An investigation into the biocatalytic and antimicrobial properties of *Eucomis autumnalis* Mill. bulbs

submitted in fulfilment of the requirements for the degree

MAGISTER SCIENTIAE AGRICULTURAE

by

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LIST OF ABBREVIATIONS

ANOVA: analysis of variance
BL: brassinolide
BR: brassinosteroid
CS: castasterone
DC: dielectric constant
GA: gibberellic acid
GM: genetic manipulation
H_2O: water
H_2SO_4: sulphuric acid
IAA: indole-3-acetic acid
KNO_3: potassium nitrate
KOH: potassium hydroxide
LSD: least significant difference
MAO: monoamine oxidase
MeOH : methanol
$MgSO_4$,: magnesium sulphate
MIC: minimum inhibitory concentration
MRSA: methicillin-resistant *Staphylococcus aureus*
NaOCl: sodium oxychloride
NBA: nutrient broth agar
NP/PEG: Natural product - polyethylene glycol reagent
PCA: plate count agar
PDA: potato dextrose agar
ppm: parts per million
PR: pathogenesis related
P-TLC: preparative thin layer chromatography
Q-TLC: qualitative thin layer chromatography
RF: retention factor
TA: technical agar
TDZ: thidiazuron
TE: teesterone

TLC:	thin layer chromatography
TMV:	tabacco mosaic virus
UV:	ultra violet
v/v:	volume per volume

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CHAPTER 1

INTRODUCTION AND RATIONALE FOR THE STUDY

Eight hundred to 850 million people in the developing world, of which 200 million are children, are chronically undernourished while an estimated 1 to 1.5 billion people, worldwide, do not receive sufficient quantities of nutrients that are needed on a daily basis (http://www.biotechknowledge.monsanto.com/BIOTECH/knowcenter.nsf/). Add to these statistics the estimated growth in world population over the next two decades, if current predictions prove true, and a grim picture emerges.

In 2002 the World Food Summit recommitted itself to halve the number of hungry people by the year 2015 (http://www.biotechknowledge.monsanto.com/BIOTECH/knowcenter.nsf/). How honourable this objective might be, the finding of a solution is not evident as it implies considering a number of factors including a) the economic status of individuals as determined by employment and minimum wages and b) ways and means to improve agricultural productivity. In both instances a considerable amount of research is inevitable. Further, population growth is a relative uncertain factor that has to be considered. The following quotation reflects the uneasiness that pertains in this regard:

"If current predictions of population growth prove accurate and patterns of human activity on the planet remain unchanged, science and technology may not be able to prevent either irreversible degradation of the environment or continued poverty for much of the world" (Joint statement by the U.S. National Academy of Sciences and the Royal Society of London, 1992).

It is predicted that population growth will occur, in large measure, in developing countries where poverty is rife. The challenge for science is to address the need for adequate food provision and a sustainable future for agriculture. The following quotation provides some hope, but also involves a warning:

"Disaster resulting from an insufficient capacity to supply food has been averted, at least for the present, through agronomic and genetic improvements. However, the price has been the uncertainty of our ability to continue such improvements" (Swaminathan, 1993).

The problem for the future seems to be related to the fact that a solution for increased food production can probably only be obtained in three possible ways, namely a) through expansion of arable land, b) by increasing irrigation practices or c) by increasing harvestable yields through the improvement of technology. However, according to Penning de Vries (2001) severe soil erosion, especially in Africa, is minimizing the number of acreage available for cultivation, leaving an almost impossible task of increasing the amount of arable land. Further, most of the irrigatable soil on the planet is probably already utilized and chances for expansion seem slim. This leaves the increase of crop yields on currently available land as the only and most likely alternative (Heidhues, 2001).

To obtain the latter goal, future agricultural research will have to focus on certain key areas. These include a) improved disease and pest control either through conventional breeding for resistance against specific diseases or by improving chemical control methodology and technology, e.g. by finding new effective but cheaper products for application by farmers in the developing world (Nelson, 2001) and b) by applying natural biostimulants from plants either as a seed treatment or a foliar spray or both (Roth *et al*., 2000). Especially the development of natural products to achieve this goal has gained support in the recent past (Schnabl *et al*., 2001). Previous studies showed significant increases in wheat yield when grown in mixed stands with corn cockle. One such biostimulatory substance isolated from the corn cockle, agrostemmin, increased grain yields when applied to both fertilized and unfertilized areas (Schnabl *et al*., 2001). Chopped alfalfa also had a stimulatory effect on the growth of a number of vegetables and the active substance was later identified as triacontanol (Putnam & Tang, 1986). Saponins isolated from crude mungbean extracts were found to increase germination and also enhance the vegetative growth of cultivated mungbeans (Chou *et al*., 1995). The effective application of this knowledge can be instrumental in increasing crop yields and contributing towards food security in especially developing countries.

Underlying the need to develop new and cheaper natural products is the fact that the lack of an efficient integrated disease-weed-pest management system has been identified as one of the main reasons for inadequate food production in Africa and other developing countries. Further, in developed countries increased resistance by consumers to purchase plant products grown from either genetially manipulated crops or crops treated with synthetic chemicals is currently experienced. Legislation restricting the use of many synthetic crop protectants in recent years as well as the banning of copper containing synthetic pesticides in Europe, has led to increased organic farming practices (Rizvi & Rizvi, 1992). This means that indispensable tools used in crop production systems may be eliminated without existing

alternatives. This prompted research activities towards developing natural products as alternative crop protectants in recent years and accelerated the search for natural chemicals from plants, also known as green chemicals (Gorris & Smid, 1995).

Isolation and purification of active compounds from plants, however, may place them in the same category as synthetic chemicals in terms of production costs and even their impact on the environment. The application of crude plant extracts may be a feasible alternative (Gorris & Smid, 1995) due to the general view that it is biodegradable and environmentally safe compared to traditional synthetic agri-chemicals. However, the effective application of crude extracts in agricultural practices has only been established in a few cases, emphasizing the necessity for additional research.

Eksteen *et al*. (2001) screened various indigenous South African plants for antifungal activity and reported that *Eucomis autumnalis* showed above average antifungal activity against all the test organisms used, and also compared well to the synthetic fungicide used as positive control. *Eucomis autumnalis* is a bulbous plant that occurs naturally along the eastern parts of South Africa. It is used by the indigenous people of South Africa for medicinal purposes such as to cure gastro-intestinal illnesses in infants and children (Hutchings, 1989). A decoction of the bulb is also used for a wide variety of ailments including urinary diseases, stomach ache, fever, colic, flatulence, syphilis and to facilitate childbirth (Hutchings, 1996; van Wyk *et al*., 1997). The plant has also been reported to possess analgesic and antimicrobial activities (Masika *et al.*, 1997), supplying a rationale for this study.

In chapter 2 a comprehensive literature review on natural product research is presented while the methods applied in this study are outlined in chapter 3. Chapter 4 deals with the extraction procedure and *in vitro* screening of the crude extract for biostimulatory and antimicrobial properties while chapters 5 and 6 outlines the antimicrobial activity and phytotoxicity of the crude extract *in vivo* as well as the activity directed isolation, purification and identification of active substances, respectively. In conclusion, a general discussion of the findings of this study as well as recommendations for future research is supplied in chapter 7.

CHAPTER 2

LITERATURE REVIEW

2.2 INTRODUCTION

The agricultural practice relies heavily on the use of synthetic chemical crop protectants for the control of insect pests and diseases and annual sales of these chemicals contribute greatly towards the economy of a country. It is estimated that the European agricultural industry utilizes about 350 million kilograms of active ingredients on pest control per annum, of which fungicides make out the largest proportion averaging about 2.2 kg/ha (Gorris & Smid, 1995). Recently most of the copper containing synthetic pesticides have been banned in Europe and priority has been given to organic farming practices, including the application of natural plant extracts in both the agricultural and health sectors (Rizvi & Rizvi, 1992).

Plants have been known for centuries to contain active substances with medicinal, including antimicrobial, properties (Gorris & Smid, 1995). However, according to Hostettman and Wolfender (1997), less than 10% of the higher plant species on earth have been tested for biological activity, and in most cases, only for one type of activity. Bioactive compounds from plants, known as phytochemicals, are starting to play an increasingly important role in the existence of man and in different areas (Nigg & Seigler, 1992). Taking this into account, the plant kingdom can be seen as a largely untapped source of phytochemicals. According to Van Wyk *et al.* (1997), approximately 30 000 different plant species, of which about 3000 are used for their medicinal properties, are found in South Africa. These plants have been used mainly by the indigenous peoples of the country as a source of natural remedies against common diseases. A strong local market in the informal sector, using plant crude extracts for medicinal purposes, is already established (Alkofahi *et al.,* 1990; Van Wyk *et al.*, 1997).

Research programmes screening for antimicrobial properties in crude plant extracts with the aim to develop natural products continued since early times, but has only come to the fore in recent years (Borris, 1996). Over the past twenty years renewed interest in the active substances from these plants, especially by large pharmaceutical companies, has been shown (Borris, 1996). A number of bioactive components have been isolated from species belonging to the Fabaceae family (Harborne, 1994). Many of these components sprouted from the biological and ecological interaction existing between a

plant and its environment and almost all are referred to as secondary metabolites since they are not synthesized within the primary metabolic pathways.

Plant roots, for example, are regarded as under-utilized and under-explored sources of secondary metabolites in terms of its potential to be utilized as pharmaceuticals, agrichemicals, flavours and fragrances on an industrial scale. Roots of numerous plant families accumulate and/or synthesize a wide variety of products ranging from the sweet tasting glycyrrhizic acid of liquorice (*Glycyrrhiza glabra*) to the emetic and expectorant principles of ipeac (*Cephaelis ipecachuana*; Flores *et al*., 1987).

Historically, roots have economic value as a food source. However, several important secondary metabolites are produced in the roots of some plants and in developing countries the medicinal properties of these plants are exploited by the indigenous people on a small industrial scale. Importantly, scientific data on the roots are far less available than that from other plant parts. This emphasizes the immense biochemical potential still waiting for thorough exploration. One possible reason for roots being less studied than other plant parts might be the fact that the collection process leads to the destruction of vegetation; an aspect not always acknowledged by traditional healers making a living from plant roots (Flores *et al*., 1987). The fact that medicinal plants form the backbone of traditional medicine in developing countries, and are also utilized in developed countries, places pressure on natural plant populations (Wiley & Chichester, 1994). For the ecological conservation of nature, it seems extremely important that plants possessing above average potential to be developed into natural products should be identified and their agronomical properties determined in order to evaluate their potential to be developed into alternative crops.

Due to human health and environmental protection considerations, resistance by consumers to purchase plant products grown from synthetically manipulated crops is also increasing worldwide. This prompted the accelerated search for natural chemicals from plants, also known as green chemicals (Gorris & Smid, 1995), with the aim of developing natural products. Although these products will constantly have to be subjected to tests in order to determine its environmental and human safety as well as its economic viability, it is generally accepted that they are biodegradable and therefore pose less of a threat to the environment than synthetic chemicals. Because of possible toxic properties, not all green chemicals will be good candidates for practical use, and not all plants known to contain 'green chemicals' will qualify to be introduced in the agricultural practice. Politics and public opinion are generally in favour of the use of green chemicals. Purification, of these compounds, however, might

place natural products in the same category as synthetic chemical compounds in terms of production costs. The use of crude plant extracts may be an economically feasible alternative, but it is often found that the efficiency of crude extracts is relatively low (Gorris & Smid, 1995).

Be it as it may, crop and food protectants from natural origin are often considered as potentially safe sources of antimicrobials and/or pesticides. However, it must be acknowledged that their effective application in practice have only been established in a few cases emphasizing the necessity for additional research. When a plant is considered as a source for one or other natural product, a thorough evaluation with respect to its economic potential as well as its possible side effects on humans, animals and plants remain important aspects to be researched and considered before a final decision on its application potential can be reached. This applies for crude and semi-purified extracts as well as for purified compounds (Gorris & Smid, 1995).

At this point it must be acknowledged that plants currently play an important role as a source of novel, biologically active compounds and this is well documented (Recio *et al*., 1989; Hostettman & Wolfender, 1997; Eksteen *et al*., 2001). From a research point of view, different scientific disciplines such as chemistry, biochemistry, microbiology, entomology, virology, botany and the different agricultural disciplines have the joint responsibility to further investigate the sustainable use of natural plants (Van Der Watt, 1999).

In this monograph a crude bulb extract of *Eucomis autumnalis* was screened for biostimulatory and antifungal activity. Ault (1995) found that *E. autumnalis* could be successfully propagated *in vitro*. On the one hand this must be regarded as important in the case where natural vegetation is intended to be used as the source for a natural product. On the other hand this may implicate the potential of the source plant to be developed into an alternative crop with secondary economic value to farmers propagating it. All these aspects were considered in this study.

2.2 *Eucomis autumnalis* Mill.

2.2.1 Distribution in South Africa

Eucomis autumnalis occurs naturally along the eastern parts of South Africa, including the Eastern Cape, Free State, Lesotho, Kwa-Zulu Natal, Mphumalanga and Gauteng as well as the eastern parts of the North West province and the southern parts of the Limpopo Province (Van Wyk *et al.*, 1997). The plant is also a very popular ornamental plant found in gardens throughout this region.

2.3.2 Botanical description

E. autumnalis is a bulbous plant. The plant has long broad leaves with wavy margins and numerous small, yellowish-green flowers borne on a thick central stalk (**figure 2.1**). Above the flowers a rosette of green leaves is found. This characteristic gives the flower cluster the appearance of a pineapple (Van Wyk *et al.*, 1997), hence the common name pineapple lily or pineapple flower (**figure 2.2**).

Figure 2.1: *Eucomis autumnalis* (Van Wyk *et al.*, 1997).

Figure 2.2: The flower cluster of *Eucomis autumnalis* (Van Wyk *et al.*, 1997).

2.3.3 Medicinal uses

E. autumnalis is used by the Zulu people of South Africa for medicinal purposes such as to cure gastro-intestinal illnesses in infants and children (Hutchings, 1989). The plant has also been reported to possess antimicrobial and analgesic activities (Masika *et al.*, 1997) and shows high inhibition of prostaglandin-synthesis (Jager *et al.,* 1996). Recently Van Wyk *et al*. (1997) also reported anti-inflammatory, antispasmodic, anti-pyretic and purgative activities of *E. autumnalis*. A decoction of the bulb is also used for low backache, to aid in the healing of fractures and to assist in postoperative recovery. Decoctions of the bulb (**figure 2.3**) are frequently administered as enemas. Terpenoids present in *E. autumnalis* are known to be beneficial in the treatment of different wounds, including burn wounds. (Van Wyk *et al.,*1997).

Figure 2.3: The bulb of *Eucomis autumnalis* (Van Wyk *et al.* 1997).

2.3.4 Known active ingredients

E. autumnalis contains benzopyrones such as autumnariol and autumnariniol as well as steroidal terpenoids such as eucosterol. Several homoisoflavonoids, of which eucomnalin and 3,9-dihydroeucomnalin are the best known, have also been isolated from *Eucomis autumnalis* (Van Wyk *et al.* 1997).

2.4 Economically important fungal and bacterial plant pathogens in South Africa included in this study

In light of the antimicrobial properties of a crude *E. autumnalis* extract that was investigated in this study against plant pathogenic bacteria and fungi, a short discussion on the pathogens used in the screening procedure is supplied.

2.4.1 Fungal pathogens

2.4.1.1 *Fusarium oxysporum* (Slechtend. Fr.)

Fusarium oxysporum and *F. equiset*i are probably South Africa's most widely distributed cosmopolitan fungi, and is mostly associated with organic matter and plant debris. These fungi are frequently found in maize fields where they can exist as both saprophytes and parasites (Rheeder & Marasas, 1998). This pathogen is also well known for causing the abscission of avocado flowers (Thomas *et al.*, 1994) and as a pathogen of wheat causing diseases such as head blight (Boshoff *et al*., 1998). According to Boshoff *et al.* (1998), head blight caused by *F. oxysporum* reached epidemic proportions on irrigated wheat along the Orange River in the Northern Cape during the early 1990's. Toxigenic *Fusarium* species are currently still viewed as a major threat to the quality of both grain and grain products. The presence of mycotoxins in feed has been linked with livestock toxicoses, food refusal and poor reproductivity. Grain containing mycotoxins may thus be downgraded or rejected entirely by the industry.

According to Herbert & Marx (1990), Fusarium wilt or Panama disease, (caused by *F. oxysporum*), resulted in significant damage to banana plantations in the past. If not controlled efficiently, Fusarium wilt can destroy a whole banana plantation in a relatively short period of time. Furthermore *F. oxysporum* also causes dry rot, stem-end rot and wilt of potatoes. Fusarium dry rot is mainly a post harvest disease and can become a major problem when infected potatoes are stored (Venter & Steyn, 1998). The pathogen also causes wilt disease in tomatoes (Uys *et al*., 1996). Fusarium wilt was found to occur most predominantly where tomatoes are cultivated throughout the year and was isolated in complex with nematodes.

F. oxyspurum also infects medicago, especially in the roots and crown (Lamprecht *et al.*, 1990). In other studies isolations were made from discoloured tissue of grapevines showing dieback symptoms in the winter rainfall region of South Africa. One of the predominant fungal species designated as parasites were *F. oxysporum* (Ferreira *et al*., 1989).

2.4.1.2 *Botrytis cinerea* Pers.:Fr

Blossom-end rot, caused by *Botrytis cinerea* is a post harvest disease of pear fruit that normally occurs late in the storage period. In South Africa, this disease caused substantial losses during the period 1984-1988. *Botrytis cinerea* enters the flower receptacle or mesocarp tissue of immature pear fruit

from stamens and sepals via vascular tissue and becomes latent in these tissues. *B. cinerea* has also been associated with vascular tissue in raspberry, grape and black current by plugging the xylem and restricting hyphal growth. It is, however, not a true vascular pathogen in these fruits (De Kock & Combrink, 1994).

In other studies Serfontein and Knox-Davies (1990) found that *B. cinerea* caused flower head blight in *Leucadendron discolor*, resulting in unopened dead flower buds during the early flowering stage. This fungus is also a pathogen of other protea species causing forms of tip blight and is a troublesome pathogen of peas, causing grey mold, and in severe cases the wilting and drying of foliage (Baard & Los, 1989). According to Fourie and Holtz (1998), *B. cinerea* is one of the major pathogens responsible for post harvest decay of stone fruit in the Western Cape Province of South Africa. The disease commonly occurs on plum blossoms in local stone fruit orchards and plays an important part in blossom blight and was also identified as one of the pathogens causing dieback in grapevines (Ferreira *et al*., 1989).

2.4.1.3 *Mycosphaerella pinodes* (Berk. & Blox.) Vestergr

Mycosphaerella pinodes is an important and widespread disease of pea (*Pisum sativum*). According to Allard *et al*. (1992) all aerial parts of the pea plant is susceptible to infection by *Mycosphaerella pinodes.* The fungus infects the pea at seedling stage, affecting the stem, and at later stages of growth where mainly the leaves are affected (Ryan *et al.*, 1984; Hagedorn, 1984), this foliar infection is commonly known as Black Spot or Ascochyta Blight (Kraft *et al*., 1997).

2.4.1.4 *Sclerotium rolfsii* Sacc

Sclerotium rolfsii is a residual fungus that inhabits plant residue in soil, from where it infects susceptible hosts. The fungus is responsible for Basal stem rot in wheat seedlings (Scott, 1990). Soilborne diseases such as Sclerotium rot caused by *S.rolfsii* are important root diseases of tomatoes (Uys *et al*., 1996).

2.4.1.5 *Rhizoctonia solani* Kühn

Root rot caused by *R. solani* is one of the most important root diseases found on tomatoes (Uys *et al*., 1996). Appart from that, the fungus also causes major diseases such as leaf spot on tobacco as well as hypocotyl rot and leaf blight in beans (Meyer *et al*., 1990). According to Carling *et al.* (1996) as well

as Opperman and Wehner (1993) *R. solani* is an important pathogen of wheat causing crater disease especially in Tanzania and on the Springbok flats in South Africa.

2.4.1.6 *Verticillium dahliae* Kleb

Verticillium wilt is currently the most important disease causing losses to cotton crops in South Africa. During the 1994/1995 season, 81 000 ha of cotton were cultivated under both dryland and irrigation conditions. A year later 20 000 ha of this land cultivated with cotton were infested with the wilt pathogen *V. dahliae*. Irrigated cotton is severely affected by this disease, especially in soils with a high clay or silt content (Swanepoel & De Kock, 1996). The epidemical occurrence of *Verticillium* wilt in cotton in South Africa can probably be ascribed to agricultural practices followed by farmers in the problem areas. Many farmers plant wheat that is able to reduce the soil inoculum but fail to control weeds which can act as hosts to the disease. This can be detrimental since vegetables such as potatoes, tomato, beetroot, eggplant and sweet potatoes are all susceptible to *Verticillium* wilt, causing an increase in inoculum and a decrease in production (Swanepoel & De Kock, 1996).

Tomato is a high potential crop grown in seven of the nine provinces of South Africa (Uys *et al*., 1996). A survey conducted in all the main tomato growing regions of South Africa showed that wilt disease occurred in all the regions and *V. dahliae* were one of the predominant fungi causing the disease in many crops. Knowledge of the losses caused by tomato diseases is essential when prioritizing and developing disease management strategies. One of these strategies includes the use of natural fungicides.

2.4.1.7 *Botryosphaeria dothidea* (Moug.:Fr.) Ces & De Not

Pistachio nut cultivation is a developing industry in the Northern Cape area of South Africa with enormous economical potential for the country. *Botryosphaeria dothidea* has been identified recently as a major cause of basal cankers and the discoloration of xylem and phloem in the stems of pistachio trees and poses a major threat to the industry. The appearance of this pathogen on adult pistachio trees justified the need for establishing a disease management programme for pistachio in South Africa (Swart & Blodgett, 1998). *Botryosphaeria dothidea* is also known to cause canker and dieback in *Pinus* and *Eucalyptus* species. This is caused by fast spreading endophytic infections with the potential of enormous economic losses in the dynamic forestry industry of South Africa (Smith *et al*., 1996).

2.4.1.8 *Pythium ultimum* Trow

Wheat is one of the world's most important food crops and is widely cultivated. This means that wheat is subjected to infection by a wide range of pathogens. One of the most important of these pathogens is *P. ultimum* causing root rot and damping off in wheat. *Pythium ultimum* is also known for causing damping off in alfalfa (lucern; Eksteen *et al*., 2001).

2.4.2 Bacterial pathogens

2.4.2.1 *Clavibacter michiganense* subsp. *michiganense*

Clavibacter michiganense is a bacterial pathogen that causes bacterial canker and wilt in tomatoes (Agrios, 1997; Trench *et al.*, 1992). It usually occurs in the summer and is also a known pathogen of ornamental plants (Sidorovich, 1986). Most producers follow a regular spray programme that contains copper based fungicides such as copper oxychloride, copper ammonium carbonate or cupric hydroxide. These chemicals are usually alternated in the spray programme with other fungicides to reduce the incidence of bacterial leaf spot diseases (Uys *et al.*, 1996).

2.4.2.2 *Pseudomonas syringae* pv. *syringae* (Pseudomonadaceae)

Pseudomonas syringae causes bacterial cankers and gummosis in stone fruit trees in all the major fruit growing areas of the world (Agrios, 1997). It has also been found to affect apple, pear, citrus, ornamentals, vegetables and some small grains (Agrios, 1997). The disease caused by this pathogen is kown by names such as bud blast, blossom blast, die back, spur blight and twig blight. *P. syringae* is also responsible for bacterial brown spot in beans (Trench *et al*., 1992). Trench *et al*. (1992) identified *P. syringae* as the causal agent of blister bark on apples, bacterial canker on peaches, plums and apricots, blossom blast on pears, bacterial blight on peas and ice nucleation on wheat.

2.4.2.3 *Erwinia carotovora* subsp. c*arotovora* (Enterobacteriaceae)

This bacterium is part of the "carotovora" or "soft rot" group, specifically causing soft rot in a number of fleshy fruits, vegetables (Agrios, 1997) and ornamentals (Vanneste *et al.*, 1998; Sidorovich, 1986). These diseases occur worldwide mainly in fleshy storage vegetables, but it is also known to cause huge damage of crops in the field and in transit. Well known potato diseases caused by *E. carotovora* include Blackleg, Erwinia wilt and Post-harvest soft rot (Sinden *et al*., 1993; Trench *et al*., 1992). *Ervinia carotovora* pv. *carotovora* is also the causal agent of bacterial soft rot of onion (Alice & Sivaprakasam, 1995) and tobacco as well as tomato pith necrosis (Aysan *et al.,* 2000).

2.4.2.4 *Agrobacterium tumefaciens* (Rhizobiaceae)

Agrobacterium tumefaciens introduces the Ti-plasmid (T-DNA) into cells, transforming these normal cells into tumor cells in a short period of time. This leads to the formation of galls in the stems and roots of different crops. The best known disease caused by this pathogen is crown gall, found on many woody plants, primary stone fruits (Trench *et al*., 1992), pome fruits, grapes and also on willows (Agrios, 1997).

2.4.2.5 *Ralstonia solanacearum (Pseudomonas solanacearum)* (Pseudomonadaceae)

Ralstonia solanacearum (*P. solanacearum*) is an important pathogen causing bacterial wilt on vegetable crops such as pepper, potato and tomato (Abd El Ghafar, 1998; Ishikawa *et al*., 1996; Trench *et al*., 1992), as well as carrots and onions (Lemaga *et al*., 2001). *Ralstonia solanasearum* is also responsible for causing bacterial rot on ginger (Lemaga *et al*., 2001).

2.4.2.6 *Xanthomonas campestris pv. phaseoli* (Pseudomonadaceae)

Xanthomanas *species are well known bacteria causing leaf spots, fruit spots and blights of annual and perennial plants, as well as vascular wilts and citrus canker. One of the most important disaeases caused by* X. campestris pv. phaseoli *is common blight also known as bacterial blight on beans (Agrios, 1997; Trench* et al., *1992; Ohlander, 1980). Another important disease caused by* X.campestris *pv*. phaseoli *is bacterial leaf blight or green gram (Marimuthu & Kandaswany, 1980).*

2.4 The potential use of natural antimicrobial products in the agricultural industry

Plants are a major source of products already used in the so-called speciality chemical industry. Secondary metabolites extracted from plants are valued from several dollars per pound (e.g. the insecticide pyrethrum), to several thousand dollars per pound (jasmine oil), emphasizing the huge economic potential for natural agrichemicals (Flores *et al*., 1987). Both biotic and abiotic stress conditions that have to be dealt with in the agricultural industry, as well as the effect of synthetic chemicals on the environment, supplies the rationale for investigating the application potential of natural products on a larger scale that is currently the case.

One of the biotic stress factors that have to be dealt with is that of fungi growing on stored grain. The presence of these fungi can result in reduction in quantity and quality leading to severe economic losses for the farmer. In addition, many fungal species produce mycotoxins that are highly toxic to animals

and humans. Traditionally synthetic fungicides (mainly low-molecular-weight organic acids) have been used for the preservation of stored grain. However, many disadvantages are associated with the use of these products and there is a worldwide trend in limiting their usage in grain and foodstuffs.

It is for this reason that considerable time and effort has been devoted to the search for new or alternative antimicrobials over the past twenty years. Indications are that natural plant extracts may provide an alternative to these preservatives and many antimicrobials occurring naturally in plants have already been identified. For example, plant species belonging to the family Asteraceae contain a diversity of acetelynic compounds. Three important biological features of these compounds are: (1) they show activity against fungi, bacteria and nematodes, (2) most of the biologically active compounds are found in the roots and (3) polyacetylene production may be elicited by fungal pathogen infection (Flores *et al*., 1987).

A large number of Indian medicinal plants are attributed with antimicrobial actions and a screening programme was launched to investigate these claims. A number of plant extracts were screened for antibacterial and antifungal properties and selected for further testing (Naqvi *et al.*, 1991). In other studies Anaya *et al.* (1995) used fungi, seeds and insects to assess activity of extracts and isolates from Mexican plants, in research based on the vast diversity of Mexican flora and its potential as a source of useful natural products. They found that allelochemicals could modify cellular structure and activities including respiration and division.

2.5 Phytochemicals in plants with biostimulatory or inhibitory properties

2.5.1 General remarks

In light of the fact that the potential biostimulatory properties of a crude *E. autumnalis* bulb extract was also investigated in this study, a short review on the regulatory role that known phytochemicals play in controlling growth in plants, as well as its application potential in the agricultural industry, is supplied.

