Investigation of environmentally friendly postharvest treatments to extend the storage life of soft citrus cv. Clementine

by

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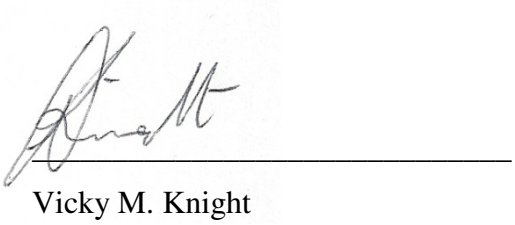
DECLARATION

I, the undersigned, hereby declare that this thesis, submitted for the degree MSc (Agric) in Plant Pathology, is my own and original work except where acknowledged. This work has not been submitted for a degree at any other tertiary institution.



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List of abbreviations

a*:	Chromacity coordinate (redness or greenness)
ACP:	Anaerobic compensation point
ARS-USDA:	Agricultural Research Service of the United States Department of Agriculture
b*:	Chromacity coordinate (yellowness or blueness)
BOPP:	Bioriented polypropylene
BCA:	Biocontrol agent
CA:	Controlled atmosphere
CGA:	Citrus Growers' Association
CO_2:	Carbon dioxide
CRD:	Completely randomized design
GC:	Gas chromatograph
GRAS:	Generally regarded as safe
HR:	Hypersensitive response
HSE:	Heat shock element
HSF:	Heat shock factor
HSP:	Heat shock protein
HWD:	Hot water dip
HWRB:	Hot water rinsing and brushing
IDM:	Integrated disease management
L*:	Chromacity coefficient (lightness)
LOL:	Lowest oxygen limit
LP:	Lipopeptide
LSD:	Least square difference
m:	Mass of a substance or product
MA:	Modified atmosphere
MAP:	Modified atmosphere packaging
O_2:	Oxygen
PAL:	Phenylalanine ammonia-lyase
PPECB:	Perishable Products Export Control Board
PR-proteins:	Pathogenesis related-proteins
SA:	South Africa
SAR:	Systemic acquired resistance
TSS:	Total soluble solids
UK:	United Kingdom
USA/US:	United States of America/United States
UV:	Ultraviolet
VOC:	Volatile organic compound

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CHAPTER 1:

General introduction

The quantity and quality of food that is available for human consumption is a global concern. In developing countries, there is an urgent need for larger quantities of highly nutritious food to feed the growing population and to combat malnutrition (World Food Summit, 1996). In developed countries on the other hand, the trend is shifting towards healthy lifestyles and the consumption of fresh, wholesome and nutritious food (Spadaro and Gullino, 2004). Fresh fruit and vegetables have therefore become an important commodity worldwide and retaining quality and freshness became a minimum requirement.

Apart from global food security challenges, fruit export accounts for 45% of all agricultural products traded in South Africa (Ortmann, 2005). Citrus fruit were ranked the third most valuable export commodity in SA in 2009, following grapes and maize (FAO Statistics, 2009). Consumer demand is shifting towards the easy peeling, seedless varieties and is evident from the increase in Clementine exports (Bijzet, 2006). Clementine (*Citrus reticulata* Blanco) is the most important species within the group of citrus fruit known as the mandarins or easy peelers. Therefore, effective management of citrus and also Clementine export is of economical importance.

Various factors can influence citrus fruit during the different stages within the supply chain (Narayanasamy, 2006). The major postharvest problems associated with citrus fruit are: desiccation, decay, overall deterioration of fruit quality and physiological disorders such as chilling injury (Mukhopadhyay, 2004). The main focus of this thesis was on the control of postharvest diseases, however, overall fruit quality was considered throughout.

Worldwide, the decay of citrus fruit during storage and transport are mainly caused by *Penicillium digitatum* (Pers.: Fr.) Sacc. (green mould) and *Penicillium italicum* Wehmer (blue mould). Citrus green and blue mould accounts for up to 90% of decay occurring during transport, storage and marketing (Agrios, 2005). Current postharvest control measures rely mainly on the use of synthetic fungicides like Imazalil and Thiabendazole, however, fungicide resistancehas been detected in pathogen populations in citrus packhouses

worldwide (Holmes and Eckert, 1999; Torres *et al.,* 2007; Usall *et al.,* 2008). Fungicide usage also holds potential risks to human and environment health and therefore there is an urgent need to find alternative control measures for the current fungicides.

Biocontrol potentially offers a more environmentally friendly alternative and is also well suited to the postharvest environment. In comparison to field applications, postharvest conditions are less variable and the target application is more concentrated (Wisniewski *et al.,* 2007). *Bacillus subtilis,* a Gram-positive bacterium, has been reported successful against various postharvest pathogens (Pusey and Wilson, 1984; Arrebola *et al.,* 2010). Yeasts as antagonists in biocontrol have also been reported in numerous studies (Castoria *et al.,* 1997; El-Ghaouth *et al.,* 2000; Karabulut and Baykal, 2003). *Candida sake* (CPA-1) and *C. saitoana* in particular have shown success in the control of postharvest *Penicillium* rots (El-Ghaouth *et al.,* 2000; Usall *et al.,* 2001). "Biocontrol cocktails" have been patented by El-Ghaouth *et al.* (2002) and involve the use of various enzymes, biomolecules, as well yeast and bacterial genera listed in the patent, in different combinations with one another. One such formulation, Innovacure, was investigated in this thesis. A formulation of the bacterial antagonist *B. subtilis* PPCB002 was also investigated, as well as the combination of the two biocontrol formulations.

Heat treatments have also proven successful in reducing postharvest diseases by disinfecting the fruit surface (Pavoncello *et al.,* 2001) and increasing the fruit's defence reactions (Mukhopadhyay, 2004). The mechanism behind this increased host resistance is not well understood, however research have shown that heat shock proteins (HSP) (Polenta *et al.,* 2007; Promyou, *et al.,* 2008), pathogenesis-related proteins (Romero *et al.,* 2006) and other secondary metabolites such as phytoalexins (Lafuente *et al.,* 2001), phenolics (Rivero *et al.,* 2001) and antioxidants are involved. Therefore, these compounds or enzymes can serve as markers for detecting increased resistance in fruit towards a wide range of stress factors (Lafuente *et al.,* 2001). Hot water dips (35°C) are currently used in some South African citrus packhouses, but mainly to increase the efficacy of fungicides (Lezar, K., April 2009, pers. comm.).

Another major obstacle associated with the postharvest life of citrus is desiccation and is caused by long periods in cold storage (Crisoto *et al.,* 2002; Korsten and Janisiewicz, 2002; Artés-Hernàndez *et al.,* 2004; Meng *et al.,* 2008). However, extending the storage life of fresh produce depends fundamentally on effective refrigeration and maintenance of the cold

chain (Harris, 1988). Modified atmosphere packaging (MAP) can reduce desiccation, since the fruit is protected from continuous air flow and high relative humidity can be maintained within the packaging. This technology is based on modification of the gaseous environment surrounding the fruit. The modified atmosphere not only reduces the respiration rate of fruit (Burdon, 1997), but also that of pests and pathogens (Shellie, 2002), leading to maintenance of fruit quality and reduction in decay. This technology can also easily be combined with other treatments in an integrated disease management (IDM) program and success has been reported when used in combination with biological control (Sivakumar *et al.,* 2007).

When it comes to the proper management of postharvest diseases and disorders, an integrated disease management (IDM) approach has always been most effective. Various studies report success with IDM programs involving biocontrol combinations (Droby *et al.,* 1998; Obagwu and Korsten, 2003; Spadaro and Gullino, 2004), physical or non-chemical treatments, such as generally regarded as safe (GRAS) compounds, natural compounds, MAP and heat treatments (Rodov *et al.,* 2000; Palou *et al.,* 2001; El-Ghaouth *et al.,* 2002; Sivakumar and Korsten, 2006; Montesinos-Herrero and Palou, 2010). In this thesis, the following postharvest treatments were investigated in different combinations with one another:

1. **Biocontrol**

- Bacterial antagonist formulation: *Bacillus subtilis* PPCB002
- Yeast antagonist formulation: Innovacure, which consisted of *Candida saitoana*, lysozyme and sodium bicarbonate
- Combination of bacterial and yeast formulations

2. **Heat treatments**

- Various hot water dip regimes

3. **Modified atmosphere packaging**

- Three different MAP materials: two bioriented polypropylene packaging and one biodegradable packaging types with different perforations

The aim of this thesis was to develop an effective IDM program, using the above mentioned alternative postharvest treatments, to reduce *Penicillium* decay in Clementine mandarins while still maintaining overall fruit quality.

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CHAPTER 2:

Literature review

Postharvest aspects of citrus fruit, with special reference to soft citrus

Citrus is regarded as the most important fruit crop in terms of international fresh produce trade (Dugo and Di Giacomo, 2002; Montesinos-Herrero and Palou, 2010). South Africa (SA) is a major role player in the global citrus industry and is ranked the 2nd largest exporter of citrus fruit overall and 5th largest exporter of soft citrus in particular (CGA, 2010)

. However, since consumer preference is leaning towards the easy-peeling, seedless varieties (Chao, 2005; Bijzet, 2006) soft citrus exports are on the increase (Bijzet, 2006; PPECB, 2007). Maintaining shelf life of the more vulnerable mandarin varieties throughout the export chain is therefore of economical importance to SA.

The main export markets of SA soft citrus are the United Kingdom (UK) (45%) and northern Europe (19%). Other important or emerging markets include Russia (9%), the Middle East (7%), America (USA) (7%) and Asia (5%) (CGA, 2010). Expanding to these markets require movement of crops over long distances in cold storage and extended periods of time, which makes it challenging to provide good quality produce on the market (Brecht *et al.,* 2003; Korsten, 2006). South Africa also competes strongly with some southern (Argentina, Peru, Uruguay, Chile and Brazil) and northern hemisphere countries (Spain, Italy, Turkey, Israel, Morocco and Egypt) for the same export markets, since the seasons overlap. For this reason, it is crucial to provide the export markets with excellent quality fruit and minimise postharvest losses.

The main obstacles associated with citrus export include: general deterioration of fruit quality, desiccation, chilling injury and decay (Mukhopadhyay, 2004). The *Penicillium* rots are responsible for a significant percentage of postharvest losses in citrus (Agrios, 2005; Leelasuphakul *et al.,* 2008) and current commercial practices rely greatly on the use of synthetic fungicides. Aside from the human and environmental health hazards associated

with the use of large volumes of fungicides, various pathogens are becoming increasingly resistant to these chemicals (Holmes and Eckert, 1999; Usall *et al.,* 2008).

In an attempt to find suitable alternatives to the use of fungicides, an array of different postharvest treatments have been investigated to reduce postharvest losses in citrus (El-Ghaouth *et al.,* 2000; Obagwu and Korsten, 2003; Sivakumar *et al.,* 2007; Montesinos-Herrero and Palou, 2010). In this thesis the following treatments were investigated for their ability to reduce *Penicillium* decay, as well as their effect on fruit quality: modified atmosphere packaging (MAP), heat treatments and biological control. This literature review will therefore deal with these treatments in detail (2.4). However, in order to understand the effects of these treatments on fruit quality and disease development, a review of the host (2.1), pathogen (2.2) and current postharvest practices (2.3) was included. These discussions will refer to citrus fruit overall with special reference to soft citrus were applicable, since many facets are generic for different citrus types and varieties.

2.1 Aspects regarding citrus fruit production

2.1.1 Taxonomy and botany of citrus fruit

The *Citrus* L. genus belongs to the orange sub-family Aurantioideae, of the family Rutaceae (Dugo and Di Giacomo, 2002). Most of the species belonging to this sub-family are evergreen, aromatic trees or shrubs, which bear a specific type of fruit known as a hesperidium (Bijzet, 2006). The tribe Citreae is one of two within Aurantioideae and contains the sub-tribe Citrinae, which includes all the citrus species of commercial importance (Dugo and Di Giacomo, 2002).

Citrus varieties of agronomical importance can be divided into the following groups: sweet oranges, lemons, grapefruits, limes, sour oranges, pummelos, citrons and mandarins. The mandarins (*Citrus reticulata* Blanko), or easy peelers can be further subdivided into five groups: Satsuma, Clementine, tangerines, Mediterranean mandarins and different hybrids of these (Dugo and Di Giacomo, 2002; Bijzet, 2006). Citrus taxonomy is complex and opinions differ regarding its parentage and nomenclature. However, throughout this dissertation the Clementine mandarin will be referred to as *C. reticulata* (Bijzet, 2006).

The hesperidium fruit type is described as a berry with a leathery rind (Bijzet, 2006). Mandarins have an oblate shape (Dugo and Di Giacomo, 2002) and Clementine in particular

is smaller, with a thin, smooth rind and has a deep orange internal and external colour (Bijzet, 2006). All citrus fruit consist of three layers: the epi-, meso- and endocarp (Dugo and Di Giacomo, 2002). The epicarp is the thin, roughly-textured and brightly coloured outer layer. It is called the flavedo, due to the high level of flavonoid production within this part of the peel. Flavonoids have antioxidant activity and are especially abundant during the growth stages of fruit and leaves. They play an important role in plant defence reactions (Del Rio and Ortuño, 2004). The flavedo also contains numerous oil glands, which produce essential oils that are responsible for the characteristic citrus smell. It is postulated that the contents of the oil glands also play a role in disease susceptibility of the fruit (Dugo and Di Giacomo, 2002).

The second layer is the mesocarp or albedo, which is the white, spongy tissue underneath the flavedo. In mandarins, the albedo is very thin (Dugo and Di Giacomo, 2002). The epi- and mesocarp together comprise the pericarp or the fruit peel. The endocarp consists of the individual segments, derived from individual locules in the ovary (Bijzet, 2006) (usually 8-12), that are enclosed by a membrane and vascular bundles. The locules or segments contain the juice vesicles and seeds (Dugo and Di Giacomo, 2002).

2.1.2 Citrus cultivation

Currently, commercial citrus production occurs within the two subtropical bands 20-40° north and south of the equator (Dugo and Di Giacomo, 2002). China is the world's largest producer of soft citrus, followed by Spain, Brazil and Japan (CGA, 2010). In SA, citrus is produced in areas with mild winters and low levels of frost. Clementine grows best in a Mediterranean climate, as can be found in the Eastern Cape midlands and coastal areas. Other areas of production include the Western Cape and southern Kwazulu-Natal, as well as the warmer subtropical areas of Mpumalanga and Limpopo. However skin blemishes and poor colour development can be problematic in these subtropical areas (Terblance, 1999; Bijzet, 2006). Soft citrus plantings, which are predominantly Clementine, cover 8.5% (4690 ha) of the total area planted with citrus in SA (CGA, 2010).

It takes about six to 12 months from fruit set to maturation (Baldwin, 1993). During the first two stages of fruit growth (Stage I and II), intense cell division and cell enlargement occur within developing fruitlets (Dugo and Di Giacomo, 2002). Factors during these stages (i.e. temperature, light, humidity) will determine the final quality of the harvested product

(Murata, 1997). The final, fruit maturation and ripening stage (Stage III) is associated with various biochemical and physiological changes in the fruit (Dugo and Di Giacomo, 2002). After a certain level of maturity is reached, fruit ripening sets in (Terblanche, 1999).Citrus is harvested at commercial maturity, since it will not mature and ripen sufficiently if it has been harvested at immature stages (Baldwin, 1993). Section 2.1.3 of this literature review will deal with the physiological processes and quality aspects associated with fruit maturation and ripening in more detail.

Citrus for the fresh-fruit market is manually harvested in SA, by means of snap-picking (Kruger and Penter, 2006). Harvesting of Clementine starts from week 17 with the cultivar 'Marisol'. The cultivar 'Nules' follows and 'Clemlate' ends the season off around week 32 (Bijzet, 2006), however, 'Nules' Clementine or 'Clemenules' is the most important Clementine cultivar worldwide (Valencia-Chamoro *et al.,* 2011). Fruit shouldn't be harvested when wet, since it creates favourable conditions for germination of pathogen spores (Narayanasamy, 2006). Keeping fruit in the shade during picking (Burdon, 1997) and reducing field heat as soon as possible after harvest, will reduce the fruit's respiration rate (Timmer *et al.,* 2000) and subsequently the risk of postharvest disease development. Such practices will also help maintain the delicate flavour and texture of soft citrus (PPECB, 2007).

2.1.3 Fruit ripening and quality

Ripening is in essence a senescence process and is strictly regulated by plant growth regulators. Ethylene is a key hormone in ripening and its production differs among different fruit. Physiologically, citrus is classified as a non-climacteric fruit. A sharp peak in respiration, or a sudden burst of ethylene is therefore not observed (except during wounding), as in climacteric fruit. Instead, a gradual decline in the fruit's respiration rate and increase in ethylene production is observed from maturation to ripening (Tucker, 1993).

Fruit maturity and ripening involves a change in fruit colour, softening, a decrease in acidity, increase in sugar content and the development of a characteristic flavour. The change in colour from green to yellow and orange is also known as colour break. This process is the result of chloroplast breakdown and increased carotenoid production in the flavedo (Baldwin, 1993). In warmer subtropical areas, the external colour may be green, while fruit are ripe internally and in these cases the fruit are picked green and degreened artificially. This

practice is used in commercial packhouses where fruit colour development is unsatisfactory (Porat *et al.,* 1999; Mukhopadhyay, 2004; Kruger and Penter, 2006). Even though citrus fruit cannot be artificially ripened with ethylene as with climacteric crops, colour break will occur in response to externally applied ethylene (Tucker, 1993). Citrus responds to high ethylene levels by increased chlorophyllase activity (Murata, 1997).

Internal physiological changes associated with fruit maturation and ripening involve a decrease in organic acids and an increase in sugars. Brix (°Bx) or total soluble sold (TSS) content is a measure of the total sugar content, however, 15-30% of the Brix reading includes other compounds, such as lipids and pectin (Baldwin, 1993). Sugars in mandarins range from 1-2.3% glucose, 1-2.8% fructose and 2-6% sucrose. Among these, sucrose levels in particular increase during ripening (Baldwin, 1993). A minimum Brix reading of 9-11°B for early to late season Clementine is required for export (PPECB, 2010). The total acidity is mainly composed of citric acid in oranges and mandarins. Acids decrease faster than sugars during storage. Total acidity levels lower than 0.8% leads to a poor, bland taste and leaves fruit more susceptible to postharvest pathogens (Chahidi *et al.,* 2008). Since external colour is not a good indicator of maturity, the Brix to acid or titrable acidity (TA) is used as a maturity index instead (Tucker, 1993) and a minimum of 8 is required for export (PPECB, 2010).

Although the sugar to acid ratio is the main determinant of fruit taste, phenolic compounds also play a role. Aroma is perceived through the interaction of various volatile compounds with sugars, acids and phenolics (Tucker, 1993). During maturation and ripening, certain volatile compounds accumulate in the pulp and juice to eventually give the characteristic flavour of citrus fruit (Dugo and Di Giacomo, 2002). Metabolites such as acetaldehyde, ethanol and ethyl acetate on the other hand, are associated with “off-flavour” tastes and aroma (Shellie, 2002).

Decay, sensory quality, mechanical damage, physiological disorders and general deterioration of fruit directly affect consumer acceptance and therefore the value of harvested produce (Abbott, 1999). Maintaining fruit quality should therefore be kept in mind when selecting disease management strategies.

2.2 Postharvest diseases of citrus

In comparison to other subtropical fruit, citrus stores relatively well due to its slow respiration rate after harvest (Murata, 1997). However citrus fruit have high sugar, water and acid content and are normally kept at high relative humidity (RH) to reduce desiccation, making them very susceptible to fungal diseases. Furthermore, postharvest handling practices may cause micro cracks and wounds, creating entry points for postharvest pathogens (Agrios, 2005).

In developing countries postharvest diseases and disorders of fresh produce can lead to losses of up to 25% (Narayasanamy, 2006), often due to a lack of cold chain facilities (Zhou *et al.*, 2007). According to El-Ghaouth *et al.* (2002b), postharvest losses can account for 10 - 50% of total losses, depending on the available infrastructure and the type of commodity. Despite the sophisticated infrastructure in the US, postharvest diseases accounted for up to 6% losses in oranges during 2005 citrus season (ARS-USDA, 2005). In addition, acceptable levels of decay for exporting citrus are very low. According to the PPECB export standards, a maximum of 1.5% decay is allowed per consignment for export SA soft citrus to the US and other export markets (PPECB, 2010).

Postharvest pathology or 'market pathology' is defined as the "*science of, and practices for, the protection of harvested produce during harvesting, packing, transporting, processing, storing and distribution*" (Narayanasamy, 2006). In many cases diseases are initiated in the field already but will only manifest after harvest when the fruit's natural defences decline and conditions are more favourable for disease development (Dugo and Di Giacomo, 2002). In other cases, infection occurs somewhere in the food chain, in which case pathogens from adjacent infected fruit enter through wounds on the healthy fruit, or infect them directly (Agrios, 2005).

According to Dugo and Di Giacomo (2002), the following pathogens most commonly cause postharvest disease in citrus: *Alternaria citri* Penz. (Mussat.), *Botrytis cinerea* (De Bary) Whetzel, *Geotrichum candidum* Link, *Penicillium digitatum* (Pers.: Fr.) Sacc., *Penicillium italicum* Wehmer and *Phytophthoraparasitica* Breda de Haan. According to Manicom (2006), *G. candidum*, *Diplodia natalensis* Pole-Evans and *Trichoderma viride* Pers.may also be important postharvest pathogens, however they are less serious than the *Penicillium* spp. Citrus green and blue mould, caused by *Penicillium digitatum* and *P. italicum* respectively, are the most commonly occurring and destructive postharvest diseases

of citrus (Timmer *et al.,* 2000; Agrios, 2005; Leelasuphakul *et al.,* 2008). *Penicillium digitatum* is more prevalent than *P. italicum,* however, both pathogens occur in all citrus growing regions (Timmer *et al.,* 2000; Manicom, 2006). The focus of this thesis was on both *Penicllium* species.

2.2.1 Green- and blue mould decay in citrus

The genus *Penicillium* was first described by Link in 1809 as moulds that produce "*brush-like sporulating structures*" (Ramirez, 1982). Both *P. italicum* and *P. digitatum* fall under the kingdom Mycota, phylum Ascomycota, class Eurotiomycetes, order Euriotales, family Euroticeae (Onions and Brady, 1987), genus *Penicillium*, subgenus *Penicillium,* section Cylindrosporum Pitt. and series *Italica* (Pitt, 1979). *Penicillium* species are grouped mainly based on differences in morphology and branching patterns of their reproductive structures (penicilli or conidiophores). Species within the *Italica* series are all terverticillate and have bell-shaped phialides, from which cylinders of conidia are produced (Pitt, 1979).

Mycelial colonies of *Penicillium* spp. vary in morphology, from floccose to a dense network of hyphae, which are narrow, septate and brightly coloured to colourless. The penicillus or conidiophore is the reproductive structure and sprouts from a fertile hypha (Pitt, 1979). At the tips of the conidiophores, the conidiogenous phialides occur, giving rise to the haploid infectious propagules, called conidia (Fig. 2.1).

Conidia or spores are small, unicellular propagules that vary in shape and outer texture. Most conidia are pigmented green or blue, for protection against UV light. *Penicillium digitatum* produce disordered chains of yellow-green to olive conidia, which vary in shape and are about 4 - 7 x 6 - 8µm in size, which is the largest among all *Penicillium* species (Pitt, 1979). Spores of *Penicillium italicum* are smaller (2 - 3 x 3 - 5µm), have a green to greyish colourand are cylindrical-elliptical in shape (Timmer *et al.,* 2000).

Penicillium is a terrestrial fungus that is prevalent in the atmosphere, soil and on plant surfaces. The genus is commonly associated with dead or dying plant material where it lives saprophytically, performing the very important role of nature's decomposers. However many *Penicillium* spp., such as *P. digitatum* and *P. italicum*,successfully evolved to survive on living tissue causing disease in its host, additional to its ability to function saprophytically on decomposing plant material (Moss, 1987).

Penicillium is a mesophyllic organism and therefore, optimum growth occurs at temperatures of 20 - 30°C (Moss, 1987; Pitt, 1979). According to Zhang and Swingle (2005), *P. digitatum* grew best at 25°C, with little to no growth occurring at 30 and 35°C. Conidia and especially ascospores are able to survive exposure to higher temperatures (Moss, 1987; Pitt, 1979). However, elevated temperatures may affect sporulation (Zhang and Swingle, 2005). *Penicillium digitatum* is more prevalent than *P. italicum,* however *P. italicum* are reportedly able to grow at 5°C (Moss, 1987; Pitt, 1979) and can grow faster than *P. digitatum* at temperatures below 10°C (Timmer *et al.,* 2000; Manicom, 2006). Therefore, *P. italicum* is often dominant in long term cold storage (Murata, 1997).

The disease cycle is the same for the two pathogens (Fig. 2.1) and can be completed within three to five days at 25°C (Holmes and Eckert, 1999 and 1995).

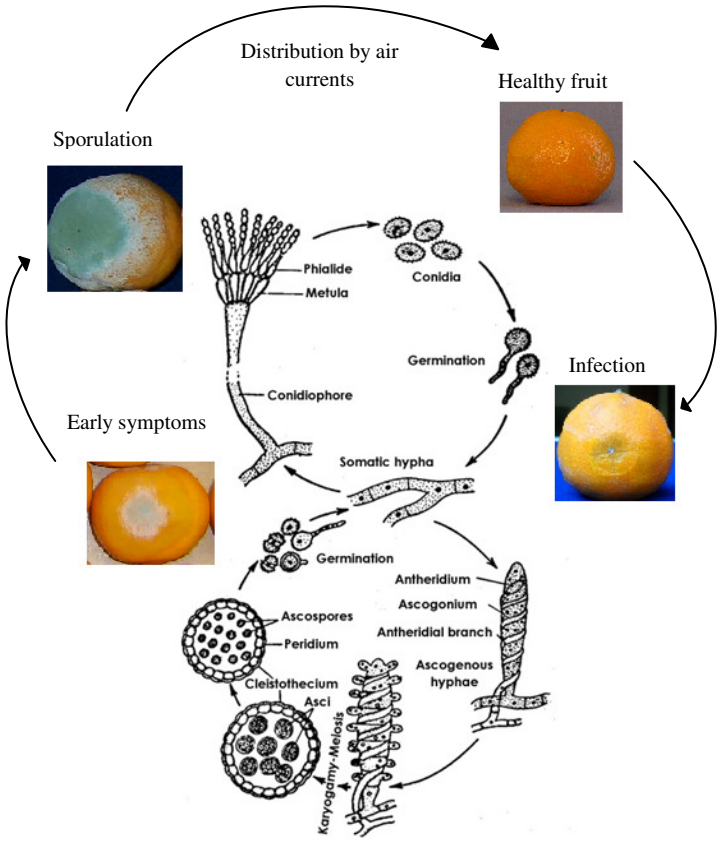

Figure 2.1: Disease cycle of green and blue mould in citrus fruit. Life cycle of *Penicillium* spp. retrieved from http://www.fao.org/inpho/.

