EFFECTIVENESS OF PRE- AND POSTHARVEST SILICON AND PHOSPHOROUS ACID APPLICATIONS IN INHIBITING *PENICILLIUM DIGITATUM* ON CITRUS FRUIT

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DECLARATION

I, Nopayi Mkhize, declare that

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ABSTRACT

Citrus is, by tonnage, globally the most-produced fruit. Although technological advances have greatly improved storage life and quality of citrus, postharvest decay remains a major problem. *Penicillium digitatum* (green mold) and *P. italicum* (blue mold) are the most economically important postharvest pathogens. Over the years, fungicides belonging to the benzimidazole, thiabendazole (TBZ), benomyl, and imidazol (IMZ) groups have been used extensively to control these diseases; however, the development of fungicide-resistant strains of the pathogens together with the withdrawal of effective chemicals from the market has led to the search for more integrated methods of disease control.

Silicon and phosphorus are able to trigger some 'systemic responses' that enhances fruit resistance to pathogen attack. The aim of this study was to ascertain the changes in biochemical composition of fruit after application of these two chemicals with the intention to improve the current understanding of their mechanisms of action. A proper understanding of these mechanisms could allow for the manipulation of fruit metabolism to improve the level of disease control.

Two orange cultivars ('Delta' Valencia and 'Washington' navel) as well as one lemon cultivar ('Eureka') from Ukulinga Research Farm, Pietermaritzburg, were used. Fruit were treated both, pre- and postharvest with three different concentrations of potassium silicate (Si, 1250mg ℓ^{-1}, 2675mg ℓ^{-1} and 5350mg ℓ^{-1}) and one concentration of phosphorous acid (P, 500mg ℓ^{-1}) as well as a combination of each of the Si concentrations together with P (1250 + 500, 2675 + 500 and 5350+ 500 mg ℓ^{-1}). For the pre-harvest experiment, trees bearing fruit were treated by a soil drench around the base of the trunk with 5 ℓ treatment solution. This treatment was carried out for four consecutive weeks leading up to harvest. As a postharvest treatment, fruit were immersed in treatment solutions for a period of 90 s. Control fruit as well as control trees were treated with water. Following these applications, fruit were inoculated with a 1 x 10^4 mℓ spore suspension of *P. digitatum*, stored at 5.5^{o}C, and sampled for

biochemical analysis ten days later. Petroleum jelly was applied over the area where the peel tissue had been sampled in order to prevent fruit desiccation and allow for disease monitoring. Disease lesion size (mm), as well as total rind phenolic and flavonoid concentrations were determined. Results were compared by analysis of variance (ANOVA) followed by Fisher's Protected Least Significant Difference test ($P< 0.05$) using GenStat® Version 14.

When applied at the lowest concentration (1250 mg ℓ^{-1}), the Si treatment provided the most effective disease inhibition, as these fruit developed the smallest average lesion size. The two higher Si concentrations (2675mg ℓ^{-1} and 5350mg ℓ^{-1}) were not significantly different ($P>0.05$) from each other and the control. Phosphorous acid provided less disease control than all other treatments and the control. Although the treatment combinations did not have a synergistic effect on disease suppression, they delayed disease onset and sporulation compared with the treatments alone. There were significant differences in the level of disease inhibition achieved by the treatments, but differences in phenolic and flavonoid concentration between treatments were not consistently significant; it can, however, be concluded that there was a correlation between disease control and increased rind phenolics. Increasing the concentration of Si did neither result in a significant increase in the level of disease control, nor in an increased production of rind phenolics or flavonoids. Separate Si and P treatments proved to be more effective in hindering disease spread than the combination of these treatments. Further research is required to fully understand the biochemical changes that these chemicals induce and to determine which mode of action they follow.

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DEDICATION

To my mother Zodwa, my grandmother mak'Nene, my grandfather Khabazela, my aunts Ntombi, Themba, Solo, Zanele, Sbongilie, Manto and Zilungile; without you I would not be half the woman I am today

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CHAPTER ONE - INTRODUCTION

Although technological advances have greatly improved storage life and quality of citrus fruit, postharvest decay remains a major problem. Penicillium rot (green and blue mold) caused by *Penicillium digitatum* (Pers.:Fr.) Sacc. (*P. digitatum*) and *Penicillium italicum* Wehmer, (*P. italicum*) respectively, account for a large percentage of postharvest fruit loss throughout the world (Ballester *et al.*, 2010). *Penicillium digitatum* and *P. italicum* are, therefore, the most economically important postharvest pathogens of citrus (Brown, 1985). Currently, there is an overwhelming reliance on synthetic fungicides to maintain fruit quality during the postharvest life of fruit. Fungicides belonging to the benzimidazole, thiabendazole (TBZ), benomyl and imidazol (IMZ) groups have been used extensively to control these diseases (Yoshioka *et al.*, 2010).

Over the past decades, however, consumer awareness of health and environmental issues associated with use of fungicides has led to the withdrawal of several important *Penicillium*-controlling fungicides; generally, there is a demand for produce to contain lower fungicide residues than the officially set standard (Ballester *et al.*, 2010). In addition, most of the remaining permitted fungicides have been rendered almost ineffective by fungicide-resistant strains of the pathogens they are to curtail. Fungicide resistance of *Penicillium* was first discovered in the early 1970s following the registration and extensive use of the systemic fungicide benomyl (Methyl 1-(butylcarbamoyl)-2-benzimidazolecarbamate, Damicone and Smith, 2009). The search for alternative or integrated methods of disease control has, therefore, become a priority (López- García *et al.,* 2003).

Amongst the alternative methods to control *Penicillium* biological control, using ‘generally recognized as safe’ (GRAS) substances, hot water treatments and ultra-violet and x-ray irradiation have been studied; however, results of these control methods have been variable (Adaskareg, 2005).

Numerous studies have also been initiated to gain insight into the mechanisms of fungicide resistance of *P. digitatum*. In several of these studies fungicide resistance has been correlated with point mutations in the β-tubulin gene that ultimately results in altered amino acid sequences at the fungus-fungicide binding site (Sánchez-Torres, 2011). Hamamoto *et al.* (2000) reported that in *P. digitatum* a unique sequence in the 126bp promoter region of CYP51, was found to be repeated five times in the isolates that exhibited fungicide resistance, while this sequence was present only once in sensitive strains of the pathogen. Understanding such resistance mechanisms is an important step in the quest to formulate new effective control measures against *P. digitatum*.

A different strategy that has recently received widespread popularity is the induction of disease resistance. This approach aims to increase the fruit's natural defence mechanisms resulting in the induction of pathogenesis-related (PR) proteins and in the accumulation of phytoalexins (Ballester *et al.*, 2010). Increasing the fruit's own natural defence system has the potential to reduce the use of postharvest fungicides that are applied to protect the fruit during its postharvest shelf life. The genus *Citrus* has been shown to produce a variety of coumarins that accumulate in fruit tissue following infection by phytopathogenic fungi (Del Rio *et al.*, 2004). The nature of phytoalexin production and the rate at which these substances accumulate are largely host-specific and depend on the pathogen genotypes (Ortoño *et al.*, 2011). Although research has shown that certain citrus flavonoids may have anti-fungal agents against *Penicillium spp.*, little is known about other secondary compounds involved (Ortoño *et al.*, 2011).

In mature citrus fruit, resistance to *Penicillium* rot can be elicited by application of physical, chemical, or microorganism antagonistic treatments; however, the efficacy of such treatments has been variable, often depending on cultivar and fruit maturity (Ballester *et al.*, 2010). Silicon (Si) has been studied extensively as an alternative to certain fungicides.

Research conducted has shown that an increase in Si concentration in certain tissues can reduce disease incidence and increase yield in rice, sugarcane and several other cereal crops (Datnoff *et al*., 2001); however, very few experiments have aimed to study the use of Si as an elicitor of induced disease resistance. Understanding the mechanisms underlying the biochemical and molecular basis of induced resistance would assist in improving overall efficiency of induced resistance (Ballester *et al.*, 2010). The aim of this study was therefore to determine the effectiveness of three different concentrations of Si and one concentration of phosphorus acid in controlling *Penicillium* and establish the changes in biochemical composition of citrus fruit after application of these two chemicals. This was done with the intention of improving the current understanding of how these chemicals interact with the fruit mechanisms.

CHAPTER TWO: LITERATURE REVIEW

2.1 Introduction

Citrus fruit are produced globally, with more than 20 countries producing an excess of 0.5 million tons in 2012 (www.faostat.fao.org/site/339/default.aspx). Oranges, lemons, mandarins and other 'soft citrus' belong to the family Rutaceae, genus *Citrus*, and are well- known for their nutritive and therapeutic value (Ladaniya, 2008). Citrus fruits are categorized as non-climacteric, because of the absence of an autocatalytic surge in ethylene production during ripening (Périn *et al.*, 2000), and have a relatively longer shelf-life than other tropical and subtropical fruits such as bananas, mangos and litchis. Citrus fruit contain high concentrations of ascorbic acid (vitamin C), as well as vitamins of the B complex, flavonoids and carotenoids. Flavonoids, especially those found in Sweet oranges, *Citrus sinensis* (*L*.), and grapefruit, *Citrus paradisi* Macfad, have been shown to improve blood circulation and have anti-allergenic, anti-carcinogenic and anti-viral properties in mice (Tripoli *et al*., 2007). Some citrus species are also high in fibre, pectin and trace elements (Ladaniya, 2008; Gorinstein *et al.*, 2001).

In 2011, South Africa became the world's third largest exporter of fresh citrus fruit, with Spain and Turkey in first and second position, respectively; overall South Africa was ranked 13th in the world in terms production. China is the world's biggest citrus producer, followed by Brazil and India, respectively (Citrus Growers Association, 2014).

Citrus producers, in different regions, face various postharvest challenges. In most developed countries, postharvest losses of fresh fruit range from 5-10%, while in developing and under-developed countries such losses range from 25-30% (Ladaniya, 2008). Postharvest losses are mainly caused by improper fruit handling, fruit senescence, physiological disorders and, most importantly, postharvest diseases. Postharvest infection

results in the development of off-flavours and ultimately leads to fruit decay, which makes fruit unsuitable for both the fresh fruit as well as the processing markets.

Plant diseases such as sour rot and diplodia stem end rot caused by the fungal pathogens *Galactomyces citri-aurantii* and *Diplodia natalensis*, respectively, are problematic in fruit produced in the warmer, more humid regions of the world (Brown, 1994b). *Penicillium italicum* is prevalent in fruit stored for prolonged periods, but the most destructive postharvest pathogen in citrus is *P. digitatum*. This fungus infects fruit through injuries; the available moisture and nutrients at injury points stimulate spore germination leading to infection (Brown *et al*., 2000). Infection can occur through individual oil glands of the exocarp (flavedo) and also through deep puncture wounds that extend into the mesocarp (albedo). These wounds are mostly unavoidable and are not easily detected during grading. They may occur at any point from harvesting, through twigs and thorns in the tree canopy, or during transportation to or out of the packhouse, as well as through nails or wooden splinters on pallet boxes (Brown *et al*., 2000).

Previously, fungicides have provided effective control of many postharvest diseases. Fungicides belonging to the benzimidazole, thiabendazole (TBZ), as well as to the benomyl and imidazol (IMZ) groups have been used extensively in commercial packhouses to control postharvest citrus decay. These groups are systemic fungicides that act on specific target sites; however, mutations in the corresponding genes of pathogens can result in the development of resistance (López-García *et al*., 2003).

The development of fungicide-resistant pathogens, together with the withdrawal of effective chemicals from the market due to human health and environmental concerns, has led to the search for more integrated methods of disease control. Therefore, understanding plant - microbe interactions is important as this may provide insights into ways of developing new *Penicillium* control measures.

2.2 Importance of Citrus Trade in South Africa

South Africa, with 54% of the country's total citrus production being exported, is the third largest citrus exporter, with only Spain and Turkey producing higher citrus volumes for the export industry. The South African citrus industry is a well-established, more than 300 year-old industry and is made up of about 1 300 export farmers and 2 200 smaller farmers. Citrus fruit are cultivated on over 58 000 ha of the country, making citrus the second largest earner of foreign exchange through agricultural exports. The industry generates well in excess of R3 billion in annual revenue (South African Fruit Farmers Association, 2010). In the 2012 season the main market for South Africa's orange exports was Northern Europe which absorbed 23 % of total orange exports. This is followed by The Middle East which accounts for 20 %, then by Russia, South East Asia and South Europe. 41% of the countries lemon exports in 2012 was absorbed by the Middle East with Northern Europe, Russia, South East-Asia and the United Kingdom accounting for the other 49% of the country's total lemon exports.

The Citrus Growers' Association is the main organization that regulates both, domestic and international handling and marketing of fruit produced within South Africa. Within the country, produce is distributed through agents, wholesalers, retailers, hawkers and institutional buyers (Ladaniya, 2008); orange production (mainly Valencias and navels) makes up 2/3 of the SA citrus production (Figure 2.1).

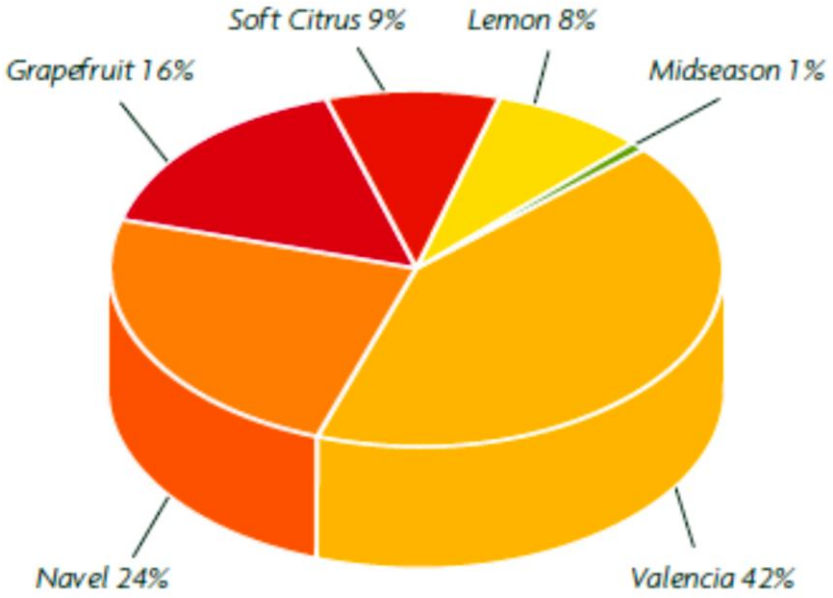

Figure 2.1: Breakdown of the different groups of citrus fruit that made up total citrus production in South Africa in 2011 (Source: Citrus Growers Association of Southern Africa, 2013).

2.3 Economically Important Citrus Species and Cultivars in South Africa

2.3.1 Valencia oranges (*Citrus sinensis* L.)

Known as “The King of Juice Oranges” Valencias are commercially the most commonly produced sweet oranges. Valencias originated in China and are thought to have been introduced to Europe by Portuguese and Spanish explorers (Khan, 2009). Valencias feature a good internal fruit quality and high climatic adaptability; these characteristics have made the fruit popular with growers. Valencias are characterized by their smooth, thin skin (Figure 2.2), sharp flavour and high juiciness and are regarded as commercially seedless - features that make the fruit excellent for both, the processed and fresh fruit markets (Khan, 2009). Internally, Valencia fruit have bright, yellowish to pale orange flesh.

