Synergistic effects of mixtures of the kresoxim-methyl fungicide and medicinal plant extracts *in vitro* and *in vivo* against *Botrytis cinerea*

by

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ABSTRACT

Synergistic effects of mixtures of the kresoxim-methyl fungicide and medicinal plant extracts *in vitro* and *in vivo* against *Botrytis cinerea*

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Key words: Apples, *Botrytis cinerea*, Grey mould, Kresoxim-methyl, Medicinal plants, Plant extracts, Strobilurin, Synergism.

The fungus *Botrytis cinerea* is an opportunistic pathogen on a wide variety of crops, causing a disease known as grey mould through infections via wounds or dead plant parts. Synthetic fungicides for controlling this disease are fast becoming ineffective due to the development of resistance. This, coupled with consumers world wide becoming increasingly conscious of potential environment and health problems associated with the build up of toxic chemicals, (particularly in food products), have resulted in pressure to reduce the use of chemical pesticide volumes as well as its residues.

An emerging alternative to random chemical synthesis is the study and exploitation of naturally occurring products with fungicidal properties. One group of compounds known as strobilurins produced by *Strobilurus* species, woodland basidiomycete fungi, is a good example of this phenomenon. Plants produce an enormous array of

secondary metabolites, and it is commonly reasoned that a significant part of this chemical diversity serves to protect plants against plant pathogens. A problem with plant-produced compounds as potential fungicides is that in the natural state, they are generally only weakly active compared to synthetic fungicides.

There have been reports on the uses of mixtures of synthetic fungicides for the control of plant pathogenic fungi. 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 provide an important precedent for the idea of synergism. For this study we hypothesize that the addition of plant extracts may enhance the antifungal efficacy of the synthetic strobilurin fungicide, kresoxim-methyl against *B. cinerea.* We selected South African medicinal plant species such as *Artemesia afra, Elyptropappus rhinocerotis, Galenia africana, Hypoxis hemerocallidea, Siphonochilus aetheopicus, Sutherlundia frutescence, Tulbaghia violacea* and *Tulbaghia alliaceae* for this study.

For the *in vitro* study, indigenous medicinal plant extracts were prepared at two-fold dilution concentrations and combined with kresoxim-methyl at concentrations of 0.25 and 0.5% (w/v). The *B. cinerea* mycelial plug assays showed potent antifungal inhibitory effects with the plant extract and kresoxim-methyl mixtures. Further analyses of the mixtures indicate synergistic effects between the fungicide and plant extracts. I surmise that these *in vitro* effects are also achievable *in vivo.* Combinations of these agents represent an attractive avenue for the development of new management strategies for controlling *B. cinerea* in the future.

A second study was conducted to analyse the final dose rates for synergistic reactions for combinations of kresoxim-methyl and medicinal plant extracts against *B. cinerea in vivo*. A series of two fold concentrations of medicinal plant extracts were combined with kresoxim-methyl to conduct decay inhibition studies on Granny Smith apples. Synergistic effects were observed for many of the kresoxim-methyl and plant extract combinations. I therefore came to the conclusion that indigenous South African plant species produce modulaters that potentiate the activity of fungicides. Whether these synergistic effects are due to the inhibition of fungal multi-drug resistant pumps require further studies at the molecular level. However, these inhibitory effects are likely to be advantageous for developing fungicide formulations 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 also ensures a reduction of the chemical residue on the fruit.

DECLARATION

I declare that "Synergistic effects of the mixtures of kresoxim-methyl fungicide and medicinal plant extracts *in vitro* and *in vivo* 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.

Full name:_____ Date:_____

Signed:_____

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Literature Review

Abstract

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rates for both components utilized in combinations or alone at normal rates. These studies provide an important precedent for the idea of synergism. For this study, it is hypothesized that the addition of plant extracts may enhance the antifungal efficacy of the synthetic strobilurin fungicide namely kresoxim-methyl, against *B.cinerea.* We selected South African medicinal plant species such as *Artemesia afra, Elyptropappus rhinocerotis, Galenia africana, Hypoxis hemerocallidea, Siphonochilus aetheopicus, Sutherlundia frutescence, Tulbaghia violacea* and *Tulbaghia alliaceae* for this study.

The pathogen *Botrytis cinerea*

The fungus *Botrytis cinerea* Pers.:Fr of *Botryotinia fuckeliana* (De Barry) Whetzel is pathogenic on a wide variety of crop plants (Schoonbeek *et al.,* 2001). The name *Botrytis* is derived from βοτυρυς, the Greek word for grape, since the fungus produces spores like bunches of grapes (Fig. 1). *B. cinerea* is the asexual stage anamorph of *Botrytonia fuckeliana*, which is the teleomorphic stage of the pathogen. The asexual stage of *B. cinerea* is classified in the genus *Botrytis*, which belongs to the family *Moniliaceae.* All pathogenic *Botrytis* species are necrotrophic, since plant cells are actively killed during pathogenesis (Prins *et al.*, 2000).

This fungus is very common in nature (Ellis, 2004). *B. cinerea* has a broad host range and can grow as a saprophyte (Schoonbeek *et al.*, 2001). The plant pathogen infects the fruits, flowers and/or green tissues of at least 235 plant species (Jarvis, 1997). Many of these are ornamental plants namely: anemone, begonia, calendula, chrysanthemum, dahlia, dogwood, fuchsia, geranium, hawthorn, heather, hydrangea, marigold, pansy, periwinkle, petunia, rose, snapdragon, sunflower, sweet peat, violet, zinnia to name a few. Among vegetables and fruit, *B. cinerea* can infect asparagus,

bean, beet, carrot, celery, chicory, crucifers, eggplant, endive, grape, lettuce, onion, pepper, potato, raspberry, rhubarb, rutabaga, shallot, strawberry, tomato, turnip, and others (Jarvis, 1997). Under favourable climatic and physiological conditions, it is capable of growing on all species of dicotyledons. *Botrytis* infections are favoured by cool, rainy spring and summer weather usually around 15°C. It can be particularly damaging when rainy weather continues over several days (Jarvis, 1997).

Several of these plant hosts of *B. cinerea* produce defense compounds belonging to various chemical classes, for example, phytoalexins, that act as constitutive or inducible chemical barriers. Phytoalexins are antimicrobial compounds of low molecular weight that are both synthesised by and accumulated in plant parts after exposure to microorganisms (Paxton, 1981). Considerable evidence supports the view that accumulation of phytoalexins at the site of attempted infection is one mechanism by which plants resist disease (Darvill and Albersheim, 1984). *B. cinerea* is able to withstand toxic effects of plant defense compounds with varying structures and mechanisms. The broad host range of *B. cinerea* most likely implies that the fungus possesses an arsenal of complementary infection factors. These include toxins, other hydrolytic enzymes, and mechanisms to cope with plant defense mechanisms (Prins *et al.* 2000). Phytotoxic compounds that play a role in pathogenesis are oxalic acid and botrydial (Colmenares *et al.* 2002). Fungal enzymes that may be involved in infection and tissue colonization are cell wall degrading enzymes such as endo- and exopolygalacturonases, endo- and exopectate lyases, pectin lyases, pectin methylesterases, and, furthermore, proteases and phenoloxidases. The broad host specificity of *B. cinerea* may relate to the presence of multiple, degenerate gene families encoding some of these enzymes. The presence of multiple

functional homologues could allow breakdown of cell wall tissues from various hosts under different conditions (Ten Have *et al.,* 1998). In contrast to many (hemi) biotrophic pathogens, cell death incited by *B. cinerea* might facilitate growth of the pathogen, for example by release of nutrients (Govrin and Levine, 2000). This strategy is especially effective since it operates in concert with enzymes involved in protection of the pathogen against oxidative stress such as peroxidase, laccases, catalases, gluthatione-S-transferase, glutathione peroxidase, and superoxidase dismutase (Gil-ad *et al.*, 2000).

The ability to withstand toxic effects of plant defence compounds that act as constitutive or inducible chemical barriers can also contribute to the virulence of fungi (Osbourn, 1999). *B. cinerea* posseses specific enzymes that degrade particular toxic plant compounds, such as α-tomatinase, that inactivates tomatin from tomato, leaves and a laccase-like enzyme that inactivates resveratrol from grapevine (Adrian *et al.,* 1998). The pathogen also possesses non-specific detoxification mechanisms such as glutathione-S-transferases and efflux pumps that can prevent the accumulation of multiple plant defense compounds in cells of the pathogen (Del Sorbo *et al.,* 2000). These non-specific mechanisms may be of particular relevance to *B. cinerea* since it has to cope with many, chemically unrelated, plant defense compounds in a broad host range (Schoonbeek, 2004).

Life cycle of *Botrytis cinerea*

The disease induced by the fungus *B. cinerea* in fruits (Fig. 1) is mainly described as grey mould and causes serious economic losses (Agrios, 1997; Schoonbeek *et al.*, 2001). The most favourable conditions for growth development of the fungus are warm temperatures and high humidity levels. On table grapes for example, at any time during the growing season it is most destructive after a rainfall period accompanied by warm temperatures either at flowering or late in the growing season. The disease spreads easily late in the season via airborne spores that are produced in abundance from infected berries. The disease develops as a moist rot on the berries and other fleshy parts of the vine, and at its latest stages is visible as a grey felt-like mat of spores (Schoonbeek *et al.*, 2001).

In wet conditions *B. cinerea* hyphae grow in young leaves to produce a characteristic V-shaped arc of dead brown tissue often with a yellow margin extending from the edge of the leaf into the main veins. Hail, insect or mechanical damage can allow entry of the pathogen *B. cinerea* into leaves resulting in irregular-shaped areas of dead tissue. During favourable infectious periods *B. cinerea* infects young succulent shoots causing soft brown spots. Infected shoots often break at the nodes revealing brown discoloration of the internal tissues. Shoots can be girdled at the point of infection causing their extremities to break, or wilt and die. When infection occurs at the base, entire shoots are killed (Built and Dubos, 1988). Individual flowers may carry latent infection and show signs of infection. Infected inflorescences develop brown, musty rot patches along the peduncle (main stalk) until the inflorescences completely rott (Built and Dubos, 1988). Latency and infection of

inflorescences are not necessarily sequential events, and can occur under different weather conditions. An early sign of infection in maturing grape berries is the formation of small circular water-soaked spots, which appear as distinct clear patches on red grapes. When rubbed, the skin over these areas cracks and slips freely revealing the inner pulp. This is known as the "slippery-skin" stage. Gradually berries go brown and soft especially after rain or high humidity. The characteristic grey masses of mycelium and conidia appear along the cracks in the skin. In compact bunches the rot can spread rapidly from berry to berry until entire bunches are rotted and covered with grey mould (Agrios, 1997; Built and Dubos, 1988).

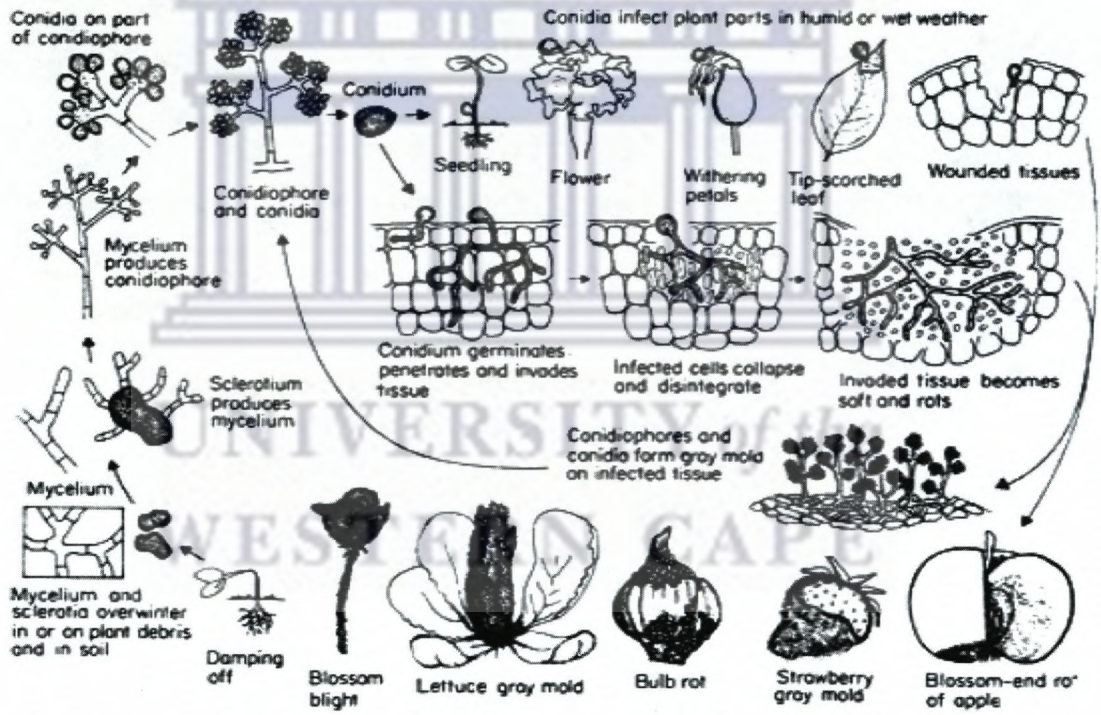

Fig. 1. Overview of the life cycle of *Botrytis cinerea*, adapted from Agrios (1997).

Disease control of *B. cinerea*

B. cinerea is an ubiquitous pathogen present in many agricultural crops in subtropical and temperate regions. Infections in crops require a minimal amount of

inoculum potential and, therefore, stringent disease control is required. Several approaches can be employed to limit crop losses caused by *B. cinerea* and related pathogens. These include breeding of plant varieties with reduced susceptibility, culture practices, biological control, and chemical control.

Plant breeding. Susceptibility and resistance to crop plants to *B. cinerea* appears to be a polygenic trait. This implies a considerable variation in *Botrytis* susceptibility amongst different varieties, which cannot be attributed to a single characteristic. A common strategy to breed for resistance is to cross susceptible crop plants with resistant relatives, to introduce quantitative trait loci associated with *B. cinerea* resistance. Another approach is the generation of transgenic crops that overproduce phytoalexins with a known activity against *B. cinerea*. Transgenic tobacco expressing the grapevine stilbene synthase produced resveratrol and displayed increased resistance (Hain *et al.*, 1993). This approach may also be valid for other crops, although transgenic expression of this gene in tomato did increase resistance to *Phytophtora infestans* but not to *B. cinerea* (Schoonbeek, 2004).

Culture methods. Good agricultural practices are very important in control of grey mould. A common practice is sanitisation to reduce sources of inoculum. This can be achieved by starting with clean material and keeping/ remaining pruned plant material away from the crop. This practice is particularly useful in greenhouses. Another important practice is to reduce the length of leaf wetness periods, which is essential for spore germination and penetration. This can be achieved by increasing plant distance, trimming of the canopy, ventilation, thereby controlling of temperature and humidity (Schoonbeek, 2004).

