BIOSTIMULATORY AND ANTIMICROBIAL PROPERTIES OF *Tulbaghia violacea* (Harv.) EXTRACTS

BY

Leeto Nteso

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Bloemfontein
South Africa

Promoter: Prof. J.C. Pretorius (Ph.D.)
Co- Promoter: Dr. G.P. Potgieter (Ph.D.)

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DEDICATION

This dissertation is dedicated to my loving wife ‘Makhang ‘Mareitumetse Nteso for the lonely four years that she sacrificed during this research, looking after the family with no financial support, and to my four daughters: Reitumetse, Nthabiseng, Palesa and Lineo. I have set a pace for you girls, aim higher than this.

DECLARATION

I declare that the dissertation submitted by me for the degree Doctor of Philosophy at the University of the Free State, South Africa is my own independent work and has not previously been submitted by me to another University. I furthermore concede copyright of the dissertation in favour of the University of the Free State.

Signed in Bloemfontein, South Africa.

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Leeto Nteso

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CHAPTER 1

INTRODUCTION AND RATIONALE FOR THE STUDY

Plant diseases cause large yield losses throughout the world and all important food crops are attacked with disastrous consequences for food security. In many cases, plant diseases may be successfully controlled with synthetic fungicides, but this is costly to African peasantry and often has disadvantages and side effects on the ecosystem (Steward and Krikorian, 1971; De Neergaard, 2001). It is, however, an established fact that the use of synthetic chemical pesticides provides many benefits to crop producers (Hannaway, 2001). These benefits include higher crop yields, improved crop quality and increased food production for an ever increasing world population. Despite the latter, synthetic pesticides may pose some hazards to the environment, especially when improperly used by farmers in developing countries who lack the technical skill of handling them, and who fail to adopt to this technology easily (Steward and Krikorian, 1971). This may result in undesirable residues left in food, water and the environment, toxicity to humans and animals, contamination of soils and groundwater and may lead to the development of crop pest populations that are resistant to treatment with agrochemicals (Hannaway, 2001). Especially sulphur and copper containing synthetic fungicides as well as the Bordeaux mixture are toxic to mammals, wildlife and many beneficial insects (Marr *et al.,*1997).

In Africa and the Near East, obsolete pesticides have become a source of great environmental concern. Some stocks are over 30 years old and are kept in poor conditions because of inadequate storage facilities and lack of staff trained in storage management. The Food Agricultural Organization (FAO) estimated that developing countries are holding stocks of more than 100 000 tonnes of obsolete pesticides, of which 20 000 tonnes are in Africa. Many of these chemicals are so toxic that a few grams could poison thousands of people or contaminate a large area. Most of these

pesticides were left over from pesticide donations provided by foreign aid programmes. In the absence of environmentally sound disposal facilities, stocks are constantly increasing (Alemayehu, 1996).

Obsolete pesticide stocks are potential time bombs. Leakage, seepage and various accidents related to pesticides are quite common and widespread. Storage conditions rarely meet internationally accepted standards. Many pesticide containers deteriorate and leak their contents into the soil, contaminating groundwater and the environment. Most stores are in the centres of urban areas or close to public dwellings. According to the World Health Organization (WHO) there are 25 million cases of acute occupational pesticide poisoning in developing countries each year (Alemayehu, 1996).

As a result of the problems outlined above, farmers in developing countries and researchers alike are seeking less hazardous and cheaper alternatives to conventional synthetic pesticides (Marr *et al.,* 1997). One such alternative, that prompted this study, is the use of natural products from plants to control plant diseases in crops as part of an organic approach to Integrated Pest Management (IPM) programmes.

Justification for pursuing this alternative was found in a statement published by the Environmental Protection Agency (EPA) regarding the advantages of natural products from plants in the control of plant diseases (Deer, 1999):

"Natural products from plants have a narrow target range and highly-specific mode of action; show limited field persistence; have a shorter shelf life and present no residual threats. They are often used as part of Integrated Pest Management (IPM) programmes; are generally safer to humans and the environment than conventional synthetic chemical pesticides and can easily be adopted by farmers in developing countries who traditionally use plant extracts for the treatment of human diseases."

A further rationale for exploring the use of plant extracts or natural products as biological pesticides more extensively can be found in the plant itself. Plants have evolved highly specific chemical compounds that provide defence mechanisms against attack by disease causing organisms, including fungal attack, microbial invasion and viral infection (Cowan, 1999). These bioactive substances occur in plants as secondary metabolites, and have provided a rich source of biologically active compounds that may be used as novel crop-protecting agents (Cox, 1990). In nature some plants have the potential to survive very harsh environmental conditions (biotic and abiotic). This has initiated the postulate that such plants might be utilized as sources for the development of natural products to be applied in agriculture by man as natural herbicides, bactericides, fungicides or products with bio-stimulatory properties in crude or semi-purified form.

As in pharmacology, biochemicals isolated from higher plants may contribute to the development of natural fungicides or bactericides for the agricultural industry in three different ways (Cox, 1990): (1) by acting as natural antimicrobial pesticides in an unmodified state (crude extracts), (2) by providing the chemical 'building blocks' necessary to synthesize more complex compounds and (3) by introducing new modes of pesticidal action that may allow the complete synthesis of novel analogues, to counter the problem of resistance to currently used synthetic products by bacterial and fungal pathogens.

It is quite common that a natural compound isolated from a plant may be of great biological interest but may not be sufficiently robust for use (Steglich *et al.*, 1990). Subsequently, a need for the modification of natural products into synthetic analogues, that will give the desired effect, may still exist. One can, for example, isolate a natural compound with promising antimicrobial activity and, by introducing a stable chemical structure with higher activity synthetically, develop a commercial product (Steglich *et al.*, 1990). The alternative that is now more vigorously being pursued in organic farming systems is the application of the plant material itself in a

natural form. However, to date the use of natural plant extracts as pesticides to control pathogens in crops are not widespread while synthetic chemical pesticides still remain the major tool in pest management systems. It is, nevertheless, expected that some biological pesticides will be commercialized in future and that the use of a combination of synthetic chemicals and natural products in IPM programmes will become more popular (Fugimori, 1999).

Another related area of organic farming systems is the potential to apply natural plant extracts as either plant growth regulators or natural herbicides. A plant growth regulator is an organic compound, either natural or synthetic, that modifies or controls one or more specific physiological processes within a plant (Lemaux, 1999). If the compound is produced within the plant it is called a plant hormone e.g. auxins, gibberellins, cytokinins, abscisic acid and ethylene. A plant growth regulator is also defined by the EPA as any substance or mixture of substances that accelerates or retards the rate of growth or maturation, or otherwise alters the behaviour of plants or their produce through physiological action (Lemaux, 1999).

Many natural plant compounds have been identified that affect the growth and development of plants. These include compounds such as amino acids, caffeine, fatty acids, flavonoids, lactones, quinones, steroids and various sulphur compounds (constituents of garlics) (Roberts and Hooley, 1988). The potential therefore exists to apply a plant extract as a foliar spray in order to stimulate growth in crop plants and hence increase yields. A principal objective of the agricultural and horticultural industries (Roberts and Hooley, 1988) is to manipulate plant growth and development in such a way that the quantity or quality of a crop is increased. An elevated interest therefore exists to identify natural plant compounds with the ability to manipulate plant growth and development over a short period, e.g. a growing season. An additional consideration is that plants whose extracts, for example show antimicrobial and/or bio-stimulatory properties, could be cultivated as alternative agricultural crops for serving as sources of active compounds in the production of natural pesticides or plant growth regulators.

In this study the antimicrobial (antifungal and antibacterial) as well as bio-stimulatory properties of *Tulbaghia violacea* (wild garlic) extracts were explored. Research carried out with crude and semi purified extracts of several species of *Allium sativum* (garlic) and plants of the *Alliaceae* family, that is plants related to *Tulbaghia violacea,* has revealed their antimicrobial potential (Rimands, 1987; Garcia and Garcia, 1988; Mosch and Zeller, 1989; Tariq and Magee, 1990; Challem, 1996; Kim *et al.*, 2001; Mehrabian and Larry, 2001). Although most researchers have concentrated their studies in the medical and health sciences in the past, it has become important to explore the application potential of plant extracts in the agricultural sciences more extensively, with the emphasis on plant protection. The latter is especially, but not exclusively, important in an African context where financial constraints call for the development of cheaper analogues.

In summary the objectives of this study were to:

- determine the *in vitro* antibacterial and antifungal properties of crude organ extracts of *Tulbaghia violacea* (Chapter 3),
- determine the *in vitro* bio-stimulatory properties of crude organ extracts of *T. violacea* under laboratory conditions (Chapter 3),
- evaluate the *in vivo* antifungal activity of the crude organ extracts under greenhouse and field conditions (Chapter 4 and 5),
- isolate, purify and identify the active chemical compound(s) responsible for the above properties of *T. violacea* (Chapters 6).

