Synergistic Effects of Mixtures of Fungicides and Medicinal Plant Extracts Against *Botrytis cinerea*

by

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BACKGROUND INTELLECTUAL PROPERTY

Synergistic Effects of Mixtures of Fungicides and Medicinal Plant Extracts Against *Botrytis cinerea*

1. Part of the data presented in this thesis form the background intellectual property for the project titled, "**Indigenous Botanical Adjuvant Technologies (iBATECH)**" funded by the **Innovation Fund** on behalf of the **South African Department of Science and Technology (DST)**.

2. Elements of the data described in this thesis form an integral part of the information that received **Provisional Patent Registration** from the **Companies and Intellectual Property Registration Office (CIPRO)** of the **South African Department of Trade and Industry (DTI)**.

DECLARATION

I declare that "*Synergistic Effects of Mixtures of Fungicides and Medicinal Plant Extracts Against Botrytis cinerea*" is my own work, that it has not been submitted for any other degree or examination in any other university, and that all the sources I have used or quoted have been indicated and acknowledged by complete references.

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ABSTRACT

We hypothesize that South African medicinal plants contain compounds that can act in synergism with synthetic antifungal compounds. Four fungicides - Sporekill™, Rovral™, Terminator™ and Teldor™ at doses 0.1, 0.2, 0.4 and 0.8 mL L^{-1} and plant species *Galenia africana*, *Elytropappus rhinocerotis* and *Tulbaghia violacea* were tested alone and in different combinations for their potency (efficacy) on radial growth inhibition of *Botrytis cinerea* strains on potato dextrose plates. Four doses of plant extract for each of the respective plant species were used. A total of 48 combinations were tested for each strain. Mixtures of plant extracts were far more effective in controlling strains compared to the individual components alone, representing significant levels of *in vitro* synergistic interactions. Combinations of these components represent an attractive future prospect for the development of new management strategies for controlling *B. cinerea*. Since the *in vitro* tests of these mixtures showed inhibitory activity, the mixtures were tested for activity in assays on Granny Smith apples. *In vitro* tests can be used to screen mixtures to obtain information on their inhibitory activity on a pathogen, however, the environmental conditions of the fruit and the ability of the pathogen to grow into the fruit cannot be simulated *in vivo*. A series of two-fold doses of medicinal plant extracts were combined with fungicides to conduct decay inhibition studies. The incidence of gray mold was significantly reduced by mixtures of plant extracts and fungicides. Under conditions similar to those in commercial storage, a drench treatment with *G. africana* and Rovral™ significantly ($p=0.05$) inhibit gray mold on the apples and was more effective than the plant extract and fungicide alone. The treatments exerted synergistic effects and were markedly better than the components applied alone. The wound colonization assay was used for optimal decay control. In a drench, much higher volumes of the treatments are used to ensure that the components of the suspension are deposited evenly over the entire fruit surface. Drenching of fruit to apply other

chemicals is an established practise in the pome (fleshy) fruit industry, and simplifies the commercial application of the mixtures, as no additional infrastructure at commercial packing houses will be required. This approach not only makes it possible to reduce fungicide concentrations while maintaining adequate decay control, but also ensures a reduction of the chemical residue on the fruit.

Key words: *Botrytis cinerea,* synergistic, antagonistic, post-harvest control, medicinal plant species, plant extracts, fungicides, low doses.

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cv	Cultivar
MIC(s)	Minimum inhibitory concentration(s)
µg	Microgram
mL	Milliliters
L	Liters
g	Gram
%	Percentage
°C	Degrees Celsius
mg	Milligram
mm	Millimeters
d	days
ppm	Parts per million
nm	Nanometers
hrs	Hours

CHAPTER 1

GENERAL INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

Fruit are among the most important foods of humans as they are nutritive and indispensable for the maintenance of health (Shahi *et al*., 2003). South Africa`s climate and soil conditions provide ideal conditions for many varieties of fruit to be grown. Citrus, deciduous and subtropical fruit are all grown throughout most of the country (Polderdijk, 2006). Deciduous fruit includes grapes, apples, pears and stone fruits. Apples constitute the bulk of deciduous fruit in South Africa, *i.e*., in 2000, apples made up the largest percentage of the deciduous fruit crop (43%). The South African fruit industry is orientated to produce for local consumption as well as export to Europe and North America. Fruit is exported in refrigerated containers or in bulk shipments. Cold storage conditions favor pathogen development and physiological breakdown of the commodity unless managed correctly (Korsten, 2006). Gray mold, caused by *Botrytis cinerea* Pers ex Fr., is regarded as the one of the most economically important post-harvest pathogens of apples that causes significant losses (Sommer, 1982; Rosenberger, 1990). The ability of gray mold to develop under conditions prevailing during cold storage, transport and marketing makes its control a challenge (Ogawa *et al*., 1995). Pre- and post-harvest applications of synthetic fungicides are frequently used to control post-harvest rots. However, these products involve risks for consumers' health (Caffarelli *et al*., 1999) and for onset of pathogen-resistant strains (Spotts and Cervantes, 1986; Guizzardi *et al*., 1995; Stehmann and De Waard, 1996). Therefore, new strategies have been proposed to reduce or replace chemicals to control post-harvest diseases (Wilson and Wisniewski, 1994; El Ghaouth, 1997).

In particular, the use of natural plant extracts seems to be effective in reducing the incidence of post-harvest fungal pathogens of different fruits, both in small-scale experiments and in semi-commercial conditions (Wilson and Wisniewski, 1994; Jijakli *et al*., 1999; Lima *et al*., 1999). Natural plant extracts have become important since they are perceived as being environmentally safer and more acceptable to the general public. Several limitations affect the commercial applicability of plant extracts. When used as a stand-alone treatment, none of the extracts clearly and consistently offer an economically sufficient level of disease control that will warrant their acceptance as a viable alternative to synthetic fungicides. The inconsistency and insufficient commercial efficacy of plant extracts is partly attributed to their inability to control previously established infections (Droby, 2001). Successful commercial control of post-harvest diseases of fruits must be extremely efficient, in the range of 95-98%. In order to develop new and highly effective biofungicides, it is imperative to make their yield and activities more reliable, especially when combining them with low doses of fungicides in integrated control programmes (Chand-Goyal and Spotts, 1997; Ippolito *et al*., 1998; Droby *et al*., 2002).

1.2 Synergistic Interactions of Fungicides in Mixtures

Since the 1970s, fungicides with systemic and site-specific activity, sometimes called modern fungicides, have been used often. Modern fungicides have a relatively low environmental toxicity index as compared to conventional fungicides, and can be applied at lower rates of active ingredients. Hence, they contribute to environmental safe agriculture. On the other hand, modern fungicides have caused resistance problems (Leroux and Descotes, 1996). Modern fungicides are mostly single-site inhibitors. Countermeasures to avoid or delay the risk of resistance development are largely based on the use of fungicide mixtures (Hayashi, *et al*., 2003).

There are three main reasons why different fungicides are combined in mixtures as part of a treatment programme: (i) to widen the spectrum of antifungal activity to control several diseases occurring simultaneously in a crop; (ii) to exploit additive and synergistic interactions between fungicides, by which the overall combined activity is increased and the concentrations of the compounds in the mixture reduced without loss of activity; and (iii) to delay the selection process of resistant strains in a pathogen population to one component of the mixture (Gisi, 1996). The properties (i and iii) of an arbitrary mixture can be tested by biological methods, whereas to measure synergism special mathematical analysis is needed (Kosman and Cohen, 1996).

To proof the occurrence of a synergistic effect between pesticides, biological evidence alone is not enough, because the observed efficacy of a mixture should be compared with its expected efficacy. Several methods have been developed to assess synergistic interaction between pesticides. Only two basic approaches are commonly used: the Abbott (also known as the Colby) method (Abbott, 1925; Colby, 1967), which is usually applied for mixtures whose constituents produce their effects in different modes, and the Wadley method (Wadley, 1945; Tammes, 1964), which is applied for mixtures in which the components have similar modes of action (Kosman and Cohen, 1996).

1.3 Definitions and Classifications

To make the definition of synergism more accurate and less ambiguous, the term: “expected effect of the mixture” is used instead of “sum of the effects of the individual components”. According to Gowing (1959) “the expected effect” is a simple summation of the effects of the materials acting alone, whereas according to Wadley (1945) and Colby (1967) it is a value that should be calculated by specific formulas.

The methods of Abbott (Abbott, 1925; Colby, 1967) and Wadley (Wadley, 1945; Tammes, 1964) were originally developed for insecticides, but were extensively studied with fungicides as well (Gisi, 1996). Responses measured with fungicides usually include diseased leaf area in both the greenhouse and the field. Percent survival of germlings and linear growth of mycelial mats *in vitro* may also be used. Choosing either method depends on the possible type of action of the components in the mixture. Two types are usually considered: different and similar action (Wadley, 1945; Gowing, 1960; Levy *et al*., 1986; Cohen and Levy, 1990; Gisi, 1991).

Different action occurs when each pesticide affects a different physiological activity or vital system in the pest or pathogen (Wadley, 1945). In this case, the Abbott procedure was proposed to determine synergism. Similar action occurs when the same vital systems in the pest are affected by both pesticides. When this is the case, one component of the mixture could be replaced by the other at a response-equivalent dose. The Wadley method is appropriate to calculate synergism when chemicals can be substituted for one another at a fixed ratio. Otherwise, synergism analysis becomes complex, and "it seems unlikely that synergism will be found under such conditions" (Wadley, 1945).

1.4 Determination of Synergism

Determination of a synergistic (or antagonistic) property of a mixture requires the comparison of the experimental results with a reference model that represents the joint action of the mixture constituents under the assumption of the absence of either synergism or antagonism. The activity of a mixture is first measured experimentally, and then it must be compared with its calculated expected activity to achieve an indication of the extent of pesticide interaction. Thus, the decision about synergism is entirely dependent on the assessed value of the expected response of the mixture (Kosman and Cohen, 1996).

There are several methods of estimating the extent of interaction of fungicides in mixtures. The two major used are those by Abbott (Abbott, 1925; Colby, 1967) and Wadley (Wadley, 1945; Wadley, 1967). The expected efficacy of a mixture, expressed as percent control ($\%C_{exp}$), can be predicted by the Abbott formula (Levy *et al*., 1986):

$$\%C_{exp} = (A + B) - (AB/100)$$

in which *A* and *B* are the control levels given by the single fungicides. If the ratio between the experimentally observed efficacy of the mixture (C_{obs}) and the expected efficacy of the mixture (C_{exp}) is greater than 1, synergistic interactions are present in the mixture. The Abbott method is used without mathematical transformations to estimate the interaction for single concentrations of a mixture, provided the control levels of the single components are not higher than about 70%. Synergistic interactions always decrease rapidly with increasing control levels of the single components (Samoucha and Cohen, 1984; Levy *et al*., 1986; Samoucha and Gisi, 1987) and may be almost zero at high control levels.

In the Wadley approach (Gisi *et al*., 1985; Levy *et al*., 1986), dose-response curves of the single components, *A* and *B*, and the mixture, *A* + *B*, are constructed. With a logit-log (or more appropriately a probit-log) transformation, the dose-response curves are linearized by regression and then used to calculate the EC (effective concentration) values for different control levels, e.g., EC_{50} or EC_{90}. When *a* and *b* are the absolute amounts of the components in the mixture, the expected effective concentration (EC_{exp}) at any control level can be calculated (Gisi *et al*., 1985) as

$$EC_{90exp} = (a + b)/[(a/EC_{90A}) + (b/EC_{90B})]$$

Synergistic interactions are present if the ratio of the expected and the observed EC values is greater than 1. The Wadley approach can be used for estimation of interactions at any fungicide concentration, and its reliability is not dependent on the

disease-control level. In single experiments, statistical procedures are available for dose-response relationsm, but not for calculation of synergistic interactions. Synergy ratios calculated on the basis of EC_{50} and EC_{90} values in most cases are very similar to each other. Because statistical confidence levels of synergy ratios are missing, ranges rather than precisely fixed values may be defined to quantify antagonistic, additive, and synergistic interactions (Table 1-1), also taking into account the degree of variation in biological responses to antifungal compounds (Gisi, 1996).

Table 1-1. Suggested terminology for levels of interaction[a] in fungicide mixtures when the Wadley approach is used for quantification.

Level of Interaction	Mathematical Definition[b]	Biological Response[c]	Biological Response[d]
<1.0	Antagonistic		
1.0	Additive		
>1.0	Synergistic		
<0.5		Antagonistic	
0.5-1.5		Additive	
>1.5		Synergistic	
<0.7			Antagonistic
0.7-1.3			Uncertain
>1.3-2.0			Weakly synergistic
>2.0			Strongly synergistic

[a]Ratio between expected and observed EC (effective concentration) values. [b] According to Levy *et al*. (1986), and De Waard and Gisi (1995). [c] According to Gisi *et al*. (1985). [d] This definition avoids the artificial case of additive interaction and is based on the examples given by Kosman and Cohen (1996). The biological response definitions cannot be used for the Abbott approach.

1.5 Examples of Synergistic Interactions in Mixtures

Reports on synergism in mixtures of commercial fungicides are rare. The classical example is the synergism in mixtures of phosphoramidate and phosphorothiolate fungicides to *Pyricularia oryzae*. The synergism has been ascribed to inhibition of phosphoramidate metabolism by a phosphorostrobin (Qo site inhibitor) and antimycin A (Qi site inhibitor), and can be synergized in *B. cinerea*, *Cochliobolus miyabeanus*, *Monilinia fructicola*, and *P. oryzae* by salicylhydroxamic acid that inhibits the cyanide-insensitive respiration pathway (Hayashi *et al*., 2003).

Extensive attempts have been made to increase the efficacy of *bio-control* systems for the management of post-harvest disease by either improving the fitness and antagonistic traits of *bio-control* agents (Chalutz and Droby, 1997), or by combining other disease-control practices with the biological-control approaches (Zhou *et al*., 1999). One of these strategies is to combine a *bio-control* agent with a fungicide. Control of *Penicillium expansum* on apples by *Crytococcus laurentii* HRA5 was improved by combining it with thiabendazole (TBZ) in a semi-commercial trial (Chand-Goyal and Spotts, 1997). More recently, Sugar and Spotts (1999) reported that with artificially inoculated pears, the addition of 100 µg/mL TBZ to the biofungicides Bio-Save® 110 and Aspire® resulted in a significant improvement in blue mold control compared with the biofungicides used alone. In packinghouse trial combinations of biofungicides, Bio-Save® 110 or Aspire® with 100 µg/mL TBZ (about 17.6% of the label rate) provided control of blue mold and gray mold of pears similar to that of TBZ alone used at the label rate (569 µg/mL) (Zhou *et al*., 2002).

Lorito *et al*. (1993) tested the antifungal activity of two chitinolytic enzymes, endochitinase and chitobiosidase, from *Trichoderma harzianum* strain PI, against nine different fungal species. Spore germination and germ tube elongation of all chitin-containing fungi (except the producing fungus) were inhibited, and the degree of inhibition was proportional to the level of chitin in the cell wall of the test fungi. The

endochitinase inhibited the growth and development of these fungi more than the chitobiosidase, however, the synergistic interaction of the two chitinolytic enzymes, endochitinase and chitobiosidase, resulted in greater inhibition than that of either enzyme used singly.

In a follow-up study, the same group (Lorito *et al*., 1994) purified two more chitinolytic enzymes from the culture filtrate of *T*. *harzianum* strain PI. These two enzymes, β-1,3-glucosidase and N-acetyl-β-glucosaminidase, were tested against *B*. *cinerea* and their antifungal activity compared with that obtained for an endochitinase and a chitobiosidase, also purified from *T*. *harzianum* strain PI. The four cell wall-degrading enzymes also were tested as a mixture containing two, three, or all four, in all possible combinations. A synergistic inhibitory effect was detected on both spore germination and germ tube elongation of *B*. *cinerea* when two, three, or all four enzymes were used together. The highest level of antifungal activity was obtained when a mixture containing all four enzymes was applied. This suggested that the synergistic antifungal interaction reported earlier for two chitinolytic enzymes (Lorito *et al*., 1993) was dramatically improved when one, two, or more fungal cell wall-degrading enzymes, with different substrate specificities, were added to the mixture (Lorito *et al*., 1996).

1.6 Synergism in Mixtures of Different and Identical Modes of Action

1.6.1 Different Modes of Action

Synergistic interactions are well documented for mixtures containing the systemic phenylamide fungicide oxadixyl, the contact fungicide mancozeb, as well as the systemic fungicide cymoxanil against *Phytophthora* and *Plasmopara* (Gisi *et al*. 1985; Grabski and Gisi, 1987; Samoucha and Gisi, 1987; Samoucha and Cohen, 1988). Synergy ratios vary within a large range (*e.g*., 1.4 to 5.3). Mixtures of the triazole fungicide cyproconazole and the chlorophenyl fungicide chlorothalonil tested against *Mycosphaerella arachidis* on peanuts, produce strong synergistic interaction at ratios 1:7

and 1:10, whereas at ratios below 1:2 and above 1:20 the mixtures may be additive or antagonistic. Fungicides with different modes of action may affect the fungus at different biochemical sites and developmental stages, resulting in combined and synergistic activity of the mixture. The components preferably should be applied simultaneously for maximum expression of synergistic interactions.

1.6.2 Identical Modes of Action

All triazole fungicides have the same biochemical mode of action (inhibition of C14 demethylation in sterol biosynthesis), but differ significantly in their spectrum of activity and, therefore, are often combined in mixtures. Mixtures of cyproconazole or flutriafol and other triazoles were tested against three important pathogens of wheat: *Erysiphe graminis*, *Leptosphaeria nodorum*, and *Mycosphaerella graminicola*. Synergistic interaction occurred in many fungicide combinations, whereas in others the biological response was uncertain. Generally, the level of synergistic interaction was not as pronounced as in mixtures of fungicides with different modes of action. Synergistic interactions were most pronounced against *E. graminis*. Obviously, there are important differences among triazole fungicides that allow synergistic interactions, including different systemic effects and uptake into the fungus and plant as well as different physicochemical properties (*e.g.*, water solubility and log *p*) and rates of metabolism (Gisi, 1996).

1.7 Bio-Control Agents (Microbes and Plants)

Gliocladium virens is used for biological control of many diseases under controlled conditions, including *B. cinerea*. The control mechanisms are probably not competition for nutrients or space nor are they related to hyperparasitism, but more likely toxins are produced. Di Pietro *et al*. (1993) claimed enzyme activity is involved in the control mechanism. In experiments with spore germination of *B. cinerea* as the target process,

they combined purified gliotoxin (G) and endochitinase (E) in different ratios and found increased activity with the mixture compared to the single components alone. When the results of Di Pietro *et al*. (1993) are analyzed according to the Abbott approach, very strong synergistic interactions are found for certain G+E combinations, especially when control levels are low. The mixture provides maximum synergy for G+E = 0.75 + 50 mg/liter (synergy ratio=38). When the same results were analyzed according to Wadley, synergy ratios were less pronounced and were between 1.1 and 1.5. The synergistic interaction between the antifungal toxin and the enzyme was described for the pathogen *in vitro*. Whether the reported synergy levels are expressed at the disease level under greenhouse or field conditions is unknown. Synergy found experimentally for natural product combinations may represent an important principle of stability in co-evolution of microbial communities in nature (Gisi, 1996).

1.8 Mechanisms of Synergism and Antagonism

The mechanisms involved in interactions of components of a mixture may be classified as pseudo- or true synergism or antagonism (De Waard and Gisi, 1995). In the case of pseudosynergism, the interaction influences the performance of the parent fungicide by affecting its distribution on the plant surface or uptake into plant tissue. It may also result from the presence of mixed pathogen populations or from populations with both fungicide-sensitive and -resistant subpopulations. In the case of true synergism, the components of a mixture directly react with each other, or the companion compound influences the physiology of the pathogen in such a way that the toxicity of the parent fungicide is changed.

Reactions between compounds in mixtures containing sterol demethylation inhibitors (DMIs) have not been adequately described. The only example is imazalil, which can be protonated at low pH, resulting in reduced accumulation of the fungicide in mycelium and decreased antifungal activity (Siegel *et al*., 1977). Activity of most DMIs

depends not on activation or detoxification reactions in plant pathogens (De Waard, 1994), but rather mostly on direct inhibition of their target enzyme, sterol 14α-demethylase. This probably explains why interactions based on interference with either one of these processes have not yet been reported. In fungi, phosphorothiolate fungicides induce mixed-function oxidases that also may have affinity to DMIs. Hence, these fungicides may antagonize the toxicity of DMIs by interfering with binding to sterol 14α-demethylase (Sugiura *et al*., 1993). Mixtures of sterioisomers of cyproconazole or tebuconazole display synergistic fungicidal activity. This can be ascribed to preferential binding of the most active isomer in these mixtures to the P-450 moiety of sterol 14α-demethylase, whereas the less active isomers may saturate other P-450s (Stehmann and De Waard, 1995).

1.8.1 Accumulation Mechanism

Accumulation of DMI fungicides in fungal mycelium is the result of two processes: passive influx and active efflux (De Waard and Van Nistelrooy, 1984). Passive influx occurs by diffusion and is probably determined by partitioning of DMIs between the extracellular medium and intracellular compartments of mycelium. Active efflux from the mycelium into the surrounding medium is an energy-dependent process. It does not operate in the absence of a carbon source in the medium, at low temperatures, under anaerobic conditions, or in the presence of various metabolic inhibitors. The efflux has an inducible character, because at particular DMI concentrations accumulation appears to be transient (De Waard, 1996).

1.8.2 Multidrug Resistance

The tenet that microorganisms develop resistance against antimicrobial compounds is well established (Marchetti *et al.*, 2000). One of the major modes by which pathogens develop resistance is through the development or enhancement of biochemical

mechanisms for the removal of antibiotics that have entered the cells of the organism. Thus, resistant microorganisms possess efficient systems known generally as multidrug resistant pumps (MDR-pumps) (Morel *et al.*, 2003). The survival of microorganisms in natural environments is favoured by the capacity to produce compounds toxic to competing organisms, and the ability to resist the effects of such toxic compounds. Both factors contribute to a competitive advantage of organisms in ecosystems. Most organisms have evolved active transport mechanisms by which endogenous toxicants can be secreted. Two major classes of transport proteins are the ATP-binding cassette (ABC) and the major facilitator superfamily (MFS) transporters. Members of both families can be regarded as a "first-line defense barrier" in survival mechanisms. In plant pathogens, these transporters can play an essential role in protection against plant defense compounds during pathogenesis. ABC and MFS transporters can play a major role in fungicide sensitivity and resistance (Schoonbeek *et al.*, 2000b).

A mechanism which may play a pivotal role in resistance to fungicides in *B. cinerea*, is decreased accumulation of the compound in mycelial cells due to energy-dependent efflux. Accordingly, the driving force behind the energy–dependant efflux of the fungicides can be the ABC transporters. Members of the MFS transporters may have similar functions (Paulsen *et al*., 1996). Some of these transporters can be regarded as fungicide pumps, which may account for the multidrug resistance of *B. cinerea* (Schoonbeek *et al.*, 2001). ABC transporters can move toxins from the inner leaflet of the membrane to the outer environment of cells thereby, reducing accumulation of the compound in cells (Holland and Blight, 1999). MFS transporters prevent accumulation of toxic compounds in cells through the process of the "proton-motive force" over membranes (Hayashi *et al.*, 2002). Therefore, the transporters in filamentous fungi play a role in the protection of *B. cinerea* to fungitoxic compounds (Schoonbeek *et al*., 2000a).

Modulators of the activity of ABC tranporters have been reported as synergists of drugs and fungicides against MDR tumour cells of mammals and DMI-resistant fungi, respectively. Resistance to drugs is often mediated by overproduction of specific ABC transporters, resulting in reduced drug accumulation in cells. Modulators inhibit ABC transporter activity, which traps the drugs inside the cells and drug resistance is neutralized. A natural function of fungal ABC transporters is to provide protection against plant defense products during pathogenesis (Hayashi *et al.,* 2003).

The ongoing efforts against microbial resistance is at a point where there is potential for plant-based compounds to be developed as MDR pump inhibitors to enhance the activity of their own natural antimicrobial compounds (Morel *et al*., 2003). Physiological functions of ABC and MFS transporters include maintenance of cell membrane integrity, cellular iron homeostasis, uptake of nutrients, presentation of antigenic peptides, and secretion of mating factors and enzymes. A widely described function is efflux of endogenous toxic compounds (Schoonbeek, 2004) - this can be deduced from the finding and report, which stated that they have been looking for plant products that do not themselves possess antimicrobial activity, but can potentiate known antibiotics by inhibiting microbial MDR pumps. MDR-mediated by active efflux has been demonstrated in azole-resistant laboratory-generated mutants of *Penicillium italicum* and *B*. *cinerea* (Stehmann and De Waard, 1995; De Waard and Van Nistelrooy, 1988; Hayashi *et al*., 2001; Vermeulen *et al*., 2001). Recently, MDR has been reported in field strains of *Penicillium digitatum* and *B*. *cinerea* (Nakaune *et al*., 1998; Chapeland *et al*., 1999).

1.9 The Plant Pathogen *Botrytis cinerea*

There are 50 species of *Botrytis*, accounting in part for the wide range of plants and plant parts affected. The pathogenic fungus, *B. cinerea* Pers.: Fr is the causal agent of gray mold. The name *Botrytis* is derived from βοτυρυς, the Greek word for grape, since

the fungus produces spores like bunches of grapes. *Botrytis cinerea* is the anamorph of *Botryotinia fuckeliana* (De Bary) Whetzel (Jarvis, 1977), which is the teleomorphic stage of the pathogen. All *Botrytis* species are necrotrophic, since plant cells are actively killed during pathogenesis (Del Sorbo *et al*., 2001).

1.9.1 Symptoms

Botrytis cinerea can be observed wherever host plants are grown from subtropical areas to temperate zones. Plants can be attacked at any stage, from seedlings to large, mature plants, but new succulent growth, freshly injured tissues and ageing or dead foliage are favoured by this disease. Botrytis usually appears as lesions on leaves and stems that quickly produce a characteristic gray/brown furry spore development which resembles a pile of ash – hence the name 'gray mold'. As the disease progresses, the lesions continue to grow and encircle stems and leaf petioles and will eventually cause plant collapse. Fungal spores can also develop on flower petals, particularly under growing conditions where condensation has been forming and humidity levels are high. Infection of flower petals leads to rapid disease development in young fruit, with the fruit tissue rapidly disintegrating into a water-soaked mass. *Botrytis* causes a soft, spongy rot with a sweet, cider-like odour. As the rot progresses, the fungus produces masses of gray spores on the surface of affected fruits. The infection may spread from fruit to fruit during storage, producing "nests" or "pockets" of decayed fruit. Small black resting bodies (sclerotia) may eventually form on infected fruit (Schoonbeek *et al.*, 2001).

1.9.2 Disease Cycle

Conidia of *B. cinerea* are spread by wind, air currents or insects. The fungus overwinters as a mycelium or sclerotia in dead crop tissue and other organic debris (Figure 1-1). In spring, conidia are formed from an overwintering mycelium or sclerotia and start epidemics (Holz, 2001). In the case of grapevine, they may infect blossoms,

colonize dead flower parts, and penetrate young grapevine berries. In the berries, the fungus may remain dormant until the fruit sugar content increases to a level that supports fungal growth (Hayashi, 2003). *Botrytis cinerea* is sometimes described as an opportunistic pathogen since infections commonly occur via wounds or previously colonized dead or senescent plant parts. Direct penetration of the intact plant cuticle is less common, but may occur under high humidity conditions and in the presence of nutrients. In the latter case, penetration is often followed by a quiescent period during which disease progress is slow or even absent (Benito *et al*., 1998; Keller *et al*., 2003). Later, depending on the climate conditions and the developmental stage of the plant, primary lesions may expand into water-soaked or necrotic lesions. On the host surface, white mycelium can be formed that turns gray with maturation of condiophores bearing asexual conidia. The common name "gray mold" relates to these disease symptoms. Rapid spore formation yields an abundant inoculum for a new infection cycle within a week (Schoonbeek, 2004).

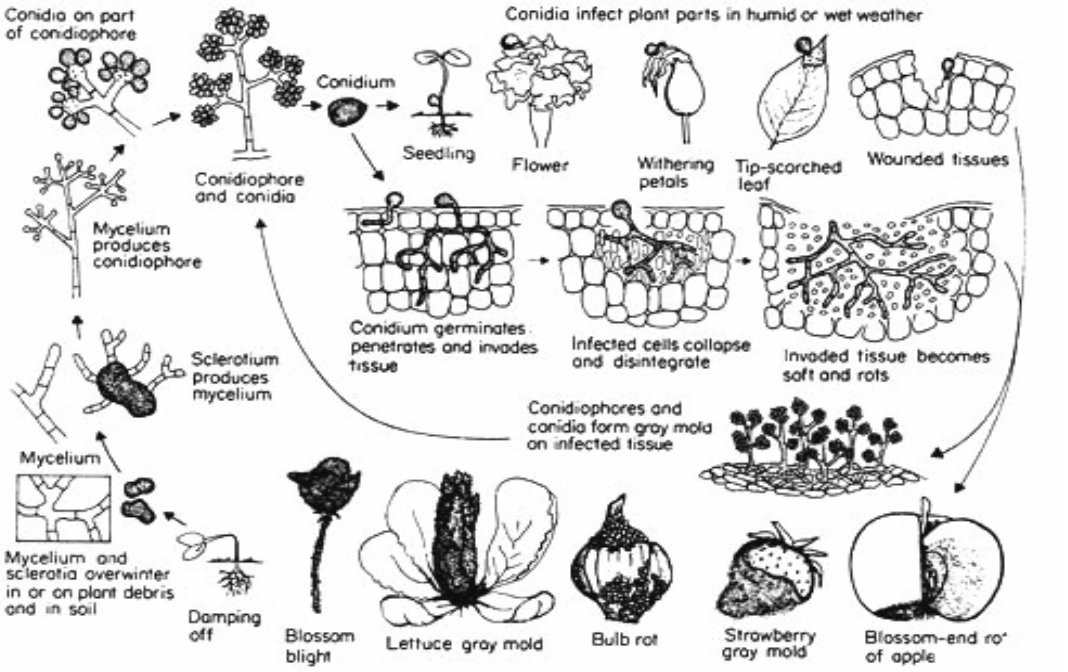

Figure 1-1. Overview of the life cycle of *Botrytis cinerea* (adapted from Agrios, 1997).

1.9.3 Disease Control Strategies

Fungicides are commonly applied as field sprays to control fruit diseases and cold chain management practices to prevent and/or control quiescent fungal infections of fruits. Despite the use of fungicides, losses of the harvested product are still recorded in countries, even with advanced cold storage facilities (Cappellini and Ceponis, 1984). In developing countries, where disease management practices and proper handling of post-harvest commodities are poor, post-harvest 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 post-harvest practices, including sanitation, careful harvesting and effective cold chain management is crucial.

1.9.3.1 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 perception that fungicides are harmful to human health and the environment has led to the implementation of more restrictive legislation dealing with permissible chemicals and residue levels. Other problems are also associated with the excessive use of fungicides, including the development of resistant strains to these fungicides (Joubert and Archer, 2000) and ecological shifts or imbalances in microbial populations, often the result of continuous fungicide use (Reimann and Deising, 2000).

The Romans have long applied elemental sulphur to control gray mold and mildew diseases in grapes. Non-systemic fungicides such as aromatic hydrocarbons (*e.g.*, chlorothalonil), dithiocarbamates (*e.g.*, thiram and mancozeb), dinitrophenols and chlorophenyls, introduced from 1950 onwards, were also used to control gray mold, but their specificity was low. The next generation of fungicides used for the control of gray

mold diseases comprised systemic fungicides such as benzimidazoles (*e.g.*, benomyl), dicarboximides (*e.g.,* vinclozolin, iprodione), triazoles (*e.g.*, tebuconazole) and *N*-phenylcarbamates (*e.g.*, diethofencarb) (Lyr, 1995). From 1995 onwards, novel classes of fungicides with specificity against *B*. *cinerea* were commercialized. These include the anilinopyrimidines cyprodinil, pyrimethanil and mepanipyrim, the phenylpyrroles fludioxonil and fenpiclonil, the hydroxyanilide fenhexamid, the quaternary ammonium compounds dimethyl didecyl ammonium chloride and didecyl dimethyl ammonium chloride (Gullino *et al*., 2000; Rosslenbroich and Stuebler, 2000; Debieu *et al*., 2001). Major problems in *B*. *cinerea* control are the low field performance of azole fungicides and related compounds (Stehmann and De Waard, 1996).

