrison) did not inhibit growth of *P. digitatum.*

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CHAPTER 5

FRUIT SURFACE COLONIZATION AND BIOLOGICAL CONTROL OF *PENICILLIUM DIGITATUM* ON VALENCIA ORANGES BY PREHARVEST YEAST APPLICATION

ABSTRACT

Preharvest application of a yeast, Isolate B13 (*Candida fermentati* (Saito) Bai.*)*, as a field spray for the control of *Penicillium digitatum*, was investigated for two seasons. In 2006, Valencia oranges at Caterall's Farm (Site 1), Richmond, KwaZulu-Natal, South Africa were sprayed with the yeast Isolate B13 at 1×10^5, 1×10^6 or 1×10^7 cells $m\ell^{-1}$, one, three or seven days before harvest, with or without the adjuvant Breakthru®. At harvest, some fruit were inoculated with *P. digitatum* by dipping wounded fruit into a suspension of conidia (1×10^4 conidia $m\ell^{-1}$) for one minute. The fruit were then stored at 24±1°C for one month. Another batch of fruit was wounded, but was not inoculated artificially. This batch was also stored at room temperature (24±1°C) at ambient relative humidity (RH) for one month. A third batch of 10 fruit were not wounded and not inoculated, and were stored in a cold room (7±1°C) at 90% RH for one month before evaluation. In 2007, an additional site, Maywood Farm (Site 2), Richmond, KwaZulu-Natal, South Africa was used, as well as the site used in 2006, Caterall's Farm (Site 1). At Site1 the yeast Isolate B13 was sprayed at 1×10^6 cells $m\ell^{-1}$, with or without Breakthru®, either one month or one day before harvest. At Site 2 the yeast Isolate B13 was sprayed at the same rate, but it was applied twice, at two months and one month before harvest, or only for one day before harvest, on separate trees. Fruit harvested from trees after one day of application of treatment were wounded. Natural inoculum was used for all trials, so conidia of *P. digitatum* were not applied artificially. Preharvest application of yeast Isolate B13 did not reduce *P. digitatum* disease incidence on unwounded Valencia oranges with natural infestation stored at 7±1°C or 24±1°C at either farms. There was no significant control of lesion diameter at 24±1°C on wounded and artificially inoculated fruit compared with the control at Site 1. Furthermore, there was no control of *P. digitatum* infection by Isolate B13 with or without the application of Breakthru®, or by Breakthru® alone, on either farm in 2007. The population of yeasts on the surface of Valencia oranges sprayed with Isolate B13 was higher than the control at harvest at both

sites. However, this higher population of yeast populations did not control the incidence of *P. digitatum*. Application of the yeast Isolate B13 as a field treatment was not effective in controlling *P. digitatum.*

5.1 INTRODUCTION

A number of researchers have reported that antagonistic microorganisms failed to control previously established infections of fruit by post-harvest pathogens (Janisiewicz, 1988; Pratella and Mari, 1993; Roberts, 1994). Earlier research in this thesis (Chapter Three) demonstrated that yeast biocontrol agents were not effective in controlling already established infections of *P. digitatum* (Pers: Fr. Sacc.). This poses a serious problem because infection of citrus fruit by *P. digitatum* often takes place in the field prior to or during harvesting processes (Green, 1932; Kavanagh and Wood, 1967; Pelser et al. 1977).

This highlights the potential for early applications of biocontrol agents. Their application in the field prior to injury that often occurs during harvest (Spotts *et al*., 1998) should provide protection against subsequent infection by *P. digitatum*. Some success has been achieved with preharvest applications of antagonistic microorganism on storage rot of fruit, e.g., the yeast *Candida oleophila* Montrocher was more effective against *Botrytis cinerea* (De Bary) Whetzel storage rots on strawberries when applied in the field at bloom, compared to treatments applied immediately after harvesting, which fail to combat latent infections (Lima *et al*., 1997).

For this approach to be successful, an important consideration in the preharvest application of microbial antagonists is the ability of the antagonists to survive in sufficiently large populations on the fruit surface after application. Yeasts may be negatively affected by pesticide sprays (Chand-Goyal and Spotts, 1996) or be washed off from the fruit surface during irrigation or rain. The antagonists must be able to tolerate low nutrient availability, UV radiation, high and low temperatures, and climatic changes (Leibinger *et al*., 1997). However, since potentially effective biocontrol yeasts were originally isolated from the fruit surfaces after or prior to harvest (Roberts, 1990; Chand-Goyal and Spotts, 1997), they should be tolerant of these conditions. Moreover, some yeast antagonists can colonize plant surfaces or wounds under unfavorable

conditions by protecting themselves with extracellular polysaccharides (Wisniewski and Wilson, 1992).

The primary objectives of this study were to assess the fruit surface colonization ability of the yeast Isolate B13 (*Candida fermentati* (Saito) Bai.) when applied prior to harvest, and to investigate the efficacy of the yeast Isolate B13 in controlling postharvest infection by *P. digitatum* when applied prior to harvest. A secondary objective was to assess the level of infection by *P. digitatum* of Valencia oranges in the field under natural conditions.

5.2 MATERIALS AND METHODS

5.2.1 *In vitro* compatibility of yeast Isolate B13 with the adjuvant Breakthru®

A loop full of the yeast Isolate B13 grown on nutrient agar plate for three days was mixed with 10 ml of undiluted Breakthru® (a silicone wetter and spreader). Sterile distilled water was used for the control (Isolate B13 only). After 10 minutes of mixing, 0.1 ml of both suspension was inoculated onto duplicate plates of potato dextrose agar (PDA) (Merck Laboratory, South Africa), amended with 0.15 g ℓ^{-1} Rose Bengal (RB) (BDN Laboratory, England). Plates were incubated at 25°C for three days under dark conditions and the growth of the yeast isolate was observed.

5.2.2 Culture preparation of the yeast Isolate B13 for the preharvest application

A formulation of the yeast, Isolate B13, was prepared by Plant Health Products (PHP)[2], Ltd. A 30 mm × 30 mm sponge containing the yeast was placed in one ℓ of sterile distilled water until most yeast cells were released into the water. Yeast concentration was determined using a haemocytometer. The concentration was adjusted to 1×10^5, or 1×10^6, or 1×10^7 cells mℓ^{-1}. Haemocytometric determination of the yeast concentrations was confirmed by dilution plating of the treatment suspension on culture plates containing 3 g of malt extract, 3 g of yeast extract, 5 g of peptone 10 g of dextrose and 20 g ℓ^{-1} agar (all from Merck Laboratory, South Africa) (according to Benbow and Sugar, 1999).

[2] Plant Health Products (Pty) Ltd., P.O. Box 207, Nottingham Road, South Africa, 3280

5.2.3 Determination of preharvest application of the yeast Isolate B13 for the control of *P. digitatum* infection of Valencia oranges during the seasons of 2006 and 2007

In the season of 2006, the study was conducted in one orchard of Valencia orange trees grown at Caterall's Farm, Richmond (29.53 S 30.17 E), KwaZulu-Natal, South Africa. Trees were over five years old. Trees for treatments with yeast and untreated control trees were selected in a randomized blocks design, with four replicates, with a single tree acting as a replicate.

The trial treatments are summarized in Table 5.1. Treatment sprays were applied seven days, three days and one day prior to fruit harvest. The yeast Isolate B13 was applied at three concentrations (1×10^5, 1×10^6, 1×10^7 cfu), with or without a silicone wetting agent, Breakthru®, which was added at a concentration of 0.125 mℓ ℓ^{-1} of water. Water alone and a Breakthru® suspension were sprayed as controls (Table 5.1).

The yeast and adjuvant suspensions were applied to the selected trees using a motorized knapsack mist blower ensuring that each tree was carefully sprayed to the point before runoff.

In the season of 2007, two similar trials were conducted in two different orchards. Site 1, Caterall's Farm, is located in the immediate vicinity of Richmond, KwaZulu-Natal, while Site 2, Maywood Farm, is located about 10 Km from Richmond, towards Pietermaritzburg. At Site 1, the citrus trees did not receive any irrigation, nor were any agrochemical sprays applied. At Site 2, the trees, over five years old, were regularly irrigated with microjet irrigation sprinklers, and were sprayed according to a scheduled programme of agrochemicals.

In the 2007 season, application of the yeast Isolate B13 was done once before harvest at Site 1 and two applications in Site 2 (once a month for two months), with or without Breakthru®, in order to provide the yeast with ample time to establish itself on the fruit surface and subsequently give protection against *P. digitatum* infection. During the 2007 season period, treatment application was extended to one month or two because previous season's results showed no difference between the treatments when applied seven, three and one day

before harvest. It was also supported by observations of *P. digitatum* on fruit or damage on orange fruit at the early green stage in the field, approximately 3-4 months prior to harvest (Figure 5.1). Yeast Isolate B13 was applied once a one month prior to harvest (27 July 2007) at Site 1 to trees different to those ones used in the 2006 season. In the second orchard (Site 2), the biocontrol agent was applied twice at monthly intervals prior to harvest (27 July 2007 and 27 August). The yeast Isolate B13 was applied in the same manner to a separate set of trees one day prior to harvest for both sites. Three trees (a tree as a replicate) per treatment were used.

Table 5.1 Trial design for preharvest application of yeast Isolate B13, at 7, 3 or 1 day prior to harvest for the control of *Penicillium digitatum* on Valencia oranges for the seasons of 2006

Treatment	**Preharvest application of B13**			**Wetter or spreader**
	7 d	**3 d**	**1 d**	**Breakthru®**
Untreated	-	-	-	-
Untreated	-	-	-	+
Pre7d+ B13	+	-	-	+
Pre7d+ B13	+	-	-	-
Pre3d+ B13	-	+	-	+
Pre3d+ B13	-	+	-	-
Pre1d+ B13	-	-	+	+
Pre1d+ B13	-	-	+	-

NB: B13= yeast isolate, Pre= preharvest application, 7d= seven days, 3d= three days, 1d= one day

Figure 5.1 Pre-harvest infection by *Penicillium digitatum* of Valencia oranges, showing field infection.

5.2.4 Assessment of preharvest application of the yeast Isolate B13 for the control of *P. digitatum* infection of Valencia oranges during the seasons of 2006 and 2007

In 2006, 30 fruit were sampled per tree from a total of four trees per treatment and were divided into three batches of 10 fruit. Ten fruit were wounded with a sterile dissecting needle (25 mm in length and 3 mm in depth) at the equator, and inoculated by dipping the wounded fruit into a suspension of *P. digitatum* (1×10^4 conidia $m\ell^{-1}$) for one minute and stored at 24±1°C for one month and lesion diameter (mm) was recorded (measured as a mean of two dimensions of horizontal and vertical diameter of the lesion). Another batch of fruit was wounded and not inoculated artificially and also stored at room temperature (24±1°C) at ambient relative humidity (RH) of 70-90% for one month. The third batch of 10 fruit were not wounded and not inoculated and were stored in a cold room (7±1°C) at 90% RH for one month before evaluation.

In 2007, the number of fruit used was similar to those described above. However, fruit that were sprayed with the yeast one day before harvest were wounded 5 times on both ends of each fruit with a sterile dissecting needle (2 mm in length and 3 mm in depth). Fruit was not artificially inoculated with *P. digitatum*. Two fruit boxes, disinfected with 70% alcohol, with five fruit per box, were used in each of the 14 treatments and placed on a bench in a complete

randomized block design (CRBD). Lesion diameter (mm) of infected wounds, (measured as a mean of the horizontal and vertical diameters of the lesion) and percentage incidence (%) of *P. digitatum* were determined one month after inoculation for each batch of fruit which was stored at room temperature (24±1°C).

5.2.5 Evaluation of colonizing capability of the yeast Isolate B13 on Valencia oranges at harvest when applied a month prior to harvest during the 2007 season

Three uniformly sized fruit from each treatment batch, as described in Section 5.2.3, were sampled at harvest to determine the number of cells of the yeast Isolate B13 (colony forming unit, CFU) on the fruit surfaces. Three fruit (whole fruit) were put together into beakers containing 900 ml of 0.05 M sterile phosphate buffer (SPB: 2.7 g $NH_2HPO_4.12H_2O$, 0.4 g $NaH_2PO_4.2H_2O$ and 8.0 g NaCl from Merck, South Africa) with 0.006% (vol/vol) Tween 20 (UniLab, Merck, South Africa). The fruit and the buffer were stirred for 10 minutes, using a magnetic stirrer and then placed in a sonicating bath for five min. A one ml sample was removed from each beaker immediately after sonication (Sonication bath: Whaledent Biosonic, Germany), and a 10 fold dilution series was made up to 10^{-5} dilution in SPB solution. Samples of each dilution were then inoculated on petri dishes containing a selective, yeast malt dextrose agar (YMDA) medium (Merck, South Africa) [(1.5 g malt extract, 1.5 g yeast extract, 2.5 g peptone, 5 g dextrose, 18 g agar, 100 mg ℓ^{-1}) and chloramphenicol (Merck, South Africa) and 50 mg ℓ^{-1} of ampicillin (Sigma, Germany) in 1 ℓ of distilled water)]. Chloramphenicol and ampicillin were incorporated to inhibit bacterial growth. Plates were incubated for 2-3 days at 25°C according to Chand-Goyal and Spotts (1994). After incubation (Incubator: Labotec, South Africa), population levels were determined using the plate dilution frequency technique of Harris and Sommers (1968).

The distinctive morphological characteristic of the yeast isolate was used to visually distinguish the yeast isolate recovered from the fruit. A pure culture of the yeast Isolate B13 was concurrently cultured to assist in identifying specific colonies. For a given treatment, only yeast species that were applied to that treatment were counted in the population sample. To determine population levels on the untreated controls, only colonies resembling those of yeast

species used in the treatments were counted, as described by Benbow and Sugar (1999). The population count trial was conducted for the season of 2007 only.

5.2.6 Statistical analysis

Lesion diameter (mm) and *P. digitatum* incidence (%) data were subjected to an analysis of variance (ANOVA) using Genstat® Executable Release 9.1 Statistical Analysis Software (Anonymous, 2006). To determine differences between treatments, Fisher's Least Significant Difference Test was used ($P<0.05$).

5.3 RESULTS

5.3.1 Compatibility of the yeast Isolate B13 with Breakthru®

Breakthru® was found to be compatible with the formulated or unformulated yeast Isolate B13 and did not affect growth of B13 on the plate compared with B13 without Breakthru®. There was no obvious difference in the growth of Isolate B13 when grown with or without Breakthru®.

5.3.2 Control of *P. digitatum* infection by the yeast Isolate B13 applied 1, 3 and 7 days prior to harvest in the 2006 season

There were no significant differences between any treatments of yeast Isolate B13 applied 1, 3 and 7 days prior to harvest for control of *P. digitatum* of Valencia oranges (Table 5.2). Breakthru® alone or with B13 did not result in better control of *P. digitatum* compared with control or B13 applied alone (Table 5.2). There were not enough fruit available to test lesion diameter at 7±1°C.

Table 5.2 Control of *Penicillium digitatum* incidence or lesion diameter by the yeast Isolate B13 and Breakthru® when applied 1, 3 and 7 days prior to harvest, in the 2006 season (fruit stored at 24±1°C)

B13/Bth concentration	Disease incidence %						Lesion diameter (mm)		
	24±1°C			7±1°C			24±1°C		
	1 d	3 d	7 d	1 d	3 d	7 d	1 d	3 d	7 d
0	12.0	13.0	13.0	12.0	12.0	12.0	100	100	100
Bth only	7.0	8.0	7.0	13.0	12.0	13.0	100	100	100
1×10^5	10.0	14.0	4.0	10.0	14.0	5.0	70	85	80
1×10^5 +Bth	6.0	25.0	20.0	5.0	25.0	19.0	100	88	86
1×10^6	5.0	15.0	4.0	5.0	15.0	5.0	93	100	91
1×10^6 +Bth	15.0	7.0	10.0	12.0	10.0	9.0	95	100	100
1×10^7	5.0	5.0	6.0	5.0	5.0	0.0	85	83	91
1×10^7 +Bth	17.0	13.0	15.0	14.0	10.0	19.0	93	94	100
P Value		0.058 NS			0.07 NS			0.065 NS	

Note:
Bth= Breakthru®

d=day

P>0.05 = Non significant (NS)

5.3.3 Control of *P. digitatum* infection by the yeast Isolate B13, applied prior to harvest in the 2007 season

There were no significant differences between any treatments of yeast Isolate B13 applied 1, 3 and 7 days prior to harvest for control of *P. digitatum* of Valencia oranges (Table 5.3). Breakthru® alone or with B13 did not significantly improve control of *P. digitatum* compared with the control or B13 applied alone (Table 5.3).

Table 5.3 Control of *Penicillium digitatum* incidence by the yeast Isolate B13 and Breakthru® when applied 1day, 1 month and 2 moths prior to harvest in the 2007 season (fruit stored at 24±1°C)

	Site 1 (Richmond Town)		**Site 2 (Maywood Farm, 10 km from Richmond Town)**	
B13/Bth concentrations	***P. digitatum* incidence (%) for the preharvest treatments after**		***P. digitatum* incidence (%) for the preharvest treatments after**	
	1 month	**1 day**	**2 month**	**1 day**
0	75	81	87	76
Breakthru® (Bth)	85	82	83	70
B13: 1×10^6	70	76	84	74
B13: 1×10^6 +Bth	73	75	80	81
B13: 1×10^7	85	91	93	77
B13: 1×10^7 +Bth	95	100	71	86
P Value	0.08 NS		0.074 NS	

Note:

Bth= Breakthru®

P>0.05 = Non significant (NS)

5.3.4 Colonization of Valencia oranges by the yeast Isolate B13 when applied as preharvest

At Site 1 yeast Isolate B13 was applied to the fruit trees one month before harvest at two concentrations, 1×10^6 or 1×10^7 cells $m\ell^{-1}$, with/without Breakthru®. The Valencia oranges treated with B13 had significantly higher numbers of yeast cells compared with the untreated control or Breakthru® alone (Table 5.4). The cell numbers of yeasts of the untreated control was not significantly higher in Site 2 compared with Breakthru® only (Table 5.4). The cell counts of yeasts with Breakthru® only application were significantly increased (Table 5.4).

At Site 2, where yeast Isolate B13 was applied to fruit trees with two applications for two months (one application per month) before harvest, all treatments at harvest had significantly higher numbers of yeast cells (Table 5.4). There was no statistical difference between treatments at 1×10^6 and 1×10^7 cells $m\ell^{-1}$ of the yeast Isolate B13 with Breakthru®, compared with the Breakthru® alone (Table 5.4). However, treatment of Valencia oranges with Isolate B13 at 1×10^7 cells $m\ell^{-1}$ without Breakthru® resulted in the highest yeast population recovery at harvest at Site 2 (Table 5.4). At Site 1 application of Isolate B13 at both concentrations, with or without Breakthru®, resulted in increased yeast populations compared to treatment with Breakthru® alone. Treatment with Breakthru® alone did not result in an increase in yeast populations compared to the water-sprayed control. At Site 2 the yeast populations after treatment with Breakthru® alone was significantly higher than the equivalent yeast population at Site 1. In general, the yeast population significantly increased with the increased applications of Isolate B13 without Bth, as shown (Table 5.4) for Site 2.

Table 5.4 Cell counts of the yeast Isolate B13 on Valencia oranges at harvest, when Isolates B13 was applied prior to harvest (in the 2007 season)

	Total B13 yeast cell counts at harvest	
Treatments	**Site 1 (in Richmond Town, received one applications of B13)**	**Site 2 (Maywood Farm, 10 km from Richmond Town, received two applications of B13)**
Untreated control	1.33×10^2 a	3.06×10^2 a
Breakthru (Bth)	5.81×10^2 a	1.73×10^4 c
B13: 1×10^6	1.02×10^3 b	3.06×10^3 b
B13: 1×10^6 +Bth	1.02×10^3 b	5.81×10^4 c
B13: 1×10^7	2.28×10^3 b	1.33×10^5 d
B13: 1×10^7 +Bth	1.33×10^3 b	5.81×10^4 c

Bth= Breakthru®

Values within a column followed by the same letter are not significantly different based on method used by Benbow and Sugar (1999).

5.4 DISCUSSIONS

Proper coverage of fruit surfaces during preharvest applications with biocontrol yeasts is crucial in order to provide protection against target organisms (Roberts, 1994). Wetters such as Breakthru® may be used during spraying for enhancing spread and thus coverage of the targeted plant material or tissue. Breakthru® was shown to be compatible with the yeast Isolate B13. Thus, Breakthru® was used as a spreader for the yeast Isolate B13 during these preharvest applications.

The yeast Isolate B13 survived on Valencia orange fruit for at least one month after application when applied once or twice (one application per month), one or two months before harvest. The cell count of yeasts was higher when Isolate B13 was applied twice, compared with only one application (Table 5.4). With a single application of the yeast Isolate B13, cell counts were not as high as those reported for *Candida laurentii* on apple fruit (final population of 1×10^5 CFU per fruit) by Benbow and Sugar (1999). At Site 2 yeast populations were higher than at Site 1. This was probably because of double applications of yeast at Site 2. However, at both farms the yeast isolate survived on the fruit at elevated levels after preharvest application, irrespective of the timing, the dose used, or the use of a wetter.

A higher yeast population was measured for fruit treated with Isolate B13. However, yeast cells were counted from the untreated fruit or the Breakthru® only treatment. These yeast cells may have been moved to the control fruit and Breakthru® treated fruit by spray drift, or rain splash, creating interplot interference. It would be necessary to design the trial differently in order to eliminate interplot interference. They may also have been part of the original epiphytic community on the fruit, as suggested by Benbow and Sugar (1999) on apple fruit preharvest yeast treatments. In our study, fruit were not sampled prior to treatment to determine which species were common epiphytes in the orchard. Fruit were also not sampled prior to treatment to determine the initial population levels of yeasts on the fruit which would have helped to determine adaptability, multiplication or relative levels of yeast populations. However, several studies have established that yeasts, including *Candida* sp., are major components of the epiphytic microbial community of mature fruit (Clark *et al*., 1954; Buhargiar and Barnett, 1971; Clark and Lorbeer, 1977).

Most fruit decay originates in wounds that develop during harvesting or transportation process (Spotts *et al*., 1998). Therefore application of antagonistic agents before the period of wounding should provide the antagonists with the opportunity to pre-emptively colonize wound sites before pathogens (Wilson and Pusey, 1985; Roberts, 1994). However, *Penicillium digitatum* incidence was not controlled by application of Isolate B13 at all concentrations, with/without Breakthru®, with all application frequencies. Similarly, preharvest application of Isolate B13 failed to inhibit *P. digitatum* lesion diameter, when tested on Valencia fruit from Site 1 in trials in 2006.

Based on these findings, and observations of many cases of early infection of orange fruit by *P. digitatum* (at the green stage, approximately 3-4 months before harvest) in the field, a second trial was conducted, with application of Isolate B13 over an extended time period; application were made one or two months before harvest, or applied one day before harvest, at two sites. However, there was no significant difference in the control of *P. digitatum* incidence as a result of applications of Isolate B13, whether applied over one or two months, or one day preharvest (Table 5.3).

Although field applications of biocontrol agents to reduce preharvest diseases have not met with much success (Peng and Sutton, 1991), some success has been reported of biocontrol using *Trichoderma harzianum* Rifai sprayed alone or in combination with dichlofluanid to control natural infestations of *B. cinerea* on apples (Tronsmo and Ystaas, 1980), and on avocado for the control of *Pseudocercospora purpurea* (Cooke) Deighton using *Bacillus subtilis* Ehrenberg (Korsten *et al*., 1997).

In our trials, when Isolate B13 was applied as preharvest treatment during seasons 2006 and 2007, no effective control of *P. digitatum* was provided by various concentrations of the yeast isolate treatments compared with the controls. This is in agreement with Korsten *et al*. (1997) who suggested that satisfactory control of plant disease through biological control is not always evident in the first season and therefore requires persistence on the part of the grower. These authors confirmed this with follow-up experiments, where biological control proved to be as effective as fungicide programmes. They also further proved that integrated control with a biocontrol agent (*B. subtilis)* was

more effective over time than commercial fungicides applied against avocado postharvest diseases.

Variability in the degree of control provided by biocontrol agents can be due to initial high levels of disease pressure (Tronsmo and Ystaas, 1980; Lonsdale, 1991), built up over time in the field. Hence, it may take time to reduce the disease to manageable levels with antagonistic organisms, such as yeasts in the orchard. Therefore, preharvest treatments with biocontrol should be tested successively over several years at more than one location for commercial acceptance of the antagonistic microorganisms for the control of *P. digitatum.*

Many researchers stress that biological control agents alone have not often provided acceptable levels of control of plant diseases, but are more effective when used in combination with other strategies (Janisiewicz and Korsten, 2002; Tian *et al*., 2002). Some strategies such as heat treatment (Kim *et al*., 1991) or silicon application have shown synergistic effects for control of fruit decay of apples (Qin and Tian, 2005).

5.5 CONCLUSIONS

The application of the yeast Isolate B13, with or without Breakthru®, at different concentrations, and different application frequencies on two citrus farms, resulted in significantly higher overall yeast cell counts on Valencia orange fruit surfaces than occurred on the control. However, none of the preharvest applications of yeast Isolate B13 reduced the incidence of *P. digitatum* disease.

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CHAPTER 6

PREHARVEST APPLICATION OF POTASSIUM SILICATE FOR THE CONTROL OF *PENICILLIUM DIGITATUM* ON ORANGES

ABSTRACT

Preharvest applications of potassium silicate (K_2SiO_3), with a concentration of 20.5% was used as a silicon source, in the preharvest experiment.for the control of *Penicillium digitatum* (Pers: Fr. Sacc), on eight years old navel and Valencia orange trees were investigated for two consecutive seasons. Each tree was drenched with 5 ℓ of K_2SiO_3 solution at concentrations of 0, 100, 500, 1000, and 10000 mg ℓ^{-1}. In the first season, K_2SiO_3 was drenched once a week or once every two weeks for four months before harvest. Water was used as the control drench. The K_2SiO_3 application during this period did not reduce the incidence of the pathogen on oranges stored at either 24±1°C or 9±1°C. However, continued application of K_2SiO_3 onto the same trees for a full year, applied once a month or once every two months, resulted in significant reductions in the incidence of *P. digitatum* decay of both varieties of oranges. Application of K_2SiO_3 at a concentration of 100 mg ℓ^{-1} or more provided good control on both oranges. There was no significant difference in the level of silicon extracted from leaves or fruit flavedo and albedo of navel and Valencia oranges. Potassium silicate has shown potential to reduce the levels of postharvest *P. digitatum* infection of navel and Valencia oranges when applied as a preharvest treatment.

6.1 INTRODUCTION

Silicon (Si) is an integral component of plants, ranging from 0.1-10.0% dry weight (Epstein, 1999). Silicon is absorbed by plants as monosilicic acid ($Si(OH)_4$) or its anion (Youshida, 1975). Recent research has established that Si can control a number of diseases of a wide range of plants (Guo *et al*., 2007; Bi *et al*., 2006; Qin and Tian, 2005; Dann and Muir, 2002 and Menzies and Belanger, 1996).

Optimization of Si nutrition can result in a positive effect on citrus seedling growth, e.g., in a greenhouse experiment, Si fertilization for 1 and 2 year-old

orange trees yielded increased shoot mass during a 6 months period (Wutscher, 1989). Matichenkov *et al*. (1999) also reported that Si optimization increased shoot and root mass of grapefruit seedlings by 20-60% and improved branching of their root systems. In field experiments, Si applications resulted in increased tree height of 14-41% and accelerated branching by 31-48% over a 6 month period (Matichenkov, 2001).