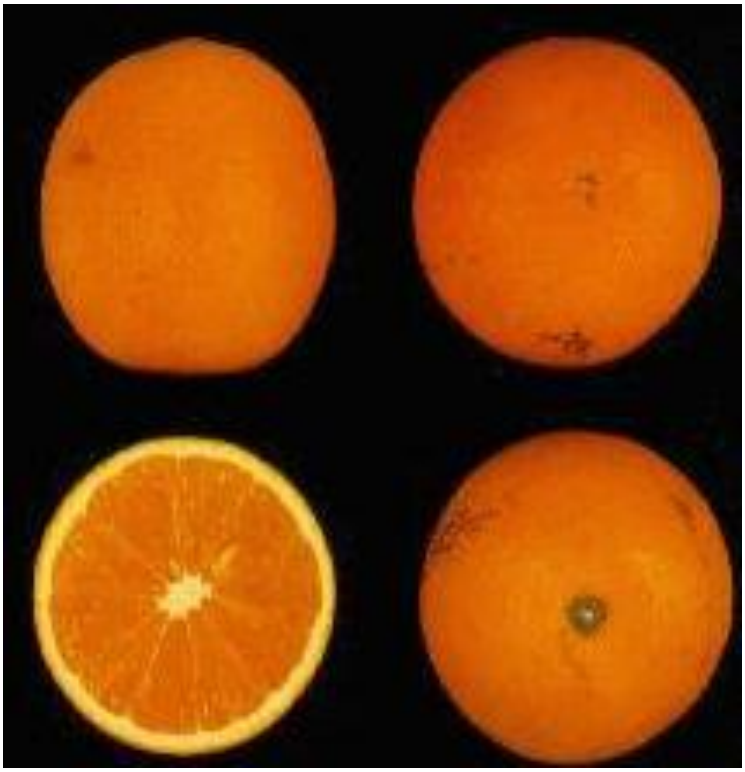

Figure 2.2: The Valencia orange is the most important commercial sweet orange used for both the fresh and processing markets (Source: http//swfrec.ifas.ufl.edu).

2.3.2 Navel oranges (*Citrus sinensis* L.)

Navel oranges are named after the protuberance at the stylar end of the fruit, which carries an embryonic fruit (Figure 2.3). These oranges are seedless with a thick albedo and sweet juicy flesh. They are mainly consumed fresh and not in the juicing industry because the juice tends to acquire a bitter taste after processing. The cultivar thrives in subtropical climates and, besides South Africa, is grown extensively in Spain, Brazil, Turkey and Morocco (Ladaniya, 2008).

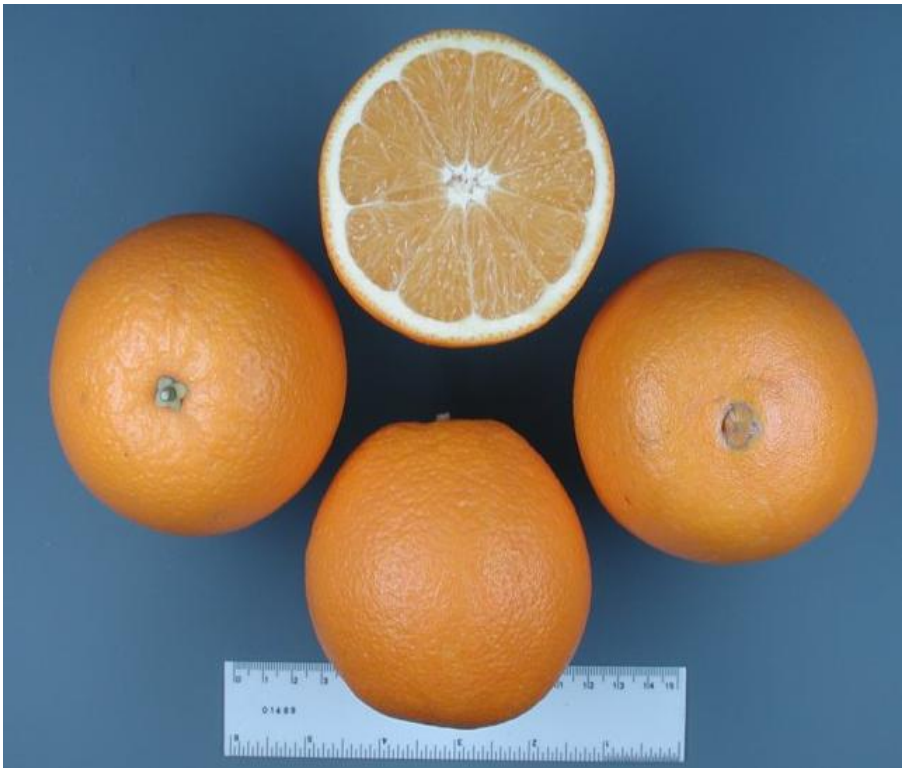

Figure 2.3: Navel oranges characterized by the growth of a bud which carriers an embryonic fruit. These oranges have a relatively thicker skin than the Valencia fruit (source: http://aggie-horticulture.tamu.edu/citrus).

2.3.3 Lemons (*Citrus* limon (L.) Burm.f.)

Lemons are thought to have originated from the Sub-Himalayan region and India (Ladaniya, 2008). Unlike oranges and mandarins, lemons are not commonly eaten fresh although they are an important component of the fresh fruit market. Lemons are mainly used for drinks, fresh juice, flavourings and medicinal purposes.

2.4 Common Postharvest Diseases of Citrus Fruit and Their Management

Several diseases affect citrus fruit that can cause postharvest losses. Penicillium rot (green and blue mold) caused by *Penicillium digitatum* (Pers.:Fr.) Sacc. and *Penicillium italicum* Wehmer, respectively, account for a large percentage of postharvest fruit loss across the world (Ballester *et al.*, 2010). Although these two pathogens are slightly different, both enter

the fruit through wounds formed on the surface and have the ability to quickly colonize the fruit. These pathogens are particularly important because they have the ability to develop fungicide-resistant strains, which are impossible to control using traditional fungicides.

2.4.1 Green mold

Green mold is caused by the fungus *Penicillium digitatum* (Pers.:Fr.) Sacc. The fungus infects fruit through injuries caused by dead wood and twigs in the canopy or during harvest. In addition, infection may occur when fruit fall onto the ground and split during the harvesting process. Even shallow injuries that involve only a few oil glands allow for infection (Brown, 1994c). The fungus can also enter the fruit through physiologically induced injuries like those associated with chilling injury. The spores contaminate packhouse equipment and accumulate in the drencher and soak tanks. They can contaminate storage rooms, transit containers and retail market places (Brown, 1994c). This means that the infection cycle can be initiated repeatedly in the packhouse, during storage and in transit.

Green mold symptoms initially appear as small soft water-soaked spots that are similar to those of sour rot (*Geotrichum citri-aurantii*) and blue mold (*P. italicum*) (Figure 2.4). The outer region of the sporulating lesion remains mycelium-free but becomes softened as the lesion continues to spread over the entire fruit. Fruit decay results in the production of large quantities of ethylene, speeding up respiration, promoting colour development and ultimately hastening senescence. During storage spores can be disseminated to healthy fruit but if these fruit are not injured infection cannot occur. Soiled fruit remain healthy but have to be cleaned before retail sale (Brown, 1994c).

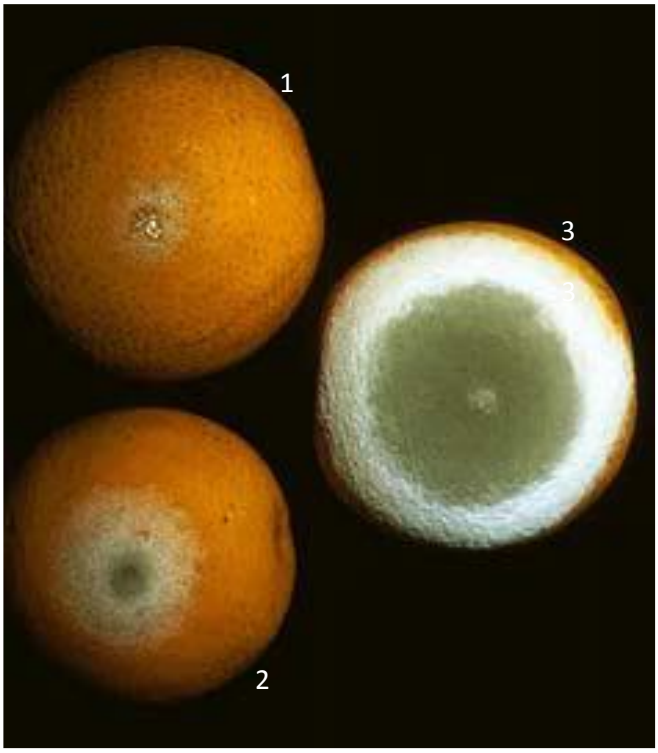

Figure 2.4: Progression of green mold (from 1-3). (1) As the lesion enlarges, white mycelium is produced on the surface of the lesion (2) and green to olive-green spores are produced at the centre of the lesion (3) (source: Brown, 1994c).

2.4.1.1 Control of green mold

Since *P. digitatum* infects fruit through wounds, fruit must be handled carefully during harvest in order to minimize injury. When fruit is graded, infected fruit must be removed promptly in order to avoid accumulation of spores in the packhouse or storage rooms. To limit the size of airborne spore populations, exhaust fans can be used to remove spores from the packhouse atmosphere (Brown, 1994c). It is also important to sanitise the packhouse, the packaging line and the washer brushes to eradicate inoculum. Solutions in drenchers and soak tanks must be treated continuously with sanitizers, such as chlorine, to prevent accumulation of green mold. Since *P. digitatum* has been known to develop resistance to postharvest fungicides, it

is important to alternate two or more unrelated fungicides to minimize the development of resistance (Palou et al., 2001)

2.4.2 Blue mold

Blue mold is caused by the fungal pathogen *Penicilium italicum* Wehmer. This disease is prevalent in fruit that have been stored for prolonged periods, because of the fungus' ability to continue development during cold storage (Brown, 1994a). The disease cycle and infection process are very similar to that of green mold, but lesions caused by *P. digitatum* expand faster than those of *P. italicum*. When infections occur simultaneously, *P. digitatum* establishes faster and, therefore, gains primary access to available resources (Plaza *et al.*, 2004), thus spreading more rapidly than *P. italicum*.

The airborne spores of the *P. digitatum* are produced in soil debris and carried by wind currents into the tree canopy. Infection, however, occurs through wounds caused by improper handling during harvest. The spores germinate and infect fruit when moisture is released from these wounds and nutrients in the fruit tissue become available to the fungus (Brown, 1994a). Like those of green mold, spores of blue mold can contaminate the packhouse equipment, soak tanks, storage rooms and transit containers (Brown, 1994a).

Blue mold is more difficult to control than green mold because of its ability to infect fruit regardless of injury. The fungus produces enzymes that soften adjacent uninfected fruit, allowing it to penetrate into fruit that initially had no wounds that served as an entry point. This ultimately results in the spread of the fungus throughout the whole packing unit. Green mold does not exhibit this "nesting" behaviour, thus infected fruit do not contaminate adjacent fruit, unless there is an injury wound or entry point allowing spores to germinate (Ladaniya, 2008).

Initial symptoms of *P. digitatum* infection appear as discoloured, soft, water-soaked spots. The disease develops slower than green mold. As the infection progresses, the lesion

enlarges and white mycelium develops at the centre of the lesion. With time, blue spores are produced, first at the centre of the lesion (Figure 2.5); these centres may become brownish. The sporulating area is often surrounded by a distinct band of white mycelium which is in turn surrounded by soft water-soaked rind tissue (Brown, 1994a).

Figure 2.5: Oranges at progressive stages (1 – 3) of blue mold infection (*Penicillium italicum*). As the disease advances blue to greyish spores are produced and a band of white mycelium surrounds the sporulating area (3) (source: Brown, 1994a).

2.4.2.1 Control of *Penicillium italicum*

Careful handling of fruit during harvesting and postharvest operations minimizes injury and reduces the risk of blue mold infection (Brown, 1994a). Strict sanitation measures should be implemented to prevent the accumulation of spores on equipment and within the packhouse atmosphere. The use of disinfectants, such as chlorine and ethanol, can help prevent build-up of inoculi. Infected fruit should be discarded far away from the packhouse and in the packing environment exhaust fans can be used to expel spores. Benomyl can be applied as a pre-harvest application three weeks prior to harvest. Postharvest, fungicides such as thiabendazole, carbendazim, thiophanate-methyl, imazalil and sodium-o-phenylphenate can be used but *Penicilium spp*. can develop resistance, if the same fungicides or one with a

similar mode of action are used repeatedly (Brown, 1985). The use of two fungicides with different modes of action combined with good sanitation practices can help in the control of blue mold. Fruit should enter the cold chain immediately after harvest to delay disease development (Brown, 1994a).

2.5 Alternative Disease Control Measures

Excessive use of synthetic fungicides has been shown to have detrimental effects on the environment and on human health. This has led to a demand for horticultural commodities free of chemical residues; however, when no or less fungicides are used, the control of both, pre- and postharvest diseases, is difficult (Bautista-Banõs *et al.*, 2006). In an effort to balance disease control and maintaining a healthy environment, scientists have intensified their search for alternative disease control strategies. These include the use of generally regarded as safe (GRAS) chemicals, the use of heat treatments, biological control agents and the induction of natural disease resistance.

2.5.1 Generally regarded as safe substances

'Generally regarded as safe' (GRAS) substances are food additives which have been designated by the United States Food and Drug Administration (FDA) as being safe under the conditions of their intended use (http://www.fda.gov). Postharvest treatments with aqueous solutions containing GRAS substances have been used as alternatives to postharvest chemicals. These GRAS substances can be used with no restrictions in the European Union as well as the United States. Compounds that fall under the GRAS classification include sodium carbonate, sodium bicarbonate, potassium sorbate and ascorbic acid. Sodium bicarbonate, in particular, has been found to be highly effective against *P. digitatum* and *P. italicum* (Montesinos-Herroro, 2009) and, as a common part of a human's daily diet (in most baked goods), faces low consumer resistance; however, owing to

the high pH of the sodium containing solution, disposal of the used solution is a major hazard, one of the main reasons why this method is not widely used (Montesinos-Herroro, 2009). The effects of sodium carbonate, sodium bicarbonate and other GRAS substances have been investigated for *Penicillium* control on citrus, but are not consistent and are highly dependent on the host species and its physical and physiological condition (Palou *et al*., 2007). Therefore, these chemicals are most effective when used in combination with other control measures**.**

2.5.2 Hot water treatment

A 'Hot water brush' treatment, used as a technique for disinfecting fruit and vegetables, was originally patented in 1999 in Israel (Israeli patent 116965). This system involves rinsing fruit with sprays of hot water, at a pre-set temperature depending on fruit species and cultivar (Bassal and El-Hamahmy, 2011; Choi *et al.*, 2011), as the produce moves along a set of brush rollers (Porat *et al*., 2000). This methodology is used as a non-chemical alternative postharvest treatment for cleaning and disinfecting citrus fruit; it is therefore particularly valuable as a "non-chemical" treatment of fruit that have been produced organically. The fruit are cleaned and disinfected, reducing potential infection, thereby maintaining quality and extending shelf life. Numerous studies have been undertaken on the effects of hot water treatment (HWT). Porat *et al.* (2000) found that HWTs for 20 s at 56°C reduced decay development by 80% on 'Star Ruby' red grapefruit. These authors also reported that the total microbial population on fruit surfaces was reduced by 76% using HWT compared with fruit rinsed and treated with tap water. Treating fruit with 59 and 62°C water seemed more effective in disinfecting fruit surfaces than using lower temperatures; however, such treatments may result in heat damage (Porat *et al.*, 2000).