Many plants produce secondary metabolites that are harmful to other species and that reduce competition in their natural habitats. This phenomenon is called allelopathy (Rice, 1974). The term allelopathy was deduced from two Latin words “allelon” and “patos” that literally mean “to suffer on each other” (Rizvi & Rizvi, 1992). Despite the meaning of the words, the authors defined allelopathy not only in terms of the possible detrimental effect one plant can have on another but also the possible

beneficiary effect through chemical substances that are released into the environment. Although allelopathy must be regarded as a rather neglected scientific discipline, the application potential of secondary plant metabolites with allelopathic properties, either in a crude or semi-purified form as commercial natural products in the agricultural industry, is recognised (Rizvi & Rizvi, 1992). It was this potential that prompted the investigation into the possible biostimulatory properties of *E. autumnalis.*

Dynamic chemical and biological evolution over millions of years has lead to the production of new end products that, from an allelopathic perspective, might be beneficial for some organisms but also detrimental to others (Rizvi & Rizvi, 1992). However, likewise toxins could have been produced in plants that are dangerous to humans and animals. Currently there is renewed interest in bioactive components, including toxins (Rizvi & Rizvi, 1992). Mid- to long-term developments likely to have a major impact on industries based on plant derived chemicals include the discovery of new secondary metabolites from unexplored plant sources. This is based on the urgent need to understand and preserve the chemical inventory of higher plants before it is lost for ever (Flores *et al*., 1987).

According to Waller (1989), allelopathic interactions between plants and other organisms may become an alternative to herbicides, insecticides and nematicides for disease and insect control. Allelochemicals are primarily secondary products of plant metabolism that may undergo a variety of reactions with plant, insect and animal species to inhibit or stimulate their growth and development. In past studies allelochemicals from *Gliricidia sepium* were extracted and the effect on the seedling growth of sorghum tested. These studies showed that seed germination and root elongation of sorghum seedlings were inhibited by various compounds in the extract (Ramamoorthy & Paliwal, 1993). A crude acetone extract of papaya seeds was shown to completely inhibit the germination of velvet leaf seeds and benzyl isothiocyanide was confirmed to be the active substance involved (Wolf *et al*., 1984). Benzyl isotiocyanate also showed strong antibacterial and antifungal properties.

Further, a field survey in apricot growing areas in India showed retardation in the germination, growth and yield of nearby wheat plants. It was noted that the magnitude of interference gradually decreased as the distance from the tree increased. Extracts of aerial parts of apricots were made and screened for their phytotoxic effect on germination and growth of wheat. Residues of light petroleum and ethyl acetate extracts showed the highest inhibition of both germination and growth of wheat seeds and

seedlings respectively. Upon further testing it was found that proanthocyanidin compounds were responsible for these activities (Rawat *et al.*, 1998).
Juglone inhibited cell elongation in the epicotyls of etiolated pea while root cell elongation was inhibited to an even greater extent. A juglone solution at a concentration of 10^{-3} M significantly decreased respiration (O_2 uptake) in roots of pea and lettuce within 30 minutes after the start of treatment, and completely reduced O_2 uptake after 2 hours. At this concentration juglone also decreased the content of total soluble protein of α-amylase activity produced by gibberellin by up to 78% in the aleuron cells of barley embryoless half seeds (Li *et al.*, 1993). Root respiration and relative plant fresh weight of *Alnus glutinosa* were reduced by a 2 x 10^{-5} M juglone treatment (Neave & Dawson, 1989).

Padhy *et al.* (2000) studied the influence of different concentrations (5, 10, 15 & 20%) of leachates of *Eucalyptis globulus* litter on seed germination, seedling growth and some physiological and biochemical aspects of finger millet *(Eleusine coracana*). They found that the leachate considerably inhibited seed germination. The longer the duration of pre-soaking of seeds in leachates, the greater was the inhibition. Increase of pre-soaking time of seeds in leachate as well as increases of leachate concentration decreased the respiration rate and catalase and alpha amylase activities, but increased peroxidase activities. Chlorophyll synthesis in leaves as well as protein carbohydrate and nucleic acid content in both shoots and roots of seedlings were also decreased with increases in leachate concentration. Yatagi & Ding (1996) found that n-hexane extracts from *Pinus massoniana* leaves greatly inhibited the growth of radicles and hypocotyls of radish (*Raphanus sativus* var. *radicula*) seeds.

However, natural secondary compounds from plants that stimulate growth in crops have also been reported in the past. Field tests over a number of years, demonstrated that wheat grain yields were increased appreciably when grown in mixed stands with corn cockle as compared to pure stands of wheat. One of the stimulatory substances isolated from the corn cockle was named agrostemmin which, when applied at the rate of 1.2 kg ha^{-1}, increased grain yields of wheat on both fertilized and unfertilized areas. Chopped alfalfa had a stimulatory effect on the growth of tomato, cucumber and lettuce. The active substance was identified as triacontanol (Putnam & Tang, 1986). p-Hydroxycinnamic acid was identified as a major compound in mungbean (*Vigna radiata* L.) plants and their rhizosphere soil. Crude mungbean saponins increased germination and enhanced growth of planted mungbeans, but the soil treatment did not increase yield (Chou *et al*., 1995).

Probably the best known growth stimulators are the phytohormones. Already in the nineteenth century the existence of growth stimulating substances in plants were suspected by Darwin and other researchers before him. It was a Danish researcher, Boysen-Jenson, that obtained the first proof in 1910. His research led to the discovery of a chemical growth stimulant that was named auxin by Fritz Went in 1926. Other growth stimulants namely cytokinins, gibberellins and ethylene were discovered later. The latter are all referred to as plant hormones since they comply with the definition of a hormone namely to be produced in one part of the plant but excert their effect elsewhere. Brassinosteroids (BRs), a new plant hormone family, was fairly recently discovered in a variety of plant species and organs (Schnabl *et al*., 2001).

2.5.2 Auxins

Auxins or indole-3-acetic acid (IAA; Mauseth, 1991) is primarily synthesized from the amino acid tryptophan and the main areas of biosynthesis are the leaf primordia and developing seeds. From here it is transported to the areas where growth is stimulated in the plant by means of a concentration gradient in the cells. According to the Cholodny-Went hypothesis, IAA is translocated away from light to the shadow side of the plant where it stimulates growth depending on its concentration (Salisbury & Ross, 1992).

Other functions of auxins include induction of length growth, stimulation of root formation and the accompanying increase in the uptake of water and minerals, apical dominance and the inhibition of leaf abscission. The hormone also influences the growth of stems in the direction of sunlight (phototropism), ensuring that the plant receives optimal quantities of light in instances where it is overshadowed by other plants.

2.5.3 Cytokinins

The first cytokinin was discovered in the 1950's and was isolated from coconut milk. All cytokinins are adenine derivates. Two natural cytokinins, zeatine and isopenthenile adenine, were discovered later. In plants, cytokinins are mainly produced in the root ends. The metabolic storage and transport of cytokinins is not yet clear, but it is suspected that transport takes place via the xylem from the roots to the aerial parts of the plant (Mauseth, 1991).

The main function of cytokinins is the stimulation of cell division in stems, and the inhibition of cell division in the roots as well as the induction of cell enlargement in leaves. High concentrations of

cytokinins are found in the endosperm that is involved in controlling the development and morphogenesis of the embryo and seed (Salisbury & Ross, 1992).

2.5.4 Gibberellins

In Japan, the fungus *Gibberella fujikuroi* causes a disease in rice called 'bakanae'. Infected plants show nominal stem elongation in comparison to uninfected rice plants. In 1926 Kurosawa proved that the reaction was repeatable when plants were treated with a filtrate of *Gibberella fujikuroi*. Subsequently, Japanese researchers concentrated on the identification and characterization of the active component that induced the elongation and in 1934 Yabuta succeeded in isolating the compound which was named gibberellic acid (GA_3). Similar physiological effects by GA_3 on other plants showed that the same chemical had to be present in higher plants. Since then different gibberilllins have been isolated, purified, characterized and their metabolic effects studied. Today at least 62 different gibberellins are known and numbered in the order of their discovery (GA_3-GA_{62}). Every plant species contains 6 to 10 different gibberellins, of which some are biologically active and others inactive (Mauseth, 1991).

Gibberellins vary in their specific activity in different species and concentrations are influenced by different environmental signals (Salisbury & Ross, 1992). The areas of biosynthesis in the plants are the young tissue of seeds and the stem apex while roots are only considered a possible area for biosynthesis. Gibberellins are transported by both the xylem and phloem of the plant. The functions of gibberellins include cell elongation, the mobilization of food reserves in the seed, the inhibition of seed formation, stimulation of flower and pollen growth and the decay of fruit.

2.5.5 Ethylene

Cousins, a ship's captain, noticed the effect of ethylene in 1910 when he found that the presence of over ripe apples in a crate of unripe bananas accelerated the ripening process. In 1934 Gane proved that ripening fruit released ethylene gas. It was however, only in the 1950's that ethylene was accepted as a natural growth regulator. Ethylene is the only plant hormone known to exist in gas form and is now known to induce the ripening process in cells. It is transported in the apoplast via intercellular spaces and released during the fruit ripening process. The gas is used in the industry for artificially ripening of climacteric fruit such as bananas, apples, mangoes and avocado's (Bennett *et al*., 2001).

Auxins stimulate ethylene production in a target area from where it diffuses quickly to adjacent areas and cause a response much faster than auxin is capable of producing (Mauseth, 1991). It therefore seems that ethylene can act as final effector of auxins. Functions of ethylene include apical dominance or inhibition of branch elongation, stimulation of stem thickness growth, stimulation of leaf and fruit abscission (Salisbury & Ross, 1992) as well as regulation of cell metabolism in the plant.

2.5.6 Brassinosteroids (BRs)

A wide range of effects in plants have been attributed to brassinosteroid (BR) activity. These include induction of resistance to microbial infection (Schnabl *et al*., 2001), cell division and cell elongation (Krizek & Mandava, 1983), hypocotyl growth, increase in leaf lamina growth and shoot apex fresh weight (Meudt *et al*., 1983). Although the BR mechanism of action has not been elucidated to date, similarities with that of other plant hormones namely cytokinins (Clouse, 1996) and auxins (Cao & Chen, 1995) have been proposed. Strong synergism between BRs and auxins has also been found in a number of studies (Arteca *et al.*, 1988). Both these hormones increase coleoptile growth, fresh weight and ethylene production. However, BRs cannot be classified as auxins, cytokinins or gibberellins but have the ability to increase the auxin sensitivity in plant tissue and to influence endogenous hormone levels. Clouse (1996) found that auxins (IAA) and BRs differ on gene expression level.

Brassinolide (BL), castasterone (CS), teesterone (TE) and 6-deoxycastasterone (6-deoxyCS) are of the best known BRs isolated from plants and all belong to the C_{28}-BRs with a 24α-methyl group. Recently it was discovered that these BRs are produced though campesterol biosynthesis (Fujioka & Sakurai, 1997; Sakurai & Fujioka, 1997). Of all the named BRs, BL is the most biologically active. As a result of this, BL is seen as the most important in terms of its role in plant growth regulation.

As different plant parts (pollen, seeds, leaves, roots and flowers) contain BRs, it seems that the biosynthesis of BRs is not limited to a specific plant organ (Fujioka & Sakurai, 1997). BRs are also widespread in the plant kingdom and not confined to specific species. The highest BR concentration is found in pollen and unripe seed. The pollen of *Helianthus annuus*, for example, contains more than 100 ng BR per gram fresh weight (Schmidt *et al*., 1997). In comparison to pollen and unripe seed, other plant parts only contain subnanogram quantities of the BRs.

When the diverse variance of the chemical structures of the A-ring, B-ring and side chains of the BRs are taken into account, it highlights the possibility that more than a hundred different BRs could be

present in the plant kingdom. It is expected that more BRs and BR related compounds will be discovered in the future. The search for more unknown BRs in plants, as well as their physiological and biochemical roles in plants, remains a challenge.

2.6 Phytochemicals with antimicrobial activity

2.6.1 General remarks

Over the past decade there has been an elevated interest in the search for antimicrobial agents of plant origin, the isolation and identification of the actual active compounds as well as the possible integration of these agents in organic crop protection and pest management programmes (Eksteen *et al.*, 2001). Despite substantial information on the antimicrobial effects of South African plant extracts on human pathogens, relatively little information is available on the efficiency of plant preparations against plant pathogens and especially fungal pathogens. However, interest in the use of plant crude extracts as ‘green chemicals’ has been shown by agrochemical companies, such as BASF, that are currently involved in extensive screening programs of plants with antimicrobial activities. One commercial preparation resulting from this programme is marketed as Milsana™, a dried extract of *Reynoutria sachalensis* (Polygonaceae), used to control powdery mildews (Gorris & Smid, 1995).

Recent studies on the antimicrobial activity of plant extracts showed the importance of natural chemicals as a possible source of non-phytotoxic, systemic and easily biodegradable alternative pesticides (Qasem & Abu-Blan, 1996). It was found that volatile materials of *Chenopodium ambrisoides* were highly toxic to *Rhizoctonia solani* while leaf extracts of *Rananculus elematis* was effective against *Alternaria tenuis*. The differences in the toxicity of different extracts may be due to their solubility in water and or the presence of inhibitors to the fungitoxic principle. Further work is needed to isolate and characterize the active agents and their modes of action, in addition to studies on economic viability and dosage of the extracts, its frequency and timing of application (Qasem & Abu-Blan, 1996).

According to Salie *et al*. (1996) the indiscriminate use of antibiotics has resulted in the emergence of a number of resistant bacterial strains. The high cost of developing new and more effective antibiotics makes the search for less expensive, alternative natural compounds imperative. Antifungal drugs are amongst the most expensive antibiotics. Especially species from the family Asteraceae show strong antifungal activity emphasizing the potential for discovering natural compounds with application

potential in the pharmaceutical industry. Moreover, aromatic plants have been used for centuries in folk medicine and food preservation, providing a range of potential compounds possessing pharmacological activities. Antimicrobial activities are mostly frequently found in the essential oil fraction (Gorris & Smid, 1995).

Recent developments in agriculture include a shift towards organic farming enterprises, especially in Europe, as a result of consumer resistance towards synthetic inorganic agrochemicals. Despite this development, the application of natural antimicrobial compounds as anti-infective agents in the agricultural industry, remains a rather neglected research field (Gorris & Smid, 1995).

2.6.2 Antibacterial phytochemicals against human and plant pathogens

A variety of phytochemicals have been studied for their antibacterial activity and potential usefulness against infectious diseases in humans. Over the past decade, flavonoids have been identified as the active antibacterial substances in a variety of plant extracts. Catechins (Toda *et al*., 1991) chalcones (Szajda & Kedzia, 1991), isoflavanones (Iinuma, *et al*., 1992), flavanones and flavanols (Kuruyanagi, *et al*., 1999) have all been reported to possess antibacterial activity. However, in some cases the test concentrations were relatively high making it difficult to assess the pharmaceutical value of these extracts in view of the very low MIC's required by the pharmaceutical industry.

Five flavonoids, namely rutin, neohesperidin, hyperoside, cactichin and ferulic acid, were recently isolated and identified from *Carpobrotus edulis* leaves that were individually or collectively responsible for the *in vitro* antibacterial activity against eleven human pathogens (Van der Watt & Pretorius, 2001). The collective action of these flavonoids extracted from the same plant points towards the broad spectrum anti-infective potential of the crude extract from this plant against human bacterial pathogens.

B) Rutin and its derivatives are combined with alkaloids for the treatment of senile cerebral defects (Vlahov, 1992). It also relieves micro trauma on tissue (Smith *et al*., 1998) and can help to improve the effectiveness of vitamin C as well as to prevent bleeding by strengthening veins (Kinghorn & Balandrin, 1993). Although rutin is relatively abundant in plants, only a small number of single species contain quantities sufficient for industrial extraction (Bruneton, 1995). This highlights a common problem and that is the effort that has to be put into identifying the

potential source or sources of a promising compound in terms of its industrial exploitation potential, especially in the event where the original source (wild or food plant) is considered for the compound to maintain its natural status.

Neohesperidin is less common than its unsaturated homologue, naringin, because most plant families accumulate its C-alkyl derivatives (Markham, 1982) and nothing is known about its antibacterial properties. The report on the antibacterial activity of neohesperidin (Van der Watt & Pretorius, 2001) against two gram (+) (*Staphylococcus epidermidis* and *S. aureus*) and two gram (-) bacteria (*Moraxella catarrhalis* and *Pseudomonas aeruginosa*) can therefore be regarded as important new information.

Catechin was described by Bruneton (1995) as a condensed form of tannin. It can be used to protect the skin layers against fluid losses and also has a vasoconstricting effect on small veins (Jansman, 1993). It kills bacteria by directly damaging the cell membrane, confirming that catechin is an antibiotic. It also shows a synergistic effect when combined with known antibiotics. Further, the findings of other authors on the antifungal activity of catechin (del Rio *et al.*, 2000) as well as the first indications that catechin and its derivatives can be synthesized (Coetzee *et al.*, 2000) needs to be mentioned.

Fukunaga *et al.* (1989) reported that the antibacterial activity of purified compounds is sometimes lower in comparison to that of the crude or even semi-purified fractions from which it was isolated. The authors speculated that this could be due to the possibility that these compounds function in synergy, either together or with a range of other compounds. It is well known that medicinal plant extracts are used either as complex mixtures or as pure compounds depending on their therapeutic indices.

This does not mean that there is no need to isolate and study the constituents of plant extracts separately. A number of reasons can be mentioned why constituents of an extract should be investigated separately after isolation and purification but, the determination of its toxicity and dosage are probably the two most important. Moreover, according to Eloff (1998), the possibility exists that natural antimicrobial components in plants can inhibit the growth of bacteria by means of unknown mechanisms other than that of known antibiotics, and for this reason the search for new antibiotics must continue.

Methicillin-resistant *Staphylococcus aureus* (MRSA), resistance of which is due to α-lactamase production, was isolated in the early 1960s. Since resistance due to penicillin-binding protein 2', so-called intrinsic resistance (Utsui & Yokota, 1985) was acquired, *S. aureus* multi-resistant to various antibiotics has been emerging worldwide as one of the major nosocomial pathogens in hospitals (Mulligan, et al., 1993) Potent anti-MRSA activity was recently found in phytoalexins isolated from *Sophora exigua*, and they were chemically characterized as hydroxyflavones (Iinuma et al., 1994). Tsuchiya *et al.* (1996) isolated differently substituted flavanones from *Sophora exigua* (collected in NE Thailand), *S. leachiana* (from the USA) and *Echinosophora koreensis* (collected in Korea), and studied their antibacterial activity against methicillin-resistant *Staphylococcus aureus* (MRSA). The minimum inhibitory concentrations (MICs) of phytochemical flavanones to clinical isolates of MRSA were determined by a serial agar dilution method.

From the selected data presented it can be concluded that the development of a natural product, containing a range of flavonoids or other active substances and producing a remedy with broad spectrum anti-biotic activity through a synergistic effect, seems achievable. However, identification of suitable natural plant sources, as well as economical issues pertaining to the isolation, purification and quality control of compounds, will have to be considered carefully.

The development and practical implementation of plant derived antimicrobials in the crop production industry, in relation to both food preservation and crop protection, must be regarded as at least as important as the need to develop natural products for the pharmaceutical industry. Interest in the use of chemicals of plant origin, especially as crude plant extracts, is increasing and larger agrochemical industries are partaking in this development because of the increased awareness of dangers involved in the use of particularly copper containing synthetic agrochemicals. However, very little information on the application of natural products as anti-infective agents in the crop production industry is currently available in the literature and this remains a rather unexplored research field.

2.6.3 Phytochemicals with antifungal activity

Interestingly, far less reference in the literature towards the antifungal activity of plant extracts against human and plant fungal pathogens could be traced compared to the reference towards bacterial pathogens. However, according to Ushiki *et al*. (1996) most plants exhibit inhibitory and stimulatory biochemical interactions with other plants and microorganisms. Especially through root exudates,

higher plants are able to affect the micro flora in the rhizosphere. The production of biologically active substances by higher plants, that prevent phytopathogens from infecting crops, have been reported and many plant species that have been widely used for medicinal purposes, also exert an allelopathic effect.

More examples of screening against a limited number of pathogens include the work of Alavez *et al.* (2000). The roots of the tropical tree *Lonchocarpus oaxacensis* afforded the 3-hydroxyflavanones jayacanol and mundulinol, as well as two flavanones, mundulin and minimiflorin (Alavez *et al*., 2000). The antifungal activity of the four isolated compounds was tested against the wood rotting fungus *Postia placenta*, but only jayacanol was active. Two novel antibacterial (against *Staphylococcus aureus*) and antifungal (against *Candida albicans*) prenylated flavanones were isolated from *Eysenhardtia texana* (Texas, USA) and their structures established (Wächter *et al.*, 1999). Both these compounds were shown to inhibit the growth of *S. aureus* and *C. albicans* at a concentration of 100 µg ml^{-1}. Activity-guided fractionation of a methanol-dichloromethane extract of the aerial parts of *E. texana* led to the isolation and identification of 4',5,7-trihydroxy-8-methyl-6-(3-methyl-[2-butenyl])-(2S)-flavanone, 4',5,7-trihydroxy-6-methyl-8-(3-methyl-[2-butenyl])-(2S)-flavanone and 4',5-dihydroxy-7-methoxy-6-(3-methyl-[2-butenyl])-(2S)-flavanone on the basis of spectral data.

Analysis of phenolic compounds in *Olea europaea* revealed the presence of tyrosol, catechin, p-coumaric acid, rutin [rutoside], luteolin and oleuropein, while the principal compounds in *Citrus* spp. were flavonones and flavones (del Rio *et al.*, 2000). In both genera, the concentrations varied according to the species, although all acted as antifungal agents and were capable of inhibiting the growth of phytopathogenic fungi. It was possible to stimulate the biosynthesis and/or accumulation of these phenolic compounds in both *O. europaea* and *Citrus* spp. by treatment with 0.3% Brotamax, while 100 ppm benzylaminopurine [benzyladenine] had the same effect in *Citrus* spp. Increased polyphenol levels lead to improved defense mechanisms in both genera. For example, treated tangelo Nova fruit showed greater resistance to *in vivo* infection by *Phytophthora citrophthora*, since the constitutive polyphenolic compounds at the fruit surface provided a first line of defense. Treatment of plants considered as potential source plants for the isolation of flavonoids on an industrial scale with Brotomax, in light of indications that synthesis of phenolic compounds could be induced by this chemical *in vivo*, can be a tool to enhance the sustainability of such a venture. This might be especially important in the event where natural plants are considered as sources of flavonoids on an industrial scale and in light of the fact that chemical synthesis of these anti-infective compounds is not yet established.

The growth of *Rhizoctonia solani* was found to be inhibited by seaweed extracts (Barreto *et al.*, 1997). Further, many essential oil components have a fungistatic effect (Gorris & Smid, 1995). The *in vitro* mycelial growth of *Aspergillis ochraceus* and *A. niger* was inhibited by the essential oils of Oregano and Thyme. It was also found that wheat grains treated with an essential oil isolated from Oregano showed no infestation by fungal pathogens after two days of incubation on potato dextrose agar, while an essential oil from Thyme showed a very low level of infestation. Carvone is the main essential oil in caraway (*Carum carvi* L.) seeds and has been commercialised as Talent™ for inhibiting the sprouting of potato tubers in the Netherlands. Along with other monoterpenes, carvone also has a strong antifungal effect (Gorris & Smid, 1995). However, essential oils from Oregano and Thyme caused, to a certain extent, a reduction in the germination of wheat seed emphasizing that thorough investigations on many fronts are necessary to ascertain the application potential of natural products. On the other hand, exposure of tulip bulbs to these essential oils for up to three weeks showed no phytotoxic effects that would adversely affect its potential marketing quality (Gorris & Smid, 1995).

Lastly, an approach that needs special attention in future is the induction of natural resistance of crops to fungal diseases by means of natural plant derived compounds. Induced isoflavonoids, known as phytoalexins, are formed in leaves as a result of fungal inoculation and also have antifungal properties (Harborne, 1991). Phytoalexins is a term used to denote defensive substances produced by plants in response to an infecting pathogen (Brooks & Watson, 1985). Typical features of phytoalexin response to the infection of resistant potato cultivars by *Phytophtora infestans* include: (a) the inhibition of the development of the fungus on the tubers; (b) the tissue layers infected by the fungus suffer necrosis, during which fungi toxic phytoalexins are produced; (c) the defence reaction is limited to the invaded tissue as well as neighbouring tissues; (d) resistant and susceptible hosts show similar basic responses, but differ in the speed of phytoalexin formation and (e) the sensitivity of the host cell that determines the speed of reaction of the host to the challenge by the infecting organism is specific, and genotypically determined.

Another defense mechanism against fungal attacks in plants is via the induction of pathogenesis related-(PR)-proteins, such as chitinase, peroxidase, lypoxygenase and β-1,3-glucanase, by brassinosteroids in the apoplast of leaf cells (Schnabl *et al*., 2001). Studies with plant extracts containing brassinosteroids resulted in an enhanced resistance of plants in various pathogen systems such as gray mould in tomatoes, powdery mildew in cucumbers and tobacco TMV. Indications are that treatment with brassinosteroids seemed to sensitize the plants to respond earlier and with a higher

impact to pathogen attack, thus supporting the role of brassinosteroids in biotic stress tolerance (Schnabl *et al*., 2001).

2.7 Closing remarks

In this study extracts from bulbs of *Eucomis autumnalis* were screened *in vitro* (chapter 4) in crude and semi-purified forms against plant bacterial and fungal pathogens as well as for possible biostimulatory activity. Subsequently, a crude extract was tested *in vivo* (chapter 5) for antifungal activity using *Mycosphaerella pinodes*, that causes Ascochyta blight on peas, as a screening system. An attempt was also made to isolate, purify and identify the groups of chemical compounds responsible for the antifungal activity (chapter 6).

CHAPTER 3

GENERAL MATERIAL AND METHODS

3.4 INTRODUCTION

In this section only those methods that are applicable to more than one chapter will be discussed. Methods and experimental layouts that are applicable to a specific chapter will be dealt with in that chapter.

3.5 MATERIALS

3.5.1 Plant material

Bulbs of the test plant, *Eucomis autumnalis*, were collected at the Blyde River canyon in Mpumalanga, South Africa during 1999. The material was dried in an oven for 21 days at 60 °C. It was subsequently ground to a powder by means of a Retsch SM 2000 mill, equipped with a fine sieve, and stored at 4°C in airtight Scott Duran bottles.

3.5.2 Other material

Aluminium thin layer chromatography plates (Silica Gel 60 F254; 20 x 20 cm) was purchased from Merck (Germany), preparative thin layer chromatography plates (Silica gel + indicator, 1mm, G 1510/LS 254; 20 x 20 cm) were from Schleicher and Schuell (Germany) or Sigma (Germany). Most of the organic solvents used were from Merck (Germany) including methanol, hexane, diethyl ether, ethanol, chloroform, butanol, acetone, hydrochloric acid, sulfuric acid and glacial acetic acid.

Agar used for biotests, namely PDA (potato dextrose agar), Technical agar (TA) and nutrient broth agar (NBA) as well as packaging material for column chromatography (Silica Gel 60; particle size 0.063-0.200 mm; 70-230 mesh ASTM) was also supplied by Merck (Germany). Other chemicals used are mentioned in separate chapters.

3.6 METHODS

3.6.1 PREPARATION OF A CRUDE EXTRACT

One kilogram of dried *E. autumnalis* bulbs was powdered using a Retch SM2000 cutting mill, placed in large five liter glass bottles, covered with 100% methanol and extracted on a roller mill overnight. The first extraction was decanted, the methanol replaced twice and three extractions performed in total. The suspensions from three extractions were pooled and vacuum filtered through a double layer of Whatman filter paper (No. 1 and 3) by means of a Buchner funnel attached to an Erlenmeyer flask equipped with a vacuum pump. The filtrate was subsequently filtered for a second time by gravity through a single sheet of Whatman No. 1 filter paper. Most of the methanol was removed from the extract by means of vacuum distillation at 45°C, using a Buchi Rotary Evaporator (Bibby Stirilin LTD, England) equipped with a cooled Liebig condenser. Each sample was extracted twice and subsequently concentrated to dryness by means of a Speedvac concentrator. The extract was stored at –20°C after determining the dry matter yields.

Techniques to obtain semi-purified extracts by means of liquid-liquid extraction with organic solvents with different DC-values, hexane, diethyl ether, ethyl acetate and dichloro methane, as well as other chromatographic techniques will be discussed in the applicable chapters (see chapters 4 and 5).