In recent years, research into the role of Si in the control of plant fungal disease has shown promising results (Belanger *et al*., 1995; Menzies and Belanger, 1996). Use of Si in hydroponic growth media can reduce the severity of powdery mildew and Pythium root rot of cucumbers (Menzies *et al*., 1991a, b; Cherif *et al*., 1994). Application of Si into potting mixes or soils reduced rice blast disease (Seebold *et al*., 2001) and fungal infections in peas caused by *Mycosphaerella pinodes (*Berk. and A. Bloxam) Vestergr (Dann and Muir, 2002).

Although Si applications have resulted in improved citrus seedling development under greenhouse and field conditions (Wutscher, 1989), to date no information is available on root application of K_2SiO_3 for the control of postharvest *P. digitatum* of citrus fruit.

The objective of this study was to investigate whether K_2SiO_3 preharvest applications could control *P. digitatum* infection of navel and Valencia oranges.

6.2 MATERIALS AND METHODS

6.2.1 Use of potassium silicate for the control of *P. digitatum* on navel and Valencia oranges by field drench application for four months before harvest

A liquid formulation of dissolved potassium silicate (K_2SiO_3), with a concentration of 20.5% provided by PQ Silicas[3] was used as a silicon source in the preharvest experiment.

[3] PQ Silicas South Africa (Pty) Ltd, 169 Tedstone Road, Wadeville, 1407, P. O. Box 12062, Gauteng, South Africa.

In 2006, a trial was conducted at Ukulinga (29.36 S 30.24 E), Research Farm (University of KwaZulu-Natal, Pietermaritzburg) on the control of *P. digitatum* of navel and Valencia orange fruit, commencing four months before harvest. Each treatment was replicated three times. A single tree (eight old) served as a replicate. Each tree was drenched with 5 ℓ of 20.5% of K_2SiO_3 solution once a week or once every two weeks at different concentrations as given in Table 6.1. Water was used as the control drench. The potassium silicate had a pH of 10 to 12). Treatments provided in Table 6.1 were laid out in a complete randomized block design (CRBD). At harvest fruit from navel and Valencia orange trees were divided into two groups, with each group having 36 fruit per tree. Thirty six of these oranges were wounded (25 mm in length and 3 mm in depth) at one site at the equator with dissecting needle) and were dipped for one minute in a suspension of *P. digitatum* conidia at a concentration of 1×10^4 conidia $m\ell^{-1}$ as used previously. Eighteen of the 36 fruit were kept at room temperature (24±1°C) and the other 18 fruit were placed in cold storage (9±1°C) with a relative humidity of 90%. Similarly, the second group of 36 oranges was not wounded. However, all fruit were dipped for one minute into a suspension of *P. digitatum* conidia at a concentration of 1×10^4 conidia $m\ell^{-1}$. Eighteen of the 36 fruit were kept at room temperature (24±1°C) and the other 18 fruit were placed in cold storage (7±1°C) with a relative humidity of 90%. Eighteen fruit, placed into three boxes, were used per treatment for each storage condition in a CRBD. Percentages of navel or Valencia oranges with visible *P*. *digitatum* infection (%) were recorded after one month of storage, either at room temperature or cold storage (9±1°C).

Table 6.1 Treatments for control of *Penicillium digitatum* by field drench application of potassium silicate for four months before harvest, on navel and Valencia oranges

Treatment	K_2SiO_3 (mg ℓ^{-1})	Application frequency
T1 (control)	0	0
T2	100	1*
T3	100	2*
T4	500	1
T5	500	2
T6	1000	1
T7	1000	2
T8	10000	1
T9	10000	2

Key: 1* = once a week, 2* = once every 2 weeks in season 2006 for four months.

6.2.2 Further determination of the efficacy of potassium silicate for the control of *P. digitatum* on navel and Valencia oranges by field drench application for 11 months before harvest

In the 2007 season, the trial design was the same as described in Section 6.2.1. Treatments were continued for the entire growing season on the same experimental trees except application of K_2SiO_3 was made once a month or once every two months instead of once a week or once every two weeks as in 2006 season. At harvest, fruit samples were collected but not wounded and not inoculated artificially because the trial depended on natural infection. The same sample sizes, storage conditions, design and assessment parameters were followed as in 2006, as described in Section 6.2.1.

6.2.3 Silicon and potassium extraction from navel and Valencia orange leaves and fruit tissues from field silicon pre-treated trees

Ten leaf samples per tree adjacent to the fruit were collected according to Vock *et al*. (1997). Three fruit samples per tree were also collected where the flavedo (peel) was separated and the fruit albedo (pith) was also separated from the

same fruit. The method and procedure for the determination of silicon as described by Kanamugire (2007) was followed, with some modification.

6.2.3.1 Extraction solution

A 15% solution of NaOH was made up by dissolving NaOH (Merck Laboratory, South Africa) pellets (150 g) in a one ℓ volumetric flask with twice-deionised water. Concentrated HCl (Merck Laboratory, South Africa) (54 mℓ) was diluted in a one ℓ volumetric flask with twice-deionised water. The solutions were used as Si extraction reagents.

6.2.3.2 Extraction procedure

The plant samples were dried at 70°C for 24 h in an oven (Labotec South Africa). Samples were milled and sieved through a 5 mm size sieve. One gram of oven dried plant material was ashed overnight at 650°C. The ashed material was transferred to a nickel crucible. To this, 5 mℓ of 15% NaOH was added and evaporated at a low heat on a hot plate until dried. A little deionised water was added to dissolve the sample which was then transferred into a 100 mℓ volumetric flask containing 40 mℓ of 0.06N HCl and topped up with deionised water to 100 mℓ. Inductively Coupled Plasma (ICP) analysis was performed, in order to determine the concentration of Si and potassium (K) from the plant material. Silicon standards of 0, 0.1, 1, 5, 10, 20, 50 mg ℓ^{-1} were prepared from a 1000 mg ℓ^{-1} of $(NH_4)_2SiF_6$ (in H_2O) (Merck Laboratory, South Africa) and used for a standard curve. Similarly, for determination of the concentration of potassium (K) from plant material the same extract prepared for the Si extraction was used. Potassium standards of 0, 0.1, 1, 5, 10, 20, 50 mg ℓ^{-1} were prepared from a 1000 mg ℓ^{-1} of KNO_3 (in HNO_3 0.5 mol ℓ^{-1}) (Merck Laboratory, South Africa) used for a standard curve. A Varian 720-ES ICP-OES was used according to the operating conditions outlined in Table 6.2. The analytical wavelength (nm) was set at Si (251.611 nm) and for K (766.491 nm).

Table 6.2 Experimental conditions for ICP-OES

ICP Spectrometer	Varian 720-ES ICP-OES
Power (kw)	1.00
Argon Plasma Flow (ℓ min^{-1})	15.0
Auxiliary Argon Flow (ℓ min^{-1})	1.50
Photomultiplier (V)	800
Integration time (sec)	1
Nebuliser (kPa)	240

6.2.4 Evaluation of the effect of potassium silicate on total soluble solids of navel and Valencia oranges

Total soluble solids (TSS) were determined by squeezing juice directly onto a refractometer (Atago, Japan). Three fruit were used per K_2SiO_3 treatment and one reading was taken from each navel or Valencia orange 11 months after treatment with K_2SiO_3 at mature stage of the fruit for the seasons 2007.

6.2.5 Statistical analysis

Data were subjected to an analysis of variance (ANOVA) using Genstat® Executable Release 9.1 Statistical Analysis Software (Anonymous, 2006). To determine differences between treatments, Fisher's Least Significant Difference Test was used ($P<0.05$).

6.3 RESULTS

6.3.1 Effect of field drench applications of potassium silicate four months before harvest for the control of *P. digitatum* of navel and Valencia oranges

When applied for four months prior to harvest, none of the K_2SiO_3 treatments, at any concentration, or at any frequency of application, caused a significant reduction in the severity of *P. digitatum*, compared with the control treatment on navel and Valencia oranges (Tables 6.3 and 6.4).

Table 6.3 Effect of four months potassium silicate field applications on severity of *Penicillium digitatum* (%) on navel oranges in the 2006 season one month after harvest

Treatments	K_2SiO_3 (mg ℓ^{-1})	Frequency of application	Severity of *P. digitatum* (%) on unwounded fruit		Severity of *P. digitatum* (%) on wounded fruit	
			24±1°C	9±1°C	24±1°C	9±1°C
T1(Control)	0	0	11	0	100	83
T2	100	1*	0	0	85	93
T3	100	2*	3	2	98	84
T4	500	1	0	3	97	86
T5	500	2	13	11	91	85
T6	1000	1	14	12	100	88
T7	1000	2	6	5	99	93
T8	10000	1	0	0	97	94
T9	10000	2	13	3	95	87
P Value			0.054 NS	0.062 NS	0.058 NS	0.060 NS

Note: Fifty four fruit per treatment were used for each wounded and unwounded.

Key: 1*: Weekly application
2*: 2 weekly application
NS= Non significant at (P≥0.05).

Table 6.4 Effect of four months potassium silicate field applications on severity of *Penicillium digitatum* (%) on Valencia oranges in the 2006 season, one month after harvest

Treatments	K_2SiO_3 (mg ℓ^{-1})	Frequency of application	Severity of *P. digitatum* (%) on unwounded fruit		Severity of *P. digitatum* (%) on wounded fruit	
			24±1°C	9±1°C	24±1°C	9±1°C
T1 (Control)	0	0	0	0	100	98
T2	100	1*	0	0	94	97
T3	100	2*	2	2	100	100
T4	500	1	0	3	97	93
T5	500	2	6	0	98	95
T6	1000	1	0	0	93	91
T7	1000	2	0	0	97	99
T8	10000	1	0	10	96	94
T9	10000	2	0	0	100	91
P Value			0.060 NS	0.056 NS	0.062 NS	0.071 NS

Note: Fifty four fruit per treatment were used for each wounded and unwounded.

Key: 1*: Weekly application
2*: 2 weekly application
NS= Non significant (P≥0.05).

6.3.2 Effects of potassium silicate field applications for 11 months preharvest, in the 2007 season, on the severity of *P. digitatum* on navel oranges, stored at 24±1°C or 9±1°C for one month

Navel orange fruit harvested from trees drenched at a concentration of 100 mg ℓ^{-1} once a month, and fruit from trees drenched at a concentration of 1,000 mg ℓ^{-1} applied either once a month or once every 2 months, showed reduced levels of *P. digitatum* compared with fruit from trees treated with K_2SiO_3 concentrations at 100 mg ℓ^{-1} applied once every two months, 500 mg ℓ^{-1} applied at both frequencies and 10,000 mg ℓ^{-1} applied once a month or the control

(0 mg ℓ^{-1}) (a detailed statistical analysis of the data in Figure 6.1 is provided in Appendix 6A).

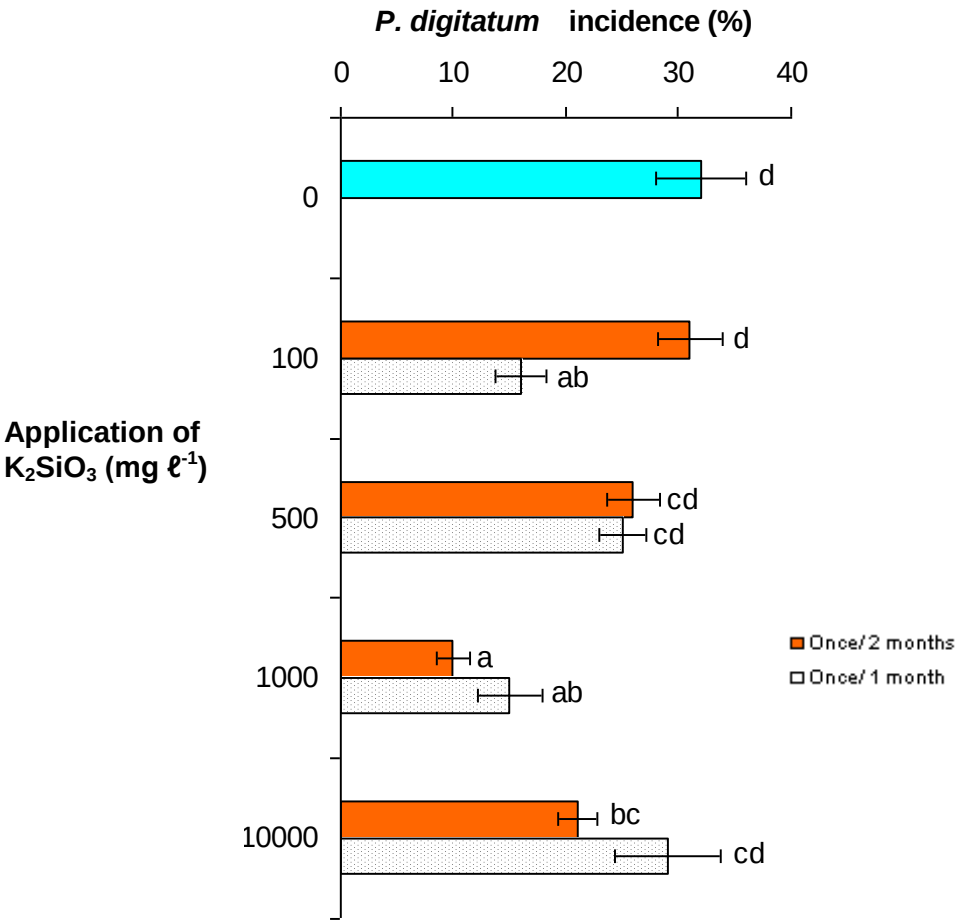

Figure 6.1 Effect of preharvest potassium silicate applications for 11 months on the incidence of *Penicillium digitatum* on navel oranges stored at 24±1°C, in the 2007 season. Treatments with different letters differ significantly (P≤0.05).

When stored at 9±1°C navel oranges treated with K_2SiO_3 at all concentrations applied, developed significantly lower incidences of *P. digitatum* compared with the untreated control (Figure 6.2) (a detailed statistical analysis is presented in Appendix 6B).

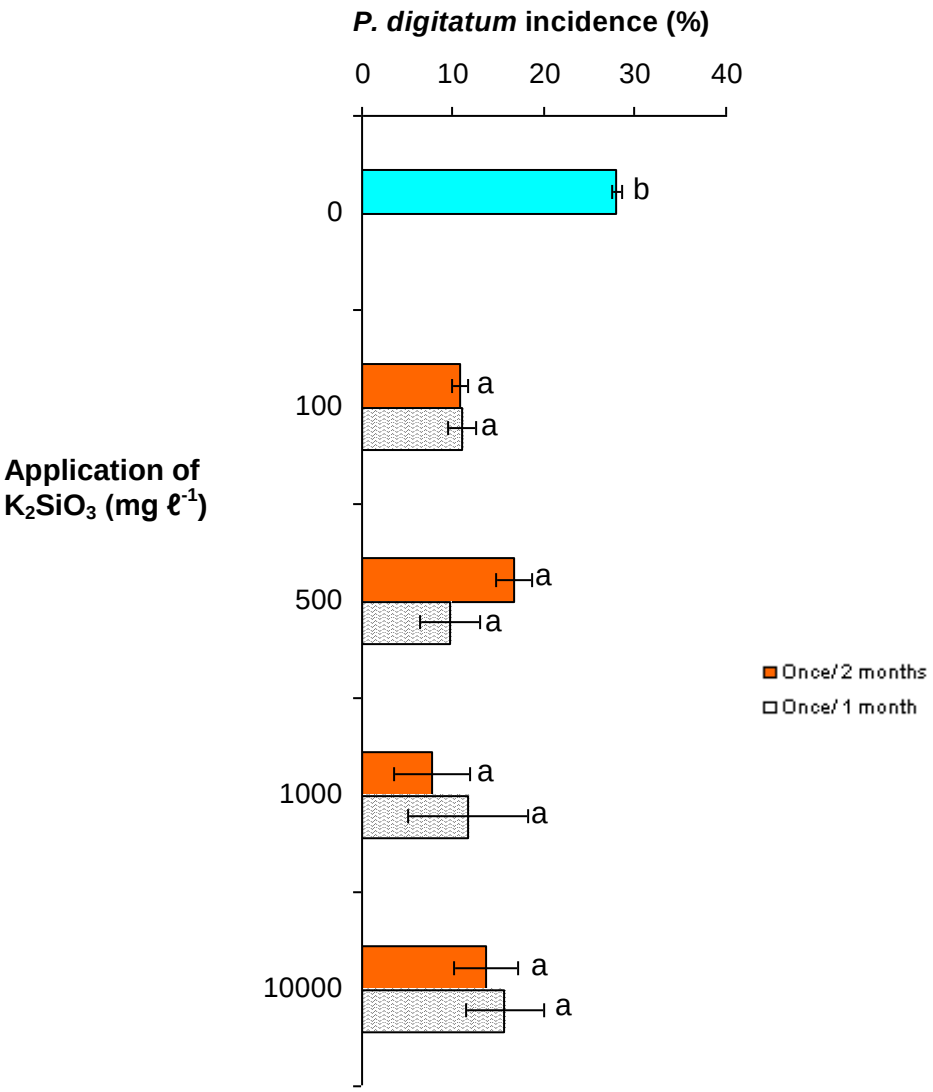

Figure 6.2 Effect of preharvest potassium silicate applications for 11 months on the incidence of *Penicillium digitatum* on navel oranges stored at 9±1°C, in the 2007 season. Treatments with different letters differ significantly (P≤0.05).

6.3.3 Effect of Potassium silicate field application for eleven months before harvest on the levels of *P. digitatum* developing on Valencia oranges, stored at 24±1°C or 9±1°C, in the 2007 season

Valencia oranges harvested from trees drenched with potassium silicate at all concentrations applied with exception of 10000 mg ℓ^{-1} applied once every month, developed significantly lower incidences of *P. digitatum* compared with the untreated control (a detailed statistical analysis of the data in Figure 6.3 is presented in Appendix 6C).

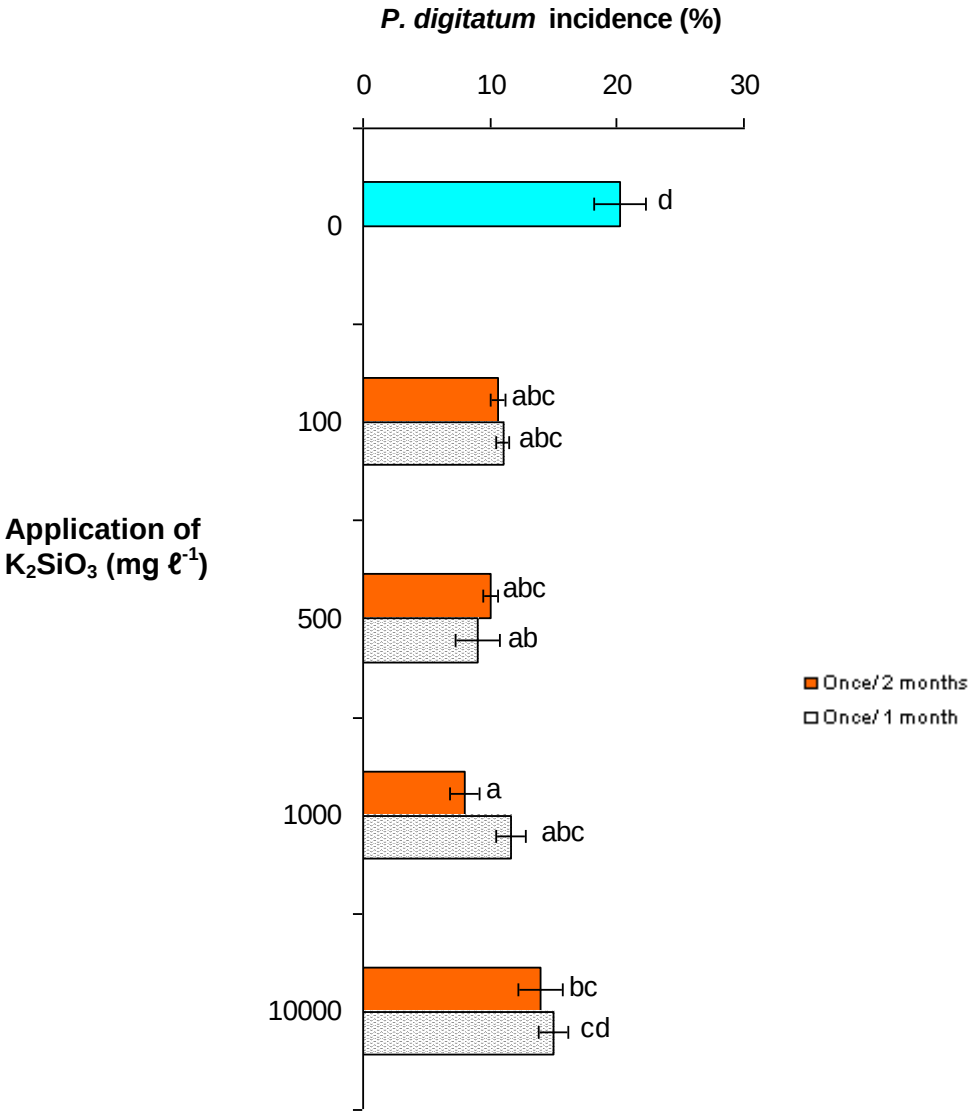

Figure 6.3 Effect of preharvest potassium silicate applications for 11 months on the incidence of *Penicillium digitatum* on Valencia oranges, stored at 24±1°C, in the 2007 season. Treatments with different letters differ significantly (P≤0.05).

Valencia oranges harvested from trees drenched with potassium silicate, at all concentrations and frequencies of application, developed significantly fewer cases of *P. digitatum* compared with the untreated control, when stored at 9±1°C (a detailed statistical analysis of the data in Figure 6.4 is provided in Appendix 6D).

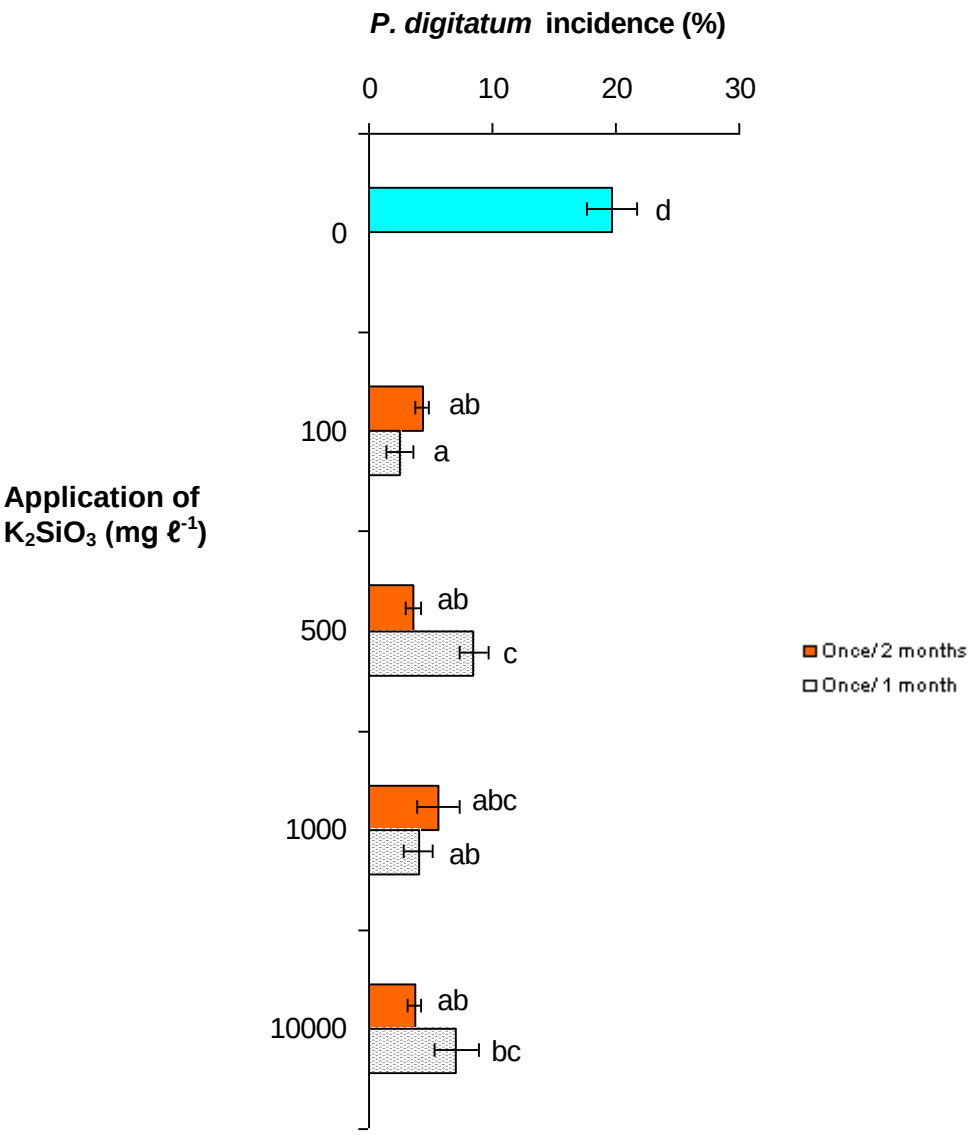

Figure 6.4 Effect of preharvest potassium silicate applications for 11 months on the incidence of *Penicillium digitatum* on Valencia oranges stored at 9±1°C, in the 2007 season. Treatments with different letters differ significantly (P≤0.05).

6.3.4 Effect of preharvest application of potassium silicate for 11 months before harvest on levels of silicon extracted from navel and Valencia oranges leaves and fruit tissues from mature fruit, in the 2007 season

There was no significant difference in the levels of extracted Si at all drenching concentrations and application frequencies compared with the untreated control, for leaves, fruit flavedo or albedo of navel or Valencia oranges (Figure 6.5). There was no significant difference in the levels of extracted K at all

drenching concentrations and application frequencies compared with the control for leaves of navel orange (Table 6.5).