2.5.3 Biological control

Biological control is defined as "the use of live predatory insects, entomopathogenic nematodes or microbial pathogens to suppress populations of different pests or pathogens"

(Pal and McSpadden Gardener, 2006) it thereby reduces the adverse effects caused by destructive pathogenic organisms (Terry and Joyce, 2004). The use of microbial antagonists to control postharvest fruit rot has been proven a promising alternative to fungicides (Usall *et al.*, 2008). Currently, five recognized modes of action are known to be involved in biological control (Pal and McSpadden Gardener, 2006):

1. hyperparasitism - the pathogen is directly attacked by a specific biological control agent (BCA) that kills it or its propagules
2. antibiotic-mediated suppression - the use of microbe-produced toxins that have the ability to poison or kill other microorganisms
3. lytic enzyme mediated suppression - the use of enzymes, produced by the BCA, that can hydrolyze structural components of pathogens, such as chitin, proteins, cellulose, hemicellulose, and DNA
4. Competition between pathogens and non-pathogens - limited nutrient resources result in reduced disease incidence and severity
5. Induction of systemic and/or local resistance in the plant host - mediated by either salicylic acid (SA), which is produced following pathogen infection and leads to the expression of pathogenesis-related (PR) proteins or jasmonic acid (JA) and/or ethylene produced following applications of some non-pathogenic rhizobacteria

The downfalls of biological control agents lie in their inconsistency. Often consistent control of the pathogen cannot be achieved by using only one microbial antagonists; only when used as part of an integrated approach to disease management does this methodology become effective (Usall *et al.*, 2008). A further disadvantage of the use of BCAs is their extreme specificity of action; one BCA cannot provide the broad spectrum control that certain fungicides allow for. In addition to their variability in efficiency, BCAs cannot control existing infections; their effects are not curative but only protective and tend to diminish as the fruit ripen (Usall *et al.*, 2008). To improve efficacy the use of mixtures of antagonists with different

modes of action, genetically manipulating promising strains and the use of BCAs together with other control measures can improve efficiency. For example, the use of *Candida famata* strain 43E together with 0.1g TBZ/L provided significantly better *Penicillium* control than when either of these control measures was used alone (Ladaniya, 2008). Yeasts actively compete for resources with the pathogens by rapidly colonizing infected wounds utilizing the nutrients that the fungus needs for successful infection. There are several commercially registered BCAs, such as Aspire™, a biological control product which contains *Candida oleophila*. This yeast has the ability to quickly colonise injuries on the fruit surface and is compatible with certain fungicides. Combining Aspire™ with TBZ can effectively control green and blue mold as well as sour rot in citrus (Brown, 1994d, Brown *et al*., 2000).

2.6. Disease Resistance

Increasing the fruit's own, natural defence system has the potential to reduce infection or spreading of the pathogen, thereby decreasing the amount of fungicides necessary to protect fruit from spoilage during postharvest handling and storage. Physical, chemical and biological treatments can be used to elicit resistance; however, the efficacy of such treatments is often variable, depending on factors such as fruit maturity and cultivar (Ballester *et al.*, 2010).

2.6.1 Natural disease resistance

During fruit development and especially after harvest, natural disease resistance (NDR) steadily declines leaving fruit more susceptible to pathogen attack (Terry and Joyce, 2004). Most postharvest diseases caused by fungi are initiated in the field or during harvesting and postharvest handling when fruit are wounded. The infection remains dormant but as the NDR diminishes, quiescent infections are able to spread. The combination of declining NDR, enhanced nutritional requirements of the established pathogen, as well as the ripening process of fruit resulting in the softening of fruit tissue, permits an increase in disease incidence (Terry and Joyce, 2004).

2.6.2 Induced resistance

The importance of induced and acquired disease resistance was documented as early as in 1933. Only recently the potential of exploiting these phenomena for plant protection has been recognised (Terry and Joyce, 2004). Improving plant protection through the use of pre-formed and/or inducible defence mechanisms (acquired resistance, AR) is a useful strategy for combating pathogen attack, especially in Integrated Pest Management (IPM) systems, because such induced mechanisms enhance the crop's NDR (Febres *et al.*, 2009). Induced resistance elicitors can be classified as biological, chemical or physical elicitors. Such compounds may induce locally, or systemically acquired resistance (SAR) and induced systemic resistance (ISR).

The systemic resistance provided by SAR and ISR are distinctly different types. The two differ in the type of inducing agents and the host signalling pathways that subsequently result in resistance (Hammerschmidt, 2007). Systemic acquired resistance is generally characterised by the accumulation of plant response proteins (PR proteins) following pathogen challenge. This accumulation often results in the formation of localised necrosis, also known as the 'hypersensitive response'. It has been reported that SAR is dependent on salicylic acid signalling. On the contrary, ISR is not associated with the expression of PR proteins and shows no formation of local necrotic lesions; however, this resistance involves the ethylene as well as the jasmonic acid pathway (Hammerschmidt, 2007).

2.6.2.1 Elicitors of induced resistance

Inducers of ISR or SAR can be biological, natural/ synthetic chemicals or minerals (Terry and Joyce, 2004). Biological microbes induce defence reactions and boost general defence mechanisms in the host plant following pathogen attack. Chemical activators change plant – pathogen interactions so that they resemble that of incompatible interactions and, thus, induce defence-related mechanisms prior to or after pathogen attack; natural and mineral inducers play an important role in the regulation of stress responses and plant developmental processes (Terry and Joyce, 2004).

2.6.2.1.1 Biological inducers

The use of avirulent or slightly modified strains of pathogenic or saprophytic micro-organisms to induce SAR in vegetative host tissues is an aspect of biological control which has been researched previously (Terry and Joyce, 2004). A large number of antagonistic micro-organisms have been shown to possess biological control activity and, therefore, these organisms have been developed and commercialized as BCA (Danielson, 2008).

2.6.2.1.2 Natural resistance inducers

The plant growth regulators salicylic acid (SA) and jasmonic acid (JA) in the form of methyl jasmonate (MJ), as well as the glucose biopolymer chitosan (Hadwiger, 2013) are amongst the most intensively studied natural inducers of resistance. Salicylic acid, as an endogenous signalling molecule, induces or enhances photosynthesis, stomatal conductance, transpiration, disease resistance, seed germination and, ultimately, crop yield. Salicylates have been shown to delay ripening and maintain postharvest quality of fruit through inhibition of ethylene biosynthesis (Terry and Joyce, 2004). Chitosan, produced by the deacetylation of chitin, induces the accumulation of chitinases, proteinase inhibitors, phytoalexins and promotes lignification in treated fruit and vegetables (Hadwiger, 2013). This has been reported to delay ripening and also limit fungal infection through direct antifungal activity and/or stimulation of postharvest resistance responses in plant tissue (Terry and Joyce, 2004). The inconsistent ability to confer disease resistance to different crops and their possible incompatibility, specifically of SA, with IPM strategies due to phytotoxicity (Terry and Joyce, 2004) has previously posed problems. Basic and applied research into the mode of action associated with natural disease inducers is, therefore, essential to acquire a better understanding and ultimately facilitate the utilization of natural disease resistance elicitors.

2.6.2.1.3 Synthetic chemical inducers

Synthetic elicitors are able to confer broad spectrum efficiency against several different pathogens in a variety of crops, such as tobacco, cucumber and banana. The first synthetic

chemicals shown to induce SAR in many horticultural commodities included 2, 6 – Dichloroisonicotinic acid (INA; CGA 41396) and its methyl ester; however, due to challenges associated with phytotoxicity the compound is not used commercially (Terry and Joyce, 2004). Despite having the potential to successfully suppress disease occurrence in several commercially important postharvest diseases, their *modus operandi* remains poorly elucidated. Although there have been some positive results, the effectiveness of chemical elicitors varies greatly, depending on the plant pathogen interaction and the environment (Terry and Joyce, 2004).

Another synthetic elicitor isacibenzolar (benzo-(1,2,3)-thiadiazole-7-carbothioic acid S-methyl ester; ASM; BTH; CGA 245704; Bion^{TM}; Actigard^{TM}) (Terry and Joyce, 2000). Acibenzolar has been proven to be one of the most effective synthetic SAR activators discovered so far. It is similar to INA in that it acts downstream of SA and also induces the accumulation of the same SAR genes and PR proteins. However, unlike INA, acibenzolar is not phytotoxic and has been shown to be effective in both monocotyledons such as wheat and dicotyledons like tobacco, *Arabidopsis thaliana* and *Cucumis sativus* (Terry and Joyce, 2004). A previous study by the same authors (Terry and Joyce, 2000) demonstrated that a single or multiple foliar treatments with 0.25 - 2.00 mg mℓ$^{-1}$ acibenzolar during anthesis delayed the development of grey mold disease on harvested strawberry cvs. ‘Andana’ and ‘Elsanta’ by about 1.2-fold in fruit stored at 5 ◦C (Terry and Joyce, 2000), indicating that acibenzolar could be valuable in the commercial management of several postharvest diseases. Although several studies have demonstrated the positive effects of acibenzolar, some have shown the chemical to be inefficient in eliciting defence against powdery mildew, *Sphaerotheca fuliginea*, in *Cucumis sativus*. This was regardless of whether the chemical was applied before or after artificial inoculation with the pathogen (Terry and Joyce, 2004). This and other similar results suggest that the timing of acibenzolar application and the developmental stage of the plant may be important in determining its efficacy (Terry and Joyce, 2004).

2.6.2.1.4 Mineral inducers

Silicon

Silicon is one of the most extensively studied mineral resistance inducer. Although not considered an essential nutrient, Si has been shown alleviate both biotic and abiotic stress in several important crops (Epstein, 2009; Hammerschmidt, 2005; Keeping and Reynolds, 2009; Liang *et al.*, 2003 and Zhu *et al.*, 2004). This mineral nutrient has also been proven to enhance growth, development and yield of rice (*Oryza sativa*), sugarcane (*Saccharum officinarum*), several cereals and a number of dicotyledons (Datnoff *et al.*, 2001). Although Si has been shown to reduce disease incidence and increase yield, little information exists on the use of Si as a tool for integrated disease management (Datnoff *et al.*, 2001). Since Si has been shown to control a number of economically important fungal diseases of rice (e.g., neck blast and brown spot) with the same efficacy as fungicides, fungicide application rates could possibly be reduced or the use of fungicides even eliminated if Si is applied (Datnoff *et al.*, 2001). By application of Si, the development of fungicide-resistant strains of pathogens may be better manageable. The reduction of the frequency of fungicide application would translate to reduced costs as well as reduced environmental pollution, as Si sources have residual activity that persists over time, implying that monthly or even yearly fungicide application would not be necessary (Datnoff *et al.*, 2001).

Phosphorous acid

Phosphonate salts have been shown to also confer broad spectrum resistance to a range of different plant hosts, such as pepper (*Capsicum annum*), grapevine (*Vitis vinifera*), rice (*Oryza sativa*) and barley (*Hordeum vulgare*). In cucumbers (*Cucumis sativus*) the induction of resistance mediated by phosphoric acid was associated with a rapid accumulation of superoxide and hydrogen peroxide followed by localised cell death. Application of phosphonate as K_3PO_4 to the primary and secondary leaf of barley was reported to result in a significant increase in activities of phenyl alanine ammonia lyase (PAL), peroxidases and lipoxygenase in the second leaf (Reignault and Walters, 2007). The activity of these enzymes

increased even more following pathogen attack. Phosphates are well-known to exhibit powerful antifungal activity and some fungicides, such as fosetyl-al, have been shown to have both, a direct effect on the pathogen and an indirect effect on the plant defence system through stimulation of such host defences (Reignault and Walters, 2007).

2.7 Biochemical Compounds Involved in Disease Control

2.7.1 Phenols

Phenols are secondary metabolic products, which are widely distributed in plants (Ma *et al.*, 2009; Xu *et al.*, 2008), but are generally not considered important for cell growth and development (Ladaniya, 2008). These compounds do, however, play an important role in plant defence, flower and fruit colouring, flavour and plant hormonal balance. They are organic aromatic compounds, which contain one, or more hydroxyl (OH^-) groups attached to a benzene ring (Michalak, 2006). Simple or monocyclic phenols are synthesised via the shikimic acid pathway that results in the formation of aromatic compounds, tannins, coumarines and lignins (Michalak, 2006; Ladaniya, 2008). Citrus fruit have been found to possess numerous preformed antifungal substances of a phenolic nature (Afek *et al.*, 1999).

Plant phenolic levels tend to increase following infection by pathogens. In citrus fruit, in particular, the increase in phenolic levels is immediately followed by lignification of wounds (Ladaniya, 2008). The oxidation of phenols by polyphenol oxidases results in production of phenols that are more potent antifungal agents than non–oxidized phenols. Oxidized phenols inhibit pectolytic enzymes, which are necessary for the invasive ability of fungal pathogens. Elevated levels of polyphenol oxidase render the environment unfavourable for the pathogen. Phenols also seem to play a role in the tolerance towards chilling injury (Mathaba et al., 2008).

2.7.2 Naringin and Hesperidin

Hespiridin, naringin and neohespiridin are the major flavonoid glycosides found in citrus; the flavonoid concentration peaks during the early stages of fruit development and steadily declines towards fruit maturity (Tripoli *et al.*, 2007). Naringin, the most prominent flavonoid in grapefruit and shaddock oranges (*Citrus maxima* Merr.), is soluble in water and has a bitter taste, which has been attributed to the structure of the disaccharide moiety (Tripoli *et al.*, 2007). The hesperidin concentration varies with citrus cultivar with the highest amount of crude hesperidin found in clementines and the lowest in navel oranges; unlike naringin, hesperidin has no taste (Tripoli *et al.*, 2007). These compounds act as antioxidants Flavonoids have been shown to improve blood circulation, have anti-allergenic, anti-carcinogenic and anti-viral properties in mice (Tripoli *et al.,* 2007). Flavonoids also have antioxidant properties, which have the potential to treat certain disorders (Gorinstein *et al.*, 2001); these compounds also have antimicrobial activity.

2.8 Justification of the Study

It has been suggested that plant activators can be a more durable form of resistance since they operate through the induction of multiple methods of control within the host tissue and not necessarily on the plant pathogen (Reglinski *et al.*, 2007). Although considerable effort has been placed on researching different aspects of induced resistance, it is still commercially underutilized as a tool to manage diseases. This can be attributed to the fact that there are still many gaps in the understanding of the phenomenon (Reglinski *et al.*, 2007). The variable efficacy of identified inducers (across pathogen and crop species) is a major limiting factor of their commercialization. The activation of vital enzymes in the phenyl propanoid pathway and the synthesis of some secondary metabolites as well as any factor that limits the production of such enzymes require investigation, as this could ultimately compromise the NDR of the host tissue (Terry and Joyce, 2004).