3.6.2 GENERAL STEPS FOLLOWED DURING BIOTESTS

3.6.2.1 Preparation of the agar

Applicable volumes of distilled water and agar powder were mixed in concentrations as specified by the suppliers, autoclaved at 121 °C for 20 minutes and cooled to 45 °C in a water bath. To maintain a sterile environment all work was carried out in a laminar flow cabinet sterilized with 80% (v/v) ethanol. After opening the bottle containing the autoclaved agar, the neck of the bottle was flamed to ensure that it stayed sterile. To prevent further contamination, desired volumes of the agar was swiftly transferred to Petri dishes under sterile conditions. Petri dishes were rotated until the entire surface was covered and left to set. To insure complete setting of the agar, plates were stored in a laminar flow cabinet overnight, subsequently placed upside down in plastic bags and stored at 4 °C to prevent condensation on the agar. One of the plates was incubated at 35 °C for 24 hours to identify any possible contamination. In instances where contamination occurred, the process was repeated.

Stock cultures of plant pathogenic bacteria were maintained on different nutrient agar slants (Biolab) at 4 ^{o}C and prepared for antibacterial tests by allowing it to grow for 24 hours at 37 ^{o}C prior to inoculation. Cultures were left overnight and subsequently diluted 1:100 with fresh sterile agar broth to obtain 10^6 cfu ml^{-1} (Meyer & Afolayan, 1995).

3.3.2.2 Screening the crude extract or semi-purified components for antibacterial activities

Plant pathogenic bacteria were pure isolates provided by the Department of Plant Sciences (Plant Pathology), University of the Free State. These included *Clavibacter pv michiganense, Pseudomonas syringae pv syringae, Erwinia carotovora* subsp. *carotovora, Agrobacterium tumefaciens, Ralstonia solanacearum and Xanthomonas campestris pv. phaseoli* while one sensitive human pathogen, *Moraxella catarrhalis* was used as a test organism.

A modified agar diffusion method (Villar *et al.*, 1986; Rios *et al.*, 1988) was used for screening both crude and semi-purified extracts for antibacterial activity. This method is widely used for antibiotic susceptibility testing as well as for screening plant extracts (Kavanagh 1975; El-Abyad *et al*. 1990; Irobi & Daramola 1993). To obtain the desired test concentration of 1 mg ml^{-1}, the dried crude extract or semi-purified fractions were dissolved in a 10% (v/v) solution of DMSO in water. To optimize diffusion of the extract into the agar medium, the inoculated agar plates were kept at a low temperature (4 ^{o}C) for 1-2 hours prior to incubation for 24 hours at 37 ^{o}C in the dark (Rios *et al.*, 1988). All experiments were repeated twice and every extract was tested in duplicate. At the end of the incubation period, inhibition zones were measured with a transparent ruler on two axes at right angles to each other to give two readings from each test. The measurement included the assay hole (March *et al*. 1991; Pfaller *et al*. 1992).

Plate count agar (PCA) was used in the bacterial screening tests. The bacterial suspensions were streaked evenly on the agar plates with a sterile cotton bud. Holes of 6 mm in diameter were made in the agar and in the centre of each test plate by means of a sterile cork borer. Fifty µl crude extract or semi-purified fraction was transferred to the holes in the agar representing 50 µg of extract per hole. After equilibration at room temperature (Vanden Berghe & Vlietinck 1991), the plates were incubated for 24 h at 37 ^{o}C in the dark.

3.3.2.3 Screening the crude extract or semi-purified components for antifungal activities

Eight common South African plant fungal pathogens, selected from different taxonomic groups, were chosen to screen for antifungal activity of crude, semi-purified and purified extracts. These pathogens included *Botrytis cinerea* Pers.:Fr. (Hyphomycetes), *Fusarium oxysporum* Schlechtend.:Fr. (Hyphomycetes), *Mycosphaerella pinodes* (Berk. & Blox.) Vestergr. (Ascomycetes), *Sclerotium rolfsii* Sacc. (Agonomycetes), *Rhizoctonia solani* Kühn (Agonomycetes), *Verticillium dahliae* Kleb. (Hyphomycetes), *Botryosphaeria dothidea* (Moug.:Fr.) Ces & De Not. (Loculoascomycetes) and *Pythium ultimum* Trow (Oömycetes). All pathogens were initially grown on PDA (potato dextrose agar) for 8 days before being used to inoculate MEA (malt extract agar) plates.

A modified agar diffusion method (Rios *et al.*, 1988) was used for determining the inhibition of mycelial growth by the extracts. Two percent malt extract agar (20 g malt extract; Difco) and 15 g technical agar (Difco) in 900 ml sterile distilled water) was prepared, autoclaved for 20 minutes at 121 °C and cooled to 45 °C in a water bath. Three hundred µl of a 33% (m/v) Streptomycin solution was added to the basal medium for controlling bacterial growth. Each extract was dissolved in 100 ml sterile distilled water and amended in the agar to yield a final concentration of 1 mg ml^{-1}. The medium was poured into 90 mm plastic Petri dishes and left to set. The centre of each test plate was subsequently inoculated with a 5 mm plug of each of the pathogens grown for 8 days on malt extract agar. Plates were incubated at 25 ± 2 °C.

Radial growth was determined after 72 h by calculating the mean of two perpendicular colony diameters on each replicate (Villar *et al.*, 1986). A plate containing only the basal medium served as control. Additionally one plate containing 1 µg/ml carbendazim/difenoconazole (Eria$^{@}$ - 187.5 g/l EC) was used as a standard fungicide against each test organism separately to determine the effectiveness of the extract treatments by comparison. Each treatment was performed in triplicate.

3.3.3 SEPARATION OF POLAR AND NON-POLAR FRACTIONS OF THE METHANOLIC CRUDE EXTRACT BY MEANS OF LIQUID-LIQUID EXTRACTION

Liquid-liquid extraction was performed on the crude bulb extract with the following five solvents in a volume ratio of 2:1(solvent/tissue) and of increasing polarity (dielectric constant; DC) in the order *n*-

hexane (DC = 1.9), diethyl ether (DC = 4.3), chloroform (DC = 4.8), ethyl acetate (DC = 6.0) and methylene chloride (DC = 8.9) to separate the active compounds (Harwood & Moody, 1989). Fractionation with each solvent was repeated ten times by shaking vigorously on a mechanical shaker for 10 min. The five solvent extractives were collected separately and subsequently evaporated to dryness under vacuum at 35 ºC or below, depending on the solvent being removed, using a Büchi Rotary Evaporator. Each fraction was tested separately for antimicrobial activity.

The successful separation of compounds by liquid-liquid fractionation was tested by means of qualitative thin layer chromatography (Q-TLC, see 3.3.4) and illustrated as a TLC profile. All fractions were tested for antifungal activity (see 3.3.2.3). Only the most active fraction(s) was purified further using column chromatography and preparative thin layer chromatography (see 3.3.5 and 3.3.6).

3.3.4 QUALITATIVE THIN LAYER CHROMATOGRAPHY (Q-TLC)

Aluminium plates, covered with fluorescent Silica 60 (F 150/LS 254: 0.1 mm), was cut according to required measurements and used for Q-TLC separation of compounds in the fractions obtained by means of liquid-liquid extraction as well as column- and preparative chromatography. In the case of column chromatography fractions 10 µg of only every third fraction was transferred to the baseline of a TLC plate in order to obtain a profile of the compounds in each fraction. Chloroform : Methanol : Water (each ratio described separately in chapter 6) was used as mobile phase to develop the TLC-plate in a pre-conditioned glass development tank.

After the separation of compounds was completed, the front line was marked and the plates dried by a flow of warm air using an ordinary hair dryer. Subsequently, the plates were treated with 10% (v/v) ethanolic sulphuric acid (H_2SO_4) and allowed to develop for 5 minutes at 100 ºC in an oven. Dark spots that developed left a profile that, together with Rf-values, was used to characterize each fraction. In the case of column chromatography fractions, those with similar profiles were pooled, tested for antifungal activity (see 3.3.2.3) and stored for further purification.

In certain instances, standard colouring reagents (Wagner & Bladt, 1996), together with fluorescence under UV-light at 254 or 365 nm were used to develop Q-TLC plates in order to identify specific groups of components. The details of these procedures are presented in chapter 6.

3.3.5 COLUMN CHROMATOGRAPHY

The bioactive diethyl ether fraction obtained through liquid-liquid extraction (see 3.6 and 6.2.2.1), was fractionated further by means of column chromatography. Silica gel 60 was used as the stationary phase and prepared by adding 350 ml of the mobile phase (Chloroform : MeOH : H_2O; 80:20:10) to 150 g of the Silica powder. The Silica paste was then transferred to a glass column and allowed to settle by compaction. Subsequently, the mobile phase was slowly added, allowed to run through the system and left to equilibrate for a minimum of four hours before separation of the active fraction commenced. After the equilibration period, 5 ml of the active fraction was carefully placed on the surface of the stationary phase and the mobile phase allowed to flow by gravitation. The volume of fractions that was required was regulated at 5 ml by using a Gilson (model FC-203 B; USA) fraction collector (**Figure 3.1**). Record of the total volume of mobile phase used, as well as the flow rate per minute, was kept.

Chloroform : MeOH : H_2O in three different ratios and in order of increasing polarity were used as mobile phases in an effort to elude all active compounds in the bioactive diethyl ether fraction from the column. Firstly, 1200 ml (80:20:10), followed by 650 ml (60:40:10) and 350 ml (40:60:10) were used for this purpose. To ensure that all compounds were removed from the stationary phase, 350 ml 100% MeOH was used and the eluate collected as one final fraction.

Column dimensions were as follows:

Bed volume (πr^2 x column length): 1093 ml

Mass of extract loaded: 2000 mg

Fraction volume = 5 ml

Flow rate: 1.67 ml min^{-1}

Number of fractions collected: 340

Figure 3.1: **a.** Glass column containing the Silica gel, mobile phase and semi-purified fraction. **b.** Gilson (model FC-203 B; USA) fraction collector with test tubes collecting 5 ml fractions.

3.3.6 PREPARATIVE THIN LAYER CHROMATOGRAPHY (P-TLC)

Preparative thin layer chromatography (P-TLC) was performed on 20 x 20 cm Silica gel F1500/LS (1 mm) plates. Not more than 25 mg of a column chromatography fraction were dissolved in approximately 500 µl of a Chloroform : methanol : water (40 : 60 : 10) solvent system and loaded onto the plate by streaking evenly over the baseline with the aid of a glass capillary tube. Between applications the extract was dried by the flow of warm air using a hairdryer. This was repeated until all of the dissolved compounds were loaded. The plates were developed in a saturated chamber in the same solvent system that served as the mobile phase.

Detection of compounds was done under UV-light at 254 and 365 nm. Individual compounds were isolated by scraping off the detected zones of the absorbent layer from the plates and transferred to Eppendorff vials. The compounds with a RF-value above 0.5 were recovered from the silica by elution with ethanol, and those with an RF value below 0.5 were recovered from the silica by eluting with methanol (Ouelette, 1992). The Silica was removed by centrifuging at 12000 rpm for five minutes, the supernatant decanted and tested for antifungal activity. Only the active isolated compounds were again tested for purity in an original analytical TLC system using Silica gel 60 F_{254}-aluminium pre-coated plates with chloroform : methanol : water (40 : 60 : 10 ; Wagner & Bladt, 1996) as solvent system. Both the methanol and ethanol were evaporated under reduced pressure at 35 ^{o}C in a rotary evaporator. These compounds were then again tested for antifungal activity (see 3.3.2.3) by using the most susceptible fungus, *Sclerotium rolfsii* at a concentration of 0.5 mg ml^{-1}. The chemical groups to which the isolated compounds belonged were identified by means of standard colouring reagents (Wagner & Bladt, 1996).

3.3.7 STATISTICAL ANALYSIS OF DATA

Analysis of variance (ANOVA) was performed on the data, using the STATYSTICA for Windows: StatSoft Inc., statistical analysis program. LSD (least significant difference) procedure for comparison of means (Steele & Torrie, 1980 ; Mason *et. al.*, 1989) was applied to separate means ($P<0.05$). Treatments differing significantly were indicated in the tables by designating different sets of letters.

CHAPTER 4

PRELIMINARY SCREENING OF AN *Eucomis autumnalis* CRUDE BULB EXTRACT FOR BIOSTIMULATORY AS WELL AS ANTIMICROBIAL ACTIVITY TOWARDS PLANT PATHOGENS

4.1 INTRODUCTION

No organism exists alone, but constantly interacts with other organisms and its environment (Seigler, 1995). The fundamental aspects of many of these interactions are chemical in nature. Allelochemicals from plants are known to influence other plants (Putnam & Tang, 1986; Waller, 1989; Rizvi & Rizvi, 1992) as well as microbial organisms in many ways and these chemicals can virtually all be classified as secondary metabolites. The role of these compounds as "chemical messengers" has proven important in our understanding of many ecological problems (Seigler, 1995) but many are used by man as pharmaceuticals, spices, fragrances, pesticides, poisons, hallucinogens, stimulants or colouring agents (Luckner, 1990).

Additionally, chemical compounds with antibacterial properties are found in a variety of plants, of which many are utilized as medicinal plants (Naqvi *et al.*, 1990 ; Rizvi & Rizvi, 1992 ; Ushiki *et al.*, 1996 ; Rabe & Van Staden., 1997 ; Mathega & Meyer, 1998). Although not as frequent, some plants also possess strong antifungal properties (Naqvi *et al.*, 1990 ; Ushiki *et al.*, 1996 ; Zhang, 1997). Isolated and purified allelochemicals responsible for these antimicrobial properties have the potential to be developed into natural products and utilized commercially in the prevention and control of plant diseases in the agricultural industry.

Many of the secondary compounds found in plants are also known for its biostimulatory or growth regulatory properties. Growth stimulants also include phytohormones such as indole acetic acid (IAA), gibberellic acid (GA), kinetin and cytokinin. A fairly recent discovery is the new phytohormone group known as brassinosteroids (BS) that not only act as growth regulators but also increase the natural resistance of plants to pathogen attacks by inducing pathogenesis related (PR) proteins such as chitinase, peroxidase and β-1,3-glucanase (Roth *et al.*, 2000). Moreover, products containing brassinosteroids (e.g. ComCat, a natural product from plant origin) show high activity towards the manipulation of plant growth and development (Roth *et al.,* 2000). Potentially, the use of biostimulants in organized agriculture has many advantages (Winson & Kell, 1997). Extracts from yeast cells (Hasal

et al., 1992) and alginate beads (Saucedo *et al*., 1989; Ruggeri *et al.*, 1991) have been used successfully as biostimulants in achieving higher growth yields (Cachon *et al.*, 1995).

In many screening procedures it was found that secondary metabolites from plants inhibited germination, root elongation and hypocotyl growth (Ramamoorthy & Paliwal, 1993; Reinhardt *et al.*, 1994; Yatagi & Ding., 1996; Rawat *et al.*, 1998; Padhy *et al*., 2000). However extracts from *Lupinus* seed have shown both inhibiting and stimulating effects on seedling growth (El-Daly & Soliman, 1998). Other allelochemicals are known to stimulate germination and growth (Putnam & Tang, 1986; Chou, *et al.*, 1995) while yet others have a direct effect on metabolism (e.g. respiration ; Anaya *et al.,* 1995). Juglone, found in walnut trees (Neave & Dawson 1989; Li *et al.*, 1993), as well as leachates from *Eucalyptus* leaves (Padhy *et al*., 2000) is known for inhibiting respiration.

Eucomis autumnalis is a medicinal plant used by the Zulu people of South Africa. This plant, although toxic, is used to cure gastro-intestinal illnesses in infants and children (Hutchings, 1989) and has been reported to possess antimicrobial and analgesic activities (Masika *et al.*, 1997). It also shows high inhibition of prostaglandin-synthesis (Jager *et al.,* 1996). Recently Van Wyk *et al*. (1997) also reported anti-inflammatory, antispasmodic, anti-pyretic and purgative activities of *E. autumnalis*. However, as far as could be ascertained, no research has been done in the past on the antimicrobial potential of this plant towards plant pathogens. Especially the latter has prompted further studies on *E. autumnalis.* Moreover, the plant is easily propagated *in vitro* (Ault, 1995) and this point towards its potential to be developed as an alternative crop in the event of new uses being found, an aspect that will also be considered in this monograph.

In this chapter, a crude extract from the bulb of *Eucomis autumnalis* was screened for biostimulatory activity by screening its effect on the respiration rate of a monoculture yeast cells as well as on germination and seedling growth (in terms of coleoptile and root growth) of Cress seeds.

Additionally, the crude extract was screened for antimicrobial properties against known fungal and bacterial plant pathogens. The fungitoxic properties of the extract was tested against eight fungal pathogens namely *Botrytis cinerea* Pers.:Fr. (Hyphomycetes), *Fusarium oxysporum* Schlechtend.:Fr. (Hyphomycetes), *Mycosphaerella pinodes* (Berk. & Blox.) Vestergr. (Ascomycetes), *Sclerotium rolfsii* Sacc. (Agonomycetes), *Rhizoctonia solani* Kühn (Agonomycetes), *Verticillium dahliae* Kleb.

(Hyphomycetes), *Botryosphaeria dothidea* (Moug.:Fr.) Ces & De Not. (Loculoascomycetes) and *Pythium ultimum* Trow (Oömycetes).

The six plant pathogenic bacteria used to screen the crude extract for antibacterial activity included *Clavibacter michiganence* subsp. *michiganense, Pseudomonas syringae* pv. *syringae, Erwinia carotovora* subsp. *carotovora, Agrobacterium tumefaciens, Ralstonia solanacearum and Xanthomonas campestris* pv. *phaseoli* while one sensitive human pathogen*, Moraxella catarrhalis,* was used as test organism.

4.2 MATERIALS AND METHODS

4.2.5 Preparation of the crude extract

See chapter 3; 3.3.1.

4.2.6 Respiration rate determination using a monoculture yeast cells

The respiration rate, or manipulated changes thereof, is often used to indicate the manipulation potential of a chemical on the metabolism of living organisms or even tissue (Seigler, 1998). An increase in the respiration rate can be indicative of either a chemical or a natural plant extract's potential to act as a biostimulatory agent. A decrease in the respiration rate in the presence of a chemical or test solution, on the other hand, can be indicative of the underlying potential of the chemical or test solution to inhibit the production of ATP and subsequently growth and development.

The effect of the crude extract of *E. autumnalis* on the respiration rate of a monoculture yeast cells was tested at the same concentration range [0 (distilled water control), 0.05, 0.5, 5 and 50 mg l^{-1}) as for germination and seedling growth (see 4.2.3). ComCat®, a commercial biostimulant and natural product from plant origin with growth and yield enhancing properties (Agraforum, Germany) was used as a second control at the optimum concentration of 0.5 mg l^{-1} (suggested by the manufacturer) in order to assess the effectiveness of the *E. autumnalis* crude extract in manipulating the respiration rate of the yeast cells by comparison. ComCat® has been demonstrated in the past to increase the respiration rate of a monoculture yeast cells (Agraforum, Germany ; personal communication).

The respiration rate of the monoculture yeast cells was determined by means of a specially designed respirometer (Fig. 4.1). The apparatus was designed to contain exactly 70 ml of any test solution together with the monoculture yeast cells when completely filled. The short bulged "arm" acts as a reservoir for the yeast cells and also allows for the equipment to be filled with test solution by defying the difference in column lengths between the short and long "arms".

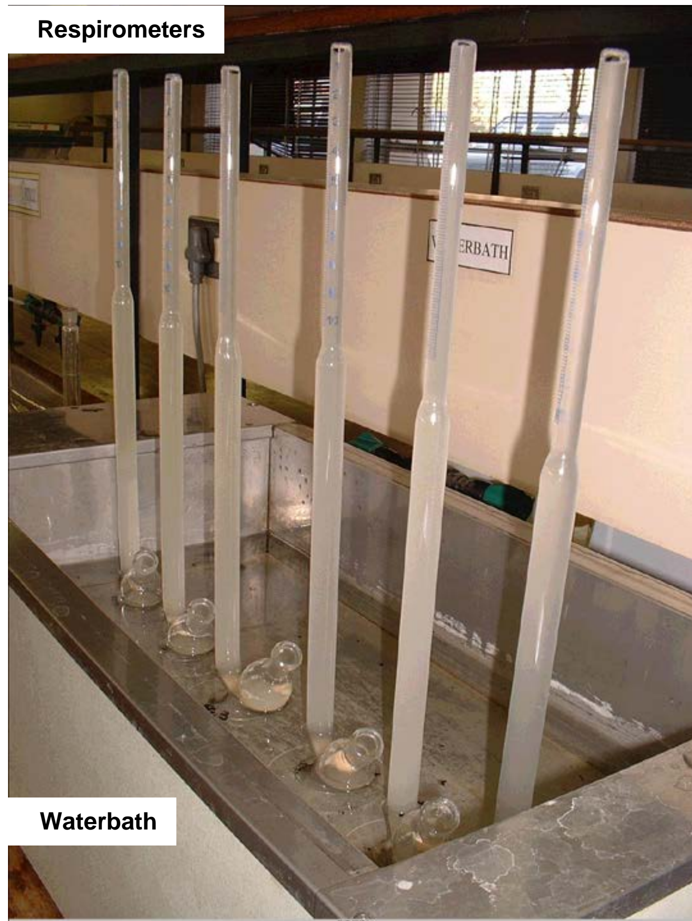

Figure 4.1: Specially designed respirometer manufactured for the purpose of determining the respiration rate of a monoculture yeast cells by measuring the CO_2 release over time in $cm^3 \ min^{-1}$.

To 70 ml of any given test solution, 0.35 g glucose was added to reach a final concentration of 5 g l^{-1} (0.5%; m/v). Eight grams of dry bakers yeast was placed in the reservoir of the respirometer followed by the careful addition of the 70 ml glucose enriched test solution until the whole apparatus was filled. Care was taken that no air bubbles were trapped in the solution by tilting the respirometer sideways. Subsequently, the apparatus with it's contents was placed in a waterbath, at 29 ºC and the volume CO_2

released determined in cm^3 every 30 minutes over a period of three hours by directly reading from the calibrated section of the long "arm". Each treatment was performed in triplicate. Means were calculated and converted to a percentage of the water control.

4.2.7 Germination and seedling growth test

The effect of the crude *E. autumnalis* extract on the germination of Cress seed and seedling growth was tested at a concentration range of 0 (distilled water control), 0.05, 0.5, 5.0 and 50.0 mg l^{-1}. ComCat®, was again used as a second control at a concentration of 0.5 mg l^{-1} in order to assess the effectiveness of the *E. autumnalis* crude extract by comparison.

Two hundred ml of each solution in the concentration range was added to a separate Erlenmeyer flask. Twenty Cress seeds per replicate were spaced evenly on a sheet of germination paper 50 mm from the top, covered with a second sheet of paper and moistened with distilled water. Both sheets of paper, containing the seeds, were subsequently rolled up together longitudinally, placed upright with the long end in one of the flasks and incubated at 25 ºC. This allowed for the germination paper to remain moistened at all times and for the seeds to be in constant contact with the different concentrations of the crude extract. The percentage seed that germinated as well as root and coleoptile lengths were determined at 24, 48, 72 and 96 hours of incubation. Each treatment was performed in triplicate and the means calculated.

4.2.8 Screening for antibacterial activity

See chapter 3 (3.3.2 and 3.3.2.2).

4.2.5 Screening for antifungal activity

See chapter 3 (3.3.2 and 3.3.2.3).

4.2.7 Statistical analysis of data

See chapter 3 (3.3.7)

4.4 RESULTS

4.4.1 Biostimulatory properties of a crude *E. autumnalis* bulb extract

4.4.1.1 Effect of the crude bulb extract on the respiration rate of a monoculture yeast cells

Compared to the water control, ComCat® significantly increased (50%) the respiration rate of a monoculture yeast cells while the low concentrations (0.05, 0.5 and 5 mg l^{-1}) of an *E. autumnalis* bulb extract had no effect (Fig. 4.2). However, a high concentration (50 mg l^{-1}) of the extract more than doubled (113%) the respiration rate of the yeast cells. All differences were statistically significant except the differences in respiration rates obtained with the extract in the lower concentration range.

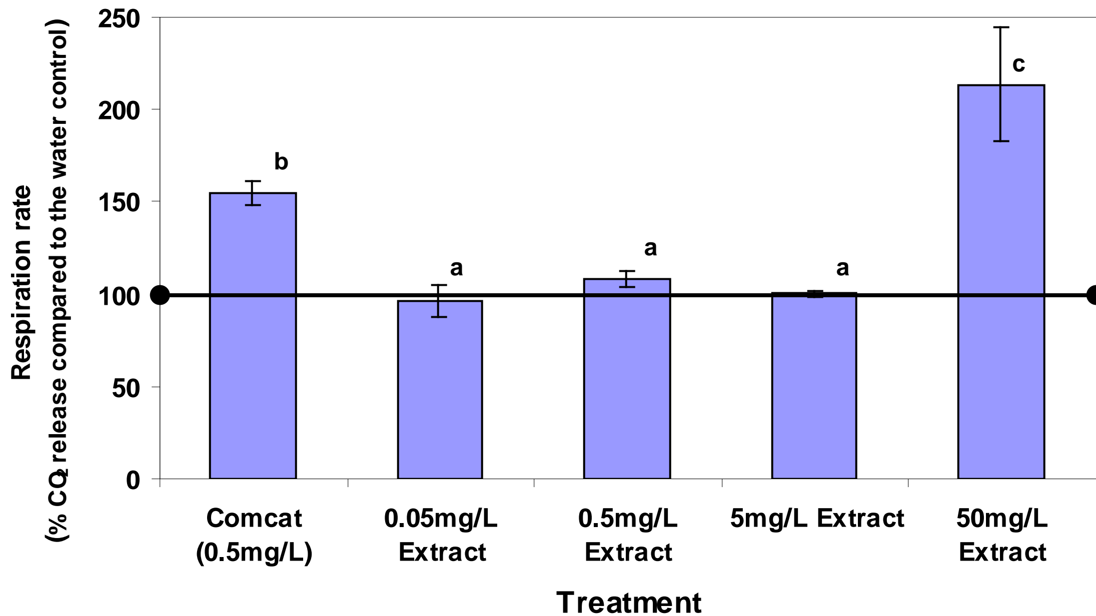

Figure 4.2: Effect of an *E. autumnalis* crude bulb extract, at different concentrations, on the respiration rate of a monoculture yeast cells measured over 3 hours. The line (●—●) represents the respiration rate obtained with the water control. (LSD was calculated at the $P < 0.05$ level; different letters on bars indicate statistical significant differences; the vertical bar = Standard Error).

4.4.1.2 Effect of the crude extract on seed germination

After the 96 hour incubation period, statistically significant differences were observed between the germination of non-treated (control) and ComCat® treated (0.5 mg l^{-1}) Cress seeds as well as germination of seeds treated with the lower concentration range of the *E. autumnalis* extract (Fig. 4.3). ComCat® stimulated the germination of Cress seeds while the *E. autumnalis* crude extract had neither a stimulatory nor an inhibitory effect

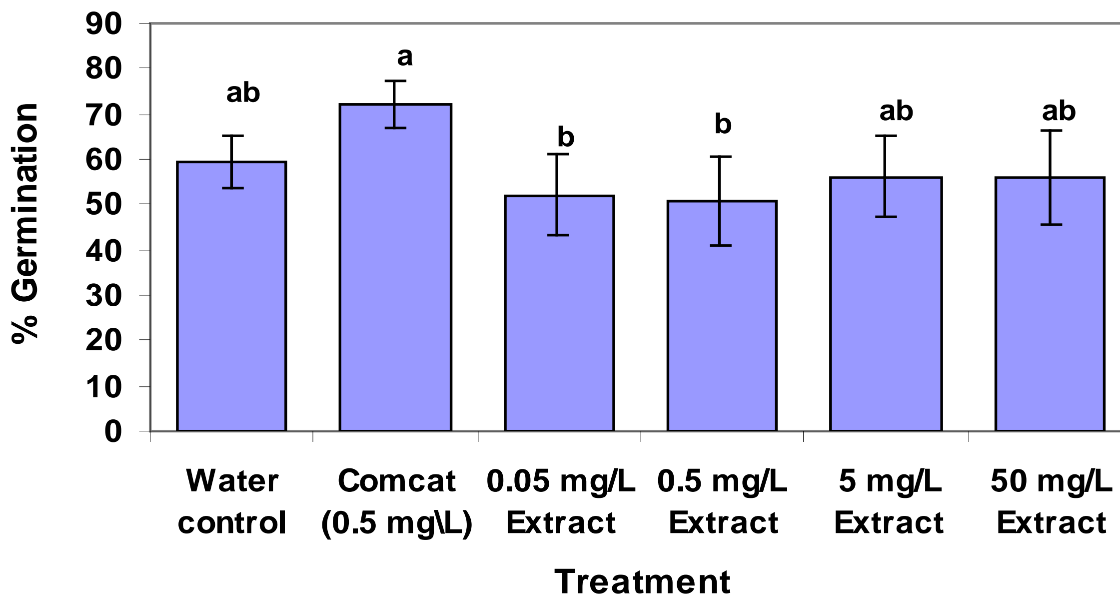

Figure 4.3: Effect of an *E. autumnalis* crude bulb extract, at different concentrations, on the percentage germination of Cress seeds over a 96 hour incubation period. (LSD was calculated at the $P < 0.05$ level. Different letters on bars indicate statistical significant differences. The vertical bar = Standard Error).

