Identification of citrus (*Citrus sinensis*) Postharvest Pathogens from Ethiopia and their Control

By

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Submitted in partial fulfilment of the requirements for the degree of

Ph.D. in Plant Pathology

to the Faculty of Natural and Agricultural Sciences

University of Pretoria

2006

DECLARATION

I, the under signed hereby declare that the work reported herein is the result of my original research findings.

Name: **Sissay Bekele** **Signature & date:** _____

ACKNOWLEDGMENTS

I sincerely wish to express my deepest gratitude and appreciation to my supervisor Prof. Lise Korsten, and co- supervisor Dr. Thierry Regnier without whose academic support and continued guidance throughout this work would not have made this study possible.

I am very much grateful to all the staff members and students of Plant Pathology Laboratories, Department of Microbiology and Plant Pathology, University of Pretoria for encouragement, support and assistance. In particular, I am indebted to Mrs Amelita Lombard and Karin Zeeman who have given me the confidence and training to work in the ISO IEC 17075 accredited laboratories. I am also grateful to Mrs Daleen Muller for her unchanged smile and respect in due course of repeated enquiry in the process of learning.

Special thanks must go to Colonel Teshome Ayalew and W/o Yigardu G/micheal family for their unreserved support and family care throughout my studies abroad.

I would like to express my greatest appreciation to Dr. Seifu G/mariam from Melkassa Agricultural Research Institute, Nazareth for his guidance and sharing of ideas during my field survey. People who are too many to mention were key informants while collecting medicinal plants from and around citrus farms of Ethiopia are greatly acknowledged.

I am deeply grateful to all the institutions who were directly or indirectly involved in this work. To mention: Upper Awash Agro Industry Enterprise, Horticulture Development Enterprise, Ethiopian

Fruit and Vegetables Marketing Corporation (Ethfruit), Ethiopian Coffee and Tea Authority library Addis Ababa, Hursso Cadet Training Center citrus farm, Toni citrus farm (Alemaya University), Tisabalima Amhara Regional Estate Development Farm, Methahara Sugar Estate citrus farm, Ethiopia. Faculty of Agriculture and Biotechnology Institute, Pretoria University, Letaba Estate fruit farms, Johan Kellerman (Crock Valley citrus packhouse) and Kieth Lesar (Citrus Research Institute, Nelspruit) South Africa are greatly acknowledged.

I would like to thank the Agricultural Research Training Programme, Alemaya University for granting me a study fellowship through the World Bank Fund. I would like to extend my thanks to the Department of Microbiology and Plant Pathology, University of Pretoria for a kiss of life while doing my final research.

Above all, I thank God for all power He gave me day and night to work. …. ***Oh God, you are my lamp who turns my darkness into light. You armed me with strength and make my way perfect and enable me to stand on the height!***

Samuel 22: 15 Vs 3

DEDICATION

This thesis is dedicated to my wife W/o Tsigreda Gebremicheal, my children: Friezer, Bisirat, Biruktawit and the lovely young Yabsira Sissay for their enormous love, commitment and courage while I was away during my study. I don't have a substitute to express my feelings for what you mean to me.

CONTENT

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SUMMARY

From a world prospective, the continuous application of chemical pesticides has serious long-term effects on human health and environmental pollution, and can result in resistant pathogen strains. However, postharvest diseases cause major losses on the markets and need to be controlled effectively. The search for biopesticides using microbial antagonists and natural plant products has subsequently become more important as viable alternatives to control postharvest diseases. Currently, little information exists in terms of citrus production practices, disease management measures and postharvest losses in Ethiopia. The aim of this study was therefore to determine what the current situation in the country is in terms of production, disease management and postharvest disease incidence, disease management practices in Ethiopia and to develop an effective and safe disease control strategy for the industry. Citrus production in Ethiopia is mainly done by Government enterprises with little technical expertise. Disease control strategies are ineffective with postharvest losses exceeding 46%. The most important postharvest pathogen identified was *Penicillium digitatum*. In development of biopesticides, three yeast antagonists [*Cryptococcus laurentii* (strain MeJtw 10-2 and strain TiL 4-3) and *Candida sake* (TiL 4-2)] and plant leaf extracts of *Acacia seyal* and *Withania somnifera* were found to have some potential to

control *Penicillium* in *in vitro* and *in vivo* trials and ensure fruit quality. The modes of action of the yeast antagonists were not based on antibiosis. Instead, it involved competitive colonization where the antagonists inhibited *P. digitatum* spore germination and reduced mycelial growth by 75-100%. Extracts from the two plant species showed broad-spectrum antimicrobial activity against a range of several fungal and bacterial pathogens. The semi-commercial application of the antagonists and plant extracts improve fruit quality and the integration of these biopesticides were found effective in semi commercial trials and may provide a commercial solution for the citrus industry.

CHAPTER ONE

GENERAL INTRODUCTION

Citrus (*Citrus sinesis* L.) is one of the major commercial fruit crops that is widely consumed both as fresh fruit or juice. Its global demand is attributed to its high vitamin C content and its antioxidant potential (Gorinstein *et al*., 2001). Citrus is mainly cultivated in the subtropical and tropical regions of the world between 40 ^{o} north and south latitude in over 137 countries on six continents (Ismail and Zhang, 2004). Brazil is the largest producer followed by the United States of America (USA), China and Mexico. Spain, USA and South Africa are the largest exporter countries followed by Turkey and Morocco (Citrus Commodity Notes, 2005). Citrus is an important fruit crop in international trade next to grapes requiring excellent quality and shelf life attributes.

Unfortunately, citrus is attacked by several plant pathogens that affect its fruit quality. In developing countries, where protection and proper handling of fresh fruit is inadequate, losses during transit and storage can represent in excess of 50% of the harvested crop (Eckert and Ogawa, 1985; Wisniewski and Wilson, 1992). Major postharvest losses have been recorded on the export markets associated with a range of pathogens. These include green and blue mould caused by *Penicillium* spp., gray mould caused by *Botrytis cinerea* Pers ex Fr (Agrios, 1997), Alternaria rot caused by *Alternaria citri* Elli and Pierce (Whiteside *et al*., 1988), anthracnose caused by *Colletotrichum gloeosporioides* Penz (Davies and Albrigo, 1994), Aspergillus rot caused by *Aspergillus niger* Van Tiegh, brown rot caused by *Phytophthora parasitica* Dast. (syn. *P. nicotianae* Breda de Haan), Diplodia stem-end rot caused by *Diplodia natalensis* Evans (Brown, 1994), sour rot caused by *Geotrichum candidum* Link ex Pers (Howard, 1936) and Trichoderma rot caused by *Trichoderma viride* Pos ex Gray (Whiteside *et al.*, 1988). However, it is often reported that the importance and impact of these pathogens on the citrus industry differ from country to country. Therefore, it is important for a country to first determine the spectrum and relevant importance of the pathogens involved in postharvest decay.

In Ethiopia, where agriculture constitutes more than 85% of the national income, citrus production is relatively small and was traditionally done for local consumption (Lipsky, 1962). Currently, citrus production in Ethiopia has expanded (FAO, 2004) with some private, association and government farms producing for local and export markets. Upper Awash

Agro-Industry is the largest Government owned enterprise that produces commercial tropical fruits and vegetables in the country. Although there are not much comprehensive data available for postharvest losses in Ethiopia, estimates by Eyob (1997) showed that more than 50% of the fresh fruit produced are lost postharvestly.

In order to reduce postharvest losses, synthetic fungicides are applied either pre- or postharvestly. However, the application of synthetic chemical compounds to control postharvest diseases often result in chemical residues on food that may affect human health (Norman, 1988) and development of resistant pathogens (Wilson and Wisniewski, 1989). Therefore, the development and use of alternative postharvest control options involving biological agents or natural plant extracts have become important since it is perceived as being environmentally safer and more acceptable to the general public (Janisiewicz and Korsten, 2002).

The citrus phylloplane harbour a large population of microorganisms adapted to survive and effectively compete in this environment (Janisiewicz and Korsten, 2000). Microorganisms identified as antagonistic fungal, yeast and bacterial species have been studied and evaluated for their potential in biocontrol programs (El-Ghaouth, *et al*., 2002; Janisiewicz and Korsten, 2002). Some antagonists have been commercialized for control of postharvest diseases of fruit such as those registered in South Africa for fruit disease control i.e. *Bacillus subtilis* (Avogreen) for the control of pre-and postharvest disease of avocado and *Cryptococcus albidus* (Yieldplus) for the control of postharvest diseases of apples and pears. Other commercial products such as *Pseudomonas syringae* (BioSave 110 and 111) to control *Geotrichum candidum* on pome fruit and citrus; *Candida oleophila* (Aspire ™) to control Penicillium decay on citrus and pome fruits have been registered by Ecogen Inc. in the USA (Shachnai *et al*., 1996). The search for new antagonists is however a continuous process and one can accept a significant growth in this market as new and more effective biocontrol agents are accepted onto the market.

However, biological control on its own is often less effective compared to commercial fungicides (Leverentz *et al*., 2003) or provides inconsistent levels of control. Therefore, to achieve a similar and consistent level of efficacy, the use of microbial antagonists integrated with commercial chemicals (Droby *et al*., 1998), hot water (Obagwu and Korsten, 2003), chloride salts (Wisniewski *et al*., 1995), carbonate salts (Obagwu and Korsten, 2003), natural

plant extracts (Obagwu, 2003) and other physical treatments such as curing and heat treatments (Ikediala *et al*., 2002) have been used.

Plant extracts have long been used traditionally for control of plant diseases (Ark and Thompson, 1959). However, the actual use of these products in plant disease control is still lacking (Obagwu, 2003). Woody plants and shrubs, particularly of the tropical flora, provide a potential source of naturally produced inhibitory chemicals (Kubo and Nakanishi, 1979). Recently, volatile chemicals (Poswal, 1996; Obagwu, 2003; Dudareva *et al*., 2004; Singh *et al*., 2004), and essential oils (Plaza *et al.* 2004) from plant extracts were successful in controlling microbial diseases of some agricultural crops, stored fruits vegetables and food commodities. Mammed (2002) reported the strong anti-fungal activity of a non-identified plant species "muka ajua" of Ethiopia and has since been used for grain preservation in storage.

According to our knowledge, no survey on the identification of indigenous postharvest pathogens of high value crops such as cotton, coffee and citrus has been made in Ethiopia. Abate (1995) already reported this lack of information. The present study is therefore designed to identify the postharvest pathogens of sweet orange (*Citrus sinensis* L.) in Ethiopia, to determine the disease incidence caused by postharvest pathogens on citrus in storage and to screen and identify microbial antagonists and natural plant extracts for control of postharvest fruit decay. This study will also focus on the efficacy of the products under semi-commercial conditions and to determine the mode of action of the products in order to further register it for commercial use.

The objectives of this study were therefore to:

1. Determine the occurrence, distribution and disease incidence of major postharvest pathogens associated with decay of sweet orange.

2. Identify potential microbial antagonists from Ethiopian citrus and determine their efficacy under laboratory and packhouse conditions.

3. Select potential tropical plant extracts and determine their efficacy as disease control products under laboratory and packhouse conditions.

4. Evaluate microbial antagonists integrated treatment under *in vitro* condition as a control strategy option for postharvest citrus disease control.

5. Determine the mode of action of developed bio-pesticides.

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CHAPTER TWO

LITERATURE REVIEW

2.1 INTRODUCTION

Citrus (*Citrus sinesis* L.) is one of the most important fruit crops known by humans since antiquity and is a good source of vitamin "C" with high antioxidant potential (Gorinstein *et al*., 2001). Citrus originated from south-eastern Asia, China and the east of Indian Archipelago from at least 2000 BC (Swingle, 1943; Webber *et al*., 1967; Gmitter and Hu, 1990). The fruit has been introduced to the new world *via* the great trade routes of Africa to the eastern Mediterranean basin by the Arab traders while the crusaders brought the fruit to Italy, Spain and Portugal around 1000 AD (Scora, 1975). The fruit was introduced further to the western hemisphere by Columbus on his second voyage in 1493 (Samson, 1980) and the planting material to the Cape in South Africa by a Dutch merchant in 1654 (Oberholzer, 1969). Currently, citrus is cultivated in the subtropical and tropical regions of the world between 40^{o} north and south latitude in over 137 countries on six continents and generates about 105 billion US dollar per year in the world fruit market (Ismail and Zhang, 2004). In Ethiopia, although the introduction, production and consumption of citrus as a horticultural crop is very recent (Seifu, 2003), the current production and area coverage has increased through private, association and government firms to meet the local and export demands.

As with other fruits, citrus is attacked by several pre- and/or postharvest pathogens that affect fruit quality. Green and blue mould infections caused by *Penicillium* spp. (Droby *et al*., 1989), anthracnose caused by *Colletotrichum gloeosporioides* Penz (Whiteside *et al*., 1988; Davies and Albrigo, 1994), and sour rot caused by *Geotrichum candidum* Link ex Pers (Howard, 1936; Whiteside *et al*., 1988; Chalutz and Wilson, 1990) are some of the major postharvest problems that cause market losses. In developing countries, where protection and proper handling of fresh fruit is inadequate, losses during transit and storage are even greater mounting up to about 50% of the harvested crop (Wisniewski and Wilson, 1992). In Ethiopia, although there are not much comprehensive data available, estimates by Eyob (1997) showed that more than 50% of the fresh fruit produced is lost postharvestly.

Currently, to minimize losses caused by citrus fruit pathogens, synthetic chemicals are applied either pre- or postharvestly. However, the application of synthetic chemicals to control

postharvest diseases often result in chemical residues on food that may affect human health (Norman, 1988). In addition, the development of chemical resistant strains may result in reduced efficacy of synthetic chemicals (Janisiewicz, 1987; Wilson and Wisniewski, 1989).

Development and use of alternative postharvest control options involving biological agents are critically important (Conway *et al*., 1999; El-Ghaouth *et al*., 2000; Korsten *et al*., 2000; Janisiewicz and Korsten, 2002). Moreover, natural plant extracts may provide an environmentally safer, cheaper and more acceptable disease control approach (Kubo and Nakanishi, 1979; Dixit *et al*., 1995; Wilson *et al*., 1997).

This chapter briefly reviews postharvest diseases generally, with particular emphasis on those important in Ethiopia. Non-chemical control options that have been studied so far and/or are currently in use are also reviewed. The possible mode of action of biopesticides is also reported. The future use of biocontrol agents from an Ethiopian perspective is also discussed.

2.2 World citrus production, consumption and marketing

Of the total world citrus production, sweet orange (*C. sinensis*) constitute the most important proportion accounting for more than two thirds of global area coverage (FAO, 2004). Currently, ten species of edible citrus are known of which eight are commercially cultivated and five are of great economic importance (Salunkhe and Desai, 1984). Annually, more than 104 million tons of citrus are produced and about 15 million tons are traded (FAO, 2004). In Africa, the total surface area under citrus production is 1.3 million hectares, of which, 44 000 ha is in South Africa and 4 500 ha in Ethiopia (Table 2.1). Despite its recent introduction to Ethiopia (Seifu, 2003), citrus farming is scattered throughout the country (Lipsky, 1962; FAO, 1965).

2.3 Citrus fruit diseases

Postharvest losses and decay of citrus fruits can be traced to infections that occur either between flowering and fruit maturity or during harvesting and subsequent handling and storage activities. Preharvest infections are mainly caused by fungal pathogens such as *Phytophthora* spp. *Colletotrichum gloeosporioides* (Penz.) Penz. and Sacc. in Penz, *Botrytis cinerea* Pers ex Fr, *Diplodia natalensis* Pole-Evans, *Phomopsis citri* Faw, and *Alternaria citri* Ellis and Pierce (Browning *et al*., 1995; El-Ghaouth *et al*., 2002).

Table 2.1 **A comparison of Ethiopian and South African citrus production area and volumes compared to the rest of the continent and the world for the period 1985-2004**

Country	Total area harvest (ha)	(%) Growth of total area harvest	Total production Mt/year	(%) Growth of production	Reference
World	4 908 106 – 7 090 356	30.8	64 053 474 –103 685 840	37.6	FAO, 2004
Africa	1 009 277 – 1 325 135	23.84	6 821 085 – 11 088 509	38.5	″ ″
South Africa	35 400 – 69 200	48.8	706 228 – 1 712 149	58.75	″ ″
Ethiopia	3 115 – 4 800	35.1	23 600 – 29 800	20.8	CACCE, 2003; FAO, 2004

Legend: CACCE = Central Agricultural Census Commission of Ethiopia.

FAO = Food and Agriculture Organization.

Stem-end fruit infections caused by *Diplodia*, *Phomopsis*, and *Alternaria* spp. remain quiescent until the fruit becomes senescent during prolonged storage (Salunkhe and Desai, 1984; El-Ghaouth *et al*., 2002). Infections initiated by *Phytophthora* spp. occur during wet periods before harvest, while *B. cinerea* infections can occur in the orchard and during storage (Batta, 2004). On the other hand, postharvest infections that occur through surface wounds inflicted during harvest and subsequent handling are mainly caused by pathogens such as *Penicillium digitatum* Sacc, *Penicillium italicum* Wehmer, *Geotrichum citri-aurantii* (*syn. G. candidum* Link ex Pers), and *Trichoderma viride*.

Among the wound pathogens, green mould (*P. digitatum*) and blue mould (*P. italicum*) account for most of the decay of citrus fruit worldwide (Plaza *et al*., 2003). Sour rot caused by *G. citri-aurantii* is the most rapidly spreading postharvest disease and can be severe on fruit stored at temperatures above 10 °C (El-Ghaouth *et al*., 2002). Some diseases such as algal disease (algal climb), canker and insect damage caused by thrips, which cause superficial (rind blemish) problems, do not affect yield or juice quality but may affect market appeal (Whiteside *et al*., 1993). In addition to these, fruit infections triggered by insect, mite and fungal attacks could be more intense and difficult to control in humid lowland areas of the tropics (Samson, 1980). Worldwide, postharvest losses of fruits and vegetables have been estimated to be 25% (Wisniewski and Wilson, 1992). In developing countries, where protection and proper handling of fruit is lacking or minimal, the losses can be as high as 50% (Coursey and Booth, 1972). In Ethiopia, such an estimate is considered conservative (Eyob, 1997). However, a higher percentage of what could be expected because of poor handling practices, lack of cool storage facilities and insufficient postharvest treatments (Eyob, 1997). A summary of the major citrus postharvest diseases, causal agent, infection type and site and spread of citrus disease infection is depicted in appendix I table 1).

2.3.1 Major citrus postharvest diseases epidemiology and control

2.3.1.1 Green mould

Over 99 species of *Penicillium* have been described (Carlos, 1982). Conidia of *P. digitatum*, the causal agent of green mould, are produced in chains and may vary in size (4 -7 x 6 – 8 µm) and shape (Fig. 2.1a and b) (Carlos, 1982). Colonies on artificial media are similar in appearance to the mould that develops on infected fruit.

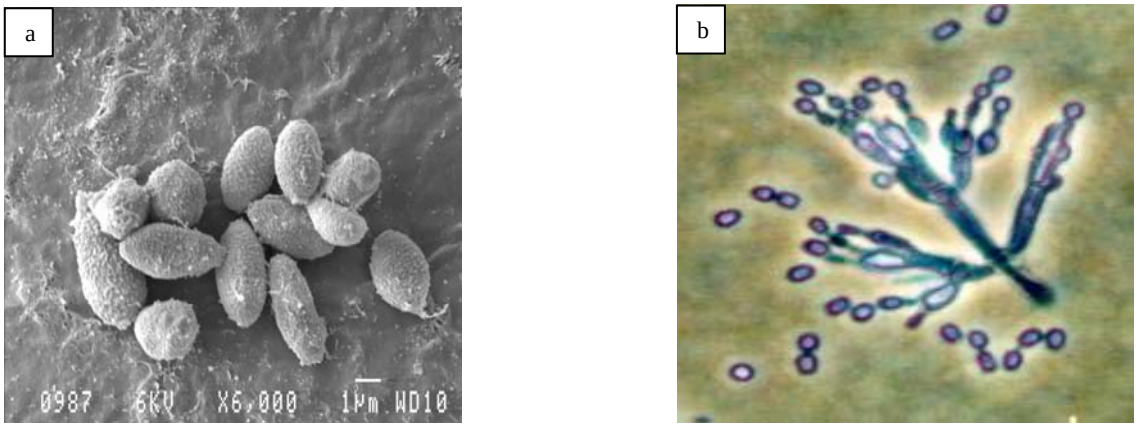

Fig. 2.1.Reproductive structures of *Penicillium digitatum,* a) Spores, (b) Conidiophore bearing spores producing phialides (Courtesy: Morgan, 2006).

2.3.1.1.1 Symptoms

Moisture plays an important role in enhancing spore growth and development. The initial symptom of green mould appears as a soft, watery, slightly discoloured spot with 6 – 12 mm diameter initially similar to sour rot and blue mould infections (Brown, 1973). Spores from the surface of infected fruit, air, field, packing area, storage room, transport containers and market places are the source of infection. The lesion diameter enlarges to 2-4 cm within 24-36 h at room temperature and the decay soon involves the juice vesicles. In five to six days, olive green spores are produced following the appearance of white mycelium around the rind encompassing the entire fruit (Fig.2.2).
(http://www.sardi.sa.gov.au/pages/horticulture/citrus/hort_citp_postpacksanitation.htm)

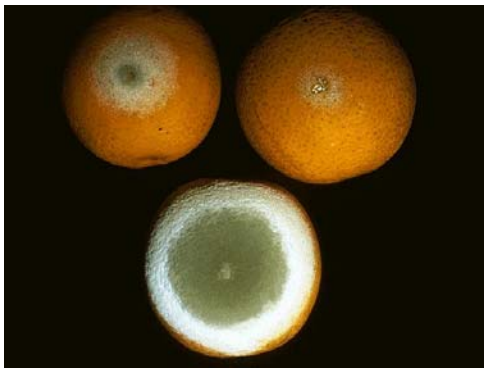

Fig. 2.2. Citrus green mould on fruit

2.3.1.1.2 Disease cycle and epidemiology

Green mould survives in the orchard from season to season primarily as conidia. Infection is initiated by airborne spores, which enter the rind through mechanical injuries (Kuramoto, 1979). Nutritionally, the pathogen is a necrotroph, which require nutrients only for germination around the wound site (Janisiewicz *et al*., 2000). A minor injury to the oil glands during harvesting and transportation promotes infection (Brown, 1973). In packed containers, the fungus doesn't usually spread from decayed fruit to adjacent intact healthy fruit. Instead, the infection and sporulation cycle can be repeated many times through the season in a packinghouses and inoculum pressure increases as the picking season advances, if precautions are not taken (Janisiewicz and Korsten, 2002). Contamination spread when spores detach from diseased fruit during the opening of packing cartons. Green mould develops most rapidly at temperatures near 24 °C and more slowly above 30 °C and below 10 °C. Rotting is almost completely inhibited at freezing temperature (0-1 °C) (Plaza *et al*., 2004).

2.3.1.2 Sour rot

Endomyces geotrichum Butler and Petersen (anamorph, *Geothricum candidum* Link ex Pers.) the causal agent for sour rot presents some conidia of 2 - 8 x 3 - 50 µm diameter (Fig. 2.3a). The fungus grows rapidly on potato dextrose agar, producing a dull gray-white colony with chains of arthrospores (Fig. 2.3b) (Butler and Eckert, 1962).

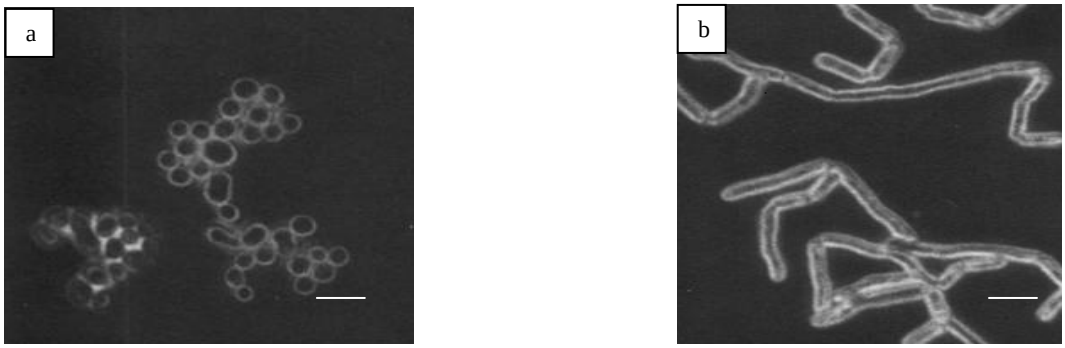

Fig. 2. 3. Reproductive structure of *Geotrichum candidum*, a) Conidia and b) Chains of arthrospores appearing dull gray white colony (bar = 1µm).

2.3.1.2.1 Symptoms

Citrus sour rot infection has the most unpleasant smell of all decays known. The initial symptoms of sour rot infections are similar to those of green and blue moulds. The cuticle is more susceptible to handle as compared to the lesions formed by *Penicillium*-induced moulds (Sommer and Ewards, 1992). The fungus degrades the rind, segment walls, and juice vesicles into a slimy, watery mass. At high relative humidity, the lesions may be covered with a

yeasty, sometimes wrinkled layer of white or cream-coloured mycelium (Baudoin and Eckert, 1982) (Fig.2.4).

Fig. 2. 4. Sour rot infection caused by *Geotrichum candidum*.

2.3.1.2.2 Disease cycle and epidemiology

The pathogen occurs commonly in soils and is windborne or splash borne to surfaces of fruit within the tree canopy. As fruits mature, they become more susceptible to sour rot infection (Baudoin and Eckert, 1982). Disease development depends on high humidity and temperature above 10 ºC, with the optimum range being 25-30 °C. Spores-laden watery debris from infected fruits and orchard soils may contaminate dip thanks, drenchers, washer brushes, belts and spread to other fruits on the packing line. Upon infection, the sour odour associated with the advanced stages of sour rot attracts flies (*Drosophila* spp.), which can disseminate the fungus and cause other injured fruit to become infected.

2.3.1.3 Brown Rot

Phytophthora spp. [*Phythophthora nicotianae* Van Breda de Hann (syn. =*P. parasitica* Dast.)] is the causative agent of citrus brown rot, which develops mainly on fruits growing near the ground (Timmer and Menge, 1988).

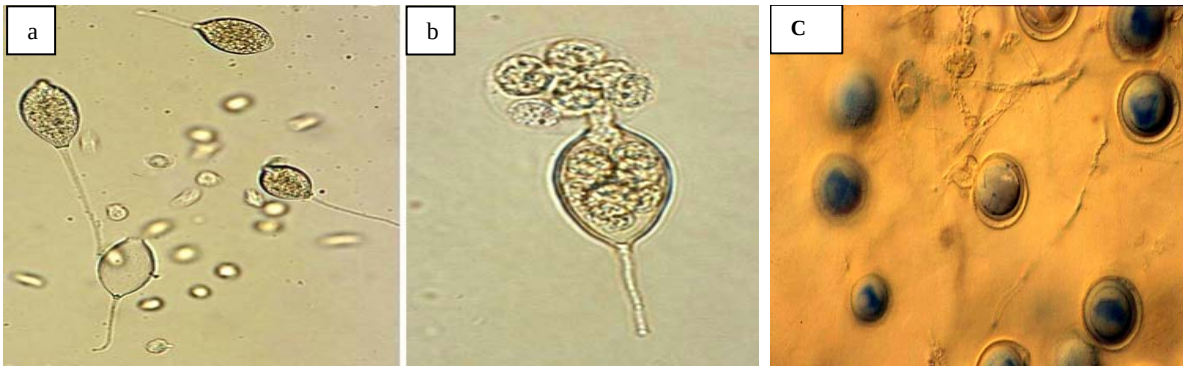

Fig. 2.5. Sporangia and zoospores of *Phytophthora* spp. a) Sporangia and zoospores b) a sporangium releasing zoospores, and c) Oospores of *Phytophthora* spp. in a culture plate (Courtesy: Babadoost, 2006).

2.3.1.3.1 Symptoms

Phytophthora infection may cause various disease symptoms on the mature fruit, trunk and root of citrus trees. Infection on fruit starts when *Phythophthora* spores from the soil splash onto the tree during rainstorms and infections develop under continual wet conditions. Initially, the firm, leathery lesions have a water-soaked appearance, but they soon turn soft and have a tan to olive brown colour and pungent odour (Fig.2.6). On the tree trunk and roots, shelling and scaling of the bark and development of lesions and gumming are common symptoms.

Fig. 2.6. *Phytophthora* spp. infection (Brown rot) on fruit (Courtesy: Futch and Timmer, 2001).

2.3.1.3.2 Disease cycle and epidemiology

Phytophthora spp. are present in almost all citrus orchards (Ann *et al*., 2004). Under moist conditions, the fungi produce large numbers of zoospores, which are splashed by rain or irrigation water onto the tree trunks, and low hanging fruits. The pathogen then develops rapidly under moist, cool conditions. On fruit, the infection progresses over the surface, but not beyond the albedo (Fig.2.6). Infected fruits in the early stage of disease development may go unnoticed at harvest and infect other fruit during storage.

2.3.2 Postharvest citrus disease control

Fungicides are commonly applied as field sprays to control fruit diseases and cold chain management practices applied to prevent and/or control quiescent fungal infections of fruits. Despite the use of fungicides, the losses of up to 20% of the harvested product are still recorded in countries even with advanced cold storage facilities (Cappellini and Ceponis, 1984). In developing countries, where the disease management practices and proper handling of postharvest commodities are poor, postharvest losses of fruits and vegetables are rated to about 50% (Eckert and Ogawa, 1985). To minimize losses and improve the shelf life of fruits and vegetables, the application of good pre- and postharvest practices including sanitation, careful harvesting and effective cold chain management practices are crucial.

2.3.2.1 Chemical control

Currently, 23 million kg of fungicides are applied annually to protect crops against diseases and pests throughout the world. Of this, about 26% of crop protectants are used in Europe, North and South America, Oceania and Asia (Tripathi and Dubey, 2004), while Africa constitutes the rest of chemical marketing and use. The application and marketing of fungicides in the USA have been reduced by 1.3% and 6% respectively (Tripathi and Dubey, 2004).

The perception that pesticides are harmful to human health and the environment has lead to the implementation of more restrictive legislation dealing with allowable chemicals and residue levels. Other problems associated with excessive use of pesticides are the development of resistant strains to tiabendazole (Timmer and Duncan, 1999), imazalil (Bus *et al*., 1991; Eckert *et al*., 1994; Timmer and Duncan, 1999) and benomyl (Bus *et al*., 1991). In addition to these, an ecological shift or imbalance in microbial populations is often the result of continuous pesticide use (Reimann and Deising, 2000). The major groups of commercial pesticides, their use and reported pathogen resistance development are summarized in appendix 1 table 2.

2.3.2.2 Non-chemical disease control strategies

The development of alternative postharvest disease control options using either microbial agents (Conway *et al*., 1999; El-Ghaouth *et al*., 2000; Korsten *et al*., 2000; Janisiewicz and Korsten, 2002; Pang *et al*., 2002; Ismail and Zhang, 2004) or natural plant products (Kubo and Nakanishi, 1979; Dixit *et al*., 1995; Wilson *et al*., 1997; Obagwu and Korsten, 2003) have become more important as successful commercial applications have gained ground. Biopesticides (microbial agents and natural plant materials) have the potential to be more environmentally safe and more acceptable by the general public for human use.

2.3.2.2.1 Cultural and physical requirements

Cultural and physical activities represent non-chemical strategies that require manipulation of the environment to decrease disease pressure. In citrus field management systems, soil drainage improvement, use of ridges (to allow air movement and draining in the juvenile phase of crop growth), use of block-raising techniques for better spacing and removal of the inoculum sources are amongst the most prominent practices involved in cultivation of citrus (Dixon, 1984).

At fruit harvesting, maximum care is required to prevent punctures, bruises, and abrasions on fruit rind. Harvesting by clipping reduces the possibility of inflicting wounds as compared to pulling (Claypool, 1983). Citrus fruit subjected to dehydration at low relative humidity after harvest is prone to stem-end rind breakdown, a physiological injury which can predispose fruit to decay (Wardowski and Brown, 2001). Therefore, temperature and humidity management in the postharvest arena is crucial to avoid deterioration of produce and the initiation of infection. The relative humidity (RH) of fruits kept in pallet boxes should be between 90% to 98%, whereas in fibreboard cartons between 85-90% to prevent carton deterioration (Wardowski and Brown, 2001). Effective sanitation practices during pre- and postharvest handling can greatly reduce the incidence of decay. Separation of sound fruits from the decayed ones in storage or distribution or repack centres reduces possible sources of inoculum and prevents contamination (Wardowski and Brown, 2001).

2.3.2.2.2 Bio-pesticides

Bio-pesticides are the new generation crop protectants based on naturally occurring microbial communities on plant surfaces and use of extracts from plant materials.

Microbial pesticides are antagonistic microorganisms, which are screened and developed for their antipathogenic activity. Antagonistic microorganisms can be collected from several sources such as dead arthropods, disease suppressive soils, and healthy plants in epidemic areas. However, epiphytic microflora derived from the commodity to be protected is the most adequate candidates (Wilson and Wisniewski, 1989). In various ways, viruses, bacteria, fungi and micro-fauna have all been observed to give some level of disease control. However, the greatest interest is directed at the use of bacteria and fungi to control soil borne, leaf and fruit diseases (Whipps and McQuilken, 1993). These probably may be attributed to the easy manipulation of the microbial strains as required.

Several species of bacteria and yeasts have been reported to reduce fungal decay of pome fruits (Janisiewicz, 1985; Mercier and Wilson, 1994; Janisiewicz *et al*., 2000), apple (Janisiewicz, 1988; Roberts, 1990; Vero *et al*., 2002; Spadaro *et al*., 2002; Batta, 2004), grape fruit (Droby *et al*., 2002), avocado (Korsten and De-Jager, 1995; Demoz and Korsten, 2006), pear (Zhang *et al*., 2005) and mango (Korsten *et al*., 1991; Koomen and Jeffries, 1993; Govender and Korsten, 2006). Currently, several antagonists have been registered in South Africa for control of postharvest diseases of avocado such as *Bacillus subtilis* (Avogreen) and pome fruit *Cryptococcus albidus* (YieldPlus) (Janisiewicz and Korsten, 2002). Other

commercial products such as *Pseudomonas syringae* (BioSave 110 and 111) to control *Geothricum candidum* and *Candida oleophila* (Aspire ™) to control penicillium on citrus and pome fruit have been registered by Ecogen Inc. in the USA (Shachnal *et al*., 1996). Biopesticides currently registered for commercial use are summarized in appendix 1 table 3.

In citrus, several bacteria such as *Bacillus* spp. have been reported to reduce postharvest decay (Huang *et al*., 1992; Obagwu and Korsten, 2003). The citrus phylloplane contains a complex and diverse population of microorganisms adapted to survive by competition. The use of such organisms could provide alternatives to the use of fungicides (Janisiewicz and Korsten, 2002).

The disease control mechanisms of biopesticides include multiple modes of actions [production of antibiotics (Fravel, 1988), induction host resistance (Droby *et al*., 2002; Poppe *et al*., 2003), synthesis of phytoalexins and/or the accumulation of an extra cellular matrix (Janisiewicz, 1988; Lima *et al*., 1998; Chan and Tian, 2005), competition for nutrients and space (Janisiewicz *et al*., 2000), siderophores production and direct interaction with the pathogen (Neilands, 1981; Schwyn and Neilands, 1987; Buyer *et al*., 1989), and/or volatile production (Fravel, 1988)] are involved. Mode of actions of some microbial antagonists are depicted in appendix 1, table 4. Although several modes of action have been described for biopesticides, all mechanisms have not been fully elucidated (El-Ghaouth *et al*., 2002). It is therefore essential to elucidate the mode of action of each and single new biopesticide. Competition for nutrients, space and induction of host resistance are mechanisms demonstrated by many researchers (Janisiewicz and Korsten, 2000, 2002; Porat *et al*., 2002; Plaza *et al*., 2004) and are currently used as a major criterion for selection of new biocontrol agents for postharvest applications.

An important consideration in pre- and postharvest application of biocontrol agents is the ability of the microorganism to survive at sufficient population levels on fruit surfaces after application and rapid colonization of wound sites by organisms competing with the pathogen for nutrients and/or space (Janisiewicz *et al*., 2000). In order to be a successful competitor at the wound site and colonize the area, the antagonist must have the ability to adapt more effectively than the pathogen to various environmental conditions such as low concentrations of nutrients, varying range of temperatures and pH (Janisiewicz *et al*., 2000; Nunes *et al*., 2001). During the last decade research on citrus biocontrol focused on microorganisms colonizing the wound site and competing with pathogens for nutrients. Among these are

Cryptococcus infirmo-miniatus, *Rhodotorula glutinis* (Chand-Goyl and Spotts, 1996), *Cryptococcus laurentii* (Roberts, 1990) and *Candida oleophila* (Hofstein *et al*., 1994) all effective against *Penicillium expansum* and *Botrytis cinerea* (causal agents of blue mould and gray moulds, respectively). *Debaryomces hansenii* (Chalutz and Wilson, 1990) has also been developed against green and blue moulds as well as sour rot.

On the other hand, the induction of host resistance is one of the mechanisms involved via the activation of the key regulatory enzyme, phenylalanine ammonia lyase (PAL) and/or peroxidase (PO) towards the synthesis of soluble and/or insoluble phenolics, respectively (Harborne, 1964; Porat *et al*., 2002; Poppe *et al*., 2003). Citrus peel produced a secondary metabolite, citral, which is believed to influence fruit resistance to disease attack (Rodov *et al*., 1995). Application of antagonists and/or natural plant products on citrus fruits could involve a series of reaction steps, which could alter the amount and activity of citral.

Therefore, understanding the mode(s) of action of effective biocontrol agents is important both for improving their performance through the development of formulations enhancing the expression of useful traits, and to establish screening criteria for searching for new potential antagonists. The general outline for antagonist development and registration for use is described in figure 2.5.

2.3.2.2.3 Plant extracts as a biological control

The use of plant extracts has long been identified as a traditional means to control plant diseases (Ark and Thompson, 1959; Cowan, 1999). However, the actual use of these products in plant disease control has only recently become an important field of study (Obagwu, 2003). The family of higher plants and shrubs, particularly of tropical flora has been shown to provide potential source of naturally produced inhibitory chemicals (Kubo and Nakanishi, 1979). The natural products of plant extracts such as volatile chemicals (Wilson *et al*., 1987; Dixit *et al*., 1995; Poswal, 1996; Dudareva *et al*., 2004), essential oils (Reuveni *et al*., 1984; Tiwari *et al*., 1988; Poswal, 1996; Meepagala *et al*., 2002; Singh *et al*., 2004) and phenolic compounds (Harborne, 1964; Regnier and Macheix, 1996; Tripathi *et al*., 2002) has been used successfully to control postharvest diseases of some agricultural crops, stored fruits, vegetables and food commodities. Moreover, the anti-fungal properties of garlic (*Allium sativum* L) have also been reported (Bisht and Kamal, 1994; Obagwu *et al*. 1997; Sinha and Saxena, 1999; Obagwu, 2003) to control fungal infestations.

Mammed (2002) has reported the strong anti-fungal activity of a non-identified plant species "muka ajua" of Ethiopia, which has been used in grain storage. Further studies made on the natural products of Ethiopian medicinal plants (Dagne and Abate, 1995) indicated the potential use of tropical flora as a useful source for selecting natural plant products.

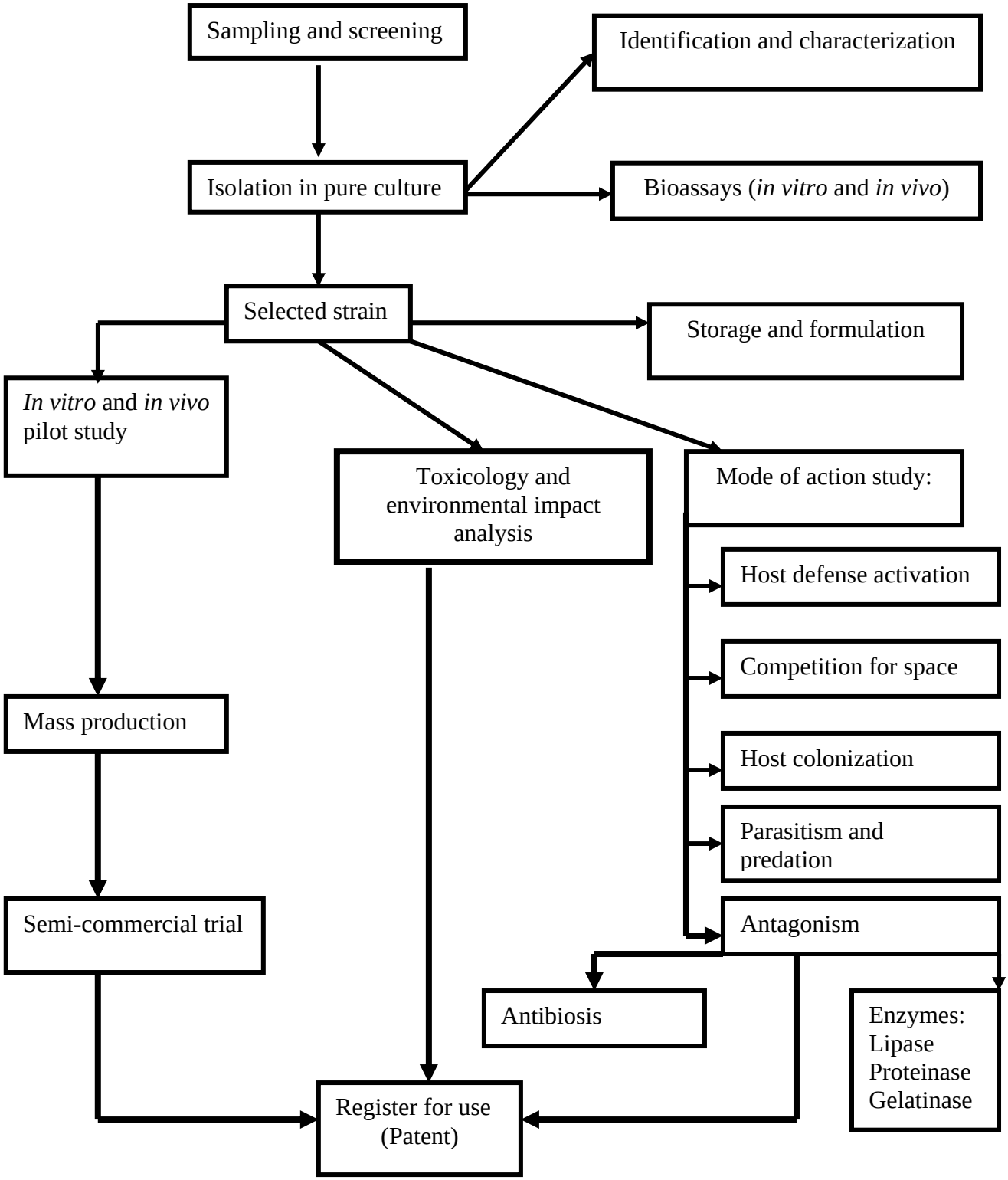

Fig. 2.5. A general outline in selection, screening and development of microbial pesticides.

The anti-helimentic activity of *Hagenia abyssinica* (Bruce) Gmel. (*Rosaceae*), anti-tumour and anti-malarial activity of *Brucea antidysenterica* Mill. (*Simaroubaceae*) (Kupchan *et al*., 1973; Phillipson and Wright, 1991), anti-leukaemic activity of *Maytenus ovatus* Loes. (*Celastraceae*) (Kupchan *et al*., 1973), analgesic and antipyretic activity of *Teclea nobilis* Delile (*Rutaceae*) and *Taverniera abyssinica* A. Rich. (*Leguminosae*) (Mascolo *et al*., 1988), antimicrobial activity of *Premna schimperi* Engl. (*Verbenaceae*) against *Staphylococcus aureus* (Habtemariam *et al*., 1993) and mulluscicidal activity of *Phytolacca dodecandra* (Lemma, 1965) are some of the many reported activities among Ethiopian flora.

Numerous antimicrobial and antifungal compounds exist naturally in plants. Plant phenolics are a diverse and abundant group of naturally occurring plant substances produced by wide range of plants (Cowan, 1999). They are characterized by the possession of aromatic rings that bear hydroxyl constituent including their functional derivatives. Most phenolic compounds are derived biosynthetically from 5-dehyderoquinate via the shikimic acid pathway or from acetate via polyketide metabolism (Fig. 2.7) (Harborne, 1964). Woody plants can synthesize and accumulate in their cells a great variety of secondary metabolites including low molecular weight phenolics (hydroxybenzoic and hydroxycinnamic acids, acetophenones, flavonoids, stilbenes and lignans (and oligo- and polymeric forms (hydrolysable and condensed cell-bound tannins and lignins) (Fig.2.6) (Harborne, 1964). The most abundant phenolics with high biomass are derived from phenylpropanoid and flavonoid biosynthesis pathways (Harborne, 1964; Robinson, 1980).

The biological significance of phenolic compounds can largely be attributed to their chemical property and reactivity (Cutler and Hill, 1994). They are generally present in the cell as glycosides or esters and are thus fairly polar (Harborne, 1964). They provide pigmentation, protection, structural support to cell wall and act as regulators of growth and development (Harborne, 1964; Robinson, 1980; Larson, 1988). Phenolic compounds make land plants adapt to UV light and ozone toxicity (Larson, 1988). Other inhibitory, stimulating and/or synergistic effects of phenolic compounds on biochemical or physiological processes and phototropism reactions mediated by phenolic photoreceptors (Towers and Abeysekera, 1984) have also been reported. Many phenolic compounds inhibit enzyme activities in a specific or non-specific manner, notably oxidative phosphorylation, ATPases and membrane transport processes (McClure, 1979). Plant phenolics play an important role as protective agents against animals and pathogens (Swain, 1977; Harborne, 1985). Toxic and inhibitory effects of

phenolic compounds on cellular processes have also been observed against animals and pathogens.

Postharvest application of plant extracts on fruits has been reported to induce host resistance by altering the metabolic pathways to synthesize more phenolics in the system (Porat *et al*., 2002; Poppe *et al*., 2003; Porat *et al*., 2003). The phenolic compounds accumulated in the peel tissue have high biological activity because of their tendency towards spontaneous or enzymatic oxidation (McClure, 1979). Many phenolics exhibit toxic or inhibitory properties after oxidation to the reactive quinine form (Baranov, 1979).

2.4 Integrated control options and strategies

Biological control alone is often less effective compared with commercial fungicides or provide inconsistent control (Janisiewicz *et al*., 1992; El-Ghaouth *et al*., 2002; Leverentz *et al*., 2003). Therefore, to achieve a similar level of efficacy provided by conventional chemicals, the use of microbial antagonists integrated with commercial chemicals (Korsten, 1993; Droby *et al*., 1998), hot water (Korsten *et al.,* 1991; Pusey, 1994; Auret, 2000; Nunes *et al*., 2002; Palou *et al*., 2002; Obagwu and Korsten, 2003), chloride salts (McLaughlin *et al*., 1990; Wisniewski *et al*., 1995), carbonate salts (Smilanick *et al*., 1999; El-Ghaouth *et al*., 2000; Palou *et al*., 2001; Palou *et al*., 2002; Obagwu and Korsten, 2003) and/or with natural plant extracts (Vaugh *et al*., 1993; Mattheis and Roberts, 1993; Wilson *et al*., 1997 and Obagwu *et al*., 997; Obagwu, 2003), other physical treatments such as curing and heat treatments (Leverentz *et al*., 2000; Ikediala *et al*., 2002; Plaza *et al*., 2003) provide a potential effective alternative treatments.