Resistance mechanisms are commonly based on modifications of the target site of the fungicides in the pathogen, or detoxification of the compound by enzymes from the pathogen. Mutants resistant to one compound from a chemical class of compounds usually display cross-resistance to compounds that have the same mode of action or the same functional group. Resistance to chemically unrelated compounds can be based on multidrug resistance (MDR). In *B*. *cinerea*, several genes involved in different mechanisms of fungicide resistance have been described. *Botrytis cinerea* strains with multidrug resistance to various classes of chemicals have only been identified in France (Chapeland *et al*., 1999).

1.9.3.2 Plant Breeding

To the farmer, especially the resource-poor, the use of resistant cultivar(s) is the least expensive of known disease control alternatives, it requires no specific action to achieve control and stands ready to challenge the disease pathogen whenever it appears. Moreover, no added production costs or need for timely decisions when to apply alternate control practices during the growing season are required. Besides being

economically advantageous, the development and use of crop cultivars resistant to diseases is environmentally sound (Poswal *et al*., 1993).

Little attention has been given to the development of resistance to pathogens in post-harvest commodities. Few plant breeding programs ever consider this type of resistance. The vegetative plant body and fruit that produce seed have probably evolved resistance mechanisms under different selection pressures. A primary function of the vegetative part of a plant is the production of reproductive structures (seeds, tubers, *etc*.). Accordingly, vegetative plant resistance would be directed primarily toward microorganisms and insects that inhibit fecundity. Resistance in the fruit, on the other hand, would be toward microbes and insects that reduce seed survival and dispersal. If resistance in vegetative plant parts and fruits evolved different selection pressures, different mechanisms of resistance might be expected. Therefore, selecting for resistance to vegetative pathogens may not impart resistance to post-harvest diseases. Also, resistance of fruits and vegetables in the field may not relate to post-harvest resistance (Wilson and Wisniewksi, 1989). Daubeny & Pepin (1977) studied 116 genetically diverse strawberry clones and found that field resistance to *B. cinerea* was not necessarily related to post-harvest resistance to *B*. *cinerea* or *Rhizopus*. Plant breeders need to recognize that resistance to post-harvest diseases may be distinct from field resistance in developing breeding programmes to use such resistance (Wilson and Wisniewski, 1989).

Cold storage (-0.5°C for 2 weeks) of table grapes affects berry characteristics and decreases the resistance level of the berry skin. For the structural bunch parts, *B*. *cinerea* conidia and germlings have different survival periods on tissues of the various parts (Coertze and Holz, 1999). On the table grape cultivar, Dauphine, the incidence of *B*. *cinerea* declines at the base of the pedicel, but on the wine grape cultivar, Merlot, the incidence remains high throughout the season. The angle formed between the fruit and the pedicel is also a morphological factor that correlates with resistance. The berry

attachment to the pedicel shows three possible positions of the berry relative to the pedicel, which may change during growth development and is characteristic of the cultivar.

Up to 3 weeks after fruit set, attachment is wide-angled. From the third to seventh week, each cultivar adopts its final structure, the angle formed being either acute, 90° or obtuse. An acute angle may contribute to disease development in that it provides a suitable site for spore accumulation. Likewise, a loose attachment between berry and pedicel, seen as a wide or narrow area of cleavage, may foster spore colonization. Certain cultivars, such as Alphonse Lavalee, with a high potential for phytoalexin elicitation and a relatively thick skin, are nonetheless highly susceptible to gray mold. The loose attachment of the berry to the pedicel and the acute angle formed may explain this apparent discrepancy (Coertze and Holz, 1999).

The fact that resistance in commercial *V. vinifera* cultivars is not determined by a sole factor that can be readily selected for an in-breeding programme, hampers the development of *B. cinerea* resistant cultivars. Grapes highly resistant to *B. cinerea* are found in *V. labrusca*, *V. labrusca* cross *V. vinifera* hybrids, or other complex hybrids (Jarvis, 1997; Diaz *et al*., 2002).

1.9.3.3 Non-Chemical Disease Control Strategies

The development of alternative post-harvest disease control options using either microbial agent (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 Nakansihi, 1979; Dixit *et al*., 1995; Wilson *et al*., 1997; Obagwu and Korsten, 2003) has become more significant as successful commercial applications have gained ground. Bio-pesticides (microbial agents and natural plant

materials) have the potential to be more environmentally safe and more acceptable by the general public for human use.

Bio-pesticides are the new-generation crop protectants based on naturally occurring microbial communities on plant surfaces and use of extracts from plant materials. Micobial pesticides are antagonistic microorganisms, which are screened and developed for their antipathogenic activity. Antagonistic microorganisms can be collected from several sources such as dead anthropos, 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 may probably 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), including 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 post-harvest diseases of avocado such as *Bacillus subtlis* (Avogreen) and pome fruit *Cryptococcus albidus* (YieldPlus^{TM}) (Janisiewicz and Korsten, 2002). Other commercial products such as *Pseudomonas syringae* (BioSave 110 and 111) to control *Geothricum candidum* and *Candida oleophila* (Aspire^{TM}) to control penicillium on citrus and pome fruit have been registered by Ecogen Inc. in the USA (Shachnal *et al*. 1996). Two strains of *Bacillus pumilus* and *Bacillus amyloliquefaciens* were recognized to be effective against *B*.

cinerea in pears and tomatoes (Mari and Guizzardi, 1998). The fruit 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 mechanism of bio-pesticides include production of antibiotics with multiple modes of actions (Fravel, 1988), such as induction of host resistance (Droby *et al*., 2002; Poppe *et al*., 2003), synthesis of phytoalexins and/or the accumulation of an extracellular matrix (Janisiewicz, 1988, Lima *et al*., 1998; Chan and Tian, 2005), competition for nutrients and space (Janisiewicz *et al*., 2000), siderophore production and direct interaction with the pathogen (Neilands, 1981; Schwyn and Neilands, 1987; Buyer *et al*., 1989), and production of volatile compounds (Fravel, 1988). Although several modes of action have been described for bio-pesticdes, all the mechanisms have not been fully clarified (El Ghaouth *et al*., 2002). It is therefore essential to elucidate the mode of action of each single new bio-pesticide. Mechanisms such as competition for nutrients, space and induction of host resistance have been demonstrated by many researchers (Janisiewicz and Korsten, 2000, 2002; Porat *et al*., 2002; Plaza *et al*., 2004) and are currently used as major criteria for the selection of new bio-control agents for post-harvest applications.

An important consideration in pre- and post-harvest application of bio-control agents is the ability of the microorganisms to survive at sufficient population levels on fruit surfaces after application and rapid colonization of wound sites by organisms 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 temperature and pH (Janisiewicz *et al*., 2000; Nunes *et al*., 2001). During the last decade research on deciduous fruit bio-control focused on microorganisms colonizing the wound site and

competing with pathogens for nutrients. Among these are *Cryptococcus infirmo-miniatus*, *Rhodotorula glutins* (Chand-Goyal and Spotts, 1996), *Cryptococcus laurentii* (Roberts, 1990) and *Candida oleophila* (Hofstein *et al*., 1994) all effective against *P. expansum* and *B. cinerea* (causal agents of blue molds and gray molds, respectively). *Debaryomyces hansenii* (Chalutz and Wilson, 1990) has also been developed against green and blue molds 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). Therefore, understanding the mode(s) of action of effective bio-control 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 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 be a 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 (Harbourne, 1964; Regnier and Macheix, 1996; Tripathi *et al*., 2002) have been used successfully to control post-harvest diseases of some agricultural crops, stored fruits, vegetables and food commodities. Moreover, the antifungal 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. Wilson *et al*. (1997) tested extracts of more than 300 species of plants belonging to 43 families against *B*. *cinerea* using the multiwell-microtiter plate assay. Of the extracts tested, approximately 5% showed significant fungicidal activity. Sholberg and Shimizu (1991) reported decay inhibition in strawberry and peach fruit by hinokitiol, an antifungal compound derived from the trunk of Japanese cypress trees. Hinokitiol was also found to be effective when incorporated into a film wrap. This compound, at a concentration ranging from 19 to 44 µg/ml inhibited spore germination of several post-harvest pathogens, including *B*. *cinerea*, *Monilinia fructicola*, and *Penicillium expansum* (El Ghaouth and Wilson, 1995).

Control of post-harvest decay was also reported with chitosan (El Ghaouth *et al*., 1992a). Chitosan, a β-1,4-glucosamine polymer, is a natural constituent of the cell wall of many fungi and is produced commercially from chitin of arthropod exoskeletons through a deacetylation process that provides sufficient free amino groups to render the polymer readily soluble in diluted organic acids. Chitosan is known to form a semi-permeable film (Averbach, 1978) that inhibits the growth of a number of pathogenic fungi including post-harvest pathogens (Allan and Hadwiger, 1979; El Ghaouth *et al*., 1992a). When applied as a coating, chitosan controlled post-harvest decay as well as delayed ripening of strawberry, bell pepper, tomato, and cucumber fruit (El Ghaouth *et al*., 1992b). The coating provided a greater barrier for the efflux of CO_2 than for the influx of O_2 (El Ghaouth *et al*., 1992c). The control of decay by chitosan appears to be due to its antifungal eliciting properties.

1.9.3.4 Cultural and Physical Requirements

Cultural and physical activities represent non-chemical strategies that require manipulation of the environment to decrease disease pressure. A common practice is sanitation to reduce sources of inoculum. This can be achieved by starting with clean material and keeping pruned plant material away from the crop. Another important

practice is to reduce the length of leaf wetness periods, which is essential for spore germination and penetration. This can be done by increasing plant distance, trimming of the canopy, ventilation, and control of temperature and relative humidity (Schoonbeek, 2004). During 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). Separation of sound fruits from the decayed ones in storage or repack centres reduces possible sources of inoculum and prevent contamination (Wardowski and Brown, 2001).

1.9.3.5 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 (Kortsen *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), with natural plant extracts (Vaugh *et al*., 1993; Mattheis and Roberts, 1993; Wilson *et al*., 1997; Obagwu *et al*., 1997; Obagwu, 2003), and other physical treatments such as curing and heat treatments (Leverentz *et al*., 2000; Ikediala *et al*., 2002; Plaza *et al*., 2003) provide potential effective alternative treatments.

1.10 South African Medicinal Plant Species

Indigenous medicinal plants are used by more than 60% of South Africans in their health care needs or cultural practices. Approximately 3,000 species are used by an estimated 200,000 indigenous traditional healers (Van Wyk *et al*. 1997).

1.10.1 *Elytropappus rhinocerotis*

1.10.1.1 Description

Elytropappus rhinocerotis (L.f.), less commonly known as renosterbos and belonging to the family Asteraceae, is a single-stemmed shrub up to about 2 m high (Figure 1-2). Although renosterbos is not showy or beautiful, it is an interesting and important component of our indigenous flora. It is the dominant member and the namesake of the threatened vegetation type renosterveld. The plant renosterbos should not be confused with the veld type renosterveld which consists of a typical assemblage of shrubs, geophytes and grasses.

The very old branches are gnarled and the bark is smooth and grayish. Older branches are bare of leaves, but bear many thin, whip-like twigs which are held erect and covered with tiny, triangular leaves pressed tightly to the stem. In between the leaves a layer of fine white hairs, imparting a woolly appearance, is visible, giving the plant an overall grayish look. Large numbers of flowerheads (capitula) are borne towards the ends of the twigs, but are very small and inconspicuous so that many people do not discern when a bush is in flower. When the seeds are shed, they become more obvious as the pale brown chaffy bracts around each flowerhead open up to give the plant a brownish, fluffy appearance. Shoot growth happens in summer, flowering in early winter and seeds are shed in late winter. Plants are fast-growing and thought to be quite long-lived (Van Wyk *et al*., 1997).

1.10.1.2 Distribution and Habitat

The natural range of renosterbos is wider than one would expect, as it occurs outside of renosterveld vegetation. Although it is widespread in the Cape Floristic Region, renosterbos also occurs throughout Namaqualand and as far as the Richtersveld. It is also found as far as the great escarpment around Molteno in the Eastern Cape, and in

the southern parts of the Eastern Cape to East London. Populations in the interior are usually restricted to high-altitude areas (Van Wyk *et al*., 1997). It readily tolerates both frost and snow, but in humid areas may be susceptible to fungal infections. In the higher rainfall parts of its range it is confined to fine-grained soils such as those derived from shale or granite, but in more arid areas the soil type appears to have less effect (Van Wyk *et al*., 1997).

Figure 1-2. *Elytropappus rhinocerotis* (L.f.) Less (Photo taken by Nicola Bergh, SANBI, September 2006).

1.10.1.3 Ecology

The plant has an extremely deep taproot which can grow to well over 6 m. It`s ability to grow in very arid areas is probably due to the taproot accessing ground water. It is flammable, but killed by fire, so it is dependent on its seed for regeneration. It thus has to produce many seeds, thousands of which are borne on each twig. After being shed in winter, seeds become dormant and do not germinate until at least the following year, or several years later. This may be an adaptation to enhance dispersal. Seeds are dispersed by the wind, having a feathery appus to catch the breeze. Germination and

establishment are facilitated by disturbance in the form of fire or clearing of vegetation. Until the taproot is established, the seedlings are extremely sensitive and are killed by drought or shade. Shading from other plants, especially grasses, would have prevented this plant from reaching large densities under natural conditions. Although it is reported to be unpalatable to stock, it has its own specialist browser, the small flightless renosterbos locust *Lentula ontusifrons*. This insect is confined to the Eastern Cape and eats only renosterbos. Several other insects appear to have associations with renosterbos. Many of these are small and not well known. Scale insects form galls, spittle bugs are often found on the twigs, and small flies parasitize the embryos.

1.10.1.4 Cultural Aspects

Renosterbos is commercially important as it is unpalatable to stock and can become a serious weed on farmlands. There is evidence that renosterbos has encroached on worked lands since settled agriculture has been practised in the Cape, reducing the commercial value of these areas. Many farmers, especially in the Eastern Cape, regard the species as a major weed and in the 1920s renosterbos was thought to be an invasive alien, imported from the East Indies in empty wine casks. Eradication strategies and research into its bio-control were explored, but the main reason for its encroachment seems to be that of overgrazing, especially immediately after burning which removes the grasses which would otherwise shade out renosterbos seedlings and reduce their numbers. Renosterbos is a poor competitor against established vegetation, but is opportunistically able to take advantage of disturbance, especially fire (Levyns, 1929).

1.10.1.5 Medicinal Uses

Watt & Breyer-Brandwijk (1962) report on the medicinal properties of renosterbos. Infusions of the young branches in alcohol are a traditional Cape remedy, thought to be beneficial in the treatment of stomach ailments, including indigestion, dyspepsia,

stomach cancer and a lack of appetite. The powdered twigs were used to treat children with diarrhea (Cillie, 1962). It was also formerly used to treat sheep suffering from *krimpsiekte* - a syndrome associated with chronic cardiac glycoside poisoning. The preparations are also said to induce sweating and the plant has been used in the treatment of influenza and fever. The active medicinal ingredient appears to be a chemical called rhinoterotinoic acid which was isolated from renosterbos and found to have significant anti-inflammatory activity. Renosterbos is likely to be an unpleasant medicine as the plant is bitter and strongly astringent as well as being resinous (Van Wyk *et al*., 1997).

1.10.2 *Tulbaghia violacea*

1.10.2.1 Description

Tulbaghia species Harv. (Alliaceae) (wild garlic, “wilde knoffel”) is a fast-growing, bulbous plant that reaches a height of 0.5 m (Figure 1-3). The leaves are long, narrow, strap-like, and slightly fleshy and smell strongly of garlic when bruised. They grow from fat, tuberous roots which spread to form clumps of plants. The pinkish mauve, tubular flowers, clustered into umbels of up to twenty flowers, are held above the leaves on a tall flower stalk, and appear over a long period in summer (January to April). They, too, smell of garlic when picked. The triangular capsules of the fruit are grouped into a head and, when ripe, they split to release the flattened, hard, black seeds (Van Wyk *et al*., 1997).

1.10.2.2 Distribution

This drought-resistant plant stretches from the Eastern Cape, KwaZulu-Natal and Limpopo, to as far north as Zimbabwe (Watson and Dallwitz, 1992).

Figure 1-3. *Tulbaghia violacea* Harv. (Photo taken by Shireen Harris, Free State National Botanical Garden).

1.10.2.3 Ecology

Most of the species of *Tulbaghia* are adapted for moth pollination and have dull flowers that become sweetly scented at night. *Tulbaghia violacea* seems likely to be pollinated by butterflies and bees as they are scented during the day (Van Wyk *et al*., 1997).

1.10.2.4 Cultural Uses

This attractive plant is ideal for the herb garden, as both the leaves and flowers can be used in salads and other dishes. The smell repels fleas, ticks and mosquitoes when crushed on the skin.

1.10.2.5 Medicinal Uses

The crushed leaves may be used to help cure sinus headaches and to discourage (by their strong smell) moles from the garden. The fresh bulbs are boiled in water and the decoctions are taken orally to clear up coughs and colds. The bulb has been used as a remedy for pulmonary tuberculosis and to destroy intestinal worms. Wild garlic may prove to have the same or similar antibacterial and antifungal activities as has been

scientifically verified for real garlic (Bruneton, 1995). It is claimed that the leaves are used to treat cancer of the oesophagus (Van Wyk *et al.,* 1997). Zulus use the leaves and flowers as spinach and as a hot, peppery seasoning with meat and potatoes. They also use the bulb to make an aphrodisiac medicine. Wild garlic is a very good snake repellent and for this reason, Zulus plant it around their homes (Kubec *et al.,* 2002).

1.10.3 *Galenia africana*

1.10.3.1 Description

Galenia africana L., commonly known as "kraalbos" or "geelbos" belonging to the family Aizoaceae (Kellerman *et al.,* 1988). "Kraalbos" is an aromatic, woody perennial sub-shrub, growing 0.5-1 m high, having oppositely arranged green leaves (5 cm long and hairless) which turn yellow with age (Figure 1-4). Inflorescence is borne at the ends of the twigs and is 30-100 mm long, with many small yellow flowers. The flowers (appearing during October-December) are about 1.5 mm in diameter, yellowish green and borne in large loose heads (Leroux *et al.,* 1994).

Figure 1-4. *Galenia africana* L. (Photo taken by F. Vries, September 2007).

1.10.3.2 Distribution

Wide distribution on dry flats and lower slopes from the Northern Cape and Namaqualand to Uniondale, the Karoo and Eastern Cape Province; often on disturbed ground and road verges.

1.10.3.3 Ecology

Galenia africana is an active invader, and is especially abundant in disturbed areas around kraals, along roads and on trampled veld. This plant is not only an indicator of disturbance, but is also a pioneer plant, being the first perennial to regrow after soil disturbances. Alternatively, it can be the only remaining species after the veld has been heavily overgrazed. The system of nomadic migration between winter and summer rainfall regions results in hungry animals having to eat the only available plants along the way. However, such plants are often undesirable. The extensive farming practices sometimes compel stock to remain in and around pens for protracted periods, and when finally put out to graze, the famished animals are highly susceptible to poisoning. The luxuriant seedlings which appear after even light rains are highly favoured by livestock that are in a poor condition (Van Aardt, 2004).

1.10.3.4 Poisoning in Sheep

The plant has been associated with liver damage and severe ascites, a condition referred to as "*waterpens*" in sheep and goats. "Waterpens" is characterised by the development of an atrophic or hypertonic arrhasis of the liver (Watt and Breyer-Brandwijk, 1962). The marked liver lesions in sheep and occasionally in goats with "kraalbos" have led farmers and researchers to believe that the plant is primarily hepatotoxic to livestock. It was suggested that the plant contains an unidentified toxin responsible for severe hepatic damage and ascites (Watt and Breyer-Brandwijk, 1962). Apart from weight loss, the habitus and appetite of sheep suffering from "waterpens" remain fair up to the terminal stages of the disease, after which the animals become

apathetic and recumbent, and eventually die. At necropsy, the liver (Figure 1-5) is always affected. Depending on the stage of the disease, the organ can either be smaller than normal or enlarged, the colour may range from a grayish-blue to a yellowish-brown and the morphology of the liver can be unaltered or distorted by nodular hyperplasia, atrophy and/ or hypertrophy of certain parts (Kellerman *et al.,* 1988).

Figure 1-5. Abdominal dropsy causes severe losses through the excessive consumption of Kraalbos (Galenia africana). Photo was taken by M.P. Van Aardt (Elsenburg).

1.10.3.5 Medicinal Uses

A decoction of *G. africana* is used as a lotion for wounds in man and animal (Watt and Breyer-Brandwijk, 1962). The Hottentots chew the plant to relieve toothache and it is used in the treatment of venereal diseases, lotion for skin diseases and for the relief of inflammation of the eyes. An ointment, made by frying the herb with *Cyanella lutea, Lobostemon fruiticosus, Melianthus major, Melianthus cosmosus," Tiendaegeneesblare"* and *"Jakkalsoorblare"* in butter, was used as a dressing for wounds, especially wounds on the legs of women. In syphilis, the external lesions were washed with a decoction of the plant and *Lobostemon fruiticosus, Melianthus major* and *Melianthus cosmosus* and

for lupus, a decoction of the plant with *Melianthus major, Melianthus cosmosus "Berglelie"* (Watt and Breyer-Brandwijk, 1962).

1.11 Research Study Hypothesis

We hypothesize that South African medicinal plants contain compounds that can act in synergism with synthetic antifungal compounds. Natural plant extracts have become important since it is perceived as being environmentally safer and more acceptable to the general public. Several limitations affect the commercial applicability of plant extracts - when used as a stand-alone treatment, none of the extracts clearly and consistently offer an economically sufficient level of disease control that will warrant their acceptance as a viable alternative to synthetic fungicides. The inconsistency and insufficient commercial efficacy are attributed, in part, to the inability of the extracts to control previously established infections (Droby, 2001). Successful commercial control of post-harvest diseases of fruits must be extremely efficient, in the range of 95-98%. In order to develop new and highly effective biofungicides, it is necessary to make their activity more reliable by combining them with low doses of fungicides in integrated control programmes (Chand-Goyal and Spotts, 1997; Ippolito et al., 1998; Droby et al., 2002).

Plants produce an enormous array of secondary metabolites, and it is commonly accepted that a significant part of this chemical diversity serves to protect plants against microbial pathogens (Dixon, 2001). These antimicrobial plant substances are classified as phytoanticipins, which are compounds that are present constitutively or phytoalexins, whose levels increase strongly in response to microbial invasion. In several well-documented cases, mutant plants that lack the ability to produce a particular phytoalexin had considerably higher levels of sensitivity to microbial pathogens, for example, mutant oats that lack saponin avenacin A-1 became sensitive to a range of fungal pathogens (Papadopoulou *et al*., 1999). Plant compounds are routinely classified as "antimicrobial"

on the basis of susceptibility test that produce mean inhibitory concentrations (MICs) in the range of 100 to 1000 µg/ml, orders of magnitude weaker than those of typical antibiotics produced by bacteria and fungi (MIC 0.01 to 10 µg/ml) (Tegos *et al*., 2002). A compound that is synthesized in response to pathogen invasion and is required to protect the plant from that pathogen, but that shows little activity in an *in vitro* susceptibility test is not necessarily an antimicrobial. Such a substance might have a regulatory function, indirectly increasing the level of resistance of the plant. This analysis suggests that we lack a solid rationale for making a functional assignment for the vast majority of plant compounds that have been classified as antimicrobials (Tegos *et al*., 2002).

Recent work however with berberine, a cationic alkaloid, offered a possible explanation for the apparent ineffectiveness of plant antimicrobial compounds (Stermitz *et al*., 2000; Stermitz *et al*., 2001). Berberine is a weak antimicrobial produced by a wide variety of plant species. It is an amphipathic cation that resembles quaternary ammonium antiseptics in its chemical properties and possibly in its mechanism of action as well. The likely targets of berberine are the cytoplasmic membrane and deoxyribo nucleic acid (DNA), into which it intercalates (Jennings and Ridler, 1983). It was found that the medicinal plant species *Berberis* produces 5'-methoxyhydnocarpin-D, which acted in synergy with berberine (Stermitz *et al*., 2000; Stermitz *et al*., 2001). Berberine, accumulates in the cells of microbial pathogens, and the accumulation is driven by the membrane potential (Severina, *et al*., 2001). This finding provides an important precedent for the idea that synergistic interaction among different compounds (antimicrobial or not) explains the frequent failures to isolate single active substances from medicinal plants (Tegos *et al*., 2002).

1.12 Aims and Objectives to Test the Hypothesis

The present study is designed to examine the efficacy of ethanolic plant extracts and commercial fungicides, alone and combined, against *B. cinerea in vitro*, and to determine if plant extract-fungicide combinations interact synergistically to suppress *B. cinerea* in storage. The specific aims of the study are to determine the efficacy of:

1. Ethanolic plant extracts and commercial fungicides at different concentrations alone or combined on mycelium growth of B. cinerea in vitro.
2. Plant extract-fungicide combinations in experiments on wounded apples.
3. Plant extract-fungicide combinations in drench trials.

1.13 References

Abbott, W.S., 1925. A method of computing effectiveness of an insecticide. J. Econ. Entomol. 18:265-267.

Agrios, G. N., 1997. Plant Pathology 4th ed, Academic Press. New York.

Allan, C. R., Hadwiger, L. A., 1979. The fungicidal effect of chitosan on fungi of varying cell wall composition. Exp. Mycol. 3: 285-287.

Ark, P. A., Thompson, J. P., 1959. Control of certain diseases of plants with antibiosis from garlic (*Allium sativum* L.). Plant Dis. Rep. 43: 276-282.

Auret, E. A., 2000. Control strategies for citrus postharvest diseases. MSc thesis, University of Pretoria, Pretoria, South Africa.

Averbach, B. L., 1978. Film-forming capability of chitosan. Pages 199-207 in: Proceedings of the 1st International Conference on chitin and chitosan, R. A. Muzzareli and E. R. Pariser, eds. MIT, Cambridge, Massachusetts, USA.

Batta, Y. A., 2004. Postharvest biological control of apple gray mould by *Trichoderma harzianum* Rifai formulated in an inverted emulsion. Crop Prot. 23: 19-26.

Benito, E. P., Ten-Have, A., Van`t Klooster, J. W., Van Kan, J. A. L., 1998. Fungal and plant gene expression during synchronized infection of tomato leaves by *Botrytis cinerea*. Eur. J. Plant Pathol. 104: 207-220.

Bisht, S. S., Kamal, B. B., 1994. Garlic extract – an effective anti-fungal treatment for the control of storage rot of apple. Proc. Natl. Acad. Sci. India 64: 233-234.

Bruneton, J. 1995. Pharmacognosy, Phytochemistry, Medicinal Plants. Paris: Lavoisier Publishing.

Buyer, J., Sikora, L. J., Chaney, R. L., 1989. A new growth medium for the study of siderophore-mediated interactions. Biol. Fertil. Soils 8: 97-101.

Caffarelli, V., Rapagnani, M. R., Letardi, A., Triole, L., Santaroni, P., and Lancia, B., 1999. Pesticide residues in horticultural product and carcinogenic risk for consumers. In: Del Re AAM, Brown CD, Capri E, Errera G, Evans SP and Trevisan M (eds) Human Enviromental Exposure to Xenobiotics (pp 665-669) La Goliardica Pavese, Pavia (Italy)

Cappellini, R. A., Ceponis, M. J., 1984. Postharvest losses of fresh fruits and vegetables. In; Moline H. E., (Ed.), Postharvest pathology of fresh fruits and vegetables: Postharvest losses in perishable crops. UC, Berekeley, Agriculture Experimental Station, pp. 24-30.

Chalutz, E., Wilson, C. L., 1990. Postharvest *bio-control* of green and blue moulds and sour rot of citrus fruit *Debaryomyces hansenii*. Plant Dis. 74: 134-137.

Chalutz, E., Droby, S., 1997. Biological control of postharvest disease. *In* Plant-microbe interactions and biological control. *Edited by* G.J. Boland and L.D Kuykendall. Marcel Dekker, Inc., New York. Pp. 157-170.

Chand-Goyal, T., Spotts, R. A., 1996. Control of postharvest pear diseases using natural saprophytic yeast colonists and their combination with a low dosage of thiabendazole. Postharvest Biol. Technol. 7: 51-64.

Chand-Goyal, T., Spotts, R. A., 1997. Biological control of postharvest diseases of apple and pear under semi-commercial and commercial conditions using three saprophytic yeasts. Biol. Contr. 10: 199-206.

Chan, Z., Tian, S., 2005. Interaction of antagonistic yeasts against postharvest pathogens of apple fruit and possible mode of action. Postharvest Biol. Technol. 36: 215-223.

Chapeland, F., Fritz, R., Lanen, C., Gredt, M.,, Leroux, P. 1999. Inheritance and mechanisms of resistance to anilinopyrimidine fungicides in *Botrytis cinerea* (*Botryotinia fuckeliana*). Pestic. Biochem. Physiol. 64:85-100.

Cillie', A. M. 1992. Kruie op Witblits, Rate, Resepte en Feite. Unpublished notes, Worcester Museum.

Claypool, L. L., 1983. Biological and cultural aspects of production and marketing of fruits. In: O`brien M, Cargell, B., Fridley, R. B. (Eds.), Priciple and practices for harvesting and handling of fruits and nuts. Avi publishing company, Inc., USA, pp. 15-45.

Coertze, S., Holz, G., 1999. Surface colonization, penetration and lesion formation on grapes inoculated fresh or after cold storage with single airborne conidia of *Botrytis cinerea*. Plant Dis. 83: 917-924.

Cohen, Y., Levy, Y., 1990. Joint action of fungicides in mixtures: Theory and practice. Phytoparasitica 18: 159-169.

Conway, W. S., Janisiewicz, W. J., Klein, J. D., Sams, C. E., 1999. Strategy for combining heat treatment, calcium infiltration, and biological control, to reduce postharvest decay of gala apple. HortScience 34: 700-704.

Colby, S.R., 1967. Calculating synergistic and antagonistic responses of herbicide combinations. Weed Sci. 15:20-22.

Cowan, M. M., 1999. Plant products as antimicrobial agents. Clin. Microbiol. Rev. 12 (4): 564-582.

Daubeny, H. A., Pepin, H. S., 1977. Evaluation of strawberry clones for fruit rot resistance. J. Am. Soc. Hortic. Sci. 102: 431-435.

Debieu, D., Bach, J., Hugon, M., Malosse, C., Leroux, P., 2001. The hydroxyanilide fenhexamid, a new sterol biosynthesis inhibitor fungicide efficient against the plant pathogenic fungus *Botryotinia fuckeliana* (*Botrytis cinerea*). Pest Manag. Sci. 57: 1060-1067.

Demoz, B. T., Korsten, L., 2006. *Bacillus subtilis* attachment, colonization, and survival on avocado flowers and its mode of action on stem-end rot pathogens. Biol. Contr. 37: 68-74.

Del Sorbo, G., Schoonbeek, H-j., De Waard, M. A., 2001. Fungal transporters involved in efflux of natural toxic compounds and fungicides. Fungal Genet. Biol. 30:1-5.

De Waard, M.A., Van Nistelrooy, J.G.M., 1984. Differential accumulation of fenarimol by a wild-type isolate and fenarimol-resistant isolates of *Pencillium italicum*. Neth. J. Plant Pathol. 90: 143-153.

De Waard, M.A., Van Nistelrooy, J.G.M., 1988. Accumulation of SBI fungicides in wild-type and fenarimol-resistant isolates of *Penicillium italicum*. Pestic. Sci. 22: 371-382.

De Waard, M. A., 1994. Resistance to fungicides which inhibit sterol 14α-demethylation, an historical perspective. BCPC Monogr 60: 3-10.

De Waard, M.A., Gisi, U., 1995. Synergism and antagonism in fungicides. Pages 565-578 in: Modern Selective Fungicides. 2nd ed. H. Lyr, ed. Gustav Fischer, Jena, Germany.

De Waard, M. A., 1996. Synergism and antagonism in fungicide mixtures containing sterol demethylation inhibitors. Phytopathology 86: 1280-1283.

Diaz, J., Ten Have, A., Van Kan, J. A., 2002. The role of ethylene and wound signaling in resistance of tomato to *Botrytis cinerea*. Plant Physiol. 129: 1341-1351.

Di Pietro, A., Lorito, M., Hayes, C.K., Broadway, R.M., Harman, G.E., 1993. Endochitinase from *Gliocladium virens*: Isolation, characterization, and synergistic antifungal activity in combination with gliotoxin. Phytopathology 83: 308-313.