4.4.1.3 Effect of the crude extract on seedling growth

4.4.1.3.1 Seedling growth measured in terms of coleoptile length

No coleoptile growth occurred during the first 24 hours of incubation, where after the coleoptile length increased in a linear fashion over the following 48 hours (Table 4.1). During this time no differences in coleoptile length between the untreated control seedlings or those treated with either ComCat® or the

E. autumnalis crude extract were observed. However, between 72 and 96 hours of incubation coleoptile growth accelerated in the ComCat® treated as well as the water control seedlings. In comparison, coleoptile growth in seedlings pretreated with the *E. autumnalis* crude extract was slightly lower than that of the control seedlings. The latter applied for all of the different concentrations of the plant extract. However, none of the observed differences were statistically significant indicating that the *E. autumnalis* crude extract had neither a stimulatory nor an inhibitory effect on the coleoptile growth of Cress seedlings.

Table 4.1: The effect of a crude *E. autumnalis* bulb extract on coleoptile growth (mm) of Cress seedlings over a 96 hours incubation period.

Treatment	**24 Hours**	**48 Hours ***	**72 Hours ***	**96 Hours ***
Control (water)	0.00 ± 0.00 **a**	5.35 ± 0.53 **a**	23.65 ± 3.41 **a**	43.89 ± 7.48 **ab**
ComCat (0.5 mg l^{-1})	0.00 ± 0.00 **a**	7.85 ± 0.49 **ab**	24.87 ± 0.97 **a**	46.57 ± 1.13 **b**
Extract (0.05 mg l^{-1})	0.00 ± 0.00 **a**	9.13 ± 1.43 **b**	28.35 ± 3.55 **a**	39.18 ± 2.55 **ab**
Extract (0.5 mg l^{-1})	0.00 ± 0.00 **a**	7.62 ± 0.45 **ab**	24.40 ± 2.90 **a**	33.02 ± 3.40 **a**
Extract (5 mg l^{-1})	0.00 ± 0.00 **a**	7.67 ± 1.10 **ab**	24.73 ± 1.57 **a**	36.78 ± 1.44 **ab**
Extract (50 mg l^{-1})	0.00 ± 0.00 **a**	8.71 ± 0.59 **b**	28.52 ± 1.84 **a**	40.87 ± 2.60 **ab**

*****Values designated with different letters differed significantly ($P<0.05$) according to the Least Significant Difference (LSD) statistical procedure.

4.4.1.3.2 Seedling growth measured in terms of root length

No significant differences in root growth were observed in Cress seedlings between the water control and the seedlings grown from seeds pretreated both with ComCat® (0.5 mg l^{-1}) and a concentration range of an *E. autumnalis* crude bulb extract over the first 48 hours of incubation (Table 4.2). However, root growth increased significantly in seedlings from seeds pretreated with ComCat® over the last 48 h of a 96 h incubation period compared to that of the water control. In seedlings grown from seeds pretreated with different concentrations of an *E. autumnalis* crude extract, root growth was significantly inhibited especially during the latter stage of the incubation period.

Table 4.2: The effect of a crude *E. autumnalis* bulb extract on root growth (mm) of Cress seedlings over a 96 h incubation period.

Treatment	24 Hours *	48 Hours	72 Hours *	96 Hours *
Control (water)	5.42 ± 0.91 **c**	16.86 ± 3.39 **a**	43.08 ± 5.62 **b**	59.63 ± 2.67 **b**
ComCat (0.5 mg l^{-1})	4.69 ± 0.54 **bc**	18.88 ± 2.06 **a**	58.03 ± 2.30 **c**	69.51 ± 1.51 **b**
Extract (0.05 mg l^{-1})	3.66 ± 0.85 **bc**	16.07 ± 1.43 **a**	32.67 ± 4.67 **ab**	41.09 ± 4.50 **a**
Extract (0.5 mg l^{-1})	2.72 ± 0.07 **a**	15.50 ± 1.33 **a**	29.95 ± 3.50 **a**	38.24 ± 5.90 **a**
Extract (5 mg l^{-1})	2.93 ± 0.20 **ab**	15.63 ± 1.17 **a**	35.43 ± 3.10 **ab**	47.25 ± 2.36 **a**
Extract (50 mg l^{-1})	3.39 ± 0.45 **ab**	16.56 ± 1.14 **a**	33.78 ± 3.26 **ab**	43.27 ± 2.44 **a**

*Values designated with different letters differed significantly ($P<0.05$) according to the Least Significant Difference (LSD) statistical procedure.

4.4.2 Antifungal properties of a crude *E. autumnalis* bulb extract

The mycelial growth of all eight plant pathogenic fungi tested was inhibited by the crude *E. autumnalis* extract at a concentration of 1 mg ml^{-1} (Table 4.3), but to different degrees. The mycelial growth of *Verticillium dahliae* (53%) and *Botrytis cinerea* (40%) were least inhibited by the crude extract while the standard fungicide showed 100% inhibition in these cases. The extract significantly outperformed the standard fungicide regarding the mycelial growth inhibition of *Rhizoctonia solani* (73%) and *Pythium ultimum* (85%) and, although not statistically significant, also that of *Sclerotium rolfsii* (86%). The standard fungicide failed to inhibit the mycelial growth of *Pythium ultimum.* Mycelial growth inhibition of both *Fusarium oxysporum* and *Botryosphaeria dothidea* by the crude extract was >70% which paralleled inhibition by the standard fungicide.

Table 4.3 : Percentage *in vitro* mycelial growth inhibition of plant pathogenic fungi by a crude bulb extract of *Eucomis autumnalis* as well as by a standard broad spectrum fungicide (carbendazim/difenoconazole).

Fungus	**% Inhibition by crude extract (1 mg ml^{-1})**	**% Inhibition by a **standard fungicide (1 µg ml^{-1})**
Botryosphaeria dothidea (Moug.:Fr.) Ces & De Not.	77 ± 1**c***	83 ± 2**b**
Botrytis cinerea Pers.: Fr.	40 ± 9**f**	100 ± 0**a**
Fusarium oxysporum Schlechtend.: Fr.	73 ± 2**c**	74 ± 2**c**
Mycosphaerella pinodes Berk. & Blox.	85 ± 4**b**	70 ± 4**cd**
Pythium ultimum Trow.	85 ± 5**b**	0 ± 0**g**
Rhizoctonia solani Kühn	73 ± 2**c**	44 ± 2**f**
Sclerotium rolfsii Sacc.	86 ± 8**b**	64 ± 3**d**
Verticillium dahliae Kleb.	53 ± 9**e**	100 ± 0**a**

*****Values designated with different letters differed significantly ($P<0.05$) according to the Least Significant Difference (LSD) statistical procedure.

**Standard fungicide = carbendazim/difenoconazole

4.4.3 Antibacterial properties of a crude *E. autumnalis* bulb extract

The crude *E. autumnalis* extract totally inhibited the growth of the very sensitive gram positive test organism, *Moraxella catharralis*. However, none of the plant pathogenic bacteria tested showed any sensitivity towards the extract (results not shown), indicating that the *E. autumnalis* extract does not possess antibacterial properties towards plant pathogenic bacteria.

4.5 DISCUSSION

The initial aim of the screening procedure followed in this chapter was to ascertain whether a crude *E. autumnalis* bulb extract possessed inherent biostimulatory and/or antimicrobial properties. This was necessary in order to establish a rationale for further investigation of the inherent property of the extract with the highest application potential in terms of possible natural product development. To evaluate the biostimulatory potential of the extract, two biotests were used: 1) the effect of the extract on the respiration rate (Seigler, 1998) of a monoculture yeast cells (Agraforum, Germany) and 2) the stimulatory or inhibitory effect of the extract on both the germination of Cress seed as well as seedling growth (Agraforum, Germany).

Only the highest concentration (50 mg ml^{-1}) of the crude *E. autumnalis* bulb extract tested, increased the respiration rate of the yeast cells significantly as compared to both the untreated and ComCat® treated controls. As acceleration of the respiration rate in cells by external factors can indicate either a positive (stimulatory) or a negative (inhibitory) influence (Seigler, 1998) on tissue, it was important to conduct the second biotest. All of the concentrations tested for the crude *E. autumnalis* bulb extract had no effect on either the germination of Cress seed or seedling coleoptile growth. However, all concentrations tested had a significant inhibitory effect on seedling root growth. The latter is an indication of the negative effect the crude bulb extract had on vegetative growth of seedlings and possibly can have on older plants. Clearly, an extract from E. *autumnalis* bulbs has no potential as a biostimulatory agent. However, the inhibitory effect of this bulb extract on plant growth might be indicative of inherent herbicidal potential in *E. autumnalis* that will have to be considered in a follow-up study.

Despite the criticism (Eloff, 1998) against the *in vitro* screening of a plant extract for antibacterial and antifungal activity using the plate diffusion method (Daniels, 1965; Towers, 1984), the technique is in essence a qualitative procedure with the aim to merely detect activity as an indication of the inherent potential of the plant from which the extract was prepared. The initial screening of an *E. autumnalis* bulb extract for antimicrobial activity, by measuring the zones of inhibition, was compared to the inhibition by a commercially available bactericide and fungicide under the same conditions.

Naqvi *et al.* (1991) reported that plant extracts showing strong antibacterial properties often do not show antifungal properties. The opposite seems to apply for plants that have strong antifungal properties but not showing any antibacterial activity, as was seen for the crude *E. autumnalis* bulb extract. The crude extract, at a concentration of 1 mg ml^{-1}, inhibited the *in vitro* mycelial growth of seven of the eight plant pathogenic fungi tested by between 50 and 86%, as did a whole plant extract (Eksteen *et el*., 2001). This is significant in light of the experience that mycelial growth inhibition by fungicides is more difficult to accomplish than inhibition of spore germination. This is also an important step towards finding "green chemicals" with strong antifungal activity for organically cultivated crops (Gorris & Smid, 1995).

Sclerotium rolfsii, Pythium ultimum and *Mycosphaerella pinodes* were most sensitive to treatment by the crude *E. autumnalis* bulb extract in terms of mycelial growth inhibition as compared to inhibition by the broad spectrum standard fungicide, carbendazim/difenoconazole (Eksteen *et al.*, 2001), that was applied as a control to assess the efficiency of the *E. autumnalis* bulb extract with regards to mycelial growth inhibition by comparison. Using a broad spectrum fungicide is convenient to the farmer but, the fact that carbendazim/difenoconazole failed to inhibit the mycelial growth of *Pythium ultimum* emphasises the importance of choosing specific fungicides for specific pathogens when the need arises. *Botrytis cinerea* and *Botryosphaeria dothidia* were the least affected by the *E. autumnalis* extract. However, these two fungi were completely controlled by the standard fungicide, showing that fungi are probably affected in different ways by various chemicals. Although the mycelial growth of *Rhizoctonia solani* was only moderately affected by the extract (73% inhibition), the standard fungicide was much less (44%) effective. In the case of *Fusarium oxysporum* the *E. autumnalis* extract was just as effective as the standard fungicide in inhibiting mycelial growth . The results presented in this chapter clearly showed that the crude *E.autumnalis* bulb extract possessed neither inherent biostimulatory nor antibacterial activity against a series of plant pathogenic bacteria. However, on grounds of the *in vitro* antifungal activity data presented, the crude *E. autumnalis* bulb extract seemed to be quite efficient in terms of broad spectrum plant fungal disease control as compared to the standard used as a positive control. Subsequently, the latter property of the crude extract was applied in an attempt to purify the active antifungal components in the crude extract by means of activity directed isolation techniques (chapter 6). In addition, in an attempt to verify the *in vitro* antifungal data under *in vivo* conditions using a living plant system, as well as the possible phytotoxic effect of the crude extract on the plant, the pea pathogen *Mycosphaerella pinodes* was chosen (chapter 5).

CHAPTER 5

In vivo CONTROL OF *Mycosphaerella pinodes* ON PEA LEAVES BY A CRUDE BULB EXTRACT OF *Eucomis autumnalis*

5.2 INTRODUCTION

Due to consumer resistance to the use of synthetic chemicals in agriculture, the search for natural products to be applied in organic farming systems either as herbicides, fungicides or pesticides has become a priority (Duke *et. al.*, 1995; Ushiki *et. al.*, 1996; Lovang & Wildt-Persson, 1998). Compounds of plant origin are generally preferred since they are regarded as environmentally safer being biodegradable (Ganesan & Krishnaraju, 1995). This has led to renewed interest in the screening of extracts from wild plants for its application potential in agriculture over the past ten to twenty years (Poswal *et al*., 1993). Although the *in vitro* screening of extracts by means of different biotests is an important first step in identifying plants with application potential in agriculture, *in vivo* confirmation of this potential is essential in the search for plant derived preparations and its commercialization (Gorris & Smid, 1995). *In vitro* mycelial growth inhibition of seven plant pathogenic fungi by a whole plant extract of *Eucomis autumnalis,* that compared favourably with a broad spectrum fungicide, was previously reported (Eksteen *et al.,* 2001).

This chapter reports on the potential of a crude extract from the *E. autumnalis* bulb to prevent the infection of pea (*Pisum sativum*) leaves by *Mycosphaerella pinodes* (Berk. & Blox.) Vestergr. (Ascomycetes) *in vivo*. *Mycosphaerella pinodes* is one of the most important, widespread (Lawyer, 1984), aggressive (Wroth, 1998) and frequent (Hagedorn, 1984) fungi infesting peas. The fungus infects pea seedlings as they emerge causing girdling stem lesions which reduce field populations and increase lodging (Ryan *et al*., 1984). Later, it also causes necrotic lesions on leaflets and stipules and, in exceptional circumstances, abscission of the leaflets (Hagedorn, 1984). According to Allard *et al*. (1992) all aerial parts of the pea plant is susceptible to infection while growth, yield and seed quality is adversely affected. Foliar disease caused by the fungus *M. pinodes* is commonly referred to as Black Spot or Ascochyta Blight which is serious as it can reduce yield and quality of peas grown for human and animal consumption (Kraft *et al*., 1997).

Phytotoxicity data on the crop and information on the potential of the crude *E. autumnalis* bulb extract to prevent fungal infection *in vivo*, is regarded as a second step towards seeking a rationale for large scale greenhouse and field trials as well as the isolation, purification and identification of the active substances involved. The latter are seen as essential steps towards developing a natural fungicide from the plant.

5.2 MATERIALS AND METHODS

5.3.1 Plant material

One hundred *Pisum sativum* cv. Mohanderfer seeds, obtained from a local seed merchant, were sown 2 cm from the surface in twenty pots at 5 seeds per pot using Bainsvlei soil and applying a standard NPK fertilizer mixture. The plants were allowed to grow for 4 weeks in a greenhouse while maintaining the soil at field capacity. After 4 weeks, two fully expanded leaflets of the same age were removed carefully from the fourth nodes of each plant and used for monitoring the potential of an *E. autumnalis* crude bulb extract to control Ascochyta blight *in vivo*.

5.3.2 Preparation of crude bulb extracts

A crude extract of the *E. autumnalis* bulbs were prepared as previously described in chapter 3 (see 3.3.1).

5.3.3 Isolation of *Mycosphaerella pinodes*

Ascochyta blight, *Mycosphaerella pinodes* (Blerk & Blox) Vestergr., was isolated from diseased leaves and stems of various winter cultivars of field pea at the time of senescence. Collections of the infected plant material were made from the central and south eastern pea-growing areas of Ethiopia. Pieces of the diseased tissues were surface sterilized for 1 minute in 96% (v/v) ethanol, three minutes in a 3.5% (v/v) NaOCl solution (Moussart *et al.*, 1998) and 30 seconds in 96% (v/v) ethanol. The tissues were subsequently aseptically transferred to corn meal agar amended with streptomycin (0.3 ml L^{-1}) in 9 cm Petri-dishes and incubated at 20 ± 1 °C in a growth chamber.

Isolates initially obtained from the plant material were then grown on Coon's medium (Ali *et al*., 1978) consisting of 4 g maltose, 2 g KNO_3, 1.2 g $MgSO_4$, 2.7 g KH_2PO_4 and 20 g agar. Cultures were incubated for 14 days to obtain pycnidiospores. To obtain an isolate derived from a single uninucleate cell, a suspension of pycnidiospores was streaked on 15% water agar, incubated overnight at 20 ± 1 °C and examined under a dissecting microscope (80x magnification). A germ tube arising from one cell of a pycnidiospore was severed and transferred to Coon's agar (Clulow *et al*, 1991). Six isolates of *M. pinodes* were obtained. All isolates from a single-spore and cultures were maintained on Coon's agar slants and stored in the dark at 5 °C.

5.3.4 Preparation of a *Mycosphaerella pinodes* spore suspension

Oat meal agar was prepared by gently heating 30 g of oats in one liter distilled water for 1 hour, stirring frequently, and subsequently filtered through a fine sieve upon which the volume was readjusted to one liter. Twenty grams of technical agar and 0.1 g Keltane AP was added to the filtrate to yield a 2% (m/v) agar concentration. The agar was autoclaved for 15 min., poured into Petri dishes and allowed to cool off before inoculating three oatmeal plates with *M. pinodes* mycelia. Plates were incubated in a 12 hour photoperiod incubator at 20 °C for 14 days, to ensure the production of picnidiospores.

To prepare the inoculum (spore suspension), sterile distilled water was added to the 14 day old cultures dislodging spores gently with a sterile glass rod. The suspension was subsequently filtered through four layers of cheese cloth in order to remove the mycelia and the concentration of pycnidiospores determined by means of a haemacytometer. The picnidiospore concentration was adjusted to 1x 10^5 spores per ml (Nasir & Hoppe, 1997) with sterile distilled water prior to inoculation of pea leaves.

5.3.5 *In vivo* assessment of crude extract phytotoxicity

Pea seeds were planted in plastic pots in Bainsvlei soil and grown in a glasshouse (minimum temperature 18 ^{o}C). Four weeks after planting, when the leaflets on the third and fourth nodes were fully expanded, 3 fourth node leaflets per replicate were removed from the plants and placed on Schleicher and Schull No. 595 filter paper, moistened with 4 ml of sterile distilled water, in 9 cm Petri dishes. Thirty µl of each of a 0.25. 0.5, 1.0 and 2.0 mg ml^{-1} solution of the crude extract were placed separately on each of three leaves per Petri dish and replicated three times. Treatment of the leaves with water and a standard fungicide (1 µg ml^{-1} carbendazim/difenoconazole) served as controls. Petri dishes

containing the treated leaflets were incubated at 20 ºC in a day/night incubator programmed for a 16 h day while 2 ml sterile distilled water was added daily to keep the filter paper moistened. Six days after treatment, possible phytotoxicity symptoms were assessed on a six-category scale (Table 2 ; Clulow *et al*., 1991) using a stereo microscope.

5.3.6 *In vivo* assessment of crude extract antifungal properties

Fourth node pea leaflets were obtained and sustained on moist filter paper in Petri dishes as described for the phytotoxicity assessment test. *In vivo* control of *M. pinodes* spore infection of the leaves by different concentrations (0.25, 0.5, 1.0 and 2.0 mg ml^{-1}) of the *E. autumnalis* crude bulb extract was followed in two ways namely, a) by inoculating the leaves with 15 µl of a spore suspension (1 x 10^5 spores ml^{-1} ; Nasir & Hoppe, 1997) 30 minutes before applying the different concentrations of the crude extract separately and b) by applying the crude extract at different concentrations prior to incubation of the leaves with the spores. A standard fungicide, carbendazim/difenoconazole, currently used against Ascochyta blight in peas (Bretag *et al*., 1995 ; Moussart et al., 1998), as well as leaves inoculated only with the spore suspension, served as controls. Three leaves per Petri dish represented a replicate and the experiment was performed in triplicate. Petri dishes containing the differently treated leaves were incubated at 20 ºC, the optimal temperature for *M. pinodes* spore germination (Roger *et al*., 1999) in a day/night incubator as illumination is necessary for spore germination (Roger & Tivoli, 1996). After six days of incubation the foliar lesions were measured and leaf damage compared to that of the controls.

5.3.7 Statistical analysis of data

See Chapter 3 (3.3.7)

5.4 RESULTS

The rather strong *in vitro* mycelial growth inhibition of the fungal plant pathogen *Mycosphaerella pinodes*, (Ascomycetes) confirmed the controlling ability of the crude *E. autumnalis* bulb extract. A 2 mg ml^{-1} concentration of the bulb extract inhibited the *in vitro* mycelial growth of *M. pinodes* completely (see chapter 4).

The *in vivo* phytotoxicity rating (Table 5.1) of the crude *E. autumnalis* bulb extract, in terms of its interaction with and potential to induce necrosis in pea leaves, revealed that the crude extract was not phytotoxic even at the highest concentration tested (Table 5.2; Figure 5.1) and the symptomless effect of the extract was similar to that of the water and standard fungicide controls. All extract solutions and controls differed significantly from the leaf necrosis induced by the *M.pinodes* spore suspension.

Table 5.1: Macroscopic characteristics of foliar phytotoxicity reaction categories

Scale categories	Characteristics
0	Symptomless
1	< 5 necrotic lesions
2	Numerous (> 5) necrotic lesions
3	< 50% of inoculated area necrotic
4	50 – 100% of inoculated area necrotic
5	Necrosis spreading beyond inoculated area

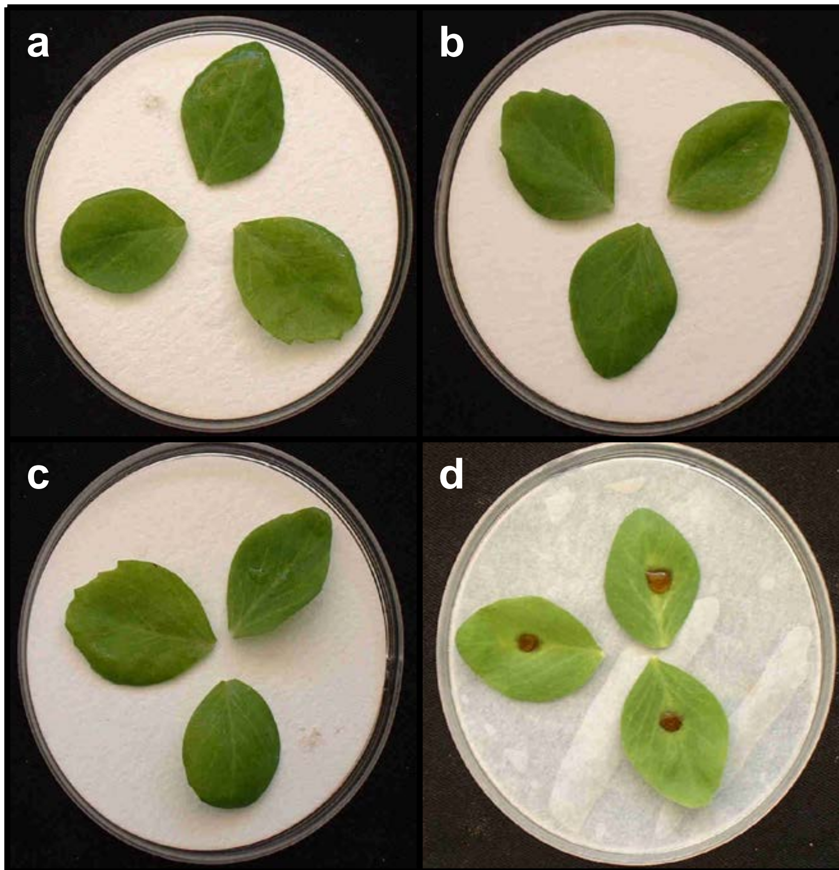

Figure 5.1: Possible phytotoxic lesions of (a) a 2 mg ml^{-1} *Eucomis autumnalis* crude bulb extract, (b) water control, (c) a standard fungicide control and (d) *Mycosphaerella pinodes* spores on pea (*Pisum sativum*) leaves.

Table 5.2: Mean foliar phytotoxicity symptom rating on a six-category scale (0 – 5 ; see Table 5.1) following direct inoculation of fourth node pea leaflets with different concentrations of a crude *Eucomis autumnalis* bulb extract.

Foliar treatment	**Rating**
Crude bulb extract only: 2 mg ml^{-1}	0 ± 0**b**
1 mg ml^{-1}	0 ± 0**b**
0.5 mg ml^{-1}	0 ± 0**b**
0.25 mg ml^{-1}	0 ± 0**b**
Standard fungicide only	0 ± 0**b**
Water control	0 ± 0**b**
Spore suspension only	4.2 ± 0.8**a**

*Values designated with different letters differed significantly (*P<0.05*) according to the Least Significant Difference (LSD) statistical procedure.

The results in table 5.3 showed that the lowest concentration of the *E. autumnalis* bulb extract that was able to inhibit *M. pinodes* spore germination completely and to prevent the infection of pea leaves with Ascochyta blight *in vivo*, was 1 mg ml^{-1}. This was true where the pea leaves were inoculated with *M. pinodes* spores both before (Figure 5.2) and after (Figure 5.3) treatment with the bulb extract and the statistical analysis revealed highly significant differences from the lower extract concentrations (0.25 and 0.5 mg ml^{-1}) as well as the control leaves that were inoculated with the spore suspension only. Interestingly, the 1 mg ml^{-1} concentration of the extract also differed significantly from the standard fungicide (1 µg ml^{-1} carbendazim/difenoconazole) treated leaves prior to spore inoculation in terms of the induction of necrotic lesions.

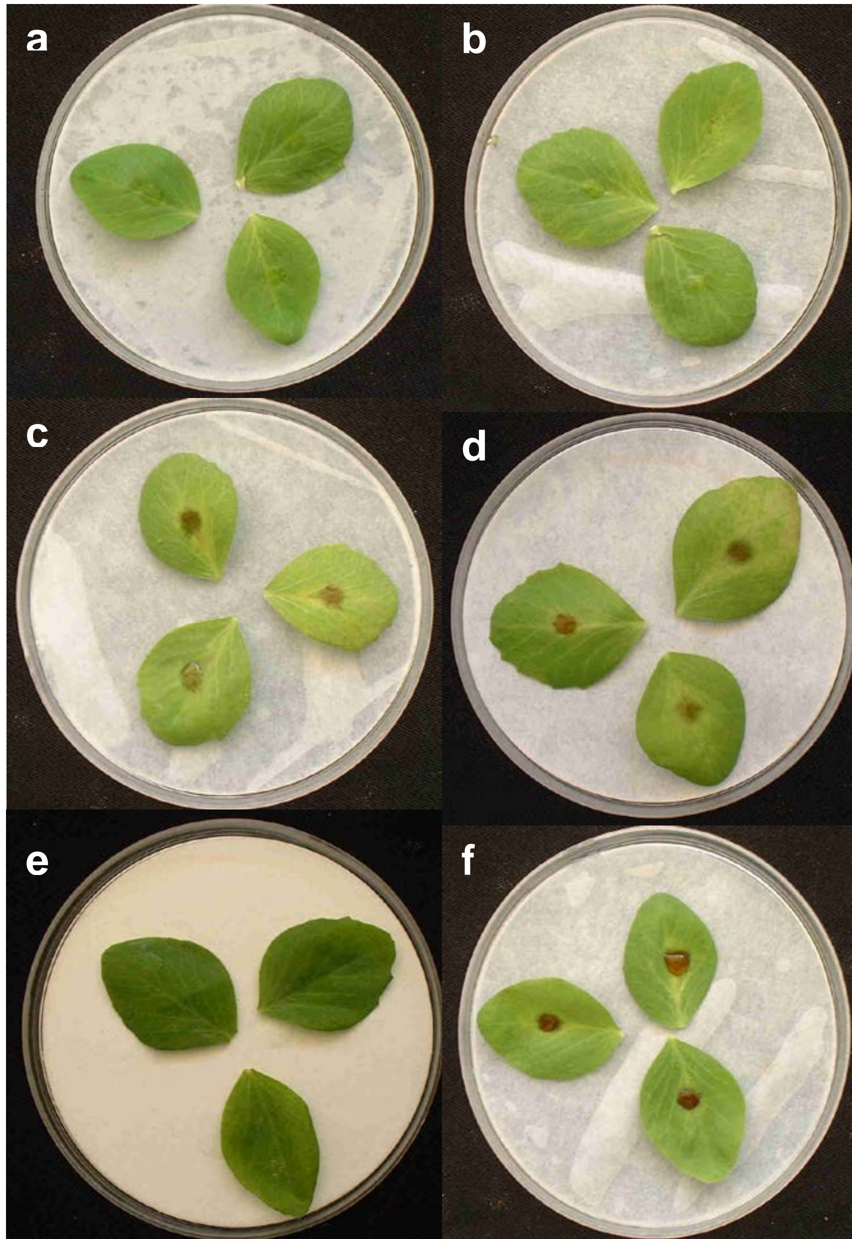

Figure 5.2: *In vivo* control of spore infection by *Mycosphaerella pinodes* in pea (*Pisum sativum*)leaves by different concentrations of an *Eucomis autumnalis* crude bulb extract when the leaves were treated 30 minutes before spore inoculation. [(a) 2mg ml^{-1} crude bulb extract, (b) 1 mg ml^{-1} crude bulb extract, (c) 0.5 mg ml^{-1} crude bulb extract, (d) 0.25 mg ml^{-1} crude bulb extract, (e) control 1: standard fungicide (1 µg ml^{-1}) and (f) control 2: *M. pinodes* spore inoculation only].