2.5 Postharvest disease control in Ethiopia

In Ethiopia, except for indigenous practices conducted by local people such as in North Wollo (Tisabalima, Wurgessa and Woldya), plant disease control practices entirely depend on the use of chemical pesticides applied during disease outbreaks. Relatively, high volume of chemical pesticides is utilized by Government owned citrus farms for which the annual expense for fertilizer and pesticides is estimated to be of 35% of the gross income (Appendix 1 table 5).

The use of biopesticides applied pre-and postharvestly to control fruit disease is a new technology not currently in commercial use. Therefore, the outcomes of this study will provide a base line of information for scientists in the country.

2.6 CONCLUSION

Like many other fresh fruits and vegetables, citrus are susceptible to a number of decay causing organisms. Chemical pesticides have traditionally been used to control diseases. The major problem being loss of their efficacy, alternative control options with biopesticides that showed good control have to be selected for postharvest application. The tropical flora and fauna is highly diverse and potentially useful for the search of biocontrol agents. Thus, the future can be upheld with this strategy to control pre- and postharvest diseases of crops in general, and citrus fruits in particular.

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CHAPTER THREE

CITRUS (*CITRUS SINENSIS*) DISEASE SURVEY: KNOWLEDGE, ATTITUDE AND MANAGEMENT PRACTICES IN ETHIOPIA

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Submitted to Tropical Research Journal for publication as:
Sissay B. Mekbib, Thierry J.C. Regnier and Lise Korsten. 2006. Citrus (*Citrus sinensis*) disease survey: knowledge, attitude and management practices in Ethiopia

Abstract

Pre- and postharvest practices ultimately determine fruit quality. A survey of actual management practices and growers' perception of effective postharvest citrus disease management strategies in Ethiopia was conducted from August 2003–February 2004. A total of 24 questionnaires were used to interview farm experts and horticultural researchers. Citrus pests and pathogens were equally identified for high preharvest fruit disease incidence. Methidathion (Propoxur, BPMC, China) and Diazinon (BASF, Germany) insecticides are currently used as predominant control methods before harvest. Most respondents spray crops only once disease and/ or pest symptoms have been observed. A high incidence (46.7%) of postharvest fruit infection was recorded from a fruit storage house in Addis Ababa. *Penicillium digitatum* Sacc. was identified as a major citrus postharvest pathogen. Although the fresh fruit market in Ethiopia has a high turn over, improvements in field production practices and general hygiene in storage facilities and packing houses are crucial to improve quality for the local and export markets.

***Key words*:** Postharvest; Fruit quality; *Penicillium* decay

3.1 INTRODUCTION

Citrus (*Citrus sinensis* L.) is a high value crop grown in the tropical and subtropical regions of the world. In world trade, citrus generates about 105 billions USD/ year (Ismail and Zhang, 2004). Currently, citrus is cultivated in more than 130 countries (Ismail and Zhang, 2004) with Brazil, China and the USA being the biggest producers and Spain, USA and South Africa the most important exporters (Citrus Commodity Notes, 2005).

Ethiopia is a relative small newcomer in citrus trade (Seifu, 2003), and the area under production has increased over the past 20 years from 3 115 ha to 4 500 ha in 2004 (Table 2). Ethiopia had been known to export citrus from the 1960's to the Middle East and Western Europe (New, 1984). However, over the past 30 years export volumes have dropped due to poor quality delivered onto the market, which is mainly due to lack of improved production practices and technology transfer has hampered industry growth (Harris, 1985).

Harvested fruits are highly perishable due to release of heat from respiration, consequently losing moisture. This characteristic may detract the appearance, weight and marketability of fruit and could make them susceptible to attack by postharvest pathogens while in storage.

In developing countries, where disease control and proper handling of fresh fruit is inadequate, losses during transit and storage have been reported to be as high as 50% of the harvested crop (Wisniewski and Wilson, 1992). In Ethiopia, no comprehensive study has been done dealing with the incidence and identity of the pathogens.

In this study, we report on a questionnaire to determine the level of knowledge, attitude and disease control management practices on citrus farms in Ethiopia and the incidence of postharvest diseases and its main causal organism.

3.2 MATERIAL and METHODS

3.2.1 Field survey: area description

A field survey was conducted between August 2003 and February 2004 in the major citrus production areas of Afar, Somali, Oromia and Amhara Regional States of Ethiopia representing about 70% of the industry. Farms were selected based on location and/ or production importance. Specific locations of places are indicated on a map (Fig. 3.1).

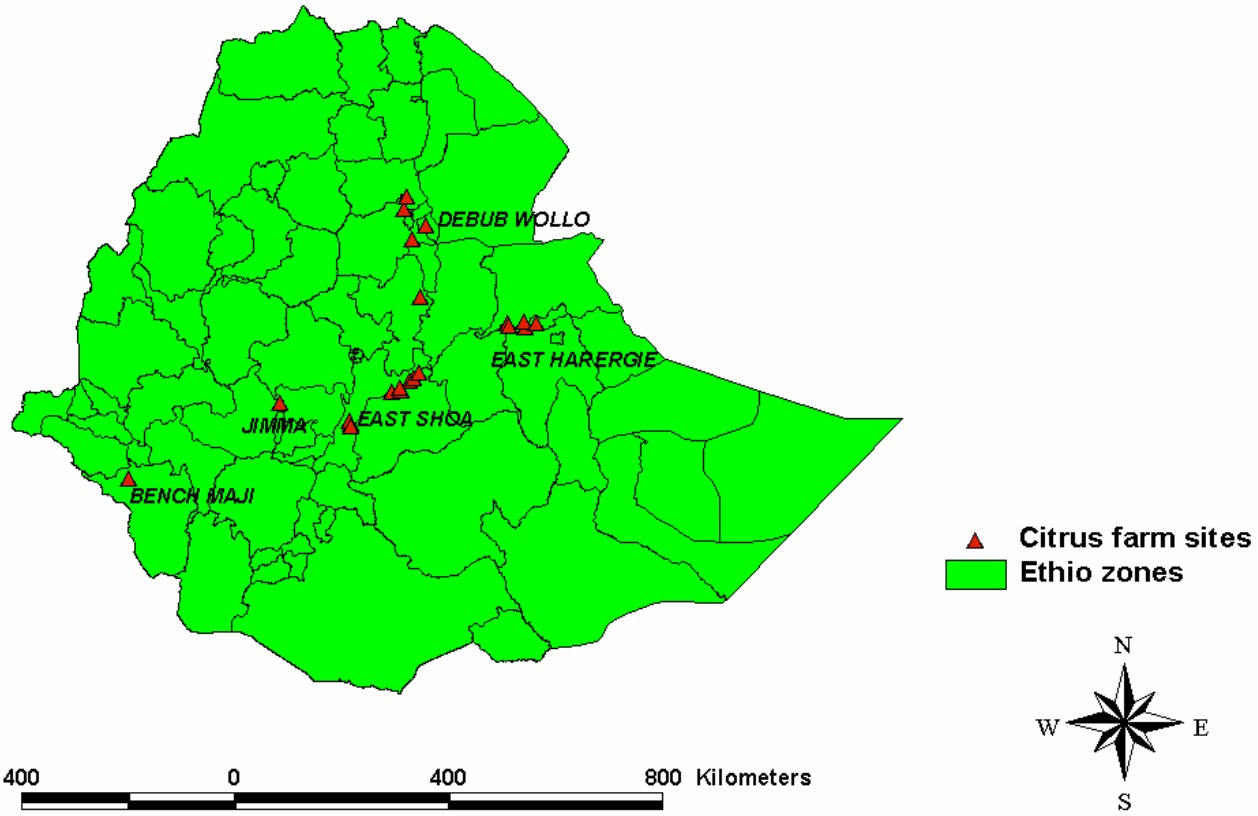

Fig. 3.1. Major citrus production regions in Ethiopia.

In total, 24 crop protection and unit farm managers were selected for a personal interview. The questionnaire has been compiled in two parts. The first part deals with preharvest citrus disease management practices containing four sections. Section 1 deals with geographical aspects of the production regions i.e. mean annual, maximum and minimum temperatures during summer and winter, water source (rainfall and or irrigation), average rainfall per year, altitude and average relative humidity (RH). Section 2 covered the history of the citrus farm and orchards, farm size, cultivars planted, stalk and scion source, orchard age, ownership of the farm, flora composition around the citrus farm, soil type and application of fertilizer. Section 3 included preharvest epidemiology and disease management practices referring to disease type manifestation, occurrence, severity and control practices. The fourth section referred to farm input, cost and production statistics including labour, pesticide and fertilizer expenses. The average annual citrus production income and loss due to postharvest pathogens

was also assessed in this section. The second part of the questionnaire dealt specifically with postharvest citrus (sweet orange) fruit handling and disease management practices covering three sections. Section 1 referred to environmental conditions at harvest (average daily maximum and minimum temperature, RH), harvesting practices, persons involved in picking of fruits, material used for picking and transporting fruits and storage temperature. Section 2 covered fruit transportation and duration, handling, cold storage conditions, types of postharvest diseases, incidence and control strategies. The third section dealt with marketing, estimated production gain or loss per year both in local and export markets.

3.2.2 Postharvest pathogen survey and disease incidence determination

Freshly harvested citrus fruits, 70 in a box were collected from eight citrus farms, Error Gota, Zeway and Shewarobit Prison farms, Methahara, NuraEra, Ghibe, Jarri Children Village, and Tisabalima citrus farms and nine local markets: Harrar, DireDawa, Nazareth, Addis Ababa (Etfruit) houses and private greengrocers. The experiment was done in triplicate and repeated twice. Samples were kept at room temperature (25 ºC) under regulated moisture (> 85% RH) for 30 days. Fruit evaluation for disease development was done after five days and thereafter every two days. Infection rate was recorded and percentage disease incidence was determined according to Vero *et al*. (2002). Percentage disease incidence (% Inc) was defined as:

$$\% \text{ Inc} = \frac{\text{Number of fruits decayed}}{\text{Number of total fruits}} \times 100$$

Potential pathogens were isolated, preliminarily identified and identity confirmed for pathogenicity by Dr. Amare Ayalew, Plant Pathology division, Department of Plant Science, Alemaya University, Ethiopia. Samples were freeze dried for three days and stored at room temperature until further use.

3.2.3 Statistical analyses

For statistical analyses and data interpretation, questionnaires collected from areas were combined for each question and descriptive statistical analyses for the main parameters were done to give an over view of citrus cultivation and disease management practices in Ethiopia. Data obtained from the postharvest disease incidence experiment were subjected to ANOVA analyses using Fisher's protected test at $P < 0.05$ using SAS software (version 8.2), 2001.

3.3 RESULTS and DISCUSSION

Categories of information and the representative answer given are indicated in (Appendix 1 table 5). The number of questionnaires completed per citrus farm and/or enterprises represent seven for Upper Awash Agro Industry, six for Horticulture Development Enterprise, two for Prison citrus farms. The rest all completed one questionnaire each.

3.3.1 Citrus farms in Ethiopia: location, climate and area coverage

Citrus producing areas surveyed in Ethiopia are indicated in Table 3.1. Sweet orange cultivation covers 82% (1 732.51 ha) of the total citrus area surveyed in the country (Table 3.2). Of these, Government ownership represented 97% of citrus farms cultivated. Upper Awash Agro Industry Enterprise owned the largest area under production (Table 3.2) and was the major marketing company in the country (78 805 tonnes/ year) (Upper Awash Agro-Industry Enterprise, 2000). Individual and association farm holdings accounted for only 2.6 and 0.4% share of the total holdings, respectively. In total, production and area harvest of citrus has increased in Ethiopia by 35.1% (Table 2.1).

Citrus farms surveyed in this study can be classified in to three agro climatic belts according to Mersha (2000) classification:

1= Single growing seasons (SS) referring to warm arid low lands (600-900 m.a.s.l.) with long growing season (LGS),

2= Two growing seasons (TS) (901-1500 m.a.s.l.) with tepid to cool areas and bimodal but distinct rainfall patterns,

3= Merging growing seasons (MS) (1501-1800 m.a.s.l.) with moist mid highlands having long and continuous rainy seasons (Table 3.1).

All citrus farms, which reported to have a lower temperature range between mid May and October (Federal Democratic Republic of Ethiopia Metrology Institute, 2004), exhibited high incidence of disease. According to the survey made, all citrus producing regions have long growing seasons with minimal precipitation and ground water supplies (Table 3.1). The period of rainy season and precipitation volumes varies from area to area. According to the climatic information obtained from Federal Democratic Republic of Ethiopia Meteorology Institute (2004), the annual rainfall of the major citrus producing areas ranges between 25-350 ml and the minimum temperature ranges from 10 ^{o}C at Degaga to 19 ^{o}C at DireDawa with the maximum temperature from 23 ^{o}C at Tulubollo to 34 ^{o}C at Methahara. The monthly mean rainfall is relatively higher at TuluBolo from June to mid-September and lower in December at Methahara and Ghibe. The climatic norm of an area is very important for disease

management and product improvement. According to Davies and Albrigo (1994), the optimum growth temperature necessary to induce flowering in citrus is between 13-24 ºC with a tolerance range of ± 3 ºC. Long wet rainy seasons (7-9 months/year) favour the development of high disease pressures and increase the volume of citrus loss at a preharvest level. Tepi and Bebeka are high altitude areas (Table 3.1) with high moistures for longer extended periods between March and December, and unlike other citrus farms, they have currently shifted to the production of coffee (Seifu, 2003).

3.3.2 Cultivation and preharvest disease management practices

3.3.2.1 Citrus cultivars

Different varieties of sweet oranges [Valencia (35.8%), Washington navel (23.9%), Hamlin (19.4%), Pineapple (7.5%), Shamuti (4.5%), Jaffa (1.5%), Robbins blood (1.5%), and other unknown cultivars (5.9%)] were described. According to the survey, Valencia was identified as the dominant variety followed by Washington navel and Hamlin. More than 70% of the total citrus area surveyed constituted old orchards (>20 years of age) and the rest represented areas with younger plantings (<20 years of age) of Valencia cultivars. However, the original source of old orchards scion and rootstock combinations is unknown and more than 84% of the respondents didn't have available information. The lack of information in this regard complicated management and breeding programs aimed at improving citrus production.

3.3.2.2 Citrus farm cultural practices and preharvest disease prevalence

Citrus farming in Ethiopia is a mixed agriculture (Seifu, 2003). Many crops, vegetables and other non-citrus trees are grown in and around citrus farms. Vegetables (mainly tomato and onions), fruits (mainly banana, papaya and avocado), and maize were reported as crops integrated in almost all citrus farms. During production, 40% of respondents differentiated between citrus pests, diseases and disorders and if assessed only do so by physical inspection. Insect pests were reported equally important to diseases (Fig. 3.2). Virus problems (13.6%), prevalence of nematodes (12.1%) and mole rats (1.5%) were also reported to cause dieback at Error Gota, Toni, Hursso and Tisablalima farms. The use and application of expert knowledge and scientific technology for the identification of diseases and disorders was found to be very limited. The development and use of diagnostic techniques for accurate identification is therefore crucial for more effective disease management strategies and to improve production practices.

Table 3.1 Major citrus farms in Ethiopia in terms of location and climatic information

	Farm name	Specific location	Distance from Addis Ababa in (km)	Altitude (m.a.s.l.) in (m)	Relative Humidity (RH)	Soil type and nutrient status	Chemical pesticides use	Fertilizer use
1	**Upper Awash Agro Industry (UAAI)***							
	i) Tibila							
	a) Degaga unit farm	N:8.428 E:39.415	135EN	1201-1500	nd	Loam and sandy soil	Methidathion, Diazinon and Metalaxyl	DAP and UREA
	b) Menberhiwot unit farm	N:8.470 E:39.589	149EN	1201-1500	nd	nd	Methidathion, Diazinon and Metalaxyl	DAP and UREA
	c) Tifsihtegenet unit farm	N:8.470 E:39.589	157EN	1201-500	nd	nd	Methidathion, Diazinon and Metalaxyl	DAP and UREA
	ii) Merti Jeju citrus farm							
	a) Merti unit farm	N:8.623 E:39.722	183EN	901-1200	nd	nd	Methidathion, Diazinon and Metalaxyl	DAP and UREA
	b) Jeju unit farm	N:8.514 E:39.569	172EN	1201-1500	nd	nd	Methidathion, Diazinon and Metalaxyl	DAP and UREA
	iii) Nura Era	N:8.670 E:39.779	178EN	901-1200	44.3	Clay, loam and sandy	Methidathion, Diazinon and Metalaxyl	DAP, UREA and manure
	iv) Awara Melka	N:8.763 E:39.877	198EN	601-900	nd	nd	Methidathion, Diazinon and Metalaxyl	DAP and UREA
2	**Horticulture Development Enterprise (HDE)***							
	i) Zeway citrus farm	N:7.899 E:38.731	165S	1501-1800	52	Sandy and loam soil	Methidathion	DAP and UREA
	ii) Gibe citrus farm	N:8.248 E:37.540	185S	901-1200	nd	Vertisol	Methidathion and Diazinon	DAP and UREA

Table … continued

	iii) Errer citrus farm							
	a) Fetuli unit farm	N:9.616 E:41.395	567E	901-1200	nd	Sandy and loam	Methidathion and Medapozoil	DAP and UREA
	b) Errer unit farm	N:9.575 E:41.384	563E	901-1200	nd	Sandy and loam	Methidathion and Medapozoil	DAP and UREA
	c) Gota unit farm	N:9.550 E:41.389	571E	901-1200	nd	Sandy and loam	Methidathion and Medapozoil	DAP and UREA
	iv) Ellen	N:9.527 E:41.662	556E	901-1200	nd	nd	Methidathion and Medapozoil	DAP and UREA
3	**Prison citrus farms***							
	i) Zeway	N:7.945 E:38.712	158S	1501-1800	52	Sandy and loam***	Mancozeb and Bayleton	DAP,UREA and manure
	ii) Shewarobit	N:10.002 E:39.899	225N	1201-1500	44.5	nd	Mancozeb and Bayleton	UREA
4	Abadir citrus farm (Methahara sugar estate)*	N:8.763 E:39.877	116E	901-1200	43.9	Vertisol, alluvial and sandy soil****	Methidathion and Lambdacyhalothrin	DAP and ASN
5	Ethioflora citrus farm**	N:7.868 E:38.726	165S	1501-1800	nd	Sandy soil	Diazinon and Tiodan	DAP,UREA and manure
6	Hursso citrus farm* Tony citrus farm*	N:9.614 E:41.643	560E	901-1200	nd	Sandy and loam	Mancozeb, Diazinon and Sumathion	DAP,UREA and manure
7		N:9.592 E:41.862	531E	901-1200	42.2	Sandy and loam	Methidathion and Sumathion	DAP and UREA
8	Jarri children village citrus farm*	N:10.973 E:39.771	440N	1501-1800	nd	Clay and loam*****	No application	DAP,UREA and manure

Table … continued

9	Amhara Regional State Development Enterprise (ARSDE) (Tisabalima citrus farm)**	N:11.459 E:39.628	450N	1501-1800	nd	Clay and sand soil	No application of pesticide. Instead, cattle urine with plant decoction were used	DAP, UREA and manure
10	Merssa citrus farm**	N:11.668 E:39.663	490N	1501-1800	nd	Clay and sand soil	Mancozeb and Malathion	Manure and seeding a legume (Lab-lab)
11	Kersa citrus farm, Bati*	N:11.190 E:40.000	417NE	1501-1800	47.3	Clay and sand soil	Malathion	Manure and mulching

Legend: * = Government ownership, ** = Private ownership, N = North, S = South, E = East; W = West, NE = Northeast, SE = Southeast, SW = Southwest, SE = Southeast, (% RH) = Percentage Relative Humidity, nd = Data not available.

*** = Soil deficient in Nitrogen and Phosphorus but high with Potassium, **** = Deficient in Iron and Zinc, ***** = deficient in nitrogen, DAP = Diammonium Phosphate.

Table 3.2 Summary of major citrus farms in Ethiopia and area coverage under sweet orange cultivation

	Citrus farms/Enterprises	Total citrus area coverage (ha)	Total citrus area coverage (%)	Sweet orange area coverage (ha)	Sweet orange area (%) as compared to the total
1	Upper Awash Agro Industry Enterprise (UAAIE)	1 496.83	71.2	1 181.25	68.2
2	Horticulture Development Enterprise (HDE)	259.66	12.3	222. 61	12.84
3	Prison citrus farms	57	2.7	39.75	2.29
4	Methahara Sugar state (Abadir) citrus farm	140	6.7	133	7.67
5	Ethioflora	2	0.095	1.5	0.12
6	Hursso, military training camp citrus farm	41.22	1.96	20	1.15
7	Toni, Alemaya University farm	10.5	0.49	9.5	0.54
8	Jarri children village citrus farm, Hike, Wollo	20	0.95	15	1.15
9	Amhara Regional State Development Enterprise farm, Tisabalima, citrus farm, Wollo (Association)	8.5	0.4	8	0.46
10	Merssa private citrus farms (individuals)	52	2.47	41	2.36
11	Ghion Hotel citrus farm, Kersa, Bati	3.5	0.17	3.2	0.18
12	Coffee Plantation Development Enterprise (CPDE)*	11.4	0.54	10.4	0.65
Total		**2 102. 61**		**1 732. 51**	

Legend: ha = Hectare, % = Percentage, * = The citrus farms currently shifted to coffee production.

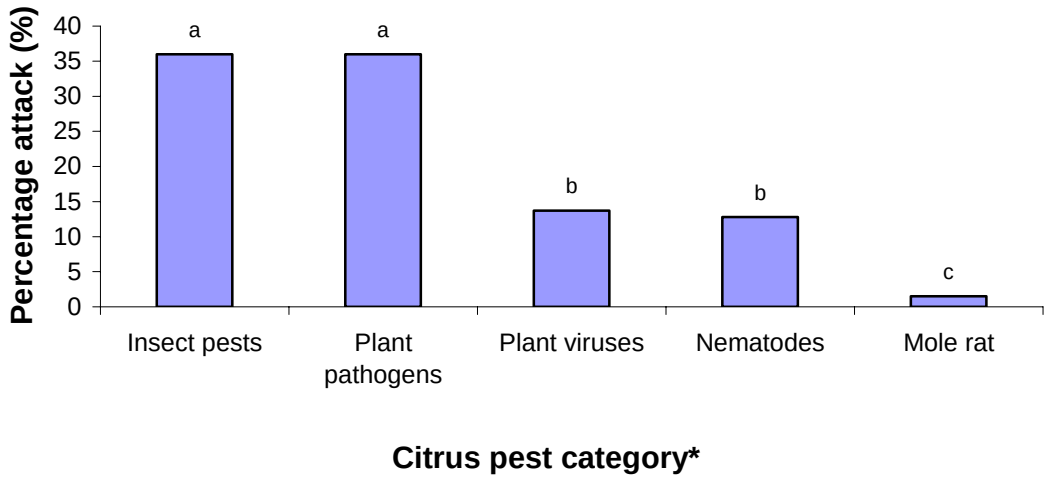

Fig. 3.2. Preharvest citrus pest category and percentage attack as identified by Ethiopian citrus producers.

Insect pests and microbial pathogens are equally important to cause high rates of disease incidence. Mediterranean fruit fly (*Ceratitis capitatia* Wiedemann), false codling moth (*Cryptophlebia leucotreta* Meyr) and thrips (*Scirtothrips aurantii* Faure) are the most important insect pests reported. Plant Pathogens such as *Phytophthora*, *Penicillium* and *Colletotrichum* spp. mostly from all citrus farms and *Phaeoramularia angolensis* in particular from Ghibe and Tisabalima, were reported. A high rate (>70%) of citrus tree dieback combined with soil borne pathogens (mainly *Phytophthora* spp.) was reported from all citrus farms in Ethiopia.

Similar reports by Seifu (2003) indicated a high severity of soil borne diseases caused by *Phytophthora* spp. The general yield loss impact of *Phytophthora* spp. in all citrus growing regions of the world has also been reported by Graham and Menge (1999). Intrinsic factors such as inadequately selected seeds and seedlings and/or inappropriate use of cultural practices such as irrigation systems (Fig. 3.3) as well as adverse edaphic conditions may increase the rate of infection and spread of the disease in an orchard (Salerno and Cutuli, 1981). According to Ippolito *et al*. (1996) poor root stock combinations attributed to high levels of gummosis and phytophthora root rotting.

Fig. 3.3. Improper use of double ring basin irrigation system.

The traditional method of irrigation in Ethiopia is the double ring basin system. The improper use of the system (Fig. 3.3) creates a direct contact between tree bark and surface water. This practice resulted in an increased phytophthora infection and eventually tree dieback (Caruso and Wilcox, 1990; Oudemans, 1999). According to Salerno and Cutuli (1981), improvement in the proper use of irrigation methods and selection of disease resistant rootstock can reduce the risk of infestation by soil borne pathogens and should be implemented.

High infestation of citrus leaf and fruit spot diseases caused by mainly *Phaeoramularia angolensis* Cavalho and Mendes was found in this survey as reported from Ghibe and Tisabalima citrus farms. This result is in agreement with previous similar reports, which showed the high incidence of the disease at Ghibe citrus farm (Mohammed, 1995) and Northwest of Ethiopia (Dessalegn and Girma, 2002). Although the detailed information about the inoculum source and disease cycle of the pathogen is not known, wind-borne conidia infect fruits and/or planting materials such as seedlings. Windbreak trees planted around the periphery of the farms are thought to be the potential sources of the pathogen (Whiteside *et al*., 1988). Although the application of this practice seems important from an agro-ecological point of view and pest trap, field disease control by sanitation and clearing of inoculum source is important (Sierra *et al*., 1993). The disease remains a major problem in the citrus production regions of Ethiopia and fruits were often observed with black stony centres, which are unmarketable.

3.3.2.3 Citrus insect pests

Red scale (*Aonidiella aurantii* Maskell), leaf minor (*Phyllocnistis citrella* Stainton), Mediterranean fruit fly (*C. capitatia*), false codling moth (*C. leucotreta*), thrips (*S. aurantii*), aphids (*Toxoptera citricidus* Kirkaldy), and bud mite (*Eriophyes sheldoni* Ewing) were identified as major pests on all citrus farms of Ethiopia. In this survey, scale insects, leaf minor and fruit fly were found to be the most important problems and cause more than 50% preharvest fruit damage. Attacks by thrips (mostly at UAAIE farms (16.6%)), bud mite (14.2%), aphids (7.1%), cotton cushion insects, (*Icerya purchasi* Maskell) (9.5%) and orange dogs (2.4%) have also been found from Toni, Tisabalima and Shoarobit farms.

The start of insect attack and extent of damage in citrus orchards were found to vary depending on orchard/tree age and maturity. In the current study, 40% respondents indicated initial infestation of scale insects and fruit fly attacks during fruiting and fruit repining stages. Citrus cultivation and regular monitoring of farm practices from land preparation to fruit maturity and harvesting will provide sufficient information to control infections of citrus (Taylor, 1996).

3.3.2.4 Chemical pesticides and use in citrus preharvest disease control

About 80% of the citrus farms surveyed applied commercial chemicals (pesticides) as a major means of pest control. Different pesticides [Methidathion (Propoxur, BPMC, China), Diazinon (BASF, Germany), Metalaxyl (Syngenta Phils. Inc., Switzerland), Mancozeb (Leads Agri Product Corp., China), Lambdacyhalothrin (Syngenta Phils. Inc., Switzerland), Thiophanate Methyl (Bayer Phils. Inc., Germany), Malathion (Zagro Corp, Singapore)] were applied during the initial observation of pests and/or diseases in the orchard. Of these, Methidathion was the most widely applied chemical followed by Diazinon for pest control. The application of chemical pesticides only during the first observation of the pest may lead to ineffective control and can result in build up of inoculum over time and disease outbreaks in the area (Fry, 1977). To reduce the risks associated with the ineffective application of chemicals and its environmental and health considerations require the investigation into alternative natural plant products (Tripathi and Dubey, 2004), microbial antagonists (Droby *et al*., 1991) and the application of improved pre- and postharvest sanitary practices (Sierra *et al*., 1993; Wilson *et al*., 1995).

3.3.2.5 Citrus farm irrigation and production practices

Moisture is a limiting factor for good quality citrus production. The annual rainfall recorded on citrus farms in Ethiopia is range between 25-350 mm (FDREMI, 2004). In almost all citrus farms, the surrounding rivers were used for irrigation purposes. Except Tisabalima and Bati (Kerssa) where irrigation schedules were reported to be twice per week, the rate of surface water application to the rest of the farms averaged twice per month (1.5l/sec). Application of surface water at longer intervals creates moisture stress during early spring while the tree is at the flowering stage (Directorate Communication National Department of Agriculture, 2000). This could result in excessive drop of flowers and fruit-lets, and result in a smaller crop with fruit having a more acidic taste (DCNDA, 2000). Drought followed by good rains could produce out-of season flowering and fruit setting. As observed from the survey, in saturated and poorly drained soils on citrus farms like Degaga (Appendix 1 table 5), such conditions have reportedly contributed to root rotting and tree die back, which ultimately resulted in total yield loss.

3.3.3 Fruit harvesting and postharvest handling practices

Traditionally, in all citrus farms, fruit is harvested manually by hand picking, tree shaking, long-stick pulling and dropping to the ground (Fig. 3.4).

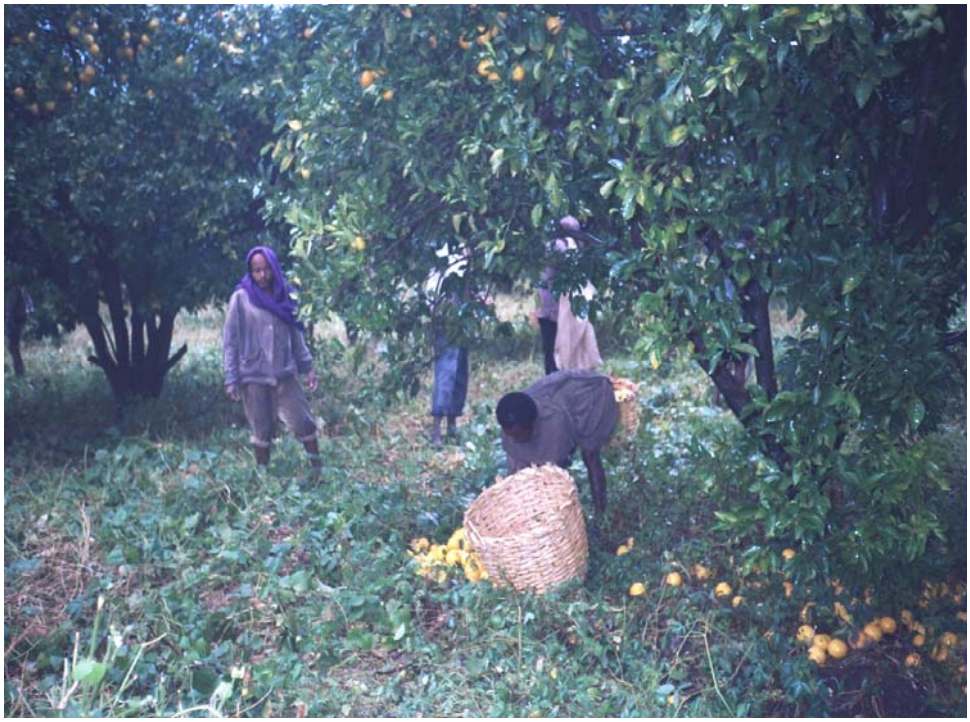

Fig. 3.4. Traditional fruit collection

Although citrus is harvested year round in Ethiopia, the peak harvesting seasons are form June to December at UAAIE and from April to August in other farms (Table 3.3). Human labour

and open private trucks are the major method of fruit transportation in almost all farms. Etfruit trucks with cooling unit facilities maintained at 4-7 °C were used at UAAIE and HDE farms to transport fruit to Addis Ababa markets. Once harvested, fruits are stored temporarily at room temperature (18-25 ºC) for about two weeks in untidy storage houses in Addis Ababa without air conditioner or other cooling facilities. Fruits were handled during distribution to buyers by jolting down crates on rough surfaces. Such handling practices of freshly picked fruits disrupt fruit physiology and may induce ethylene production, which ultimately increases fruit senescence.

3.3.4. Citrus postharvest disease incidence survey and pathogen identity

Penicillium. digitatum was identified as the major citrus postharvest pathogen followed by *Colletotrichum gloeosporioides* and *Geotrichum candidum* (data not shown) in both market and farm fruit collections (Fig. 3.5 and 3.6). All fruits collected from farms exhibit some degree of postharvest disease development, which ranged between 5.7- 28.5%. Low rate of disease incidence (5.7%) was observed on fruits from NuraEra whereas a higher disease incidence (26.5%) was found on fruits from Jarri children's village, South Wollo (Fig. 3.5).

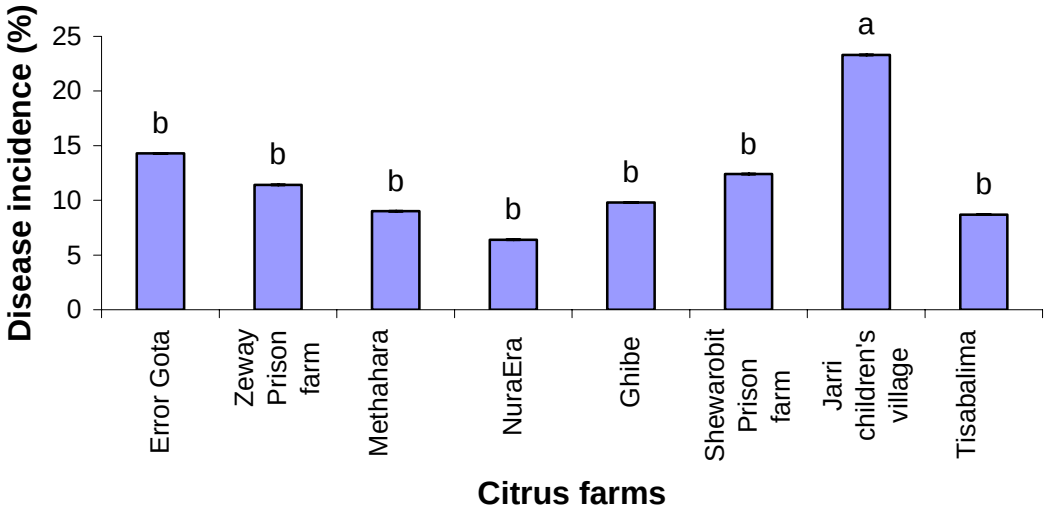

Fig. 3.5. Postharvest disease incidence of fruits collected directly from citrus farms in Ethiopia. Bars with the same letter are not significantly different (P <0.05) according to Fisher's protected LSD test and t- grouping.

Table 3.3 Peak harvesting seasons of citrus in Ethiopia based on a 2003-2004 survey

Farm units	Enterprise	Season											
		Jan.	Feb.	Mar.	Apr.	May	Jun.	Jul.	Aug.	Sept.	Oct.	Nov.	Dec.
Nura-Era	UAAIE[a]	x	x	-	-	-	x	x	x	x	x	x	x
Tibilla	UAAIE[a]	-	-	-	-	-	-	x	x	x	-	-	-
Merti-Jeju	UAAIE[a]	-	-	-	-	-	-	x	x	x	x	x	-
Awara-Melka	UAAIE[a]	-	-	-	-	-	-	x	x	x	x	x	x
Zeway	HDE[b]	-	-	-	x	x	x	x	x	-	-	-	-
Zeway	Prison farm[c]	-	-	-	x	x	x	x	x	-	-	-	-
Errer Gota	HDE[b]	-	-	-	-	-	-	-	-	-	x	x	x
Fetuli	HDE[b]	x	x	-	-	-	-	-	-	x	x	x	x
Methahara	MSE[d]	-	-	-	-	-	-	-	-	x	x	-	-
Ethioflora	Private	-	-	-	-	-	x	x	x	-	-	-	-
Toni	Alemaya University	-	-	-	-	-	-	-	-	x	x	x	x
Hursso	Defence force	-	-	-	-	-	-	-	-	x	x	x	x
Shewarobit	Prison farm[c]	-	-	-	-	-	-	x	x	x	-	-	-
Kerssa, Bati	Ghion Hotel	-	-	-	-	-	x	x	x	x	-	-	-
Jarri Children's Village	S W A O[e]	-	-	-	-	-	-	-	x	x	x	-	-
Tisabalima	ARSAF[f]	-	-	-	-	-	-	-	x	x	x	-	-
Merssa	Private[g]	x	x	x	x	-	-	-	-	-	-	-	-
Ghibe	HDE[b]	-	-	-	-	x	x	x	x	-	-	-	-

Legend: X = Peak harvesting time

- = Not peak harvesting time

[a] = Upper Awash Agro Industry Enterprise

[b] = Horticultural Development Enterprise

[c] = Government prison farms

[d] = Methahara Sugar Estate

[e] = South Wollo Agricultural Office

[f] = Amhara Regional State Association Farm

g = Private individual holdings

Fruits collected from markets had similar postharvest disease development that ranged between 5-46.7%. Significantly (P <0.05) higher rates of disease incidence (46.7%) was exhibited on Tibila pineapple fruits obtained from Addis Ababa Etfruit market whereas the lowest rate (5%) was observed on fruits collected from Addis Ababa greengrocer (Fig. 3.6).

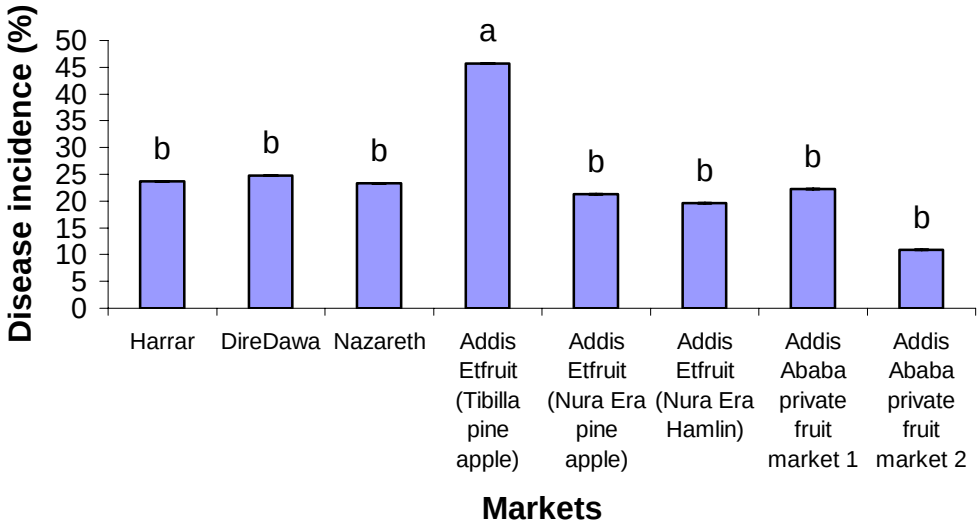

Fig. 3.6. Postharvest disease incidence of citrus fruits collected from fresh produce markets in Ethiopia. Bars with the same letter are not significantly different (P <0.05) according to Fisher's protected LSD test and t-grouping.

High postharvest disease incidence of fruit decay reported indicate that pre-and postharvest handling practices were inadequate in this sector. In the process of harvesting, fruit dropping to the ground by tree shaking was found to be common harvesting practices, which includes plugging create fruit punctures which could be contaminated by mould and sour rot pathogens (Kanopacka and Plocharski, 2004). Careful harvesting by hand picking with gloves, placing fruit in bags, protecting fruits from direct sun after harvest and avoiding jolting of bins over rough roads could decrease fruit contamination and postharvest decay.

3.3.5 Fresh produce and marketing

More than 98% of the fruit produced in Ethiopia are supplied to the nearest local markets. Most of the produce on the local market is supplied by UAAIE and HDE farms, which is marketed to Addis Ababa, Nazareth, Methahara, Diredawa and Harar. The fruit produce from Toni, Hursso and Errer Gota is supplied to the eastern capital

cities (Dire Dawa, Harar) and to a lesser extent to Addis Ababa. Fruits from the North Central Ethiopia citrus farms (Merssa, Tisabalima and Jarri) are marketed to Dessie, Woldya and Mekele towns. A very small proportion of fruit produce (2%) from the central east parts of Ethiopia (UAAIE, Tonni, Hursso and Error Gota) is exported to the neighbouring countries i.e. Djibouti and Somalia. Movement of produce across the border is mainly by private dealers using railway transport and trucks where there are no cooling facilities except spraying water manually over the surface of the fruit during the long hours (10-12 hr) of travel, which could also be at night.

CONCLUSION and RECOMMENDATION

The wide range of altitudes, climates and soil types in Ethiopia allowed for effective production of a variety of crops. Citrus is one of the high value crops cultivated in the country next to coffee in terms of local production. However, the production and export of citrus fruit to international markets is minimal and/or non-existent. Pre- and postharvest practices attribute to quality deterioration of citrus fruit. Improvements in the following practices are therefore crucial:

1. Selection of known disease resistance root stock varieties,
2. Improvement in field sanitation and controlling of weeds, infected trees and/or other crops growing around or in citrus farms. Cultivation of legumes (*Lablab purpureus* L.) (*Fabaceae*) under the orchard after land clearing during the onset of the second harvesting season (e.g. at Tisabalima, South Wollo), control weed growth and ameliorate soil nitrogen,
3. Development of appropriate alternative irrigation practices to replace the currently used double ring basin system,
4. Maintaining the water balance between onset of flowering, fruiting and ripening of fruit to avoid saltiness and fruit burst,
5. Improvement in harvesting practices and in fruit handling, and the subsequent training of these workers in best practices,
6. Although fruit volumes on the Ethiopian local fruit market is fast moving, the use of cold storage and clean packinghouse facility is important to retain quality,
7. Establishment or upgrading of centres for disease and pest identification and control studies, and

8. Careful integration of production and marketing for local and export products to improve the Ethiopian citrus industry.

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CHAPTER FOUR

DEVELOPING ETHIOPIAN MICROBIAL BIOCONTROL AGENTS FOR CITRUS POSTHARVEST DISEASE CONTROL

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Submitted to Biocontrol Journal for publication as:
Sissay B. Mekbib, Thierry J.C. Regnier and Lise Korsten. 2006. Efficacy and mode of action of yeast antagonists selected from Ethiopia for control of *Penicillium digitatum* on citrus

Abstract

An alternate to chemical pesticides, microbial antagonists are used to control pre- and postharvest decay of fruits and vegetables. In this study, three yeast antagonists [two strains of *Cryptococcus laurentii* (MeJtw10-2 and TiL4-3) and one strain of *Candida sake* (TiL4-2)] isolated from twig and leaf surface of an orange tree controlled citrus green mould by 70-95% with higher broad spectrum activity against sour rot and anthracnose caused by *Geotrichum candidum* and *Colletotrichum gloeosporioides*. Possible mode of action of these yeast antagonists [antibiosis, volatiles, phenolics, enzyme production assays, competition for nutrients and space] were studied. All antagonists showed no antibiosis effect against the tested pathogens. Some activity of enzyme production was exhibited by antagonist MeJtw10-2 (*C. laurentii*) unlike antagonist TiL4-2 (*C. sake*) and TiL4-3 (*C. laurentii*). All antagonists showed significant (P <0.05) inhibition of *P. digitatum* spore germination both in cylinder insert and direct well contact experiments. *In vitro* dual culture experiments exhibited 75-100% control of *P. digitatum* spore growth by fast colonization and competition effect on solid medium. All antagonists showed the production of extracellular matrix, which enhanced their rapid colonization on fruit wound site during infection. Germination of *P. digitatum* conidia was significantly (P <0.05) inhibited when pathogens and antagonists were in physical contact. These results may indicate that competition for nutrients is one of the modes of action of these potential antagonists against the pathogen, *P. digitatum*.

Key words: Yeast antagonists; Cylinder insert; *Cryptococcus laurentii*, *Candida sake*

4.1 INTRODUCTION

Biological control with plant extracts and microbial agents has been explored as an alternative to the use of synthetic chemicals for managing postharvest diseases of fruits and vegetables (Wilson and Wisniewski, 1989). Several species of bacteria and yeasts have been already reported to reduced fungi decay of pome fruits (Janisiewicz, 1985; Janisiewicz, 1988; Vinas *et al*., 1998; Mercier and Wilson, 1994; Spadaro *et al*., 2002; Batta, 2004), grape fruit (Droby *et al*., 2002), avocado (Korsten and De-Jager, 1995, Demoz and Korsten, 2006), mango (Korsten *et al*., 1991; Govender and Korsten, 2006), citrus (Obagwu and Korsten, 2003).

The success of some of these microbial antagonists in laboratories and large-scale studies has stimulated the interest of several workers in the development of biological products for postharvest application. Currently, some antagonists such as *Bacillus subtilis* (Avogreen) have been registered in South Africa for the control of pre- and postharvest diseases of avocado (Janisiewicz and Korsten, 2002). Other antagonists such as *Cryptococcus albidus* (YieldPlus) for the control of postharvest diseases of apples and pears, *Pseudomonas syringae* (BioSave 110 and 111) for the control of *Geothricum candidum* on pome and citrus, and *Candida oleophila* (Aspire ^{TM}) for the control of penicillium decay on citrus have been registered by Ecogen Inc. in the USA (Shachnal *et al*., 1996).

Biocontrol systems of antagonistic microorganisms have involved various modes of actions by competition for nutrients and space and/ or induction of host resistance mechanisms. Therefore, the selection and development of microbial antagonists for postharvest application involve the use of in *vitro* and *in vivo* experimental trials including pilot studies.

Because of its fauna and flora diversity and endemism, tropical environments appears to be an ideal source of microbial antagonists (Tewoldebirhan, 1991), which could help the upcoming industry to establish potential biocontrol with no environmental and health problems. The objectives of the present study were to search for effective antagonists from different citrus production regions of Ethiopia, and to investigate *in vitro* and *in vivo* modes of action, recovery and compatibility.

4.2 MATERIAL and METHODS

4.2.1 Sample collection

Samples were collected from healthy looking citrus orchards where disease pressure was supposed to be high. Ten citrus trees per farm and 10 samples from each of the vegetative parts of each tree (leaves, twigs and fruits) were collected from 20 citrus farm units of Ethiopia. Samples collected were kept in brown paper bags and transferred to the Plant Pathology Laboratory, Alemaya University, Ethiopia for preliminary screening. Samples were processed immediately or kept in cooler boxes until use.

4.2.2 Pathogen

Penicillium digitatum Sacc. was used as a test pathogen in the bioassay procedure to select the potential antagonists. The test pathogen was originally isolated from infected citrus fruit obtained from Toni farm, DireDawa, Ethiopia and the pathogen identity was confirmed by Dr. Amare Ayalew (Pathology Division, Plant Science Department, Alemaya University). Once maintained on Potato Dextrose Agar (PDA) (Biolab, Johannesburg, South Africa), the culture was placed under UV light for 7-14 days at 25 °C to sporulate. A conidial suspension of the pathogen was prepared by adding 20 ml of sterilized distilled water onto the surface of the PDA culture plate and spores were harvested by gentle swabbing. Spore concentration was standardized to 1 x 10^5 spores ml^{-1} prior to use using a haemocytometer.