It is the air-borne conidia that are the infective propagules and they can only infect fruit through small wounds or natural openings in the epidermis (Timmer *et al.,* 2000; Agrios,

2005). A single fruit can be covered with up to two billion green or blue spores within three to five days at 25°C (Holmes and Eckert, 1995). These spores are easily dispersed when fruit are handled and will then settle on healthy fruit again. During high RH conditions, other opportunistic fungi can also invade rotten areas and form secondary infections. If the RH is low fruit will become mummified (Timmer *et al.,* 2000). *Penicillium italicum* fruit infections commonly lead to nesting or pockets of diseased fruit when fruit are stored together in picking crates or boxes (Timmer *et al.,* 2000; Manicom, 2006). Although *Penicillium* is classified as an Ascomycete, the sexual stage or sclerotoid cleistothecia as first described by Brefeld, are rarely seen and have never been observed for many *Penicillium* spp. (Onions and Brady, 1987).

The first symptoms of infection is known as the pin-hole stage and appears as soft, water-soaked areas that develop into lesions with diameters of 6 - 12mm (Fig. 2.1B). Softening of tissue is caused by the enzyme pectin transeliminase (Moss, 1987). Within 24 - 36 h at 24°C, the lesion size can increase to 40mm and also extend inwards towards the juice vesicles. At this stage white, powdery mycelium becomes apparent on the fruit's surface (Timmer *et al.,* 2000; Manicom, 2006). In the case of a *P. digitatum* infection, olive-green spores are initially produced from the centre of the lesion. A large zone of softened tissue, covered with white mycelia, surrounds the olive-green sporulating zone (Fig. 2.2D). In the case of *P. italicum* lesions, blue spores are produced and the sporulating area and is surrounded by only a thin strip of white mycelia. A large zone of softened tissue surrounds this lesion (Fig. 2.2A and C) (Timmer *et al.,* 2000; Manicom, 2006).

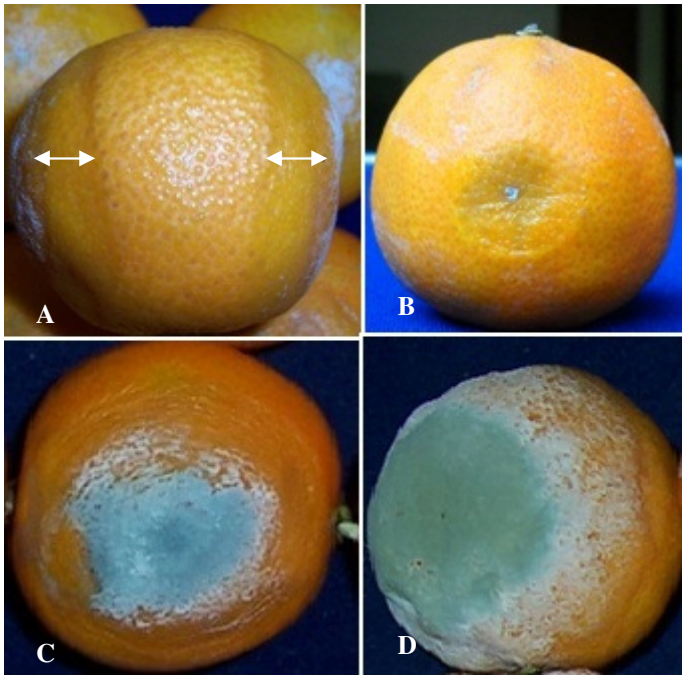

Figure 2.2: Symptoms of green (*Penicillium digitatum*) and blue mould (*P. italicum*) infections on artificially infected 'Nules' Clementine. (A) Arrows indicate soft water-soaked areas reflecting *P. italicum* infection; (B) initial stage or "Pinhole stage" of *P. digitatum* infection; advanced stages of (C) *P. italicum* and (D) *P. digitatum* infection.

2.3 Commercial postharvest practices

Apart from the postharvest practices and fungicide applications, preventative control measures also play an important role in the control of postharvest *Penicillium* decay. These control measures include proper sanitation in the packhouse and orchard, as well as careful harvesting and handling practices (Timmer *et al.,* 2000; Kruger and Penter, 2006; Manicom, 2006). It is also essential that only quality fruit are harvested, packed and stored (Korsten, 2006). Postharvest practices are applied after fruit has been harvested and includes all the steps in the food chain, including storage, transport, distribution, retail, export and import of fruit (Hewett, 2006; Likar and Jevšnik, 2006). The following section will briefly describe the standard operations of SA citrus packhouses, however, these practices vary among packhouses.

Upon arrival, the fruit receive a fungicide drench, before entering the pack-line. This drench normally contains 500µg/ml Imazalil in the form of an emulsifiable concentrate (EC), for the control of green and blue mould (Erasmus *et al.,* 2011). Citrus fruit with poor colour are then sent to the degreening chambers. Degreening involves exposing fruit to 2uL/L

ethylene and 0.3% CO_2 for 72 h at 95% RH and 23°C (Barry and Van Wyk, 2006). These conditions vary among packhouses (Grierson, 2004). Degreening of citrus increases their susceptibility to infection. Conditions in degreening chambers also favour spore germination and appressoria formation (Mukhopadhyay, 2004).

The fruit then enters the pack line, by being dumped directly into a chlorine bath. This will provide a cushioning effect upon dumping, rinse off dirt and disinfect fruit. However, if not properly chlorinated the water bath can be a source of fungal inoculum. As the fruit move along the pack line, it is sized and cleaned with brushes and a high pressure sprayer, which is disinfected with Sporekill® or chlorine. After cleaning, the fruit passes through a drying tunnel and diseased or blemished fruit are removed (Kruger and Penter, 2006).

Fungicide is applied to the fruit as a dip or spray. The fungicide dips contain Imazalil sulphate (500ppm), Guazitine at 1000ppm, CitriCure and UltraCure (Guazitine formulations) at 1000ppm and 2,4-D sodium salt (Deccomone) at 250ppm (Kruger and Penter, 2006). Imazalil is effective against green and blue mould, while Guazitine targets *G. candidum* (sour rot). The fungicide 2,4-D is applied for calyx retention and thereby reduce a site of entry for latent pathogens such as *D. natalensis* and *G. citricarpa*. Thiabendazole is also applied to control latent infections. In some SA citrus packhouses the fungicide bath is heated to 35°C for increased fungicide efficacy (Lezar, K., April 2009, pers. comm.). Various studies have reported improved efficacy of heated fungicide solutions, thereby allowing lower levels of chemicals to be used (Smilanick *et al.,* 1997; Lurie, 1998; Torres *et al.,* 2007).

After fungicide application, the fruit passes through another drying tunnel and are then waxed. Wax are applied to protect fruit from desiccation (Harris, 1988) and it also contains fungicides, including Imazalil EC at 3000ppm, Thiabendazole (Tecto 500) at 4000ppm, Guazitine (Deccowax/Citriwax) at 3000ppm and 2,4-D sodium salt at 250ppm. The fruit are once again dried, graded and sized, using electronic weight sensor technology. Fruit are then packed in boxes and transported to large distributers (Kruger and Penter, 2006). Fruit should never be packed if any free water remains on the fruit's surface, since it promotes the occurrence of fungal infections (Brecht *et al.,* 2003).

After packing, fruit may be kept in cold storage or might be transported directly to local distributers or containers for export. Commercial storing conditions for soft citrus are 3.5°C at 95% RH however greener fruit are often kept at 11°C to promote colour break.

Reaching the optimal storage temperature as soon as possible after harvest and maintaining the cold chain throughout distribution and export are crucial in retaining the quality of soft citrus (PPECB, 2007). However, citrus remains a subtropical fruit and extended periods in cold storage have negative impacts on fruit quality (Chalutz*et al.,* 1985), such as chilling injury (Burdon, 1997; Mukhopadhyay, 2004), desiccation (Harris, 1988) and general deterioration of fruit quality. Some *Alternaria* spp. and *Penicillium* spp. are also able to adapt and survive cold storage temperatures and infect the more vulnerable fruit (Mukhopadhyay, 2004). Fruit destined for the USA and other export destinations are subjected to a phytosanitary cold quarantine treatment for control of the Mediterranean fruit fly (*Ceratitis capitata*) (Porat *et al.,* 2000b). It is required that fruit be kept at -0.6°C for a minimum of 22 days before shipping (Barry and Van Wyk, 2006), which may cause chilling injury on exported citrus (Kruger and Penter, 2006).

As discussed above, synthetic fungicides are greatly relied on for the management of citrus postharvest diseases. However, apart from the health and environmental risks associated with the use of synthetic fungicides, pathogen resistance towards products such as Thiabendazole and Imazalil has been reported in citrus packhouses worldwide (Holmes and Eckert, 1999; Torres *et al.,* 2007; Usall *et al.,* 2008). In spite of all these preventative and postharvest control measures, citrus green and blue mould remain a serious postharvest problem in citrus (Shellie, 2002).

2.4 Alternative postharvest treatments

Alternative postharvest treatments for the control of postharvest fruit diseases include physical treatments, biocontrol, as well as the use of low-toxicity compounds also known as ‘soft chemicals’. Biocontrol and soft chemicals have been found effective against *Penicillium* spp. in various studies and is discussed in more detail in section 2.4.2 of this Chapter. Physical treatments against *Penicillium* decay include irradiation (D’hallewin *et al.,* 2000; Montesinos-Herrero and Palou, 2010), heat treatments (section 2.4.1) and manipulated storage conditions such as refrigeration, controlled atmosphere (CA) and modified atmosphere packaging (MAP) technology (section 2.4.3). According to Fallik (2004), heat treatments are the most promising among them.

2.4.1 Heat treatments

According to Fallik (2004), the efficacy of heat treatments against fruit decay was first reported in a study by Fawcett (1922) for brown rot in citrus. In the years to follow, heat treatments were mainly used for insect pest control. However inexpensive chemicals, such as ethylene dibromide and methyl bromide led to the replacement of heat treatments in pest disinfestations and quarantine treatments. Since the use of these chemicals has been prohibited, heat treatments are applied on some commodities. The increasing demand by consumers for pesticide-free fresh produce has also led to a renewed interest in heat treatments as alternative postharvest treatment for disease control (Lurie, 1998).

2.4.1.1 Types of heat treatments

There are three types of heat treatments: hot air, vapour heat and hot water treatments. Hot air treatments may be applied with or without humidity, with continuous or static airflow. Vapour heat is the exposure of fruit to vapour saturated air at 40 - 50°C and is used for insect disinfestations. Hot water and hot air treatments have also been found effective against fungal pathogens (Lurie, 1998). Thermal curing is the application of water saturated air at lower temperature ranges (34 - 36°C) but for longer periods of time (48 - 72 h) (Rodov *et al.,* 2000; Pavoncello *et al.,* 2001). Thermal curing is effective against citrus postharvest pathogens, however long exposure times make it impractical and reportedly, detrimental to fruit quality (Porat *et al.,* 2000b; Rodov *et al.,* 2000). According to Fallik (2004), hot water dips and hot water rinsing and brushing (HWRB) are the two most important postharvest heat treatments for fresh produce.

Hot water dips (HWD) are quick (50 - 53°C for 2 - 3 min.) and therefore more convenient than other heat treatments (Rodov *et al.,* 2000). Heat is more effectively transferred in water and therefore, shorter exposure times are required (Lurie, 1998). Hot water dips are also easier to implement into current infrastructure and they are more economical than vapour heat or hot air systems. Recent advances in postharvest heat treatments include the HWRB technology, introduced in 1996. This system is intended for incorporation into a commercial pack line for various crops. In such a system, the fruit move along a set of brushes and are exposed to a hot water rinse at 48 - 63°C for 10 - 25 s (Fallik, 2004).

2.4.1.2 Effect of heat treatments on Penicillium decay

Several postharvest heat treatments have been reported to control green and blue mould in citrus. However, research findings vary slightly regarding optimal heat treatment regimes. Table 2.1 lists the effects of some short-term heat treatments (HWD and HWRB) on *P. digitatum* and *P. italicum*. Heat treatments that reportedly resulted in damage to the fruit were not included in this table.

Table 2.1: Efficacy of postharvest heat treatments against *Penicillium digitatum* and *P. italicum* in citrus

Type of heat treatment	**Temperature**	**Exposure time**	**Effective against green- and blue mould Yes (Y)/No (N)**	**Cultivar**	**Reference**
HWD	45°C	2.5 min.	Y*	Clementine	Larriguadierre *et al.* (2002) in Fallik (2004)
HWD	45°C	2.5 min.	N	Clementine	Palou *et al.* (2002)
HWD	40 - 60°C	20s and 40s	N	Clementine	Torres *et al.* (2007)
HWD	45 or 50°C	1 or 2.5 min.	N	Clementine	Palou *et al.* (2002)
HWD	50°C and lower	2.5 min.	N	Valencia	Palou *et al.* (2001)
HWRB	52°C	10s	N (Also *A. citri*)	Oroblanco	Rodov *et al.* (2000)
HWD	52°C	2 min.	Y (Also *A. citri*)	Oroblanco	Rodov *et al.* (2000)
HWD	52°C	2 min.	Y (Also *A. citri*)	Satsuma	Hong *et al.* (2007)
HWD	52 - 53°C	2 min.	Y	Eureka	Nafussi *et al.* (2001)
HWRB	53°C	20s	N (*P. digitatum*)	Star Ruby	Pavoncello *et al.* (2001)
HWD	53°C	2 min.	Y	Marsh, Oroblanco, Eureka	Rodov *et al.* (1995)
HWD	53°C and 55°C	2.5 min.	Y	Valencia	Palou *et al.* (2001)
HWD	55°C	1 min.	N (Also *A. citri*)	Satsuma	Hong *et al.* (2007)
HWD	50 - 55°C	3 min.	Y	Fortune	Schirra and D'hallewin (1997)
HWRB	56°C	10s	Y (Also *A. citri*)	Oroblanco	Rodov *et al.* (2000)
HWRB	56°C	20s	Y (*P. digitatum*)	Minneola	Porat *et al.* (2000a)
HWRB	56°C	20s	Y (*P. digitatum*)	Star Ruby, Shamouti, Minneola	Porat *et al.* (2000a)
HWRB	59°C	20s	Y (*P. digitatum*)	Star Ruby grapefruit, Shamouti, Minneola	Porat *et al.* (2000a)
HWRB	60°C	10s	Y (Also *A. citri*)	Oroblanco	Rodov *et al.* (2000)
HWD	60°C	20s	Y (Also *A. citri*)	Satsuma	Hong *et al.* (2007)
HWRB	62°C	20s	Y (*P. digitatum*)	Star Ruby,Shamouti, Minneola	Porat *et al.* (2000a)
HWRB	62°C	20s	Y (*P. digitatum*)	Star Ruby	Pavoncello *et al.* (2001)

*Where no organism is indicated the treatment was effective or ineffective against both *P.digitatum* and *P. italicum*

Table 2.1 indicates that the lower heat treatments (lower than 50°C) were mostly ineffective against *P. digitatum* and *P. italicum* decay in Clementine. Treatments higher than this temperature, with longer exposure times (2 - 3 min.) seemed to be more effective against these pathogens overall. The HWRB treatments are effective at shorter exposure times, since the temperature range of these treatments are higher. From Table 4.1 and as reported by Palou *et al.* (2002), the heat treatment intensity required for effective control of green and blue mould is higher in Clementine than for other citrus cultivars.

2.4.1.3 Effect of heat treatments on fruit quality

Heat treatments not only have an effect on decay, but also on fruit physiology and quality. In some commodities heat treatments delay the ripening processes and in other commodities enhance it (Lurie, 1998). Heat treatments involving long exposure times (e.g. thermal curing) have been found to affect the TSS:TA ratio in fruit, while shorter treatments such as HWD and HWRB do not have a significant effect on these parameters (Porat *et al.,* 2000a and b; Rodov *et al.,* 2000; Hong *et al.,* 2007).

Heat treatments involving long exposure times are often associated with moisture loss in fruit (Schirra and D'hallewin, 1997; Porat *et al.,* 2000b). In contrast, short heat treatments can reduce moisture loss and improve the overall appearance of fruit (Rodov *et al.,* 1995; Schirra and D'hallewin, 1997; Porat *et al.,* 2000a). It has been described that the elevated temperatures cause the wax platelets to melt and evenly coat the fruit. Moisture loss in these fruit will not only be reduced, but fruit will appear shinier and possible entry sites for invading pathogens will also be blocked (Schirra and D'hallewin, 1997; Porat *et al.,* 2000a). However, the effect of heat treatments on moisture loss in fruit varies and depends on the cultivar and treatment (Hong *et al.,* 2007). Rodov *et al.* (2000) reported that combining heat treatments with Thiabendazole and plastic liners or individually sealed fruit resulted in effective control of *Penicillium* decay as well as reduced moisture loss. Therefore it may be beneficial to combine heat treatments with strategies to reduce desiccation.

The most effective heat treatment regime for a particular commodity is often very close to the temperature range that can be detrimental to its quality (Palou *et al.,* 2001).Temperatures above 54°C for 3 min. severely damaged the epicuticular wax layer and off-flavours were detected in Fortune mandarins (Schirra and D'hallewin, 1994). Mandarins in particular are more delicate and stress conditions can easily affect their flavour or aroma

(Shi *et al.,* 2005). The effect of heat treatment on fruit quality depends on various factors, including preharvest conditions, fruit type, cultivar, age and type of heat treatment regimen applied (Lurie, 1998). Heat treatments should therefore be managed very carefully.

2.4.1.4 Mode of action of heat treatments

Disinfection of fruit surfaces from fungal spores have been described as the major mode of action of heat treatments against postharvest decay (Pavoncello *et al.,* 2001; Fallik, 2003). However, Nafussi *et al.* (2001) reported that heat treatments only have a fungistatic effect on *Penicillium* conidia and that germination recurs within 48 h (Nafussi *et al.,* 2001). However, by inhibiting sporulation (Nafussi *et al.,* 2001) and reducing initial disease pressure (Hong *et al.,* 2007) heat treatments can reduce the rate of disease development throughout the storage period.

Research has also shown that heat treatments may enhance host resistance towards pathogen attack. In response to stress treatments, plants are able to launch an array of defence reactions, through process known as the hypersensitive response (HR) (Van Loon *et al.,* 1998; Agrios, 2005). The HR is a localised reaction that triggers various structural (Agrios, 2005) and biochemical processes (Lafuente *et al.,* 2001; Oostendorp *et al.,* 2001; Sala *et al.,* 2005) involved in induced host resistance. According to Polenta *et al.* (2007), Klein and Lurie (1992) reported that exposure of plant tissues to a certain stress factor will not only protect it against higher levels of the applied stress, but also against other stress factors.

Various studies have linked the HR with protection against decay (Porat *et al.,* 2000a; Pavoncello *et al.,* 2001; Zhang and Swingle, 2005) and some regard it as the major mode of action (Schirra *et al.,* 2000; Nafussi *et al.,* 2001). The type and intensity of heat treatment affects the timing and intensity of the plant's defence reactions and will determine whether infection will occur or not (Ballester *et al.,* 2006). Analysis of these biochemical reactions can be used to identify heat treatment regimes that maximise resistance. It is however important to consider the effect of these heat treatments on fruit quality as well (Polenta *et al.,* 2006 and 2007), since a so-called "cross-over" point is reached, above which the heat treatment becomes destructive rather than protective (Florissen *et al.,* 1996).

2.4.1.5 Heat shock proteins (HSP)

The HSP are synthesised in all living tissue in response to a 5 - 10°C rise in temperature, but are also found in cells under normal conditions at significantly lower levels (Polenta *et al.,* 2007). Their production can also be triggered by other stress factors such as anaerobic and chemical stress (Florissen *et al.,* 1996) and non-pathogen attack (Pavoncello *et al.,* 2001). The name "heat shock protein" is therefore a misnomer, since it is also produced in response to other stress factors.

Heat shock proteins are chaperone proteins (Promyou, *et al.,* 2008; Al-Whaibi, 2011) involved in the folding and assembly of other proteins. Apparently these proteins can also recover partially denatured proteins and remove fully denatured proteins (Sevillano *et al.,* 2009). The main function of HSP is therefore to maintain proteins in their functional form, thereby allowing normal cell functions to continue (Pavoncello *et al.,* 2001). The HSP have also been reported to assist in the export of pathogenesis-related (PR) proteins during pathogen infection (Pavoncello *et al.,* 2001) and are involved in the HR in incompatible reactions, where they have a signalling function (Garavaglia *et al.,* 2009).

The HSP are a diverse group of proteins and can be grouped in five families (Table 2.2), based on their molecular mass: the 100 kDa, 90 kDa, 70 kDa, 60 kDa (Wang *et al.,* 2004) and the small HSP (sHSP) (15-40 kDa) (Polenta *et al.,* 2007).

Table 2.2: The different heat shock protein (HSP) families and their functions

Heat shock protein (kDa)	**Function**	**Reference**
100	Constitutively produced, however increased production in response to stress. Degradation and disassembly of non-functional and harmful proteins	Wang *et al.* (2004)
90	Responsible for folding, assembling, transporting and activation of signal transduction proteins. Reducing mutation, by maintaining mutant proteins in their wild-type conformation. Necessary for the hypersensitive response (HR) occurring in non-host resistance - R gene expression are dependent on HSP90	Garavaglia *et al.* (2009)
70	Protects heat sensitive proteins from becoming permanently denatured. Necessary for the hypersensitive response (HR) occurring in non-host resistance	Garavaglia *et al.* (2009) Garavaglia *et al.* (2009)
60	Prevents aggregation of non-functional proteins, refolding of proteins, aid in cell death	Al-Whaibi (2011)
27	Microfilament stabilisation, prevents cell death	Al-Whaibi (2011)
21	Only expressed in response to and are therefore involved under temperature stress conditions	Al-Whaibi (2011)

18.1	Prevents thermal aggregation of proteins	Lee and Vierling (2000)
15-40	Highly relevant and abundant in plants where it mainly provides a chaperone function	Polenta *et al.* (2007) and Lee and Vierling (2000)

On a molecular level, the heat shock response is fundamentally regulated by heat shock factors (HSF), also known as heat stress transcription factors. These factors recognise conserved heat shock promoter elements (HSE), which in turn have their binding sites on the promoter site of HSP genes or genes related to heat stress (Morimoto, 1998).

2.4.1.6 Other compounds involved in induced host defence reactions

In addition to HSP production, the HR further leads to the activation and translocation of molecules such as phenolics (Agrios, 2005), phytoalexins (Lafuente *et al.,* 2001) and pathogenesis-related proteins (PR) (Oostendorp *et al.,* 2001). These compounds are not only antimicrobial, but are also signalling molecules that trigger activation of various systemic defence reactions throughout the plant. This process is known as systemic acquired resistance (SAR) (Agrios, 2005).

Phenolic compounds play a significant role in SAR (Ballester *et al.,* 2006). Phenylalanine ammonia-lyase (PAL), a key enzyme in the phenolic biosynthesis pathway, is highly correlated with induced defence reactions in host plants. The role of PAL was reported in protection against chilling injury in 'Fortune' mandarins (Lafuente *et al.,* 2001). Its activity can be triggered by a variety of factors, including wounding or mechanical damage (Tucker, 1993; Sala *et al.,* 2005), UV radiation (Tucker, 1993) and hydrogen peroxide (Ben-Yehoshua *et al.,* 2008), which can be sensitised by a number of chemical or microbial elicitors (Van Loon *et al.,* 1998).

The PR-proteins also play a significant role in the onset of SAR (Van Loon *et al.,* 1998; El-Ghaouth *et al.,* 2003). The most well-known PR proteins include those with β-1,3-glucanase and chitinase activity (Kauffmann *et al.,* 1987; Collinge *et al.,* 1993). These enzymes are able to break down chitin and β-1,3-glucan, which are the major constituents of fungal cell walls (Kauffmann *et al.,* 1987). A HWRB treatment at 62°C for 20 s caused an increase in HSP, chitinase and β-1,3-glucanase activity in 'Star Ruby' grapefruit, which further increased the fruit's resistance towards *P. digitatum* infection. However, according to the authors the main mode of action was removal of spores from fruit surfaces (Pavoncello *et al.,* 2001).

Studies suggest that heat treatments should be applied in combination with other postharvest treatments to improve persistence and efficacy. The combination of sodium carbonate and bicarbonate (Palou *et al.,* 2001; Palou *et al.,* 2002) and biocontrol (Torres *et al.,* 2007) in combination with HWD have shown synergism and effective control of green and blue mould in Clementine specifically. Such combinations may also reduce the effective intensity of the applied heat treatment, as well as the risk of rind damage to the fruit (Palou *et al.,* 2001). However, modelling the physiological responses of fruit to different treatment regimes is still lacking and is necessary for successful incorporation of heat treatments into IDM programs (Lurie, 1998).

2.4.2 Biological control

Biological control or biocontrol of plant diseases is defined by Cook and Baker (1983) as "*the reduction of the amount of inoculum or disease-producing activity of a pathogen accomplished by or through one or more organisms other than man*". This definition includes the use of antagonistic microorganisms, cross-protection, genetic manipulation and practices that induce host resistance. Biocontrol agents (BCA) or antagonists are living organisms that have the ability to kill, inhibit growth or reduce disease producing processes of the target pathogen. There are four main mechanisms by which BCA antagonise plant pathogens: antibiosis, competition, parasitism or inducing resistance in the plant hosts. Some organisms exhibit a combination of these mechanisms (Zhou *et al.,* 2007). Biocontrol of plant diseases mostly employ the use of microorganisms as antagonists, while insect pests are commonly combated with other predatory insects (Cook and Baker, 1983).

Postharvest applications of biocontrol is especially promising since conditions such as temperature and humidity are controlled, thereby reducing the inconsistent performance often experienced with preharvest applications thereof. The target application is also more concentrated in the case of postharvest applications and is not sprayed over the whole tree as with field applications (Wisniewski *et al.,* 2007). Less extensive toxicological and environmental impact studies are required to commercialise a BCA in comparison with a new fungicide, making it cheaper to develop (El-Ghaouth *et al.,* 2000). However, according to Korsten (2006), there are certain shortcomings in SA legislation, which complicates commercialisation of biopesticides. It is also difficult to find a potential BCA, because they need to be safe, effective, economic (Gabler *et al.*, 2006) and adapted to the harsh conditions

that are experienced during cold storage and CA or MAP (Sivakumar *et al.,* 2007; Schotsmans *et al.,* 2008). It is a time consuming process, involving various screening procedures and *in vivo* bioassays (Obagwu, 2003; Havenga, 2004; Zhou *et al.,* 2007). Biocontrol also lack curative action, since the organism needs time to colonise wound sites and produce antifungal compounds (El-Ghaouth *et al.,* 2003; Obagwu and Korsten, 2003; Leelasuphakul *et al.,* 2008). Biocontrol agents are therefore not effective against quiescent infections and the level of control achieved by biocontrol alone is not comparable to those of commercial fungicides (El-Ghaouth *et al.,* 2002b).

Nonetheless, various biofungicides have been developed for the control of postharvest diseases on fruit. Aspire (*Candida oleophila* Montrocher strain 1-182, Ecogen Inc., Langhorne, PA) (Droby *et al.,* 1998) and Biosave-100 and -110 (*Pseudomonas syringae* van Hall strains ESC30 and ESC11, Ecoscience Corp., East Brunswick, NJ) are registered in the US for the control of citrus postharvest diseases (Janisiewicz and Jeffers, 1997). Avogreen® was the first registered biopesticide in SA, used for control of Cercospora spot on avocado (Korsten and De Jager, 1995; Havenga *et al.,* 1999) and Yield Plus (*Cryptococcus albidus*) for control of postharvest diseases of pome fruit (De Koch, 1998). Some products that are applied preharvest can help reduce postharvest *Penicillium* infections, e.g. the heat-tolerant yeast formulations, ProYeast-ST or ProYeast-ORG (AgroGreen) (Kurtzman and Droby, 2001; Wisniewski *et al.,* 2007). Only the BCA *Bacillus subtilis*, as well as a biocontrol mixture of the yeast *Candida saitoana* and lysozyme (Biocure or Innovacure), will be reviewed here.