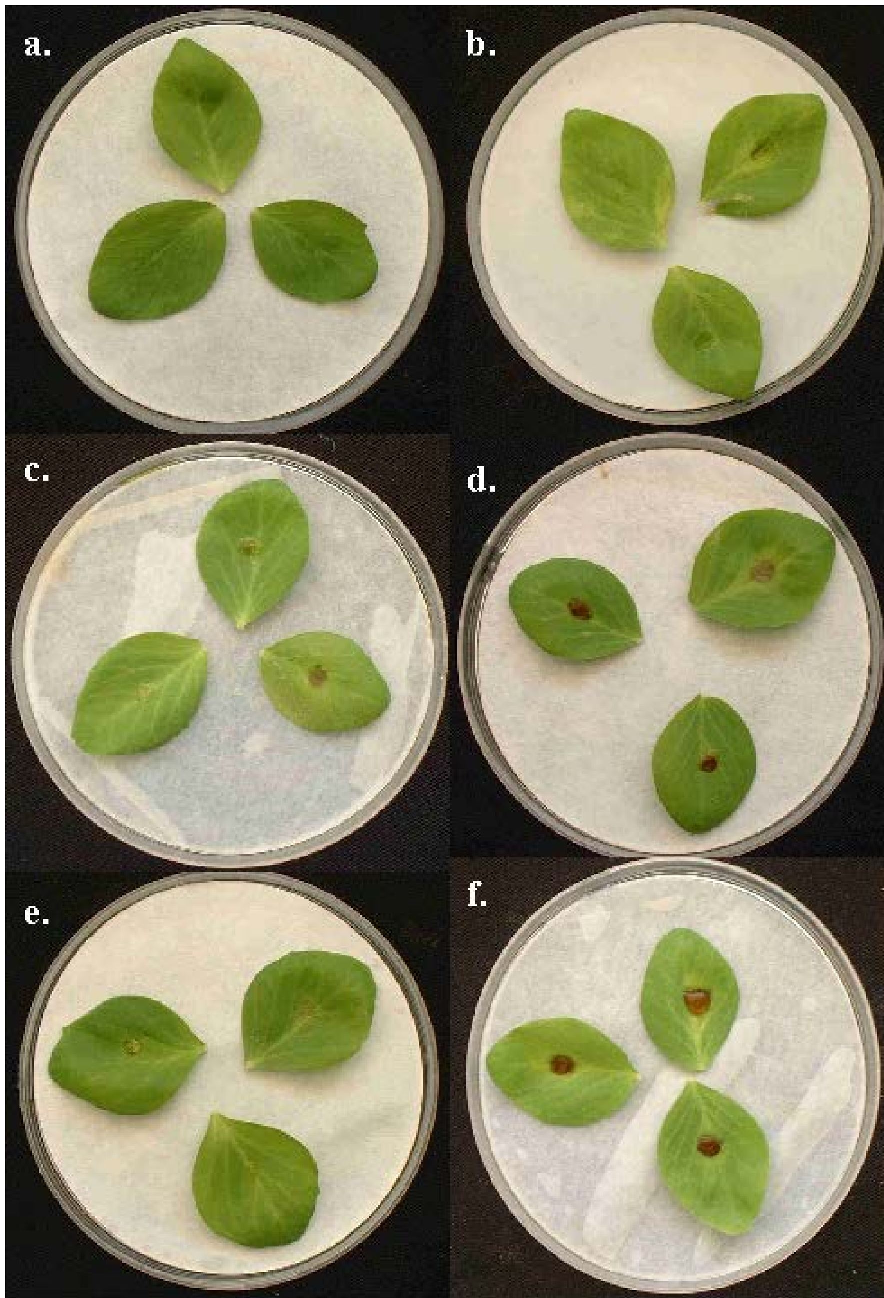

Figure 5.3: *In vivo* control of spore infection by *Mycosphaerella pinodes* in pea (*Pisum sativum*)leaves by different concentrations of an *Eucomis autumnalis* crude bulb extract when the leaves inoculated with spores 30 minutes before treatment with the extract. [(a) 2mg ml^{-1} crude bulb extract, (b) 1 mg ml^{-1} crude bulb extract, (c) 0.5 mg ml^{-1} crude bulb extract, (d) 0.25 mg ml^{-1} crude bulb extract, (e) control 1: standard fungicide (1 µg ml^{-1}) and (f) control 2: *M. pinodes* spore inoculation only].

Table 5.3: Mean lesion size following direct inoculation of fourth node pea leaflets with a *Mycosphaerella pinodes* spore suspension either before or after treatment with different concentrations of a crude *Eucomis autumnalis* bulb extract.

Foliar treatment	**Mean lesion size (mm)**
Crude bulb extract followed by spore inoculation:	
2 mg ml^{-1}	0 ± 0**a**
1 mg ml^{-1}	0 ± 0**a**
0.5 mg ml^{-1}	6.6 ± 1.8**c**
0.25 mg ml^{-1}	8.4 ± 3.0**d**
Spore inoculation followed by crude bulb extract:	
2 mg ml^{-1}	0 ± 0**a**
1 mg ml^{-1}	0 ± 0**a**
0.5 mg ml^{-1}	6.4 ± 1.9**c**
0.25 mg ml^{-1}	8.2 ± 1.3**d**
Standard fungicide followed by spore inoculation	0 ± 0**a**
Spore inoculation followed by standard fungicide	2.8 ± 1.7**b**
Spore inoculation only	9.4 ± 1.70**d**

*Values designated with different letters differed significantly ($P<0.05$) according to the Least Significant Difference (LSD) statistical procedure.

5.5 DISCUSSION

A crude bulb extract of *E. autumnalis*, at a concentration of 1 mg ml^{-1}, inhibited the *in vitro* mycelial growth of seven of the eight plant pathogenic fungi tested by between 50 and 86%, in agreement with Eksteen *et el*. (2001) for a whole plant extract. This is significant in light of the experience that mycelial growth inhibition by fungicides is more difficult to accomplish than inhibition of spore

germination. The three fungal pathogens most sensitive to *in vitro* mycelial growth inhibition by the extract, of which growth was inhibited by >80%, were *M. pinodes*, *P. ultimum* and *S. rolfsii*. *Mycosphaerella pinodes* was chosen as test organism for assessing the potential of the crude *E. autumnalis* bulb extract to control Black Spot or Ascochyta Blight infection in pea leaves *in vivo* under controlled conditions.

The lowest concentration (MIC) of the bulb crude extract that completely prevented the *in vivo* infection of pea leaves by *M. pinodes* picnidiospores, when the leaves were inoculated with spores both before and after treatment with the extract, was 1 mg ml^{-1}. Although the necrotic lesions on pea leaves treated with concentrations lower than 1 mg ml^{-1} (0.25 and 0.5 mg ml^{-1}) were smaller than those measured on control leaves inoculated with spores only, the differences were not statistically significant. However, it will be worthwhile to consider testing a concentration range between 0.5 and 1 mg ml^{-1} as the MIC seems to lie within this range. Although *Eucomis* species are known to be poisonous (Watt & Breyer-Brandwijk, 1963), the crude *E. autumnalis* bulb extract showed no phytotoxic reaction on the leaves even at the highest concentration (2 mg ml^{-1}) applied.

Experimental *in vivo* results on the potential of plant extracts to control fungal infections of crops, obtained under controlled conditions, contribute to the assessment of the application potential of the extract in organised agriculture. Ascochyta blight can develop within days under favourable moisture and temperature conditions and currently available fungicides must be applied before the pathogen invades host tissue to ensure successful control (Roger *et al*., 1999). The present study confirmed that a crude bulb extract of *E. autumnalis*, at a concentration as low as 1 g L^{-1}, has the potential to be applied as both a preventative or corrective agent against infection of pea plants by *M. pinodes* spores. Additionally, the extract showed significant potential to inhibit secondary mycelial growth of *M. pinodes*. Although more *in vivo* testing is necessary on other diseases, including foot rot caused by soil or seed borne *M. pinodes* spores (Moussart *et al*., 1998) and other fungal pathogens, indications are that the extract possesses significant potential as a corrective broad spectrum antifungal agent. The latter is enhanced by the findings of Moussart *et al*. (1998) as well as Dey and Singh (1994) that *M. pinodes* is non-systemic.

Effective use of the present results requires additional information under field conditions and an indication of the abundance of inoculum that the extract is capable of controlling. These findings could

be of value for the use of a crude *E. autumnalis* bulb extract in an integrated pest management (IPM) system to minimize crop losses caused by Ascochyta Blight. This would also be in line with objectives of evaluating natural plant products for their potential in IPM systems (Rios *et al*., 1988 ; Lydon & Duke, 1989 ; Simmonds *et al*., 1992), especially at the resource-poor farmers' level (Poswal *et al*., 1993). In this light, and from an agricultural perspective, *E. autumnalis* also has the potential to be developed as a new or alternative crop with a further impact on the agricultural economics of this country. However, from an academic perspective, isolation, purification and identification of the active substance(s) involved are also important to eventually elucidate the mechanism of action of the active ingredient(s) (See chapter 6).

CHAPTER 6

ISOLATION AND PURIFICATION OF ACTIVE SUBSTANCES WITH ANTIFUNGAL PROPERTIES FROM A CRUDE BULB EXTRACT OF *Eucomis autumnalis*

6.1 INTRODUCTION

Crop protection relies on the availability and use of a range of chemical protectants, required to control the vast range of micro-organisms that could spoil agricultural products. As a result of recent changes in legislation, restricting the use of many synthetic crop protectors world wide, research activity has been directed towards the development of alternative natural crop protectants (Gorris & Smid, 1995). Screening for the potential of plant extracts in this regard, is becoming a priority for the future (Van Wyk *et al*., 1997). Also taking into account the problems accompanying the use of agri-chemicals, it becomes evident that more attention should be given to isolating active ingredients from plants with the aim of developing natural products, as well as to determine their modes of action (Van der Watt & Pretorius, 2001).

It is well known that plant extracts are used for therapeutic purposes as either complex mixtures or pure compounds depending on their therapeutic indices. It is, therefore, necessary to determine the chemical composition of the complex mixture so as to identify the compounds responsible for the antifungal or any other bioactivity. Isolation and identification of the active constituents, or at least the chemical groups to which they belong, is essential for the study of their toxicity, stability and effects on the metabolism and physiology of a crop as well as for dosage purposes and for structure-activity investigations (Aquino *et al*., 1995) in order to elucidate the mechanism of action. However, separating one or more active compounds from a substantial number of other molecules in a plant crude extract, can be a formidable task. Nevertheless, great success has already been achieved by using existing separation and purification techniques. The separation of a crude extract into fractions by means of liquid-liquid extraction and column chromatographic techniques form a major part of the initial separating procedures. This is followed with either preparative thin layer or HPLC separation techniques. A biotest is needed to keep track of the positioning of a specific active substance in the separated fractions during the extraction process (Cannel, 1998) and this is referred to as activity directed separation. In this study an anti-fungal biotest was used to follow the fractioning of active substances with antifungal properties in a bulb extract of *E. autumnalis.*

The crude extract of *E. autumnalis* bulbs proved to have strong *in vitro* (chapter 4) as well as strong *in vivo* (chapter 5) antifungal properties while showing no phytotoxic effect on the pea leaves used in the latter biotest. In this chapter semi-purified fractions of *E. autumnalis* were prepared by means of different chromatography techniques, the anti-fungal properties of each fraction verified by means of a biotest and the active substances purified and identified at least to the level of the chemical groups to which it belonged.

6.2 MATERIALS AND METHODS

6.2.1 MATERIALS

All organic solvents used (see 3.2.2), were of the purest grade available and supplied by Merck (Germany)

6.2.3 METHODS

6.2.3.1 Fractionation of components from a crude bulb extract of *E. autumnalis* by means of liquid-liquid extraction

See chapter 3 (3.3.3).

All fractions were subsequently tested for antifungal activity (see 3.3.2.3). Only the fraction showing the highest bioactivity was then purified further by using column chromatography and preparative thin layer chromatography (see 3.3.6).

6.2.3.2 Further fractioning of only the most bioactive liquid-liquid extraction using column chromatography

See chapter 3 (3.3.5).

6.2.3.3 Qualitative thin layer chromatography (Q-TLC)

Every third fraction collected in separate test tubes after separating compounds from the bioactive diethyl ether extraction using column chromatography was transferred to the baseline of a fluorescent Silica Gel 60 TLC plate by means of a capillary tube (± 10µg of each; see 3.3.4). This was done to

obtain a profile of the components isolated by means of column chromatography to a) determine which fractions contained similar molecules and b) to monitor the successfulness of the fractioning process. Since different mobile phases were used during the column chromatography fractioning process, each eluate was treated separately as explained above.

The mobile phase used to develop the plates was Chloroform : MeOH : H_2O (80:20:10). After the components were allowed to separate on the plates, the frontline was marked and the plates coloured with 10% (v/v) ethanolic H_2SO_4 (see 3.6). Those fractions with similar TLC profiles were pooled and are hence referred to as column fractions. Combined column fractions were subsequently tested for antifungal activity against the most susceptible fungus *Sclerotium rolfsii.*

6.2.3.4 Preparative thin layer chromatography (P-TLC)

See Chapter 3 (3.3.6).

6.2.3.5 Spray reagents used for identification of the groups of chemicals to which each active compound belongs

Different spray reagents (Wagner & Bladt, 1996) were used to test for different chemical groups to which active components belonged after separating the components by means of P-TLC. The compounds were placed on separate Q-TLC plates, developed and treated with different spray reagents (11 in total) in order to identify chemical groups.

6.2.3.5.1 Anthraglycosides

Solvent system: Ethyl acetate : methanol : water (100 : 13.5 : 10).

Spray reagent: Potassium hydroxide (KOH).

The plate was sprayed with 10 ml of a 5% (v/v) ethanolic solution of KOH. Evaluation was done in both the visible and UV (365 nm) region with and without warming. Yellow, red and red-brown colours depicted the presence of this group.

6.2.2.5.2 Cardiac glycosides

Solvent system: Ethyl acetate : methanol : water (100 :13.5 :10)

Spray reagent: Kedde reagent

Five ml freshly prepared 3% (v/v) ethanolic 3,5-dinitrobenzoic acid was mixed with 5 ml of 2 M NaOH. The plate was sprayed with 5-8 ml of the reagent and evaluated under visible light.

6.2.2.5.3 Bitter principles including terpenoids

Solvent system: Ethyl acetate : methanol : water (100 :13.5 :10)

Spray reagent: Vanillin-sulphuric acid reagent

Solution I: 1% (m/v) ethanolic vanillin

Solution II: 10% (v/v) ethanolic sulphuric acid

The plate was sprayed with 10 ml of solution I, followed immediately by 10 ml of solution II. After heating at 110°C for 5 minutes, the plate was evaluated in visible light. Depending on the structure of the molecule, the fluorescence differ from red-violet, brown-red, blue-green, blue and grey or red-grey.

6.2.2.5.4 Alkaloids

Solvent system: Ethyl acetate : methanol : water (100 :13.5 :10)

Spray reagent: Dragendorff reagent

Solution (a): 0.5 g basic bismuth nitrate was dissolved in 10 ml glacial acetic acid and 40 ml water and prepared while heating. This solution was then filtered.

Solution (b): 8 g of potassium iodide was dissolved in 30 ml of water.

Stock solution: Solutions (a) and (b) were mixed 1:1

Spray solution: 1 ml stock solution was mixed with 2 ml glacial acetic acid and 10 ml water.

The plate was sprayed and viewed in visible light.

6.2.2.5.5 Phenolic compounds

Solvent system: Ethyl acetate : methanol : water (100 :13.5 :10)

Spray reagent: Ammonium vanadate /θ-anisidine spray reagent

Solution (a): saturated, aqueous ammonium vanadate

Solution (b): 0.5g θ-anisidine was dissolved in 2 ml H_3PO_4. This solution was then diluted to 100 ml with ethanol and filtered. The plates were sprayed with solution (a), and while still wet, they were then sprayed with solution (b) and heated at 80 ^{o}C. Observation was done in visible light.

6.2.2.5.6 Saponins

Solvent system: Ethyl acetate : methanol : water (100 :13.5 :10)

Spray reagent: 20 ml of an ethanolic ammonium-III-chloride solution was prepared by adding 4 g of the ammonium-III-chloride to 20 ml of ethanol. The TLC plate was sprayed with the colouring reagent and heated for 5 min at 100 ºC and viewed in visible light. Under these circumstances the molecules coloured grey to purple brown. Fluorescence under UV light at 365 nm developed yellow, orange, brown, blue or green spots that all indicated the presence of saponins.

6.2.2.5.7 Essential oils

Solvent system: Toluene : ethyl acetate (93 : 7)

Spray reagent: Vanillin-sulphuric acid (as in section 6.2.2.5.3)

Evaluation was done in visible light.

6.2.2.5.8 Valepotriates

Solvent system: Toluene : ethyl acetate (93 : 7)

Spray reagent: Hydrochloric acid-glacial acetic acid

The plate was sprayed with a mixture of eight parts of concentrated hydrochloric acid and two parts of glacial acetic acid. After spraying, the plate was heated at 110ºC for 10 min. Evaluation was done in visible light and under UV light at 365 nm.

6.2.2.5.9 Coumarins

Solvent system: Toluene : ethyl acetate (93 : 7)

Spray reagent: Potassium hydroxide (KOH) reagent

The plate was sprayed with about 10 ml of a 10% ethanolic solution of KOH. Evaluation was done in UV light at 365 nm, before chemical treatment and after treatment as well as in visible light with and without and warming.

6.2.3.5.10 Flavonoids

Solvent system: Ethyl acetate : methanol : water (100 :13.5 :10)

Spray reagent: Natural product / polyethylene glycol reagent (NP/PEG) was used.

The plate was sprayed with 1% (m/v) methanolic diphenylboricacid-β-ehtylaminoacid, followed by 5% (m/v) ethanolic polyethylene glycol-4000 and viewed under UV light at 365 nm.

Flavonoids produce orange, orange-yellow, blue or yellow-green colours when fluorescing under 365 nm UV light.

6.2.3.5.11 Steroids

Solvent system: Ethyl acetate : methanol : water (100 :13.5 :10)

Spray reagent: The Lieberman-Buchard solution was used.

The plate was sprayed with a solution consisting of 5 ml H_2SO_4, 5 ml acetic acid and 50 ml ethanol. The plate was viewed under UV light at 365 nm and evaluated for compounds fluorescing violet, orange, purple, pink-brown, purple-brown or brown.

6.2.3 Summary of the order in which techniques were applied to obtain data regarding the antifungal properties of *Eucomis autumnalis*

a) Liquid-liquid extraction was performed on the crude extract in order to separate the main components on the basis of polarity as well as to determine in which fraction the activity was situated.

b) Qualitative TLC separationand visualising the plate with 10% ethanolic H_2SO_4, was performed on the bioactive fraction only in order to obtain a Q-TLC profile of the active fraction for comparison with preparative TLC profiles.

c) Compounds in the active diethyl ether liquid-liquid extraction was further separated by means of column chromatography and the Q-TLC profiles of every third fraction obtained in order to establish which column fractions showed similar profiles. Those with similar TLC profiles were combined and referred to as column fractions.

d) Each column fraction was tested for antifungal activity.

e) The most active column fraction was purified further by separating the components by means of preparative TLC

f) All the compounds isolated by means of P-TLC were tested for antifungal activity. Subsequently, the chemical groups to which only the active compounds belonged were identified by using different spray reagents (Wagner & Bladt, 1996).

6.5 RESULTS

6.5.1 Antifungal activity of semi-purified fractions obtained by means of liquid-liquid extraction of the crude bulb extract of *E. autumnalis*

The diethyl ether fraction clearly showed the highest antifungal activity (82.97% growth inhibition of the test organism *Sclerotium rolfsii*; Table 6.1). Subsequently, only this fraction was purified in further steps. Although the chloroform, ethyl acetate and methylene chloride extractions also showed some antifungal activity, this was too low to justify further purification of these fractions.

Table 6.1: Antifungal activity of semi-purified fractions obtained by means of liquid-liquid extraction of the crude *E. autumnalis* bulb extract tested against the test organism *Sclerotium rolfsii* at a concentration of 0.5 mg ml^{-1}. The total mass of all compounds recovered in each fraction is indicated.

Fraction	% Inhibition	Recovery (g)
Hexane	15.66	2.5128
Diethyl ether	82.97	3.2317
Chloroform	33.29	0.5473
Ethyl acetate	41.15	0.5674
Methylene chloride	42.08	0.9502

6.5.2 Q-TLC profile of components in the semi-purified fractions of a crude *E. autumnalis* bulb extract obtained by means of liquid-liquid extraction

The Q-TLC plate (Figure 6.1) of semi-purified liquid-liquid fractions of an *E. autumnalis* bulb extract showed subtle differences in terms of compound profiles as the Rf-values of different compounds were surprisingly close. In the case of the hexane and diethyl ether, as well as the ethyl acetate and methylene chloride fractions, differences in profiles were more accentuated. The very similar compound profiles of the diethyl ether (DC = 4.3) and chloroform (DC = 4.8) fractions can be attributed to the fact that the DC-values of these two solvents are very close. However, in terms of antifungal activity (Table 6.1), the diethyl ether fraction clearly outperformed all other fractions indicating that most of the active substance(s) was extracted at a DC-value of 4.3. The highest recovery of compounds was also achieved with the latter solvent.

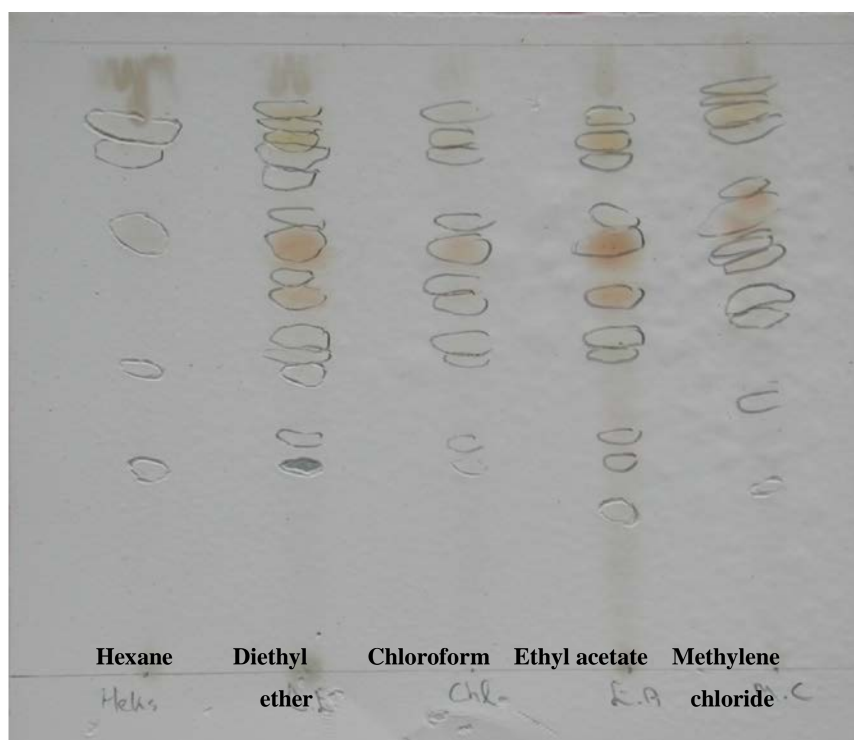

Figure 6.1: Qualitative TLC profiles of components in the crude *E. autumnalis* extract, fractionated by means of liquid-liquid extraction. The organic solvents were used in order of ascending polarity. Mobile phase: Chloroform : Methanol : Water (80:20:10). Stationary phase: Silica gel 60. The plate was visualised with 10% (v/v) ethanolic H_2SO_4.

Despite the fairly high antifungal activity measured in the ethyl acetate and methylene chloride fractions, only the diethyl ether fraction was purified further by means of column chromatography using different mobile phases (see 6.2.2.2).

6.5.3 Q-TLC profiles and antifungal activity of column chromatography fractions obtained from the active diethyl ether extraction of an *E. autumnalis* bulb extract

Four combined column chromatography fractions (combined on grounds of similarities in their Q-TLC profiles; Figure 6.2) were obtained with the first mobile phase (Chloroform : MeOH : H_2O; 80:20:10). These fractions showed very similar antifungal activity (Table 6.2) albeit much lower than that of the semi-purified diethyl ether extract.

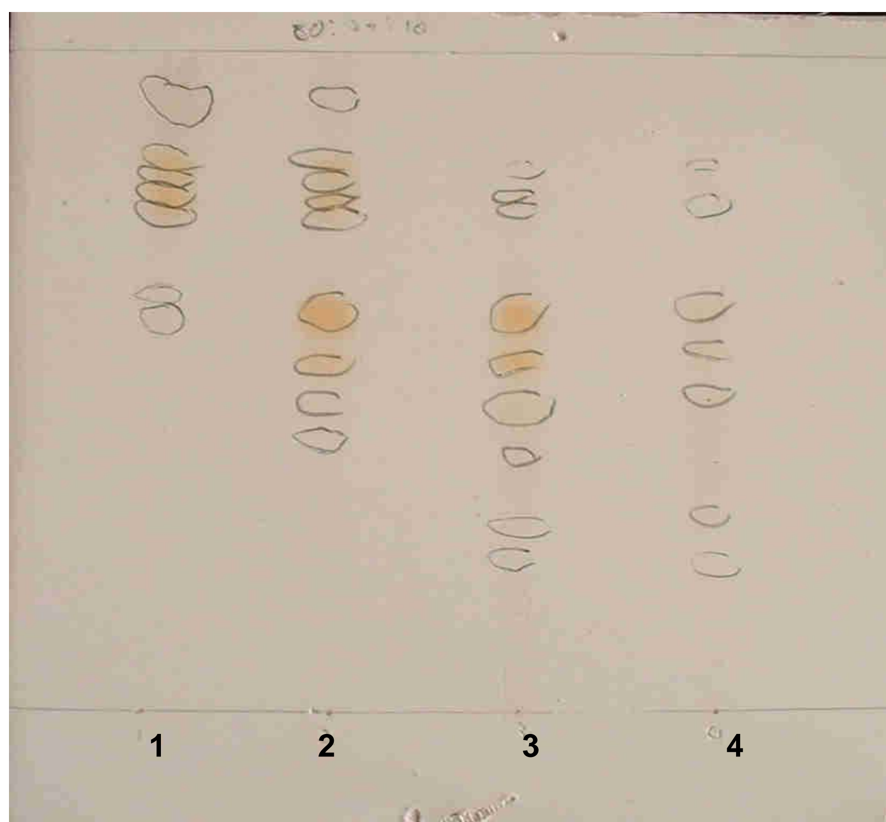

Figure 6.2: Qualitative TLC profile of combined column chromatography fractions of the bioactive diethyl ether extraction eluded from the column using Chloroform : MeOH : H_2O (80:20:10) as mobile phase. Stationary phase: Silica gel 60. The plate was visualised with 10% ethanolic H_2SO_4.