4.2.3 Antagonists

Isolation and screening of the antagonists was done according to Reyes *et al*. (2004) with slight modifications. Samples (fruit, leaves and twigs) were dipped in a sterile jar (1L) with 400 ml Ringer's solution containing a standard concentration of *P. digitatum* (1 x 10^5 spores ml^{-1}) and left on a rotary shaker at 150 *rpm* for ten minutes. Serial dilutions of the wash water were plated out on PDA and Standard 1 Nutrient Agar (STD-1 NA) (Biolab, Johannesburg) to get individual colonies. Culture plates were incubated at 25 ºC for 48-72 h and evaluated for antagonist activity against *P. digitatum*. Fast colonizing microbial isolates without antibiosis effect *in vitro* were randomly selected from culture plates, purified and preserved for further *in vivo* trials. Potential isolates were taken to Plant Pathology Laboratories, (Pretoria University, South Africa) for further analyses, identification and mode of action study. Samples were brought into the country following standard quarantine requirements according to the national legislation and germ-plasm transfer agreements, (import permit no. P0017192).

4.2.4 *In vivo* antagonist screening assay

Twenty boxes of fresh citrus (*Citrus sinensis* L) fruits with 80 oranges in a box were collected from Crocodile Valley packhouse (Nelspruit, Mpumalanga, South Africa). Fruits were disinfected with 1% sodium hypochlorite for 2 min and air dried prior to wounding. The fruits were wounded on both sides with a picture hook. Wounds were made to a depth of ca 3mm into the fruit rind. An overnight culture of antagonist cell suspension grown in Nutrient Broth (NB) (Biolab, Johannesburg) was standardized to 10^8 cells/ ml^{-1} using the Petroff Hauser counting chamber. Under preventive application, 40µl of the antagonist cell suspension was inoculated onto the wounded area, 12 hours prior to the application of the pathogen, *P. digitatum*. Wound inoculation of *P. digitatum* alone or with NB served as a positive or negative control, respectively. Three fruits per isolates were used and the experiment was repeated twice. Treated fruits were stored at 25 ºC for 7–14 days and moisture was maintained between 80–90% relative humidity (RH). Fruits were evaluated every two days for disease development and data recorded.

4.2.5 *In vitro* antagonist screening assay for broad spectrum activity

In vitro screening of antagonists for broad-spectrum activity was done according to the method described by Spadaro *et al*. (2002) with slight modifications. Three postharvest fungal pathogens: *P. digitatum* (UPPed-1), *G. candidum* (UPGec-1) and *Colletotrichum gloeosporioides* Penz (UPCog-1) obtained from the culture collection of Plant Pathology Laboratories, (University of Pretoria) were used as a test pathogens. Fungal cultures grown on Malt Extract Agar (MEA) (Merck, Johannesburg) for seven days at 25 ºC were placed under UV light for 7-14 days until sporulation. A standard concentration (10^5 spores ml^{-1}) of each pathogen suspension were prepared in Ringer's solution and used immediately in the subsequent trials. Eighteen potential antagonists selected from the *in vivo* experiments on citrus in section 4.2.4 were used. The growth rates of antagonists were tested on four different solid media (PDA, MEA, STD-1NA and citrus peel-agar). For the citrus peel agar medium preparation, 5% v/v of citrus peel were homogenised and filtered through Whatman no. 1 and added to 20g l^{-1} of Agar-Agar (pH 5.5). A standard concentration of antagonist suspension (10^8 cells ml^{-1}) determined using Petroff Hauser counting chamber was used in the challenge test against the three pathogens. Ten micro litres of antagonist cell suspension was streaked on one side of MEA medium in 90 mm Petri dishes, 2mm from the border and three streaks per plate was made at each opposite side at equal distance from the centre. The same volume of pathogen suspension was put at the centre and plates were air dried and incubated at 25 ºC for seven days. Percentage growth inhibition of the pathogen was calculated according to

Skidmore (1976) using the formula: (C-r) x 100/C, where, C = growth diameter of pathogen alone, r = growth diameter of pathogen grown with an antagonist.

4.2.6 Antagonist identification

Microbial antagonists selected for their postharvest decay control potential were further screened and identified according to the method described by Droby *et al*. (1989). Rose Bengal chloramphenicol agar medium was used as a selective medium to distinguish potential antagonists in their respective categories as bacteria and/ or yeasts. Further identification of potential isolates was done using the API system (I D 32 C Biomerieux, USA). Cultural and microscopic characteristics of isolates were used to confirm identification. Isolates were maintained in 20% glycerol at -70 ºC and routinely grown on their respective media, STD 1-NA for bacterial and MEA for yeast isolates.

4.2.7 *In vivo* antagonist's activity and disease incidence against *Penicillium digitatum*

Potential antagonists selected for their best performances were further evaluated for disease incidence reduction on citrus fruit. Fruit and inocula preparation was done as described in section (4.2.4). Inoculated fruits were incubated at 7 ºC for 30 days and fruits were evaluated for disease development by the end of the incubation period, and the data was recorded. The experiment was done twice. Percentage of disease incidence reduction or percentage of intact fruit appearance was calculated using the formula described by Vero *et al*. (2002).

4.2.8 Mechanisms of biocontrol:

4.2.8.1 Antibiosis assay

A streak assay was done as described by Poppe *et al*. (2003). STD 1- NA, MEA and PDA, separately mixed with 10g l^{-1} orange flavedo tissue powder, were used as growth media. An agar disk (4 x 4mm) from a seven days old culture of *P. digitatum* was placed at the centre of a Petri dish containing 20 ml agar medium per Petri dish.. An overnight grown antagonist broth culture was streaked on three sides of the plate at equal distances from the centre. Plates were then incubated at 25 ºC for seven days and evaluated for formation of an inhibition zone. Five plates were used per treatment and the experiment was repeated twice.

Culture preparation for *in vitro* antibacterial assay was done according to (Castoria *et al*., 2001). A loop full of actively growing antagonists was inoculated into a flask containing 50 ml NB and kept overnight on the shaker at 170 rpm. Separate flasks were prepared for further inoculation assays and Thin Layer Chromatographic study of the culture filtrate active

component. The antagonist suspension was centrifuged at 5000 x g for 10 minutes and the culture filtrate was transferred into another sterilized tube. Total soluble phenolics were extracted as follows: two fold volumes of ethyl acetate was added into the culture filtrate, vortexed for 30 seconds, and left to settle for one minute. The organic phase containing the ethyl acetate and the soluble phenolics was transferred to a clean Eppendorf tube. The extraction was repeated three times. The combined supernatants were left to dry under an air vacuum chamber and re-dissolved with one ml of distilled water.

Total soluble phenolics compounds were quantified using the Folin Ciocaleteu's Phenol reagent (Sigma) (Bray and Thorpe, 1954). A comparative study was performed on the culture filtrates by TLC on pre-coated Silica Gel 60 (Merck, 60F254) using chloroform/methanol/ethyl acetate/acetone and water (55:20:20:5:3.5) as a separation solvent system. The TLC plate was loaded with 20 µl of each sample. Sterile broth culture were used as negative control and standard chemicals such as isoferulic (Sigma), *P*-coummaric acid (Sigma), novobiocin, cyclohexamide and chloramphenicol (CAPS Pharmaceuticals, SA) were used as positive controls.

4.2.8.1.1 Antibacterial assay

The TLC plates prepared as described in section (4.2.7.1) were covered with a nutrient agar and used to test the antibacterial activity of antagonists further. Two millilitres of an overnight grown indicator bacterium (10^8 cell ml^{-1}), in this case, *Erwinia carotovora,* mixed with equal volume of molten STD1- NA (50 °C) and 0.1 ml of 2% (w/v) 2.3.5-triphenyltetrazolium chloride (Sigma) was over laid on the TLC plate and left to solidify. Solidified plates were incubated overnight at 25 ºC and antibacterial activity evaluated. Appearance of red pigmentation on the plate indicates growth of bacteria and the formation of clear zone on the other hand showed pathogen growth inhibition.

4.2.8.1.2 Antifungal assay

The TLC plates prepared as described in (4.2.7.1) were also used for antifungal activity assay. A *P. digitatum* spore suspension (10^5 spore ml^{-l}) was prepared in glucose minimal salt medium and sprayed directly onto the dried TLC plate. Plates were incubated in moist atmosphere (>90RH) for 2-3 days at 25 ºC. The presence of fungitoxic activity of antagonists was noted by the formation of clear zone around the pathogen spore.

4.2.8.2 Volatile production assay

Fifty microliters of a suspension of each isolate (10^8 spores ml^{-1}) prepared as described in section (4. 2.7.1) was spread plated on 90-mm Petri dishes containing 20 ml aliquots of MEA. Another set of plates containing the same quantity of MEA was inoculated with *P. digitatum* (10^5 spores ml^{-1}) by centrally placing 10µl of spore suspension in the Petri dish. Once the surface dried, the lids were removed and the yeast plates were placed open ended on the fungal plates and sealed with parafilm. Plates were incubated at 25 ºC for 7-14 days and the fungal colony diameter was measured. The control consisted of MEA plates streaked with sterile distilled water instead of yeasts. Each treatment was replicated four times and the experiment was repeated once. Data obtained were statistically analysed.

4.2.8.3 Antagonist enzyme activity assay

Potential antagonists selected for their efficacy were evaluated *in vitro* for production of different enzymes using different synthetic media. Chitinolytic activity was determined according to the method described by Frandberg and Schnurer (1994). Specific media were used to determine the production of amylase, lipase, proteinase, and gelatinase (Norris and Ribbons, 1971). Each specific medium was autoclaved for 20 minutes at 121 ºC and culture plates were prepared for streak inoculation. In all cases, four replicate plates were inoculated for each isolate and the experiment was repeated three times.

4.2.8.4 *In vitro* yeast antagonist-pathogen interaction

The possible interaction of yeasts with the hyphae of *P. digitatum* was assessed using the method described by Chan and Tian (2005) with slight modifications. Petri dishes (90 mm in diameter) containing each 20 ml of MEA amended with 0.5% citrus juice (v/v) was used as an assay medium. Ten micro litres of the pathogen suspension (10^5 spores ml^{-1}) were placed on the centre of the plate. After 12 h of incubation at 25 ºC, 50µl of each yeast cell suspension (1 x 10^8 cells ml^{-1}) were placed at the margin of the fungal inoculum. The dual cultures were incubated at 25 ºC for 5-7 days and plates were evaluated for antagonist–pathogen direct interaction and data was recorded. Experiments were repeated twice.

4.2.8.5 Competition for nutrients and space: cylinder insert trials

The method described by Janisiewicz *et al*. (2000) was used to evaluate the effects of nutrient depletion by antagonists on the germination and growth of *P. digitatum* conidia. Potato Dextrose Broth (PDB) (Oxoid, Johannesburg) (20% or 40%) and orange peel extract (OPE) (0.5 and 5%) diluted in physiological solution was used as source of nutrients (Poppe *et al*.,

2003). Standard concentrations (1 x 10^8 CFU ml^{-1}) of each antagonist (MeJtw 10-2, TiL4-2, and TiL4-3) were dispensed in the wells of culture plates (0.6 ml per well). The pathogen, *P. digitatum* suspension in a physiological solution (10^5 spores ml^{-1}) were dispensed inside the cylinder inserts (0.4 ml per cylinder) and placed in the wells. Plates were incubated at 25 ºC for 24hr. After incubation, membranes from the cylinder inserts were removed accordingly, blotted with sterilized tissue paper, and cut with a sterilised scalpel. A quarter of a membrane was transferred to a glass slide, stained with lactophenol blue solution (Fluka, Switzerland) and mounted for light microscopy (Zeiss, Germany) to observe spore and/or conidia germination. The percentage of germinating conidia on the membranes was scored using four classes: 1= no germination, 2= germ tube <2x conidia size, 3= germ tube 2 to 4x conidia size; 4= germ tube >4x conidia size. Hundred conidia per treatment were counted (Janisiewicz *et al*., 2000). Each experiment was carried out twice with four wells per treatment.

After 24 h incubation and removal of the cylinders, two parallel experiments: turbidimetric growth measurements of antagonist populations in the wells were done at 640nm. A quarter of insert membrane was removed, blotted dry on sterilized surface, cut, transferred to MEA plates and incubated at 25 ºC for a period of two weeks. Plates were evaluated for pathogen-antagonist growth and percentage growth diameter of the pathogen and/or the antagonist was recorded and pathogen growth inhibition rate was statistically computed. The trial was also carried out without cylinders, in which the standard concentration of spore suspension was added directly to the well containing the standard concentration of the antagonist to study the direct interaction between the pathogen and antagonist. Evaluation was done by estimating the rate of spore germination in 100µl suspension using the germinating rate scale described above (Meziane *et al*., 2006).

4.2.9 Effect of antagonists culture filtrate on pathogen conidial germination

To determine the effect of antagonists (MeJtw 10-2, TiL 4-2 and TiL 4-3) on spore germination and germ tube elongation of *P. digitatum*, the method described by Castoria *et al*. (2001) and Spadaro *et al*. (2002) were used. The culture filtrate prepared as described in section 4.2.7.1 was used in this trial. Treatment combinations prepared as heated and not heated culture filtrates were used with or without PDB and/or the pathogen, *P. digitatum*. The application of cyclohexamide (0.1%) (Sigma, Germany) with or without *P. digitatum* spores and PDB with *P. digitatum* spores were regarded as a positive and negative control, respectively.

4.2.10 Minimum *in vitro* inhibitory antagonist concentration against *Penicillium digitatum*

To determine the optimal antagonist concentration at which effective inhibition of pathogens could be achieved, the checkerboard-type titration technique (Korsten, 1993) was used. Fresh Valencia fruits of more or less the same size and maturity were used as described in section 4.2.4. Squares (5 mm x 5mm), spaced 5 mm apart, were drawn with water proof ink in five vertical and five horizontal rows on one side of the fruit. Each square was prick-wounded to a depth of 3 mm using picture hook. As determined in the preliminary experiment, a range of antagonist and pathogen concentrations from 1 x 10^5 to 10^8 spores ml^{-1}, with the application of 40µl suspension were used accordingly. The higher concentration (1 x 10^8 cells ml^{-1}) of antagonists applied to the first vertical row from the left and the 10^7 concentration to the second, 10^6 to the third, 10^5 to the fourth row, respectively. The last vertical row served as a control and received 40µl of sterilized Ringer's solution. Fruit were left to air dry at room temperature for 12 h prior to the application of various concentrations of the pathogen, *P. digitatum.* Each square in the top horizontal row was pipette inoculated with 40µl of the 10^8 pathogen spore suspension, successively in the following rows. The last horizontal row (lower bottom) received 40µl of sterilized Ringer's solution only. Four fruits were used for each antagonist-pathogen treatment combination and the experiment was repeated three times. Inoculated fruits were kept in cardboard boxes and incubated at 25 ºC and >85% RH for seven days. Fruits were evaluated for disease development and data was statistically computed.

4.2.11 Colonization and attachment study using *scanning electron microscopy*

Surface colonization and attachment of antagonists at wound sites were determined according to Usall *et al*. (2001). A uniform 3 x 3 mm wound was made at four sites around the equator of fruit using a picture hook. Thirty micro litres of antagonists (C-20, C-28 and C-47) suspension at 1 x 10^8 cells ml^{-1} were pipetted into each wound site prior to the application of the pathogen. The same volume of *P. digitatum* suspension at 1 x 10^5 spores ml^{-1} was inoculated, separately into each wound. The separate application of antagonists and/ or the pathogen alone were regarded as a control. The experiments were done in triplicate. Inoculated fruits were either used immediately for scanning electron microscopy (SEM) evaluation or incubated. Fruits were placed at ambient temperature into 400mm x 300mm x 100mm plastic tray wrapped with a high density polyethylene sleeve to maintain high relative humidity (>85% RH). Samples taken by the time of inoculation, 6, 12, 24 and 48 hours were used. The peel tissue from wounds on the surface of citrus fruit was cut (4 x 4 mm) and fixed by immersion in 2.5% glutaraldehyde in 0.075 M phosphate buffer at pH 7.0 for 24 h at room

temperature. Samples were rinsed for 1 h (four or five changes) with 0.075 M sodium phosphate buffer (pH 7. 2) and dehydrated in a series of ethanol concentrations before critical point drying. Dried tissues were mounted on aluminium stubs, coated with gold-palladium, and observed at 6kv with a scanning electron microscope (Joel JSM 840, Tokyo, Japan).

4.2.12 *In vitro* integrated treatment of antagonists with plant extracts and commercial chemicals

Various strength of treatment combinations (10^{-1}, 10^{-2} and 10^{-3}) of fresh and six months old plant extract preparations preserved at 4 ºC and commercial chemicals [Prochloraz (AgrEvo, South Africa) (450 g L^{-1}), Guazatine (Rhone-Poulenc, France) (200 g L^{-1}), RSAF-1 (2-furaldehyde) (Illovo, South Africa) (7 %, v:v), Ultracure (Natural Crop Protection, South Africa) (210 g L^{-1}) Quatrokill (N, N Didecyl-N, N- dimethyl ammonium chloride) (Hyper Agrochemicals (Pty) Ltd., Johannesburg) (1.3 g L^{-1}), and Imazalil (Sanachem., Johannesburg) (1.35 g L^{-1})] were used in this trial. Yeast antagonist cultures grown overnight in NB and standardized to 10^6 spores/cells ml^{-1} using a haemacytometer (Janisiewicz *et al*., 2000) and preserved at 4 ºC in an ice box prior to use. One millilitre of antagonists suspension were transferred onto 14 ml molten MEA or STD-1 NA medium in a tube before rotating and poured into a Petri dish and mixing it gently by swirling before solidification. Four plugs (5mm diameter) were punched from actively growing cultures on agar plates to prepare agar wells. Each well was 30 mm from each other and two mm from the edge of the plate. Forty microliters of a plant extract and/ or industrial chemical were transferred into agar wells and plates were incubated at 25 ºC for 48-72 h. Sterilized distilled water alone was regarded as a negative control. The experiment was done in triplicate and repeated twice.

4.2.13 Statistical analyses

Fruit disease incidence data were analysed using analysis of variance (ANOVA) using Fisher's protected LSD test (P <0.05) and t- grouping with SAS (version 8.2) 2001. The inhibition rate of pathogen spore germination were analysed using the non-parameteric Kruskall Wallis test followed by Man-Whitney test at P<0.05 with SAS (version 8.2) 2001.

4.3 RESULTS

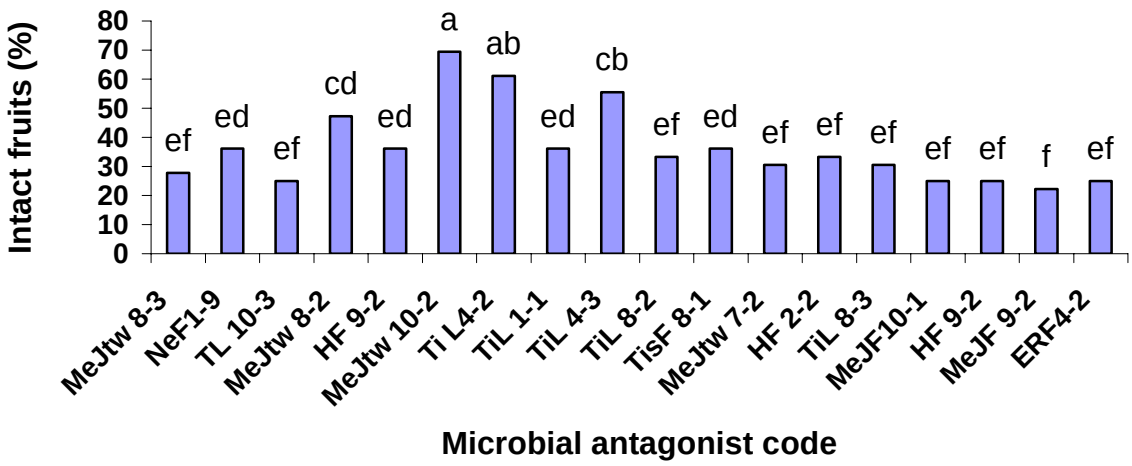
4.3.1 *In vivo* screening and selection of potential antagonists

Of the 242 microbial epiphytes preliminary isolated from leaf, fruit and twig washes of citrus, 18 potential antagonists were selected for further evaluation (Fig.4.1). Four strains: MeJtw 8-

2, MeJtw 10-2, TiL4-2, and TiL4-3 showed the highest rate of disease incidence reduction (between 50-75%).

4.3.2 *In vitro* antagonists screening assay for broad-spectrum activity

All antagonists showed some degree of antagonistic activity against all three tested pathogens (Fig. 4. 2, 3, 4). About 55.6% of the antagonists exhibited a growth inhibition rate between 30-95%. Six isolates [HF 8-2, MeJtw 10-2, TiL 4-2, TiL 4-3, TiL 1-1 and TiL 8-2] showed high growth inhibition (60-90%) against *P. digitatum* (Fig. 4. 2).

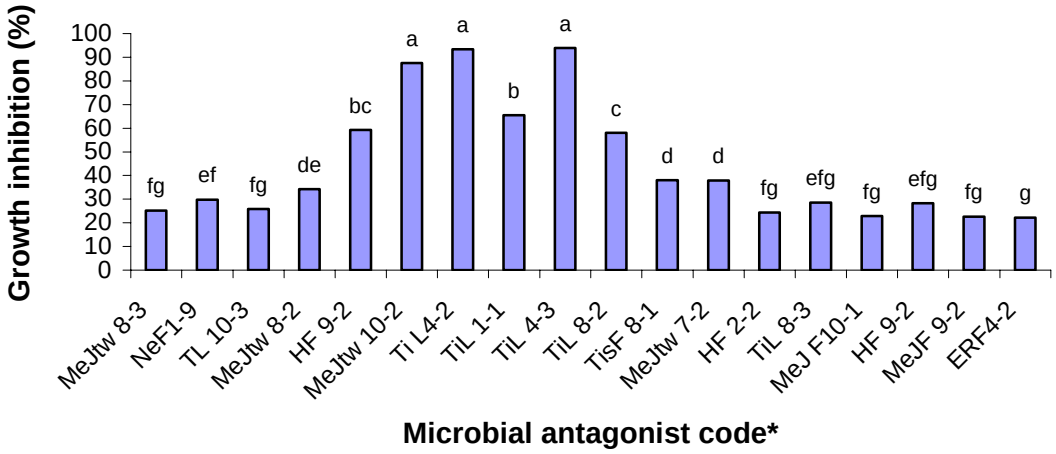


Legend: *= Bars represent percentage intact fruits. Bars with similar letters are not significantly different according to Fisher's protected LSD and t- grouping. Codes given to microbial antagonists referred as follows: MeJtw 8-3= MertiJeju twig sample isolate number 8-3, NeF1-8= NuraEra fruit sample isolate number 1-8, MeJtw 8-2= MertiJeju twig sample isolate number 8-2, HF8-2= Hursso fruit sample isolate number 8-2, MeJtw 10-2= MertiJeju twig sample isolate number 10-2, TiL4-2= Tibila leaf sample isolate number 4-2, TiL1-1= Tibila leaf sample isolate number 1-1, TiL4-3= Tibila leaf sample isolate number 4-3, TiL8-2= Tibila leaf sample isolate number 8-2, TisF8-1= Tisabalima leaf sample isolate number 8-1, MeJtw 7-2= MertiJeju twig sample isolate number 7-2, HF2-2= Hursso fruit sample isolate number 2-2, TiL8-3= Tibila leaf sample isolate number 8-3, MeJF10-1= MertiJeju fruit sample isolate number 10-1, HF8-2= Hursso fruit sample isolate number 8-2, MeJF8-2= MertiJeju fruit sample isolate number 8-2, and ERF4-2= Error Gota fruit sample isolate number 4-2.

Fig. 4.1. *In vivo* microbial antagonist screening assay on citrus fruit for control of *Penicillium digitatum*.

Isolates [MeJtw 10-2, TiL 4-2, TiL 4-3 and HF 2-2] against *G. candidum*, and isolates MeJtw 8-2, Mejtw10-2, TiL 4-2, TiL 4-3, MeJF10-1 and ERF 4-2 against *C. gloeosporioides* showed 30-35% growth inhibition, respectively. Three isolates with code MeJtw 10-2, TiL 4-2 and

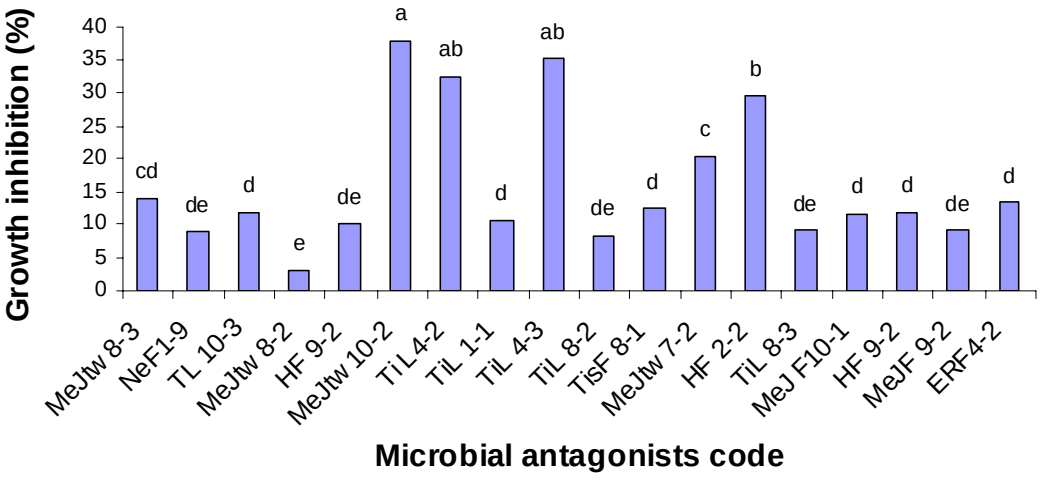
TiL 4-3 showed high growth inhibition rate with broad-spectrum activity against the three tested pathogens and were identified as yeasts.



Legend: *= Description refers to figure 1. Bars represent antagonists growth inhibition activity against *Penicillium digitatum*.

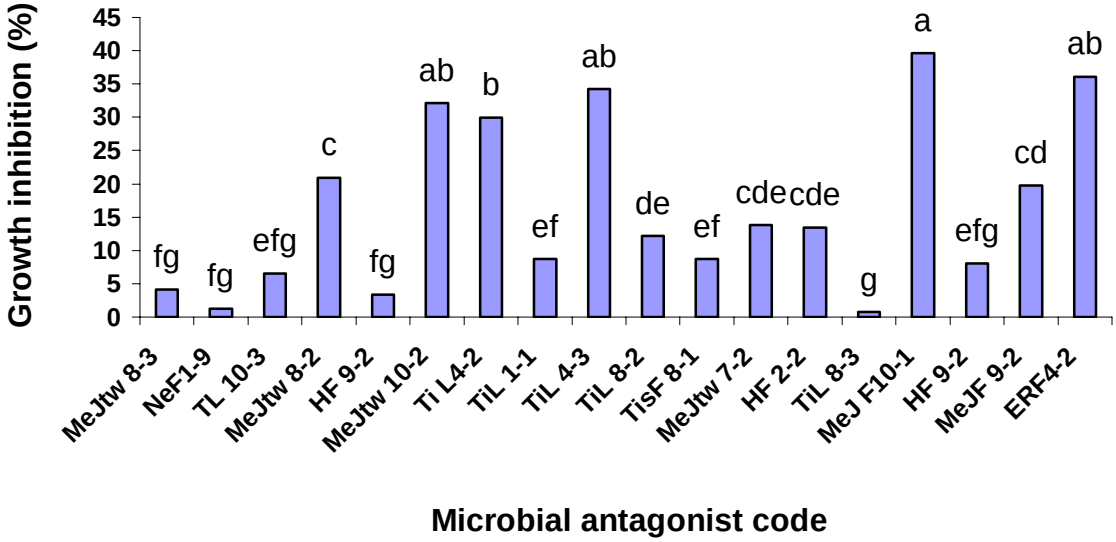
Fig. 4. 2. *In vitro* antagonist activity assay against *Penicillium digitatum*.

Further identification of yeasts with API® C identification system showed that isolate MeJtw 10-2 as *Cryptococcus laurentii*, isolate Til4-2 as *Candida sake* and isolate TiL4-3 as another strain of *Cryptococcus laurentii*.



Legend: *= Description refers to figure 1. Bars represent antagonists growth inhibition activity against *Geotrichum candidum* Link ex Pers.

Fig. 4. 3. *In vitro* antagonistic activity against *Geotrichum candidum*.

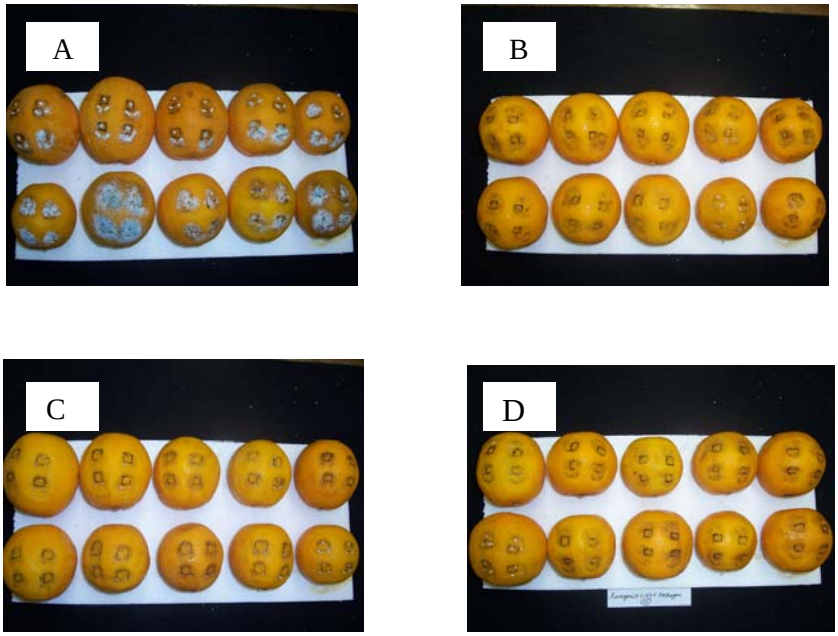


Legend: *= Description refers to figure 1. Bars represent antagonists growth inhibition activity against *Colletotrichum gloeosporioides* Penz.

Fig. 4. 4. *In vitro* antagonists assay against *Colletotrichum gloeosporioides*.

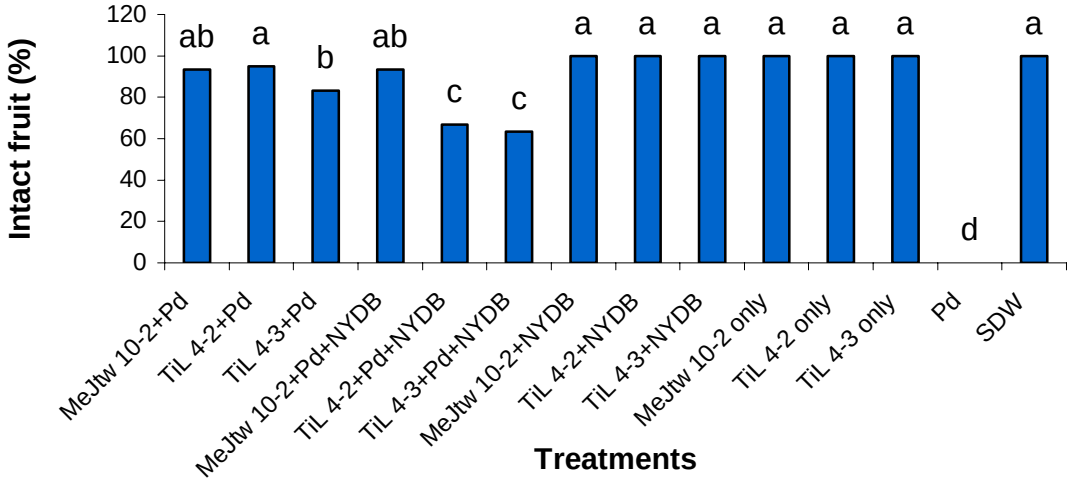
4.3.3 *In vivo* antagonists activity and disease incidence against *Penicillium digitatum*

From the *in vitro* experiments conducted in section 4.3.2, three antagonists [MeJtw 10-2, TiL 4-2 and TiL 4-3] were selected for their broad spectrum and overall effective antifungal activity. Fast and competitive colonisation of antagonists correlated directly with the high percentage of intact fruit. Wound application of antagonists against *P. digitatum* showed a significant ($P < 0.05$) rate of disease incidence reduction by (60-90%) on fruits incubated at 7 ºC for 30 days (Fig. 4. 6). A higher rate of disease incidence reduction was observed by antagonist MeJtw 10-2 (*C. laurentii*) (>85%). Antagonist TiL4-3 (strain of *C. laurentii*) and TiL4-2 (*C. sake*) showed 65-85% disease incidence reduction unlike antagonist MeJtw10-2 (Fig. 4. 6). No significant (P <0.05) changes were observed in the reduction of disease incidence by the addition of NYDB to antagonist MeJtw10-2 (*C. laurentii*). The rate of fruit infection increased significantly (P <0.05) with the addition of NYDB to a treatment combination with TiL4-2 (*C. sake*) and/ or TiL4-3 (*C. laurentii*) (Fig. 4. 5 and 6). Fruits remained 100% intact with antagonist treatments alone and with or without a NYDB combination (Fig. 4. 5 and 6).



Legend: Treatment codes given are described as follows: A= *Penicillium digitatum* infection (control), B= MeJtw 10-2 + *Penicillium digitatum*, C= TiL 4-2 + *Penicillium digitatum*, D= TiL 4-3 + *Penicillium digitatum*.

Fig. 4. 5. Isolates: MeJtw 10-2 (*Cryptococcus laurentii*), TiL 4-2 (*Candida sake*) and TiL4-3 (*Cryptococcus laurentii*) activity *in vivo* against *Penicillium digitatum*.

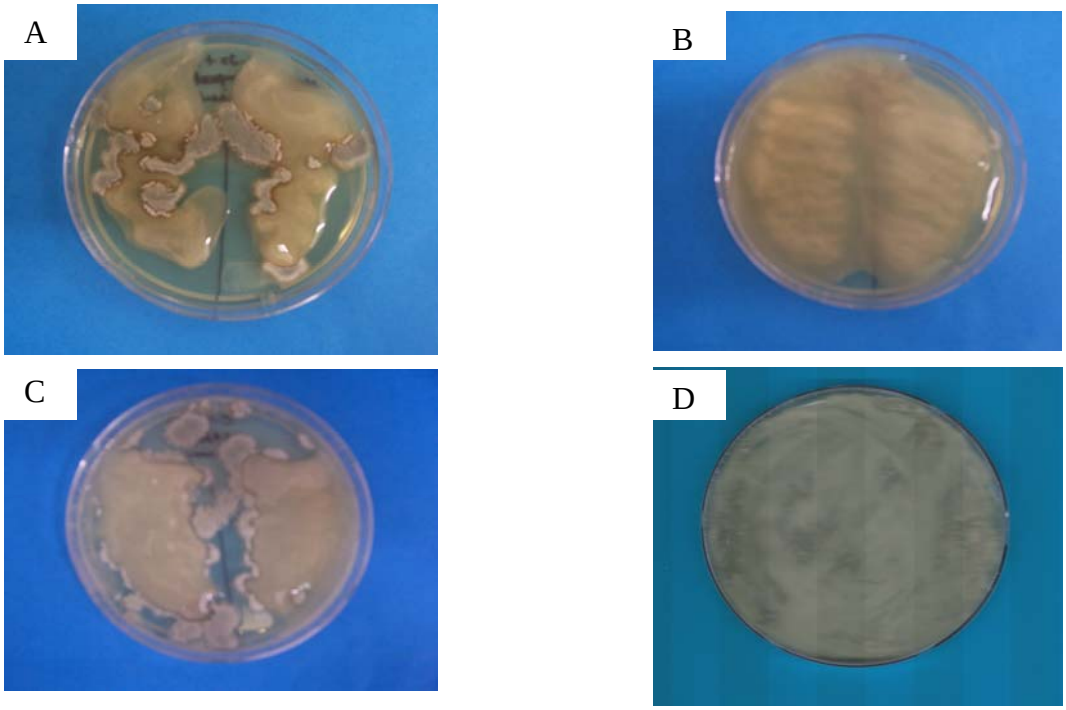


Legend: Bars with the same letter are not significantly different (P <0.05) according to Fisher's LSD test and t- grouping. Designated codes are referred as follows: MeJtw 10-2 = *Cryptococcus laurentii*, TiL 4-2 = *Candida sake*, TiL 4-3 = *C. laurentii*, Pd = *Penicillium digitatum* and SDW = Sterilised distilled water.

Fig. 4.6. *In vivo* yeast antagonists activity against *Penicillium digitatum*.

4.3.4 Antibacterial and antifungal assay

In the dual culture assay, all potential antagonists had no antibiosis activity against *P. digitatum*. Instead, rapid surface colonization activity on medium was noticed. (Fig. 4. 7).



Legend: Pictures from A-D depict antagonists colonization effect over the growth of *Penicillium digitatum*. A= activity of antagonist MeJtw (*Cryptococcus laurentii*) (80% efficacy) B= activity of antagonist TiL4-2 (*Candida sake*) (100% efficacy), C = activity of antagonist TiL4-3 (C*ryptococcus laurentii*) (85% efficacy) against the growth of *Penicillium digitatum* and D= growth of *Penicillium digitatum* alone (control).

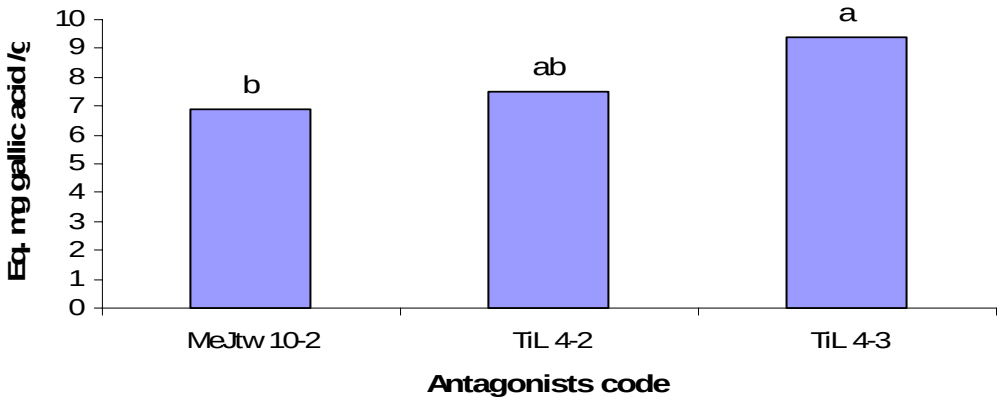
Fig. 4. 7. *In vitro* yeast antagonists activity on MEA plate against *Penicillium digitatum*.

4.3.5 Antagonist volatile production assay

None of the three potential antagonists produced volatiles (P <0.05) as compared to the control.

4.3.6 Antagonist total phenolic content determination

The antagonists exhibited production of some phenolic compounds between 6 – 10 equivalent mg Gallic acid/g dry weight (Fig. 4. 8). A strain of *C. laurentii* (TiL4-3) contained relatively higher amounts of phenolic compounds (Fig. 4. 9).



Legend: Bars with the same letter are not significantly different ($P < 0.05$) according to Fisher's protected LSD test and t- grouping.

Fig. 4. 10. Antagonists total phenolics content determination.

4.3.7 Antagonists enzyme activity assay

Among the potential antagonists tested, one isolate *C. laurentii* (MeJtw10-2) showed some degree of extra cellular amylase, lipase and proteinase activity unlike *C. sake* (Til4-2) and *C. laurentii* (TiL4-3)] isolates (Table 4.1).

Table 4.1 Enzymatic activities of three yeasts, *in vitro*

Yeast antagonist code	Chitinolytic activity	Extracellular amylase activity	Lipase activity	Proteinase activity	Gelatinase activity
MeJtw 10-2	-	+	+	+	-
TiL 4-2	-	-	-	-	-
TiL 4-3	-	-	-	-	-

Legend: + = activity present
- = no activity

4.3.8 *In vitro* competition for nutrients and space

Conidia of *P. digitatum* germinated at various concentrations of MEB and OPE within the first 24 h (Table 4.2a). At the higher concentration of the OPE, more conidia germinated. On the other hand, almost no conidia germinated in Ringer's solution (Table 4.2a). The antagonists prevented germination of conidia in all treatment combinations with 20 and 40% of MEB and 0.5 and 5% of OPE. All antagonists greatly reduced conidia germination in the higher OPE concentrations (Table 4.2a). Antagonists MeJtw10-2 (*C. laurentii*) and Til4-2 (*C.*

sake) showed a higher reduction rate of conidia germination compared to isolate Til4-3 (*C. laurentii*).

Cylinder insert membranes that moved from the original treatment to new wells containing the corresponding growth medium but without antagonists resulted in germination of all conidia in 5% OPE and the majority of the conidia in 0.5% OPE and 20 and 40% of MEB in the second 24 h incubation period (Table 4.2b).

Table 4. 2a Percentage germination of *Penicillium digitatum* conidia on Polytetra-fluoroethylene membranes

Treatment	Germinating rating scale*			
	1	2	3	4
Control:				
Ringer 's solution	98^{b}	2^{k}	0^{k}	0^{g}
20% MEB	19^{k}	21^{c}	23^{b}	37^{d}
40% MEB	10^{l}	5^{h}	27^{a}	58^{c}
0.5% orange peel extract (OPE)	9^{m}	11^{f}	17^{c}	63^{b}
5% OPE	0^{n}	3^{j}	9^{d}	88^{a}
With antagonists:				
Ringer's solution + antagonist MeJtw 10-2	100^{a}	0^{m}	0^{k}	0^{g}
20% MEB + antagonist MeJtw 10-2	95^{e}	5^{h}	0^{k}	0^{g}
40% MEB + antagonist MeJtw 10-2	96^{d}	4^{i}	0^{k}	0^{g}
0.5% OPE + antagonist MeJtw 10-2	91^{f}	7^{g}	2^{i}	0^{g}
5% OPE + antagonist MeJtw 10-2	98^{b}	1^{l}	1^{j}	0^{g}
Ringer's solution + antagonist TiL 4-2	100^{a}	0^{m}	0^{k}	0^{g}
20% MEB + antagonist TiL 4-2	97^{c}	3^{j}	0^{k}	0^{g}
40% MEB + antagonist TiL 4-2	98^{b}	2^{k}	0^{k}	0^{g}
0.5% OPE + antagonist TiL 4-2	98^{b}	2^{k}	0^{k}	0^{g}
5% OPE + antagonist TiL 4-2	97^{c}	3^{j}	0^{k}	0^{g}
Ringer's solution + antagonist TiL 4-3	100^{a}	0^{m}	0^{k}	0^{g}
20% MEB + antagonist TiL 4-3	68^{i}	27^{b}	4^{g}	1^{f}
40% MEB + antagonist TiL 4-3	78^{g}	16^{e}	3^{h}	3^{e}
0.5% OPE+ antagonist TiL 4-3	65^{j}	29^{a}	6^{e}	0^{g}
5% OPE + antagonist TiL 4-3	76^{h}	18^{d}	5^{f}	1^{f}

Legend: *Germinating rating scale: 1= no germination; 2= germ tube <2x conidia size; 3= germ tube 2 to 4x conidia size; 4= germ tube >4x conidia size: 100 conidia per treatment were counted. Code given to antagonists referred as follows: MeJtw 10-2 (*Cryptococcus laurentii*) = Merti-Jeju farm twig sample 10-2, TiL 4-2 (*Candida sake*) = Tibila farm leaf sample 4-2, TiL 4-3 (*Cryptococcus laurentii*) = Tibila farm leaf sample 4-3. Means with the same letter in the column are note significantly different (P <0.05) according to Duncan's Multiple Range test and grouping.

Table 4. 2b Percentage germination of *Penicillium digitatum* conidia on Polytetrafluoroethylene membranes in cylinders

Original treatment	Germinating rating scale*			
	1	2	3	4
Control:				
Ringer 's solution	96^{a}	2^{k}	2^{m}	0^{p}
20% MEB	0^{m}	4^{j}	14^{h}	82^{d}
40% MEB	0^{m}	0^{l}	11^{j}	88^{c}
0.5% OPE	0^{m}	0^{l}	7^{l}	93^{b}
5% OPE	0^{m}	0^{l}	0^{n}	100^{a}
With antagonists:				
Ringer's solution + antagonist MeJtw 10-2	64^{c}	18^{e}	12^{i}	6^{m}
20% MEB + antagonist MeJtw 10-2	18^{g}	23^{a}	30^{d}	29^{k}
40% MEB + antagonist MeJtw 10-2	12^{j}	21^{b}	31^{c}	36^{g}
0.5% OPE + antagonist MeJtw 10-2	19^{f}	19^{d}	24^{f}	38^{f}
5% OPE+ antagonist MeJtw 10-2	0^{m}	0^{l}	0^{n}	100^{a}
Ringer's solution + antagonist TiL 4-2	76^{b}	12^{h}	8^{k}	4^{n}
20% MEB + antagonist TiL 4-2	11^{k}	19^{d}	37^{a}	33^{i}
40% MEB + antagonist TiL 4-2	13^{i}	23^{a}	26^{e}	38^{f}
0.5% OPE + antagonist TiL 4-2	9^{l}	11^{i}	34^{b}	46^{e}
5% OPE+ antagonist TiL 4-2	0^{m}	0^{l}	0^{n}	100^{a}
Ringer's solution + antagonist TiL 4-3	54^{d}	20^{c}	23^{g}	3^{o}
20% MEB + antagonist TiL 4-3	31^{e}	17^{f}	26^{e}	26^{l}
40% MEB + antagonist TiL 4-3	14^{h}	21^{b}	24^{f}	31^{j}
0.5% OPE + antagonist TiL 4-3	18^{g}	16^{g}	31^{c}	35^{h}
5% OPE + antagonist TiL 4-3	0^{m}	0^{l}	0^{n}	100^{a}

Legend: For germinating rating scale refer to table 4.2a above. Further analysis of antagonist impact on *Penicillium digitatum* conidia germination was assessed by inserting the cylinder membranes to the new wells containing the same growth medium (Ringer's solution, 20% of MEB, 40% of MEB, 0.5% OPE and 5% of OPE) as used for original treatment to each antagonist. But, this time, the medium used was without antagonists. Plates were incubated for additional 24 h at 25 ºC. Means with the same letter in the column are note significantly different (P <0.05) according to Duncan's Multiple Range test and grouping.

4.3.9 Turbidimetric measurements of antagonists growth in different growth mediums

All three antagonists showed a higher rate of population growth in 5% OPE in the first 24 and second 48 h of incubation (Fig. 4. 10, 11, 12). Antagonist Til4-2 (*C. sake*) exhibited a higher rate of population growth than the two *C. laurentii* strains (MeJtw10-2 and TiL4-3) (Fig. 4.10, 11, 12).