2.4.2.1 Bacterial antagonist, Bacillus subtilis

The antifungal properties of *Bacillus* spp.are well established and the use of *Bacillus subtilis* (Ehrenberg) Cohen to control postharvest fruit diseases was first studied by Pusey and Wilson (1984), against brown rot of stone fruit (Pusey and Wilson, 1984). The species of *Bacillus* are Gram-positive, endospore-forming bacteria, of the family Bacillaceae under the domain Eubacteria (Cook and Baker, 1983). These bacteria occur abundantly and saprophytically in the soil (Chaurasia *et al.,* 2003), as well as on the phyllo- and fructoplane (Janisiewicz and Korsten, 2002). Endospores are the survival structures of this organism and these are resistant to high and low temperatures, UV irradiation, desiccation and organic solvents (Emmert and Handelsman, 1999; Leelasuphakul, 2008), making it more adaptable to conditions that usually prevail during cold storage of fruit. These characteristics also make

the organism suitable for stable, powder formulations (Emmert and Handelsman, 1999; Ongena and Jacques, 2007). The endospores and vegetative state of *B. subtilis* have shown antifungal activity towards *Pencillium* spp. (Leelasuphakul *et al.,* 2008).

Antibiosis is the main antimicrobial mechanism of the *Bacillus* spp. (McKeen *et al.,* 1986; Spadaro and Gullino, 2004). A wide range of antimicrobial compounds are produced by *Bacillus* spp. and of these the lipopeptides, or antibiotics, are the most important (Spadaro and Gullino, 2004; Touré *et al.,* 2004; Leelasuphakul *et al.,* 2008). The lipopeptidesare highly diverse, but can be divided into three main families: surfactin, iturin and fengycin (or plipastatin). Compounds from all three families are produced by *B. subtilis,* and each have different functions and target organisms (Ongena and Jacques, 2007). The general mechanism behind the antimicrobial activity of lipopeptides is to alter the lipopeptide composition of cell membranes of pathogens and therefore their permeability (Chaurasia *et al.,* 2005). Effects of these antibiotics on antagonised fungi can be seen as swelling of mycelia and irregular shaped spores (Leelasuphakul *et al.,* 2008). Inhibition of conidia formation and deformation of mycelia have also been reported for various plant pathogens (Chaurasia *et al.,* 2005). The surfactin and fengycin type lipopeptides can also act as elicitors of induced host defense reactions (Ongena and Jacques, 2007) or SAR.

Apart from the lipopeptides, *B. subtilis* also produces antifungal enzymes such as chitinase and β-1,3-glucanase and antifungal volatiles, also known as volatile organic compounds (VOC) (Leelasuphakul *et al.,* 2008). The VOC are effective against cyanobacteria, protozoa, plants, as well as some fungi and bacteria (Fiddaman and Rossal, 1993). According to Leelasuphakul *et al.* (2008), the effect of the antifungal enzymes and VOC are insignificant in comparison to the lipopeptides, however these antifungal compounds are involved in the SAR in host plants (Fiddaman and Rossal, 1993). In contrast, Obagwu (2003) reported that the efficacy of various *B. subtilis* strains against green and blue mould in Valencia was not due to antibiotic production and that other compounds or factors presented the major mode of action.

Various *B. subtilis* strains have been found successful against green mould in mandarins (Leelasuphakul *et al.,* 2008), Valencia (Obagwu and Korsten, 2003) and gray mould in apple (Touré *et al.,* 2004). Although many studies report success with *B. subtilis* against postharvest pathogens *in vitro,* similar results are not always observed during semi-

commercial trials (Korsten *et al.,* 1993; Obagwu, 2003; Havenga, 2004) and therefore more research is necessary on the formulation of biocontrol products.

2.4.2.2 Candida saitoana and biocontrol cocktails

Yeasts are unicellular fungi that are able to colonise and proliferate in a wide variety of ecological niches, due to their adaptability to different carbon sources. Most are saprophytic mesophiles, although some can be pathogenic and endure extreme temperatures (Deak, 2006). Yeasts antagonists have also been reported in numerous biocontrol studies (Castoria *et al.,* 1997; El-Ghaouth *et al.,* 2000; Schisler *et al.,* 2011). Competition is reportedly the main mode of action, however antifungal hydrolases such as chitinase and β-1,3-glucanase have also been reported to be involved (El-Ghaouth *et al.,* 2003). Furthermore, yeast antagonists have reportedly been able to induce host defence reactions (Castoria *et al.,* 1997; El-Ghaouth *et al.,* 1998 and 2003), as well as parasitise on fungal hyphae (El-Ghaouth *et al.,* 1998; Pimenta *et al.,* 2010).

Various studies reported success with the use of *Candida* spp. against postharvest diseases of fruit. *Candida saitoana* Nakasa and Suzuki was found to reduce gray mould in apples (El-Ghaouth *et al.,* 2003). *Candida sake* (CPA-1) and *C. saitoana* have shown success in the control of postharvest *Penicillium* rots (El-Ghaouth *et al.,* 2000; Usall *et al.,* 2001). *Candida pelliculosa* was found effective against grey mould on tomato (Dal Bello *et al.,* 2008). As mentioned, *C. oleophila* 1-182 have been developed into the commercial postharvest biocontrol product, Aspire (Droby *et al.,* 1998) and is registered for use in the US against *Botrytis* spp.and *Penicillium* spp. on citrus and pome fruit.

"Biocontrol cocktails" have been patented by El-Ghaouth *et al.* (2002a) and involves the use of various enzymes, biomolecules, as well yeast and bacterial genera listed in the patent, in different combinations with one another. According to the authors, the efficacy of the yeast *C. saitoana* against *P. digitatum* infections in citrus could be increased by the addition of the antifungal enzymes lysozyme and lyticase. Disease control comparable to Imazalil could be obtained with the *C. saitoana* ($1x10^8$ cfu/ml) and lysozyme (0.01%) combination against natural infections of citrus (El-Ghaouth *et al.,* 2002a). This combination was formulated into the product Innovacure by Neova™ Technologies Inc. (Peardonville Road, Abbotsford BC, Canada). The formulation is also known as Biocure and may also contain sodium bicarbonate (Wisniewski *et al.,* 2007). In addition to biocontrol cocktails,

biocontrol combinations with physical and chemical treatments have also been found highly effective against various postharvest fruit diseases (Janisiewicz and Conway, 2010).

2.4.3 Modified atmosphere packaging

Fruit is packaged for ease of handling, to protect them from distribution and storage factors and to provide information about the product it contains. Packaging is designed to the crop's requirements and should allow adequate ventilation (Burdon, 1997). Citrus is conventionally packaged in Telescopic and open style cartons (Kruger and Penter, 2006). As mentioned, desiccation is a major problem in exported citrus. Moisture loss of 5 - 10% can render a fruit commercially unacceptable (Tucker, 1993). Furthermore, moisture loss leads to rapid fruit quality deterioration since ripening and deterioration are triggered by water loss (Chien *et al.,* 2007). Plastic liners and wax coatings are normally used to reduce moisture loss in citrus, however waxes and coatings often result in poor flavour due to the build-up of fermentative metabolites (Obenland *et al.,* 2008). Rodov *et al.* (2000) reported that plastic liners are more successful than wax in reducing moisture loss, but that decay may be enhanced by the humid conditions inside these plastic liners. Modified atmosphere packaging (MAP) is not only effective against moisture loss (Sivakumar and Korsten, 2006), but may also have an effect on postharvest pathogens (Shellie, 2002). Nevertheless, this technology has not been used commercially for citrus fruit yet (Murata, 1997).

According to Schotsmans *et al.* (2008), the effects of controlled atmosphere (CA) and MAP conditions on retention of fruit quality have been realised first in the experiments by Kidd and West (1927). The potential of MAP as quarantine treatment for control of insects and postharvest pathogens had only been discovered much later, in experiments by Morgan and Gaunce (1975) (Schotsmans *et al.,* 2008). Controlled atmosphere and MAP technology are based on modification of the gas composition surrounding fruit, in packages or chambers. In the case of MAP, a selective barrier can manipulate the movement of O_2 and CO_2 through the packaging in such a manner that O_2 levels are slightly lowered, while CO_2 levels build up. This leads to reduced ethylene production and sensitivity (Burdon, 1997) and an overall reduction in the respiration rate of fruit. Moisture loss is also reduced and subsequently, overall fruit quality is maintained (Chien *et al.,* 2007). Fruit are also protected from bruising and contamination with dirt and pathogen inocula (Serry, 2010).

As mentioned, the gas composition within the MAP not only affects the physiological processes of the fruit itself, but also that of pests and pathogens (Shellie, 2002). Oxygen concentrations of 3 - 4 kPa and lower restrict most fungal growth. When fruit are transferred back to room temperature, lesions that develop are smaller, since initial infection was delayed (Brown, 1992). According to Shellie (2002), *P. digitatum* growth was inhibited by O_2 concentrations lower than 0.05 - 0.1 kPa, however growth resumed once levels reached 0.25 kPa. These conditions are quite anoxic for most fungi. It was however found that in cold storage, the insecticidal and antimicrobial effects of CA or MAP was due to the high CO_2 levels and build up of carbonic acid, rather than the lowered O_2 levels. Since low temperatures are associated with low respiratory rates, O_2 demand is lower and CO_2 can build up. This in turn leads to the build-up of carbonic acid, which can lead to interference with essential metabolic pathways (Schotsmans *et al.,* 2008).

However, citrus is a non-climacteric fruit, therefore high CO_2 levels do not have a significant inhibitory effect on the fruit's respiration rate (Murata, 1997). Further, excessive CO_2 and low O_2 concentrations inside the packaging can activate anaerobic respiration processes (Sivakumar and Korsten, 2006), which can lead to the accumulation of fermentation products such as ethanol and acetaldehyde (Murata, 1997). Citrus and particularly mandarins are very sensitive to anaerobic stress and the development of fermentative metabolites or off-flavour volatiles (Murata, 1997; Obenland *et al.,* 2008). Apart from poor taste, ultra-low O_2 levels caused rind breakdown in 'Rio Red' grapefruit after the fruit was transferred to normal air. Nelson (1933) in Shellie (2002) described this disorder as "brown spot" or "storage spot". However, these conditions had no significant effect on TA, TSS, or fruit colour (Shellie, 2002).

Nevertheless, many studies have focused on CA and MAP technology, since it is a cheap and easy treatment for reducing moisture loss and extending shelf life in cold stored fruit (Sivakumar and Korsten, 2006). Some studies have also reported success with MAP (Murata, 1997; Sivakumar *et al.,* 2007; Serry, 2010), shrunk seal-packaging (Rodov *et al.,* 2000) and edible coatings (Chien *et al.,* 2007) in reducing decay and deterioration of fruit quality in various citrus cultivars. Anaerobic treatments are also investigated for its potential as a pest quarantine treatment (Shellie, 2002). It is however essential that the MAP isperfectly suited to the specific commodity, since it can be detrimental to the product if extreme gas compositions develop in the packaging (Brecht *et al.,* 2003).

Modified atmosphere packaging is usually selected according to the levels of O_2 and CO_2 that a particular commodity can tolerate. It is known as the anaerobic compensation point (ACP) or the lowest oxygen limit (LOL) of the particular product, i.e. the minimum O_2 concentration at which the fruit can be stored without inducing fermentation (Brecht *et al.,* 2003). It will depend on the respiration rate of the fruit and its maturity level, the perforation size of the packaging (i.e. the rate of gas exchange) and the optimal storage temperature of the fruit (Burdon, 1997). According to Zhang *et al.* (2008), the recommended MAP conditions for citrus are 5 - 10% O_2 and 0 - 4.5% CO_2. For Satsuma mandarins an O_2 concentration of 8 - 12% and a CO_2 concentration of 0 - 2% are recommended (RH 83 - 90%; 1 - 4°C) (Murata, 1997).

The performance of MAP will also depend on the initial fruit quality and ability to maintain the cold chain. Diseased or damaged fruit will respire at different rates than intact, healthy fruit. Fluctuating temperatures will influence the gaseous atmosphere inside the packaging. This is because the packaging material and the respiration rate of the fruit are affected by fluctuating temperatures (Brecht *et al.,* 2003; Zhang *et al.,* 2008). This may lead to gas compositions that favour growth of pathogens and are detrimental to the fruit (Brecht *et al.,* 2003). Fluctuating temperatures further cause condensation, which promotes fungal growth. Modelling of the fruit's respiration rate, its LOL or rate of CO_2 production at fluctuating conditions will further aid in selecting the most suitable MAP (Brecht *et al.,* 2003).

Controlled atmosphere and MAP conditions can also influence the survival and efficacy of BCA (Schotsmans *et al.,* 2008). According to Sivakumar *et al.* (2007), CO_2 concentrations above 10% affected the performance of the bacterium *B. subtilis*, while temperature did not have a significant effect on the organism's efficacy. *Bacillus subtilis* is an aerobic organism, but is able to grow under anaerobic conditions. However, under low O_2 conditions it has to compete with yeasts and other anaerobes (Sivakumar *et al.*, 2007). Yeast growth is stimulated by high CO_2 levels (Karabulut and Baykal, 2004) and yeast antagonists are therefore more likely to survive under MAP conditions.

Modified atmosphere packaging can easily be combined with other postharvest treatments (Sivakumar and Korsten, 2006; Sivakumar *et al.,* 2007). However, only when the MAP is suited to the LOL or ACP of a particular commodity under various conditions, will it add value to an IDM program. Since citrus is sensitive to anaerobic stress, the possibility of

incorporating ethylene and acetaldehyde absorbing properties in the film as discussed by Murata (1997), should perhaps be further explored, in order to reduce the development of off-flavours.

2.4.4 Integration of treatments

For an alternative postharvest treatment to replace the synthetic fungicides currently used on a commercial scale, it will have to exhibit nearly complete curative and protective control of postharvest diseases. No alternative control measure achieved comparable success up to now (Conway *et al.,* 2007). Combinations of treatments have shown more promise (Burdon, 1997). They complement one another and thereby provide more consistent control (Conway *et al.,* 2007). The use of more complex control strategies also reduce the risk of resistance building up in pathogen populations (El-Ghaouth *et al.,* 2002b).

The 'hurdle effect' was first introduced by Leistner in 1978 and is the application of hurdles or treatments to fresh or minimally processed food in order to reduce decay, maintain microbial safety as well as overall quality and taste. The main hurdles applied in such an approach are: temperature, pH, water activity, redox potential, preservatives and biocontrol agents. Hurdle technology can be developed for each commodity and involves the strategic selection of different treatments (Leistner, 2000). Various integrated programs have been successful in reducing *Penicillium* decay and maintaining fruit quality in citrus. Such integrated studies include the combination of physical treatments (Montesinos-Herrero and Palou, 2010), edible coatings (Valencia-Chamoro *et al.,* 2011) or biocontrol (El-Ghaouth *et al.,* 2002) with GRAS compounds; heat treatments with sodium bicarbonate and biocontrol have also been investigated by various studies (Palou *et al.,* 2001 and 2002; Obagwu and Korsten, 2003; Torres *et al.,* 2007; Usall *et al.,* 2008); heat treatments and plastic packaging (Rodov *et al.,* 2000); ethylene and 1-MCP, as well as HWD's with Imazalil (Smilanick *et al.,* 1997).

2.5 Conclusion

The aim of this project was to combine a pre-storage heat treatment, with a suitable MAP type and biocontrol product to reduce *Penicillium* decay and maintain overall fruit quality (Fig. 2.3). According to Wisniewski *et al.* (2007), the *C. saitoana* based product

Innovacure or Biocure provides good eradicative control. In addition, HWD disinfects fruit surfaces, has a fungistatic effect on pathogen inoculum and could possibly induce host defence reactions. The BCA *B. subtilis* and *C. saitoana* are able to colonise the fructoplane and exhibit antagonism towards fungal pathogens throughout the storage period. Modified atmosphere packaging reduces desiccation and maintains fruit quality, which will increase overall resistance in fruit towards postharvest pathogens.

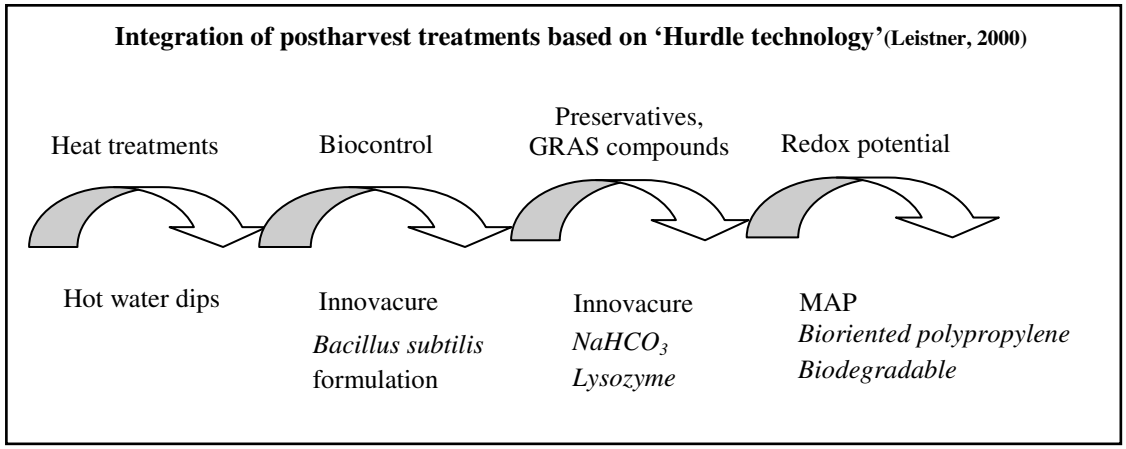

Figure 2.3: A schematic representation of a proposed integrated disease management (IDM) program for improving the storability of Clementine mandarins, according to the 'Hurdle technology' approach as described by Leistner (2000).

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CHAPTER 3

Effect of hot water dips on defence reactions in Clementine mandarins

Abstract

Postharvest heat treatments have reportedly been successful in triggering host defence reactions in fruit. Heat shock proteins (HSP) are involved in this process and were therefore used as marker molecules to find optimal hot water dip (HWD) treatments for Clementine mandarins. The enzyme phenylalanine ammonia-lyase (PAL) and phenolic compounds are also involved in host defence reactions and their response to the different HWD was also monitored. 'Nules' Clementine from two different growing seasons (2009-2010) and production areas (Western Cape and Eastern Cape) were exposed to a range of HWD treatments. Peel samples were taken throughout the storage period and induced HSP production was detected using gel electrophoresis and Western Blotting. According to the results, a HWD at 52°C for 5 min. allowed maximum elicitation of 70 kDa HSP production in 'Nules' Clementine, without damaging the fruit. However, HWD regimes exceeding 55°C resulted in peel damage. Results further indicated that the enzyme PAL was also involved in the heat shock response and that increased HSP production was associated with reduced PAL activity.

3.1 Introduction

Host plants have a range of natural defence mechanisms to protect them against environmental stress factors and pathogen attack (Oostendorp *et al.,* 2001). Owing to the growing interest in more environmentally friendly treatments for managing postharvest diseases of fruit, research on inducing host defence reactions to protect them against pathogen attack has increased (Schirra *et al.,* 2000). A variety of elicitors have been successful in inducing host resistance (El-Ghaouth *et al.,* 2002; Liu *et al.* 2005; Badawy and Rabea, 2009), including heat treatments and in particular HWD. Many regard the major mode of action of heat treatments to be the removal of pathogen inocula and/or inhibition of spore germination on fruit surfaces (Pavoncello *et al.,* 2001; Fallik, 2004). However, others regard induced host

resistance to be an important mechanism of action as well (Schirra *et al.,* 2000; Nafussi *et al.,* 2001). Various studies have linked HWD with induced host defence reactions and protection against decay (Pavoncello *et al.,* 2001; Zhang and Swingle, 2005) and chilling injury (Sabehad *et al.,* 1996; Porat *et al.,* 2000).

In order to find the correct heat treatment regime for a particular commodity, the treatment intensity needs to be determined at which host defence reactions are maximised without irreversibly damaging the fruit (Polenta *et al.,* 2007). The exact way in which heat treatments affect host defence reactions is not well understood. However pathogenesis related (PR) proteins (e.g. chitinases and β-1,3-glucanases) (Romero *et al.,* 2006) and heat shock proteins (HSP) (Polenta *et al.,* 2007) are known to be involved. The HSP are synthesised in all living tissue in response to a 5 - 10°C rise in temperature. These proteins are however also found in cells under normal conditions, but at significantly lower levels (Polenta *et al.,* 2007). Heat shock protein production can reportedly also be triggered by other stress factors such as anaerobic and chemical stress (Florissen *et al.,* 1996), as well as non-pathogen attack (Pavoncello *et al.,* 2001). The HSP are chaperone proteins (Promyou, *et al.,* 2008; Al-Whaibi, 2011) and their main function is to maintain enzymes and proteins in their functional form (Pavoncello *et al.,* 2001). These proteins have also been reported to assist in the export of PR-proteins during pathogen infection (Pavoncello *et al.,* 2001) and are also involved in the hypersensitive response in incompatible reactions, where they have a signalling function (Garavaglia *et al.,* 2009).

In addition to HSP production, other compounds such as phenolics (Agrios, 2005), phytoalexins (Lafuente *et al.,* 2001) and PR-proteins (Oostendorp *et al.,* 2001) are also activated in response to stress factors. These compounds are not only antimicrobial, but are also signalling molecules that trigger various systemic defence reactions throughout the plant. This process is known as systemic acquired resistance (SAR) (Oostendorp *et al.,* 2001). Phenolic compounds play a significant role in SAR (Ballester *et al.,* 2006). Phenylalanine ammonia-lyase (PAL) is a key enzyme in the phenolic biosynthesis pathway and its activity is correlated with induced defence reactions in host plants (Ben-Yehoshua *et al.,* 2008).

The aim of this chapter was to find an optimal HWD regime for 'Nules' Clementine that induces host defences without damaging the fruit. The 70 kDa HSP was selected as biochemical marker for detecting induced defence reactions, because it is known to be involved in the heat shock response in plants (Lee and Vierling) and citrus in particular

(Garavaglia *et al.,* 2009). In addition, the effect of HWD treatment on total phenolic content (TPC) and PAL activity were also investigated, since these molecules also play a role in host defence reactions.

3.2Materials and methods

Fruit

Disease- and blemish-free 'Nules' Clementine fruit of the 2009 and 2010 seasons were received from the Kirkwood area in the Eastern Cape (EC) as well as the Citrusdal and Piketberg areas in the Western Cape (WC). Fruit from the EC were received earlier (April), while fruit from the WC were received later in the season (June). Fruit from different areas were used for different experiments. The fruit were harvested at commercial maturity and did not undergo any postharvest treatments such as degreening or fungicide dips. The fruit were transported to the University of Pretoria at ambient temperature within 48h after harvest.

Determining the time of peak heat shock protein production

This trial was conducted during 2009 and repeated in 2010 with fruit from the EC. The following heat treatment regimes were included in the 2009 trial: a) freshly harvested fruit (no heat treatment); b) 52°C for 5 min.; c) 55°C for 2 min.; and d) 60°C for 30 s. The repeat experiment in 2010 excluded the 55°C for 2 min. and 60°C for 30 s HWD regimes (c and d), since rind breakdown was observed with the highest temperature HWD (c) and reduced HSP production was detected above 52°C for 5 min. Instead, two HWD treatments at 35°C for 10 min. and 50°C for 5 min. were included. Samples were taken directly after treatment and stored at -19°C. The rest of the fruit were kept at 4°C and samples were taken at two-, four-, eight- and 14 day intervals. In total, 25 fruit were allowed per treatment and three to five tissue samples of three gram each were taken randomly from each treatment.

Heat treatment regimes that induce maximum heat shock protein production

This experiment was performed in 2010 with fruit from the WC and included the following hot water dip treatments: 25°C (control), 35°C for 10 min., 45°C for 10 min., 48°C for 7 min., 50°C for 5 min., 52°C for 5 min., 52°C for 3 min. After treatment, fruit were dipped in tap water (18°C) for ca. 10 min. to reduce pulp temperatures. The fruit were kept at

4°C and samples were taken four days after heat treatment application as described previously.

Analysis of heat shock proteins

Peel tissue samples of 3g each were homogenised in 7ml of an aqueous buffer: 100mM Tris-HCl (Merck, Johannesburg, South Africa (SA)), pH 8; 1mM EDTA (British Drug Houses Chemicals Ltd., Poole, England); 1mM PMSF (Roche, Johannesburg) and centrifuged at 10 000g and 4°C for 30 min. After centrifugation, the supernatant was filtered through large 55mm Whatman® filter paper discs (Whatman International Ltd., England). In the 2010 WC experiments an acetone precipitation step was performed according to ThermoScientific Doc. No. TR0049 (www.piercenet.com) to concentrate the protein extracts. Protein content determination was performed as described in the Lowry protein method (Lowry, 1951) and 70µg of protein were separated on a 12% SDS-PAGE gel, using the Hoefer® miniVE system (Hoefer Scientific instruments, United States of America (USA)). The separated proteins were transferred to a BioTrace™ PVDFmembrane (Pall Corporation, USA), using the Trans-Blot® SD electrophoretic transfer cell (Biorad, USA). Detection of HSP was done using the monoclonal anti-heat shock protein 70 primary and an anti-mouse IgG peroxidase conjugated secondary antibody, both from Sigma (Sigma-Aldrich, St. Louis, USA). The 1-Step™ TMB-blotting substrate (Thermo Scientific, Rockford, USA) for horseradish peroxidase was used as described in the manufacturer's instructions to visualise protein bands. All analyses were repeated at least once for confirmation.

Phenylalanine ammonia-lyase activity: Investigation of TPC and PAL activity followed the same approach as for HSP. However, only the 2010 experiments were evaluated and analysis was not performed for all time intervals. Three replicates of ten fruit each were used per treatment. The analysis of PAL activity was done according to Jiang *et al.* (2001), with minor modifications. Peel tissue was grinded in liquid nitrogen to a fine powder. Three gram of powdered peel tissue was grinded with 10 ml ice-cold acetone and filtered through a Whatman no.1 filter paper disc using a Buchner funnel. The filtered powder was left to air dry. The 0.3g of powdered peel tissue were homogenised in 15ml borate buffer pH 8.8, containing 0.074% EDTA and 35uL/100ml b-Mercaptoethanol (Merck), using the Ultra Turrax®T18 Basic (IKA®Works, Brazil), at 6000rpm and 25°C. The macerated samples were then centrifuged for 25 min. at 7000rpm and 20°C, with the Eppendorf 5804R (Merck). The supernatant was then filtered, using 55mm Whatman filter

paper discs and a filter paper disc supporter. To the remaining 100ml of borate buffer, 0.05g L-Phenylalanine (Merck) was added to make up the reaction buffer. Five hundred microlitre of the final, filtered supernatant was then transferred to clean test tubes, in triplicate. Two millilitre of the reaction buffer was also added to each tube. The tubes were incubated at 37°C for 30 min., after which the absorbance was read at 290nm, using the SPECTRAmax Plus 384 (Molecular Devices, Sunnyvale, USA).