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CHAPTER 2
LITERATURE REVIEW

2.1 Introduction

It is estimated that there are more than 250 000 higher plant species on earth (Cox, 1990; Cowan, 1999) offering a vast, virtually untapped, reservoir of bioactive chemical compounds with many potential uses, including their application as pharmaceuticals and agrochemicals. It is generally assumed that natural compounds from plants pose less risk to animals and humans and are more environmentally friendly than their synthetic counterparts (Johnson, 2001). However, besides the potential of exploiting compounds from plants as natural products, plants also contain bioactive compounds that play important roles in natural defence mechanisms. These have developed during evolution to protect plants against herbivorous mammals as well as insects and can therefore be poisonous to the environment.

Research in the fields of pharmacology and plant pathology has revealed antimicrobial activity in extracts of many plants. Many organizations and institutions in different countries in the world are currently concentrating on natural plant product research. For example, the aim of the Agrochemical Discovery and Development Program of the National Centre for Natural Products Research in the USA is to identify lead compounds for the development of environmentally benign and toxicologically safe pest management agents (Borris, 1996). This programme is done in collaboration with scientists in the Natural Products Utilization Research unit of the United States Development Agency (USDA) agricultural research service. Emphasis is on the discovery and development of compounds that are useful in the control of pests affecting small niche crops. The research centre is devoted to improving agricultural productivity through the discovery, development and commercialization of agrochemicals derived from natural plant compounds (Johnson, 2001).

According to the Natural Antifungal Crop Protectants research agency (Hall, 2002) spoilage and plant pathogenic fungi are responsible for some 20% loss of the potential global plant production for food and non-food use. The very large amount of chemical crop protectants used to control these losses can be detrimental to both the environment and human health. Therefore research has been initiated to develop and implement non-synthetic crop protectants using natural antifungal agents (green chemicals) or antifungal metabolites from plants. These natural crop protectants will be designed for use on food or non-food crops vulnerable to fungal deterioration (Hall, 2002). From an agronomic perspective, a secondary aim of research on natural plant products is to cultivate bioactive plants, as alternative agricultural crops, to serve as sources for the bioactive compounds.

Meanwhile some South African plants are currently being screened at the University of the Free State, South Africa (Pretorius *et al.,* 2002a) for antibacterial, antifungal, herbicidal and bio-stimulatory activities. Preliminary results have revealed bio-stimulatory activity in some crude extracts and, when applied under green house and field conditions, increased the level of resistance to pathogens in these crops. Additionally, yield increases were observed in these crops. Within the same screening programme certain plants with significant antimicrobial activities have been identified during preliminary laboratory screening procedures. *Tulbaghia violacea* was one of these identified South African plant species and hence this extensive study on the plant was undertaken.

2.2 Biology of *Tulbaghia violacea*

2.2.1 Introduction

Tulbaghia violacea is known as wild garlic in English, ‘wilde knoffel’ in Afrikaans and ‘isihaqa’ in Zulu and is one of 24 species of *Tulbaghia* found in South Africa (Eliovson, 1973). Americans refer to it as society garlic and the name stems from the fact that, although its taste is close to that of real garlic, it is supposed not to leave behind

embarrassing bad-breath odours. Thus, one could cook with this plant; then venture out in polite society without the bad odours being detected (Neal, 1998; Faucon, 2000). The name *Tulbaghia* was coined (Burbidge, 1978) by Linnaeus in 1771 as a tribute to Van Tulbagh who was at that time governor of the Cape colony and who was responsible for sending much South African plant material to Europe for identification. The species name "*violacea*" comes from the Latin for "violet" (Faucon, 2000). It belongs to the family *Alliaceae* (*Lilliaceae*).

2.2.2 Country of origin and distribution

Tulbaghia violacea originated in South Africa and grows in the Eastern Cape, Natal, Northern Transvaal and as far north as Zimbabwe (Eliovson, 1973). It is grown as an ornamental in botanical gardens and in home gardens all over southern Africa and it is cultivated in some overseas countries such as the USA and the UK (Burbidge, 1978).

2.2.3 Uses

According to Bannoche Gardens (2001), *Tulbaghia violacea* has been used in foods as a garlic replacement. The Zulu people eat the green parts of the plant as peppery spinach and the bulb is used as an emetic as well as a love potion. *Tulbaghia violacea* is also used for the treatment of infant and mother in the case of a depressed fontanelle, as a remedy for pulmonary tuberculosis and as an antihelminthic. The freshly harvested bulbs are boiled in water and the decoctions are either taken orally or as an enema for stomach problems. The Zulu people also regard this species as a snake repellent (Eliovson, 1973; Burton, 1990). Wild garlic is traditionally (Van Wyk *et al.,* 1997) used for fever and colds, but also for asthma and tuberculosis. The leaves are used to treat cancer of the oesophagus.

2.2.4 Botanical description

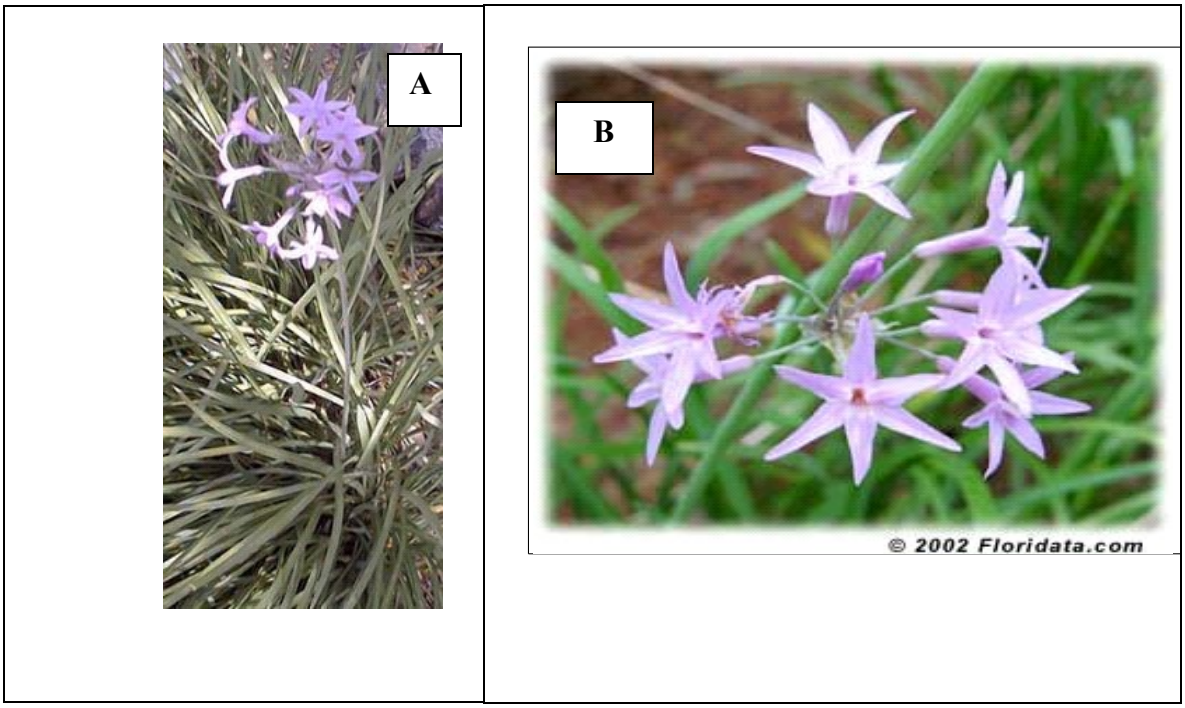

Figure 2.1: Morphological structure of *T. violacea*. **A.** Clump and grass-like foliage. **B.** The flower heads borne on long, slender solitary stalks.

Tulbaghia violacea is a spreading, vigorous, clump-forming perennial herb with grass-like foliage and corm-like rhizomes. It usually emits a strong smell of garlic, especially when damaged, which makes it impossible to use as a cut flower. Its rootstock is a corm or a rhizome, bearing many thick roots (Leistner, 2000). The leaves are deciduous or greyish green to evergreen depending on the climate. They are narrow, linear, frequently distichous and numerous per shoot. They form a thick, erect taft at the base of the plant with a long sheathing neck and grow up to 30 cm long (Eliovson, 1973; Leistner, 2000; Fig. 2.1).

Inflorescence consists of 3-40 flowered umbels. The flower heads are borne on long, slender, solitary stalks that stand well above the foliage. The stalked flowers are tiny and grouped in umbels at the tips of the stems (Fig. 2.1). Each tiny flower consists of a fat tube opening into six tiny segments and has an upstanding crown at the centre.

The plant's mature height is 30 to 60 cm. The flowers are of a lilac purple or violet colour (Eliovson, 1973; Horticopia, 2002).