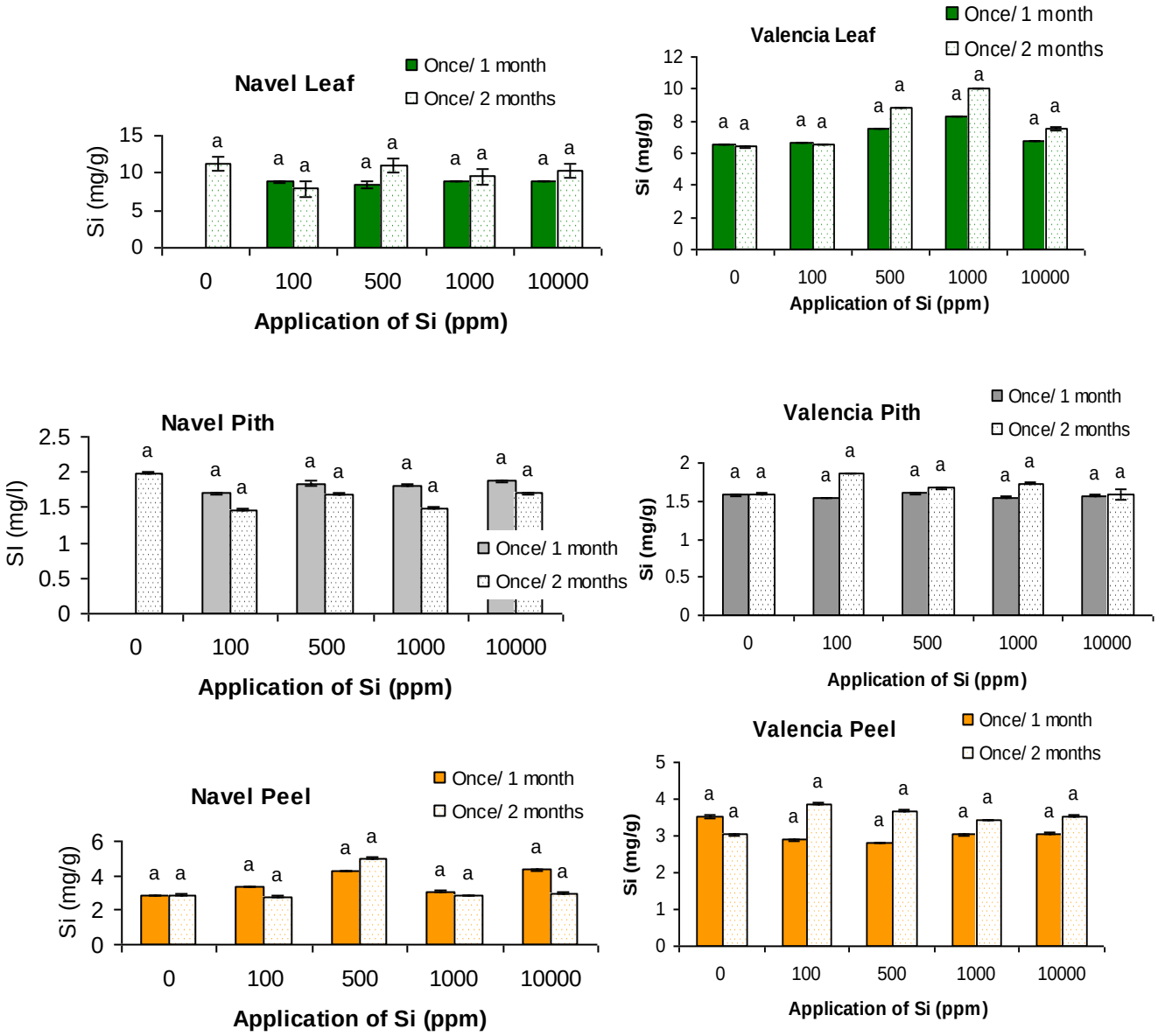

Figure 6.5 Quantity of Si extracted from leaves and fruit tissue (peel and pith) of navel and Valencia oranges at maturity after 11 months of preharvest applications of potassium silicate in the 2007 season.

Table 6.5 Effect of preharvest applications of potassium silicate for 11 months before harvest on potassium extraction from navel tree leaves when fruit were mature, in the 2007 season

Treatments	K_2SiO_3 (mg ℓ^{-1})	Frequency of application	K extracted (mg/g plant leaf)
T1 (Control)	0	0	193.9
T2	100	1*	215.5
T3	100	2*	238.1
T4	500	1	185.5
T5	500	2	240.1
T6	1000	1	204.7
T7	1000	2	219.7
T8	10000	1	229.7
T9	10000	2	190.9
P Value			0.884 NS

NS= Non significant (P≥0.05).

Key: 1*: Once/ 2 monthls application
2*: Once/ 1 month application

6.3.4 Effect of preharvest application of potassium silicate on total soluble solids of navel and Valencia oranges

Total soluble solids extracted from navel and Valencia oranges after 11 months of treatment of the trees with various application levels of K_2SiO_3 did not differ statistically between treatments with and without K_2SiO_3 (Table 6.6).

Table 6.6 Effect of preharvest application of potassium silicate for 11 months before harvest on total soluble solids of navel and Valencia oranges at fruit maturity stage for 2007 season

Treatments	K_2SiO_3 (mg ℓ^{-1})	Frequency of application	% Total soluble solids	
			Navel	Valencia
T1 (Control)	0	0	9.1	9.8
T2	100	1*	9.0	9.9
T3	100	2*	8.3	9.6
T4	500	1	9.2	9.9
T5	500	2	8.9	9.4
T6	1000	1	9.5	10.0
T7	1000	2	9.0	9.3
T8	10000	1	9.1	9.6
T9	10000	2	9.0	9.9
P Value			0.478	0.296
			NS	NS

NS= Non significant (P≥0.05).

Key: 1*: Once/ 2 monthls application
2*: Once/ 1 month application

6.4 DISCUSSION

Potassium silicate was not effective in reducing the incidence of *P. digitatum* of navel and Valencia oranges when drenched for only four months before harvest (Tables 6.3 and 6.4). However, K_2SiO_3 was generally effective in reducing the incidence of *P. digitatum* on both orange varieties, when drenched once a month or once every two months for the whole growing season in 2007 on the same trees which received K_2SiO_3 treatments for four months in 2006 (Figures 6.1; 6.2; 6.3 and 6.4). The ineffectiveness during the first season of K_2SiO_3 application was probably due to the short period of time for the K_2SiO_3 to be taken up by the trees.

Application of potassium silicate as a preharvest treatment on Valencia oranges generally resulted in a decreased incidence of *P. digitatum,* at most

concentrations tested, compared with navel oranges that received similar treatments, at 24±1°C as shown in Figures 6.1 and 6.3, respectively. This result seems to indicate that K_2SiO_3 reduces the incidence of *P. digitatum* on Valencia oranges better than on navel oranges. Plant responses to Si applications may be different depending on the crop genotype, as suggested by Rodrigues *et al*. (2001).

Application of potassium silicate at all concentrations as a preharvest treatment on navel and Valencia trees resulted in a significant decrease in the incidence of *P. digitatum* on navel and Valencia oranges, when stored at 9±1°C (Figures 6.2 and 6.4). This finding indicates that oranges can be protected from *P. digitatum* infection during shipping to distant markets of four to six weeks by preharvest application of K_2SiO_3.

A further finding was on the effect of wounding before inoculation with *P. digitatum* on navel and Valencia fruit from trees that received K_2SiO_3 preharvest application. Normally wounds occur during harvesting. The wounds inflicted artificially on fruit here were substantial, in order to replicate a worst case scenario (usually such wounded fruit would be picked up on packhouse sorting line). Orange fruit that received preharvest treatment with K_2SiO_3, and were then wounded and then inoculated with *P. digitatum* did not develop less disease than the untreated control oranges. On unwounded fruit (but with already infected with latent infections) of navel and Valencia oranges, fruit from trees that received K_2SiO_3 preharvest applications did not develop significantly fewer infections (Tables 6.3 and 6.4).

Incidence of *Penicillium digitatum* on Valencia oranges was reduced by K_2SiO_3 applications at concentrations as low as 100 mg ℓ^{-1}, if applied once every two month for an entire growing season. This result established that levels of control of *P. digitatum* are not affected by the levels of Si applied within the range tested (100-10,000 mg ℓ^{-1}). Achieving good levels of control of *P. digitatum* with lower level of K_2SiO_3 application would make it easier and more economical to apply the product in the field. Potassium silicate costs R120.00 per hectare and citrus trees will require four applications per season (Paterson, pers. comm. 2008).

Potassium silicate applications in our experiments had no effect on total soluble solids in navel and Valencia oranges. However, other researchers, such as Ayres (1996), have shown that applications of soluble silicon increased the sugar content in sugar beet and sugar cane.

It has been reported that Si accumulation on the epidermal tissues of the shoot (leaves) causes epidermal thickening of the cellulose layer (Epstein, 1999; Matichenkov *et al*., 1999). In this study, the level of Si extracted from navel and Valencia orange leaves and particularly the fruit flavedo and albedo did not significantly differ compared with the untreated control. The results demonstrated that the theory that the application of soluble silicon to crops reduces disease because of a physical barrier may not be a viable theory. An induced resistance response enhanced or primed by K_2SiO_3 may have contributed to reduced incidence of *P. digitatum.* This theory is supported by Liang *et al*. (2005) who reported that continuous root-applied Si enhanced resistance to infection by powdery mildew. Other researchers have also suggested that Si stimulates host resistance mechanisms against pathogen, such as enhancing the production of phenolic compounds in crops, e.g., cucumbers and barley (Carver *et al*., 1987; Menzies *et al*., 1991b; Cherif *et a*l., 1992; Fawe *et al*., 1998). Liang *et al*. (2003) found that drenching cucumber with Si significantly enhanced enzyme activity in the roots of salt-stressed plants compared to Si-deprived plants. The same authors also showed that the Si effect was time-dependent and became more effective with extended, long-term trials.

The effect of potassium (K) in disease control is well documented (Schneider, 1985). For example, fertilization with K demonstrated reduced *Fusarium* yellow disease of celery caused by *Fusarium oxysporum* (Schneider, 1985); reduced *Sphaerotheca fuliginea* in cucumber plants (Reuveni *et al*., 2000) and controlled gray mold in table grape (Karabulut *et al*., 2005). However, there was no significant difference in the levels of K extracted from treated and untreated plants in these trials (Table 6.5). Therefore, it is unlikely that potassium alone was responsible for the reduced incidence of *P. digitatum* in this trial.

A body of evidence is available for the physical role of silicon versus diseases (Epstein, 1999; Matichenkov *et al*., 1999), as well as evidence of enhanced development (speed and level) of systemic acquired resistance (SAR) and induced systemic resistance (ISR), and accelerated accumulation of phytoalexins (Carver *et al*., 1987; Menzies *et al*., 1991b; Cherif *et a*l., 1992; Fawe *et al*., 1998; Liang *et al*., 2003). Applications of soluble silicon have the potential to reduce levels of a range of diseases (Belanger *et al*., 1995; Menzies and Belanger, 1996; Liang *et al*., 2005), and to provide for relief of abiotic stresses (Liang *et al*., 2003). We therefore propose that this is the role played by preharvest potassium silicate application. This is, to the best of our knowledge, the first report of the beneficial effect of K_2SiO_3 as a preharvest treatment for the control of *P. digitatum* on citrus fruit.

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CHAPTER 7

PREVENTATIVE AND CURATIVE EFFECT OF POTASSIUM SILICATE POSTHARVEST TREATMENT FOR THE CONTROL OF *PENICILLIUM DIGITATUM* ON LEMONS

ABSTRACT

Postharvest application of potassium silicate (K_2SiO_3) reduced disease lesion diameter caused by *P. digitatum* on wounds of lemon fruit at 24±1°C. Fruit treated with potassium silicate at a concentration of 100,000 mg ℓ^{-1} developed the smallest lesion diameter when the potassium silicate was applied as a preventative treatment (3 hours before inoculation with *P. digitatum*) or curatively (inoculated with *P. digitatum* 3 hours before treatment). Scanning electron microscopy revealed that growth of *P. digitatum* conidia was significantly inhibited or restricted up to 10 days when K_2SiO_3 was applied preventatively into the wounds of lemons. This study demonstrated the potential of K_2SiO_3 for the control of *P. digitatum* infection on lemons when applied as a postharvest treatment. Scanning electron microscopy studies confirmed that K_2SiO_3 applications to lemon wounds stopped germination of conidia of *P. digitatum*.

7.1 INTRODUCTION

Several additives have potential for disease control, such as silicates (Belanger *et al*., 1995), carbonates and bicarbonates (Smilanick *et al*., 1999; Conway *et al*., 2007; Janisiewicz *et al*., 2008; Smilanick *et al*., 2008), chitosan (El-Ghaouth *et al*., 1992) and various acids (Sholberg, 1998). Food additives that control post-harvest diseases would be ideal because they may be applied to the food system without restriction (Maga and Tu, 1994).

Carbonic acid salts, such as sodium carbonate (soda ash) and sodium bicarbonate (baking soda) have been shown to control plant diseases (Palou *et al*., 2001). Treatments of citrus in solutions of sodium carbonate and sodium bicarbonate reduce the incidence of postharvest green moulds (Palou *et al*., 2001). Good control of green mould of oranges has also been achieved in South Africa using both salts (Lesar, 2007). Sodium carbonate has shown the potential to control green mould when applied long after pathogen inoculation

(Smilanick *et al*., 1995) and are being used in the California citrus industry (Smilanick *et al*., 1999). Sodium silicate and calcium chloride have been shown to control green and blue moulds of Clementine mandarins (Ligorio *et al*., 2007).

Several researchers have reported that silicon applications reduce plant diseases when applied as a fertilizer, using soil or foliar applications (Menzies *et al*., 1991a; Menzies *et al*., 1992; Cherif *et al*., 1994; Belanger *et al*., 1995; Menzies and Belanger, 1996; Rodrigues *et al*., 2003). Silicon applications for control of postharvest fungal infections have also shown promising results. For example, application of silicon dioxide and sodium silicate reduced the severity of pink rot of Chinese cantaloupe caused by *Trichothecium roseum* (Pers.) Link. (Guo *et al*., 2007); Bi *et al.* (2006) used sodium silicate to control pink rot caused by *Alternaria alternata* (Fr.) Keissl. *Fusarium* spp. and *Trichothecium roseum* on Hami melons (*Cucumis melo* L. var. *inodorus* Jacq.). Qin and Tian (2005) found that silicon in the form of sodium metasilicate reduced infection development caused by *Penicillium expansum* (Link) Thom. and *Monilinia fructicola* (G. Winter) Honey of sweet cherry fruit and Biggs *et al*. (1997) also found silicon to control *M. fructicola* on peach fruit.

The mechanisms by which silicon provides protection to plants against fungal pathogens are not yet fully elucidated. Several studies have revealed that deposition of the silicon in the cell wall around infection sites provides a physical barrier to pathogen penetration (Heath and Stumpf, 1986; Carver *et al*., 1987; Datnoff *et al*., 1997). However, potassium silicate may also be effective by catalyzing or accelerating the rapid release of biochemical defense products such as phenolic compounds and pathogenesis-related proteins in infected plants (Cherif *et al*. 1992; Epstein, 1999).

There does not appear to be any other reports documenting the effect of silicon salts on the postharvest control of *P. digitatum* on citrus fruit. The objective of this study was to investigate the value of applying K_2SiO_3 as a postharvest treatment in order to manage *P. digitatum*.

7.2 MATERIALS AND METHODS

7.2.1 Dissolved potassium silicate

A liquid formulation of dissolved potassium silicate (K_2SiO_3), with a concentration of 20.5%, provided by PQ Silicas[4], was used as the silicon source in this postharvest experiment.

7.2.2 Evaluation of the preventative activity of potassium silicate against *P. digitatum* as a postharvest treatment on lemons

Freshly harvested lemons (ten fruit per treatment) were washed in 70% alcohol for one minute, and then air dried. Each fruit was wounded (25 mm in length and 3 mm in depth) at the equator, and then treated with 100 µl of potassium silicate solution at concentrations of 10, 100, 1000, 10000 and 100,000 mg ℓ^{-1}. After the wound site had dried for three hours, each wound was inoculated with 100 µℓ of conidia of *P. digitatum* suspension containing a concentration of 1×10^4 conidia $m\ell^{-1}$. Wounds inoculated with the same amount of *P. digitatum* conidia served as a control. Fruit were kept at room temperature (24±1°C). Two boxes, with five fruit per box, were used per treatment and placed on a bench in a randomized block design (RBD). Lesion diameter (mm) of each infected wound was determined 10 days after inoculation. Lesion diameter was measured by taking the mean of the horizontal and vertical diameters of each lesion. Data were subjected to an analysis of variance (ANOVA) using Genstat® Executable Release 9.1 Statistical Analysis Software (Anonymous, 2006). To determine differences between treatments, Fisher's Least Significant Difference Test was used ($P<0.05$).

7.2.3 Determination of the curative activity of potassium silicate against *P. digitatum* as a postharvest treatment on lemons

Similar procedures as in Section 7.2.2 were followed, except individual wounded fruit were first inoculated with 100 µl of *P. digitatum* conidia at a concentration of 1×10^4 conidia $m\ell^{-1}$. After the wound site had dried for three hours, each fruit wound was treated with 100 µℓ solution of potassium silicate at the concentrations described in Section 7.2.2. Wounds inoculated with the same amount of *P. digitatum* conidia served as a control. Fruit were

[4] PQ Silicas South Africa (Pty) Ltd, 169 Tedstone Road, Wadeville, 1407, P. O. Box 12062, Gauteng, South Africa.

kept at room temperature (24±1°C). Two boxes, each with five fruit per box, were used per treatment and placed on a bench in a RBD. Lesion diameter (mm) of each infected wound was determined 10 days after inoculation, as described in Section 7.2.2. Data were subjected to an analysis of variance (ANOVA) and least significant differences were determined as described above.

7.2.4 Observations of *in vivo* interaction of potassium silicate and *P. digitatum* in lemon wounds using scanning electron microscopy

Lemon fruit were wounded, then treated with K_2SiO_3 at a concentration of 100,000 mg ℓ^{-1}, (this concentration was chosen because it provided complete control of infection by the pathogen) and were then inoculated with *P. digitatum,* as described in Section 7.2.2. Ten days after inoculation, tissue from the treated (Figure 7.1A and B), and the control wounds, was excised. The tissue samples were fixed overnight in 3% glutaraldehyde in a 0.05 M sodium cacodylate buffer (pH 7.2). Residual fixative was removed with repeated sodium cacodylate buffer washes. The samples were subsequently dehydrated in an alcohol series (10 minutes each in 30%, 50%, 70%, 80%, 90%, and 3 × 10 minutes in 100%) in a fume cupboard. The specimens were then transferred into critical point drier baskets under 100% alcohol and placed in a pre-cooled Hitachi HCP- 2 critical point drier. Following critical point drying (CPD) and gold-palladium sputter-coating (Polaron Equipment Limited ESEM, coating unit E5100), the samples were viewed in an ESEM (Philips, FEI XL 30) at an accelerating voltage of 15 keV. Samples from five fruit from each treatment were viewed; five fields of observation were taken from each fruit sample tissue. For the evaluation criteria of the effects of potassium silicate on *P. digitatum*, counts of germinated, ungerminated or partially germinated conidia of *P. digitatum* were recorded for the interaction tests of *P. digitatum* with potassium silicate. For each treatment five fields from five fruit were viewed and counts were taken.

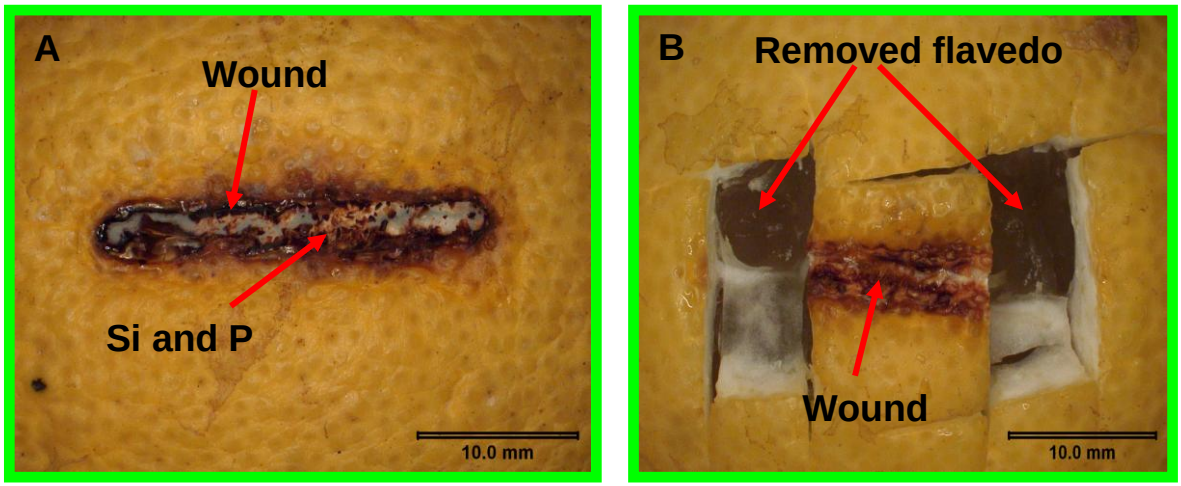

Figure 7.1 (A) Visual effects of a potassium silicate solution at 100,000 mg ℓ^{-1} on the development of *Penicillium digitatum* (P) on lemon. In (A), no infection of *P. digitatum* developed in the wounded lemon fruit surface. The second picture (B) shows how skin samples were excised from the wounded fruit of to be used for electron microscope studies

7.3 RESULTS

7.3.1 Preventative activity of potassium silicate against *P. digitatum*, when applied as a postharvest treatment on lemons three hours before pathogen inoculation

Application of K_2SiO_3 at all concentrations tested resulted significantly in smaller lesion diameters (Figure 7.2). Increasing the concentration of K_2SiO_3 from 10 to 100 mg ℓ^{-1} reduced lesion diameters from 17.17 mm to 10.85 mm, but the difference was not statistically different. As the K_2SiO_3 concentration was increased from 10,000 to 100,000 mg ℓ^{-1}, the lesion diameter was significantly reduced from 23.00 mm to 7.09 mm (Figure 7.2). However, the lesion diameter that developed after treatment of the fruit with K_2SiO_3 at 1,000 was not significantly different to the treatment with 10,000 mg ℓ^{-1} (a detailed statistical analysis of the data in Figure 7.2 is provided in Appendix 7A).

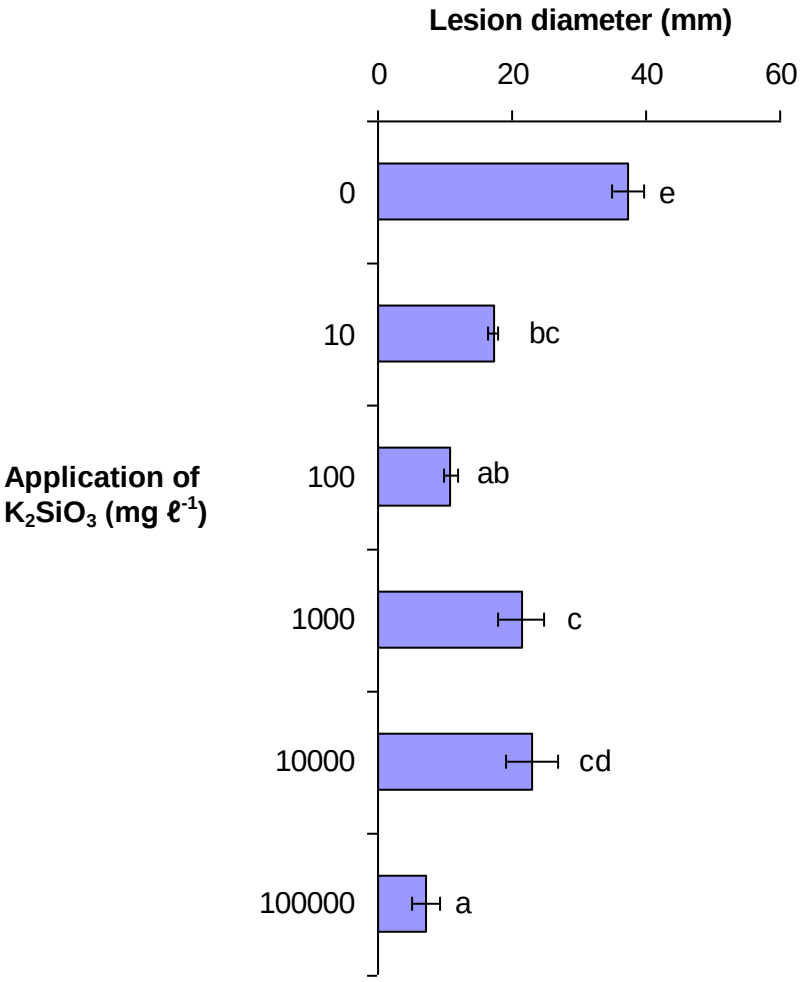

Figure 7.2 Lesion diameter as an indicator of postharvest control of *Penicillium digitatum* on lemons treated preventatively with various concentrations of potassium silicate.

7.3.2 Curative activity of potassium silicate against *P. digitatum,* applied as a postharvest treatment on lemons three hours after pathogen inoculation

When potassium silicate was applied curatively to lemons at 10 and 100,000 mg ℓ^{-1} it reduced the lesion diameter significantly. However, the higher concentration was more effective, and it caused a significantly smaller lesion diameter than the lesion diameter that resulted from the application of 10 mg ℓ^{-1} potassium silicate. Curiously, when potassium silicate was applied curatively at 100, 1000 and 10,000 mg ℓ^{-1} the control provided was not significant (a detailed statistical analysis of the data in Figure 7.3 is provided in Appendix 7B).

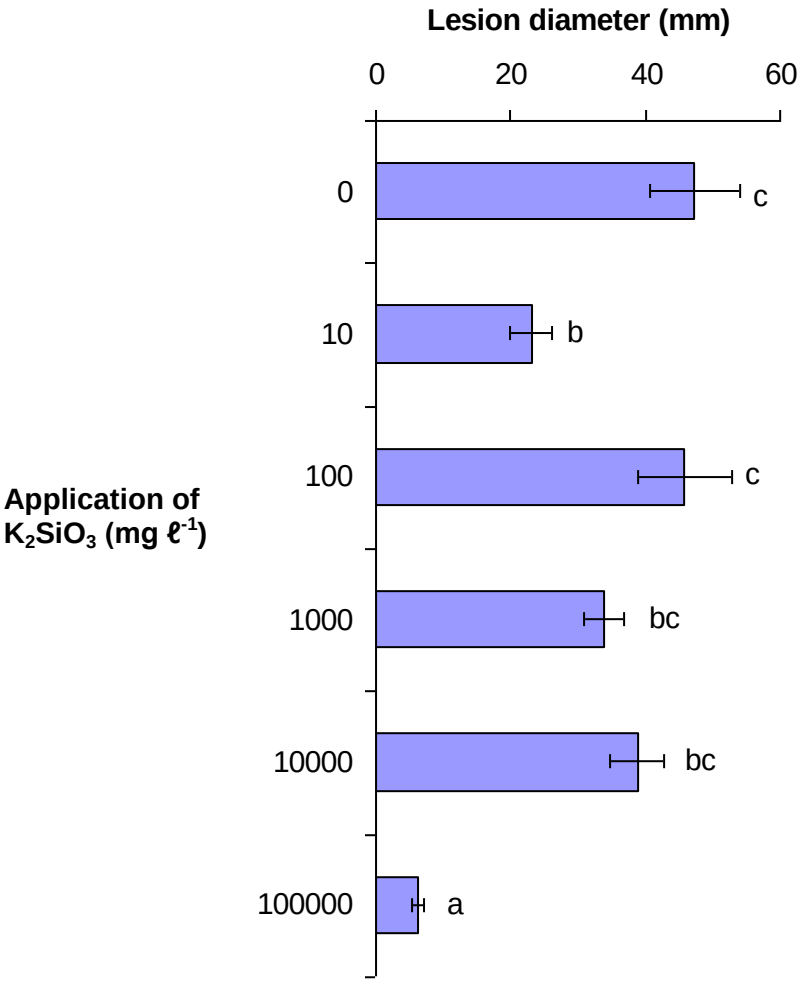

Figure 7.3 Postharvest control of *Penicillium digitatum* on lemons treated curatively with various concentrations of potassium silicate

7.3.3 Visual observation of wounds of lemons inoculated with *P. digitatum*

Lemons inoculated with *P. digitatum* showed visible green conidia of *P. digitatum* and /or softening of the fruit surface (Figure 7.4).

Figure 7.4 Wounded lemons infected with *Penicillium digitatum* (P), 10 days after inoculation.