According to Hayashi (2003), the main strategies to reduce grey mould by culture methods under green house and field conditions are:

i. Reduction of the humidity by ventilation, lowering of the water supply, and temperature control.
ii. Decrease of inoculum by removing dead, decayed, or infected materials,
iii. Reduction of wounding by birds, insects, fungal infection, hail, and frost,
iv. Reduction of crop density in order to create an unfavourable microclimate for *B. cinerea,*
v. Limited nutrient conditions, and
vi. The use of UV films in greenhouse to prevent induction of conidia formation.

Consequently, in many crops culture practices cannot provide sufficient disease control.

Biological control and induced resistance. Biological control is based on the application of competitive or parasitic micro-organisms which may compete for space or nutrients, may produce antagonistic antibiotics or may hyperparasitize mycelium (Jakab *et al.*, 2001). For example, in practice successful results have been obtained with *Ulocladium atrum* which antagonize *B. cinerea* by competing for nutrients. The micro-organisms can also exert their function indirectly by stimulation of plant responses. Various signaling pathways are involved in the activation of induced resistance, such as systemic acquired resistance or induced systemic resistance. These pathways depend to different degrees on signaling molecules such as salicylic acid, ethylene, and jasmonic acid. The induction of systemic resistance can also be triggered by application of salicylic acid or its homologue, benzothiadiazole and β-

aminobutyric acid (Jakab *et al.*, 2001). Many microbial species have been investigated for biological control of *B. cinerea*. Although there are many successful reports, biological control is not yet a reliable method for *B. cinerea* control in practice. A general drawback of microbial biocontrol agents is that they do not maintain their activity in crops under field conditions that favour *B. cinerea*.

Chemical control. *B. cinerea* is one of the first recorded targets of chemical disease control ever. The Romans already applied elemental sulphur to control grey mould and mildew diseases in grapes. Fungicides are the main strategy for chemical control of gray mould (Nicot *et al.*, 2000). In 1950 non-systemic fungicides were introduced, but their specificity was low. From 1995 onwards, novel classes of fungicides with specificity against *B. cinerea* were commercialized. Resistance development to fungicides is due to the emergence of fungicide resistant mutants in wild-type populations upon selection pressure of fungicides in space and time (Schoonbeek, 2004). Chemical control is therefore of utmost importance, but have suffered in different crops due to the development of fungicide resistance in *B. cinerea* (Schoonbeek *et al.*, 2001).

Several chemical classes of novel botryticides, with specificity against *B. cinerea* have been developed since the 1950's when conventional fungicides such as aromatic hydrocarbons and dithiocarbons and dithiocarbamates, were introduced. These fungicides have a specific mode of action and have no systemic activity in plants (Hayashi, 2003). Although conventional fungicides such as chlorothalonil, dichlofluanid, or thiram are still used to control *B. cinerea*, most of these compounds are weak botryticides. Systemic fungicides such as benzimidazoles and

dicarboximides have also been used for grey mould control since the 1970's. During the last decade anilinopyrimidines, fenhexamid, fluazinam, phenylpyrroles, and strobilurins were introduced as new botryticides. However, their efficacy against *B. cinerea* is hampered by rapid emergence of resistance (Hayashi, 2003).

Chemical fungicides either kill the fungus itself (fungicidal products) or stop its growth (fungistatic products). In both cases, the fungicide attacks the biological structure (for example, the cell wall) or biological function (for example, protein synthesis) of the fungus. Over time, natural selection frequently occurs, with resistant strains of the fungus surviving and eventually replacing the strains that are susceptible to the fungicide. The fungicides become progressively less effective and must eventually be either modified or replaced by an entirely new fungicide.

Historically, chemical fungicides have proved they can be non-specific and therefore can act on organisms other than the target fungus, including other naturally occurring beneficial organisms. Because of their chemical nature, they may also be toxic and non-biodegradable. Chemical residues can build up in the soil and throughout the food chain. Consumers worldwide are becoming increasingly conscious of the potential environment and health problems associated with the build-up of toxic chemicals, particularly in food products. This has resulted in a growing consumer pressure to reduce the use of chemical pesticides. As a result, "organic" products or those resulting from sustainable production programmes- produced without the aid of chemicals- are increasingly perceived as more-healthy, more desirable and of premium value (Built and Dubos, 1988).

Strobilurin fungicides

Discovery of the fungicidal effects of lime-sulphur and Bordeaux mixture more than 100 years ago was the precursor of modern fungicide development. An emerging alternative to random chemical synthesis is the study and exploitation of naturally occurring products with fungicidal properties to identify new lead molecules (Ypema and Gold, 1991).

Several compounds have been isolated from inconspicuous woodland basidiomycetes. The best-known examples are oudemansin and strobilurin A produced by *Oudemansiella mucida* (Schrader: Fries) Höhnel and *Strobilurus tenacellus* (Pers. ExFr.). These compounds, strobilurins, presumably inhibit the establishment of competing fungi on substrates utilized by *O. mucida* and *S. tenacellus*. Several agrochemical companies have developed synthetic strobilurins as lead molecules (Ypema and Gold, 1991). In recent years strobilurins have become some of the most important active ingredients among fungicides used worldwide (Sauter, 2000). As with substances found in nature, strobilurins act as fungicides and offer effective and environmentally safe protection against almost all fungal diseases affecting cereals, fruit and vegetables, thus ensuring good crop yields. Strobilurins and other novel active ingredients play increasingly important roles in managing the development of fungicide resistance. They have a suppressive effect on other fungi, reducing competition for nutrients; they inhibit electron transfer within mitochondria and disrupt the metabolic processes of the target fungi (Bartlett, 2002). Well-known strobilurins are azoxystrobin, kresoxim-methyl, picoxystrobin, pyraclostrobin and trifloxystrobin.

The strobilurin fungicide, kresoxim-methyl, is the culmination of the use of synthetic methods to modify the chemical structure, and to improve the fungicidal activity and environmental stability of the naturally occurring strobilurin A. Special chemical and physical properties of kresoxim-methyl have demonstrated protective, post infection, and antisporulant activity against economically important fungal diseases. It is nontoxic to plants, mammals, birds, insects, and earthworms. The compound is nonirritating to eyes and skin, nonmutagenic, and nonsensitizing. It protects both upper and lower leaf surfaces from infection, and provides protection mainly on fungi that complete their life cycles deeper in the leaf or plant tissue. It has the greatest activity on fungal development stages that occur or are initiated on or just below the leaf surface (Ypema and Gold, 1991).

Many fungi have developed resistance to fungicides that once controlled them due to the fact that many fungicides are single-site inhibitors of fungal metabolism. Kresoxim-methyl offers an effective resistance management tool, because its efficacy against target pathogens is not affected by occurrence of strains resistant to other fungicides. Whether this resistance will ultimately affect fungicide performance depends on many factors, such as the relative fitness of these resistant strains, the reproduction and dispersal rate of the target fungus, the biochemical and biological mode of action of the fungicide, and the application regimes.

Strobilurin*s* prevent energy generation in the cell. This is carried out by mitochondria-small organelles, which act as power stations for the cell. Strobilurins act by competing with a natural molecule (co-enzyme Q) for a binding site on a protein known as cytochrome b. When the strobilurin is bound, the co-enzyme Q

cannot react-energy produced at this site is blocked (Holloman and Wood, 2003). Therefore kresoxim-methyl was produced and modified and specifically inhibits respiration by binding to the mitochondrial bc1 complex subsequently blocking electron transfer and ATP synthesis. Subsequently it is equally active in mitochondrial preparations from *B. cinerea* (Ypema and Gold, 1991).

Strobilurins represent the last currently known chemical structure that can provide any post-infection control (Koeller *et al.*, 2002). The fungicides arrest fungal development by interfering with a single metabolic pathway in the fungus, but resistance can develop if the target fungus develops mechanisms for bypassing the blocked pathway, as described above. In contrast, multi-site inhibitors disrupt several metabolic pathways in fungi, thereby making it difficult for the fungus to circumvent the action of the fungicide. The contact fungicides exhibit multi-site inhibition and they prevent spores from germinating on the surface of susceptible tissue, be it leaves or fruits. Once spores are allowed to germinate and to infect tissue beneath the surface, contact fungicides can no longer control the infections. The new strobilurins can however stop the fungus after the tissue has been infected. Therefore, these fungicides are effective when applied on post-infections in the absence of resistance.

Fungicide mixtures and synergism

Since the 1970's, fungicides with systemic and site-specific activity, sometimes called modern fungicides, have been used. Often modern fungicides have a relatively low environmental toxicity as compared to conventional fungicides, and can be applied at lower rates of active ingredients. Hence, they contribute to environmentaly safe agriculture. On the other hand, modern fungicides have caused resistance

problems (Leroux and Descotes, 1996). Countermeasures to avoid or delay the risk of resistance development are largely based on the use of fungicide mixtures. The main purpose of mixtures is extending the anti-fungal spectrum of commercial products. Mixtures can also extend the spray interval time. The use of mixtures, which show synergistic interaction may result in a reduction in amount of active ingredients and can also act as a countermeasure against resistance (Hayashi, 2003). By analogy, the combination of fungicidal derivatives with other antifungal or antimicrobial agents could also represent a possible approach (Marchetti *et al.*, 2000).

The process in which the presence of one chemical enhances the effects of the second is called a synergistic effect or synergy, and the chemical are sometimes described as showing synergism (Oxford English Dictionary). Potentiation is the enhancement of the action of one chemical by the presence of a second. It is not uncommon for the effects of two chemicals on an organism to be greater than the effect of each chemical individually, or the sum of the individual effects. The most obvious cases were observed for phenylamide fungicides in mixtures with mancozeb and cymoxanil against *Phytophthora infestants* (Mont) de Barry and *Plasmopara viticola* Berl and de Toni (Gisi *et al.,* 1985). Experimental synergists that show activity in *in vitro* experiments have been described frequently. The classical example is the synergism in mixtures of phosphoramidate and phosphorothiolate fungicides to *Pyricularia oryzae* Cav. The synergism has been ascribed to inhibition of phosphoramidate metabolism by a phosphorothiolate. The fungitoxic activity of mitochondrial bc1 complex inhibitors such as metominostrobin (Qo site inhibitor), and antimycin A (Qi site inhibitor) can be synergized in *B. cinerea, Cochliobolus miyabeanus* (Ito and Kurib.), *Monilinia fructicola* Wint. and *P. oryzae* by

salicylhydroxamic acid that inhibits the cyanide-intensive respiration pathway (Hayashi *et al.,* 1996). Mixtures of stereoisomers of the sterol biosynthesis inhibitors cyproconazole or tebuconazole also display synergism (Fuchs, 1998). This has been ascribed to the binding of the most active isomer to the $P450_{14DM}$ target site, whereas the less active isomers may saturate other P450s (Hayashi, 2003).

Synergistic interactions between components in a mixture can relate to one of the following mechanism: (a) non-mediated diffusion across the plasma membrane, (b) carrier-mediated transport across the plasma membrane, (c) energy-dependant efflux from the fungal cell, (d) 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 (Balan *et al.,* 1997). Synergism may be classified as pseudo-or true synergism. Pseudo-synergism occurs with compounds that affect the distribution of active ingredient on the plant surface, prevent run-off from leaves, or stimulate the uptake into plant tissue. True synergism is the case if components of a mixture directly react with each other, or if one of the compounds influences the physiology of the pathogen in such a way that it enhances the toxicity of the other compound (De Waard, 1996). Synergy is a frequent phenomenon in fungicide mixtures. Its magnitude depends on the ratio of the components in the mixture and their modes and mechanisms of action. It is affected by the sensitivity of microorganisms to fungicides and the composition of populations. Synergy may reduce the selection process of resistant subpopulations and allow a longer duration of fungicidal activity. Rates of fungicides can be reduced using synergistic mixtures without loosing efficacy. Synergistic interactions are most pronounced when components are used in split applications. Synergy may occur between antifungal

compounds of different natures and sources (including natural products) and between fungicides, herbicides, and insecticides with different or identical modes of action and in different formulations. Synergy may occur at the enzyme, fungus, disease, and/or epidemic level(s), depending on the type and timing of the fungicidal applications. Synergy may be expressed in different intensities during the stages of the disease cycle. The mechanisms of synergy are speculative and may be due to a combination of effects rather than to a single specific effect; decreased aggressiveness of the pathogen and increased concentration of components at the target site may be major effects (Gisi, 1996).

Since the advent of single-site inhibitory fungicides, there has been increased interest in the use of fungicide combinations for controlling plant-pathogenic fungi (Lorbeer, 1996). In recent years, combinations have been reported as synergistic when dosage rates of one or both of the components can be reduced without loss of effective control of both the target pathogen. Most modern fungicides are single-site inhibitors. 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 compared to the control achieved by either of the components utilized in combination or alone at normal rates. Such combinations also may simultaneously reduce the potential for development of resistance by the target pathogen to either component (Lorbeer, 1996).

Fungicides are often combined in mixtures for three main reasons: (1) to widen the spectrum of antifungal activity to control several diseases occurring simultaneously in a crop; (2) to exploit additive and synergistic interactions between

fungicides, by which the overall activity is increased and the concentrations of the compounds can be reduced without loss of activity; and (3) to delay the selection process of resistant individuals in a pathogen population to one component of the mixture. Many fungicide mixtures have been used for disease control for a long period of time without knowledge of the superior performance of the mixture compared with components used alone.

Fungicide mixtures are widely used in commercial products. The main advantages of mixtures are that they can extend the antifungal spectrum of the single products and delay resistance development to the individual component. Fungicide mixtures may also display a synergistic interaction by which the amount of active ingredients can be reduced (De Waard, 1987). If a synergist could annul the mechanism of resistance to a particular fungicide, the synergistic activity of a mixture would be limited to the fungicide-resistant subpopulation of a pathogen. Such synergists need not necessarily be fungitoxic by themselves and could be useful as an anti-resistance strategy (Hayashi, 2003). Deployment of mixtures of a multi-site inhibitor with a site-specific fungicide, are probably the best strategy to lower the risk of resistance development and widen the fungicidal spectrum (Hayashi, 2003).

Multidrug resistance in *Botrytis cinerea*

It is very well documented that micro-organisms through the years have proven to become resistant against antimicrobial compounds (Marchetti *et al.*, 2000). One of the major modes by which pathogens develop resistance is through the development or enhancement of methods for the removal of antibiotics that have entered the cells

of the organism. Thus, resistant microorganisms posses efficient systems known generally as multidrug resistant pumps (MDR-pumps) (Morel *et al.*, 2003).