Si and phosphorous acid seem to trigger a response that enhances phenol production and therefore improves resistance to pathogen attack in the fruit (Maksimovic *et al.*, 2007). Maksimovic *et al.* (2007) showed that the resistance of cucumber to powdery mildew following of Si application was largely due to an enhanced production of phenolic compounds. However, not much is known about the possible mode(s) of action of these inducers of induced resistance.

2.9 Hypothesis

Three concentrations of Si (1250, 2675 and 5350 mg ℓ^{-1}) and one concentration of PA (500 mg ℓ^{-1}) were evaluated with respect to their efficacy to reduce disease severity in 'Eureka' lemons, 'Washington' navels and 'Delta' Valencia oranges inoculated with *Penicillium digitatum.* The differences in rind phenolic and flavonoid concentration following application of Si and PA was evaluated and compared in inoculated, wounded and unwounded fruit. The timing of Si and PA application (pre- or postharvest) was also assessed. The hypothesis was that pre- or postharvest treatment of fruit with Si and PA will reduce the incidence of *Penicillium digitatum* and increase phenolic and flavonoid content in treated fruit, thus potentially increasing the natural resistance of the fruit to future pathogen attack.

The objectives of this study were therefore:

- to assess the level of effectiveness of both Si and PA in controlling or reducing disease severity of inoculated fruit
- to quantify the changes in phenolic and flavonoid content in the fruit peel that occur following application of these two mineral elicitors in inoculated, un-inoculated and wounded fruits
- to determine whether increasing treatment dosage can be correlated with increased changes in biochemical composition and the level of disease control achieved

- to determine the effectiveness of both, Si and PA, across three different cultivars of *Citrus*
- to determine the optimal time for the application of Si and PA (pre- or postharvest) to induce resistance to *Penicillium*

An understanding of the above-mentioned factors is essential, as the ultimate driving force for the adoption of induced resistance as a pathogen control measure will be the effectiveness and availability of reliable resistance inducers.

CHAPTER THREE: MATERIALS AND METHODS

3.1 Plant Material

The experiment was conducted during the 2010 and 2011 seasons. Mature fruit of *Citrus sinensis* cvs 'Delta' Valencia and 'Washington' navel orange, and *Citrus limon [L.] Burm.* cv 'Eureka' lemon, were harvested from a citrus orchard at the University of KwaZulu-Natal Research Farm, Ukulinga (29°36' S; 30°24' E; 775 m asl), in Pietermaritzburg. Healthy fruit where selected for the experiment on the basis of uniformity in shape, colour and size.

3.2 Chemicals

A water-soluble silica liquid formulation with a concentration of 20.5% potassium silicate (AgriSil™ K50™, PQ Corporation Fertilizer Group) was used as the Si source for both, the pre- and the postharvest experiments. Three experimental concentrations (1250, 2675 and 5350 mg Si ℓ^{-1}) were chosen. The systemic water-soluble liquid fungicide Phosguard400 SL (Ocean Agriculture (PTY) Ltd) was used as a PA source. Phosguard is routinely used in citrus for the control of Phytophthora root and crown rot; a concentration of 500 mg ℓ^{-1} was used in this study according to the manufacturer's recommendation.

3.3 Fungal Isolation and Maintenance

3.3.1 *Penicillium digitatum* isolation

Spores of the pathogen were cultured from conidia of infected navel oranges obtained from Ukulinga Research Farm, Pietermaritzburg, by plating the conidia onto potato dextrose agar (PDA) amended with 0.15 g ℓ^{-1} of Rose Bengal. Plates were incubated at 25°C for 7-10 days. Pure cultures were then sub-cultured on malt extract agar (MEA) plates after identification and verification of *P. digitatum* conidia using a compound microscope.

3.3.2 Culture purification

To ensure that a pure, contamination-free culture would be obtained, 10 x 10 mm blocks of agar were cut out from the agar plates and placed onto a fresh PDA plate. A sterile glass ring was then placed around the block and the plate was sealed and incubated at 21°C for another five days. Since the pathogen is filamentous, it grew and crept under the ring leaving any bacterial or yeast contamination behind. A 10 x 10 mm block was then cut from the growth outside the ring, transferred onto a new plate, and sealed with Parafilm™.

3.3.3 Culture maintenance

Since cultures only needed to be preserved for 18 months, 10 x 10mm blocks of fungi were cut with a sterile scalpel from the growing edge of the colony. These blocks were then transferred into McCartney bottles containing 6mℓ of distilled water. The lids were tightly screwed on and the bottles stored at room temperature. To revive the cultures the blocks of agar were transferred onto fresh PDA plates using a pair of sterile forceps and subsequently incubated at 21°C.

3.3.4 Inoculum standardization

Determination of the concentration of the inoculum is important because using too little inoculum may cause no reaction, while too much inoculum may result in severe symptoms, thus skewing the data. A conidial suspension was prepared by washing a 10-day-old culture of *P. digitatum* into McCartney bottles containing sterile distilled water. The conidial concentration was adjusted to 1×10^4 mℓ$^{-1}$ using a haemocytometer.

3.4 Pre- and Postharvest Treatment of Fruit

3.4.1 Pre-harvest treatment application

For the pre-harvest experiment conducted over two seasons, 2010 and 2011, ‘Eureka’ lemon, ‘Washington’ navel and ‘Delta’ Valencia trees were treated by drenching 5 ℓ treatment

solution once a week for four consecutive weeks leading up to harvest around the base of the trunk (Table 3.1). Each treatment was replicated three times in order to accommodate both treatments, pre- and postharvest, within the same orchard. At harvest, 20 fruit were picked from each of the three trees for each treatment so that there would be a total of 60 fruit per treatment. The fruit were immediately transported to the laboratory for processing. Upon arrival, 30 fruit were randomly selected from the original sample size of 60 fruit per treatment. These 30 fruit were divided into three groups, each consisting of 10 fruit for each of the eight treatments. Each individual fruit was treated as a replicate. The first group of fruit was wounded, as it has been reported that simply wounding the fruit is sufficient to trigger phytoalexin accumulation (Lagrimini *et al.*, 1993), possibly setting off induced resistance responses. The second group of fruit was inoculated with a 1×10^4 conidia $m\ell^{-1}$ spore suspension of *P. digitatum*. The third group of fruit was left unwounded and un-inoculated. Each fruit was placed into a brown paper bag in order to limit cross-contamination between replicates, then placed in clearly labelled boxes and stored at 5.5°C in order to simulate current commercial storage conditions.

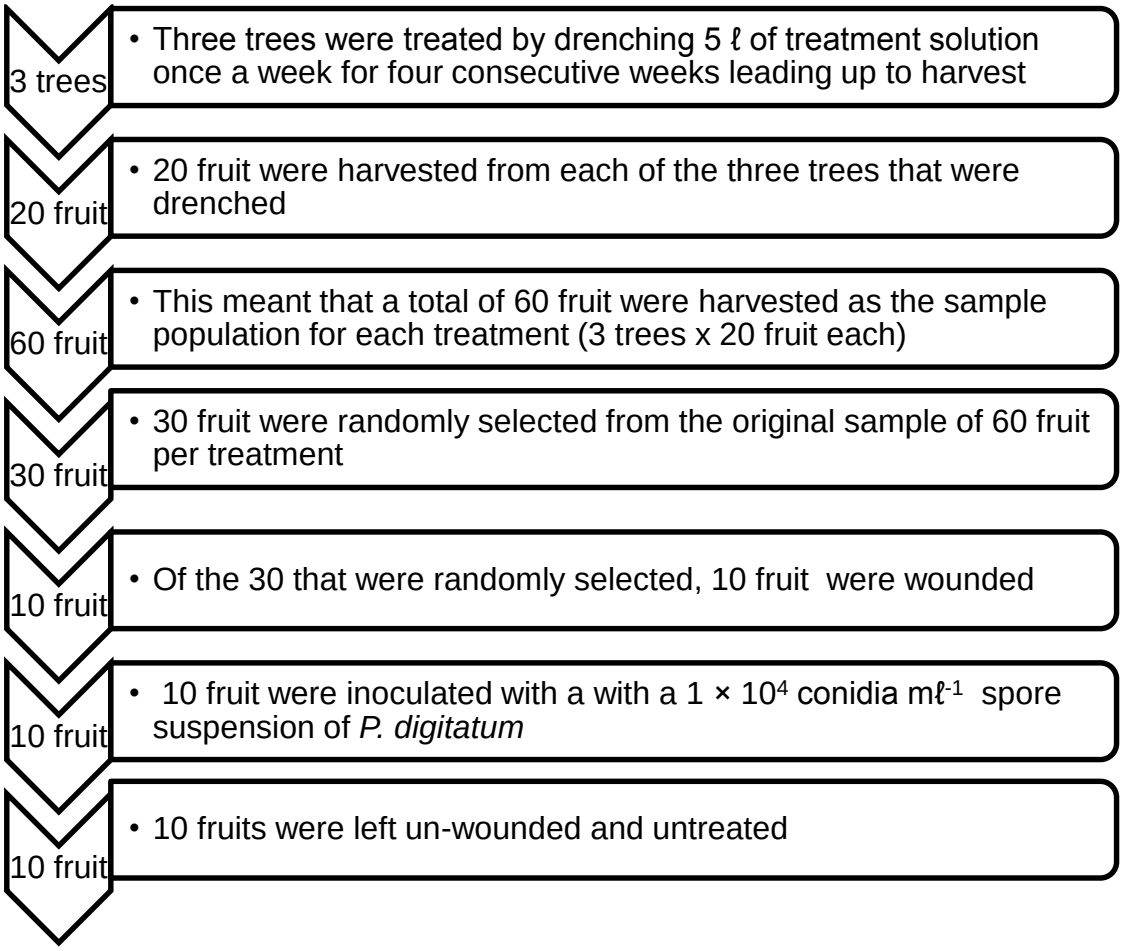

Figure 3.1: Experimental design for the pre-harvest experiment conducted during the 2010-2011 season. Layout was applied for each of the seven treatments and the control

3.4.2 Postharvest treatment application

Fruit for the postharvest experiment were treated with one of the seven treatments (Table 3.2) by immersion into the particular solution (at room temperature) for 90 s and then left to air dry for 180min. Thereafter, fruit were divided into three groups as outlined for the pre-harvest fruit. Following inoculation or wounding, fruit were packaged and stored as described earlier (Section 3.4.1).

Table 3.1: Treatment combinations and concentrations used during the 2010 and 2011 harvest seasons for pre- and postharvest experiments conducted using fruit harvested from Ukulinga Research Farm

Treatments		**Concentration (mg ℓ^{-1})**
1.	S1 (Si Concentration 1)	1250
2.	S2 (Si Concentration 2)	2675
3.	S3 (Si Concentration 3)	5350
4.	P (phosphorous acid)	500
5.	S1+ P	1250 + 500
6.	S2 + P	2675 + 500
7.	S3 + P	5350 + 500
8.	Control	0

3.5 Inoculation of fruit

Fruit were inoculated along the equatorial region (figure 3.2) using an inoculation tool immersed in the conidial suspension. Treatment was carried out by puncturing fruit on one side along the equator with a clean inoculation tool.

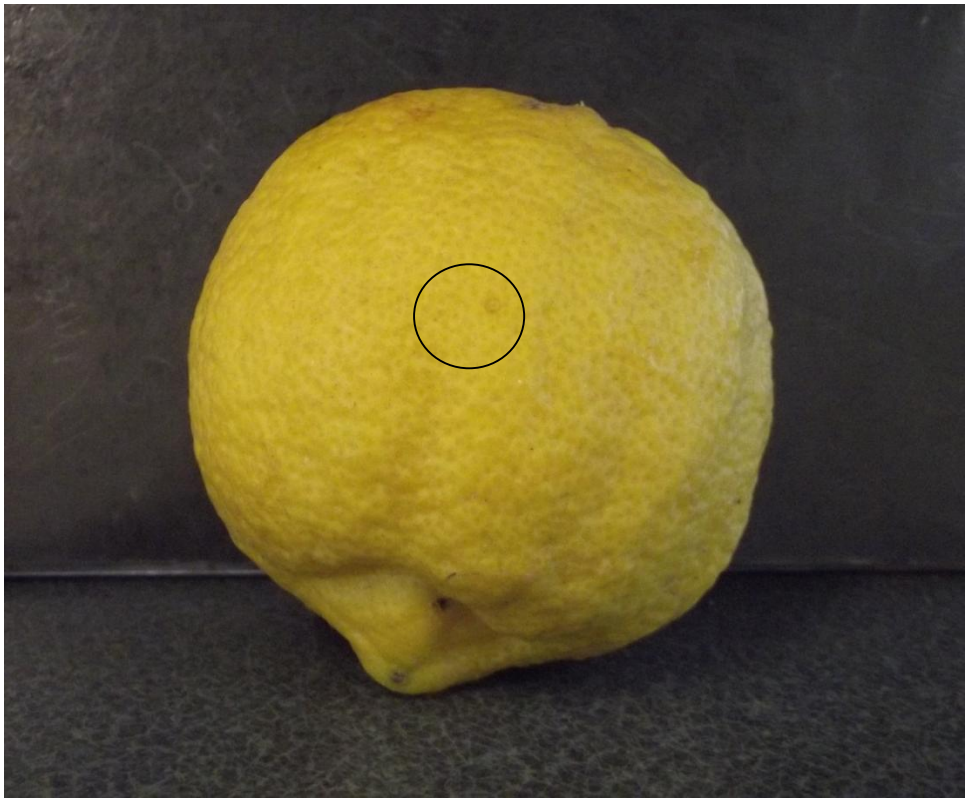

Figure 3.2: Puncture in ‘Eureka’ lemon used to introduce *P. digitatum* pathogen into the fruit

3.6 Fruit sampling and disease rating

For both, pre- and postharvest experiments, all fruit were sampled once, 10 days post inoculation (10 DPI). Fruit were sampled using a modified method of Blakey *et al.* (2010), whereby small discs of fruit flavedo (and parts of the albedo) tissue were removed with a 30mm diameter fruit borer as shown in figure 3.3. This section only was sampled because

the flavedo tissue is considered to be a major barrier for pathogen invasion (Afek et al., 1999).

The area where the flavedo tissue had been removed was sealed using petroleum jelly in order to prevent dissection and fruit shrinkage, thus allowing the fruit to be used for diseases progress evaluation. Immediately after removal, samples were shock-frozen in liquid nitrogen and stored at -10°C until lyophilisation. Once completely dry, samples were ground into a fine powder using a coffee grinder, then stored at -10°C for future phytochemical analyses.

Since fruit were stored at 5.5°C, symptoms only became apparent 7-10 days post-inoculation. Disease progression was monitored by measuring the disease lesion size (in mm) using digital callipers 7, 14, 21 and 28 days post-inoculation.

Figure 3.3: Eureka lemon with flavedo tissue removed after sampling and petroleum jelly applied to seal sampled areas in order to prevent desiccation

3.7 Phenolic extraction

Phenolic compounds were extracted according to the method reported by Abeysinghe *et al.* (2007), with slight modifications. Ground tissue (0.5 g DM) was weighed into test tubes and Phytochemicals were extracted with 5 mℓ 1.2 M HCl in 80% methanol/water, the tube capped and the solution vortexed for 1 min. Samples were then placed in a hot water bath at 90°C for 3 h, with vortexing at regular intervals to release the bound phenolics. Thereafter, samples were allowed to cool down to room temperature and the sample volume adjusted to 10 mℓ with methanol before being centrifuged at 10,000g for 5 min to remove the solid fraction. The supernatant was used for determination of total phenolics and total flavonoids concentration.

