# CHAPTER ONE

## GENERAL INTRODUCTION

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

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

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

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

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

Plant surfaces harbor a large population of microorganisms that are well adapted to colonizing that particular niche (Janisiewicz and Korsten, 2000). The inhibitory activities of some of these microorganisms play an important role in the natural control of numerous plant diseases. Many microorganisms with antagonistic properties have been identified, evaluated and registered for commercial use on fruits. One such example is *Bacillus subtilis* (Avogreen) registered in South Africa by the University of Pretoria according to

the Fertilizer, Farm Feeds, Agricultural and Stock Remedies Act of 1947 (Act 36 of 1947). Other biocontrol agents for control of fruit diseases have been registered in other countries such as the *Pseudomonas syringae* (BioSave 110) and *Pichia guilliermondii* (Aspire) marketed by Village Farms LLC and Ecogen Inc. respectively. However, there are obviously untapped pools of microorganisms of which many more beneficial microorganisms are yet to be discovered. The search for new microorganisms with antagonistic properties is therefore a continuous process.

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

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

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

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

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

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

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

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

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# CHAPTER TWO

# LITERATURE REVIEW

## 2.1. Introduction

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

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

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

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

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

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

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

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

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

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

## 2.2. Postharvest Diseases

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

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

### 2.2.1. Citrus Black Spot

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

#### Symptoms

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

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

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

Table 2.3 Continued

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

Table 2.3 Continued

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

Table 2.3 Continued

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

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

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

**Disease cycle and epidemiology**

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

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

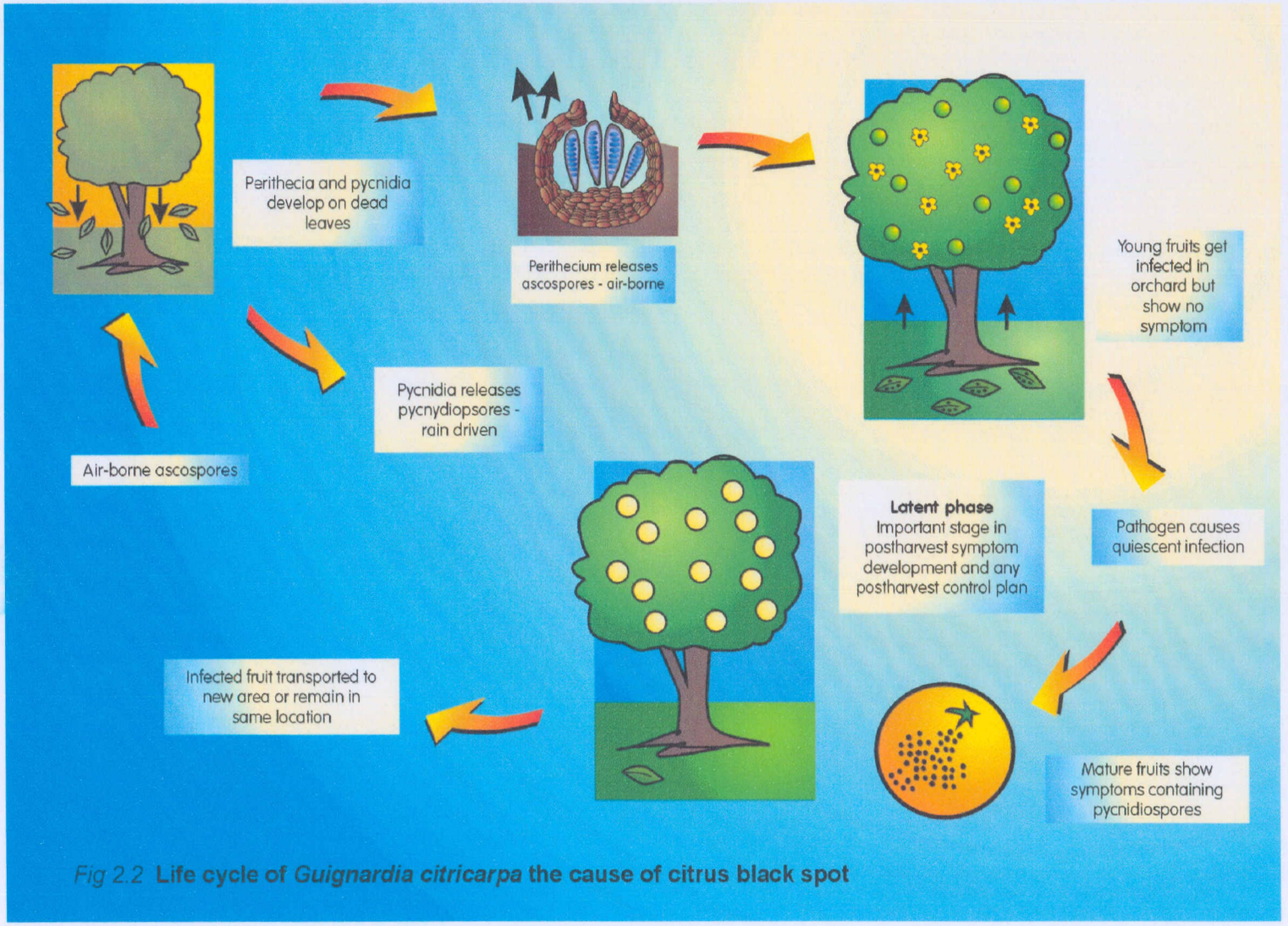


*Fig 2.2* **Life cycle of *Guignardia citricarpa* the cause of citrus black spot**