Survival of micro-organisms 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 "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 big role in resistance to fungicides in *B. cinerea*, is decreased accumulation of the compound in mycelial cells due to energy-dependant efflux. Subsequently the driving force behind the energy –dependant efflux of the fungicides can be the ATP-binding cassette (ABC) transporters. Members of the major facilitator superfamily (MFS) transporters may have similar functions. They are membrane bound proteins. These transporters moderate the activity of several classes of toxic compounds. Some of these transporters can be regarded as fungicide pumps, which may account for the multi-drug resistance of *B. cinerea* (Schoonbeek *et al.*, 2001). ABC transporters use the energy of the ATP hydrolysis to transport compounds over membranes. They can move toxins from the inner leaflet of the

membrane to the outer environment of cells thereby reducing accumulation of the compound in the cells. 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 ATP-binding cassette (ABC) tranporters have been reported as synergists of drugs and fungicides against multi-drug resistant (MDR) tumour cells of mammals and demethylation inhibitor (DMI)-resistant fungi, respectively. Resistance to drugs can be 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 nutralized. 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 one can see that there is potential for plant-based compounds to develop 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 and cellular iron homeostasis, import 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 posses antimicrobial activity but can potentiate known antibiotics by inhibiting microbial MDR pumps.

Selected South African medicinal plant species.

Artemesia afra. *Artemisia afra* Jacq. (wormwood, wildeals) (*Asteraceae*) is a highly aromatic, erect multi-stemmed perennial shrub of up to two meters in height (Van Wyk *et al.,* 1997). The feathery leaves are finely divided and usually have a grayish-green colour, due to presence of fine hairs, up to 80mm long x 40mm wide. Flowers are borne along the branch ends. They are pale yellowish and inconspicuous. Aerial parts are deciduous in regions experiencing cold winters and the branches die back but rapidly regenerate from the base. The parts used are mainly the leaves and sometimes the roots. Wildeals as it is commonly known or African wormwood is widespread in all provinces of South Africa except the Northern Cape, also Lesotho, Swaziland and northwards into tropical Africa; usually in mountain habitats along forest margins and stream-sides.

"Wildeals" is one of the most widely used traditional medicines in South Africa. Numerous ailments are treated with it. These include mainly coughs, colds and influenza (Watt and Breyer- Brandwijk, 1962) – but also fever, loss of appetite, colic, headache, earache, malaria and intestinal worms, amongst others (Watt and Breyer-Brandwijk, 1962). The most common practice is to insert fresh leaves into the nostrils to clear blocked nasal passages. The roots, known as "inyathelo", are used to treat colds and fever (Van Wyk *et al.,* 1997).

Volatile oils, which contains mainly 1,8-cineole, *-thyjone, *-thyjone, camphor and borneol, has definite antimicrobial and anti-oxidative properties (Graven, 1992). Toxic and hallucinogenic effects have been associated with thujone, so that overdoses or continued use over long periods are potentially harmful (Arnold, 1989). Also

present are terpenoids of the eudesmadien-and germacratien types, as well as courmarins and acetylenes (Van Wyk *et al.,* 1997), but the contribution of these compounds to biological activity is not known. Pharmacological aspects of wildeals are decongestant and antibacterial effects of volatile oils are well known (Bruneton, 1995). In addition, narcotic, analgesic and antihistamine activity have been demonstrated in preliminary tests (Hutchings, 1996).

Elytropappus rhinocerotis. *Elytropappus rhinocerotis* (Lf) Less (*Asteraceae*) (Renosterbos) is a shrub, which is extremely common on the Cape coastal forelands. It is an erect bushy shrub of up to one meter in height. The minute, grayish-green leaves are tightly grouped on the thin stems. The tiny flower heads are inconspicuous, with a single floret in each. Together with several other fynbos species, it also occurs in isolated interior populations, confined to mesic high-altitude sites separated by large tracts of arid country (Levyns, 1935). Renosterveld, a distinctive veld type in some parts of the Western and Eastern Cape provinces, is named after this dominant and invasive species (Van Wyk *et al.,* 1997).

Infusions of the young branches in brandy or wine are a traditional Cape medicine for indigestion, dyspepsia, ulcers and stomach cancer (Watt and Breyer- Brandwijk, 1962). It may also be taken as tonic to improve a lack of appetite and as a stomach bitter (Cillie', 1992). Some reports claim it to have been a popular remedy during the 1918 influenza epidemic and that it stimulates perspiration (Watt and Breyer-Brandwijk, 1962). Some of the activity of the medicine may be due to rhinocerotinoic acid, a labdane diterpenoid which has been isolated from *E.*

rhinocerotis (Van Wyk *et al.,* 1997). Rhinocerotinoic acid has significant anti-inflammatory activity but tested negatively as an anti-arthritic.

Galenia africana. *Galenia africana* L., commonly known as "kraalbos" or "geelbos" belonging to the family Aizoaceae, is a dominant plant throughout Namaqualand and the Clanwilliam areas. In recent years *G. africana* has become more widespread in the Western and Southern Karoo (Kellerman *et al.,* 1988). "Kraalbos" is an aromatic, woody perennial sub-shrub, growing 0.5-1m high, having oppositely arranged green leaves (5cm long and hairless) which turn yellow with age. Inflorescence is born at the ends of the twigs and is 30-100mm long, with many small yellow flowers. The flowers are about 1.5mm in diameter, yellowish green and born in large loose heads. According to farmers, if the plant is green, it is palatable and not poisonous, but if it is yellow and dry it is poisonous and non-palatable (Le Roux *et al.,* 1994).

G. 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. Local farmers claim that during the summer months the "kraalbos" is highly poisonous. This is the time when the plant is dry, woody with yellow leaves and it is these leaves, which are highly toxic. They claim that the leaves contain a high level of acidic material and when eaten by animals the acids "eat away" the stomach lining. As the acids eat away the stomach lining it becomes thinner and water permeates through this thinner lining. This is the time that the

animal is unable to stand up again after lying down due to abdominal distension (Kellerman *et al.,* 1988).

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 veneral diseases, a decoction a 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).

Due to the severe drought and overgrazing, "kraalbos" is almost the only pasture plant on the farms in Namaqualand. Animals are forced to browse the plant and are more prone to develop clinical disease. 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 heptotoxic 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 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 un-altered or distorted by nodular hyperplasia, atrophy and/ or hypertrophy of certain parts (Kellerman *et al.,* 1988).

Hypoxis hemerocallidea. *Hypoxis hemerocallidea* (Fisch. and C. A. Mey) (African potato) *(Hypoxidaceae)* are tuberous perennials with long, strap-shaped leaves in three distinct groups, up to 30cm long x 3.2cm in width, folded from the midrib, distinctly ribbed, glabrous on the upper surface, and softly pilose on the margin and lower surface. It has yellow, star-shaped flowers. The species can be distinguished by the size, shape of the flowers. *H. hemerocallidea* has broad, slightly hairy leaves which are arranged one above the other to form three distinct groups spreading outwards from the centre of the plant. The tuberous rootstock (corm), which is dark brown or black on the outside and yellow within when freshly cut, is used. Its geographical distribution is common in grassland areas of the Eastern Cape Province, KwaZulu/Natal, Mpumalanga, Northern Province, Gauteng, Swaziland and Lesotho (Van Wyk *et al.,* 1997).

As traditional remedies, aqueous infusions are given to sickly children as a tonic, and to adults for dizziness, mental and bladder disorders, while fresh juice is applied to burn wounds (Buck, 1996). The stem and leaves are mixed with other ingredients to treat prostate problems (Pujol, 1990). Traditional users are also said to include

testicular tumors, prostate hypertrophy and urinary infections (Albrecht, 1996). *H. hemerocallidea* extracts have been used for some years in Europe as a remedy for BPH (benign prostatic hyperplasmia), bioactivity being ascribed to the sterol component. Additional claims, based on hypoxoside activity, have been made for its medical benefits in the treatment of cancer, HIV-AIDS and inflammation (Albrecht, 1996). Taken orally, this species is reputed to cause purging. In view of the sterol content of this species, its use during pregnancy should be undertaken with caution. The activity of the active ingredient against prostratic adenoma is ascribed to phytosterol glycosides, mainly β-sitosterol. Pumpkin oil, which contains high levels of phytosterols, is marketed in Europe for treatment of benign prostate hypertrophy (Bruneton, 1995). Anti-cancer, anti-HIV and anti-inflammatory activity is ascribed to rooperol (the aglycone of hypoxoside, which is the 4,4′-diglucoside) and the compound has showed promising results in clinical trials (Albrecht, 1996). The activity of sitosterol is ascribed to enzymatic effects (inhibition of 5*-reductase) or to decreased binding of dihydrotesterone within the prostate (Bruneton, 1995). Rooperol was shown to have several biological activities. It is markedly antimutagenic and cytotoxic to cancer cells (Albrecht, 1996).

Siphonochilus aethiopicus. *Siphonochilus aethiopicus* (Schweif.) BL Burt (*Zingiberaceae*), (wild ginger) is a deciduous plant with large, hairless leaves developing annually from a small, distinctive, cone-shaped rhizome. The spectacular flowers appear at ground level in early summer, from the end of October to early December. They are broadly funnel-shaped, pink and white in colour with a small yellow blotch in the middle (Smith., 1997; Onderstal., 1978; Gordon-Gray, 1989). Most plants are bisexual, and they have much larger flowers than female plants

(Gordon-Gray, 1989). The small, berry-like fruits are borne below or above the ground. The leaves and rhizomes have a smell similar to that of real ginger, *Zingibar officinale.* The rhizome, which is cone-shaped and narrows to a tapering point, is dug up and sold. Rhizomes harvested during the growing season will have roots on them and are used as such. Those taken during the dormant period, when the plants are leafless, have no roots on them. Wild ginger has a restricted distribution area, namely Mpumulanga and the Northern Province and has become extinct in KwaZulu-Natal (Van Wyk *et al.,* 1997).

The rhizomes of wild ginger are used for colds (to clear nasal passages), coughs, influenza and hysteria (Watt and Breyer-Brandwijk, 1962). It may also be taken for pain (Watt and Breyer-Brandwijk, 1962), including the treatment of asthma and dysmennorhoea. It is one of the most coveted medicinal plants in South Africa. The rhizomes have a characteristic pungent smell. Its use in the treatment of malaria and to relieve menstrual pains has also been reported in ethnobotanical literature.

It contains a volatile oil with *-terpineol (a natural antiseptic) and various other monoterpenoids, but the main compound is a highly characteristic sesquiterpenoid (Van Wyk *et al.,* 1997). The similarity between wild ginger and true ginger appears to be superficial only, as none of the terpenoids of ginger oil are present in the essential oil of *Siphonochilus.* The monoterpenoids and sesquiterpenoids in the oil are most likely responsible for the reported benefits in colds and influenza. Volatile oils are generally used for their decongestant, antiseptic and diuretic effects (Bruneton, 1995). The hydrodistilled oils of the roots and rhizomes were analyzed using GC-MS. Seventy compounds were identified in the root oils, which represents

88% of the total oil composition while 60 compounds (88% of total composition) were identified for the oils obtained from rhizomes. With the exception of minor quantitative variationsl the essential oil of the roots and rhizomes are virtually identical in composition. Essential oils, which are generally used for their decongestant properties, provide some rationale for the use of wild ginger in the traditional treatment of flu and coughs.

Sutherlandia frutescens. *Sutherlandia frutescens* (L.) R. Br. (*Fabaceae*) (Cancer bush) is an attractive small shrub of up to a meter in height. Leaves are slightly to densely hairy, often giving their plant a silvery appearance. Each leaf is divided into numerous small leaflets. The large red flowers are followed by characteristic bladder like, papery pods. The six species of *Sutherlandia* are difficult to tell apart and some are likely to be combined (Phillips and Dryer, 1934). The leaves are mainly used but all above ground parts are usually included. S. *frutescens* is one of five currently recognized *Sutherlandia* species, all of which are confined to Southern Africa, but are difficult to distinguish because they often grade into each other, and some botanists consider them to be merely different forms of a single large and variable species. Three of the species, namely *S. frutescens, S. microphylla* and *S. tomentosa* are probably, used interchangeably as kankerbos (Phillips and Dryer, 1934). The genus is restricted to Southern Africa, and occurs in South Africa, Botswana and Namibia. *S. frutescens* is widely distributed and shows remarkable regional variations. Some species have become popular garden plants in many parts of the world (Schrire and Andrews, 1992).

Cancer bush is an old Cape remedy for stomach problems and internal cancers (Dykman, 1891; Rood, 1994). It is said that to be a useful bitter tonic and a good general medicine. According to tradition, the virtues of the plant extend to include remedies for colds, influenza, chicken-pox, diabetes, varicose veins, piles, inflammation, liver problems, backache and rheumatism (Dykman, 1891; Rood, 1994). It is used as a bitter tonic or blood purifier. For the treatment of eye infections and wounds, as a douche for prolapse of the uterus.

Sutherlandia seeds contain canavanine, a non-protein α-amino acid (Bell, 1978). The plant is rich in amino acids and pinitol, but has small amounts of saponins and no alkaloids (Van Wyk *et al.*, 1997). Canavanine has antitumourigenic properties (Rood, 1994) and it is possible that some other amino acids are responsible for the reported benefits in treating cancer. It is also possible that the mechanism is one, which acts on the immune system but this is pure speculation. Research into anticancer and immunomodulatory activity of this species is currently in progress (Charlson, 1980). The presence of pinitol explains the traditional anti-diabetic use.

***Tulbaghia violacea* and *Tulbaghia alliacea*.** *Tulbaghia violacea* Harv. (Alliaceae) (wild garlic) is a small bulbous herb, with long, narrow, hairless leaves arising from several white, fleshy bases. The attractive mauve, pale purple or white little flowers occur in groups of about ten or more at the tip of a slender stalk (Van Wyk *et al.*, 1997). Indigenous to Natal, Transvaal and the Eastern Cape region in South Africa where it grows in rocky grasslands. The evergreen leaves of *T. violacea* exhibit a garlic-like smell when bruised and have been used in some cultures as a substitute for garlic and chive. The plant is known by several common names

including "society garlic", "sweet garlic" and "wild garlic". These names originated from the belief that, in spite of its garlic-like flavour, the consumption of *T. violacea* is not accompanied by the development of bad breath as is the case with the consumption of domesticated garlic, *Allium sativum. L* (Watson and Dallwitz, 1992).

T. violacea has been associated with the treatment of fever and colds, asthma, tuberculosis, and gastrointestinal ailments. *T. alliacea* (Lf) was an early Cape remedy for fever (Forbes, 1986) and is also used as a purgative and for fits, rheumatism and paralysis (Watt and Breyer-Brandwijk, 1962). Extracts are administered as enemas for stomach problems. The leaves have been used to treat cancer of the oesophagus (Watson and Dallwitz, 1992). Freshly harvested bulbs are boiled in water and the decoctions either taken orally or as an enema. The leaves may be eaten as vegetables (Van Wyk *et al.,* 1997).

However, extensive consumption of this plant has been associated with a variety of undesirable symptoms; such as abdominal pain, inflammation, and gastroenteritis. It has also been reported that society garlic deters moles, and that the Zulus of South Africa grow this plant around their homes to repel snakes (Kubec *et al.,* 2002). It is known for its antiseptic properties. Wild garlic may prove to have the same or similar antibacterial and antifungal activities as has been scientifically verified for real garlic (Bruneton, 1995). The latter also decreases blood cholesterol and has antihypertensive and antiplatelet effects (Van Wyk *et al.,* 1997).