Dixit, S. N., Chandra, H., Tiwari, R., Dixit, V., 1995. Development of botanical fungicide against blue mold of mandarins. J. Stored Prod. Res. 31(2): 165-172.

Dixon, R. A., 2001. Natural products and plant disease resistance. Nature 411: 843-847.

Droby, S., 2001. Enhancing *bio-control* activity of microbial antagonists of postharvest diseases. Enhancing *Bio-control* agents and handling Risks. M. Vurro *et al.* (Eds) IOS Press.

Droby, S., Cohen, A., Weiss, B., Horev, B., Chalutz, E., Katz, H., Keren-Tzur, Shachnai, S., 1998. Commercial testing of Aspire: a yeast preparation for the biological control of postharvest decay of citrus. Biol. Contr. 12: 97-100.

Droby, S., Vinokur, V., Weiss, B., Cohen, L., Daus, A., Goldschmidt, E. E., Porat, R., 2002. Induction of resistance to *Penicillium digitatum* in grape fruit by the yeast *bio-control* agent *Candida oleophila*. The Am. Phytopathol. Soc. 92(4): 393 – 399.

Dudareva, N., Pichersky, E., Gershenzon, J., 2004. Biochemistry of plant volatiles. Plant Physiol. 135: 1893-1902.

Eckert, J. W., Ogawa, J. M., 1985. The chemical control of postharvest diseases: subtropical and tropical fruits. Ann. Rev. Phytopathol. 23: 421-454.

El Ghaouth, A., Arul, J., Grenier, J., Asselin, A., 1992a. Potential use chitosan in postharvest preservation of fruits and vegetables. Pages 440-452 in: Advances in Chitin and Chitosan, J. B. Brines, P. A. Sandford, and J. P. Zikakis eds. Elsevier Appl. Sci.

El Ghaouth, A., Arul, J., Grenier, J., Asselin, A., 1992b. Antifungal activity of chitosan on two postharvest pathogens of strawberry fruits. Phytopathology 82: 398-402.

El Ghaouth, A., Arul, J., Asselin, A., Benhamou, N., 1992c. Antifungal activity of chitosan on postharvest pathogens: Induction of morphological and cytological alterations in *Rhizopus stolonifer*. Mycol. Res. 96:769-779.

El Ghaouth, A., 1997. Biologically-based alternatives to synthetic fungicides for the control of postharvest diseases. J. Ind. Microbiol. Biotechnol. 19: 160-162.

El Ghaouth, A., Wilson, C. L., 1995. Biologically-based technologies for the control of postharvest diseases. Postharvest news and information 6: 5-11.

El Ghaouth, A., Smilanick, J. L. Wilson, C. L., 2000. Enhancement of the performance of *Candida saitoana* by the addition of glycolchitosan for the control of postharvest decay of apple and citrus fruit. Postharvest Biol. Technol. 19: 103-110.

El Ghaouth, A., Wilson, C. L., Wisniewski, M., Droby, S., Smilanick, J. L., Korsten, L., 2002. Biological control of postharvest disease of citrus fruits. In: Gnanamanickam, S. S. (Ed.), Biological control of crop diseases, Mared Dekker, Inc., New York, pp. 289-312.

Fravel, D. R., 1988. Role of antibiosis in the control of plant diseases. Ann. Rev. Phytopathol. 26: 75-91.

Gisi, U, Binder, H., Rimbach, E., 1985. Synergistic interactions of fungicides with different modes of action. Tran. Brit. Mycol. Soc. 85: 299-306.

Gisi, U., 1991. Synergism between fungicides for control of *Phytophthora*. Pages 361-372 in: *Phytophthora*. J.A. Lucas, R.C. Shattock, D.S. Shaw, and L.R. Cooke, eds. Cambridge University Press, Cambridge.

Gisi, U. 1996. Synergistic interaction of fungicides in mixtures. Phytopathology 86: 1273-1278.

Govender, V., Korsten, L., 2006. Evaluation of different formulations of *Bacillus licheniformis* in mango packhouse trials. Biol. Contr. 37: 237-242.

Gowing, D.P., 1959. A method of comparing herbicides and herbicide mixtures at the screening level. Weed Sci. 7: 66-76.

Gowing, D.P., 1960. Comments on tests of herbicide mixtures. Weed Sci. 8: 379-391.

Grabski, C., Gisi, U., 1987. Quantification of synergistic interactions of fungicides against *Plasmopara* and *Phytophthora*. Crop Prot. 6:64-71.

Gullino, M. L., Leroux, P., Smith, C. M., 2000. Uses and challenges of novel compounds for plant disease control. Crop Prot. 19: 1-11.

Guizzardi, M., Caccioni, D.R.L., Pratella, G. C., 1995. Resistance monitoring of *Monilinia laxa* (Aderh. *et* Ruhl.) Honey to benzimidazoles and dicarboximides in postharvest stage. J. Plant Dis. Prot. 102: 86-90.

Harborne, J. B., 1964. Biochemistry of phenolic compounds. Academic Press, London. pp. 295-361.

Hayashi, K., Schoonbeek, H., Sugiura, H., De Waard, M. A., 2001. Multidrug resistance in *Botrytis cinerea* associated with decreased accumulation of the azole fungicide oxpoconazole and increased transcription of the ABC transporter gene *BcatrD*. Biochem. Physiol. 70: 168-179.

Hayashi, K., Schoonbeek, H., De Waard, M. A., 2002. *Bcmfs1*, a novel MFS transporter from *Botrytis cinerea*, provides tolerance to the natural toxic compounds camptothecin and cercosporin and DMI fungices. Appl. Environ. Microbiol. 68: 4996-5004.

Hayashi, K., 2003. ABC and MFS transporters from *Botrytis cinerea* involved in sensitivity to fungicides and natural toxic compounds. Doctoral Thesis.

Hayashi, K., Schoonbeek, H., De Waard, M. A., 2003. Modulators of membrane drug transporters potentiate the activity of the DMI fungicide oxpoconazole against *Botrytis cinerea*. Pest Manag. Sci. 59:294-302.

Hofstein, R., Fridlender, B., Chalutz, E., Droby, S., 1994. Large scale production and pilot testing of biological control agents for postharvest diseases. In: Wilson, C. L., Wisniewski, M. E.

(Eds.), Biological control of postharvest diseases-Theory and practice. CRC Press, Boca Raton, FL, pp. 89-100.

Holland, I. B., Blight, M. Am., 1999. ABC-ATPases, adaptable energy generators fuelling transmembrane movement of a variety of molecules in organisms from bacteria to humans. J. Mol. Biol. 293: 381-399.

Holz, G., 2001. The occurrence and control of *Botrytis cinerea* (grey mould) in grapes: A South African perspective. September Winelands 1-14.

Ikediala, J. N., Hansen, J. D., Tang, J., Drake, S. R., Wang, S., 2002. Development of a saline water immersion technique with RF energy as a postharvset treatment against codling moth in cherries. Postharvest Biol. Technol. 24: 209-221.

Ippolito, A., Nigro, F., Romanazzi, G., Campanella, V., 1998. Field application of *Aureobasidium pullulans* against *Botrytis* storage rot of strawberries. In: Bertolini P, Sijmons PC, Guerzoni ME and Serra F (eds) Workshop on 'Non Conventional Methods for the Control of Post-harvest Disease and Microbiological Spoilage' (COST 914-COST 915), October 9-11, 1997 (pp 127-133) Bologna, Italy.

Ismail, M., Zhang, J., 2004. Postharvest citrus disease and their control. Outlooks Pest Manag. 1(10): 29-35.

Janisiewicz, W. J., 1985. Biological control of postharvest disease of pome fruits. Phytopathology 77: 481-485.

Janisiewicz, W. J., 1988. *Bio-control* of postharvest diseases of apples with antagonist mixtures. Phytopathology 78: 194-198.

Janisiewicz, W. J., Tworkoski, T. J., Sharer, C., 2000. Characterization of the mechanism of biological control of postharvest diseases on fruit with a simple method to study competition for nutrients. Phytopathology 90: 1196-1200.

Janisiewicz, W. J., Korsten, L., 2000. Control of postharvest diseases and microbial spoilage of vegetables by application of microbes. In: Bartz, J. (Ed.), The physiology and microbiology of vegetables after harvest. Chapter 23, Marel Dekker, New York.

Janisiewicz, W. J., Korsten, L., 2002. Biological control of postharvest diseases of fruits: Ann. Rev. Phytopathol. 40: 411-444.

Janisiewicz, W. J., Usall, J., Bors, B., 1992. Nutritional enhancement of biological control of blue mould on apples. Phytopathology 82: 1364-1370.

Jarvis, W. R., 1977. *Botryotinia* and *Botrytis* species: taxonomy, physiology and pathogenicity: a guide to the literature. Vol. 15. 195 pages. Canada Department of Agriculture, Harrow.

Jennigs, B. R., Ridler, P. J., 1983. Interaction of chromosomal strains with DNA. An electrofluorescence study. Biophys. Struct. Mech. 10: 71-79.

Jijakli, M.H., Lepoivre, P., Grevesse, C., 1999. Yeast species for *bio-control* of apple postharvest diseases: An encouraging case of study for practical use. In: Upadhyay RK and Mukerji KG (eds) Biotechnological Approaches in Biocontrol of Plant Pathogens. Kluwer Academic/Plenum Publishers, New York (pp 31-49).

Joubert, S., Archer, E., 2000. The critical importance of preventive *Botrytis* control in red wine cultivars. Wynboer March, 1-6.

Keller, M., Viret, O., Cole, F. M., 2003. *Botrytis cinerea* infection in grape flowers: Defense reaction, latency and disease expression. Phytopathology 93: 316-322.

Kellerman, T. S., Coetzer, J. A. W., Naude, T. W., 1988. Plant poisoning and mycotoxicoses of livestock in Southern Africa. Oxford University Press, Cape Town.

Koomen, I., Jeffries, P., 1993. Effects of antagonistic microorganisms on the postharvest development of *Collectotrichum gloeosporiodes* on mango. Plant Pathol. 42: 230-237.

Korsten, L., Van Harmelen, M. W. S., Heitmann, A., De Villiers, E. E., De-Jager, E. S., 1991. Biological control of postharvest mango diseases. South African Mango Growers Association Yearbook, 11: 65-67.

Korsten, L., De-Jager, E. S., 1995. Mode of action of *Bacillus subtilis* for control of avocado postharvest pathogens. South African Avocado Growers` Association Year Book 18: 124-130.

Korsten, L., De-Jager, E. E., Paul, I., Obagwu, J., El-Ghaouth, A., 2000. Alternative control of citrus Postharvest disease. Poster presented at the International Society of citriculture at Orlando, Florida, USA, 3-7, December 2000.

Korsten, L., 2006. Advances in control of postharvest diseases in tropical fresh produce. Int. J. Postharvest Technol. Innov. 1 (1): 48-61.

Korsten, L., 1993. Biological control of avocado fruit diseases. PhD Thesis, University of Pretoria, Pretoria, South Africa.

Kosman, E., Cohen, Y., 1996. Procedures for calculating and differentiating synergism and antagosim in action of fungicide mixtures. Phytopathology 86: 1255-1264.

Kubec, R., Velisek, J., Musah, R. A., 2002. The amino acid precursors and odor formation in society garlic *Tulbaghia violacea* (Harv). Phytochem. 60: 21-25.

Kubo, I., Nakanishi, K., 1979. Some terpenoid antifeedants from tropical plants. In: Geissbuhaler, H., Pergamon (Eds), Press, Oxford. Pp. 284-294.

Leroux, P. M., Kotze, C. D., Nel, G. P., Glen, H. F., 1994. Bossieveld. Grazing plants of the Karoo and Karoo-like areas. Bulletin 428. Directorate of Agricultural Information, Pretoria. South Africa.

Leroux, P., Descotes, A., 1996. Resistance of *Botrytis cinerea* to fungicides and strategies for its control in the Champagne vineyards. Proceedings of Brighton Crop Protection Conference – Pests and Diseases, BCPC, Surrey, UK, pp. 131-136.

Leverentz, B., Janisiewicz, W. J., Conway, W. S., Saftner, R. A., Fuchs, Y., Sams, E., E., Camp, M. J., 2000. Combining yeasts or a bacterial biocontrol agent and heat treatment to reduce postharvest decay of 'Gala' apples. Postharvest Biol. Technol. 21: 87-94.

Leverentz, B., Conway, W. S., Janisiewicz, W. J., Saftner, R. A., Camp, M. J., 2003. Effect of combining MCP treatment, heat treatment, and biocontrol on the reduction of postharvest decay of 'Golden Delicious' apples. Postharvest Biol. Technol. 27: 221-233.

Levy, Y., Benderly, M., Cohen, Y., Gisi, U., Bassand, D., 1986. The joint action of fungicides in mixtures: Comparison of two methods for synergy calculations. EPPO Bull. 16:651-657.

Levyns, M.R., 1929. Veld-burning experiments at Ida's Valley, Stellenbosch. Trans. R. Soc. S. Afr. 17(2): 61-95.

Lima, G., De Curtis, F., Castoria, R., De Cicco, V., 1998. Activity of the yeasts *Cryptococcus laurentii* and *Rhodotorula glutaris* against postharvest rots on different fruits. Biocontr. Sci. Technol. 8: 257-267.

Lima, G., Arru, S., De Curtis, F., Arras, G., 1999. Influence of antgonist, host fruit and pathogen on the biological control of postharvest fungal diseases by yeasts. J. Ind. Microbiol. Biotechnol.23: 223-229.

Lorito, M., Di Pietro, A., Hayes, C. K., Woo, S. L., Harman, G. E., 1993. Antifungal, synergistic interaction between chitinolytic enzymes from *Trichoderma harzianum* and *Enterobacter cloacae*. Phytopathology 83: 721-728.

Lorito, M., Peterbauer, C., Hayes, C. K., Harman, G. E., 1994. Synergistic interaction between fungal cell wall-degrading enzymes and different antifungal compounds enhances inhibition of spore germination. Microbiol. 140: 623-629.

Lorito, M., Peterbauer, C., Sposato, P., Mach, R. L., Strauss, J., Kubicek, C. P. 1996. Mycoparasitic interaction relieves binding of the Cre1 carbon catabolite repressor protein to promoter sequences of the *ech-42* (endochitinase-encoding) gene in *Trichoderm harzianum*. Proc. Natl. Acad. Sci. USA 93: 14868-14872.

Lyr, H., 1995. Modern selective fungicides: Properties, applications, mechanisms of action. 2nd rev. and enl. ed. 595 pages. Gustav Fischer, Jena; New York.

Marchetti, O., Moreillon, P., Glauser, M.P., Bille, J., Sanglard, D., 2000. Potent synergism of the combination of fluconazole and cyclosporine in *Candida albicans*. Antimicrob. Agents Chemother. 44: 2373-2381.

Mari, M., Guizzardi, M., 1998. The postharvest phase: Emerging technology for the control of fungal diseases. Phytoparasitica 26: 59-66.

Mattheis, J., Roberts, R., 1993. Fumigation of sweet cherry (*Prunus avium* 'Bing') fruit with low molecular weight aldehydes for postharvest decay control. Plant Dis. 77:810-814

McLaughlin, R. J., Wisniewski, M. E., Wilson, C., Chalutz, E., 1990. Effect of inoculum concentration and salts solutions on biological control of postharvest diseases of apples with *Candida* sp. Phytopathology 80: 456-461.

Meepagala, K. M., Sturtz, G., Wedge, D. E., 2002. Antifungal constituents of the essential oil fraction of *Artemisia drencunculus* L. var. *dracunculus*. J. Agric. Food Chem. 50: 6989-6992.

Morel, C., Stermitz, F. R., Tegos, G., Lewis, K., 2003. Isoflavones as potentiators of antibacterial activity. J. Agric. Food. Chem. 51: 5677-5679.

Nakaune, R., Adachi, K., Nawata, O., Tomiyama, M., Akutsu, K., Hibi, T., 1998. A novel ATP-binding cassette transporter involved in multidrug resistance in the phytopathogenic fungus *Penicllium digitatum*. Appl. Environ. Microbiol. 64: 3983-3988.

Neilands, J. B., 1981. Microbial iron compounds. Ann. Rev. Biochem. 50: 715-731.

Nunes, C., Usall, J., Teixido, N., Miro, M., Vinas, I., 2001. Nutritional enhancement of biocontrol activity of *Candida sake* (CPA-1) against *Penicillium expansum* on apples and pears. Eur. J. Plant Pathol. 107: 543-551.

Nunes, C., Usall, J., Teixido, N.,Abadias, M., Immaculada, V., 2002. Improved control of postharvest decay of pears by the combination of *Candida sake* (CPA-1) and ammonium molybdate. Phytopathology 3 (92): 281-287.

Obagwu, J., 2003. Developing biopesticides for control of citrus fruit pathogens of importance in global trade. PhD thesis, University of Pretoria, Pretoria, South Africa

Obagwu, J., Korsten, L., 2003. Integrated control of citrus green and blue moulds using *Bacillus subtilis* in combination with sodium bicarbonate or hot water. Postharvest Biol. Technol. 28: 187-194.

Obagwu, J., Emechebe, A. M., Adeoti, A. A. 1997. Effect of extracts of garlic (*Allium sativum* L.) bulb and neem (*Azadirachta indica* Juss) seed on the mycelial growth and sporulation of *Colletotrichum capsici*. J. Agri. Technol. 5: 51-55.

Ogawa, J. M., Zehr, E. I., Bird, G. W., Ritchie, D. F., Uriu, K. Uyemoto, J. K. (Eds), 1995. Compendum of stone fruit disease. APS, St. Paul, Minnesota, p. 98.

Palou, L., Smilanick, J. L., Usall, J., Virias, I., 2001. Control of postharvest blue and green moulds of roanges by hot water, sodium carbonate and sodium bicarbonate. Plant Dis. 85: 371-376.

Palou, L., Usall, J., Munoz, J. A., Smilanick, L., Vinas, I., 2002. Hot water, sodium carbonate, and sodium bicarbonate for the control of postharvest green and blue molds of clementine mandarins. Postharvest Biol. Technol. 24:93-96.

Pang, X., Qun Zhang, Z. Q., Huang Xue, M., 2002. Biological control of postharvest diseases of fruits and vegetables. J. Trop. Subtrop. Bot. 10: 186-192.

Papadopoulou, K., Melton, R. E., Legett, M., Daniel, M. J., Osburn, A. E., 1999. Compromised disease resistance in saponin-deficient plants. Proc. Natl. Acad. Sci. USA 96: 12923-12928.

Paulsen, I. T., Brown, M. H., Skurray, R. A., 1996. Proton-dependent multidrug efflux systems. Microbiol. Rev. 60: 575-608.

Plaza, P., Usall, J., Torres, R., Lamarca, N., Asensio, A., Vinas, I., 2003. Control of green and blue mould by curing on oranges during ambient and cold storage. Postharvest Biol. Technol. 28: 195-198.

Plaza, P., Usall, J., Teixido, N., Vinas, I., 2004. Effect of water activity and temperature on competing abilities of common postharvest citrus fungi. Int. J. Food Microbiol. 90: 75-82.

Polderdijk, J.J., Van Dyk, F. E., Ferreira, D., Guis, G. W., Keller, S., 2006. Chapter 11: Fruitful – Integrated supply-chain information systems for fruit produce between South Africa and The Netherlands. Agro-food chains and networks for development 129-140.

Poppe, L., Vanhoutte, S., Hofte, M., 2003. Modes of action of *Pantoea agglomerans* CPA-2, an antagonist of postharvest pathogens on fruits. Eur. J. Plant Pathol. 109: 963-973.

Porat, R., McCollum, T. G., Vinokur, V., Droby, S., 2002. Effects of various elicitors on the transcription of a β-1,3-endoglucanase gene in citrus fruit. J. Phytopathol. 150:70-75.

Poswal, M. A. T., Akpa, A. D., Alabi, O., 1993. Cultural control of pests and diseases: Prelude to integrated pest management practices for resource-poor farmers in Nigerian agriculture. J. Sustain. Agric. 3: 5-47.

Poswal, M. A. T., 1996. Antifungal activity of natural plant oils against *Alternaria citri*, the navel-end rot pathogen. Proc. Int. Soc. Citri. 478-481.

Pusey, P. L., 1994. Enhancement of biocontrol agents for postharvest diseases and their integration with other control strategies. In: Wilson, E. L., Wisniewski, M. E. (Eds.), Biological control of postharvest diseases CRC press, Bock Raton, pp.77-88.

Regnier, T., Macheix, J. J., 1996. Changes in wall-bound phenolic acids, phenylalanine and tyrosine ammonia-lyases, and peroxidases in developing durum wheat grains (*Triticum turgidum* L. var. *Durum*). J. Agric. Food Chem. 44: 1727-1730.

Reimann, S., Deising, H. B., 2000. Fungicides: risk of resistance development and search for new targets. Arch. Phytopathol. Plant Prot. 33: 329-349.

Reuveni, R., Fleischer, A., Putievski, E., 1984. Fungistatic activity of essentials oils from *Ocimum basilicum* chemotypes. Phytopathology 10: 20-22.

Roberts, R. G., 1990. Postharvest biological control of gray mould of apple by *Cryptococcus laurentii*. Phytopathology 80: 526-530.

Rosenberger, D.A., 1990. Gray mold. In: Jones, A.L., Aldwinckle, H.S., (Eds.), Compendium of Apple and Pear Diseases. APS Press, St. Paul, MN, USA, pp. 55-56.

Rosslenbroich, H. J., Stuebler, D., 2000. *Botrytis cinerea*-history of chemical control and novel fungicides for its management. Crop Prot. 19: 557-561.

Samoucha, Y., Cohen, Y., 1984. Synergy between metalaxyl and mancozeb in controlling downy mildew in cucumbers. Phytopathology 74: 1434-1437.

Samoucha, Y., Cohen, Y., 1988. Synergistic interactions of cymoxanil mixtures in the control of metalaxyl-resistant *Phytophthora infestans* of potato. Phytopathology 78: 636-640.

Samoucha, Y., Gisi, U., 1987. Use of two-and three-way mixtures to prevent buildup of resistance to phenylamide fungicides in *Phytophthora* and *Plasmopara*. Phytopathology 77: 1405-1409.

Schoonbeek, H., Del Sorbo, G. and De Waard, M. A. 2000a. Fungal Transporters Involved in Efflux of Natural Toxic Compounds and Fungicides. Fungal Genet. Biol. 30: 1-15.

Schoonbeek, H., Del Sorbo, G. and De Waard, M. A. 2000b. The ABC transporter BcartB affects the sensitivity of *Botrytis cinerea* to the phytoalexin resveratrol and the fungicide fenpiclonil. Mol. Plant-Microbe. Interact. 14: 562-571.

Schoonbeek, H., Del Sorbo, G., De Waard, M. A., 2001. The ABC transporter BcatrB affects the sensitivity of *Botrytis cinerea* to the phytoalexin resveratrol and the fungicide fenpiclonil. Mol. Plant-Microbe Interact. 14: 562-571.

Schoonbeek, H., 2004. ABC transporters from *Botrytis cinerea* in biotic and abiotic interactions. Doctoral thesis.

Schwyn, B., Neilands, J. B., 1987. Universal chemical assay for the detection and determination of siderophores. Anal. Biochem. 160: 47-56.

Severina, I. I., Muntyan, M. S., Lewis, K., Skulachev, V. P., 2001. Transfer of cationic antibacterial agents berberine, palmatine and benzalkonium through bimolecular planar phospholipids film and *Staphylococcus auereus* membrane. IUBMB-Life Sc. 52: 321-324.

Shachnal, A., Chalutz, E., Droby, S., Cohen, L., Weiss, B., Daus, A. Q., Katz, H., Bercovitz, A., Keren-Tzur, M., Binyamini, Y., 1996. Commercial use of Aspire^{TM} for the control of postharvest decay of citrus fruit in the packinghouses. Proc. Inter. Soc. Citri. 2: 1174-1177.

Shahi, S. K., Patra, M., Shukla, A. C., Dikshit, A., 2003. Use of essential oil as botanical-pesticide against post harvest spoilage in *Malus pumilo* fruits. BioControl 48: 223-232.

Sholberg, P. L., Shimizu, B. N., 1991. Use of the natural plant product, hinokitiol, to extend shelf-life of peaches. J. Can. Insti. Food Sci. Technol. 2: 273-276.

Siegel M.R., Kerkenaar, A., Kaars Sijpesteijin, A., 1977. Antifungal activity of the sysmemic fungicide imazalil. Neth. J. Plant Pathol. 83: 121-133.

Singh, G., Marimuthu, P., Catalan, C., de-Lampasona, M. P., 2004. Chemical, antioxidant and antifungal activities of volatile oil of black pepper and its acetone extract. J. Sci. Food Agric. 84: 1878-1884.

Sinha, P., Saxena, S. K., 1999. Inhibition of fruit rot fungus and fruit fly by leaf extracts of onion and garlic. Indian J. Agric. Sci. 69: 651-653.

Smilanick, J. L., Margosan, D. A., Mlikota, F., Usall, J., Micheal, I. F., 1999. Control of citrus green mould by carbonate and bicarbonate salts and the influence of commercial postharvest practices on their efficacy. Plant Dis. 83: 139-145.

Sommer, N.F., 1982. postharvest handling practices and postharvest disease of fruit. Plant Dis. 66: 357-364.

Spadaro, D., Vola, R., Piano, S., Gullino, M. L., 2002. Mechanisms of action and efficacy of four isolates of the yeast *Metschnikowia pulcherrima* active against postharvest pathogens on apples. Postharvest Biol. Technol. 24: 123-134.

Spotts, R. A., Cervantes, L. A., 1986. Populations, pathogenicity, and benomyl resistance of *Botrytis* sp., *Penicillium* spp., and *Mucor piriformis* in packhouses. Plant Dis. 70: 106-108.

Stehmann, C., De Waard, M. A., 1995. Accumulation of tebuconazole by isolates of *Botrytis cinerea* differing in sensitivity to sterol demethylation inhibiting fungicides. Pestic. Sci. 45: 311-318.

Stehmann, C., De Waard, M. A., 1996. Sensitivity of populations of *Botrytis cinerea* to triazoles, benomyl and vinclozolin. Eur. J. Plant Pathol. 102:171-180.

Stermitz, F. R., Beeson, R. D., Mueller, P. J., Hsiang, J.-F., Lewis, K., 2001. *Staphylococcus aureus* MDR efflux pump inhibitors from a *Berberis* and a *Mahonia* (*sensu strictu*) species. Biochem. Syst. Ecol. 29: 793-798.

Stermitz, F. R., Lorenz, P., Tawara, J. N., Zenewicz, L., Lewis, K., 2000. Synergy in a medicinal plant: antimicrobial action of berberine potentiated by 5`-methoyhydnocarpin, a multidrug pump inhibitor. Proc. Nal. Acod. Sci. USA 97: 1433-1437.

Sugiura, H., Hayashi, K., Tanaka, T., Takenaka, M., Uesugi, Y., 1993. Mutual antagonism between sterol demethylation inhibitors and phosphororthiolate fungicides on *Pyricularia oryzae* and the implication for their mode of action. Pestic. Sci. 39: 193-198.

Tammes, P.M.L., 1964. Isoboles, a graphic representation of synergism in pesticides. Neth. J. Plant Pathol. 70: 73-80.

Tegos, G., Stermitz, F. R., Lomovskaya, O. and Lewis, K. 2002. Multidrug pump inhibitors remarkable activity of plant antimicrobials. Antimicrob. Agents Chemother. 46:3133-3141.

Tiwari, R., Mishra, D. N., Upadhyay, P. S., 1988. Efficacy of some plant volatiles for the control of black mould of onion caused by *Aspergillus niger* Van Tiegh during storage. Natl. Acad. Sci. Lett. 11: 345-347.

Tripathi, P., Dubey, N. K., Pandey, V. B., 2002. Kaemferol: the antifungal principle of *Acacia nilotica* Linn. Del. Indian Bot. Soc. 81: 51-54.

Tripathi, P., Dubey, N. K., 2004. Exploitation of natural products as an alternative strategy to control postharvest fungal rotting of fruit and vegetables. Postharvest Biol. Technol. 32: 235-245.

Van Aardt, M. P., 2004. Plant poisoning in Namaqualand. Elsenburg, Springbok Veterinary Services.

Vaugh, S. F., Spencer, G. F., Shasha, S., 1993. Volatile compounds from raspberry and strawberry fruit inhibit postharvest decay fungi. J. Food Sci. 58: 793-796.

Van Wyk, B-E., Van Oudtshoorn, B. & Gericke, N., 1997. Medicinal plants of South Africa. Briza Publications, Pretoria.

Vermeulen, T., Schoonbeek, H., De Waard, M. A., 2001. The ABC transporter BcartB from *Botrytis cinerea* is a determinant of the activity of the phenylpyrride fungicide fludioxonil. Pest Manag. Sci. 57: 393-402.

Vero, S., Mondino, P., Burgueno, J., Soubes, M, and Wisniewski, M., 2002. Characterization of biocontrol activity of two yeast strains from Uruguay againsy blue mould of apple. Postharvest Biol. Technol. 26: 91-98.

Wadley, F.M., 1945. The evidence required to show synergistic action of insecticides and a short cut in analysis. ET Circ. 223. U.S. Department of Agriculture, U.S. Government Printing Office, Washington, DC.

Wadley, F.M., 1967. Experimental statistics in Entomology. Graduate School Press, Washington State University, Pullman.

Wardowski, W. F., Brown, G. E., 2001. Postharvest decay control recommendations for Florida citrus fruit. Cooperative Extension Service, Institute of Food and Agricultural Science, University of Florida, pp. 1-7.

Watson, L., and Dallwitz, M. J. 1992 Ecology and evolutionary biology conservatory. 'The families of flowering plants: descriptions, illustrations, identification, and information retrieval.' Version: 19th August 1999.

Watt, J.M. & Breyer-Brandwijk, M.G., 1962. Medicinal and poisonous plants of southern and eastern Africa, edn 2. Livingstone, Edinburgh.

Whipps, J. M., McQuilken, M. P., 1993. Aspects of biocontrol of fungal plant pathogens. In: Jones D. G. (Ed.), Exploitation of microorganisms. Chapman and Hall, London, pp. 45-79.

Wilson, C. L., Franklin, J. D., Pusey, P. L., 1987. Biological control of *Rhizopus* rot of peach with *Entobacter cloacae*. Phytopathololology 27: 425-441.

Wilson, C. L., Wisniewski, M. E., 1989. Biological control of postharvest diseases of fruits and vegetables: An emerging technology. Ann. Rev. Phytopathol. 27:425 – 441.

Wilson, C. L., Wisniewski, M. E., 1994. Biological control of postharvest diseases of fruits and vegetables – theory and practice. CRC Press, Boca Raton, Florida, 182 pp

Wilson, C. L., Solar, J. M., El Ghaouth, A., Wisniewski, M. E., 1997. Rapid evaluation of plant extracts and essential oils for antifungal activity against *Botrytis cinerea*. Plant Dis. 81: 204-210.

Wisniewski, M. E., Droby, S., Chalutz, E., Eilam, Y., 1995. Effect of Ca+2 and Mg+2 on Botrytis cinerea and Penicillium expansum in vitro and on the biocontrol activity of Candida oleophila. Plant Pathol. 44: 1016-1024.

Zhang, H., Zheng, X., Fu, C., Xi, Y., 2005. Postharvest biological control of gray mould rot of pear with Cryptococcus lauerntii. Postharvest Biol. Technol. 35: 79-86.

Zhou, T., Northover, J., and Schneider, K. E., 1999. Biological control of postharvest diseases of peach with phyllosphere isolates of Pseudomonas syringae. Can. J. Plant Pathol. 21:375-381.

Zhou, T., Northover, J., Schneider, K. E. Lu, X., 2002. Interactions between Pseudomonas syringae MA-4 and cyprodinil in the control of blue mold and gray mold of apples. Can. J. Plant. Pathol. 24: 154-161.