Table 6.2: Antifungal activity of combined column chromatography fractions obtained from the active diethyl ether extraction of an *E. autumnalis* bulb extract using Chloroform : MeOH : H_2O (80:20:10) as mobile phase. The fractions were tested against the fungus *Sclerotium rolfsii* at a concentration of 0.5 mg ml^{-1}. The total mass of all compounds recovered in each fraction is indicated.

Combined column chromatography fraction No.	% Inhibition	Recovery (g)
1	57.16	0.2887
2	54.63	0.5760
3	51.36	0.1338
4	54.45	0.0632

Subsequently, compounds were eluded from the Silica column using the slightly more polar ratio of Chloroform : MeOH : H_2O (60:40:10) as mobile phase. On the basis of similarities in Q-TLC profiles (Figure 6.3), five combined fractions were obtained with the latter mobile phase. Of these, fraction 5 showed the highest antifungal activity (91.95%; Table 6.3) against *S.rolfsii* while fraction 7 (63.24%) was more active than the rest, including the first four fractions eluded with the less polar mobile phase.

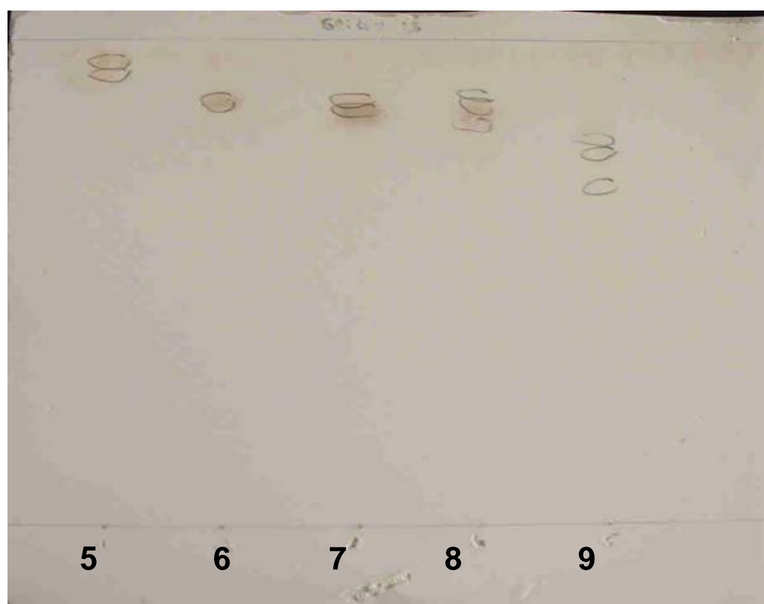

Figure 6.3: Qualitative TLC profile of combined column chromatography fractions of the bioactive diethyl ether extraction eluded from the column using Chloroform : MeOH : H_2O (60:40:10) as mobile phase. Stationary phase: Silica gel 60. The plate was visualised with 10% ethanolic H_2SO_4.

Table 6.3: Antifungal activity of combined column chromatography fractions obtained from the active diethyl ether extraction of an *E. autumnalis* bulb extract using Chloroform : MeOH : H_2O (60:40:10) as mobile phase. The fractions were tested against the fungus *Sclerotium rolfsii* at a concentration of 0.5 mg ml^{-1}. The total mass of all compounds recovered in each fraction is indicated.

Fraction No.	**% Inhibition**	**Recovery (g)**
5	91.95	0.0273
6	56.60	0.0553
7	63.24	0.1510
8	41.21	0.0580
9	53.11	0.0165

The previously mentioned solvent Chloroform : MeOH : Water ratio of 60:40:10 eluded most of the compounds from the column and none was recovered with the 40:60:10 mobile phase ratio. However, one single compound was finally eluded from the column with pure methanol (Figure 6.4). The antifungal activity of this compound (Table 6.4) was rather low (45.3% inhibition). From the TLC profile it was clear that all components were successfully eluded from the column.

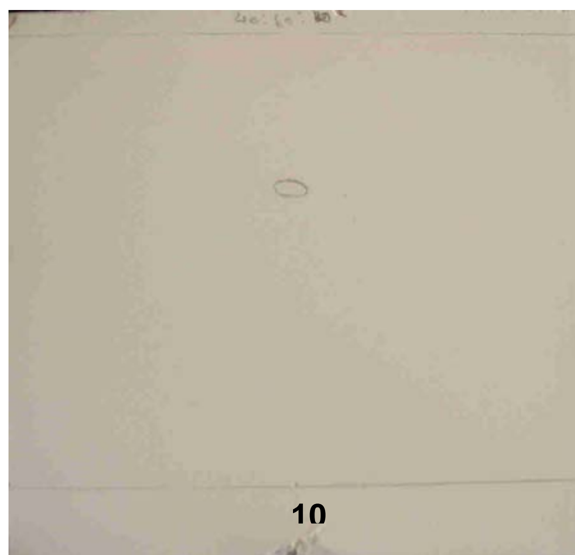

Figure 6.4: Qualitative TLC profile of combined column chromatography fractions of the bioactive diethyl ether extraction eluded from the column using pure methanol as mobile phase. Stationary phase: Silica gel 60. The plate was visualised with 10% ethanolic H_2SO_4.

Table 6.4: Antifungal activity of the final column chromatography fraction obtained from the active diethyl ether extraction of an *E. autumnalis* bulb extract using pure methanol as mobile phase. The fraction was tested against the fungus *Sclerotium rolfsii* at a concentration of 0.5 mg ml^{-1}. The total mass of the single compound recovered is indicated.

Fraction No.	% Inhibition	Recovery (g)
1	45.3	0.0058

6.5.4 Components isolated from the bioactive column chromatography fraction No.5 by means of preparative TLC (P-TLC)

After P-TLC was performed on column fraction 5, four semi-purified fractions (a, b, c and d) were obtained. Fractions a, b and d each yielded one compound (numbered 1, 2 and 6 respectively) while extraction (c) yielded three compounds (numbered 3, 4 and 5; Figure 6.5). Compounds 1, 2, 3 and 6 showed fluorescent rings indicating that the fractions might not be 100% pure.

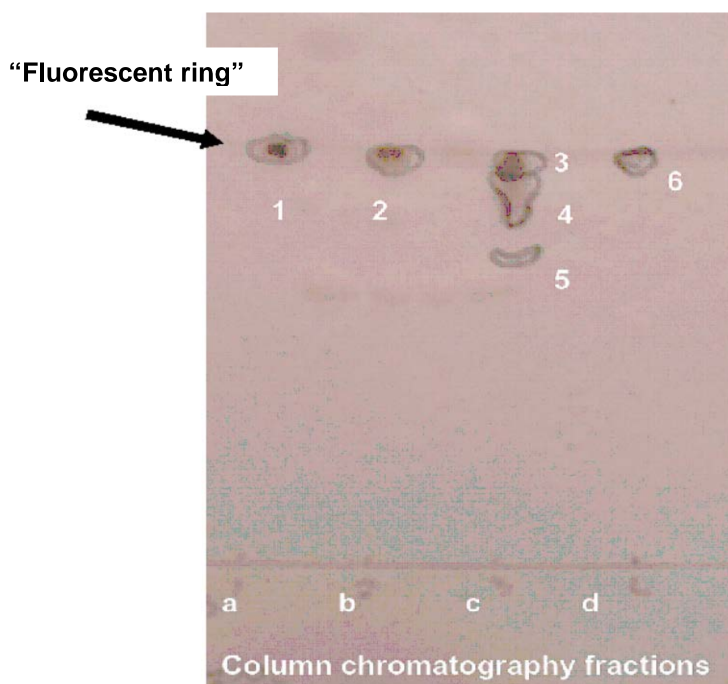

Figure 6.5: Q-TLC profile of six antifungal compounds isolated from column chromatography fraction no. 5 by means of preparative thin layer chromatography (P-TLC).

Testing of the six fractions with colour reagents to identify the chemical groups they belong to produced diverse results. The compounds were tested for eleven chemical groups. The results in table 6.5 show that compound 1 tested positive for three groups namely the Atracenes, Flavonoids and Steroids, while compound 2 tested positive for all chemical groups tested. Compound 3 tested positive for four of the groups while compounds 4 and 5 only tested positive for the flavonoid group. Compound 6 tested positive for the flavonoid and steroid groups only. Interestingly, all fractions tested positive for the flavonoid group that leaves a strong indication that the anti-infective components in *Eucomis autumnalis* are flavonoids.

Table 6.5: Identification of chemical groups to which 6 compounds isolated from *Eucomis autumnalis* bulbs belong, by using specific colouring agents.

Compound Number	**Anthra glycosides**	**Terpenoid**	**Saponin**	**Flavonoid**	**Steroid**
1	+			+	+
2	+	+	+	+	+
3		+	+	+	+
4				+	
5				+	
6				+	+

6.6 DISCUSSION

In this chapter an attempt was made to isolate and purify the active antifungal compounds from the bulbs of *E. autumnalis*. Five liquid-liquid extractions were obtained from the crude bulb extract of which the diethyl ether extraction showed the highest antifungal activity. Only the latter fraction was purified further in order to identify the chemical groups to which active compounds belonged. Ten combined fractions were obtained from the diethyl ether extraction after column chromatographic separation using different mobile phases. Fraction 5 showed the highest anti-fungal activity against the test organism (*Sclerotium rolfsii*). Subsequently, active components in fraction 5 were separated by means of preparative TLC and the chemical groups to which they belonged identified by means of standard colouring reagents (Wagner & Bladt, 1996) as well as fluorescence under UV light.

Due to some compounds having very similar Rf-values, and subsequently tested positive for more than one chemical group, it was clear that all components in fraction 5 were not separated in pure form with this procedure. However, all active compounds in this fraction tested positive for flavonoids, strongly indicating the presence of this chemical group. Flavonoids are known for their strong antifungal (Picman *et al*., 1995; Ragasa *et al*., 1997; Li *et al*., 1999) as well as antibacterial (Tsuchiya *et al.*, 1996; Kuruyanagi *et al*., 1999) properties.

Del Rio *et al.* (2000) reported on the antifungal activity of crude extracts from different *Citrus* spp. and implicated flavonoids to be the principal compounds capable of inhibiting the growth of plant pathogenic fungi. Some of these *Citrus* spp. are grown in Cuba and Spain for the production of flavonoids on an industrial scale (Reynaldo *et al*., 1999). Beno-Moualem *et al.* (2001) also described the important role flavonoids play in the defence system of avocado fruits against attack by fungal pathogens. Other studies also showed flavonoids to posses strong antiviral and antifungal activity including those isolated from leaf extracts of *Ozomothamus leptophyllus*, a native plant of New Zeeland (Wood *et al*., 1999), and those isolated from the aerial parts of *Eysehardtia taxicana* (Wächther *et al.,* 1999). *Terminalia alata* is a medicinal plant known for containing high quantities of flavonoids (Srivastava *et al*., 2001) and extracts of this plant showed very high antifungal activity.

Flavonoids are the largest group of naturally occurring phenolic phytochemicals and more than 5000, of which many are common in higher plants (Harborne, 1991), have been described. The range of known flavonoids is therefore vast and these are frequently referred to by trivial names which generally relate to plant origin, and which can be very confusing (Robards & Antolovich, 1997). The simplest compounds in this group consist of a 15C-skeleton structured as two phenyl rings (A- and B-rings), connected by a 3C-bridge or C-ring (C6-C3-C6) (Iwashina, 2000). The three-carbon bridge between the phenyl groups is commonly cyclized with oxygen, and flavonoids are conveniently grouped into classes according to the degree of non-saturation and degree of oxidation of the C-ring (Robards & Antolovich, 1997).

The most common of these are the anthocyanins, flavones and flavonols. Other groups include the chalcones, flavanones, dihydroflavonols, catechins, leucoanthocyanidins, aurones, isoflavones, isoflavonoids, biflavonyls, xanthones and the condensed tannins. However, variation in molecular structure due to substitution with additional hydroxyl, methoxyl, methyl or glycosyl groups as well as

aromatic and aliphatic acids, sulphate, prenyl, methelenedioxyl or isoprenyl groups attaching to the flavonoid nucleus, contribute to further diversity within the flavonoid group (Iwashina, 2000).

Additional structural complexity is introduced by the common occurrence of flavonoids as glycosides in which one or more of the flavonoid hydroxyl groups are bound to a sugar or sugars by an acid-labile hemi-acetal bond (Robards & Antolovich, 1997). In principle any of the hydroxyl groups can be glycosylated leading to the immense complexity of, for example a common flavonoid such as kaempferol that may occur in nature in 214 different glycosidic forms (Robards & Antolovich, 1997). Moreover, most of the flavonoid compounds reported in the literature are glycosides of a relatively small number of flavonoid aglycones which often are accumulated in the vacuoles of plant cells. Of the several hundred flavonoid aglycones (approximately 200 flavones and 300 flavonols) that have been isolated from plants (Wollenweber & Jay, 1980), only eight are distributed widely namely the anthocyanins kaempferol, quercitin and luteolin, the flavones myricetin and apigenin and the flavonols pelargonidin, delphinidin and cyanidin (Siegler, 1995).

Many other biological activities of flavonoids have been reported including antiulcer-, antihepatotoxic- (Vogel, 1982; Beier & Nigg, 1992), insect repelling- (Hedin & Waage, 1986), antiviral- (Vlietinck *et al.*, 1986), anti-inflammatory-(Vogel, 1982), anti-oxidant- (Khushbaktova *et al.*,1996), free radical scavenging- (Mathiesen *et al.*, 1995), hypercholesterolemia- (Monforte *et al.*, 1995), anti-HIV- (Lin *et al.*, 1997), anti-diabetic- (Yoshikawa *et al.*, 1998), anti-hepatotoxic- (Shirwaikar *et al.*, 1995) and anticancerous (Cassady *et al.*, 1990) properties. Moreover, functions of flavonoids in plants may include serving as visual attractants for pollination, as pigments for light absorbance and as antioxidants. However, flavonoid aglycones probably play major roles as internal physiological regulators, including growth regulation and chemical messengers in plants (Siegler, 1995). Certain flavonoids can interrupt the electron transport chain of respiration and photosynthesis (Arntzen *et al.*, 1974). Quercitin, morin, myricetin and fisetin are active inhibitors of lipid peroxidation in the presence of ferrous iron (Siegler, 1995). Glycosides absorb harmful frequencies of UV-light and prevent destruction of important compounds in plants, e.g. the chlorophyll of photosystem II, as well as permit entry of useful wavelengths (Stafford, 1991).

What makes these phytochemicals remarkable from an industrial perspective is their vast abundance in both edible and non-edible plants as well as their potential to be developed into natural products. It is estimated that about 2% of all carbon photosynthesized by plants, amounting to about 1 x 10^9 tons per

annum, is converted into flavonoids or closely related compounds (Robards & Antolovich, 1997). With this vast amount of naturally occurring flavonoids being available, and in light of the diverse activities that have been identified for this group of compounds to date, the time is ripe to consolidate and integrate existing knowledge of this group of phytochemicals and to evaluate its industrial application potential.

Early suggestions that the flavonoids were involved in disease resistance in plants as well as the association of increased endogenous flavonoid synthesis with the early stages of infection (Robards & Antolovich, 1997), must be regarded as unexplored opportunities due to the lack of follow-up tests to evaluate the application potential of these compounds in agriculture. The diversity of the flavonoids in agronomic lines and their roles in plant resistance suggest that they could be exploited for increased food production (Robards & Antolovich, 1997).

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Further, flavonoids possess characteristics that make them ideal chemotherapeutic agents including (a) their widespread occurrence in plants, (b) their species specific distribution, (c) the fact that they are easy to detect, chromatograph and identify, (d) their relative stability *in vivo* as well as after isolation and (e) the indication that both their biosynthesis and accumulation are independent of environmental influence (Markham, 1989).

A substantial pool of information on the toxic effects of flavonoids to micro-organisms has been published to date. Numerous studies have indicated that flavonoids may be useful food compounds and confirmed the rather high potential of this group of chemicals as anti-infective agents. However, most of the research involved *in vitro* studies that make it difficult to draw definite conclusions about the usefulness of flavonoids, although the *in vivo* studies that have been performed do give a hopeful picture for the future (Nijveldt *et al.*, 2001). However, much more research, especially clinical trials, is needed concerning the possible side effects of natural products, with concentrated flavonoid content, to humans and animals. Field trials to evaluate the performance of flavonoids as anti-infective agents under different soil and environmental conditions for the crop production industry, as well as possible side effects, also seem to be a neglected research field. The latter approach to commercializing the use of flavonoids in the agricultural industry, might be the swiftest way to the industrialization of this unique group of chemicals.

Further studies on bulb as well as above ground part extracts of *Eucomis autumnalis* will be required in order to identify and determine the chemical structure of each of the active components, implicated as flavonoids in this study. Additional *in vivo* tests with purified substances, in a glasshouse or in the field, will also be necessary in order to determine the viability of these green chemicals as possible natural fungicides.

CHAPTER 7

GENERAL DISCUSSION

The initial aim of this study was to screen a crude bulb extract of *Eucomis autumnalis* for inherent qualities, including bio-stimulatory and antimicrobial properties, and to establish a protocol for further studies in the event of any such activities being observed. Depending on the outcome, an evaluation of *E. autumnalis* as natural source of compounds with the potential of being applied in the agricultural industry for the mentioned activities, as well as its potential to be developed as an alternative crop, was anticipated.

A crude bulb extract of *E. autumnalis* was screened for bio-stimulatory activity by following its effect on the respiration rate of a monoculture yeast cells [chapter 4; often used to indicate the manipulation potential of a chemical on the metabolism of living organisms (Seigler, 1998)] A commercial bio-stimulant and natural product from plant origin, ComCat®, with bio-stimulatory and yield increasing properties, was used as second control together with distilled water in order to assess the effectiveness of the extract in manipulating the respiration rate of the yeast cells. Compared to the water and ComCat® controls, a high concentration of the bulb extract significantly increased the respiration rate of the yeast cells while lower concentrations had no effect. This confirmed that the crude bulb extract possessed bio-stimulatory activity, but implicated either inhibitory or stimulatory activity (Agraforum, Germany – personal communication).

In order to distinguish between the two possibilities, the influence of the crude extract on the germination of Cress seeds and subsequent seedling growth were also tested using the same concentrations for the extract as well as the positive control as were used during the respiration tests. ComCat® stimulated the germination of Cress seeds while the *E. autumnalis* crude extract had no statistically significant effect on germination. Further, while ComCat® had a stimulatory effect on both coleoptile and root growth of Cress seedlings, the crude bulb extract showed neither a stimulatory nor an inhibitory effect on coleoptile growth but clearly inhibited root growth. Although at a rather high concentration, it is concluded that a crude *E. autumnalis* bulb extract possesses potential to be utilized, along with other plant extracts (Neave & Dawson, 1989), as a possible natural herbicide (Waller,

1989). The latter was not pursued further in this study as subsequent screening of the crude extract showed much higher potential for antimicrobial activity at much lower concentrations of the extract. Although screening of the crude bulb extract showed significant antibacterial activity against the gram-positive human bacterium *Moraxella catharrhalis*, no inhibition of six plant pathogenic bacteria was observed. However, the extract showed potent antifungal activity against eight plant pathogenic fungi of economic importance in South Africa confirming the broad spectrum potential of the extract as well as its potential to be developed as a natural fungicide. The opposite, namely plant extracts showing antibacterial but not antifungal properties, was previously reported (Naqvi *et al*., 1991).

Especially the significant mycelial growth inhibition achieved with the extract, generally regarded as more difficult to accomplish than inhibition of spore germination (Naqvi *et al*., 1991), prompted a further study. Although the mycelial growth of *Verticillium dahliae* and *Botrytis cinerea* was least affected by the extract, that of *Sclerotium rolfsii, Rhizoctonia solani, Mycosphaerella pinodes* and *Pythium ultimum* was inhibited more effectively by the crude extract than by the broad spectrum standard fungicide used as a positive control. The control fungicide showed highest activity against four of the organisms, but this was only statistically significant for *Botrytis cinerea* and *Botryosphaeria dothidea*.

Subsequently, before attempting to purify active antifungal compounds from the bulb extract, it was necessary to ascertain whether the crude extract, being poisonous to humans (Watt & Breyer-Brandwijk, 1963), had any phytotoxic effect on plant material and whether spore germination of fungi could be inhibited *in vivo*. The acquirement of phytotoxicity data and information on the potential of a crude extract to prevent fungal infection *in vivo*, is seen as the next step towards developing a natural fungicide. For this purpose the phytotoxicity of the extract as well as the inhibition of *Mycosphaerella pinodes* spores on four-week-old pea (*Pisum sativum*) leaflets were used in screening tests and compared to water and a standard fungicide (carbendazim/difenoconazole) control.

Six days after treatment, the *in vivo* phytotoxicity rating of the crude *E. autumnalis* bulb extract, in terms of its interaction with and potential to induce necrosis in pea leaves, revealed that the extract showed no phytotoxic reaction on the leaves even at the highest concentration applied. Moreover, the *in vivo* germination of *M. pinodes* spores was inhibited completely by the extract preventing infection of pea leaves with Ascochyta blight, at a concentration as low as 1 mg ml^{-1}, both when the pea leaves were inoculated with *M. pinodes* spores before and after treatment with the extract. The extract, in both

cases, was more effective against spore germination than the standard fungicide suggesting that a crude bulb extract of *E. autumnalis*, at the indicated concentration, has the potential to be applied both as a preventative or corrective agent against infection of pea plants by *M. pinodes* spores. This supplied the rationale for attempting to isolate the antifungal active compounds from *E. autumnalis* bulbs by means of standard chromatographic techniques and to determine the chemical group(s) to which the active compound(s) belonged.

Activity directed fractionation (Cannel, 1998) of the crude extract by means of liquid-liquid extraction, using organic solvents in order of increasing polarity and *Sclerotium rolfsii* as test organism, yielded five fractions. Of these the diethyl ether fraction, showing the highest antifungal activity, was further fractionated by means of column chromatography using different mobile phases. Every third column fraction was spotted on a Q-TLC plate and the compounds separated in order to obtain a profile of each. Those fractions with similar Q-TLC profiles were combined yielding ten column fractions. Only fraction 5, showing the highest antifungal activity, was further fractionated by means of preparative thin layer chromatography (P-TLC) yielding four (a, b, c, and d) bioactive semi-purified fractions, compounds or groups of compounds. From fractions a, b and d one compound each (numbered 1, 2 and 6 respectively) were isolated while fraction c yielded three compounds (numbered 3, 4 and 5). Compounds 1, 2, 3 and 6 showed fluorescence rings under UV-light indicating that 100% purity have not been obtained. The latter was confirmed as four of the six compounds tested positive for more than one chemical group using standard colouring reagents (Wagner & Bladt, 1996). However, all six compounds tested positive for flavonoids, strongly indicating the presence of this chemical group.

Flavonoids are generally present in the leaf cuticle and epidermis cells of plants, defending it against the dangerous effects of UV-radiation and anti-microbial infections (Bruneton, 1995). Different flavonoids and other phenolic compounds react with free radicals to prevent the degeneration of membranes by preventing the action of free radicals on membrane phospholipids (Bruneton, 1995). The functionality of flavonoids in plants is as diverse as the more than 5000 flavonoids that have been identified. Inter alia, the knowledge gained by studying flavonoids has proven useful for examination of many taxonomic problems at the genus and species level for several reasons: 1) most plants contain several major flavonoid compounds (usually glycosides) that tend to differ in the various taxa (forms, subspecies, varieties or species) that have been examined, 2) analysis of compound mixtures is reasonably simple and techniques for identification of the compounds are well established and 3) the

flavonoid compounds present in hybrids often represent an additive combination of those of the parental types (Siegler, 1995).

With regard to the latter use of flavonoid content data, the systematics of the genus *Baptisia* (Fabaceae), for example, has been studied extensively and almost all species of the genus have distinctive complements of flavonoid glycosides. Flavonoid chemistry was especially useful for analysis of the many hybrid forms encountered; some of which involved as many as four different species. In general it was possible to recognize hybrid plants because they simply had additive patterns (i.e. the hybrids often had a combination of all the compounds found in the putative parental types (Siegler, 1995). A further interesting feature regarding the functionality of flavonoids is the allelopathic effect observed in plants involved with nodulation by *Rhizobium* bacteria. The 3-*0*-glycosides of 10 anthocyanidins and flavonols, of which the most important are delphinidin, petunidin and malvidin, are involved with the stimulation of *Rhizobium leguminosarum* during nodulation of the black bean, *Phaseolus vulgaris* (Hungria *et al*., 1991a). Three other flavonoids, eriodictyol, naringenin and 7-*0*-glycoside, or genistin, are the most active compounds from root exudates of the black bean (Hungria *et al*., 1991b). Tsai and Phillips (1991) also reported on the stimulating effect of quercitin 3-*0*-galactoside from alfalfa seeds on spore germination of two fungi, *Glomus etunicatum* and *G. macrocarpum*, responsible for the formation of vesicular-arbuscular-mycorrhizal interactions.

Since flavonoids are phenolic compounds, they react with proteins and can thus interact with enzymes and biological processes in the cells. This consequently makes them toxic to some micro-organisms and possibly also to some animals (Markham, 1989; Grayer, 1989). Although toxicity to micro-organisms forms the basis for the exploitation of naturally occurring flavonoids as anti-infective agents, toxicity to animals might be a concern in terms of the possible side effects natural flavonoid pharmacological and veterinarian products might have on humans and animals respectively. However, the application potential of this group of naturally occurring phytochemicals as anti-infective drugs on an industrial scale in the pharmaceutical and agricultural industries needs to be explored more intensively in future.

The term ‘anti-infective’ literally refers to the prevention of infection in plants, animals or humans by either 1) being toxic to the source of infection (bacterium; fungus; virus; parasite) or by 2) preventing infectious toxins from the source to exert its influence on a cell, tissue or organ level through disruption of its mechanism of action. However, toxicity to the source of infection might also imply toxicity to

cells, tissues or organs of recipient organisms and needs to be dealt with in any case where chemicals, including chemicals from natural sources, are applied to plants, animals or humans.

The recent work of Srivastava *et al.* (2001) lead to the isolation of three new glycoside compounds from *Terminalia alata* [*T. elliptica*] roots that showed antifungal activity against the plant pathogen *Aspergillus niger* and the human pathogen *Candida albicans* at extremely low concentrations of between 25 and 32 ppm. The three new glycosides identified were: 1) 3,3'-di-O-methylellagic acid 4-O-β-D-glucopyranosyl-(1,4)-β-D-glucopyranosyl-(1,2)-α-L-arabinopyra-noside (Figure 7.1), 2) 5,7,2'-tri-Omethylflavanone 4'-O-α-L-rhamnopyranosyl-(1,4)-β-D-glucopyranoside (Figure 7.2) and 3) 2-α,3-β,19-β,23-tetrahydroxyolean-12-en-28-oic acid 3-O-β-D-galactopyranosyl-(1,3)-β-D-glucopyranoside-28-O-β-D-glucopyranoside (Figure 7.3). Compound 1 was a glycoside of an ellagic acid, whereas compounds 2 and 3 were a flavanone glycoside and a triterpene saponin, respectively. Reports, such as this of Srivastava *et al.* (2001), on the discovery of new flavonoids are always welcomed. However, depending on the economic importance of a single pathogen, it is advisable that initial screening revealing activity rather be followed up by a more comprehensive study on a range of pathogens than to test activity against a single pathogen.

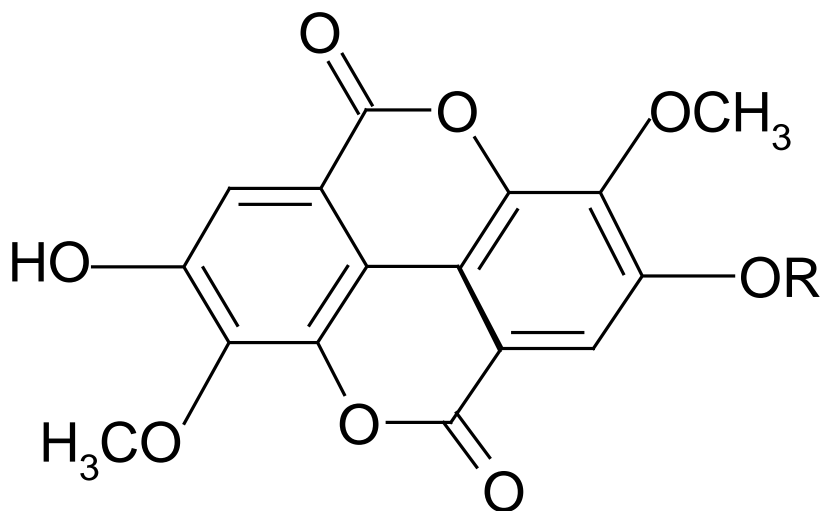

Figure 7.1: **Compound 1**: 3,3'-di-O-methylellagic acid 4-O-β-D-glucopyranosyl-(1,4)-β-D-glucopyranosyl-(1,2)-α-L-arabinopyranoside [**R** = β-D-gluc (1 →4)-β-D-gluc- (1 →2)-α-L-ara]. A glycoside of an ellagic acid (Srivastava *et al.,* 2001).