2.2.5 Cultivation

Tulbaghia violacea is very easy to grow and will endure neglect for many years. The plants form clumps that do not need to be divided unless the flowers show signs of deterioration. Eliovson (1967) classified *T. violacea* as both a summer and a drought-resistant bulb. It is able to thrive in quite poor soil or in ordinary garden soil, but grows more successfully in good loam soil mixed generously with compost. Drainage should be good, particularly in areas of winter rainfall. The plants require moisture at regular intervals throughout the year, but more particularly during summer. They are hardy and may be grown in cold places. Even if the foliage is sometimes affected by the frost, it will recover again in the spring (Eliovson, 1973). It blooms mostly in spring, summer and autumn and with occasional blossom during the rest of the year. Propagation is by seeds or divisions (Faucon, 2000).

2.3 Antimicrobial properties of plant extracts

Several researchers have explored the antimicrobial potential of different plant organ extracts including roots, stems, leaves, flowers, fruits and seeds of many flowering plant species (*Angiospermae*). In some cases the whole shoot system or aerial and below soil parts extracts were tested separately, while in other cases the whole plant extracts were screened for antimicrobial activity. Different solvent systems were used, ranging from non-polar to more polar solvents, while both *in vitro* and *in vivo* antimicrobial bio-tests were employed in activity directed screening and purification procedures.

For example, Pretorius *et al.* (2002a) performed a wide search for South African plant species with fungitoxic properties against plant pathogens of economic importance in agriculture. Thirty-nine plant species, representing 20 families from the subclasses

Rosidae, Asteridae, Commelinidae and *Liliidae* were collected from the Blyde River Canyon Nature Reserve, Mpumalanga, South Africa. Crude extracts were prepared and bio-assayed, at equal concentrations, for their antifungal potential by determining the inhibitory effects on the mycelial growth of seven economically important plant pathogenic fungi. Statistically, significant differences between plants and plant parts were observed as well as the resistance of different fungi to treatment with different plant extracts. The most significant broad spectrum mycelial growth inhibition was obtained with extracts from two species of the subclass *Liliidae,* namely *Aristea ecklonii* and *Agapanthus inapertus*. The crude extract of *A. ecklonii* performed best of all extracts as it totally inhibited the mycelial growth of all seven of the plant pathogenic test organisms and outperformed the inhibition by a broad spectrum synthetic fungicide (carbendazim/difenoconazole). Crude extracts of *A. inapertus* showed complete inhibition of four and strong inhibition of the remaining three plant pathogenic fungi.

The antibacterial and antifungal activities of 38 plants belonging to 17 families were tested by Ghosh *et al*. (2000). The solvent extracts of different morphological parts of these plants were tested against 14 bacterial and 18 fungal strains. Out of these, *Alpinia mutica, Cephalandra indica, Croton bonplandianum, Curcuma amada, Holarrhena antidysenterica, Moringa oleifera* and *Zingiber spectabile* were found to contain antimicrobial properties (Ghosh *et al.,* 2000). Khan and Omoloso (2002) studied the antibacterial and antifungal activities of methanol extracts of *Harpullia petiolaris* leaves, stems, root barks and heartwoods (collected from Papua New Guinea). Antibacterial activities were found to be highest in fractions of root bark, petrol and dichloromethane fractions of stem bark, the petrol fraction of heartwood and the butanol fraction of leaves. Antifungal activity was only observed in the petrol fractions of the root bark and stem heartwood.

Ethanol and water extracts of leaves, flowers, shoots, bark and fruits of 30 herbal and woody plant species were tested for *in vitro* growth inhibition of *Erwinia amylovora* by Krupinski and Sobiczewski (2001) using an agar diffusion method. Active extracts

were found in 23 species, while in 13 of these the active substances were found for the first time. The highest growth inhibition of this bacterium was recorded for extracts of *Aloe arborescens*, *Juglans regia, Rhus typhina* [*R. hirta*], *Salvia officinalis* and *Satureja hortensis*. In almost all cases ethanol appeared to be a better solvent of active plant substances against *E. amylovora* than water.

Plant seeds also contain compounds with antimicrobial properties. Seed extracts of 50 plant species, belonging to different families, were evaluated for their ability to inhibit the growth of *Trichoderma viride in vitro* (Bharathimatha *et al.,* 2002). Of the various seed extracts, that of *Harpullia cupanioides* (Roxb.), belonging to the family *Sapindaceae*, displayed very high antifungal activity. The seed extract of *H. cupanioides* strongly inhibited the growth of *Rhizoctonia solani, Curvularia lunata* [*Cochliobolus lunatus*], *Colletotrichum musae* and *Alternaria alternata* and retained its antifungal activity even after heating at 100 °C for 10 minutes or autoclaving at 121 °C for 20 minutes.

Rodriguez and Montilla (2002) reported on the *in vitro* and *in vivo* antimicrobial effect of a *Citrus paradisi* seed extract (Citrex) on *F. oxysporum lycopersici* causing tomato wilt. Five treatments were evaluated: (i) immersion of plant roots in a solution of Citrex before transplant; (ii) weekly application to the foliage; (iii) weekly application to the soil; (iv) weekly application to the foliage and to the soil and (v) immersion of plant roots at transplant plus weekly application to the soil. The control was infested soil without application of the product. Treatments (i) and (iii) reduced wilting by 85%, indicating that it is possible to control soilborne pathogens with the *C. paradisi* seed extract.

The antimicrobial potential of plant extracts has also been reported against the highly resistant fungi that cause soil-borne damping-off disease in plants. The efficacy of crude, boiled water and acetone extracts of 17 plant species against *Rhizoctonia solani,* causing wet root rot disease in chickpeas, was evaluated under laboratory conditions (Kane *et al*., 2002). The crude, boiled water and acetone extracts of *Allium*

sativum, the crude and boiled water extract of *Eucalyptus* sp., as well as the boiled water and acetone extracts of *Zingiber officinale* caused 100% inhibition of the mycelial growth of the pathogen. In the same year Prabha *et al.* (2002) reported on the antifungal properties of extracts from *Foeniculum vulgare, Coriandrum sativum, Trigonella foenum-graecum, Anethum graveolens* and *Cuminum cyminum* against three fungi, including *Fusarium oxysporum.* All extracts showed a relatively greater inhibitory effect on *F. oxysporum,* with stem extracts from *A. graveolens* exhibiting complete growth suppression, while the mycelial growth of the other two fungi was also inhibited significantly.

In search of antimicrobial activity of plant extracts, the essential oils of *Callistemon lanceolatus* [*Callistemon citrinus*], *Citrus medica, Eclipta alba, Hyptis suaveolens* and *Ocimum canum* [*O. americanum*] were tested by Om *et al*. (2001) against *Rhizoctonia solani,* the cause of damping-off disease of tomato and chilli (*Capsicum annuum*). The essential oils of *Citrus medica, E. alba* and *O. canum* completely inhibited the growth of the fungus within 24 hours. The essential oils of *C. lanceolatus* and *O. canum* controlled the damping-off disease of tomato and chilli to a lesser extent.

Solunke *et al.* (2001) demonstrated the potential of plant extracts to be applied in integrated pest management programmes. The authors conducted a study to manage Sclerotium rot (*Sclerotium rolfsii [Athelia rolfsii]*) of potato using a commercial fungicide, carbendazim, separately and together with plant extracts. Sensitivity of fungal isolates (SRP-1, SRP-2, SRP-3 and SRP-4) against carbendazim was determined beforehand. Subsequently, aqueous extracts of *Azadirachta indica, Allium cepa, Glossocardia bosvallea [G. bosvallia]* and *Vinca rosea [Catharanthus roseus]* were mixed with carbendazim in solution and used to treat potato slices. Based on the minimum inhibitory concentration of carbendazim against the four isolates, SRP-4 appeared to be tolerant while SRP-1 was sensitive. The percentage control efficacy (PCE) of carbendazim alone and in mixture with plant extracts were tested against the tolerant isolate, SRP-4. Using carbendazim in combination with

plant extracts increased its PCE. Application of carbendazim along with *A. indica* and *G. bosvallea* recorded a PCE of 100%. Carbendazim with *V. rosea* and with *A. cepa* recorded PCE's of 90.77 and 80.53% respectively. The results showed that it is possible to reduce the selection pressure of carbendazim when combined with plant extracts.

Investigations by Ioannou *et al.* (2002) in Cyprus showed that plant extracts may replace methyl bromide fumigation in the control of soil-borne diseases in greenhouse tomato. Solarization treatments over 2 to 6 weeks reduced *Fusarium* populations by 70-99%. Among soil treatments, Nemacur, Nemaclean and combined soil amendments with organic matter, chitin and Acidam (sulfur plus *Thiobacillus sp.*) significantly reduced nematode (*Meloidogyne* sp.) and *Fusarium* populations while increasing the yield by up to 35%. The authors concluded that short-term solarization treatments (2-4 weeks), combined with nematicides or certain soil amendments, could provide a sustainable alternative to methyl bromide fumigation, which will incidentally be phased out for the greenhouse tomato industry of Cyprus by 2005.