Analysis of total phenolic content: Fruit peel tissue was sampled and extracted as described for analysis of PAL activity. To the final, filtered supernatant of the peel tissue extract, acetic acid (Merck) was added to the concentration of 2.6% of the total volume. One volume of diethyl ether (Saarchem, Wadeville, SA) was also added and the mixture was mixed well. The mixture was left to separate for a few minutes, after which the clear, top layer was transferred to clean beakers. The liquid was left to evaporate. To the evaporated samples, 100 µL methanol (Saarchem) was added to resuspend the remaining compounds. The resuspended samples were analysed by adding 175µL distilled H_2O, 25µL Folin Ciocalteu reagent (Sigma Aldrich), 50µL 20% Na_2CO_3 (Merck) and 5µL of each sample, to a separate well in a microtitre plate. After incubation at 40°C for 30 min., the samples were analysed using the Multiskan Ascent® plate reader (ThermoLabsystems, USA) at 690nm (Sivakumar *et al*., 2005).

The effect of heat treatments on external appearance or epicuticular wax layer

Random fruit samples were selected from the 2009 HWD experiment (control fruit; 52°C for 5 min.; 55°C for 2 min.; 60°C for 30 s). Peel samples were excised and suspended for 14 days in fixing solution, which consisted of 2.5% gluteraldehyde in 0.075M phosphate buffer pH 7.4 (Merck). After fixation, samples were rinsed three times in 0.075M phosphate buffer for 15 min. The samples were then fixed in 0.5% osmium tetroxide for one hour, after which samples were rinsed three times in distilled water. Dehydration of the samples was then done by placing samples in a series of ethanol solutions for 10 min. each (50%, 70%, 90%, 100%, 100%, 100%). Samples were further dehydrated using a Hitachi 840 HCP-2 critical point dryer. Dehydrated samples were then mounted on aluminium stubs and coated with gold palladium using an Eiko IB-3 ion coater. The prepared samples were viewed with the Hitachi 840 JEOL scanning electron microscope operating at 5 kV (Silimela and Korsten, 2007).

Statistical analysis

Statistical analysis for TPC and PAL activity was performed using GenStat Discovery Edition 3 software (www.vsni.co.uk). For the EC experiment, two-way analysis of variance (ANOVA) for a completely randomised design (CRD) was performed. One-way analysis for a CRD was performed for the WC experiment. Significant differences between treatment means were determined using Duncan's Multiple range test at the 5% level of significance. Significant differences were indicated with a different letter.

3.3 Results

Heat treatment regimes that induce maximum heat shock protein production

The western blots of 2009 EC experiments (Fig. 3.1) showed protein bands within the 70 kDa range, which indicates 70 kDa HSP production in these samples. The HSP production could therefore be detected directly after treatment (Day 0) in samples exposed to all three HWD. Four days after heat treatment, the 52°C for 5 min. treatment resulted in maximum HSP production, while production decreased for the higher-temperature treatments. No HSP production was visible after eight days in cold storage.

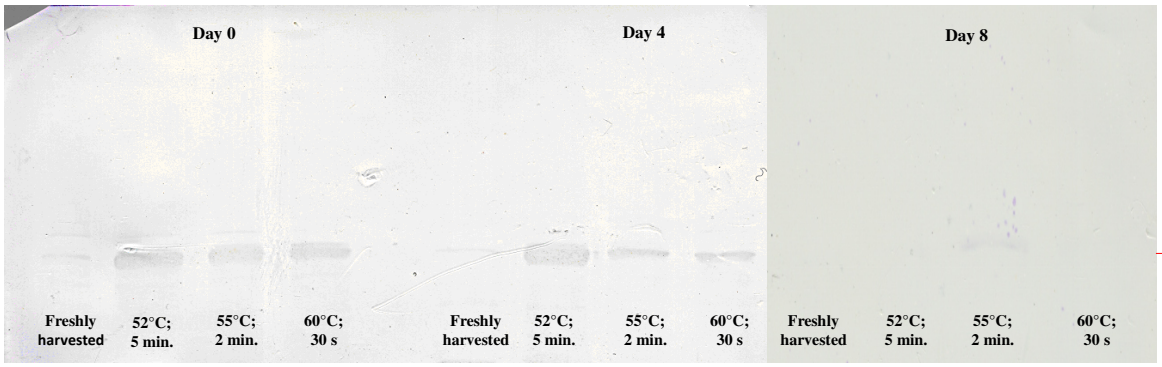

Figure 3.1: Western blot analysis to detect 70 kDa heat shock protein production in 'Nules' Clementine from the Eastern Cape (2009), in response to various hot water dip regimes.

In contrast, no bands were visible when samples were taken directly after treatment (Day 0) in the 2010 EC experiment (Fig. 3.2). A significant band was detected two days and four days after heat treatment at 52°C for 5 min. Four days after heat treatment, fruit exposed to 50°C for 5 min. also showed a feint band within the same range and after 14 days, this band had become the most prominent. A decreased intensity of the band associated with the higher-temperature treatment (52°C for 5 min.) was observed.

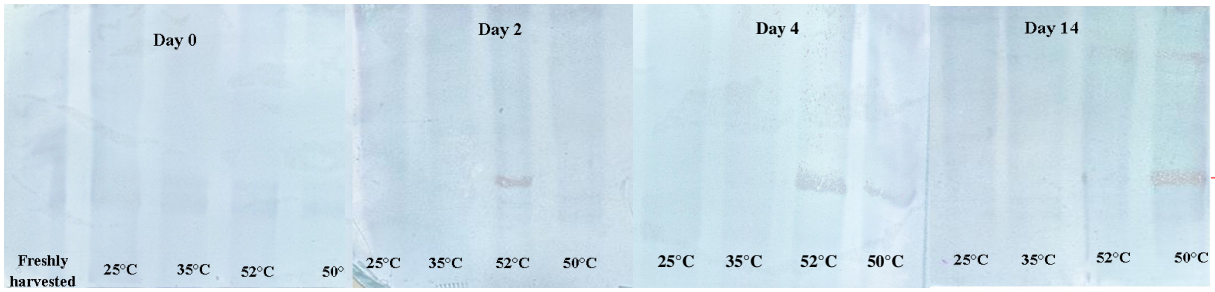

Figure 3.2: Western blot analysis to detect 70 kDa heat shock protein production by 'Nules' Clementine from the Eastern Cape (2010), in response to various hot water dip regimes. Treatments: 25°C (25°C for 10 min.); 35°C (35°C for 10 min.); 52°C (52°C for 5 min.) and 50°C (50°C for 5 min.).

Lower-intensity HWD regimes did not induce HSP production in the WC fruit. Maximum 70kDa HSP production was triggered by heat treatments higher than 50°C and more so by the 52°C for 5 min. heat treatment (Fig. 3.3). However, the 70 kDa HSP bands were less pronounced in comparison with experiments with fruit from the EC (Fig 3.1 and 3.2), even at the optimum heat treatment regimes (50-52°C for 5 min.).

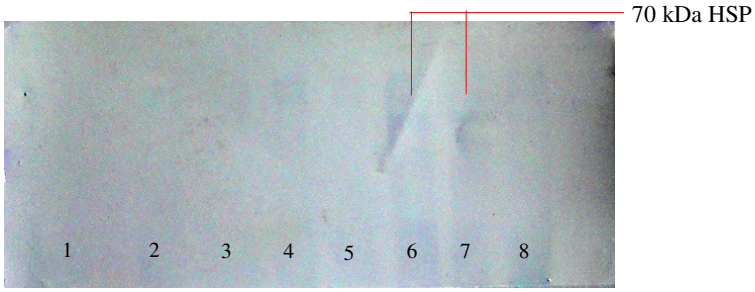

Figure 3.3: Western blot analysis to detect 70 kDa heat shock protein production in 'Nules' Clementine from the Western Cape (2010), in response to various hot water dip regimes. Lane 1: Untreated fruit; lane 2: 25°C for 10 min. (negative control); lane 3: 35°C for 10 min.; lane 4: 45°C for 10 min.; lane 5: 48°C for 7 min.; lane 6: 50°C for 5 min.; lane 7: 52°C for 5 min.; lane 8: 52°C for 3 min.

Phenylalanine ammonia-lyase activity and total phenolic content

Results indicated that PAL and TPC for each treatment did not fluctuate significantly throughout the storage period (data not shown). Therefore, average values for PAL activity and TPC are given over the entire storage period. Peel samples exposed to 52°C for 5 min. showed significantly higher PAL activity in comparison to other HWD (Fig. 3.4). In contrast, TPC was lowest upon exposure to 52°C for 5 min. HWD however none of the treatments differed significantly from the 25°C control treatment (Fig. 3.5).

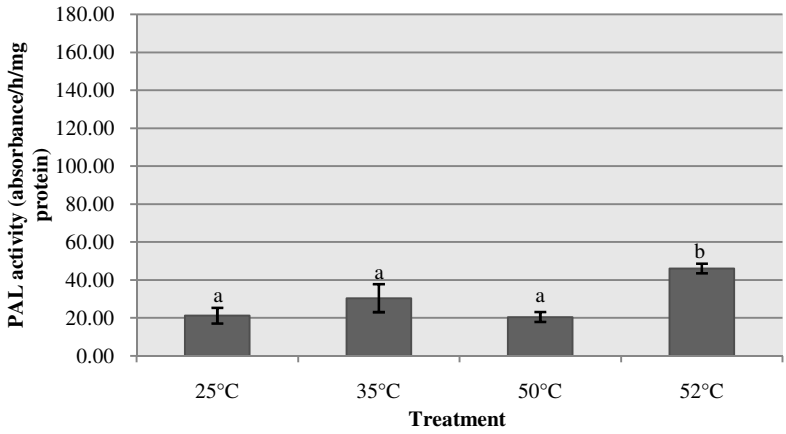

Figure 3.4: Phenylalanine ammonia-lyase (PAL) activity in 'Nules' Clementine from the Eastern Cape (2010), in response to various hot water dip treatments. Treatments: 25°C (25°C for 10 min.); 35°C (35°C for 10 min.); 50°C (50°C for 5 min.); 52°C (52°C for 5 min.). Columns with the same letter do not differ significantly with respect to PAL activity, according to Duncan's Multiple range test at the 5% level of significance.

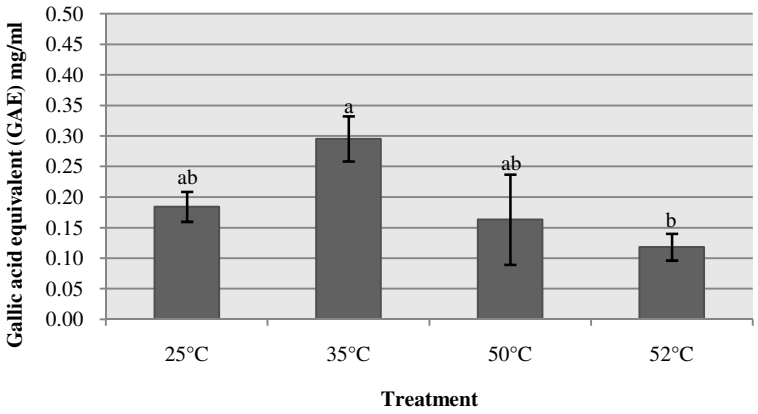

Figure 3.5: Total phenolic content (TPC) in 'Nules' Clementine from the Eastern Cape (2010). In response to various hot water dip treatments. Treatments: 25°C (25°C for 10 min.); 35°C (35°C for 10 min.); 50°C (50°C for 5 min.); 52°C (52°C for 5 min.). Columns with the same letter do not differ significantly with respect to TPC, according to Duncan's Multiple range test at the 5% level of significance.

In the WC trials, PAL activity was not significantly affected by increasing temperatures (Fig. 3.6), whereas TPC increased as the HWD temperature increased (Fig. 3.7).

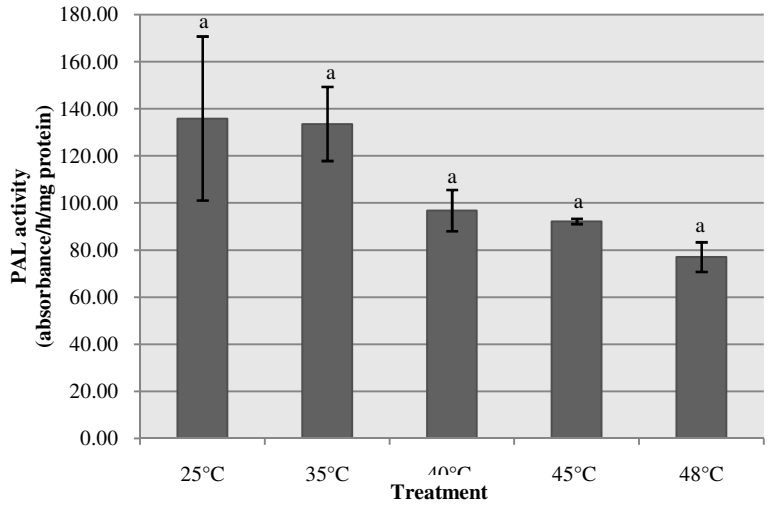

Figure 3.6: Phenylalanine ammonia-lyase (PAL) activity in 'Nules' Clementine from the Western Cape (2010), in response to different hot water dip treatments. Treatments: 25°C (25°C for 10 min.); 35°C (35°C for 10 min.); 40°C (40°C for 10 min.); 45°C (45°C for 10 min.); 48°C (48°C for 7 min.). Columns with the same letter do not differ significantly with respect to PAL activity, according to Duncan's Multiple range test at the 5% level of significance.

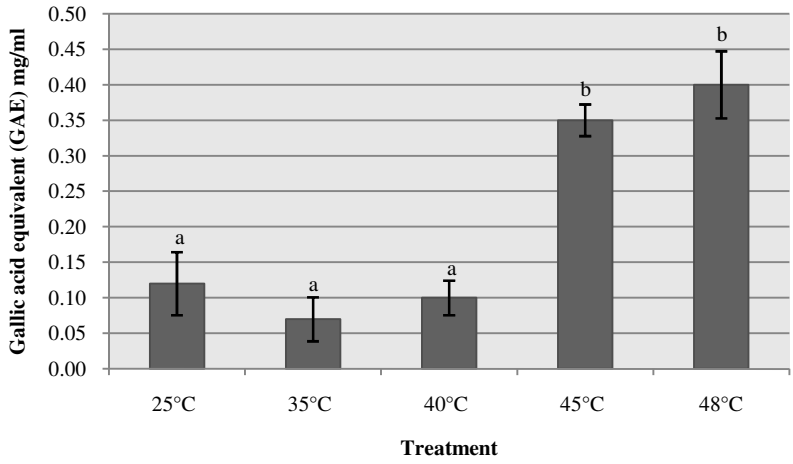

Figure 3.7: Total phenolic content (TPC) in 'Nules' Clementine from the Western Cape (2010), in response to different hot water dip treatments. Treatments: 25°C (25°C for 10 min.); 35°C (35°C for 10 min.); 40°C (40°C for 10 min.); 45°C (45°C for 10 min.); 48°C (48°C for 7 min.). Columns with the same letter do not differ significantly with respect to TPC, according to Duncan's Multiple range test at the 5% level of significance.

The effect of hot water dips on external appearance and epicuticular wax layer

Rind breakdown was observed only in fruit subjected to 60°C for 30 s (Fig. 3.8). No damage was observed at lower temperature ranges, even with longer exposure times (e.g. 50-52°C for 5 min.).

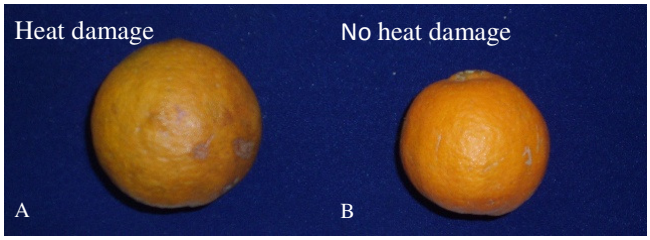

Figure 3.8: Effect of hot water dips on external appearance of 'Nules' Clementine. Heat damage was observed in fruit subjected to the highest heat treatment regime (A). A: 60°C for 30 s; B: 55°C, 2 min.

Electron micrographs of peel tissue subjected to 60°C for 30 s showed dark areas on the peel surface. Upon further magnification, a disrupted epicuticular wax layer was observed (Fig. 3.9 A and B), while lower-heat treatment regimes showed normal distribution of wax platelets (Fig. 3.9 C and D).

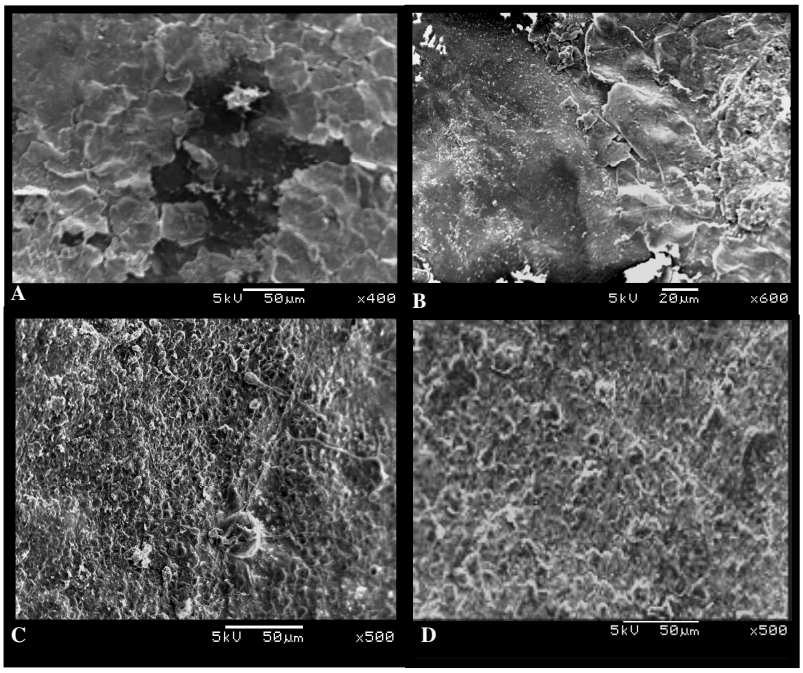

Figure 3.9: Scanning electron micrographs of peel sections of 'Nules' Clementine subjected to different hot water dip regimes: (A and B) 60°C for 30 s; (C); no heat treatment (D) 55°C for 2 min.

3.3 Discussion

Heat shock proteins are produced constitutively in all living tissues at low levels. However, an increase in production can occur upon exposure to different stress treatments (Polenta *et al.,* 2007). This explains why a feint 70 kDa HSP band was initially visible in freshly harvested fruit during the 2009 experiment with fruit from the EC. Some inconsistency exists regarding the time of peak HSP production. According to Gurley (2011) and Garavaglia *et al.* (2009), the heat shock response peaks one to two hours after treatment exposure. However, Pavoncello *et al.* (2001) and Sabehat *et al.* (1996) reported maximum 70 kDa HSP production between one day and four days after heat treatment, followed by a rapid decline in HSP production. Variability in the time of HSP production was also observed in this study. During the 2009 experiment, all of the HWD regimes elicited HSP production directly after treatment. However, this was not observed during the following season. This variation in timing could be attributed to the fact that milder treatments were applied during the 2010 experiments in comparison to the previous year. Seasonal differences could also have played a role in the variation in heat shock response in these two experiments (Wang *et al.,* 2004; Polenta *et al.,* 2007). Regardless of this difference in timing, HSP production peaked four days after exposure to a HWD at 52°C for 5 min. in both seasons.

Results further indicated that the 60°C for 30 s HWD resulted in peel damage and are therefore not recommended for Clementine mandarins. Maximum HSP production was observed for heat treatments higher than 50°C and more so by the 52°C for 5 min. heat treatment. The range of HWD regimes that maximise HSP production without damaging fruit is very narrow. According to Pavoncello *et al.* (2001), high temperatures with shorter exposure times can induce HSP production as well as reduced temperatures with longer exposure times. However, the WC 2010 experiment showed that heat treatments lower than 50°C (48°C for 7 min.; 45°C for 10 min.; 35°C for 10 min. and 25°C 10 min.) did not induce 70 kDa HSP production, even with longer exposure times. Fruit from the WC did not respond as intensely to the 52°C for 5 min. and 50°C for 5 min.treatments as fruit from the EC. This variation in heat shock response could be ascribed to different environmental conditions in the two growing areas. It must also be noted that in the EC experiments pulp temperatures were not reduced directly after heat treatment application, which could have altered the heat shock response of these fruit. Therefore, if heat treatments are to be used on a commercial scale, designs of processing lines will need to be modified to properly control

heat treatment application, since small fluctuations in heat treatment application will affect the heat shock response in fruit (Saltveit, 2000).

Heat treatments had a significant effect on PAL activity and TPC in the 2010 EC trial. However, these parameters did not fluctuate significantly throughout the storage period (data not shown). Various studies have reported that the time of peak PAL activity can vary depending on the commodity, cultivar and storage conditions (El-Ghaouth *et al.,* 2003; Lafuente *et al.,* 2003; Sala *et al.,* 2005). Results from this trial further indicated that PAL activity developed concomitantly with HSP production, where a 52°C for 5 min. HWD also resulted in maximum PAL activity.

According to the results of the WC trial, PAL activity was unaffected by HWD treatment lower than 50°C. Similarly, the HSP analyses of this study revealed that these HWD regimes did not induce HSP production. Therefore, these results indicate that the enzyme PAL and the 70kDa HSP respond similarly to HWD treatments in this study. However, overall PAL activity was much lower in the EC, than in the WC trials. In contrast, overall HSP production was much more pronounced in the EC than in the WC trials. According to Saltveit (2000) and Campos-Vargas *et al.* (2005) plants respond preferentially to heat than to other stress factors. As a result, protein production is redirected towards HSP, bringing other biosynthetic systems e.g. phenylpropanoid pathway to a halt (Saltveit, 2000). This would explain why overall PAL activity was low when HSP production was most pronounced (i.e. EC trial) and vice versa. These results therefore indicate that PAL activity and HSP respond similarly to HWD treatment, however, increased HSP production is associated with reduced PAL activity.

The enzyme PAL is responsible for converting phenylalanine into phenolic compounds (Berlin and Widholm, 1977). Therefore, TPC is expected to be positively correlated with PAL activity. However, results of the EC and WC trials indicated that there was no correlation between PAL activity and TPC. Choi *et al.* (2011) reported that heat treatments may liberate low-molecular weight phenoliccompounds in citruspeels,, thereby explaining the higher TPC in peel tissue exposed to higher-heat treatment regimes in the WC trial. Differences in environmental factors, treatment applications and physiological maturity of the peel tissue could have contributed to the variation in TPC in WC and EC trials. In addition, the phenyl-propanoid pathway in which PAL is involved not only leads to the

production of phenolics, but also to other secondary metabolites such as phytoalexins and lignins (El-Ghaouth *et al.,* 2003).

3.5 Conclusion

According to the results of this study, HWD regimes above 50°C were successful in eliciting 70 kDa HSP production in 'Nules' Clementine. It was found that temperatures above 55°C resulted in peel damage, even at shorter exposure times. Variability in the heat shock response was observed, which could be attributed to differences in environmental conditions prevailing during the season, the harvesting date and/or treatment applications. Nonetheless, the optimal HWD regime could be identified as 52°C for 5 min., since it allowed maximum HSP elicitation without damaging the fruit. However, the role of increased HSP production in disease control still needs to be determined. The enzyme PAL was also involved in the heat shock response, since its reaction to HWD treatment followed a similar trend as observed for HSP in this study. However, results indicated that increased production of HSP was associated with reduced PAL activity. No correlation could be drawn between TPC and PAL activity or HSP production in this study.

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CHAPTER 4:

Biocontrol of green and blue mould in Clementine mandarins

Abstract

The efficacy and mode of action of a potential biocontrol agent (*Bacillus subtilis* PPCB002) against green- (*Penicillium digitatum*) and blue mould (*P. italicum*) pathogens of citrus was investigated. Although *in vitro* studies indicated that PPCB002 was effective against *Penicillium* pathogens through a variety of mechanisms, *in vivo* experiments with artificially inoculated fruit did not show similar efficacy. Therefore, the commercially formulated biocontrol product, Innovacure, was included in further investigations. The Innovacure formulation and/or its combination with PPCB002 successfully reduced disease incidence and severity of both pathogens. In some instances the reduction in disease was comparable to the commercial treatment. However, due to slight variability and lack in curative action at ambient temperature, it is recommended that future studies be continued with Innovacure in combination with more effective biocontrol formulations, antifungal compounds and/or other disease control strategies.

4.1 Introduction

Biocontrol has been found to be a promising alternative treatment to the use of synthetic fungicides for the control of postharvest diseases of fruit (Droby *et al.,* 1998; El-Ghaouth *et al.,* 2000; Korsten, 2004; Yu *et al.,* 2007 and Wisniewski *et al.,* 2007). However there are also various obstacles associated with the use of biocontrol products, such as variability in performance and lack of curative action (El-Ghaouth *et al.,* 2003; Obagwu and Korsten, 2003; Leelasuphakul et al 2008). Nonetheless, various biofungicides have successfully been developed for the control of postharvest diseases of fruit (Droby *et al.,* 1998; El-Ghaouth *et al.,* 2000; Torres *et al.,* 2007).

Biocontrol agents (BCA) also herein referred to as antagonists, are living organisms (Cook and Baker, 1983; Zhou *et al.,* 2007) or the product of a living process (Wisniewski *et al.,* 2007), that are able to kill or inhibit the growth of target pathogens. For the control of postharvest pathogens of fruit, antagonistic microorganisms such as yeasts and bacteria are

normally used (Cook and Baker, 1983). The bacterium *Bacillus subtilis* (Ehrenberg) Cohen have been thoroughly investigated for use as a BCA. This antagonist has been found effective against various postharvest pathogens of fruit, through the production of a wide spectrum of antimicrobial compounds (Pusey and Wilson, 1984; Leelasuphakul *et al.*, 2008; Arrebola *et al*., 2010a). Yeasts as antagonists in biocontrol have also been reported in numerous studies, but competition is reportedly their major mode of action (Castoria *et al.,* 1997; El-Ghaouth *et al.,* 2000; Karabulut and Baykal, 2003). *Candida sake* Berkh. (CPA-1) (Usall *et al.,* 2001), *C. oleophila* Montrocher (1-182) (Droby *et al.,* 1998) and *C. saitoana* Nakasa and Suzuki (El-Ghaouth *et al.,* 2000) have previously shown success in the control of postharvest *Penicillium* rots.

Biocontrol have often been reported to be inconsistent and ineffective as a stand-alone treatment (Droby *et al.,* 1998; El-Ghaouth *et al.,* 2003; Obagwu and Korsten, 2003; Leelasuphakul *et al.,* 2008). The use of "biocontrol cocktails" has been investigated and patented by El-Ghaouth *et al.* (2002) and involves the use of various enzymes, biomolecules, as well yeast and bacteria in different combinations with one another. Improved efficacy and curative action can be achieved with these combinations. Due to this multifaceted mode of action, biocontrol cocktails can be a more sustainable alternative than the use of one-directional fungicides (El-Ghaouth *et al.,* 2002).