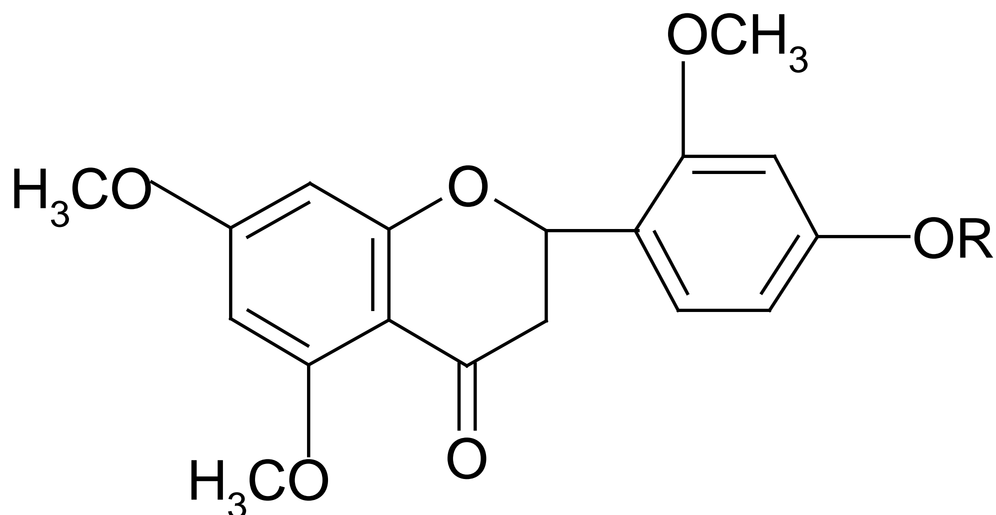

Figure 7.2: **Compound 2**: 5,7,2'-tri-Omethylflavanone 4'-O-α-L-rhamnopyranosyl-(1,4)-β-D-glucopyranoside. [**R** = (α-L-rham-(1 → 6)-β-D-gluc]. A flavanone glycoside (Srivastava *et al.*, 2001).

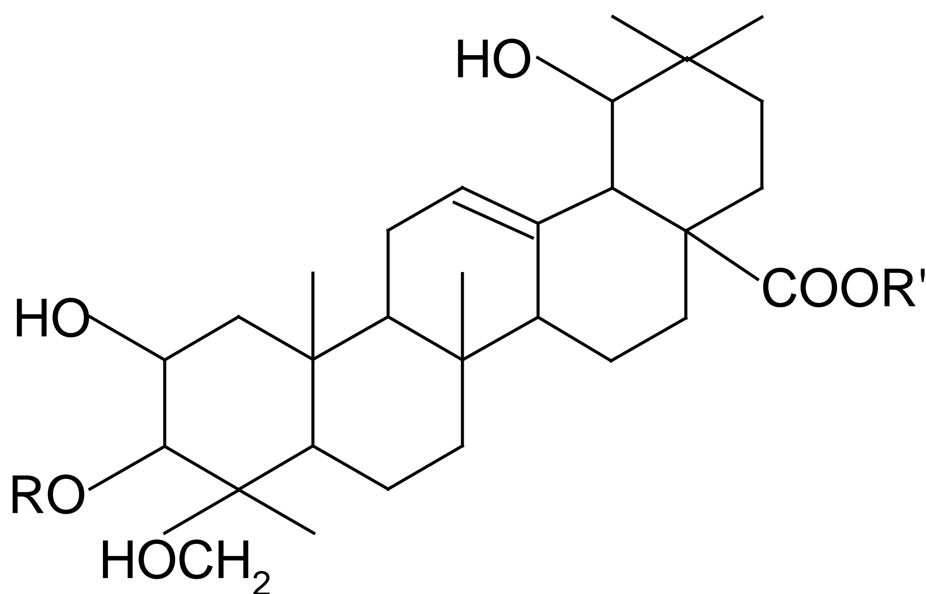

Figure 7.3: **Compound 3**: 2-α,3-β,19-β,23-tetrahydroxyolean-12-en-28-oic acid 3-O-β-D-galactopyranosyl-(1,3)-β-D-glucopyranoside-28-O-β-D-glucopyranoside [**R** = β-D-gal-(1 → 3)-β-D-gluc; **R'** = β-D-gluc]. A triterpene saponin (Srivastava *et al.,* 2001).

Another example of screening against a single pathogen comes from the work of Picman *et al*. (1995). Twenty-five flavonoids were tested for their effects on the mycelial growth of the plant pathogen *Verticillium albo-atrum*. MIC values for the most active compounds, flavone and flavanone, were 1

and 5 ppm, respectively. Some compounds were inactive at the highest concentration tested (200 ppm). Despite the limited information presented by Picman *et al*. (1995) in terms of broad spectrum control, an important observation was that active compounds did not show a common substitution pattern. Unsubstituted flavonoids were stronger mycelial growth inhibitors and, in most cases, increasing the number of substitutions (hydroxylation, methoxylation and glycosylation) resulted in loss of antifungal activity. This is important information for pesticide companies interested in mimicking natural compounds by chemical synthesis.

More examples of screening against a limited number of pathogens include the work of Alavez *et al.* (2000) and Wächter *et al.* (1999). The roots of the tropical tree *Lonchocarpus oaxacensis* afforded the 3-hydroxyflavanones jayacanol and mundulinol, as well as two flavanones, mundulin and minimiflorin (Alavez *et al.,* 2000). The antifungal activity of the four isolated compounds was tested against the wood rotting fungus *Postia placenta*, but only jayacanol was active. Two novel antibacterial (against *Staphylococcus aureus*) and antifungal (against *Candida albicans*) prenylated flavanones were isolated from *Eysenhardtia texana* (Texas, USA) and their structures established (Wächter *et al.,* 1999). Both these compounds were shown to inhibit the growth of *S. aureus* and *C. albicans* at a concentration of 100 µg ml^{-1}. Activity-guided fractionation of a methanol-dichloromethane extract of the aerial parts of *E. texana* led to the isolation and identification of 4',5,7-trihydroxy-8-methyl-6-(3-methyl-[2-butenyl])-(2S)-flavanone, 4',5,7-trihydroxy-6-methyl-8-(3-methyl-[2-butenyl])-(2S)-flavanone and 4',5-dihydroxy-7-methoxy-6-(3-methyl-[2-butenyl]) -(2S)-flavanone on the basis of spectral data.

A crude extract from a New Zealand native plant, *Ozothamnus leptophyllus*, was shown to possess antifungal activity (Wood *et al*., 1999). Flavonoids, mainly chalcones in the leaf exudate, were isolated and identified. Interestingly, these flavonoids are excreted by glandular trichomes and form quasi-crystalline deposits on the abaxial side of the leaves. The compound responsible for most of the antimicrobial activity was 2', 4', 6'-trihydroxy-chalcone (Figure 7.4), inhibiting the growth of the dermatophytic fungus *Trichophyton mentagrophytes*, and also proved to have antiviral activity against *Polio virus* and *Herpes simplex* 1. What is especially interesting about the source plant *Ozothamnus leptophyllus*, is that flavonoids could be obtained from it without even making extracts of the plant parts but merely by washing the crystalline flavonoid deposits from the leaves. Theoretically the process might be much cheaper and time sparing than harvesting plant parts on a large scale and preparing extractants from it. Whether the amounts of flavonoids that could be retrieved in this way will be sustainable on an industrial scale remains to be seen.

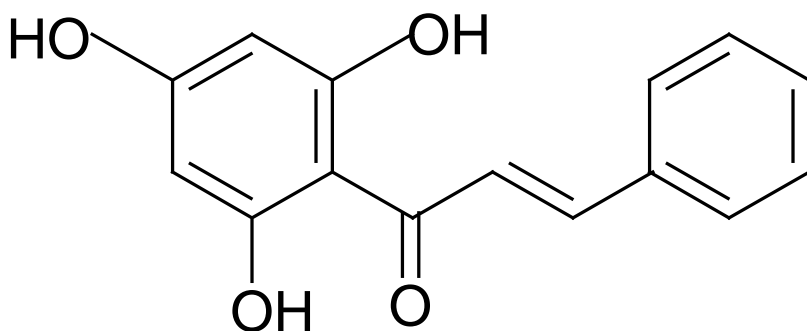

Figure 7.4: 2', 4', 6'-Trihydroxy-chalcone that inhibits the growth of the dermatophytic fungus *Trichophyton mentagrophytes* and also proved to have antiviral activity against *Polio virus* and *Herpes simplex* 1 (Wood *et al*., 1999).

Analyses of phenolic compounds in *Olea europaea* revealed the presence of tyrosol, catechin, p-coumaric acid, rutin [rutoside], luteolin and oleuropein, while the principal compounds in *Citrus* spp. were flavanones and flavones (Del Rio *et al.,* 2000). In both genera, the concentrations varied according to the species, although all acted as antifungal agents and were capable of inhibiting the growth of phytopathogenic fungi. It was possible to stimulate the biosynthesis and/or accumulation of these phenolic compounds in both *O. europaea* and *Citrus* spp. by treatment with 0.3% Brotamax, while 100 ppm benzylaminopurine [benzyladenine] had the same effect in *Citrus* spp. Increased polyphenol levels lead to improved defense mechanisms in both genera. For example, treated tangelo Nova fruit showed greater resistance to *in vivo* infection by *Phytophthora citrophthora*, since the constitutive polyphenolic compounds at the fruit surface provided a first line of defense. Treatment of plants considered as potential source plants for the isolation of flavonoids on an industrial scale with Brotomax, in light of indications that synthesis of phenolic compounds could be induced by this chemical *in vivo*, can be a tool to enhance the sustainability of such a venture. This might be especially important in the event where natural plants are considered as sources of flavonoids on an industrial scale and in light of the fact that chemical synthesis of these anti-infective compounds is not yet established (Del Rio *et al*., 2000).

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It is interesting to note that many plants possess effective defense mechanisms against bacterial attacks while the same cannot be said for fungal attacks (Naqvi *et al*., 1991). In light of some indications that flavonoids might play a role in plants as part of a natural defense mechanism against both bacterial and fungal diseases, a rationale for further investigation in this field, as well as the application potential of

these flavonoids in the agricultural industry in its broadest context, is well established and should be pursued with more zest.

Further, the application potential of flavonoids as anti-infective agents in the agricultural industry needs serious consideration. For the moment the prospect to apply flavonoid rich plant extracts as natural plant pathogenic bactericides or fungicides seems to be an avenue that can be pursued much quicker than its possible application as pharmaceuticals. This is due to the lengthy and sometimes difficult clinical trials that have to be performed on animals or humans as well as the identification of side effects on many fronts. In contrast, crude or semi-purified plant extracts as well as purified flavonoids can be tested on crops in either glasshouse or field trials by using rather simple *in vivo* techniques in much shorter time. Most registration offices, e.g. the EPA in the USA, require statistical data from three seasons before registration of a new product can be considered for the agricultural industry. However, in some cases, more than one trial in the same year but at different locations is accepted to make up for the three-season requirement. It therefore seems that natural product researchers should be encouraged to endeavour accelerated research on natural products for the agricultural, and especially the crop production industry. The advantages of natural products are numerous including their biodegradability and the widely accepted notion that they are environmentally friendly, to mention only two.

In this study both the bio-inhibitory and antifungal properties of an *E. autumnalis* crude extract were shown. Non-withstanding the fact that its bio-inhibitory potential should be exploited in future research in terms of the possible development of a natural herbicide, only the antifungal activity was used in this study for the activity directed isolation of the active compounds involved. An important outcome in this regard is that a crude bulb extract inhibited a broad spectrum of economic important plant fungal pathogens *in vitro* while its *in vivo* inhibitory potential of *Mycospaerella pinodes* spores were also confirmed. Further, even the highest concentration tested showed no signs of phytotoxicity on pea (*Pisum sativum*) leaves. In future research a variety of crop-pathogen *in vivo* systems should be investigated in order to confirm the potential of developing a natural fungicide using *E. autumnalis* as source plant.

It is further suggested that a follow-up study should be undertaken to identify all the active ingredients, down to its molecular structure, by purifying the active compounds to its purest form while considering to start with a large amount of fresh material due to the low recovery of active compounds during the

purification process. Lastly, from an agronomic perspective, the potential for *E. autumnalis* to be developed as an alternative crop, in the event that its use as a source for the active antifungal ingredients can be economically justified, has been established in this study. Future agronomic studies, including the application of irrigation scheduling and different fertilizer regimes, should be undertaken in an attempt to explore ways and means of obtaining the highest possible yield of the plant while the antifungal activity is either not affected or improved. As harvesting of bulbs is a destructive method, the antifungal potential of the above ground parts should also be investigated. In the event that the above ground parts show promise as source of the antifungal compounds, the economic potential of the plant will be elevated as it is a perennial.

SUMMARY

Over the past number of years a renewed interest in the search for natural alternative products, with the aim to replace existing synthetic antimicrobial, herbicidal and pesticidal chemicals in organic crop production systems, was shown. *Eucomis autumnalis*, a medicinal plant found in South Africa, was used in this study to determine the biostimulatory and antimicrobial properties of a crude bulb extract as well as to evaluate the potential to develop a natural product from this plant. Preliminary screening procedures showed that the crude bulb extract possessed no biostimulatory, but indeed antimicrobial properties.

After confirming the *in vitro* antifungal properties of the crude extract on a number of fungal pathogens, its *in vivo* activity against *Mycosphaerella pinodes*, causing Ascochyta blight on peas (*Pisum sativum)*, was also confirmed. Using pea leaves, it was also shown that the crude bulb extract had no phytotoxic effect on the plant tissue even at the highest concentration applied. The collective *in vitro* and *in vivo* data obtained supplied the rationale for attempting to isolate and purify the active substance(s) responsible for the antifungal activity.

By means of activity directed liquid-liquid extraction, using a series of organic solvents with increasing polarity, most of the antifungal activity was contained in a diethyl ether extractant. Column chromatographic separation of compounds in the active diethyl ether extractant yielded ten combined fractions of which only one showed significant anti-fungal activity against the test organism *Sclerotium rolfsii*.

Subsequently, six active components were isolated from the latter fraction by means of preparative TLC and the chemical groups to which they belonged identified using standard colouring reagents as well as fluorescence under UV light. Due to some compounds having similar Rf-values and tested positive for more than one chemical group, it was clear that all in this fraction were not separated in pure form. However, all active compounds in this fraction tested positive for flavonoids, strongly indicating the presence of this chemical group. Flavonoids are known for their strong antifungal properties.

It was concluded from this study that the bulbs of *E. autumnalis* possess significant antifungal activity to warrant a further investigation into the possibility to utilize this plant in developing a natural

fungicide with application potential in agriculture. However, purification of the active substance(s) as well as the elucidation of its(their) molecular structure(s) will be important in order to investigate a possible mechanism of action.

KEY WORDS: Natural products, alternative crops, *Eucomis autumnalis*, antifungal properties, active ingredients, flavonoids

OPSOMMING

Oor die afgelope tien jaar was daar ‘n oplewing in die soeke na natuurlike alternatiewe produkte om bestaande sintetiese antimikrobiese, onkruiddodende en insekdodende middels in organiese plantproduksie stelsels te vervang. *Eucomis autumnalis*, ‘n medisinale plant wat weidverspreid in Suid Afrika voorkom, is in hierdie studie gebruik om vas te stel of daar enige biostimulerende of antimikrobiese eienskappe in ’n ru-ekstrak van die bolle voorgekom het asook om die potensiaal vir die plant om in ’n natuurlike produk ontwikkel te word, te evalueer. ’n Voorlopige studie het aangedui dat die ru-ekstrak oor geen biostimulerende eienskappe beskik nie maar wel oor betekenisvolle antimikrobiese eienskappe.

Nadat die *in vitro* antifungale eienskap van die ru-ekstrak teen ’n verskeidenheid van plantpatogeniese swamme bevestig is, is die *in vivo* aktiwiteit teen *Mycosphaerella pinodes*, wat swartroes op ertjies (*Pisum sativum)* veroorsaak, ook bevestig. Daar is ook aangetoon dat die ru bol-ekstrak, selfs teen die hoogste konsentrasie toegedien, geen fitotoksiese effek op die blaarweefsel van ertjies gehad het nie. Die kollektiewe *in vitro* en *in vivo* data wat bekom is het die rasionaal verskaf vir ’n verdere poging om die aktiewe antifungale komponent(e) te isoleer en te suiwer.

Deur aktiwiteitsgerigte vloeistof-vloeistof ekstraksie, met behulp van ’n reeks organiese oplosmiddels met toenemende polariteit, is die meeste antifungale aktiwiteit in diëtieleter gekonsentreer. Kolom chromatografiese skeiding van die komponente in die aktiewe diëtieleter ekstrak het tien gekombineerde fraksies opgelewer waarvan slegs een betekenisvolle antifungale aktiwiteit teen die toetsorganisme, *Sclerotium rolfsii*, getoon het. Ses aktiewe komponente is voorts deur middel van preparatiewe dunlaagchromatografie uit laasgenoemde fraksie geïsoleer en die chemiese groepe waaraan hulle behoort is met behulp van standaard kleurreagense asook fluoressensie onder UV-lig geïdentifiseer. Aangesien die Rf-waardes van sommige komponente baie ooreengestem het en sommiges positief getoets het vir meer as een komponent, was dit duidelik dat aktiewe bestanddele nie in suiwer vorm bekom was nie. Maar, alle fraksies het positief getoets vir flavonoïede wat sterk gedui het op die teenwoordigheid van hierdie chemiese groep. Flavonoïede is bekend vir hul sterk antifungale eienskappe.

Deur hierdie studie is tot die gevolgtrekking gekom dat die bolle van *E. autumnalis* oor genoegsame antifungale aktiwiteit beskik om ‘n verdere studie te onderneem ten einde die moontlikheid om die

plant te gebruik vir die ontwikkeling van ‘n natuurlike fungisied, wat in die landboupraktyk toegepas kan word, te ondersoek. Daar word ook aanbeveel dat verdere suiwering van die aktiewe komponent(e) asook vasstelling van die chemiese struktuur daarvan uitgevoer moet word ten einde moontlike aksiemeganismes te ontsyfer.

SLEUTELWOORDE: Natuurlike produkte, alternatiewe gewasse, *Eucomis autumnalis*, antifungale eienskappe, aktiewe bestandele, flavonoïede

REFERENCES

Abd El Ghafar N.Y. 1998. Control of potato bacterial wilt using crop rotation. *Annals of Agricultural Science* 43(2): 575-587.

Agrios G.N. 1997. Plant Pathology. Academic Press Ltd. London.

Alavez S.D., Reyes C.R., Jimenez E.M., Gomez G.F., Chavez U.I. and Sousa S.M. 2000. Flavanones and 3-hydroxyflavanones from *Lonchocarpus oaxacensis*. *Phytochemistry.* 55(8): 953-957.

Ali S.M., Nitschke L.F., Dube A.J., Krause M.R. and Cameron B. 1978. Selection of pea lines for resistance to pathotypes of *Ascochyta pinodes*, *Ascochyta pisi* and *Phoma medicaginis* var. *pinodella*. *Australian Journal of Agricultural Research* 29: 841-849.

Alice D. and Sivaprakasam K. 1995. Antibiotics and garlic clove extract - inhibitory agents of cell wall degrading enzymes. *Hindustan Antibiotics Bulletin* 37(1-4): 44-47.

Alkofahi A.S., Abdelaziz A., Mahmoud I., Abuirjie M., Hunati A. and el-Oqla A. 1990. Cytotoxicity, mutagenicity and antimicrobial activity of forty Jordanian medicinal plants. *International Journal of Crude Drug Research* 20(2):139-144.

Allard B., Bill L. and Touraud G. 1992. L'Anthracnose du pois: revue bibliographique et synthèse. *Agronomie* 13: 5-24.

Anaya A.L., Hernandes-Bautista B.E., Pelayo-Benavides H.R., Calera M., Fernandes-Luiselli E., Inderjid (ed.), Dakshini K.M.M. (ed.) and Einhellig F.A. 1995. Allelopathy in Mexican plants. More recent studies. *Allelopathy: Organisms, processes and applications.* ACS Symposium Series, 582:224-241.

Aquino R., De Simone F., De Tommasi N. and Pizza C. 1995. In**:** Studies in Natural Products Chemistry. Volume 17: Structure and Chemistry (Part D); Atta-ur Rahman (ed.); Elsevier. pp. 113-152.

Arntzen C.J., Falkenthal S.V. and Bobick S. 1974. Inhibition of photophosphorylation by kaempferol. *Plant Physiology* 53: 304-306.

Arteca R. N., Bachman J. M. and Tsai D. S. 1988. Fusicoccin, an inhibitor of brassinosteroid-induced ethylene production. *Physiologia Plantarum.* 74 (4): 631-634.

Ault J.R. 1995. *In vitro* propagation of *Eucomis autumnalis, E. comosa* and *E. zambesiaca* by twin-scaling. *Horticultural Science* 30(7): 1441-1442.

Aysan Y., Yildiz N., Albajes R. and Sekeroglu E. 2000. Effect of plant extracts on tomato stem necrosis. *IOBC-WPRS Working Group "Integrated Control in Protected Crops, Mediterranean Climate". Proceedings of the meeting, Antalya, Turkey, 24-28 April 2000. Bulletin-OILB-SROP.* 23(1) 59-62.

Baard S.W. & Los. O. 1989. Two newly reporteddiseases of peas in South Africa. *Phytophylactica*. 21:307-309.

Barreto M., Straker C.J. and Critchley A.T. 1997. Short note on the effects of ethanolic extracts of selected South African seaweeds on the growth of commercially important pathogens, *Rhizoctonia solani*, Kühn and *Verticillium* sp. *South African Journal of Botany* 63(6):521-523.

Beier R.C. and Nigg N. 1992. In: Phytochemical Resources for Medicine and Agriculture; H.N. Nigg; D. Seigler; (eds.), Plenum Press: New York. pp.247-367.

Bennett A. B., Smith G. M. and Nichols B. G. 2001. Regulation of climacteric respiration in ripening avocado fruit. *Plant Physiology* 83(4): 973-976.

Beno-Moualem D., Vinokur Y. and Prusky D. 2001. Cytokinins increase Epicatchin content and fungal decay resistance in Avocado fruits. *Journal of Plant Growth Regulation* 20:95-100

Borris R.P. 1996. Natural products research: Perspectives from a major pharmaceutical company. *Journal of Ethnopharmacology* 51:29-38

Boshoff W.H.P., Pretorius Z.A. and Swart W.J. 1998. Fusarium species in wheat grown from headblight infected seed. *South African Journal of Plant and Soil* 15(1):46-47.

Bretag T.W., Price .V. and Keane P.J. 1995. Importance of seed borne inoculum in the etiology of the ascochyta blight complex of field peas (*Pisum sativum* L.) grown in Victoria. *Australian Journal of Experimental Agriculture* 35: 525-530.

Brooks C.J.W. and Watson D.G. 1985. Phytoalexins. *Natural Product Reports*. 427-459.

Bruneton J. 1995. Pharmacognosy, Phytochemistry, Medicinal plants. Lavoisier Publishing Inc. New York. USA.

Cachon R., Catte M., Nomme R., Prevost H. and Divies C. 1995. Kinetic behaviour of *Lactococcus lactis* ssp *diacetylactis* immobilized in calcium alginate gel beads. *Processes in Biochemistry* 30(6):503-510.

Cannel R.J.P. 1998. How to approach the isolation of a natural product. In: Methods in Biotechnology Vol. 4: Natural Products Isolation. R.J.P. Cannel (ed.), Human Press Inc. Totowa. New Jersey.

Cao H. P. and Chen S. K. 1995. Brassinosteroid-induced rice lamina joint inclination and its relation to indole-3-acetic acid and ethylene. *Plant Growth Regulation* 16(2): 189-196.

Carling D.E., Meyer L. and Brainard K.A. 1996. Crater disease of wheat caused by Rhizoctonia solani AG-6. *Plant Disease* 80(12):1429.

Cassady J.M., Baird W.M. and Chang C. 1990. Natural products as a source of potential cancer chemotherapeutic and chemo-preventative agents. *Journal of Natural Products* 53: 23-41.

Chou C.H., Waller G.R., Cheng C.S., Yang C.F. and Kim D. 1995. Allelochemical activity of naturally occurring compounds from mungbean (*Vigna radiata* L.) plants and their surrounding soil. *Botanical bulletin of Academia Sinica* 36(1):9-18.

Clouse S. D. 1996. Molecular genetic studies confirm the role of brassinosteroids in plant growth and development. *Plant Journal* 10(1): 1-8.

Clulow S.A., Lewis B.G. and Matthews P. 1991. A Pathotype Classification for *Mycosphaerella pinodes*. *Journal of Phytopathology* 131: 322-332.

Coetzee, J., Mciteka, L., Malan, E and Ferreira, D. 2000. Structure and synthesis of the first procassinidin dimers based on epicatechin, and gallo- and epigallo-catechin. *Phytochemistry* 53(7): 795-804.

Daniels F. 1965. A simple microbial method for demonstrating phytotoxic compounds. *Journal of Investigative Dermatology* 44:259.

De Kock L. & Combrink J.C. 1994. Blossom-end rot caused by *Botrytis cinerea.* Acta Horticulturae. 368:667-672.

Del Rio J.A., Arcas M.C., Botia J.M. Baidez A.G., Fuster M.D.and Ortuno A.M. 2000. Involvement of phenolic compounds in the antifungal defence mechanisms of *Olea europaea* L. and *Citrus sp. Recent Research Developments in Agricultural and Food Chemistry.* 4(2):331-341.

Dey S.K. and Singh G. 1994. Seed borne infection of *Ascochyta rabei* in Chickpea and its transmission to aerial plant parts. *Phytoparasitica* 22: 31-37.

Duke S.O., Abbas H.K., Inderjit (ed.), Dakshini K.M.M. (ed.) and Einhellig F.A. 1995. Natural products with potential use as herbicides. Allelopathy: - organisms, -processes and –applications. American Chemical Society, Washington, USA. pp. 348-362.

Eksteen D., Pretorius J.C., Nieuwoudt T.D. and Zietsman P.C. 2001. Mycelial growth inhibition of plant pathogenic fungi by extracts of South African plant species. *Annals of Applied Biology* 139: 243-249.

El-Abyad M.S., Morsi N.M., Zaki D.A. and Shaaban M.T. 1990. Preliminary screening of some Egyptian weeds for antimicrobial activity. *Microbios* 62: 47-57.

El-Daly F.A. and Soliman M.H. 1998. Allelopathic potentiality of Lupinus seed extract on yield and chemical composition of soybean seeds. *Egyptian Journal of Physiological Sciences* 21(3):369-381.

Eloff J.N. 1998. Which extractant should be used for the screening and isolation of antimicrobial components from plants? *Journal of Ethnopharmacology* 60: 1-8.

Ferreira J.H.S., Matthee F.N. and Thomas A.C. 1989. Fungi associated with Dieback and pruning wounds of grapevines in South Africa. *South African Journal for Enology and Viticulture* 10(2):62-66.

Flores H.E., Hoy M.W. and Pickard J.J. 1987. Secondary metabolites from root cultures. *Trends in Biotechnology* 5:64-69.

Fourie J.F. and Holtz G. 1998. Effects of fruit and pollen exudates on growth of *Botrytis cinirea* and infection of plum and nectarine fruit. *Plant Disease* 82(2):165-170.

Fujioka S. and Sakurai A. 1997. Biosynthesis and metabolism of brassinosteroids. *Physiologia Plantarum* 100(3): 710-715.

Fukunaga, T., Nishiya, K., Kajikawa, I., Takeya, K and Itokawa, H. 1989. Studies on the constituents of Japanese mistletoes from different host trees and their antimicrobial and hypotensive properties. *Chemical and Pharmaceutical Bulletin* 37(6): 1543-1546.

Ganesan T. and Krishnaraju J. 1995. Antifungal properties of wild plants-II. *Advances in Plant Sciences* 8: 194-196.

Gorris L.G.M. and Smid E.J. 1995. Crop protection using natural antifungal compounds. *Pesticide Outlook* 6: 20-24.