Similarly, F*usarium* wilts caused by the fungal pathogen *Fusarium oxysporum*, are some of the most widespread and destructive diseases of many major ornamental and horticultural crops (Bowers and Locke, 2000). Presently, preplant soil fumigation and fungicide applications are used to control wilt diseases. However, methyl bromide, the major fumigant used, is scheduled to be phased out, because it was defined by the Montreal Protocol of 1991 as a chemical that contributes to the depletion of the ozone layer (Bowers and Locke, 2000). Due to the environmental and safety concerns associated with pesticides, Bowers *et al.* (2000) investigated the effect of several formulated plant extracts and essential oils on soil populations of *F. oxysporum* and disease control in the greenhouse as an alternative component in integrated control strategies. Treatment of the soil with 10% aqueous emulsions of the formulated extracts of a chilli pepper extract and essential oil of mustard mixture, a cassia tree extract and clove oil, reduced populations of *Fusarium* by 99.9, 96.1 and 97.5% respectively, three days after soil treatment. The same formulations also

suppressed disease development in the greenhouse and resulted in an 80 to 100% plant stand after six weeks. The observed reductions in the pathogen population in soil and the increase in plant stand in the greenhouse indicated that these natural plant products may play important roles in future biologically based management strategies for control of *Fusarium* wilt diseases.

Recently two new liquid formulations of the resistance-inducing *Reynoutria sachalinensis* extract, namely Milsana VP 1999 and Milsana VP 2000/VP 2001, were studied in Greece and Germany for their impact on the infection of cucumber by powdery mildew (*Sphaerotheca fuliginea*) as well as on yield. In four small-scale trials, it was shown that Milsana effectively controlled powdery mildew when applied at 7-day intervals and both highly and less susceptible cultivars could be effectively protected. A statistically significant increase in yield was observed in cucumbers treated with Milsana formulations, which was at least as high as that obtained by fungicide applications. From the present study, it can be concluded that Milsana reduces powdery mildew severity even in highly susceptible cucumber cultivars. Thus, Milsana can substantially contribute to the management of cucumber powdery mildew in organic or integrated farming systems (Petsikos *et al.,* 2002).

Additionally, plant extracts with antibacterial properties can also provide an alternative to certain antibiotics. Zeller *et al.* (2002) reported on such alternatives to the antibiotic streptomycin for the control of fire blight on pome fruits, caused by *Erwinia amylovora,* which is of great economic importance for German and European fruit producers. An antagonistic preparation, BIOPRO, showed a control efficacy of up to 60% and the plant extract from *Hedera helix* revealed a high efficacy in the field in combination with a low concentrated copper compound and a metal salt. The control of fire blight was comparable with the antibiotic streptomycin under artificial and natural infection conditions.

Extracts from the neem tree (*Azadirachta indica*) have been studied intensively in the past. Neem is perhaps the most useful traditional medicinal plant in India and each

part of the tree has some medicinal property that has made it commercially exploitable (Kausik *et al.,* 2002). Extracts from the leaves and seed kernels of the neem tree (Ume *et al.,* 2001) were tested for antifungal activity against the plant pathogenic fungus *Sclerotium rolfsii* [*Corticium rolfsii*]. All the extracts showed some effect against different growth stages of the fungus, but the effects were fungistatic rather than fungitoxic. The non-polar extracts of the seed kernels were reported to be more effective than those rich in polar terpenoids such as azadirachtin and an aqueous leaf extract was also more effective than the kernel-derived material.

Subsequently, Amadioha (2002) evaluated the antifungal activities of the different extracts of *A. indica*. The oil extract from seeds as well as water and ethanol leaf extracts of the plant were effective in reducing the radial growth of *Cochliobolus miyabeanus* in culture and in controlling the spread of brown spot disease in rice. However, the oil extract was found to be the most effective, followed by the ethanol leaf extract, in inhibiting the growth of the pathogen *in vitro* and in controlling the development of the disease *in vivo*. The oil and ethanol extracts compared favourably with carbendazim (Bavistin) at 0.1% a.i. and had the potential to control the brown spot disease of rice in the field. Additionally, Bohra and Purohit (2002) studied the effects of the aqueous extracts of 17 plant species collected from Rajasthan, India on a toxigenic strain of *Aspergillus flavus*. The neem extract recorded the highest mycelial growth inhibition of the fungus.

In fact, what is extremely promising from a natural product development perspective is that many plant extracts compare favourably with commercially synthesized fungicides. For example, leaf extracts of *Azadirachta indica, Atropa belladonna, Calotropis procera, Ocimum basilicum, Eucalyptus amygdalina, Ailanturs excelsa* [*Ailanthus excelsa*] and *Lantana camara,* at different concentrations, were compared to the fungicides Bavistin [carbendazim], Dithane M-45 [mancozeb], captan, thiram and Topsin M [thiophanate-methyl] at standard concentrations against *F. oxysporum* inducing fenugreek wilt under green house conditions. Seeds of fenugreek were separately soaked overnight in each leaf extract as well as the fungicide solutions

(Gupta and Bansal, 2003). All the leaf extracts, except that of *Ailanturs excelsa,* significantly inhibited the mycelial growth of *Fusarium* compared to the untreated control. Maximum germination of fenugreek seeds was observed with Bavistin (93.33%), followed by *Atropa belladonna* (90.66%), *Azadirachta indica* (87.99%), *L. camara* (87.99%), *C. procera* (85.99%), *O. basilicum* (85.33%) and *E. amygdalina* (82.40%). Dithane M-45 (80.00%), Captan (86.66%), thiram (86.66%) and Topsin M (86.66%) were on par with the leaf extracts.

Equally promising was the results of a study conducted to determine the inhibitory effect of 58 plant extracts on spore germination and the effective control of grape downy mildew (*Plasmopara viticola*). Among the plant extracts, those of *Chloris virgata, Dalbergia hupeana, Pinus massoniana, Paeonia suffruticosa* and *Robinia pseudoacacia* inhibited spore germination of the pathogen significantly. An *in vivo* leaf disc test showed that the infected leaf discs, treated with these five plant extracts, exhibited no disease symptoms. Their effects were the same or better than that of the traditional fungicide, liquid Bordeaux (Chen *et al.,* 2002).

Pandey *et al.* (2002) similarly compared the antifungal potential of leaf extracts from 49 angiosperms, collected in Uttar Pradesh, India with commercial fungicides, by screening them against *Helminthosoporium sativum* [*Cochliobolus sativus*]. The leaf extract of *Mangifera indica* completely inhibited the mycelial growth of the test fungus while four plant species, *Allium sativum, Azadirachta indica, Lawsonia inermis* and *Matricaria chamomila* [*Chamomilla recutita*] showed more than 90% inhibition. On assaying different parts of *Mangifera indica,* the leaf and seed extracts were found to possess the highest activity and, together with a leaf extract of *Matricaria chamomile*, performed better than the commercial fungicides.

Kishore *et al.* (2002) reported on the antimicrobial activity of aqueous leaf extracts from *Lawsonia inermis* and *Datura metel* against *Mycosphaerella berkeleyi* causing late leaf spot in groundnuts (*Arachis hypogaea*). Field experiments were conducted at ICRISAT, Patancheru, India during 1999 and 2000 using a susceptible groundnut

cultivar (TMV2). The *D. metel* extract continuously reduced disease progress up to 115 days after sowing while the severity of late leaf spot at harvest was significantly less than that of the controls. The *L. inermis* extract was slightly less effective, containing disease progress up to 95 days after sowing, but disease severity was also considerably less than that of the untreated and the positive chlorothalonil treated controls. Pod yields in plots sprayed with *L. inermis* and *D. metel* extracts were 20.0 and 48.3% higher, respectively, than in the control plots.

Pretorius *et al.* (2002b) performed a similar study on the control of black spot (Ascochyta blight) in pea leaves, caused by *Mycosphaerella pinodes,* by a crude bulb extract of *Eucomis autumnalis*. The fourth internode leaves were removed from 4-week-old pea (cv. Mohanderfer) plants, placed on moist filter paper in Petri dishes and inoculated with an *M. pinodes* spore suspension before and after treatment with the extract. The crude extract prevented *M. pinodes* spore infection of the leaves when the leaves were inoculated with spores both before and after treatment with the extract, confirming complete inhibition of spore germination. The crude *E. autumnalis* extract showed no phytotoxic effect on the leaves even at the highest concentration applied.

From the literature it is interesting to note that plant extracts seem to have a high application potential in controlling a wide range of plant diseases on an equally wide range of crops. For example, Leksomboon *et al.* (2001) demonstrated the potential of leaf and other aqueous extracts of *Hibiscus sabdariffa, Psidium guajava, Punica granatum, Spondias pinnata* and *Tamarindus indica* in controlling citrus canker (*Xanthomonas axonopodis pv. citri*) infesting *Citrus aurantiifolia* under both laboratory and field conditions in Thailand during 2000. Spraying with leaf extracts of *T. indica* resulted in the lowest citrus canker incidence (48%) noted under greenhouse conditions. Under field conditions, the number of diseased leaves and disease incidence was greatly reduced compared to the untreated control, after spraying with *T. indica* aqueous extracts.