3.8 Phytochemical analyses

3.8.1 Determination of total phenolic concentration

The phenolic concentration was determined according to Abeysinghe *et al.* (2007) with slight modification according to Mathaba and Bertling (2013). Total flavedo phenolics were analysed using a modified colorimetric Folin-Ciocalteu method. Four millilitres of distilled water and 0.1mℓ of properly diluted flavedo extract were placed into a test tube. Folin-Ciocalteu reagent (0.5mℓ) was added to the solution and allowed to react for 3 min; thereafter the reaction was neutralized with 1mℓ saturated sodium carbonate. The absorbance of the solution was read after 2 h at 760 nm, using a spectrophotometer. Chlorogenic acid was used as the standard and the phenolic concentration was expressed as mg chlorogenic acid equivalents x 100 g DW^{-1}.

3.8.2 Determination of total flavonoid concentration

To determine total flavonoid content the method described by Abeysinghe *et al.* (2007), with some modifications, was used. Properly diluted flavedo extract (0.1 mℓ) was added to a glass

test tube containing 3.5 mℓ of absolute ethanol. Thereafter, 4 mℓ of 90% di-ethylene glycol was added and thoroughly mixed; the colour reaction was initiated by adding 0.1 mℓ of 4 M sodium hydroxide. Absorbance at 420 nm was read after an incubation period of 10 minutes at 40°C using a spectrophotometer. Rutin was used as the standard and total flavonoid content was expressed as mg rutin equivalent (RE/100 g DM).

3.9 Statistical analyses

Data were subjected to analysis of variance (ANOVA) using GenStat® Version 14 (VSN International, Hemel Hempstead,UK). Mean separation was done using Fishers Protected Least Significant Difference test in GenStat at the 5% level of significance.

CHAPTER FOUR: RESULTS

4.1 Effects of Si and phosphorous acid on disease progression

Fruit from the three cultivars used in this study ('Eureka' lemons; 'Washington' navel and 'Delta' Valencia oranges) were treated pre- and postharvest with three different concentrations of Si (S1= 1250, S2= 2675, S2= 5350 mg ℓ^{-1}), one concentration of PA (P= 500 mg ℓ^{-1}) and combinations of each of the Si and PA treatments. Fruit were then inoculated with suspension of 10^4 mℓ^{-1} *P. digitatum* spore and disease progress was monitored over 28 days (Figure 4.1.1). The effectiveness of the pre- and postharvest treatment application and the overall effect of the treatments on each cultivar was compared (Figure 4.1.2). The fruits response to each of the treatments was assessed (Figure 4.1.3 and 4.1.4).

In all three cultivars, disease symptoms appeared 7 days post inoculation. 'Eureka' fruit had a higher disease incidence 14, 21 and 28 days post-inoculation than navel and Valencia oranges (Figure 4.1.1). This pattern was consistent in both, pre- and postharvest treated fruit (Figure 4.1.2).

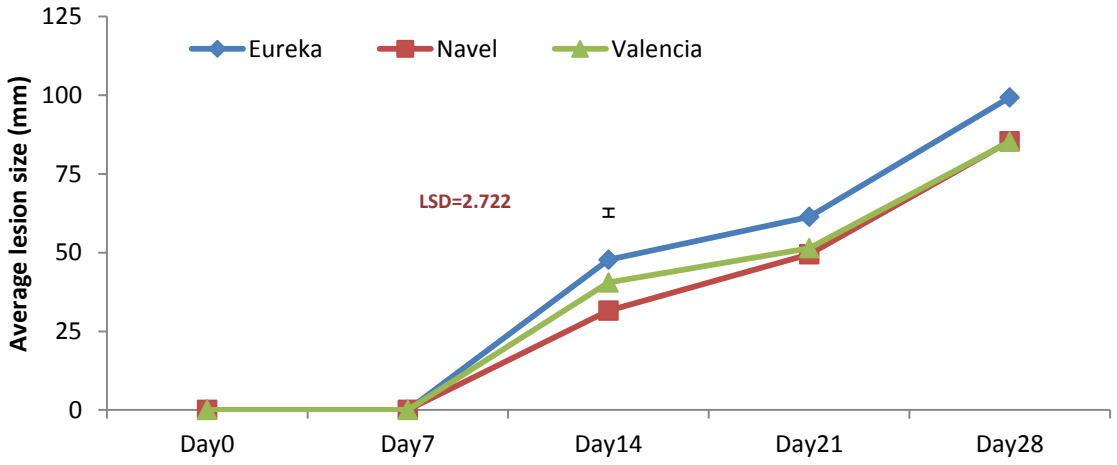

Figure 4.1.1: Average disease lesion size on 'Eureka' lemons; 'Washington' navel and 'Delta' Valencia oranges inoculated with a 1×10^4 conidia mℓ^{-1} *P. digitatum* spore suspension after treatment application. Lesions measured 7, 14, 21 and 28 days post-inoculation ($P< 0.05$).

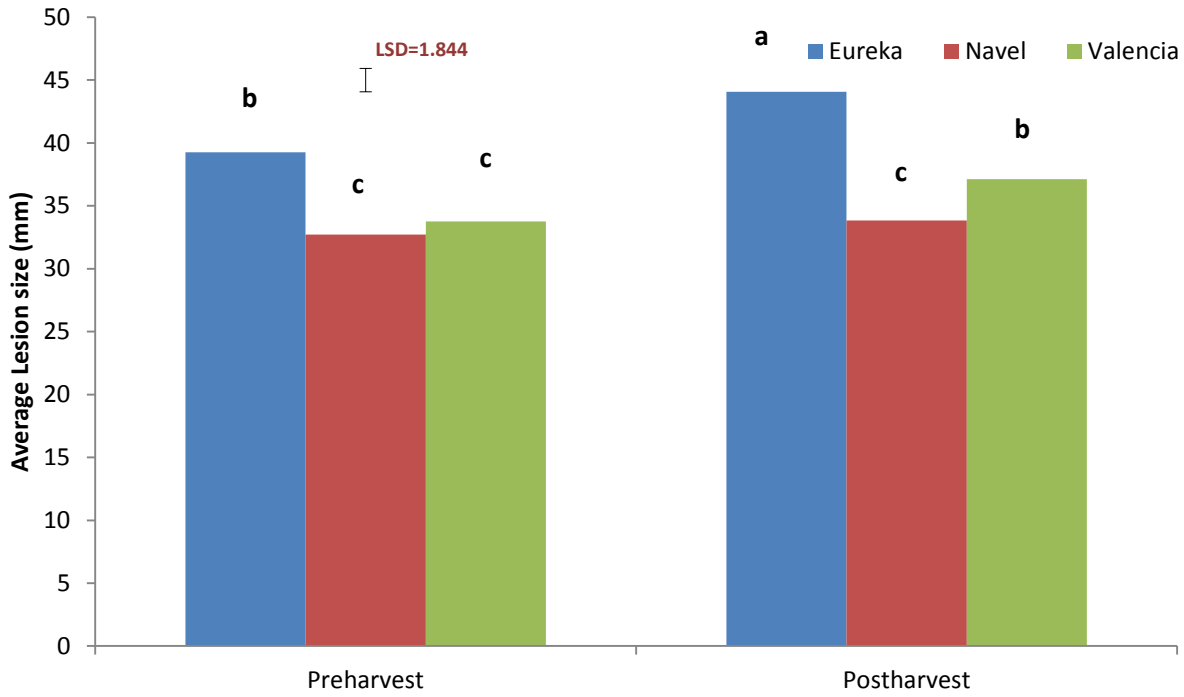

Figure 4.1.2: Overall effect of pre- and postharvest silicon and phosphorous acid treatments on average disease lesion size in 'Eureka' lemons, 'Washington' navel and 'Delta' Valencia oranges inoculated with a 1×10^4 *P. digitatum* conidia $m\ell^{-1}$ spore suspension. Experiment was conducted over two seasons, 2010 and 2011 ($P< 0.05$).

In all three cultivars, in the pre-harvest experiment, Si was the most effective in reducing disease symptoms at the lowest concentration (treatment S1) compared with the higher Si concentrations (S2 and S3). Treatment with P alone was the second most effective. Fruit treated with S1 and P as standalone treatments outperformed higher concentrations of Si (S2 and S3) as well as fruit treated with combination treatments (S1+P, S2+P and S3+P) and the control (figure 4.1.3).

Combining the most effective treatments (S1 and P) (figure 4.1.3) did not result in the expected synergistic effect; fruit treated with S1+P had an average lesion size not significantly different from fruit treated with the control treatment. Although not statistically

significant, in the case of ‘Eureka’ lemons, combining the higher concentrations of Si (S2 and S3) with P increased the severity of disease symptoms with fruit exhibiting a higher average disease lesion size (42.9 and 43.9mm, respectively) than control fruit (40.5mm).

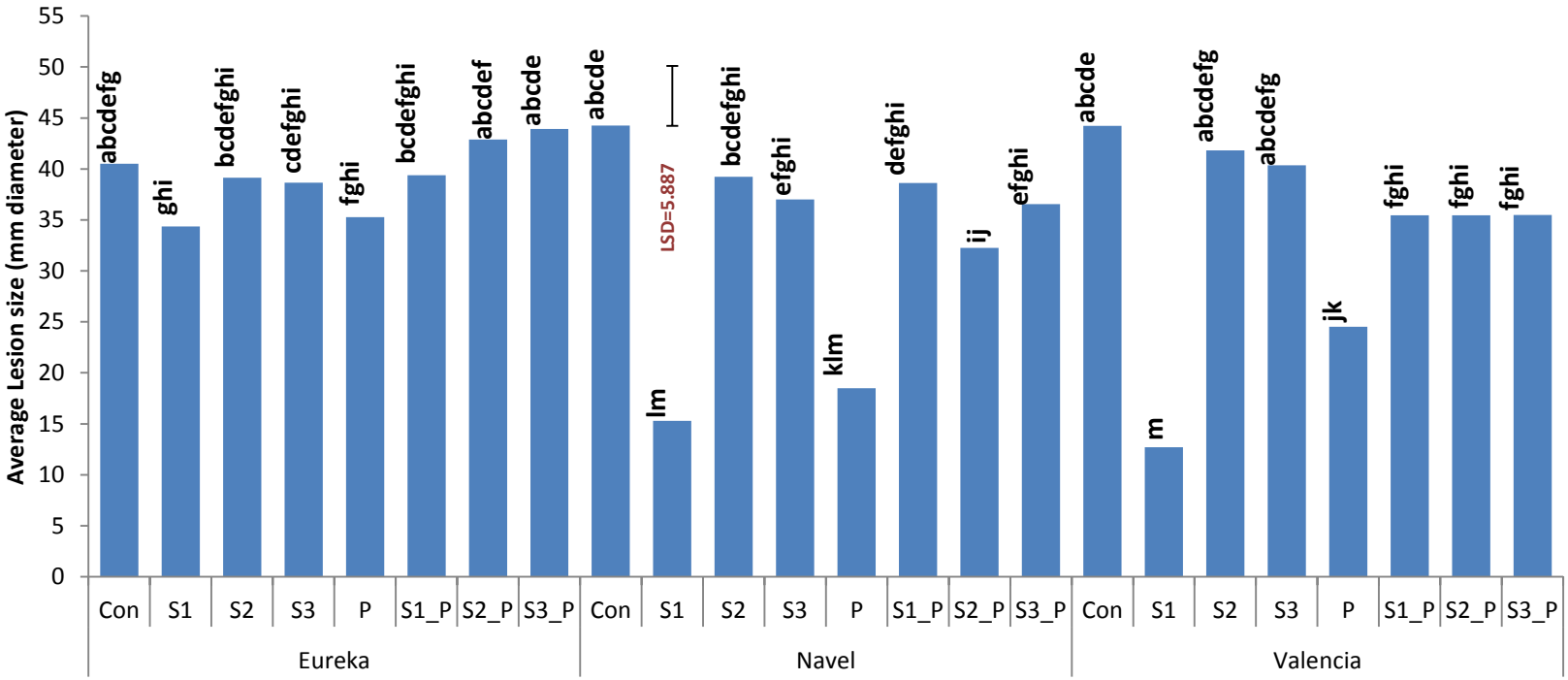

Figure 4.1.3: Average disease lesion size in inoculated 'Eureka'; 'Washington' navel and 'Delta' Valencia fruit treated pre-harvest with three different concentrations of Si (S1= 1250, S2= 2675, S3= 5350 mg ℓ^{-1}), one concentration of Phosphorous acid (PA= 500 mg ℓ^{-1}) and combinations of each of the Si and PA treatment. Once treated fruit were inoculated with 1×10^4 *P. digitatum* conidia mℓ^{-1}spore suspension, disease severity was determined 28 days post-inoculation. The experiment was run over two seasons, 2010 and 2011($P< 0.05$).

In the postharvest experiment, treatment of navel and Valencia oranges with the lowest Si concentration produced significant better disease control than other treatments, while for ‘Eureka’ such treatments were not significantly different form each other. Once again, standalone treatments with higher concentrations of Si and combination treatments of S+P did not result in the reduction of disease severity and in some cases resulted even in an incidence that was not statistically different than that of fruit in the control groups (figure 4.1.4).

Treatment of navel fruit with P also resulted in a reduction in disease symptoms. Although treatment of Valencia fruit with P resulted in a reduction in disease severity compared with control fruit, there was no statistical difference between the treatment combinations of P and Si and the individual P, S1, S2 or S3 treatments (figure 4.1.4).

‘Eureka’ lemons exhibited a higher level of disease incidence than navel and Valencia oranges. Treatments S1 and P did not result in a marked reduction of disease severity when compared to the control, a result similar to what was observed with navel and Valencia fruit (figure 4.1.4).