7.3.4 Scanning electron microscope observations of wounds of lemon inoculated with *P. digitatum*

Scanning electron microscope observations showed hyphal growth (Figures 7.5A, B and C), and conidia of *P. digitatum* developing in wounds of lemon fruit causing decay (Figure 7.5D).

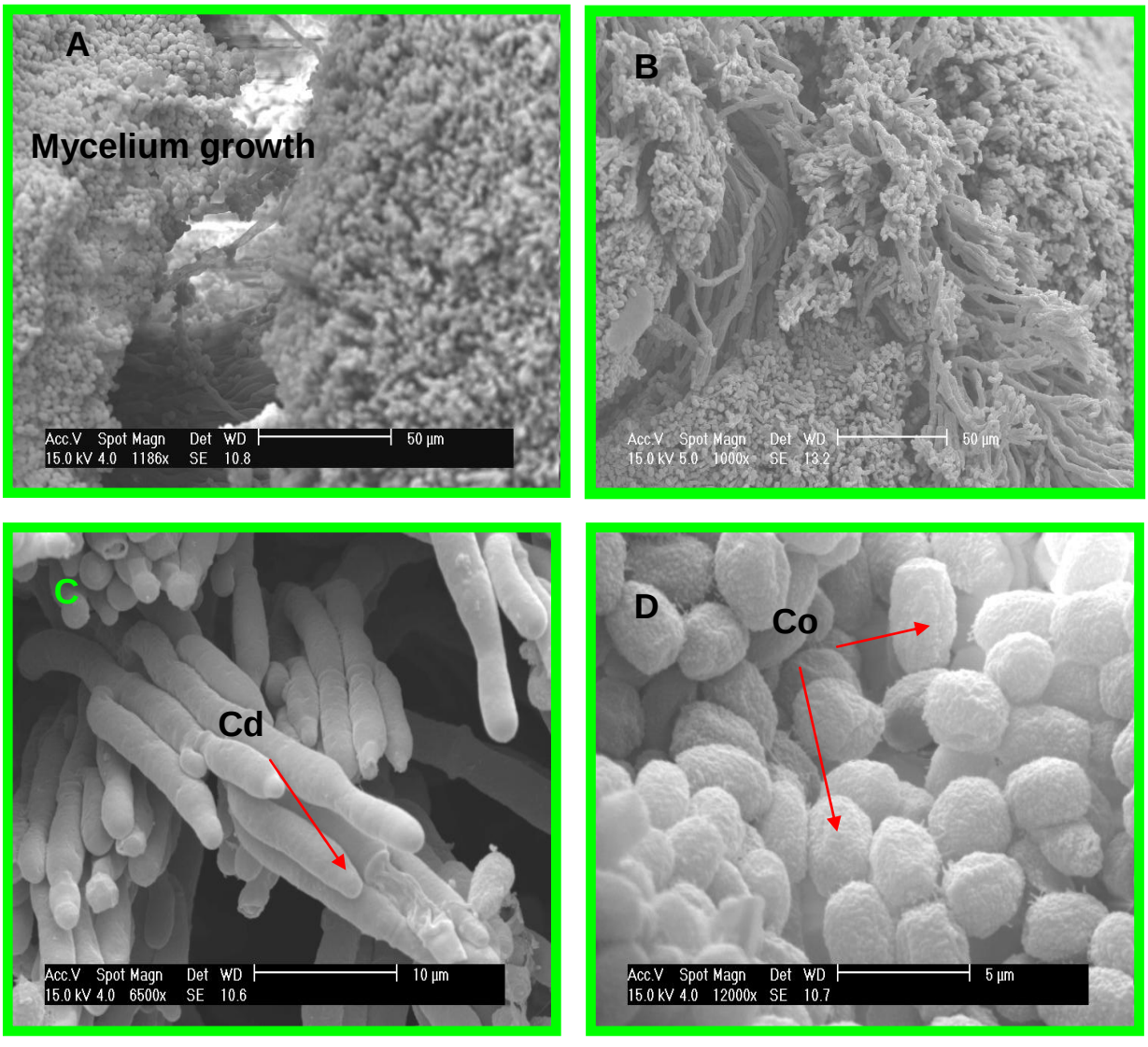

Figure 7.5 Scanning electron micrograph of untreated (control) lemons: (A and B) mycelia of *Penicillium digitatum* in the wounds of lemon; (C) mycelia and conidiophores of *P. digitatum* in wounds of lemon; (D) dense production of conidia of *P. digitatum* in lemon wounds. Cd= Conidiophores; Co= conidia.

7.3.5 Visual observation of wounds of lemons treated with potassium silicate and inoculated with *P. digitatum*

When potassium silicate applied to wounds of lemons three hours before inoculation with *P. digitatum,* little visible growth of *P. digitatum* developed, nor did softening of the wounded fruit surface occur (Figure 7.6).

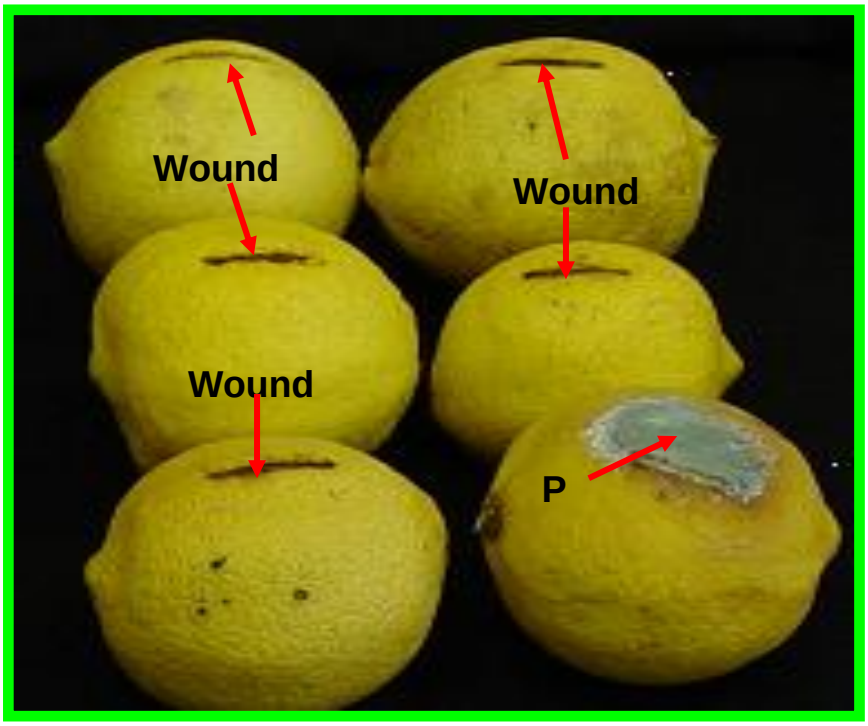

Figure 7.6 Postharvest control of *Penicillium digitatum* on lemons treated preventatively with potassium silicate at a concentration of 100,000 mg ℓ^{-1} 10 days after inoculation with the pathogen.

7.3.6 Scanning electron microscope observations of the interaction between potassium silicate and *P. digitatum* in lemon wounds

Inhibition of conidial germination of *P. digitatum* was observed as a result of the pre treatment with K_2SiO_3 at 100,000 mg ℓ^{-1} into wounds of lemons. Deposition of the K_2SiO_3 was evident in wounds of the fruit (Figures 7.7A and B). Complete inhibition of conidial germination of *P. digitatum* was observed (Figures 7.7A and B) in 19 cases out of 25 observations (Table 7.1). In 6 cases out of the 25 observations there was restricted germination of conidia (Figures 7.7C and D) (Table 7.1). This contrasted markedly with rapid germination of conidia and subsequent vigorous growth by the fungus on the control wounds (Figure 7.5). The most important outcome was that no mycelial growth was observed on any wounded lemons treated with K_2SiO_3 solutions and then inoculated with *P. digitatum* (Figure 7.7 and Table 7.1), compared to the dense mycelium that developed on the control wounds (Figure 7.5 and Table 7.1).

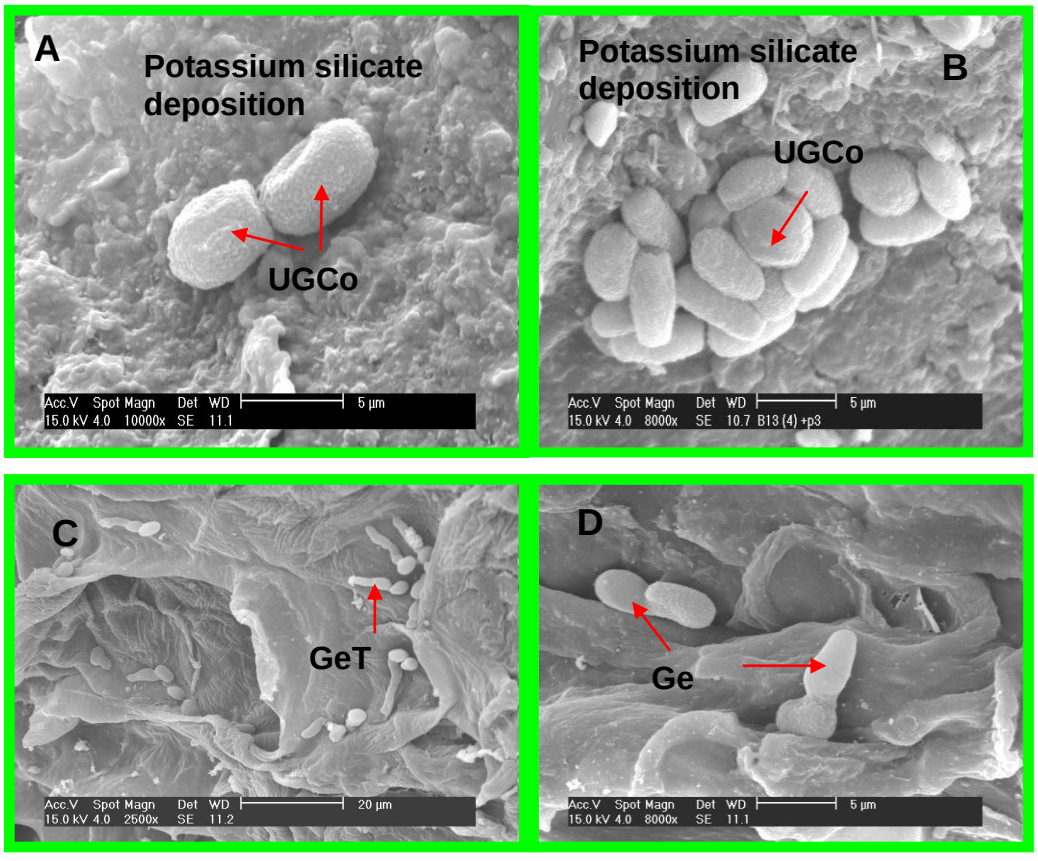

Figure 7.7 Scanning electron micrograph of the interactions of potassium silicate and *Penicillium digitatum* in wounds of lemons. In (A) and (B) inhibition of germination of conidia of *P. digitatum* (conidia on top of K_2SiO_3 depositions in fruit wounds); In (C) and (D): partial germination of conidia of *P. digitatum* or germ tube forming in the fruit mesocarp.

Co= Conidium, GCo= Germinating Conidium, UGCo= Ungerminated Conidium, GeT= Germ Tube.

Table 7.1 Number of germinated, inhibited and cessation of germination of *P. digitatum* conidia observed using scanning electron microscope observations of the interaction between potassium silicate and *P. digitatum* and *P. digitatum* alone in lemon wounds

Treatment	Germinated conidia (mycelia, hyphae)	Germination inhibited (germ tube formation)	Germination stopped (remain as conidia)
P. digitatum	25 b	0 a	0 a
P. digitatum + K_2SiO_3	0 a	6 b	19 b
P Value	<0.001	<0.001	<0.001
LSD(0.05)	0.46	1.4	2.3
CV %	2.500974	32.2749	28.00

7.4 DISCUSSION

Potassium silicate was effective in preventing or suppressing *P. digitatum* infection of lemon fruit (Figures, 7.2, 7.3, 7.6, 7.7 and Table 7.1). Lesion diameters of *Penicillium digitatum* on fruit treated with K_2SiO_3 were significantly smaller than the control when wounded lemons were treated three hours before inoculation. Similar results were reported with *A. alternata*, *Fusarium* spp., and *T. roseum* on Hami melons by Bi *et al*. (2006) and with *P. expansum* and *M. fructicola* on sweet cherry fruit by Qin and Tian (2005). This suppression could have been due to induction of phenolic compounds produced within the three hour period of time before inoculation of *P. digitatum*. This suggestion is supported by work of Bi *et al*. (2006) on melons and by Qin and Tian (2005) on sweet cherry fruit. The efficacy was influenced by the applied concentration of K_2SiO_3 in which application of the highest concentration (100,000 mg ℓ^{-1}) resulted in the smallest lesion development (7.09 mm), although it was not significantly different than the lesion diameter of 10.85 mm provided when K_2SiO_3 was applied at 100 mg ℓ^{-1} (Figure 7.2). This finding is in agreement with the observations of Menzies *et al*. (1991b), who found that powdery mildew caused by *Sphaerotheca fuliginea* (Schltdl.) Pollacci on cucumber plants was reduced significantly with an increasing concentration of K_2SiO_3 in nutrient solutions.

Effects of K_2SiO_3 applied to wounds of lemon preventatively at 100,000 mg ℓ^{-1} on *P. digitatum* control were observed with ESEM (Figure 7.1). Observations showed complete inhibition of conidial germination in the presence of K_2SiO_3 deposits (Figure 7.7A and B) and this was also supported by observation of 19 out of 25 cases that were viewed, which showed complete inhibition of conidial germination of *P. digitatum* (Table 7.1). This inhibition could be due to a direct K_2SiO_3 effect on conidia and subsequently affect conidial germination as a result of fungicidal effect as reported by Bekker *et al*. (2006) on several phytopathogenic fungi from avocado. These authors showed that soluble potassium silicate suppressed fungal growth effectively *in vitro* (plate test) of several phytopathogenic fungi from avocado and the effect was largely a fungicidal effect.

Germination of conidia on the albedo of fruit (Figures 7.7C and D) was severely restricted, compared with the water-treated control, where normal conidial germination, germ tube growth, hyphal growth and sporulation were obvious (Figure 7.5). This could have been due to fungistatic properties of K_2SiO_3 in the fruit mesocarp where K_2SiO_3 deposition was not evident (Figures 7.7C and D). Conidial germination was inhibited even when K_2SiO_3 deposits were not in direct contact with the *P. digitatum* conidia (Figures 7.7A and B), compared to obvious deposition of K_2SiO_3, where germination of conidia was inhibited completely. Similar results were found by Qin and Tian (2005) with potassium silicate applications where conidial germination or restricted conidial germination or complete inhibition of mycelial growth of *P. expansum* and *M. fructicola* was found in wounds of sweet cherry fruit.

The reduction in lesion diameter was concentration dependent, where the best prevention was achieved at the highest concentration of K_2SiO_3. This could have been related to the fungistatic properties of K_2SiO_3, which inhibited conidial germination or delayed germination, as seen in the ESEM study. Results supporting this were reported by Bowen *et al*. (1992), who found that sodium silicate at lower concentrations mildly promoted conidial germination and germ tube development of *Uncinula necator* (Schwein.) Burrill. The same author noted that it is not unusual for a chemical to enhance fungal growth at lower concentrations and yet to be fungistatic at higher concentrations. It should be realized that potassium silicate solutions are highly alkaline (pH of 9-12) and

it is therefore possible that their fungistatic activity was due to strong alkalinity. Tissue browning of lemon wounds was observed after K_2SiO_3 treatment and could possibly be in line to the alkaline condition created by K_2SiO_3 around the site of treatment.

The mechanisms involved in the inhibition or restriction of *P. digitatum* conidial germination on lemon wounds as the result of potassium silicate treatment could be due to accumulation of K_2SiO_3 on wounded fruit forming a physical barrier (Heath and Stumpf, 1986; Carver *et al*., 1987; Datnoff *et al*., 1997) as shown in Figures 7.7A and B or its ability to induce defence responses (Cherif *et al*. 1992; Epstein, 1999) on the lemons or of its fungistatic properties (Bekker *et al*., 2006), or a combination of all three modes of action. It was shown that K_2SiO_3 applications can prevent *P. digitatum* infection in the postharvest situation. Its ability to suppress pathogen infection of wounded lemons was confirmed by ESEM studies. This potential of K_2SiO_3 as a postharvest treatment of fruit could be extended to the treatment of other fruit for the control of other postharvest fungal diseases.

7.5 CONCLUSION

This study has demonstrated the potential of K_2SiO_3 for controlling *P. digitatum* infection of lemons when applied preventatively or curatively as a postharvest treatment. ESEM studies confirmed that germination and development of conidia of *P. digitatum* were inhibited by K_2SiO_3 treatment on lemons.

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CHAPTER 8

INTEGRATED CONTROL OF *PENICILLIUM DIGITATUM* USING A HOT WATER DIP, POTASSIUM SILICATE AND YEAST ISOLATE B13

ABSTRACT

The use of a hot water dip, potassium silicate (K_2SiO_3) and yeast Isolate B13 (*Candida fermentati* (Saito) Bai.) treatments, alone or in combination, were investigated for their capacity to reduce the development of *Penicillium digitatum* (Pers. Fr.) Sacc. On Valencia oranges during postharvest storage *In vivo* studies indicated that a hot-water dip at 50-56°C for 45-180 seconds, reduced disease development in inoculated wounds to less than 20% compared with control fruit treated with tap water (60%), without causing any rind injuries. Yeast Isolate B13 was found to be compatible with commercial waxes and its growth was not affected by K_2SiO_3 postharvest applications. A stand-alone treatment or combinations of a hot-water dip and K_2SiO_3, combined with yeast Isolate B13, applied under commercial packhouse conditions have shown potential to reduce postharvest losses to *P. digitatum*. In trials carried out at two commercial packhouses, control of the pathogen by hot-water, K_2SiO_3 and the yeast Isolate B13 were superior or equivalent to that achieved with imazalil.

8.1 INTRODUCTION

The valuable effect of pre-storage hot water immersion treatments in preventing development of postharvest diseases has been applied to various fruit and vegetables (Hara *et al.,* 1996; Lurie, 1998; Schirra *et al*., 2000; Fallik, 2004). Hot water treatments are relatively easy to use, require short treatment periods, and can make use of reliable monitoring of fruit and water temperatures (Couey, 1989; Lurie, 1998). According to Tsang *et al*. (1995), hot water units are easily assembled, simple to operate, and affordable.

Postharvest hot water treatments have been investigated for the control of postharvest infection of citrus fruit and recommendations are provided by numerous authors (Barkai-Golan and Phillips, 1991; Lurie, 1999; Porat *et al.,* 2000; Palou *et al*., 2001; Smilanick *et al*., 2003). For example, immersion of

citrus fruit in 50-53°C water for 2-3 minutes controls *Penicillium* and/or *Alternaria* spp., which causes infection in oranges, lemons, and grapefruit (Couey, 1989; Schirra *et al*., 1997*;* Nafussi *et al.,* 2001). Palou *et al*. (2001) showed that hot water controlled blue mould at 50-55°C when applied for 150 seconds. However, physiological responses of different citrus fruit to heat treatments may differ with growing location and season (Hara *et al*., 1996; Schirra *et al.*, 1997).

Considerable laboratory success has been reported with antagonistic microorganisms for the control of postharvest diseases, and much information is now available regarding postharvest biocontrol antagonists (Wilson *et al*., 1996), including our recent research with yeast Isolate B13 (*Candida fermentati* (Saito) Bai.) for the control of *Penicillium digitatum* (Pers: Fr. Sacc.), presented in Chapter Three. Antagonistic yeasts isolated from fruit surfaces have shown activity against a number of postharvest pathogens on a variety of fruit (Wilson and Chalutz, 1989; MacLaughlin *et al.*, 1990; Roberts, 1990; Janisiewicz, 1994; Chad-Goyal and Spotts, 1997; El-Ghaouth *et al*., 1998). Presently, the yeast *Candida oleophila* Montrocher is available commercially as Aspire®. Acceptance of antagonistic biocontrol agents as an alternative to synthetic fungicides will depend on their commercial results (El-Ghaouth *et al*., 2000).

The objectives of this study were:
Firstly to conduct an unreplicated, observational trial (Rayner, 1967) over a wide range of temperature x exposure period combinations, in order to identify a narrower range of temperature x exposure period combinations that would control *Penicillium digitatum* without causing damage to rind quality of Valencia oranges.

Secondly, to conducted a replicated, clinical trial in this narrower range of temperatures x exposure period combinations in order to find the best combination of temperature x exposure period that would control *P. digitatum* infection on Valencia oranges without causing rind damage. These tests have not been dealt in great detail by other researchers (see Table 1.3) and to the best of our knowledge no research on this field has been conducted in South Africa. Research has shown that physiological responses of citrus to heat

treatments vary with citrus variety, cultivar, growing location, and even the season (Hara *et al*., 1996; Schirra *et al.*, 1997).

Thirdly, the study aimed to investigate the effects of hot water, the yeast Isolate B13 and potassium silicate treatments, alone or in combination, in trials conducted at three commercial packhouses for the control of *P. digitatum* of Valencia oranges. Semi-commercial trials are important because commercialization of antagonistic biocontrol agents always depend on their performance under commercial conditions (El-Ghaouth *et al*., 2000). Acceptance of novel treatments by farmers usually depends on positive large scale experimental results. These experiments have not been conducted by other researchers. In particular, the application of potassium silicate as a postharvest treatment on citrus is new approach. Similarly, the combination of hot water, potassium silicate and a yeast biocontrol agent has not been tested previously.

8.2 MATERIALS AND METHODS

8.2.1 Fruit

Mature, fully coloured Valencia oranges from Caterall's farm in Richmond (29.53 S 30.17 E), KwaZulu-Natal, South Africa, were harvested from the field and were used in this experiment before any other postharvest treatments were applied. The fruit was stored for three days at 9±1°C and 90% relative humidity (RH) before use.

8.2.2 Observational Trial 1: hot water treatments to determine a safe temperature x exposure time for Valencia oranges

Tap water was heated to test temperatures in a 100 ℓ water tank invented as novel hot water bath with an electronic control unit to maintain water at a constant temperature for the duration of experiment. Temperature controlled for hot water bath using a full proportional integral derivative (PID) temperature controller. The PID controller used has an accuracy of less than 0.2% of the displayed temperature value, plus one digit, with a sampling time of 250mS. The components of the hot water bath include: a temperature controller with full PID functions, with solid state relay (SSR) output to control the heating element, a pressure switch, a circulating pump, the heating element (immersion type)

indicating lamps, fuses, a main switch and a contactor. The water bath was insulated, and water was pumped around the bath at 30 L s^{-1} to keep an even distribution of water temperature. The unit was built in-house by the Department of Plant Pathology, University of KwaZulu-Natal, Pietermaritzburg, South Africa. Valencia oranges were placed in stainless steel wire baskets to immerse them in the water tank.

In the first experiment, the tested temperatures were 20 (control), 50, 52, 54, 56, 58, 60, 62, 64, 66, 68, 70 and 80°C (±0.1°C). For each temperature, the Valencia oranges were exposed for a period of 20, 30, 45, 60, 75, 90, 105, 120 and 180 seconds. Each treatment was applied to three oranges as a single replicate (Rayner, 1967). The treated fruit were then air dried, placed in open carton board boxes and stored at 24±1°C. After seven days of storage, the fruit were classified into one of two categories: (1) no visible rind damage, which were marked as “NO”; (2) presence of slight or moderate rind blemishes or severe rind injury, marked as “YES”.

8.2.3 Observational Trial 2: hot water treatments followed by cold water dip for the control of rind injury of Valencia oranges

In the second experiment, a similar experimental set up was followed as for the first experiment, as described in Section 8.2.2. Again an unreplicated, observational trial was conducted (Rayner, 1967). The only difference was that, following the hot water treatment, the Valencia oranges were immediately immersed for 1 minute into tap water cooled to 1°C in a hydro-cooling tank. The experiment was conducted to determine whether the cold water treatment could stop the heat energy damaging the fruit rind. Each treatment was applied to three oranges as a single replicate.

8.2.4 Replicated Trial 1: efficacy of hot water treatments for the control of *P. digitatum* infection of Valencia oranges

Valencia oranges were harvested and stored as described in Section 8.2.1. These oranges were wounded 5 times with a sterile dissecting needle (2 mm deep × 2 mm in diameter) at two sites around the stem ends. The wounded fruit were dipped for 1 minute into a *P. digitatum* conidial suspension of 1×10^4 conidia $m\ell^{-1}$. The inoculated Valencia oranges were air dried on a laboratory bench at 24±1°C. After 24 hours, the fruit were immersed in hot

water for the test temperatures and time periods of 50-70^{0}C x 20-180 seconds, as described in Section 8.2.2. Fruit that had been dipped in tap water (20°C) or were not treated at all served as controls. Each treatment was applied to 10 oranges, independently, as ten replicates. Treated fruit was air dried, placed in open carton board boxes and kept at 24±1°C. After two weeks of storage the percentage incidence of *P. digitatum* infection on the fruit was determined. In some treatments, some fruit had to be discarded because of fruit fly infestations.

8.2.5 *In vitro* compatibility of yeast Isolate B13 with commercial wax

A loop full of yeast Isolate B13 grown on a nutrient agar plate for three days was added into 10 mℓ of a commercial wax (Polygreen®). Sterile distilled water was used in place of the wax for the control. After ten minutes, a 0.1 mℓ of suspension was plated in duplicate onto potato dextrose agar (PDA) amended with Rose Bengal. Plates were incubated at 25°C for three days and the growth of the yeast isolate was observed.

8.2.6 *In vitro* compatibility of the yeast Isolate B13 with potassium silicate

A loop full of the yeast Isolate B13, taken from a discrete colony grown on a nutrient agar plate for three days, was added into 10 mℓ of K_2SiO_3 diluted with sterile distilled water to concentrations of 10, 100, 1,000, 10,000 and 100,000 mg ℓ^{-1}. Sterile distilled water was used for the control (without K_2SiO_3). Ten minutes later 0.1 mℓ of suspension was plated onto nutrient agar plates in duplicate. The plates were incubated at 25°C for three days and growth of the yeast Isolate B13 with and without K_2SiO_3 was observed.

8.2.7 Preliminary investigation of the ability of yeast Isolate B13 to control *P. digitatum*, *Phytophthora* rot and sour rot of Valencia oranges at the Katopé packhouse

At the Katopé packhouse (28.53 S 30.17 E), Richmond, KwaZulu-Natal, Valencia oranges were submerged in a 24,000 ℓ capacity water tank containing sodium hypochlorite at pH 7.2. Fruit were then lifted out of the solution and moved onto a conveyer belt for washing. The fruit then passed over foam-rubber rollers for drying and then onto a moving-belt sorting table, where sub-standards fruit were removed.

Twenty-five Valencia oranges with four replicates were used for each treatment. Treatments included three concentrations of the antagonistic yeast Isolate B13 at 1×10^5, 1×10^6 and 1×10^7 cells $m\ell^{-1}$. Oranges were treated by dipping them into a tank for 1 minute. There were two controls: an untreated control, i.e., with no yeast Isolate B13 and no fungicide; and a fungicide control, with imazalil 1-[2-(2,4-dichlorophenyl)-2-(2-propenyloxy) ethyl]-1H-imidazole at 500 mg ℓ^{-1} (670 g of the chemical in 1000 ℓ of water). Oranges were treated with the fungicide by dipping them for one minute into a 200 ℓ capacity tank heated to 37°C. After treatment all fruit were passed through a high-velocity air drier operating at 32°C, waxed, and dried again by passing them through a high-velocity air drier. Rollers were washed extensively between applications of different treatments.