An approach to search for plants with antimicrobial activity against plant pathogens that often results in success is to exploit indigenous knowledge on medicinal plants and to screen these known plants for likely candidates. The rationale behind this approach is to screen traditional medicinal plants known in a specific area for their antimicrobial properties instead of randomly choosing potential candidates from the long list of currently known flowering plants, conifers, ferns or bryophytes. Rajiv *et al.* (2002) conducted a study to screen for the most effective extracts out of 15 medicinal plants against *Helminthosporium nodulosum* [*Cochliobolus nodulosus*]) causing blight in finger millet. These included *Impatiens balsamina, Solanum nigrum, Tagetes erecta, Allium sativum, Azadirachta indica, Datura metel, Emblica officinalis* [*Phyllanthus emblica*], *Eucalyptus citriodora, Euphorbia pulcherrima, Lantana camara, Mentha arvensis, Mimosa pudica, Nerium indicum* [*N. oleander*], *Ocimum sanctum* [*O. tenuiflorum*] and *Ricinus communis.* Extracts were sprayed on the potted finger millet plants at 15, 30, 45, 60 and 75 days after sowing. Crude extracts of *S. nigrum* and *I. balsamina* showed the highest mycelial growth inhibition, followed by *T. erecta.* Overall, the crude extract of *S. nigrum* recorded the best result *in vitro* but was found inferior to the *I. balsamina* extract in *in vivo* tests (Rajiv *et al.,* 2002).

A similar approach was followed by Morais *et al.* (2002) in screening crude extracts of known medicinal plants against bacterial pathogens of tomato. The antibacterial activity of crude extracts from 45 medicinal plants was tested against *Xanthomonas campestris pv. vesicatoria* [*X. vesicatoria*], *Ralstonia solanacearum* and *Clavibacter michiganense subsp. michiganense* [*C. michiganensis subsp. michiganensis*]. Some assays were also performed to verify the capability of these plant extracts to show antibiosis. Five of the 45 extracts showed significant activity against the test bacteria confirming the potential of using either these extracts or active substances contained in them as natural products under field conditions.

Finally, an aspect that needs special reference is that the antimicrobial efficacy of plant extracts compare favourably with that of biological agents in controlling plant diseases. Devanath *et al.* (2002) studied the *in vitro* sensitivity of *Ralstonia*

solanacearum, causing bacterial wilt of ginger towards biotic antagonists, plant extracts and chemicals. The efficacy of five fungal antagonists (*Trichoderma harzianum, T. viride, T. koningii, Aspergillus terreus* and *Gliocladium virens*), two bacterial antagonists (*Pseudomonas fluorescens* and *Bacillus subtilis*), extracts of three medicinal plants (*Psidium guajava, Aloe vera* [*A. barbadensis*] and *Datura stramonium*) and two chemicals (200 ppm streptocycline and 0.25% Blitox [copper oxychloride 50]) was assessed *in vitro* against *R. solanacearum.* The aqueous extract of *Aloe vera* was found to be the most effective in suppressing the growth of the pathogen, followed by the extract of *Psidium guajava* and the bacterial antagonists *Pseudomonas fluorescens* (Devanath *et al*., 2002).

In fact, research into antimicrobial activities of plant extracts has offered some potential solution to disease control in the absence of effective chemical control. For example, few commercial fungicides have been effective in inhibiting teliospore germination of *Tilletia indica*, the causal agent of Karnal bunt of wheat. This disease is becoming more widespread, in part, because of the lack of effective chemical control. Various extracts of native plants from Sonora, Mexico were evaluated to determine their antifungal activity against *T. indica*. Dichloromethane and methanol extracts were incubated with the fungus to measure inhibition of mycelial growth. Dichloromethane extracts from *Chenopodium ambrosiodes* and *Encelia farinosa* reduced radial mycelial growth significantly, but total inhibition occurred at a relatively high concentration of 500 mg ml^{-1} of the dichloromethane extract from *Larrea tridentata*. Teliospores subjected to treatment with the latter plant extract showed no viability when transferred to fresh culture media confirming its potential to be applied as controlling agent for *T. indica* (Rivera *et al.,* 2001).

2.4 Economically important plant pathogenic fungi used as test organisms in this study

Fungi are the most important plant pathogens (Parry, 1990). It is estimated that more than 8000 species of fungi can cause diseases in plants. All plants are prone to

parasitic fungi that can attack one specific or many kinds of plants (Agrios, 1997). The following fungi cause diseases and are of economic importance in crop plants and were used for the *in vitro* anti-fungal bioassay of the extracts of *T. violacea.*

2.4.1 *Botrytis cinerea Pers.: Fr.* (*Hyphomycetes*)

Botrytis cinerea causes *Botrytis* grey mould, blights, wilting and leaf spots in a wide range of plant species in temperate zones and in many economically important field cultivated crops such as vegetables, ornamentals, bulbs and fruits (Wheeler, 1969; Parry, 1990; Agrios, 1997; Alfonso *et al.,* 2000; Shafia *et al.,* 2001). The pathogen also causes serious losses in greenhouse cultivated tomato through infection of flowers and stem wounds (Eden *et al.,* 1996; Shafia *et al.,* 2001).

The disease is most common on leaves, where grey-brown irregular lesions can be seen early in the season. It also causes bleached lesions on stems, usually associated with some form of damage such as cracks or broken branches. During humid weather, typical grey mould may be seen on leaf and stem surfaces. Pest damage on pods frequently results in *Botrytis* infection where pods may decay, become dehydrated and again become covered with a grey mouldy growth (Parry, 1990).

Specific chemical control measures are seldom found for *Botrytis cinerea* alone and it is unlikely that fungicide application specifically for *Botrytis* would be economically justifiable. However, extracts of oilseed rape applied to control other more economically important diseases such as *Alternaria*, are quite effective against *Botrytis* (Parry, 1990) and strains resistant to either benzimidazoles or dicarboximide fungicides have been reported (Raposo *et al.*, 1996).

2.4.2 *Fusarium oxysporum Schlechtend. :Fr.* (*Hyphomycetes*)

Fusarium oxysporum causes wilt diseases in many annual plants. *Fusarium* wilt (*F. oxysporum f. lycopersici*) is one of the most prevalent and damaging diseases of tomato wherever tomatoes are grown intensively. The disease is most destructive in warm climates and warm, sandy soils of temperate regions (Agrios, 1997) where it may cause substantial losses, especially on susceptible varieties and under favourable weather conditions. Occasionally entire fields of tomatoes are killed or severely damaged before the crop can be harvested. It also causes damping-off and seedling blights of a wide range of crops, as well as root and stem rot of cucumber plants in greenhouses. Serious yield losses as a result of infection by this pathogen in commercial greenhouses have been reported (Wheeler, 1969; Agrios, 1997; Punja and Parker, 2000). Other *Fusarium* species attack a wide range of crops including wheat, barley, oats, rye, grasses and numerous other plant species (Parry, 1990).

The first symptoms of *Fusarium* wilt of tomatoes show as slight vein clearing on the outer, younger leaflets, followed by epinasty of the older leaves. Plants infected at the seedling stage wilt and die. Older plants in the field may wilt and die suddenly if the infection is severe (Agrios, 1997). Other symptoms involve stunting of the plants and yellowing of the lower leaves. In the case of root and foot rot, the stem base becomes brown and water-soaked (Parry, 1990; Agrios, 1997).

Chemical control may be effective when reducing early season infections arising from seed-borne inoculum, but there are no highly effective chemical control measures available (Parry, 1990). Other recommended control measures are clean cultural practice, crop rotation and development of resistant varieties as well as soil sterilization which is very expensive (Parry, 1990; Agrios, 1997).

2.4.3 *Sclerotium rolfsii Sacc. (Agonomycetes)*

Sclerotium rolfsiii causes root rot in ground-nuts, cotton, beans, potatoes and tomatoes. A first sign of infection is progressive rotting of the root system which often involves the basal portion of the stem (foot rot) and, as a result, the plant cannot

obtain the water and nutrients it needs. This gives rise to a number of symptoms in the shoot: growth is checked and the plant becomes stunted, the leaves yellow and wilt, some drop and eventually the plant collapses and dies (Wheeler, 1969).

Root diseases are among the most difficult to control. Once symptoms appear in the foliage it is usually too late to do anything. Two general methods for controlling root diseases are crop rotation and eradication by chemicals. Chemical treatment of the soil (soil sterilization) has been considered economically practical only on seedbed and glasshouse sites (Wheeler, 1969).