Tulbaghia species contains marasmicin, with decomposing products, lots of which are degradation and unstable products. Many of these compounds have also shown to possess strong antimicrobial and antifungal activity as well as antithrombotic properties (Kubec *et al.,* 2002). *T. violacea* leaves seem to posses an exceptionally powerful garlic taste. Chewing and ingestion of the leaves seems normally to be accompanied by development of a strong breath that persists for several hours. When the leaves or the rhizomes are handled with bare hands, a strong organo-sulphur scent persists on the fingers for several hours. It is assumed that the odour producing compounds are decomposition products of marasmicin (Kubec *et al.*, 2002). The characteristic smell is due to a complex process in which sulphur containing compounds are broken down enzymatically when the plant is damaged. The main sulphur –containing substance in the intact garlic plant is alliin. The garlic-like smell of wild garlic is due to two sulphur compounds, but alliin does not seem to occur in the plant (Burton and Kaye, 1992).

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CHAPTER 2

Synergistic effects of the mixtures of kresoxim-methyl fungicide and medicinal plant extracts *in vitro* against *Botrytis cinerea*

Abstract

Botrytis cinerea is pathogenic on a wide variety of crop plants, and the disease caused by this fungus is known as grey mould. The disease can be controlled by cultural practices however, in many crops cultural practices cannot provide sufficient disease control. Synthetic fungicides for control of this diseae are fast becoming ineffective due to resistance. Development of resistance to fungicides is due to the emergence of fungicide resistant mutants in wild-type populations upon selection pressure of these fungitoxic compounds in space and time. This may be particularly relevant to *B. cinerea* that has a broad host range, consequently, often resulting in resistance or minimal inhibition when singularly applied antifungal agents are used. There have been reports on the uses of mixtures of synthetic fungicides for the control of plant pathogenic fungi and to overcome resistance developmnt. 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 the strobilurin fungicide kresoxim-methyl and medicinal plant extracts. Medicinal plant extracts were prepared at concentrations of 6.25, 12.5, 25 and 50% (w/v), and combined with kresoxim-methyl at concentrations of 0.25 and 0.5%. *In vitro* assays were performed. The *B. cinerea* mycelial plug assays showed potent antifungal inhibitory effects with the plant extract and

kresoxim-methyl mixtures. Further analyses of the mixtures indicate synergistic effects between the fungicide and plant extracts. Which should be studied *in vivo*. Combinations of these agents represent an attractive perspective for the development of new management strategies for controlling *B. cinerea* in the future.

Introduction

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 MIC's in the range of 100 to 1000 µg/ml, orders of magnitude weaker that those of typical antibiotics produced by bacteria and fungi (MICs 0.01 to 10 µg/ml) (Tegos *et al.*, 2002). Berberine, a cationic alkaloid, is a weak antimicrobial produced by a wide variety of plant species. Recent work with berberine from several *Berberis* medicinal plant species offered a possible explanation for the apparent ineffectiveness of plant antimicrobials (Stermitz *et al.*, 2000a; 2000b). It was found that *Berberis* plants produce 5`-methoxyhydnocarpin-D, which acted in synergy with berberine against human bacterial pathogens (Stermitz *et al.*, 2000a; 2000b; 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 (Tegos *et al.*, 2002). 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 providing a functional role for the vast majority of plant compounds that have been classified as antimicrobials.

Until now, the global use of fungicide mixtures with a synergistic action in practice is rather limited (De Waard, 1987). The main advantages of mixtures are that they can extend the antifungal spectrum of the single products and delay resistance development to the individual components. Mixtures can also extend the spray interval time (Hayashi *et al.*, 2003). The most obvious cases were observed for phenylamide fungicide in mixtures with mancozeb and cymoxanil against *Phytophthora infestans* (Mont) de Bary and *Plasmopara viticola* Berl & de Toni (Gisi *et al.*, 1985). Experimental synergists that show activity in *in vitro* experiments have been described frequently. The classical example is the synergism in mixtures of phosphoramidate and phosphorothiolate fungicides to *Pyricularia oryzae* Cavara. Mixtures of stereoisomers of the sterol biosynthesis inhibitors cyproconazole or tebuconazole also display synergism against *Botrytis cinerea* Pers.:Fr (Stehmann and De Waard, 1995, 1996). The combination of 2-chloro-N-(4,-chlorobiphenyl-2-yl) nicotinamide and kresoxim-methyl is considered an important instrument for resistance management and is ideal for integrated pest management programmes against *Uncinula necator* (powdery mildew) in grapes (BASF).

The findings above provide an important precedent for the idea of synergistic interactions between modern fungicides applied at lower rates of active ingredient and medicinal plant extracts. The main objective of this study was to determine whether different concentrations of kresoxim-methyl and methanolic extracts of medicinal plant species indigenous to South Africa inhibit the mycelial growth of *B. cinerea*. This study also identified the lowest concentrations of the plant extracts in combination with kresoxim-methyl that produce the best inhibitory effects *in vitro*.

Materials and Methods

Pathogen preparations. *Botrytis cinerea* was isolated from the surface of 'Granny Smith' apples infected with grey mould disease and maintained on potato-dextrose agar (PDA) at 25 °C. Fresh inoculum was prepared by transferring spores from stock cultures to PDA and incubating these at 25 °C in the dark under a white fluorescent light with a 12:12 light: dark photoperiod. The *B. cinerea* was grown on the PDA for 14 days before use.

Preparation of plant extracts. The plant parts of the following 10 medicinal plant species used in traditional medicine practices in South Africa were obtained for the preparation of plant extracts: *Artemisia afra* Jacq. (fresh leaves), *Allium sativum* (bulbs), *Elytropappus rhinocerotis* (Lf) Less (fresh leaves), *Galenia africana* L (dried leaves), *Hypoxis hemerocallidea* (Fisch. And C. A. Mey) (fresh corms), *Siphonochilus aethiopicus* (Schweif.) BL Burt (fresh rhizomes + fleshy roots), *Sutherlandia frutescens* (L.) R. Br. (dried leaves), *Tulbaghia alliacea* (Lf) (fresh corms), *Tulbaghia violacea* Harv. (fresh leaves + rhizomes) *T. violacea* from the Eastern Cape (fresh leaves + rhizomes). The voucher specimens of plants were identified at the University of the Western Cape Herbarium, Bellville, South Africa.

Weighed dried leaves were powdered in a hammer mill and extracted overnight in a closed container at room temperature in methanol (MeOH) to obtain 50% (weight/volume) stock concentrations. Fresh plant material was crushed in a Waring blender and extracted overnight at room temperature in MeOH to obtain 50% (weight/volume) stock concentrations. The 50% extracts was filtered through Whatman no. 4 qualitative filter paper and stored at 0 °C until used.

In vitro assay. Preliminary *in vitro* postharvest assays were performed with the medicinal plant extracts and kresoxim-methyl on PDA against *B. cinerea* to assist in the selection of the main experimental treatments for this study. All the assays conducted with mixtures of the medicinal plant extracts and kresoxim-methyl contained an increasing dose of the extracts and two fungicide concentrations. Preliminary *in vitro* studies showed that at 0.25 and 0.5% kresoxim-methyl provide the best *in vitro* inhibitory effects with plant extracts for mycelial plug studies with *B. cinerea*. The assays included controls containing sterile water and methanol (5%, v/v) without the compounds to be tested. Treatments and controls were performed simultaneously in the main experiment. For the main experiment each of the 50% plant extract stock solutions was added in final concentrations of 6.25%, 12.5%, 25% and 50% to final concentrations of 0.25 or 0.5% (2500 or 5000 ppm) kresoxim-methyl (Stroby WG, BASF South Africa (Pty) Ltd.).

A 1ml suspension of the plant extract and kresoxim-methyl as single or mixed concentrations were poured and spread evenly onto the PDA surface and allowed to dry in a laminar flow. Each plate was inoculated with three 3-mm mycelial plugs removed from the margins of actively growing 14-day-old *B. cinerea* cultures, and placed upside down on the PDA surface. Radial growth was assessed 5 days after incubation at 22-23°C.

Statistical Analysis. The experimental design was completely randomized. Each mycelial plug in a petri dish constituted a replicate. To assess differences in the mycelial growth of *B. cinerea* among the treatments, the percentage inhibition was calculated from the radial growth as: 100 – (treatment/control) X 100. All analyses were carried out using SAS version 8.2 (SAS, 1999).

Using the data for each entry, the shortfall test statistics was employed to determine if it could be the best entry (SAS, 1992; Van Aarde, 1994). Shortfall test statistic (q_i) was estimated by the formula $q_i = [Xmax|i - Xi / Sv]$. Where:

X_i = means for entry $_i$.

$Xm_{ax|I}$ = highest other mean

S_v = standard error of an entry mean, with $_v$ degrees of freedom.

The observed significance level (SL_i) was obtained via the SAS PROBMC function, estimated by the formula $SL_i = 1\text{-}p[q > Xm_{ax|i} - X / S_v]$ where q is the Many-One t Statistic: Dunnett's One-Sided Test.

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 kresoxim-methyl and the medicinal plant extract, respectively. The synergy ratio, R, 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, expected, or antagonistic, respectively.

Results

Single and combinations of different kresoxim-methyl and plant extract concentrations inhibit the mycelial growth of *B. cinerea*. The growth of *B. cinerea* mycelium from agar plugs was influenced by a significant interaction among kresoxim-methyl by plant type by plant extract concentration (Table 1). Therefore, the inhibitory effects of kresoxim-methyl and plant extract combinations were plotted (Fig. 1.A, B, C). For interpretation of the data, inhibition above 70% was used to indicate the best inhibitory kresoxim-methyl and plant extract concentration mixtures

(Fig. 1.A, B, C, and Table 2). Shortfall tests for all 144 treatments showed that significant level values for replications of 99 treatments below 70% were all $P > 0.05$ (Table 2). In the absence of kresoxim-methyl all the plant extract concentrations from 6.25 to 50%, except for *G. africana* extracts of 12.5, 25 and 50% produce inhibitory effects below 70% (Fig.1.A). In general, the mycelium growth of *B. cinerea* decreased with increased plant extract concentrations. The 0.25% kresoxim-methyl combination with *G. africana* at 12.5, 25 and 50% showed the best inhibition (82, 87, 84%) (Fig. B). The *E. rhinocerotis* extracts perform best at concentrations of 25 and 50%, while *A. sativum*, *S. aethiopicus*, *T. alliacea*, *H. hemerocallidea* and *T. violacea* were best at 12.5 and 25%, but not at 50%. In combination with 0.5% kresoxim-methyl, the best concentrations for *T. violacea* were 6.25, 12.5, 25 and 50% (Fig. C). *A. sativum* and *T. alliacea* were also part of the best combinations with 0.5% kresoxim-methyl over all the concentrations. *Elytropappus rhinocerotis* and *G. africana* performed better at the higher concentrations of 12.5, 25 and 50%, while *A. afra* and *H. hemerocallidea*, *S. frutescense* and *T. violacea* (EC) showed better performance at lower concentrations but not at 50%.

Overall, 12.5, 25 and 50% *G. africana* extracts produced the best inhibitory effects, irrespective of the kresoxim-methyl concentrations. At higher concentrations of 25 and 50% *E. rhinocerotis* showed the best inhibitory effects in combination with kresoxim-methyl 0.25 and 0.5%. Most of the other extract concentrations in combination with kresoxim-methyl showed best inhibition levels at variable levels, especially at extract concentrations of 25% and 0.5% kresoxim-methyl. Total inhibition (100%) was observed, for example, for the combination of 0.5% kresoxim-methyl and 25% *T. alliacea*.

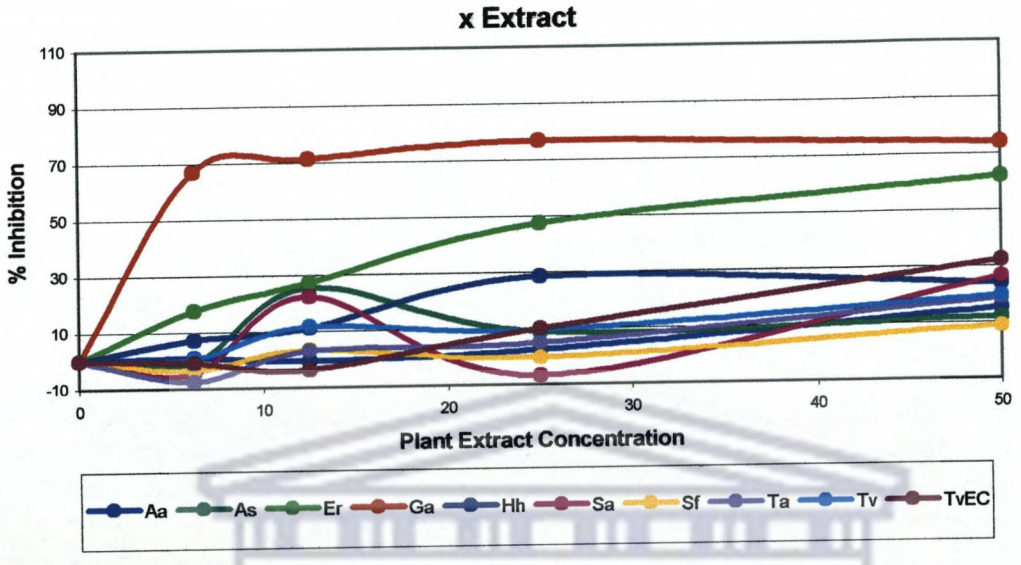
The Abbott formula showed that of the 79 mixtures in this study, 48 (61%) showed synergistic interactions that range from 1.0 to 7.9 (Table 3). All the combinations between kresoxim-methyl and *G. africana* extracts showed no synergism, with ratio's ranging between 0.5 and 0.9. *Siphonochilus aethiopicus* shows the highest synergism of 7.9 for the 0.25% kresoxim-methyl and 0.25% plant extract mixture. For this study, almost all the 50% plant extract mixtures with 0.25 and 0.5% kresoxim-methyl showed antagonistic interactions. All concentrations of *T. violacea* showed synergism ranging from 1.0- 3.4.

Table 1. Summary of the analysis of variance for mycelial growth inhibition of a *Botrytis cinerea* isolate, showing sum of squares (SS) mean squares (MS) and significant levels for main effects of kresoxim-methyl (K-M) and medicinal plant extract concentrations, and all interactions involving combinations of kresoxim-methyl and plant extracts.