CHAPTER 2

THE *IN VITRO* EFFECTS OF MIXTURES OF FUNGICIDES AND MEDICINAL PLANT EXTRACTS AGAINST *BOTRYTIS CINEREA*

2.1 Abstract

Since problems of resistance against fungicides became evident, increased attention has been paid to the possibilities of coping with resistance using mixtures of fungicides. When utilized in two-way mixtures, such fungicides may maintain or enhance the level of control of a pathogen at reduced rates for both components utilized in combinations or alone at normal rates. These studies also provide an important precedent for the idea of synergism. This underpinned our objective to test if in combination a synergistic effect will in fact occur between fungicides and South African medicinal plant extracts. Four fungicides- Sporekill™, Rovral™, Terminator™ and Teldor™ at doses 0.1, 0.2, 0.4 and 0.8 mL L^{-1} and plant species *Galenia africana*, *Elytropappus rhinocerotis* and *Tulbaghia violacea* were tested alone and in different combinations for their efficacy of radial growth inhibition of *Botrytis cinerea* strains on potato dextrose plates. Four plant extract doses for each of the respective plant species were used. A total of 48 combinations were tested for each strain. Mixtures were far more effective in controlling strains compared to the individual components alone, representing significant levels of *in vitro* synergistic interactions. Mixtures of Sporekill™ either with *G*. *africana*, *E*. *rhinocerotis* or *T*. *violacea* had synergistic effects. Synergistic interactions between Sporekill™ and plant extracts were highly dependent on the wild-type strain. Rovral™ in combination with either of the three plant species were far more efficient in inhibiting strains of *B*. *cinerea* than the components alone. Combinations of Rovral™ with *E*. *rhinocerotis* or *T*. *violacea* exerted, in some cases, an inhibitory efficacy up to 100%. The addition of

Terminator™ to samples containing any dose of the plant extracts did not significantly increase the level of inhibition for any strain tested. Teldor™-containing mixtures showed higher levels of inhibition and efficacy increased up to 100%. The effectiveness of combinations was reflected in relatively low doses of Teldor™. Combinations of these components represent an attractive perspective for the development of new management strategies for controlling *B*. *cinerea* in the future.

2.2 Introduction

The gray mold fungus *Botrytis cinerea* Pers. is one of the major post-harvest pathogens of fruit (Zheng *et al*., 2007). The fungus mainly infects fruit through wounds caused during harvesting and handling in the packing-house processing lines (Arras *et al*., 2002). Currently, the control of post-harvest molds relies mainly on the use of synthetic fungicides. Fungicide toxicity in the environment and on human health and the development of fungicide resistance by pathogens have necessitated great efforts to develop and exploit alternatives to the synthetic fungicides (Droby *et al*., 2003).

Several proposed non-fungicidal approaches, including the use of biological control with antagonistic microorganisms, heat treatment, induction of resistance and plant extracts, have been studied extensively (Zheng *et al*., 2007). Although all these alternative approaches have been shown to reduce post-harvest diseases, they do not always offer control comparable to that provided by synthetic fungicides (Wilson *et al*., 1994; Wisniewski *et al*., 2001; Janisiewicz and Korsten, 2002; Droby *et al*., 2003; Ippolito *et al*., 2005; Droby, 2006). Thus, to develop new and highly effective biofungicides it is necessary prerequisite to make their activity more reliable by combining them with other control strategies such as low doses of fungicides in integrated control programmes (Chand-Goyal and Spotts, 1997; Ippolito *et al*., 1998; Droby *et al*., 2002).

Fungicide mixtures have become widely used in recent years. Mixtures are used to broaden the spectrum of activity of a compound or to achieve higher levels of activity by means of synergistic interaction (Scardavi, 1966). The use for mixtures arose when the relatively newly developed systemic, site-specific fungicides lost their efficacy due to fungal resistance (Bashan *et al*., 1991). Field experiments have shown that mixtures of site-specific and multi-site fungicides may delay the buildup of resistant populations (Gisi *et al*., 1985; Sanders *et al*., 1985; Samoucha and Cohen, 1988). Synergistic interactions between fungicides with different modes of action have been demonstrated *in vitro* with *Phytophthora cactorum* (Lebert et Cohn) Schröter and *Phytophthora cinnamoni* Rands and *in vivo* with *Phytophthora infestans* (Mont.) de Bary on tomato and potato, *Plasmospora viticola* Berk. et Curtis ex. De Bary and *B. cinerea* Berl. et de Toni on grapevines (Gisi *et al*., 1983; Gisi *et al*., 1985; Samoucha and Cohen, 1986; Samoucha and Cohen, 1988; Samoucha *et al*., 1988). The exposure of the pathogen to sublethal concentrations of one fungicide may affect it to an extent that sublethal doses of the second fungicide will be more detrimental than in the absence of the first (Samoucha and Gisi, 1987). The observation that mixtures persist longer on crops compared with their components alone (Samoucha *et al*., 1988) provides another possible explanation for synergy under field conditions (Bashan *et al*., 1991).

Plants produce an enormous array of secondary metabolites, and it is commonly accepted that a significant part of this chemical diversity serves to protect plants against microbial pathogens (Dixon, 2001). These plant compounds are routinely classified as “antimicrobial” on the basis of susceptibility tests that produce MICs in the range of 100 to 1000 µg/ml, orders of magnitude weaker than those of typical antibiotics produced by bacteria and fungi (Tegos *et al*., 2002). Tegos *et al*. (2002) argued that a compound that is synthesized in response to pathogen invasion and is required to protect the plant from a pathogen, but that shows little activity in an *in vitro* susceptibility test is not necessarily an “antimicrobial” (Tegos *et al*., 2002). Such a compound might have a regulatory

function indirectly increasing the level of resistance of the plant. This analysis suggests that we need still to discover evidence for providing a functional role for the vast majority of plant compounds that have been classified as “antimicrobials”.

The objective of this study was, therefore, to evaluate the antifungal activity of plant extracts alone, and in combinations with fungicides against *B. cinerea* to determine the *in vitro* synergistic responses of South African medicinal plant compounds.

2.3 Materials and Methods

2.3.1 Isolation of Fungal Cultures

Botrytis cinerea strains PPRI 8506, 8507 and 7338 were obtained from the Agricultural Research Council - Plant Protection Research Institute, Mycology Division, Pretoria, South Africa. Strain PPRI 8506 was isolated from infected plums and strains PPRI 8507 and 7338 from infected apples. Inocula of *B. cinerea* were cultured on potato dextrose agar (PDA) at 25 °C.

2.3.2 Fungicides

All fungicides were purchased from pesticide distribution companies in SA. Fungicides were suspended in water (concentration is given in milliliters active ingredient per liter) and tested alone or in mixtures with plant extracts in the *in vitro* experiments. The following fungicides (technical grade) were used: Sporekill™ (didecyl dimethyl ammonium chloride, Hygrotech, 120 g/L a.i.), Rovral™ Aquaflo (iprodione, Aventis CropScience, 500 g/L a.i.), Terminator™ (dimethyl didecyl ammonium chloride, Fairhill Crop Care cc, 250 g/L a.i.) and Teldor™ 500 SC (fenhexamid, Bayer Ltd, 500 g/L a.i.). The standard recommended doses for post-harvest application of these fungicides in South Africa are: Sporekill™ 1.5 mL L^{-1}, Rovral™ 1mL L^{-1}, Terminator™ 0.8 mL L^{-1} and Teldor™ 1mL L^{-1}.

2.3.3 Plant Extraction Method

Plant material for *Galenia africana* L. (Aizoaceae) and *Elytropappus rhinocerotis* (L.f) Less (Asteraceae) were collected from Wellington and *Tulbaghia violacea* Harv. (Alliaceae) were obtained from nurseries in Stellenbosch, South Africa. Powdered, air-dried leaves of *G. africana* (500 g) and whole plants (leaves and shoots) of *E. rhinocerotis* (400 g) were used for extractions. Extractions were performed overnight in a closed container at room temperature in ethanol (EtOH: 1000 ml) analytical grade to obtain 500 and 400 mg ml^{-1} (50 and 40%) stock concentrations, respectively. Fresh, whole plants (leaves and rhizome) of *T. violaceae* (500 g) were crushed in a Waring blender and extracted overnight in a closed container at room temperature in EtOH (1000 ml) to obtain 500 mg ml^{-1} (50%) stock concentration. The extracts were filtered through a Büchner funnel (110 mm diameter) and Whatman no. 4 qualitative filter paper, and stored at 4°C until used.

2.3.4 Radial Growth Assay

The inhibitory effect of plant extracts alone and in mixtures with fungicides on *B. cinerea* radial growth was carried out in 90 mm petri plates containing 20 mL of solidified potato dextrose agar (PDA). The 50% ethanolic plant extracts were diluted in sterile distilled water in 10 mL doses of 62.5, 125.0, 250.0 and 500.0 mg mL^{-1} with fungicides at 0.1, 0.2, 0.4 and 0.8 mL L^{-1}. *E. rhinocerotis* were diluted in sterile distilled water in 10 mL doses of 50.0, 100.0, 200.0 and 400.0 mg mL^{-1} with fungicides at 0.1, 0.2, 0.4 and 0.8 mL L^{-1}. A 1-ml suspension of the treatments were spread evenly across the agar surface and allowed to dry in a laminar flow hood. A 3-mm diameter disc of inoculum of the test *Botrytis cinerea* strain, cut from the periphery of an actively growing culture on PDA plates, was placed on the inoculated agar in each petri plate and kept in the inverted position. The petri plates were kept at 25°C for 5 days. At the end of the

incubation period, growth of the pathogen was determined by measuring fungal radial growth with the aid of a vernier caliper.

2.4 Statistical Analysis

A completely randomized experimental design was used for the resulting treatments. The growth of the pathogen was determined by measuring fungal radial growth (widest axis) with the aid of a vernier caliper. One mycelia plug in a petri plate constituted a replicate. To assess differences in the mycelia growth of *B. cinerea* strains among the treatments, the percentage inhibition was calculated from the radial growth as: treatment/control X 100. All analyses were carried out using the statistical software package SAS version 8.2 (SAS, 1999). The synergistic ratio for percentage inhibition was based on the Abbott formula (Abbott, 1925) as described by Gisi (1996): expected efficacy of the mixture, $C_{exp} = A + B - (AB/100)$ in which A and B are the control levels given by the "fungicide" and the "plant extract", respectively. The synergy ratio (SR), between the observed (C_{obs}) and expected (C_{exp}) efficacies of the mixture was calculated as $SR = C_{obs}/C_{exp}$. A deviation from the C_{exp} as calculated from the SR between the expected and the observed response of the two compounds would indicate synergism, additivity, or antagonism. By definition, additive interactions are present if SR=1, synergism occurs if SR>1 and antagonism if SR<1 (Levy *et al*., 1986; De Waard and Gisi, 1995).

2.5 Results

The sensitivity of wild-type strains of *B. cinerea* to Sporekill™, Rovral™ aquaflo, Terminator™ and Teldor™ is given in Figures 2-1 to 2-3. All the strains tested varied considerably in their sensitivity to each fungicide dose tested. Sporekill™ showed the lowest inhibitory effects on the radial growth of all the strains. Rovral™ was effective in

inhibiting the radial growth of strain PPRI 8507 in a dose-dependent manner, with complete control at the higher doses of 0.4 and 0.8 mL L^{-1} (Figure 2-1). Large variation in the efficacy between Rovral™ concentrations was noted for both strains PPRI 8506 and 7338 (Figures 2-2 and 2-3, respectively). A relatively high dose (0.8 mL L^{-1}) of Rovral™ was required for 100% control of strain PPRI 7338 (Figure 2-3). Terminator™ had a relatively low inhibitory effect on all three of the wild-type strains.

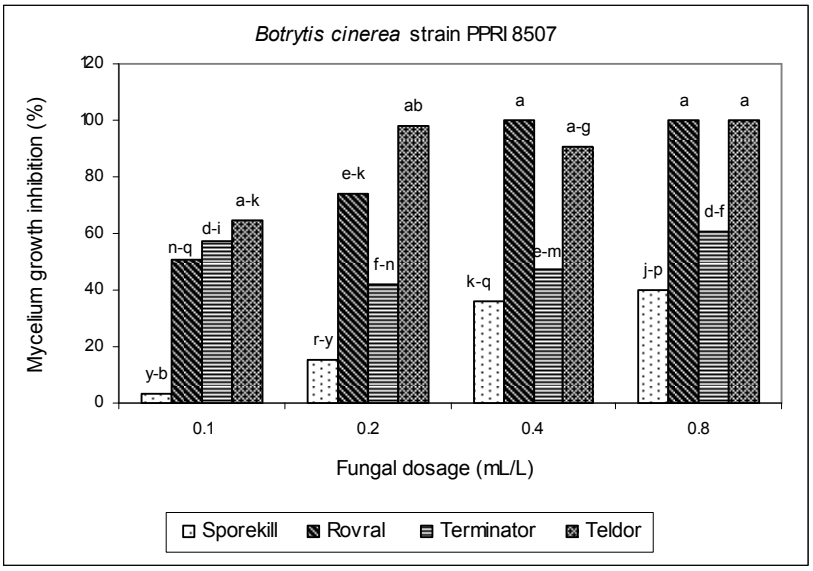

Figure 2-1. Effect of different fungicide doses 0.1, 0.2, 0.4 and 0.8 mL L^{-1} on the growth rate of strain PPRI 8507 of *Botrytis cinerea* in radial growth assays.

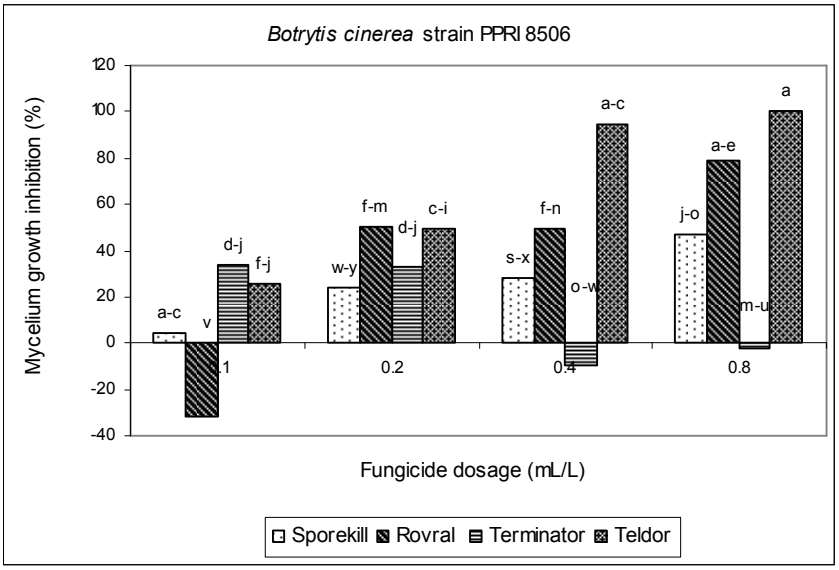

Figure 2-2. Effect of different fungicide doses 0.1, 0.2, 0.4 and 0.8 mL L^{-1} on the growth rate of strain PPRI 8506 of *Botrytis cinerea* in radial growth assays.

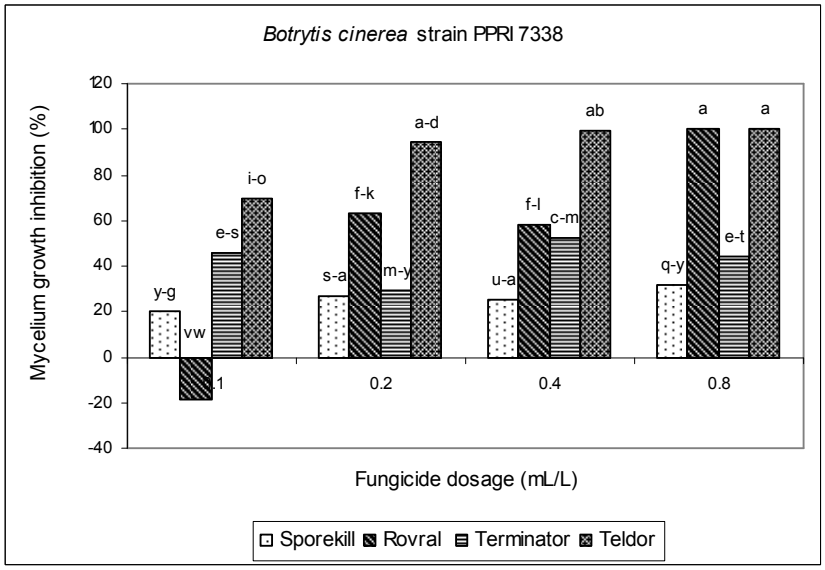

Figure 2-3. Effect of different fungicide doses 0.1, 0.2, 0.4 and 0.8 mL L^{-1} on the growth rate of strain PPRI 7338 of *Botrytis cinerea* in radial growth assays.

There was a significant linear dose-response to Teldor™, with the best inhibition occurring at the higher doses (Figures 2-1 to 2-3). Teldor™ showed the highest fungicidal activity towards the strains than Sporekill™, Rovral™ and Terminator™ in this antifungal study. Combinations of the fungicides at doses 0.1, 0.2, 0.4 and 0.8 mL L^{-1} with plant species *G*. *africana*, *E*. *rhinocerotis* and *T*. *violacea* was tested for their efficacy of radial growth inhibition of *B*. *cinerea* strains. Four plant extract doses for each of the respective plant species were used. A total of 48 combinations were tested for each strain.

Analysis of variance (ANOVA) on radial growth inhibition of each strain of *B*. *cinerea* indicated highly significant ($p < 0.0001$) interaction among plant type, extract and fungicide doses (Table 2-1). In a separate experiment with the fungicide, Teldor™ showed a significant interaction in radial growth of the wild-type strains of *B*. *cinerea* between plant extract, doses and fungicide (Table 2-2). On the basis of these significant interactions, data were summarized for individual strains and fungicides in tables to show significant differences of growth inhibition and synergistic interactions.

Table 2-1. Analysis of variance for mycelium growth inhibition of three *Botrytis cinerea* strains (PPRI 8507, 8506 and 7338), showing significant levels for main effects of Teldor™ (fenhaxamid) and medicinal plant extract doses, and all interactions involving combinations of Teldor™ and plant extracts.

		Probability>F[b]		
Source[a]	**df**	**PPRI 8507**	**PPRI 8506**	**PPRI 7338**
Fungicide	4	<.0001	<.0001	<.0001
Extract Dose (ED)*	12	0.0001	0.0024	<.0001
ED + Fungicide	48	0.0040	<.0001	<.0001

[a]Extracts from three medicinal plant species.

*ED, extract dose per plant species tested.

[b]Significant values associated with the *F* tests.

Of the 16 mixtures of Sporekill™ and *G*. *africana* tested, 15 exhibited significantly higher inhibition levels against PPRI 8507 (Table 2-3), compared to Sporekill™ alone. Only 3 of the 16 mixtures tested, showed significantly higher inhibition levels than the *G*. *africana* alone. Our results indicate that 2 of the mixtures were synergistic (SR>1.0), 4 were additive (SR=1.0) and 10 were antagonistic (SR<1.0). For combinations with *E*. *rhinocerotis*, 6 and 3 mixtures produced significantly higher radial growth inhibition levels compared to the Sporekill™ and plant extract alone, respectively (Table 2-3).

Combinations of 0.1 mL L^{-1} Sporekill™ (0.012 g/mL a.i.) and 400 mg mL^{-1} extract of *E*. *rhinocerotis* showed a synergistic interaction (SR>1.2). Combinations with *T*. *violacea* resulted in 6 and 11 mixtures with significantly higher radial growth inhibition levels compared to the fungicide and plant extract alone, respectively. In this case, 7 of the combinations produce synergistic interactions (Table 2-3).

Table 2-2. Analysis of variance for mycelium growth inhibition of three *Botrytis cinerea* strains (PPRI 8507, 8506 and 7338), showing the significant levels for the main effects of the fungicides Sporekill™ (didecyl dimethyl ammonium chloride), Rovral™ (iprodione) and Terminator™ (dimethyl didecyl ammonium chloride), and medicinal plant extract doses as well as all interactions involving combinations of the fungicides and plant extracts.

		Probability>F[b]								
		Sporekill™			Rovral™			Terminator™		
Source[a]	**df**	**PPRI 8507**	**PPRI 8506**	**PPRI 7338**	**PPRI 8507**	**PPRI 8506**	**PPRI 7338**	**PPRI 8507**	**PPRI 8506**	**PPRI 7338**
Fungicide	4	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0033	0.0024	0.0000
Extract	12	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
Plant Extract	3	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
Extract Dose (ED*)	3	0.0000	0.0011	0.0000	0.2585	0.0604	0.0444	0.0000	0.0000	0.0001
Plant ED	6	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
Fungicide x Extract	48	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
Plant ED + Fungicide	12	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
ED + Fungicide	12	0.0039	0.0000	0.0000	0.0000	0.0400	0.0000	0.0001	0.0001	0.0000
Plant ED + Fungicide	24	0.0013	0.0000	0.0000	0.0117	0.0001	0.0000	0.0000	0.0000	0.0000

[a]Extracts form three medicinal plant species.

[b]Significant values associated with the *F* tests.

*Extract dose per plant species tested.

Table 2-3. Inhibition of mycelium growth and synergy ratio (SR) between fungicide Sporekill™ (didecyl dimethyl ammonium chloride) and *Galenia africana* (Ga), *Elytropappus rhinocerotis* (Er), *Tulbaghia violacea* (Tv) plant extract doses against the PPRI 8507 strain of *Botrytis cinerea in vitro*.

Synergy Ratio[b]	Plant Species	Plant Extract Dose (mg mL^{-1})	Mycelium Growth Inhibition (%)[a]				
			Sporekill™ Dose (mL L^{-1})				
			0.0	**0.1**	**0.2**	**0.4**	**0.8**
	Control		0	3.2	15.1	35.8	40.3
	Ga	**62.5**	34.4	54.7^{f}	46.5¶	61.2^{f}	65^{f}
SR			-	1.5 +	1.0	1.0	1.1 +
	Ga	**125**	55.9	56.3¶	45.2¶	45.2¶	68.3¶
SR				1.0	0.7	0.8	0.7
	Ga	**250**	80.4	69.1¶	65.3¶	71.9¶	70
SR			-	0.9	0.8	0.8	0.8
	Ga	**500**	91.3	89.7	80.6¶	86.2¶	88.2¶
SR			-	1.0	0.9	0.9	0.9
	Er	**50**	12.5	9.1¶	14	27.5	20.3¶
SR			-	0.6	0.5	0.6	0.4
	Er	**100**	26.6	9.44^{f}	13.8	30.3	49.3§
SR			-	0.3	0.4	0.6	0.9
	Er	**200**	32.7	29.2¶	25.6	40.1	54§
SR			-	0.8	0.6	0.6	0.9
	Er	**400**	36.3	46.4¶	29.5	41.5	58.8^{f}
SR			-	1.2+	0.6	0.7	0.9
	Tv	**62.5**	-0.1	20.5^{f}	11	51.7	23.7^{f}
SR			-	6.6+	0.7	1.4+	0.6
	Tv	**125**	-3.1	6.9	11.9	29§	10¶
SR			-	34.6+	1.0	0.9	0.3
	Tv	**250**	-14.5	-6.7	8.6§	42.1§	42.4§
SR			-	0.6	3.1+	1.6+	1.3+
	Tv	**500**	-11.9	14.9§	32^{f}	14.2^{f}	23.4^{f}
Sr			-	-1.8	6.4+	0.5	0.7

[a]SR>1=Synergistic (+); SR=1=Additive; SR<1=Antagonistic; [b]Synergy ratio between expected and observed mycelium growth (SR=Exp/Obs). [¶]Mycelium growth inhibition values of the plant extract and fungicide mixtures differ significantly ($p<0.05$) from the value of the fungicide dose in the column. [§]Mycelium growth inhibition values of the plant extract and fungicide mixtures differ significantly ($p<0.05$) from the value of the plant extract doses without fungicide in the column. [f]Mycelium growth inhibition values of the plant extract and fungicide differ significantly ($p<0.05$) from the value of both the fungicide and plant extract doses in the column.

For the *B*. *cinerea* strain PPRI 8506 the 16 mixtures between Sporekill™ and extracts of *G*. *africana*, *E*. *rhinocerotis* and *T*. *violacea* resulted in 16, 14 and 8 mixtures, respectively, exhibiting significantly higher inhibition levels compared to Sporekill™ alone, while 6, 4 and 10 mixtures shows significantly higher inhibition levels compared to the respective extract alone (Table 2-4). In the mixtures with Sporekill™ at concentrations of 0.1, 0.2 and 0.4 mg mL^{-1}, 5 of *G*. *africana*, 3 of *E*. *rhinocerotis* and 5 of *T*. *violacea* concentrations produced synergistic interactions (Table 2-4). Of the 16 mixtures of Sporekill™ with *G*. *africana*, *E*. *rhinocerotis* and *T*. *violacea* 16, 12 and 9 mixtures showed excellent efficacy against PPRI 7338 (Table 2-5) compared to Sporekill™ alone. In this case, 12, 13 and 15 mixtures produced significantly higher inhibition levels than the extract alone. Combinations of Sporekill with *G*. *africana*, *E*. *rhinocerotis* and *T*. *violacea* resulted in 6, 2 and 9 mixtures, respectively, with good synergistic interactions (Table 2-5).

Radial growth inhibition of strain PPRI 8507 (Table 2-6) with the combinations of Rovral™ with *G*. *africana*, *E*. *rhinocerotis* and *T*. *violacea* resulted in 6, 5 and 6 mixtures that produced statistically similar effects reported for the fungicide. In the mixtures with Rovral™, 1 of *G*. *africana*, 1 of *E*. *rhinocerotis* and 5 of *T*. *violacea* produced synergistic interactions (Table 6). However, strain PPRI 8506 (Table 2-7), was less sensitive to Rovral™ and extracts from the respective plant species alone compared to strain PPRI 8507. The combinations of Rovral™ with *G*. *africana*, *E*. *rhinocerotis* and *T*. *violacea*, showed significantly higher radial growth inhibition to that observed for the fungicide.

Combination of Rovral™ with the plant extracts resulted in 18 mixtures with synergistic interaction (Table 2-7). Similar inhibitory responses were demonstrated for strain PPRI 7338 (Table 2-8). The combination of Rovral™ and plant extract produced 8 treatments of *G*. *africana*, 8 of *E*. *rhinocerotis* and 6 of *T*. *violacea* with synergistic effects against strain PPRI 7338 (Table 2-8). The data show that the level of inhibition by

Rovral™ and the plant extract doses alone was not as high as that of the mixtures of both products on all 3 wild-type strains.

Mixtures of Terminator™ and plant extracts were effective in inhibiting radial growth of PPRI 8507 (Table 2-9). Of the 16 mixtures tested for the respective plant species, 9 of *G. africana*, 1 of *E. rhinocerotis* and 1 of *T. violacea* resulted in statistically higher activity than the fungicide, since Terminator™ had a relatively low inhibitory effect on PPRI 8507. Terminator™ at 0.4 mL L^{-1} showed synergistic activity for strain PPRI 8507 when combined with *T. violacea* at 125 mg L^{-1} (Table 2-9). Out of the 16 mixtures of Terminator™ and *G. africana* tested, 9 exhibited significantly higher inhibition levels against PPRI 8506 (Table 2-10), compared to Terminator™ alone. Only 2 of the 16 mixtures tested, exhibited significantly higher inhibition levels than the plant extract alone.

For combinations with *E. rhinocerotis*, 6 and 11 mixtures produced significantly higher radial growth inhibition levels compared to Terminator™ and plant extract alone. Combinations with *T. violacea* resulted in 2 mixtures with significantly higher radial growth inhibition levels compared to the fungicide and plant extract alone, respectively. In the mixture with Terminator™ 1 of *G. africana* and 1 of *E. rhinocerotis* combinations produced synergistic interactions (Table 2-10). Of the mixtures of Terminator™ with *G. africana*, *E. rhinocerotis* and *T. violacea,* 9, 3 and 3 mixtures showed excellent efficacy against PPRI 7338 compare to Terminator™ alone (Table 2-11). In this case, 2, 12 and 14 mixtures resulted in significantly higher inhibition levels compared to the plant extract alone. Our results indicate that 5 mixtures of *E. rhinocerotis* and 1 of *T. violacea* were synergistic according to the synergy ratio calculation (Table 2-11). Combinations of Teldor™ with *G. africana* and *E. rhinocerotis* resulted in 8 and 4 mixtures producing significantly higher inhibition levels than the fungicide alone. In this case, 15, 11 and 16 mixtures produced significantly higher inhibition levels compare to the plant extracts

alone. Combination of Terminator™ with the plant extracts resulted in 5 mixtures with synergistic interactions (Table 2-12).

For the *B*. *cinerea* strain PPRI 8506, the 16 mixtures between Teldor™ and extracts of *G*. *africana*, *E*. *rhinocerotis* and *T*. *violacea* resulted in 7, 6 and 11 mixtures, respectively, with higher inhibition levels compared to Teldor™ alone, while 14, 16 and 16 mixtures shows significantly higher levels compared to the respective extract alone (Table 2-13). In this case, 14 of the combinations produced synergistic interactions. Combinations of Teldor™ and plant extracts were effective in inhibiting radial growth of PPRI 7338 (Table 2-14). Of the combinations, 4 of *G*. *africana*, 1 of *E*. *rhinocerotis* and 4 of *T*. *violacea* resulted in statistically higher inhibitory activity than the fungicide, while 13, 15 and 16 mixtures, respectively, showed higher inhibition levels than the plant extract alone. Teldor™ at 0.1 mL L^{-1} (0.05 g a.i.) showed synergistic interaction when combined with *T*. *violacea* at 125, 250 and 500 mg L^{-1}.

2.6 Discussion

In vitro inhibition studies of ethanolic extracts of medicinal plant species of *E*. *rhinocerotis* and *T*. *violacea* exhibit weak antifungal properties against *B*. *cinerea* strains. The strongest antifungal activity observed was with the single plant extract of *G*. *africana* at 250 and 500 mg mL^{-1} doses. However, when the plant extracts were combined with the fungicides significant inhibition in radial growth of the fungal strains was observed for the combinations, especially at lower concentrations of the fungicides. Synergistic interaction of plant extracts and fungicides in various combinations was based on the Abbott formula (Abbott, 1925) as described by Gisi (1996). Our results indicate that combinations of Sporekill™ either with *G*. *africana*, *E*. *rhinocerotis* or *T*. *violacea* had synergistic effects.

Table 2-4. Inhibition of mycelium growth and synergy ratio (SR) between fungicide Sporekill™ (didecyl dimethyl ammonium chloride) and *Galenia africana* (Ga), *Elytropappus rhinocerotis* (Er), *Tulbaghia violacea* (Tv) plant extract doses against the PPRI 8506 strain of *Botrytis cinerea in vitro*.

Synergy Ratio[b]	Plant Species	Plant Extract Dose (mg mL^{-1})	Mycelium Growth Inhibition (%)[a]				
			Sporekill™ Dose (mL L^{-1})				
			0.0	**0.1**	**0.2**	**0.4**	**0.8**
	Control		0	4.3	24.3	28.4	46.8
	Ga	**62.5**	65.7	54.2¶	63.6¶	73.7¶	66.7¶
SR			-	0.8	0.9	1.0	0.8
	Ga	**125**	73.8	80.8¶	40.7*f*	73.2¶	83.3*f*
SR			-	1.1+	0.5	0.9	1.0
	Ga	**250**	62.8	78.4§	77.2*f*	84.5*f*	80.9*f*
SR			-	1.2+	1.1+	1.2+	1.0
	Ga	**500**	82.6	89.1	90.4	85.3	92.4
SR			-	1.1+	1.0	1.0	1.0
	Er	**50**	25.8	33.6¶	23.4	54.8*f*	26.4¶
SR			-	1.2+	0.5	1.2+	0.4
	Er	**100**	28.1	37.8¶	38.6¶	34.0	62.7*f*
SR			-	1.2+	0.8	0.7	1.0
	Er	**200**	39.2	41.2¶	43.1¶	44.3¶	64.1*f*
SR			-	1.0	0.8	0.8	0.9
	Er	**400**	43.9	43¶	45.8¶	49.5¶	58.7§
SR			-	0.9	0.8	0.8	0.8
	Tv	**62.5**	3.2	29.7*f*	1.6¶	51.7*f*	43.1§
SR			-	4.0+	0.1	1.7+	0.9
	Tv	**125**	4.5	18.6*f*	14.4	31.7§	30.2*f*
SR			-	2.2+	0.5	1.0	0.6
	Tv	**250**	-5.5	5.0	-2.5¶	37.8§	50.1§
SR			-	-5.2	-0.1	1.5+	1.1+
	Tv	**500**	-4.8	9.5§	5.3¶	7¶	14.5*f*
Sr			-	-32.4	0.3	0.3	0.3

[a]SR>1=Synergistic (+); SR=1=Additive; SR<1=Antagonistic; [b]Synergy ratio between expected and observed mycelium growth (SR=Exp/Obs). ¶Mycelium growth inhibition values of the plant extract and fungicide mixtures differ significantly ($p<0.05$) from the value of the fungicide dose in the column. §Mycelium growth inhibition values of the plant extract and fungicide mixtures differ significantly ($p<0.05$) from the value of the plant extract doses without fungicide in the column. *f*Mycelium growth inhibition values of the plant extract and fungicide differ significantly ($p<0.05$) from the value of both the fungicide and plant extract doses in the column.