2.4.4 *Rhizoctonia solani Kühn (Agonomycetes)*

Rhizoctonia solani causes damping-off on a wide range of cultivated plants including cereals, potato, root and fodder crops, legumes, vegetables and ornamentals, and it also causes root rots (Wheeler, 1969; Agrios, 1997). Sheath blight, caused by *R. solani*, has become an important disease of rice in tropical Asia, especially in intensive rice cropping systems (Teng *et al.,* 1990; Savary *et al.,* 1994; Willocquet *et al.*, 1999). *Rhizoctonia solani* disease of potato (black scurf) is one of the common diseases that occur in potato production areas throughout the world (Errampalli and Johnston, 2001). This soil-borne fungus is a causal agent of seed decay, pre- and post-emergence damping off, stem canker, root rot, fruit decay and foliage disease. Moreover, it occurs worldwide and causes disease in a broad range of host plants (Keijer *et al.,* 1997) including widespread damage in glasshouse-grown lettuce and other crops (O'Neill *et al.,* 1999; Thornton *et al.,* 1999).

An early symptom is poor seedling emergence when infected seeds are planted. Seedlings that have emerged often show water soaking, browning or shrivelling of the stem tissues at soil level and they keel over and die as a result. Post-emergence damping off as a result of *R. solani* infection causes stunted growth, yellowing and wilting of the foliage. When the plants are pulled up they are found to have extensive

browning and rotting of the smaller roots, or stem lesions at soil level (Wheeler, 1969; Parry, 1990).

Control of the pathogen involves cultural and chemical control (soil sterilization) (Wheeler, 1969; Parry, 1990). Cultivars highly resistant to rice sheath blight are not currently available, and so far control of the disease has relied mainly on the use of synthetic fungicides (Savary and Mew, 1996).

2.4.5 *Botryosphaeria dothidea* (*Moug. : Fr.*) C*est DeNot* (*Loculoascomycetes*)

Botryosphaeria dothidea is especially damaging to woody plants such as *Pinus* and *Eucalyptus* (Smith *et al.,* 2001) and causes a destructive panicle and shoot blight of pistachio (*Pistacia vera*) (Ma and Michailides, 2002). It has a wide range of other host plants, including apple varieties, but it only causes serious damage to those host plants that are weakened or are under environmental stress such as drought, winter injury, sunscald, poor pruning, low or unbalanced nutrition and other plant diseases (Ellis, 2002). It also causes white rot (Bot rot) of apple fruits, as well as canker on limbs and other above-soil woody portions of the tree (Brown and Mcmanus, 2000; Ellis, 2002).

Symptoms of infection on apple trees include small, circular spots or “blisters” on the twigs that enlarge, become somewhat sunken and fill with a watery fluid. The fungus may grow rapidly through tissues to form slightly sunken, dark-coloured cankers that may extend to the cambium on very susceptible apple varieties. Under favourable conditions, several cankers may fuse to girdle and kill large limbs. Small, reddish-brown spots appear around the lenticels on the apple fruit. These enlarge and become slightly depressed. The tissues under the spots develop into a soft and mushy rot (Ellis, 2002).

There is no single effective control measure for *Botryosphaeria* blight (Ma *et al.,* 2002), except an integrated programme of cultural practices and chemical control measures. Any practice that helps to maintain trees in a healthy, vigorous condition is

critical for controlling the canker phase of the disease. The use of fungicides combined with good sanitation is beneficial for controlling the fruit rot phase of the disease. Fungicides are not effective for controlling the canker phase of the disease on weakened trees (Ellis, 2002).

2.4.6 *Pythium ultimum Trow* (*Oömycetes*)

Pythium ultimum causes diseases of germinating seeds and of seedlings, collectively known as damping-off and seedling blights. It also causes root rot in a wide range of crops as well as rot of potato tubers (Wheeler, 1969; Parry, 1990). The disease occurs in valleys and forest soils, in tropical and temperate climates and in almost every greenhouse. The pathogen affects seeds, seedlings and older plants of almost all kinds of vegetables, flowers, cereals and many fruit and forest trees (Agrios, 1997). Although the greatest damage is done to the seed and seedling during germination and either before or after emergence, the fungus also attacks the fleshy organs of plants, which then rot with resultant heavy losses during storage of these products (Agrios, 1997). Root diseases caused by *P. ultimum* and *P. aphanidermatum* have been shown to be the most common and destructive root-infecting diseases of lettuce in recirculating hydroponic systems (Utkhede *et al.,* 2000).

The damping-off symptoms caused by this pathogen vary with the age and stage of development of the plant affected. Infected seeds fail to germinate, become soft and mushy, then turn brown, shrink and finally disintegrate resulting in poor seedling stands with bare patches. Invaded areas of the seedlings become water-soaked and discoloured and the cells soon collapse. The invaded portion of the stem cannot support the plant and the seedling falls and dies. When older plants are attacked by *P. ultimum* on the rootlets, they usually show lesions on the stem that can girdle the plant, causing stunting, wilting and death (Agrios, 1997). The symptoms on fleshy organs of some vegetables, such as cucurbit fruits, potatoes and cabbage heads during storage and transit is a cottony fungus growth on the surface, while the interior turns into a soft, watery, rotten mass, called “leak” (Parry, 1990; Agrios, 1997).

Control under greenhouse conditions involves soil sterilization by steam or dry heat or with chemicals (Agrios, 1997). The use of chemically treated seeds is also important. Certain cultural practices are sometimes helpful in reducing the amount of infection such as soil drainage, crop rotation and destruction of fungal inoculum on debris. Utkhede *et al.* (2000) reported that in recirculating hydroponic systems, once the pathogen is introduced in the system, the control is very difficult and sometimes the grower is forced to destroy the crop.

2.4.7 *Mycosphaerella pinodes* (*Berk. + Blox.*) *Vestergr.* (*Ascomycetes*)

Mycosphaerella pinodes is the cause of leaf spot (*Ascochyta* blight) and foot-rot in mainly peas but may also affect beans and causes leaf spot in many other plants. It is particularly important in temperate parts of the world (Wheeler, 1969; Parry, 1990; Clulow *et al.,* 1991; Agrios, 1997). *Mycosphaerella* blight (Béasse *et al.*, 2000) is the most destructive foliar pathogen of pea in France. In the field, yield losses are large and depend on the conditions. In seven years out of ten, on average, *Mycosphaerella* blight reduced yields by 20%. According to Roger *et al.* (1999) *Ascochyta* blight occurs worldwide and causes yield losses of 30% up to over 45%.

Symptomal necrotic lesions develop on all aerial parts of the pea plant, including the pods grown from contaminated seeds. The lesions initially consist of small 'tea-leaf' shaped discrete dark brown or purple-brown patches. Lesions may enlarge and assume a concentric ring pattern (Parry, 1990; Roger *et al.*, 1999; Béasse *et al.*, 2000). Infection of stems results in small brown streaks at first, which may later coalesce, girdling the stem and giving it a blue-black colour. Lesions on pods develop in a similar way to lesions on leaves, and symptoms are always more severe on the lowest parts of the plants (Parry, 1990; Béasse, *et al.,* 2000).

Cultural control includes the use of certified seed. Debris should also be burned if possible. Chemical seed treatment and fungicide foliar sprays help to reduce the disease virulence (Parry, 1990).

2.5 Economically important plant pathogenic bacteria used as test organisms in this study

Many species of bacteria have been found to cause diseases in plants and, according to Agrios (1997), most bacteria causing plant diseases belong to the class *Schizomycetes* and orders P*seudomonadales, Eubacteriales* and *Actinomycetales*. They cause leaf spots and blights, soft rots of fruit, root and storage organs, wilts, overgrowths, scabs and cankers. The following six bacteria are of economic importance in South Africa and were grown artificially on nutrient media to test their response to the crude extracts of *T. violacea* in this study.

2.5.1 *Clavibacter michiganensis* subsp. *michiganensis* (*Smith*)

Different subspecies of *Clavibacter michiganensis* cause bacterial mosaic of wheat (*C. michiganensis* subsp. *tesselarius*), Goss's wilt and blight of maize, sorghum, sudan grass and sugarcane (*C. michiganensis* subsp. *nebraskensis*) (Maloy and Murray, 2001). Bacterial canker, caused by *C. michiganensis* subsp*. Michiganensis,* has been reported as a limiting factor in the production of tomatoes grown for processing in Michigan, USA (Hausbeck *et al*., 2000). A seedling infected with this bacterium (Hausbeck *et al.*, 2000) may wilt and die shortly after transplanting to the field or may survive to produce a stunted plant that eventually succumbs to this disease that causes serious losses for tomato growers. In wheat the symptoms are small yellow leaf spots which turn to dark brown, while in maize, sorghum, Sudan grass and sugarcane the symptoms are water-soaked, discrete streaks which appear parallel to leaf veins. These later develop into leaf blight (Maloy and Murray, 2001).