Grayer R.J. 1989. *In*: Plant Phenolics; P.M. Day and J.B. Harborne (eds.), Academic Press: London. pp. 283-324.

Hagedorn D.J. 1984. The Compendium of Pea Diseases. *The American Phytopathology Society*, Minnesota, U.S.A.

Harborne J.B. 1991. Flavonoid pigments. In: Herbivores: Their Interactions with Secondary Plant Metabolites; G.A. Rosenthal and R. Berenbaum (eds.), Academic Press: San Diego, CA. 1: 389 – 429.

Harwood L.M and Moody C.J. 1989. Experimental organic chemistry. Principles and practice. Blackwell Scientific Publishers.

Hasal P., Vojtíšek V., Čejková A., Kleczek P. and Kofroňová O. 1992. An immobilized whole yeast cell biocatalyst for enzymatic sucrose hydrolysis. *Enzyme and Microbial Technology* 14(3):221-229.

Hedin P.A. and Waage S.K. 1986. Roles of flavonoids in plant resistance to insects. *In* Flavonoids and Medicine: Biochemical, Pharmacological and Structure-Activity Relationships. V. Cody, E. Middleton Jr. and J.B. Harborne (eds.), Liss, New York, pp. 87-100.

Heidhues F . 2001. In: Crop Science: Progress and Prospects. J. Nösberger, H.H. Geiger and P.C.Struik (eds.), Cromwell Press, Trowbridge, UK.

Herbert J.A. and Marx D. 1990. Short term control of Panama disease of bananas in South Africa. *Phytophylactica* 22:339-340.

Hostettmann K. and Wolfender J.K. 1997. The search for biologically active secondary metabolites. *Pesticide Science* 51:471-482.

http://www.biotechknowledge.monsanto.com/biotech/knowcentre.nsf

Hungria M., Joseph C.M. and Phillips D.A. 1991a. Anthocyanidins and flavonols, major *nod*-gene inducers from seeds of a black-seeded common bean (*Phaseolus vulgaris* L.). *Plant Physiology* 97: 751-758.

Hungria M., Joseph C.M. and Phillips D.A. 1991b. *Rhizobium nod*-gene inducers exuded naturally from roots of common bean (*Phaseolus vulgaris* L.). *Plant Physiology* 97: 759-764.

Hutchings A. 1996. Zulu Medicinal Plants, University of Natal Press, Pietermaritzburg.

Hutchings A. 1989. A survey and analysis of traditional medicinal plants as used by the Zulu, Xhosa and Sotho. *Bothalia 19(1):111-123.*

Iinuma, M., Tanaka, T., Mizuno, M., Yamamoto, H., Kobayashi, Y. and Tonemori, S. 1992. Phenolic constituents in *Erythrina x bidwilli* and their activity against oral microbial organisms. *Chemical and Pharmaceutical Bulletin* 40: 2749 – 2752.

Iinuma, M., Tsuchiya, H., Sato, M., Yokoyama, J., Ohyama, M., Ohkawa, Y., Tanaka, T., Fujiwara, S. and Fujii, T. 1994. Flavanones with potent antibacterial activity against methicillin-resistant *Staphylococcus aureus*. *Journal of Pharmacy and Pharmacology* 46: 892 – 895.

Irobi O.N. and Daramola S.O. 1993. Antifungal activities of crude extracts of *Mitracarpus villosus* (Rubiaceae). *Journal of Ethnopharmacology* 40: 137-140.

Ishikawa R., Fujimori K. and Matsuura K. 1996. Antibacterial activity of validamycin A against *Pseudomonas solanacearum* and its efficacy against tomato bacterial wilt. *Annals of the Phytopathological Society of Japan* 62(5): 478-482.

Iwashina, T. 2000. The structure and distribution of the flavonoids in plants. *Journal of Plant Research* 113: 287 – 299.

Jager A.K., Hutchings A. and Van Staden J. 1996. Screening of Zulu medicinal plants for prostaglandin-synthesis inhibitors. *Journal of Ethnopharmacology* 52(2):95-100.

Jansman A.J.M. 1993. Tannins in Faba beans (*Vicia faba* L.): Antinutritional properties in monogastric animals; Ph D thesis, Department of Animal nutrition, Wageningen Agricultural University, Netherlands. p.12-19.

Kavanagh F. 1975. Antibiotic assays: Principles and Precautions. *Methods in Enzymology* 43: 55-69.

Khushbaktova, Z.A.; Yusupova, S.M.; Zamaraeva, M.V.; Tadzhibaeva, E.T.; Syrov, V.N.; Batirov, E-K.; Yuldashev, M.P. 1996. *Chem. Nat. Comp.* 32(3): 338-343. (Translated from Khimiya Prirodnykh Soedinenii, 1996, 32 (3): 350-356).

Kinghorn K. and Balandrin G. 1993. In: ACS Symposium series 534; Washington DC: *American. Chemical Society.* p.639-700.

Kraft J.M., Dunne B., Goulden D. and Armstrong S. 1997. The search for resistance in peas to *Mycosphaerella pinodes* http://www.nal.usda.gov/ttic/tektran/data/000008/07/000008075.html

Krizek D. T. and Mandava N. B. 1983. Influence of spectral quality on the growth response of intact bean plants to brassinosteroid, a growth-promoting steroidal lactone. I. Stem elongation and morphogenesis. *Physiologia Plantarum* 57(3): 317-323.

Kuruyanagi M., Arakawa T., Hirayama Y. and Hayashi T. 1999. Antibacterial and antiandrogen flavonoids from *Sophora flavescens*. *Journal of Natural Products* 62(12): 1594-1599.

Lamprecht S.C., Marasas W.F.O., Knox-Davies P.S. and Calitz F.J. 1990. Incidence of Fusarium species in different cropping systems of annual Medicago species and wheat. *Phytophylactica* 22: 69-76.

Lawyer A.S. 1984. Disease caused by *Ascochyta* spp. In: The Compendium of Pea Diseases. D J Hagendorn (ed.), American Phytopathology Society, St. Paul, Minnesota, U.S.A. p. 11-15.

Lemaga B., Kanzikwera R., Kakuhenzire R., Hakiza J.J. and Manzi G. 2001. The effect of crop rotation on bacterial wilt incidence and potato tuber yield. *Fifth Triennial Congress of the African Potato Association, 28 May-2 June 2000, Kampala, Uganda. African-Crop-Science-Journal*. 9(1): 257-266.

Li H.H., Nishimura H., Hasegawa K. and Mizutani J. 1993. Some physiological effects and the possible mechanism of action of juglone in plants. *Weed Researsh Tokyo* 38(3):214-222.

Li X.C., ElSohly H.N., Nimrod A.C. and Clark A.M. 1999. Antifungal activity of (-)-epigallocatechin from *Coccoloba dugandiana* against *Cryptococcus neoformans*. *Planta Medica* 65(8): 780.

Lin, Y-M., Anderson, H., Flavin, M.Y. and Pai, Y-H. S. 1997. *In vitro* anti-HIV activity of biflavonoids isolated from *Rhus succedanea* and *Garcinia multiflora*. *Journal of Natural Products* 60: 884 – 888.

Lovang U. and Wildt-Persson T. 1998. Botanical pesticides. The effect of aqueous extracts of *Melia azedarach* and *Trichilia emetica* on selected pathogens of tomato, bean and maize. Minor Field Studies International Office, Swedish University of Agricultural Sciences, No. 52. Pp. 23.

Luckner M. 1990. Secondary metabolism in plants and animals. Springer-Verlag, Berlin.

Lydon J. and Duke S.O. 1989. The potential of pesticides from plants. In: Herbs, -spices and medicinal plants: Recent Advances in Botany. Craker L.E. and Simon J.E. (eds.), *Horticulture and Pharmacology* 4:1-41.

Marimuthu T and Kandaswamy T.K. 1980. Bacterial leaf blight of green gram (*Vigna radiata* var. *aureus*) caused by *Xanthomonas phaseoli* and reaction of certain varieties to the disease. *Madras Agricultural Journal* 67(6) 413.

Markham K.R. 1989. *In*: Methods in Plant Biochemistry; P.M. Dey and J.B. Harborne (eds.), Academic Press: London. Vol. I, pp. 197-235.

Markham K.R. 1982. Techniques of Flavonoid Identification, Academic Press: London. p.82.

Martini N. and Elof J.N. 1998. The preliminary isolation of several antibacterial compounds from *Combretum erythrophyllum* (Combretaceae). *Journal of Ethnopharmacology* 62:225-263.

Masika P.J., Sondani A., Van Awerberke W. and Van Awerberke W. 1997. Preceived causes, diagnosis and treatment of babeosis and anaplasmosis in cattle by livestock farmers in communal areas of the central Eastern Cape Province, South Africa. *Journal of the South African Veternary Association* 689(2):40-44.

Mason R. L., Gunst R.F. and Hess J.L. 1989. Statistical design and analysis of experiments - with applications to engineering and science. John Wiley and Sons, Inc. Canada. Pp. 692.

Mathekga A.D.M. and Meyer J.J.M. 1998. Antibacterial activity of South African *Helichrysum* species. *South African Jounal of Botany* 64(5):293-295.

Mathiesen, Malterud, K.E. and Sund, R.B. 1995. Antioxidant activity of fruit exudate and C-methylated dihydrochalcones from *Myrica gale*. *Planta Medica* 61(6): 515-518.

Matsuda H., Yano M., Kubo M., Iinuma, M., Oyama M. and Mizuno M. 1991. *Journal of the Pharmaceutical Society of Japan* 111: 193–198.

Mauseth J.D. 1991. Botany: An introduction to plant biology. Saunders College Publishing. Chile.

Meudt W. J (ed). 1983. Strategies of plant reproduction. Allanheld, Osmun and Co. Publisher, Inc.; Towota; USA. pp. 386.

Meyer J.J.M. and Afolayan A.J. 1995. Antibacterial activity of *Helichrysum aureonitens* (Asteraceae). *Journal of Ethnopharmacology* 47: 109-111.

Meyer J.C., Van wyk R.J. and Phillips A.J.L. 1990. Rhizoctonia leaf spot of tobacco in South Africa. *Plant Pathology* 39:206-207.

Monforte M.T., Trovato A., Kirjavainen S., Forestieri A.M., Galatai E.M. and Lo Curto R.B. 1995. Biological effects of hesperidin, a citrus flavonoid, (note II): hypolipidemic activity on experimental hypercholesterolemia in rat. *Farmaco* 50: 595 – 599.

Moussart A., Tivoli B., Lemarchand E., Deneufbourg F., Roi S. and Sicard G. 1998. Role of seed infection by the Ascochyta blight pathogen of dried pea (*Mycosphaerella pinodes*) in seedling emergence, early disease development and transmission of the disease to aerial plant parts. *European Journal of Plant Pathology* 104: 93-102.

Mulligan,M.E., Murray-Leisure, K.A., Ribner, B.S., Standiford, H.C., John, J.F., Korvick, J.A., Kauffman, C.A. and Yu, V.L. 1993. Methicillin-resistant *Staphylococcus aureus*: a consensus review of the microbiology, pathegenesis and epidemiology with implications for prevention and management. *American Journal of Medicine* 94: 313 – 328.

Naqvi S.A.H., Khan M.S.Y. and Vohora S.B. 1991. Anti-bacterial, anti-fungal and anthelmintic investigations on Indian medicinal plants. *Filoterapia* Vol. LXII(2):221-228

Nasir M. and Hoppe H.H. 1997. Evaluation of varietal susceptibility in peas to *Mycosphaerella pinodes* . *Annals of Applied Biology* 18, 32-33.

Neave I.A. and Dawson J.O. 1989. Juglone reduces growth, nitrogenase activity and root respiration of antinorhizal black alder seedlings. *Journal of Chemical Ecology* 15(6):1823-1836.

Nelson R. 2001. In: Crop Science: Progress and Prospects. J. Nösberger, H.H. Geiger and P.C.Struik (eds.), Cromwell Press, Trowbridge, UK.

Nigg H.N. and Seigler D. 1992. Phytochemical Resources for Medicine and Agriculture. Plenum Press. New York.

Nijveldt, R.J., van Nood, E., van Hoorn, D.E.C., Boelens, P.G., van Norren, K. and van Leeuwen, P.A.M. 2001. Flavonoids: a review of probable mechanisms of action and potential applications. *American Journal of Clinical Nutrition* 74: 418 – 425.

Ohlander L.J.R. 1980. Research on harricot bean *Phaseolus vulgaris* L. production in Ethiopia 1972-1976. *Rapport,-Institutionen-for-Vaxtodling*. 82, 294.

Opperman L. and Wehner F.C. 1993. Survey of fungi associated with grass roots in virgin soils on the springbok flats. *South African Journal of Botany* 60(1):67-72.

Ouelette R.J. 1992. Inroduction to general, Organic and Biological Chemistry. Third edition. Macmillan Publishing Company. New York.

Padhy B., Patnaik P.K. and Tripathy A.K. 2000. Allelopathic potential of *Eucalyptus* leaf litter leaches on germination and seedling growth of finger millet. *Allelopathy Journal* 7(1):69-78.

Penning de Vries F.T.W. 2001. In: Crop Science: Progress and Prospects. J. Nösberger, H.H. Geiger and P.C.Struik (eds.), Cromwell Press, Trowbridge, UK.

Pfaller M.A., DuPont B., Kobayashi G.S., Muller J., Rinaldi M.G., Espinell-Ingroff A., Shadomy S., Troke P.F., Walsh T.J. and Warnock D.W. 1992. Standardized susceptibility testing of flucanozole: an international collaborative study. *Antimicrobial agents and Chemotherapy* 36: 1805-1809.

Picman A.K., Shneider E.F. and Picman J. 1995. Effect of flavonoids on mycelial growth of *Verticillium alboatrum*. *Biochemical Systematics and Ecology* 23(7-8): 683-693.

Poswal M.A.T., Masunga G., Javaid I. and Kwerepe B C. 1993. Potential of different toxic and medicinal plant extracts for the control of fungal plant pathogens in Botswana. *Med. Fac. Landbouww*. University of Gent. 58:3b, 1373-1381.

Putnam A.R. and Tang C. 1986. The science of alleopathy. John Wiley and Sons, Inc. New York.

Qasem J.R. and Abu-Blan H.A. 1996. Fungicidal activity of some common weed extracts against different plant pathogenic fungi. *Journal of Phytopathology* 144:157-161.

Rabe T. and Van Staden J. 1997. Antibacterial activity of South African plants used for medicinal purposes. *Journal of Ethnopharmacology* 56(1):81-87.

Ragasa C.Y., Cruz C.A., Chiong I.D., Tada M. and Rideout J.A. 1997. Antifungal flavonoids from *Waltheria americana. Philipine Journal of Science* 126(3): 243-250.

Ramamoorthy M. and Paliwal K. 1993. Alleopathic compounds in leaves of *Gliricidia sepium* (Jacq.) kunth ex Walp. and its effect on *Sorgum vulgare* L. *Journal of Chemical Ecology*. 19(8):1691-1701.

Rawat M.S.M., Pant, G., Prasad D., Joshi, R.K., Pande G.B., Pant G. and Prasad D. 1998. Plant growth inhibitors (proantocyanidins) from *Prunis armeniaca*. *Biochemical Systematics and Ecology* 26(1):13-23.

Recio M.C., Rios J.L. and Villar A. 1989. A review of some antimicrobial compounds isolated from medicinal plants reported in the literature 1978-1988. *Phytotherapy Research* 3: 117-125.

Reinhardt C.F., Meissner R. and Labuscagne N. 1994. Allelopathic interaction between *Chenopodium album* L. and certain crop species. *South African Journal of Plant and Soil*. 11(1):45-49.

Reynaldo I., Fuster M.D., Botia J.M., Garcia-Phig D., Ortuno a. and Del Rio J.A. 1999. Flavonoids found in several citrus species cultivated in Cuba and Spain for their industrial application. *Cultivos Tropicales*. 20(3): 73-75.

Rheeder J.P. and Marasas W.F.O. 1998. *Fusarium* species from plant debris associated with soils from maize production areas in the Transkei region of South Africa. *Mycopathologia* 143:113-119.

Rice E.L. 1974. Allelopathy. Academic Press. New York.

Rios J.L., Recio M.C. and Villar A. 1988. Screening Methods for natural products with antimicrobial activity: a review of the literature. *Journal of Ethnopharmacology* 23:127-149.

Rizvi S.J.H. and Rizvi V. 1992. Allelopathy: basic and applied aspects. Chapman and Hall. London.

Robards, K.; Antolovich, M. 1997. Analytical Chemistry of Fruit Bioflavonoids: A Review. *Analyst* 122: 11–34.

Roger C. and Tivoli B. 1996. Effect of culture medium, light and temperature on sexual and asexual reproduction of four strains of *Mycosphaerella pinodes*. *Mycological Research* 100 (3): 304-306.

Roger C., Tivoli B. and Huber L. 1999. Effects of temperature and moisture on disease and fruit body development of *Mycosphaerella pinodes* on pea (*Pisum sativum*). *Plant Pathology* 48: 1-9.

Roth U., Friebe A. and Schnabl H. 2000. Resistance induction in plants by a Brassinosteroid-containing extract of *Lychnis viscaria* L. *Zeitschrift für Naturforscung* 54:131-138.

Rugerri B., Sassi G., Specchia V., Bosco F. and Marzona M. 1991. Alginate beads coated with polyacylamide resin: potential as a biocatalyst. *Process Biochemistry* 26(6):331-335.

Ryan E.W., Staunton W.P. and Cassidy J.C. 1984. Disease of Vegetables, Research Reports 1983. Horticulture. Dublin, Irish Republic. *Ann Foras Taluntais*, 41-42.

Sakurai A. and Fujioka S. 1997. Studies on biosynthesis of brassinosteroids. *Bioscience, Biotechnology and Biochemistry* 61(5): 757-762.

Salie F., Eagles P.F.K. and Leng H.M.J. 1996. Preliminary antimicrobial screening of four South African Asteraceae species. *Journal of Ethnopharmacology.* 52:27-33.

Salisbury F.B. and Ross C.W. 1992. Plant Physiology. Wadsworth Publishing Company. Belmont. California.

Saucedo J.E.N., Barbotin J.N. and Thomas D. 1989. Continuous production of gibberellic acid in a fixed-bed reactor by immobilized mycelia of G*ibberella fujikuroi* in calcium alganate beads. *Applied Microbiology and Biotechnology* 30:226-233.

Schmidt J., Altmann T. and Adam G. 1997. Brassinosteroids from seeds of Arabidopsis thaliana. *Phytochemistry* 45(7): 1325-1327.

Schnabl H., Roth U and Friebe A. 2001. Brassinosteroid-induced stress tolerances of plants. *Recent Research Developments in Phytochemistry* 5: 169-183.

Scott D.B. 1990. Wheat diseases in South Africa. Department of Agricultural Development. Pretoria.

Seigler D.S. 1995. Plant Secondary Metabolism, Kluwer Academic Publishers: Boston. pp.151-185.

Serfontein S. and Knox-Davies P.S. 1990. A flower-head blight of *Leucadendron discolor* caused by *Botrytis cineria*. *Phytophylactica* 22:119-120.

Shirwaikar, A., Kumar, A.V., Krishnanand, B.R., Sreenivasan, K.K. and Vasanth-Kumar, A. 1995. Chemical investigation and antihepatotoxic activity of *Thespesia populnea*. *International Journal of Pharmacognosy* 33(4): 305-310.

Sidorovich L.E. 1986. Antibacterial activity of some preparations on causal agents of bacterial diseases of ornamentals. *Mikrobiologicheskii Zhurnal* 48(3): 75-80; 2 tab.; 6 ref.

Simmonds M.S.J., Evans H.C. and Blaney. 1992. Pesticides for the year 2000: Mycochemicals and Botanicals. In: Pest Management and the Environment in 2000. W.M. Kadir and H.S. Barlow (eds.), CAB International, Wallingford, UK. Pp. 127-164.

Sinden S.L., Kobayashi R.S., Nordeen R.O. and Owens L.D. 1993. Perspectives on controlling potato soft rot with foreign genes for antibacterial compounds. *Acta Horticulturae* 336, 79-84.

Smith G.F., Chesselet P., Van Jaarsveld E.J., Hartmann H., Hammer S., Van Wyk B., Burgoyne P., Klak C. and Kurzweil H. 1998. Mesembs of the world. Briza Publications.

Smith H., Wingfield M.J., Crous P.W. and Coutinho T.A. 1996. *Sphaeropsis sapinea* and *Botryosphaeria dothidia* endopathic in *Pinus* spp. and *Eucalyptis* spp. in South Africa. *South African Journal of Botany* 62(2):86-88.

Srivastava S.K., Srivastava, S.D. and Chouksey B.K. 2001. New antifungal constituents from *Terminalia alata. Fitoterapia* 72:106-112.

Stafford, H.A. 1991. Flavonoid evolution: An enzymic approach. *Plant Physiology* 96: 680-685.

Steele R.G and Torrie J.H. 1980. Principles and Procedures of Statistics. 2nd ed. McGraw-Hill, New York.

Swaminathan M.S. 1993. Perspectives for crop protection in sustainable agriculture. In: Crop Protection and Sustainable Agriculture. Ciba Foundation Symposium 177. Wiley, Chichester, p. 252-272.

Swanepoel A. and De Kock H. 1996. Progress with *Verticillium* wilt of cotton in the Republic of South Africa. *Proceedings of the Beltwide Cotton Conference* 1:273-275.

Swart W.J. and Blogett J.T. 1998. First report of *Botryosphaeria dothidia* basal canker of Pistacio trees in South Africa. *Plant disease* 82(8):960.

Szajda, M. and Kedzia, B. 1991. New *N*-substituted derivatives of 3-azachalcone of potential antimicrobial activity. *Die Pharmazie* 46: 745 – 746.

Thomas T.V., Eicker A. and Robbertse P.J. 1994. Possible role of fungi in negatively affecting fruitset in avocados. *South African Journal of Botany* 60(5):251-256.

Toda, M., Okubo, S., Hara, Y. and Shimamura, T. 1991. Antibacterial and bactericidal activities of tea extracts and catechins against methicillin resistant *Staphylococcus aureus. Japanese Journal of Bacteriology* 46: 839-845.

Towers G.H.N. 1984. Interaction of light with phytochemicals in some natural systems. *Canadian Journal of Botany* 62:2900-2911.

Trench T.N., Wilkinson D.J. and Esterhuysen S.P. 1992. South African Plant Disease Control Handbook. University of Natal, Pietermaritzburg.

Tsai, S.M. and Phillips, D.A. 1991. Flavonoids released naturally from alfalfa promote development of symbiotic *Glomus* spores *in vitro*. *Applied and Environmental Microbiology* 57: 1485-1488.

Tsuchiya H., Sato M., Miyazaki T., Fujiwara S., Tanigaki S., Ohyama M., Tanaka T. and Iinuma M. 1996. Comparitive study on the antibacterial activity of phytochemical flavones against methicillin-resistant *Staphylococcus aureus*. *Journal of Ethnopharmacolog*. 50(1): 27-34.

Ushiki J., Hayakawa Y. and Tadano T. 1996. Medicinal plants for suppressing soilborne plant diseases. I. Screening for medicinal plants with antimicrobial activity in roots. *Soil Science and Plant Nutrition* 42(2):423-426.

Utsui, Y. and Yokota, T. 1985. Role of an altered penicillin-binding protein in methicillin and cephem-resistant *Staphylococcus aureus*. *Antimicrobial Agents and Chemotherapy* 28: 397 – 403.

Uys M.D.R., Thompson A.H. and Holz G. 1996. Diseases associated with tomato in the main tomato-growing regions of South Africa. *Journal of the South African Society of Horticultural Science* 6(2):78-81.

Van der Watt, E and Pretorius, J.C. 2001. Purification and identification of active antibacterial components in *Carpobrotus edulis* L. *Journal of Ethnopharmacology* 76(1): 87-91.

Van der Watt E. 1999. ‘n Ondersoek na die antibakteriese eienskappe van *Carpobrotus edulis* L. *Thesis: Magister Scientae.* University of the Free State, South Africa.

Van Wyk B-E., van Oudtshoorn B. and Gericke N. 1997. Medicinal plants of South Africa. Briza Publications. South Africa. p. 32.

Van den Berghe D.A. and Vlietinck A.J. 1991. Screening methods for antibacterial and antiviral agents from higher plants. In: Methods in Plant Biochemistry, Hostettman K. (ed.), Vol 6. Assays for Bioactivity. Academic Press, London.

Vanneste J.L., Cornish A., Yu J., Voyle M.D. and Verhoyen M.N.J. 1998. A microcin produced by a strain of *Erwinia herbicola* is involved in biological control of fire blight and soft rot caused

by *Erwinia* sp. XXV International Congress, Part 3: Culture techniques with special emphasis on environmental implications. *Acta Horticulturae* 513: 39-46

Venter S.L. and Steyn P.J. 1998. Correlation between fusaric acid production and virulence of isolates of *Fusarium oxysporum* that causes potato dry rot in South Africa. *Potato Research* 41:289-294.

Villar a., Rios J.L, Recio M.C., Cortes D. and Cave A. 1986. Antimicrobial activity of bezylisquinoline alkaloids. II. Relation between chemical composition and antimicrobial activity. *Planta Medica* 52:556-557.

Vlahov G. 1992. Flavonoids in three olive (*Olea europea*) fruit varieties during maturation. *Journal of the Science of Food and Agriculture.* 8: 157-159.

Vlietinck A.J., Vanden Berghe D.A., Van Hoof L.M., Vrijsen R. and Boeyè, A. 1986. In: Plant Flavonoids in Biology and Medicine: Biochemical, Pharmacological and Structure-Activity Relationships; V. Cody; E. Middleton Jr. and J.B. Harborne (eds.),Liss: New York. pp.537-540.

Vogel G. 1982. In: Proceedings of the International Bioflavonoid Symposium, Munich, F.R.G.; L.Farkas; M. Gàbor; F. Kàllay; H. Wagner (eds.), Elsevier: Amsterdam. pp.461-474.

Wächter G.A., Hoffman J.J., Furbacher T., Blake M.E. and Timmerman B.N. 1999. Antibacterial and antifungal flavones from *Eysenhardtia texana. Phytochemistry* 52:1469-1471.

Wagner H. and Bladt S. 1996. Plant drug analysis. A Thin Layer Chromatography Atlas. Springer. New York.

Waller G.R. 1989. Biochemical frontiers of allelopathy. *Biologica Plantarum* 31(6):418-447.

Watt J.M. and Breyer-Brandwijk M G. 1963. Medicinal and poisonous plants of Southern and Eastern Africa. E and S Livingstone Ltd. London.

Wiley G. and Chichester H. 1994. Plant drug analysis. A thin layer chromatography atlas. Springer. New York.

Winson M.K. and Kell D.B. 1997. If you've got it flaunt it – rapid screening for microbial biocatalysts. *Trends in Biotechnology* 15(4):120-122.

Wolf R.B., Spencer G. and Kwolek W.F. 1984. Inhibition of Velvetleaf (*Abutilon theophrasti*) germination and growth by benzyl isothiocyanate, a natural toxicant. *Weed Science* 32:612-615.

Wollenweber E. and Jay M. 1988. In The Flavonoids: Advances in Research since 1980; J.B. Harborne (ed.), Chapman and Hall: London. pp. 233-302.

Wood J.E., Munro M.G.H., Blunt J.W., Perry N.B., Walker J.R.L. and Ward J.M. 1999. Biologically active compounds from *Ozothamus leptophyllus. New Zealand Journal of Botany.* 37(1): 167-174.

Wroth J.M. 1998. Variation in pathogenicity among and within *Mycosphaerella pinodes* populations collected from field pea in Australia. *Canadian Journal of Botany* 76:11, 1955-1966.

Yatagi M. and Ding Y. 1996. Amounts of extractives from *Pinus massonania* their chemical compositions, miticidal activities and growth regulation effects on radish seeds. *Journal of the Japan Wood Research Society* 42(12):1221-1227.

Yoshikawa, M., Shimada, H., Nishida, N., Li, Y-H., Toguchida, I., Yamahara, J. and Matsuda, H. 1998. Antidiabetic principles of natural medicines. II. Aldose reductase and alpha-glucosidase inhibitors from Brazilian natural medicine, the leaves of *Myrcia multiflora* DC. (Myrtaceae): Structures of myrciacitrins I and II and myrciaphenones A and B. *Chemical and Pharmaceutical Bulletin* 46(1): 113-119.

Zhang Q. 1997. Effects of soil extracts from repeated plantation woodland of Chinese-fir on microbial activities and soil nitrogen mineralization dynamics. *Plant and Soil* 191:205-212.