In this study two antagonists were investigated for their efficacy against the most destructive and prevalent postharvest pathogens of citrus fruit: green (*Penicillium digitatum* (Pers.) Sacc.) and blue mould (*P. italicum* Wehmer) (Timmer *et al.,* 2000; Leelasuphakul *et al.,* 2008). The bacterial antagonist *B. subtilis* PPCB002 (PPCB002) was investigated for efficacy against these pathogens *in vitro* and possible modes of action. The yeast-based product, Innovacure, was directly included in bioassays, since the efficacy and mode of action of this product have already been investigated (El-Ghaouth *et al.,* 2002). The two biocontrol formulations were also combined to determine if a synergistic effect might exist with their combination, especially since they have different modes of action.

4.2 Materials and methods

4.2.1 Fungal pathogens

Fungal isolates of *Penicillium crustosum* Thom, *P. digitatum* and *P. italicum* were taken from the fungal culture collection of Plant Pathology laboratories, Department of Microbiology and Plant Pathology, University of Pretoria (UP) or the Agricultural Research Council, Plant Protection Research Institute (Biosystematics Division: Mycology) respectively. The isolates were grown on malt extract agar (MEA, Merck, Johannesburg, South Africa (SA)). Spore suspensions were prepared by adding 5ml of sterile, distilled water (dH_2O) containing 0.02% of Tween 80 (Associate Chemical Enterprises Pty (Ltd.), Southdale, United Kingdom (UK)) to the fungal culture. Spores were liberated with a sterile spreading rod. The concentration of the spore suspensions was adjusted to 10^6 spores/mlusing a haemocytometer.

4.2.2 Isolation, identification and dual culture experiments

Identification and mode of action studies that follow refer to *B. subtilis* PPCB002, since these investigations have previously been performed for the Innovacure formulation (El-Ghaouth *et al.,* 2002). The bacterial antagonist was initially isolated from the surface of Valencia oranges (Letaba Estates, Limpopo Province, South Africa) and was identified as a possible antagonist during preliminary screening tests (Obagwu and Korsten, 2003). The antagonist was identified as *Bacillus subtilis* by Dr. E. Arrebola-Diez at the Department of Plant Pathology (UP), according to Arrebola-Diez *et al.* (2010a). Further screenings were conducted to evaluate the efficacy of PPCB002 against *P. digitatum* and *P. italicum in vitro,* by means of dual culture assays, according toYoshida *et al.* (2001) (Fig. 4.1). The BCA and the pathogens were cultured for 7 to 14 days on the same plate, which contained potato dextrose agar (PDA) (Merck). Disease suppression was expressed as percentage inhibition according to the following formula: [(R1-R2)/R1]*100, where R1 = colony diameter of the control plate and R2 = colony diameter of the test plate. Nine replicates were included per treatment.

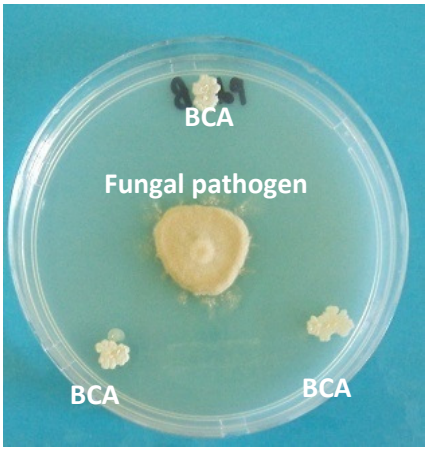

Figure 4.1: Dual culture assay method to determine the effect of diffusible substances produced by the antagonist *Bacillus subtilis* PPCB002 on *Penicillium digitatum* and *P. italicum* growth *in vitro.*

4.2.3 Mode of action of Bacillus subtilis PPCB002

Antibiotic production by *Bacillus subtilis* PPCB002

A. Sequencing the Bacillomycin gene region

The presence of genes involved in bacillomycin, iturin A and surfactine production in PPCB002, were investigated by amplification and sequencing the bacillomycin operon. Within this operon, the Bmy, ituD and lpa are key gene regions involved in the production of these antibiotics or lipopeptides. For DNA extraction, the Illustra™ Bacterial genomicPrep Mini Spin Kit (GE-Heathcare, Little Chalfont, UK) was used according to manufacturer's instructions. The following primer pairs (All from Whitehead Scientific, Johannesburg) were used to perform PCR amplification of the respective gene regions mentioned above: BmyA (f: 5' AAA GCG GCT CAA GAA GCG AAA CCC 3' and r: 5' CGA TTC AGC TCA TCG AAC AGG TAG GC 3'); ituD (f: 5' ATG AAC AAT CTT GCC TTT TTA 3' and r: 5' TTA TTT TAA AAT CCG CAA TT 3') and lpa-14 (f: 5' ATG AAA ATT TAC GGA GTA TA 3' and r: 5' TTA TAA CAG CTC TTC ATA CG 3').

The PCR reaction was performed using the GeneAmp PCR system 2400 (Applied Biosystems, Foster City, United States of America (USA)). Conditions for amplification involved an initial step at 95°C for 5 min.; followed by 35 cycles of the following conditions: 1 min. at 94°C, 1 min. at 50°C, 1.5 min. at 72°C; and a final step at 72°C for 7 min.

The resulting PCR products from each respective set of primers (either BmyA, ItuD, lpa), were purified prior to the sequencing PCR reaction, using the Invitek MSB spin

PCRapace Kit (Invitek GmbH, Berlin, Germany) and instructions was followed according to manufacturer specifications. Amplified PCR products were separated on a 1% (w/v) agarose gel (Whitehead Scientific), containing 0.01% ethidium bromide (Merck) and viewed using the ultraviolet-illuminated electrophoresis gel-documentation system (Vilber Lourmat, OmniScience, Johannesburg). A 100 base pair (bp) Hyperladder IV (Bioline, Celtic Molecular Diagnostics (Pty) Ltd., Cape Town, SA) marker was used for size comparison. The cleaned PCR products were then used for the sequencing PCR reaction using the BigDye® Terminator V 3.1 Cycle Sequencing Kit (Applied Biosystems, Warrington, UK). The PCR product was once again purified and sent to the sequencing facility at the Bioinformatics department at the UP. Sequences were edited using the Vector NTI Advanced 9.1.0 software (www.invitrogen.com/bioinformatics (2004)) and edited sequences were compared to existing sequences in GenBank, using the Blast analysis (http://www.ncbi.nlm.gov/BLAST/).

B. Thin layer chromatography (TLC), bioautography and high performance liquid chromatography (HPLC)

Lipopeptide extracts of PPCB002, *Bacillus amiloliquefaciens* PPCB004 and known Iturin (*B. subtilis* 6639 or *B. amyloliquefaciens* PPCB004) and Bacillomycin (*B. subtilis* 6614) producing organisms (Romero *et al.,* 2007) were prepared by Dr. E. Arrebola-Diez at the Department of Plant Pathology (UP), according to Arrebola *et al.* (2010b). These extracts were loaded onto Silica gel TLC plates (Merck). The plates were prepared in triplicate. The mobile phase consisted of methanol (Saarchem, Wadeville, SA), dH_2O and chloroform (Saarchem) in the ratio 6.25:1:16.25. The TLC plates and solvent were placed in a suitable tank and left for 45min. to develop and allow separation of compounds within the lipopeptide extracts. After development, the plates were left to dry, upon which the plates were sprayed with distilled H_2O to allow visualisation of spots, or areas with adsorbed lipopeptides. All the spots were marked and the Rf-values were calculated. The plates were left overnight to allow complete evaporation of the solvent. After evaporation, 50 to 60ml of sterile, molten MEA containing 2% tetrazolium salt (TTZ) (British Drug Houses (BDH), Poole, England), were inoculated with two pathogenic *Penicillium* species. The media were then poured over the TLC plates and placed inside sterile containers. The containers were sealed with Parafilm and incubated for five days at 25°C. After incubation, lipopeptides showing antifungal activity was indicated by clear inhibition zones on the developed TLC plate.

Production of extracellular enzymes

Chitinase, amylase, protease and lipase production by PPCB002 was investigated during initial screenings by Dr. E. Arrebola-Diez, as described in Havenga (2005). Five replicates were included per enzyme test.

Detection of phenolics produced by the antagonist

Phenolic extracts were prepared from the secondary metabolites of PPCB002 by Dr. E. Arrebola-Diez at the Department of Plant Pathology, UP as follows: the antagonist strains and controls were plated out on antibiotic production media (APM) broth (McKeen et al. 1986) and were incubated at 37ºC for 5 days with orbiting movement at 100rpm. The cultures were centrifuged at 2500 g for 5 min and filtrated using Whatman filter paper No 1 (Whatman International Ltd., England). The supernatant pH was adjusted to 2.5 by adding acetic acid (Merck) at 20% final concentration. The phenolic extraction was performed using one volume of diehyl ether (Saarchem). The upper layer was recovered and evaporated. The precipitates were dissolved in methanol for further analysis. The visual display of different phenolic produced was assayed on silicon TLC using ethylacetate (Merck), methanol and water in the ratio 8:1:1 as solvent with exposure of the TLC plate under UV light at 254nm. These extracts were also used to perform bioautography as described in section 4.2.3 (B).

Effect of volatile compounds produced by PPCB002 on growth of P. digitatum and P. italicum *in vitro*

The "sealed plate" method was followed, as described by Fiddaman and Rossal (1993), with slight modifications. The antagonist PPCB002 was grown on nutrient agar (NA) (Merck) and incubated at 37ºC for 24 h. Thereafter, pure colonies were transferred to an antibiotic production minimal medium prepared according to McKeen *et al.* (1986). Meanwhile, MEA plates were inoculated with spore suspensions of the fungal pathogens by pipetting 10µl of spore suspension onto the centre of the plate. The AMP medium containing PPCB002 was inverted and placed on top of the fungal culture. The two plates were sealed together with Parafilm and incubated at 25 ºC up to 10 days as shown in Fig. 4.2.

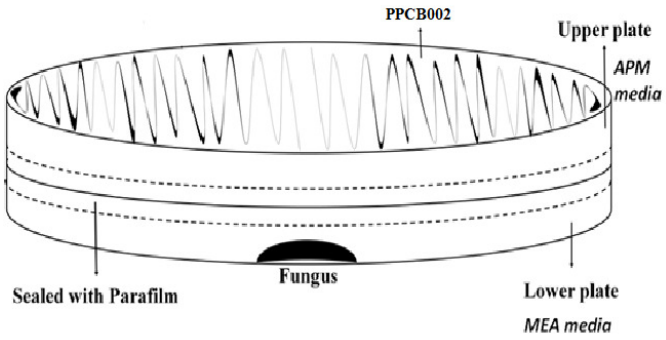

Figure 4.2: The "sealed plate" method, for determining the antifungal activity of volatiles produced by *Bacillus subtilis* PPCB002, on *Penicillium italicum* and *P.digitatum* growth. Illustration: Dr. E. Arrebola-Diez.

Inhibition of fungal growth by PPCB002 volatiles were calculated by comparing the percentage of the plate that was overgrown with the test pathogen, to that of the control. Five replicates were included per treatment.

Biofilm formation

The ability of PPCB002 to form a biofilm was compared to that of the commercial antagonist *B. subtilis* ATCC55466 (Avogreen strain) as well as another promising antagonist, *Bacillus licheniformis* B251, which are known to form biofilms. Analysis of biofilm production was performed according to Peeters *et al.* (2008). Pure liquid cultures of PPCB002, ATTC55466 and B251 were prepared by inoculating sterilised nutrient broth (NB) (Merck) with pure cultures of these bacterial antagonists and incubating overnight at 37°C on a rotary shaker. For each treatment, 24 wells of a sterile 96 well microtitre plate (Corning, New York, USA), were used. The control wells were filled with sterile NB, while other wells were filled with 100µL liquid cultures of different test organisms. The cultures were incubated at 37°C for four hours, after which the cultures were again removed and each well was washed with 100µL of sterile, physiological saline solution (PS). After washing out the unattached cells, 100µL of sterile NB was added to each well and the plate was incubated overnight at 37°C to allow biofilm formation. The overnight culture was removed and each well was again washed with 100µL PS. One hundred microliter absolute ethanol was transferred to each well and again removed after 10 min. After the ethanol was removed and the remaining ethanol was allowed to evaporate, 100µL of crystal violet (Sigma, Johannesburg) was added to each well and the plate was incubated for 20 min. The excess crystal violet was washed out after 20 min., using tap water. The plate was dried by gently dabbing on clean tissue paper. Finally 150µL 33% acetic acid was added to each well. The

samples were analysed at 595nm using the Multiskan Ascent® Plate Reader (Thermo Labsystems, USA).

4.2.4 Effect of two biocontrol products on green (*P. digitatum*) and blue mould (*P. italicum*) in Clementine

Preparation of fruit and fungal spore suspensions

Early season 'Nules' Clementine fruit from the Eastern Cape (Kirkwood) were used for biocontrol experiments. The fruit were harvested at commercial maturity and transported to the University of Pretoria, within 48h at ambient temperature. Fruit were washed for in 0.05% NaOCl (Merck)and rinsed twice with dH_2O, to disinfect fruit surfaces. The surface disinfected fruit were allowed to air dry and wounds were made on each side of the equatorial plane using a sterile wounding tool (2mm long, 1mm thick). *Penicillium digitatum* and *P. italicum* spore suspensions were prepared by flooding seven day pure cultures of these pathogens with ¼ strength Ringers (Merck) solution. Spores were liberated using a sterile spreader. Based on inoculum concentrations used in relevant publications (Usall *et al.,* 2008; Arrebola *et al.,* 2010b), the spore suspensions were adjusted to $1x10^6$cfu/ml using a haemacytometer.

Biocontrol formulations

The antagonist PPCB002 was formulated by Stimuplant (Pretoria, SA), to the concentration of $1x10^9$ cells/ml. The Innovacure formulation was produced by Neova™ Technologies Inc. (Abbotsford, Canada) and was used at a final concentration of 5 x 10^7cells/ml of *C. saitoana*. The final concentration also contained 0.01% lysozyme and 2% sodium bicarbonate.

Application of biocontrol products

In the case of preventative applications, the biocontrol formulations were applied 24 h before pathogen inoculation and in the case of curative applications, the pathogen inoculation were done 24h before application of the biocontrol formulations. For pathogen inoculations, 10µl of the pathogen spore suspensions were pipetted into each wound. Commercial biocontrol formulations were applied as follows: dipping the fruit in five litres of *B. subtilis* PPCB002 for 2 min.; spraying fruit with *C. saitoana*, using a hand held spray bottle until fruit

was completely covered; or, by dipping the fruit first in the *B. subtilis* formulation and once dry, spraying the fruit with *C. saitoana*. For the commercial control, the fruit were dipped in 500 ppm imazalil (Universal Crop Protection (Pty) Ltd., Kempton Park, SA), allowed to air-dry and then covered with Carnauba wax (FMC Technologies Inc., Cape Town, SA) using a hand held spray bottle. Half of the fruit were kept at 4°C and 80-90% RH and the other half was kept at room temperature. Disease severity was recorded after seven days at ambient temperature and 14 days in cold storage. Severity was expressed as the average lesion diameter (mm) of two lesions per fruit and a total of twenty fruit were included per treatment. The trial was repeated.

Statistical analysis

For *in vitro* work, analysis of variance (ANOVA) for a completely randomised design (CRD) was performed. Fisher's Protected Least Significant Difference (L.S.D) test was used to separate treatment means at the 5% level of significance. *In vivo* trials were analysed as a randomised-block design (RBD), with trials defined as blocks. Mean separation for the *in vivo* experiments was performed with the L.S.D test at the 1% level of significance as treatment variances were not homogenous. Significant differences were indicated with a different letter. Statistical analysis was performed using GenStat Discovery Edition 3 software (www.vsni.co.uk) and SPSS 8.0 software for Windows (SPSS Inc., Chicago, IL, USA).

4.3 Results

4.3.1 Dual culture

Results indicated that PPCB002 was able to inhibit *P. digitatum* growth by 22.7% ± 2.29 and *P. italicum* growth by 43.1% ± 2.38 after 14 days at 25°C. Therefore, these results agree with initial screening studies during which this strain was isolated as a possible antagonist. The reduction in growth by PPCB002 was also significantly more for *P. italicum* than for *P. digitatum,* according to the LSD test ($P < 0.05$).

4.2.3 Mode of action of *Bacillus subtilis* PPCB002

Antibiotic production by *Bacillus subtilis* PPCB002

A. Sequencing the Bacillomycin gene region

Gel electrophoresis of PCR amplification products indicated that the three key gene regions (*Bmy, ituD* and *lpa*) involved in the production of bacillomycin, iturin A and surfactine, were present in the genome of PPCB002 (Fig. 4.3).

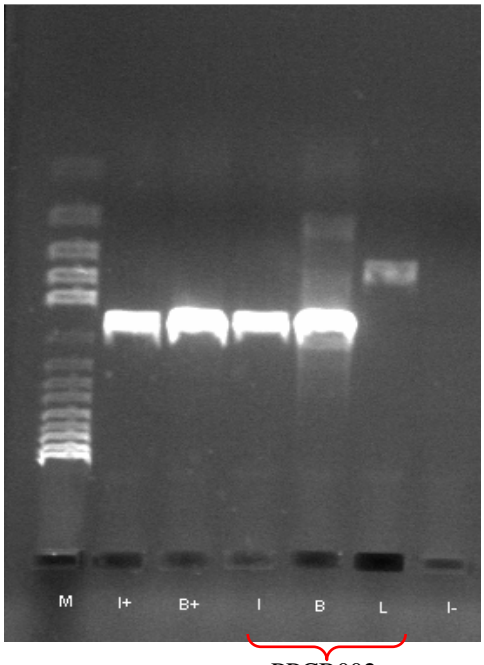

Figure 4.3: Polymerase chain reaction amplification products of different gene regions within the bacillomycin operon of *Bacillus subtilis* PPCB002. M: 100bp molecular weight marker; I+: Iturin positive control (*ituD* amplification of *B. subtilis* UMAF6639); B+: Bacillomycin positive control (*Bmy* amplification of *B. subtilis* UMAF6614); I: *ituD* amplification of PPCB002; B: *Bmy* amplification of PPCB002; L: *Lpa* amplification of PPCBOO2; I-: *ituD* amplification of Iturin negative strain.

B. Thin layer chromatography (TLC), bioautography and high performance liquid chromatography (HPLC)

The silica TLC plates (data not shown) indicated that PPCB002 lipopeptide extracts produced spots at Rf-values of 0.3, 0.7 and 0.1, which corresponds to known Rf-values of Bacillomycin, Surfactin and Fengycin respectively (Arrebola *et al.,* 2009). Bioautoghraphy results indicated that among these lipopeptides, only Bacillomycin exhibited antifungal activity towards the test pathogens *P. crustosum* (Fig. 4.4), *P. italicum* (Fig. 4.5) and *P. digitatum* (data not shown).

Figure 4.4: Absence of mycelial growth of *Penicillum crustosum* over silica TLC plate (bioautography) at the spot Rf = 0.4, which corresponds to Iturin produced by *Bacillus amyloliquefaciens* PPCB004 (1) and *B. subtilis* UMAF6639 (4). Spot Rf = 0.3 corresponds to Bacillomycin produced by *B. subtilis* PPCB002 (2) and *B. subtilis* UMAF6614 (3).

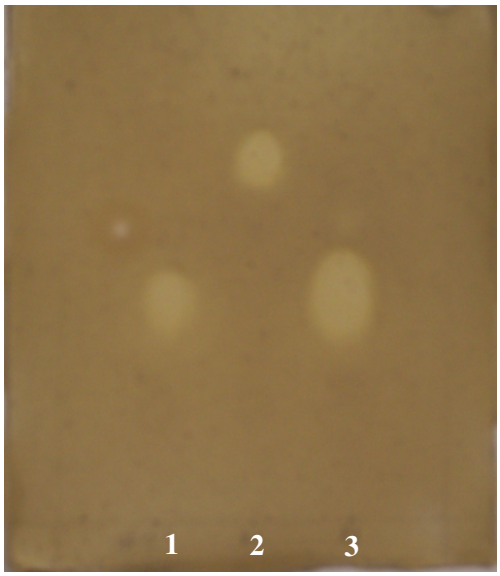

Figure 4.5: Absence of mycelial growth of *Penicillum italicum* over silica TLC plate (bioautography) at the spot Rf = 0.3, which corresponds to Bacillomycin produced by *Bacillus subtilis* UMAF6614 (1) and *B. subtilis* PPCB002 (3). An inhibition zone was also observed at Rf = 0.4, which corresponds to Iturin A production by *Bacillus amyloliquefaciens* PPCB004 (2).

The HPLC analysis of the lipopeptide extracts indicated the presence of two main groups of compounds eluded at 22.3 - 27.8 min and 87.3 - 101.6 min. These retention times corresponds to that of bacillomycin and surfactine respectively, as reported by Romero *et al.* (2007).

Production of extracellular enzymes

According to results of the antifungal enzyme culture assays, PPCB002 produced amylase and protease and did not produce any chitinase or lipase (data not shown).

Determination and quantification of phenolics produced by the antagonist

Silica TLC displayed the presence of several kinds of phenolic compounds when it was exposed to UV light. However, bioautography revealed that none of these spots or compounds resulted in inhibition zones (data no shown).

Effect of volatile compounds produced by PPCB002 on growth of P. digitatum and P. italicum *in vitro*

Volatile compounds produced by PPCB002 did not have an effect on colony diameter of *P. digitatum* (0% growth inhibition). Colony diameter of *P. italicum* was reduced by 12.10 ± 7.9% however this inhibition was non-significant in comparison to colony diameters of control plates. Therefore the volatile production from PPCB002 did not have an effect on *P. digitatum* and *P. italicum* growth *in vitro*.

Biofilm formation

Results revealed that PPCB002 was more effective in biofilm formation compared to the other antagonists tested (Fig. 4.6 and 4.7).

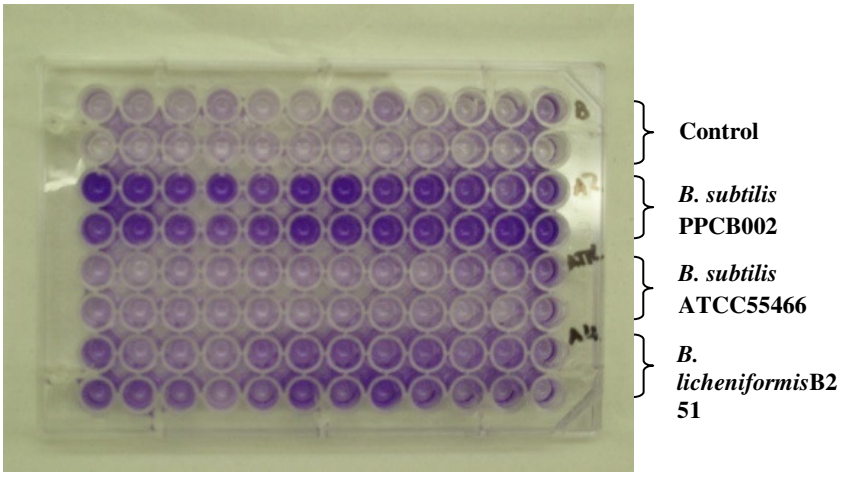

Figure 4.6: *In vitro* biofilm formation by *Bacillus subtilis* PPCB002,using the crystal violet assay. Two lanes were allocated to each organism. Purple discolouration represents biofilm formation.

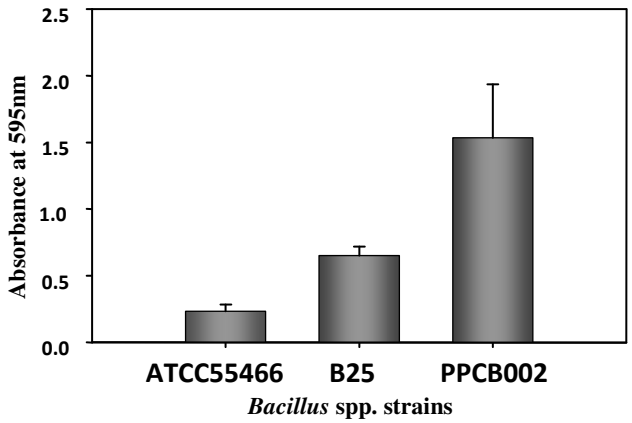

Figure 4.7: Average absorbance obtained with crystal violet assay on biofilm for *Bacillus subtilis* ATCC55466 and PPCB002, as well as *Bacillus licheniformis* B251. Error bars indicate standard deviations.

4.3.3 Effect of two biocontrol products on green- (*P. digitatum*) and blue mould (*P. italicum*) in Clementine

At ambient temperature, preventative application of Innovacure resulted in a significant reduction in disease severity of *P. digitatum* (Fig. 4.8). Disease severity for this treatment was significantly lower than that of the other biocontrol treatments and was comparable to the commercial control.

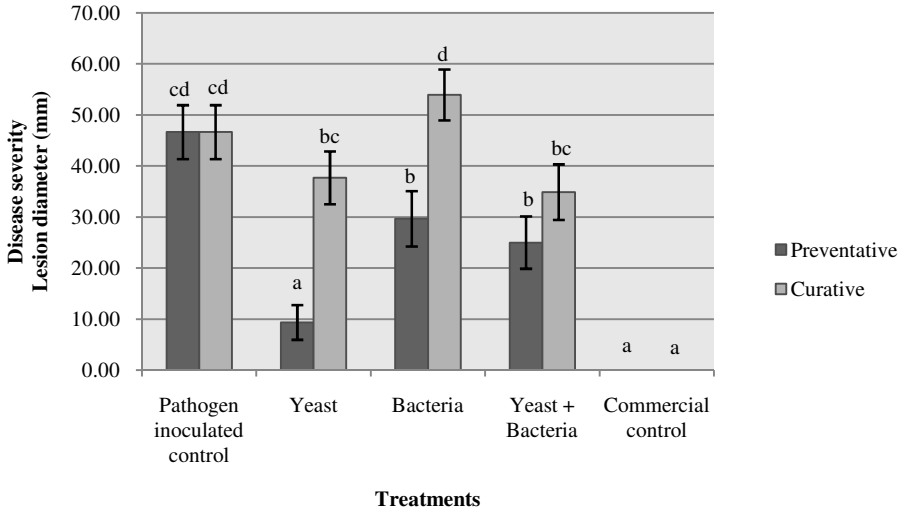

Figure 4.8: Effect of three biocontrol treatments on *Penicillium digitatum* disease severity in artificially inoculated 'Nules' Clementine after seven days at ambient temperature. Error bars indicate standard deviation. Columns with the same letter do not differ significantly with regard to disease severity according to Fisher's Protected Least Significant Difference (L.S.D) test at the 1% level of significance.

When applied preventatively, the PPCB002 formulation and combination treatment also resulted in a significant reduction in *P. digitatum* disease severity (Fig. 4.8). The rest of the treatments were not effective against *P. digitatum* when fruit are kept at ambient temperature.