In tomatoes clean cultural practice is recommended, which involves ploughing under of infected plants as well as crop rotation. Chemical control is also used (Hausbeck *et al.*, 2000).

2.5.2 *Pseudomonas syringae* subsp. *syringae* (van Hall)

Pseudomonas syringae causes bacterial canker and gummosis of stone fruit trees, blister spot of apple and pear, bacterial leaf spot and blight of celery, maize, millet, vegetables, cucumber, sunflower, peach, lucerne and a wide range of field and cash crops (Agrios, 1997; Braun *et al*., 2000; Morris *et al.*, 2000; Preston *et al.*, 2000; Maloy and Murray, 2001). Bacterial canker and gummosis of stone fruit trees occur in all major fruit growing areas of the world, and the disease is also known as bud blast, dieback, spur blight and twig blight. Yield losses vary from 10 to 75% in various parts of the world, but sometimes up to 80% (Agrios, 1997). In France some cantaloupe farmers reported 80% to 100% yield losses in 1996 and 1997 due to bacterial blight (Morris *et al.*, 2000).

The general symptoms are cankers on cherry, peach, apricot and other trees, while gum may exude from affected tissues. Infected buds are killed and flowers wilt (Agrios, 1997). Bacterial leaf spots symptoms depend on the type of plant attacked. The symptoms of bacterial leaf spot and blight of celery are rusty, brown and circular to irregular spots on leaves. Defoliation may occur. Bacterial leaf spot of sorghum show water-soaked, irregular, elliptical spots on the lower leaves with reddish hue. As they dry, they turn red at their margins and their centres become light coloured. The lesions occur on leaves, leaf sheaths and seeds (Maloy and Murray, 2001).

According to Braun *et al.* (2000), there are no bactericides available to control kernel blight of barley or of small grains caused by *P. syringae* subsp. *syringae*. Agrios (1997) recommends the use of resistant varieties and certain cultural practices, such as the use of healthy nursery trees.

2.5.3 *Erwinia carotovora* subsp. c*arotovora* (Jones)

This pathogen causes bacterial soft rots in vegetables and some annual ornamentals that have fleshy storage tissues. These include potatoes, carrots, radishes, onions, hyacinths and iris. It also infects crops with fleshy fruit such as cucumber, squash, eggplant and tomato, or those with succulent stem, stalk or leaves such as cabbage, celery, lettuce and spinach (Agrios, 1997; Zolobowska and Pospieszny, 1999; Choi and Lee, 2000). Bacterial soft rots are found all over the world and cause serious diseases of crops in the field, in transit, and especially in storage, resulting in greater total loss of produce and economic losses than any other bacterial disease (Agrios, 1997; Zolobowska and Pospieszny, 1999).

The soft-rot symptoms (Agrios, 1997) produced on fruits and other fleshy organs in the field or in storage are very similar on all the hosts. At first a small water-soaked lesion appears on the tissue that enlarges rapidly in diameter and in depth. The affected area becomes soft and mushy. The tissues within the affected region become opaque in a short time, or appear cream-coloured and slimy, disintegrating into a mushy mass of disorganized cells. A whole fruit or tuber may be converted into a soft, watery, colourless, decayed mass within a period of three to five days (Agrios, 1997; Malavolta *et al.,* 1998; Hseu *et al.,* 2001).

Chemical sprays are generally not recommended for the control of soft rots. The control of bacterial soft rots of vegetables is based almost exclusively on sanitary and cultural practices. All debris should be removed from warehouses and the walls should be disinfected with appropriate phyto-chemicals (Agrios, 1997).

2.5.4 *Agrobacterium tumefaciens* (Smith and Towsend)

Agrobacterium tumefaciens causes crown galls in many woody and herbaceous plants belonging to 140 genera of more than 60 families. It attacks pome and stone

fruit trees, brambles and grapes. Plants with crown galls and tumours on their main roots and crowns grow poorly and produce reduced yields. Severely infected plants or vines may die (Wheeler, 1969; Agrios, 1997; Stafford, 2000).

The pathogen induces localized swellings or outgrowths called galls, it has the ability to transform normal plant cells to tumour cells in a short period of time, and once the transformation to tumour cells has been completed, these cells become independent of the bacterium and continue to grow and divide abnormally even in the absence of the bacteria (Wheeler, 1969; Agrios, 1997).

Crown gall control (Agrios, 1997) is based primarily on certain cultural and sanitary practices. Susceptible nursery stock should not be planted in fields known to be infested with the pathogen. Instead, infested fields should be planted with maize or other grain crops for several years before they are planted with nursery stock. Since the bacterium enters only through relatively fresh wounds, wounding of the crowns and roots during cultivation should be avoided. Antibiotics have been used to prevent development of galls after infection (Agrios, 1997).

2.5.5 *Ralstonia solanacearum* (Smith)

Ralstonia solanacearum is the major cause of bacterial wilt that affects over 200 plant species of which many are of economic importance. It also causes bacterial wilt of many solanaceous vegetables and plants including tobacco, tomato, medicinal plants, jute, potato and eucalyptus (Stead, 1999; Coutinho *et al.,* 2000; Huang *et al.*, 2000; Mathew *et al.*, 2000; Supriadi, 2000; Lyons *et al.*, 2001; Maloy and Murray, 2001; Robertson *et al.,* 2001). It also causes brown rot in potatoes (Stead, 1999) with devastating results.

Wilt diseases are diseases of mature plants in which the plant's vascular system becomes plugged, resulting in limited water uptake and development. Wilt symptoms include leaf yellowing, necrosis leaf drooping and brownish discolouration of the

vascular tissues. If an infected stem is placed in water, white bacterial ooze emerges from the cut end of the plant (Maloy and Murray, 2001).

Bacterial wilt caused by *R. solanacearum* is very difficult to control because it has many host plants. However, strategies for controlling the disease include selection of disease-free soil, disease-free plant materials, resistant cultivars, control of nematodes, soil treatment with heat and chemicals and the adoption of strict quarantine regulations (Supriadi, 2000).

2.5.6 *Xanthomonas campestris* pv. *phaseoli* Pammel (Dowson)

Xanthomonas campestris pv. *phaseoli* causes bacterial blight of bean and is a major disease of this crop in many parts of the world (Parry, 1990; Partridge, 1997). Different subspecies of *X. campestris* cause bacterial leaf spot diseases in lucerne, regonia, celery, all types of citrus plants, cotton, cowpea, cucurbits, mango, mustard, sorghum, soybean, tomato and pepper. It also causes bacterial blight diseases in pea, cabbage, cauliflower, broccoli, onion, carrot, wheat, barley and some grass species (Zhao *et al.*, 2000; Black *et al*., 2001; Maloy and Murray, 2001; Nunez, 2002).

Bacterial blight symptoms in beans are initially visible as small water-soaked spots on leaf margins or between the veins. Spots eventually dry and turn brown; they enlarge with age and blight large areas of leaf tissue. Heavily infected leaves may become tattered when wind-whipped. In severe cases they die and remain attached to the plant. Pod infections also begin as water-soaked spots. Pod lesions may be reddish brown and slimy exudates may be present in the lesions. Pod lesions ultimately turn brown and in severe cases the entire pod shrivels and seed produced from the pods may be shrivelled, discoloured or non-existent (Partridge, 1997).

Bacterial blight of bean can be controlled through the use of pathogen-free seed and disease-tolerant varieties. At least a two-year crop rotation period should be allowed between bean crops. Weeds, volunteer beans and alternate pathogen hosts should

be eliminated from the field. Chemical control can also be used, i.e. application of copper fungicides (Partridge, 1997). Thoppil *et al.* (2001) discovered the antimicrobial activity of essential oil from *Teucrium plectranthoides.* The oil mixed with acetone at ratios of 1:0, 1:1 and 1:2 inhibited the growth of eight fungi and seven bacteria including *X. campestris*.

2.5.7 *Moraxella catarrhalis* (Frosch + Kolle)

Moraxella catarrhalis is a human pathogen generally more prevalent in the elderly and has been associated with patients with chronic obstructive pulmonary disease (COPD) (Hunter, 2001). Infection by this organism can be controlled by administration of the Co-Amoxidav drug to patients (Hunter, 2001). In this study *M. catarrhalis* was merely used as a test organism.

2.6 Bio-stimulatory and/or inhibitory properties of plant extracts

Besides compounds with direct antimicrobial properties found in plants, other natural compounds exhibit bio-stimulatory or inhibitory activity towards the growth of other plants. This phenomenon is known as allelopathy. In light of possible side effects in this regard, especially in the event where a crude extract of an identified plant showing above average antimicrobial activity is considered to be applied as a natural product in agriculture, this aspect was included in this study. In principle bio-stimulatory activity in addition to antimicrobial activity in the same plant extract can be regarded as an added bonus, especially if the stimulation is towards growth or induced resistance in treated crops. On the other hand growth inhibition or any other inhibitory activity by plant extracts on the treated crop is regarded as negative or phytotoxic activity. This aspect is elaborated in the next section.