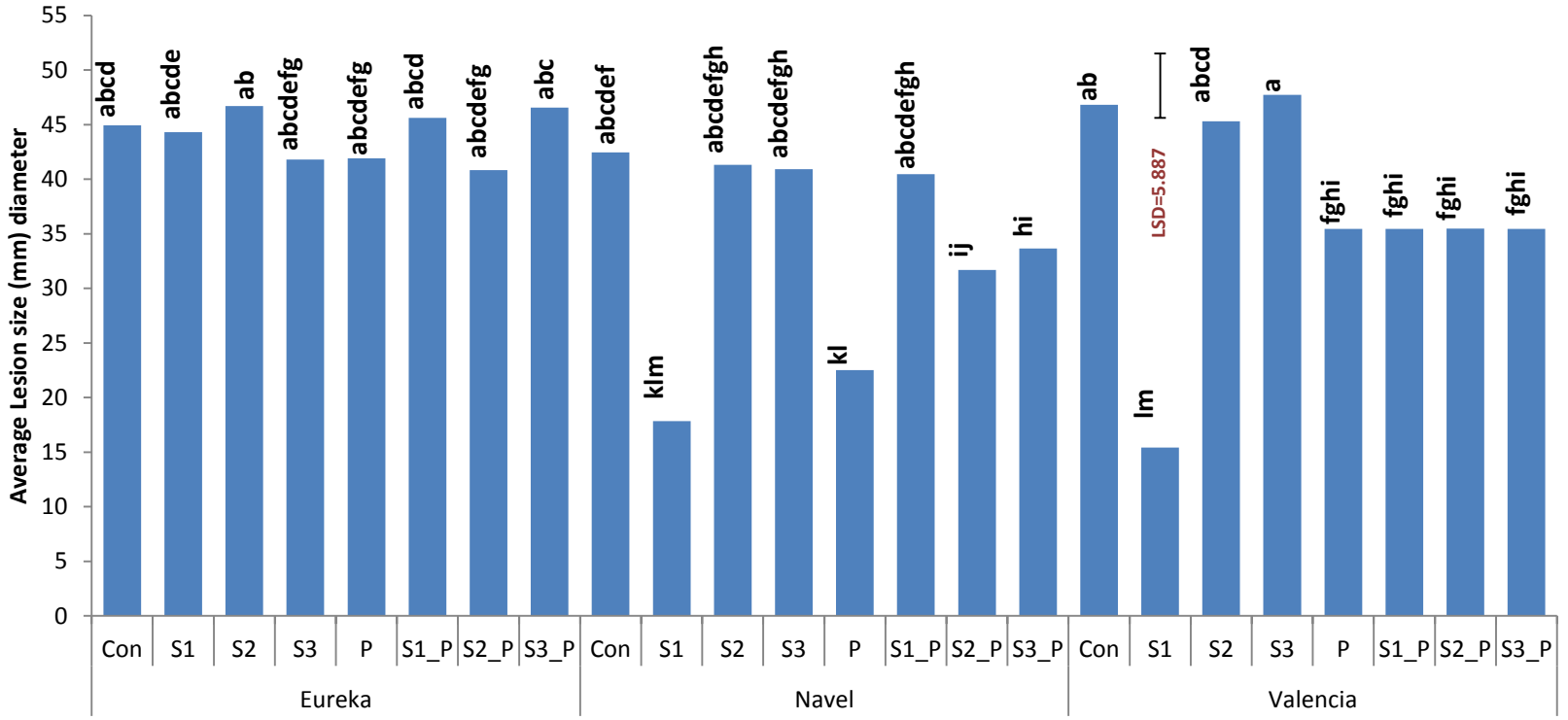

Figure 4.1.4: Average disease lesion size in inoculated 'Eureka', 'Washington' navel and 'Delta' Valencia fruit treated postharvest with three different concentrations of Si (S1= 1250, S2= 2675, S3= 5350 mg ℓ^{-1}), one concentration of Phosphorous acid (PA= 500 mg ℓ^{-1}) and combinations of each of the Si treatments with PA. Once treated fruit were inoculated with a 1×10^4 *P. digitatum* conidia mℓ^{-1} spore suspension, disease progress was monitored over 28 days. The experiment was run over two seasons, 2010 and 2011($P< 0.05$).

Figure 4.1.5: Pictorial comparison between Valencia fruit treated with two different concentrations of Si, 5350 mg ℓ^{-1} (left) and 1250 mg ℓ^{-1} (right) at 21 days after inoculation with 1x10^4 *Penicillium* spore suspension. Fruit treated with 1250 mg ℓ^{-1} Si exhibited a delay in the production of secondary hyphae

Figure 4.1.6: Valencia fruit treated with the two best-performing treatments Phosphorous acid (centre) S1 (1250 mg Si ℓ^{-1}Si, right) and control (left) 21 days after inoculation with a 1×10^4 *P. digitatum* conidia mℓ^{-1}spore suspension.

4.2 Effects of Si and PA applications on the production of phenolics in citrus flavedo

'Eureka' fruit accumulated the lowest phenolic concentration, while navel fruit accumulated the highest concentration of phenolics when treatments were applied pre-harvest (figure 4.2.1).

In all three cultivars, PA treatment seemed to induce a more positive reaction the control fruit of each respective cultivar (figure 4.2.2). Treatment of fruit with a combination of both Si (at the different levels) and P did not result in the accumulation of total phenolic acid concentrations that were significantly superior to the standalone treatments.

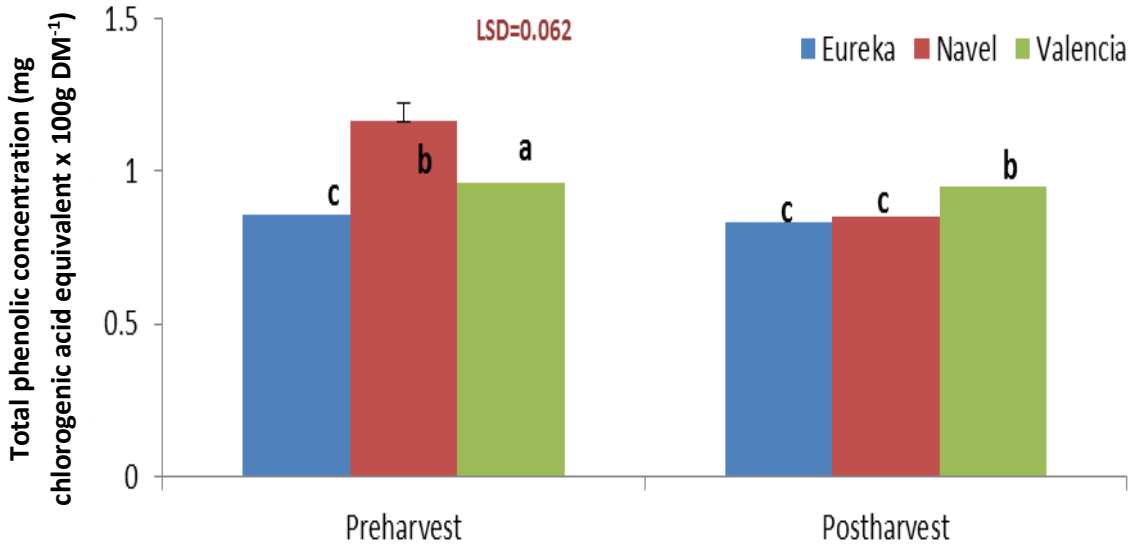

Figure 4.2.1: Comparison of overall effect of pre- and postharvest treatment applications on total phenolic concentration (mg chlorogenic acid equivalent/100g DM) in flavedo tissue of treated 'Eureka', 'Washington' navel and 'Delta' Valencia.

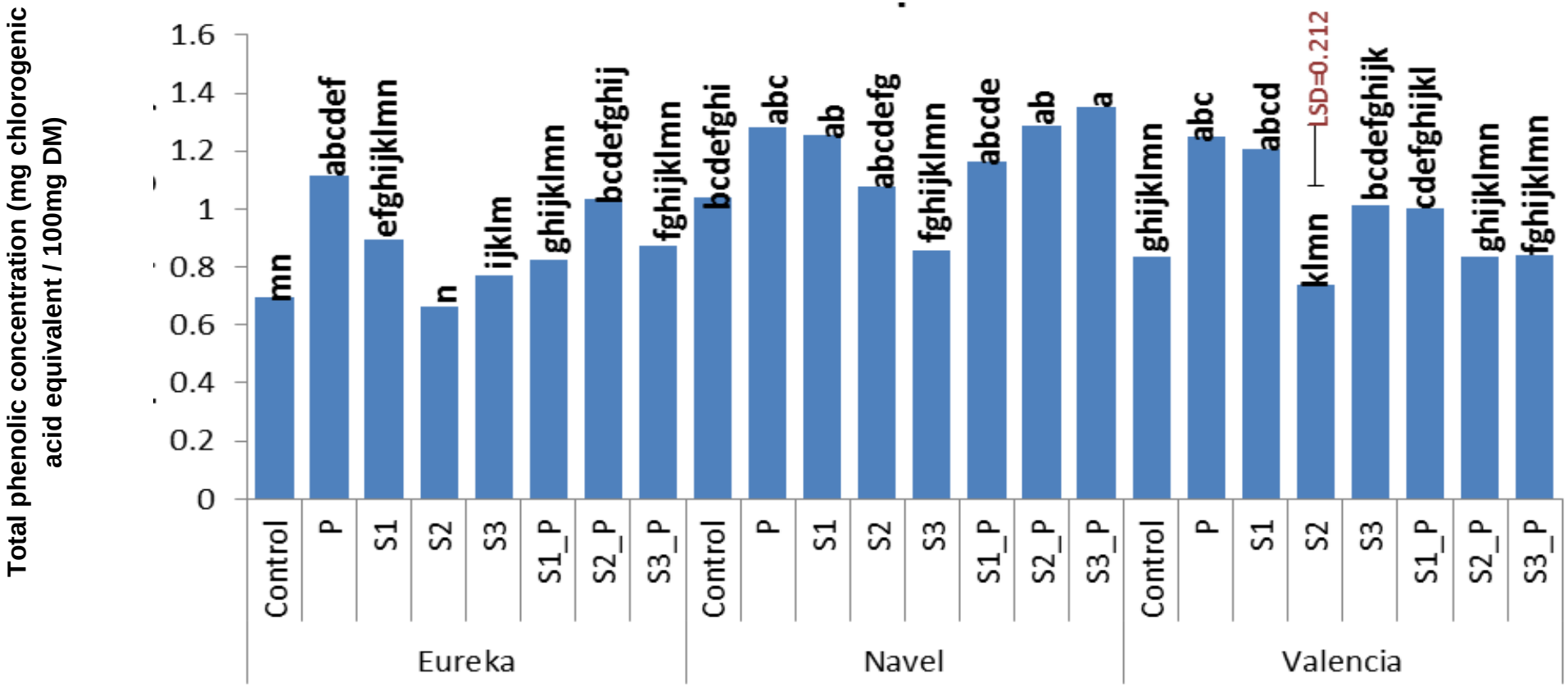

Figure 4.2.2: Total phenolic concentration (mg chlorogenic acid equivalent/100g DM) in flavedo tissue of fruit from 'Eureka' lemon, 'Washington' navels and 'Delta' Valencia orange trees treated pre-harvest by drenching with three concentrations of Si (S1= 1250, S2= 2675, S3= 5350 mg ℓ^{-1}) and one concentration of Phosphorous acid (PA = 500 mg ℓ^{-1}) and combinations of each of the Si treatments with PA ($P< 0.05$).

In the postharvest experiment fruit were treated by immersion into different concentrations of Si (S1= 1250, S2= 2675, S2= 5350 mg ℓ^{-1}) and one concentration of PA (500 mg ℓ^{-1}) and combinations of each of the Si treatments with PA.

No external rind damage, resulting from the 90 second postharvest dips, was observed in any of the fruit. Postharvest dips were, however, generally not as effective as the pre-harvest treatments in increasing flavedo phenolic acid concentrations (figure 4.2.1). Fruit treated postharvest generally accumulated lower phenolic acid concentrations in the flavedo.

In the case of 'Eureka' fruit, control fruit, treated with water only, had lower total phenolic concentrations than fruit of other treatments, except for fruit of the S1+P treatment. In navels, fruit treated with PA as a standalone treatment accumulated significantly higher phenolic levels than fruit of other treatments. The second highest rind phenolic concentration was determined for fruit treated with the lowest Si concentration (treatment S1). Treatment of fruit with the combination treatments (S1+P, S2+P and S3+P) had a particularly negative effect on rind phenolics, as these fruit exhibited a reduced total phenolics, containing significantly lower total phenolic concentrations than the control (figure 4.2.3).

In Valencia fruit no significant difference between control fruit treated and those treated with PA was observed. Fruit treated with S1 accumulated higher total phenolic levels than control fruit and all other treatments (figure 4.2.3).

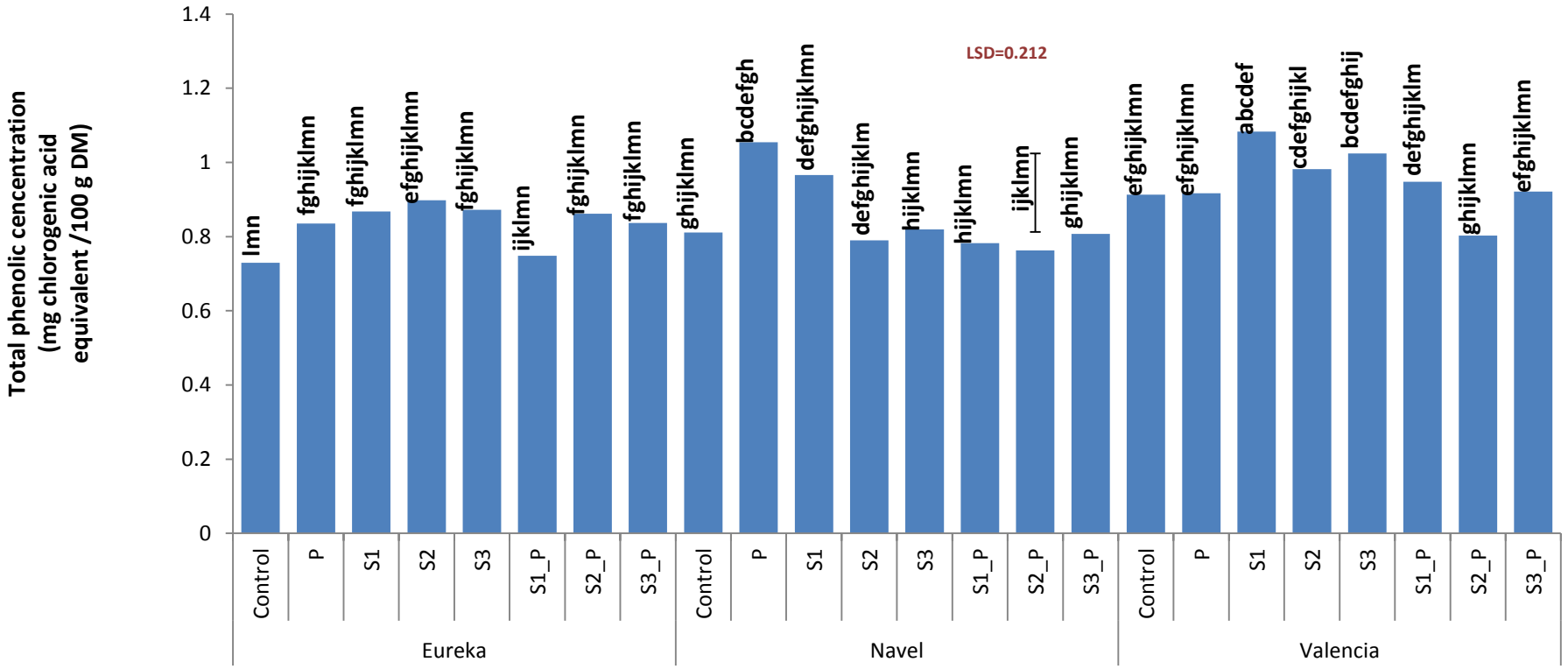

Figure 4.2.3: Total phenolic concentration (mg chlorogenic acid equivalent/100 g DM) in flavedo tissue of 'Eureka' lemons, navel and Valencia oranges treated postharvest with three different Si concentrations (S1= 1250, S2= 2675, S3= 5350 mg ℓ^{-1}) and one concentration of Phosphorous acid (PA = 500 mg ℓ^{-1}) as well as the combination of each of the Si treatments with PA ($P< 0.05$).