Both preventative and curative applications of PPCB002 was ineffective against *P. italicum* at ambient temperature (Fig. 4.9). The rest of the treatments resulted in a significant reduction in *P. italicum* severity. Curative application of Innovacure formulation did not differ significantly from its preventative application against this pathogen. None of the biocontrol treatments resulted in *P. italicum* disease reduction comparable to the commercial control at ambient temperature.

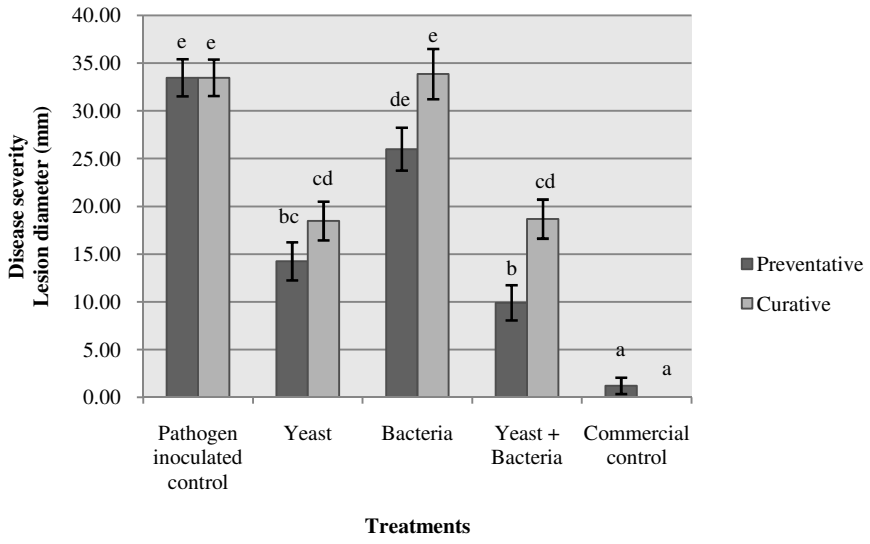

Figure 4.9: Effect of three biocontrol treatments on *Penicillium italicum* disease severity in artificially inoculated 'Nules' Clementine after seven days at ambient temperature. Error bars indicate standard deviation. Columns with the same letter do not differ significantly with regard to disease severity according to Fisher's Protected Least Significant Difference (L.S.D) test at the 1% level of significance.

In cold storage, all the biocontrol formulations significantly reduced decay in *P. digitatum*, except for the curative application of the PPCB002 formulation (Fig. 4.10). A disease severity reduction comparable to that of the commercial control was achieved by the preventative and curative application of the combination treatment, as well as Innovacure.

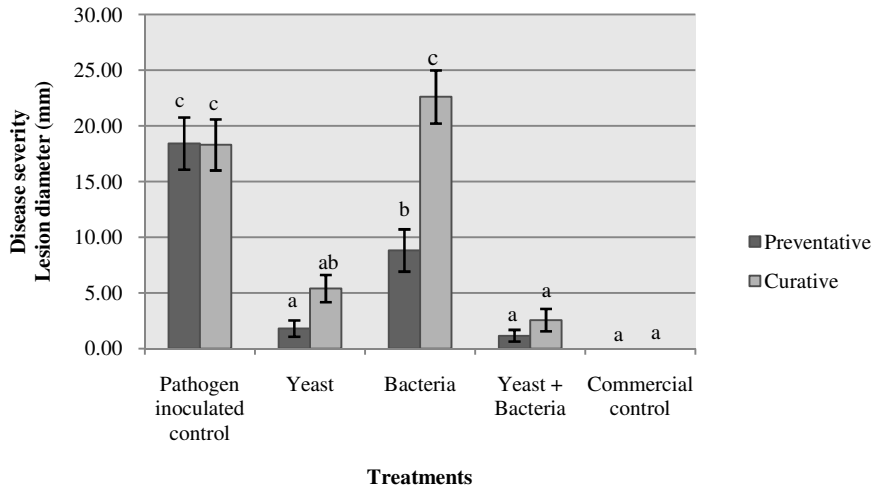

Figure 4.10: Effect of three biocontrol treatments on *Penicillium digitatum* disease severity on artificially inoculated 'Nules' Clementine after 14 days in cold storage at 4°C. Error bars indicate standard deviation. Columns with the same letter do not differ significantly with regard to disease severity according to Fisher's Protected Least Significant Difference (L.S.D) test at the 1% level of significance.

In the case of *P. italicum,* all the biocontrol formulations significantly reduced decay in cold storage, except for the curative and preventative applications of PPCB002 formulation. The rest of the treatment applications resulted in disease reduction comparable to the commercial control (Fig. 4.11), except for the curative application of Innovacure.

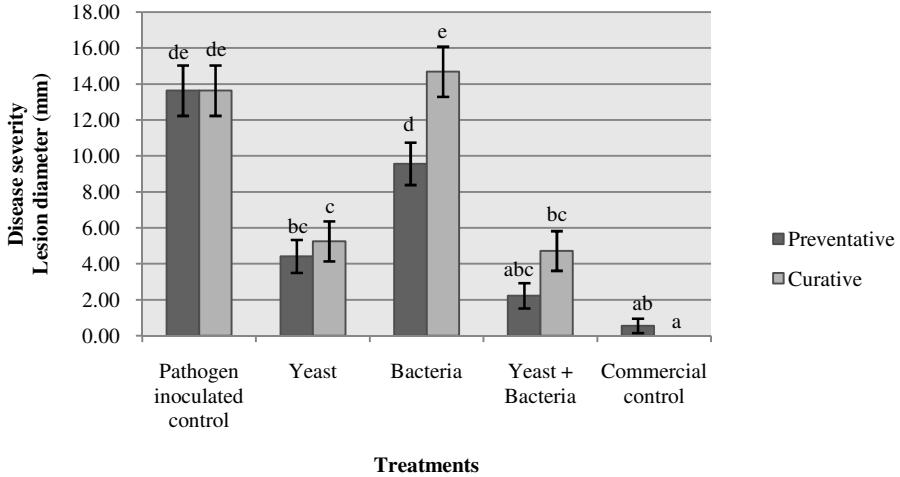

Figure 4.11: Effect of three biocontrol treatments on *Penicillium italicum* disease severity on artificially inoculated 'Nules' Clementine after 14 days in cold storage at 4°C. Error bars indicate standard deviation. Columns with the same letter do not differ significantly with regard to disease severity according to Fisher's Protected Least Significant Difference (L.S.D) test at the 1% level of significance.

4.4 Discussion

In this study, the antagonist PPCB002 was found effective against *P. digitatum* and *P. italicum in vitro*. In addition, PPCB002 was more effective against *P. italicum* than *P. digitatum*, which is not surprising since this pathogen is known to grow more vigorously (Pitt, 1979 and Moss, 1987). Dual culture assays may involve different modes of action, such as antibiosis, parasitism and competition for available nutrients. Among the antimicrobial metabolites produced by *B. subtilis*, the lipopeptides or antibiotics have been found especially useful against postharvest fungal pathogens (Moyne *et al.,* 2004; Tourè *et al.,* 2004; Ongena and Jacques, 2007). The gene regions necessary for production of the lipopeptides Bacillomycin, Iturin A and Surfactine, were found to be present in the genome of PPCB002. However, TLC and HPLC analysis revealed that only Bacillomycin and Surfactine were present in the lipopeptide extracts of PPCB002. The bioautography assays further revealed that between Bacillomycin and Surfactine, only the former exhibited antifungal activity towards *Penicillium* species. Nonetheless, Bias *et al.* (2004) reported that surfactine is important for biofilm formation.

Volatile and phenolic compounds produced by PPCB002 did not have a significant inhibitory effect on *P. italicum* and *P. digitatum in vitro*, which is in agreement with the findings of Leelasuphakul *et al.* (2008). Extracellular enzyme analysis further revealed that PPCB002 is a producer of amylase and protease. Although these enzymes are reportedly able to hydrolyse fungal cell walls (Dunne *et al.,* 1997 and Bar-Shimon *et al.,* 2004), various studies have found the inhibitory effect of hydrolytic enzymes excreted by BCA to be insignificant (Roberts and Selitrennikoff, 1988; Yehuda *et al.,* 2003; and Leelasuphakul *et al.,* 2008). It has been reported that hydrolytic enzymes rather work synergistically with other antibiotic compounds, by making the pathogen's cell walls more permeable and allowing infiltration of these fungitioxic compounds (Lorito *et al.,* 1994; Schirmböck *et al.,* 1994). The enzyme amylase is also responsible for the breakdown of complex nutrient sources such as starch (Doetcsh and Cook, 1973) enabling the antagonist to compete for available nutrient sources *in vitro*.

In the CV assay, PPCB002 was a vigorous biofilm producer *in vitro.* Biofilm formation is of cardinal importance to a potential BCA, since it allows the organism to colonise and produce antifungal metabolites on the fructoplane. From the *in vitro* studies it could therefore be concluded that PPCB002 was effective against *P. italicum* and *P.*

digitatum in vitro, mainly through Bacillomycin production and competition for available nutrients. The ability of the antagonist to form a biofilm further adds to its competitive mode of action, as well as its ability to exclude the pathogen from entry sites.

Significant disease control was achieved in some cases by preventative application of the PPCB002 formulation to artificially inoculated 'Nules' Clementine. However, the efficacy of preventative applications of PPCB002 was never comparable to the commercial control, nor was it as effective as preventative applications of the other biocontrol treatments, except in one case. Curative applications of PPCB002 were ineffective against both pathogens at ambient temperature and in cold storage. In some cases, preventative applications of Innovacure and/or the biocontrol combination treatment were also more effective than curative applications at ambient temperature. Biocontrol has often been described as an approach that does not provide curative action (El-Gaouth *et al.,* 2003; Obagwu and Korsten, 2003 and Leelasuphakul et al 2008). Nonetheless, curative application of Innovacure and the biocontrol combination treatment resulted in a significant reduction in disease severity in most cases. In cold storage, curative and preventative applications of these treatments were equally effective. The Innovacure formulation therefore exhibited curative action, agreeing with El-Ghaouth *et al.* (2002). The curative action of Innovacure could perhaps be attributed to the lysozyme and sodium bicarbonate, since these are known to hydrolyse fungal cell walls (El-Ghaouth *et al.,* 2002) and are therefore expected to have an immediate effect on fungal growth. However, curative application of Innovacure at ambient temperature was ineffective against the fast growing *P. digitatum*.

In many cases, preventative applications of Innovacure and the combination treatment resulted in disease reduction comparable to the commercial control. However, the efficacy of these treatments was not consistent. In some cases the combination treatment performed comparable or slightly better than Innovacure and vice versa. Variability in the performance of biocontrol have been reported previously (Droby *et al.,* 2009) and in particular when used on citrus fruit (Droby *et al.,* 1993 and 1998). Palou *et al.* (2002) also reported that sodium carbonate salt was less effective against green and blue mould on Clementine compared to other citrus cultivars. The authors ascribe this variation to differences in rind interactions with the salt. Such a variation could explain the variation observed with Innovacure in this study, since it contained sodium bicarbonate. Nonetheless, in all cases preventative applications of either Innovacure or the combination treatment was able to reduce decay significantly.

The PPCB002 formulation with Innovacure did not work together synergistically in this study, since efficacy was not improved by the combination of these two treatments. Combination of BCA has been reported to improve efficacy and reliability due to a multifaceted mode of action (Guetsky *et al.,* 2001). However, PPCB002 was not as effective as Innovacure as stand-alone treatment and could have affected the performance of the combination treatment as well.

4.5 Conclusion

The bacterial antagonist PPCB002 was able to inhibit *P. digitatum* and *P. italicum* growth *in vitro,* mainly through the production of Bacillomycin. The ability of the organism to produce biofilms and compete for space and nutrients were also found to be important modes of action. However, the formulated product of PPCB002 was not as effective against these pathogens when applied to artificially inoculated 'Nules' Clementine. The PPCB002 formulation also lacked curative action against both pathogens. Innovacure, as well as the combination of PPCB002 and Innovacure was more effective than the PPCB002 formulation during *in vivo* assays. Disease control was comparable to the commercial control in some cases. Although these treatments exhibited curative action, preventative applications were found to be more effective, especially when fruit are kept at ambient temperature. It is recommended that future investigations continue with Innovacure in combination with more effective biocontrol formulations or disease control strategies to improve curative action and reliability of this biocontrol product.

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CHAPTER 5:

Integrated alternative postharvest treatments to improve the storability of Clementine mandarins

Abstract

The last step in the development of an alternative disease management (IDM) programme for Clementine mandarins was the selection of a suitable modified atmosphere packaging (MAP) type and combining it with a previously selected hot water dip (HWD) and biocontrol treatment. A bioriented polypropylene (BOPP) packaging type with certain perforations was selected among three different packaging materials as the most suitable for 'Nules' Clementine. Although desiccation could effectively be reduced by this packaging type, extreme O_2 and CO_2 levels occurred when fruit were taken out of cold storage. Peel damage was observed on some fruit exposed to these conditions. Overall, alternative treatment combinations did not have a major impact on internal fruit quality. However the maturity index (MI) was affected by some treatments and co-incidentally these treatments were also associated with reduced efficacy against *Penicillium* decay. Among the different treatment combinations, HWD + biocontrol were the only treatment that could effectively reduce disease incidence of both pathogens under lower-disease pressure conditions. However, under higher-disease pressure conditions none of the alternative treatment combinations were as effective as the commercial control. These treatments lacked the strong curative effect exhibited by the commercial control. Although increased HSP production was detected by some treatment combinations, it could not be correlated with protection against decay in this study.

5.1 Introduction

Growing concern by consumers regarding food safety and environmentally friendly production practices has lead to an increased search for alternative postharvest technologies and treatments (Spadaro and Guillino, 2004). Biocontrol (Droby *et al.,* 1998; El-Ghaouth *et al.,* 2000; Korsten, 2004) and hot water dips (HWD) (Schirra and D'hallewin, 1997; Palou *et al.,* 2001 and Hong *et al.,* 2007) have shown great success against some of the major

postharvest pathogens of fruit. Unfortunately, these treatments rarely supply sufficient decay control as stand-alone treatments (Janisiewicz and Korsten, 2002; Zhang *et al.,* 2010). However, combining different alternative treatments can provide more effective disease control (Conway *et al.,* 2007; Burdon, 1997).

Integrated disease management (IDM) programmes were first introduced as a 'hurdle technology' approach in 1978. Fundamentally, this technology combines various treatments or compounds which creates 'hurdles' or obstacles against decay development, food quality deterioration and product contamination (Leistner, 2000). Various IDM programs have been successful in reducing *Penicillium* decay and maintaining fruit quality in citrus using a combination of heat treatments, sodium bicarbonate and biocontrol (Palou *et al.,* 2001 and 2002; Obagwu and Korsten, 2003; Torres *et al.,* 2007; Usall *et al.,* 2008). Success has also been achieved by combining biocontrol and GRAS compounds (El-Ghaouth *et al.,* 2002); heat treatments and plastic packaging (Rodov *et al.,* 2000); HWD with Imazalil (Smilanick *et al.,* 1997), as well as HWD with yeast antagonists (Zhang *et al.,* 2010).

The mode of action and efficacy of HWD and biocontrol products against *Penicillium* decay were reported on in Chapter 3 and 4 of this thesis with notable success. However, aside from postharvest decay, general deterioration of fruit quality and desiccation are also major obstacles associated with citrus fruit postharvest (Agrios, 2005 and Leelasuphakul *et al.,* 2008). To combat desiccation and improve overall appearance, commercial practices involve applying natural or synthetic waxes to the fruit (Harris, 1988). However Rodov *et al.* (2000) reported that plastic liners are more successful than wax in reducing moisture loss. Not only can MAP effectively reduce moisture loss (Sivakumar and Korsten, 2006), but this technology may also have an effect on postharvest pathogens (Shellie, 2002). Nevertheless, MAP have not yet been used commercially for citrus fruit (Murata, 1997), hence the need for further investigation.

The MAP technology is based on modification of the gaseous environment surrounding the fruit inside these polymer bags or films. This modified gas composition affects the respiration rate of fruit and postharvest pathogens, which in turn affects the rate of fruit quality deterioration and decay (Shellie, 2002). The type of gas conditions created relies greatly on the perforation of the packaging material, which will alter O_2 and CO_2 permeation across the film (Sivakumar and Korsten, 2006). However, the packaging material has to be selected according to the specific requirements of each cultivar, since citrus fruit does not

respond well to low O_2 conditions and is very sensitive to fermentation. In this Chapter, three different MAP materials were evaluated as packaging materials for 'Nules' Clementine. The selected MAP type was then combined with a previously selected HWD (52°C for 5 min.), as well as a biocontrol mixture consisting of yeast- (*Candida saitoana* Nakasa and Suzuki) and bacterial-based (*Bacillus subtilis* (Ehrenberg) Cohen) formulations. These treatments and their combinations were evaluated for efficacy against *Penicillium italicum* Wehmer and *P. digitatum* (Pers.: Fr.) Sacc., as well as the effect on fruit quality and host defence reactions.

5.2 Materials and methods

5.2.1 Fruit

For all trials, 'Nules' Clementine fruit from Western Cape (Citrusdal and Piketberg) area and the 2010 growing season were used. Disease and blemish-free fruit of uniform size were selected for all trials. All fruit were washed in 0.05% NaOCl (Merck, Johannesburg, SA), rinsed with distilled water (dH_2O) and then left to air-dry prior to treatment application.

5.2.2 Selection of modified atmosphere packaging

Two bioriented polypropylene packaging materials (BOPP 1 and 2) (Knilam packaging (Pty) Ltd., Westlake, South Africa (SA)) and one biodegradable packaging material (NatureFlex™ Innovia) were tested, based on the gas composition created within the packages. Packaging perforations were given as 0.00313% and 0.00565% of perforated material for BOPP 1 and 2 respectively. Ten fruit were placed in each bag after which the bags were sealed. Fruit were kept at 4°C and 80-90% relative humidity (RH) for ten days. After cold storage fruit were transferred to ambient temperature and RH (±18°C and 40- 50% RH) for ten days. The gas composition of three bags was measured daily using the PBI Dansensor CO_2/O_2 gas analyzer (Checkmate 9900, Ringsted, Denmark). For all the trials, HOBO® data-loggers (Onset®, Maryland, USA), were placed inside random bags or inside random boxes in cold storage and ambient temperature, to measure temperature and RH conditions.

5.2.3 Effect of alternative treatment combinations on fruit quality

Fruit were received within 48h after harvest and were kept at 4°C and 80-90% relative humidity for seven days before treatment. Fruit were subjected to different combinations of the following postharvest treatments: i) a hot water dip at 52°C for 5 min., ii) a mixture of

the biocontrol formulations *B. subtilis* PPCB002 (Stimuplant, Pretoria, SA) and Innovacure (Neova™ Technologies Inc., Abbotsford, Canada) and iii) packaging in the selected MAP material (BOPP 2). Pulp temperatures were reduced in fruit subjected to HWD treatment, by dipping in tap water (18°C for ca. 10 min.) directly after exposure to heat treatment. The biocontrol combination treatment was applied as described in Chapter 4 (section 4.2.4) of this thesis. For fruit quality analysis, seventy fruit were included per treatment. Ten fruit were selected randomly from each treatment and each fruit represented one replicate. The remaining fruit were used for biochemical analysis of host defence reactions.

Fruit colour and overall appearance: Peel colour was measured using a Minolta Chromameter CR-300 (Osaka, Japan) and the CIE (Commission International de l'Eclairage) *L** (lightness coefficient "lightness") a* (chroma "greenness to redness") b* (hue angle "blueness to yellowness") colour space system (Abbot, 1999). Nine replicates were used per treatment for all the external quality parameters. Colour was expressed as colour index (CI) as described by Carvalho *et al.* (2008):

$$CI = \frac{1000 \times a^*}{L^* \times b^*}$$

Weight loss and fruit firmness: The weight of each fruit was taken before and after storage and the % weight loss calculated. Fruit firmness was measured on opposite sides of the equatorial plane of each fruit, using a Chatillon digital penetrometer (John Chatillon & Sons, New Cork, USA) fitted with an 8-mm fruit tester probe and expressed in Newton (N).

pH: For all internal quality parameters, the juice of each fruit were extracted and filtered through two layers of cheesecloth. The pH of the filtered juice was determined using a pH meter (Hanna Instruments, Johannesburg). Ten replicates were included per treatment for all the internal quality parameters.

Brix: The TSS or Brix was determined with a digital refractometer (PR-100 Atago, Tokyo, Japan) and expressed as percentages.

Titratable acidity: Titratable acidity (TA) was determined by titration of juice with 0.01 M sodium hydroxide (NaOH) (Sivakumar and Korsten, 2006) and expressed as percentage citric acid per 100ml of juice, using the following calculation:

$$\text{Percentage citric acid} = \frac{\text{Titre x acid factor* x 100}}{\text{10 ml juice}}$$

*acid factor for citrus is 0.0064.

Maturity index: The maturity index (MI) is used to measure the level of maturity or ripeness of fruit and is determined by calculating the sugar to acid, or Brix to TA ratio (Tucker, 1993).

Ascorbic acid content: Ascorbic acid content was determined by means of a visual titration method with 2,6-dichlorophenol-indophenol (DCPIP). To 2 ml of the extracted juice, 8 ml of 3% (w/v) metaphosphoric acid (Merck) (HPO_3) was added. This mixture was titrated with a 0.25% (w/v) solution of the DCPlP (Sigma-Aldrich, St. Louis, USA), until the juice mixture changed to a pink colour that persisted for 15 sec. The dye strength was determined by titrating with a 1% (w/v) l-ascorbic acid (Sigma-Aldrich) standard and using the titre in the following equation:

$$\text{Dye strength} = \frac{0.5}{\text{Dye titre}}$$

The ascorbic acid content was expressed as mg of ascorbic acid per ml of dye, using the following formula:

$$\text{Mg of ascorbic acid/100ml of juice} = \frac{\text{Titre x dye strength x volume made}}{\text{Aliquot taken for estimation x juice volume}} \text{ x } 100$$

5.2.4 Effect of alternative treatment combinations on host defense reactions

Fruit were treated as described in section 5.2.3. Random samples were taken for heat shock protein (HSP), phenylalanine ammonia-lyase (PAL) and total phenolic content (TPC) analysis, after four days in cold storage (4°C and 80-90% relative humidity (RH)). Analysis was performed as described in Chapter 3. Three replicates of 10 fruit each were included per treatment for PAL and TPC analysis. The HSP analysis was repeated for confirmation.

5.2.5 Effect of alternative treatment combinations on *Penicillium italicum* and *Penicillium digitatum* disease development

A total of 1500 'Nules' Clementine were wounded once on the equatorial plane of each fruit with a sterile metal rod (1mm x 2mm). Fruit were inoculated with *P. digitatum* and *P. italicum* by dipping in spore suspensions (1 x 10^6cfu/ml) of the respective pathogens. The pathogens were isolated and spore suspensions were prepared as described in Chapter 4 (section 4.2.4). Inoculated fruit were left to air-dry for approximately 5h at ambient temperature. Fruit were then subjected to the postharvest treatment combinations as described in section 5.2.3. All fruit were kept at ambient temperature and relative humidity (approximately 18°C and 30%) for nine days. Seventy fruit were included per treatment and disease incidence was expressed as percentage of fruit infected, out of the total number of fruit. Disease severity was recorded using the following disease severity scale for each fruit: 1 = 0% of the fruit area infected (healthy fruit); 2 = 25%; 3 = 50%; 4 = 75% and 5 = 100% of fruit area infected. Disease severity was expressed as the percentage of fruit in each treatment falling in a particular severity class.

This trial was repeated during the 2011 growing season at a commercial packhouse in the Eastern Cape (Kirkwood), with fruit from this area. However, higher disease pressure conditions were applied during this trial to determine the efficacy of the alternative treatment combinations under such conditions. Fruit inoculation was done by pipetting 10µl of the respective pathogen spore suspensions at higher concentrations (2.5 x 10^6cfu/ml) into each wound. Although this concentration is higher than normally recommended for evaluating postharvest treatments for citrus postharvest diseases, efficacy with biocontrol has been reported with equally high inoculum concentrations in Arrebola *et al.*(2010). In addition, according to (Palou *et al.,* 2001) in Fischer *et al.* (2009), *Penicillium* populations in citrus packhouses can increase by 40-60% as the season progresses. The RH was also much higher in this area (ca. 70%), which contributed to disease expression. Seven replicates of ten fruit each were included per treatment. Disease incidence and severity were recorded as described above.

5.2.6 Statistical analysis

Statistical analyses were performed using GenStat Discovery Edition 3 software (www.vsni.co.uk). All trials adopted a completely randomized design (CRD) for which one-way analysis of variance (ANOVA) was performed. Mean separation of disease data for the

2010 IDM trial was done using the χ_2-test ($p < 0.05$), because all seventy fruit were pooled. For all other trials, mean separation was done using Fisher's Protected Least Significant Difference test (L.S.D) at the 1% level of significance, since treatment variances were not homogenous. Significant differences were indicated with a different letter.

5.3 RESULTS

5.3.1 Selection of MAP

In cold storage, BOPP 2 packaging provided O_2 and CO_2 concentrations closest to the optimum required by citrus (5 - 10% O_2 and 0 - 4.5% CO_2). The gas composition in the biodegradable packaging material was similar to that of BOPP 2 (data not shown), however these bags were not strong enough to hold ten fruit and was therefore excluded from further investigations. When the fruit were transferred to ambient temperature, the O_2 concentration dropped rapidly, followed by a sharp increase in CO_2 in both packaging types (Fig. 5.1).

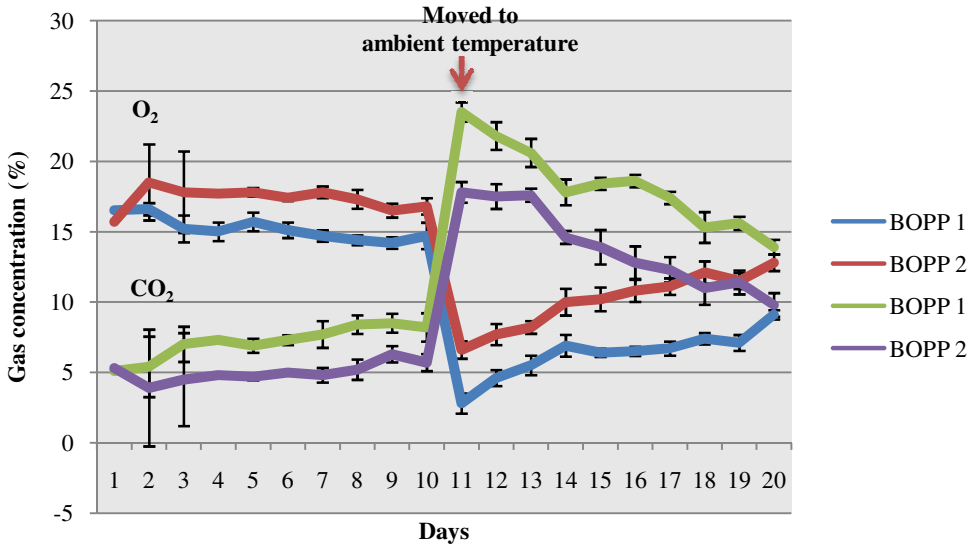

Figure 5.1: Gas evolution of O_2 and CO_2 within two bioriented polypropylene (BOPP) modified atmosphere packaging (MAP) materials, containing 'Nules' Clementine. Fruit were kept in cold storage (4°C; 80-90% RH) and at ambient temperature (approx. 18°C; 30% RH) for ten days. Error bars indicate standard deviation with regard to gas concentration on each day.