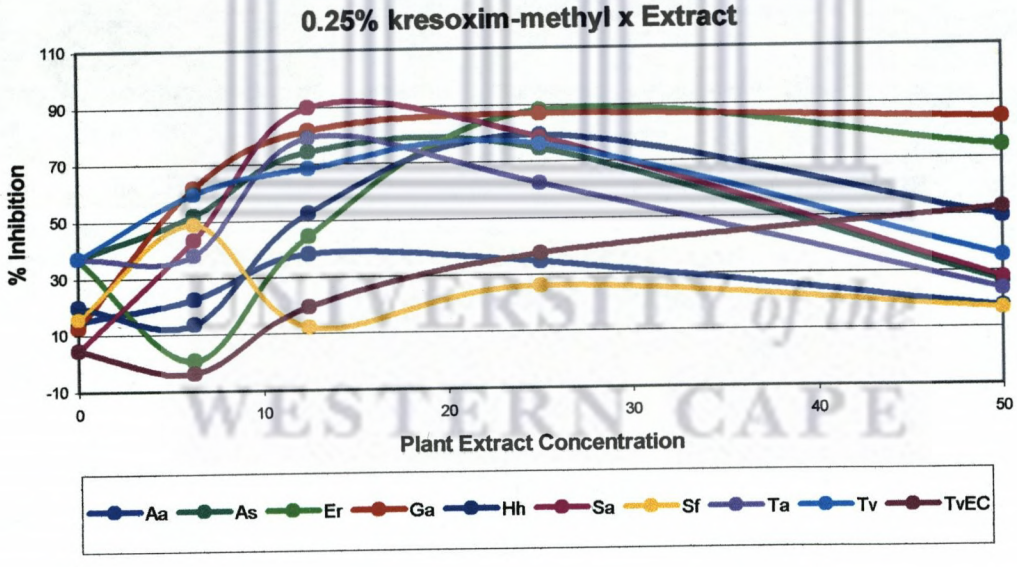
Source[a]	df	SS	MS	F-value	Pr>F
K-M	2	422324.923	211162.462	768.162	0.0000
Extract (1)	47	344437.805	7328.464	26.659	0.0000
Control vs Extracts (2)	8	44043.078	5505.385	20.027	0.0000
Plant species (3)	9	147324.712	16369.413	59.548	0.0000
Extract concentrations (4)	3	75565.273	25188.424	91.630	0.0000
3*4	27	77504.742	2870.546	10.442	0.0000
K-M x 1	94	291558.384	3101.685	11.283	0.0000
K-M x 2	16	14980.193	936.262	3.406	0.0000
K-M * 3	18	169929.126	9440.507	34.343	0.0000
K-M *4	6	35788.338	5964.723	21.698	0.0000
K-M *3*4	54	70860.728	1312.236	4.774	0.0000
Error	821	225687.207	274.893		
Corrected total	964	1284008.319			

[a] (1) = total number of 48 plant extract concentrations tested in experiment, (2) = Each plant extract tested against a control (3) = Extracts from 10 medicinal plant species, (4) = Four concentrations per plant extract tested.

Fig. 1



A



B

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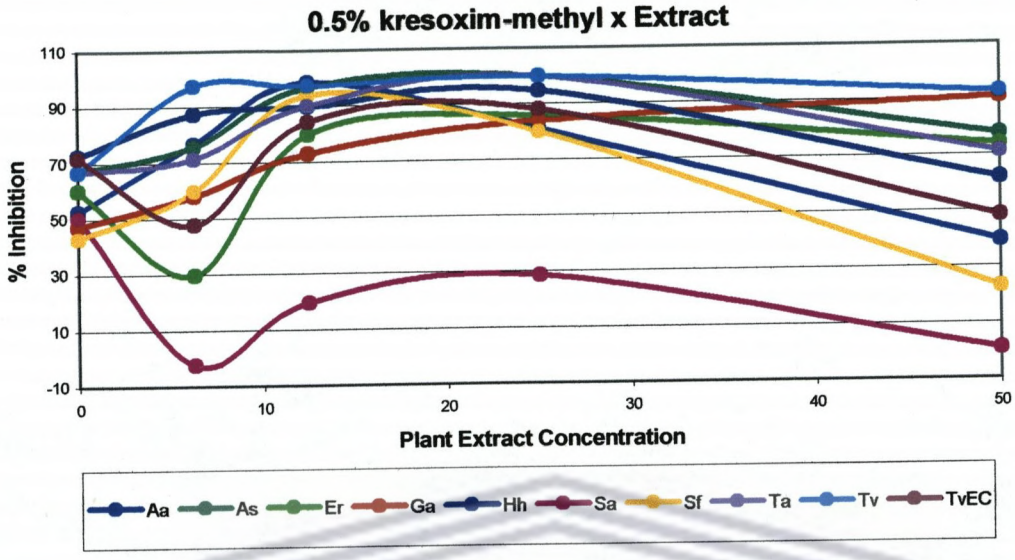
Fig. 1 (cont.)



C

Fig. 1. Effect of kresoxim-methyl fungicide concentrations (**A**) 0, (**B**) 0.25 and (**C**) 0.5 and different plant extract concentrations on the growth rate of *Botrytis cinerea* in radial growth experiments. Plant species used for extracts were: Aa = *Artemisia afra*, As = *Allium sativum*, Er = *Elytropappus rhinocerotis*, Ga = *Galenia africana*, Hh = *Hypoxis hemerocallidea*, Sa = *Siphonochilus aethiopicus*, Sf = *Sutherlandia frutescens*, Ta = *Tulbaghia alliacea*, Tv = *Tulbaghia violacea*, TvEC = *Tulbaghia violacea* (Eastern Cape).

Table 2. Employment of the shortfall test statistic (q_i) to determine the best entries for kresoxim-methyl and plant extract mixtures on the radial growth of *Botrytis cinerea* with the cut off point at 70% inhibition.

Kresoxim-methyl concentration (%)	Plant species[a]	Extract concentration (%)	no.	Inhibition (%)	Shorfall test [b]	Significance level [c]
0.5	Ta	25.0	6	100.00	0	0.9952
0.5	Tv	25.0	6	100.00	0	0.9952
0.5	As	25.0	6	99.82	0.0187	0.9948
0.5	Aa	12.5	9	98.48	0.174	0.9974
0.5	Tv	6.25	6	97.50	0.2612	0.9868
0.5	Tv	12.5	6	97.50	0.2612	0.9868
0.5	As	12.5	6	96.78	0.3358	0.9827
0.5	Hh	25.0	9	94.74	0.6023	0.9835
0.5	Sf	12.5	9	93.91	0.6967	0.9765
0.5	Tv	50.0	6	92.50	0.7835	0.9315
0.5	Ga	50.0	9	90.76	1.0572	0.9251
0.25	Sa	12.5	9	90.03	1.1413	0.9056
0.5	Ta	12.5	6	90.00	1.0447	0.8697
0.5	Hh	12.5	9	89.82	1.1645	0.8996
0.25	Er	25.0	9	88.15	1.3563	0.8396
0.5	TvEC	25.0	9	88.10	1.3619	0.8376
0.5	Hh	6.25	9	87.25	1.4589	0.7997

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Table 2 (cont.)

0.25	Ga	25.0	9	86.95	1.4931	0.7853
0.5	Er	25.0	9	85.80	1.5247	0.7245
0.5	TvEC	12.5	9	84.40	1.785	0.6413
0.25	Ga	50.0	9	84.29	1.7983	0.6340
0.5	Ga	25.0	9	83.81	1.8528	0.6038
0.25	Ga	12.5	9	81.81	2.0817	0.4723
0.5	Aa	25.0	9	80.89	2.1869	0.4138
0.5	Sf	25.0	9	80.04	2.2842	0.3608
0.5	Er	12.5	9	79.75	2.317	0.3436
0.25	Hh	25.0	9	79.30	2.3691	0.3169
0.25	Ta	12.5	6	79.29	2.164	0.3482
0.25	Sa	25.0	9	78.75	2.4314	0.2862
0.5	As	50.0	6	77.50	2.3505	0.2622
0	Ga	25.0	3	76.86	1.974	0.3066
0.5	Aa	6.25	9	76.66	2.6715	0.1840
0.25	Tv	25.0	6	76.07	2.4997	0.2025
0.5	As	6.25	6	75.71	2.5371	0.1891
0.25	As	25.0	6	74.64	2.649	0.1522
0.25	As	12.5	6	74.46	2.6676	0.1465
0.25	Er	50.0	9	74.44	2.9245	0.1058
0	Ga	50.0	3	74.00	2.2177	0.2127
0.5	Er	50.0	9	73.70	3.0093	0.0862
0.5	Ga	12.5	9	72.95	3.0953	0.0692
0.5	Hh	0	6	72.46	2.8774	0.0929

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Table 2 (cont.)

0.5	TvEC	0	6	71.75	2.9511	0.0781
0	Ga	12.5	3	71.43	2.4371	0.1450
0.5	Ta	6.25	6	71.25	3.0034	0.0688
0.5	Ta	50.0	6	71.07	3.0221	0.0657
0.25	Tv	12.5	6	68.57	3.2832	0.0328

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[a] Aa = *Artemisia afra*, As = *Allium sativum*, Er = *Elytropappus rhinocerotis*, Ga = *Galenia africana*, Hh = *Hypoxis hemerocallidea*, Sa = *Siphonochilus aethiopicus*, Sf = *Sutherlandia frutescens*, Ta = *Tulbaghia alliacea*, Tv = *Tulbaghia violacea*, TvEC = *Tulbaghia violacea* (Eastern Cape).

[b] Shortfall test statistic (q_i) estimated by the formula $q_i = [Xm_{ax|i} - X / S_v]$

[c] The observed significance level (SL_i) was obtained via the SAS PROBMC function, estimated by the formula $SL_i = 1\text{-}p[q > Xm_{ax|i} - X / S_v]$ where q is the Many-One t Statistic: Dunnett's One-Sided Test (Variance estimate is 274.8930 with 821 df).

Table 3. Employment of the synergy ratio tests to determine the best entries for kresoxim-methyl (K-M) and plant extract mixtures on the percentage inhibition of decay *in vitro* by *Botrytis cinerea*.

K-M (%)	Plant species[a]	Extract conc (%)	Decay inhibition (%)	Means followed by the same letters in the column are not significantly different at P>0.05 according to the Student t-Least Significant Difference test	Synergy Ratio [b]
0.25			14.8	KLMNOPQRSTUV	
0.25			37.0	ZABCDEFGH	
0.25			12.9	LMNOPQRSTUVW	
0.25			20.0	GHIJKLMNOPQ	
0.25			4.7	PQRSTUVWXY	
0.25			15.7	JKLMNOPQRSTU	
Mean	**17.5**				
0.5			52.4	RSTUVWXYZ	
0.5			60.0	OPQRSTUVWX	
0.5			47.1	WXYZABC	
0.5			72.5	HIJKLMNOPQ	
0.5			50.4	TUVWXYZA	
0.5			42.8	XYZABCDE	
Mean	**54.2**				
0	Aa	6.25	0.9	RSTUVWXY	
0	Aa	12.5	-0.5	TUVWXY	
0	Aa	25	2.6	QRSTUVWXY	
0	Aa	50	15.3	KLMNOPQRSTUV	
0.25	Aa	6.25	22.6	FGHIJKLMNOP	1.2

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Table 3 (cont.)

0.25	Aa	12.5	38.3	ZABCDEFG		2.3
0.25	Aa	25	34.6	ZABCDEFGHIJ		1.7
0.25	Aa	50	17.2	IJKLMNOPQRST		0.5
0.5	Aa	6.25	76.7	EFGHIJKLMNOPQ		1.4
0.5	Aa	12.5	98.5	ABC		1.9
0.5	Aa	25	80.9	BCDEFGHIJKLM		1.4
0.5	Aa	50	39.3	YZABCDE		0.6
0	As	6.25	-0.4	STUVWXY		
0	As	12.5	25.7	DEFGHIJKLMNO		
0	As	25	8.9	NOPQRSTUVWXY		
0	As	50	12.1	LMNOPQRSTUVWX		
0.25	As	6.25	52.0	TUVWXYZ		3.1
0.25	As	12.5	74.5	GHIJKLMNOPQ		1.8
0.25	As	25	74.6	GHIJKLMNOPQ		2.9
0.25	As	50	26.4	DEFGHIJKLMN		0.9
0.5	As	6.25	75.7	FGHIJKLMNOPQ		0.8
0.5	As	12.5	96.8	ABCD		1.2
0.5	As	25	99.8	AB		2.3
0.5	As	50	77.5	EFGHIJKLMNOP		1.2
0	Er	6.25	17.8	IJKLMNOPQRST		
0	Er	12.5	27.6	DEFGHIJKLMN		
0	Er	25	47.4	WXYZABC		
0	Er	50	62.0	MNOPQRSTUVW		

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Table 3 (cont.)

0.25	Er	6.25	0.7			RSTUVWXY	0.0
0.25	Er	12.5	44.7		WXYZABCD		1.0
0.25	Er	25	88.1	ABCDEFGHI			1.4
0.25	Er	50	74.4	GHIJKLMNOPQ			0.9
0.5	Er	6.25	29.6			BCDEFGHIJKL	0.4
0.5	Er	12.5	79.8	CDEFGHIJKLMN			1.0
0.5	Er	25	85.8	ABCDEFGHIJK			0.9
0.5	Er	50	73.7	GHIJKLMNOPQ			0.6
0	Ga	6.25	67.1	KLMNOPQRSTU			
0	Ga	12.5	71.4	IJKLMNOPQR			
0	Ga	25	76.9	EFGHIJKLMNOPQ			
0	Ga	50	74.0	GHIJKLMNOPQ			
0.25	Ga	6.25	61.7	NOPQRSTUVWX			0.7
0.25	Ga	12.5	81.8	ABCDEFGHIJKL			0.9
0.25	Ga	25	87.0	ABCDEFGHIJ			0.9
0.25	Ga	50	84.3	ABCDEFGHIJKL			0.9
0.5	Ga	6.25	58.1		QRSTUVWXY		0.5
0.5	Ga	12.5	73.0	HIJKLMNOPQ			0.6
0.5	Ga	25	83.8	ABCDEFGHIJKL			0.6
0.5	Ga	50	90.8	ABCDEFGH			0.7
0	Hh	6.25	7.2			OPQRSTUVWXY	
0	Hh	12.5	11.4			LMNOPQRSTUVWXY	
0	Hh	25	28.6			CDEFGHIJKLM	
0	Hh	50	24.0			EFGHIJKLMNO	

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Table 3 (cont.)