Table 2-5. Inhibition of mycelium growth and synergy ratio (SR) between fungicide Sporekill™ (didecyl dimethyl ammonium chloride) and *Galenia africana* (Ga), *Elytropappus rhinocerotis* (Er), *Tulbaghia violacea* (Tv) plant extract doses against the PPRI 7338 strain of *Botrytis cinerea in vitro*.

Synergy Ratio[b]	Plant Species	Plant Extract Dose (mg mL^{-1})	Mycelium Growth Inhibition (%)[a]				
			Sporekill™ Dose (mL L^{-1})				
			0.0	**0.1**	**0.2**	**0.4**	**0.8**
	Control		0	19.8	26.6	25.4	31.5
	Ga	**62.5**	56.4	57.9¶	50.3¶	71.3*f*	80.1*f*
SR			-	0.9	0.7	1.1+	1.1+
	Ga	**125**	66.7	81.1§	70.2¶	75.8¶	85.1*f*
SR			-	1.1+	0.8	1.0	1.1+
	Ga	**250**	83.3	81.2	85.6¶	97¶	79.1¶
SR			-	0.9	1.0	1.1+	0.9
	Ga	**500**	86.2	88.3	84.5¶	89.6	100*f*
SR			-	1.0	0.9	1.0	1.1+
	Er	**50**	9	24§	6.3¶	48.8¶	8.7¶
SR			-	0.9	0.2	1.5+	0.2
	Er	**100**	18.4	12.4	21.8	29.2	38.1¶
SR			-	0.4	0.5	0.7	0.9
	Er	**200**	15.9	21.1	36.5§	35.5§	56.8*f*
SR			-	0.6	1.0	1.0	1.3+
	Er	**400**	34.2	38.5¶	37.6	43.7¶	55*f*
SR			-	0.8	0.7	0.7	1.0
	Tv	**62.5**	-0.9	26.3§	11.6¶	43.1*f*	52.6¶
SR			-	1.4+	0.4	1.7+	1.7+
	Tv	**125**	6.2	5.4¶	20.5§	50.3¶	40.7§
SR			-	0.2	0.7	1.7+	1.1+
	Tv	**250**	-6.4	7.3§	8.2*f*	39.3*f*	65.6*f*
SR			-	0.5	0.4	1.9+	2.4+
	Tv	**500**	0.1	24.8§	8.3¶	17.7§	26.1§
Sr			-	1.2+	10.3+	0.7	0.8

[a]SR>1=Synergistic (+); SR=1=Additive; SR<1=Antagonistic; [b]Synergy ratio between expected and observed mycelium growth (SR=Exp/Obs). ¶Mycelium growth inhibition values of the plant extract and fungicide mixtures differ significantly ($p<0.05$) from the value of the fungicide dose in the column. §Mycelium growth inhibition values of the plant extract and fungicide mixtures differ significantly ($p<0.05$) from the value of the plant extract doses without fungicide in the column. *f*Mycelium growth inhibition values of the plant extract and fungicide differ significantly ($p<0.05$) from the value of both the fungicide and plant extract doses in the column.

Table 2-6. Inhibition of mycelium growth and synergy ratio (SR) between fungicide Rovral™ (iprodione) and *Galenia africana* (Ga), *Elytropappus rhinocerotis* (Er), *Tulbaghia violacea* (Tv) plant extract doses against the PPRI 8507 strain of *Botrytis cinerea in vitro*.

Synergy Ratio[b]	Plant Species	Plant Extract Dose (mg mL^{-1})	Mycelium Growth Inhibition (%)[a]				
			Rovral™ Dose (mL L^{-1})				
			0.0	**0.1**	**0.2**	**0.4**	**0.8**
	Control		0	51	73.9	100	100
	Ga	**62.5**	40.4	76^{f}	75.1§	70.3§	86.3§
SR			-	1.1+	0.9	0.7	0.9
	Ga	**125**	59.9	53.7	73.5	86.4§	90.2§
SR			-	0.7	0.8	0.9	0.9
	Ga	**250**	82.3	67	87.4	93.2	78.3¶
SR			-	0.7	0.9	0.9	0.8
	Ga	**500**	92.1	92.4¶	100¶	78.1	77.9¶
SR			-	1.0	1.0	0.8	0.8
	Er	**50**	20.5	63.3§	100^{f}	100§	100§
SR			-	1.0	1.3+	1.0	1.0
	Er	**100**	33.3	50.3	81.5§	100§	100§
SR			-	0.7	1.0	1.0	1.0
	Er	**200**	38.8	49.3	78.9§	100§	96.3§
SR			-	0.7	0.9	1.0	1.0
	Er	**400**	42.1	59.8§	70.2§	55.4¶	65.9^{f}
SR			-	0.8	0.8	0.6	0.7
	Tv	**62.5**	-15.5	71.5§	78.5§	79.8¶	90.6§
SR			-	1.6+	1.1+	0.8	0.9
	Tv	**125**	-15.6	63.3§	51.9§	80.4^{f}	100§
SR			-	1.5+	0.8	0.8	1.0
	Tv	**250**	-6.4	67.5§	64.3§	100§	81.2§
SR			-	1.4+	0.9	1.0	0.8
	Tv	**500**	-7	54.1§	64§	90.6§	86§
Sr			-	1.1+	0.9	0.9	0.9

[a]SR>1=Synergistic (+); SR=1=Additive; SR<1=Antagonistic; [b]Synergy ratio between expected and observed mycelium growth (SR=Exp/Obs). [¶]Mycelium growth inhibition values of the plant extract and fungicide mixtures differ significantly ($p<0.05$) from the value of the fungicide dose in the column. [§]Mycelium growth inhibition values of the plant extract and fungicide mixtures differ significantly ($p<0.05$) from the value of the plant extract doses without fungicide in the column. [f]Mycelium growth inhibition values of the plant extract and fungicide differ significantly ($p<0.05$) from the value of both the fungicide and plant extract doses in the column.

Table 2-7. Inhibition of mycelium growth and synergy ratio (SR) between fungicide Rovral™ (iprodione) and *Galenia africana* (Ga), *Elytropappus rhinocerotis* (Er), *Tulbaghia violacea* (Tv) plant extract doses against the PPRI 8506 strain of *Botrytis cinerea in vitro*.

Synergy Ratio[b]	Plant Species	Plant Extract Dose (mg mL^{-1})	Mycelium Growth Inhibition (%)[a]				
			Rovral™ Dose (mL L^{-1})				
			0.0	**0.1**	**0.2**	**0.4**	**0.8**
	Control		0	-31.9	50.6	49.6	78.6
	Ga	**62.5**	40.6	41.9[¶]	27.4	44.7	53.2
SR			-	1.9+	0.4	0.6	0.6
	Ga	**125**	54.6	56.2[¶]	56.7	51.8	58.2
SR			-	1.4+	0.7	0.7	0.6
	Ga	**250**	35.5	53.4[¶]	56.1	60.7	53.6
SR			-	3.6+	0.8	0.9	0.6
	Ga	**500**	69.8	83.1[¶]	69.2	73	70.4
SR			-	1.4+	0.8	0.9	0.8
	Er	**50**	-28.5	40.1[*f*]	73.5[§]	88.8[*f*]	100[¶]
SR			-	-0.6	2.0+	2.5+	1.4+
	Er	**100**	-24.7	20.5[*f*]	37.1[§]	100[*f*]	87.3[§]
SR			-	-0.3	1.0	2.7+	1.2+
	Er	**200**	-5.4	16.4[¶]	59[§]	70.7[§]	87[§]
SR			-	-0.4	1.2+	1.5+	1.1+
	Er	**400**	2.8	27[¶]	44.4[§]	18.1[¶]	47.3[*f*]
SR			-	-1.0	0.9	0.4	0.6
	Tv	**62.5**	-105.5	30.5[*f*]	33.8[§]	70.2[§]	71.5[§]
SR			-	-0.2	-22.4	-19.7	1.3+
	Tv	**125**	-101.7	22.2[*f*]	32.6[§]	43.3[§]	100[§]
SR			-	-0.1	90.5+	-26.1	1.8+
	Tv	**250**	-103.3	56.6[*f*]	52.6[§]	100[*f*]	100[§]
SR			-	-0.3	-122.3	-40.6	1.8+
	Tv	**500**	-101.3	-28.3[§]	32.7[§]	51.6[§]	89.9[§]
Sr			-	0.2	58.6+	-35.5	1.6+

[a]SR>1=Synergistic (+); SR=1=Additive; SR<1=Antagonistic; [b]Synergy ratio between expected and observed mycelium growth (SR=Exp/Obs). [¶]Mycelium growth inhibition values of the plant extract and fungicide mixtures differ significantly ($p<0.05$) from the value of the fungicide dose in the column. [§]Mycelium growth inhibition values of the plant extract and fungicide mixtures differ significantly ($p<0.05$) from the value of the plant extract doses without fungicide in the column. [*f*]Mycelium growth inhibition values of the plant extract and fungicide differ significantly ($p<0.05$) from the value of both the fungicide and plant extract doses in the column.

Table 2-8. Inhibition of mycelium growth and synergy ratio (SR) between fungicide Rovral™ (iprodione) and *Galenia africana* (Ga), *Elytropappus rhinocerotis* (Er), *Tulbaghia violacea* (Tv) plant extract doses against the PPRI 7338 strain of *Botrytis cinerea in vitro*.

Synergy Ratio[b]	Plant Species	Plant Extract Dose (mg mL^{-1})	Mycelium Growth Inhibition (%)[a]				
			Rovral™ Dose (mL L^{-1})				
			0.0	**0.1**	**0.2**	**0.4**	**0.8**
	Control		0	-18.8	62.7	58.2	100
	Ga	**62.5**	41.8	36.8¶	57.2	59.9	66.9*f*
SR			-	1.2+	0.7	0.8	0.7
	Ga	**125**	55.5	43.6¶	61.8	69	67.7¶
SR			-	0.9	0.7	0.8	0.7
	Ga	**250**	77.7	31.6*f*	62.3	56.5§	74.9¶
SR			-	0.4	0.7	0.6	0.7
	Ga	**500**	81.6	87.7¶	89.4¶	74.3	87.3
SR			-	1.1+	1.0	0.8	0.9
	Er	**50**	-21.6	50.9*f*	60.1§	60.4§	100
SR			-	-1.1	1.1+	1.2+	1.0
	Er	**100**	-9.1	29.5*f*	43.4§	88*f*	100
SR			-	-1.0	0.7	1.6+	1.0
	Er	**200**	-12.3	25.8*f*	63§	100*f*	100
SR			-	-0.8	1.1+	1.9+	1.0
	Er	**400**	12.1	37.7*f*	53.1§	16.6¶	34¶
SR			-	-8.5	0.8	0.3	0.3
	Tv	**62.5**	-59.9	48.3*f*	58.2§	43.3§	100§
SR			-	-0.5	1.4+	1.3+	1.0
	Tv	**125**	-61.1	26.6*f*	43.6§	63§	84.9§
SR			-	-0.3	1.1+	1.9+	0.8
	Tv	**250**	-62	54.2*f*	52.7§	83*f*	89.5§
SR			-	-0.6	1.3+	2.6+	0.9
	Tv	**500**	-15.4	-0.7§	45§	58.7§	80.7§
Sr			-	0	0.8	1.1+	0.8

[a]SR>1=Synergistic (+); SR=1=Additive; SR<1=Antagonistic; [b]Synergy ratio between expected and observed mycelium growth (SR=Exp/Obs). ¶Mycelium growth inhibition values of the plant extract and fungicide mixtures differ significantly ($p<0.05$) from the value of the fungicide dose in the column. §Mycelium growth inhibition values of the plant extract and fungicide mixtures differ significantly ($p<0.05$) from the value of the plant extract doses without fungicide in the column. *f*Mycelium growth inhibition values of the plant extract and fungicide differ significantly ($p<0.05$) from the value of both the fungicide and plant extract doses in the column.

Table 2-9. Inhibition of mycelium growth and synergy ratio (SR) between fungicide Terminator™ (dimethyl didecyl ammonium chloride) and *Galenia africana* (Ga), *Elytropappus rhinocerotis* (Er), *Tulbaghia violacea* (Tv) plant extract doses against the PPRI 8507 strain of *Botrytis cinerea in vitro*.

Synergy Ratio[b]	Plant Species	Plant Extract Dose (mg mL^{-1})	Mycelium Growth Inhibition (%)[a]				
			Terminator™ Dose (mL L^{-1})				
			0.0	**0.1**	**0.2**	**0.4**	**0.8**
	Control		0	57.5	41.9	47.5	61
	Ga	**62.5**	40.4	37.7	-3.4^{f}	52	39.1¶
SR			-	0.5	-0.1	0.8	0.5
	Ga	**125**	59.9	35.5^{f}	43.3	47.6	1.3¶
SR			-	0.4	0.6	0.6	0
	Ga	**250**	82.3	49.3§	59§	60.5§	51.7¶
SR			-	0.5	0.7	0.7	0.6
	Ga	**500**	92.1	63.8§	70.1^{f}	86.3§	65.2§
SR			-	0.7	0.7	0.9	0.7
	Er	**50**	20.5	21.5¶	-1.3^{f}	51.3§	52.5§
SR			-	0.3	0	0.9	0.8
	Er	**100**	33.3	-2.1^{f}	1.9^{f}	18.1¶	30.9¶
SR			-	0	0	0.3	0.4
	Er	**200**	38.8	53.1	28.2	30	39.7¶
SR			-	0.7	0.4	0.4	0.5
	Er	**400**	42.1	33.7¶	29.9	25.6¶	36.3¶
SR			-	0.4	0.5	0.4	0.5
	Tv	**62.5**	3.1	29.2^{f}	42.2§	20.5¶	5.5¶
SR			-	0.5	1.0	0.4	0.1
	Tv	**125**	-7	27.2^{f}	27.8§	56§	3.8¶
SR			-	0.5	0.7	1.3+	0.1
	Tv	**250**	-7.5	23.8^{f}	18.5^{f}	1.0¶	3.5¶
SR			-	0.4	0.5	0	0.1
	Tv	**500**	6.5	31.2^{f}	-0.7¶	-1.0¶	18.4¶
Sr			-	0.5	0	0	0.3

[a]SR>1=Synergistic (+); SR=1=Additive; SR<1=Antagonistic; [b]Synergy ratio between expected and observed mycelium growth (SR=Exp/Obs). [¶]Mycelium growth inhibition values of the plant extract and fungicide mixtures differ significantly ($p<0.05$) from the value of the fungicide dose in the column. [§]Mycelium growth inhibition values of the plant extract and fungicide mixtures differ significantly ($p<0.05$) from the value of the plant extract doses without fungicide in the column. [f]Mycelium growth inhibition values of the plant extract and fungicide differ significantly ($p<0.05$) from the value of both the fungicide and plant extract doses in the column.

Table 2-10. Inhibition of mycelium growth and synergy ratio (SR) between fungicide Terminator™ (dimethyl didecyl ammonium chloride) and *Galenia africana* (Ga), *Elytropappus rhinocerotis* (Er), *Tulbaghia violacea* (Tv) plant extract doses against the PPRI 8506 strain of *Botrytis cinerea in vitro*.

Synergy Ratio[b]	Plant Species	Plant Extract Dose (mg mL^{-1})	Mycelium Growth Inhibition (%)[a]				
			Terminator™ Dose (mL L^{-1})				
			0.0	0.1	0.2	0.4	0.8
	Control		0	33.5	33.3	-9.9	-1.9
	Ga	62.5	40.8	13.8[§]	-36.4	17.7[¶]	-6.8[§]
SR			-	0.2	-0.6	0.5	-0.2
	Ga	125	54.6	65.6[¶]	-30	13.9[§]	30.8[¶]
SR			-	0.9	-0.4	0.3	0.6
	Ga	250	35.5	28.5	33.7	17.7[¶]	14.9
SR			-	0.5	0.6	0.6	0.4
	Ga	500	69.8	55.6	63.2[¶]	57.7[¶]	85.9[¶]
SR			-	0.7	0.8	0.9	1.9+
	Er	50	-28.8	0.1[f]	-14.4[¶]	23.5[f]	3.3[§]
SR			-	0	-1.0	-0.6	-0.1
	Er	100	-24.7	-13.7[¶]	-9.5[¶]	4.2[§]	5.1[§]
SR			-	-0.8	-0.6	-0.1	-0.2
	Er	200	-5.4	6.8[¶]	22.7[¶]	-9.7	7.7
SR			-	0.2	0.8	0.6	-1.0
	Er	400	2.8	24.2	10.1	8.2	5.4
SR			-	0.7	0.3	-1.2	5.7+
	Tv	62.5	-42.3	-44.9	-40.9	-20	7.6[§]
SR			-	-8.4	-8	0.4	-0.2
	Tv	125	-50.3	-55.5	-18.9[f]	44	-10.2[§]
SR			-	-1099	75.6+	-0.7	0.2
	Tv	250	-406	-52.7	-31.7	-24.5[¶]	-39.5[¶]
SR			-	-8.1	-5.1	0.4	0.9
	Tv	500	-27.9	-21.3[¶]	-38.4	-34	15.6
Sr			-	-1.4	-2.6	0.8	-0.5

[a]SR>1=Synergistic (+); SR=1=Additive; SR<1=Antagonistic; [b]Synergy ratio between expected and observed mycelium growth (SR=Exp/Obs). [¶]Mycelium growth inhibition values of the plant extract and fungicide mixtures differ significantly ($p<0.05$) from the value of the fungicide dose in the column. [§]Mycelium growth inhibition values of the plant extract and fungicide mixtures differ significantly ($p<0.05$) from the value of the plant extract doses without fungicide in the column. [f]Mycelium growth inhibition values of the plant extract and fungicide differ significantly ($p<0.05$) from the value of both the fungicide and plant extract doses in the column.

Table 2-11. Inhibition of mycelium growth and synergy ratio (SR) between fungicide Terminator™ (dimethyl didecyl ammonium chloride) and *Galenia africana* (Ga), *Elytropappus rhinocerotis* (Er), *Tulbaghia violacea* (Tv) plant extract doses against the PPRI 7338 strain of *Botrytis cinerea in vitro*.

Synergy Ratio[b]	Plant Species	Plant Extract Dose (mg mL^{-1})	Mycelium Growth Inhibition (%)[a]				
			Terminator™ Dose (mL L^{-1})				
			0.0	**0.1**	**0.2**	**0.4**	**0.8**
	Control		0	45.4	29.6	52.1	44.5
	Ga	**62.5**	41.8	41.8	15.9[§]	37.9	46.1
SR			-	0.7	0.3	0.5	0.7
	Ga	**125**	55.5	56	35	47.7	33.9
SR			-	0.7	0.5	0.6	0.5
	Ga	**250**	77.7	65.1	56.6	70.5	60.1
SR			-	0.7	0.7	0.8	0.7
	Ga	**500**	81.6	61	66.2	81.9[¶]	75.5[¶]
SR			-	0.7	0.8	0.9	0.8
	Er	**50**	-21.6	27.3[§]	-4.4[¶]	53.1	50.1
SR			-	0.8	-0.3	1.3+	1.5+
	Er	**100**	-9.1	-1.7	-11.1[¶]	31.8	33.3[§]
SR			-	0	-0.5	0.7	0.8
	Er	**200**	-12.3	42.2[§]	26.1[§]	25.9[¶]	42.1[§]
SR			-	1.1+	1.2+	0.6	1.1+
	Er	**400**	12.1	20[¶]	23.4	-22.1	48.4
SR			-	0.4	0.6	-0.4	0.9
	Tv	**62.5**	-24.4	24.4[§]	67.7[¶]	33[§]	-44.5
SR			-	0.8	5.4+	0.8	-1.4
	Tv	**125**	8.7	16.8[¶]	34.8	58.8	36.3[§]
SR			-	0.3	1.0	1.0	0.7
	Tv	**250**	3.4	33.4[§]	7.5	41.3[§]	22.4
SR			-	0.7	0.2	0.8	0.5
	Tv	**500**	20.2	21.2	28.1	-57.8[§]	53.5
Sr			-	0.4	0.6	-0.9	1.0

[a]SR>1=Synergistic (+); SR=1=Additive; SR<1=Antagonistic; [b]Synergy ratio between expected and observed mycelium growth (SR=Exp/Obs). [¶]Mycelium growth inhibition values of the plant extract and fungicide mixtures differ significantly ($p<0.05$) from the value of the fungicide dose in the column. [§]Mycelium growth inhibition values of the plant extract and fungicide mixtures differ significantly ($p<0.05$) from the value of the plant extract doses without fungicide in the column. [ƒ]Mycelium growth inhibition values of the plant extract and fungicide differ significantly ($p<0.05$) from the value of both the fungicide and plant extract doses in the column.

Table 2-12. Inhibition of mycelium growth and synergy ratio (SR) between fungicide Teldor™ (fenhexamid) and *Galenia africana* (Ga), *Elytropappus rhinocerotis* (Er), *Tulbaghia violacea* (Tv) plant extract doses against the PPRI 8507 strain of *Botrytis cinerea in vitro*.

Synergy Ratio[b]	Plant Species	Plant Extract Dose (mg mL^{-1})	Mycelium Growth Inhibition (%)[a]				
			Teldor™ Dose (mL L^{-1})				
			0.0	**0.1**	**0.2**	**0.4**	**0.8**
	Control		0	65	97.7	91	100
	Ga	**62.5**	76.9	70.7	77.2	92	100
SR			-	0.8	0.8	0.9	1.0
	Ga	**125**	41.5	75.6	93.3§	97.4§	100§
SR			-	1.0	0.9	1.0	1.0
	Ga	**250**	44.4	92.6	69.5	96.8§	93.8
SR			-	1.1+	0.7	1.0	0.9
	Ga	**500**	58.8	100	76.5	100	100
SR			-	1.2+	0.8	1.0	1.0
	Er	**50**	1.6	83.3§	93.9§	-29.6¶	45.7*f*
SR			-	1.3+	1.0	-0.3	0.5
	Er	**100**	26.1	58.5	98.1§	29.9¶	44.4
SR			-	0.8	1.0	0.3	0.4
	Er	**200**	54	100	71.1	37.3¶	48.2¶
SR			-	1.2+	0.7	0.4	0.5
	Er	**400**	59.5	100	61.4	50.2	40.5¶
SR			-	1.2+	0.6	0.5	0.4
	Tv	**62.5**	31.8	40.8	32.5¶	69.5	96.1§
SR			-	0.5	0.3	0.7	1.0
	Tv	**125**	-10.9	41.5§	41.5*f*	58.8§	100§
SR			-	0.7	0.4	0.7	1.0
	Tv	**250**	10.9	38.3	28.6¶	41.8	96.8§
SR			-	0.6	0.3	0.5	1.0
	Tv	**500**	17.4	36	53.7	88.4§	100§
Sr			-	0.5	0.5	1.0	1.0

[a]SR>1=Synergistic (+); SR=1=Additive; SR<1=Antagonistic; [b]Synergy ratio between expected and observed mycelium growth (SR=Exp/Obs). ¶Mycelium growth inhibition values of the plant extract and fungicide mixtures differ significantly ($p<0.05$) from the value of the fungicide dose in the column. §Mycelium growth inhibition values of the plant extract and fungicide mixtures differ significantly ($p<0.05$) from the value of the plant extract doses without fungicide in the column. *f*Mycelium growth inhibition values of the plant extract and fungicide differ significantly ($p<0.05$) from the value of both the fungicide and plant extract doses in the column.

Table 2-13. Inhibition of mycelium growth and synergy ratio (SR) between fungicide Teldor™ (fenhexamid) and *Galenia africana* (Ga), *Elytropappus rhinocerotis* (Er), *Tulbaghia violacea* (Tv) plant extract doses against the PPRI 8506 strain of *Botrytis cinerea in vitro*.

Synergy Ratio[b]	Plant Species	Plant Extract Dose (mg mL^{-1})	Mycelium Growth Inhibition (%)[a]				
			Teldor™ Dose (mL L^{-1})				
			0.0	**0.1**	**0.2**	**0.4**	**0.8**
	Control		0	25.5	49.7	94.5	100
	Ga	**62.5**	-2	92.4^{f}	69.6§	79.6§	100
SR			-	3.8+	1.4+	0.8	1.0
	Ga	**125**	27.2	100^{f}	40.2	94.8§	100§
SR			-	2.2+	0.6	1.0	1.0
	Ga	**250**	24.8	57.4	57.6	57.4	100§
SR			-	1.3+	0.9	0.6	1.0
	Ga	**500**	84.9	71.1	80.3	91.3	100
SR			-	0.8	0.9	0.9	1.0
	Er	**50**	6	81^{f}	53.4§	87.1§	100
SR			-	2.7+	1.0	0.9	1.0
	Er	**100**	13.2	83.4^{f}	36.4	79.5§	100§
SR			-	2.4+	0.6	0.8	1.0
	Er	**200**	0.8	15.6	66.5	81.1	100
SR			-	0.6	1.3+	0.9	1.0
	Er	**400**	27.4	49.8	84.5¶	100	100
SR			-	1.1+	1.3+	1.0	1.0
	Tv	**62.5**	-76.4	89.5^{f}	91.9§	100§	100§
SR			-	-2.8	8.2+	1.1+	1.0
	Tv	**125**	-110.6	67.2§	96.3^{f}	100§	100§
SR			-	-1.2	-16.2	1.1+	1.0
	Tv	**250**	-61.5	73.8^{f}	94.2§	100§	100§
SR			-	-3.7	5.0+	1.1+	1.0
	Tv	**500**	-63.8	82.6^{f}	90.5§	91.9§	-30.5¶
Sr			-	-3.7	5.1+	1.0	-0.3

[a]SR>1=Synergistic (+); SR=1=Additive; SR<1=Antagonistic; [b]Synergy ratio between expected and observed mycelium growth (SR=Exp/Obs). [¶]Mycelium growth inhibition values of the plant extract and fungicide mixtures differ significantly ($p<0.05$) from the value of the fungicide dose in the column. [§]Mycelium growth inhibition values of the plant extract and fungicide mixtures differ significantly ($p<0.05$) from the value of the plant extract doses without fungicide in the column. [f]Mycelium growth inhibition values of the plant extract and fungicide differ significantly ($p<0.05$) from the value of both the fungicide and plant extract doses in the column.

Table 2-14. Inhibition of mycelium growth and synergy ratio (SR) between fungicide Teldor™ (fenhexamid) and *Galenia africana* (Ga), *Elytropappus rhinocerotis* (Er), *Tulbaghia violacea* (Tv) plant extract doses against the PPRI 7338 strain of *Botrytis cinerea in vitro*.

Synergy Ratio[b]	Plant Species	Plant Extract Dose (mg mL^{-1})	Mycelium Growth Inhibition (%)[a]				
			Teldor™ Dose (mL L^{-1})				
			0.0	**0.1**	**0.2**	**0.4**	**0.8**
	Control		0	69.6	94.6	99.2	100
	Ga	**62.5**	48.4	67.1[§]	55.4[¶]	88.4[§]	100[§]
SR			-	0.8	0.6	0.9	1.0
	Ga	**125**	88	89.1[¶]	74.3[¶]	100	100
SR			-	0.9	0.7	1.0	1.0
	Ga	**250**	84.9	74.7[¶]	82.5	87.8	100
SR			-	0.8	0.8	0.9	1.0
	Ga	**500**	84.5	85.5	88.2	100	100
SR			-	0.9	0.9	1.0	1.0
	Er	**50**	21.6	75.1[§]	80.5[§]	96.7[§]	95.7[§]
SR			-	1.0	0.8	1.0	1.0
	Er	**100**	69.4	62.3	74.7[¶]	94.9[§]	95.9[§]
SR			-	0.7	0.8	0.8	1.0
	Er	**200**	38.2	58.3[§]	63.6[*f*]	82.1[§]	87[§]
SR			-	0.7	0.7	0.8	0.9
	Er	**400**	49.8	61.7	75.6[*f*]	85[§]	90.3[§]
SR			-	0.7	0.8	0.9	0.9
	Tv	**62.5**	-4.8	66.1[§]	85.5[§]	71.6[*f*]	100[§]
SR			-	1.0	0.9	0.7	1.0
	Tv	**125**	2.5	92.9[*f*]	89.7	97.8[§]	100[§]
SR			-	1.3+	0.9	1.0	1.0
	Tv	**250**	0.1	82[§]	72.1[*f*]	74.8[*f*]	100[§]
SR			-	1.2+	0.8	0.8	1.0
	Tv	**500**	6.8	77.5[§]	73.9[*f*]	100[§]	100[§]
Sr			-	1.1+	0.8	1.0	1.0

[a]SR>1=Synergistic (+); SR=1=Additive; SR<1=Antagonistic; [b]Synergy ratio between expected and observed mycelium growth (SR=Exp/Obs). [¶]Mycelium growth inhibition values of the plant extract and fungicide mixtures differ significantly ($p<0.05$) from the value of the fungicide dose in the column. [§]Mycelium growth inhibition values of the plant extract and fungicide mixtures differ significantly ($p<0.05$) from the value of the plant extract doses without fungicide in the column. [*f*]Mycelium growth inhibition values of the plant extract and fungicide differ significantly ($p<0.05$) from the value of both the fungicide and plant extract doses in the column.

This study shows that the variations in inhibition and synergistic interactions between Sporekill™ and the plant extracts were influenced by the strain used. For instance, synergism between Sporekill™ and *G*. *africana* is more likely for strains PPRI 8506 and 7338 than for strain PPRI 8507. The efficacy of the Sporekill™ and *G*. *africana* combinations was reflected at relatively low doses of Sporekill™ to achieve inhibition above 70%. Synergy ratios of the combinations decreased with increasing amounts of Sporekill™.

The results showed that combinations of Rovral™ aquaflo with *G*. *africana*, *E*. *rhinocerotis* or *T*. *violacea* were more efficient in inhibiting strains of *B*. *cinerea* than the components alone. Combinations of Rovral™ with *E*. *rhinocerotis* or *T*. *violacea* exerted, in some cases, an inhibitory efficacy up to 100%. The relative increase in the efficacy of these combinations represents significant synergistic interaction between plant extracts and fungicides. Calculated interaction ratios ranged between 1.1 and 90.5.

The addition of Terminator™ to samples containing any dose of plant extract did not significantly increase the level of inhibition for any of *Botrytis* strain tested. In this case, according to the Abbott formula, the mixing of these components produced primarily antagonistic responses and a few showed synergism. The combination of Teldor™ with the plant extracts increased the efficacy to 100%. The effectiveness of combinations was reflected in the relatively low doses of Teldor™. This finding indicated that strong synergistic interactions occur between sublethal doses of Teldor™ and plant extracts.

Indigenous medicinal plants have played an important role in South Africa, as they are being used in the traditional treatment of various human diseases on an empirical basis (Hutchings *et al*., 1996). Our results confirm the statement made by Tegos *et al*. (2002) that plant species produced secondary metabolites that still have unknown functional assignments. “Synergy” is a popular concept in the field of herbal medicine, suggesting plant extracts contain compounds with potentiating functions (Duke and

Bogenschutz-Godwin, 1998). An important purpose for mixing plant extracts with fungicides could be to (i) provide broad antifungal coverage and (ii) to delay or reduce resistance. Stermitz *et al*. (2000 a, b) showed how two different components of the same medicinal plant, *Berberis fremontii,* can act in synergy with one compound disabling a resistance mechanism and potentiating the antibacterial activity of the antibiotic substance. *B*. *fremontii* makes an ineffective antibiotic, berberine, but when combined with 5'-methoxyhydnocarpin-D (5'-MHC), also produced by the same plant species, it becomes an effective antimicrobial agent. 5'-MHC has no antimicrobial activity on its own, but is a potent inhibitor of the NorA multidrug-resistant (MDR) pump (Tegos *et al*., 2002). Reporting on fungicide mixtures with synergistic action in practice is rather limited (Hayashi *et al*., 2003; Lorbeer, 1994).