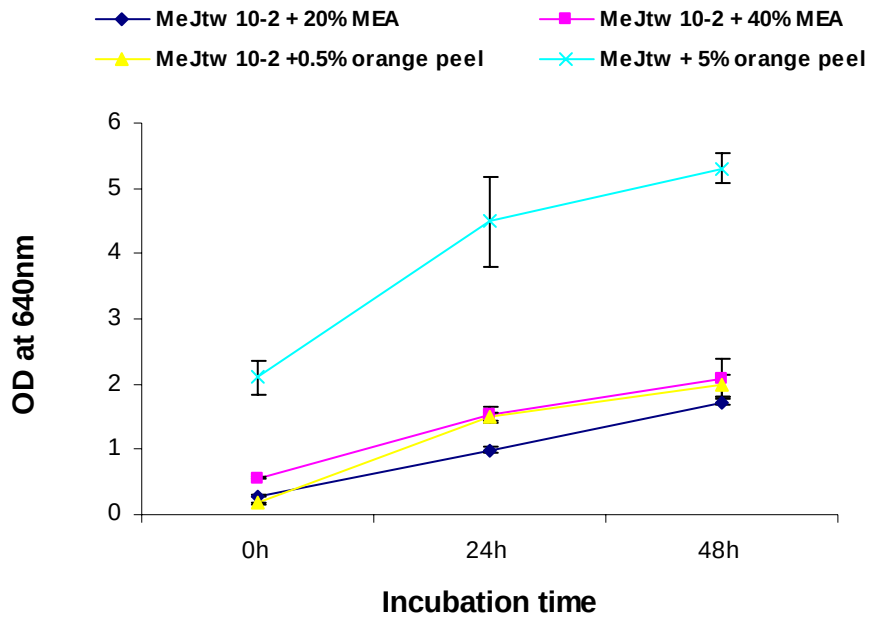


Fig. 4. 10. Turbidimetric measurement of antagonist MeJtw 10-2 (*Cryptococcus laurentii*) growth in different culture growth media.

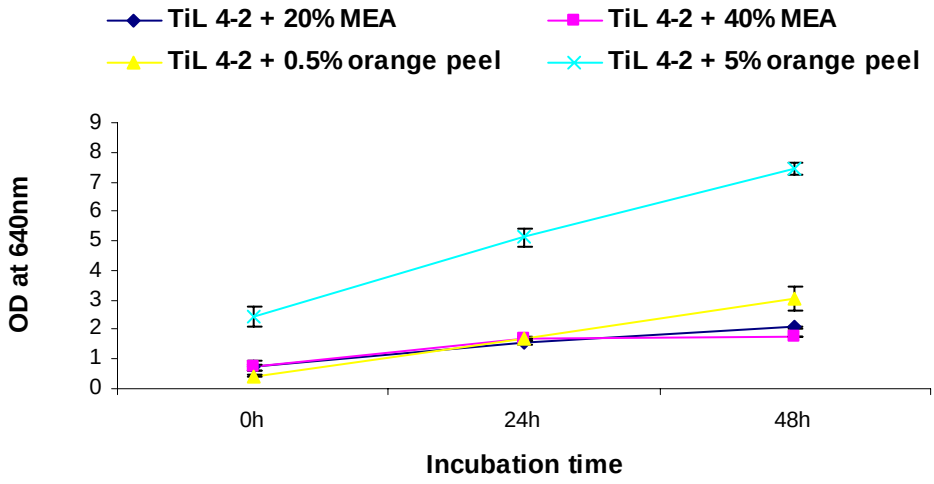


Fig. 4. 11. Turbidimetric measurement of antagonist TiL 4-2 (*Candida sake*) growth in different culture growth media.

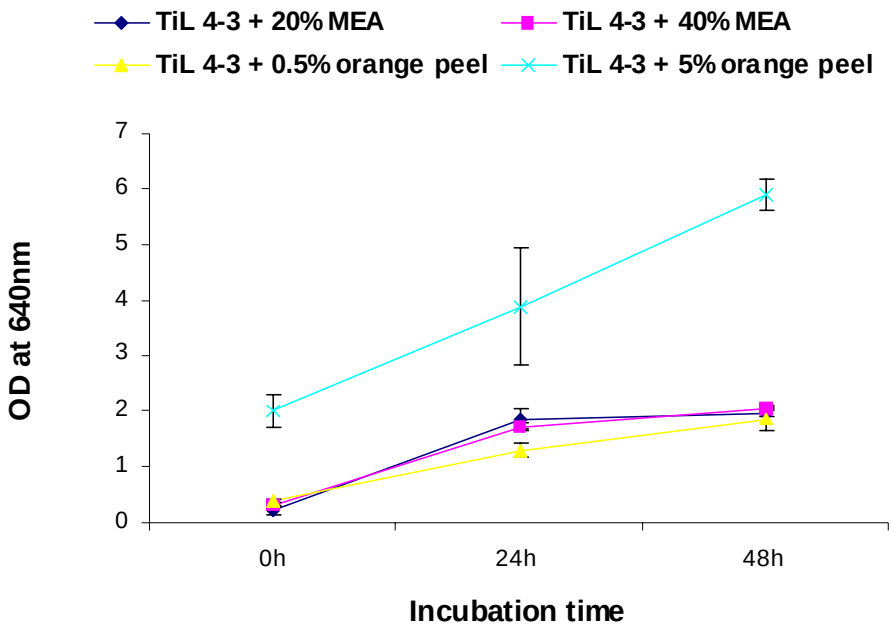


Fig. 4. 12. Turbidimetric measurement of antagonist TiL 4-3 (*Cryptococcus laurentii*) growth in different culture growth media.

4.3.10. *In vitro* study of antagonist-pathogen interaction using micro well plates

Conidia of *P. digitatum* germinated in all culture broths except in Ringer's solution when incubated for 24 and 48 h at 25 ºC. All three antagonists [MeJtw10-2 (*C. laurentii*), TiL4-2 (*C. sake*) and TiL 4-3 (*C. laurentii*) greatly decreased *P. digitatum* conidia germination for the first 24 h incubation, where the highest inhibition was exhibited by antagonists TiL4-2 (Table 4.3a). Almost all conidia of *P. digitatum* germinated in the following 24 h cycle at 25 ºC. The antagonist prevented conidia germination between 48-82% with the highest rate recorded for the antagonist TiL 4-2 (*C. sake*) (82%) and the least by antagonist TiL4-3 (*C. laurentii*) (Table 4. 3b) (48%).

Table 4. 3a Percent germination of *Penicillium digitatum* conidia in micro wells direct interaction with antagonists exposed for 24 h incubation at 25 ºC

Treatment	Germinating rating scale*			
	1	2	3	4
Control:				
Ringer ‘s solution	96^{e}	4^{i}	2^{e}	0^{e}
20% MEB	9^{l}	16^{a}	28^{a}	47^{d}
40% MEB	9^{l}	11^{e}	26^{b}	54^{c}
0.5% orange peel extract (OPE)	6^{m}	12^{d}	17^{c}	65^{b}
5% OPE	0^{n}	8^{f}	11^{d}	81^{a}
With antagonists:				
Ringer’s solution + antagonist MeJtw 10-2	100^{a}	0^{m}	0^{f}	0^{e}
20% MEB + antagonist MeJtw 10-2	92^{h}	8^{f}	0^{f}	0^{e}
40% MEB + antagonist MeJtw 10-2	94^{f}	6^{h}	0^{f}	0^{e}
0.5% OPE + antagonist MeJtw 10-2	96^{e}	4^{i}	0^{f}	0^{e}
5% OPE + antagonist MeJtw 10-2	97^{d}	3^{j}	0^{f}	0^{e}
Ringer’s solution + antagonist TiL 4-2	100^{a}	0^{m}	0^{f}	0^{e}
20% MEB + antagonist TiL 4-2	97^{d}	3^{j}	0^{f}	0^{e}
40% MEB + antagonist TiL 4-2	98^{c}	2^{k}	0^{f}	0^{e}
0.5% OPE + antagonist TiL 4-2	98^{c}	2^{k}	0^{f}	0^{e}
5% OPE + antagonist TiL 4-2	99^{b}	1^{l}	0^{f}	0^{e}
Ringer’s solution + antagonist TiL 4-3	100^{a}	0^{m}	0^{f}	0^{e}
20% MEB + antagonist TiL 4-3	86^{k}	14^{b}	0^{f}	0^{e}
40% MEB + antagonist TiL 4-3	89^{i}	11^{e}	0^{f}	0^{e}
0.5% OPE+ antagonist TiL 4-3	87^{j}	13^{c}	0^{f}	0^{e}
5% OPE + antagonist TiL 4-3	93^{g}	7^{g}	0^{f}	0^{e}

Legend: *Germinating rating scale: 1= no germination; 2= germ tube <2x conidia size; 3= germ tube 2 to 4x conidia size; 4= germ tube >4x conidia size: 100 conidia per treatment were counted. For code given to antagonists refer to table 4.2a legend. Means with the same letter in the column are note significantly different (P <0.05) according to Duncan’s Multiple Range test and grouping.

Table 4. 3b Percent germination of *Penicillium digitatum* conidia in micro wells direct interaction with antagonists exposed for additional 24 h incubation at 25 ºC

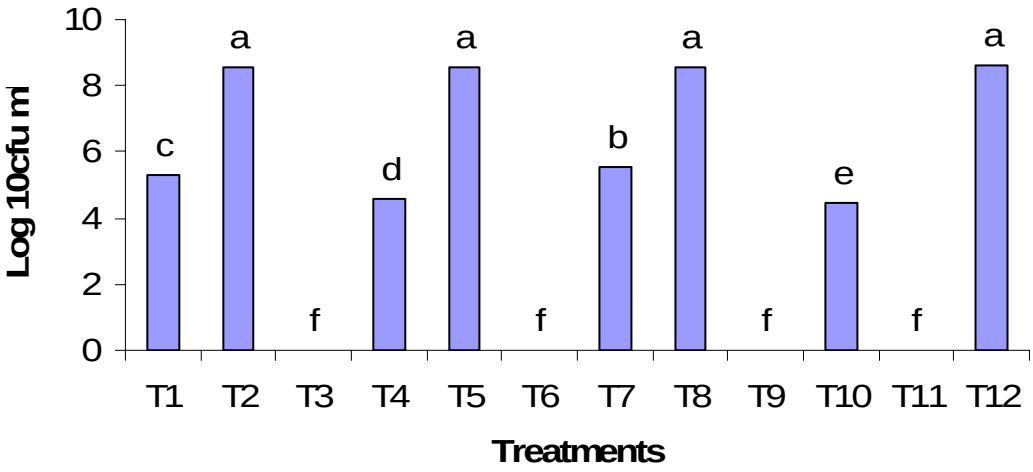
Original treatment	Germinating rating scale*			
	1	2	3	4
Control:				
Ringer 's solution	94^{b}	2^{k}	4^{m}	0^{m}
20% MEB	1^{m}	3^{j}	14^{f}	82^{d}
40% MEB	0^{n}	3^{j}	4^{m}	93^{c}
0.5% orange peel extract (OPE)	0^{n}	2^{k}	3^{n}	95^{b}
5% OPE	0^{n}	0^{l}	0^{o}	100^{a}
With antagonists:				
Ringer's solution + antagonist MeJtw 10-2	100^{a}	0^{l}	0^{o}	0^{m}
20% MEB + antagonist MeJtw 10-2	48^{l}	22^{a}	18^{c}	12^{g}
40% MEB + antagonist MeJtw 10-2	51^{j}	18^{d}	21^{a}	10^{i}
0.5% OPE + antagonist MeJtw 10-2	55^{i}	19^{c}	15^{e}	11^{h}
5% OPE + antagonist MeJtw 10-2	68^{f}	18^{d}	8^{j}	6^{k}
Ringer's solution + antagonist TiL 4-2	100^{a}	0^{l}	0^{o}	0^{m}
20% MEB + antagonist TiL 4-2	68^{f}	15^{f}	11^{h}	6^{k}
40% MEB + antagonist TiL 4-2	72^{e}	13^{g}	9^{i}	6^{k}
0.5% OPE + antagonist TiL 4-2	79^{d}	9^{h}	7^{k}	5^{l}
5% OPE + antagonist TiL 4-2	82^{c}	7^{i}	6^{l}	5^{l}
Ringer's solution + antagonist TiL 4-3	100^{a}	0^{l}	0^{o}	0^{m}
20% MEB + antagonist TiL 4-3	49^{k}	18^{d}	19^{b}	14^{f}
40% MEB + antagonist TiL 4-3	51^{j}	21^{b}	17^{d}	11^{h}
0.5% OPE+ antagonist TiL 4-3	56^{h}	16^{e}	12^{g}	16^{e}
5% OPE + antagonist TiL 4-3	61^{g}	19^{c}	11^{h}	9^{j}

Legend:*Germinating rating scale and other descriptions, see table 4. 2a legend. Means with the same letter in the column are not significantly different (P <0.05) according to Duncan's Multiple Range test and grouping.

4.3.11 Effects of the culture filtrate against spore germination of *Penicillium digitatum*

All yeast antagonists: MeJtw10-2 (*C. laurentii*), TiL4-2 (*C. sake*) and TiL4-3 (*C. laurentii*) culture suspensions amended with PDB showed significant (P <0.05) inhibition against *P. digitatum* spore germination (Fig. 4.13). A treatment combination with antagonist TiL4-2

showed 46.6% inhibition followed by MeJtw10-2 (38.3%) and TiL4-3 (35.8%) (Fig. 4.13). No inhibition was observed with autoclaved spore culture suspensions.



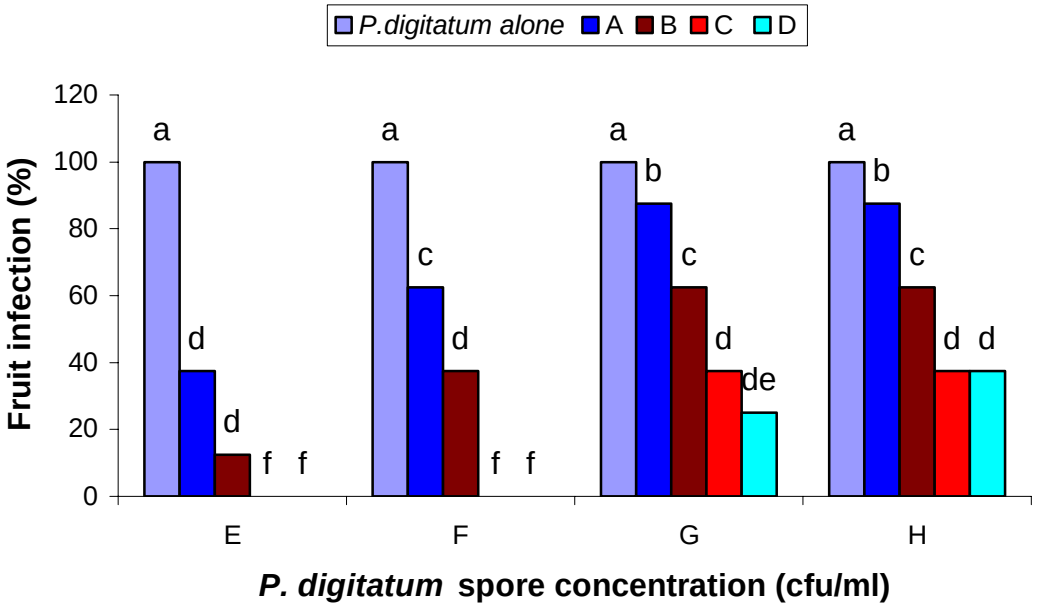
Legend: Mean values are expressed in Bars. Bars designated with the same letter are not significantly different according to Fisher's LSD test (P <0.05) and t-grouping. Treatments are described as follows: T1= Antagonist MeJtw 10-2 + PDB (Potato Dextrose Broth) (Oxoid, Johannesburg) + *P. digitatum* spore, T2= Antagonist MeJtw 10-2 boiled culture + *P. digitatum* spore, T3= Antagonist MeJtw 10-2 boiled culture alone, T4 = Antagonist TiL 4-2 + PDB (Oxoid) + *P. digitatum* spore, T5= Antagonist TiL 4-2 boiled culture + *P. digitatum* spore, T6= Antagonist TiL 4-2 boiled culture alone, T7= Antagonist TiL 4-3 + PDB (Oxoid) + *P. digitatum* spore, T8= Antagonist TiL 4-3 boiled culture + *P. digitatum* spore, T9= Antagonist TiL 4-3 boiled culture alone, T10= Cyclohexamide (Sigma, Germany) + *P. digitatum* spore, T11= Cyclohexamide alone and T12= *P. digitatum* alone.

Fig. 4. 13. *In vitro* activity of antagonists against *Penicillium digitatum* spore germination.

4.3.12 Minimum inhibitory concentration of antagonists against *Penicillium digitatum*

All spore concentrations of antagonists [10^5, 10^6, $10^7$and 10^8] significantly (P <0.05) reduced disease incidence as compared to the control (Fig. 4. 14, 15, 16). A high rate of disease incidence reduction was observed and an increased application of antagonist spore suspension was found with lower concentrations (10^5 and 10^6) of the pathogen, *P. digitatum*. Antagonist MeJtw10-2 (*C. laurentii*) showed complete control of Penicillium infection when applied at 10^7 and 10^8 spore concentrations challenged to 10^5 and 10^6 spore concentration of the pathogen. All spore concentrations of antagonist TiL4-2 (*C. sake*) [10^5, 10^6, $10^7$and 10^8] showed complete control of Penicillium spore when challenged at lower (10^5) concentration.

Antagonists at 10^8 concentration showed complete control of a pathogen challenged at 10^6 concentration (Fig. 4. 15). The antagonist TiL4-3 (*C. laurentii*) on the other hand showed complete control of *P. digitatum* fruit decay when applied at higher concentration (10^7 and 10^8) challenged against lower concentrations of the pathogen spore suspension (10^5 and 10^6), respectively.

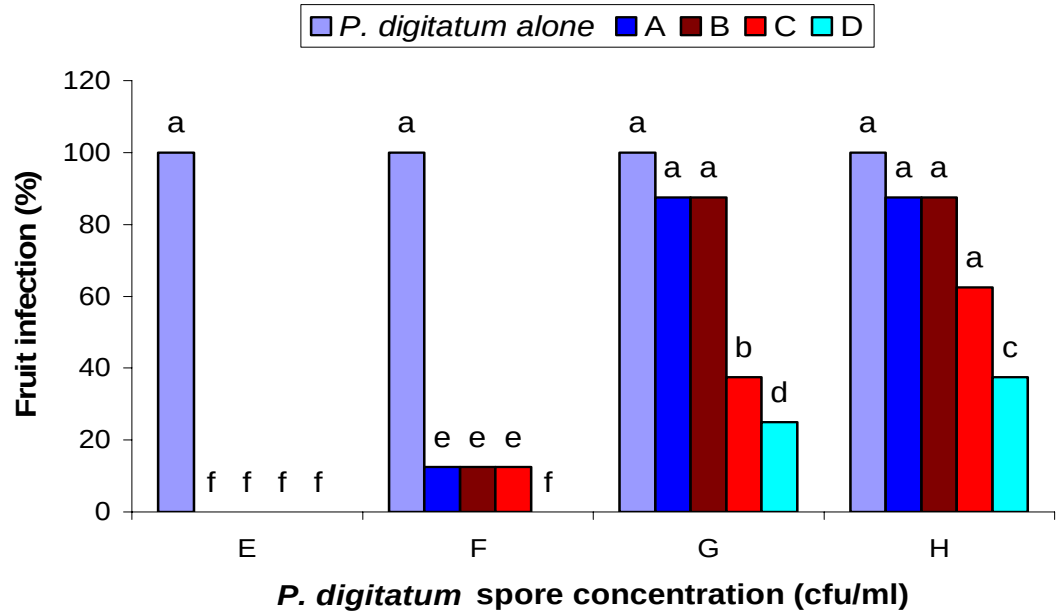


Legend: Bars represent mean of experiments. Means with the same letter are not significantly different at $P < 0.05$ using Fisher's LSD t- grouping.

Bars with different colour referred percentage intact fruit and antagonist concentrations used in the test, accordingly:
A= 1 x 10^5, B= 1 x 10^6, C= 1 x 10^7, and D= 1 x 10^8 spores ml $^{-1}$.

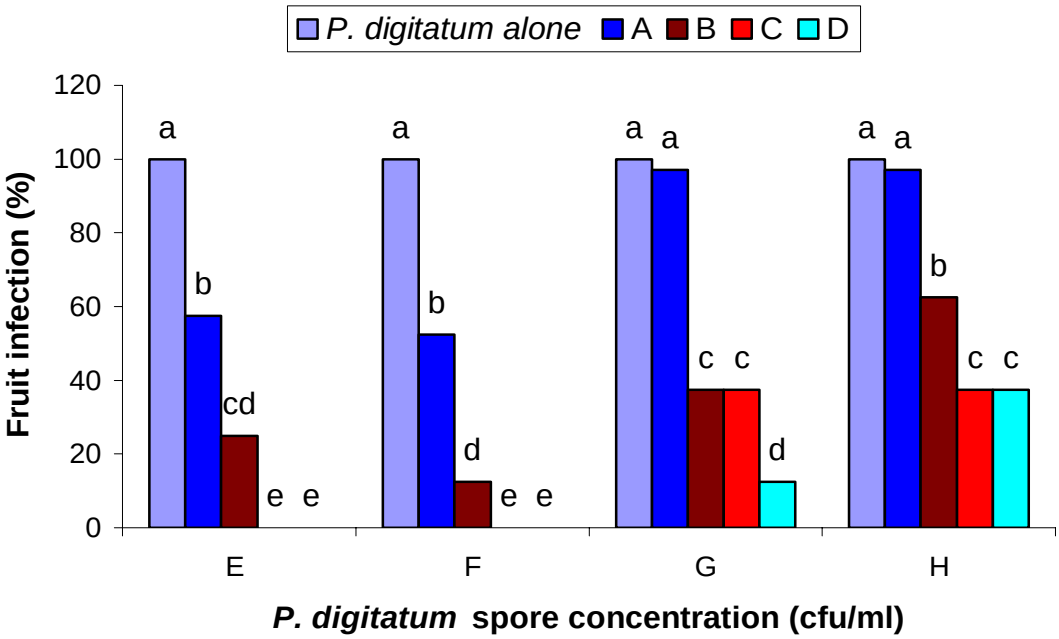
*Capital alphabets on the horizontal line of the figure referred to *P. digitatum* spore concentrations:
E= 1 x 10^5, F= 1 x 10^6, G= 1 x 10^7, and H= 1 x 10^8 spores ml^{-1}. In the preliminary experiment, all antagonists at 10^4concentration were found ineffective to the range of pathogen concentrations tested and were therefore excluded for simplicity.

Fig. 4.14. *In vivo* efficacy of MeJtw 10-2 (*Cryptococcus laurentii*) minimum inhibitory concentrations against *Penicillium digitatum* spores growth .



Legend: Bars with the same letter are not significantly different according to Fisher's LSD test (P <0.05) and t- grouping. For other letter descriptions refer the figure 4. 14.

Fig. 4. 15. *In vivo* antagonist (TiL 4-2) minimum inhibitory concentrations determination against spore germination of *Penicillium digitatum* on citrus fruit.



Legend: Means with the same small alphabet letters are not significantly different at P <0.05 using Fisher's LSD t- grouping. For the rest of letters descriptions refer the above figure 4.14.

Fig. 4. 16. *In vivo* antagonist TiL 4-3 (*C. laurentii*) minimum inhibitory concentrations determination against *Penicillium digitatum* spore growth on citrus fruit.

4.3.13 Wound site colonization and attachment of antagonists

Under SEM observations of orange fruit wounds inoculated with antagonists and the pathogen *P. digitatum*, a significant reduction of conidia germination and different mechanisms of wound healing could be seen (Fig. 4-17). Mode of actions that involved secretion of extracellular fluid and sticking of the pathogen (Fig. 4. 17A-F) were the major activities identified. All antagonists produce extracellular fluid when applied alone. The application of *P. digitatum* alone showed higher degree of conidia germination Fig. 4.17 E-G), which later changed into germ tube elongation and hyphae growth (Fig. 4.17H and I).

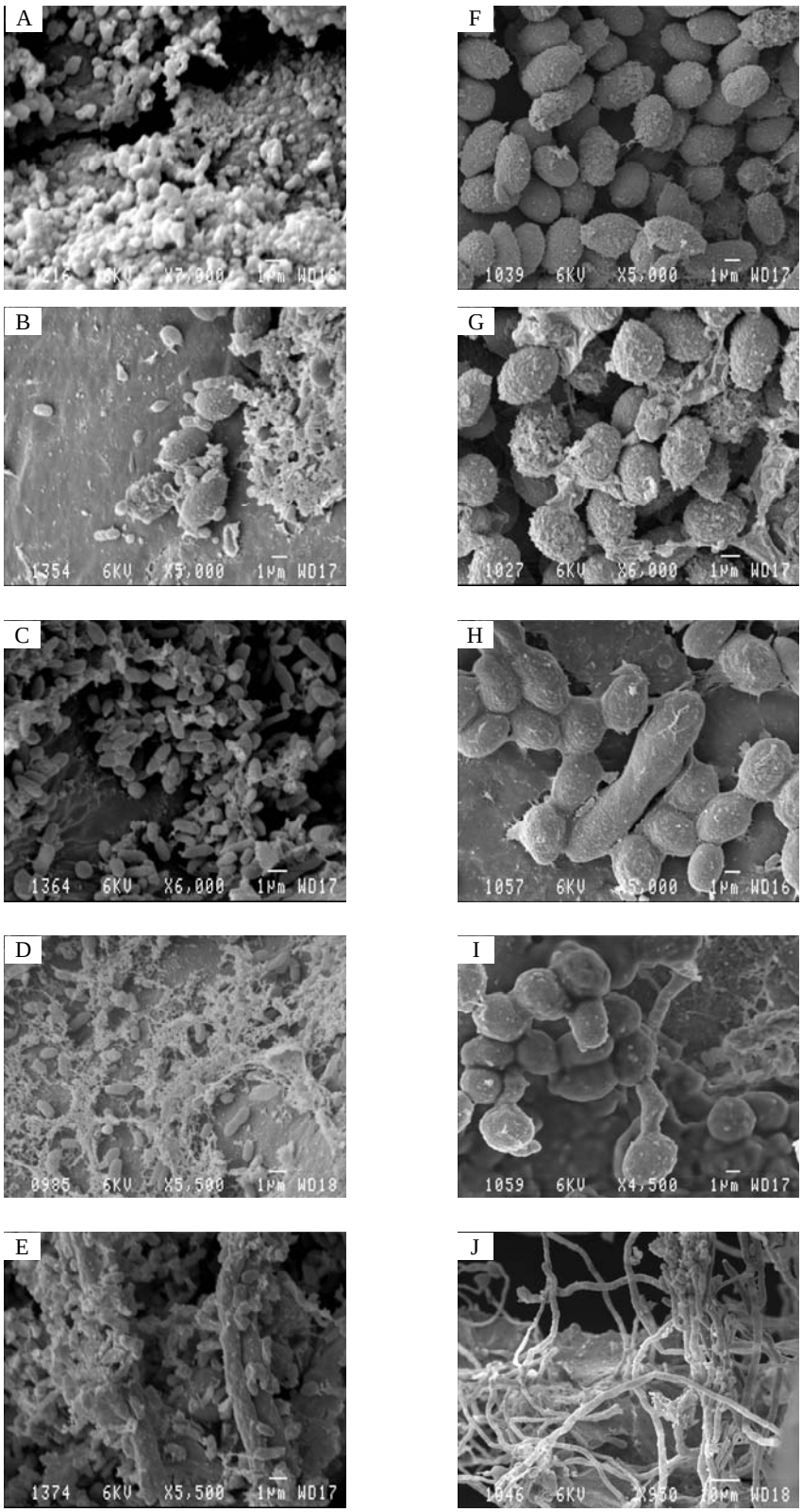


Fig. 4. 19. Antagonists mode of action against *Penicillium digitatum* on fruit wound viewed through scanning electron microscope. Images from A-F describe antagonists (MeJtw 10-2, TiL 4-2 and TiL4-3) activity at 0, 6, 12, 24 and 48h attachment against *Penicillium digitatum* at the wound site, respectively. Images from G-J refer to the development of infection by *P. digitatum* at the wound site at 0, 6, 12, 24 and 48 h of attachment.

4.3.14 *In vitro* integrated treatment between yeast antagonists, plant extracts and commercial fungicide

All antagonists showed higher rate of growth with fresh and old preparations of plant extracts at various treatment concentrations (Table 4.4). Although they exhibited high rate of recovery (data not shown here), maximum rate of antagonist growth inhibition (67%) was observed with the application of Procloraz (10^{-1} dilution) to antagonist TiL 4-2 and Guazatine (10^{-1} dilution) to antagonist MeJtw 10-2.

Table 4. 4 Integrated treatment *in vitro* between yeast antagonists, plant extracts and commercial fungicide

Treatments	Dilution	Yeast antagonists*		
		MeJtw10-2	TiL4-2	TiL4-3
Extract A fresh preparation	Control	0	0	0
	10^{-1}	++	++	++
	10^{-2}	0	+	0
	10^{-3}	0	0	0
Extract A old preparation	Control	0	0	0
	10^{-1}	++	++	++
	10^{-2}	0	0	0
	10^{-3}	0	0	0
Extract B fresh	Control	0	0	0
	10^{-1}	+	++	++
	10^{-2}	0	0	0
	10^{-3}	0	0	0
Extract B old	Control	0	0	0
	10^{-1}	+	+	0
	10^{-2}	0	0	0
	10^{-3}	0	0	0
Prochloraz	Control	0	0	0
	10^{-1}	+++	+++++	+++
	10^{-2}	++	++++	+++
	10^{-3}	++	+++	++
Guazatine	Control	0	0	0
	10^{-1}	+++++	++++	++++
	10^{-2}	++++	+++	++
	10^{-3}	+++	+	0
RSAF-1	Control	0	0	0
	10^{-1}	+	+	++++
	10^{-2}	0	0	0
	10^{-3}	0	0	0
Ultracure	Control	0	0	0
	10^{-1}	+++	+++	++
	10^{-2}	+++	++	+
	10^{-3}	+++	+	+
Quatrokill	Control	0	0	0
	10^{-1}	+++	++++	+++
	10^{-2}	++	+++	++
	10^{-3}	++	++	++
Imazilil	Control	0	0	0
	10^{-1}	++++	+++	+++
	10^{-2}	+++	++	++
	10^{-3}	+++	+	+

Legend: The formation of inhibition zone were tabulated in to the following categories: 0 = No Inhibition, + = 1-2 mm diameter, ++ = 3-6 mm diameter =, +++ = 7-10 mm diameter, ++++ = 11-14 mm diameter and +++++ = 15-18 mm diameter. * = Antagonist MeJtw10-2 (*Cryptococcus. laurentii*), TiL4-2 (*Candida sake*) and TiL 4-3 (*C. laurentii*). Extract A = *Acacia seyal* Del. Var. Seyal, B = *Withania somnifera* L. Dunal.

4.4 DISCUSSION

In this study, three potential yeast antagonists [two strains of *C. laurentii* (Megtw10-2 and TiL4-2) and one strain of *C. sake* (TiL4-3) exhibited high inhibition of *P. digitatum* growth rate. In addition, these isolates have a broad spectrum activity against *G. candidum* and *C. gloeosporioides*. Several previous reports demonstrated the potential use and application of yeast antagonists to control postharvest decay of fruits and vegetables (Wisniewski and Wilson, 1992; Janisiewicz and Bors, 1995). The successful application of *C. laurentii* on arbutus berries (Zheng *et al*., 2004), pear (Zhang *et al*., 2005a), oranges (Zhang *et al*., 2005b), apples (Roberts, 1990), strawberries, kiwi fruits and table grapes (Lima *et al*., 1998) and *C. sake* on apple (Usall *et al*., 2001), pears (Nunes *et al*., 2001) has been studied. These isolates proofed effective against a range of pathogens including *Penicillium* spp. (Teixido *et al*., 1998; Abadias *et al*., 2002; Vero *et al*., 2002; Zhang *et al*., 2003; Zhang, *et al*., 2005a), *G. candidum* (Chalutz and Wilson, 1990) and *C. gloeosporioides* (Koomen and Jeffries, 1993).

It is evident from the *in vitro* study that the selected potential antagonists did not show antibiosis or volatile production against the pathogens tested. On the other hand, i*n vivo* wound treatment application of these antagonists showed significant (P <0.05) reduction of disease incidence between 65-95% on fruits incubated at 7 ºC for 30 days. Fast colonization and competitive ability of antagonists were previously demonstrated by the non-destructive *in vitro* cylinder insert experiment (Janisiewicz *et al*., 2000). Inhibition of pathogen spore germination during the first 24 h of cylinder insert experiments, and its germination when transferred to fresh nutrient solution without antagonists confirmed that competition for nutrients and space by the antagonists were the main mode of actions which is in agreement with Janisiewicz *et al*. (2000). Reports with the application of different yeast antagonists such as *Debaryomyces hansenii* (Droby *et al*., 1989), *Pichia guilliermondi* (Arras *et al*., 1998) and *Aureobasidium pullulans* (Janisiewicz *et al*., 2000; Castoria *et al*., 2001) against *Penicillium* spp indicated similar results.

A higher rate of disease incidence reduction was observed through activity of antagonists TiL4-2 (*C. sake*) (95%) followed by *C. laurenti*, isolate MeJtw10-2 and TiL4-3 (70-90%). This result showed higher efficacy as compared to reports made by Usall, *et al*. (2001) and Vero *et al*. (2002) with *C. sake* (CPA-1) on apple (70 and 80%, respectively), Zhang *et al*. (2005) with *C. laurentii* on orange fruits with (80%) efficacy against blue mould.

The addition of NYDB as growth additive to yeast antagonists on fruit wound sites suppress ed the activity of the antagonists against *P. digitatum*. Significant (P <0.05) infection rate of fruits was observed on fruits treated with antagonists TiL4-2 (*C. sake*) and/ or Til4-3 (*C. laurentii*) using NYDB as a growth substrate medium. The application of NYDB to the antagonist MeJtw10-2 (*C. laurentii*), however did not exhibit significant (P <0.05) change in fruit decay. Unlike the report made by Nunes *et al*. (2001), this study demonstrated that the nutritional environment amended at the wound site could favour growth of a pathogen rather than the antagonists. On the other hand, this result is in agreement with the report made by Vero *et al*. (2002) indicating the growth limitation of antagonists with the addition of a nitrogen source medium as a growth substrate on apple wounds. This proofed the great potential of the yeast antagonists for their rapid colonization for space and nutrients when minimum nutrients are available at the wound site and without additional expenses.

Boiled culture filtrates of all antagonists failed to control spore germination of *P. digitatum*. It is evident from this experiment that only live cells were effective in controlling *P. digitatum* spore germination involving competition for nutrients and space rather than antibiosis. Similar reports by Droby *et al*. (1989) indicated effective competition of a yeast antagonist on grapefruits for nutrients against *P. digitatum*. This explanation for the rapid colonization of antagonists on the wound site with minimum nutrients available is also supported by the scanning electron microscope observation and *in vitro* dual culture study of antagonists on solid MEA media suggesting the production of an extracellular matrix on which they grow faster. According to Janisiewicz (1988), the rapid growth of antagonists is facilitated by the production of these extracellular polysaccharides over the surface. Chan and Tian (2005) on the other hand explained that the extracellular matrix produced by the antagonists may have a lytic effect towards the pathogen and provides higher amounts of simple carbon sources for the antagonists (Lima *et al*., 1998). Therefore, the rapid growth of the yeast antagonists without any additives at the wound site indicates their ability and considerable potential to be used as a biocontrol agent (Vero *et al*., 2002).

Results from MIC determination of antagonists demonstrated that the efficacy of the yeast antagonists depends on the inocula concentration of both the pathogen and antagonists. All antagonists effectively decreased disease incidence (100%) at concentration of 10^8 cells ml^{-1} against *P. digitatum* (10^6 spores ml^{-1}). The efficacy of antagonists however decreased as the concentration of the pathogen inoculum was increased. Antagonist TiL4-2 (*C. sake*) suppressed *P. digitatum* growth at a minimum concentration of 10^5 spores ml^{-1} of both

antagonist and pathogen, which is a better result compared to the report made by Droby *et al*. (1989).

In conclusion, although the mechanisms by which yeast biocontrol agents provide decay control are not fully understood, the mode of action of several yeast antagonists doesn't involve antibiosis as was found in this study. Instead, competition for nutrients (Benbow and Sugar, 1999; Janisiewicz *et al*., 2000) and space (Janisiewicz *et al*., 2000) at the wound site is more likely the mode of action. In this study, the rapid colonization effect of yeast antagonists through production of the extracellular matrix that sticks to the pathogens and/ or having a lytic effect against the pathogen was also found as was confirmed with the *in vitro* dual culture experiments supported by the electron microscopy study. Such peculiar characteristics of these yeast antagonists signify their great potential for industrial use in the postharvest disease control arena.

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CHAPTER FIVE

IN VITRO ANTIMICROBIAL ASSAY OF SOME MEDICINAL PLANTS FROM ETHIOPIA AGAINST PLANT AND FOOD-BORNE PATHOGENS

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Submitted to Fitoterapia for publication as:
Sissay B. Mekbib, Thierry J.C. Regnier, Catharina A.M. Zeeman and Lise Korsten. 2006. *In vitro* antimicrobial assay of medicinal plants from Ethiopia against plant and food-borne pathogens.

Abstract

Thirty-seven extracts of 23 plant species collected from three citrus growing regions of Ethiopia were screened for their activity against seven plant pathogens and five food-borne pathogens. In total, 21 extracts from 13 plant species showed some degree of antimicrobial activity to at least one pathogen. Of these, seven species, i.e. *Achyranthus aspera*., *Tribulus terrestris*, *Withania somnifera*, *Acacia seyal*, *Dolichos oliver*, *Cissus quadrangularis* and *Mirabilis jalapa* are species with no known previous reports of antimicrobial activity against the tested pathogens. The minimum inhibitory concentration value of eight selected plant extracts with antimicrobial activity against both fungal and bacterial pathogens ranged between 1:2 and 1:5 (v:v), indicating significant differences in their composition of active compounds. Thin layer chromatography was used for separation of the chemical compounds. None of the extracts inhibited *Escherichia coli* or *Erwinia carotovora*. On the other hand, three plant extracts inhibited a bacterial strain with complete resistance to all antibiotics tested. *Acacia seyal*, which demonstrated broad-spectrum antimicrobial activity, contained substantial concentrations of soluble phenolic compounds. Further determination of the active chemical ingredients is crucial for health improvement studies and postharvest disease control.

Key words: Plant extracts; Antibacterial; Antifungal; Phenolic compounds

5.1 INTRODUCTION

Plants are indispensable sources of medicinal importance used in both Western type pharmaceutical products and local medicinal preparations. The traditional use of plant material for treatment of human ailments dates back to prehistoric times (Cowan, 1999). According to the World Health Organisation, 80% of the world's population relies on traditional medicines to meet their daily health requirements (Maffi, 1999). However, from the estimated 250 000 species of higher plants described to date, only 5-15% have been studied for their potential therapeutic value (Rojas et al., 2003; Steep, 2004).

Ethiopia is a tropical country with a high floral diversity and endemism (Brenan, 1978). According to Tewoldebirhan (1991), there are about 7 000 species of higher plants in Ethiopia, of which 12% are endemic. More than 80% of the Ethiopian population depends on traditional remedies (Dawit and Ahadu, 1993), derived mainly (95%) from plant material (Dawit, 1986). The nationwide use of plants as a sole source of traditional medicine provides promising opportunities for the search of ethnobotanical specimens based on traditional knowledge.

Several researchers have studied the ethnobotanical (Dawit and Ahadu, 1993; Giday, 2001; Desissa and Binggeli, 2002), phytochemical (Abegaz and Woldu, 1991; Dagne and Abate, 1995) and antimicrobial activities (Habtemariam et al., 1993; Mammed, 2002) of a variety of medicinal plants. However, despite the broad spectrum of plants studied to date, no publications dealing with the potential of Ethiopian medicinal plants used for their antimicrobial activity could be found. The present study was aimed at screening potentially useful medicinal plants from Ethiopia for their antimicrobial potential to control major plant pathogens. In addition with the growing importance of food safety, it was decided to also evaluate the potential inhibitory activity of these extracts against major food borne pathogens.

In this chapter, we report on 37 extracts of 23 medicinal plants from three citrus growing regions of Ethiopia, which are also agriculturally important areas in terms of soil type and weather. Information about agro-ecology and cultural uses of medicinal plants are also included.

5.2 MATERIALS AND METHODS

5.2.1 Plant material

Twenty-three cultivated and wild medicinal plant spp. were collected from three citrus growing areas in Ethiopia, i.e. Somali, Oromia and Amhara Regional States between September 2002 and January 2003 (Table 5.1). Information about regionally important plants used in medicine was collected by consulting and interviewing local traditional healers. Plant samples including leaf-, stem-, root- and seed parts were collected, washed with tap water, air dried, packed into brown paper bags and transported to the herbarium of Alemaya University for identification. Identities of plant species were confirmed by Dr. Lisanework Nigatu from the Department of Plant Science (Alemaya University) and voucher specimens were stored and labelled in the collection. Dry samples were then brought to Plant Pathology Laboratories, University of Pretoria, South Africa with permit number (P0017192) for phytochemical analyses and biocontrol studies. Strict quarantine handling, processing and plant destruction protocols were followed during and after processing of samples. Plant rights and traditional knowledge have been protected within the University's ethical criteria requirements guidelines.

5.2.2 Plant material extraction

Dried, undamaged plant parts (leaves, stems, roots and seeds/fruits) were selected and reduced to powder in a Satin coffee grinder (Russell Hobbs, Germany). The powdered samples were stored at ambient temperature in glass bottles until further use. Two solvents, i.e. methanol/ acetone/ water (7:7:1 v:v) (Regnier and Macheix, 1996) with some modification and distilled water alone (Bautista-Banos et al., 2003) were used for extraction purposes. One part of the dried plant powder was suspended in 20 parts of solvent mixture followed by three successive extractions. The first and second extraction suspensions were mixed using a VM-300 vortex mixer (Labotec, Johannesburg) and placed on a rotary shaker (Stuart Scientific, United Kingdom) for 1 h at 170 rpm. Samples were centrifuged in a micro-centrifuge (Sigma, Germany) at 3913 x g for 10 min. The third extraction was placed overnight on the rotary shaker (Stuart Scientific) and centrifuged (Sigma). For each plant sample, the supernatants from three extractions were combined, concentrated under vacuum at room temperature (23 ºC) and freeze-dried. Distilled water was added to the concentrate to make up 10 ml of stock solution. The suspensions were filter sterilised

through a syringe filter (0. 22 µm pore size) into sterilised containers. Suspensions were either used immediately or kept at -4 ºC for later use.

5.2.3 Test pathogens

Three fungal pathogens [(*Penicillium digitatum* Sacc. (UPPed-1), *Geotrichum candidum* Lk ex Pers. (UPGec-1) and *Phytophthora nicotianae* Breda de Hann (UPPhn-1)], six bacterial plant pathogens [two strains each of *Erwinia carotovora* (UPErc-1 and UPErc-2) and *Xanthomonas campestris* pv. *mangiferaeindicae* (UPXac-1 and UPXac-2), and one strain each of *Pseudomonas syringae* pv. *syringae* (UPPss-1), *Ralstonia solanacearum* (UPRas-1)] and five food-borne pathogens [*Escherichia coli* (UPEsc-1), *Salmonella typhimurium* (UPSat-1), *Shigella sonnei* (UPShs-1), *Staphylococcus epidermidis* (UPSte-1) and *Streptococcus faecalis* (UPStf-1)] were obtained from the culture collection of Plant Pathology Laboratories University of Pretoria, South Africa. The pathogens were subcultured and maintained on Potato Dextrose Agar (PDA) (Biolab, Johannesburg) for fungi and Standard-1 nutrient agar (STD-1 NA) (Biolab) for bacteria. Fungal cultures were incubated for 7-14 days at 25 ºC under UV light until sporulation. Spores were harvested from the plates using a sterile swab and 20 ml of ¼ strength Ringer's solution (Merck, Johannesburg). A fungal spore concentration of 10^5 spores ml^{-1} was prepared using a haemacytometer. Agar blocks (3 x 3 mm size) from these cultures were used in all further trials. For bacteria, densities of cultures grown in Nutrient Broth (NB) (Biolab) on a rotary shaker (Stuart Scientific) for 24 h at 25 ºC were determined using a Petroff-Hauser counting chamber. A standardised concentration of 10^8 cells ml^{-1} was used in all subsequent tests.

5.2.4 *In vitro* antimicrobial assay

Two assay techniques, i.e. the agar plate (Thornberry, 1950) with slight modification and agar well diffusion assay (Rojas et al., 2003), were used to evaluate the antimicrobial activity of plant extracts against fungal and bacterial pathogens.

5.2.4.1 Agar plate technique

This method was selected to screen plant extracts for their efficacy against the fungal pathogens *P. digitatum*, *G. candidum* and *P. nicotianae*. This technique avoids volatilisation of active plant extract compounds. Aliquots of 9 ml PDA were made up in test tubes, autoclaved and cooled down to 50 ºC, after which 1 ml of plant extract was added aseptically, poured into a Petri dish (90 mm diameter) and swirled to cover the base. Fungal agar blocks (3 x 3 mm) from the cultures prepared

as described in section 2.3 were transferred to the centre of the plates. Plates were incubated at 25 ºC for 7-14 days and evaluated every two days for growth inhibition. The experiment was performed in triplicate and percentage inhibition of pathogen growth was determined according to Skidmore (1976), using the following formula: Percentage inhibition = (C - r) x 100/C, where r = fungal radial growth measured on the treated plate and C = radial growth measured on the control plate.

5.2.4.2 Agar well diffusion

This technique was used to determine the toxicity of extracts against bacterial pathogens, which multiply sufficiently to detect growth or inhibition within 24-48 h of incubation. Bacterial broth cultures were prepared to a density of 10^8 cells ml^{-1} as described in Section 2.3. Aliquots of 100 µl were spread evenly onto individual STD-1 NA agar plates. On each plate, four equidistant wells were made in the agar with a 0.5 mm diameter sterilised cork borer, 2 mm from the edge of the plate. Fifty µl of each plant extract was transferred to a respective agar well and plates were incubated at 25 ºC for 24-48 h. The same volumes of antibiotics [Streptomycin (Sigma) (0.2 mg/ml), Tetracycline (Sigma) (2%), Novobiocin (Sigma) (2%) and Rifampicin (Rolab, Johannesburg) (2%)] were used as positive controls. Extraction solvents [methanol, acetone and sterilised distilled water] were included as negative controls. Experiments were performed in triplicate. The formation of clear inhibition zones around the wells were regarded as positive results and measured in mm.

5.2.5 Determination of minimum inhibitory concentration of selected plant extracts

The minimum inhibitory concentration (MIC) of each plant extract was determined using the method described by Barbour et al. (2004). Eight plant extracts that showed a wide range of antimicrobial activity in 2.4 were used for further tests. One ml of each plant extract, prepared as described in 2.2, was serially diluted in sterile NB. The plant extract volume to broth medium ratio (v:v) was prepared at 1:2; 1:2.5; 1:3; 1:3.5; 1:4 and 1:5. Each plant extract dilution was inoculated with 20 µl of the standard concentration of pathogen inoculum prepared as described in section 2.3. Culture tubes were incubated at 25 ºC for 24 h (bacterial isolates) and 72 h (fungal pathogens) and were evaluated visually for presence or absence of growth. The lowest plant extract concentration retaining its inhibitory effect (absence of turbidity) was regarded as the MIC value of the extract. Control flasks with uninoculated medium were incubated in parallel. The extraction solvents

methanol, acetone and sterilised distilled water were regarded as negative controls, whereas antibiotics were incorporated as positive controls. Experiments were performed in triplicate.