0.25	Hh	6.25	13.7		KLMNOPQRSTUVW	0.6
0.25	Hh	12.5	52.6	RSTUVWXYZ		1.9
0.25	Hh	25	79.3	DEFGHIJKLMN		1.8
0.25	Hh	50	49.3	UVWXYZA		1.2
0.5	Hh	6.25	87.3	ABCDEFGHIJ		1.4
0.5	Hh	12.5	89.8	ABCDEFGHI		1.4
0.5	Hh	25	94.7	ABCDE		1.2
0.5	Hh	50	61.6	NOPQRSTUVWX		0.8
0	Sa	6.25	-4.6		WXY	
0	Sa	12.5	22.6		FGHIJKLMNOP	
0	Sa	25	-6.8		XY	
0	Sa	50	26.3		DEFGHIJKLMN	
0.25	Sa	6.25	43.5	WXYZABCD		3.5
0.25	Sa	12.5	90.0	ABCDEFGHI		2.3
0.25	Sa	25	78.8	DEFGHIJKLMNO		7.9
0.25	Sa	50	27.5		DEFGHIJKLMN	0.6
0.5	Sa	6.25	-2.4		UVWXY	0.1
0.5	Sa	12.5	19.6		GHIJKLMNOPQR	0.3
0.5	Sa	25	28.4		CDEFGHIJKLM	0.6
0.5	Sa	50	0.7		RSTUVWXY	0.0
0	Sf	6.25	-3.4		VWXY	
0	Sf	12.5	3.4		QRSTUVWXY	
0	Sf	25	-0.2		STUVWXY	
0	Sf	50	8.8		NOPQRSTUVWXY	

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Table 3 (cont.)

0.25	Sf	6.25	49.2		UVWXYZA		3.6
0.25	Sf	12.5	12.4			LMNOPQRSTUVW	0.6
0.25	Sf	25	26.1		DEFGHIJKLMNO		1.5
0.25	Sf	50	16.8			IJKLMNOPQRST	0.6
0.5	Sf	6.25	59.7		PQRSTUVWX		1.2
0.5	Sf	12.5	93.9	ABCDEF			1.7
0.5	Sf	25	80.0	CDEFGHIJKLMN			1.5
0.5	Sf	50	22.8			FGHIJKLMNOP	0.4
0	Ta	6.25	-7.5				Y
0	Ta	12.5	3.2				QRSTUVWXY
0	Ta	25	5.0				PQRSTUVWXY
0	Ta	50	18.6			HIJKLMNOPQRS	
0.25	Ta	6.25	38.0		ZABCDEFG		4.0
0.25	Ta	12.5	79.3	DEFGHIJKLMN			4.0
0.25	Ta	25	62.3		MNOPQRSTUVW		2.8
0.25	Ta	50	23.6			FGHIJKLMNOP	0.7
0.5	Ta	6.25	71.3		IJKLMNOPQRS		1.6
0.5	Ta	12.5	90.0	ABCDEFGHI			1.6
0.5	Ta	25	100.0	A			1.7
0.5	Ta	50	71.1		IJKLMNOPQRS		1.0
0	TvE	6.25	-0.9				TUVWXY
0	TvE	12.5	-3.6				VWXY
0	TvE	25	10.1				MNOPQRSTUVWXY
0	TvE	50	32.2		ABCDEFGHIJK		

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Table 3 (cont.)

0.25	TvE	6.25	-3.6	VWXY		0.2
0.25	TvE	12.5	19.5	GHIJKLMNOPQR		1.4
0.25	TvE	25	37.6	ZABCDEFG		1.4
0.25	TvE	50	52.4	STUVWXYZ		1.1
0.5	TvE	6.25	47.7	WXYZAB		0.9
0.5	TvE	12.5	84.4	ABCDEFGHIJKL		1.7
0.5	TvE	25	88.1	ABCDEFGHI		1.4
0.5	TvE	50	48.1	VWXYZAB		0.6
0	Tv	6.25	0.7		RSTUVWXY	
0	Tv	12.5	11.8		LMNOPQRSTUVWXY	
0	Tv	25	9.6		MNOPQRSTUVWXY	
0	Tv	50	19.6		GHIJKLMNOPQR	
0.25	Tv	6.25	59.6	PQRSTUVWXY		3.4
0.25	Tv	12.5	68.6	JKLMNOPQRST		2.4
0.25	Tv	25	76.1	EFGHIJKLMNOPQ		2.9
0.25	Tv	50	35.4	ZABCDEFGHI		1.0
0.5	Tv	6.25	97.5	ABCD		1.8
0.5	Tv	12.5	97.5	ABCD		1.5
0.5	Tv	25	100.0	A		1.6
0.5	Tv	50	92.5	ABCDEFG		1.3

[a] Plant species used for extracts were: Aa = *Artemisia afra*, As = *Allium sativum*, Er = *Elytropappus rhinocerotis*, Ga = *Galenia africana*, Hh = *Hypoxis hemerocallidea*, Sa = *Siphonochilus aethiopicus*, Sf = *Sutherlandia frutescens*, Ta = *Tulbaghia alliacea*, TvEC = *Tulbaghia violacea* (Eastern Cape),Tv = *Tulbaghia violacea*.

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Table 3 (cont.)

[b] 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 kresoxim-methyl and the plant extract, respectively. The synergy ratio, R, 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, expected, or antagonistic, respectively.

Discussion

Minimizing losses due to Botrytis grey mould depends on the availability of effective natural and synthetic fungicides. During the 1990s, new classes of fungicides have been developed in order to meet the demand for environmentally safer products, low toxicity to humans and wildlife and low residues in food. Modern fungicides include the classes of aniline-pyrimidines, phenoxyquinolines, phenylpyrroles, and strobilurins (Knight *et al.,* 1997), which are highly selective site-specific inhibitors of the metabolism of the target organisms. A disadvantage of fungicides with a specific mode of action is the high risk of resistance development (Jespers & De Waard, 1993). This has been the case for the first generation of modern fungicides (e.g. benzimidazoles, phenylamides, dicarboxinides, and sterol biosynthesis inhibitors), whose activity was significantly reduced by the development of resistance in target fungal populations. Application of the widely used azole and strobulirin fungicides for control of *B. cinerea* is also very limited.

In this study methanol extracts of medicinal plant species of *A. afra, A. sativum*, *E. rhinocerotis*, *H. hemerocallidea*, *S. aethiopicus*, *S. frutescens*, *T. alliacea*, and *T. violacea* exhibited weak antifungal properties against *B. cinerea* in the *in vitro* bioassays. The strongest antifungal activity observed was with the single plant extract for *G. africana* at 25 and 50% concentrations. However, when concentrations of the plant extracts were combined with kresoxim-methyl significant reduction in mycelium growth of the fungus was observed for almost all of the combinations. Plant extract concentrations of between 6.25 and 25% were required to obtain 90-100% inhibition in mixtures with kresoxim-methyl compared to 50% extract concentrations and single fungicide treatments. These inhibitory effects were especially significant for concentrations of *A. afra*, *E. rhinocerotis*, *G. africana*, *H.*

hemerocallidea, *S. aethiopicus*, *S. frutescens*, *T. alliacea*, and *T. violacea*. Complete inhibition of *B. cinerea* infection in mixtures was observed for *E. rhinocerotis* at 25%, *S. frutescens* at 12.5%, *T. alliacea* at 25% and *T. violacea* at 25%.

Through the Abbott method we were able to calculate mathematically the synergistic ratios for all the plant extracts at their respective concentrations and combinations with kresoxim-methyl. Except for *G. africana,* the plant extracts showed synergism. However *G. africana* had ratios ranging from 0.7 to 0.9, which leans towards synergism and one could perhaps attain synergism if another range of concentrations had been used. S. *aethiopicus* showed a potent synergistic interaction while *T. violacea* shows good synergy for all concentrations tested.

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 study clearly shows that South African indigenous plant species produced secondary metabolites that still have unknown functional assignments. Synergistic interactions between components in a mixture can relate to one of the following mechanisms (De Waard, 1985): (a) 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. "Synergy" is a popular concept in the field of herbal medicine, suggesting plant extracts contain compounds potentiating each other (Duke and Bogenschutz-Godwin, 1998). Stermitz *et al.* (2000a, 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). Resistance to chemically unrelated compounds can be based on multiple resistance or multidrug resistance. 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 a*l., 2000). Morel *et al.* (2003) found plant compounds that do not possess antibacterial activity themselves, but can potentiate known antibiotics by inhibiting microbial MDR pumps. 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 (Hayashi, *et al.*, 2001, Le Roux *et al.*, 1999). ABC transporters belong to large protein families that function as drug transporters that reduce the intracellular accumulation of fungitoxic compounds. The combined use of azoles with plant-derived nontoxic inhibitors of ABC transporters lowered the inhibitory dosage of these fungicides (Del Sorbo *et al.*, 1998). Modulators known to reduce MDRs in tumour cells synergyzed the fungitoxic activity of fungicide oxpoconazole, a sterol demethylation inhibitor against *B. cinerea* (Hayashi *et al.*, 2003). It is anticipated that gene regulation of ABC genes in fungi will be an important research topic in the near future. Inhibitors of transcription of these genes would act as strong synergists of drugs, which have lost their efficacy because of pathogen resistance. Knowledge of the ABC transporters opens possibilities of developing novel strategies for controlling plant diseases, either by modulation of transporter activity or by transgenic expression of plant active genes in crops. Knowledge of the biochemistry

and regulatory mechanism of MDR inhibitors are important to alter the secondary metabolite profiles of plants by genetic engineering in the near future.

The widely occurring plant pathogenic fungus, *B. cinerea,* infects the fruits, flowers, or green tissues of at least 235 plant species (Jarvis, 1997). The disease induced by this fungus is described as grey mould. Grey mould is a major pre-and postharvest fruit decay problem in the horticultural industry of South Africa. This requires the use of different and large numbers of antifungal compounds as part of the disease control and resistance 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 EUR 740 million. This represents 80% of the total value of exports of fresh fruit and vegetables by all the ACP countries to the EU. However, 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).

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 reduce through this technology. This technology 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 that for the development of new cash crops for low-income rural farmers by adding multiple-value to indigenous plant species.

Local manufacturing and marketing can create new opportunities for entrepreneurs that with experience and more products can sustain businesses and maintain the biodiversity and development of products within the boundaries of South Africa.

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 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 able to withstand 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*.

We therefore came to the conclusion that South African indigenous plant species produce modulators that potentiate the activity of fungicides. Whether the potentiation effects are due to inhibition of fungal MDR pumps require future studies at the molecular level. However, this specific inhibitory effect is 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 also ensures a reduction of the chemical residue on the fruit.

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CHAPTER 3

In vivo response of mixtures of kresoxim-methyl fungicide and medicinal plant extracts against *Botrytis cinerea*

Abstract

Preliminary *in vivo* studies carried out in our laboratory, regarding the inhibition of the disease-causing fungus *Botrytis cinerea*, showed antagonistic reactions when *in vitro* combinations of medicinal plant extracts (6.25 -50% w/v) and the strobilurin fungicide, kresoxim-methyl (0.25 or 0.5%), were used for *in vivo* studies on apples. The objective of this study was therefore, to assess and analyse the dose rates that produce synergistic reactions for combinations of medicinal plant extracts and kresoxim-methyl against *B. cinerea* in an apple bioassay. A final series of two fold concentrations of medicinal plant extracts ranging from 6.25 to 0.19%, were combined with 0.0005% kresoxim-methyl to conduct decay inhibition studies on Granny Smith apples. Synergistic effects were observed for many of the plant extract mixed with kresoxim-methyl. We came to the conclusion that indigenous South African plant species produce potential modulaters that potentiate the activity of fungicides. Whether these modulation effects are due to the inhibition of fungal multi-drug resistant pumps require further studies at the molecular level. However, these inhibitory effects are likely to be advantageous for developing fungicide formulations and application strategies with low toxicity effects on the environment. This approach will not only make it possible to reduce fungicide concentrations while maintaining adequate decay control, but also ensures a reduction of the chemical residue on the plant surfaces.

Introduction

A general concern for public safety and the development of resistance to many fungicides by postharvest pathogens (Bertrand and Saulie-Carter, 1978; Rosenberg and Meyer, 1981) have increased the interest in alternative methods to control fruit diseases. Control of plant diseases depends primarily upon the application of chemical fungicides, despite potentially toxic effects on humans, wildlife, and the environment (De Waard *et al.*, 1993). Increasingly, modern fungicides that are safe for humans and wildlife and have benign environmental profiles, are applied in low dosage rates, and have high specificity to target organisms, to ensure sustainable crop production (Beffa, 2004). However, the continuous appearance of new pathogen races that are resistant to these fungicides has also now become a great concern to plant pathologists and agrochemists (Delp, 1980; Staub, 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 plant pathogens (Ark and Thompson, 1959; Dixon, 2001; Fawcett and Spencer, 1970, Morrissey and Osbourne, 1999; Wilson *et a*l., 1997). A problem with plant-produced compounds as potential fungicides is that in the natural state, they are generally only weakly active compared to commercial fungicides. Recent work with berberine a cationic alkaloid from several *Berberis* medicinal plants offers a possible explanation for the apparent ineffectiveness of plant antimicrobials (Stermitz *et al.*, 2000a, b). Berberine is a weak antimicrobial produced by a wide variety of plant species. It was found that *Berberis* plants produce 5`-methoxyhydnocarpin-D, which acted in synergy with berberine against human bacterial pathogens (Stermitz *et al.*, 2000a, b; Tegos *et al.*, 2002). This demonstrates that plants produce compounds that might have a regulatory function, indirectly increasing the level of resistance of the

plant, and suggests that a solid rationale, is lacking for assigning a functional role to the vast majority of plant compounds that have been classified as antimicrobials (Tegos *et al.*, 2002).

There are many reports on the successes of fungicide mixtures for the control of plant pathogenic fungi (De Waard, 1987; Gisi *et al.*, 1985; Grabiski and Gisi, 1987; Lorbeer and Vincelli, 1990; Samoucha and Cohen, 1989). Most modern fungicides are single-site inhibitors. 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 (Lorbeer, 1996). The most obvious cases were observed for phenylamide fungicide in mixtures with mancozeb and cymoxanil against *Phytophthora infestans* (Mont) de Bary and *Plasmopara viticola* Berl & de Toni (Gisi *et al.*, 1985). Mixtures of stereoisomers of the sterol biosynthesis inhibitors cyproconazole or tebuconazole also display synergism against *Botrytis cinerea* Pers.:Fr (Stehmann and De Waard, 1995, 1996). Combinations may also reduce the potential for development of resistance by the target pathogen to either component.

Fungicide combinations and the novel regulatory functions of natural plant compounds offer the opportunity to find and validate disease control strategies with high biological activity, with low dose rate application, and to overcome pathogen resistance. In this respect, we speculate that the addition of plant extracts may enhance the efficacy of the synthetic fungicide, kresoxim-methyl (K-M) in an *in vivo* assay against *B. cinerea*, causing grey mould of apples. Plant extracts with antimicrobial activity reported in the literature are not easily accessible. We therefore selected some well-known indigenous medicinal plant species used by traditional

healers for treatment of various human ailments for centuries in South Africa (Van Wyk *et al.*, 2002). Kresoxim-methyl is a broad-spectrum synthetic fungicide from Strobulirin with a methyl methoxyiminoacetate moiety, which controls major economic fungi from all four taxonomic groups of fungi (Ammerman *et al.*, 1992). Strobulirins are fungicidal natural products found in Bacidiomycete fungi *Strobilurus tenacellus*, which inhibit the mitochodrial respiration of fungi (Anke *et al.*, 1977). Grey mould, induced by *B. cinerea* is a plant pathogenic fungus that causes major pre- and post-harvest disease on many economically important crops (Jarvis, 1997; Janisiewicz *et al.*, 1991). A heavily sporulating gray mould can develop in 2-3 days, hence the common name of this disease, gray mould. The fungus is often regarded as a wound pathogen that may penetrate through wounds or natural openings. Disease infection may occur in the field during harvesting or earlier, but development usually occurs during the post-harvest stage.