Balancing fungicidal potency and improved performance with low environmental impact remains a challenge for fungicide research. Fungicides that give low or non-detectable residues in the crop are actively sought in research programs (Knight *et al*., 1997). Compounds are selected that rapidly degrade on plant surfaces, metabolize quickly in the plant, require use at very low rates, or act indirectly by promoting the plant's defense mechanism. The replacement of metalaxyl with its R-enantiomer, mefenoxam, which allows a 50% reduction in use rates, is an innovative example of how the goal of lower use rates might be achieved (Nunniger *et al*., 1996).

Natural plant defense compounds belonging to various chemical classes that act as constitutive or inducible chemical barriers, such as stilbenes, isoflavonoids, coumarins, and sesquiterpenes have been amply described (Osbourne, 1999), however, *B. cinerea* has been found to be able to withstand toxic effects to these compounds. It is commonly accepted that a significant part of phytochemical diversity serves to protect plants against microbial pathogens (Dixon, 2001). Despite a collection of antifungal and antibacterial compounds, plant products *per se* have not been used to any significant extent in the development of antimicrobial pesticides (Duke and Bogenschutz-Godwin, 1998), and a

few with simple structures are suitable for use as leads for chemical synthesis (Knight *et al*., 1997). Since chemical defenses are rather weak when extracted from plants and tested *in vitro,* the general approach is to chemically synthesize the compounds for greater efficacy.

Morel *et al*. (2003) found plant compounds that do not possess antibacterial activity themselves, but can potentiate known antibiotics by inhibiting microbial multidrug resistant (MDR) pumps. Modulators known to reduce MDRs in tumour cells synergized the fungitoxic activity of fungicide oxpoconazole, a sterol demethylation inhibitor against *B. cinerea* (Hayashi *et al*., 2003). Whether the potentiation effects in this study are due to inhibition of fungal MDR pumps require further studies at the molecular level. However, this type of inhibitory effect is likely to be advantageous for developing new fungicide formulations and application strategies with low toxicity effects on the environment. This approach not only makes it possible to reduce fungicide doses while maintaining adequate decay control, but also ensures a reduction of the chemical residue on the fruit. To the best of our knowledge, the synergy linking between these components is reported here for the first time. The suitable use of these products as commercial products requires having them further evaluated through *in vivo* studies.

2.7 References

Abbott, W.S., 1925. A method of computing effectiveness of an insecticide. J. Econ. Entomol. 18:265-267.

Arras, G., Scherm, B., Migheli, Q., 2002. Improving biocontrol activity of *Pichia guillermondii* against post-harvest decay of oranges in commercial packing-houses by reduced concentrations of fungicides. BioControl Sci. Technol. 12: 547-553.

Bashan, B., Levy, Y., Cohen, Y., 1991. Synergistic interactions between phenylamide fungicides and respiration inhibitors in controlling phenylamide-resistant populations of *Phytophthora infestans* on potato tuber disks. Phytoparasitica 19: 73-77.

Chand-Goyal, T., Spotts, R.A., 1997. Biological control of postharvest diseases of apple and pear under semi-commercial conditions using three saprophytic yeasts. Biol. Contr. 10: 199-206.

De Waard, M. A., Gisi, U., 1995. Synergism and antagonism in fungicides. Pages 565-578 in: Modern Selective Fungicides. 2nd ed. H. Lyr, ed. Gustav Fischer, Jena, Germany.

Dixon, R. A., 2001. Natural products and plant disease resistance. Nature 411: 843-847.

Droby, S., Porat, S., Wisniewski, M., El-Ghaouth, A., Wilson, C., 2002. Integrated control of postharvest decay using yeast antagonists, hot water and natural materials. Book of Abstract 7th meeting of the WG 'Biological control of fungal and bacterial plant pathogens', Kusadasi (Turkey), 22-25 May, p.25.

Droby, S., Wisniewski, M., El-Ghaouth, A., Wilson, C., 2003. Biological control of postharvest diseases of fruit and vegetables: current achievements and future challenges. Acta Hort. 628:703-713.

Droby, S., 2006. Improving quality and safety of fresh fruits and vegetables after harvest by the use of biocontrol agents and natural materials. Acta Hort. 709: 45-51.

Duke, J. A., Bogenschutz-Godwin, M. J., 1998. The synergy principle at work in plants, pathogens, insects, herbivores, and humans (CRC, Boca Raton, FL).

Gisi, U., Binder, H., Rimbach, E., 1985. Synergistic interactions of fungicides with different modes of action. Trans. Br. Mycol. Soc. 85: 299-306.

Gisi, U., Harr, J., Sandmeier, R., Wiedmer, H., 1983. A new systemic oxazolidinone fungicide (SAN 371 F) against diseases caused by peronosporales. Meded. Fac. Landbouwwet. Gent. 48: 541-549.

Gisi, U. 1996. Synergistic interaction of fungicides in mixtures. Phytopathology 86: 1273-1278.

Hayashi, K., Schoonbeek, H., De Waard, M. A., 2003. Modulators of membrane drug transporters potentiate the activity of the DMI fungicide oxpoconazole against *Botrytis cinerea*. Pest Manag. Sci. 59: 294-302.

Hutchings, A., Scott,A. H., Lewis, G. and Cunningham. 1996. Zulu medicinal plants: an inventory. University of Natal Press, Scottsville, South Africa, pp.195-196.

Ippolito, A., Nigro, F., Romanazzi, G., Campanella, V., 1998. Field application of *Aureobasidium pullulans* against Botrytis storage rot of strawberries. In: Bertolini P, Sijmons PC, Guerzoni ME and Serra F (eds) Workshop on 'Non Conventional Methods for the Control of Post-harvest Disease and Microbiological Spoilage' (COST 914-COST 915), October 9-11, 1997 (pp 127-133) Bologna, Italy.

Ippolito, A., Schena, L., Pentimone, I., Nigro, F., 2005. Control of postharvest rots of sweet cherries by pre- and postharvest applications of *Aureobasidium pullulans* in combination with calcium chloride or sodium bicarbonate. Postharvest Biol. Technol. 36: 245-252.

Janisiewicz, W.J., Korsten, L., 2002. Biological control of postharvest diseases of fruits. Ann. Rev. Phytopathol. 40: 411-441.

Knight, S. C., Anthony, V. M., Brady, A. M., Greenland, A. J., Heany, S. P., Murray, D. C., Powell, K. A., Schultz, M. A., Spinks, C. A., Worthingtom, P. A. and Youle, D., 1997. Rationale and perspectives on the development of fungicides. Ann. Rev. Phytopathol. 35:349-372.

Levy, Y., Benderly, M., Cohen, Y., Gisi, U., Bassand, D., 1986. The joint action of fungicides in mixtures: Comparison of two methods for synergy calculation. EPPO Bull. 16:651-657.

Lorbeer, W. J., 1994. Introduction. Synergism, antagonism, and additive action of fungicide mixtures. Phytopathology 86:1261-1262.

Morel, C., Stermitz, F. R., Tegos, G., Lewis. K., 2003. Isoflavones as potentiators of antibacterial activity. J. Agric. Food Chem. 51:5677- 5679.

Nuninger, C., Watson, G., Leadbitter, N. and Ellgehausen, H. 1996. CGA 329451: Introduction of the enatiomeric form of the fungicide metalaxyl. Proc. Brighton Crop Prot. Con. Pests Dis. 1:4146.

Osbourne, A. E., 1999. Antimicrobial phytoprotectants and fungal pathogens: A commentary. Fungal Genet. Biol. 26:163-168.

Samoucha, Y., Cohen, Y., 1986. Efficacy of systemic and contact fungicide mixtures in controlling late blight in potatoes. Phytopathology. 76: 855-859.

Samoucha, Y., Cohen, Y., 1988. Synergistic interactions of cymoxanil mixtures in the control of metalaxyl-resistant *Phytophthora infestans* of potato. Phytopathology 78: 636-640.

Samoucha, Y., Gisi, U., 1987. Use of two-and three-way mixtures to prevent buildup of resistance to phenylamide fungicides in *Phytophthora* and *Plasmopara*. Phytopathology 77: 1405-1409.

Samoucha, Y., Levy, R. S., Cohen, Y., 1988. Efficacy over time of cymoxanil mixtures in controlling late blight in potatoes incited by a phenylamide-resistant isolate of *Phytophthora infestans*. Crop Prot. 7: 210-215.

Sanders, P. L., Houser, W. J., Parish, P. J., Cole, H. Jr., 1985. Reduced-rate fungicide mixtures to delay fungicide resistance and to control selected turfgrass diseases. Plant Dis. 69: 939-943.

SAS, 1991. SAS/STAT User`s guide, version 8, 1st printing, volume 2, SAS Institute Inc, SAS campus drive, Cary, North Caroline 27513.

Scardavi, A., 1966. Synergism among fungicides. Ann. Rev. Phytopathol. 4: 335-348.

Stermitz F. R., Lorenz P., Tawara J. N, Zenewicz L., Lewis K., 2000a. Synergy in a medicinal plant: antimicrobial action of berberine potentiated by 5`-methoyhydnocarpin, a multidrug pump inhibitor. Proc Nat Acad Sci USA 97:1433-1437.

Stermitz, F. R., Tawara-Matsuda, J., Lorenz, P., Mueller, P., Zenewicz, L., Lewis K., 2000b. 5`-Methoxyhydrnocarpin and pheophorbide a *Berberis* species components which potentiate berberine growth inhibition of resistant *Staphylococcus aureus*. J Nat. Prod. 63:1146-1149.

Tegos, G., Stermitz, F. R., Lomovskaya, O., Lewis, K., 2002. Multidrug inhibitors uncover remarkable activity of plant antimicrobials. Antimicrob. Agents Chemother. 46: 3133-3141.

Wilson, C. L., El-Ghaouth, A., Chalutz, E., Drody, S., Stevens, C., Liu, J. Y., Khan, V., Arul, J., 1994. Potential of induced resistance to control postharvest diseases of fruits and vegetables. Plant Dis. 78: 837-844.

Wisniewski, M., Wilson, C., El-Ghaouth, A., Droby, S., 2001. Non-chemical approaches to postharvest disease control. Acta Hort. 553: 407-412.

Zheng, X., Yu, T., Chen, R., Huang, B., Wu, V. C-H., 2007. Inhibiting *Penicillium expansum* infection on pear fruit by *Cryptococcus laurentii* and cytokinin. Postharvest Biol. Technol. 45: 221-227.

CHAPTER 3

THE *IN VIVO* EFFECTS OF MIXTURES OF FUNGICIDES AND MEDICINAL PLANT EXTRACTS AGAINST *BOTRYTIS CINEREA*

3.1 Abstract

Gray mold due to *Botrytis cinerea* is one of the major post-harvest diseases of apples in South Africa. In an attempt to evaluate alternative control measures, the synergistic effects of three fungicides, Sporekill™, Rovral™ and Terminator™, in combination with plant extracts from *Galenia africana*, *Elytropappus rhinocerotis* and *Tulbaghia violacea*, were assessed. Surface-disinfected Granny Smith apples were wounded and treated with low doses of fungicides alone or in mixtures with different doses of extracts of the plant species. More effective and prolonged control of *Botrytis* decay was always exerted by plant extracts in combination with fungicides. The combination treatments provided synergistic effects and showed markedly better efficacy, especially when inoculation was followed by a 7d incubation period at 20^{o}C. This study suggests that the combination of a plant extract with a lower dose of fungicide may be a useful alternative strategy to manage *B. cinerea* infections effectively and to reduce risks associated with extensive use of synthetic fungicides.

3.2 Introduction

Gray mold due to *Botrytis cinerea* Pers. is one of the most important post-harvest rots of apples as well as of several other fruits and vegetables (Sommer, 1982; Rosenberger, 1990). Pre- and post-harvest applications of synthetic fungicides are frequently used to control post-harvest rots. However, fungicide residue toxicity in the environment and its effect on human health is a common public concern (Spotts and Cervantes, 1986;

Guizzardi *et al*., 1995; Stehmann and De Waard, 1996; Droby *et al*., 2003). Moreover, the use of chemicals is also limited by the development of fungicide resistance as well as by low or zero-residue tolerance requirements for some export and processing markets. Since the current environment increasingly limit the use of chemicals, alternative or integrative control measures are becoming important for disease management strategies (Lima *et al*., 2006).

Plant extracts appear particularly promising in preventing fungal rots on different fruit and vegetables (Appleton and Tansey, 1975; Damayanti *et al*., 1996). However, when plant extracts are applied alone under commercial conditions they are sometimes not sufficient to control post-harvest decay satisfactorily (Droby *et al*., 1998; Lima *et al*., 2003). Integrating plant extracts with other means of control in order to make their activity more reliable may be the best option for large-scale applications. Fungicide mixtures have proven to be extremely useful (Gisi *et al*., 1985; Sanders *et al*., 1985; Samoucha and Cohen, 1988). Yet, aside from studies proposing the combination of plant extracts with small quantities of chemicals, very little research has studied the potential of this integrated approach for the concomitant control of *B. cinerea* infections.

Therefore, the objective of this study was to evaluate the compatibility of plant extracts with selected fungicides and to verify whether the combined application of plant extracts with a low dose of fungicides on apples may be an effective strategy for inhibiting *B. cinerea* infections in storage.

3.3 Materials and Methods

3.3.1 Pathogens

Botrytis cinerea strains, PPRI 8507 and 7338, were obtained from the Agricultural Research Council – Plant Protection Research Institute (ARC-PPRI), Mycology Division, Pretoria, South Africa. Strains PPRI 8507 and 7338 were isolated from infected apples.

Inocula of *B. cinerea* were cultured on potato dextrose agar (PDA) at 25°C. Conidial suspensions were prepared from 14-d old cultures by flooding the plates with sterile solution of 0.05% Tween-80 in sterile distilled water and gently scraping the mycelium with a spatula. Tween-80 breaks spore clumps into individual spores. The concentration of the resulting conidial suspension was determined using a haemocytometer and adjusted to 10^4 conidia mL^{-1}.

3.3.2 Plant Extraction Method

Plant material of *Galenia africana* L. (Aizoaceae) and *Elytropappus rhinocerotis* (L.f) Less (Asteraceae) were collected from Wellington and *Tulbaghia violacea* Harv. (Alliaceae) were from nurseries in Stellenbosch. Powdered, air-dried leaves of *G. africana* (500 g) and the whole plant of *E. rhinocerotis* (400 g) were used for extractions. Extractions were performed overnight in a closed container at room temperature in methanol (MeOH: 1000 mL, analytical grade) to obtain 500 and 400 mg mL^{-1} (50 and 40%, respectively) stock concentrations. Fresh, whole plants of *T. violaceae* (500 g) were crushed in a Waring blender and extracted overnight in a sealed container at room temperature in MeOH (1000 mL) to obtain a 500 mg mL^{-1} (50%) stock concentration. The extracts were filtered through a Büchner funnel (110 mm diameter) and Whatman no. 4 qualitative filter paper, and stored at 4°C until used.

3.3.3 Fungicides

All fungicides were purchased from suppliers in South Africa. Fungicides were suspended in water (concentration is given in milliliters active ingredient per liter) and tested alone or in mixtures with plant extracts in the experiments. The following fungicides were used: Sporekill™ (didecyl dimethyl ammonium chloride, Hygrotech, 120 g/L a.i.), Rovral™ Aquaflo (iprodione, Aventis CropScience, 500 g/L a.i.) and Terminator™ (dimethyl didecyl ammonium chloride, Fairhill Crop Care cc, 250 g/L a.i.). The standard recommended

doses for post-harvest application of these fungicides in South Africa are: Sporekill™ 1.5 mL L^{-1}, Rovral™ 1mL L^{-1} and Terminator™ 0.8 mL L^{-1}.

3.3.4 Fruit Material

Mature (commercial shipping maturity stage) apples (fruit cv. Granny Smith) were selected for uniformity of size and ripeness. Fruit were purchased from a local commercial pack house and kept at 25°C overnight prior to each experiment to adjust the fruit to room temperature. Any fruit with apparent injuries or infections were removed. Fruit were gently cleaned with 70 % ethanol for 2 min and then air-dried at room temperature prior to placing them on trays in boxes to remove any potential fungicide residues on the fruit surface from field applications.

3.3.5 Tests on Wounded Apples

The methanolic plant extracts were diluted in sterile distilled water in 10 mL doses of 1.95, 3.91, 7.81, 31.25 and 62.5 mg mL^{-1} with fungicides at 0.01 mL L^{-1}. Two wounds (3 mm diameter and 3 mm deep) were produced around the pedicel zone on each fruit using the tip of a sterile borer. Fruit wounds were treated with 20 µL plant extract doses alone and fungicides dose alone as well as mixtures of these plant extracts and fungicides, and with sterile distilled water as the control. After 2 hrs, treated wounds were inoculated with 20 µl of *B. cinerea* conidial suspensions (10^4 conidia mL^{-1}). Wetted paper towels were placed on the bottom of the boxes to prevent wounds from drying out and to increase humidity for the treatments to establish *B. cinerea* in the wounds. Trays were covered with plastic bags for 24 hrs to maintain the relative humidity at 100%. Sealed apple boxes were subsequently arranged randomly and incubated in regular atmosphere (air) storage rooms at either 20°C for 7 d or 30 d at -0.5°C. All fruit stored at 30 d (-0.5°C) were moved to 20°C for 7 d to simulate shelf-life storage. After storage, lesion size in fruits were measured. Means of lesion diameters were used to calculate the percentage inhibition (%) for each treatment by comparison with the water control as follows: Inhibition (%) =

(average of infected fruit in control – average of infected fruit in the treatment/average of infected fruit in control) x 100%. One treatment unit consisting of twenty fruit was replicated three times in the experiments. The experiments were conducted twice and the data pooled. Data presented are the percentage inhibition for each treatment.

3.3.6 Drench Treatment

The methanolic plant extracts were diluted in 5000 mL distilled water in doses of 1.95, 3.91, 7.81, 31.25, 62.5 mg mL^{-1} with fungicides at a dose of 0.01 mL L^{-1}. Apples were wounded with a nail-like pointer with one wound per apple and drenched or immersed in each of the individual treatments for 2 min with occasional agitation. Sixty apples were drench in each treatment. The treated apples were packed in cardboard boxes with the wounded side face up in the box to dry. Fruit were sprayed after 2 hrs with conidial suspensions of the *B. cinerea* strain PPRI 7338. Boxes of fruit were stored in air at -0.5°C for 30 d and moved to 20°C for 7d. The boxes were placed in the cold room according to a completely randomized design. After storage, lesion size in fruits were measured. Means of lesion diameters were used to calculate the percentage of inhibition for each treatment as described before. The experiments were conducted twice and the data pooled. Data presented are the percentage inhibition for each treatment.

3.3.7 Statistical Analysis

A series of two similar experiments were conducted on each of two strains of *B. cinerea* (PPRI 8507 and 7338). Sixty-five treatment combinations (extracts at different concentrations in combination with fungicides) were replicated at random in each of the experiments. The treatment design was a complete factorial, with the different concentrations of extracts tested in combination with each fungicide, therefore one-way analysis of variance (ANOVA) was performed, with treatment combinations as factor, on % Infection (% Control) for each experiment separately, using the General Linear Models (GLM) procedure of SAS statistical software version 9.1 (SAS Institute Inc., Cary, NC,

USA). After testing for experiment homogeneity of variance, results of experiments were also combined and investigated in one overall analysis of variance for each sources of *B. cinerea* (John & Quenouille, 1977). The Shapiro-Wilk test was performed to test for normality (Shapiro, 1965). Student's t-least significant difference was calculated at the 5% level to compare treatment means. A probability level of 5% was considered significant for all significance tests (Snedecor, 1980).

The synergy ratio (SR) for percentage inhibition was based on the Abbott formula (Abbott, 1925) as described by Gisi (1996): Expected efficacy of the mixture, C_{exp} = A + B – (AB/100) in which A and B are the control levels given by the "fungicide" and the "plant extract", respectively. The SR between the observed (C_{obs}) and expected (C_{exp}) efficacies of the mixture is calculated as SR = C_{obs}/C_{exp}. A deviation from the C_{exp} as calculated from the SR between the expected and the observed response of the two compounds would indicate synergistic, additive, or antagonistic interactions. By definition, additive interactions are present if SR=1, synergism occurs if SR>1 and antagonism if SR<1 (Levy *et al*., 1986; De Waard and Gisi, 1995).

3.4 Results

The sensitivity of *B. cinerea* strains, PPRI 8507 and 7338, to Sporekill™ (0.012 g/mL a.i.), Rovral™ (0.005 g/mL a.i.) and Terminator™ (0.0025 g/mL a.i.) doses of 0.01 mL L^{-1} each was compared in wound-inoculated trials on cv. Granny Smith apples (Figures 3-1 and 3-2). Strain PPRI 8507 was highly resistant to the Sporekill™, Rovral™and Terminator™ dose compared to strain PPRI 7338 after the 7-d (20^{o}C) and 30-d (-0.5^{o}C) + 7-d (20^{o}C) storage periods. The inhibition levels for strain PPRI 7338 in the 7-d storage trial varied from 50.3% for Sporekill™, 37.0% for Rovral™ and 28.3% for Terminator™ (Figure 3-1). After the 30+7-d storage period, strain PPRI 7338 inhibitory levels were about 11.8% for Sporekill™, 8.1% for Rovral™ and 19.3% for Terminator™ (Figure 3-2). In a drench trial, strain PPRI 7338 showed inhibitory levels that varied from 44% for Sporekill™, 20.0% for

Rovral™ and 24% for Terminator™ at dosages of 0.01 mL L^{-1} (Figure 3-3). The standard recommended doses for post-harvest application of these fungicides in South Africa are: Sporekill™ 1.5 mL L^{-1}, Rovral™ 1mL L^{-1} and Terminator™ 0.8 mL L^{-1}. Strain PPRI 8507 was not used in the drench experiment due to its high resistant levels against the fungicide doses in the wound inoculation experiments.

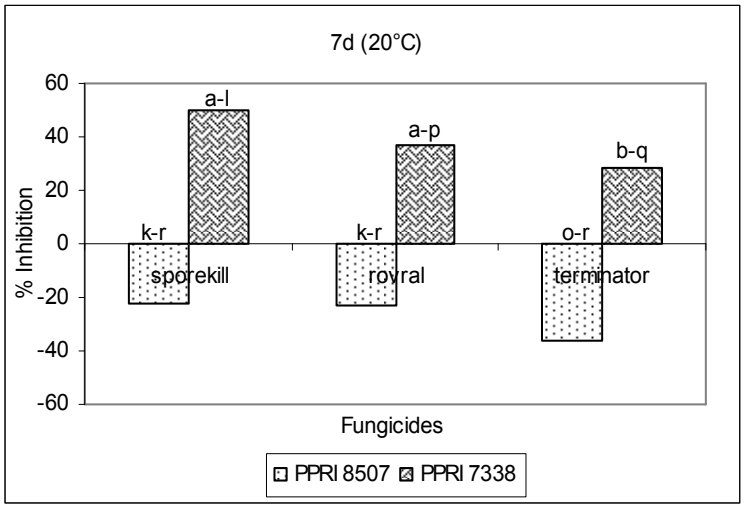

Figure 3-1. Sensitivity of *Botrytis cinerea* strains PPRI 8507 and 7338 to 0.01 mL L^{-1} Sporekill™, Rovral™ and Terminator™ after a 7-d storage of Granny Smith apples at 20°C. Fruit were wounded, inoculated with fungicides and, after 2 hrs, inoculated with conidial suspension of both strains. Values marked by the same letters are not statistically different at p=0.05, according to Student`s t-Least Significant difference.

For the wound-inoculated fungicide and plant extract combination studies, the percent reduction of Botrytis decay was determined after a 7-d (20^{o}C) incubation period with strains PPRI 8507 and 7338 (Tables 3-1, 3-2 and 3-3). A total of 54 combinations were tested for each strain. The apple wound-inoculation experiments were conducted twice and data were pooled and analysed according to the factorial design. Analysis of variance (ANOVA) showed highly significant ($p<0.0001$) interaction among plant type and

extract dose used in these trials (Table 3-1). In order to ascertain the type of interaction exerted by the combined treatments, the SR was calculated according the Abbotts formula for the *B. cinerea* strain PPRI 8507 (Table 3-2). Of the 18 mixtures of fungicides (Sporekill™, Rovral™, Terminator™) and *G. africana* extracts tested, 2 mixtures for each of the respective fungicide combination exhibited significantly (p=0.05) higher inhibition levels against strain PPRI 8507, compared to the fungicides alone. Only 3 of the mixtures tested, exhibited significantly higher inhibition levels than *G. africana* extracts alone. Our results indicate that 11 mixtures were synergistic (SR>1.0), 6 were additive (SR=1.0) and 1 were antagonistic (SR<1.0). The 18 combinations of *E. rhinocerotis* extracts with the fungicides resulted in 3 mixtures with significantly higher inhibition levels than Sporekill™ alone. In this case, 3 of the combinations produced synergistic interactions. For the 18 fungicide combinations with *T. violacea* extracts, 5 mixtures produced significantly higher inhibition levels compared to Sporekill™ and plant extract alone, respectively, and 9 combinations produced synergistic effects (Table 3-2).

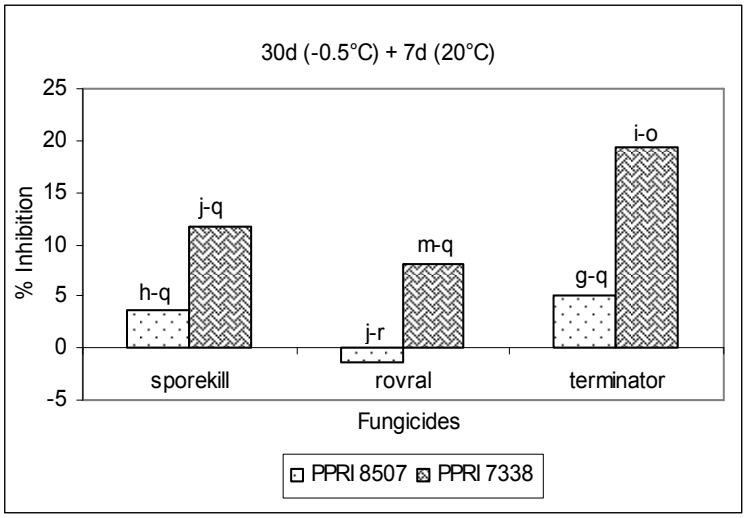

Figure 3-2. Sensitivity of *Botrytis cinerea* strains PPRI 8507 and 7338 to 0.01 mL L^{-1} Sporekill™, Rovral™ and Terminator™ after a 30-d storage of Granny Smith apples at -0.5 °C. Fruit were then moved to 20°C for 7 d to simulate shelf-life storage. Fruit were wounded, inoculated with fungicides and, after 2 hrs, inoculated with conidial suspension

of both strains. Values marked by the same letters are not statistically different at p=0.05, according to Student`s t-Least Significant difference.

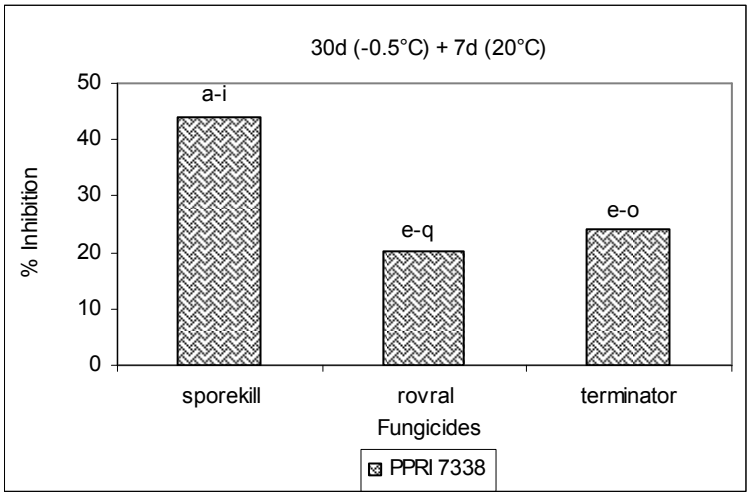

Figure 3-3. Sensitivity of *Botrytis cinerea* strains PPRI 7338 to 0.01 mL L^{-1} Sporekill™, Rovral™ and Terminator™ after a 30-d of storage of Granny Smityh apples at -0.5°C and fruit were moved to 20°C for 7 d to simulate shelf-life storage. Fruit were wounded and drenched for 2 min in fungicides with occasional agitation. After 2 hrs fruit were sprayed with conidial suspensions of *B*. *cinerea*. Values marked by the same letters are not statistically different at p=0.05, according to Student`s t-Least Significant difference.

Wounds treated with combinations of the fungicides and *G*. *africana* resulted in considerable decay control for strain PPRI 7338 (Table 3-3). Six mixtures of *G*. *africana* with Sporekill™, Rovral™ or Terminator™ exerted inhibition levels significantly higher than the fungicide alone. Our results further showed that 12 mixtures exhibited significantly higher inhibition levels than *G*. *africana* alone. The combination of *G*. *africana* and fungicide dose showed 11 mixtures with strong synergistic interactions. High inhibitory responses were observed with combinations of *E*. *rhinocerotis* (1.9 and 3.9 mg mL^{-1}) and the fungicides. Interactions between *E*. *rhinocerotis* and the fungicides showed four mixtures with synergistic effects. Similar results were also observed with combinations of *T*. *violacea* with Sporekill™, Rovral™ or Terminator™. In this case, higher inhibitory levels

of the components were evident at relatively low *T. violacea* extract dosages (1.9-7.8 mg mL^{-1}). The results showed that *T. violacea* and the fungicide combinations produced four mixtures with synergistic interactions. Synergy ratios ranged between 1.1 and 3.8 for *T. violacea* and fungicide combinations (Table 3-3).

ANOVA of experiments conducted on Granny Smith apples stored at -0.5°C for 30 d and then incubated for a period of 7 d at 20°C are presented in Table 3-4. Both plant extract and fungicide treatments were effective in controlling gray mold ($p<0.0001$) and showed a significant interaction ($p<0.0001$). Of the 15 mixtures of *G. africana* with Sporekill™, Rovral™ or Terminator™, five mixtures showed significantly higher inhibition levels than Sporekill™ and Rovral™, respectively, while two mixtures exhibited higher inhibition levels than Terminator™ alone (Table 3-5). Only five of the 15 mixtures tested, showed significantly higher inhibition levels than *G. africana* alone. Combining *G. africana* with Rovral™ or Terminator™ produced synergistic effects. Inhibition levels of the combinations increased with increasing concentrations of *G. africana*. The addition of *E. rhinocerotis* to Sporekill™, Rovral™ or Terminator™ produced two mixtures each for *E. rhinocerotis* + Sporekill™ and *E. rhinocerotis* + Rovral™ and one mixture for *E. rhinocerotis* + Terminator™ with higher inhibiton levels than the respective fungicide alone. Fungicide combinations with *E. rhinocerotis* resulted in three mixtures with synergistic interactions. The addition of *T. violacea* at 7.8 mg mL^{-1} to Sporekill™ or Rovral™ increased the levels of inhibition, but only antagonistic responses were recorded (Table 3-5).

For the 30-d (-0.5°C) + 7-d (20°C) storage experiment of strain PPRI 7338, the addition of 62.5 mg mL^{-1} *G. africana* to Sporekill™, Rovral™ or Terminator™ resulted in higher decay control levels than the fungicides alone (Table 3-6). One of the combinations of *G. africana* with Sporekill™ or Rovral™ showed each a synergistic effect. One synergistic interaction was observed between the *T. violacea* extract (7.8 mg mL^{-1}) and Sporekill™ combination (Table 3-6).

Table 3-1. Analysis of variance for percentage inhibition of wound-inoculated Granny Smith apples with conidial suspension of *Botrytis cinerea* (Strains PPRI 8507 and 7338) stored at 20°C for 7 d, showing the significant levels for main effects of the fungicides, Sporekill™ (didecyl dimethyl ammonium chloride), Rovral™ (iprodione) and Terminator™ (dimethyl didecyl ammonium chloride), and medicinal plant extract doses as well as all interactions involving combinations of fungicides and plant extracts.

		Probability>F[b]	
Source[a]	**df**	**PPRI 8507**	**PPRI 7338**
Fungicide	3	<0.0001	0.0026
Extract	2	<0.0001	<0.0001
Fungicide + Extract*	6	<0.0001	0.1493
Extract Concentrate	5	<0.0001	0.6062
Fungicide + Extract Concentrate	15	0.3777	0.3844
Extract + Extract Concentrate	10	<0.0001	0.0004
Fungicide + Extract + Extract Concentrate	30	0.0940	0.0805

[a]Extracts form three medicinal plant species.

[b]Significant values associated with the *F* tests.

*Extract dose per plant species tested.