Treated Valencia oranges were packed in plastic mesh bags and placed in a completely randomized design (CRD) on shelves at 7±1°C for two months at 90% RH. After this, they were placed on a bench in a CRD at 24±1°C for one month to simulate shelf life. The percentage of diseased fruit with visible conidia of *P. digitatum* or any other diseases were recorded for each replicate.

8.2.8 Assessment of a hot water dip, yeast Isolate B13 and potassium silicate treatments, applied alone or in combination, for the control of *P. digitatum* of Valencia oranges at the Katopé packhouse

The same experimental set up was used as described in Section 8.2.7. The differences were that hot water and/or K_2SiO_3 were added as treatments in this test, with or without the yeast Isolate B13. The trial treatments are summarized in Table 8.1. The yeast Isolate B13 was used at a concentration of 1×10^6 cells $m\ell^{-1}$. Fruit was stored at 9±1°C for one month, followed by one month at 24±1°C before evaluating incidence of diseases.

In earlier experiments, a hot water dip at 56°C for 60–180 seconds had provided good control of *P. digitatum* infection of Valencia oranges (Figure 8.2) and did not cause any damage to the fruit used. For this reason, a hot water treatment with temperature of 56°C x one minute period was chosen. Valencia oranges were therefore treated at 56°C x one minute, as described in Section 8.2.2. Other Valencia oranges were treated by dipping them into 1,000 mg ℓ^{-1} of K_2SiO_3 for one minute in a separate tank. The yeast Isolate B13

(1×10^6 cells mℓ$^{-1}$) and K_2SiO_3 (1,000 mg ℓ$^{-1}$) were combined in the same container for a combination treatment, where Valencia oranges were dipped for one minute. Valencia oranges used for the combination of all three treatments were first dipped for one minute in hot water at 56^0C, followed by dipping in a combination of the yeast Isolate B13 at 1×10^6 cells mℓ$^{-1}$ and K_2SiO_3 at 1,000 mg ℓ$^{-1}$. The normal packhouse practices, storage and assessment of the treatments were followed as described in Section 8.2.6.

Table 8.1 Treatments for screening of yeast Isolate B13, hot water, and potassium silicate, alone or in combination, for control of *Penicillium digitatum* on Valencia oranges at Katopé Packhouse

Treatment	Hot water (56°C)	K_2SiO_3	B13	Imazalil
1 (control)	No	No	No	No
2	No	No	No	Yes
3	Yes	No	No	No
4	No	Yes	No	No
5	No	No	Yes	No
6	Yes	Yes	No	No
7	Yes	No	Yes	No
8	No	Yes	Yes	No
9	Yes	Yes	Yes	No

8.2.9 Assessment of a hot water dip, yeast Isolate B13 and potassium silicate treatments, alone or in combination, for the control of *P. digitatum* of Valencia oranges at Gateway Packhouse

A similar experimental set up as described in Section 8.2.8 was followed at Gateway Packhouse, Thornville Junction, Richmond, South Africa (28.53 S 30.17 E). The trial treatments were the same, as summarized in Table 8.1. The only differences were that the water tank capacity was 30,000 ℓ, with imazalil added into it at 500 mg ℓ$^{-1}$ (670 g of the chemical in 1000 ℓ of water). A high-pressure overhead washer was in the processing line, and a high-velocity air drier operating at 32°C was not present. Similar procedures were followed for all the treatment concentrations, procedures, period of storage, and

assessment of *P. digitatum* incidence on Valencia oranges, as detailed in Section 8.2.8.

8.2.10 Assessment of a hot water dip, yeast Isolate B13 and potassium silicate treatments, alone or in combination, for the control of *P. digitatum* of Valencia oranges at Maywood Packhouse

A similar experimental set up as described in Section 8.2.8 was followed for the Maywood Packhouse, Richmond, South Africa (28.53 S 30.17 E). The trial treatments were also the same, as summarized in Table 8.1. Experimental differences included that the water holding capacity of the tank was only 10,000 ℓ, with no imazalil added into it. Valencia oranges were treated with imazalil in a separate tank at the recommended rate (670 g of the chemical in 1000 ℓ of water). Similar procedures were followed for all the treatment concentrations, procedures, period of storage, and assessment of the incidence of *P. digitatum* infection on Valencia oranges, as detailed in Section 8.2.8.

8.2.11 Statistical analysis

Data for the hot water and temperature interactions experiment for the control of *P. digitatum* was analyzed using SAS Logit Linear Regression Model procedure (SAS, 1987), with P at the 5% level (Hosmer, 1989).

Data for the commercial trials were subjected to an analysis of variance (ANOVA) using Genstat® Executable Release 9.1 Statistical Analysis Software (Anonymous, 2006). To determine differences between treatments, Fisher's Least Significant Difference Test was used ($P<0.05$).

8.3 RESULTS

8.3.1 Observational Trial 1: effects of hot water dip treatments at different temperatures and exposure periods on the quality of Valencia orange rind

No rind damage was observed on Valencia oranges when treated at temperatures of 58°C and less (regardless of exposure times). Similarly, no damage was noted at 60°C except at 180 seconds (Table 8.2) and at 62°C with an exposure time of 45 seconds or less (Table 8.2). There was no damage observed on the controls at 20°C (Table 8.2). However, Valencia oranges dipped at 62°C for a period of 180 seconds, and at 80°C for a period of 20

seconds, showed slight to moderate rind blemishes (Figures 8.1 A, B) respectively. The primary objective of this trial was to find a narrower range of temperature x time combinations that did not cause physiological damage to the fruit. The best temperature range without damage was 50-58°C at all exposure times (Table 8.2).

Table 8.2 Effect of hot water dip treatments at different temperatures and exposure periods, on the quality of rind of Valencia oranges stored at 24±1°C for 2 weeks.

Exposure	**Exposure period (seconds)**								
Temperature (°C)	**20**	**30**	**45**	**60**	**75**	**90**	**105**	**120**	**180**
20	-	-	-	No	-	-	-	-	-
50	No	No	No	No	No	No	No	No	No
52	No	No	No	No	No	No	No	No	No
54	No	No	No	No	No	No	No	No	No
56	No	No	No	No	No	No	No	No	No
58	No	No	No	No	No	No	No	No	No
60	No	No	No	No	No	No	No	No	Yes
62	No	No	No	Yes	Yes	Yes	Yes	Yes	Yes
64	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
66	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
68	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
70	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
80	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes

No= No damage
Yes= Presence of damage

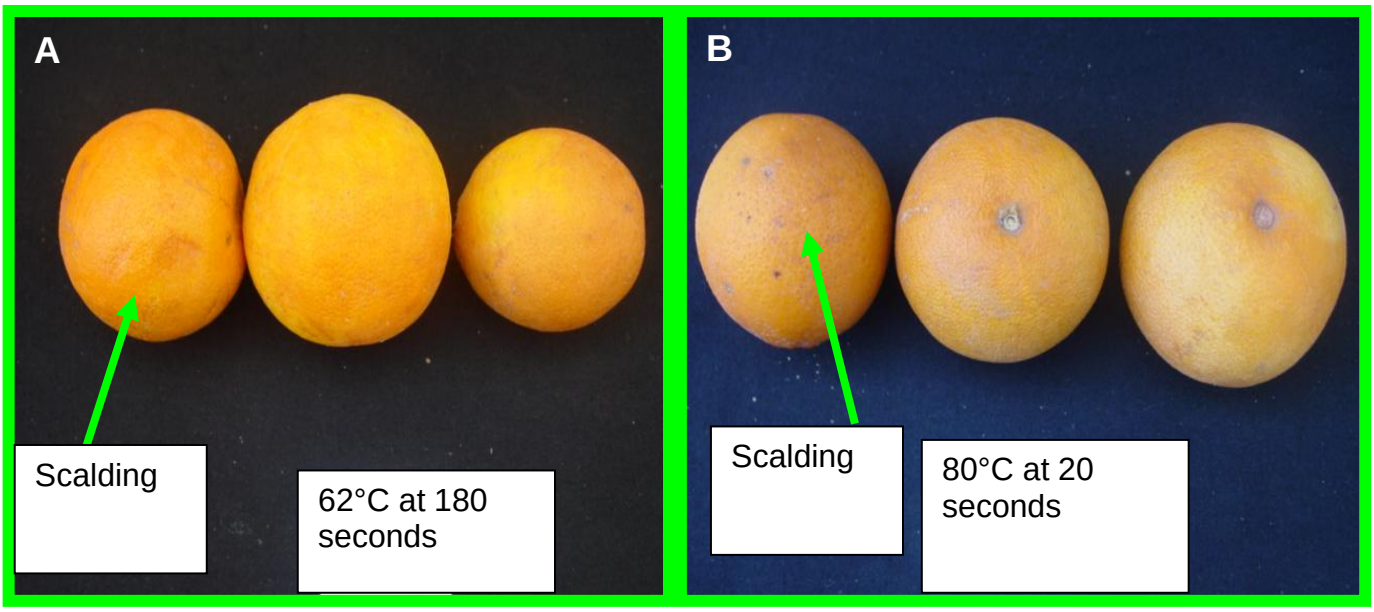

Figure 8.1 Peel scalding of Valencia oranges treated only with hot water, after two weeks of storage. Fruit were dipped into water at 62°C for 180 seconds (A), and into water at 80°C for 20 seconds (B).

8.3.2 Observational Trial 2: effects of hot water dip treatments at different temperatures and exposure periods followed by a cold water dip for one minute, on rind quality of Valencia oranges

The cold water treatment did not reduce physiological damage caused by the hot water treatment on Valencia oranges. Rind damage observed on the fruit was similar to those observed earlier with the hot water treatment at temperatures of 60°C at 180 seconds and temperatures of above 62°C and exposure period of 45 seconds, as presented in Table 8.2.

8.3.3 Replicated Trial 1: effectiveness of hot water dip treatments of Valencia oranges in the control of artificially inoculated *P. digitatum*

Temperatures from 50-60°C, for all immersion periods, consistently reduced the incidence of *P. digitatum* infection to 0-30% (Appendix 8A), compared to the treatment at 20°C that developed an incidence of 60% after two weeks (Table 8.3 and Figure 8.3A). Observations from this trial also provided further support for the earlier findings (Table 8.2) regarding the damage caused to fruit rind at specific temperature × time combinations.

Hot water immersion treatment at 66-70°C resulted in differing responses in terms of *P. digitatum* control depending on the immersion period (Table 8.3 and Figures 8.2 C and D). When fruit were exposed to higher temperatures (e.g., 70°C) for 90–180 seconds, incidence of *P. digitatum* increased to 100% (Table 8.3 and Figure 8.3D) and the rind of the Valencia oranges was severely injured. However, oranges treated at 66-70°C for shorter periods developed lower infection levels (Figure 8.3) but still showed rind damage.

Logit regression model analysis showed that significant levels of control of *P. digitatum* resulted from treatments with hot water, with the main effects of temperature and time, and their interaction effect, temperature x time, being significant at (P=0.05) =<0.0001 (a detailed statistical analysis of the data in Figure 8.2 is provided in Appendix 8A).
Interpretation of the trial is dominated by the interaction effect of temperature × time. From the Logit Regression Model equation below, the graph in Figure 8.3 was generated.

Logit (not diseased fruit) = -6.7355 + 0.1541 × (temperature, °C) +
0.1476 × exposure period, seconds) –
0.00254 × (temperature × exposure period)

The graph in Figure 8.2 showed that control of *P. digitatum* on Valencia oranges as a result of treatment with a hot water dip at temperatures of 50, 56, 60 and 70°C for 20 seconds was positive. However, at a temperature of 70°C, a dip in water for 20 seconds caused rind injury (Table 8.2). When the exposure period was increased to 45 seconds, all water temperatures of 50, 56, 60 and 70°C provided the same level of control. As the exposure time was increased to 60 seconds, water temperatures of 50, 56, and 60°C showed increased positive control, while the water temperature of 70°C at 60 seconds showed negative control. Similarly, as the exposure period increased for the water temperatures of 50 and 56°C, the rate of *P. digitatum* control was increased. At a temperature of 60°C the exposure time had little effect on control of the pathogen. However, at a water temperature of 70°C increased exposure periods resulted in increasingly poor control of the pathogen (Figure 8.2).

The optimum range of temperature x exposure period for the control of *P. di*gitatum was 50-56°C × 60-180 seconds (Figure 8.2). No rind injuries were caused at this range of temperature x exposure period (Table 8.2).

Logit (not diseased fruit) = -6.7355 + 0.1541 × (temperature, °C) +
0.1476 × exposure period, seconds) –
0.00254 × (temperature × exposure period)

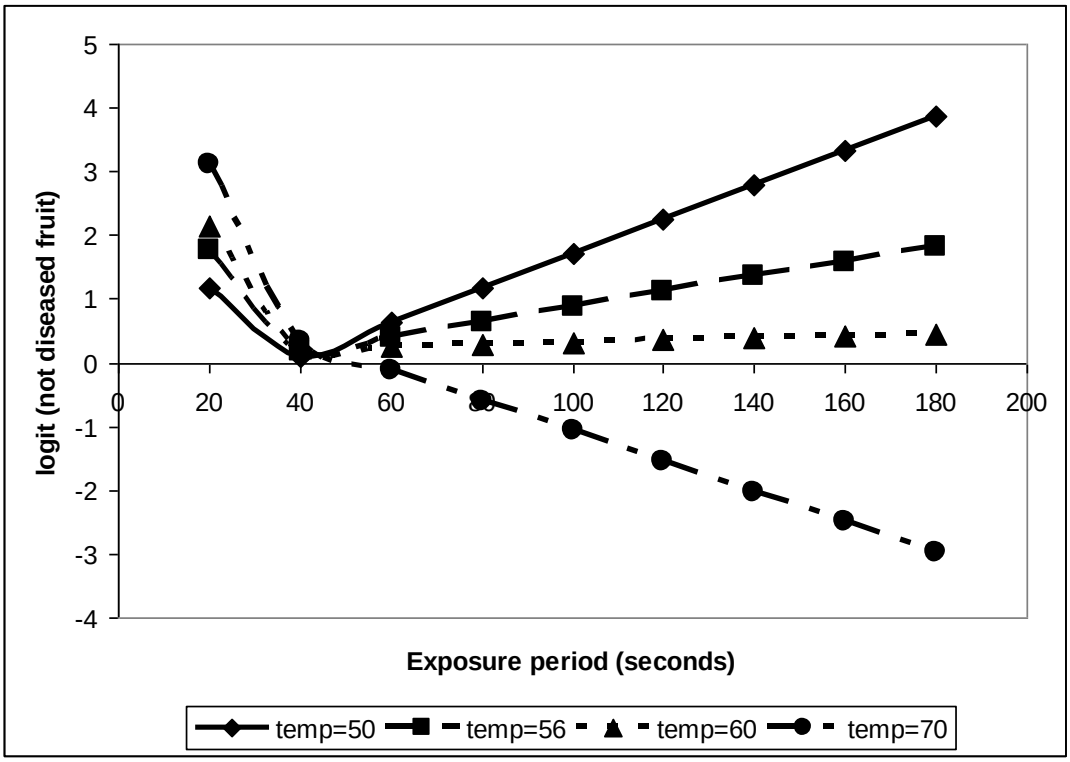

Figure 8.2 Efficacy of hot water dip treatments for the control of artificially inoculated *Penicillium digitatum* on Valencia oranges stored at 24°C for two weeks in a replicated trial.

Table 8.3 Efficacy of hot water dip treatments for the control of artificially inoculated *Penicillium digitatum* on Valencia oranges stored at 24°C for two weeks.

Exposure Temperature (°C)	**Disease Incidence (%) Exposure Period (seconds)**								
	20	**30**	**45**	**60**	**75**	**90**	**105**	**120**	**180**
20 (not dipped)				20					
20 (dipped)				60					
50	10	20	10	10	10	0	0	0	0
52	20	30	0	0	0	10	0	0	10
54	10	0	0	0	0	0	10	20	0
56	10	0	0	0	0	0	10	10	10
58	10	0	20	10	10	0	0	0	0
60	10	10	0	0	10	30	10	10	0
62	0	20	10	20	0	0	0	10	100
64	0	0	20	0	10	0	0	10	10
66	10	0	0	30	0	30	50	20	40
68	0	0	25	12.5	38	0	12.5	0	50
70	12.5	12.5	12.5	12.5	37.5	77	75	50	100

The purple block shows a zone of the temperature × exposure period combinations (52-56^{0}C × 45-75 seconds) in which disease control is 100% and rind damage is zero. It also fits into the existing exposure periods of fruit in hot water baths in packhouses in KwaZulu-Natal.

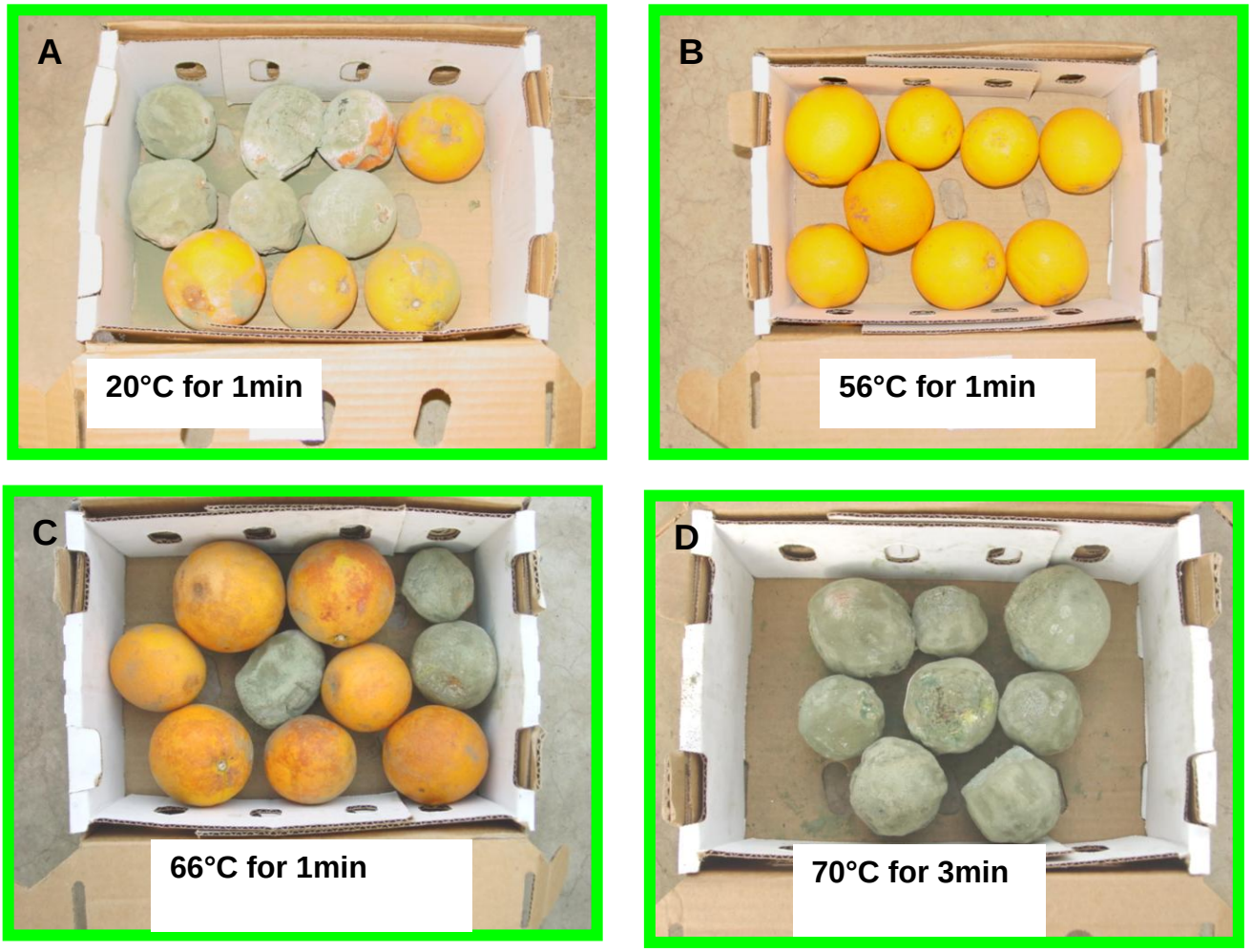

Figure 8.3 Effects of hot water dip treatments using various temperature x exposure periods for the control of *Penicillium digitatum* on Valencia oranges

8.3.4 *In vitro* compatibility of the yeast Isolate B13 with commercial wax

The three commercial waxes tested, Polygreen® (Figure 8.4 A) or Gateway wax (Quick dry poly) or Avoshine®, did not inhibit the growth of the formulated yeast Isolate B13, compared with yeast Isolate B13 without wax (Figure 8.4 B).

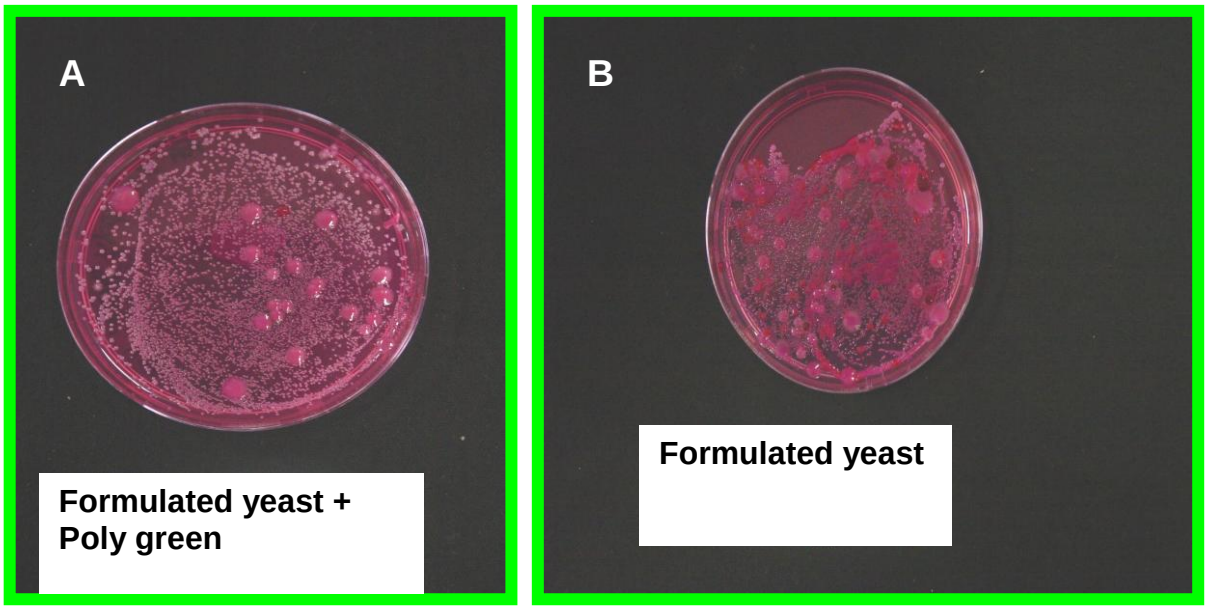

Figure 8.4 Growth of yeast Isolate B13 with (A) /without (B) commercial wax (poly green) on PDA media amended with Rose Bengal after three days of incubation at 25°C.

8.3.5 *In vitro* compatibility of the yeast Isolate B13 with potassium silicate

The yeast Isolate B13 was compatible with potassium silicate (K_2SiO_3) at concentration of 10000 mg ℓ^{-1}, when grown on agar plates with or without potassium silicate, as shown in Figure 8.5 A and 8.5 B, respectively.

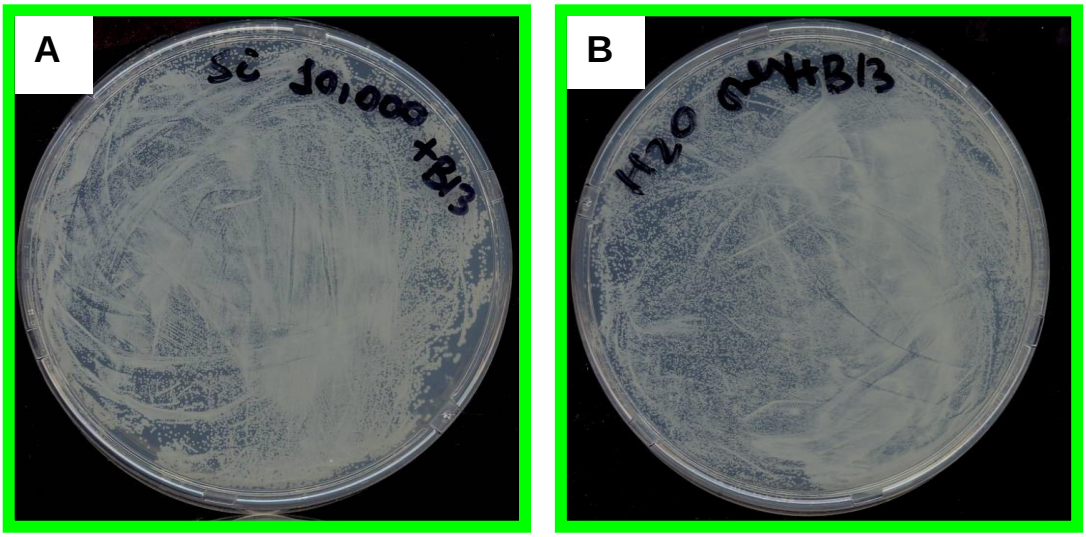

Figure 8.5 Growth yeast Isolate B13 mixed with potassium silicate (Si) at 10,000 mg ℓ^{-1} grown on agar plates (A) and growth of yeast Isolate B13, without potassium silicate on nutrient agar plates (A).

8.3.6 Preliminary evaluation of the yeast Isolate B13 for the control of natural infections of green mould decay, brown rot and sour rot of Valencia oranges at Katopé Packhouse

In the preliminary packhouse trials, commercially harvested Valencia oranges were kept for two months at 7±1°C. The fruit were not artificially inoculated with *P. digitatum,* rather depended on natural infections by the pathogen. The untreated control (no yeast and no fungicide) developed 6% *P. digitatum.* Imazalil treated fruit developed 12% *P. digitatum* (Table 8.4). The yeast Isolate B13 significantly reduced *P. digitatum* to 4%, 5% and 2% when applied at three concentrations of 1×10^5 cells $m\ell^{-1}$, 1×10^6 cells $m\ell^{-1}$, and 1×10^7 cells $m\ell^{-1}$, respectively, compared with imazalil treated fruit (Table 8.4). After storage for one month at 22±1°C, fruit treated with the yeast Isolate B13 at 1×10^6 cells $m\ell^{-1}$ had an incidence of 2% *P*. *digitatum*, which was lower than that for imazalil treated fruit (6%), but was not significantly different ($p \leq 0.05$). The yeast Isolate B13 also significantly reduced mixed infections of brown rot (caused by *Phytophthora citrophthora* (R. E. Sm. & E. H. Sm.) Leanian) and sour rot (caused by *Geotrichum citri-aurantii* (Ferraris) Butler) resulting in a 6% incidence, compared with 15% and 20% for the control and imazalil treatments, respectively, after storage for two months at 7±1°C. It also significantly reduced mixed disease incidence level after one month of storage at 22±1°C (5% compared with 20% and 24% for the control and imazalil, respectively) (Table 8.4).