5.3.2 Effect of alternative treatment combinations on fruit quality

Fruit colour and overall appearance: None of the treatments significantly affected external fruit colour (data not shown). Peel damage was observed in some treatments

however these fruit were not included in external colour evaluations, since they were not representative of the whole treatment. Different types of rind breakdown were observed on the fruit. One type included symptoms such as sunken oil glands and brown, dried out areas on the peel (Fig. 5.2 A and B). Other symptoms were much darker and affected areas did not appear as dried out (Fig. 5.2 C).

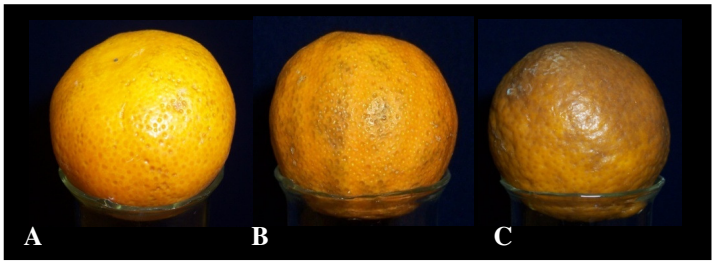

Figure 5.2: Rind breakdown observed in 'Nules' Clementine in response to various alternative postharvest treatment combinations. A: HWD + MAP; B: HWD + biocontrol; C: MAP.

Weight loss and fruit firmness: A significant reduction in desiccation was observed for all treatments with MAP (Fig. 5.3). The rest of the treatments did not significantly affect weight loss in fruit. None of the postharvest treatments significantly affected fruit firmness in comparison with the untreated control.

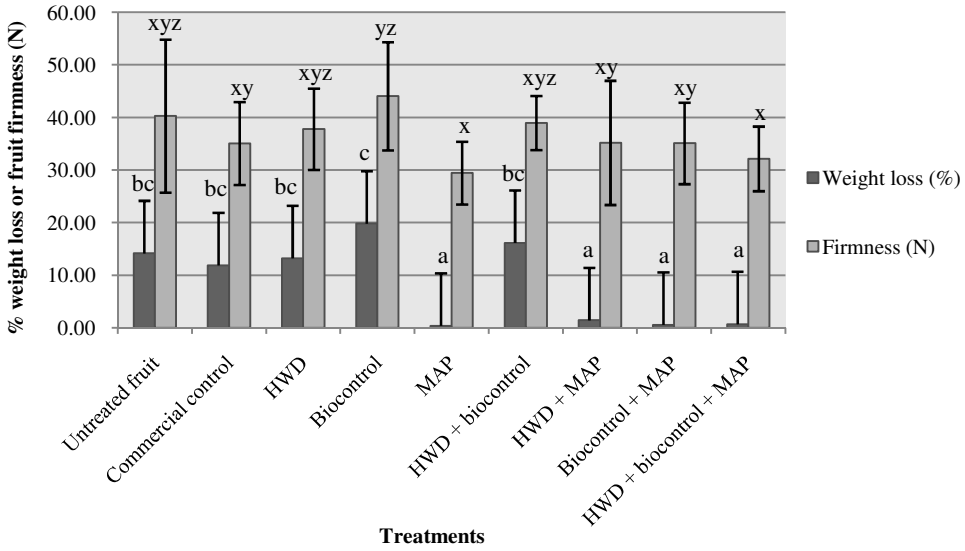

Figure 5.3: The effect of alternative postharvest treatment combinations on % weight loss and fruit firmness in 'Nules' Clementine. Error bars indicate standard deviation of treatment means. Bars with the same letter do not differ significantly with regard to weight loss or firmness, according to Fisher's protected Least Significant Difference test (L.S.D) at the 1% level of significance.

pH: Apart from the commercial control, alternative postharvest treatments did not have a significant impact on the juice pH of 'Nules' Clementine (Fig. 5.4).

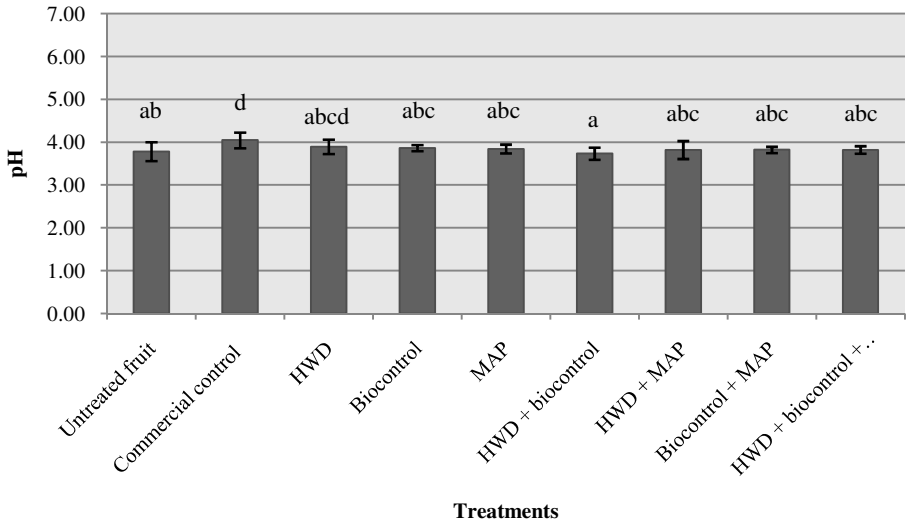

Figure 5.4: The effect of different postharvest treatments on juice pH of 'Nules' Clementine. Error bars indicate standard deviation of treatment means. Bars with the same letter do not differ significantly with regard to pH, according to Fisher's protected Least Significant Difference test (L.S.D) at the 1% level of significance.

Brix: The HWD treatment resulted in a significantly lower Brix or TSS content reading in comparison to the untreated control and the biocontrol treatments (Fig. 5.5). The rest of the postharvest treatment combinations did not differ significantly from the untreated control.

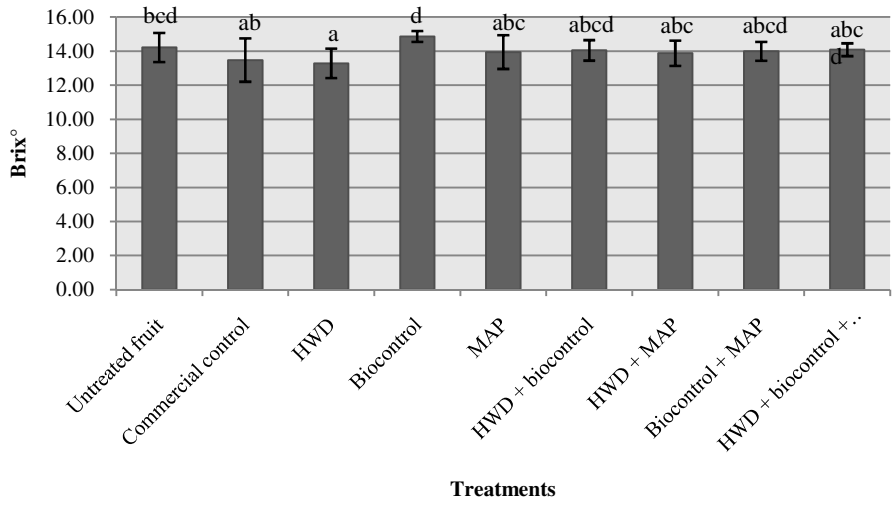

Figure 5.5: The effect of different postharvest treatments on the Brix of 'Nules' Clementine. Error bars indicate standard deviation of treatment means. Bars with the same letter do not differ significantly with regard to Brix content, according to Fisher's protected Least Significant Difference test (L.S.D) at the 1% level of significance.

Titratable acidity: The TA was significantly reduced by the biocontrol + MAP and commercial treatments, in comparison to the untreated control (Fig. 5.6). The rest of the treatment combinations did not significantly affect TA.

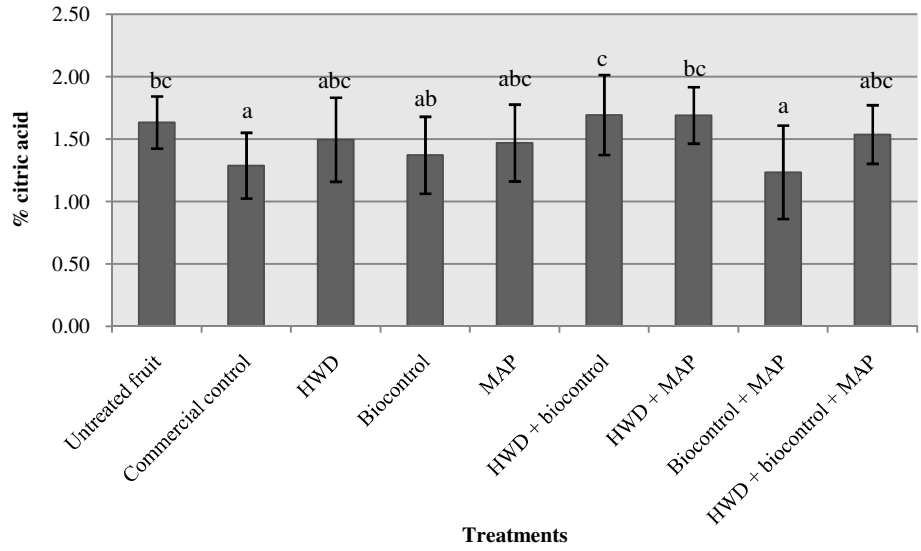

Figure 5.6: The effect of different postharvest treatments on the titratable acidity (TA), expressed as % citric acid, in 'Nules' Clementine. Error bars indicate standard deviation of treatment means. Bars with the same letter do not differ significantly with regard to TA, according to Fisher's protected Least Significant Difference test (L.S.D) at the 1% level of significance.

Maturity index: The MI of 'Nules' Clementine was significantly higher in the biocontrol and biocontrol + MAP treatments (Fig. 5.7). The rest of the treatment combinations did not significantly affect the MI in comparison to the untreated control.

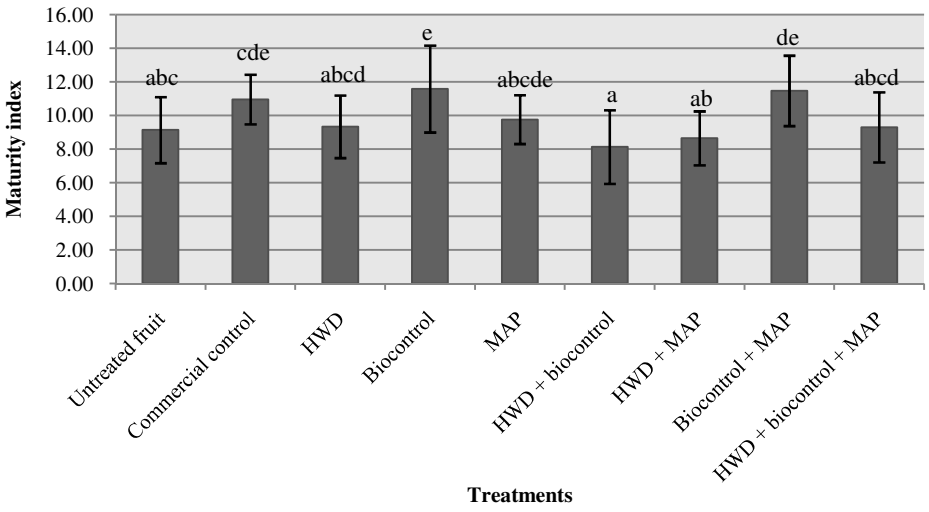

Figure 5.7: The effect of different postharvest treatments on maturity index of 'Nules' Clementine. Error bars indicate standard deviation of treatment means. Bars with the same letter do not differ significantly with regard to the maturity index, according to Fisher's protected Least Significant Difference test (L.S.D) at the 1% level of significance.

Ascorbic acid content: Fruit subjected to biocontrol + MAP and MAP treatments showed significantly higher ascorbic acid content when compared to untreated fruit. The rest of the treatment combinations did not differ significantly from the untreated control (Fig. 5.8).

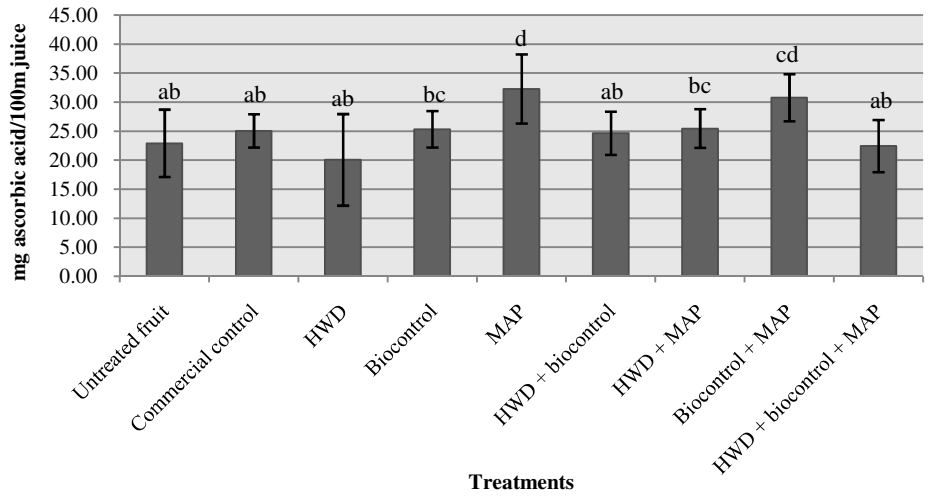

Figure 5.8: The effect of different postharvest treatments on ascorbic acid content in juice of 'Nules' Clementine. Error bars indicate standard deviation of treatment means. Bars with the same letter do not differ significantly with regard to ascorbic acid content, according to Fisher's protected Least Significant Difference test (L.S.D) at the 1% level of significance.

5.3.3 Effect of alternative postharvest treatment combinations on induced host defense reactions

Western blot analysis indicated prominent protein bands in the 70kDa range for HWD + biocontrol or MAP + biocontrol treatments (Fig. 5.9). These treatments are therefore associated with increased 70 kDa HSP production. The 70 kDa-HSP-antibody also cross-reacted with a larger HSP within the 70 – 100 kDa range. Feint bands within these ranges were also visible for some of the other treatments, including the HWD treatment. However the intensity of these bands was not comparable to that of the HWD + biocontrol or MAP + biocontrol treatments.

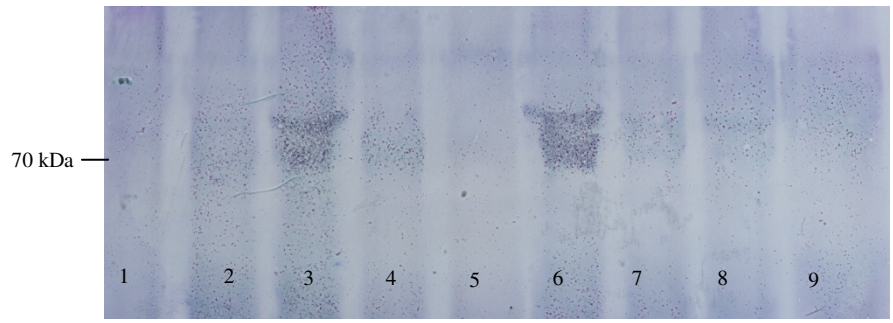

Fig. 5.9: Western blot of 2010 integrated disease management (IDM) trial, indicating differences in HSP production in response to different alternative postharvest treatment combinations. Lane 1 = Commercial control; lane 2 = MAP; lane 3 = biocontrol + MAP; lane 4 = HWD + biocontrol + MAP; lane 5 = biocontrol; lane 6 = HWD + biocontrol; lane 7 = HWD + MAP; lane 8 = HWD; lane 9 = Untreated.

Treatment combinations didn't significantly affect PAL activity in comparison to untreated fruit, except for the biocontrol treatment which resulted in significantly lower PAL activity. The TPC was significantly higher in MAP, HWD + MAP and HWD + biocontrol treatments (Fig. 5.10 and 5.11).

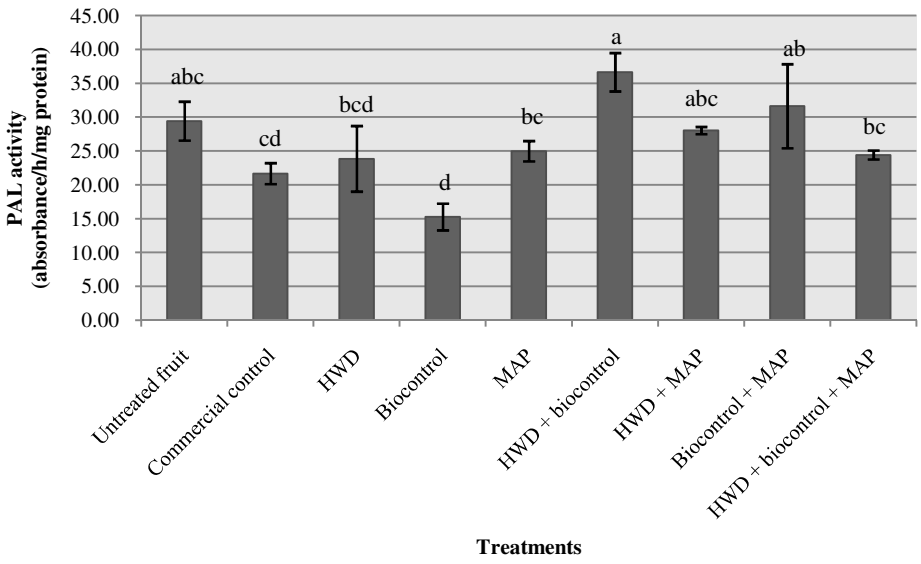

Figure 5.10: Phenylalanine ammonia-lyase (PAL) activity in 'Nules' Clementine four days after exposure to different alternative postharvest treatments. Error bars indicate standard deviation of treatment means. Bars with the same letter do not differ significantly with regard to PAL activity, according to Fisher's protected Least Significant Difference test (L.S.D) at the 1% level of significance.

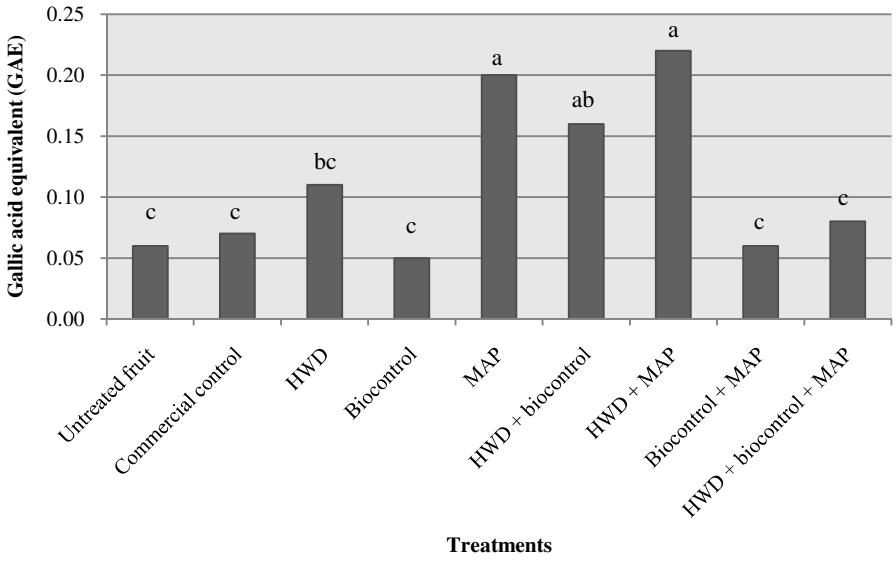

Figure 5.11: Total phenolic content (TPC) in 'Nules' Clementine four days after exposure to different alternative postharvest treatments. Bars with the same letter do not differ significantly with regard to TPC, Fisher's protected Least Significant Difference test (L.S.D) at the 1% level of significance.

5.3.4 Effect of alternative treatment combinations on *Penicillium italicum* and *P. digitatum* disease development

Disease severity followed the same trend as disease incidence in both trials, therefore, only disease incidence data is shown here. The HWD and HWD + biocontrol treatments could significantly reduce *P. italicum* disease incidence and did not differ significantly from the commercial control (Fig. 5.12). Biocontrol and MAP treatments were not effective against *P. italicum* when compared to the untreated control.

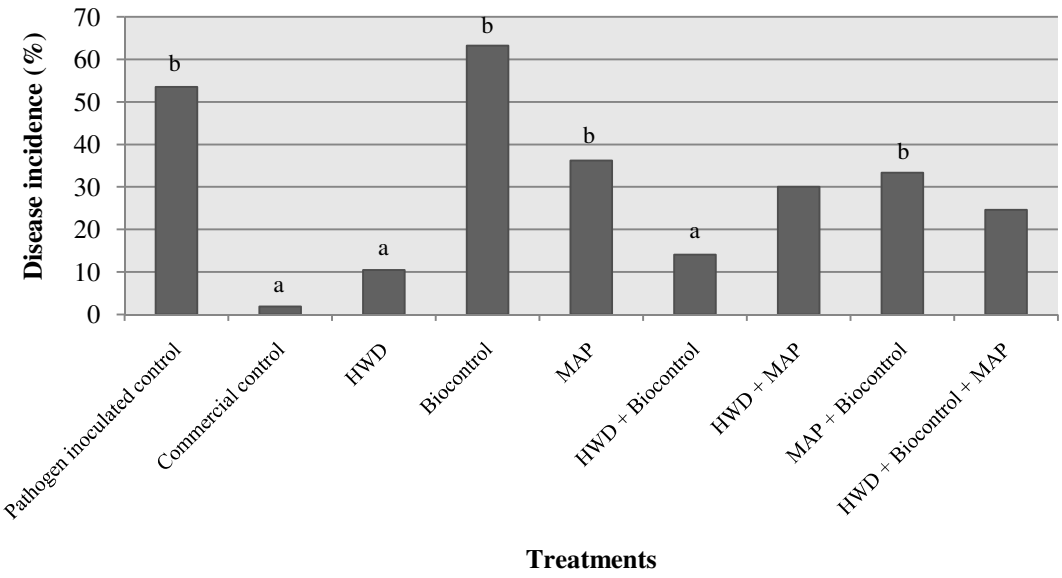

Figure 5.12: Effect of alternative postharvest treatment combinations on *Penicillium italicum* disease incidence in 'Nules' Clementine under lower-disease pressure conditions. Fruit werekept at ambient temperature and relative humidity for nine days. MAP: modified atmosphere packaging; HW: hot water dip at 52°C for 5 min.; biocontrol: combination of *Bacillus subtilis* PPCB002 and Innovacure formulation. Bars with an "a" do not differ significantly from the commercial control, while bars with a "b" do not differ significantly from the pathogen inoculated control according to the χ_2-test ($p < 0.05$).

Disease incidence of *P. digitatum* was significantly reduced by HWD + biocontrol and HWD + MAP combinations. Disease reduction by these treatments did not differ significantly from the commercial control (Fig. 5.13). The MAP and MAP + biocontrol treatments was not effective in reducing disease incidence of *P. digitatum*.

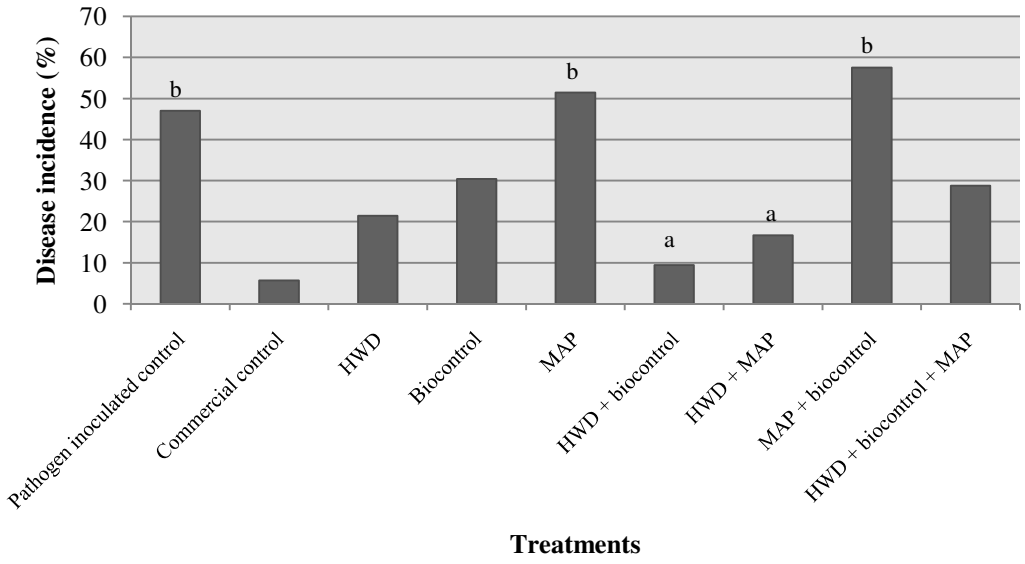

Figure 5.13: Effect of alternative postharvest treatment combinations on *Penicillium digitatum* disease incidence in 'Nules' Clementine under lower-disease pressure conditions. Fruit were kept at ambient temperature and relative humidity for nine days. MAP: modified atmosphere packaging; HW: hot water dip at 52°C for 5 min.; biocontrol: combination of *Bacillus subtilis* PPCB002 and Innovacure formulation. Bars with an "a" do not differ significantly from the commercial control, while bars with a "b" do not differ significantly from the pathogen inoculated control according to the χ_2-test ($p < 0.05$).

Under higher-disease pressure conditions, HWD + biocontrol + MAP, biocontrol + HW and HW + MAP significantly reduced *P. italicum* disease incidence (Fig. 5.14). The reduction in decay by these treatment combinations was however not comparable to that of the commercial control.

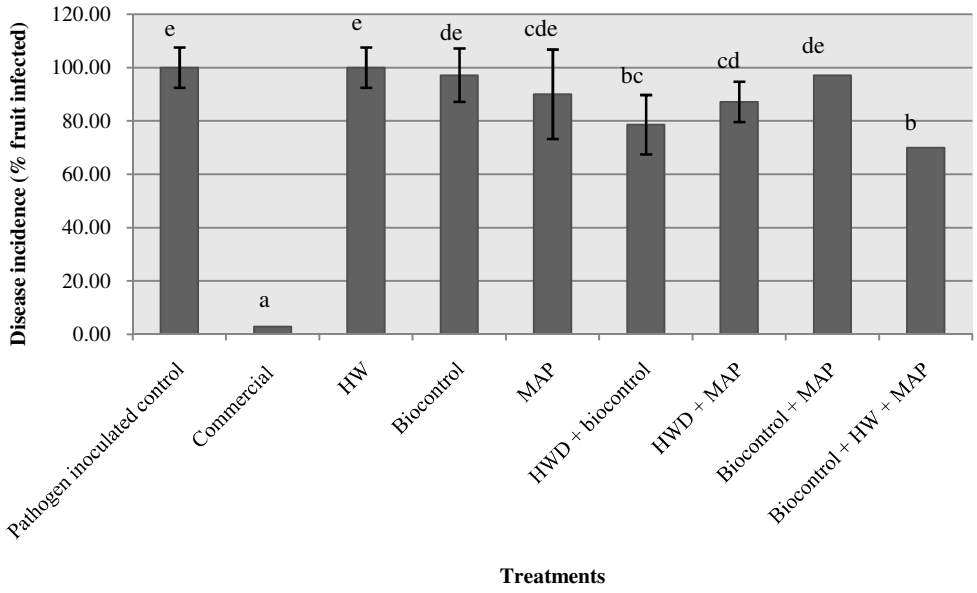

Figure 5.14: Effect of alternative postharvest treatment combinations on disease incidence of *Penicillium italicum* in 'Nules' Clementine under higher-diesease pressure conditions. Fruit were kept at ambient temperature and relative humidity for nine days. MAP: modified atmosphere packaging; HW: hot water dip at 52°C for 5 min.; biocontrol: combination of *Bacillus subtilis* PPCB002 and Innovacure formulation. Error bars indicate standard deviation of treatment means. Bars with the same letter do not differ significantly with regard to disease incidence, according to Fisher's Protected Least Significant Difference (L.S.D) test at the 1% level of significance.