5.2.6 Phytochemical analysis:

5.2.6.1 Determination of total soluble phenolics, free acids, bound ester and glycoside

Crude Extract

Crude extracts (CE), prepared as described in 5.2.2, were used to quantify the amount of total soluble phenolics. (De Ascensao and Dubery, 2003)

Extraction of free acids

To extract the free acids (FA), 1.25 ml of the CE was acidified with 25µl trifluoroacetic acid. An equal volume of diethyl ether was added and the mixture shaken and allowed to stand briefly to allow separation of fractions. The organic phase was removed and placed in a new Eppendorf tube. This procedure was repeated four times. The separated upper phase layers combined and diethyl ether evaporated under vacuum. Two hundred and fifty micro liters of methanol was added and the extracts were stored at 4 ºC until further use. (De Ascensao and Dubery, 2003)

Extraction of bound esters

Sodium hydroxide (2N) was added to plant extracts prepared as described previously (5.2.2) at the rate of 2% w/v (i.e 0.2 g/ 1.25 ml). The mix was vortexed and kept in the dark for 4 h. After 3 h, samples were kept in an icebox and an equal volume of HCl (1M) was added in order to acidify the mixture. Three-fold volume of diethyl ether was added; the mixtures shaken and the samples allowed to settle to enhance the separation of hydrolysed ester-bond phenolic compounds. The procedure was repeated three times. The organic phases were combined and diethyl ether evaporated under vacuum. Two hundred and fifty micro liters of methanol was added and the extracts were stored at 4 ºC until further use. (De Ascensao and Dubery, 2003)

Extraction of glycosides

The process involved hydrolysis of glucose-conjugated compounds. Sixty milliliter of concentrated HCl (10N) was added to an Eppendorf tube containing 1ml of the crude extract. Samples were kept in a water bath for 1 h at 96 ºC. Five hundred micro liters of diethyl ether was added while the sample was kept in the icebox. The extraction with an equal volume of diethyl ether was repeated

three times and the separated upper phase layers were combined together. The organic phases were combined and dried under vacuum. Two hundred and fifty micro liters of methanol was added and the extracts were stored at 4 ºC until further use.

The total contents of soluble phenolics in medicinal plants were determined using a modification of the Folin-Ciocalteu's Phenol reagent (Bray and Thorpe, 1954). The extracts of 37 plants were evaluated using the 96 wells ELISA-plates (Merck, Germany). In each well, 25 µl of the Folin-Ciocalteu reagent (Sigma) was added to 175:5 µl (v:v) of distilled water and the test plant extract respectively. After three min., 50 µl of 20% sodium carbonate was added into each well. Four wells were used for each sample, randomly placed on the ELISA plate, and the experiment was done in duplicate. Plates were incubated at 40 ºC for 30 min. Phenolic measurements were taken with an ELISA reader version 1.3.1 (Multiscan Ascent VI. 24 354-0973, Finland). The absorbance of a blank consisting of distilled water was subtracted from all sample readings. Data were calculated as gallic acid equivalent in µg g^{-1} using the standard curve (y= 1.3527 x + 0.0109, R^2=0.9989). (De Ascensao and Dubery, 2003)

5.2.6.2 Thin layer chromatography

The same eight plant extracts used in 2.5 were used for further evaluation. The following solvent combinations were tested to obtain the best separation of phenolic compounds: toluene/ ethyl acetate (1:1), chloroform/ methanol/ ethyl acetate/ acetone/ water (50:20:20:5:3.5), ethyl acetate/ formic acid/ water (3:1:3), butanol/ ethanol/ water (5:1:2), toluene/ acetic acid (4:1), chloroform/ ethyl acetate/ formic acid (5:4:1), butanol/ acetic acid/ water (6:1:2), acetic acid (10%), methanol/ butanol/ ethyl acetate/ dichloromethane (1:1:1:1), ethyl acetate/ acetic acid/ water (3:1:3), ethyl acetate/ acetic acid/ formic acid/ water (50:5.5:5.5:13) and chloroform/ acetone/ formic acid (9:2:1), of which, toluene/ ethyl acetate (1:1), butanol/ ethanol/ water (5:1:2), and ethyl acetate/ acetic acid/ formic acid/ water (50:5.5:5.5:13) gave the best result. The TLC plate (pre-coated aluminium, SIL G-100) was loaded with 10 µl of each sample. Spots were visualized with a CAMAT 50HZ UV lamp at 254 and 366 nm. Three separation solvent systems from the preparation described [were selected. Of these again, toluene/ ethyl acetate (1:1) was further used for separation of soluble FA, GLY and EB of *A. seyal* and *W. somnifera* plant extracts selected for their antifungal potential without antibiosis. The visibility of compounds was amplified by spraying ammonia vapour onto the plates and the R_f values of the separated spots were determined.

5.2.6.3 High performance liquid chromatography analyses

Fresh preparation and six months old extracts of *A. seyal* and *W. somnifera* prepared as described in 5.2.2 were used. Identification and quantification of individual phenolic compounds of CE, FA, GLY, EB, of the two plants extract: *A. seyal* and *W. somnifera* were done by high performance liquid chromatography (HPLC).

The samples were analysed on a Hewlett Packard HPLC equipment (Agilent 1100 series) equipped with a 20 µl loop injection valve (Agilent) and connected with a UV detector at 280, 325 and 340 nm. A Luna 3u C18 reverse phase column (250 x 4.60 mm) was used. Acetonitrile and water (pH 2.6 acidified with phosphoric acid, H_3PO_4) were used as eluents with a gradient program from 7% acetonitrile/ water at 0 minutes to 20% at 20 minutes increasing to 23% at 28 minutes, 27% at 40 minutes, 29% at 45 minutes, 33% at 47 minutes and 80% at 50 minutes. Twenty microliters of each sample [(CE, 20x; FA, 10x; Gly, 10x and EB, 10x diluted) were injected and chromatogramed at a flow rate of 1 ml min^{-1}. Data were analyzed using the Hewlett Packard software. The phenolic compounds in the extracts were identified by comparison with the reference compounds such as, gallic acid, caffeic acid, ferulic acid, syringic acid, quercetin, umbelliferone, naringin, hydroxy benzoic acid, 3,4, dihydroxy benzoic acid, sinapic acid, vanillic acid, *ρ*-coumaric acid, salicylic acid, scopoletin, catechin, kaempferol, chlorogenic acid, luteolin and fisetin.

HPLC data was analysed qualitatively by comparing the presence and absence of peaks in chromatograms obtained with the different treatments. For unidentified compounds, the area of the peak (mAU*s) was used to evaluate quantitative differences among treatments while for the known compounds; the amount (µg/ml) was used for comparison between treatments. Data from the areas of unidentified compounds was subjected to normality and homogeneity of variances tests then log (x+1) transformed prior to analysis.

5.2.7. Statistical analysis

One-way analysis of variance (ANOVA) was performed using the SAS computer program (version 8.2, 2001). Treatment means were compared with Tukey's HSD multiple range test at a 5% level of significance.

5.3 RESULTS

5.3.1 Medicinal plant species

The 37 plant extracts prepared from 23 plant species collected from Ethiopian citrus growing regions are shown in Table 5.1. From preliminary trials, methanol/ acetone/ water was identified as the best solvent system compared to aqueous extraction. The most effective plant species regarding antimicrobial activity were found in Hursso, Somali National Regional State. Plant leaves were found to be more inhibitory (44.2%), followed by stem (27.9%), root (14%) and seeds (10.8%) extracts.

Table 5.1 Plant species collected in Ethiopia, their location, plant parts used in the study and known usage as described by local healers

Plant species	Family	Plant type	Location	GPS coordinates	Altitude (m asl)	Plant part used				Local use(s) in Ethiopia*** as described by traditional healers interviewed
						Leaf	Stem	Root	Seed	
Acacia seyal Del. var. Seyal	Mimosaceae	Tree	Hursso[d]	N: 9.614 E: 41.643	1062.5	✓	✓	✓	-	Intestinal disorder, bleeding and conjunctivitis
Achyranthus aspera L.	Amaranthaceae	Herb	Errer[c]	N: 9.573 E: 41.38	996	✓	-	✓	-	Intestinal disorder (dysentery)
Agave sisalana L.	Agavaceae	Herb	Tisabalima[f]	N: 11.459 E: 39.628	1492	✓*	-	-	-	Insecticide
Artemisia afra Jacq. Ex. Willd	Compositae	Herb	Merssa[g]	N: 11.668 E: 39:.663	1602	✓	-	-	-	Hemorrhage (topical and decoction drink)
Azaridachta indica A. Juss	Meliaceae	Tree	Errer[c]	N: 9.575 E: 41.384	996	✓	-	-	-	Stomach ache (bloating) and insect repellant
Calotropis procera Ait. Dry	Asclepiadaceae	Herb	Errer[c]	N: 9.575 E: 41.384	996	✓	✓	-	-	Chronic skin infection and hemorrhage
Cissus quadrangularis L.	Vitaceae	Herb	Tisabalima[f]	N: 11.459 E: 39.628	1492	-	✓**	-	-	Insectcide and fungicidal
Convolvulus sp.	Convolvulaceae	Herb	Errer[c]	N: 9.550 E: 41.389	1084	-	✓	✓	-	Snake bite, decoction drink
Cucumis meeusei A. Rich	Cucurbitaceae	Herb	Alemaya University[h]	N: 9.00 E: 37.968	1890	-	-	✓	-	Skin burn and discharge of after birth
Dolichos oliveri Schweinf.	Fabaceae	Herb	Hursso[d]	N: 9.614 E: 41.643	1062.5	✓	-	✓	-	Epilepsy and sinus
Euphorbia abyssinica JF Geml.	Euphorbiaceae	Tree	Abomissa[e]	N: 8.491 E: 39.835	1600	-	✓	-	-	Wound healing (topical) and worm expel
Lablab purpureus L.	Fabaceae	Herb	Tisabalima[f]	N: 11.459 E: 39.628	1492	✓	-	-	-	Weed control and nitrogen fixation
Millettia ferruginea (Hochst) Baker	Papilionoideae	Tree	Tisabalima[f]	N: 11.459 E: 39.628	1492	✓	-	-	-	Insecticide
Mirabilis jalapa L.[a]	Nyctaoginaceae	Herb	Hursso[d]	N: 9.614 E: 41.643	1062.5	-	-	✓	-	TB[b], Cancer
Nicotiana tabacum L.	Solanaceae	Herb	Tisabalima[f]	N: 11.459 E: 39.628	1492	✓	✓	-	✓	Insecticide
Table … continued	Portulacaceae	Herb	Errer[c]	N: 9.573 E: 41.38	996	✓	-	-	-	Breast and knee tumours (surface application)
Ruta chalepensis L.	Rutaceae	Herb	Merssa[g]	N: 11.668	1602	✓	✓	-	-	Stomach ache and intestinal disorder

				E: 39.663						
Solanum incanum L.	Solanaceae	Herb	Alemaya University[h]	N: 9.00 E: 37.968	1890	✓	-	-	✓	TB[b]
Solanum nigrum L.	Solanaceae	Tree	Hursso[d]	N: 9.614 E: 41.643	1062.5	✓	-	-	✓	Gastritis, cancer and haemorrhage
Tribulus terrestris L.	Zygophyllaceae	Herb	Errer[c]	N: 9.573 E: 41.38	996	✓	✓	-	-	Induce uterus contraction and discharge of urine
Tagetes minuta L.	Asteraceae	Herb	Tisabalima[f]	N: 11.459 E: 39.628	1492	✓	-	-	-	Insecticide
Tamarindus indica L.	Caesalpiniaceae	Tree	Ghibe Valley[i]	N: 8.248 E: 37.540	995	✓	-	-	✓	Stomach ache and intestinal disorder
Withania somnifera L. Dunal	Solanaceae	Herb	Hursso[d]	N: 9.614 E: 41.643	1062.5	✓	✓	-	-	Epilepsy cure

Legend: [a] = Cultivated plant

[b] = Tuberculosis

[c] = East of Addis Ababa, the capital, 400 km (train) or 560 km (road)

[d] = East of Addis Ababa, 420 km (train) or 540 km (road)

[e] = South east of Addis Ababa, 160 km (road)

[f] = North east of Addis Ababa, 450 km (road)

[g] = North east of Addis Ababa, 490 km (road)

[h] = East of Addis Ababa, 500 km (road)

[i] = South west of Addis Ababa, 185 km (road)

*= Modified leaf

** = Modified stem

*** = According to traditional healers around the area

5.3.2 *In vitro* antimicrobial assay

Some degree of antimicrobial activity, at least to one pathogen, was shown by 21 extracts from 13 species. Of these extracts, 11 showed selective toxicity to fungal pathogens, while two of them inhibited bacterial growth. Eight of the extracts showed broad-spectrum activity against both fungal and bacterial pathogens (Table 5.2 and 5.3). In the *in vitro* semi-qualitative experiment, leaf and root extracts of *A. seyal*, root extracts of *M. jalapa*, leaf extracts of *T. minuta* L., leaf extracts of *W. somnifera* and seed extracts of *Solanum incanum* L. showed broad spectrum antimicrobial activity to the microbial pathogens challenged. The bacterial inhibition zones were in the range of 4-30 mm. Maximum inhibition was detected with *M. jalapa* against *S. epidermidis*. The latter pathogen was found most susceptible to over 80% of plant extracts evaluated (Table 5.2). Two species of bacterial pathogens (*E. carotovora* $_1$ and *E. coli*) were not affected by any of the plant extracts. On the other hand, some bacterial pathogens showed resistance to the antibiotics used in the control experiment. *Xanthomonas campestris*$_2$ was resistant to all antibiotics tested, while strain UPXac-1 was not inhibited by streptomycin. Similarly, *R. solanacearum* showed resistance to streptomycin, whereas *E. carotovora*$_2$, *P. syringae* and *S. sonnei* were resistant to novobiocin (Table 5.2). Sterilized distilled water, methanol and acetone did not have any inhibitory effect against the pathogens.

5.3.3 Determination of minimum inhibitory concentration of selected plant extracts

The MIC values of the eight plant extracts which showed inhibitory activity against some pathogens tested are shown in Table 5.4. The MIC values of extracts ranged between 1:1 and 1:5 (v:v) dilution ratio. The MIC of *A. seyal* ranged between 1:2 for *S. sonnei* and 1:4 for *S. epidermidis* and *X. campestris*, whereas the MIC of *W. somnifera* ranged between 1:3 for *S. epidermidis* and 1:3.5 for *S. faecalis* and *X. campestris*. Similarly, the MIC of *M. jalapa* root extract ranged between 1:2 (*S. sonnei*) and 1:5 (*S. epidermidis*).

5.3.4 Thin layer chromatography and R_f values of selected plant extracts

Of the twelve separation solvent systems evaluated, three were selected as most effective. The R_f value of these plant extracts are given in Table 5.5. Butanol/ ethanol/ water (5:1:2) resulted in a high band separation with almost all extracts except for *A. seyal*. Extracts such as *T. indica*, and *M. jalapa* showed no band separation activity to other solvent systems used. Unlike the other extracts evaluated, *T. minuta* and *S. incanum* exhibited fractional separation in the three solvent systems selected.

Table 5.2 Plant extract toxicity assay against plant and food borne bacterial pathogens tested

Plant species	Plant parts tested	Eq. mg gallic acid/g dry weight	Bacterial pathogens	Bacterial growth inhibition zone (mm)*
Acacia seyal Del. var. Seyal	Leaf	172.4	*Erwinia carotovora* $_1$	14 ± 0.7^{c}
			Pseudomonas syringae pv. *syringae*	16 ± 0.5^{e}
			Ralstonia solanacearum	15 ± 0.4^{ce}
			Shigella sonnei	06 ± 0.3^{f}
			Staphylococcus epidermidis	23 ± 0.8^{g}
			Xanthomonas campestris pv. *mangiferaeindicae*$_2$	24 ± 1.1^{g}
Acacia seyal Del. var. Seyal	Root	15.46	*E. carotovora*$_1$	13 ± 0.4^{c}
			P. syringae pv. *syringae*	13 ± 0.2^{c}
			R. solanacearum	13 ± 0.6^{c}
			S. sonnei	04 ± 0.6^{a}
			S. epidermidis	18 ± 1.0^{h}
			X. campestris pv. *mangiferaeindicae*$_2$	14 ± 0.3^{c}
Achyranthus aspera L.	Leaf	7.97	*S. epidermidis*	05 ± 0.5^{a}
Achyranthus aspera L.	Root	6.74	*S. epidermidis*	07 ± 0.5^{b}
Azadirachta indica A. Juss	Leaf	41.6	*S. epidermidis*	05 ± 0.5^{a}
Dolichos oliveri Schweinf.	Leaf	24.73	*S. epidermidis*	06 ± 0.6^{ab}
Mirabilis jalapa L.[b]	Root	28.84	*E. carotovora*$_1$	18 ± 0.2^{h}
			P. syringae pv.*syringae*	10 ± 0.3^{d}
			R. solanacearum	10 ± 0.4^{d}
			S. sonnei	08 ± 1.0^{b}
			S. epidermidis	30 ± 0.4^{i}
			X. campestris pv. *mangiferaeindicae* $_1$	20 ± 0.6^{k}
			X. campestris pv. *mangiferaeindicae* $_2$	04 ± 0.4^{a}
			S. typhimurium	15 ± 0.3^{c}
Ruta chalepensis L.	Leaf	18.62	*S. epidermidis*	07 ± 0.5^{b}
Solanum incanum L.	Leaf	17.75	*E. carotovora*$_1$	05 ± 0.5^{a}
			S. epidermidis	10 ± 0.7^{dj}
	Seed	57.80	*E. carotovora*$_1$	04 ± 0.5^{a}
			S. epidermidis	15 ± 1.1^{ce}
			X. campestris pv. *mangiferaeindicae*$_2$	17 ± 0.6^{he}
Tagetes minuta L.	Leaf	36.90	*E. carotovora*$_1$	12 ± 0.3^{i}
			e pv. *syringae*	10 ± 0.6^{dj}
			earum	09 ± 1.0^{j}

Table … continued

			S. epidermidis	16 ± 0.7[ce]
			X. campestris pv. *mangiferaeindicae*$_2$	13 ± 0.8[i]
Withania somnifera L. Dunal	Leaf	11.61	*Streptococcus faecalis*	13 ± 0.5[c]
			S. epidermidis	11 ± 0.5[d]
			X. campestris pv. *mangiferaeindicae*$_2$	16 ± 0.6[e]
Control trials with chemicals:				
Tetracycline			*E. carotovora*$_1$	9 ± 0.7[j]
			E. carotovora$_2$**	7 ± 0.9[b]
			P. syringae pv.*syringae*	17 ± 1.3[eh]
			R. solanacearum	17 ± 0.8[eh]
			X. campestris pv. *mangiferaeindicae*	18 ± 1.4[eh]
			X. campestris pv. *mangiferaeindicae*$_2$	0
			*E. coli***	7 ± 0.9[f]
			S. typhimurium	11 ± 0.4[d]
			S. sonnei	9 ± 0.8[j]
			S. epidermidis	2 ± 0.4[l]
			S. faecalis	15 ± 0.7[ce]
Streptomycin			*E. carotovora*$_1$	10 ± 0.2[j]
			E. carotovora$_2$**	10 ± 0.6[dj]
			P. syringae pv.*syringae*	6 ± 0.2[f]
			R. solanacearum	0
			X. campestris pv. *mangiferaeindicae*$_2$	0
			*E. coli***	10 ± 0.9[dj]
			S. typhimurium	17 ± 1.0[eh]
			S. sonnei	2 ± 0.3[l]
			S. epidermidis	10 ± 0.7[dj]
			S. faecalis	4 ± 0.5[a]
Novobiocin			*E. carotovora*$_1$	1 ± 0.2[m]
			E. carotovora$_2$**	0
			P. syringae pv.syringae	0
			R. solanacearum	3 ± 0.3[n]
			X. campestris pv. mangiferaeindicae	5 ± 1.0[a]
			X. campestris pv. mangiferaeindicae$_2$	0
				1 ± 0.3[m]
			ium	7 ± 0.3[b]
				0
			S. epidermidis	22 ± 0.9[g]
			S. faecalis	12 ± 0.4[c]
			E. carotovora$_1$	1 ± 0.2[m]

Table … continued

Rifampicin	*E. carotovora*	2 ± 0.2^{l}
	E. carotovora$_2$**	2 ± 0.4^{l}
	P. syringae pv. syringae	4 ± 0.3^{a}
	R. solanacearum	7 ± 0.8^{b}
	X. campestris pv. mangiferaeindicae	7 ± 0.3^{b}
	X. campestris pv. *mangiferaeindicae*$_2$	0
	*E. coli***	5 ± 0.7^{a}
	S. typhimurium	14 ± 0.6^{c}
	S. sonnei	3 ± 0.3^{n}
	S. epidermidis	30 ± 0.6^{i}
	S. faecalis	10 ± 0.6^{dj}

Legend: * = Numerical data represent the means ± SE of bacterial pathogen inhibition zones. In a column, means followed by the same letter are not significantly different at the 5% level of Tukey's HSD. Strains resistant to all of the tested plant extracts are indicated only in the control trials.

** = Strains resistant to all of the plant extracts. Their inhibition indicated only in the control trials with antibiotics.

0 = No inhibition

^{b} = Maximum inhibition.

Table 5. 3 Plant extract toxicity assay against *Penicillium digitatum, Phytophthora nicotianae* and *Geotrichum candidum*

Plant species	Plant parts tested	Eq. mg gallic acid/g dry weight	Fungal pathogens inhibited by plant extracts	Fungal growth inhibition
Achyranthus aspera L.	Root	6.74	*P. nicotianae*	+
Azadirachta indica A. Juss	Leaf	41.6	*P. digitatum*	+++
Cissus quadrangularis L.	Modified leaf	10.27	*P. nicotianae*	++
Dolichos oliveri Schweinf.	Leaf	24.73	*P. digitatum*	+++
			G. candidum	+++
			P. nicotianae	++
Dolichos oliveri Schweinf.	Root	12.54	*P. nicotianae*	+++
Nicotiana tabacum L.	Stem	12.36	*P. digitatum*	+++
Nicotiana tabacum L.	Seed	11.00	*P. nicotianae*	++
Ruta chalepensis L.	Leaf	18.62	*P. digitatum*	++
Solanum incanum L.	Leaf	17.75	*P. nicotianae*	++
Solanum incanum L.	Seed	57.80	*P. digitatum*	++++
			G. candidum	++++
			P. nicotianae	+++
Solanum nigrum L.	Seed	22.58	*P. nicotianae*	+++
Tribulus terrestris L.	Leaf	17.87	*P. digitatum*	+++
			G. candidum	++
Tamaridus indica L.	Leaf	20.37	*P. digitatum*	++++
Tamarindus indica L.	Seed	44.2	*P. digitatum*	++++
			G. candidum	+++
			P. nicotianae	++++
Withania somnifera L. Dunal	Stem	6.95	*P. nicotianae*	+

Legend: * =Antimicrobial activities of plant extracts is expressed by "+" sign depending on the strength of fungal growth inhibition.
+ = Inhibition present; ++ = Strong inhibition; +++ = Very strong inhibition; ++++ = Exceptional inhibition of the fungal pathogens. Strains resistant to all of the tested plant extracts are indicated only in the control experiment (Table 2b).
* = Strain UPEcr-1; *** = Resistant strain to all of the antibiotics tested, but significantly inhibited by plant extracts.

Table 5.4 Minimum inhibitory c ns of the most efficacious plant extracts evaluated against twelve-test pathogens

Plant extracts solvents[b] and/or antibiotics[c]	Part used	Minimum inhibitory concentration values of plant extracts to twelve different test pathogens[a]		
		Bacterial food-borne pathogens	Bacterial plant pathogens	Fungal pathogens

		Ec	***Ss***	***Se***	***Sf***	***St***	***Erc***	***Ps***	***Rs***	***Xcm***	***Pd***	***Gc***	***Pn***
Acacia seyal Del. var. Seyal	leaf	NE	1:2	1: 4	NE	NE	1:3.5	1:3.5	1:3.5	1:4	NE	NE	NE
Withania somnifera L. Dunal	leaf	NE	NE	1:3	1:3.5	NE	NE	NE	NE	1:3.5	NE	NE	NE
Tagetes minuta L.	leaf	NE	NE	1:3.5	NE	NE	1:3	1:2.5	1:2.5	1:3	NE	1:2.5	NE
Dolichos oliver Schweinf	leaf	NE	NE	1:2.	NE	NE	NE	NE	NE	NE	1:3	1:2.5	1:2
Mirabilis jalapa L.	root	NE	1:2	1:5[d]	NE	1:3	1:3.5	1:2.5	1:2.5	1:3.5	NE	NE	NE
Solanum incanum L.	seed	NE	NE	1:3	NE	NE	NE	NE	NE	1:3	1:3.5	1:3	1:2.5
Tamaridus indica L.	seed	NE	NE	NE	NE	NE	NE	NE	NE	NE	1:2	1:2	1:3
Azadirachta indica A. Juss	leaf	NE	NE	1:2	NE	NE	NE	NE	NE	NE	1:2	NE	NE
Controls:													
Sterilized distilled water	-	NE	NE	NE	NE	NE	NE	NE	NE	NE	NE	NE	NE
Methanol	-	NE	NE	NE	NE	NE	NE	NE	NE	NE	NE	NE	NE
Acetone	-	NE	NE	NE	NE	NE	NE	NE	NE	NE	NE	NE	NE
Tetracycline	-	1:2	1:2	NE	1:3	1:2.5	1:2	1:3.5	1:3.5	1:3.5	NE	NE	NE
Streptomycin	-	1:2.5	NE	1:2.5	1:2	1:3	1:2.5	1:2	NE	NE	NE	NE	NE
Novobiocin	-	NE	NE	1:4	1:2.5	1:2.5	NE	NE	NE	1:2	NE	NE	NE
Rifampicin	-	1:2	NE	1:5	1:2	1:3	NE	NE	1:2	1:2	NE	NE	NE

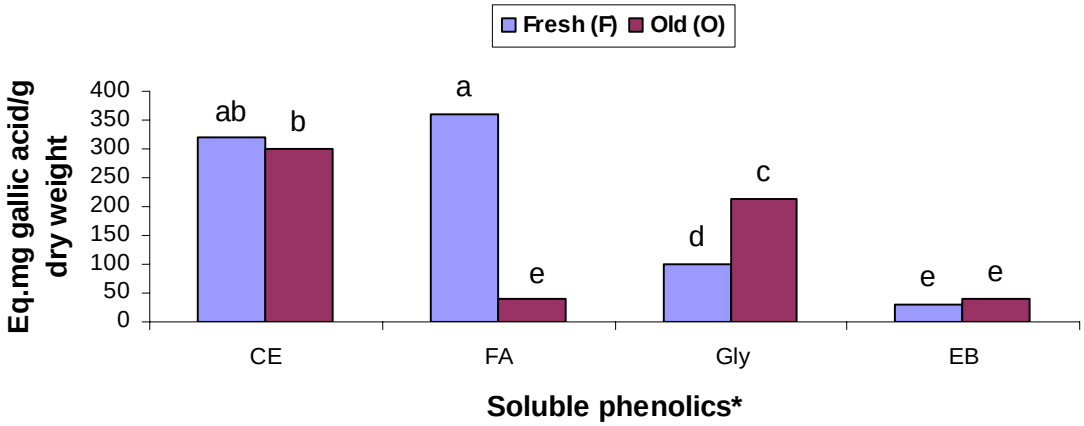
Legend: [a,] =Food-borne and plant pathogens: *Ec = Escherichia coli, Ss = Shigella sonnei, Se = Staphylococcus epidermidis, Sf = Streptococcus faecalis St = Salmonella typhimurium, Erc = Erwinia carotovora,* (UPErc-1). *Ps = Pseudomonas syringae* pv. *syringae, Rs = Ralstonia solanacearum, Xcm = Xanthomonas campestris pv. mangiferaeindicae* (UPXac-1), Pd = *Penicillium digitatum, Gc = Geotrichum candidum*, and *Pn = Phytophthora nicotianae*.

[b] = Sterilized distilled water, methanol and acetone used as negative control.

[c] =Tetracycline, Streptomycin, Novobiocin and Rifampicin as positive controls.

[d] = A plant extract and an antibiotics with higher dilution ratio of MIC efficacy, [e] = NE, not effective.

Thin layer chromatography analyses of fresh and old preparations of *A. seyal* and *W. somnifera* showed significant (P <0.05) variation in their phenolic contents (Fig. 5.1 and 5.2). Fresh preparations of *A. seyal* extracts showed significantly (P <0.05) higher concentrations of free acid (FA) and glycoside (Gly) phenolics than the old preparations (Fig. 5. 1). All fresh preparations of *W. somnifera* extracts showed high concentrations of crude extract (CE), free acids (FA), glycosides (Gly) and ester bound (EB) phenolics content unlike the old preparations (Fig. 5.2). The TLC analyses of *A. seyal* and *W. somnifera* showed the presence of high concentration of Gallic, Ferulic and Syringic acid compounds as a principal component of the phenolic compounds (Fig. 5.3).



Legend: *= Quantification of total soluble phenolic compounds in CE= crude extract, FA= free acid, Gly.= glycoside, and EB= ester bound compounds. Bars with similar letters are not significantly different at Fisher's protected LSD (P <0.05) analysis and t-grouping.

Fig. 5.1. Quantification of total soluble phenolic compounds in fresh and old preparations of *Acacia seyal* extracts.

Table 5.5 Chromatography analysis (R_f values) of plant extracts in selected thin layer chromatography solvent systems

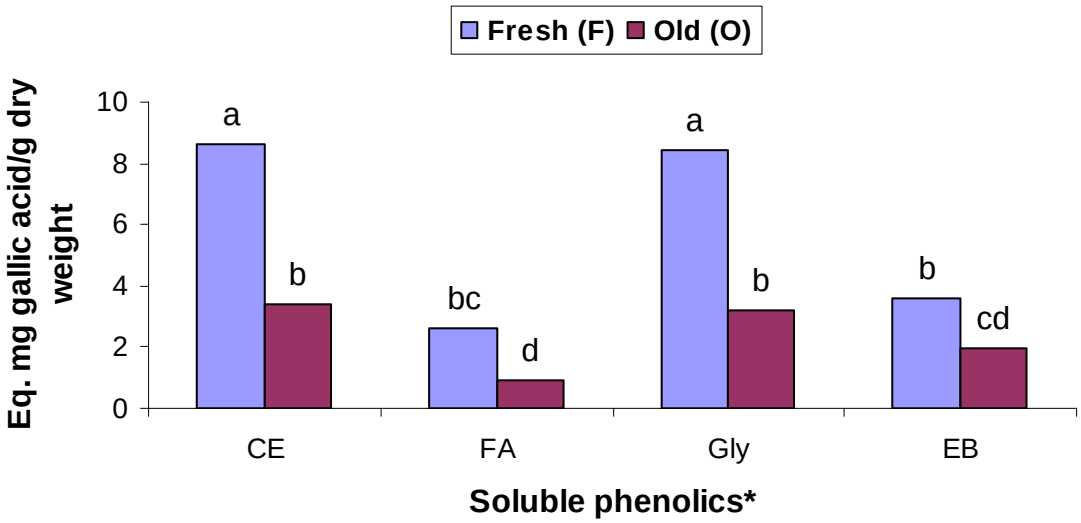
Separation solvent system	R_f values of plant extract compounds Plant material code[a]							
	H_1	I_1	K_1	Q	V_1	X_2	Z	ZA
Toluene/ ethyl acetate (1:1)*	nd	nd	nd	0.27g, 0.33	0.05, 0.4	nd	nd	0.39
Chloroform/ ethanol/ ethyl acetate/ acetone/ water (50:20:20:5:3.5)	nd	nd	nd	nd	nd	nd	nd	nd
Ethyl acetate/ formic acid/ water (3:1:3)	nd	nd	nd	nd	nd	nd	nd	nd
Butanol/ ethanol/ water (5:1:2)*	0.03a, 0.09b, 0.16c, 0.23d, 0.28e, 0.3, 0.34h, 0.36, 0.42j, 0.55k, 0.69, 0.78L	nd	0.03a, 0.08m, 0.14, 0.2p, 0.23d, 0.29f, 0.34h, 0.41, 0.46, 0.53, 0.66n, 0.78L	0.02, 0.07, 0.1, 0.16c, 0.22, 0.32i, 0.45, 0.55k, 0.62, 0.73	0.03a, 0.09b, 0.15, 0.21, 0.32i, 0.42j, 0.52, 0.6, 0.68, 0.78L	0.11o, 0.15, 0.16c, 0.2p	0.03a, 0.11o, 0.19, 0.28e	0.08m, 0.12, 0.2p, 0.27g
Toluene/ acetic acid (4:1)	nd	nd	nd	nd	nd	nd	nd	nd
Chloroform/ ethyl acetate/ formic acid (5:4:1)	nd	nd	nd	nd	nd	nd	nd	nd
Butanol/ acetic acid/ water (6:1:2)	nd	nd	nd	nd	nd	nd	nd	nd
Acetic acid (10%)	nd	nd	nd	nd	nd	nd	nd	nd
Methanol/ butanol/ ethyl acetate/ dichloromethane (1:1:1:1)	nd	nd	nd	nd	nd	nd	nd	nd
Ethyl acetate/ acetic acid/ water (3:1:3)	nd	nd	nd	nd	nd	nd	nd	nd
Ethyl acetate/ acetic acid/ formic acid/ water (50:5.5:5.5:13)*	0.54, 0.66n, 0.74	0.63	0.76	0.65, 0.84, 0.92	0.29f, 0.42j, 0.55k, 0.66n, 0.75	nd	nd	0.93
Chloroform/ acetone/ formic acid (9:2:1)	nd	nd	nd	nd	nd	nd	nd	nd

Legend: [a] = Ratio of front compound migration on TLC in a separation solvent system. The R_f values followed by similar letters may indicate similar compounds in each plant extract.

* = Selected solvent systems for high separation of plant extract compounds to determine the R_f value of bands on the chromatogram developed.

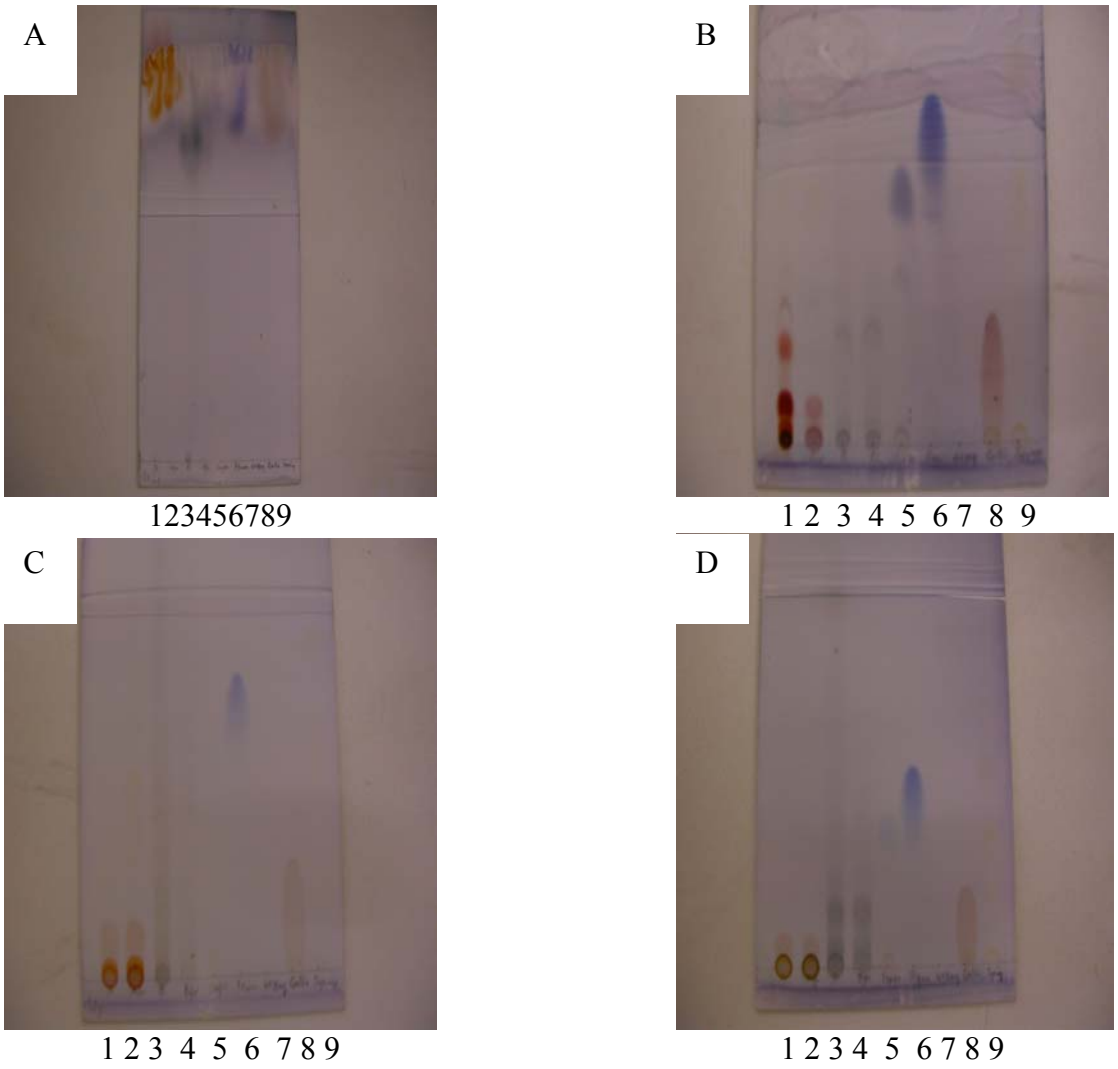
H_1 = *Withania somnifera* L. Dunal; I_1, *Acacia seyal* Del. var. Seyal; K_1, *Dolichos oliveri* Schweinf; Q, *Tagetes minuta* L.; V_1, *Solanum incanum* L.; X_2, *Tamardius indica* L.; Z =*Mirabilis jalapa* L.; *Azadirachta indica* L.

nd = Not determined.



Legend: * = For description refer to figure 5.1.

Fig. 5.2. Quantification of total soluble phenolic compounds in fresh and old preparations of *W. somnifera* extracts.



Legend: The labelled TLC plates are described as follows: A = crude extract (CE), B=free acid (FA), C= glycoside (Gly) and D = ester bound (EB) phenolics. Each plate lane number represented [1= *A. seyal* (fresh) extract, 2 = *A. seyal* (old) extract, 3 = *W. somnifera* (fresh) extract, 4 = *W. somnifera* (old) extract, and standard chemicals [5 = iso-ferulic, 6 = *p*-coumaric, 7 = 4H benzoic acid, 8 = gallic acid and 9 = synergic acid] as a reference compounds, respectively.

Fig. 5. 3. Thin layer chromatography of fresh and old preparations of *Acacia seyal* and *Withania somnifera* leaf extracts.

5.3.5 High performance liquid chromatography of fresh and old plant extracts

High performance liquid chromatography separation, identification and quantification of fresh and old extracts of *Acacia seyal* are depicted in table 5.6–5.9 and *Withania somnifera* in table 5.10–

5.13, respectively. Except the concentration of glycoside and ester bound phenolic compounds of *Acacia seyal* and *W. somnifera* extracts, no significant (P <0.05) variation was observed in CE, FA, and EB phenolics concentrations of fresh and old extract preparations of both plants. *Acacia seyal* extracts exhibited a diverse group of phenolic compounds [gallic acid, 3, 4 Dihydroxy benzoic acid, ferulic acid, caffeic acid, *p*-coumaric acid and salicylic acid]. Higher concentrations of gallic acid were obtained from both fresh (758.05 mg/ml) and old (948.73 mg/ml) preparations of *A. seyal* extracts (Table 5.9).

Table 5.6 Fresh and six month old preparations of *Acacia seyal* crude extract active compounds separation, identification and quantification using high performance liquid chromatography

HPLC peak (code)	Crude extract preparations											
	Fresh						Six months old					
	Retention time	Area of the peak	Height of the peak	Max. absorption (nm)	Compound name	[ ] (mg/ml)	Retention time	Area of the peak	Height of the peak	Max. absorption (nm)	Compound name	[ ] (mg/ml)
A	4.16o ± 0.01	22.36j ± 0.00	2.03j ± 0. 00	340	nd	nd	4.12o± 0.01	25.42k ± 0.01	1.94j ± 0.01	280	nd	nd
B	4.81n± 0.01	13.41l± 0.01	1.64k ± 0.01	280	nd	nd	6.13n ± 0.02	6.08mn ± 0.02	0.61l ± 0.02	280	Gallic acid	1.21 ± 0.01
C	6.22m ± 0.02	3.73o ± 0.01	0.54m ± 0.01	280	Gallic acid	1.01± 0.01	11.22m ± 0.01	3.96n ± 0.02	0.33n ± 0.01	280	3,4D	1.55 ± 0.01
D	11.23l ± 0.01	5.62n ± 0.01	0.44n ± 0.01	280	3,4 D	2.21 ± 0.01	15.87l ± 0.01	183.18d ± 0.01	22.66d ± 0.01	280	nd	nd
E	15.9k ± 0.01	33.02i ± 0.02	3.31h± 0.00	280	nd	nd	16.24k± 0.01	128.27g ± 0.14	16.07g ± 0.02	280	nd	nd
F	16.27j ± 0.01	138.2f ± 0.02	16.79d ± 0.01	280	nd	nd	24.16j ± 0.01	151.18f ± 0.01	17.09e ± 0.01	280	nd	nd
G	24.24i ± 0.01	155.02e ± 0.01	17.37c ± 0.00	280	nd	nd	26.34i ± 0.01	56.12i ± 0.02	3.52h ± 0.01	340	nd	nd
H	26.51h± 0.01	55.93h ± 0.01	3.47g ± 0.01	280	nd	nd	27.98h ± 0.01	6.44m ± 0.02	0.36m ± 0.01	280	Ferulic acid	1.11 ± 0.01
I	27.54g ± 0.01	19.12k ± 0.01	0.67l ± 0.01	325	Ferulic acid	0.91 ± 0.01	28.62g ± 0.01	877.92a ± 0.02	86.1a ± 0.02	280	nd	nd
J	28.73f ± 0.01	682.9a ± 0.02	65.78a ± 0.01	280	nd	nd	30.18f ± 0.01	178.53e ± 0.02	16.09f ± 0.02	280	nd	nd
K	30.32e ± 0.01	178.15d ± 0.01	16.62e ± 0.00	280	nd	nd	32.08e ± 0.01	8.42lm ± 0.02	0.67k ± 0.01	280	nd	nd
L	35.44d ± 0.01	216.7b ± 0.01	18.33b ± 0.00	280	nd	nd	35.28d ± 0.01	351.95b ± 0.07	29.23b ± 0.01	280	nd	nd
M	36.57c ± 0.17	68.88g ± 0.01	2.06i ± 0.01	280	nd	nd	36.47c ± 0.01	105.64h ± 0.02	2.40i ± 0.01	280	nd	nd
N	38.61b± 0.01	11.96m ± 0.01	0.53m ± 0.01	280	Salicylic acid	6.25 ± 0.01	38.38b ± 0.01	10.41l ± 0.49	0.38m ± 0.02	280	Salicylic acid	6.31 ± 0.02
O	41.18a ± 0.01	208.04c ± 0.01	16.31f ± 0.00	280	nd	nd	40.98a ± 0.01	326.26c ± 0.02	25.66c ± 0.02	280	nd	nd

Legend: In each column, means with the same letter are not significantly different. Unidentified chemical compounds in the phenolics family are designated by (nd) = not determined.

Table 5.7 Fresh and six month old preparations of *Acacia seyal* leaf extracts free acid active ingredients separation, identification and quantification of phenolic compounds using high performance liquid chromatography

HPLC peak (code)	Crude extract preparations											
	Fresh						Six months old					
	Retention time (RT)	Area of the peak	Height of the peak	Max. absorption (nm)	Compound name	[ ] (mg/ ml)	Retention time (RT)	Area of the peak	Height of the peak	Max. absorption (nm)	Compound name	[ ] (mg/ ml)
A	3.54a ± 0.01	61.63o ± 0.01	4.83j ± 0.04	280	nd	nd	3.54w ± 0.02	67.99v ± 0.04	4.97o ± 0.02	280	nd	nd
B	6.34b ± 0.02	644.25a ± 0.04	124.65a ± 0.02	280	Gallic acid	62.46a ± 0.03	6.11v ± 0.02	79.08t ± 0.02	3.8t ± 0.02	280	Gallic acid	60.63
C	11.51c ± 0.02	40.02v ± 0.02	1.75t ± 0.02	280	3,4 D	2.33d ± 0.03	11.12u ± 0.02	135.31m ± 0.02	6.27h ± 0.02	280	3,4 D	5.37
D	12.43d ± 0.01	19.55x ± 0.02	2.18s ± 0.01	325	nd	nd	12.05t ± 0.02	171.38k ± 0.02	5.79l ± 0.02	280	nd	nd
E	15.26e ± 0.02	102.44j ± 0.01	8.33g ± 0.01	280	nd	nd	15.27s ± 0.02	92.76r ± 0.02	6.47g ± 0.02	280	nd	nd
F	15.91f ± 0.03	465.12c ± 0.01	29.09c ± 0.02	280	nd	nd	15.37s ± 0.04	829.47a ± 0.02	77.14a ± 0.02	280	nd	nd
G	16.26g ± 0.01	292.22d ± 0.02	19.62d ± 0.02	280	nd	nd	16.26r ± 0.02	223.43g ± 0.02	15.89c ± 0.02	280	nd	nd
H	16.36h ± 0.03	545.45b ± 0.02	41.57b ± 0.02	280	nd	nd	16.65q ± 0.02	260.43d ± 0.02	18.78b ± 0.02	280	nd	nd
I	18.19i ± 0.02	44.21s ± 0.02	2.75q ± 0.02	280	nd	nd	17.92p ± 0.02	61.17x ± 0.02	5.87k ± 0.02	280	Nd	nd
J	19.32j ± 0.02	60.85p ± 0.02	2.64r ± 0.01	280	Caffeic acid	1.39e ± 0.02	18.59o ± 0.02	67.04w ± 0.02	4.38s ± 0.02	325	Caffeic acid	280 ± 0.02
K	21.73k ± 0.01	183.29g ± 0.02	4.57l ± 0.02	280	nd	nd	21.86n ± 0.02	84.68s ± 0.02	4.82p ± 0.02	280	nd	nd
L	24.18l ± 0.02	85.13l ± 0.01	4.32m ± 0.02	280	nd	nd	24.21m ± 0.02	233.64f ± 0.02	5.64m ± 0.02	280	nd	nd
M	25.85m ± 0.02	38.65w ± 0.01	3.04o ± 0.02	325	P-coumaric acid	5.74c ± 0.04	25.68l ± 0.02	75.47u ± 0.02	5.38n ± 0.02	325	P-coumaric acid	3.56 ± 0.03
N	26.25n ± 0.02	154.12h ± 0.02	7.21h ± 0.02	280	nd	nd	26.03k ± 0.02	131.83o ± 0.02	7.64f ± 0.02	280	nd	nd
O	27.02o ± 0.02	185.61f ± 0.02	8.95f ± 0.02	325	Ferulic acid	1.12f ± 0.01	27.04j ± 0.02	187.75j ± 0.02	10.26e ± 0.02	325	Ferulic acid	4.2 ± 0.02
P	27.57p ± 0.02	42.47u ± 0.02	5.27i ± 0.01	280	nd	nd	27.59i ± 0.02	390.56b ± 0.02	13.13d ± 0.02	280	nd	nd
Q	28.62q ± 0.02	224.82e ± 0.01	19.38e ± 0.03	280	nd	nd	29.21h ±0.02	105.09q ± 0.02	5.81l ± 0.02	280	nd	nd
R	29.55r ± 0.02	80.82n ± 0.02	2.75q ± 0.02	280	nd	nd	30.72g ± 0.02	129.31p ± 0.02	4.74q ± 0.02	280	nd	nd
S	32.09s ± 0.02	46.03r ± 0.02	2.17s ± 0.02	280	nd	nd	32.14f ± 0.02	133.21n ± 0.02	6.11i ± 0.02	280	nd	nd
T	35.27t ± 0.01	87.29k ± 0.02	4.64k ± 0.03	280	nd	nd	35.33e ± 0.03	257.78e ± 0.02	5.93j ± 0.02	280	nd	nd
U	36.34u ± 0.02	55.72q ± 0.02	2.84p ± 0.02	340	nd	nd	36.4d ± 0.02	215.42h ± 0.02	5.93j ± 0.02	280	nd	nd
V	38.11v ± 0.03	42.83t ± 0.01	1.15u ± 0.01	280	Salicylic acid	6.23b ± 0.03	38.98c ± 0.01	208.44i ± 0.02	3.65v ± 0.03	280	Salicylic acid	2.58
W	40.97w ± 0.03	82.21m ± 0.02	2.64r ± 0.03	280	nd	nd	41.06b ± 0.02	314.35c ± 0.02	4.69r ± 0.02	280	nd	nd
X	45.43x ± 0.01	104.83i ± 0.02	3.66n ± 0.02	280	nd	nd	45.82a ± 0.02	148.29l ± 0.02	3.72u ± 0.02	280	Nd	nd

Legend: In each column, means with the same letter are not significantly different. Unidentified chemical compounds in the phenolics family are designated by (nd) = not determined.