Table 8.4 Control of natural infection of *Penicillium digitatum* and *Phytophthora* and sour rot incidence (%) of Valencia oranges under commercial packhouse condition with the yeast Isolate B13 after two months at 7±1°C and one month 22±1°C.

Treatment	7± 1°C		22 ± 1°C	
	P. digitatum	Other pathogens	*P. digitatum*	Other pathogens
Untreated control	6.0 ab	15.0 cd	6.0	24.0 b
Imazalil	12.0 c	20.0 d	5.0	20.0 b
B13: 1 X 10^5	4.0 a	8.0 ab	4.0	7.0 a
B13: 1 X 10^6	5.0 a	12.0 bc	7.0	7.0 a
B13: 1 x 10^7	2.0 a	6.0 a	2.0	5.0 a
P value	0.017***	0.006****	NS (0.06)	0.001****
LSD	4.5984	5.3921	-	5.392
CV %	30.84	17.19	27.16	16.65

Other pathogens = *Phytophthora* rot and sour rot

*= 0.05 significantly, **= 0.01 highly significant, ****=0.001 very highly significant, NS= Non Significant

8.3.7 Effects of hot water dip, yeast Isolate B13 and potassium silicate treatments, alone or in combination, on the control of *P. digitatum* of Valencia oranges at Katopé Packhouse

All treatments provided significantly better control of *P. digitatum* infection, compared with imazalil treated oranges. None of the treatments were significantly different from the untreated control (a detailed statistical analysis of the data in Figure 8.6 is provided in Appendix 8B),

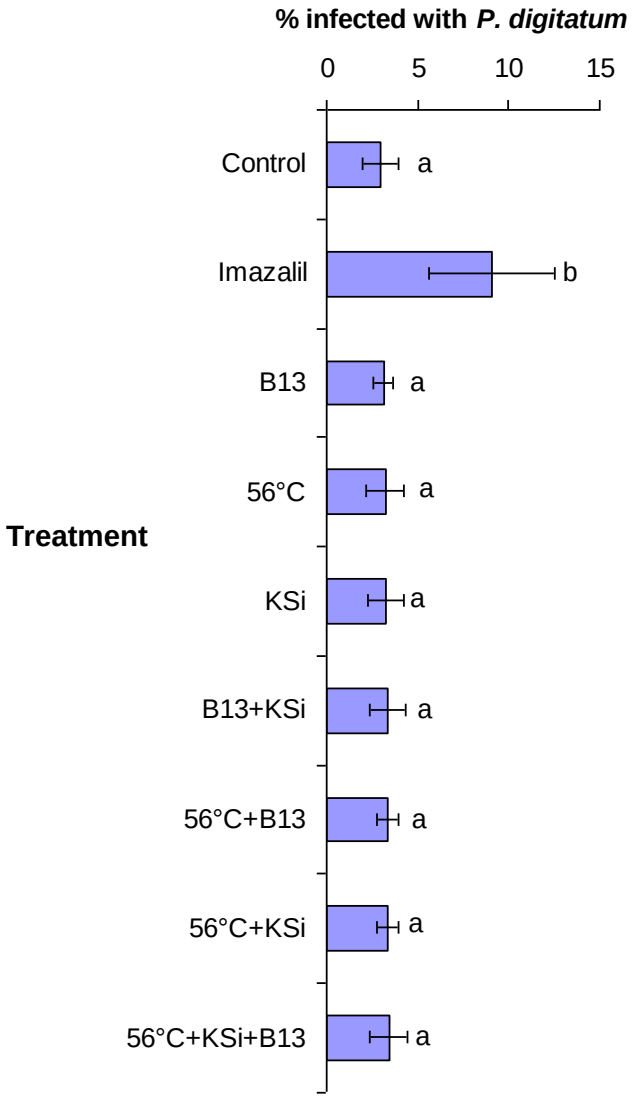

Figure 8.6 Incidence of *Penicillium digitatum* on Valencia oranges after treatment with hot water, and/or yeast Isolate B13 or K_2SiO_3 (KSi), alone or in combination, at the Katopé packhouse

8.3.8 Effects of a hot water dip, yeast Isolate B13 and potassium silicate treatments, alone or in combination, on the control of *P. digitatum* of Valencia oranges at Gateway Packhouse

There was no significant difference in incidence of *P. digitatum* as a result of the treatments applied, and the untreated control, on Valencia oranges (a detailed statistical analysis of the data in Figure 8.7 is given in Appendix 8C).

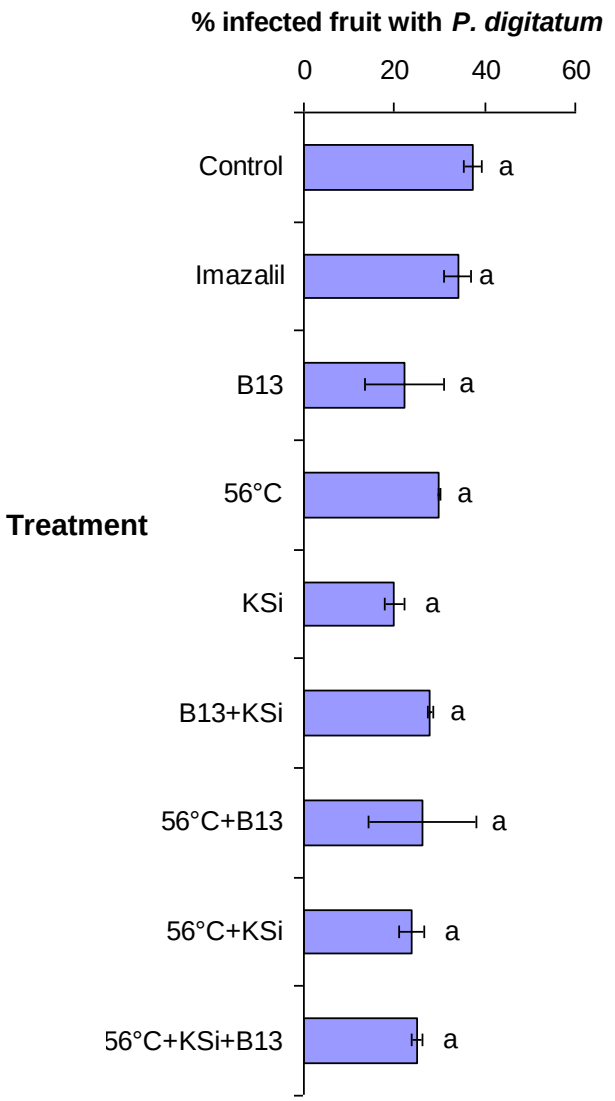

Figure 8.7 Incidence of *Penicillium digitatum* on Valencia oranges after treatment with hot water, and/or yeast Isolate B13 and/or K_2SiO_3 (KSi), alone or in combination, at the Gateway packhouse.

8.3.9 Effects of a hot water dip, yeast Isolate B13 and potassium silicate treatments, alone or in combination, for the control of *P. digitatum* of Valencia oranges at the Maywood Packhouse

The incidence of *P. digitatum* on Valencia oranges was significantly reduced by all treatments, compared with the untreated control (a detailed statistical analysis of the data in Figure 8.8 is presented in Appendix 8D).

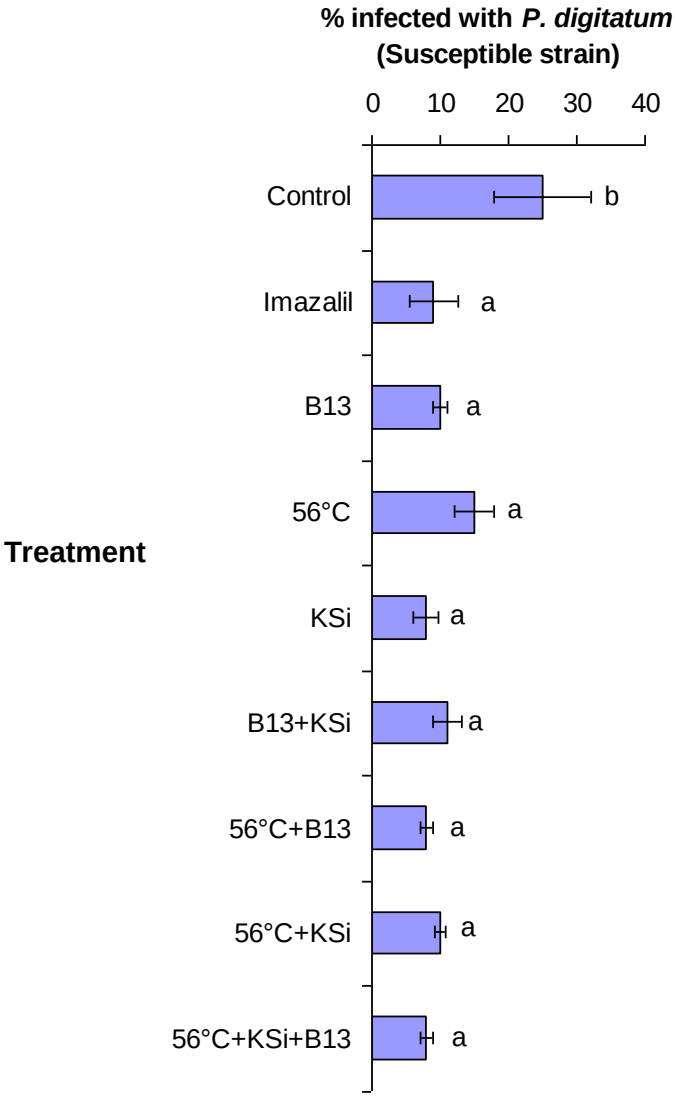

Figure 8.8 Incidence of *Penicillium digitatum* on Valencia oranges after treatment by hot water, and/or yeast Isolate B13, and/or K_2SiO_3 (KSi), alone and in combination, at the Maywood packhouse.

8.4 DISCUSSION

"Increased public awareness has, in recent years, brought about a renewed interest in the use of non-chemical treatments for the protection of fresh produce" (Fallik *et al*., 2000). Postharvest heat treatment has been demonstrated to control or delay pathogen development in different fruit while maintaining quality (Lurie, 1998). Here we have shown that treating artificially inoculated Valencia oranges at a temperature of 50- 56°C for 60-180 seconds successfully controlled *P. di*gitatum (Figure 8.2) without causing rind injury (Table 8.2). Smoot and Melvin (1963) found that immersion of oranges in water

at 53°C controlled *P. digitatum* of artificially inoculated oranges. Houck (1967) as well showed that a hot water dip at 52°C was able to control *P. digitatum* on Eureka lemon, which falls within temperature range of our results.

Porat *et al.* (2000) reported similar findings to ours, using hot water brushing (HWB) treatment, to reduce decay development by rinsing and brushing the fruit 24 hours after artificial inoculation with a *P. digitatum* conidial suspension on 'Minneola' tangerines, Shamouti' oranges and 'Star Ruby' red grapefruit. When treated at 56, 59 and 62°C for 20 seconds, disease incidence was 20%, 5% and less than 1%, respectively, of the disease incidence on untreated fruit or fruit treated with tap water.

Smilanick *et al*. (2003) also reported a significant reduction of *P. digitatum* decay of lemons and Valencia oranges using a brief hot water drench treatment.

Rind injury on Valencia oranges did not occur at a water temperature of 56°C and exposure periods of 45-180 seconds in this experiment. Similar test on lemons carried on by Schirra and D'hallewin (1997) found that hot water treatment was injurious on Fortune mandarins dipped at 56°C, which could be due to the thinner peel layer of Fortune mandarin compared to Valencia orange. The different effects of similar temperature x time treatments on fruit rind quality of different citrus varieties indicate that different cultivars of citrus fruit respond to hot water treatment differently. The efficiency of hot water treatment is dependent on the temperature used x time of exposure, as shown graphically and statistically and in Figure 8.2 and Appendix 8A, respectively. Palou *et al*. (2001) reported that a hot water treatment failed to control *P. digitatum* after two weeks of storage. In contrast, these results have shown that the hot water treatment provided control for up to four weeks.

In the Observational Trial 2, the use of cold water treatment following hot water treatment did not cause a reduction of rind injury of Valencia oranges, and was therefore discarded as a viable treatment.

At higher temperatures, from 66°C-70°C, Valencia oranges were severely injured and *P. digitatum* control was actually reduced. For example, Valencia

oranges treated at 70°C for 180 seconds were all infected by the pathogen (100% incidence) (Table 8.3). The higher disease incidence that occurred in this treatment indicate that the mycelium of *P. digitatum* survived treatment with high temperature water, and that hot water treatments at high temperatures were ineffective in killing conidia of *P. digitatum.* Hot water treatment at the higher temperatures also caused damage to the fruit rind, which may have increased the susceptibility of the tissue to the pathogen. Karabulut *et al*. (2002) also showed that hot water treatments were not able to kill conidia of *P. expansum* Link of apples.

The hot water treatment showed that the pathogen was not controlled by heat energy because the treatments at the highest temperature resulted in 100% incidence of disease. In contrast brief treatments of 30-120 seconds at 50-56°C resulted in high level of disease control. We concluded that induced resistance must be triggered by the heat shock of these treatments, but if the heat level was too high or far too long, then the mechanism of induced resistance was damaged or diabled.

These findings are in agreement with those of Ben-Yehoshua *et al*. (1998) and Schirra *et al*. (2000) who reported that the mode of action of heat treatment on citrus fruit is via induction of resistance; which results in increased lignin formation, release of heat shock related proteins and phytoalexin production against green mould caused by *Penicillium digitatum.* Similarly, Nafussie *et al*. (2001) showed that after hot water treatment of lemons, lignin-like materials were produced at the pathogen inoculation site, followed later by an accumulation of phytoalexins.

In preliminary packhouse trials, commercially harvested Valencia oranges were kept for two months at 7±1°C. The fruit were not artificially inoculated with *P. digitatum,* the outcome of the trial depending upon natural infections by *P. digitatum.* The untreated control (no yeast and no fungicide) developed 6% *P. digitatum.* Imazalil treated fruit developed 12% *P. digitatum*, possibly due to the elimination of naturally competing organisms from the fruit surface compared to the untreated control (Table 8.4). The yeast Isolate B13 resulted in reduced mixed infection by providing control of infection by multiple pathogens after one month of storage at 22±1°C, with a 5% incidence compared with 20% and

24% for the control and imazalil treated fruit, respectively, (Table 8.4). This result indicated that the yeast Isolate B13 may control postharvest pathogens other than just *P. digitatum (*e.g., *Phytophthora citrophthora* R.E. Sm. and E.H. Sm.) Leonian. and *Endomyces geotrichum* E.E. Butler and L.J. Petersen.).

In semi-commercial scale packhouse trials at Katopé, Gateway and Maywood packhouses, a hot water dip, yeast and/or K_2SiO_3 treatments, and their combinations, were compared. Oranges were stored at 24±1°C for one month under natural infections of the pathogen. Results from the Katopé packhouse showed that the untreated control (no yeast and no fungicide) fruit developed significantly less *P. digitatum* infections than imazalil treated fruit (Figure 8.6). This may have been due to imazalil eliminating natural competing organisms from the fruit surface compared with the untreated control. Alternatively there may have been high levels of *P. digitatum* inoculum in the imazalil tank. This finding was in agreement with the preliminary screening trial performed in the same packhouse.

Penicillium digitatum was not significantly controlled by applied treatments at the Gateway packhouse (Figure 8.7). This was probably because of a delay between initial infections and the application of treatments because the fruit was kept for longer than five days in the packhouse before the treatments were applied.

In the Maywood packhouse trial all treatments significantly reduced infection by the pathogen. The imazalil treatment also significantly reduced the *P. digitatum* infection of the fruit (Figure 8.8). Clearly the inoculum of *P. digitatum* at this packhouse was susceptible to imazalil.

In the preliminary commercial trial at the Katopé packhouse, the level of control provided by the yeast Isolate B13, when used alone, was equal or superior to the level of control provided by commercially recommended fungicides, which differs with reports by Droby *et al*. (1993; 1998). However, in subsequent trials in different packhouses, there was a varying level of control provided by the treatments that can be attributed to differences in fruit quality, inoculum density, pathogen susceptibility, and the time between initial infection and the application of treatments, as suggested by Droby *et al*. (1993).

8.5 CONCLUSIONS

In *in vivo* studies, carried out by dipping Valencia oranges 24 hours after inoculation with *P. digitatum* conidial suspension, hot water dip treatments at 50-56°C for 45-180 seconds reduced disease development, when compared with fruit treated with tap water, without causing any rind injuries.

Treating Valencia orange fruit with hot water, a yeast (Isolate B13) and K_2SiO_3 in combination resulted in control of *P. digitatum* comparable with imazalil, in two commercial packhouses.

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CHAPTER 9

CONTROL OF POSTHARVEST *PENICILLIUM* SPP. OF LITCHI FRUIT USING MICROBIAL ANTAGONISTS AND POTASSIUM SILICATE

ABSTRACT

Twenty three yeast and 13 *Bacillus* isolates were obtained from litchi fruit surfaces, and tested with 10 yeast and 10 *Bacillus* isolates that provided good control of *Penicillium digitatum* of citrus fruit (Chapter Three). These isolates were screened for antagonism against postharvest *Penicillium* sp. on litchi fruit. In a preliminary study, six yeast and three *Bacillus* isolates reduced the percentage surface area of litchi fruit infected to ≤50%, when applied three hours pre-inoculation. Further screening of the best six yeast and three *Bacillus* isolates, showed that four yeast isolates (YL4, YL10, YLH and B13), when applied 3 hours prior to inoculation with *Penicillium* sp., reduced infection on litchi fruit to ≤40%, compared with an untreated control that had 96.7% infection. The application of isolates to litchi fruit did not produce a curative action against *Penicillium* spp. when applied 3 hours post-infection. Postharvest application of potassium silicate (K_2SiO_3) significantly reduced severity of already established *Penicillium* spp. on litchi fruit at all concentrations tested. Control was achieved at concentrations as low as 10 mg ℓ^{-1} but the best control was achieved at 1,000 mg ℓ^{-1} of K_2SiO_3 solution. The best yeast isolates were superior to all the *Bacillus* isolates, and provided good preventative control of *Penicillium* spp., while K_2SiO_3 provided good preventative and curative control.

9.1 INTRODUCTION

Litchi (*Litchi chinensis* Son.) is grown as a commercial fruit crop in Australia, China, India, Israel, South Africa, South East Asia, Taiwan, USA and Vietnam (Underhill *et al*., 1997). Litchi fruit are susceptible to postharvest infection by microorganisms such as bacteria and filamentous fungi (Lonsdale, 1988). Losses are estimated to be 20-30% of the harvested fruit. In severe cases losses can reach 50% before consumption (Jiang *et al*., 2001). *Penicillium* spp. are the major postharvest fungal pathogens of litchi (Figures 9.1B). The

pathogen is not well controlled by sulphur fumigation (Kremer-Koehne and Lonsdale, 1990; Jacobs and Korsten, 2004). Furthermore, the sulphur causes undesirable residue (Kremer-Koehne, 1993), changes fruit taste (Lonsdale and Kremer-Koehne, 1991), and constitutes a potential health hazard for consumers and workers (Koening *et al.,* 1983). To date, there are no registered chemicals for the control of postharvest diseases of litchi fruit.

The absence of effective chemical controls, together with strict regulatory control of chemical residues that enforced by importing countries (Wilson *et al*., 1991), as well as public pressure and concern over food safety, have all increased the incentive to find alternative control methods (Holmes and Eckert, 1999). Biological control has been proposed as an alternative to fungicides, and some success has been accomplished by utilizing antagonistic biological agents (McLaughlin *et al.*, 1992). Limited research in the area of biological control of postharvest pathogens has been encouraging. Korsten (2004) has reported some success in developing biological control agents for postharvest disease control of litchi fruit. However, the researcher only tested *Bacillus* sp., and no formulated product of *Bacillus* is yet available for testing. Furthermore, application of silicon has not been attempted to address the problem.

The objectives of this study were therefore: (1) to isolate yeast and *Bacillus* strains antagonistic to *Penicillium* spp.; (2) to investigate their efficacy in controlling infection by *Penicillium* spp. on litchi; and (3) to investigate the use of K_2SiO_3 for control of *Penicillium* spp. on litchi.

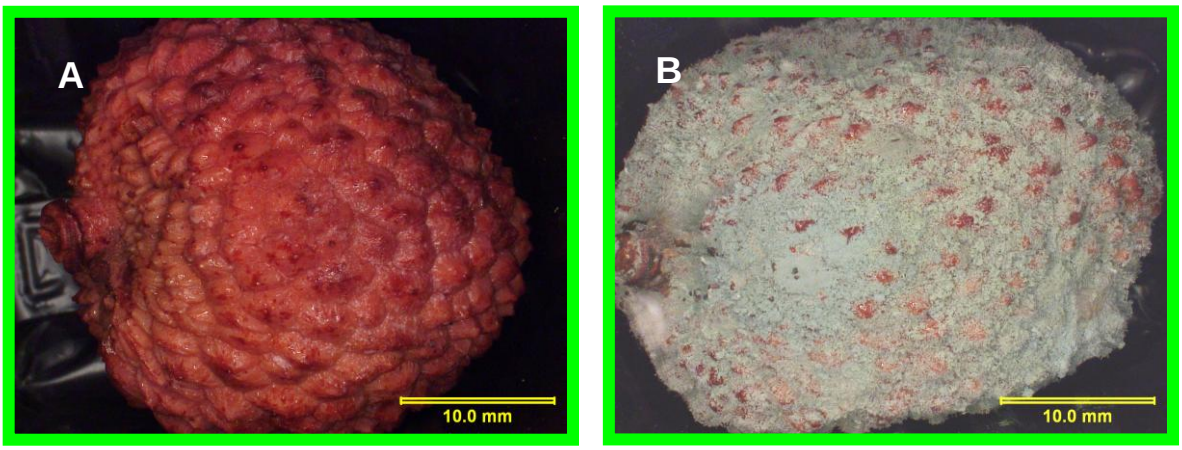

Figure 9.1 Healthy litchi fruit (A) and conidia of a *Penicillium* sp. covering a litchi fruit (B)

9.2 MATERIALS AND METHODS

9.2.1 Fruit used for isolation of potential antagonists

Litchi fruit were obtained from Fruit and Veg City or from Pick'n Pay stores in Pietermaritzburg, KwaZulu-Natal, South Africa. Undamaged fruit were either used immediately, or after storage for 2-3 days at room temperature, or after storage in a cold room at 8°C for 5-7 days.

9.2.2 Isolation of a *Penicillium* sp. from litchi fruit

An isolate of a *Penicillium* sp. was isolated from infected litchi fruit by directly plating conidia onto potato dextrose agar (PDA) amended with 0.15 g ℓ^{-1} of Rose Bengal (RB). Plates were incubated at 25°C for 10 days. Pure cultures of an isolate of *Penicillium* sp. was established by sub-culturing onto malt extract agar (MEA) plates, after identification of conidia under a compound microscope. The pathogen was maintained in double autoclaved, distilled water with regular transfers onto fresh litchi fruit to maintain the aggressiveness of the isolate. A conidial suspension was prepared for inoculation by washing 10 day old cultures of *Penicillium* sp. on MEA medium, with sterile distilled water.

9.2.3 Isolation of antagonistic yeasts and *Bacillus*

Bacillus and yeast isolates were recovered from the surface of 3-5 mature litchi fruit. This was done by placing the fruit in 250 mℓ Erlenmeyer flasks containing 100 mℓ sterile distilled water plus 25% Ringer's Solution and shaking in a water bath (G.F.L. 1083, Labortechnik, Germany) at 120 rotations per minute (rpm) for 1 hour. Fruit were removed and the liquid suspension was used to make a serial dilution of 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4} of the rinsing water before plating. An aliquot of 0.2 mℓ of each dilution was plated onto PDA agar amended with 0.15 g ℓ^{-1} of RB for recovery of yeast isolates. The resulting plates were incubated at 25°C for 3 days. Pure cultures of yeast isolates were made by sub-culturing from discrete colonies on the plates. For isolation of *Bacillus* spp. similar serial dilutions were used, but were heat treated at 80°C for 15 minutes, in a water bath to eliminate non-spore-forming microbes. Aliquots of 0.2 mℓ were then poured onto tryptone soy agar (TSA) plates. Plates were incubated for three days at 28°C, after which representative colonies were arbitrarily selected and streaked onto fresh TSA plates to obtain single colonies.

9.2.4 Preliminary screening of antagonistic yeast and *Bacillus* isolates against *Penicillium* sp. on litchi fruit

A total of 23 yeasts and 13 *Bacillus* isolates were obtained from litchi fruit. The 10 most effective yeast and 10 most effective *Bacillus* isolates obtained from citrus fruit in earlier research (Chapter Three) were also assessed for their preventative action against the selected *Penicillium* sp. on litchi.See Appendix 9A for details of the isolates used.

The litchi fruit were treated by dipping them into 70% alcohol for one minute, dried, and then dipped into a suspension of yeast or *Bacillus* cells (1×10^8 cells $m\ell^{-1}$) for 1 minute. Three hours after drying, each fruit was sprayed with one mℓ of a conidial suspension of *Penicillium* sp. at 1×10^4 conidia $m\ell^{-1}$, adjusted using a haemocytometer. Control fruit were sprayed with 1 mℓ of sterile distilled water. Fruit were kept at room temperature (24±1°C) for 10 days. One plastic tray with five fruit per treatment was used. Treatments were placed on a bench in a completely randomized block design (CRBD). Fruit were examined for percentage surface area of the fruit covered by mycelium and/or conidia *Penicillium* sp. initial screening was based on observation by making a subjective estimation visual estimation (using visual rating scale) 10 . Yeast and *Bacillus* isolates that reduced the percentage of fruit surface area infected by *Penicillium* to ≤50% were chosen for further screening.

9.2.5 Evaluation of preventative action of the six best yeast and three best *Bacillus* isolates against *Penicillium* sp. on litchi fruit

A total of six yeasts and three *Bacillus* isolates were assessed for their preventative action against *Penicillium* spp. on litchi fruit.

Similar procedures as described in Section 9.2.4 were followed, however, litchi fruit were sprayed with a suspension of conidia of *Penicillium* sp. (1×10^4 conidia ml^{-1}) 24 hours after the fruit had been treated by dipping them into a cell suspension of the test organism (yeast or *Bacillus* at 1×10^8 cells ml^{-1}). Fruit sprayed with the same amount of distilled water served as a control. Fruit were kept at 24±1°C. Two trays, with five fruit per tray, were used per treatment and placed on a bench in a CRBD. Fruit were evaluated for percentage fruit surface area covered by mycelium and/or conidia *Penicillium* sp. by making a subjective

estimation based on a visual rating scale 10 days after inoculation with the pathogen.

9.2.6 Determination of the curative action of the six best yeasts and three best *Bacillus* antagonists against *Penicillium* sp. on litchi fruit

Similar procedures as described in Section 9.2.4 were followed; however, litchi fruit were dipped in a *Penicillium* conidial suspension (1×10^4 conidia mℓ$^{-1}$) before treatment with each antagonist. After the fruit had dried for three hours, it was sprayed with one mℓ of the test organisms (yeast or *Bacillus* at 1×10^8 cells mℓ $^{-1}$) per fruit. Fruit sprayed with the same amount of distilled water served as a control. Fruit were kept at 24±1°C. Two trays, with five fruit per tray, were used per treatment and placed on a bench in a CRBD. Fruit were evaluated for percentage fruit surface area covered by mycelium and/or conidia *Penicillium* sp. by making a subjective estimation based on a visual rating scale 10 days after inoculation with the pathogen.