To investigate the possible effect of fruit wounding on rind phenolics, fruit were artificially wounded and compared with fruit inoculated with the pathogen as well as with those that were left unwounded. In navel fruit from trees treated with Si and or PA pre-harvest, wounding induced a significant accumulation of total phenolics compared with inoculated fruit. This effect was, however only visible as a trend in the navel postharvest treatment as well as in the 'Eureka' and Valencia treatments. In all three cultivars and in both, pre- and postharvest experiments, fruit that were left unwounded and un-inoculated accumulated - or had a tendency to accumulate - lower levels of phenolics than the wounded and the inoculated fruit (figure 4.2.4).

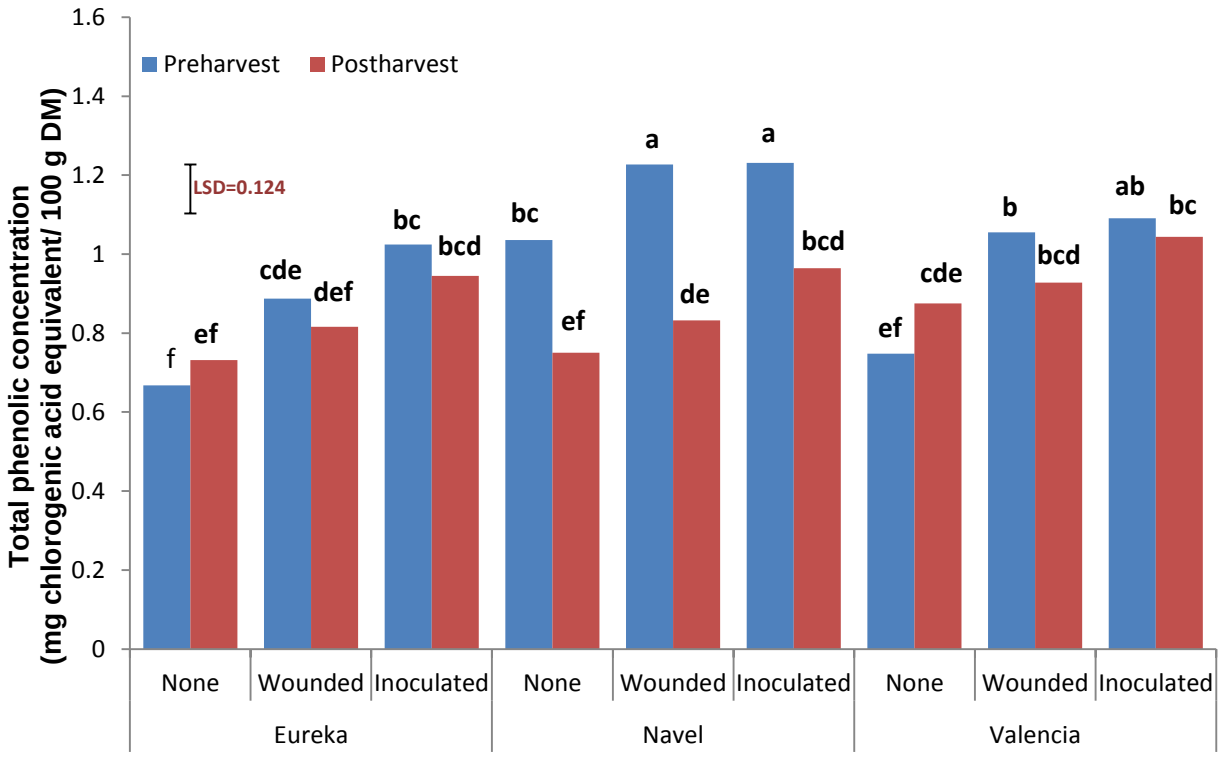

Figure 4.2.4: Effects of wounding or inoculating the fruit on total phenolic acid accumulation (mg chlorogenic acid equivalent/ 100 g DM) in flavedo tissue of 'Eureka' lemons treated pre- or postharvest with Si or PA ($P < 0.05$)

4.3 Effects of Si and PA applications on production of flavonoids in citrus flavedo

When ‘Eureka’ fruit were treated pre-harvest with PA or S1 rind flavonoid concentrations were significantly higher than those of the control and all other treatments (figure 4.3.1). Increasing Si concentration from 2675 (S2) to 5350 mg ℓ^{-1} (S3) did not result in a significant increase in total flavonoids (figure 4.3.1).

Unlike in ‘Eureka’ lemons, in navel oranges treated pre-harvest, treatment S2 increased rind flavonoids most, followed by treatment S1. Fruit treated with PA, S3, S2+PA and S3+PA had total flavonoid levels that were significantly lower than those of the control (figure 4.3.1).

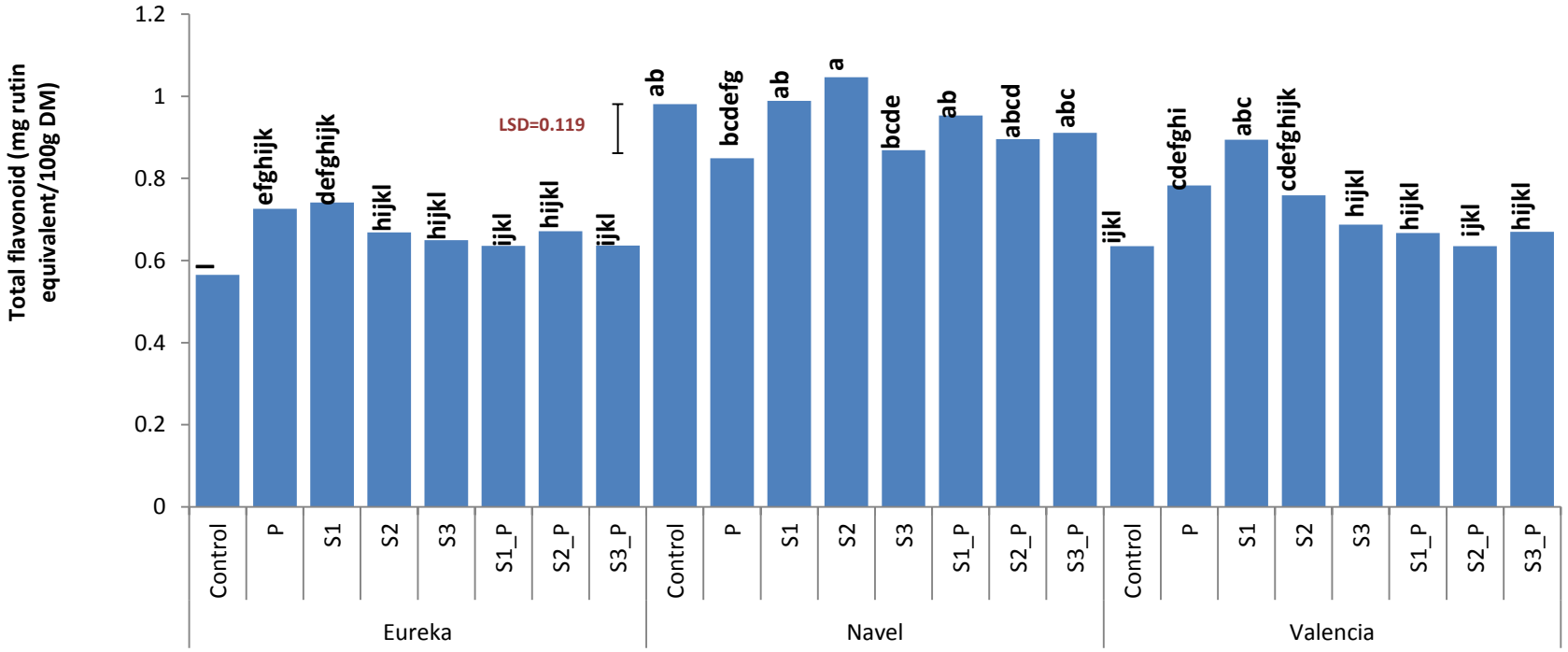

Figure 4.3.1: Total flavonoid concentration (mg rutin equivalent/100 g DM) in flavedo tissue of 'Eureka' lemon, 'Washington' navel and 'Delta' Valencia oranges from trees treated pre-harvest. Trees were treated by drenching with one of three different concentrations of Si (S1= 1250, S2= 2675, S3= 5350 mg Si ℓ^{-1}) and one concentration of Phosphorous acid (P= 500 mg ℓ^{-1}) and combinations of each of the Si treatments with Phosphorous acid ($P< 0.05$).

Overall, flavonoids in navel flavedo increased most following pre-harvest tree treatment application, while postharvest Valencia flavedo responded most positively to the fruit treatments (figure 4.3.2).

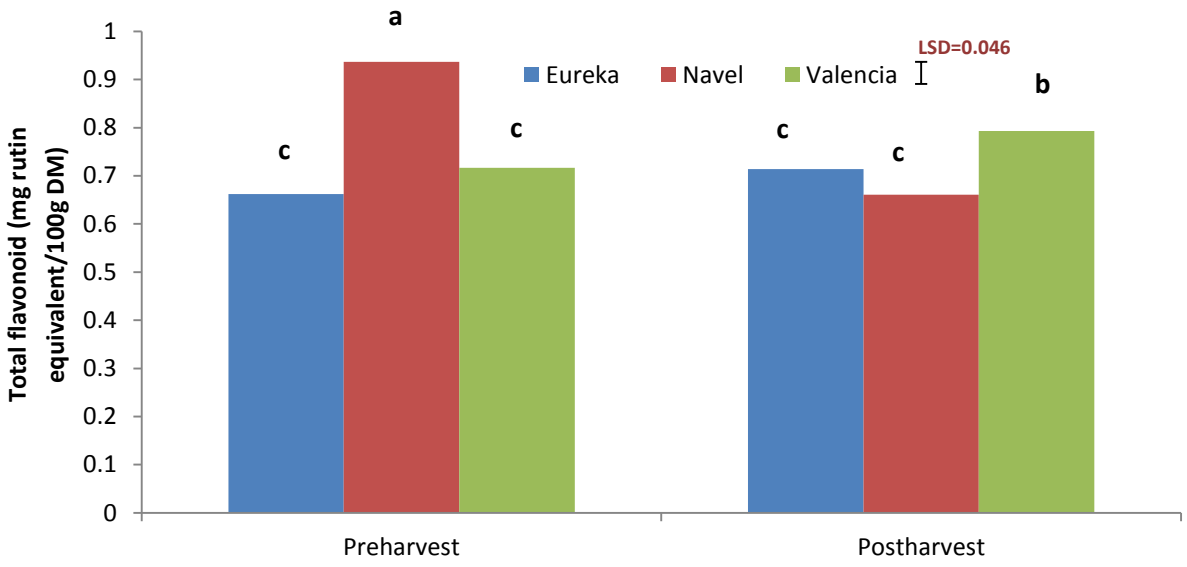

Figure 4.3.2: Comparison of overall effect of pre- and postharvest treatment applications on total flavonoid concentration (mg rutin equivalent/100g DM) in flavedo tissue of treated 'Eureka' lemons, 'Washington' navels and 'Delta' Valencia oranges

As with the pre-harvest trial, postharvest treatment of Eureka fruit with S1 and P as standalone treatments resulted in the highest accumulation of total flavonoids. There was a slight difference between Eureka fruit treated with treatments S2, S2+P and the control fruit treated with water (figure 4.3.3).

In the navel fruit, postharvest treatment with treatment S1+P proved to be the best treatment whilst there was no significant difference between fruits treated with treatments P, S1 and S2 (figure 4.3.3). In Valencia fruit the highest concentration of Si (treatment S3), applied as a standalone treatment, produced the best results.

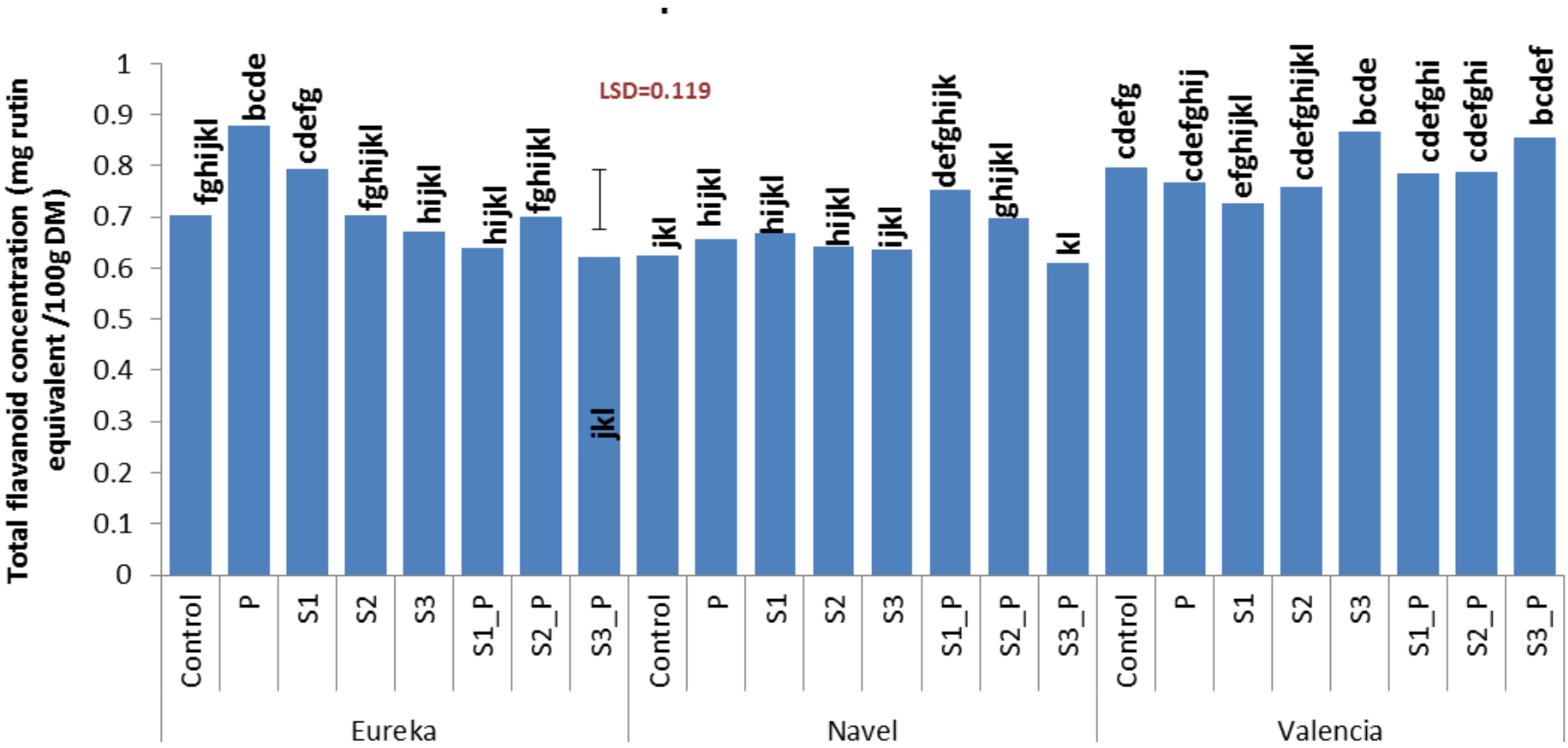

Figure 4.3.3: Total flavonoid concentration (mg rutin equivalent/100 g DM) in flavedo tissue of 'Eureka' lemons, 'Washington' navel and 'Delta' Valencia oranges. Fruit were treated by subjecting them to 90 second postharvest dips in one of three different concentrations of Si (S1= 1250, S2= 2675, S3= 5350 mg ℓ^{-1}) and one concentration of Phosphorous acid (PA= 500 mg ℓ^{-1}) and combinations of each of the Si treatments with Phosphorous acid.