Table 5.8 Fresh and six month old preparations of *Acacia seyal* leaf extract glycoside active ingredients separation, identification and quantification using high performance liquid chromatography

HPLC peak (code)	Crude extract preparations											
	Fresh						Six months old					
	Retention time (RT)	Area of the peak	Height of the peak	Max. absorption (nm)	Compound name	[ ] (mg/ ml)	Retention time (RT)	Area of the peak	Height of the peak	Max. absorption (nm)	Compound name	[ ] (mg/ ml)
A	6.1t± 0.02	7.74t ± 0.02	1.08t ± 0.02	280	nd	nd	4.3j ± 0.01	244.95d ± 0.04	4.74f ± 0.02	280	nd	nd
B	11.21s ± 0.02	42.6s1± 0.02	2.84r ± 0.01	280	3,4 D	1.67d ± 0.02	6.03i ± 0.02	20.46j ± 0.03	1.34j ± 0.02	280	Gallic acid	23.74 ± 0.01
C	15.89r ± 0.02	812.95i ± 0.02	86.56f ± 0.03	280	nd	nd	15.92h ± 0.02	1694.77a ± 0.02	209.18a ± 0.02	280	nd	nd
D	16.23q ± 0.02	625.46j ± 0.02	66.94h ± 0.03	280	nd	nd	-	-	-	-	-	-
E	16.63p ± 0.02	331.85o ± 0.01	29.14l ± 0.04	280	nd	nd	-	-	-	-	-	-
F	19.16o ± 0.02	51.14r ± 0.02	2.64s ± 0.03	280	Caffeic acid	6.36b ± 0.02	-	-	-	-	-	-
G	21.99n ± 0.02	53.24q ± 0.02	4.57q ± 0.02	280	nd	nd	-		-	-	-	-
H	24.15m ± 0.02	1135.75g ± 0.03	121.19d ± 0.01	280	nd	nd	-	-	-	-	-	-
I	25.28l ± 0.02	418.91n ± 0.02	21.77o ± 0.02	280	P-coumaric acid	4.73c ± 0.03	-	-	-	-	-	-
J	25.75k ± 0.02	484.16l ± 0.01	32.13k ± 0.01	280	nd	nd	-	-	-	-	-	-
K	26.35j ± 0.03	603.09k ± 0.02	39.22j ± 0.02	340	nd	nd	26.06g ± 0.02	61.81f± 0.04	5.87e ± 0.01	280	Nd	nd
L	27.63i ± 0.03	239.79p ± 0.02	16.64p ± 0.02	280	Ferulic acid	1.06e ± 0.06	-	-	-	-	-	-
M	28.53h ± 0.04	13958.32a ± 0.02	1230.15a ± 0.03	280	nd	nd	28.69f ± 0.02	1420.74b ± 0.02	144.65b ± 0.03	280	nd	nd
N	30.16g ± 0.02	1229.36f ± 0.02	99.92e ± 0.02	280	nd	nd	30.28e ± 0.03	28.67i ± 0.02	2.65h ± 0.02	280	nd	nd
O	35.18f ± 0.03	13621.36b ± 0.02	954.11b ± 0.02	280	nd	nd	32.19d ± 0.02	52.31g ± 0.02	4.37g ± 0.03	280	nd	nd
P	36.43e ± 0.01	4644.51d ± 0.02	84.41g ± 0.02	280	nd	nd	35.39c ± 0.02	259.29c ± 0.02	21.45c ± 0.04	280	nd	nd
Q	38.15d ± 0.02	430.45m ± 0.02	25.69m ± 0.02	280	Salicylic acid	13.17a ± 0.02	36.59b ± 0.01	44.34h ± 0.03	1.43i ± 0.03	280	nd	nd
R	40.92c ± 0.02	9982.26c ± 0.06	656.39c ± 0.02	280	nd	nd	41.11a ± 0.02	177.11e ± 0.03	14.44d ± 0.02	280	nd	nd
S	43.08b ± 0.01	2016.62e ± 0.05	48.79i ± 0.02	280	nd	nd	-	-	-	-	-	-
T	47.23a ± 0.02	1130.93h ± 0.02	24.84n ± 0.03	280	nd	nd	-	-	-	-	-	-

Legend: In each column, means with the same letter are not significantly different. Unidentified chemical compounds in the phenolics family are designated by (nd) = not determined.

Table 5.9 Fresh and six month old preparations of *Acacia seyal* leaf extracts ester bound phenolic compounds active ingredients separation, identification and quantification using high performance liquid chromatography

HPLC peak (code)	Crude extract preparations											
	Fresh						Six months old					
	Retention time (RT)	Area of the peak	Height of the peak	Max. absorption (nm)	Compound name	[ ] (mg/ml)	Retention time (RT)	Area of the peak	Height of the peak	Max. absorption (nm)	Compound name	[ ] (mg/ml)
A	6.35o ± 0.02	6052.87a ± 0.02	1131.75a ± 0.03	280	Gallic acid	758.05a ± 0.03	6.34o ± 0.02	7497.46a ± 0.02	1390.06a ± 0.02	280	Gallic acid	948.73 ± 0.93
B	11.09n ± 0.02	4.33o± 0.02	0.32o± 0.01	280	3, 4 D	1.68d ± 0.01	11.09n ± 0.01	3.54o ± 0.02	0.37o ± 0.02	325	3, 4 D	1.35 ± 0.04
C	15.90m ± 0.02	404.57g ± 0.03	33.51g ± 0.01	280	nd	nd	15.87m ± 0.02	2313.66b ± 0.03	270.64b ± 0.02	280	nd	nd
D	16.64l ± 0.02	608.59e ± 0.02	54.1e ± 0.02	280	nd	nd	16.24l ± 0.02	214.71k± 0.02	17.07k ± 0.02	280	nd	nd
E	19.91k± 0.02	25.53n ± 0.02	2.88 m± 0.02	340	Caffeic acid	9.23b ± 0.02	18.84k ± 0.02	8.78 n± 0.02	0.85n ± 0.02	340	Caffeic acid	9.91 ± 0.03
F	24.16j ± 0.02	192.48l ± 0.02	21.32i ± 0.02	280	nd	nd	24.16j ± 0.02	280.86j ± 0.02	28.12g ± 0.02	280	nd	nd
G	25.79i ± 0.02	270.20h ± 0.02	22.97h ± 0.02	280	nd	nd	26.34i ± 0.03	31.12m ± 0.02	1.64m ± 0.02	280	nd	nd
H	27.61h ± 0.02	39.83m ± 0.01	2.66 n± 0.02	340	Ferulic acid	0.72e ± 0.55	27.55h ± 0.02	78.61l ± 0.03	5.29l ± 0.02	340	Ferulic acid	1.16 ± 0.12
I	28.61g ± 0.01	1027.02c ± 0.02	98.8c ± 0.01	280	nd	nd	28.60g ± 0.02	1330.92d ± 0.03	124.58d ± 0.02	280	nd	nd
J	30.16f ± 0.01	201.05k ± 0.03	18.31j ± 0.02	280	nd	nd	30.16f ± 0.02	281.15i ± 0.03	24.06i ± 0.03	280	nd	nd
K	31.74e ± 0.02	568.67f ± 0.02	48.26f ± 0.02	280	nd	nd	31.73e ± 0.02	679.64f ± 0.02	59.34f ± 0.02	280	nd	nd
L	35.24d ± 0.02	1333.35b ± 0.02	108.52b ± 0.02	280	nd	nd	35.24d ± 0.02	1834.86c ± 0.02	148.03c ± 0.03	280	nd	nd
M	38.72c ±0.02	246.65i ± 0.03	17.64k ± 0.02	280	Salicylic acid	3.04c ± 0.03	38.74c ± 0.02	347.67h ± 0.02	24.34h ± 0.03	280	Salicylic acid	4.11 ± 0.02
N	40.93b ± 0.01	850.33d ± 0.03	61.67d ± 0.02	280	nd	nd	40.96b ± 0.02	1172.71e ± 0.03	83.86e± 0.03	280	nd	nd
O	43.92a ± 0.02	238.06j ± 0.02	12.97l ± 0.02	280	nd	nd	43.93a ± 0.02	381.36g ± 0.02	17.67j ± 0.02	280	nd	nd

Legend: In each column, means with the same letter are not significantly different. Unidentified chemical compounds in the phenolics family are designated by (nd) = not determined.

Table 5.10 Identification and quantification of fresh and six months old crude extracts of *Withania somnifera* using high performance liquid chromatography

HPLC peak (code)	Crude extract preparations											
	Fresh						Six months old					
	Retention time (RT)	Area of the peak	Height of the peak	Max. absorption (nm)	Compound name	[ ] (mg/ ml)	Retention time (RT)	Area of the peak	Height of the peak	Max. absorption (nm)	Compound name	[ ] (mg/ ml)
A	2.09g ± 0.03	2.39g± 0.02	0.46e ± 0.03	280	nd	nd	3.79e ± 0.02	57.19a ± 0.02	1.87a ± 0.02	280	nd	nd
B	3.76f ± 0.02	41.49b ± 0.02	1.85b ± 0.04	280	nd	nd	9.10d ± 0.02	1.69d ± 0.02	0.26d ± 0.02	280	nd	nd
C	5.31e ± 0.02	198.65a ± 0.03	30.89a ± 0.02	280	nd	nd	-	-	-	-	-	-
D	6.36d ± 0.02	2.54f ± 0.04	0.48e ± 0.03	280	nd	nd	-	-	-	-	-	-
E	26.36c ± 0.02	5.54d ± 0.04	0.69c ± 0.02	280	nd	nd	28.72c ± 0.02	3.66c ± 0.02	0.31c ± 0.02	280	nd	nd
F	35.29b ± 0.03	5.44e ± 004	0.35f ± 0.04	280	nd	nd	35.65b ± 0.02	11.21b± 0.02	0.40b ± 0.02	280	nd	nd
G	40.96a ± 0.02	18.94c ± 0.02	0.56d ± 0.03	280	nd	nd	41.16a ± 0.02	3.65c ± 0.02	0.30c ± 0.01	280	nd	nd

Legend: In each column, means with the same letter are not significantly different. Unidentified chemical compounds in the phenolics family are designated by (nd) = not determined.

Table 5.11 Identification and quantification of free acid by high performance liquid chromatography from fresh and six months old crude extracts of *Withania somnifera*

HPLC peak (code)	Crude extract preparations											
	Fresh						Six months old					
	Retention time (RT)	Area of the peak	Height of the peak	Max. absorption (nm)	Compound name	[ ] (mg/ml)	Retention time (RT)	Area of the peak	Height of the peak	Max. absorption (nm)	Compound name	[ ] (mg/ml)
A	3.52k ± 0.02	52.28d ± 0.02	4.56c ± 0.02	280	nd	nd	3.53j ± 0.02	50.28d ± 0.02	4.54c ± 0.02	280	nd	nd
B	5.29j ± 02	11.31h± 0.02	1.82f ± 0.02	280	nd	nd	6.34i ± 0.02	2.00j ± 0.02	0.39g ± 0.02	280	nd	nd
C	11.32i ± 0.03	3.56k ± 0.02	0.44i ± 0.02	280	3, 4 D	1.39 ± 0.01	11.13h ± 0.01	5.36h ± 0.02	0.53f ± 0.02	280	3, 4 D	2.01± 0.02
D	20.86h ± 0.02	672.16a ± 0.03	5.21b ± 0.03	280	nd	nd	18.18g ± 0.02	512.59a ± 0.02	5.19b ± 0.02	280	nd	nd
E	23.54g ± 0.03	77.17c ± 0.02	2.12d ± 0.02	280	nd	nd	20.87f ± 0.02	494.57b ± 0.02	5.42a ± 0.02	280	nd	nd
F	27.43f ± 0.02	99.39b ± 0.02	9.42a ± 0.02	325	nd	nd	23.01e ± 0.02	72.92c ± 0.02	2.99d ± 0.02	280	nd	nd
G	29.25e ± 0.01	28.43e ± 0.01	1.95e ± 0.02	280	nd	nd	27.78d ± 0.03	2.38i ± 0.02	0.25i ± 0.02	325	nd	nd
H	33.19d ± 0.3	5.31j ± 0.02	0.35j ± 0.01	280	nd	nd	32.06c ± 0.03	5.64g ± 0.02	0.29h ± 0.02	280	nd	nd
I	38.37c ± 0.02	6.37i ± 0.02	0.45i ± 0.02	280	Salicylic acid	6.09 ± 0.02	38.37b ± 0.02	18.31e ± 0.01	0.31h ± 0.02	280	Salicylic acid	6.91± 0.02
J	39.79b ± 0.02	11.67g ± 0.02	0.94g ± 0.01	280	nd	nd	39.78a ± 0.02	10.55f ± 0.02	0.74e ± 0.01	280	nd	nd
K	43.55a ± 0.02	15.41f ± 0.02	0.78h ± 0.02	280	nd	nd	-	-	-	-	-	-

Legend: In each column, means with the same letter are not significantly different. Unidentified chemical compounds in the phenolics family are designated by (nd) = not determined.

Table 5.12 Fresh and six months old preparations of *Withania somnifera* extract glycoside phenolic compounds active ingredients separation, identification and quantification using high performance liquid chromatography

HPLC peak (code)	Crude extract preparations											
	Fresh						Six months old					
	Retention time (RT)	Area of the peak	Height of the peak	Max. absorption (nm)	Compound name	[ ] (mg/ml)	Retention time (RT)	Area of the peak	Height of the peak	Max. absorption (nm)	Compound name	[ ] (mg/ml)
A	4.04g ± 0.02	129.97b ± 0.01	2.33a ± 0.01	340	nd	nd	3.7 ± 0.01	71.83 ± 0.02	2.68 ± 0.01	340	nd	nd
B	6.05f ± 0.02	15.29d ± 0.02	2.12b ± 0.03	280	nd	nd	5.11 ± 0.01	24.58 ± 0.02	1.52 ± 0.02	280	nd	nd
C	11.21e± 0.01	2.08g ± 0.02	0.31f ± 0.02	280	3, 4 D	8.09	10.97 ± 0.02	3.74 ± 0.01	0.45 ± 0.03	280	3, 4 D	1.46 ± 0.02
D	19.24d ± 0.03	3.75f ± 0.02	0.43g ± 0.01	325	Caffeic acid	5.66	-	-	-	-	nd	nd
E	35.63c ± 0.02	24.02c ± 0.02	0.77d ± 0.01	280	nd	nd	-	-	-	-	nd	nd
F	37.37b ± 0.02	164.21a ± 0.02	1.26c ± 0.02	280	nd	nd	36.92 ± 0.03	7.77 ± 0.02	0.57 ± 0.01	280	nd	nd
G	40.15a ± 0.03	10.59e ± 0.02	0.59e ± 0.02	280	nd	nd	39.75 ± 0.02	8.19 ± 0.02	0.56 ± 0.02	280	Salicylic acid	5.86 ± 0.03

Legend: In each column, means with the same letter are not significantly different. Unidentified chemical compounds in the phenolics family are designated by (nd) = not determined.

Table 5.13 Fresh and six months old preparations of *Withania somnifera* extracts ester bound phenolic compounds active ingredients separation, identification and quantification using high performance liquid chromatography

HPLC peak (code)	Crude extract preparations											
	Fresh						Six months old					
	Retention time (RT)	Area of the peak	Height of the peak	Max. absorption (nm)	Compound name	[ ] (mg/ ml)	Retention time (RT)	Area of the peak	Height of the peak	Max. absorption (nm)	Compound name	[ ] (mg/ ml)
A	3.52k ± 0.01	39.44a ± 0.02	4.20a ± 0.01	340	nd	nd	3.53i ± 0.02	41.56a ± 0.02	4.43a± 0.02	280	nd	nd
B	6.32j ± 0.02	1.88k ± 0.02	0.35f ± 0.02	280	Gallic acid	0.00	6.36h ± 0.03	22.76b ± 0.02	4.44a ± 0.02	280	Gallic acid	0.00
C	7.87i ± 0.02	6.53h ± 0.01	1.07c ± 0.02	280	nd	nd	7.87g ± 0.02	7.61e ± 0.01	1.26b ± 0.01	280	nd	nd
D	11.08h ± 0.01	9.63f ± 0.02	1.08c ± 0.01	280	3, 4 D	3.81c± 0.01	11.08f ± 0.01	11.34d ± 0.01	1.21c ± 0.01	280	3, 4 D	2.90a ± 0.03
E	18.19g ± 0.02	9.75e ±0.02	1.07c ± 0.02	280	nd	nd	18.22e ± 0.01	5.63h ± 0.02	0.61d ±0.02	280	Vanilic acid	1.28b ± 0.02
F	19.01f ± 0.01	12.43d ± 0.02	1.46b ± 0.02	325	Caffeic acid	9.54a ±0.02	-	-	-		-	-
G	25.29e ± 0.02	8.49g ± 0.02	0.86d ± 0.02	280	nd	nd	-	-	-		-	-
H	27.84d ± 0.01	1.92j ± 0.02	0.19g ± 0.02	340	Ferulic acid	0.00	27.51d± 0.02	4.95i ± 0.01	0.44e ± 0.02	325	Ferulic acid	0.00
I	33.13c ± 0.02	16.89b ± 0.02	0.75e ± 0.03	340	nd	nd	31.92c ± 0.02	7.44f ±0.02	0.40f ± 0.02	280	nd	nd
J	39.81b ± 0.02	13.5c ± 0.02	1.02c ± 0.06	280	Salicylic acid	6.42b ± 0.02	35.97b ± 0.02	12.82c ± 0.02	0.38f ± 0.02	280	nd	nd
K	41.91a ± 0.01	3.96i ± 0.02	0.29f ± 0.01	325	nd	nd	41.19a ± 0.01	6.44g ± 0.02	0.44e± 0.01	280	nd	nd

Legend: In each column, means with the same letter are not significantly different. Unidentified chemical compounds in the phenolics family are designated by (nd) = not determined.

5.4 DISCUSSION

Plants have great potential to synthesize aromatic substances, most of which are phenolics and their oxygen-substituted derivatives (Cowan, 1999). The search for potential ethnobotanical compounds from plant material requires intensive *in vitro* screening of plant extracts. In this study, 37 extracts from 23 plant species collected from three citrus growing regions of Ethiopia were screened for their antimicrobial activity. Twenty-one plant extracts from 13 species (56%) showed some degree of antimicrobial activity to at least one of the pathogens challenged. Seven of these species [*A. aspera*, *T. terrestris*, *W. somnifera*, *A. seyal*, *D. oliver*, *C. quadrangularis* and *M. jalapa*] were, to our knowledge, not previously reported for their ethnobotanical potential. According to Rojas et al. (2003), this report indicates the high therapeutic potential of tropical flora where numerous species are yet to be documented and investigated.

Some plant extracts demonstrated strong selective antifungal and antibacterial activities, which may indicate their potential as antimicrobial products. *In vitro* tests showed eight of these extracts [leaf extracts of *D. oliveri*, *T. minuta*, *R. chalepensis*, *S. incanum* and *A. indica*; seed extracts of *S. incanum* and root extracts of *A. aspera* and *A. seyal*] demonstrated antimicrobial activity to both fungal and bacterial pathogens. A further nine [leaf extracts of *T. terrestris* and *T. indica*; stem extracts of *N. tabacum* and *W. somnifera* and *C. quadrangularis*; seed extracts of *S. nigrum*, *N. tabacum* and *T. indica* and root extracts of *D. oliver*] exhibited selective antifungal activity only, and four [leaf extracts of *A. aspera*, *W. somnifera*, *A. seyal* and root extracts of *M. jalapa*] showed selective antibacterial activity. The plant extracts tested in this study were highly effective against the Gram-positive bacterium *S. epidermidis* compared to the Gram-negative bacteria. Differences in the antimicrobial effect of the plant extracts tested against Gram-positive and Gram-negative bacteria may be due to differences in permeability barriers. Similar reports indicate susceptibility of the Gram-positive bacterium *S. epidermidis* to other plant extracts such as *Cordia curassavica, Lantana achyranthifolia* and *Lippia graveolens* (Hernandez et al., 2003) and seed extracts of *Syzygium jambolanum* (Chandrasekaran and Venkatesalu, 2004). In this study, the inhibition halo formed by the root extract of *M. jalapa* showed high inhibitory activity against *S. epidermidis*. The inhibitory activity found in this study was more pronounced than that reported by Hernandez et al. (2003) when he evaluated certain plants for their antimicrobial activities against several bacterial pathogens. The inhibitory effect of *M. jalapa* was at a similar level of effectiveness as Rifampicin.

The antimicrobial activity of plant extracts depends on the type and amount of phenolics present in the plant tissue and the pathogen's inherent resistance (Martini et al., 2004). Quantitative

information obtained from the Folin-Ciocalteu method provides information about the amount of soluble phenolics in the plant extract. *A. seyal*, unlike any other plant extract tested, had a high content of equivalent mg Gallic acid/g dry weight both in fresh and old extract preparations. The result is also supported by HPLC analyses due to the presence of high concentration of gallic acid, para-coumaric acid, ferulic acid, caffeic acid, 3,4 dihydroxy benzoic acid and salicylic acid. This may attribute to its strong antimicrobial activity as determined when oxidized to natural aromatic polymer compounds (cinnamic acid derivatives) to inhibit auto-oxidation of oils and fats in the host tissue (Cowan, 1999). It could be due to better extraction by the methanolic solvent system as compared to water (data not indicated here) (Ozkan et al., 2004).

Although there was a significant (P <0.05) difference in the phenolic concentration of fresh and old preparations of *W. somnifera* extracts, the total phenolic concentration was very low unlike *A. seyal* extracts. A result from HPLC analysis has also supported this fact that phenolic compounds are present at very low concentrations. According to Rahman et al. (1991), the majority of compounds in *W. somnifera* extracts are withanolides, glycowithanolides with a very low proportion of alkaloids (0.2%). These results validate the importance of other compounds in plant extracts antipathogenic activity.

The R_f value of the selected plant extracts depicted on the TLC chromatogram correspond with the value of different phenolics that may be involved in the antipathogenic activity of the plant material (Block et al., 1958; Smith, 1960). Plant extracts that exhibit broad-spectrum *in vitro* activity against microbial pathogens, i.e. *W. somnifera*, *A. seyal* and *M. jalapa*, showed no visible band formation in one or more of the separation solvent systems under 254 or 366 nm. According to Harborne (1964), measurements of the ultraviolet absorption spectrum may be affected by etherification and/ or glycosylation of the hydroxyl group to detect phenolics under given UV light spectra. Alternatively, this could be an indication for the presence of protein conjugated antimicrobial compounds with non-specific and/ or synergistic interactions in the system (Cowan, 1999).

The MIC value of the eight plant extracts selected in this study ranged between 1:1 and 1:5 indicating the strength of their active compounds. According to Cruickshank and Perrin (1964), toxic phenolic compounds present in such low concentrations may have a stimulatory effect on pathogen growth. In this study, some plant extracts were ineffective against some of the test pathogens used. Amongst these, *E. coli* and one strain of *E. carotovora* (UPErc-2) proved highly resistant to all plant extracts tested. This characteristic may be attributed to their similar replication

origin, being under the same family, Enterobacteriaceae (Takeda et al., 1982). Similar results were reported for *E. coli* by Hernandez et al. (2003), which described possible development of resistance by the bacteria. To our knowledge, resistance development by *E. carotovora*$_2$ has not been reported in previous studies. On the other hand, the *X. campestris*$_2$, which showed resistance to all antibiotics tested, was significantly inhibited by *A. seyal*, *W. somnifera*, *T. minuta* and *M. jalapa*. To our knowledge this is also the first report of antimicrobial activity of these plant extracts against the pathogen. Although the dilution ratio and antimicrobial efficacy varies from one plant to another, about 65% of the plant extracts were found effective against several bacterial strains screened.

Preliminary *in vivo* tests with some selected plant extracts showed remarkable control of fruit decay due to *P. digitatum* in South Africa (data not included in this study), which may indicate the promising potential of the plant extracts for postharvest disease control, especially for the citrus industry. Future research advances on this aspect is important to determine the active chemical compounds of these plant extracts for commercial use.

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CHAPTER SIX

CONTROL OF *PENICILLIUM DIGITATUM* GROWTH ON CITRUS FRUIT USING TWO PLANT EXTRACTS AND THEIR MODE OF ACTION

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Submitted to Journal of Phytoparasitica for publication as:
Sissay B. Mekbib, Thierry J.C. Regnier, and Lise Korsten. 2006. Control of *Penicillium digitatum* growth on citrus fruit using two plant extracts and their mode of action.

Abstract

Extracts from two plant species *Withania somnifera* and *Acacia seyal* selected from Ethiopia were used in this study to evaluate their potential as a natural biopesticide and to study their mode of action. Ethanolic extracts of these plants were tested *in vivo* on citrus fruits for their efficacy to control *Penicillium digitatum* when applied as a spray and wound application. Up to 70% of wound inoculated fruits did not develop decay symptoms for up to 21 days of storage at 25 ºC and >85%RH. Soluble phenolic concentrations, which inversely correlated with an increase of cell wall bound phenolics was found in treated fruit. Scanning electron microscopy revealed deposition of crystal plant material sticking to the pathogen and around the wound site. The application of plant extracts increased the epiphytic background total microbial population but decreased diversity.

***Key words*:** Soluble phenolics; Insoluble phenolics; Postharvest disease; Host resistance; Ferulic acid

6.1 INTRODUCTION

Pre- and postharvest pathogens negatively affect the quality of citrus fruits (Eckert and Brown, 1986). Mould decay caused by *Penicillium digitatum* Sacc is the main postharvest disease affecting fruit quality and is initiated through injuries before or during harvesting, packing and processing (Eckert and Brown, 1986). The importance and impact of wound pathogens may differ from country to country. In countries where protection and proper handling of fresh fruit is inadequate, losses during transit and storage may be as high as 50% of the harvested crop (Tripathi and Dubey, 2004). Chemical control with imazalil, quazatine and thiabindazole remains the main options to reduce postharvest diseases (Poppe *et al*., 2003). The commercial use of postharvest fungicides has become restricted because of public health concerns (Unnikrishnan and Nath, 2002), development of pathogen resistance (Fogliata *et al*., 2001; Dianz *et al*., 2002) and environmental issues (Janisiewicz and Korsten, 2002). This effect instigated the search for natural control options using plant extracts and/or microbial antagonists.

The potential of plant extracts for control of plant diseases have long been recognized (Ark and Thompson, 1959). There are about 250 000 species of higher plants of which only 5-15% have been studied for their therapeutic value (Rojas *et al*., 2003). The use of plants for human disease control attracts more attention compared to its use in plant and animal disease control (Hernandez *et al*., 2003; Newton *et al*., 2002; Cano and Volpato, 2004). In crop protection studies, various natural plant products have been identified and used to control postharvest diseases of fruits and vegetables.

The use of volatile compounds [*Hinokitiol* (*β*-thujaplicin) from the roots of *Hiba arboruitae* (Japanese cypress) against *Botrytis cinerea* Pers ex Fr and *Alternaria alternate* (Fr.) Keissler on eggplant and pepper fruits (Fallik and Grinberg, 1992), strawberry volatiles against postharvest fungal pathogens (Vaugh *et al*., 1993; Moline *et al*., 1997; Droby *et al*., 1999); glucosinolates from mustard and horseradish against microbial pathogens (Ishiki *et al*., 1992), citral against *P. digitatum*, *Penicillium italicum* Wehmer and *Geotrichum candidum* Link ex Pers (Klieber *et al*., 2002) and garlic against citrus green and blue moulds (Obagwu and Korsten, 2003)] are indications of the potential use of plant extracts for plant disease control.

The activity of natural plant products on the host tissue may involve direct interaction with the pathogen or induction of host resistance (Capdeville *et al*., 2002; Porat *et al*., 2002). The

mechanism involved in the former direct host reaction however is less understood (Porat *et al*., 2002). Host resistance induction on the other hand may involve several complex mechanisms including hypersensitive responses, build up of cell wall barriers, increase production of phytoalexins, accumulation of pathogenesis-related (PR) proteins, and fungal cell wall hydrolases (El-Ghaouth *et al*., 2002; Porat *et al*., 2002).

In this particular study, the antifungal activity and mode of action of two selected plant extracts *Withania somnifera* L. Dunal (code H_2), *Acacia seyal* Del var. Seyal (code I_1) were studied for preventive application against *P. digitatum* decay on citrus. Information of these plants for their use in plant disease control in general and postharvest use in particular is lacking. Limitations in the natural distribution of plants and/or the youngness of the field towards making use of plants for postharvest disease control may hinder their use.

The aim of this study was to evaluate the efficacy of the two selected plant extracts by wound and spray treatment applications and to investigate the mode of action involved in the healing mechanisms of the fruit wound against green mould. The non-target effect of the plant extracts on the citrus micro-biota was also evaluated.

6.2. MATERIALS AND METHODS

6.2.1. Fruits

Untreated freshly harvested Valencia fruits were collected from Rustenburg citrus packhouse, Northwest Province, South Africa. Fruits were surface sterilized with sodium hypochlorate (1%) for two minutes and air-dried before use.

6.2.2. The pathogen

The pathogen, *P. digitatum* was obtained from the culture collection of Plant Pathology Laboratories, University of Pretoria, South Africa and its pathogenicity was confirmed. The pathogen was grown on Potato Dextrose agar (PDA) (Biolab, Merk, Johannesburg, South Africa) at 25°C. Ten to twenty milliliters of sterilized distilled water was added to the surface of a 14 day old culture, surface rubbed with a glass rod and the collected spore concentration was determined using a haemacytometer. A conidial suspension (10^5 conidia ml^{-1}) was prepared (Janisiewicz *et al*., 2000) and used immediately and/ or stored in the fridge (0-4°C) until further use.

6.2.3. Plant materials

Two species of plant samples *A. seyal* and *W. somnifera* were collected from Ethiopia, prepared, imported (Permit No. P0017192) and processed as described in chapter 5.

6.2.4. Plant extraction

A methanol/ acetone/ water (7:7:1, v:v) solvent was used as extraction system (Regnier and Macheix, 1996). Three successive extractions were conducted from the dried plant powder (1:20 w/v). The 1st and 2nd extraction suspension were mixed with a vortex (VM-300) and placed on a rotary shaker for one hour at 170 rpm. Samples were cold centrifuged in a micro-centrifuge (Denver instrumental Company, USA) at 3913 x g for 10 minutes. The 3rd extraction was placed over night on the rotary shaker and centrifuged as described above. The combined supernatants were concentrated to 1 ml under vacuum and freeze-dried for 48h. Tubes were refilled uniformly with sterilized distilled water to a volume of 10 ml and re-sterilized using a hypodermic syringe driven filter paper (0.22 µm pore size). Samples were either immediately used or kept in the refrigerator at 4 ºC ± 1°C until further use.

6.2.5. *In vivo* antifungal assay

In vivo preventive antifungal activities of plant extracts were tested using the method described by (Poppe *et al*., 2003), with some modifications. Wound (3 x 3 mm) and/ or spray applications of extracts were applied to the fruit 12 h prior to challenging inoculation with the pathogen. Each fruit was wounded on the opposing sides of the fruit on the middle between the stem and styler end of the fruit. Ten percent of the original concentration of the plant extracts was used indiscriminately in all trials. The pathogen concentration was standardized at 10^5 conidia ml^{-1}. For the fruit wound (FW) experiment the following treatments were included: FW only, wounding followed by application of *P. digitatum* (10^5 spore ml^{-1}) (30µl) only, wounding followed by *W. somnifera* extract (30µl) only, wounding followed by *A. seyal* extract (30µl) only, wounding followed by *W. somnifera* challenged with *P. digitatum* after 12 h of application of the extract and wounding followed by ethanolic extract of *A. seyal* challenged with *P. digitatum* after 12 h of application of the extract. Wounding followed by the application of commercial chemicals [decodone (Greifswald, Germany) and thiabendazole (Tecto 90, Johannesburg) 1000ppm for 30 sec] challenged with *P. digitatum* was included for comparison.

For spray experiment the following treatments were included: fruit surface spraying with *P.*

digitatum (10^5 spore ml^{-1}) only, surface spraying with *W. somnifera* extract only, surface spraying with *A. seyal* extract only, surface spraying with *W. somnifera* followed by drying and spraying with *P. digitatum* (10^5 spore ml^{-1}) after 12 h of application of the extract and surface spraying with *A. seyal* and challenged with *P. digitatum* after 12 h of application of the extract. Spray application of commercial chemicals [decodone and thiabendazole, 1000ppm for 30 sec] followed by the application of *P. digitatum* was included for comparison.

For each of the wound and spray treatment, twenty fruits were used and the experiment was done in triplicate and repeated once. Treated fruits were packed in boxes and incubated at 25 ºC with >85% RH for 21 days. Evaluation was done every two days and data was recorded as number of lesions developing. Efficacy of treatment application was determined according to Vero *et al*. (2002).

6.2.6. Non-target effect of plant extracts on orange surface microbial flora

The non-target effect of the plant extracts on the natural fruit micro-flora was evaluated by determining the total microbial count and the population of bacteria, yeast and mycelial fungi. The natural microflora background was determined on freshly harvested Valencia fruits and on fruits spray treated with extracts as described in section 2.5 and stored for 21 days at 25 ºC. Nine fruits were randomly selected from three boxes per treatment before and after 21 days of storage. Each fruit was placed in 500 ml Ringer's (Merck, South Africa) and sonicated for 30 sec. The wash water was filter sterilized with a membrane (0.45 µm pore size) under vacuum. A filter membrane was placed in 10 ml Ringer's and serially diluted. A 100µl of each diluted sample was spread plated on three different media [PDA, Standard 1 Nutrient agar (STD-1 NA) and Malt Extract Agar (MEA), each of which were amended with 0.002 g L^{-1} of rifampicin, cyclohexamide to discriminate growth of bacteria and fungi respectively]. Dilution plates were done in triplicate and plates were incubated at 25 ºC for two weeks. Total colony counts (cfu ml^{-1}) were computed using the following formula and log transformed for analysis (Zhang *et al*., 2005).

$$N = \frac{\sum C}{(n_1 + 0.1 * n_2)d}$$

Where, $\sum C$, is the some of colonies counted on all plates retained
n_1, is the number of plates retained in the first dilution
n_2, is the number of plates retained in the second dilution
d, is the dilution factor corresponding to the first dilution

6.2.7. Induce resistance study:

6.2.7.1. Orange peel powder preparation

Two fruit samples were randomly picked from each treatment before and after treatments and used for orange peel preparation. Forty six samples were used from the treatment side (ts) and untreated controlled side (cs) of the fruit. Samples were freeze-dried for 48 h, reduced to powder, sieved with a strainer (0.05 µm pore size) and kept in sterilized Scott bottles for subsequent use.

6.2.7.2. Extraction of soluble phenolic compounds

Two successive citrus peel soluble phenolic tests were conducted before and after treatment application using dichloromethane and petroleum ether as extraction solvents according to Kim *et al*. (1991) with slight modifications. One milliliter of dichloromethane was poured in an Eppendorf tube containing 0.05 g of orange peel collected from the previously described treatments. The sample was mixed with a vortex for 1 min and centrifuged (Centronix, 1236) for 10 minutes at 3913 x g. The supernatant was transferred to a new Eppendorf tube and the extraction repeated once. One milliliter of petroleum ether was added to the remaining peel residue, mixed and centrifuged as described. The extraction procedure was repeated once. The supernatant was dried under vacuum and 500µl methanol was added to stock the final volume. The residual extract was either stored at 4 ºC or used immediately for subsequent extraction of cell-bound phenolics.

6.2.7.3. Extraction of wall-bound phenolic compounds

Residual peel powders obtained from section 2.7.2 were used for extraction of non-soluble phenolic compounds using blowing Pasteur pipettes. A Pasteur pipette was modified to a blowing apparatus by gentle flame heating the tip and simultaneous mouth air blowing into it. The tip was sealed and cooled in air. One millilitre of 0.05 N NaOH transferred into a blowing Pasteur Pipette was mixed with 0.01g of peel powder and the pipette was sealed before transfer into a water bath (95 ºC) for one hour. Pipettes were removed from the water bath, kept on ice for 10 minutes before the tips were opened and the contents transferred into an Eppendorf tube. Sixty millilitres of concentrated HCL (10M) was added to reduce the pH to ± 5. Samples were centrifuged in a micro centrifuge at 3913 x g (Denver Instrumental Company, USA) for two minutes and the supernatant was transferred into a new Eppendorf tube. One millilitre of diethyl ether was added to the remaining residue, vortexed and centrifuged for two minutes. The supernatant was transferred into the tubes containing the

concentrated suspension and extraction with diethyl ether was repeated four times. The combined supernatants were reduced into dryness under vacuum and 250 µl methanol was added to stock the final volume for subsequent use.

6.2.7.4. Quantification of total phenolics

The concentration of total soluble and/ or wall-bound phenolics was determined using the Folin-Ciocalteu method as described in chapter 5, section 5.2.6.1.

6.2.8. Plant extracts activity against *Penicillium digitatum* on citrus peel

Surface attachment and colonization of the pathogen were determined according to Chan and Tian (2005). Treatment combinations included in this experiment were: fruit wound only, wounding followed by *P. digitatum* only, wounding followed by *A. seyal* extract and *P. digitatum* and wounding followed by *W. somnifera* and *P. digitatum*. Control experiments included plant extracts applied to the fruit wound without the pathogen and the pathogen on its own. For each treatment six fruits were used and four fruits were used at random for scanning electron microscope (SEM) preparation. Wound lesions were cut transversely into four slices (4 x 4 mm) after 0, 12, 24, and 48 h of treatment application on fruit wounds. The cut peel tissue was fixed, mounted and viewed as described in chapter 4, section 4.2.11.

6.2.9. Statistical analyses

Data was analyzed using the SAS computer program (version 8.1, 2002). Differences between means were tested using least significant differences and treatment means were compared with Fisher's protected LSD test (P <0.05) and t- grouping. To determine the microflora population on treated and untreated fruit surfaces, the cfu ml^{-1} of fruit wash data were transformed to logarithms to improve the homogeneity of variances (Zhang *et al*., 2005).

6.3 RESULTS

6.3.1. *In vivo* antifungal activity of plant extracts

Wound application of *W. somnifera* and *A. seyal* extracts against the pathogen showed significant reduction of disease incidence by 70-75 %, respectively (Table 6.1). Spray application of plant extracts on the other hand exhibited 100 % protection against the postharvest pathogen *P. digitatum* (Table 6. 2).

Table 6.1 *In vivo* antifungal activity of plant extracts with wound treatment

Treatments*	(%) Disease incidence	(%) Intact fruit**
Fruit wound only	10	90[a]
Wound application of extract *W. somnifera* only	0	100[a]
Wound application of extract *A. seyal* only	0	100[a]
Wound inoculation of *P. digitatum* only	100	0[d]
Wound inoculation of extract *W. somnifera* followed by *P. digitatum*	30	70[b]
Wound inoculation of extract *A. seyal* followed by *P. digitatum*	25	75[b]
Wound application of decodone followed by *P. digitatum*	30	55[c]
Wound application of thiabendazole followed by *P. digitatum*	25	70[b]

Legend: * = Mean of sample size for each treatment done in triplicate.

** =Values in the same column followed by different superscripts are significantly different (P <0.05).

Table 6.2 *In vivo* antifungal activity of plant extracts on citrus with spray treatment

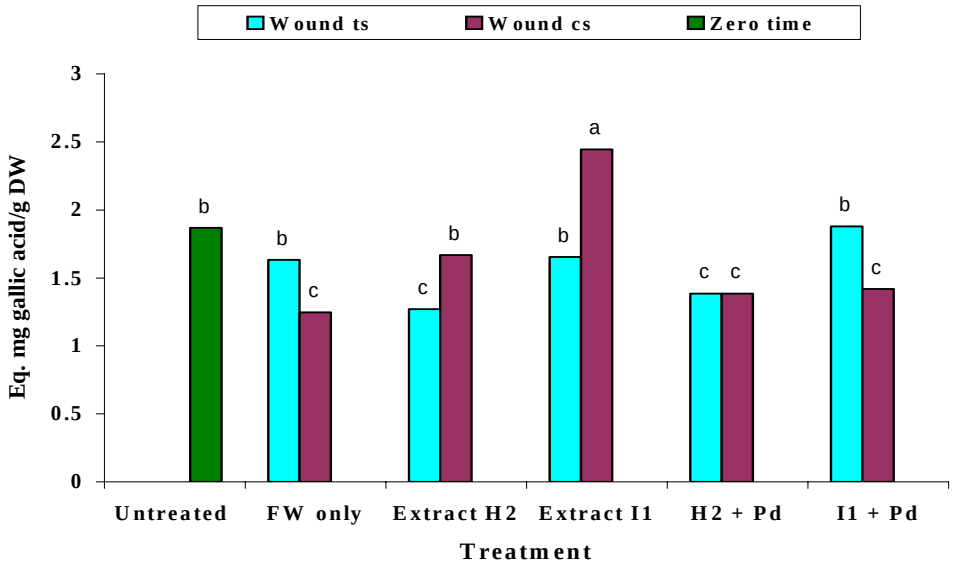
Treatments*	Disease incidence (%)	Intact fruit** (%)
Spray application of *P. digitatum* only	60	40[b]
Spray application of extract *W. somnifera* only	0	100[a]
Spray application of extract *A. seyal* only	0	100[a]
Spray application of extract *W. somnifera* followed by *P. digitatum*	0	100[a]
Spray application of extract *A. seyal* with *P. digitatum*	0	100[a]
Spray application of decodone followed by *P. digitatum*	0	100[a]
Spray application of thiabendazole followed by *P. digitatum*	0	100[a]

Legend: * = Mean of sample size for each treatment done in triplicate.

** = Values in the same column followed by different superscripts are significantly different (P <0.05).

6.3.2 Quantification of total soluble phenolics

Wound treated oranges with extract *A. seyal* showed significant increase in the concentration of total soluble phenolics around the control side (cs) of the rind. In other wound treatments [(cs) of fruit wound (FW) alone, treated side (ts) of extract *W. somnifera* alone, (ts) and (cs) of extract *W. somnifera* + *P. digitatum* (Pd) treated fruits showed significant decrease in their total soluble phenolics concentration (Fig. 6.1)].



Legend: Bars represent ± SE of the means. Bars with the same letter are not significantly (P <0.05) different according to Fisher's protected LSD and t-grouping. Abbreviations/ words used for the various treatments are indicated as follows:

□ (ts) = treated side of a fruit

□ (cs) = Control, untreated side of a fruit

FW only = fruit wound only

Extract H_2 = *Withania somnifera* L. Dunal extract treatment

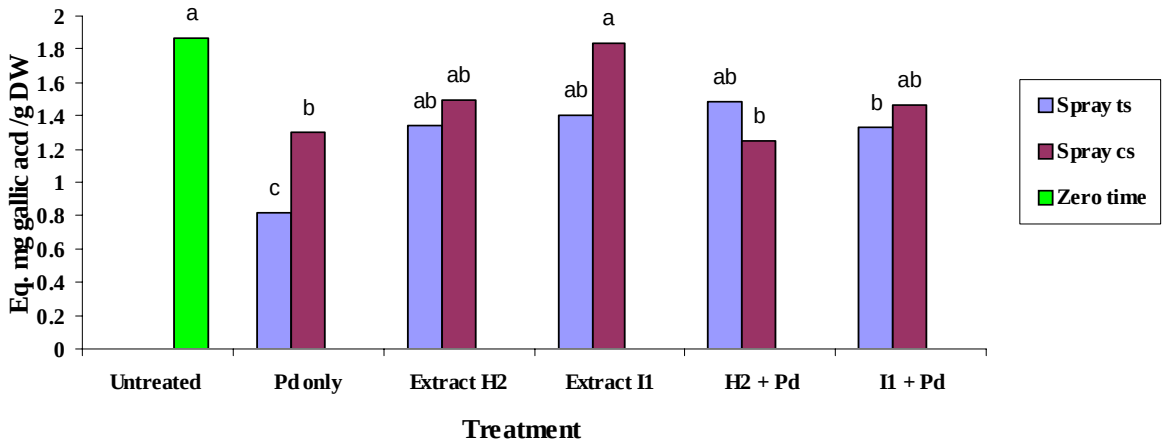
Extract I_1= *Acacia seyal* Del. var. Seyal extract treatment

H_2 + Pd = *Withania somnifera* L. Dunal extract followed by *Penicillium digitatum*.

I_1 + Pd = *Acacia seyal* Del. var. Seyal extract followed by inoculation with *P. digitatum*.

Fig. 6.1. Soluble phenolic concentrations in wound treated orange peels using plant extracts.

Spray treated fruits exhibited no significant increase in their total soluble phenolics concentration. Treated (ts) and control sides (cs) of Pd, (cs) of extract H_2 + Pd and (cs) of extract I_1 + Pd treated fruit rinds showed significant (P <0.05) decrease in the amount of total soluble phenolics (Fig. 6.2).