9.2.7 Determination of the curative activity of potassium silicate against *Penicillium* sp. as a postharvest treatment on litchi fruit

Litchi fruit, naturally infected with *Penicillium* sp*.,* were used (Figure 9.2). Fruit were dipped for 1 minute in potassium silicate (K_2SiO_3) solutions at concentrations of 10, 100, 1,000, and 10,000 mg ℓ$^{-1}$. Fruit dipped in sterile distilled water served as control. Fruit were kept at 24±1°C. Two trays, with five fruit per tray, covered with a plastic bag, were used per treatment and placed on a bench in a CRBD. Fruit were evaluated for percentage fruit surface area covered by *Penicillium* sp. by making a subjective estimation based on a visual rating scale 10 days after treatment.

Figure 9.2 Litchi fruit infected with *Penicillium* spp.

9.2.8 Statistical analysis

All data were subjected to an analysis of variance (ANOVA) using Genstat® Executable Release 9.1 Statistical Analysis Software (Anonymous, 2006). To determine differences between treatments, least significant differences were determined at $P<0.05$.

9.3 RESULTS

9.3.1 Preliminary screening of antagonistic yeasts and *Bacillus* isolates against *Penicillium* sp. on litchi fruit applied preventatively

From a total 23 yeast and 13 *Bacillus* isolates, plus the 10 yeast isolates and 10 *Bacillus* isolates from citrus (Chapter Three) (for source of the fruit see Appendix 9A), only six yeast and three *Bacillus* isolates reduced *Penicillium* development on litchi fruit to ≤50%, compared to the untreated control that developed 96% infection (Table 9.1).

Table 9.5 Severity of *Penicillium* sp. infection of litchi fruit as affected by antagonistic yeasts or *Bacillus* isolates, applied preventatively

Isolate and Control	Isolate type	% surface area of fruit covered by *Penicillium sp.*
BL4	*Bacillus*	50
BL6	*Bacillus*	50
FMV110	*Bacillus*	44
B13	Yeast	49
Grape	Yeast	45
YL4	Yeast	33
YL7	Yeast	48
YL 10	Yeast	35
YLH	Yeast	50
Others (45 isolates)	*Bacillus* and yeasts	>55
Water (control)	*Penicillium sp.* only	96

9.3.2 Further screening of the six best antagonistic yeasts and three best *Bacillus* isolates against *Penicillium* sp. on litchi fruit, when applied preventatively

Four yeast isolates, YL4, YL10, B13 and YLH, were highly effective at reducing the severity of *Penicillium* spp. infection on litchi fruit (Figure 9.3 and Figure 9.4 for yeast isolates, YL4, YL10 and YLH). Yeast isolates, YL7, Grape, and the *Bacillus* isolates, BL4, BL6 and FMV110 significantly reduce the severity of *Penicillium* sp. on litchi fruit compared with the untreated control (a detailed statistical analysis of the data in Figure 9.3 is provided in Appendix 9B).

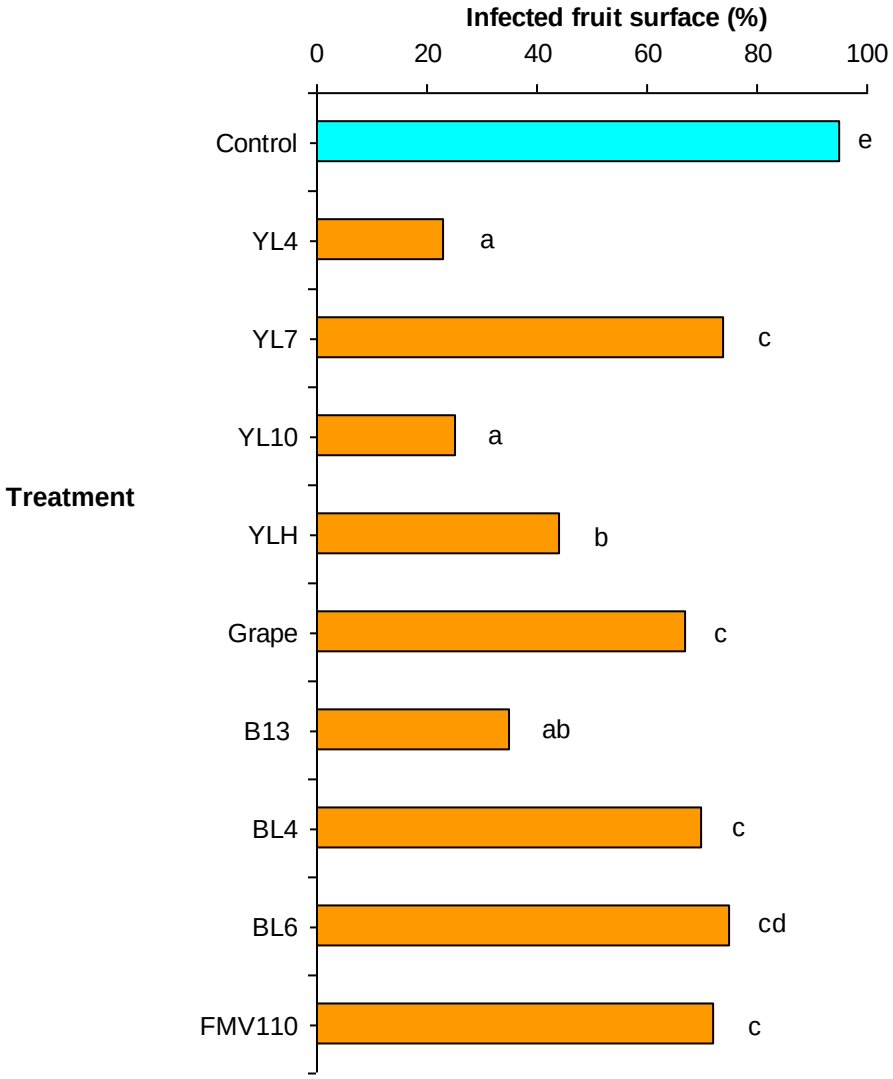

Figure 9.3 Effectiveness of antagonistic yeast and *Bacillus* isolates, applied preventatively, for the reduction of Penicillium infection of litchi fruit

Figure 9.4 Control of *Penicillium* sp. on litchi fruit by antagonistic yeast isolates, applied preventatively: (A) Untreated control (inoculated with *Penicillium* sp. only). (B), (C) and (D) show fruit dipped in yeast isolates YL4, YL10 and YLH (1×10^8 cells $m\ell^{-1}$), respectively, and subsequently sprayed with 1 mℓ of *Penicillium* sp. (1×10^4 conidia $m\ell^{-1}$).

9.3.3 Screening of antagonistic yeasts and *Bacillus* isolates against *Penicillium* sp. on litchi fruit, when applied curatively

Yeast isolates YL4 and YL10 highly significantly provided curative control of *Penicillium* sp. infection more successfully than any of the other treatments. The yeast isolate YLH, YL7 and *Bacillus* isolates BL4 and FMV110 did not provide curative control of *Penicillium* sp. Infection (a detailed statistical analysis of the data in Figure 9.5 is presented in Appendix 9C).

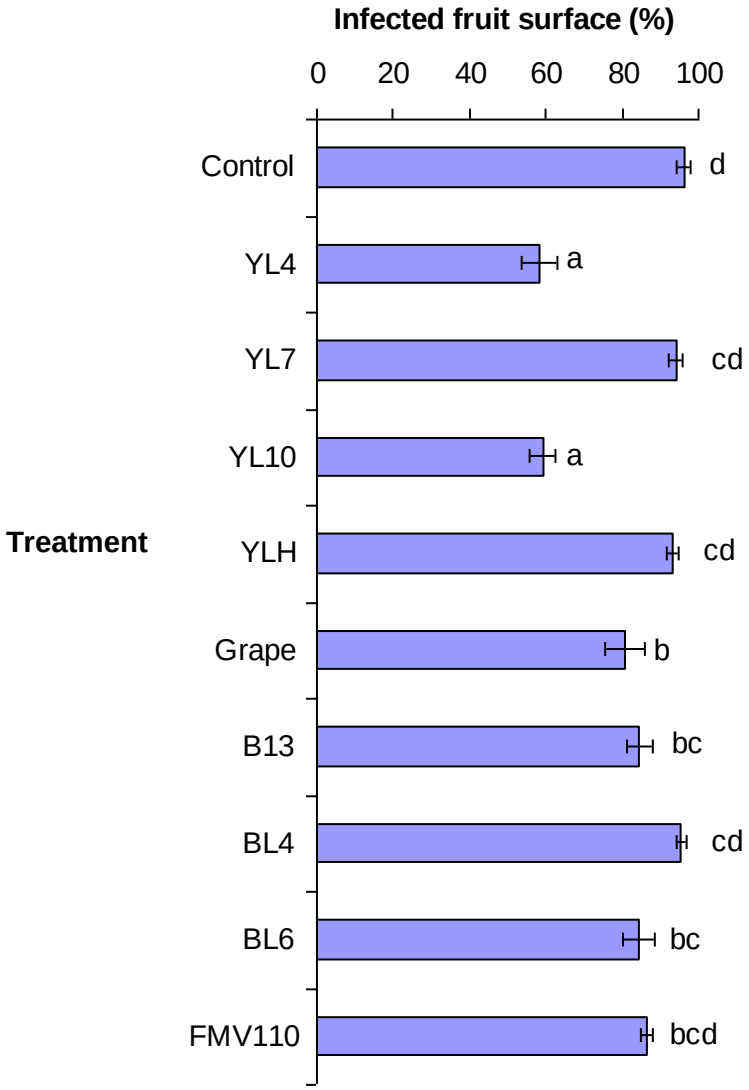

Figure 9.5 Effectiveness of antagonistic yeast and *Bacillus* isolates in reducing the severity of *Penicillium* infection of litchi fruit, when applied curatively, three hours after the pathogen.

9.3.4 Effect of potassium silicate as a postharvest curative treatment for the control of *Penicillium* sp. on litchi fruit

When potassium silicate (K_2SiO_3) was applied curatively for the control of *Penicillium* sp. infection on litchi fruit it was effective at all concentrations tested (Figure 9.6). Increasing the concentration of K_2SiO_3 from 10 mg ℓ^{-1} to 100 mg ℓ^{-1} did not reduce the severity of the pathogen (Figure 9.6). Increasing the concentration of K_2SiO_3 from 100 mg ℓ^{-1} to 1,000 mg ℓ^{1} did not significantly reduced infection severity. Increasing the concentration of K_2SiO_3 from 1,000 mg ℓ^{-1} to 10,000 mg ℓ^{-1} did not further reduce infection severity (a detailed statistical analysis of the data in Figure 9.6 is provided in Appendix 9D).

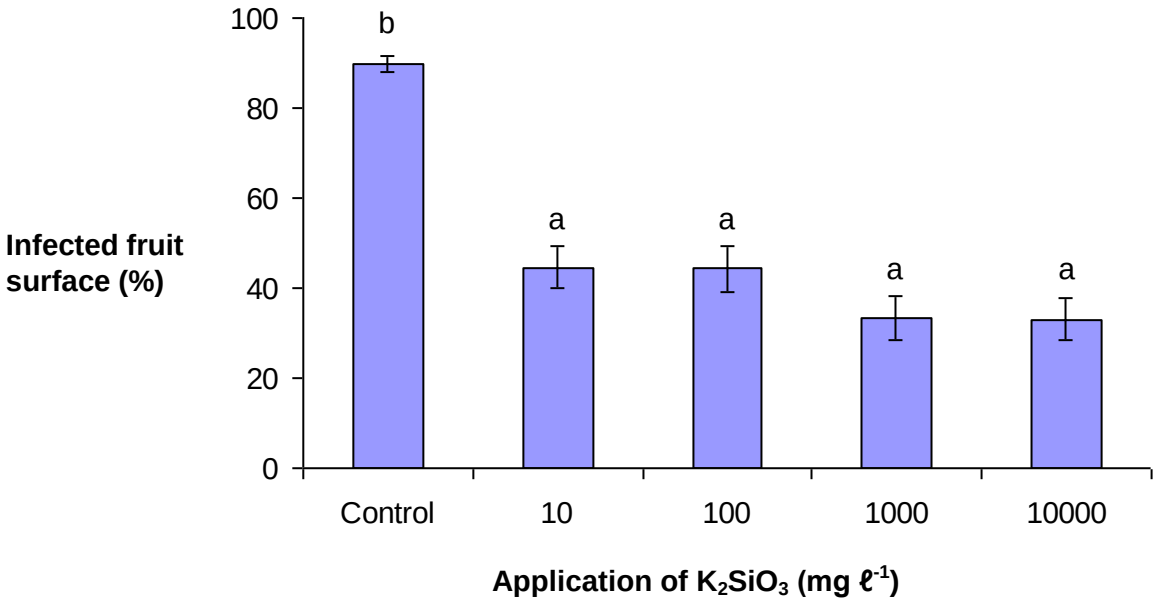

Figure 9.6 Effectiveness of potassium silicate applied curatively against *Penicillium* sp. of litchi fruit.

9.4 DISCUSSION

The major objective of this study was to isolate yeasts and *Bacillus* spp. antagonistic to *Penicillium* sp. on litchi fruit and to assess their potential for the biological control of this pathogen. This approach has been reported by others (Korsten *et al*., 1993; Korsten, 2004; Sivakumar *et al*., 2008). However, it is the first study in which both yeasts and *Bacillus* have been isolated in South Africa and their antagonistic activity evaluated against *Penicillium* sp. Yeast isolates and *Bacillus* spp. were isolated from the surface of litchi fruit in South Africa, and were then screened for inhibitory activity against *Penicillium* sp. on both artificially inoculated and naturally infected fruit.

In the initial screening 6 yeasts and 3 *Bacillus* isolates reduced infection by *Penicillium* sp. on litchi fruit to <50%. In a subsequent screening, all six yeast and three *Bacillus* isolates significantly reduced the fruit surface area infected by *Penicillium* sp. on litchi to <50% when applied preventatively (Figure 9.3). However, only four yeast isolates (YL4, YL10, Grape and B13) and one *Bacillus* isolate (BL6) provided curative control of *Penicillium* sp. on litchi fruit. The yeast isolates YL4, YL10 and B13 exhibited the highest antagonistic activity, providing the best control both preventatively and curatively (Figures 9.3 and 9.4).

The curative effect of these yeast isolates on litchi contradicts the findings of Qing and Shiping (2000) who found that there was no effective curative control of *Rhizopus* by the yeast *Pichia membranaefaciens* Hansen when the yeast was applied to nectarines 24-48 hours after inoculation with *Rhizopus*. Similarly, de Capdeville *et al*. (2002) showed that selected antagonistic yeasts reduced the progress of disease more effectively when applied to apples 24 - 96 hours before inoculation with *Penicillium expansum* (Link) Thom than when applied 24 hours after inoculation. It is also a different outcome than our earlier research on citrus (Chapter Three), where biocontrol by yeast isolates for the control of *P. digitatum* of navel and Valencia oranges and lemons was only preventative and not curative.

Potassium silicate was effective in suppressing *Penicillium* sp. infection of litchi fruit in the postharvest situation. Postharvest silicon application has also been reported to control *Alternaria alternata*, *Fusarium* spp., and *Trichoderma roseum* on Hami melons by Bi *et al*. (2006) and to control *P. expansum* and *Monilinia fructicola* (Wint.) Honey on sweet cherries by Qin and Tian (2005). This suppression could be due to induction of phenolic compounds by K_2SiO_3, as determined with the treatment of melons (Bi *et al*., 2006), and on sweet cherries (Qin and Tian, 2005). Control of *P. digitatum* on lemons, using K_2SiO_3 as a postharvest treatment, was recorded in earlier research (Chapter Seven).

The reduction in disease severity was concentration dependent, and the best suppression was achieved at higher concentrations of K_2SiO_3. This finding agrees with results of Menzies *et al*. (1991) who found that powdery mildew caused by *Sphaerotheca fuliginea* (Schltdl.) Pollacci on cucumbers was better controlled with increasing concentration of K_2SiO_3 in nutrient solutions. This could be related to the fungistatic properties of K_2SiO_3. Bowen *et al*. (1992) found that sodium silicate at lower concentrations mildly promoted conidial germination and germ tube development of *Uncinula necator* (Schwein.) Burrill. It should be recognized that K_2SiO_3 solutions are highly alkaline (pH of 9-12) and it is therefore possible that their fungistatic activity is due to strong alkalinity. The mechanisms involved in the suppression of *Penicillium* sp. infection could be due to either (1) direct contact of K_2SiO_3 with *Penicillium* sp.; (2) with the silicon acting as a physical barrier (Carver *et al*., 1987; Datnoff *et al*., 1997); (3) the soluble silicon acting as a priming agent to enhance host plant

defence responses (Cherif *et al*. 1992; Epstein, 1999); (4) or because of its fungistatic properties, Bekker *et al*., 2006); or a combination of the four putative modes of action.

This was a preliminary study and therefore there is a need for further research on the integrated control of *Penicillium* sp. of litchi:

- To investigate the modes of action of antagonistic yeasts by observing surface colonisation by yeast isolates using scanning electron microscopy.
- To investigate possible mode of action by observing the interaction of *Penicillium* sp. and potassium silicate on the fruit using scanning electron microscopy.
- Because infection of the fruit by *P. digitatum* often occurs in the field before or during harvest (Biggs, 1995), it may be important to apply biocontrol agents early. i.e., their greatest value may be realized by application in the field prior to wounding that often occurs during harvest.
- A postharvest hot water treatment may be effective against *Penicillium* spp. on litchi fruit when applied alone, or as a part of an integrated control package, combined with a biocontrol yeast and potassium silicate in a single treatment.

9.5 CONCLUSIONS

Out of the 33 yeasts and 23 *Bacillus* isolates screened, 6 yeasts and 3 *Bacillus* isolates caused a reduction of ≥50% in the severity of *Penicillium* sp. infection, when applied preventatively as postharvest treatments on the surface of litchi fruit. None of the citrus-sourced antagonists were effective on litchi fruit.

The best yeast isolates, YL4 and YL10, were superior to all the *Bacillus* isolates tested, and provided good control of *Penicillium* sp., both preventatively and curatively (three hours).

This study also demonstrated the potential of K_2SiO_3 for preventative and curative action against *Penicillium* spp. on litchi fruit.

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CHAPTER 10
THESIS OVERVIEW

The purpose of this overview is to look back at the objectives presented in the introduction, to review progress made towards these objectives, to identify future research needs and then recommend future research directions. Finally, it is to make recommendations to the citrus industry based on the research outcomes of this thesis. The overview is not to be viewed as a scientific paper, but rather to clarify the objectives and outcomes of this thesis, and as an industry advisory document.

Research Objectives and Outcomes

The research results are summarized in the following points with respect to the specific objectives referred at the beginning of the thesis:

Objective 1: To investigate the effect of the number of conidia of *P. digitatum* on its pathogenicity on citrus fruit.

> *Penicillium digitatum* suspension at concentrations of 1, 10, 20, 50, 100, 500, 1×10^3, 1×10^4, and 1×10^5 conidia ml^{-1} caused equally significant navel orange fruit decay.

Objective 2: To evaluate the efficacy of the fungicide imazalil to control *P. digitatum.*

> An isolate of *P. digitatum,* isolated from an infected orange fruit, showed resistance to imazalil (the industry standard postharvest fungicide). Subsequent research showed that 10 out of 10 isolates of *P. digitatum* from KwaZulu-Natal packhouses were resistant to imazalil. This situation poses a serious threat to the citrus industry in South Africa (Chapter 2.3.2).

Objective 3: To isolate yeast and *Bacillus* strains antagonistic to *P. digitatum* and to investigate their efficacy in controlling infection by the pathogen under *in vivo* conditions on citrus fruit.

a) A total of 60 yeast and 92 *Bacillus* isolates were obtained from the surfaces of several citrus varieties from various orchards in South Africa.

b) Ten yeast and 10 *Bacillus* isolates reduced infection by *P. digitatum* by more than 50% when applied three hours before inoculation.

c) Two yeast isolates, B13 and Grape (both isolates of *Candida fermentati* (Saito) Bai.), provided good preventive action to navel and Valencia oranges, and lemons, when applied 48 hours prior to inoculation with the pathogen

d) The two yeast isolates, B13 and Grape, did not produce any curative action to lemons and Valencia oranges against *P*. *digitatum* when applied three hours post infection,

e) Two yeast isolates, B13 and Grape, were superior to all the *Bacillus* isolates.

Objective 4: To investigate the modes of action of effective antagonist yeast B13 for the control of *P. digitatum*, by observing fruit surface colonization using scanning electron microscopy.

a) Environmental scanning electron microscopy (ESEM) studies showed effective colonization of lemon wounds by yeast Isolate B13.

b) Scanning electron microscopy observations demonstrated that yeast Isolate B13 inhibited or restricted conidial germination of *P. digitatum*, when it was applied preventatively.

Objective 5: To assess the ability of yeast Isolate B13 to colonize citrus fruit surfaces and to assess its efficacy in controlling postharvest infection by *P. digitatum*, when applied prior to harvest.

a) Spraying citrus trees with yeast Isolate B13 a few months (1-2 months) or few days (1, 3 and 7 days) before harvest was not effective in providing preventative control of *P. digitatum* of Valencia oranges.

b) The population of the yeast Isolate B13 on sprayed Valencia oranges was higher than the untreated control at harvest at two locations. However, this higher population of the yeast Isolate B13 did not reduce the incidence of *P. digitatum*.

Objective 6: To investigate the effects of preharvest applications of potassium silicate for the control of *P. digitatum* infection of navel and Valencia oranges.

Regular potassium silicate drenches in the field resulted in a significant preventative control of *P. digitatum* infection, on both navel and Valencia oranges.

Objective 7: To investigate the effects of applying potassium silicate as a postharvest treatment in order to manage *P. digitatum.*

Potassium silicate as a postharvest resulted in preventative and curative control of *P. digitatum*.

Objective 8: To evaluate the use of a hot water dip as a stand-alone treatment under laboratory conditions.

Hot-water dip treatments at 50-56°C for 60-180 seconds (in increments of 15 seconds) significantly reduced pathogen development in inoculated wounds of Valencia oranges, without causing any rind damage.

Objective 9: To investigate the individual or combined effects of hot water, the yeast Isolate B13 and potassium silicate treatments in three commercial packhouses for the control of *P. digitatum* of Valencia oranges.

The individual use of the yeast Isolate B13, a hot water dip and application of potassium silicate as postharvest treatments provided excellent control of *P. digitatum* in the packhouses, superior or equivalent to the control provided by the fungicide imazalil. Their combinations did not improve their effectiveness against the pathogen.

Objective 10 (a): To isolate yeast and *Bacillus* isolates antagonistic to *Penicillium* sp. of litchi fruit.

A total of 23 yeast and 13 *Bacillus* isolates were obtained from litchi fruit surfaces.

Objective 10 (b): 10to investigate their efficacy in controlling the pathogen under *in vivo* conditions on litchi fruit.

i. Six yeast and three *Bacillus* isolates reduced infection by *Penicillium* sp. (≥50% reduction), when applied three hours before inoculation.

ii. Four yeast isolates, YL4, YL10, YLH and B13, provided good preventative control of *Penicillium* when applied to litchi fruit three hours prior to inoculation with the pathogen. However, YL4 and YL10 were consistently more effective.

iii. The yeast Isolate B13 isolated from oranges also showed good biocontrol activity against *Penicillium* sp. of litchi fruit, but was not effective as isolates YL4 or YL10.

iv. Two of the yeasts isolates (YL4 and YL10) provided curative action against *Penicillium* infection of litchi fruit.

v. The yeast isolates were superior to all *Bacillus* isolates, when applied to litchi fruit, before or after artificial inoculation with *Penicillium* sp.

Objective 11: To investigate the effectiveness of applying potassium silicate as a postharvest treatment in order to manage *Penicillium* sp. of litchi fruit.

Application of potassium silicate as a postharvest treatment for the control of the pathogen resulted in a reduced level of disease severity, when applied preventatively or curatively, on naturally infected litchi fruit.

Future Research

The following research studies would complement this body of research.

- Investigation of the sensitivity of *Penicillium* pathogens of citrus in South African packhouses to imazalil and other fungicides, such as thiabendazole (TBZ) and sodium ortho-phenylphenate (SOPP), because the findings of this research would reflect the need to tackle the issue with appropriate resources.

- Continue screening yeast isolates for biological control, an addition to consolidating research involving Isolate B13.

- Conduct toxicological study of Isolate B13 to enable it to be released to the citrus industry for routine postharvest treatment of citrus fruit.

- Continue preharvest applications of yeast Isolate B13 in citrus orchards for more than one season for the control of *P. digitatum* and other postharvest pathogens. Multi-season applications may be needed before this approach starts to control the pathogen.

- Use of DNA fingerprinting to track the colonization and performance of Isolate B13 in the field, associated with the proposed research above.

- Investigate whether the catalysis of enhanced levels of systemic acquired resistance (SAR) is responsible for the preventative and curative activity of potassium silicate against *P. digitatum* of citrus and litchi fruit.

- Investigate the use of potassium silicate added to the hot water dips at reduced concentrations. This may reduce any browning effect on the fruit and reduce the cost of treatment, without compromising postharvest disease control.

- Evaluate the use of a hot water dip and a hot water dip plus potassium silicate treatment of litchi fruit for the control of *Penicillium* disease.

- Investigate whether hot water treatment triggers and enhances the level of systemic acquired resistance (SAR) in the citrus fruit for the control of *P. digitatum*.

- Investigate whether Isolate B13, a hot water dip and/or potassium silicate treatments may control blue mould of citrus caused by *P. italicum*.

- Investigate the possibility of using hot water treatment for the control of black spot and fruit fly.

- Conduct more commercial trials on the use of yeasts, hot water dips and potassium silicate in South Africa and other parts of the world, to provide further data as basis for the global adoption of these techniques as treatments of citrus, litchi and other fruit crops.

Relevance of the Research to the Citrus Industry

Penicillium digitatum causes a serious disease of citrus fruit (Figure 10.1A). It is present wherever citrus fruit are grown, such as in the field (Figures 10.1B and C), on equipment (Figure 10.1D), in storage rooms (Figure 10.1E), on packhouse lines (Figure 10.1F) and in the market place.

The scale of losses to *P. digitatum* is significant because firstly, as little as one conidium of the pathogen can cause citrus fruit to be unmarketable. Because the pathogen is present almost everywhere, it is not possible to grow and process citrus fruit that are completely free of the inoculum of *P. digitatum*. Secondly, mutant strains of *P. digitatum* have developed resistance to imazalil (Figure 10.1E) and other key postharvest fungicides. As such, the citrus industry needs non-chemical control options in order to combat this disease.

We have developed a range of control options that are effective against *P. digitatum* of citrus fruit, specifically, biological control using yeast isolates, pre- and postharvest applications of potassium silicate, and hot water dips.

Figure 10.1 Presence of *P. digitatum* in various places where citrus is handled (A-F).

Proposed Practices to Minimize *P. digitatum* Incidence in the Citrus Industry

The following control measures are proposed as practical control options that will reduce the incidence of *P. digitatum*.

Preharvest

Sanitation practices, combined with pre-harvest application of potassium silicate (10^3 mg ℓ^{-1} × 2 months × 12 months).