Diagram courtesy of Prof. J.M. Kotzé

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

### 2.2.2. The penicillia

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

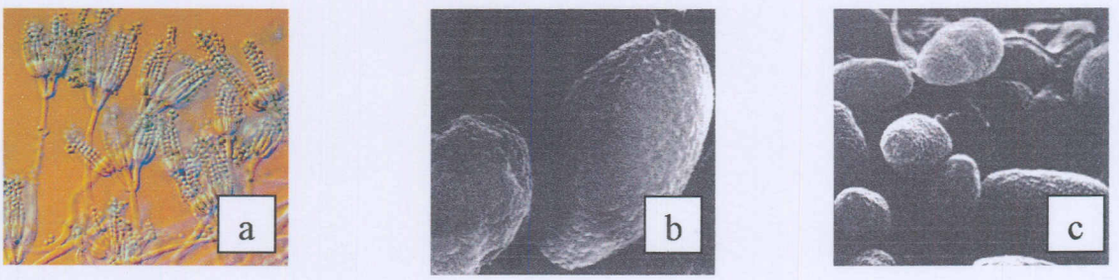


Fig.2.3 Reproductive structures of *Penicillium*, a) Conidiophores bearing spore producing phialides, b) *Penicillium digitatum* spores, c) *Penicillium italicum* spores (Carlos, 1982; Anonymous, 1999).

#### 2.2.2.1. *Penicillium digitatum* (green mold)

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

**Symptoms**

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

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

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

**Disease cycle and epidemiology**

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

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

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

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

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

### 2.2.2.2. *Penicillium italicum* (blue mold)

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

**Symptoms**

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

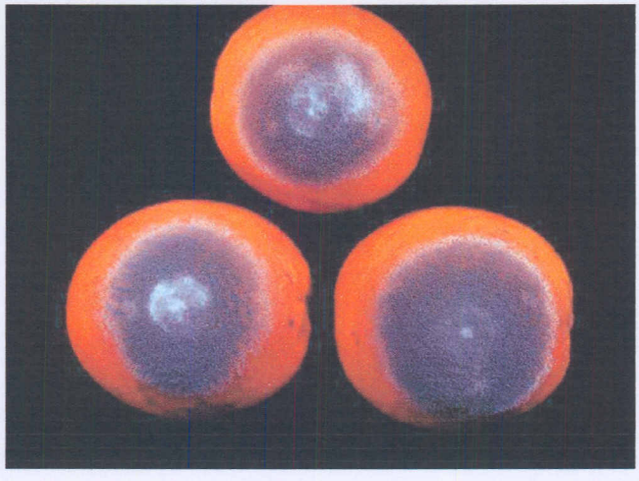

Fig. 2.5 Symptoms of citrus blue mold caused by *Penicillium italicum*

**Disease cycle and epidemiology**

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

## 2.3. Control of Postharvest Diseases

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

### 2.3.1. Chemical control

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

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

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

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

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

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

### 2.3.2. Biological control with microbial antagonists

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

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

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

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

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

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

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

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

Antibiosis, generally defined as antagonism mediated by specific or non-specific metabolites of microbial origin, by lytic agents, enzymes, volatile compounds, or other toxic substances (Jackson, 1965), is a common phenomenon responsible for the biocontrol activity of many organisms developed as biocontrol agents such as *Pseudomonas*, *Bacillus, Trichoderma* spp (Alabouvette and Lamanceau, 1999). Many phenotypically identical microorganisms are capable of producing vastly different kinds of secondary metabolites, each of which could be highly target specific (Cutler, 1986). Such metabolic compounds may include alcohols and acetic acids (Atlas and Bartha, 1998), ammonium (Fravel, 1988), and antibiotics (Mc Keen *et al.*, 1986; Atlas and Bartha, 1998), which may be inhibitory to other micoorganisms and so give them a competitive advantage over organisms with no such capabilities.

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

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

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

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

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

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

### 2.3.3. Plant extracts

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

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

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

**Plant secondary metabolites**

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

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

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

**Extraction techniques**

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

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

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

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

### 2.3.4. The use of inorganic salts in postharvest disease control

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

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

### 2.3.5. Heat treatment

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

### 2.3.5. Integrated control

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

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

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

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

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

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

## 2.4. Food-Borne Pathogens

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

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

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

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

**Conclusion**

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

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