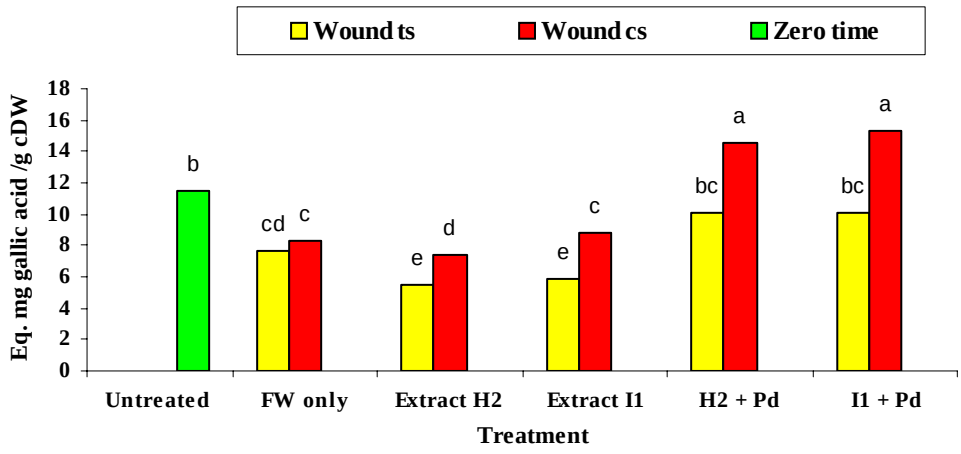


Legends: Bars represent ± SE of the means. Bars with the same letter are not significantly (P <0.05) different according to Fisher's protected LSD and t- grouping. For designated codes given to treatments refer to figure 6.1.

Fig. 6.2. Soluble phenolic concentrations in spray treated orange peels using plant extracts.

6.3.3. Quantification of total cell wall-bound phenolics

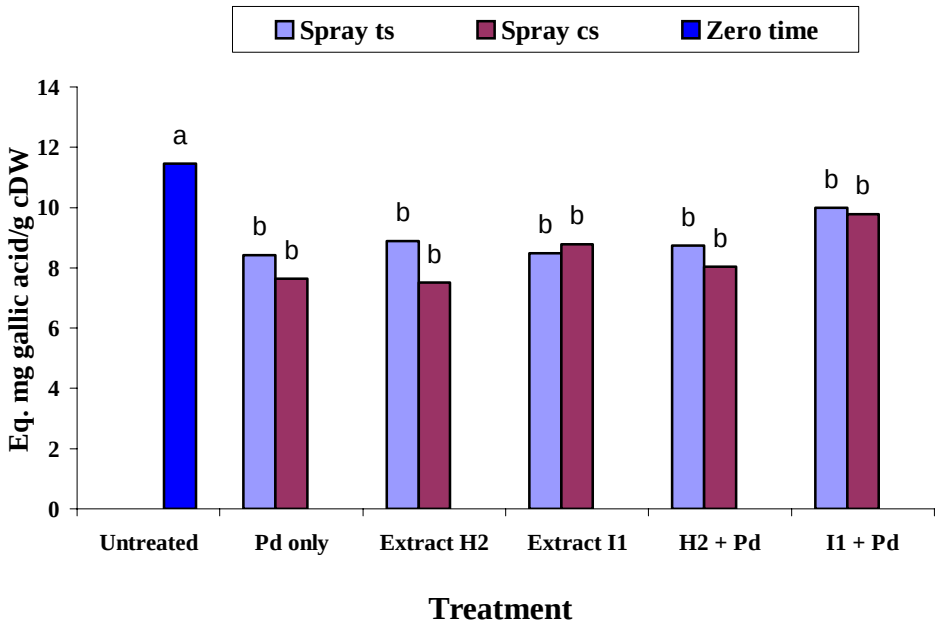
Wound and spray applications of treatments showed significant difference in cell wall-bound phenolics concentration of treated fruits (Fig. 6. 3 and 6. 4). Wound treated oranges with extract H_2 + Pd and extract I_1 + Pd showed significant increase (P <0.05) in their total insoluble phenolic concentrations at the control side (cs) of the orange rind. The concentrations decreased significantly (P <0.05) in the treated (t) and control (c) side of (FW), *W. somnifera* and *A. seyal* extracts alone treated fruits (Fig. 6. 3).



Legends: Bars represent ± SE of the means. Bars with the same letter are not significantly (P <0.05) different according to Fisher's protected LSD and t- grouping. For designated codes given to treatments refer to figure 6.1.

Fig. 6.3. Insoluble (cell wall-bound) phenolic concentrations in wound treated orange peels using plant extracts.

Spray applications of Pd alone, *W. somnifera* extract alone, *A. seyal* extract alone and *W. somnifera* extract followed by challenge treatment with Pd showed significant decrease in the total insoluble phenolic concentrations at both sides of the fruit treated (ts and cs). Spray application of *A. seyal* extract challenged with Pd did not exhibit any significant increase in the total insoluble phenolic concentration as compared to the control (Fig. 6.4).



Legend: Bars represent ± SE of the means. Bars with the same letter are not significantly (P <0.05) different according to Fisher's protected LSD and t-grouping. For designated codes given to treatments refer to figure 6.1.

Fig. 6. 4. Insoluble (cell wall-bound) phenolics concentration in spray treated orange peels using plant extracts.

6.3.4 Non-target effect of plant extracts on orange micro-flora

The post-treatment effect of plant extracts on the total microbial flora is depicted in (Table 6. 3-4). Wound applications of *W. somnifera* alone, and wound and spray applications of extract I_1 alone showed a positive impact in augmenting the growth of yeasts (Table 6. 4). The percentage growth of moulds increased significantly with wound applications of the pathogen (Pd) (Table 6. 3). Spray applications of the pathogen (Pd) showed a positive effect on increasing the total bacteria and mould counts (Table 6. 4). Preventive wound applications of *W. somnifera* and preventive wound and spray applications of *A. seyal* extracts against the pathogen, *P. digitatum* exhibited an increase in total bacteria count (Table 6. 3-4). These population shifts were significant for treatments to be further evaluated in integrated trials.

6.3.5 Effect of plant extracts against *Penicillium digitatum* on citrus

Scanning electron microscope (SEM) examination of wound treated orange peels with preventive application *A. seyal* and *W. somnifera* depicts a complex set of mode of actions against *P. digitatum* (Fig.6. 5A-P). The mechanism involved showed direct reaction of the plant extract with the pathogen by sticking and/ or deposition of crystal like substances around the wound site (Fig. 6. 5I-P). Control experiments showed fungal mass deposition around the wound site of infected fruit (Fig. 6. 5E-H).

Table 6. 3 The non-target effect assessment of the two plant extracts (*Acacia seyal* Del. var. Seyal (I_1) and *Withania somnifera* Dunal (H_2) on orange wound treated fruit surface microflora

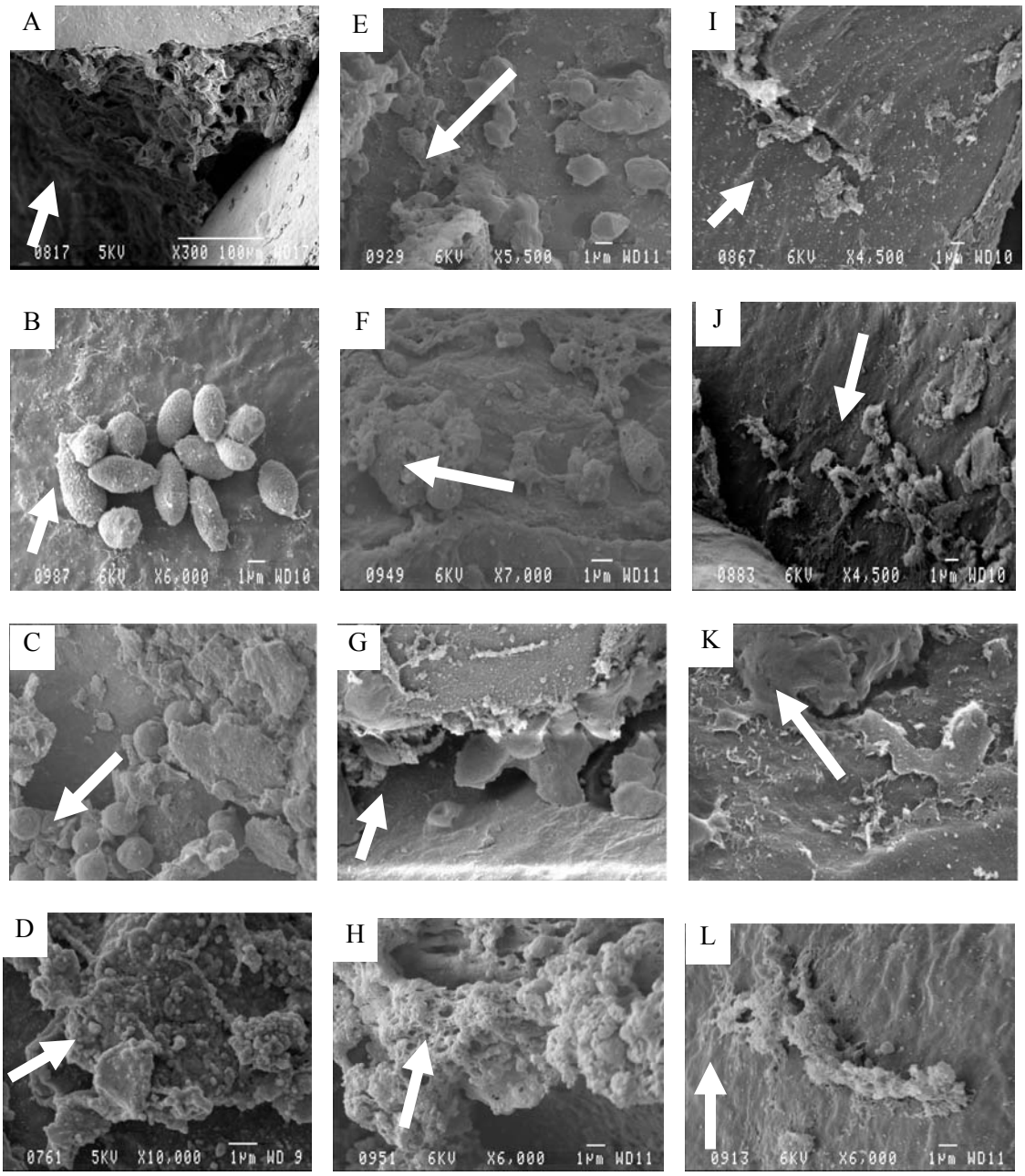
Treatments	Treatment application					
	Total microbial count (Log $_{10}$ cfu ml $^{-1}$)					
	STD-1NA		PDA		MEA	
	Bacteria	Mould	Yeast	Mould	Yeast	Mould
Untreated control	$5.02^{a} + 0.04$	$3.87^{b} + 0.10$	$3.07^{b} + 0.04$	$3.86^{b} + 0.11$	$3.90^{b} + 0.11$	$4.25^{a} + 0.03$
Extract H_2 alone	$3.14^{c} + 0.07$	$2.51^{cd} + 0.07$	$3.31^{a} + 0.02$	$2.56^{c} + 0.13$	$4.05^{a} + 0.06$	$2.61^{bc} + 0.03$
Extract I_1 alone	$3.21^{c} + 0.11$	$2.40^{d} + 0.09$	$3.36^{a} + 0.07$	$2.83^{c} + 0.15$	$4.15^{a} + 0.06$	$2.71^{b} + 0.05$
Pd alone	$2.47^{d} + 0.12$	$4.24^{a} + 0.83$	$2.70^{c} + 0.07$	$4.67^{a} + 0.13$	$2.33^{c} + 0.03$	$4.28^{a} + 0.02$
Extract H_2 + Pd	$4.53^{b} + 0.21$	$2.22^{e} + 0.09$	$3.38^{a} + 0.07$	$2.76^{c} + 0.12$	$4.09^{a} + 0.03$	$2.55^{c} + 0.04$
Extract I_1 + Pd	$4.44^{b} + 0.22$	$2.64^{c} + 0.04$	$3.40^{a} + 0.08$	$2.70^{c} + 0.16$	$4.08^{a} + 0.03$	$2.69^{b} + 0.10$

Legend: [a] = Untreated zero time fruit wash regarded as a control. Treated fruits incubated at 25^{o}C for 3 weeks. Relative humidity (RH) maintained between 80-90%. H_2 = *Withania somnifera* L. Dunal; I_1 = *Acacia seyal* Del. var. Seyal, Pd = *Penicillium digitatum.* Means in each column with the same letter are not significantly different by Fisher's protected LSD and t- grouping (P<0.05).

Table 6. 4 The non-target effect of the two plant extracts *Acacia seyal* Del. var. Seyal (I_1) and *Withania somnifera* Dunal (H_2) on spray treated orange fruit surface microflora.

Treatments	Treatment application					
	Total microbial count (Log $_{10}$ cfu ml $^{-1}$)					
	STD-1NA		PDA		MEA	
	Bacteria	Mould	Yeast	Mould	Yeast	Mould
Untreated stored	$5.13^{a} + 0.07$	$3.89^{a} + 0.10$	$3.08^{c} + 0.03$	$3.19^{b} + 0.09$	$3.90^{b} + 0.11$	$4.22^{a} + 0.09$
Extract H_2 alone	$3.39^{d} + 0.12$	$2.41^{c} + 0.07$	$3.54^{b} + 0.05$	$2.60^{de} + 0.07$	$4.11^{a} + 0.02$	$2.89^{d} + 0.06$
Extract I_1 alone	$3.70^{c} + 0.06$	$2.37^{c} + 0.12$	$3.51^{b} + 0.02$	$2.71^{cd} + 0.11$	$4.17^{a} + 0.05$	$2.90^{d} + 0.04$
Pd alone	$4.21^{b} + 0.06$	$2.17^{d} + 0.05$	$2.87^{d} + 0.09$	$4.72^{a} + 0.06$	$3.17^{c} + 0.08$	$3.26^{b} + 0.02$
Extract H_2 + Pd	$3.44^{e} + 0.04$	$2.60^{b} + 0.03$	$3.59^{ab} + 0.06$	$2.81^{c} + 0.07$	$4.20^{a} + 0.07$	$2.19^{e} + 0.06$
Extract I_1 + Pd	$4.29^{b} + 0.03$	$2.58^{b} + 0.04$	$3.69^{a} + 0.13$	$2.55^{e} + 0.04$	$4.19^{a} + 0.03$	$3.04^{c} + 0.03$

Legend: For designated abbreviations refer the legend description in table 6. 3.



Legend: Preventive application of plant extracts. Images from **A-D** showed fruit wound lesion with or without *Penicillium digitatum* application: **A**= just after wounding, **B**= just after *P. digitatum* application, B= 6 h later, **C**= 12 h later, **D**= 24 h later; **E-H** showed wound + *A. seyal* extract + *P. digitatum* application: **E**= just after application, **F**= 6 h later, **G**= 12 h later, and **H**= 24 h later against the pathogen. Spore growth inactivation by sticking together and flooding the surface seems the major mode of action of *A. seyal* extract. Images **I-L** showed wound + *W. somnifera* extract + *P. digitatum* reaction: **I**= just after application, **J**= 6 h later, **K**= 12 h later, and **L**= 24 h later against the pathogen. Spore growth inactivation by deposition of substances around glandular openings seems the major mode of action of *W. somnifera* extract.

Fig. 6.5. Mode of action study of plant extracts on Valencia oranges.

6.4 DISCUSSION

This study reports on two plant extracts for their postharvest disease control efficacy and the mechanism involved in host resistance induction. Leaf extracts from *W. somnifera* and *A. seyal* exhibited 70-75% *in vivo* inhibitory efficacy against the postharvest fruit pathogen, *P. digitatum*. These plant species were initially selected for their broad spectrum antimicrobial activity against human and plant pathogens. Comparative *in vitro* studies with these plant extracts showed better performance as compared to commercial chemicals.

In this study, all fruit spray treated with plant extracts and wound application of the two extracts on their own showed 100% protection of the orange fruit against *P. digitatum*. This report indicates similar results as described by Porat *et al*. (2003) with the application of elicitors. Reports on the traditional use of *W. somnifera* for control of human ailments in Ethiopia (Demissew, 1989; Bekele, 1993; Desissa and Binggeli, 2002), India (Bhatia *et al*., 1987) and *A. seyal* in East Africa (Duke, 1983) are indicators for safe and potential use of these plant extracts for postharvest disease control.

Wound and/ or spray application of a plant extract alone and/ or preventive application against the pathogen *P. digitatum* showed a change in the total phenolics concentration of orange peels as compared to the control. A decrease or an increase in the total soluble phenolics concentration of a plant tissue indicates host defence reaction system involving certain mode of action against the pathogen. According to Robards and Antolovich (1997), any environmental stimuli applied on the host tissue may increase the total soluble phenolics concentration through phenylpropanoid pathway. Treatment side (ts) and control sides (cs) of wound inoculated fruits with plant extracts exhibited significant change in the total soluble phenolics concentration. Wound application of extract (I_1) alone showed significant increase in the total soluble phenolics concentration in the (cs) of an orange rind. According to Cheng and Breen (1991), this reaction could show the high potential of the plant material in induction of the key enzyme phenyl alanine lyase (PAL) activity towards the synthesis of soluble phenolics. On the other hand, in treatment side (ts) of a fruit with extract H_2 alone, (ts) and (cs) of extract H_2 + Pd treated fruits, the concentration of soluble phenolics were found decreased. In this interaction, the host defence mechanisms against the pathogen involved another mechanism other than oxidation of soluble phenolics (Harborne, 1964). As reported by Cruickshank and Perris (1964), phenolic compounds at low concentrations do not have any inhibitory effect on plant pathogens instead they have a stimulatory effect on host

defence mechanism to build up the lignified tissues of the wall. A decrease in the total soluble phenolics concentration of an orange peel and healing of the wound surface involved a synthesis of cell wall bound phenolics that could serve as a physical and biological barrier to invading pathogens. The stimulatory reaction involve induction of a key enzyme (PAL) in the phenylpropanoid pathway to synthesis ferulic acid, a lignin monomer that conjugated with glucose to form a cell wall bound phenolics, lignin (Cruickshank and Perris, 1964). Lignin, as a major cell wall component of a plant tissue builds up cell wall barriers and increase host resistance. Induced defence reactions of a fruit can be restricted to tissues close to the wound site of the stimulus or can be spread or expressed throughout the neighbouring tissues (El-Ghaouth *et al*., 2002). Significant increase in the total cell wall bound phenolics concentration was exhibited on the control side (cs) of an orange rind with preventive application of extract H_2 + (Pd) and I_1 + (Pd). In other wound treatment combinations, the total insoluble phenolics content was significantly decreased.

Images viewed through SEM showed two possible modes of actions that could be involved in the defence mechanism of the host. Deposition of crystal-like substances on the wound side and direct interaction of the extract with the pathogen by sticking the spores together were identified as possible mechanisms observed in the healing process of an infected fruit. The mode of action shown by accumulation of crystals around the wound site is a similar mechanism as described by Porat *et al*. (2002). The other mechanism involved with direct reaction to the pathogen by sticking indicates their putative involvement in the physical and biochemical defence responses against the pathogen. The latter mechanism however is the first to be reported.

The non-target effect of the plant extracts on the orange fruit micro-flora showed a general trend of decrease in microbial diversity while favouring surface colonization by yeasts and bacteria. Wound and/ or spray application of extracts (H_2 and I_1) in combination with *P. digitatum* showed establishment of yeast and bacterial population on the surface of the fruit. Reports by Leben *et al*. (1965) showed similar results of plant extracts effect in enhancing growth of epiphytic yeasts and bacterial strains. The abundance of epiphytic micro flora on the peel of citrus fruits confirms the importance of natural protection against microbiological alterations by natural antagonists, which are capable of competing for nutrients and space (Arras, 1996; Janisiewicz *et al*., 2000). The mode of actions exhibited by these plant extracts is desirable for postharvest application. Further semi-commercial studies are recommended for verification of the product for commercial use.

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CHAPTER SEVEN

SEMI COMMERCIAL EVALUATION OF PLANT EXTRACTS ON QUALITY RETENTION IN *CITRUS SINENSIS*

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Submitted to Postharvest Biology and Technology Journal for publication as:
Sissay B. Mekbib, Dharini Sivakumar, Thierry J.C. Regnier and Lise Korsten, 2006. Developing Ethiopian microbial biocontrol agents for citrus postharvest disease control.

Abstract

Six postharvest treatments with extracts of *Acacia seyal* Del. var. Seyal and *Withania somnifera* L. Dunal were tested using artificial wounding or dip applications on citrus (*Citrus sinensis* L.). Quality retention effects of extracts were studied with the application of extracts as either a pre-wax, combined with wax or as a plant extract dip alone. Chlorine washed and commercial chemical treated fruits were included as comparative controls. Fruit were stored for 50 days at 25 ºC and 75% RH or at 7 ºC and 80-95% RH to simulate domestic and export conditions. Fruit quality were assessed for incidence of decay, physico-chemical and sensory parameters. Canonical variate analysis of data indicate that *A. seyal* and *W. somnifera* extracts applied as a pre-wax treatment or combined with wax or using the extract alone resulted in more fruit that retained the colour of the skin, odour/ smell and flavour with overall acceptability when kept at 7 ºC and 80-95% RH for 50 days. Fruits were also assessed for disease development but overall natural infection was too low to see any significant effect. The two plant extracts have potential as a safe, cost-effective alternative for protecting the fruit without affecting the quality during long-term storage.

Key words: Plant extracts; Postharvest treatments; Physico-chemical; Sensory evaluation.

7.1 INTRODUCTION

Citrus (*Citrus sinensis* L.) fresh fruit is one of the major export crops in global trade. Citrus is cultivated in the subtropical and tropical regions of the world in 137 countries and on six continents (Salunkhe and Desai, 1984; Ismail and Zhang, 2004). Annually, more than 104 million tons of citrus fruit are produced of which 15 million tones end up in global trade (FAO, 2004). The storage life of citrus is limited to a maximum of eight weeks at low temperature (Mukhopadhyay, 2004), and inferior quality is often observed on 9-25% of the product at export destinations due to postharvest pathogens (*Penicillium digitatum* Sacc., *Geotrichum candidum* Link and *Colletotrichum gloeosporioides* (Penz.) and physiological disorders (peel pitting and browning) (Klieber *et al*., 2002; Alferez *et al*., 2005). Chemical fungicides used to control postharvest diseases are increasingly being lost to the export sector due to increased requirements of more stringent maximum residue levels and re-registration requirements for pesticides (Plaza *et al*., 2004). Further concern over build up of pathogen resistance and negative impact on environmental health (Brown, 1977; Eckert and Ogawa, 1985; Vero *et al*., 2002) necessitate the search for alternative control options.

Application of plant extracts and essential oils have been extensively evaluated for postharvest application on fruits (Dudareva *et al*., 2004; Tripathi and Dubey, 2004). Saks and Barkai-Golan (1995) reported that application of *Aloe vera* L. Webb and Berth gel on wounded grapefruit reduced green mould decay by 75%, six days after inoculation with *P. digitatum*. The essential oil cumin from *Cuminum cyminum* L. Cumin has also been reported to protect citrus fruits from *P. digitatum* (Yigit *et al*., 2000).

Natural plant extracts can successfully replace synthetic fungicides to control postharvest decay if they are applied during the packhouse operation without additional expenditure on new equipment. *Acacia seyal* Del. var. Seyal and *Withania somnifera* L. Dunal are indigenous plants in Ethiopia used as traditional medicines (Demissew, 1989; Bekele, 1993). As shown in chapter 6, extracts from *A. seyal* and *W. somnifera* showed a broad spectrum *in vitro* antimicrobial activity against food borne and plant pathogens. *In vivo* application of these extracts showed up to 75 % reduction of *P. digitatum* incidence when kept for 21 days under simulated export conditions.

In order to make the application efficient in this study, the plant extract was incorporated in to

the commercial wax formulation or were applied prior to wax application to protect the fruit during storage and transportation. The objective of this study was to evaluate the efficacy of extracts from two indigenous Ethiopian plants *A. seyal* and *W. somnifera* on decay control and quality retention of citrus fruits at long-term cold and room temperature storages.

7.2 MATERIALS and METHODS

7.2.1 Fruit collection

Fifty four boxes of Valencia oranges, each containing 88 fresh fruits were randomly collected from J. M. du Toit citrus packhouse (Tzaneen, Limpopo Province, South Africa). Fruit were transported during the winter at 18 ºC to the Plant Pathology Laboratories, University of Pretoria for immediate treatment.

7.2.2 Plant material extraction

Two plant species *A. seyal* and *W. somnifera* collected from Metahara and Hursso, Ethiopia, were air-dried and undamaged leaf parts of these plants were powdered in a blender (Russell Hobbs) and stored at 18 ºC in amber bottles until further use. One part of the dried plant powder was suspended in 20 parts (w/v) methanol solvent mixture [(methanol/ acetone/ water) (7:7:1)] followed by three successive extractions as described in chapter 5 section 5.2.2. The combined supernatants were concentrated to dryness under vacuum at 25 ºC and equal volume of distilled water as to the original extraction solvent system was added to make the final stock solution. The suspension were then filter sterilised using 0.45µm pore size (Sartorius, Germany) into sterilized Schott bottles and stored at 4 ± 1°C until further use.

7.2.3 Postharvest treatments:

7.2.3.1 Wound treatment

In vivo antifungal activities of *A. seyal* and *W. somnifera* against *P. digitatum* were tested using the method described by Poppe *et al*. (2003), with some modifications. Wound (3 x 3 mm) applications of extracts were applied 12 h prior to the inoculation of the pathogen. The culture of *P. digitatum* collected from the culture collections of Plant Pathology laboratories, University of Pretoria, South Africa were used. In order to avoid a variable inoculum pressure, the pathogen concentration was standardized to 10^5 conidia ml^{-1} using a haemacytometer (Janisiewicz *et al*., 2000) and preserved at 4 ºC in an ice box prior to use. Six treatment combinations indicated in table 7.1 were used.

Table 7.1 Plant extracts treatment combinations for wound application on fruit

Code	Treatment description
1	Fruit wound + *A. seyal* + *P. digitatum*
2	Fruit wound + *W. somnifera* + *P. digitatum*
3	Commercial packing line treatment [(Dipping fruit in chlorine water (Sodium hypochlorite, 250 ppm) for two minutes, spore kill (12% didecyl dimethyl ammonium chloride) (Hygrotech (Pty) Ltd., Johannesburg) (900-1400 ppm) for brief time spray (30 seconds), quattro kill (N, N Didecyl-N, N-dimethyl ammonium chloride) (Hyper Agrochemicals (Pty) Ltd., Johannesburg) (1300ppm) at 45 ºC for five minutes, imazalil (Sanachem, Johannesburg) (1350ppm) for brief time spray (30 seconds), air drying for two minutes and waxing with Citrosol (100 000 ppm) (Brenntag, Germany) for two minutes, drying and packing.
4	Untreated not wounded
5	Wound only
6	Wound + *P. digitatum*

Wound inoculation of the pathogen alone was regarded as a negative control. The application of commercial chemicals was regarded as a positive control. Fruit wounding alone was included to confirm the effect of wound treatments. Ten fruits per treatment and four wounds (3 x 3 mm diameter) per fruit were used. Fruits wounded aseptically with picture hooks (3 x 3 mm) were inoculated with 30 µl of the crude plant extract, air dried for 12 h and inoculated with the same volume of the pathogen, *P. digitatum*. Treated fruits were kept for 21 days in citrus boxes at 8 ºC with a relative humidity of >85% (RH) to simulate export conditions. Evaluation of fruits for disease development was done weekly and percentage disease incidence was computed. The experiment was repeated twice.

7.2.3.2 Fruit dipping

For each treatment, a total of 528 fruits were randomly selected. In each treatment application, fruits were dipped in treatment suspensions for two minutes and air-dried for 10 minutes. Fruits were subjected to either one of the following dip postharvest treatments (Table 7.2).

Table 7.2 Plant extracts treatment combinations for dip application on fruit

Code	Treatment combinations
1	*A. seyal* leaf extract application followed by air drying and waxing with Citrosol
2	*W. somnifera* leaf extract application followed by air drying and waxing with Citrosol
3	Combined treatment of *A. seyal* leaf extract incorporated in the commercial waxing
4	Combined treatment of *W. somnifera* leaf extract incorporated in the commercial waxing (Brenntag)
5	Treatment with *A. seyal* leaf extract alone
6	Treatment with *W. somnifera* leaf extract alone
7	Washing in commercial chlorine alone
8	Commercial packing line treatment as described in the previous experiment, subsection 7.2.3.1 (Table7.1)
9	Untreated control

Fruits were dipped in treatment suspensions for two minutes and air-dried for 10 min. A set of 44 fruits were packed in commercial cardboard boxes (300 x 400mm) and stored at 25 °C and 75% RH and a replicate set were kept at 7 ºC and 80-90% RH for 50 days simulating local and export conditions, respectively. The fruits were then evaluated for overall quality retention and organoleptic parameters.

7.2.4 Fruit quality

Postharvest fruit quality was assessed for incidence of browning on a 1-5 rating hedonic scale, where: 1= very poor, 2= poor, 3= fair with limited acceptability, 4= good, and 5= excellent (Alferez *et al*., 2005). Fruit firmness was measured with a penetrometer (Magness-Taylor penetrometer test) equipped with a six mm diameter plunger capable of penetrating through the peel into the pulp (Abbott, 1999). Ten fruits were taken at random from the different postharvest samples, and firmness was measured on opposite sides of each fruit (Sivakumar *et al*., 2005). Fruit percentage weight loss was calculated out of a hundred by subtracting treated

stored fruit weight from untreated fresh fruit weight measurement before storage. Total Soluble Solids (TSS) was determined trice using fruit juice and a hand-held refractometer (Atago, Japan, Brix 0-30%). Results were expressed as percentages of TSS. Titratable acidity (TA) was also determined by titrating 10 ml of the sample filtrate against 0.1 M NaOH with phenolphthalein as indicator. The turning point was taken as the sudden change of the solution to a slight pink colour, with acidity expressed as percentage citric acid equivalent (Schirra *et al*., 2004).

7.2.5 Sensory evaluation

For sensory evaluation, fruit samples removed from cold storage were kept at room temperature (25 ºC). A set of 10 fruit per treatment was placed on white plates and immediately presented to a taste panel of six panellists familiar with the quality and sensory parameters of citrus fruit. The qualitative analysis based on quality parameters (Table 7.3) was done according to Varela *et al*. (2005).

Table 7.3 Sensory attributes selected for descriptive analysis

Attribute	Associate descriptor
Smell	Total intensity of smell
Freshness	Smell of fresh oranges
Colour	Natural colour of the peel, flavedo and edible portion and presence of browning
Appearance	Condition of a fruit whether it is fresh, shriveled, firm or soft
Flavour	Total intensity of flavour during the first chewing
Sweetness	Taste of the fruit: sweet, bitter or sourness

Quality assessment values were given for each treatment using a hedonic scale structured from 1 to 5 (Srinivasa *et al*., 2004), where 1 meant very poor, 2 meant poor, 3 meant fair with limited acceptability, 4 meant good and 5 meant excellent. Prior to the evaluation procedure, the panel was trained with attribute descriptor by profiling fruit sections to associated parameters. Replicate samples were pooled together according to their storage temperature and fifty fruits per sample were used. Each sample was identified by a random three-digit code. The order of presentation of the samples on the plates was randomised for each panellist. Fruits were displayed in lightened room on big dining table using white plates and

panelists were provided with knife and tissue papers for cutting and cleaning; and glass of water for mouth rinsing between samples. Evaluation of samples from both temperature regimes was done at different times for reliability and validity of results.

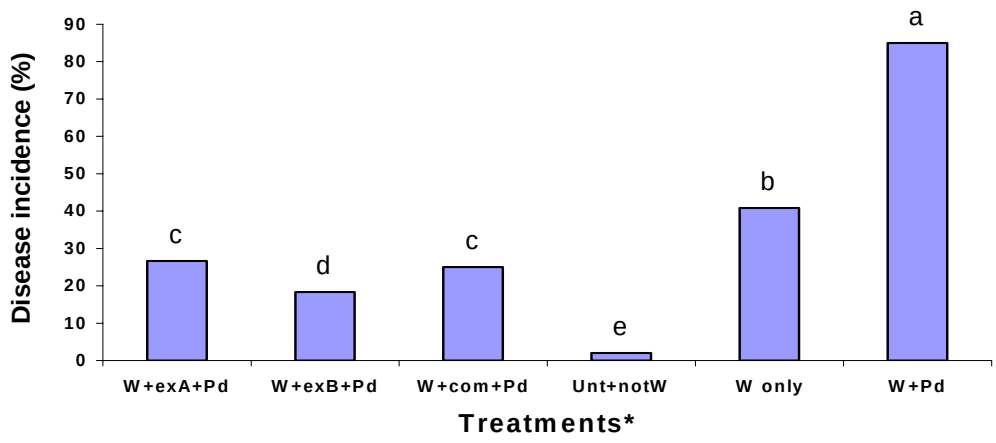
7.2.6 Statistical analysis

The experiments were in a completely randomised design and were carried out twice during the 2004 and 2005 growing seasons. Analysis of variance was used to test for differences between treatments. Treatment means were separated using Fisher's protected t-test least significant difference (LSD) at the 5% level of significance (Snedecor and Cochran, 1980). Data were analysed using the statistical program GenStat for Windows (2004). Multivariate canonical variate analysis (CVA) was used as a useful statistical tool to identify differences between groups of individuals (treatments). It summarises and analysed information contained in the different independent variables, and maximized variation between the groups of individuals while minimising variation within the groups of original variables. Comparisons between the samples and storage conditions and determination of the extent of variation observed in the results were also accounted.

7.3 RESULTS

7.3.1 Postharvest disease incidence and browning evaluation

In vivo wound treated fruits with *A. seyal* and / or *W. somnifera* showed significant (P <0.05) reduction of *P. digitatum* incidence by more than 75% comparable to the effect of commercial chemical treatments when kept for 21 days under simulated export conditions (Fig. 7.1). In fruits subjected to postharvest dip treatments, decay was not observed on fruits held at 7 ºC and 80-90% RH. Incidence of chilling was 6% instead, in untreated fruits stored at this temperature (Table 7.4). Higher incidence of fruit decay (24-36%) was observed on fruits stored at ambient (25 ºC) temperature and 75% RH (Table 7.5). Fruits subjected to a pre-wax application with plant extracts showed relatively higher incidence of browning at 25 ºC (Table 7.5). Significant changes (P <0.05) in firmness and weight loss was observed in extract treated fruits kept at 7 and 25 ºC (Table 7.4 and 7.5). The plant extract *A. seyal* alone or used as a pre-wax application showed significantly (P <0.05) higher retention of fruit firmness at 7 ºC and similar treatments revealed lower firmness at 25 ºC. On the other hand, combined application of *A. seyal* or *W. somnifera* extracts with wax enabled the fruit to retain firmness both at 25 ºC and 7 ºC. Pre-wax application of *A. seyal* extract or *A. seyal* extract alone retained fruit firmness better than the commercially adopted treatment kept at 7 ºC storage.



Legend: Each bar represents treatment means. Means with the same letter are not significantly different by Fisher's protected test at (P <0.05). *Treatment applications are described as follows: W+ exA + Pd = Fruit wound + *A. seyal* extract + *P. digitatum*; W+ exB + Pd = Fruit wound + *W. somnifera* extract + *P. digitatum*; W + com + Pd = Fruit wound + Commercial chemical treatment + *P.digitatum*; Un + not W = Untreated and not wounded fruit; W only = Wounded fruit only; W+Pd = Wounded fruit + *P. digitatum*

Fig. 7.1 *In vivo* wound treatment evaluation of *Acacia seyal* and *Withania somnifera* efficacy against *Penicillium digitatum* on citrus.

A non-significant variation in TSS was observed in fruits subjected to pre-wax application or combined application with plant extracts or plant extracts alone at 25 ºC (Table 7.5). Untreated and chlorine washed fruits showed a significant (P <0.05) increase in TSS unlike other postharvest treatments. Fruits subjected to commercial treatment with waxing showed a significant (P <0.05) decrease in SS and TA levels. Separate application of *A. seyal* or *W. somnifera* extracts resulted in significant (P <0.05) decrease in TA levels in fruits stored at 25 ºC.

7.3.2 Quality assessment and analysis for sensory attributes

Mean separation analyses of sensory parameters showed significant (P <0.05) differences between different types of postharvest treatments with plant extracts alone, or the combination of treatments or with pre-wax treatments and plant extracts (Table 7.6). For further analyses, CVA was carried out to evaluate the differences among the six sensory parameters used and

to show the relative contribution of each variable to the sensory quality on citrus with respect to the different postharvest treatments. The CVA plot axis CA 1 accounts for 61% of the variance and CA 2 for 19% (Fig. 7.2). Together, they account for nearly 80% of the total variance observed. The figure shows six well-separated groups corresponding to samples from different postharvest treatments and storage conditions. The untreated control at 7 ºC is situated to the lower left side of the plot, untreated control and chlorine washed fruit appeared to the lower middle of the plot. The fruit held at 7 ºC and subjected to pre-wax application with of *A. Seyal* or *W. somnifera*, *A. seyal* alone, combined application of wax with *A. seyal* or *W. somnifera* and commercially adopted treatment appeared at the middle left side of the plot. The pre-wax with *W. somnifera*, combined application of wax with *W. somnifera* and the commercially adopted treatment held at 25 ºC were grouped together towards the upper middle part of the plot. The variates responsible for the sensory characters were flavour (r = -0.899), odour (r = -0.789), appearance (r = -0.738), and flavedo colour (r = -0.636). The variate mostly responsible for this was skin colour (r = 0.708). The fruit pre-waxed with *W. somnifera*, combined application with wax and *W. somnifera* and the commercially adopted treatment held at 25 ºC revealed more over matured orangish colour. In this evaluation, pre-wax application of *A. seyal* and *W. somnifera*, combined application of wax with *W. somnifera*, and plant extracts *A. seyal* or *W. somnifera* alone retained the quality of the fruit at ºC.

Table 7.4 Effect of semi-commercial application of plant extracts (*Acacia seyal* Del.var.Seyal and *Withania somnifera* L. Dunal) on postharvest decay control and overall quality retention of citrus fruits during long-term (50 days) cold storage (7 °C)

Postharvest treatments	Penicillium decay incidence (%)	Chilling effect (%)	Weight loss (%)	Firmness (N)	Soluble solids concentration (%)	Titratable acidity (%)
Pre-wax application of A. seyal extract	0.00	0. 00	$0.01^{d} \pm 0.0$	$38.54^{a} \pm 0.4$	$12.66^{c} \pm 0.3$	$1.28^{e} \pm 0.1$
Pre-wax application of *W. somnifera* extract	0.00	0. 00	$0.01^{d} \pm 0.0$	$34.79^{cd} \pm 1.0$	$13.47^{ab} \pm 0.3$	$1.37^{bcd} \pm 0.0$
A. seyal extract + wax mix	0.00	0. 00	$0.00^{d} \pm 0.0$	$36.53^{abc} \pm 0.6$	$13.47^{ab} \pm 0.2$	$1.40^{abc} \pm 0.0$
W. somnifera extract + wax mix	0.00	0. 00	$0.02^{c} \pm 0.0$	$35.80^{bc} \pm 1.4$	$13.21^{abc} \pm 0.3$	$1.35^{cd} \pm 0.0$
A. seyal extract only	0.00	0. 00	$0.00^{d} \pm 0.0$	$38.45^{a} \pm 0.9$	$13.32^{ab} \pm 0.4$	$1.34^{de} \pm 0.1$
W. somnifera extract only	0.00	0. 00	$0.01^{d} \pm 0.0$	$32.69^{e} \pm 1.0$	$13.44^{ab} \pm 0.2$	$1.41^{ab} \pm 0.0$
Control						
Untreated	$10..5^{a} \pm 1..3$	$6.67^{a} \pm 2.08$	$0.06^{a} \pm 0.0$	$33.51^{de} \pm 2.0$	$13.74^{a} \pm 0.2$	$1.35^{d} \pm 0.0$
Chlorine washed only	0.00	0. 00	$0.04^{b} \pm 0.0$	$32.88^{de} \pm 0.6$	$13.51^{ab} \pm 0.6$	$1.47^{a} \pm 0.1$
Commercial	0.00	0. 00	$0.01^{d} \pm 0.0$	$36.99^{ab} \pm 2.4$	$13.09^{bc} \pm 0.3$	$1.40^{bcd} \pm 0.1$

Legend: [x] Means in each column followed by the same letter are not significantly different at $P < 0.05$ by Fisher's protected least significant test. Relatively high incidence (10.5%) of fruit decay was observed in untreated fruits. Chilling injury column indicates incidence of chilling injury-affected fruits only in untreated fruits. Abbreviations described as follows: *A. seyal* = *Acacia seyal* Del. var Seyal, *W. somnifera* = *Withania somnifera* L. Dunal.

Table 7.5 Effect of semi-commercial application of plant extracts (*Acacia seyal* Del.var.Seyal and *Withania somnifera* L. Dunal) on postharvest decay control and overall quality retention of citrus fruits at long-term room (25 °C) temperature storage

Postharvest treatments	**Penicillium decay incidence (%)**	**Browning effect (%)**	**Weight loss (%)**	**Firmness (N)**	**Soluble solids concentration (%)**	**Titratable acidity (%)**
Pre-wax application of *A. seyal* extract	0.00	$6.3^{a} \pm 2.3$	$0.11^{b} \pm 0.0$	$29.68^{cde} \pm 0.7$	$13.21^{bc} \pm 0.6$	$1.43^{ab} \pm 0.1$
Pre-wax application of *W. somnifera* extract	0.00	$6^{a} \pm 1.0$	$0.12^{b} \pm 0.0$	$26.57^{ef} \pm 1.1$	$13.41^{bc} \pm 0.3$	$1.41^{abc} \pm 0.1$
A. seyal extract + wax mix	0.00	0. 00	$0.11^{b} \pm 0.0$	$36.07^{a} \pm 1.7$	$13.87^{ab} \pm 0.6$	$1.40^{abc} \pm 0.0$
W. somnifera extract + wax mix	0.00	$2.33^{b} \pm 2.3$	$0.11^{b} \pm 0.0$	$34.98^{ab} \pm 2.5$	$13.40^{bc} \pm 0.1$	$1.30^{cde} \pm 0.1$
A. seyal extract only	0.00	0. 00	$0.12^{b} \pm 0.0$	$27.67^{def} \pm 0.9$	$13.71^{ab} \pm 0.8$	$1.21^{e} \pm 0.1$
W. somnifera extract only	0.00	0. 00	$0.12^{b} \pm 0.1$	$30.77^{cd} \pm 1.6$	$13.13^{bc} \pm 0.3$	$1.16^{e} \pm 0.1$
Control						
Untreated	$36^{a} \pm 3.0$	$0.3^{bc} \pm 0.6$	$0.17^{a} \pm 0.1$	$29.77^{cde} \pm 1.8$	$14.43^{a} \pm 0.4$	$1.52^{a} \pm 0.1$
Chlorine washed only	$9^{b} \pm 2.4$	0. 00	$0.11^{b} \pm 0.0$	$32.24^{bc} \pm 1.9$	$14.34^{a} \pm 0.0$	$1.38^{bcd} \pm 0.1$
Commercial	0.00	$5.33^{a} \pm 0.0$	$0.15^{a} \pm 0.0$	$25.93^{f} \pm 3.0$	$12.93^{c} \pm 0.2$	$1.25^{de} \pm 0.1$

Legend: [x] Means in each column followed by the same letter are not significantly different at $P < 0.05$ by Fisher's protected least significant test. High incidence (36%) of fruit decay was observed in untreated fruits. Chilling injury column indicates incidence of chilling injury-affected fruits. For abbreviations, see table 7.4 legend.

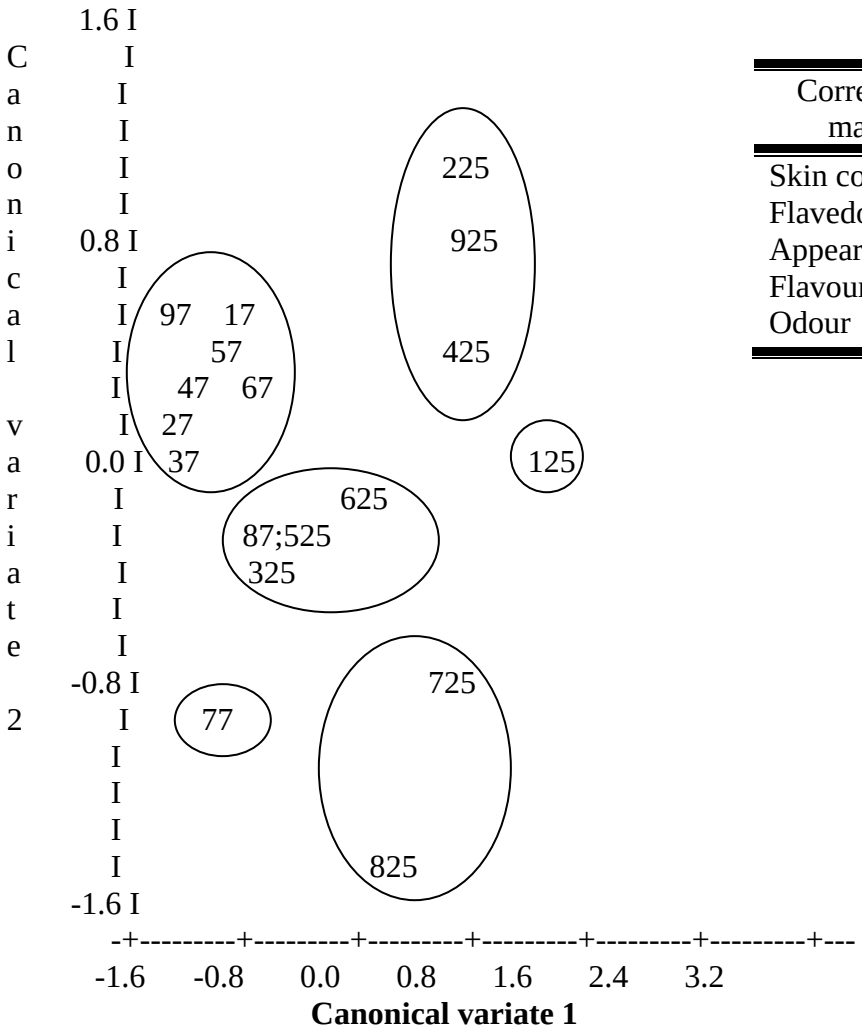
Table 7.6 Sensory evaluation of fruits treated with plant extract treatment combinations and stored at 7 and 25 °C for 50 days

Treatments	Sensory evaluation parameters (7 °C storage temperature)		Sensory evaluation parameters (25 °C storage temperature)			
	Skin colour	Odour/Smell	Skin colour	Appearance	Flavour	Odour/ Smell
Pre-wax application of *A. seyal*	4.3ab	3.8abc	2.7ab	2.5ab	2.2b	2.8ab
Pre-wax application of *W. somnifera*	4.2abc	4.7a	3.8a	3.3ab	2.3b	2.8ab
A. seyal + wax mix	4.0abc	4.3ab	3.5ab	3.5a	4.0a	4.0a
W. somnifera + wax mix	4.2abc	4.0abc	3.5ab	3.0ab	2.7ab	3.0ab
A. seyal extract alone	4.2abc	3.7bc	3.7a	3.5a	4.0	4.0a
W. somnifera extract alone	4.2abc	4.2abc	3.5ab	3.2ab	3.3ab	3.5a
Untreated control	3.3c	4.0abc	2.8ab	2.3ab	3.2ab	3.0ab
Chlorine washed	3.5bc	3.3c	2.3b	2.2b	3.5ab	3.2ab
Commercial line treatment	4.5a	4.3ab	3.3ab	3.3ab	2.2b	2.0b

Legend: Means of the same letter are not significantly different by Fisher's protected test at $P < 0.05$. Sensory parameters, which showed no significant differences, are avoided for simplicity. Postharvest fruit quality was assed using 1 –5 rating hedonic scales, where: 1= very poor, 2 = poor, 3 = fair with limited acceptability, 4 = good, and 5 = excellent. For abbreviations see table 7.4 legend.