Novel modes of action are necessary to overcome resistance to existing products. It offers today the opportunity to rapidly find and definitively validate target(s) of a new fungicide. Preliminary *in vivo* studies carried out in our laboratory, upon the inhibition of the disease causing fungus *Botrytis cinerea*, showed antagonistic reactions when *in vitro* combination of concentrations between medicinal plant extracts (6.25 -50% w/v) and the strobilurin fungicide, K-M (0.25 or 0.5%) were used for *in vivo* studies on apples. The objective of this study was therefore, to assess and analyse the dose rates that produce synergistic reactions for combinations of medicinal plant extracts and K-M against *B. cinerea* in an apple bioassay.

Materials and Methods

Pathogen inoculum. The *B. cinerea* strain used in this study was isolated from the surface of 'Granny Smith' apples infected with gray mold disease and maintained on potato-dextrose agar (PDA) at 25 °C. Fresh inoculum was prepared by transferring spores from stock cultures to PDA and incubating these at 25 °C in the dark under a white fluorescent light with a 12:12 light: dark photoperiod. Conidial suspensions of *B. cinerea* were prepared from spores harvested from 14-d-old cultures with 10-ml sterile distilled water containing 0.05% (v/v) Tween 80. Spore counts were made with a haemocytometer and suspensions were adjusted with sterile distilled water to 1×10^4 spores/ml.

Preparation of plant extracts. The plant parts of the following 10 medicinal plant species used in traditional medicine practices in South Africa were obtained for the preparation of plant extracts: *Artemisia afra* Jacq. (fresh leaves), *Elytropappus rhinocerotis* (Lf) Less (fresh leaves), *Galenia africana*L. (dried leaves), *Hypoxis hemerocallidea* (Fisch. And C. A. Mey) (fresh corms), *Siphonochilus aethiopicus* (Schweif.) BL Burt (fresh rhizomes + fleshy roots), *Sutherlandia frutescens*(L) R. Br. (dried leaves), *Tulbaghia alliacea* (Lf) (fresh corms), *Tulbaghia violacea* Harv. (fresh leaves + rhizomes). The voucher specimens of plants were identified in the University of the Western Cape Herbarium, Bellville, South Africa.

Weighed dried leaves were powdered in a hammer mill and extracted overnight in a closed container at room temperature in methanol (MeOH) to obtain 50% (weight/volume) stock concentrations. Fresh plant material was crushed in a Waring blender and extracted overnight at room temperature in MeOH to obtain 50%

(weight/volume) stock concentrations. The 50% extracts was filtered through Whatman no. 4 qualitative filter paper and stored at 0 °C until used.

Postharvest assays on apples. A series of preliminary in vivo postharvest assays were performed with the medicinal plant extracts and K-M on the apple cultivar, Granny Smith against *B. cinerea* to assist in the development of the main experimental hypotheses. All the assays conducted with mixtures of the medicinal plant extracts and kresoxim-methyl contained an increasing dose of the extracts and a given amount of fungicide. The assays included controls containing sterile water and methanol (5%, v/v) without the compounds to be tested. Treatments and controls were performed simultaneously in an experiment. For the main experiment each of the 50 % plant extract stock solutions was added in final concentrations of 0.19, 0.39, 0.78, 1.56, 3.13 and 6.25% to prepare mixtures with a final concentration of 0.0005% (5 ppm or 5 mg/L) kresoxim-methyl (Stroby WG, BASF South Africa (Pty) Ltd.).

Fruits were removed from cold storage, surface-sterilized with 70% EtOH for 2 min then air-dried. Each apple was wounded (5 mm in diameter and 3 mm in depth) three times halfway between the calyx and the stem end. A 20-µl drop of each treatment was placed in the wounds and allowed to dry for two hours before application of a 20-µl conidial suuspion. To assess differences in the mycelial growth of *B. cinerea* among the treatments the percentage inhibition were calculated: 100 – (treatment/control) X 100. Suspensions of *B. cinerea.* Fruits were stored in commercial cardboard boxes at 20 °C in a high humidity (95% RH) walk-in incubator. Diameter of *B. cinerea* decay lesions was determined after a 7-d incubation period. Percentage inhibition among the treatments was calculated on the basis of lesion diameter as follows: % = 100 – (treatment/control) X 100.

Data analyses. For the main experiment each treatment was performed on three apples, each with triplicate wounds. Percentage inhibition was determined for each replicate and averaged for the three apples. Data for lesion diameter and percentage decayed fruit were subjected to a standard analysis of variance. The analysis of variance was performed using SAS version 8.2 (SAS 1999). Student t-Least Significant Difference was calculated at the 5% significant level to compare treatment means of lesion diameter and percentage inhibition.

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 K-M and the medicinal plant extract, respectively. The synergy ratio, R, 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, expected, or antagonistic, respectively.

Results

In order to interpret the results for the postharvest *in vivo* interactions between mixtures of fungicides and medicinal plant extracts the results of the lesion diameters and percentage inhibition are reported for this study. Significant interactions were detected for lesion diameter and percentage inhibition of gray mold between plant extracts by extract concentrations by K-M or extract concentrations by K-M as well as plant extracts by K-M and plant extracts by extract concentrations, with K-M having a greater affect in its respective interactions (Table 4 and 5).

The 0.0005% K-M concentration selected through preceding apple assays resulted in smaller lesions (11.8 mm), which were 57.4% smaller than that of the water control (27.8 mm) (Table 6 and 7). Statistically, decay development for the K-M treatment was significantly less (P=0.05) than for the water control, while decay for the methanol control was not significantly different from either K-M or water control.

Of the 48 extract concentrations from the eight medicinal plant species used alone, 37 showed significant larger lesions of decay compared to the K-M treatment. Lesion diameters varies from 20.9 to 33.9 mm compared to the water control, 27.8 mm and K-M, 11.8 mm. Percentage inhibition varied from 16.9 to 24% for the extract concentrations used alone compared to the 57.4% K-M control. Decay development for the rest of the 11 plant extracts used alone varied from 3.9 to 21.2 mm for lesion diameter and the inhibition levels from 27.9 to 86.0%, which was not significantly different from the K-M treatment.

For the 48 treatments where plant extracts and K-M were combined, 17 of the mixtures were significantly more, inhibitory (0.0 – 2.5 mm or 90.9 –100%) to *B. cinerea* infection. This includes the following plant species and extract concentrations: *A. afra* (1.56%); *E. rhinocerotis* (0.19, 0.39%); *G. africana* (6.25%); *H. hemerocallidea* (0.19, 0.39, 1.56, 3.13%); *S. frutescens* (0.19, 0.39, 0.78%); *T. alliacea* (0.19, 0.39%); *T. violacea* (0.19, 0.39, 0.78, 3.13%).

The Abbott formula showed that of the 48 mixture treatments, 28 showed synergistic interactions that range from 1.0 – 1.8. Of the 17 mixtures with significantly higher percentages of inhibition than the K-M treatment, 14 showed synergistic effects that range from 1.0 – 1.5, while three showed potential additive

responses. The 14 treatments include the following plant species (concentrations and synergy ratio): *A. afra* (1.56%, 1.1); *E. rhinocerotis* (0.39%, 1.4); *H. hemerocallidea* (0.19%, 1.4; 0.39%, 1.1; 1.56%, 1.7; 3.13% 1.8); *S. frutescens* (0.19%, 1.4; 0.39%, 0.39; 0.78%, 1.4); *T. alliacea* (0.19%, 1.4; 0.39%, 1.2); *T. violacea* (0.19%, 1.4; 0.78%, 1.0; 3.13%, 1.5).

Table 4. Summary of the analysis of variance for lesion diameter at 7 days after inoculating wounded Granny Smith apples with conidia of *Botrytis cinerea*, showing sum of squares (SS) mean squares (MS) and significant levels for main effects of kresoxim-methyl (K-M) and medicinal plant extract concentrations, and all interactions involving combinations of kresoxim-methyl and plant extracts.

Source[a]	df	SS	MS	F-value	Pr>F
Treatment	98	31938.566	325.904	10.93	<0.0001
Extract (E)	7	1027.436	146.776	4.92	<0.0001
Extract Concentration (EC)	5	1059.674	211.935	7.11	<0.0001
E*EC	35	4610.265	131.722	4.42	<0.0001
K-M	1	20627.682	20627.682	692.10	<0.0001
E*K-M	7	811.794	115.971	3.89	0.0005
EC*K-M	5	934.471	186.894	6.27	<0.0001
E*EC*K-M	35	2291.821	65.481	2.20	0.0004
Contrast					
Methanol+Control vs E	1	193.718	193.718	6.50	0.0115
Methanol vs Control	1	0.259	0.259	0.01	0.926
Control: Water vs K-M	1	381.444	381.444	12.80	0.0004

[a] Treatment = total number of 99 treatments, (E) = eight medicinal plant extracts tested. (EC) = five extracts concentrations per plant species.

Table 5. Summary of the analysis of variance for inhibition (%) of gray mold on Granny Smith apples inoculated with conidia of *Botrytis cinerea*, showing sum of squares (SS) mean squares (MS) and significant levels for main effects of kresoxim-methyl (K-M) and medicinal plant extract concentrations, and all interactions involving combinations of kresoxim-methyl and plant extracts.

Source[a]	df	SS	MS	F-value	Pr>F
Treatment	98	414454.153	4229.124	10.93	<0.0001
Extract (E)	7	13332.638	1904.663	4.92	<0.0001
Extract Concentration (EC)	5	13750.972	2750.194	7.11	<0.0001
E*EC	35	59825.588	1709.302	4.42	<0.0001
K-M	1	267677.280	267677.280	692.10	<0.0001
E*K-M	7	10534.335	1504.905	3.89	0.0004
EC*K-M	5	12126.261	2425.252	6.27	<0.0001
E*EC*K-M	35	29740.061	849.716	2.20	0.0003
Contrast					
Methanol+Control vs E	1	2513.802	2513.802	6.50	0.0115
Methanol vs Control	1	3.363	3.363	0.01	0.926
Control: Water vs K-M	1	4949.851	4949.851	12.80	0.0004

[a] Treatment = total number of 99 treatments, (E) = eight medicinal plant extracts tested. (EC) = five extracts concentrations per plant species.

Table 6. Lesion diameter of gray mold on Granny Smith apples inoculated with conidia of *Botrytis cinerea*, and the relative level of synergism of mixtures containing 0.0005% (5ppm) of kresoxim-methyl (K-M) and different concentrations of medicinal plant extracts.

Treatment[a]				
K-M	Plant species	Extract Conc (%)	Lesion diam. (mm)	Means followed by the same letters in the column are not significantly different at P=0.05 according to the Student t-Least Significant Difference test
N	Control	0	27.8	A B C D E F G H[b]
Y	Control	0	11.8	R S T U V W X Y Z
N	Methanol	0	20.1	F G H I J K L M N O P Q R
N	Aa	0.19	32.4	A B
N	Aa	0.39	24.7	A B C D E F G H I J K L M
N	Aa	0.78	24.3	A B C D E F G H I J K L M
N	Aa	1.56	19.4	H I J K L M N O P Q R S T
N	Aa	3.13	31.6	A B C
N	Aa	6.25	33.9	A
Y	Aa	0.19	12.8	Q R S T U V W X Y
Y	Aa	0.39	3.3	Z A B
Y	Aa	0.78	7.6	X Y Z A B
Y	Aa	1.56	1.8	B
Y	Aa	3.13	17.6	K L M N O P Q R S T
Y	Aa	6.25	14.0	P Q R S T U V W X
N	Er	0.19	14.1	P Q R S T U V W X
N	Er	0.39	24.9	A B C D E F G H I J K L M
N	Er	0.78	30.4	A B C D
N	Er	1.56	3.9	Z A B

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Table 6 (cont.)

N	Er	3.13	29.5	A B C D E
N	Er	6.25	31.6	A B C
Y	Er	0.19	0.0	B
Y	Er	0.39	1.1	B
Y	Er	0.78	6.2	X Y Z A B
Y	Er	1.56	13.2	Q R S T U V W X Y
Y	Er	3.13	20.3	F G H I J K L M N O P Q R
Y	Er	6.25	17.3	L M N O P Q R S T U
N	Ga	0.19	24.8	A B C D E F G H I J K L M
N	Ga	0.39	22.3	D E F G H I J K L M N O P
N	Ga	0.78	27.3	A B C D E F G H I
N	Ga	1.56	17.3	L M N O P Q R S T U V
N	Ga	3.13	11.2	S T U V W X Y Z A
N	Ga	6.25	7.2	X Y Z A B
Y	Ga	0.19	3.7	Z A B
Y	Ga	0.39	8.1	W X Y Z A B
Y	Ga	0.78	8.2	W X Y Z A B
Y	Ga	1.56	7.7	W X Y Z A B
Y	Ga	3.13	4.9	Y Z A B
Y	Ga	6.25	1.0	B
N	Hh	0.19	25.4	A B C D E F G H I J K L
N	Hh	0.39	19.8	H I J K L M N O P Q R S
N	Hh	0.78	29.5	A B C D E
N	Hh	1.56	27.8	A B C D E F G H
N	Hh	3.13	28.7	A B C D E F G
N	Hh	6.25	20.9	E F G H I J K L M N O P Q
Y	Hh	0.19	2.1	B
Y	Hh	0.39	1.1	B
Y	Hh	0.78	4.0	Z A B

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Table 6 (cont.)

Y	Hh	1.56	1.7	B
Y	Hh	3.13	1.7	B
Y	Hh	6.25	8.3	W X Y Z A B
N	Sa	0.19	28.8	A B C D E F
N	Sa	0.39	26.6	A B C D E F G H I J
N	Sa	0.78	20.0	G H I J K L M N O P Q R S
N	Sa	1.56	21.2	E F G H I J K L M N O P Q
N	Sa	3.13	18.8	I J K L M N O P Q R S T
N	Sa	6.25	23.1	C D E F G H I J K L M N O
Y	Sa	0.19	14.5	O P Q R S T U V W X
Y	Sa	0.39	4.6	Y Z A B
Y	Sa	0.78	7.6	X Y Z A B
Y	Sa	1.56	6.8	X Y Z A B
Y	Sa	3.13	5.1	Y Z A B
Y	Sa	6.25	19.3	H I J K L M N O P Q R S T
N	Sf	0.19	25.3	A B C D E F G H I J K L
N	Sf	0.39	26.3	A B C D E F G H I J K
N	Sf	0.78	24.4	A B C D E F G H I J K L M
N	Sf	1.56	25.4	A B C D E F G H I J K L
N	Sf	3.13	26.8	A B C D E F G H I
N	Sf	6.25	23.3	C D E F G H I J K L M N O
Y	Sf	0.19	2.5	A B
Y	Sf	0.39	0.0	B
Y	Sf	0.78	1.9	B
Y	Sf	1.56	8.6	V W X Y Z A B
Y	Sf	3.13	3.8	Z A B
Y	Sf	6.25	18.0	J K L M N O P Q R S T
N	Ta	0.19	23.2	C D E F G H I J K L M N O
N	Ta	0.39	21.1	E F G H I J K L M N O P Q

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Table 6 (cont.)