Data of decay development for strain PPRI 7338 on apples drenched with the fungicide and plant extract combinations are shown in Table 3-7. An extract dose of *G. africana* at 62.5 mg mL^{-1} was effective in inhibiting gray mold when combined with Sporekill™, Rovral™ and Terminator™ compared to the fungicides alone. Combinations of *G. africana* and Rovral™ exerted an inhibitory efficacy of 91.9 % and also represented a synergistic interaction. Synergistic interactions between *E. rhinocerotis* and Sporekill™ or Terminator™ were also observed. *Tulbaghia violacea* at 31.3 and 62.5 mg mL^{-1} produced synergistic activity when combined with Rovral™ (Table 3-7).

Table 3-2. Percentage decay on Granny Smith apples stored at 20°C for 7d after inoculation with conidia of *Botrytis cinerea* strain PPRI 8507, and the synergy ratios of mixtures containing Sporekill™ (didecyl dimethyl ammonium chloride), Rovral™ (iprodione) or Terminator™ (dimethyl didecyl ammonium chloride) and *Galenia africana* (Ga), *Elytropappus rhinocerotis* (Er) or *Tulbaghia violacea* (Tv) plant extract doses.

Synergy Ratio[a]	Plant Species	Plant Extract Dose (mg mL^{-1})	% Inhibition of Decay[b]			
			Water Control	Sporekill™ 0.01 mL L^{-1}	Rovral™ 0.01 mL L^{-1}	Terminator™ 0.01 mL L^{-1}
	Control		0	-22.2	-22.8	-36.2
	Ga	**1.9**	-3.2	-26.3	-33.7	-28.7
SR			-	1.0	1.3 +	0.7
	Ga	**3.9**	-18.1	-39.6	-19.8	-28.1
SR			-	0.9	0.4	0.5
	Ga	**7.8**	-29.1	-13.7	-29.1	32.8[f]
SR			-	0.2	0.5	-0.4
	Ga	**15.6**	-20.8	-13.1	-12.3	76.9[f]
SR			-	0.3	0.3	-1.2
	Ga	**31.3**	44.8	35.3[¶]	33.6[¶]	81.9[f]
SR			-	1.1 +	1.0	3.3 +
	Ga	**62.5**	75.5	33.1[¶]	80.5[f]	100[f]
SR			-	0.5	1.2 +	1.5 +
	Er	**1.9**	-3.8	1.6[f]	-14.8	-11.2
SR			-	-0.1	0.5	0.3
	Er	**3.9**	-2.9	11[f]	-36.3	17.2[f]
SR			-	-0.4	1.4 +	-0.4
	Er	**7.8**	-17.2	7.1[f]	-29.3	-21.6
SR			-	-0.2	0.7	0.4
	Er	**15.6**	-18.2	9.8[f]	-42.5	-21.4
SR			-	-0.2	0.9	0.4
	Er	**31.3**	-11.2	-12.0	-42.2	-18.1
SR			-	0.3	1.2 +	0.4
	Er	**62.5**	-6.8	-15.2	-40.9	-34.4
SR			-	0.5	1.3 +	0.8
	Tv	**1.9**	-25.8	19.9[f]	-25.9	-32.2
SR			-	-0.4	0.5	0.5
	Tv	**3.9**	-25.6	-7.6	-20.7	-19.3
SR			-	0.1	0.4	0.3

Continued/…

Table 3-2. (Continued).

Synergy Ratio[a]	Plant Species	Plant Extract Dose (mg mL^{-1})	% Inhibition of Decay[b]			
			Water Control	Sporekill™ 0.01 mL L^{-1}	Rovral™ 0.01 mL L^{-1}	Terminator™ 0.01 mL L^{-1}
	Tv	**7.8**	-4.9	7.9^{f}	-29.3	-12.3
SR			-	-0.3	1.0	0.3
	Tv	**15.6**	-27.5	12.4^{f}	-29.3	-26.3
SR			-	-0.2	0.5	0.4
	Tv	**31.3**	-35.3	21.7^{f}	-34.8	-23.3
SR			-	-0.3	0.5	0.3
	Tv	**62.5**	-29.5	27.4^{f}	-15.5	-22.2
SR			-	-0.5	0.3	0.3

[a]The synergism ratio for percentage inhibition was based on the Abbott formula (Abbott, 1925) as described by Gisi (1996): Expected efficacy of the mixture, C_{exp} = A + B – (AB/100) in which A and B are the control levels given by fungicide and the plant extract, respectively. The synergy ratio, SR, between the observed, C_{obs}, and expected, C_{exp}, efficacies of the mixture is calculated as $R=C_{ob}/C_{exp}$. If R is greater than, equal to, or less, than 1, then interaction between compounds is synergistic, additive, or antagonistic, respectively. SR>1=Synergistic (+); SR=1=Additive; SR<1= Antagonistic. [b]Decay inhibition mean values of the plant extract and fungicide combinations: [¶]significantly different ($p<0.05$) from the value of the fungicide dose (0.01 mL L^{-1}) in the column; [§]significantly different ($p<0.05$) from the respective values of the plant extract doses without fungicide in the same row; [f]significantly different ($p<0.05$) from the value of both the fungicide and plant extract doses in the column.

Table 3-3. Percentage decay on Granny Smith apples stored at 20°C for 7d after inoculation with conidia of *Botrytis cinerea* strain PPRI 7338, and the synergy ratios of mixtures containing Sporekill™ (didecyl dimethyl ammonium chloride), Rovral™ (iprodione) or Terminator™ (dimethyl didecyl ammonium chloride) and *Galenia africana* (Ga), *Elytropappus rhinocerotis* (Er) or *Tulbaghia violacea* (Tv) plant extract doses.

Synergy Ratio[a]	Plant Species	Plant Extract Dose (mg mL^{-1})	% Inhibition of Decay[b]			
			Water Control	Sporekill™ 0.01 mL L^{-1}	Rovral™ 0.01 mL L^{-1}	Terminator™ 0.01 mL L^{-1}
	Control		0	50.3	37	28.3
	Ga	**1.9**	30.3	82.1^{f}	92.9^{f}	55.4^{f}
SR			-	1.3 +	1.7 +	1.1 +
	Ga	**3.9**	-25.7	59.4^{f}	60.7^{f}	52.7^{f}
SR			-	1.6 +	2.9 +	5.3 +
	Ga	**7.8**	53.6	75.5^{f}	80.6^{f}	87.6^{f}
SR			-	1.0	1.1 +	1.3 +
	Ga	**15.6**	68.9	100^{f}	100^{f}	100^{f}
SR			-	1.2 +	1.2 +	1.3 +
	Ga	**31.3**	100	100	100¶	100¶
SR			-	1.0	1.0	1.0
	Ga	**62.5**	100	100¶	91.8¶	100¶
SR			-	1.0	0.9	1.0
	Er	**1.9**	70	62¶	76.6^{f}	-4.4
SR			-	0.7	0.9	-0.1
	Er	**3.9**	20.1	86.1^{f}	28.7§	18.9
SR			-	1.4 +	0.6	0.4
	Er	**7.8**	0.3	75^{f}	23.7§	29.1^{f}
SR			-	1.5 +	0.6	1.0
	Er	**15.6**	-21.5	-24.5	72.1^{f}	17.5
SR			-	-0.6	3.1 +	1.4 +
	Er	**31.3**	32.1	-18.6	12.3	-7.9
SR			-	-0.3	0.2	-0.2
	Er	**62.5**	15.6	-15.2	16.5§	-3.1
SR			-	-0.3	0.4	-0.1
	Tv	**1.9**	53	6.2	74.2^{f}	32.7¶
SR			-	0.1	1.1 +	0.5
	Tv	**3.9**	47.1	15.2	52.7^{f}	54.3^{f}
SR			-	0.2	0.8	0.9

Continued/…

Table 3-3. (Continued).

Synergy Ratio[a]	Plant Species	Plant Extract Dose (mg mL^{-1})	% Inhibition of Decay[b]			
			Water Control	Sporekill™ 0.01 mL L^{-1}	Rovral™ 0.01 mL L^{-1}	Terminator™ 0.01 mL L^{-1}
	Tv	**7.8**	-10.4	73.2^{f}	83.8^{f}	79.1^{f}
SR			-	1.6 +	2.8 +	3.8 +
	Tv	**15.6**	41.8	26.4	-0.5	37.6¶
SR			-	0.4	0	0.6
	Tv	**31.3**	-29.4	43.8§	36.8§	75^{f}
SR			-	1.2 +	2.0 +	10.4 +
	Tv	**62.5**	5.1	22.9§	45.3^{f}	35.3^{f}
SR			-	0.4	1.1 +	1.1 +

[a]The synergism ratio for percentage inhibition was based on the Abbott formula (Abbott, 1925) as described by Gisi (1996): Expected efficacy of the mixture, C_{exp} = A + B – (AB/100) in which A and B are the control levels given by fungicide and the plant extract, respectively. The synergy ratio, SR, between the observed, C_{obs}, and expected, C_{exp}, efficacies of the mixture is calculated as $R=C_{ob}/C_{exp}$. If R is greater than, equal to, or less, than 1, then interaction between compounds is synergistic, additive, or antagonistic, respectively. SR>1=Synergistic (+); SR=1=Additive; SR<1= Antagonistic. [b]Decay inhibition mean values of the plant extract and fungicide combinations: [¶]significantly different ($p<0.05$) from the value of the fungicide dose (0.01 mL L^{-1}) in the column; [§]significantly different ($p<0.05$) from the respective values of the plant extract doses without fungicide in the same row; [f]significantly different ($p<0.05$) from the value of both the fungicide and plant extract doses in the column.

Table 3-4. Analysis of variance for percentage inhibition after storage at -0.5°C for 30 d and then at 20°C for 7 d of wounded Granny Smith apples inoculated with *Botrytis cinerea* (Strains PPRI 8507 and 7338), showing the significant levels for main effects of the fungicides, Sporekill™ (didecyl dimethyl ammonium chloride), Rovral™ (iprodione) and Terminator™ (dimethyl didecyl ammonium chloride), and medicinal plant extract doses as well as all interactions involving combinations of the fungicides and plant extracts.

		Probability>F[b]		
		Wound-Inoculated Test		Drench Test
Source[a]	**df**	**PPRI 8507**	**PPRI 7338**	**PPRI 7338**
Trial	1	0.5957	<0.0001	0.0608
Treatment	64	<0.0001	<0.0001	<0.0001
Trial*Treat	64	<0.0001	<0.0001	0.5748

[a]Extracts form three medicinal plant species.

[b]Significant values associated with the *F* tests.

*Extract dose per plant species tested.

Table 3-5. Percentage decay on Granny Smith apples stored at -0.5°C for 30 d and then at 20°C for 7 d after inoculation with conidia of *Botrytis cinerea* strain PPRI 8507, and the synergy ratios of mixtures containing Sporekill™ (didecyl dimethyl ammonium chloride), Rovral™ (iprodione) or Terminator™ (dimethyl didecyl ammonium chloride) and *Galenia africana* (Ga), *Elytropappus rhinocerotis* (Er) or *Tulbaghia violacea* (Tv) plant extract doses.

Synergy Ratio[a]	Plant Species	Plant Extract Dose (mg mL^{-1})	% Inhibition of Decay[b]			
			Water Control	Sporekill™ 0.01 mL L^{-1}	Rovral™ 0.01 mL L^{-1}	Terminator™ 0.01 mL L^{-1}
	Control		0	3.7	-1.3	5.1
	Ga	**1.9**	-10.3	9.8[f]	-6.3	5.1[§]
SR			-	-1.6	0.5	-1.1
	Ga	**3.9**	25.5	8.7[¶]	5.3[¶]	NT
SR			-	0.3	0.2	-
	Ga	**7.8**	21.0	NT	9.8[¶]	-0.3
SR			-	-	0.5	0
	Ga	**15.6**	50.9	50.9[¶]	52[f]	NT
SR			-	1.0	1.0	-
	Ga	**31.3**	55.8	56.2[f]	72.9[f]	53.1[¶]
SR			-	1.0	1.3 +	0.9
	Ga	**62.5**	85.1	75.4[¶]	63.6[¶]	95.5[f]
SR			-	0.9	0.7	1.1 +
	Er	**3.9**	-9.5	10[f]	0	NT
SR			-	-1.8	0	-
	Er	**7.8**	2.6	8.1[f]	NT	NT
SR			-	1.3 +	-	
	Er	**15.6**	-1.1	NT	-4.9	14[f]
SR			-	-	2.0 +	3.5 +
	Er	**31.3**	-4.5	NT	2.7[f]	NT
SR			-	-	-0.5	-
	Er	**62.5**	-2.3	NT	4[f]	NT
SR			-	-	-1.1	-
	Tv	**7.8**	-9.5	15.6[f]	4.2[f]	NT
SR			-	-2.9	-0.4	-
	Tv	**31.3**	2.9	-1.3	-6.5	-5.8
SR			-	-0.2	-4.0	-0.7
	Tv	**62.5**	-11.2	NT	1.9[f]	NT
SR			-	-	-0.2	-

Table 3-5. (Continued).

[a]The synergism ratio for percentage inhibition was based on the Abbott formula (Abbott, 1925) as described by Gisi (1996): Expected efficacy of the mixture, C_{exp} = A + B – (AB/100) in which A and B are the control levels given by fungicide and the plant extract, respectively. The synergy ratio, SR, between the observed, C_{obs}, and expected, C_{exp}, efficacies of the mixture is calculated as $R=C_{ob}/C_{exp}$. If R is greater than, equal to, or less, than 1, then interaction between compounds is synergistic, additive, or antagonistic, respectively. SR>1=Synergistic (+); SR=1=Additive; SR<1= Antagonistic. [b]Decay inhibition mean values of the plant extract and fungicide combinations: [¶]significantly different ($p<0.05$) from the value of the fungicide dose (0.01 mL L^{-1}) in the column; [§]significantly different ($p<0.05$) from the respective values of the plant extract doses without fungicide in the same row; [ƒ]significantly different ($p<0.05$) from the value of both the fungicide and plant extract doses in the column. NT=Not tested.

Table 3-6. Percentage decay on Granny Smith apples stored at 20°C for 7 d after inoculation with conidia of *Botrytis cinerea* strain PPRI 7338, and the synergy ratios of mixtures containing Sporekill™ (didecyl dimethyl ammonium chloride), Rovral™ (iprodione) or Terminator™ (dimethyl didecyl ammonium chloride) and *Galenia africana* (Ga), *Elytropappus rhinocerotis* (Er) or *Tulbaghia violacea* (Tv) plant extract doses.

Synergy Ratio[a]	Plant Species	Plant Extract Dose (mg mL^{-1})	% Inhibition of Decay[b]			
			Water Control	Sporekill™ 0.01 mL L^{-1}	Rovral™ 0.01 mL L^{-1}	Terminator™ 0.01 mL L^{-1}
	Control		0	11.8	8.1	19.3
	Ga	**1.9**	21.1	24[f]	3	7.3
SR			-	0.8	0.1	0.2
	Ga	**3.9**	12.3	41.3[f]	-2.8	NT
SR			-	1.8 +	-0.1	-
	Ga	**7.8**	10.2	NT	8.3[¶]	NT
SR			-	-	0.5	-
	Ga	**15.6**	39.9	NT	42.5[f]	NT
SR			-	-	0.9	-
	Ga	**31.3**	57.7	40.4[¶]	81.3[f]	37.7[¶]
SR			-	0.6	1.3 +	0.6
	Ga	**62.5**	95.1	95.6[¶]	73.2[¶]	93[¶]
SR			-	1.0	0.8	1.0
	Er	**3.9**	15.9	12.8[¶]	2.1	NT
SR			-	0.5	0.1	-
	Er	**7.8**	8.8	10.7[§]	NT	15.5[§]
SR			-	0.5	-	0.6
	Er	**15.6**	8.4	NT	4.4	4.9
SR			-	-	0.3	0.2
	Er	**31.3**	5.7	NT	-0.4	NT
SR			-	-	0	-
	Er	**62.5**	7.0	NT	8.8[f]	NT
SR			-	-	0.6	-
	Tv	**7.8**	8.5	33.3[f]	6.5	7.7
SR			-	1.7 +	0.4	0.3
	Tv	**31.3**	26.9	11.0	3.3	0.7
SR			-	0.3	0.1	0
	Tv	**62.5**	20.1	NT	13.6	NT
SR			-	-	0.5	-

(Continued/…)

Table 3-6. (Continued).

[a]The synergism ratio for percentage inhibition was based on the Abbott formula (Abbott, 1925) as described by Gisi (1996): Expected efficacy of the mixture, C_{exp} = A + B – (AB/100) in which A and B are the control levels given by fungicide and the plant extract, respectively. The synergy ratio, SR, between the observed, C_{obs}, and expected, C_{exp}, efficacies of the mixture is calculated as $R=C_{ob}/C_{exp}$. If R is greater than, equal to, or less, than 1, then interaction between compounds is synergistic, additive, or antagonistic, respectively. SR>1=Synergistic (+); SR=1=Additive; SR<1= Antagonistic. [b]Decay inhibition mean values of the plant extract and fungicide combinations: [¶]significantly different ($p<0.05$) from the value of the fungicide dose (0.01 mL L^{-1}) in the column; [§]significantly different ($p<0.05$) from the respective values of the plant extract doses without fungicide in the same row; [ƒ]significantly different ($p<0.05$) from the value of both the fungicide and plant extract doses in the column. NT=Not tested.

Table 3-7. Percentage decay on Granny Smith apples stored at -5°C for 30 d and then at 20°C for 7 d after drenching of fruit with mixtures containing Sporekill™ (didecyl dimethyl ammonium chloride), Rovral™ (iprodione) or Terminator™ (dimethyl didecyl ammonium chloride) and *Galenia africana* (Ga), *Elytropappus rhinocerotis* (Er) or *Tulbaghia violacea* (Tv) plant extract doses against PPRI 7338 strain of *Botrytis cinerea*.

Synergy Ratio[a]	Plant Species	Plant Extract Dose (mg mL^{-1})	% Inhibition of Decay[b]			
			Water Control	Sporekill™ 0.01 mL L^{-1}	Rovral™ 0.01 mL L^{-1}	Terminator™ 0.01 mL L^{-1}
	Control		0	44	20.2	24
	Ga	**1.9**	-35.9	25.1[§]	9	4
SR			-	1.1 +	-1.1	-1.2
	Ga	**3.9**	-28.9	17.6[§]	-27.7	NT
SR			-	0.6	9.7 +	-
	Ga	**7.8**	-41.9	NT	-33.2	NT
SR			-	-	2.5 +	-
	Ga	**15.6**	-7.5	NT	-6.3	NT
SR			-	-	-0.4	-
	Ga	**31.3**	82.9	25.4	63.1[¶]	4.1
SR			-	0.9	0.7	0
	Ga	**62.5**	83.8	77[¶]	91.9[*f*]	66.3[¶]
SR			-	0.9	1.1 +	0.8
	Er	**3.9**	30	-1.8	-45.9	NT
SR			-	0	-1.0	-
	Er	**7.8**	-37.8	25.3[§]	NT	-32.9
SR			-	1.1 +	-	7.0 +
	Er	**15.6**	-7.4	NT	-48.4	-39.3
SR			-	-	-3.4	-2.1
	Er	**31.3**	3.4	NT	-62.4	NT
SR			-	-	-2.7	-
	Er	**62.5**	-16.3	NT	-48.2	NT
SR			-	-	-6.7	-
	Tv	**7.8**	19.2	53.6[*f*]	35.5[*f*]	18.3
SR			-	1.0	1.0	0.5
	Tv	**31.3**	-6.6	-5.6	32.8[*f*]	-20
SR			-	-0.1	2.2 +	-1.1
	Tv	**62.5**	-13.4	NT	43.1[*f*]	13.8
SR			-	-	4.5 +	0

(Continued/…).

Table 3-7. (Continued).

[a]The synergism ratio for percentage inhibition was based on the Abbott formula (Abbott, 1925) as described by Gisi (1996): Expected efficacy of the mixture, C_{exp} = A + B – (AB/100) in which A and B are the control levels given by fungicide and the plant extract, respectively. The synergy ratio, SR, between the observed, C_{obs}, and expected, C_{exp}, efficacies of the mixture is calculated as $R=C_{ob}/C_{exp}$. If R is greater than, equal to, or less, than 1, then interaction between compounds is synergistic, additive, or antagonistic, respectively. SR>1=Synergistic (+); SR=1=Additive; SR<1= Antagonistic. [b]Decay inhibition mean values of the plant extract and fungicide combinations: ¶significantly different ($p<0.05$) from the value of the fungicide dose (0.01 mL L^{-1}) in the column; §significantly different ($p<0.05$) from the respective values of the plant extract doses without fungicide in the same row; *f* significantly different ($p<0.05$) from the value of both the fungicide and plant extract doses in the column. NT=Not tested.

3.5 Discussion

In the present study, extracts of indigenous medicinal plants were tested on apples in combination with fungicidesat low dosage rates to verify whether combination applications could be a viable strategy to control *B. cinerea* infections and decay development in storage. This hypothesis was confirmed since several combination treatments provided more effective control of *B*. *cinerea* infections compared to plant extracts and fungicides applied alone. Single extracts of the medicinal plant species, *E*. *rhinoceoris* and *T*. *violacea* exhibited weak or no antifungal properties against *B*. *cinerea* strains in this study compared to *G. africana*. The strongest antifungal activity observed was with a single plant extract dose of 31.3 and 62.5 mg mL^{-1} of *G*. *africana*.

Mixtures between *G*. *africana* and Sporekill™, Rovral™ or Terminator™ exerted, in some cases, a control efficacy up to 100 %. The relative increase in control efficacy of mixtures represents in certain cases a strong synergistic interaction between the individual compounds. Gisi *et al*., (1985), Grabski & Gisi (1985; 1987) and Samoucha & Cohen (1984; 1986) showed synergistic interactions between fungicides with different modes of action. Reporting on fungicide mixtures with synergistic action in practice is rather limited (Lorbeer, 1996; Hayashi *et al*., 2003). The synergistic activity between plant extracts and

fungicides is of particular interest if we consider that the fungicides used in this study are among the few other fungicides currently sanctioned for post-harvest protection of apples and other fruit in several countries and that the spread of resistant strains of *B*. *cinerea* often makes their activity inconsistent.

Botrytis cinerea strain PPRI 8507 showed high levels of resistance to the fungicides and plant extracts. However, growth of these strains were inhibited when the fungicides were combined with plant extracts, especially evident after the 7-d (20°C) incubation period assessments of Botrytis decay levels on the fruits. Results of the 30-d (-0.5^{o}C) + 7-d (20^{o}C) storage period showed that Botrytis decay development were inhibited at the low temperature storage period, but a general increase in decay was observed after the 7-d shelf-life storage period. This study recognized that the low fungicide dosage of 0.01 mL L^{-1} was an unrealistic application for the long-term storage period of 37 d. However, the study objective to observe synergistic interactions was still achieved in many of the combination treatments. The standard recommended doses for post-harvest application of these fungicides in South Africa are Sporekill™ 1.5 mL L^{-1}, Rovral™ 1mL L^{-1} and Terminator™ 0.8 mL L^{-1}. Thus, reduction of the recommended fungicide dosage to at least 50 % or 25 % should be considered in future studies. Apples exported from South Africa must have a storage potential of at least 4 weeks/30 d at low temperature. Storage ability, therefore, plays an integral role in the future commercial success of the mixtures. This study also showed a trend in that *G*. *africana* extracts showed synergistic effects at higher concentration levels between 15.6 and 62.5 mg mL^{-1}, while *E*. *rhinocerotis* and *T*. *violacea* showed synergistic effects between 1.9 and 7.8 mg mL^{-1}.

The study further demonstrates that secondary metabolites produced by South African indigenous medicinal plant species still have “unknown functional assignments” a statement made in the studies of Tegos *et al*. (2002). “Synergy” is a popular concept in the field of herbal medicine, suggesting plant extracts contain compounds that potentiate the effects of each other (Duke and Bogenschultz-Godwin, 1998). Synergistic interactions

between components in a mixture can relate to one of the following mechanisms: (a) simple (non-mediated) diffusion across the plasma membrane, (b) carrier-mediated transport to the target site, (e) activation, (f) detoxification, (g) affinity for the target site, (h) circumvention of the target site, and (i) compensation of the target site (De Waard, 1985, 1997). Stermitz *et al*. (2000 a, b) showed how two different components of the medicinal plant, *Berberis fremontii* can act in synergy, with one compound disabling a resistance mechanism and potentiating the antibacterial activity of the antibiotic substance. *Berberis fremontii* makes an ineffective antibiotic berberine, but when combined with 5'-methoxyhydnocarpin-D (5'-MHC, also produced by the same plant species) it becomes an effective antimicrobial agent. 5'-MHC has no antimicrobial activity on its own, but is a potent inhibitor of the NorA multidrug resistant (MDR) pump (Tegos *et al*., 2002).

Minimizing losses due to *Botrytis* gray mold depends on the availability of effective natural and synthetic fungicides. Reducing the concentration of fungicides in the post-harvest drench treatment of apples could result in substantially lower fungicide residues on apple fruit. In addition, the use of sanitizers such as Sporekill™ and Terminator™, would also have additional advantages in food safety (Zhou *et al*., 2001). Natural defense compounds belonging to various chemical classes that act as constitutive or inducible chemical barriers, such as stilbenes, isoflavonoids, coumarins, and sesquiterpenes have been described (Osbourne, 1999), but *B. cinerea* has been found to be resistant to the toxic effects of these compounds. It is commonly accepted that a significant part of phytochemical diversity serves to protect plants against microbial pathogens (Dixon, 2001). However, despite a collection of antifungal and antibacterial compounds, plant products *per se* have not been used to any significant extent in the development of antimicrobial pesticides (Duke, 1990), and a few with simple structures are suitable for use as leads for chemical synthesis (Knight *et al*., 1997). Chemical synthesis is required since chemical defenses are rather weak when extracted from plants and tested *in vitro* and *in vivo*.

In conclusion, the combination of medicinal plant extracts with low doses of fungicides in a post-harvest environment is an interesting control strategy from an economic as well as technical point of view. We recommend that in studies to commercialise such a strategy application rates of fungicides at 25% and 50% of the commercial rates in combination with the plant extracts be considered. Such combinations could display several positive effects, such as greater decay control and longer storability of fruits, reduction of risks for fungicide resistance in the pathogen, reduction of risks for accumulation of toxic residues in fruit and increase interest for large-scale application of plant extract-fungicide products.

3.6 References

Abbott, W.S., 1925. A method of computing effectiveness of an insecticide. J. Econ. Entomol. 18:265-267.

Appleton, J.A., Tansey, M.R., 1975. Inhibition of growth of 200 pathogenic fungi by garlic extract. Mycologia 67: 882-885.

Damayanti, M., Susheela, K.H., Sharma, G.J. 1996. Effect of plant extracts and systemic fungicide on the pineapple fruit-rotting frungus. Ceratostis Paradoxa. Cytobios. 86: 155-165.

De Waard, M. A., 1985. Fungicide synergism and antagonism. Fungicides for Crop Protection BCPC Monograph 31, BCPC, Surrey, UK, pp. 89-95.

De Waard, M. A., 1997. Significance of ABC transporters in fungicide sensitivity and resistance. Pestic. Sci. 51:271-275.

De Waard, M. A., Gisi, U., 1995. Synergism and antagonism in fungicides. Pages 565-578 in: Modern Selective Fungicides. 2nd ed. H. Lyr, ed. Gustav Fischer, Jena, Germany.

Dixon, R. A., 2001. Natural products and plant disease resistance. Nature 411:843- 847.

Droby, S., Cohen, A., Weiss, B., Horev, B., Chalutz, E., Katz, H., Keren-Tzur, M., Shachnai, A., 1998. Commercial testing of Aspire: a yeast preparation for the biological control of postharvest decay of citrus. Biol. Contr. 12: 97-100.

Droby, S., Wisniewski, M., El Ghaouth, A., Wilson, C., 2003. Biological control of postharvest diseases of fruit and vegetables: current achievements and future challenges. Acta Hort. 628: 703-713.

Duke, J. A., Bogenschultz-Godwin, M. J., 1998. The synergy principle at work in plants, pathogens, insects, herbivores, and humans. CRC, Boca Raton, FL.

Duke, S. O., 1990. Natural pesticides from plants, p. 511-517. In: J. Janick and J. E. Simon (eds.), Advances in new crops. Timber Press, Portland, OR.

Gisi, U., Binder, H., Rimbach, E., 1985. Synergistic interactions of fungicides with different modes of action. Trans. Br. Mycol. Soc. 85: 299-306.

Gisi, U. 1996. Synergistic interaction of fungicides in mixtures. Phytopathology 86: 1273-1278.

Grabski, C., Gisi, U., 1985. Mixtures of fungicides with synergistic interactions for protection against phenylamide resistance in *Phytophthora*. BCPC monograph 31: 315 - 318.

Grabski, C., Gisi, U., 1987. Quantification of synergistic interactions of fungicides against *Plasmopara* and *Phytophthora*. Crop Prot. 6, in press.

Guizzardi, M., Caccioni, D.R.L., Pratella, G.C., 1995. Resistance monitoring of *Monilinia laxa* (Aderh. et Ruhl) honey to benzimidazoles and dicarboximides in postharvest stage. J. Plant Dis. Prot. 102: 86-90.

Hayashi, K., Schoonbeek, H-J., De Waard, M., 2003. Modulators of membrane drug transporters potentiate the activitiy of the DMI fungicide oxpoconazole against *Botrytis cinerea*. Pest Manag. Sci. 59:294-302.

John, J.A., Quenouille, M.H., 1977. Experiments: Design and Analysis, 2nd Edition. The Garden City Press Limited, Letchworth, Hertfordshire.

Knight, S. C., Anthony, V. M., Brady, A. M., Greenland, A. J., Heany, S. P., Murray, D. C., Powell, K. A., Schultz, M. A., Spinks, C. A., Wor thingtom, P. A. and Youle, D. 1997. Rationale and perspectives on the development of fungicides. Ann. Rev. Phytopathol. 35:349-372.

Levy, Y., Benderly, M., Cohen, Y., Gisi, U., Bassand, D., 1986. The joint action of fungicides in mixtures: Comparison of two methods for synergy calculation. EPPO Bull. 16:651-657.

Lima, G.,De Curtis, F., Castoria, R., De Cicco, V., 2003. Integrated control of apple postharvest pathogens and survival of biocontrol yeasts in semi-commercial conditions. Eur. J. Plant Pathol. 109: 341-349.

Lima, G., De Curtis, F., Piedimonte, D., Spina, A.M., De Cicco, V., 2006. Integration of biocontrol yeast and thiabendazole protects stored apples from fungicides sensitive and resistant isolates of *Botrytis cinerea*. Postharvest Biol. Technol. 40: 301-307.

Lorbeer, W. J., 1996. Introduction. Synergism, antagonism, and additive action of fungicide mixtures. Phytopathology 86:1261-1262.

Osbourne, A. E., 1999. Antimicrobial phytoprotectants and fungal pathogens: A commentary. Fungal Genet. Biol. 26:163-168.

Samoucha, Y., Cohen, Y., 1984. Synergy between metalaxyl and mancozeb in controlling downy mildew in cucumbers. Phytopathology 74: 1434-1451

Samoucha, Y., Cohen, Y., 1986. Efficacy of systemic and contact fungicide mixtures in controlling late blight potatoes. Phytopathology 76: 855-859.

Samoucha, Y., Cohen, Y., 1988. Synergistic interactions of cymoxanil mixtures in the control of metalaxyl-resistant *Phytophthora infestans* of potato. Phytopathology 78: 636-640.

Sanders, P. L., Houser, W. J., Parish, P. J., Cole, H. Jr., 1985. Reduced-rate fungicide mixtures to delay fungicide resistance and to control selected turfgrass diseases. Plant Dis. 69: 939-943.

SAS, 2000. SAS/STAT Users Guide, Version 8, First Edition, Volume 2. SAS Institute Inc., Cary, NC, USA.

Shapiro, S.S., Wilk, M.B., 1965. An analysis of variance test for normality (complete samples), Biometrika 52: 591-611.

Sommer, N.F., 1982. postharvest handling practices and postharvest disease of fruit. Plant Dis. 66: 357-364.

Snedecor, G.W., Cochran, W.G., 1980. Statistical Methods, 7th ed. The Iowa State University Press, Ames.

Spotts, R.A., Cervantes, L.A., 1986. Population, pathogenicity and benomyl resistance to *Botrytis* spp., *Penicillium* spp., and *Mucor puriformis* in packinghouse. Plant Dis. 70: 106-108.