Among the different alternative treatment combinations, only the biocontrol treatment could significantly reduce *P. digitatum* disease incidence under higher-disease pressure conditions (Fig. 5.15). This reduction in disease incidence was however not comparable to that of the commercial control.

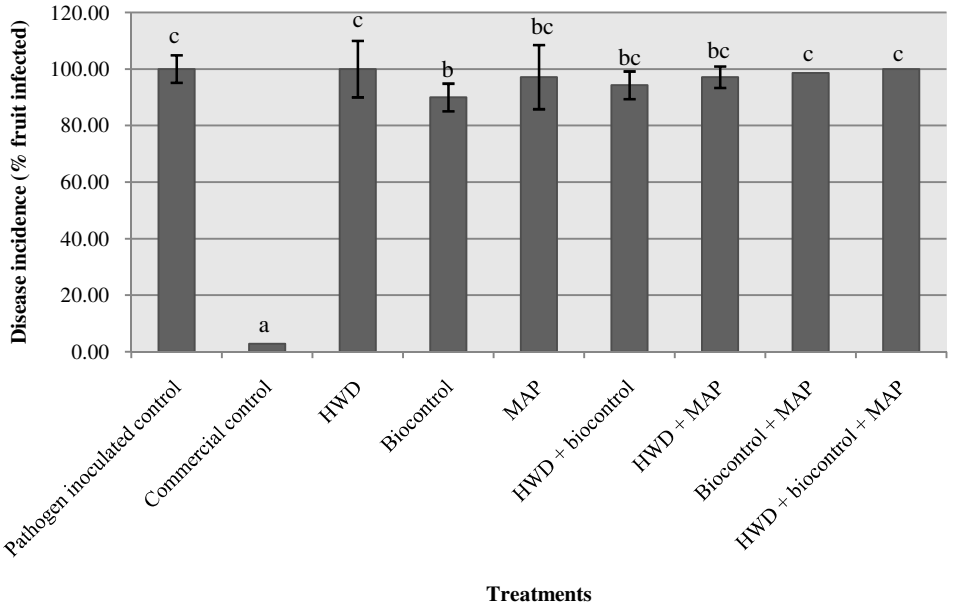

Figure 5.15: Effect of alternative postharvest treatment combinations on disease incidence of *Penicillium digitatum*in 'Nules' Clementine under higher-disease pressure conditions. Fruit were kept at ambient temperature and relative humidity for nine days. MAP: modified atmosphere packaging; HW: hot water dip at 52°C for 5 min.; biocontrol: combination of *Bacillus subtilis* PPCB002 and Innovacure formulation. Error bars indicate standard deviation of treatment means. Bars with the same letter do not differ significantly with regard to disease incidence, according to Fisher's Protected Least Significant Difference (L.S.D) test at the 1% level of significance.

Reduced sporulation in both pathogens was observed for MAP treatments (Fig. 5.16).

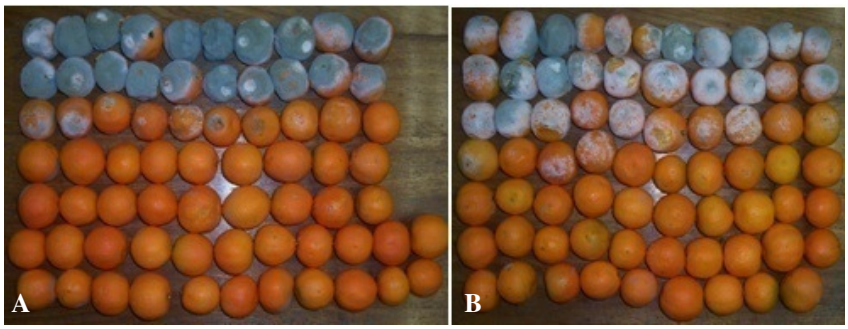

Figure 5.16: A reduction in sporulation of *Penicillium digitatum* by modified atmosphere packaging (MAP). *Penicillium digitatum* inoculated control (A) and treatment with MAP and biocontrol (B).

5.4 DISCUSSION

Results of the MAP trial revealed that the gas composition in both BOPP packaging types did not fluctuate greatly in cold storage. However when the fruit were transferred to ambient temperature, the O_2 concentration decreased drastically and a sharp rise CO_2 concentration was observed (BOPP 1: 15.3%; BOPP 2: 11.5%). Brecht *et al.* (2003) and Zhang *et al.* (2008) attributed a similar trend to the modification of film properties and fruit respiration ratethat rapidly increased when fruit were moved to ambient temperature (Brecht *et al.,* 2003; Zhang *et al.,* 2008). Gas equilibrium could not be reached in either of the BOPP bags after 10 days. The O_2 levels continued to increase steadily, while CO_2 levels continued to decrease after the fruit had been transferred to ambient temperature. Fruit in both packaging types were therefore exposed to extreme O_2 and CO_2 conditions during this period. According to Zhang *et al.* (2008), the recommended MAP conditions for citrus are 5 - 10% O_2 and 0 - 4.5% CO_2. Although the CO_2 concentration remained high in BOPP 2 (between 5 - 10%), the O_2 concentration was above the 10% limit after storage. This was not the case with BOPP 1, which resulted in extreme CO_2 and O_2 concentrations throughout storage. Also, since gas equilibrium has not been reached after 10 days, the CO_2 in BOPP 2 was expected to reach the acceptable limit over time. Therefore, BOPP 2 was selected for incorporation into the IDM program.

In this study, MAP and treatment combinations with MAP, reduced desiccation by nearly 100%. In contrast, the untreated control and waxed commercial control resulted in more than 10% weight loss. The reduction in weight loss provided by MAP is beneficial, considering moisture loss of between 5-10% can render a fruit commercially unacceptable (Tucker, 1993). Interestingly, HWD treatments did not significantly increase desiccation. According to Porat *et al.* (2000), increased moisture loss due to heat treatments is mostly associated with longer exposure periods. Fruit firmness was not significantly affected by any of the treatment combinations in this study.

Some of the fruit exposed to treatment combinations with MAP (BOPP 2) exhibited rind breakdown symptoms. Rind breakdown on citrus fruit in response to high CO_2 conditions was also reported by Ke and Kader (1990) in Petracek *et al.* (1998) and by Nelson (1933) in Shellie (2002), who called it “brown spot” or “storage spot”. However, when MAP was combined with biocontrol, no rind breakdown was observed. Therefore, the biocontrol products somehow reduced rind breakdown due to MAP conditions, even though gas

composition was not significantly affected by biocontrol treatment (data not shown). A HWD treatment at 52°C for 5 min. was not detrimental to peel tissue of 'Nules' Clementine in this case. However, when this HWD was combined with biocontrol and/or a MAP, rind breakdown was observed in some fruit. These symptoms were however different from storage spot mentioned earlier. Nevertheless, none of the treatment combinations had a significant effect on the colour of unaffected fruit (data not shown), which is in agreement with the findings of Shellie *et al.* (2002) and Luengwilai *et al.* (2007).

In this study, fruit treated with biocontrol and biocontrol + MAP had significantly higher MI levels. Brix was only affected significantly by the HWD treatment and was therefore not the reason for increased MI in these treatments. In the case of biocontrol + MAP, the increased MI could be ascribed to a significant reduction in TA, however this was not the case for biocontrol. A significant reduction in TA and increase in pH was also observed in fruit that had undergone commercial treatment. It has been reported that acidity levels lower than 0.8% leads to a poor, bland taste and increased susceptibility to pathogen attack (Chahidi *et al.,* 2008). Imazalil application provided sufficient control of *P. digitatum* and *P. italicum* regardless of the reduced TA. Co-incidentally biocontrol and biocontrol + MAP were ineffective against *P. digitatum* and *P. italicum* in all but one of the cases. Therefore, in addition to the variability of biocontrol performance and the humid conditions within the MAP, these treatments also led to a significant increase in the MI of fruit.

None of the alternative postharvest treatment combinations negatively affected ascorbic acid content in this study. The MAP and biocontrol + MAP treatments resulted in significantly higher levels of ascorbic acid in comparison to the untreated control. According to Lee and Kader (2000), ascorbic acid losses are accelerated by desiccation. Therefore, MAP retained and improved ascorbic acid content in 'Nules' Clementine in this study. Fermentation was also not triggered by MAP treatment or combinations with it, since there was no significant increase in production in any of the metabolites associated with fermentation (data not shown). Therefore, aside from the rind breakdown observed for some of the treatment combinations, BOPP 2 MAP type does not affect the taste and flavour parameters of 'Nules' Clementine. These results are in agreement with the findings of Luengwilai *et al.* (2007).

Investigation of host defence reactions showed that HWD treatment did not have a significant impact on 70kDa HSP production in comparison to the HWD + biocontrol or

biocontrol + MAP treatments. In addition, another protein within the 70 - 100 kDa range was also observed in response to these treatments. Although HSP are normally produced in response to heat treatments, other mild stress factors are also known to induce HSP production (Pavoncello *et al.,* 2001; Polenta *et al.,* 2007). These stress factors include anaerobic stress (Florissen *et al.,* 1996) and *B. subtilis* lipopeptides within the fengycin and surfactin groups (Ongena and Jacques, 2007). Furthermore, the findings in Chapter 3 also showed that fruit from the Western Cape area did not react as strongly to HWD treatment as fruit from the Eastern Cape. Since HWD treatments were applied in a similar manner and fruit from the same growing area were used for this IDM trial, the same reaction to HWD treatment is expected. Although HWD, biocontrol and MAP treatments could not elicit HSP production as stand-alone treatments, the combination of HWD with either MAP or biocontrol was able to elicit HSP production. Therefore, these treatments somehow interacted to induce HSP production within the 70 – 100 kDa range.

Biochemical analysis further indicated that the PAL activity and TPC could not be correlated with each other, which is in agreement with the findings of Chapter 3. However, in contrast to the findings of Chapter 3, PAL activity and HSP production did not respond similarly to the HWD treatments in this study. Since PAL activity can be elicited by a wide variety of triggers (Saltveit, 2000; Lafuente *et al.,* 2001; 2003; El-Ghaouth *et al.,* 2003), variability in the response to different postharvest treatment combinations is to be expected.

Results with artificially inoculated fruit showed that some alternative postharvest treatments were effective against *P. digitatum* and *P. italicum* under lower-disease pressure conditions. The HWD + biocontrol treatment was as effective as the commercial control against both pathogens under these conditions. The synergistic effect between HWD and biocontrol has been reported by Obagwu and Korsten (2003). However, the same synergistic effect was not observed against *P. italicum*,since the efficacy of the HWD treatment did not differ significantly from the HWD + biocontrol treatment. The MAP and MAP + biocontrol treatments were ineffective against both pathogens under lower-disease pressure conditions. Overall, biocontrol and MAP treatments were associated with variability and poor disease control. Although biocontrol could significantly reduce *P. digitatum* decay in one case, it was ineffective in all the other cases. Variability in performance has often been ascribed to biocontrol systems (Droby *et al.,* 1998; Wisniewski *et al.,* 2007). Similarly, the HWD + MAP performed well against *P. digitatum,* but not against *P. italicum.* Decay can be enhanced by the high humidity conditions of MAP (Rodov *et al.,* 2000; Brecht *et al.,* 2003).

Notwithstanding its poor efficacy, sporulation was reduced by MAP and the packaging had the added benefit of reducing moisture loss.

Under higher-disease pressure conditions the alternative postharvest treatment combinations showed reduced efficacy against both pathogens. Although some of the treatment combinations were able to significantly reduce disease incidence, it was not comparable to the commercial control which resulted in nearly 100% reduction in disease incidence under these conditions. Commercially used chemicals such as imazalil and thiabendazole are fungitoxic and therefore exhitit strong curative action. The systemic nature and efficacy of imazalil residues on fruit surfaces also provides residual protection against decay (Siegel and Ragsdale, 1978; Usall *et al.,* 2008). Since treatments were applied curatively in this case, it can be concluded that Imazalil was able to reduce the higher inoculum load and control pre-existing infections. The alternative treatment combinations were not as effective and lacked similar curative action. Biocontrol has often been described as an approach that does not provide curative action (El-Ghaouth *et al.,* 2003; Obagwu and Korsten, 2003; Leelasuphakul et al 2008). Nafussi *et al.* (2001) also reported that heat treatments mostly have a fungistatic effect on fungal spores. Other modes of action were therefore responsible for the reduction in decay under lower-disease pressure conditions. Induced HSP production was observed for HWD + biocontrol and biocontrol + MAP treatments. The HWD + biocontrol treatment could also effectively reduce decay of both pathogens under lower-disease pressure conditions. However, biocontrol + MAP treatment was not effective in reducing *Penicillium* decay, therefore induced HSP production can not necessarily by correlated with protection against decay in this case.

5.5 Conclusion

Among the MAP materials tested in this Chapter, a BOPP 2 packaging type which consisted of 0.00565% of perforated material, was selected as the most suitable for 'Nules' Clementine. However, fluctuating temperatures influenced the gas composition within the packaging and peel damage was observed in some fruit. Nonetheless, desiccation was reduced by 100%. Due to the humid conditions created within the packaging, MAP was not effective against *Penicillium* decay as a stand-alone treatment. Biocontrol was also ineffective and inconsistent in comparison to the commercial control. However when MAP or biocontrol was combined with a HWD at 52°C for 5 min., disease control comparable to

the commercial control could be achieved under lower-disease pressure conditions. However, under higher-disease pressure conditions alternative treatment combinations did not show similar efficacy, due to lack of strong curative action as exhibited by the commercially used chemicals. Induced HSP production could not be correlated with protection against decay in this case, because treatments that resulted in induced HSP production was not always associated with a reduction in decay. In addition, the fungistatic nature of heat treatments could also have played a role in protection against decay under lower-disease pressure conditions. Although none of the alternative treatment combinations had a major impact on internal fruit quality, results indicated that effective alternative treatment combinations were associated with maintenance of internal fruit quality or resulted in a delay ripening processes.

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CHAPTER 6:

General discussion

Reports of fungicide resistance in pathogen populations and concerns regarding human and environmental health associated with fungicide usage have accentuated the need for alternative strategies to control postharvest fruit diseases. This thesis focussed on the development of a novel integrated disease management (IDM) approach to reduce postharvest diseases and increase the marketable produce of Clementine mandarins. The alternative postharvest treatments evaluated in this programme included a pre-storage hot water dip treatment (HWD), biocontrol and modified atmosphere packaging (MAP). Initially, different intensities, formulations and packaging types of the respective treatment strategies were evaluated as stand-alone treatments. Finally, selected treatments were applied in different combinations with one another in the IDM trials. Treatments were evaluated based on efficacy against the most prevalent and destructive postharvest pathogens of citrus, *P. digitatum* (Pers.: Fr.) Sacc.and *P. italicum* Wehmer. The effect of these treatments on overall fruit quality and host defence reactions was also investigated.

The first alternative treatment investigated was heat treatments. Recent research has shown that heat treatments are not only able to reduce or inactivate pathogen inoculum on fruit surfaces, but are also able induce host defence reactions (Schirra *et al.,* 2000). Since HSP are known to be involved in this process (Polenta *et al.,* 2007), they were used as marker molecules to find an optimal HWD regime for Clementine mandarins. The enzyme phenylalanine ammonia-lyase (PAL) and various phenolic compounds are also involved in host defence reactions and their response to the different HWD was also monitored. Results indicated that a HWD at 52°C for 5 min. allowed maximum elicitation of the 70 kDa HSP in 'Nules' Clementine, without damaging the fruit. This HWD was therefore selected for further investigations in the IDM trials (Chapter 5). Time of peak HSP production occurred within four days after exposure to heat treatment. Some variability in the heat shock response was however observed in this study and could be ascribed to differences in production area, growing season and/or treatment application. It was also concluded that HWD regimes exceeding 55°C resulted in peel damage and is therefore not recommended for 'Nules' Clementine.

Results further indicated that PAL activity and HSP responded similarly to HWD treatments. However, when HSP production was most pronounced, reduced levels of PAL activity was observed. Saltveit (2000) and Campos-Vargas *et al.* (2005) reported that plants respond preferentially to heat than to other stress factors and therefore increased HSP production can lead to inhibition phenolpropaniod biosynthesis. No correlation could however be drawn between PAL activity and TPC. Differences in environmental factors, treatment applications and physiological maturity of peel tissue could have affected TPC and its response to HWD treatment. In addition, the phenyl-propanoid pathway in which PAL is involved not only leads to the production of phenolics, but also to other secondary metabolites such as phytoalexins and lignins (El-Ghaouth *et al.,* 2003).

In Chapter 4, a promising strain of *Bacillus subtilis* (Ehrenberg) Cohen (PPCB002) was investigated and was able to inhibit growth of *P. digitatum* and *P. italicumin vitro.* The major mode of action of PPCB002 could be established as antibiosis and in particular, the production of Bacillomycin. This organism was also able to compete for space and nutrients through colonisation of surfaces (biofilm formation). However, during *in vivo* trials with 'Nules' Clementine a PPCB002 formulation did not show similar efficacy against these pathogens. According to various authors, the formulation of biocontrol products can lead to loss of antagonistic properties (Korsten *et al.,* 1993; Havenga *et al.,* 1999; Torres *et al.,* 2007). Subsequently, a yeast-based biocontrol product, Innovacure (*Candida saitoana* Nakasa and Suzuki) was included in the bioassays with 'Nules' Clementine. The two biocontrol formulations were also combined to determine if any synergistic effect resulted from their combination. The Innovacure formulation, as well as the Innovacure + PPCB002 combination treatments could successfully reduce disease incidence and severity of both pathogens. Control comparable to the commercial treatment could be achieved in some cases. The Innovacure + PPCB002 combination treatment was selected for inclusion in the IDM trials. Since the metabolism and mode of action of these products or biocontrol agents are different, their combination was expected to be more resilient under a variety of circumstances and in combination with other postharvest treatments.

Three different MAP was investigated for reducing desiccation in 'Nules' Clementine (Chapter 5). Selection of MAP was based on the gas composition created within the packaging. The biodegradable MAP type was not suitable for packing fruit since it was easily ruptured. The gas composition in both bioriented (BOPP) packaging types changed drastically with fluctuating temperatures. This trend has been ascribed to modification of

film properties and the respiration rate of the fruit (Brecht *et al.,* 2003; Zhang *et al.,* 2008). Subsequently, fruit in both packaging types were exposed to extreme O_2 and CO_2 conditions (O_2: >10% and CO_2: <4.5%) for a certain period (Zhang *et al.,* 2008). However, the O_2 concentration in the BOPP 2 packaging was above the 10% limit after storage and the gradually declining CO_2 concentration was expected to reach the acceptable limit over time. Therefore, BOPP 2 was selected for further investigation in the IDM program.

The best-performing alternative treatments were applied in different combinations with one another in the IDM trials (Chapter 5). Although treatments with MAP resulted in nearly 100% reduction in decay, rind breakdown symptoms were observed on some fruit exposed to BOPP 2 and combinations with it. "Brown spot" or "storage spot" on citrus fruit in response to high CO_2 conditions was also reported by Ke and Kader (1990) in Petracek *et al.* (1998) and by Nelson (1933) in Shellie (2002). However, when MAP was combined with biocontrol, no rind breakdown was observed. A HWD treatment at 52°C for 5 min. was not detrimental to peel tissue of 'Nules' Clementine in this case. However, when HWD was combined with MAP or biocontrol, peel damage was also observed on some fruit.

Aside from rind breakdown, none of the treatment combinations had a radical effect on fruit quality. Interestingly however, fruit treated with biocontrol and biocontrol + MAP had significantly higher MI levels. Co-incidentally biocontrol and biocontrol + MAP were also ineffective against *P. digitatum* and *P. italicum* in all but one of the cases. An increased MI in fruit is associated with an increase in sugar content and/or decrease in acidity, which increases their susceptibility to pathogen attack (Agrios, 2005; Chahidi *et al.,* 2008). Poor disease control by these treatment combinations could therefore be attributed to higher MI levels in fruit.

Investigation of host defence reactions showed that HWD treatment did not have a significant impact on 70kDa HSP production in comparison to the HWD + biocontrol or biocontrol + MAP treatments. Apart from heat treatments, the HSP also respond to other stress factors. According to Florissen *et al.* (1996) andOngena and Jacques (2007), both biocontrol and MAP treatments are able to trigger HSP production, which explains why increased HSP production was detected with these treatment combinations. Furthermore, the findings of Chapter 3 showed that fruit from the Western Cape area did not react as strongly to HWD treatment. In contrast to the findings of Chapter 3, PAL activity and HSP production did not respond similarly to the HWD treatments in the IDM trial. Since PAL

activity can be elicited by a wide variety of triggers (Saltveit, 2000; Lafuente *et al.,* 2001; 2003; El-Ghaouth *et al.,* 2002), variability in the response different postharvest treatment combinations is to be expected.

Results of the two IDM trials indicated that some alternative postharvest treatments were effective against *P. digitatum* and *P. italicum* under lower-disease pressure conditions. The HWD + biocontrol treatment was as effective as the commercial control against both pathogens under lower-disease pressure conditions. In contrast to Obagwu and Korsten (2003), there was no synergistic effect involved between these treatments, since their combination did not always result in an improvement in disease control. Overall, biocontrol and MAP treatments were associated with variability and poor disease control. Variability in performance has often been ascribed to biocontrol systems (Droby *et al.,* 1998; Wisniewski *et al.,* 2007). Decay could also have been enhanced by the high humidity conditions within MAP (Rodov *et al.,* 2000; Brecht *et al.,* 2003).

Under higher-disease pressure conditions the alternative postharvest treatment combinations showed reduced efficacy against both pathogens. Although some of the treatment combinations were able to significantly reduce disease incidence, it was not comparable to the commercial control which continued to achieve nearly 100% reduction in disease. The alternative treatment combinations lacked strong curative action as exhibited by Imazalil in this case (Siegel and Ragsdale, 1978; Usall *et al.,* 2008). Biocontrol has often been described as an approach that does not provide curative action (El-Ghaouth *et al.,* 2003; Obagwu and Korsten, 2003; Leelasuphakul et al 2008). Nafussi *et al.* (2001) also reported that heat treatments mostly have a fungistatic effect on fungal spores. Other modes of action were therefore responsible for the reduction in decay under lower-disease pressure conditions. However, treatments which resulted in induced HSP production did not always result in reduction in decay. Therefore, induced HSP production could not be correlated with protection against decay in this case.

In conclusion, a BOPP MAP with 0.00565% of perforated material is not recommended for Clementine mandarins, since it resulted in poor disease control and rind breakdown on fruit in this study. A HWD at 52°C for 5 min. followed by an application of Innovacure (*C. saitoana,* 5 x 10^7 cfu/ml) and PPCB002 formulations (1 x 10^9 cfu/ml), resulted in most effective and reliable disease control among the various alternative treatment combinations tested. This treatment combination relied on the following mechanisms of

action: the fungistatic effect of the HWD, the antagonistic effect of the biocontrol formulations and maintenance of overall fruit quality and/or reduction in maturity index. However, peel damage was observed on some fruit exposed to this treatment combination. In addition, the biocontrol treatment was associated with variability and poor disease control in the IDM trial. It is therefore recommended that future studies focus on either the HWD or Innovacure treatment and combine these with other, more effective biocontrol formulations or disease control strategies.

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SUMMARY

The aim of this thesis was to develop an effective integrated disease management (IDM) program to reduce *Penicillium digitatum* (Pers.: Fr.) Sacc. and *P. italicum* Wehmerdecay in Clementine mandarins, while still maintaining overall fruit quality. Three alternative treatments were investigated for inclusion into the IDM programme: hot water dips (HWD), biocontrol and modified atmosphere packaging (MAP).

In Chapter 3, the effect of hot water dips on host defence reactions was investigated in order to improve host resistance to green- (*P. digitatum*) and blue mould (*P. italicum*) in fruit. The 70 kDa heat shock protein (HSP) was used as target molecule to detect increased defence reactions in fruit subjected to various HWD regimes. The effect of these heat treatments on total phenolic content (TPC) and phenylalanine ammonia-lyase (PAL) activity was also investigated. Maximum 70kDa HSP production occurred in fruit subjected to 52°C for 5 min. four days after exposure to heat treatment. Treatments exceeding 55°C resulted in peel damage. Results further indicated that the enzyme PAL was also involved in the heat shock response and that increased HSP production was assiociated with reduced PAL activity. Although increased HSP production was induced by some treatment combinations in the IDM trial, it could not be correlated with protection against decay in this study.

The efficacy and mode of action of the bacterial biocontrol agent, *Bacillus subtilis* (Ehrenberg) Cohen strain PPCB002 (PPCB002), against green- and blue mould was investigated in Chapter 4. Although *in vitro* studies indicated that PPCB002 was effective against *Penicillium* pathogens through a variety of mechanisms, similar efficacy could not be achieved during *in vivo* experiments with artificially inoculated 'Nules' Clementine. Therefore, a commercially formulated product, Innovacure (*Candida saitoana* Nakasa and Suzuki), was included in further investigations. The Innovacure formulation, as well as its combination with PPCB002 could successfully reduce disease incidence and severity of both pathogens. The combination of the two biocontrol formulations was selected for incorporation into the IDM trial, since the two products or organisms have different modes of action.

Finally, a suitable MAP type was selected in Chapter 5 and combined with the previously selected hot water dip (HWD) and biocontrol treatments. A bioriented

polypropylene (BOPP) packaging type with 0.00565% perforated material was selected among three different packaging materials as the most suitable for Clementine. Although desiccation could effectively be reduced by this packaging type, it was associated with high levels of decay. Under fluctuating conditions, extreme O_2 and CO_2 levels also developed within the packaging which resulted in peel damage on some fruit. This MAP type is therefore not recommended for Clementine.

A HWD at 52°C for 5 min. combined with a mixture of PPCB002- and Innovacure-formulations, was effective against green and blue mould under lower-disease pressure conditions. However, rind breakdown was detected on some of the fruit subjected to this treatment combination. In addition, the biocontrol treatment showed variability in performance and lack of curative action, especially at ambient temperature (Chapter 3). It is therefore recommended that future studies continue with either the HWD treatment or the Innovacure formulation, in combination with other, more effective biocontrol formulations and/or other disease control strategies.

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