N	Ta	0.78	30.8	A B C D
N	Ta	1.56	24.0	B C D E F G H I J K L M
N	Ta	3.13	22.7	D E F G H I J K L M N O P
N	Ta	6.25	30.2	A B C D
Y	Ta	0.19	0.0	B
Y	Ta	0.39	1.1	B
Y	Ta	0.78	6.5	X Y Z A B
Y	Ta	1.56	7.4	X Y Z A B
Y	Ta	3.13	11.1	T U V W X Y Z A
Y	Ta	6.25	8.7	U V W X Y Z A B
N	Tv	0.19	23.4	C D E F G H I J K L M N
N	Tv	0.39	14.9	N O P Q R S T U V W X
N	Tv	0.78	16.4	M N O P Q R S T U V W
N	Tv	1.56	30.0	A B C D
N	Tv	3.13	24.6	A B C D E F G H I J K L M
N	Tv	6.25	22.1	D E F G H I J K L M N O P
Y	Tv	0.19	0.0	B
Y	Tv	0.39	1.9	B
Y	Tv	0.78	0.0	B
Y	Tv	1.56	5.0	Y Z A B
Y	Tv	3.13	0.0	B
Y	Tv	6.25	20.9	E F G H I J K L M N O P Q

[a] N = No, Y = Yes, Aa = *Artemisia afra*, Er = *Elytropappus rhinocerotis*, Ga = *Galenia africana*, Hh = *Hypoxis hemerocallidea*, Sa = *Siphonochilus aethiopicus*, Sf = *Sutherlandia*

Table 7. Percentage inhibition of gray mold decay on Granny Smith apples inoculated with conidia of *Botrytis cinerea*, and the relative level of synergism of mixtures containing 0.0005% (5ppm) of kresoxim-methyl (K-M) and different concentrations of medicinal plant extracts.

Treatment[a]					
K-M	Plant species	Extract Conc (%)	Decay inhibition (%)	Means followed by the same letters in the column are not significantly different at P=0.05 according to the Student t-Least Significant Difference test	Synergy ratio R[b]
N	Control	0	0.0	V W X Y Z A B	
Y	Control	0	57.4	C D E F G H I J K	
N	Methanol	0	27.4	K L M N O P Q R S T U V W	
N	Aa	0.19	-16.9	A B	
N	Aa	0.39	11.2	P Q R S T U V W X Y Z A B	
N	Aa	0.78	12.4	P Q R S T U V W X Y Z A B	
N	Aa	1.56	30.0	I J K L M N O P Q R S T U	
N	Aa	3.13	-13.8	Z A B	
N	Aa	6.25	19.2	B	
Y	Aa	0.19	53.9	D E F G H I J K L	1.3
Y	Aa	0.39	88.0	A B C	1.3
Y	Aa	0.78	72.6	A B C D E	1.1
Y	Aa	1.56	93.6	A	1.1
Y	Aa	3.13	36.6	I J K L M N O P Q R	0.8
Y	Aa	6.25	49.6	E F G H I J K L M	0.7
N	Er	0.19	49.1	E F G H I J K L M	
N	Er	0.39	10.4	P Q R S T U V W X Y Z A B	
N	Er	0.78	-9.6	Y Z A B	

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Table 7 (cont.)

N	Er	1.56	86.0	A B C	
N	Er	3.13	-6.2	X Y Z A B	
N	Er	6.25	-13.9	Z A B	
Y	Er	0.19	100.0	A	0.9
Y	Er	0.39	96.0	A	1.4
Y	Er	0.78	77.5	A B C D E	1.6
Y	Er	1.56	52.3	D E F G H I J K L	0.4
Y	Er	3.13	26.9	K L M N O P Q R S T U V W	0.5
Y	Er	6.25	37.4	H I J K L M N O P Q	0.9
N	Ga	0.19	10.6	P Q R S T U V W X Y Z A B	
N	Ga	0.39	19.7	M N O P Q R S T U V W X Y	
N	Ga	0.78	1.6	T U V W X Y Z A B	
N	Ga	1.56	37.6	G H I J K L M N O P Q	
N	Ga	3.13	59.2	B C D E F G H I J	
N	Ga	6.25	74.1	A B C D E	
Y	Ga	0.19	86.7	A B C	1.3
Y	Ga	0.39	70.9	A B C D E F	0.9
Y	Ga	0.78	70.3	A B C D E F	1.2
Y	Ga	1.56	72.2	A B C D E F	0.8
Y	Ga	3.13	82.4	A B C D	0.7
Y	Ga	6.25	96.3	A	0.7
N	Hh	0.19	8.4	Q R S T U V W X Y Z A B	
N	Hh	0.39	28.5	J K L M N O P Q R S T U	
N	Hh	0.78	-6.1	X Y Z A B	
N	Hh	1.56	-0.1	U V W X Y Z A B	
N	Hh	3.13	-3.4	V W X Y Z A B	
N	Hh	6.25	24.6	L M N O P Q R S T U V W X	
Y	Hh	0.19	92.5	A	1.4
Y	Hh	0.39	95.9	A	1.1

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Table 7 (cont.)

Y	Hh	0.78	85.6	A B C	1.7
Y	Hh	1.56	94.0	A	1.7
Y	Hh	3.13	94.0	A	1.8
Y	Hh	6.25	70.0	A B C D E F	0.9
N	Sa	0.19	-3.9	W X Y Z A B	
N	Sa	0.39	4.1	S T U V W X Y Z A B	
N	Sa	0.78	27.9	J K L M N O P Q R S T U V	
N	Sa	1.56	23.8	L M N O P Q R S T U V W X	
N	Sa	3.13	32.4	I J K L M N O P Q R S T	
N	Sa	6.25	16.8	N O P Q R S T U V W X Y Z	
Y	Sa	0.19	47.8	E F G H I J K L M N	0.9
Y	Sa	0.39	83.3	A B C D	1.4
Y	Sa	0.78	72.8	A B C D E	0.9
Y	Sa	1.56	75.4	A B C D E	0.9
Y	Sa	3.13	81.6	A B C D	0.9
Y	Sa	6.25	30.3	I J K L M N O P Q R S T U	0.3
N	Sf	0.19	8.9	Q R S T U V W X Y Z A B	
N	Sf	0.39	5.1	R S T U V W X Y Z A B	
N	Sf	0.78	12.2	P Q R S T U V W X Y Z A B	
N	Sf	1.56	8.6	Q R S T U V W X Y Z A B	
N	Sf	3.13	3.6	T U V W X Y Z A B	
N	Sf	6.25	16.1	N O P Q R S T U V W X Y Z	
Y	Sf	0.19	90.9	A B	1.4
Y	Sf	0.39	100.0	A	1.6
Y	Sf	0.78	93.1	A	1.4
Y	Sf	1.56	69.1	A B C D E F G	1.1
Y	Sf	3.13	86.2	A B C	1.4
Y	Sf	6.25	35.3	I J K L M N O P Q R S	0.5
N	Ta	0.19	16.6	N O P Q R S T U V W X Y Z	

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Table 7 (cont.)

N	Ta	0.39	24.0	L M N O P Q R S T U V W X		
N	Ta	0.78	-11.1	Y Z A B		
N	Ta	1.56	13.6	P Q R S T U V W X Y Z A		
N	Ta	3.13	18.2	M N O P Q R S T U V W X Y		
N	Ta	6.25	-8.7	Y Z A B		
Y	Ta	0.19	100.0 A			1.4
Y	Ta	0.39	96.2 A			1.2
Y	Ta	0.78	76.6 A B C D E			1.7
Y	Ta	1.56	73.4 A B C D E			1.0
Y	Ta	3.13	60.2 B C D E F G H I			0.8
Y	Ta	6.25	68.8 A B C D E F G H			1.4
N	Tv	0.19	15.8	O P Q R S T U V W X Y Z		
N	Tv	0.39	46.4 E F G H I J K L M N O			
N	Tv	0.78	40.8 F G H I J K L M N O P			
N	Tv	1.56	-8.24	Y Z A B		
N	Tv	3.13	11.2	P Q R S T U V W X Y Z A B		
N	Tv	6.25	20.5	M N O P Q R S T U V W X Y		
Y	Tv	0.19	100.0 A			1.4
Y	Tv	0.39	93.2 A			0.9
Y	Tv	0.78	100.0 A			1.0
Y	Tv	1.56	81.9 A B C D			1.7
Y	Tv	3.13	100.0 A			1.5
Y	Tv	6.25	24.5	L M N O P Q R S T U V W X		0.3

[a] N No, Y = Yes, Aa = *Artemisia afra*, Er = *Elytropappus rhinocerotis*, Ga = *Galenia africana*, Hh = *Hypoxis hemerocallidea*, Sa = *Siphonochilus aethiopicus*, Sf = *Sutherlandia frutescens*, Ta = *Tulbaghia alliacea*, Tv = *Tulbaghia violacea*

[b] Synergy ratio R = Observed percentage inhibition /Expected percentage inhibition (Abbott, 1925).

Discussion

Indigenous medicinal plants have played an important role in South Africa, as they are used in the traditional treatment of various human diseases on an empirical basis (Hutchings *et al.*, 1996). In this study single extracts of the medicinal plant species: *A. afra*, *E. rhinocerotis*, *G. africana*, *H. hemerocallidea*, *S. aethiopicus*, *S. frutescens*, *T. alliacea*, and *T. violacea* exhibit weak or no antifungal properties against *B. cinerea* in the apple bioassay. The strongest antifungal activity observed with a single plant extract was for 6.25% *G. africana*. However, when low concentrations of the plant extracts were combined with a subinhibitory concentration of K-M, synergistic and additive interactions were observed for almost all of the combinations. The synergistic effects were especially observed for the lowest concentrations of *A. afra*, *E. rhinocerotis*, *G. africana*, *H. hemerocallidea*, *S. aethiopicus*, *S. frutescens*, *T. alliacea*, and *T. violacea*. Complete inhibition of *B. cinerea* infection in mixtures was observed for *E. rhinocerotis* at 0.19%, *S. frutescens* at 0.39%, *T. alliacea* at 0.19% and *T. violacea* at 0.19, 0.78 and 3.13%. This experiment shows that subinhibitory concentrations of a fungicide and very low concentrations of plant extracts act in synergy. Higher plant extract concentrations in preliminary studies showed antagonistic reactions.

This study shows that South African indigenous medicinal plant species produce secondary metabolites that still have unknown functional assignments. "Synergy" is a popular concept in the field of herbal medicine, suggesting plant extracts contain compounds potentiating each other (Duke and Bogenschutz-Godwin, 1998) in reference to chapter 2. Stermitz *et al.* (2000a, 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. *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). Synergistic interactions between components in a mixture can relate to one of the following mechanisms: (a) 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). This is in keeping refernce with chapter 2.

The widely occurring plant pathogenic fungus, *B. cinerea* infects the fruits, flowers, or green tissues of at least 235 plant species (Jarvis, 1997). The disease induced by this fungus is described as grey mould. Grey mould is a major pre-and postharvest fruit decay problem in the horticultural industry of South Africa. This requires the use of different and a large number of antifungal compounds as part of the disease control and resistance 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 EUR 740 million. This represents 80% of the total value of exports of fresh fruit and vegetables by all the ACP countries to the EU. However, 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). Balancing fungicidal potency and improved performance with low environmental impact

remains a challenge for fungicide research as discussed in chapter 2. 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).

Minimizing losses due to *Botrytis* grey mould depends on the availability of effective natural and synthetic fungicides. During the 1990s, new classes of fungicides have been developed in order to meet the demand for environmentally safer products, low toxicity to humans and wildlife and low residues in food. The modern fungicides include the classes of aniline-pyrimidines, phenoxyquinolines, phenylpyrroles, and strobilurins (Knight *et al.*, 1997), which are highly selective site-specific inhibitors of the metabolism of target organisms. A disadvantage of fungicides with a specific mode of action is the high risk of resistance development (Jespers and De Waard, 1993). This has been the case for the first generation of modern fungicides (e.g. benzimidazoles, phenylamides, dicarboxinides, and sterol biosynthesis inhibitors), whose activity was significantly reduced by the development of resistance in target fungal populations. Application of the widely used azole and strobulirin fungicides for control of *B. cinerea* is also very limited.

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 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*.

Resistance to chemically unrelated compounds can be based on multiple resistance or multidrug resistance. 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). Morel *et al.* (2003) found plant compounds that do not possess antibacterial activity themselves, but can potentiate known antibiotics by inhibiting microbial MDR pumps. 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 (Hayashi, *et al.*, 2001, Le Roux *et al.*, 1999). ABC transporters belong to large protein families that function as drug transporters that reduce the intracellular accumulation of fungitoxic compounds. The combined use of azoles with plant-derived nontoxic inhibitors of ABC transporters lowered the inhibitory dosage of these fungicides (Del Sorbo *et al.*, 1998). Modulators known to reduce MDRs in tumour cells synergyzed the fungitoxic activity of fungicide oxpoconazole, a sterol demethylation inhibitor against *B. cinerea* (Hayashi *et al.*, 2003). We anticipate that gene regulation of ABC genes in fungi will be an important research topic in the near future. Inhibitors of transcription of these genes would act as strong

synergists of drugs, which have lost their efficacy because of pathogen resistance. Knowledge of the ABC transporters open possibilities of developing novel strategies for controlling plant diseases, either by modulation of transporter activity or by transgenic expression of plant active genes in crops. Knowledge of the biochemistry and regulatory mechanism of MDR inhibitors are important to alter the secondary metabolite profiles of plants by genetic engineering in the near future.

Reporting on fungicide mixtures with synergistic action in practice is rather limited (Hayashi *et al.*, 2003; Lorbeer, 1996). 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 reduce through this technology. This technology 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 that for the development of new cash crops for low-income rural farmers by adding multiple-value to indigenous plant species. Local manufacturing and marketing can create new opportunities for entrepreneurs that with experience and more products can sustain businesses and maintain the biodiversity and development of products within the boundaries of South Africa.

I therefore came to the conclusion that South African indigenous plant species produce modulators that potentiate the activity of fungicides *in vivo*. Whether the inhibition of fungal MDR pumps are as a result of potentiation effects, future studies at the molecular level will be required. However, this specific inhibitory effect is

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 also ensures a reduction of the chemical residue on the fruit.

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