Stehmann, C., De Waard, M.A., 1996. Sensitivity of population of *Botrytis cinerea* to triazoles, benomyl and vinclozolin. Eur. J. Plant Pathol. 102: 171-180.

Stermitz, F. R., Lorenz, P., Tawara, J. N., Zenewicz, L., Lewis, K., 2000a. Synergy in a medicinal plant: antimicrobial action of berberine potentiated by 5`- methoyhydnocarpin, a multidrug pump inhibitor. Proc. Nat. Acad. Sci. USA 97: 1433-1437.

Stermitz, F. R., Tawara-Matsuda, J., Lorenz, P., Mueller, P., Zenewicz, L., Lewis, K., 2000b. 5`-Methoxyhydrnocarpin and pheophorbide a *Berberis* species components which potentiate berberine growth inhibition of resistant *Staphylococcus aureus*. J. Nat. Prod. 63: 1146-1149.

Tegos, G., Stermiz, F. R., Lomovskaya, O., Lewis, K., 2002. Multidrug inhibitors uncover remarkable activity of plant antimicrobials. Antimicrob. Agents Chemoth 46:3133-3141.

Zhou, T., Chu, C. L., Liu, W.T., Schneider, K.E., 2001. Postharvest control of blue mold and gray mold on apples using isolates of *Pseudomonas syringae*. Can. J. Plant Pathol. 23: 246-252.

CHAPTER 4

SUMMARY, CONCLUSIONS AND RECOMMENDATIONS

4.1 Introduction

The widely occurring plant pathogenic fungus, *Botrytis cinerea* is the causal agent of gray mold and infects fruits, flowers and green tissues of at least 235 plant species (Jarvis, 1997). Gray mold is one of the major pre-and postharvest fruit decay problems in the horticultural industry of South Africa. The control of gray mold disease and circumvention of resistance of the pathogen requires the use of a large number of different antifungal compounds as part of the management strategies to export quality horticultural produce to the European Union (EU), which represents one of South Africa's main export sectors with a total value of approximately € 740 million. Regulatory authorities in the EU countries have imposed rules, aimed at reducing the total volume of agrochemicals applied (Hirst, 1992). According to current EU regulations, some 400 important chemical compounds are to be effectively withdrawn from use on export crops through either total withdrawal or adoption of very low maximum residue limits (MRLs). Fungicides that give low or non-detectable residues are, therefore, actively sought in research programs especially compounds that rapidly degrade on plant surfaces, metabolize quickly in the plant, require use at very low rates, or act indirectly by promoting the plant defense mechanisms (Knight *et al*., 1997).

Plant extracts appears particularly promising in preventing fungal rots on different fruits and vegetables (Appleton and Tansey, 1975; Damayanti *et al*., 1996). However, plant extracts applied alone under commercial conditions are sometimes not sufficient to control post-harvest decay satisfactorly (Droby *et al*., 1998; Lima *et al*., 2003). Integrating plant extracts with other means of control in order to make their activity more reliable may

be the best option for large-scale application of extracts. Synergistic fungicide mixtures have proven to be extremely useful in this regard (Gisi *et al*., 1985; Sanders *et al*., 1985; Samoucha and Cohen, 1988). Notwithstanding studies proposing the combination of plant extracts with small quantities of chemicals, very little research has studied the potential of this integrated approach for the concomitant control of *B. cinerea* strains.

Plants produce an enormous array of secondary metabolites, and it is commonly accepted that a significant part of this chemical diversity serves to protect them against microbial pathogens (Dixon, 2001). These antimicrobial plant substances are classified as phytoanticipins, which are compounds that are present constitutively or phytoalexins, whose levels increase strongly in response to microbial invasion. Plant compounds are routinely classified as "antimicrobial" on the basis of susceptibility tests that produce minimal inhibitory concentrations (MICs) in the range of 100 to 1000 μg mL^{-1}, orders of magnitude weaker than those of typical antimicrobials produced by bacteria and fungi (MIC 0.01 to 10 μg mL^{-1}) (Tegos *et al*., 2002). A compound that is synthesized in response to pathogen invasion and is required to protect the plant from a pathogen, but that shows little activity in an *in vitro* susceptibility test is not necessarily an antimicrobial. Such substances might have a regulatory function, indirectly increasing the level of resistance of the plant. This analysis suggests that we lack a solid rationale for making a functional assignment for the vast majority of plant compounds that have been classified as antimicrobials.

Recent work, however, with berberine - a cationic alkaloid - offered a possible explanation for the apparent ineffectiveness of plant antimicrobial compounds (Stermitz *et al*., 2000 a; b). Berberine is a weak antimicrobial produced by a wide variety of plant species. It is an amphipathic cation that resembles quaternary ammonium antiseptics in its chemical properties and possibly in its mechanism of action as well. It was found that the medicinal plant species *Berberis* produce 5'-methoxyhydnocarpin-D, which acted in synergy with berberine (Stermitz *et al*., 2000 a; b; Tegos *et al*., 2002). Berberine,

accumulates in the cells of microbial pathogens, and the accumulation is driven by a membrane potential. The finding provides an important precedent for the idea that synergistic interactions among different compounds may explain the frequent failures to isolate single active substances from medicinal plants.

4.2 *In Vitro* and *In Vivo* Synergistic Studies

In considering the aforementioned factors, we hypothesize that South African medicinal plants contain compounds that can act in synergism with synthetic antifungal compounds. To test our hypothesis about the synergistic potential of South African medicinal plants *in vitro*, combinations of four fungicides, Sporekill™, Rovral™, Terminator™ and Teldor™ at dosages 0.1, 0.2, 0.4 and 0.8 mL L^{-1} with plant extracts of *G*. *africana*, *E*. *rhinocerotis* and *T*. *violacea* were tested for inhibitory effects on the radial growth of *B*. *cinerea* strains (PPRI 8507, 8506, 7338) inoculated on potato dextrose agar plates. Dose ranges for *G*. *africana* and *T*. *violacea* were 62.5, 125, 250 and 500 mg mL^{-1}; for *E*. *rhinocerotis* 50, 100, 200 and 400 mg mL^{-1}. The *in vivo* efficacies of the three fungicides, Sporekill™, Rovral™ and Terminator™ at doses of 0.01 mL L^{-1} alone and in combination with the different extract concentrations (1.95 to 62.5 mg mL^{-1}) of the plant species *G*. *africana*, *E*. *rhinocerotis* and *T*. *violacea* were assessed on cv. Granny Smith apples. After inoculation, the apples were stored for either 7 d at 20°C before decay assessment or 30 d at -0.5°C and moved to 15°C for 7 d. The percentage inhibition (%) for each treatment was calculated by comparison with a water control as follows: Inhibition (%) = (average of infected fruit in control – average of infected fruit in the treatment/ average of infected fruit in control) x 100. The synergistic ratio for percentage inhibition was based on the Abbott formula (Abbott, 1925) as described by Gisi (1996): expected efficacy of the mixture, Cexp = A + B – (AB/100) in which A and B are the control levels given by the “fungicide” and the “plant extract”, respectively. The synergy ratio (SR), between the observed (C_{obs}) and expected (C_{exp}) efficacies of the mixture is calculated as SR = C_{obs}/C_{exp}.

The results of the *in vitro* studies showed that mixtures between the fungicides and plant extracts were highly effective in reducing *Botrytis* radial growth compared to the individual compounds and several synergistic interactions were observed for the combination treatments. In contrast to the *in vitro* bioassays, the fungicide dose had to be reduced from 0.1 mL L^{-1} to 0.01 mL L^{-1} for the apple bioassays to obtain fruit decay levels of about 50% in order to observe improved inhibition levels and to calculate synergistic interactions. A series of lower two-fold plant extract doses from 1.95 to 62.5 mg mL^{-1} were combined with the fungicides since the plant extract doses of 125 to 500 mg mL^{-1} induced antagonistic interactions (high decay levels). Once the appropriate dose combinations were identified and applied, Botrytis decay was significantly reduced by mixtures of plant extracts and fungicides with several incidences of synergistic interactions, especially after the 7-d-20°C incubation period.

This study also showed that the *B. cinerea* strain PPRI 8507 expressed higher levels of resistance to the fungicides and plant extracts compared to strain PPRI 7338. Results of the 30-d storage period at -0.5°C showed that Botrytis decay development were primarily inhibited by the low temperature conditions, however, a general increase in decay was observed at the end of the 7-d (20°C) shelf-life storage period. Apples exported from South Africa must have a storage potential of at least 4 weeks (30 d) at low temperature. Therefore, testing storage effects plays an integral part in the success of the mixtures. Synergistic interactions were still observed at the end of the shelf-life storage period. This study also demonstrated a trend in that *G. africana* extracts showed synergistic effects at doses of 15.6 and 62.5 mg mL^{-1}, while *E. rhinocerotis* and *T. violacea* showed synergistic effects at lower doses of 1.9 and 7.8 mg mL^{-1}. A commercial-simulated laboratory drench trial showed that *G. africana* and Rovral™ combinations significantly inhibit Botrytis decay on apples, again with several synergistic interactions.

4.3 Preliminary Table Grape Field Trial

Field trials were conducted in the table grape vineyard at Nietvoorbij, Stellenbosch, South Africa cv. Waltham Cross in 2005/06 season. The experiments were planned as a complete randomised design with three random replicates. Sprays against *Botrytis* were applied at flowering, bunch closure, veráison and harvest. Pre-harvest spray applications of fungicides and plant extracts alone and in mixtures were applied with a mistblower (nozzle size 4). Fruit bunches at optimum ripeness were harvested into plastic picking lugs (27 X 35 X 53 cm) in the morning. Loose berries, small clusters, and damaged or rotten berries were removed. Eight packed commercial cardboard boxes with 5-8 bunches covered with or without SO_2 sheets were stored for 30 d at -0.5°C in regular atmosphere (RA) followed by 7 d shelf life at 15°C. At the end of the 30 d storage period, four cartons of each treatment were removed from -0.5°C for evaluation and four moved to storage at 15°C for 7 d to simulate shelf life. Bunches were evaluated for berry decay. Berry decay was identified as *B. cinerea* through signs of sporulation and the surface of the discoloured flesh that detached easily when touched. The percentage decay was calculated by the weight of decayed berries as a proportion of the cluster weight per carton.

% *Botrytis* decay control was calculated by expressing the percentage infection as a percentage of the water control such that decay control of the water control equals zero (% *Botrytis* decay control = 100 - (%infection/ %infection of the water control)*100). One-way analysis of variance was performed on percentage *Botrytis* infection (% *Botrytis* decay control) for each experiment separately, using the general linear models (GLM) procedure of SAS statistical software version 8.01. Results of experiments were also combined and investigated in one overall analysis of variance. The Shapiro-Wilk test was performed to test for normality (Shapiro, 1965). *Botrytis* infection % was transformed Ln(X+1) to obtain normality (Snedecor, 1980). Student's t-least significant difference was calculated at the 5% level to compare treatment means. A probability level of 5% was considered significant for tests. Table 4-1 summarizes the effects of the different treatments on

Botrytis decay development in table grapes. The fungicide Flint™ was applied in combination with plant extracts at half its commercially recommended dose. Rovral™ and Teldor™ doses of 0.01mL L^{-1} were applied in combination with plant extracts.

Table 4-1. Results of a preliminary field trial to evaluate control of *Botrytis cinerea* gray mold on table grapes cv. Waltham Cross stored in the presence or absence of sulfur dioxide (SO_2) at -0.5°C for 30 d and then 7 d at 15°C sprayed with mixtures containing Flint™ (trifloxystrobin), Teldor™ (fenhexamid) or Rovral™ (iprodione) and *Galenia africana*, *Tulbaghia violacea* or *Elytropappus rhinocerotis* plant extracts at different doses.

Treatment	Dose	Botrytis Decay Control (%)			
		30 Days -0.5°C + SO_2	7 Days 15°C + SO_2	30 Days -0.5°C - SO_2	7 Days 15°C - SO_2
***G. africana* (Ga)**	20 mg mL^{-1}	100^{a}	-258.7^{a}	38.6^{ab}	16.1^{d}
***T. violaea* (Tv)**	150 mg mL^{-1}	100^{a}	-93.5^{a}	55.7^{ab}	78.3^{ab}
***E. rhinocerotis* (Er)**	250 mg mL^{-1}	100^{a}	-1145.7^{b}	-84.1^{bc}	80.3^{ab}
Flint™[§]	15 g 100 L^{-1}	100^{a}	100^{a}	54.6^{ab}	93.5^{a}
Teldor™/Rovral™[§]	1 mL L^{-1} / 1 mL L^{-1}	100^{a}	100^{a}	47.7^{ab}	96.2^{a}
Ga + Teldor™/Rovral™	3.9 + 0.01 mL L^{-1}	100^{a}	-43.5^{a}	100^{a}	65.4^{bc}
Tv + Teldor™/Rovral™	31.3 + 0.01 mL L^{-1}	100^{a}	-1435^{a}	45.5^{ab}	92.2^{a}
Er + Teldor™/Rovral™	15.6 + 0.01 mL L^{-1}	100^{a}	-417.4^{a}	73.9^{ab}	49.9^{c}
Ga + Flint™	3.9 mL L^{-1}+ 7.5 g 100L^{-1}	100^{a}	100^{a}	-194.3^{c}	77.9^{ab}
Tv + Flint™	31.3 mL L^{-1}+ 7.5g 100L^{-1}	100^{a}	100^{a}	85.2^{ab}	67.9^{bc}

[§]Commercial dosages of fungicides Flint™, Teldor™ and Rovral™.

[a-d]Mean values followed by different letters within columns are significantly different ($p<0.05$), according to the Student's t Least Significant Different test.

In hindsight, it must also be recognized that the low fungicide dosage of 0.01 mL L^{-1} for the table grape trial was an unrealistic low concentration rate for a field application. The standard recommended doses for application of these fungicides in South Africa are 1

mL L^{-1} for Teldor™ and Rovral™. Reducing the recommended fungicide dose to at least 50 % or 25 % should, therefore, be considered in future studies. Botrytis decay did not develop during the low storage temperature (-0.5°C) conditions in the presence of SO_2 for all the treatments, except the control. The presence of SO_2 at the 7-d storage period seems to have had a negative effect in the Teldor™/Rovral™ and plant extract combination treatments compared to decay control effects in the absence of SO_2. Mixtures of Flint™ and plant extracts showed 100% decay control during the 7-d shelf-life storage at 15°C in the presence of SO_2 compared to the results in the absence of SO_2. Future *in vivo* laboratory and field trials will also assist in analysing results with more tangible conclusions in terms of storage time, temperature and SO_2 effects.

4.4 Preliminary Mode of Action Study

Morel *et al*. (2003) described plant compounds that do not possess antimicrobial activity themselves, but can potentiate known antimicrobials by inhibiting microbial multidrug resistant (MDR) pumps. A common mechanism of MDR is the overexpression of energy-dependent multidrug efflux pumps, also known as multidrug transporter proteins or P-glycoproteins (Del Sorbo *et al*., 2000). Recent data suggest that fungicide-resistant mutants and field strains of *B. cinerea* display MDR related to overproduction of specific ATP-binding cassette (ABC) transporters (Le Roux *et al*., 1999; Hayashi, *et al*., 2001). ABC transporters belong to a large protein family of drug transporters that reduce the intracellular accumulation of fungitoxic compounds. The combined use of fungicides with plant-derived non-toxic inhibitors of ABC transporters lowered the inhibitory dosage of these fungicides (Del Sorbo *et al*., 1998). Modulators known to reduce MDRs in tumour cells synergise the fungitoxic activity of the fungicide oxpoconazole, a sterol demethylation inhibitor against *B. cinerea* (Hayashi *et al*., 2003). Inhibitors of transcription of these genes would act as strong synergists of antimicrobials, which have lost their efficacy because of pathogen resistance. Based on the information above, we therefore

hypothesise that the synergistic effects observed between South African medicinal plant extracts and the fungicides are due to the inhibition of ABC transporter genes in the *B. cinerea* strains.

Harvested, germinating inocula of the *B. cinerea* wild-type (PPRI 7338) and ΔBcartrB mutant (obtained from Dr. Maarten de Waard, Wageningen University, The Netherlands) were exposed to plant extracts from *G. africana* (33 mg mL^{-1}), *E. rhinocotis* (7.8 mg mL^{-1}), and *T. violacea* (15.6 mg mL^{-1}), and the fungicide Rovral™ (0.01 mL L^{-1}) as well as combinations of the compounds for 60 minutes. The samples were then pelleted by centrifugation, flash frozen in liquid nitrogen and freeze-dried overnight. RNA was isolated from the freeze-dried *B. cinerea* wild-type and ΔBcartrB mutant samples treated with the different fungicide-plant extract mixtures using The One Step RNA Reagent (Biobasic Inc.) and the RNeasy Mini Kit (QIAGEN).

Total genomic DNA was isolated from *B. cinerea* and used as template to produce a probe for use in Northern blotting. The gene *Bcatr*B was selected to be the first gene of interest since it has been implicated in pathogen sensitivity to the plant defense compound, resveratol (Schoonbeek *et al*., 2001). The primers YP25 (5'–GACTCTCCACTCACCACAAGT-3') and YP31 (5'–AGACCCAGACACTTTACTCGCGG-3') were used to PCR amplify a 722 bp DIG-labelled *Bcatr*B-specific DNA probe according to the manufacturer's instructions (Roche Diagnostics, South Africa, Pty, Ltd). Five micrograms of total RNA was electrophoresed under denaturing conditions on a 1.2% agarose gel. The RNA was transferred to Hybond™ + nylon membrane by capillary transfer. The DIG Northern Starter kit (Roche Diagnostics, South Africa, Pty, Ltd) was used according to the manufacturer's instructions. Selected RNA samples were subjected to a two-step RT-PCR to determine whether *BcatrB* was being expressed. Contaminating DNA was removed from the samples using the DNA-free kit (Ambion) according to the manufacturer's instructions. Two micrograms of RNA was used as template for cDNA synthesis using the Retroscript Reverse transcriptase PCR kit (Ambion) according to the

manufacturer's instructions. Four microliters of the RT reaction were used as template for specific amplification of *Bcatr*B using primers YP25 and YP31. PCR products were separated by electrophoresis on a 1% agarose gel.

Neither of the two RNA isolation kits used consistently produced high quality RNA in sufficient quantities for Northern blotting. Hence, the influence of the fungicide-plant extract formulations on RNA quality was investigated. However, RNA quality and quantity were not appreciably improved by any of the changes made to the formulations. Further investigations into the influence of the formulations are required and other RNA isolation kits may have to be evaluated. Also, Inconclusive results were obtained from the Northern blots due to the high background hybridisation obtained. Figure 4-1 shows one of these blots, where faint positives were observed in lanes 4 and 6 which corresponded to treatment of the wild-type fungus with plant extracts from *G. africana* (33 mg mL^{-1}); and *E. rhinocerotis* (7.8 mg mL^{-1}), respectively. The band for the *E. rhinocerotis* extract is slightly more intense than that for the *G. africana* extract. However, no conclusions can be made until the Northern blot process can produce a clear result with minimal background hybridisation. Extensive troubleshooting was conducted to improve all aspects of RNA handling/isolation and Northern blotting. However, the intense background could not be eliminated.

The inconclusive Northern blot results prompted an investigation into *Bcatr*B expression in the wild-type fungus and Δ*Bcatr*B mutant using PCR. Figure 4-2 clearly shows that *Bcatr*B is not expressed in the wild-type control (lanes 2, 3, 4 and 6) or the Δ*Bcatr*B mutant treated with Rovral™ (0.01 mL L^{-1}) (lane 11), while it is expressed in response to treatment with Rovral™ (0.01 mL L^{-1}) (lanes 5, 7, 8 and 10). Despite the slow progress using the Northern blot technique, a result was obtained via RT-PCR. Although, not a quantitative result, it can be concluded that the *Bcatr*B gene in the wild-type fungus is functional and is induced in response to the fungicide, Rovral™. Taking into account the *in vivo* study results, the Rovral™ dosage of 0.01 mL L^{-1} was selected since it did not

completely inhibit fungal growth. Schoonbeek *et al*. (2001) found that the expression of *Bcatr*B is upregulated by treatment of *B. cinerea* germlings with the grapevine phytoalexin resveratrol and fungicide fenpiclonil. Therefore, it can be concluded that the expression of *Bcatr*B does indeed relate to the efflux of fungicides and thus decreases sensitivity of the fungal cells to lower Rovral™ doses. Subsequent combination of this Rovral™ dose with plant extracts increases the inhibitory efficacy against the fungicide. Further studies may elucidate the importance of plant extract compounds as inhibitors of *Bcatr*B and other ABC transporters.

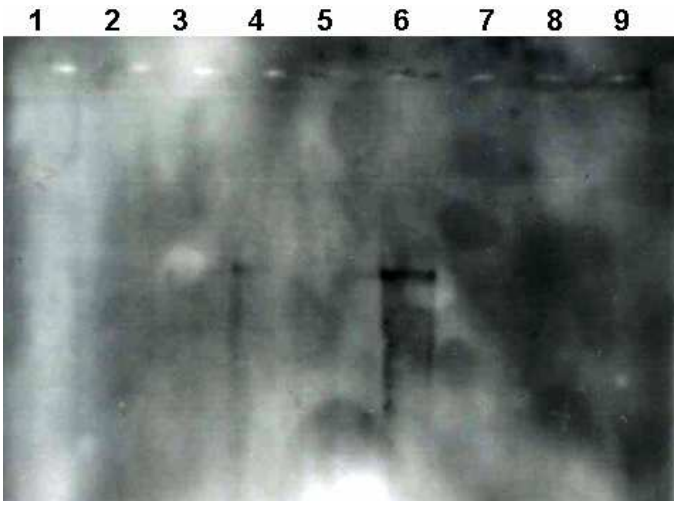

Figure 4-1. Northern analysis of *Bcatr*B expression in wild-type *Botrytis cinerea* (PPRI 7338) treated with different plant extract-fungicide formulations. Lanes **1:** water-treated control; **2:** methanol (0.01 mL L^{-1}); **3:** Rovral™ (0.01 mL L^{-1}); **4:** *Galenia africana* (33 mg mL^{-1}); **5:** *Tulbaghia violacea* (15.6 mg mL^{-1}); **6:** *Elytropappus rhinocerotis* (7.8 mg mL^{-1}); **7:** Rovral™ (0.01 mL L^{-1}) + *G. africana* (33 mg mL^{-1}); **8:** Rovral™ (0.01 mL L^{-1}) + *T. violaceae* (15.6 mg mL^{-1}); **9:** Rovral™ (0.01 mL L^{-1})+ *E. rhinocerotis* (7.8 mg mL^{-1}).

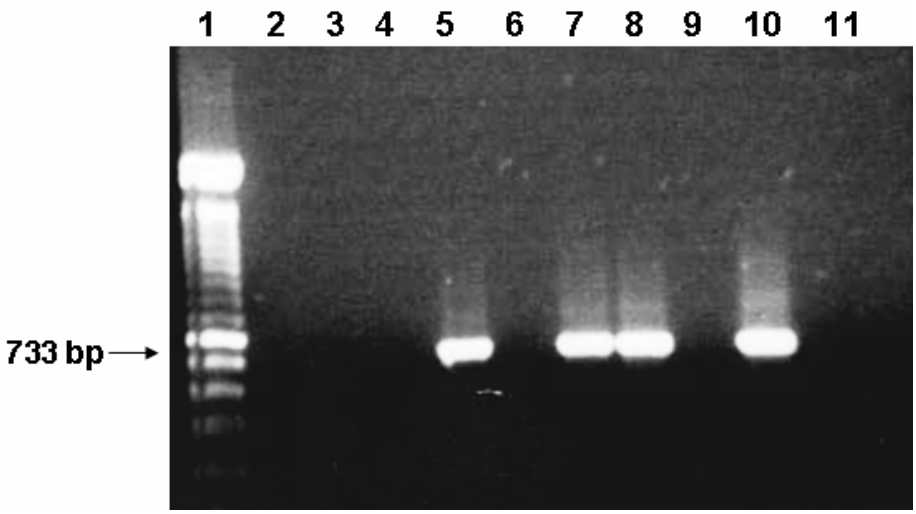

Figure 4-2. RT-PCR analysis of *Bcatr*B expression in *Botrytis cinerea* wild-type (PPRI 7338) and Δ*Bcatr*B mutant. Lanes **1:** 100 bp marker; **2:** wild-type treated with water, no DNase treatment and no RT control; **3:** wild type treated with water, DNase-treated and no RT control; **4:** wild-type treated with Rovral™ (0.01 mL L^{-1}), DNase-treated and no RT control; **5:** wild-type treated with water, DNase-treated RT-PCR; **6:** wild-type treated with water, no DNase treatment and no RT control; **7:** wild-type treated with water, DNase treated RT-PCR; **8:** wild-type treated with Rovral™ (0.01 mL L^{-1}), DNase-treated RT-PCR; **9:** wild-type treated with Rovral™ (0.01 mL L^{-1}), DNase-treated and no RT control; 1**0:** wild-type treated with Rovral™ (0.01 mL L^{-1}), DNase-treated RT-PCR; **11:** Δ*Bcatr*B mutant treated with Rovral™ (0.01 mL L^{-1}), DNase-treated, RT-PCR. Lanes with the same description refer to independently isolated RNA samples.

4.5 Conclusions

- This study showed that alcohol extracts from South African medicinal plant species produce compounds that potentiate the activity of fungicides *in vitro* and *in vivo*.
- Doses of the fungicides and plant extracts in the *in-vivo* studies had to be lowered in order to observe synergistic interactions.

- Storage temperatures and shelf-life incubation periods influenced Botrytis decay levels on fruit.
- Whether the potentiation effects are due to the inhibition of fungal multidrug resistant (MDR) pumps will require further studies at the molecular level.
- Knowledge of the ABC transporters open possibilities for developing novel strategies to control plant diseases, either by modulation of transporter activity or by transgenic expression of plant active genes in crops.
- Understanding of the biochemistry and regulatory mechanism of MDR pump inhibitors are important to alter the secondary metabolite profiles of plants by genetic engineering in the near future.
- Overall, the specific inhibitory effects of plant extracts are likely to be advantageous for developing new fungicide formulation and application strategies with low toxicity effects on the environment. This approach not only makes it possible to reduce fungicide concentrations while maintaining adequate decay control, but could also result in a reduction of the chemical residue on the fruit.

4.6 Recommendations

- The fungicide resistant characteristics of *B. cinerea* strain PPRI 8507 discovered in this study should make it suitable for further fungicide potentiation and MDR analyses experiments.
- The role of the fungicide mode of action when combined with plant compounds should be considered in future studies.
- Natural botanical products with fungicide potentiation effects will provide a competitive advantage to the South African deciduous fruit industry when the pesticide inputs and fungal resistance levels are effectively reduced through this

technology. This initiative will have a general positive impact on overall food safety and the environment.

- It is also evident that potential economic benefits associated with this technology relate to the domestication and conservation of indigenous, drought-tolerant plant species, particularly for the development of new cash crops for low-income rural farmers by adding multiple-values to indigenous plant species.
- Local manufacturing and marketing of plant extract products can create new opportunities for entrepreneurs to sustain businesses within the boundaries of South Africa.

4.7 References

Abbott, W.S., 1925. A method of computing effectiveness of an insecticide. J. Econ. Entomol. 18:265-267.

Appleton, J.A., Tansey, M.R., 1975. Inhibition of growth of 200 pathogenic fungi by garlic extract. Mycologia 67: 882-885.

Damayanti, M., Susheela, K.H., Sharma, G.J. 1996. Effect of plant extracts and systemic fungicide on the pineapple fruit-rotting frungus. Ceratostis Paradoxa. Cytobios. 86: 155-165.

Del Sorbo, G., Ruocco, M., lorito, M., Scula, F., Zoina, F., Andrade, A. C., De Waard, M. A., 1998. Potential for exploitation of ATP-binding cassette transporters in biological control. IOBC/wprs Bulletin 21: 241-246.

Del Sorbo, G., Schoonbeek, H-J., De Waard M. A., 2000. Fungal transporters involved in efflux of natural toxic compounds and fungicides. Fungal Genet. Biol. 30:1-15.

Dixon, R. A., 2001. Natural products and plant disease resistance. Nature 411: 843-847.

Droby, S., Cohen, A., Weiss, B., Horev, B., Chalutz, E., Katz, H., Keren-Tzur, M., Shachnai, A., 1998. Commercial testing of Aspire: a yeast preparation for the biological control of postharvest decay of citrus. Biol. Contr. 12: 97-100.

Gisi, U., Binder, H., Rimbach, E., 1985. Synergistic interactions of fungicides with different modes of action. Trans. Br. Mycol. Soc. 85: 299-306.

Gisi, U. 1996. Synergistic interaction of fungicides in mixtures. Phytopathology 86: 1273-1278.

Hayashi, K., Schoonbeek, H., Sugiura, H., De Waard, M. A., 2001. Multidrug resistance in *Botrytis cinerea* associated with decreased accumulation of the azole fungicide oxpoconazole and increased transcription of the ABC transporter gene BcatrD. Pestic. Biochem. Physiol. 70:168-179.

Hayashi, K., Schoonbeek, H., De Waard, M. A., 2003. Modulators of membrane drug transporters potentiate the activity of the DMI fungicide oxpoconazole against *Botrytis cinerea*. Pestic. Manag. Sci. 59:294-302.

Hirst, P., 1992. Pesticide reduction programs in Denmark, The Netherlands and Sweden. Inter. Eviron. Affairs 4:234-253.

Jarvis, W. R., 1977. *Botryotinia* and *Botrytis* species: taxonomy, physiology, and pathogenicity: a guide to the literature. Vol.15. Canada Department of Agriculture, Harrow.

Knight, S. C., Anthony, V. M., Brady, A. M., Greenland, A. J., Heany, S. P., Murray, D. C., Powell, K. A., Schultz, M. A., Spinks, C. A., Worthingtom, P. A., Youle, D., 1997. Rationale and perspectives on the development of fungicides. Ann. Rev. Phytopathol. 35:349-372.

Le Roux, P., Chapeland, F., Desbrosses, D., Gredt, M., 1999. Patterns of crossresistance to fungicides in *Botryotinia fuckeliana* (*Botrytis cinerea*) isolates from French vineyards. Crop Prot. 18:687-697

Lima, G.,De Curtis, F., Castoria, R., De Cicco, V., 2003. Integrated control of apple postharvest pathogens and survival of biocontrol yeasts in semi-commercial conditions. Eur. J. Plant Pathol. 109: 341-349.

Morel, C., Stermitz, F. R., Tegos, G., Lewis. K., 2003. Isoflavones as potentiators of Antibacterial activity. J. Agric. Food Chem. 51:5677-5679.

Samoucha, Y., Cohen, Y., 1988. Synergistic interactions of cymoxanil mixtures in the control of metalaxyl-resistant *Phytophthora infestans* of potato. Phytopathology 78: 636-640.

Sanders, P. L., Houser, W. J., Parish, P. J., Cole, H. Jr., 1985. Reduced-rate fungicide mixtures to delay fungicide resistance and to control selected turfgrass diseases. Plant Dis. 69: 939-943.

Schoonbeek, H., Del Sorbo, G., De Waard, M. A. 2001. The ABC transporter BcatrB affects the sensitivity of Botrytis cinerea to the phytoalexin resveratrol and the fungicide fenpiclonil. Mol. Plant. Microbial Inter. 14:562-571.

Shapiro, S.S., Wilk, M.B. 1965. An analysis of Variance Test for Normality (complete samples), Biometrika 52, 591-611.

Snedecor, G.W., Cochran, W.G.,1980. Statistical Methods, 7th Edition, The Iowa State University Press, Ames.

Stermitz, F. R., Lorenz, P., Tawara, J. N., Zenewicz, L., Lewis, K., 2000a. Synergy in a medicinal plant: antimicrobial action of berberine potentiated by 5`- methoyhydnocarpin, a multidrug pump inhibitor. Proc. Nat. Acad. Sci. USA 97: 1433-1437.

Stermitz, F. R., Tawara-Matsuda, J., Lorenz, P., Mueller, P., Zenewicz, L., Lewis, K., 2000b. 5`-Methoxyhydrnocarpin and pheophorbide a *Berberis* species components which potentiate berberine growth inhibition of resistant *Staphylococcus aureus*. J. Nat. Prod. 63: 1146-1149.

Tegos, G., Stermiz, F. R., Lomovskaya, O., Lewis, K., 2002. Multidrug inhibitors uncover remarkable activity of plant antimicrobials. Antimicrob. Agents Chemoth 46:3133-3141.