In ‘Eureka’ fruit the only significant difference in flavonoid concentration was observed between fruit treated pre- or postharvest and inoculated (figure 4.3.4).

Higher flavonoid concentrations were found in the rind of navel fruit from pre-harvest treated trees than in postharvest treated fruit, with a lower flavonoid concentration in the flavedo of fruit that were neither wounded nor inoculated than in pre-harvest treated wounded and in pre-harvest treated, inoculated fruit. Postharvest treated navel fruit showed no significant difference in flavonoid concentration between fruit whether being wounded, inoculated or not treated at all (figure 4.3.4).

In Valencia fruit only non-treated fruit showed a difference in pre- and postharvest flavonoid concentrations, with wounding and inoculation resulting in no difference in flavonoid concentration of pre- and postharvest treatments (figure 4.3.4).

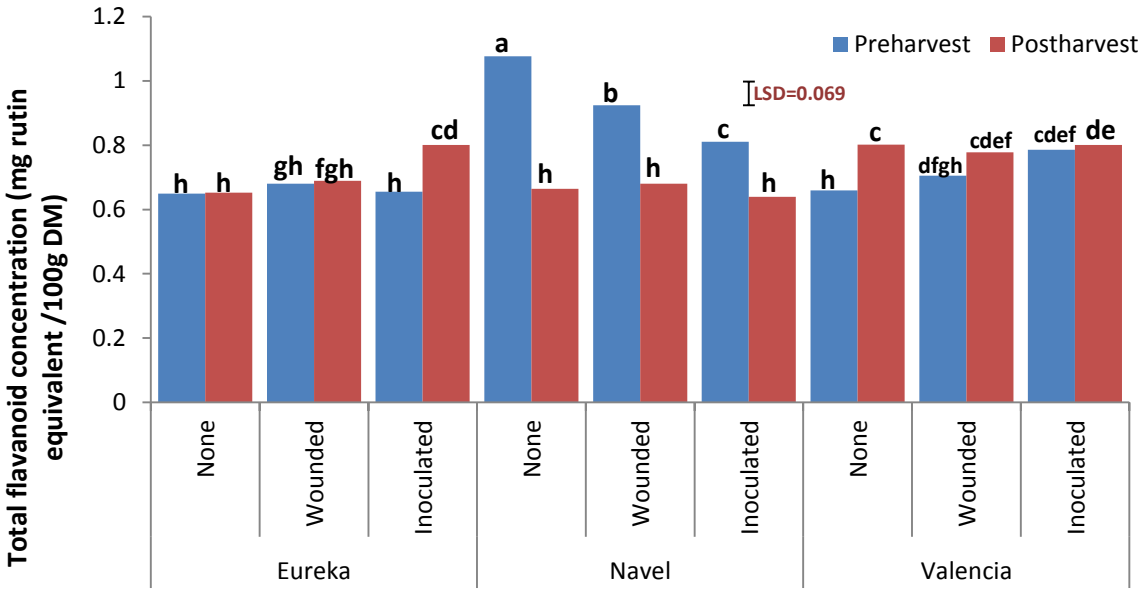

Figure 4.3.4: Effects of wounding, inoculating or leaving fruit unwounded and uninoculated on total flavonoid acid accumulation (mg rutin equivalent/100 g DM) in flavedo tissue of treated ‘Eureka’ lemons, ‘Washington’ navel oranges and ‘Delta’ Valencia oranges

CHAPTER FIVE: DISCUSSION

5.1 Effects of Si and phosphorous acid on disease incidence

Being responsible for about 90% of production losses during postharvest handling and storage (Macarisin *et al.*, 2007) *P. digitatum* is undoubtedly the most economically devastating pathogen of citrus fruit. The prevalence of the fungus despite fungicide application and implementation of various control strategies makes the disease particularly devastating. The fungus is particularly difficult to control as the development of resistant germplasm has not been successful yet and environmentally safe, but still efficient alternatives are absent (Macarisin *et al.*, 2007).

Although Si is not considered to be an essential element in all higher plants, it has been proven to play numerous roles in plant growth and development in a variety of species. Silicon has been shown to alleviate stress caused by abiotic factors, such as manganese toxicity and sodium chloride stress and also shown to protect plants against various diseases such as anthracnose (*Colletotrichum gloeosporioides*) in avocados, pink rot (*Trichothecium roseum*) in melons (Guo *et al*., 2007) and rice blast (*Magnaporthe grisea*) (Ashtiani *et al.*, 2012).

Of the eight treatments used in this study, the lowest Si concentration (S1= 1250 mg ℓ^{-1}) was most effective in restricting disease progression in all citrus types evaluated (Figure 4.2 and 4.3). These results are in agreement with reports by Agarie *et al.* (1998), Bekker *et al.* (2007), Liang *et al.* (2008), Cai *et al.* (2009) and Epstein (2009) who documented that Si plays an important role in inducing stress resistance in a variety of agricultural crops; however, high Si concentrations can also exacerbate disease incidence, as visible in the S2 and S3 treatments (S2= 2675, S3= 5350 mg ℓ^{-1}) that resulted not in reduced, but, in certain instances, an increased disease lesion size. Besides Si, PA also reduced disease incidence,

allowing the suggestion that the combination of these treatments could have an additive effect. Contrary to this expectation, however, treatment combinations (S1+P, S2+P and S3+P) did not have any synergistic effect on disease suppression, as the average lesion size of fruit treated with these treatment combinations was not significantly different ($P > 0.05$) to the control.

Treating fruit with the lowest Si concentration (S1) also resulted in a delayed onset of disease symptoms. Disease lesions in fruit treated with S1 were generally smaller compared with those of other treatments. Although there was establishment of primary hyphae, a delay in the production of the characteristic olive green spores was observed in S1 compared with fruit in the control treatment (Figure 4.1.5 and 4.1.4). This is in agreement with Hammerschmidt (1999) who reported that in cases where disease resistance was not induced within the host tissue, rapidly spreading secondary hyphae developed after the establishment of the primary hyphae; however, in plants where resistance has been induced, the hyphae stop development at the primary hyphae stage. This has been attributed to the glucanases or chitinases properties of the PR proteins typical of SAR following pathogen attack. These enzymes block the development of fungal oomycetes by hydrolase action on fungal cell walls or by other enzyme activity (Hammerschmidt, 1999). It has also been suggested that there may be changes in cell wall chemistry resulting in the inability of the pathogen to shift from primary to secondary hyphae production due to the inactivity of polygalacturonase, resulting in an inability to break down components of cell walls in induced tissue (Hammerschmidt, 1999).

Liang (2003) reported that drenching cucumber plants with Si significantly enhanced the enzyme activity in roots of salt-stressed plants compared with Si-deprived plants. Most importantly, the benefits of Si were more pronounced in extended or long-term experiments (Liang, 2003). In the current study, trees were drenched once a week for four consecutive weeks with 5ℓ Si and / or PA solution prior to harvest. Therefore, increasing the frequency of Si application or extending it over the entire season may be more beneficial resulting in better

disease control. The lack of disease control observed following pre- and postharvest Si and P treatments (Figure 4.1.4) is consistent with reports by Abraha *et al.* (2010) who found that Si application did not significantly reduce disease incidence in both, navel and Valencia oranges, when trees were drenched once a month for four months prior to harvest.

According to Abraha *et al.* (2010) Si treatments result in a more effective reduction in disease incidence in both citrus types, lemons and oranges, when trees are drenched once a month throughout the entire growing season (Abraha *et al.,* 2010). This may be due to relatively longer periods of availability of plant-accessible Si within the soil allowing for its accumulation in plant tissue. With regard to pre-harvest Si application, it can be concluded that it may not be the concentration or level of Si applied but rather the frequency of application being important; therefore, lower levels of Si distributed evenly throughout the entire season could not only be more effective but also economically sound. Tesfay *et al.* (2010) reported that avocado fruit mesocarp is able to absorb Si postharvest from the treatment solution and these authors confirmed the deposition of Si between the cell wall and cell membrane by transmission electron microscopy. Besides the Si concentration applied pre-harvest, the period of time that fruit spend in the treatment solution may also be an important factor in determining the amount of Si the fruit is able to absorb. A period of 90s submersion in the solution may not be sufficient to allow for adequate absorption of both, Si and phosphorous acid into the plant. This could explain the lack of disease control observed when Si was used as a postharvest treatment instead of a pre-harvest treatment. To confirm such an assumption, determination of the Si concentration in the citrus rind would be required in a manner similar to Kaluwa (2010) who used scanning electron microscopy to determine Si levels in various avocado tissues following Si fruit applications. Unfortunately, due to time constraints such determination was omitted in our experiments.

For both pre– and postharvest experiments, Valencia oranges demonstrated a slightly greater degree of disease control than navels and ’Eureka’ lemons. This suggests that Si has

a greater potential to reduce disease occurrence in Valencia oranges than in the other citrus types investigated. This observation was not un-expected since plant responses to Si application, or any other treatment, may vary depending on several factors such as genotype, cultivar and fruit maturity (Montesinos-Herrero *et al.*, 2009).

5.2 Effects of Si and phosphorous acid (PA) application on the production of phytochemicals in citrus flavedo

One of the main objectives of this study was to investigate the ability of pre- and postharvest Si and PA application to enhance rind phenolic and flavonoid concentrations in order to reduce disease severity in citrus fruit inoculated with *Penicillium digitatum.* The application of Si has been previously demonstrated to enhance phenols and flavonoids in plants (Maksimovic *et al.*, 2007). In this study, 1250 mg ℓ^{-1} Si increased the rind flavonoid and phenolic concentration (figure 4.2.2, 4.2.3 and 4.3.1). These results correspond with those reported by Bekker *et al.*, (2007) who found that the concentration of phenolics was enhanced in avocado trees following Si application.

When polyphenol accumulation was compared between the lemons and navel and Valencia oranges, it was found that lemons accumulate the lowest levels of phenolics and flavonoids. This is contrary to findings by Gorinstein *et al.* (2001) that lemons have significantly higher rind polyphenol concentration than oranges. 'Eureka' lemons used in this study were less resistant to pathogen attack and had correspondingly lower total phenolic and flavonoid concentrations than the two orange types. This may be attributed to the quality of lemon fruit used, their maturity and/or other pre-harvest orchard conditions.

Flavanone concentrations in the fruit have been found to be dependent on the particular specie and also the specific cultivar (Ortuño *et al.*, 2006). Flavonones, such as hesperidin and naringin and the polymethoxy flavones nobiletin, have been demonstrated to reduce the radial growth of *P. digitatum* when added to PDA medium. Ortuño *et al.* (2006) found that 100 hours post culturing, the growth of the fungus was inhibited by up to 75%. In addition to

this, ultrastructural modifications of the hyphae cell wall were observed when *P. digitatum* was cultured in the presence of nobiletin; individual cells also had a smaller cytoplasmic density when compared with the control (Ortuño *et al.*, 2006); This could be the possible reason why fruit was found to contain higher concentrations of both, phenolics and flavonoids, had a lower disease incidence. The fact that these compounds are mainly found in the flavedo, and to a lesser extent the albedo, substantiates the idea that they play a role in the protection of fruit against attack by pathogens (Ortuño *et al.*, 2006), providing a "first resistance" barrier to invading fungi.

The occurrence of wounds during fruit harvesting and postharvest handling cannot be eliminated; once obtained, the damage can only be minimized through careful handling. Previous studies have suggested that fruit that are wounded prior to or during harvest are "primed" such that they will recognize and react to subsequent fungal infection more effectively (Laggrimini *et al.*, 2003). Contrary to this hypothesis, artificially wounding fruit did not result in a marked increase in production of phenolics and total flavonoids compared with the non-wounded control. Although not statistically significantly different ($P>0.05$), there was a tendency towards higher flavonoid concentration in wounded fruit. This could indicate that fruit reacted to the threat of pathogen invasion and the fruit produced biochemical compounds to counteract the fungal attack.

CONCLUSION

South Africa ranks as the world's third largest exporter of fresh citrus fruit, behind Spain, and is ranked 13th in the world in terms of total citrus production (Siphugu, 2011). The Global Agricultural Information Network reported that Valencia exports from South Africa increased in 2010 to 697,500 T, navels increased to 343,500 T and lemons increased to 144,000 T (Siphugu, 2011). Because the industry is largely export-orientated, the production of high quality, disease-free fruit is essential. *Penicillium digitatum* is undoubtedly the most economically devastating pathogen of citrus fruit. Growers are heavily reliant on synthetic fungicides for the control of this pathogen, but the fungus' ability to develop fungicide-resistant strains is a constant threat, not only to the South African Citrus industry but to growers all over the world. This has ultimately resulted in the search for alternative control measures.

Plants have been shown to produce a broad range of secondary metabolites that are toxic to pathogens. In the region of 10 000 secondary plant metabolites have been shown to have anti-pathogenic properties and there could be many more metabolites which have not yet been identified (Tripathi and Dubey, 2004). Several studies have investigated the potential role of phenolic compounds as phytoalexins, but these have been few. The majority of studies seem to indicate that flavedo tissue has the highest defence potential (Macarisin *et al.*, 2007). It remains unclear, whether the response to pathogen attack leads to tolerance or resistance mechanisms. It could be possible to use plant phytoalexins effectively; they are alternatives to chemical pesticides protecting agricultural plant produce not only safer for the environment, but also for the human health than synthetic fungicides, because of the natural origin of these compounds.

Both, Si and PA, have the potential to induce resistance in plants (Palou *et al*., 2007). Although not conclusive, the results of the present study do not rule out the potential of Si and PA to improve fruit quality through induced disease resistance. The integration of pre-

and postharvest application of mineral fungicides that improve postharvest fruit quality through the stimulation of secondary metabolites could ultimately reduce grower's heavy reliance upon fungicides for decay control. The study also revealed the potential of Si to increase phenolics and flavonoids in citrus fruit. Similar results have previously been reported in avocado by Bekker *et al.* (2007) and Tesfay *et al.* (2011) who associated the improvement in fruit quality with increased phenolic concentration in the fruit following treatment with Si. Continuous consolidated research efforts will be pivotal in the search for alternative control measures to *P. digitatum.*

RECOMMENDATIONS

In this study 'Eureka' lemons consistently had a higher average disease lesion size and overall lowest phenolic concentration than navel and Valencia oranges, regardless of the time of treatment application (pre- or postharvest). This indicates that treatments were ineffective in this citrus cultivar and, although navel and Valencia fruit did react to the treatments applied, other mineral agents would have to be identified for use on lemons.

When using a combination of treatments, it is important to ensure that these treatments are compatible. The use of Si and PA together, in some instances, resulted in disease lesion sizes that where higher than that of control fruit, which were treated with only water, indicating a certain degree of incompatibility of these treatments.

Not all phenolics or all flavonoids present in the fruit play a role in disease suppression and inducement of natural defence systems; hence, it would be useful to identify specific flavonoids and phenolics that do influence disease suppression and subsequently monitor the concentration of these specific compounds in relation to disease incidence.

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