W. somnifera and commercially adopted treatment appeared at the middle left side of the plot. The pre-wax with *W. somnifera*, combined application of wax with *W. somnifera* and the commercially adopted treatment held at 25 ºC were grouped together towards the upper middle part of the plot. The variates responsible for the sensory characters were flavour (r = -0.899), odour (r = -0.789), appearance (r = -0.738), and flavedo colour (r = -0.636). The variate mostly responsible for this was skin colour (r = 0.708). The fruit pre-waxed with *W. somnifera*, combined application with wax and *W. somnifera* and the commercially adopted treatment held at 25 ºC revealed more over matured orangish colour. In this evaluation, pre-

wax application of *A. seyal* and *W. somnifera*, combined application of wax with *W. somnifera*, and plant extracts *A. seyal* or *W. somnifera* alone retained the quality of the fruit at 7 ºC.



Correlation matrix	(r) value
Skin colour	0.708
Flavedo colour	-0.636
Appearance	-0.738
Flavour	-0.899
Odour	-0.769

Legend: The first CV (horizontal axis) mainly contrasts with cold (7 ºC) and room (25 ºC) temperatures. The second CV (vertical axis) contrasts mainly to temperature and varies mostly between 2 and 9 or 7 and 8 treatments. Numbers are designated for each treatment in accordance with storage temperature used. Two digits for cold and three digits for room temperature storages are given. The first digit represents a treatment order from (1-9) and the next digit (s), 7 for cold temperature and 25 for room temperature storages, respectively. Cold storage (7 °C) treatments designation represented by the following order as follows: 17- pre-wax application of *A. seyal*; 27 pre-wax application of *W. somnifera*; 37- *A. seyal* + wax mix; 47- *W. somnifera* + wax mix; 57- *A. seyal* extract alone; 67- *W. somnifera* extract alone; 77- Untreated control; 87- Chlorine washed; 97- Commercial line treatment. Room temperature (25 ºC) storage treatments designations represented in the following order: 125- pre-wax application of *A. seyal*; 225 pre-wax application of *W. somnifera*; 325- *A. seyal* + wax mix; 425- *W. somnifera* + wax mix; 525- *A. seyal* extract alone; 625- *W. somnifera* extract alone; 725-Untreated control; 825-Chlorine washed; 925-Commercial line treatment.

Fig. 7.2. Sensory evaluation canonical variate analyses.

7.4 DISCUSSION

It is evident from this study that the two selected plant extracts, *A. seyal* and *W. somnifera* reduce disease incidence and retained the overall quality of citrus when used as a postharvest decay control protective agent during long term cold (7 ºC) and ambient (25 ºC) temperature storages. Pre-wax, wax-mix and/or *A. seyal* and *W. somnifera* extracts alone resulted in significant disease incidence reduction and quality retention of citrus fruits stored under simulated export conditions. These results were comparable and often better than the commercial chemical treatments. This is the first report where these plant extracts were used in citrus postharvest trials and showed potential to retain quality and prevent decay.

Higher incidence of postharvest *Penicillium* decay (36%) was detected in untreated fruits kept at long-term ambient temperature unlike other treatment coatings. The separate application of *A. seyal* and/or *W. somnifera* alone and/or in combination with wax showed significant reduction of *Penicillium* disease incidence, which could involve either the suppression of spore germination and/or the inhibition of mycelial growth. Browning was detected in some treatments such as pre-wax applications of *A. seyal* (6.3%), wax-mix and/or pre-wax application of *W. somnifera* (2.33-6%), untreated fruits (0.3%) and commercial chemical treated fruits (5.33%) stored at room temperature. According to Petracek *et al*. (1998), high temperature storage of waxed fruits stimulates postharvest browning by decreasing peel gas permeability and desiccation.

Realtively higher incidence of *Penicillium* decay (10%) and browning (chilling injury) (6.7%) was detected in untreated orange fruits kept at long-term cold storage. According to Biolatto *et al*. (2005) development of peel pitting on untreated fruits at long-term cold storage are associated with the accumulation of aldehydes and alcohol produced by anaerobic respiration. The chilling effect was not detected in the plant extract or commercial wax treated fruits stored at the same temperature. Postharvest treatments have been known to reduce fruit chilling injury incidences i.e. ethylene degreening prior to cold storage (Grierson, 1974), waxing (Davis and Harding, 1959) and fungicide application (Schiffman-Nadel *et al*., 1972; Petracek *et al*., 1998; Schirra *et al*., 2004). In this study, the postharvest application of plant extracts showed a similar effect in inhibiting pitting and fruit decay, which signifies their commercial value as a postharvest treatment option.

Commercial chemical treated fruits showed a decrease in percentage concentration of SS, TA, fruit firmness and augmenting weight loss on fruits stored at room temperature. According to DeEll *et al*. (2001), firmness depends on cell size, cell wall thickness and strength, turgor pressure and the manner in which cells bind together. In this particular experiment, the low percentage concentration of acidity, SS, firmness and percentage weight loss in commercial chemical treated fruits were associated with waxing (Davis *et al*., 1967; Hagenmaier and Baker, 1993; Hagenmaier, 2002). It has been reported by Ben-Yehoshua *et al*. (1994) that waxing of fruits results in the build up of high carbon dioxide and low oxygen concentrations, which help delay the rate of respiration, senescence and resulting in firm fruits as observed at low storage temperatures. However, an increase in carbon dioxide or ethylene within the wax layer could cause anaerobic stress and result in less firm fruits as studied in apples (Knopacka and Plocharski, 2004) with off flavour fruits like banana (Satyan *et al*., 1992), kiwifruit (Marsh *et al*., 2004) and grape fruits (Biolatto *et al*., 2005; Shi *et al*., 2005) as observed with commercial treatments kept at 25 ºC storage conditions.

Application of *A. seyal* and/ or *W. somnifera* plant extracts with different treatment combinations on citrus fruit showed a significant effect on fruit quality retention as evaluated with flavour, odour, flavedo colour and overall appearance in sensory parameters. These results confirm the data obtained from the physicochemical analysis. It is therefore evident from this study that the application of *A. seyal* and *W. somnifera* extracts would have an effect on the complex biochemical changes associated with ripening but the mechanism of the effect on these changes has not been determined.

This study showed that *A. seyal* and *W. somnifera* can potentially be used as an alternative to synthetic fungicides and waxes to retain fruit quality. Since these plants are used in traditional healing of human aliments, i.e. *W. somnifera* in India (Bhatia *et al*., 1987) and Ethiopia (Demissew, 1989; Bekele, 1993), *A. seyal* in Ethiopia and tropical Africa countries (Duke, 1983; Bekele, 1993), and it could therefore represent a novel postharvest treatment. Further testing of these extracts developed during the current study could be recommended commercially as a safe method for quality retention and postharvest decay control of citrus.

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CHAPTER EIGHT

GENERAL DISCUSSION AND CONCLUSION

In developing countries, where protection and proper handling of fresh fruit are inadequate, losses during transit and storage account for over 50% of the harvested crop (Wisniewski and Wilson, 1992). In this study it was found that actual losses recorded in Ethiopia in citrus storage was 46.7%. This decay was mostly caused by *Penicillium* species, particularly by *Penicillium digitatum* Sacc., the causal agent of citrus green mould. This disease is of economic importance in all citrus producing regions of the world and is mostly related to poor handling and storage practices (Eckert and Eaks, 1989). To prevent or minimise such losses, synthetic chemicals are applied either pre- or postharvestly. However, the application of these chemicals may result in chemical residues on food that affect human health (Roistacher *et al*., 1960; Matsumura, 1972; Houck, 1977; Koeman, 1978; Norman, 1988) and can lead to build up of pathogen resistance or environmental pollution (Janisiewicz, 1987; Wilson and Wisniewski, 1989). The use of biocontrol agents to manage postharvest decay of fruit has been explored as an alternative to synthetic fungicides (Wilson and Wisniewski, 1989; Benbow and Sugar, 1999) and several commercial products are now available (Bull *et al*., 1997; Droby *et al*., 1998; Janisiewicz and Korsten, 2002). The choices of using natural plant products and/or the development of natural microbial antagonists thus could minimise environmental risks.

Results obtained in the present study showed that the selected plant extracts and yeast antagonists have desirable characteristics for postharvest applications to control *P. digitatum* on citrus. From a total of 23 plant species and 242 potential microbial isolates of three citrus growing regions in Ethiopia, screening for their antimicrobial activity yielded two superior plant species [*Acacia seyal* Del. var. Seyal and *Withania somnifera* L. Dunal] and three yeast antagonists [MeJtw 10-2 (*Cryptococcus laurentii* (Kufferath) Skinner, TiL4-2 (*Candida sake*) and TiL4-3 (*C. laurentii*)].

Application of *A. seyal* and/or *W. somnifera* plant extracts with different treatment combinations on citrus fruit showed a significant effect on fruit quality retention as evaluated with flavour, odour, flavedo colour and overall appearance in sensory parameters. These results confirm the data obtained from the physicochemical analysis and show the potential effect of these plant extracts involving complex biochemical changes associated with ripening

and fruit quality. This is the first report where plant extracts from *A. seyal* and *W. somnifera* are described to be used as an alternative to synthetic fungicides and waxes to retain fruit quality. The commercial use of these plant extracts can result in a safe method to protect the citrus from postharvest decay and could represent a novel postharvest treatment. These products are used in traditional healing of human ailments [*W. somnifera* in India (Bhatia *et al*., 1987) and Ethiopia (Demissew, 1989; Bekele, 1993), *A. seyal* in Ethiopia and other tropical African countries (Duke, 1983; Bekele, 1993)]. *In vivo* tests with some selected plant extracts showed remarkable control of fruit decay due to *P. digitatum* in South Africa, which may indicate the promising potential for postharvest disease control, especially for the citrus industry. In addition, the plant extracts provided a shiny gloss to the fruit surface and prevented desiccation, suggesting a potential replacement for wax. Future research advances on this aspect would contribute to determining the active chemical compounds of these plant extracts for commercial use as postharvest applications. In order to test the potential application of these extracts against other pathogens, several fungal and bacterial spp. were inhibited by the extracts. The effective control on important food borne pathogens such as *Staphylococcus*, *Salmonella* and *Shigella* spp., previously associated with citrus and other fruits and vegetables, could also make the commercial product more acceptable for other disease control strategies.

In this study, three potential yeast antagonists [two strains of *C. laurentii* (MeJtw10-2 and TiL4-2) and one of *C. sake* (TiL4-3)], exhibiting the best inhibition of *P. digitatum* and broad-spectrum activity against *Geotrichum candidum* (Link ex Pers) and *Colletotrichum gloeosporioides* Penz., were identified. The potential use and application of yeast strains without antibiosis activity have been demonstrated by many workers to control postharvest decay of fruits and vegetables (Wilson and Wisniewski, 1989, Wisniewski and Wilson, 1992, Janisiewicz and Bors, 1995). It is evident from the *in vitro* study of this experiment that the selected potential antagonists did not show any antibiosis or volatile production against any of the pathogens tested. The isolates also showed a significant rate of disease incidence reduction (70-100%) on fruits incubated at 7 ºC and 25 ºC for >30 days. The application of antagonist TiL4-2 (*C. sake*) suppressed *P. digitatum* growth at a minimum concentration (10^5 spores ml^{-1}) of both antagonist and pathogen, which is a more effective control than previous reports made by Droby *et al*. (1989). The rapid growth of the yeast antagonists without any additive at the wound site indicates their ability and considerable potential use as a biocontrol agent (Vero *et al*., 2002). This would require further commercial testing upon product formulation and registration according to Act 47, 2000 of the Republic of South Africa.

Excluding antibiosis as potential mode of action at the initial screening stages is important when selecting a natural antagonist for postharvest disease control. Although the mechanisms by which yeast biocontrol agents provide decay control are not fully understood, the mode of action of several yeast antagonists was shown in this study not to involve antibiosis. The mechanism involved was found to be competition for nutrients (Benbow and Sugar, 1999; Janisiewicz *et al*., 2000) and space (Janisiewicz *et al*., 2000) at the wound site. In this study, the fast colonisation effect of yeast antagonists by producing extracellular matrix that sticks to the pathogens was evident. This was confirmed by the *in vitro* dual culture experiments supported by electron microscope results. The fast recovery and compatibility of yeast antagonists integrated with plant extracts *in vitro* and *in vivo* treatments showed potential for industrial application to substitute chemical pesticides.

The search for potential antagonists from specific geographic areas based on their distinct mode of actions other than antibiosis against the range of pathogens is crucial for selection of and development of antagonists for postharvest application. The future search and development of biopesticides therefore can be upheld with this strategy to control pre- and postharvest diseases of citrus in particular and other crops in general.

Suggestions for future studies:

The out comes of this study can provide an effective alternation for pesticides. In order to develop these products the following needs to be done:

1. Commercial evaluations of various treatment formulations of *A. seyal* and *W. somnifera* extracts and its assessment under export conditions and overseas.
2. Semi-commercial and commercial evaluations of various yeast antagonist treatment formulations under simulated and export conditions.
3. Evaluate product consistency by repeating semi commercial and commercial trials.
4. Evaluate the efficacy of both plant extracts and yeast antagonists on other crops.
5. Product registration and commercialisation.

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Appendix 1

Table 1 Major citrus postharvest diseases: typical symptoms on fruit, infection type, infection site and spread of infection with possible control strategies involved

Disease	Causal agent	Typical symptoms on fruit	Infection type	Infection site	Spread to adjacent fruit	General control strategies	Reference
Anthracnose	*Colletotrichum gloeosporioides* (Penz) Sacc.	-Initially are silvery gray and leathery, and later the infected ring becomes brown to grayish black and softens as the rot progresses. The pathogen grows and sporulates in deadwood on the trees, with water transmitting spores to the immature fruit surface by forming appressoria. The structure remain latent, do not cause decay prior to harvest.	Quiescent, infective pathogen.	Injured rind.	Yes.	-Improved cultural practices such as removal of dead wood and twigs to reduce inocula. -Proper handling of fruits at harvest to minimize injury. -Cooling of fruits immediately after harvest at or below 10 ^{o}C. -Application of sanitary measures by removing infected fruits.	Brown, 1994.

Table … continued

Black rot (stem-end rot)	*Alternaria citri* Ellis and Pierce -Cause premature coloring of a fruit on the tree (the most typical characteristic of the disease) and it causes fruit drop early in the season. -The fungus may cause stem-end rot infection of Valencia and grapefruits in long term cold storage	-Late infected fruit appear sound on the exterior and may escape. the attention of graders. -Some times exhibits external symptoms at the blossom end (top), but is more often found in the core of the fruit bottom. -Infected fruit appear to have a dray, black, decayed area at or near the stylar or stem end.	Quiescent, infective pathogen.	Natural openings at the stem-end.	No.	-Delay harvesting time until the infected fruit drop. -Application of postharvest treatments which delay fruit button (calyx) senescence may delay black rot development.	Brown, 1994. Browning *et al.*, 1995.
Black spot	*Guignardia citricarpa* Kiely	-Variable in symptoms it may appear as hard freckle or virulent (spreading) spots.	Quiescent, infective pathogen.	Intact or injured rind.	Yes.		Kotze, 1993.

Table… continued

Blue mould	*Penicillium italicum* Wehmer -Common in cold seasons.	-The decay first appears as watery discoloured spots that can easily punctured. -The white mycelium soon produces a mass of powdery blue coloured sporulating area surrounded by a white margin.	Active, wound pathogen.	Injured rind.	Yes.	-Mould sporulating may be inhibited by approved fungicide treatment. -Sanitation in the handling, packing and storage operations is very important. -Tests for pathogen resistance in the packinghouse- -Repacking *Penicillium* infected fruit is important and storage of packed fruits at or below 4.4 ^{o}C delay mould development.	
Greasy spot	*Mycosphaerella citri* Whiteside	-Form necrotic specks on fruit rind between epidermis and oil glands -The lesions are pink at first and become brown or black with rind blotch in 3 to 6 months time.	Quiescent, infective pathogen.	Intact or injured rind.			Whiteside *et al*., 1993. Browning *et al*., 1995.

Table… continued

Green mould	*Penicillium digitatum* Sacc. -These moulds are most common in a cold seasons.	-First appears as watery discoloured spots that are easily punctured by finger pressure and later as white fungal mycelium producing a mass of powdery olive green or light to bright blue spores surrounded by a large white margin. -Finally, the decayed fruit becomes soft, shrunken, and shrivelled and entirely covered with spores.	Active wound pathogen.	Injured rind.	No.	-Minimize scratches, punctures and plugging ensuring careful harvesting and handling. -Sanitary practices must be applied to avoid resistant strains to fungicides. -Remove all debris and decayed fruit from the packing site. -Application of disinfectants. -Application of tests periodically to detect resistant strains. -Application of approved fungicides before or after harvest provide control of moulds. -At packhouse and transition store fruits at or below 4.4 ^{o}C.	Brown, 1994. Browning *et al.*, 1995.

Table … continued

Lime Anthracnose	*Gloeosporium limetticola* Clausen	Young fruits attacked by a disease usually shed. -Fruits infected later develop corky lesions that vary from slightly sunken spots to deep cankers over much of the surface but lack yellow haloes unlike canker.	Quiescent, infective pathogen.	Intact or injured rind.			Whiteside *et al*., 1993. Browning *et al*., 1995.
Sour rot	*Geotrichum candidum* Lk ex Pers (*Endomyces geotrichum*)	-Slightly raised, water-soaked, clear to yellow initial lesions, which are confusing with those of Penicillium moulds are developed. -At high relative humidity, yeasty layer may cover the	Active, wound pathogen.	Injured rind.	Yes.	-Minimize scratches, punctures and plugging ensuring careful harvesting and handling. -Avoid harvesting fruits with high peel moisture early in the morning. -Avoid fruit contact with soil during harvest.	Brown, 1994; Wills *et al*., 1998.

Table … continued

		lesion and produces sour odour that attracts fruit flies which may enhance the spread of the fungus. -The infection quickly spreads into a soft decaying area favoured by moderate temperature 27 ^{o}C.				-Immediate cooling of picked fruits to below 10 ^{o}C will delay decay development. -Application of adequate sanitary practices (soak tanks with chlorine at proper pH). -Application of disinfectants. -Application of mixtures of fungicides.	
Stem-end rot	*Diplodia natalensis* P. Evans (syn. *Botryodiplodia theobromse* Pat.; *Physalospora rhodina* Berk and Curt	-Initially, decay occur at both ends of the fruit. -In infected fruit, lesions appear as dark discoloration	Quiescent, infective pathogen.	Natural openings at the stem-end.	No.	-Improve cultural practices such as removing dead trees, wood. -Harvesting by clipping rather than pulling.	Brown, 1994.

Table continued

		within 1-2 weeks during storage time. -Development of sour fermented odour as the fruit becomes black.				-Remove some buttons (sepal base) that harbour pathogen. -Spot picking for natural colours and delaying harvest until more colour develops (reduce degreening time). -In packhouse increase humidity to 90 – 95%. -Maintaining of temperature at 82- 84 ^{o}F and ethylene formation at 1 – 5 ppm during degreening and storage.	
Stem-end rot	*Phomopsis citri* Faw -The fungi colonize dead twigs and wood on the tree where spores are dispersed by rain and wind to fruit. -Decay occurs after harvest when the fungus grows from the calyx (button) into the fruit.	-Decay appears as a buff coloured to brown, leathery, pliable area encircling the button or stem-end of the fruit. -Infection spreads through the core in a nearly even rind pattern from the stem-end to the surrounding.	Quiescent, infective pathogen.	Injured rind.	Yes.	-Improved cultural practices such as removal of dead wood and twigs to reduce inocula. -Proper handling of fruits at harvest to minimize injury. -Cooling of fruits immediately after harvest at or below 10 ^{o}C. -Application of sanitary measures by removing infected fruits.	Whiteside *et al.*, 1988.

Table … continued

Trichoderma rot	*Trichoderma viride* Pos ex Gray. -The fungus is ubiquitous in soil growing on dead twigs. -Spores disseminated by contact with soil and / or infected wood. -The fungus mycelia are white and the conidia are globose with rough texture.	-Infection may be at any location of fruit rind. -Infected fruits develop cocoa brown colour with leathery and pliable appearances. -Decay on the fruit starts at the stem-end or stylar end. -Rotted fruits characteristically produce coconut odour.	Quiescent, infective pathogen.	Injured rind.	No.	-Improved cultural practices such as removal of dead wood and twigs is required to reduce inocula. -Proper handling of fruits at harvest to minimize injury. -Cooling of fruits immediately after harvest at or below 10 ^{o}C. -Application of sanitary measures by avoiding infected fruits.	Whiteside *et al.*, 1988.

Appendix 1

Table 2 Chemicals used as postharvest fungicides on citrus fruit and other crops

Name and formulation	Pathogens controlled	Host	Remarks	Extracted
Alkaline inorganic salts				
sodium tetraborate (borax)	*Penicillium* spp	Citrus	Only reasonably effective; Problem with residues	Willis *et al*., 1998
sodium carbonate	*Penicillium* spp	Citrus	Only slightly effective	
sodium hydroxide	*Penicillium* spp	Citrus	Only slightly effective	
Ammonia and aliphatic amines				
ammonia gas	*Penicillium*, *Diplodia*,	Citrus	Good for fumigation of degreening	
sec-butylamine	*Rhizopus*		and storage rooms	
	Penicillium, stem-end rots	Citrus	Good control as dip or fumigant	
Aromatic amines				
dichloran	*Rhizopus*, *Botrytis*	Stone fruits, carrot, sweet potato	Very effective	
Benzimidazoles				
benomyl, thiabendazole,	*Penicillium* spp	Citrus	Effective at low concentration;	
thiophanate methyl	*Colletotrichum* and other	Banana, apple,	resistance problem; residue	
carbendazim	fungi	pear, pineapple, stone fruit	tolerance 0-10µg/g	

Table … continued

Triazoles			
imazalil	*Penicillium*, stem-end rots	Citrus	Effective against benzimidazole-resistant strains and at low concentration
prochloraz	*Penicillium* spp	Citrus	Effective against benzimidazole-resistant strains
guanidine			
guazitine	*Penicillium*, *Geotrichum*	Citrus	Effective against benzimidazole-resistant strains
Hydrocarbons and derivatives			
biphenyl	*Penicillium*, *Diplodia*	Citrus	Smell unpleasant
methyl chloroform	*Penicillium*, stem end rots	Citrus	Inhibits spore germination only
Oxidising substances			
hypochlorous acid	Bacteria, fungi build up in wash water	Produce	Good sterilant, no penetration of injury sites, corrosive to metal
iodine	Bacteria, fungi	Citrus, grapes	Staining problem, expensive
nitrogen trichloride	*Penicillium* spp	Citrus, tomato	Hydrolyses to hypochlorous acid
Organic acids and aldehydes			
dehydroacetic acid	*Botrytis* and other fungi	Strawberry	Dip not accepted by industry
sorbic acid	*Alternaria*, *Cladosporium*	Fig	Sterilant for picking boxes, storage rooms
formaldehyde	Fungi		
Phenols			

Table … continued

o-phenylphenol	*Penicillium* spp	Citrus	Causes fruit injury
sodium o-phenylphenate	*Penicillium*, bacteria and other fungi	Produce	pH control needed to prevent injury; residue tolerance 10-12µg/g
Salicylanilide	*Penicillium*, *Phomopsis*, *Nigrospora*	Citrus, banana	Slight control
Sulphur (inorganic)			
sulphur dust	*Monilinia*	Peach	nd
lime-sulphur	*Sclerotinia*		
sulphurdioxide gas, bisulphate	*Botrytis*	Grapes	Sulphur dioxide gas needs moisture to be effective;
Sulphur (organic)			
captan	Storage rots	Various produce	Nd
thiram	*Cladosporium*, crown and stem-end rots	Strawberry, banana	Nd
ziram	*Alternaria*, crown and stem-end rots	Banana	Nd Nd
thiourea	*Penicillium* spores	Citrus	Toxic to man
thioacetamide	*Diplodia*		Nd

Legend: Nd = not determined

Appendix 1

Table 3 Microbial antagonists registered as biopesticide for control of fungal diseases

Species name	Type	Target pathogen	Product name	Manufactured (Country)	Extracted from
Bacteria					Montesinos, 2003
Bacillus poplliae	I	*Popilla japonica*	_	_	
B. thuringiensis var. *aizawai*	I	*Galleria melonella*	_	_	
B. thuringiensis var. EG2348	I	*Lymantria dispar*	_	_	
Burkholderia cepacia	F	Soil borne fungi, nematodes	_	_	
Pseudomonas fluorescens	F	Soil borne fungi	_	_	
P. syringae ESC- 10, ESC-11	F	Postharvest Fungi	_	USA	
P. chlororaphis	F	Soil borne fungi	_	_	
P. aureofaciens Tx-1	F	Antracnose, soil borne	_	_	
*Pseudomonas aeruginosa**	F	-Downy mildew of Grape, cucumber, pumpkin, pepper and melon; root rot by *Pythium spp.*; late blight of potato by *Phytophthora infestans.* -To control *Geotrichum candidum* infection on pome and citrus.	Biosave 110, 111	USA	Shachnal *et al*., 1996 Montesinos, 2003

Table … continued

Bacillus subtilis	F	Pre-and postharvest disease of avocado.	Avogreen	South Africa	Janisiewicz and Korsten, 2002; Montesinos, 2003
B. subtilis FZB24	F	Soil borne fungi	_	_	Motesinose, 2003
B. subtilis GB03	F	Soil borne and wilt	_	_	
B. subtilis GB07	F	Soil borne fungi	_	_	
Streptomyces griseoviridis K61	F	*Phythium, Fusarium, Botrytis, Alternaria, Rhizoctonia* and *Phytophthora* sp.	Mycostop	Kemira Argo of Finland	
S. lydicus	F	Soil borne fungi.	_	_	
Agrobacterium radiobacter K84, K1026	B	Crown gall *A. tumefaciens.*	_	_	
Ralstonia solanacearum non-pathogenic	B	Pathogenic *R. solanacearum*	_	_	
Pseudomonas fluorescens A506	B	Frost damage, fire blight (*Erwinia amylovora*).	_	_	
Pseudomonas syringae pv. *tagetis*	H	*Cirsium arvense*	_	_	
Xanthomonas campestris pv. *poae*	H	*Poa annua*	_	_	

Table … continued

Fungus biopesticides					
Trichoderma polysporum, T. harzianum	F	Soil borne fungi	_	_	Montesinos, 2003
T. harzianum KRL-AG2	F	Soil borne fungi	_	_	
T. harzianum	F	Foliar fungi	_	_	
T. harzianum, T. viride	F	Various	PlantShield	BioWorks, Inc.in Geneva	
T. viride	F	Phythium, Rhizocotonia, Fusarium and Botrytis	_	_	
T. lignorum	F	Vascular wilt	_	_	
Trichoderma spp	F	Soil borne	_	_	
Ampelomyces quisqualis M-10	F	Powdery mildew	_	_	
Talaromyces flavus V117b	F	Soil borne fungi	_	_	
Gliocladium virens GL-21	F	Pythium, Rhizocotonia and Sclerotinium sp. (Soil borne fungi).	SoilGuard	Cerit, USA	(http//www.cfgrower.com/tips/oct/biological.html) Montesinos, 2003
G. catenulatum	F	Soil borne fungi	_	_	Montesinos, 2003
Fusarium oxysporum non-pathogenic	F	Pathogenic Fusarium	_	_	
Pythium oligandrum	F	*Phytium ultimum*	_	_	
Phlebiopsis gigantean	F	Heterobasidium	_	_	

Table … continued

Coniothyrium minitans	F	*Sclerotinia sclerotiorum*			Montesinos, 2003
Candida oleophila 1- 182	F	Penicilium decay on citrus and pome fruits (postharvest decay).	Aspire ™	USA	Shachnal *et al*., 1996 Montesinos, 2003
Cryptococcus albidus	F	Postharvest disease of apples and pears (postharvest decay).	Yield plus	USA	Shachnal *et al*., 1996 Montesinos, 2003
Phytophthora palmivora MWV	H	*Morrenia odorata*	_	_	Montesinos, 2003
Colletotrichum gloeosporioides	H	Cuscuta and various	_	_	
C. gloeosporioides f. sp. *malvae*	H	*Malva pulsilla*	_	_	
C. g. f. sp. *aeschynomene*	H	*Curty indigo*	_	_	
C. coccodes	H	*Abutilon theophrasti*	_	_	
C. truncatum	H	*Sesbania exalta*	_	_	
Aiternaria cassia	H	*Senna obtusifolia*	_	_	
Viruses .					
Pine sawfly NPV	I	*Diprion similes*	_	_	
Heliothis NPV	I	*Helicoverpa zeae*	_	_	
Gypsy moth NPV	I	*Lymantria dispar*	_	_	
Tussok moth NPV	I	*Orgyia pseudotsugata*	_	_	
Mamestria brassicae NPV	I	*Heliothis*	_	_	
Spodoptera exigua virus	I	*S. exigua*	_	_	
Bacteriophage of *P. tolaasii*	F	Bacterial rot of mushroom	_	_	

Legend: B = bactericide; F = fungicide; H = herbicide; I = insecticide; N = nematicide.
* A winner of the 2004 Presidential Green Chemistry Challenge Award.
_ = status not known.

Appendix 1

Table 4 Microbial antagonists and their mode of action on fruits

Microbial antagonists	Pathogen	Mode of action	Compound/ Metabolite produced	Commodity/ fruit	References
Bacteria					
*Bacillus subtilis**	*Penicillium digitatum*	-Antibiosis -Competition for nutrients and space	Iturin -	Peaches Avocado	Pusey and Wilson, 1984; Demoz and Korsten, 2006
*Burkolderia (Pseudomonas) cepacia**	*P. digitatum*	-Antibiosis -Competition for nutrients and space.	Pryrrolnitrin	Apple, pears and citrus	Smilanick and Denis-Arrue, 1992.
P. syringae (ESCO-10 and ESC-11)*	*P. digitatum*	-Antibiosis -Competition for nutrients and space	Syringomycin	Citrus	Bull *et al.*, 1997.
Entrobacter cloacae	*P. digitatum*	-Competition for nutrients and space	-	Citrus and pome, peach.	Wilson *et al.*, 1987.
Yeasts					
Pichia guilliermondii Wicker*	*P. italicum*	-Competition for nutrients and space -Directly parasitizing the pathogen	-	Citrus	Arras *et al.*, 1998.
*Candida saitoana**	*P. italicum*	-Competition for nutrients and space. -Directly parasitizing the pathogen when co cultured with *Botrytis cinerea.*	-	Citrus/apple	El-Ghaouth *et al.*, 2000.
*Debaryomyces hansenii**	*P. italicum*	-Competition for -Directly parasitizing co cultured with *Botrytis cinerea.*	-	Citrus	Droby *et al.*, 1989.

Table … continued

Cryptococcus laurentii	*P. italicum*	-Competition for nutrients and space.	-	Citrus/ apple	Roberts, 1990.
Aureobasidium pullulans (de Bary) Arnaud	*P. italicum*	-Competition for nutrients and space	-	Citrus	Janisiewicz *et al*., 2000.
Sporobolomyces roseus	*P. italicum*	-Competition for nutrients and space	-	Citrus	Janisiewicz, 1994.

Legend: * = antagonists with multiple mode of actions.

- = status not known.

Table 5 Categories of information gathered in the informally structured questionnaire on citrus cultivation, pre- and postharvest disease control practices in Ethiopia

Category information	**Information requested**	**Dominant Response**
Geographical aspects	Summer and winter temperature	Table 2
	Water source relation: Rainfall	79.2%
	Irrigation	100%
	Altitude	Table 2
	Humidity	Table 2
History of citrus farm/ orchards	Ownership: Government	97%
	Private (Individual)	2.6%
	Private (Association)	0.4%
	Farm size (ha)	Table 3
	Cultivar variety:	
	Valencia	35.8%
	Washington navel	23.9%
	Hamlin	19.4%
	Pineapple	7.5%
	Shamuti	4.5%
	Jaffa	1.5%
	Robbins blood	1.5%
	Unknown cultivars	5.9%
	Scion/ root stock sources move in:	
	From certified growers (California (USA), Israel and Asmara (Erteria)	21.1%
	From local growers (UAAIE, Ghibe, Error)	36.8%
	Material source unknown	42.1
	Orchard establishment, age in:	
	Old orchards (>20 years)	70%
	Young orchards (<20 years)	30%
	Type of crop used in	Cereals, vegetables, fibre crops, oil crops
	Soil type and nutrient status	Table 2
	Fertilizer used	Table 2
Preharvest epidemiology and disease/ pest management practices	Disease type:	
	Gumosiss (bark irruption)	In all citrus farms (>70%)
	Leaf and fruit spot	Ghibe citrus farm
		Tisablaima association citrus farm
	Insect pests	Reported 50% fruit damage
	Nematodes	12.1%
	Control measures:	Mostly by UAAIE, Hursso, Ghibe and Error Gota farms
	Chemical spray (Insecticides)	
	Plant decoction with animal urine and planting of a legume (*Lablab purpureus* L.) between citrus trees	Tisabalima association farm

Average input cost statistics in major Government citrus farms as compared to annual gross income	Labour cost	7.76%
	Agrochemicals (Pesticides and Fertilizers)	38.3%
Fruit harvesting	Peak time of harvest	Table 4
	Harvesting temperature	Ambient average temperature (18-25 °C)
	Harvesting techniques	Hand picking, tree shaking and pulling with long stick
	Persons involved in fruit picking	Temporary workers
Fruit transportation and storage facility	Fruit storage facility after picking	None
	Means of transportation	Open private and air-conditioned Etfruit trucks.
	Storage facility, general imperession	Untidy and with no temperature control in private and Etfruit storage houses
	Postahrvest disease incidence	Fig. 10 and 11
	Pathogens	*Penicillium* spp.
	Disease control methods	Sorting out and remove decayed fruits
Fruit marketing	Local markets	Towns around farms and Addis Ababa, Harar and DireDawa
	Export markets	Djibouti and Somalia
Others	Pack house facilities	None
	Overall farm experience on pre- and posthavest disease control activities:	Involved cultural practices such as field sanitation and use of pesticides in Government farms and animal urine + plant decoction in private farms.

APPENDIX 1I

QUESTIONNAIRE: I

Code "A" 2003/04

PREHARVEST CITRUS DISEASE MANAGEMENT PRACTICES ASSESSMENT

This questionnaire was translated to the local language (Amharic) for fieldwork

SITE: Citrus Production units in Ethiopia

Brief description: **Date:** _____

This questionnaire was designed as part of a PhD study that will focus on citrus fruit diseases and its control in Ethiopia. The first part of the questionnaire deal with pre-and postharvest factors, citrus diseases, crop management, and the second section dealing with fruit handling, storage, distribution and marketing. Therefore, we are kindly requesting your sincere response in replaying to the questions. Your input and time is much appreciated. All data will be held confidential and will only be used for research purposes.

Thank you!

Region: _____

Farm Name: _____

Farm Address: _____

I. Geographical aspects

1. What is the average summer and winter temperatures? (mark the applicable answer with an "X")

1-a: Summer (day)

15-20^{o}C	21-25^{o}C	26-30^{o}C	>30^{o}C

1-b: Summer (night)

<10^{o}C	10-15^{o}C	16-20^{o}C

1-c: Winter (day)

<10^{o}C	10-15^{o}C	16-20^{o}C	21-25^{o}C

1-d: Winter (night)

<10^{o}C	10-15^{o}C	16-20^{o}C

2. Water source used in the farm

Rainfall	irrigation	Both

3. How often do you irrigate your orchards?

Twice in a week	Once in a week	Twice in a month	Once in a month	Any other

4. What is the average rainfall per a year in ml?

<250ml	250-500ml	501-750ml	751-1000ml	>1001

5. What is the general altitude in ft above sea level?

0-300	301-600	601-900	901-1200	1201-1500	1501-1800	>1800

6. What is the average humidity of the farm?

<30%	31- 50%	51-70%	71-90%	>90%

II. History of citrus farm and its orchards

7. How big is a farm in hectares?

_____

8. How many types of sweet orange cultivars are produced on the farm? Can you name them and put in order of their importance in terms of area planted percentage composition?

9. From where did you purchase the planting material?

Certified growers	Local growers	Any other source. Name the name

10. How was the citrus seedlings/trees produced?

By seeding	By grafting

11. How old are the orchards?

0-10 years	10-20 years	20-30 years	>30 years

12. What is the ownership status of the citrus plantation site

Government	Association	Private (own)

13. Are there any other crops growing in/ or around the citrus farm?

Yes	No

14. For question number 12 above, if your answer is yes, what type of crop is it?

15. What is the soil type of the farm?

16. Have you ever determined the nutrient status of the soil?

Yes	No

17. For the above question number 16, if your answer is yes, are their deficient chemical elements identified so far? List their names.

18. For question number 16, if your answer is no, how did you managed diseases and or disorders associated with mineral deficiencies?

_____
_____
_____

19. Do you use a fertilization program?

Yes	No

20. For question number 18, if your answer is yes, what type of fertilizer do you applied?

_____
_____
_____
_____
_____

21. Referring to question number 19, how often do you apply fertilizer to a farm with in a year?

_____
_____
_____

III. Pre-harvest Epidemiology and Disease management practices

22. Do you have problems of diseases on your citrus trees?

Yes	No

23. Which part of the tree is attacked with the most common diseases?

Root	Stem	Leaf	Fruit

24. Which type of infection is most prevalent? Put in order (1-4) according to their importance

Fungal infection	Bacterial infection	Virus infection	Nematode attack	Insect problems

25. If the disease has a microbial origin, which type? Can you name/ describe the type of disease and its pathogen in order of its importance?

_____
_____
_____

26. If insects are important, what type of insects?
Write their names in order of importance

a)_____
b)_____
c)_____
d)_____
e)_____
f)_____

27. If nematodes are present, which type? Mention by name according to their importance.
    a) _____
    b) _____
    c) _____

28. Referring to question number 25, when do you think does the fungal infection start to appear on the orchards?

a

At grafting	At flowering	Just at fruiting	At fruit ripening	

b

At grafting	At flowering	Just at fruiting	At fruit ripening	

c

At grafting	At flowering	Just at fruiting	At fruit ripening	

d

At grafting	At flowering	Just at fruiting	At fruit ripening	

e

At grafting	At flowering	Just at fruiting	At fruit ripening	

29. With reference to question 26, when do you think does insect problem start to appear on the orchard?

a

At grafting	At flowering	Just at fruiting	At fruit ripening	

b

At grafting	At flowering	Just at fruiting	At fruit ripening	

c

At grafting	At flowering	Just at fruiting	At fruit ripening	

d

At grafting	At flowering	Just at fruiting	At fruit ripening	

e

At grafting	At flowering	Just at fruiting	At fruit ripening	

f.

At grafting	At flowering	Just at fruiting	At fruit ripening	

g.

At grafting	At flowering	Just at fruiting	At fruit ripening	

30. Referring to question number 24, when do you think does the respective virus infection start to appear on the orchards?

a

At grafting	At flowering	Just at fruiting	At fruit ripening	

b

At grafting	At flowering	Just at fruiting	At fruit ripening	

c

At grafting	At flowering	Just at fruiting	At fruit ripening	

d

At grafting	At flowering	Just at fruiting	At fruit ripening	

e

At grafting	At flowering	Just at fruiting	At fruit ripening	

31. Referring to question number 24, when do you think does the respective nematode infection start to appear on the orchards?

a

At grafting	At flowering	Just at fruiting	At fruit ripening	

b

At grafting	At flowering	Just at fruiting	At fruit ripening	

c

At grafting	At flowering	Just at fruiting	At fruit ripening	

d

At grafting	At flowering	Just at fruiting	At fruit ripening	

e

At grafting	At flowering	Just at fruiting	At fruit ripening	

32. How do you control disease? Explain your experience on the farm.

_____
_____
_____
_____
_____
_____
_____

33. Did you apply pesticides on your farm?

Yes	No	

34. Referring to the above question number 25, if you have applied pesticides, mention their names and application involved.

Name the commercial pesticides	What concentrations are being applied?	When do you start spraying pesticides?	How often do you spray during the growing season	What is the type of insect controlled?

III. Input costs and production statistics

35. How many workers are there in the farm?

_____
_____
_____

36. What is the average working hours of the farm worker per day?

_____
_____

37. What is the average salary of a farm worker per month?

_____
_____

38. What is the average input invested for pesticides purchase per year?

_____
_____
_____
_____

39. What is the average input invested for fertilizer purchase per year?

_____
_____
_____
_____
_____

QUESTIONNAIRE: II **Code "B" 2003/04**

Postharvest citrus (sweet orange) fruit handling and disease management practices

SITE: _____ **Date:** _____

Name: _____

Region: _____

Packhouse/Market Name and address: _____

I. Fruit harvesting

1. What is the daily temperature of a farm?

<10°C	10-15 °C	16-20 °C	21-25 °C	>25°C

2. When is the peak time for harvesting fruit?

Jan.	Feb.	May	Apr.	Ma.	Jun.	Jul.	Au.	Sep.	Oct.	Nov.	Dec.

3. What is the average temperature in the region?

a) Day time

<10°C	10-15 °C	16-20 °C	21-25 °C	>25°C

b) Night time

<0 °C	0-5 °C	6-10 °C	11-15 °C	>15 °C

4. What is the average relative humidity (RH)?

<29	30-40	41-50	51-60	61-70	71-80	>81

5. How do you pick fruit from the orchard?

Hand picking with gloves	Without gloves	By pulling with long sticks	By climbing in the tree

Describe if you have another method of harvesting _____

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6. Who are picking your fruits?

Farm workers	Retailing market dealers	Part time workers

Mention if there are any _____

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7. Referring to question number 5, how many fruits are harvested at a time in a day?_____

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8. Where do you put fruits while collecting?

In a sack (1/2 a quintal size	In plastic crates	Openly on the ground/soil

Mention if there is any fruit collection method

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9. At what temperature do you store fruits?

<0 ^{o}C	0-5 ^{o}C	6-10 ^{o}C	11-15 ^{o}C	>15 ^{o}C

Mention if there is any

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II. Fruit Transportation

10. How do you transport fruits from farm to packhouse?

By vehicle	By cart	By human labor

Explain if there are any other methods used in your farm to transport fruits?

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11. For how long do you store fruit in the packhouse?

<6hrs	A day (24hrs)	48hrs	A week	More than a week	We don't store fruits at all

12. Storage conditions of fruits during transit?

<0 ^{o}C	0-5 ^{o}C	6-10 ^{o}C	11-15 ^{o}C	>15 ^{o}C

III. Postharvest Diseases

13. Do you have problems of postharvest diseases in the storage /packhouses?

Yes	No

14. For the above question (9), if your answer is no, what do you think the case is? May you explain the detail?

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15. For question (13) above, if your answer is yes, what are the major diseases associated? May you write down their names in order of importance?

a)_____

b)_____

c)_____

d)_____

e)_____

f)_____

16. Referring to question number 15, how do you control postharvest diseases and their dissemination? Explain your experience?

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17. Do you apply commercial chemicals to control postharvest citrus diseases?

Yes	No

18. For the above question (17) if your answer is yes, mention the type of chemical applied and how frequently used?

Chemical name	Formulation	Application (how frequently used)	Remarks

19. For the same question number 17, if your answer is no, why? May you explain the reason?

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20. Referring to question 18, the spray of chemicals, by what equipment and is the machine calibrated?

Yes	No

21. For question number 20, if your answer is no, what is the hindrance?

a) lack of knowledge

b) lack of training

c) lack of economy

d) mention if there are any other factors

22. How far is the average distance to your local market? Name market places and their distance from the farm packhouse.

Name of market places	Approximate distance (km)	Safety measures taken to keep quality of fruits	Remarks

23. How do you transport the packed fruit to the local market?

By vehicle	On the back of animals	By human labor

Mention if there are any means of fruit transportation:

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24. Do you have international market access for the fruit produced and or its product?

Yes	No

25. For the above question (24), if your answer is yes, where? Mention the name of the country according to their market importance?

Country	Fruit market	fruit product	Remarks

24. How do you transport the packed fruit to the international market?

By air	By ship	By train

25. What is the transit temperature used during export? If others, give

4°C	7 °C	10 °C	>11 °C	

26. Do you apply chemicals to control decay of fruits during transit?

Yes	No

27. For the above question number 26, if your answer is yes, what chemicals do you use? List the name of commercial chemicals applied?

Chemical name	Formulation	Application (how frequently used)	Remarks

28. For question number 24, if your answer is no, what is hindrances to export?

a) lack of knowledge, know how, contact
b) quality guarantee because of diseases and associated problems
c) mention if encountered other factors

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29. Write any experiences of your farm (cultural, physical, biological or a combination of them) in postharvest disease handling and management practices to control citrus fruit disease.

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IV. Fruit Price and marketing

30. What is the average price of fruit per kilogram in the local market?

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31. What is the average price of fruit per kilogram in the export market?

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APPENDIX III

SENSORY EVALUATION FORM

Name _____

Date _____

Time _____

Parameters	A	B	C	D	E	F	G	H	I
Skin colour									
Colour of the ediable portion									
Colour of the flavedo									
Appearance									
Flavour									
Odour or smell									
Juiciness									
Sweetness									
Sour or Bitterness									
Overall acceptability									

Attribute	Definition
Smell	Total intensity of smell
Fresh	smell of fresh oranges
Flavour	Total intensity of flavour during the first chewing
Sweetness	Sweet taste
Bitterness or sourness	bitter or sourness
Appearance	whether it is fresh, shriveled, firm, soft
Colour	natural colour of orange or presence of browning.

Hedonic scale 1-5, where,

1=very poor, 2 poor, 3 fair, limited acceptability, 4- good, 5= excellent

Signature of the participant

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Identification of citrus (*Citrus sinensis*) Postharvest Pathogens from Ethiopia and their Control

By

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SUMMARY

From a world prospective, the continuous application of chemical pesticides has serious long-term effects on human health and environmental pollution, and can result in resistant pathogen strains. However, postharvest diseases cause major losses on the markets and need to be controlled effectively. The search for biopesticides using microbial antagonists and natural plant products has subsequently become more important as viable alternatives to control postharvest diseases. Currently, little information exists in terms of citrus production practices, disease management measures and postharvest losses in Ethiopia. The aim of this study was therefore to determine what the current situation in the country is in terms of production, disease management and postharvest disease incidence, disease management practices in Ethiopia and to develop an effective and safe disease control strategy for the industry. Citrus production in Ethiopia is mainly done by Government enterprises with little technical expertise. Disease control strategies are ineffective with postharvest losses exceeding 46%. The most important postharvest pathogen identified was *Penicillium digitatum*. In development of biopesticides, three yeast antagonists [*Cryptococcus laurentii* (strain MeJtw 10-2 and strain TiL 4-3) and *Candida sake* (TiL 4-2)] and plant leaf extracts of *Acacia seyal* and *Withania somnifera* were found to have some potential to

control *Penicillium* in *in vitro* and *in vivo* trials and ensure fruit quality. The modes of action of the yeast antagonists were not based on antibiosis. Instead, it involved competitive colonization where the antagonists inhibited *P. digitatum* spore germination and reduced mycelial growth by 75-100%. Extracts from the two plant species showed broad-spectrum antimicrobial activity against a range of several fungal and bacterial pathogens. The semi-commercial application of the antagonists and plant extracts improve fruit quality and the integration of these biopesticides were found effective in semi commercial trials and may provide a commercial solution for the citrus industry.