Sanitation of infected fruit **Potassium-silicate application**

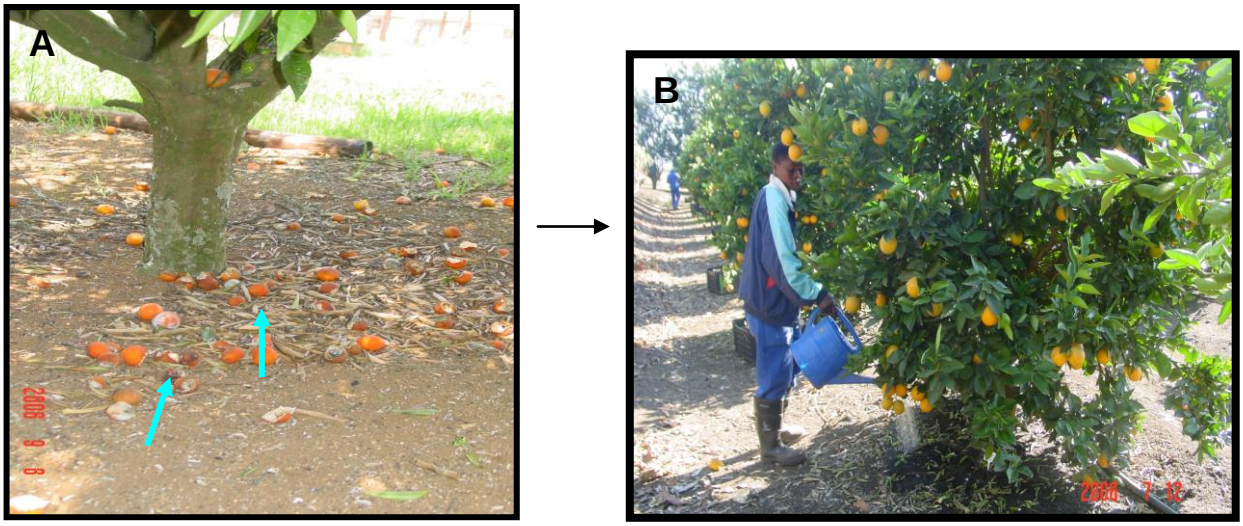

Figure 10.2 (A) *Penicillium digitatum* infected fruit on the ground under tree (indicated by arrow) and (B) drenching an orange tree with potassium silicate solution.

Postharvest

Postharvest treatments of citrus fruit with a hot water dip containing potassium silicate, followed by a spray application of Isolate B13.

Hot water bath at 55-56°C for 45-60 seconds and/or potassium silicate added to the dip.

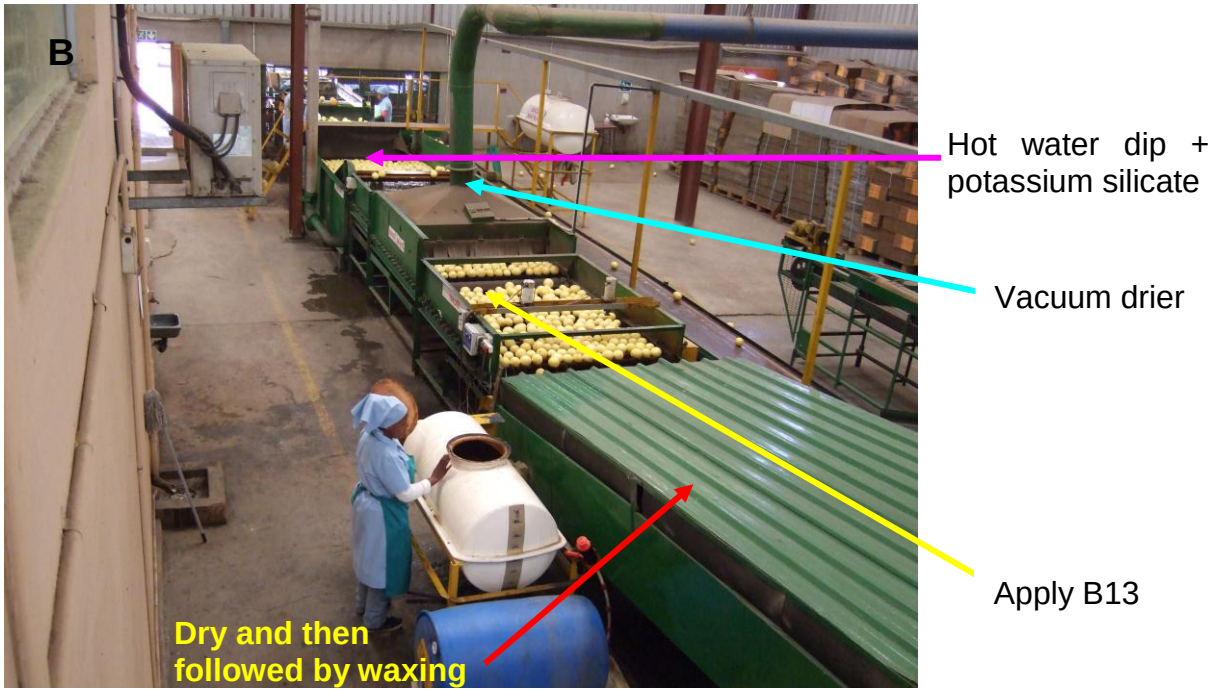

Figure 10.3 (A) Treating fruit in hot water bath and (B) Flow diagram of Proposed Postharvest Handling of Citrus Fruit to Control *Penicillium digitatum* and Other Postharvest Pathogens (Brown and Sour Rots).

Flow Chart for the Postharvest Practices to Minimize *P. digitatum* Disease in the Citrus Packhouse

The following flow chart represents typical packhouse facility for the washing, waxing, and sorting of citrus. We propose the addition of the two control measures developed as part of this thesis at two points in the flow chart, namely, the hot water bath, and ULV spray to deposit biocontrol agent after the first drying station. This would not disrupt the current system of handling citrus fruit. The costs of the interventions we propose would also be relatively low, requiring only ULV spray unit and an additional drying unit. As soon as the potassium silicate and Isolate B13 are registered for the postharvest use on citrus, their application in packhouse handling of citrus could be implemented.

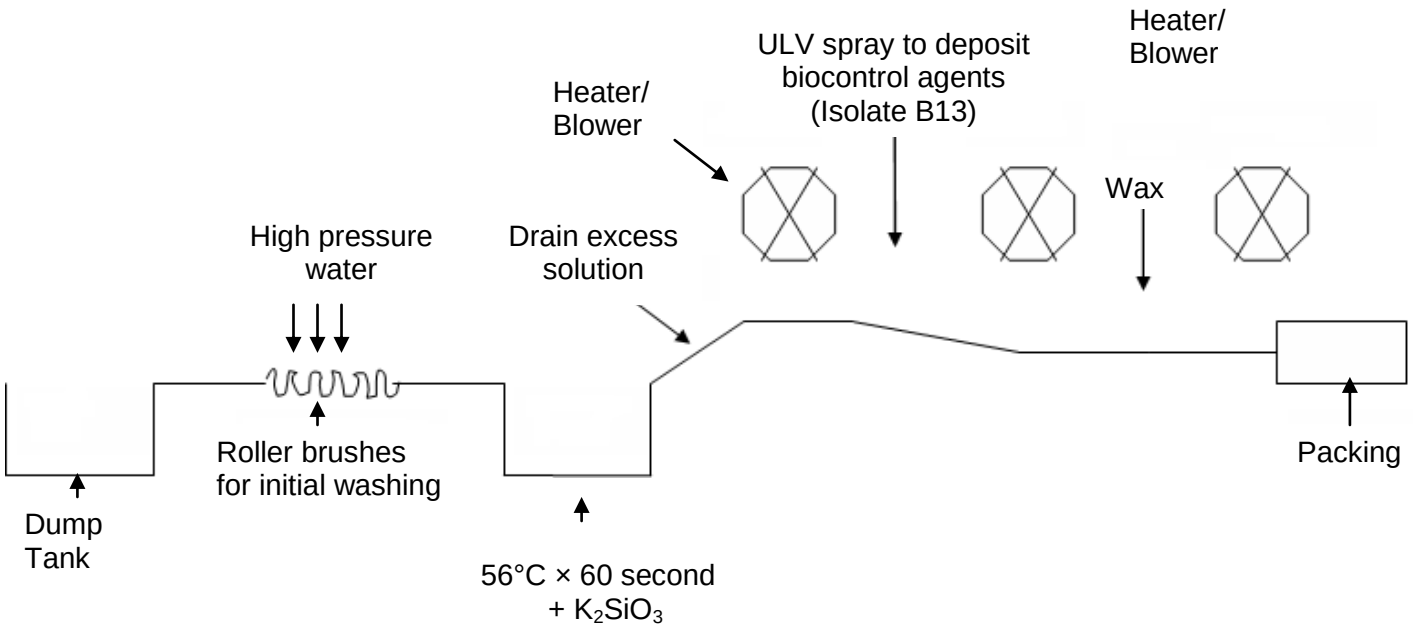

Figure 10.4 Flow chart of Proposed Postharvest Handling of Citrus Fruit to Control *Penicillium digitatum* and Other Postharvest Pathogens (Brown and Sour Rots); two changes are proposed to existing packlines, shown in colour. All other steps are are already in place.

Appendices

Appendix 2A: Pathogenicity of an isolate of *P. digitatum* on navel oranges

Number of *P. digitatum* conidia inoculated per dose of 1 ml	Lesion diameter (mm)
0	23.3 a
1	57.5 bc
10	47.2 b
20	61.5 bc
50	69.3 bc
100	66.9 bc
500	69.5 c
1000	65.3 bc
10000	75.5 c
100000	76.0 c
P Value	<0.001
LSD	19.98
CV %	34.8

Means followed by the same letter are not significantly different

Appendix 3A Location and fruit source for isolation of yeast and *Bacillus* isolates

Yeast and *Bacillus* names	**Isolate type**	**Fruit used for isolation**	**Location fruit obtained**
B3	*Bacillus*	Navel oranges	Thornville, KwaZulu-Natal
B6	*Bacillus*	Valencia oranges	Thornville, KwaZulu-Natal
B7	*Bacillus*	Navel oranges	Richmond, KwaZulu-Natal
B8	*Bacillus*	Valencia oranges	Thornville, KwaZulu-Natal
B9 (1)	*Bacillus*	Rough Lemon	Richmond, KwaZulu-Natal
B9 (2)	*Bacillus*	Rough Lemon	Richmond, KwaZulu-Natal
GW1	*Bacillus*	Granadilla fruit	Nelspruit, Mpumalanga
Papaya	*Bacillus*	Papaya fruit	Nelspruit, Mpumalanga
S1-1	*Bacillus*	Valencia orange	Nelspruit, Mpumalanga
S1-2	*Bacillus*	Valencia orange	Nelspruit, Mpumalanga
B-a	Yeast	Valencia oranges	Pietermaritzburg, KwaZulu-Natal
B13	Yeast	Valencia oranges	Thornville, KwaZulu-Natal
EP	Yeast	Mandarins	Hilton, KwaZulu-Natal
Grape	Yeast	Grapefruit	Nelspruit, Mpumalanga
GR1	Yeast	Rough lemon	Nelspruit, Mpumalanga
Lemon P	Yeast	Eureka lemon	Hilton, KwaZulu-Natal
ON3	Yeast	Navel orange	Richmond, KwaZulu-Natal
RG2	Yeast	Rough lemon	Nelspruit, Mpumalanga
SPL	Yeast	Valencia oranges	Pietermaritzburg, KwaZulu-Natal
UL3	Yeast	Eureka lemon	Hilton, KwaZulu-Natal
Others (132 isolates)	*Bacillus,* yeasts	Various citrus fruit	KwaZulu-Natal, Mpumalanga

Appendix 3B: Preventative treatments of Valencia oranges (three fruits per treatment) with 10 yeast and 10 *Bacillus* isolates to control *Penicillium digitatum*

** Wald tests for fixed effects ***

Fixed term	Wald statistic	d.f.	Wald/d.f.	Chi-sq prob
* Sequentially adding terms to fixed model				
Variety	181.16	19	8.63	<0.001
* Dropping individual terms from full fixed model				
Variety	181.16	19	8.63	<0.001

Standard error of differences: Average 10.97

Average variance of differences: 120.3

Duncan's multiple range test

Identifier	Mean	
Grape	24.17	|
EP	27.39	||
B3	28.55	||
GR1	30.76	|||
RG2	30.96	|||
B13	31.39	|||
ON3	32.06	|||
B-a	32.49	||||
Lemon P	33.36	|||||
B7	35.82	|||||
SPL	36.96	|||||
B9 (2)	37.04	|||||
UL3	39.24	||||
GW 1	39.84	|||||
Papaya	40.25	|||||
S1 1	41.84	||||
S1 2	42.16	|||
B6	44.11	||
Inoculated	45.20	||
B8	48.58	|
B9 (1)	48.94	|

Appendix 3C: Preventative treatments of navel oranges with 10 yeast and 10 *Bacillus* isolates against *Penicillium digitatum*

** Wald tests for fixed effects ***

Fixed term	Wald statistic	d.f.	Wald/d.f.	Chi-sq prob
* Sequentially adding terms to fixed model				
Variety	134.05	19	6.38	<0.001
* Dropping individual terms from full fixed model				
Variety	134.05	19	6.38	<0.001

Standard error of differences: Average 10.97

Average variance of differences: 120.3

Duncan's multiple range test

Identifier	Mean				
B13	0.00	|			
Grape	0.00	|			
SPL	1.74	|			
B-a	2.96	|			
RG2	6.11	|			
GR1	18.28		|		
B3	18.92		|		
B9 (2)	18.93		|		
GW 1	19.41		|		
Lemon P	21.08		|		
B7	22.12		| |		
EP	28.11			| |	
ON3	28.47			| |	
B6	29.88			|	
B8	30.71			|	
S1.1	34.84			| |	
B9 (1)	38.83				| |
UL3	44.50				| |
S1.2	46.97				|
Papaya	62.04				|
Inoculated	68.41				|

Appendix 3D: Preventative treatments of Valencia oranges with 10 yeast and 10 *Bacillus* isolates aginst *Penicillium digitatum*

```
*** Wald tests for fixed effects ***

   Fixed term              Wald statistic      d.f.    Wald/d.f.    Chi-sq prob

* Sequentially adding terms to fixed model

   Variety                      291.23           19        13.87      <0.001

* Dropping individual terms from full fixed model

   Variety                      291.23           19       13.87      <0.001


Standard error of differences:     Average        7.878

Average variance of differences:                  62.24
```

```
  Duncan's multiple range test

      Identifier        Mean

           Grape          2.75 | |
           EP             6.22   |
           Lemon P       19.33     |
           RG2           22.71     |
           GR1           24.31     |
           B13           25.25     |
           B-a           33.16       |
           ON3           38.04       | |
           UL3           40.42         |
           SPL           41.23         | |
           GW1           46.53           | |
           Papaya        49.79             | |
           S1.2          51.57             | |
           B9(1)         53.44               |
           S1.1          53.47               |
           B7            54.07               |
           B8            55.68               | |
           Inoculated 60.41                  | |
           B3            62.95                   | |
           B6            64.48                   | |
           B9(2)         67.96                     |
```

Appendix 3E: Preventative treatments of lemons with 10 yeast and 10 *Bacillus* isolates against *Penicillium digitatum*

*** Wald tests for fixed effects ***

Fixed term	Wald statistic	d.f.	Wald/d.f.	Chi-sq prob
* Sequentially adding terms to fixed model				
Variety	422.79	19	21.14	<0.001
* Dropping individual terms from full fixed model				
Variety	422.79	19	21.14	<0.001

Standard error of differences: Average 8.575

Average variance of differences: 77.68

Duncan's multiple range test

Identifier	Mean				
Grape	0.00	|			
ON3	0.00	|			
Ep	0.00	|			
B13	0.00	|			
B-a	1.27	|			
RG1	3.38	|			
SPL	3.55	|			
RG2	3.98	|			
B9(1)	10.92		|		
Lemon P	11.66		|		
GW1	16.92		|		
B7	39.95			|	
S1.2	44.34			|	
Papaya	50.70				|
S1.1	53.81				| |
B9(2)	54.02				| |
B6	56.62				| |
B8	57.02				| |
UL3	57.95				|
Inoculated	58.16				|
B3	59.10				|

Appendix 3F: Dose effects of two yeast isolates, B13 and Grape, on the control of *P. digitatum*, applied preventatively on lemons

Yeast isolate concentration (cells per ml)	**Lesion diameter (mm)**	
Control	75.365	d
B13($1x10^5$)	12.1406	ab
B13($1x10^6$)	8.76375	ab
B13($2.5x10^6$)	6.90625	ab
B13($1x10^7$)	11.2956	ab
B13($1x10^8$)	0.00	a
Grape($1x10^5$)	33.2644	bc
Grape($1x10^6$)	19.9575	ab
Grape($2.5x10^6$)	21.1106	ab
Grape($1x10^7$)	9.51625	ab
Grape($1x10^8$)	23.4613	ab
P Value	<0.001	
LSD	30.371	
CV %	25.3	

Means followed by the same letter are not significantly different

Appendix 6A: Effect of preharvest potassium silicate applications for 11 months on the incidence of *Penicillium digitatum* on navel oranges stored at 24±1°C, in the 2007 season.

Application of K_2SiO_3 (mg ℓ^{-1})	Frequency of application	*Penicillium digitatum* incidence (%)
0	0	32 d
100	1*	31 d
100	2*	16 ab
500	1	26 cd
500	2	25 cd
1000	1	10 a
1000	2	15 ab
10000	1	21 bc
10000	2	29 cd
P Value		0.0002
LSD		8.5515
CV %		21.89

Means followed by the same letter are not significantly different

Key: 1*: Once/ 2 monthls application
2*: Once/ 1 month application

Appendix 6B: Effect of preharvest potassium silicate applications for 11 months on the incidence of *Penicillium digitatum* on navel oranges stored at 9±1°C, in the 2007 season.

Application of K_2SiO_3 (mg ℓ^{-1})	**Frequency of application**	***Penicillium digitatum* incidence (%)**
0	0	28.0 b
100	1*	10.7 a
100	2*	11.0 a
500	1	16.7 a
500	2	9.7 a
1000	1	7.7 a
1000	2	11.7 a
10000	1	13.7 a
10000	2	15.7 a
P Value		0.0264
LSD		10.392
CV %		43.74

Means followed by the same letter are not significantly different

Key: 1*: Once/ 2 monthls application
2*: Once/ 1 month application

Appendix 6C: Effect of preharvest potassium silicate applications for 11 months on the incidence of *Penicillium digitatum* on Valencia oranges stored at 24±1°C in the 2007 season.

Application of K_2SiO_3 (mg ℓ^{-1})	**Frequency of application**	***Penicillium digitatum* incidence (%)**
0	0	20.3 d
100	1*	11.0 abc
100	2*	10.7 abc
500	1	9.0 ab
500	2	10.0 abc
1000	1	11.7 abc
1000	2	8.0 a
10000	1	15.0 cd
10000	2	14.0 bc
P Value		0.0062
LSD		5.5438
CV %		26.52

Means followed by the same letter are not significantly different

Key: 1*: Once/ 2 monthls application
2*: Once/ 1 month application

Appendix 6D: Effect of preharvest potassium silicate applications for 11 months on the incidence of *Penicillium digitatum* on Valencia oranges stored at 9±1°C, in the 2007 season.

Application of K_2SiO_3 (mg ℓ^{-1})	**Frequency of application**	***Penicillium digitatum* incidence (%)**
0	0	19. 7 d
100	1*	3.0 a
100	2*	4.0 ab
500	1	9.0 c
500	2	4.0 ab
1000	1	4.0 ab
1000	2	5.7 abc
10000	1	7.0 bc
10000	2	4.0 ab
P Value		0.0098
LSD		3.86405
CV %		33.60188

Means followed by the same letter are not significantly different

Key: 1*: Once/ 2 monthls application
2*: Once/ 1 month application

Appendix 7A: Lesion diameters as an indicator of postharvest control of *Penicillium digitatum* on lemons treated preventatively with various concentrations of potassium silicate

Application of K_2SiO_3 (mg ℓ^{-1})	**Lesion diameter (mm)**
0 (control)	37.36 d
10	17.34 bc
100	10.69 ab
1000	21.81 c
10000	23.81 c
100000	7.17 a
P Value	0.001
LSD	6.764
CV %	14.00

Means followed by the same letter are not significantly different

Appendix 7B: Lesion diameters as an indicator of postharvest control of *Penicillium digitatum* on lemons treated curatively with various concentrations of potassium silicate

Application of K_2SiO_3 (mg ℓ^{-1})	**Lesion diameter (mm)**
0 (control)	47.62 d
10	23.04 b
100	45.74 d
1000	33.96 c
10000	38.90 cd
100000	6.24 a
P Value	0.001
LSD	8.343
CV %	17.00

Means followed by the same letter are not significantly different

Appendix 8A: Statistical analysis for efficacy of hot water dip treatment in the control of artificially inoculated *Penicillium digitatum* on Valencia oranges stored at 24 °C for 2 weeks.

The LOGISTIC Procedure

Analysis of Maximum Likelihood Estimates

Parameter	DF	Estimate	Standard Error	Wald Chi-Square	Pr > ChiSq
Intercept	1	-6.7355	1.8855	12.7614	0.0004
Temperature_	1	0.1541	0.0316	23.8075	<.0001
Exposure_period	1	0.1476	0.0238	38.3327	<.0001
Temperatu*Exposure_p	1	-0.00254	0.000385	43.4651	<.0001

Appendix 8B: Control of *Penicillium digitatum* incidence on Valencia oranges by hot water, the yeast Isolate B13 and/or K_2SiO_3 treatments (KSi), alone or in combination, at Katopé Packhouse

Treatments (Imazalil, B13, Hot water, Potassium silicate and their combinations)	**% infected fruit with *Penicillium digitatum***
0 (Control)	2.00 a
Imazalil	9.67 b
B13	3.00 a
56°C	2.00 a
KSi	2.00 a
B13+KSi	3.00 a
56°C+B13	3.00 a
56°C+KSi	2.00 a
56°C+KSi+B13	2.00 a
P Value	0.001
LSD	2.448295
CV %	44.80895

Means followed by the same letter are not significantly different

Appendix 8C: Control of *Penicillium digitatum* incidence on Valencia oranges by hot water, the yeast Isolate B13 and/or K_2SiO_3 (KSi) treatments, alone or in combination, at the Gateway packhouse

Treatments (Imazalil, B13, Hot water, Potassium silicate and their combinations)	**% infected fruit with *Penicillium digitatum***
0 (Control)	37.37
Imazalil	34.00
B13	22.12
56°C	30.00
KSi	20.02
B13+KSi	27.98
56°C+B13	26.23
56°C+KSi	23.94
56°C+KSi+B13	25.01
P Value	0.3754 (NS)
LSD	15.482
CV %	32.93

NS= Non Significant at P≤0.05

Appendix 8D: Control of *Penicillium digitatum* incidence on Valencia oranges by hot water, the yeast Isolate B13 and/or K_2SiO_3 (KSi) treatments, alone or in combination, at the Maywood packhouse

Treatments (Imazalil, B13, Hot water, Potassium silicate and their combinations)	**% infected fruit with *Penicillium digitatum***
0 (Control)	25.07 b
Imazalil	9.06 a
B13	10.01 a
56°C	14.97 a
KSi	7.96 a
B13+KSi	11.02 a
56°C+B13	7.99 a
56°C+KSi	9.98 a
56°C+KSi+B13	7.99 a
P Value	0.0176
LSD	9.0787
CV %	45.78

Means followed by the same letter are not significantly different

Appendix 9A. Litchi trials: Details of yeast and *Bacillus* isolates screened for antagonism of *Penicillium* spp.

Yeast and *Bacillus* names	**Isolate type**	**Fruit used for isolation**	**Location fruit obtained**
B3	*Bacillus*	Navel oranges	Thornville, KwaZulu-Natal
B6	*Bacillus*	Valencia oranges	Thornville, KwaZulu-Natal
B7	*Bacillus*	Navel oranges	Richmond, KwaZulu-Natal
B8	*Bacillus*	Valencia oranges	Thornville, KwaZulu-Natal
B9 (1)	*Bacillus*	Rough Lemon	Richmond, KwaZulu-Natal
B9 (2)	*Bacillus*	Rough Lemon	Richmond, KwaZulu-Natal
GW1	*Bacillus*	Granadilla fruit	Nelspruit, Mpumalanga
Papaya	*Bacillus*	Papaya fruit	Nelspruit, Mpumalanga
S1-1	*Bacillus*	Valencia orange	Nelspruit, Mpumalanga
S1-2	*Bacillus*	Valencia orange	Nelspruit, Mpumalanga
B-a	Yeast	Valencia oranges	Pietermaritzburg, KwaZulu-Natal
B13	Yeast	Valencia oranges	Thornville, KwaZulu-Natal
EP	Yeast	Mandarins	Hilton, KwaZulu-Natal
Grape	Yeast	Grapefruit	Nelspruit, Mpumalanga
GR1	Yeast	Rough lemon	Nelspruit, Mpumalanga
Lemon P	Yeast	Eureka lemon	Hilton, KwaZulu-Natal
ON3	Yeast	Navel orange	Richmond, KwaZulu-Natal
RG2	Yeast	Rough lemon	Nelspruit, Mpumalanga
SPL	Yeast	Valencia oranges	Pietermaritzburg, KwaZulu-Natal
UL3	Yeast	Eureka lemon	Hilton, KwaZulu-Natal
YL4	Yeast	Litchi	Fruit and Veg city, Pietermaritzburg
YL7	Yeast	Litchi	Fruit and Veg city, Pietermaritzburg
YL 10	Yeast	Litchi	Pick’n Pay, Pietermaritzburg
YLH	Yeast	Litchi	Pick’n Pay, Pietermaritzburg
Others (45 isolates)	*Bacillus*, yeast	Litchi	KwaZulu-Natal

Appendix 9B: Effectiveness of antagonistic yeast and *Bacillus* isolates, applied preventatively for the reduction of *Penicillium* infection of litchi fruit

Treatments (yeast and *Bacillus* isolates)	**Infected fruit surface with *Penicillium* spp. (%)**
0 (Control)	91.30 e
YL4	24.81 a
YL7	70.37 c
YL10	23.33 a
B13	34.81 ab
Grape	65.93 c
BL4	69.07 c
BL6	74.81 cd
FMV110	71.48 c
YLH	46.67 b
P Value	0.001
LSD	12.295
CV %	40.07

Means followed by the same letter are not significantly

Appendix 9C: Effectiveness of antagonistic yeast and *Bacillus* isolates, applied curatively for the reduction of *Penicillium* infection of litchi fruit (three hours)

Treatments (yeast and *Bacillus* isolates)	**Infected fruit surface with *Penicillium* spp. (%)**
0 (Control)	96.11 d
YL4	58.15 a
YL7	94.07 cd
YL10	59.26 a
YLH	93.15 cd
B13	84.63 bc
Grape	80.74 b
BL4	95.56 cd
BL6	84.44 bc
FMV110	86.30 bcd
P Value	0.001
LSD	10.107
CV %	22.65

Means followed by the same letter are not significantly different

Appendix 9D: Effectiveness of potassium silicate applied curatively against *Penicillium* spp. of litchi fruit

Application of K_2SiO_3 (mg ℓ^{-1})	**Infected fruit surface with *Penicillium* spp. (%)**
0 (Control)	89.74 b
10	44.63 a
100	44.26 a
1000	33.15 a
10000	33.07 a
P Value	0.001
LSD	12.459
CV %	47.25

Means followed by the same letter are not significantly different