Allelopathy (Delabays and Mermillod, 2002) is a chemical process whereby certain plants release natural compounds into the environment that can either stimulate or inhibit the growth and development of surrounding plants. In nature the latter is more

likely, as a form of competition for growing space, where allelochemicals are released from the roots of one plant and absorbed by the roots of surrounding plants preventing it from growing in the same area. According to Delabays and Mermillod (2002) there are several ways in which an allelopathic plant can release its protective chemicals. These include (a) volatilization where a chemical is released in the form of a gas through the leaves and, on absorption, sensitive surrounding plants are stunted or die, (b) leaching of allelochemicals from decomposing abscised leaves preventing surrounding plants to establish in the same area and (c) exudation of allelochemicals into the soil through the roots preventing surrounding plants to prosper.

A study directed towards identifying bio-stimulatory properties in plant extracts was performed by Cruz *et al.* (2002a) by treating the roots of bean, maize and tomato with an aqueous leachate of *Callicarpa acuminata* and following the *in vitro* effects on radicle growth, protein expression, catalase activity, free radical production and membrane lipid peroxidation in the roots. The aqueous extract of *C. acuminata* inhibited the radicle growth of tomato but had no effect on root growth of maize or beans. However the expression of various proteins in the roots of all treated plants was observed. In treated bean roots the expression of an 11.3-kDa protein by leachate showing a 99% similarity with subunits of an alpha-amylase inhibitor found in other beans was induced. In treated tomato an induced 27.5kDa protein showed 95% similarity to glutathione-S-transferases of other Solanaceae. Spectrophotometric analysis and native gels revealed that catalase activity was increased two-fold in tomato roots and slightly in bean roots while no significant changes were observed in treated maize roots. Luminol chemiluminescence levels, a measure of free radicals, increased four-fold in treated tomato roots and two-fold in treated bean roots. Oxidative membrane damage in treated roots, measured by lipid peroxidation rates revealed almost a three-fold increase in peroxidation in tomato while no effect was observed in maize or beans (Cruz *et al.,* 2002a).

The significance of this study lies in the fact that various metabolic events can be manipulated in plants by treatment with certain plant extracts. What has to be

established by researchers is whether these altered metabolic events contribute towards positive or negative physiological changes within the treated plants. And the rationale for this type of research lies in the search for natural allelochemicals to be applied in sustainable weed and pest as well as disease control management systems (Singh *et al.,* 2001). According to Singh *et al.* (2001), allelochemicals isolated from some plants show strong bio-herbicidal activity at high concentrations but at low concentrations these extracts can promote crop seed germination and seedling growth, hence showing a potential to be applied as bio-stimulatory agents or growth promoting substances in agriculture.

It is therefore imperative that research in this regard should concentrate on both the inhibitory and stimulatory effect of plant extracts on seed germination, seedling growth and the physiology of other test plants in order to verify the action at hand (Khan *et al.*, 2001; Ameena and George, 2002; Cheng *et al.*, 2002; Cruz *et al.*, 2002b; Duary, 2002; Obaid and Qasem, 2002; Qasem, 2002).

Extensive research has been done in the past to study the inhibitory bioactivity of extracts from many plant species on weeds (Wu *et al*., 2002). For example, extracts of *Wedelia chinensis* reduced seed germination, inhibited seedling growth, resulted in yellowing leaves and reduced resistance to disease in weeds such as *Cyperus difformis, Paspalum thunbergii, Alternanthera sessilis* and *Cynodon dactylon* at relative low concentration of 0.4 g fresh weight/ml water. This study confirmed the potential of *W. chinensis* extracts sprayed before crop emergence to control the germination of weed seeds (Nie *et al.,* 2002). Another study showed that a sorghum extract reduced seed germination and seedling growth of the weed *Trianthema portulacastrum* substantially at high concentrations (75-100%) but, promoted shoot length of the weed at low concentrations (25%) (Randhawa *et al.,* 2002).

Similar contrasting results were reported for the effects of extracts from eight lucerne cultivars on seed germination as well as on root and hypocotyl development of lettuce seedlings (Tran and Tsuzuki, 2002). Extracts from some lucerne cultivars had a

stimulatory effect in terms of seed germination as well as root and hypocotyl growth, whereas others showed the direct opposite effect, confirming that crop plants can also be affected by plant extracts aimed at controlling weed growth.

Singh *et al.* (2003) confirmed this phenomenon by showing that aqueous leaf leachates of *Eucalyptus citriodora* inhibited the germination and seedling growth of all test crops (*Vigna radiata, V. mungo,* and *Arachis hypogaea*) investigated. Further, in their study of bioactivity of plant extracts, Deena *et al.* (2003) demonstrated the inhibitory effect of leaf, stem and root leachates from *Andrographis paniculata* on germination and seedling growth in rice. From this it became clear that the bio-activities of plant extracts are unpredictable and may give different and often contrasting results with regard to inhibition or promotion of growth and development in other plants. The reaction of crops to treatment with plant extracts may depend on the interaction between different types of plant species or even on the concentration of the extracts (Channal *et al.,* 2002a).

From an agricultural perspective, plant extracts containing growth promoting substances have always been of interest to the research community in terms of the role they could play in addressing future food security issues. The ideal break-through would be to identify a plant or plants that contain bio-stimulatory substances promoting growth and yields in agricultural and horticultural crops, as well as resistance to pathogens. Numerous plant species more or less adhering to this description of an “ideal candidate,” were identified through research. Channal *et al.* (2002b) reported on the seed germination as well as seedling growth enhancement of sunflower and soyabean by leaf extracts from three out of the seven tree species (*Tectona grandis, Tamarindus indica, and Samanea saman*) investigated. Similar effects were reported by Terefa (2002) for *Parthenium hysterophorus* extracts on tef (*Eragrostis tef*) and by Neelam *et al.* (2002) for *Leucaena leucocephala* extracts on wheat (*Triticum aestivum*). However, none of these studies revealed that treatment with the different plant extracts had any effect on the final yields of the crops under investigation.

In this regard, a report by Ferreira and Lourens (2002) demonstrating the effect of a liquid seaweed extract (now trading as a natural product under the name Kelpak) on improving the yield of canola, must be regarded as significant. Kelpak, applied singly or in combination with the herbicide clopyralid at various growth stages of canola (*Brassica napus*), cultivars Monty and Dunkeld, were assessed in a field experiment conducted in Langgewens and Tygerhoek, South Africa during 1998-99. Foliar application of 2 litres Kelpak/ha, applied at the four-leaf stage, significantly increased the yield of the crop in Langgewens. In Tygerhoek, application at the same rate significantly increased canola yields when applied at either the three- or five-leaf growth stage, but the highest yield was obtained by treatment with Kelpak at the three-leaf growth stage.

Research into the allelopathic activity of plant extracts has resulted in the identification of active ingredients responsible for both inhibition or stimulation of either seed germination or seedling growth. In this respect Chung *et al.* (2002) reported on the inhibitory effect of ferulic, p-hydroxybenzoic, p-coumaric and m-coumaric acids isolated from three rice cultivars on the growth of barnyard grass. This suggested that these compounds may be, at least, a key factor in rice allelopathy on barnyard grass, and the information presented may contribute to the development of natural herbicides.

Similar active allelopathic compounds were isolated by Sasikumar *et al.* (2002). In their study on the allelopathic effects of *Parthenium hysterophorus* leachates on cowpea, pigeonpea, greengram, blackgram and horsegram, the authors reported significant seed germination inhibition for all test crops. Gas chromatographic analysis showed the presence of phenolic acids, namely caffeic, p-coumaric, ferulic, p-hydroxybenzoic and vanillic acids in the leachates from different plant parts (leaf, stem, flower, and root) of *P. hysterophorus*. A mixture of allelopathic compounds in bioassays significantly inhibited the germination and vigour index of all test crops. However, leachates from flowers had no inhibitory effect on the germination of blackgram and greengram seeds.

Kato and Kawabata (2002) isolated a growth-inhibiting compound from the acetone extract of thirty-day-old lemon balm (*Melissa offinalis*) shoots by means of silica gel column chromatography. This uncharacterized compound inhibited the growth of cress seeds at concentrations higher than 0.3 µg $ml.^{-1}$ Iqba *et al.* (2002) showed that living buckwheat reduced weed biomass compared to plots without buckwheat. A laboratory study revealed that root exudates from buckwheat (collected from Aomori, Japan) suppressed root and shoot growth of the weeds: *Trifolium repens, Brassica juncea, Amaranthus palmeri, Echinochloa crus-galli* and *Digitaria ciliaris* but also that of lettuce and reduced weed dry weight. Fagomine, 4-piperidone and 2-piperidinemethanol were isolated from a chloroform extract and identified as the active ingredients.

Several allelochemicals have also been characterized from the *Helianthus annuus* that inhibit seed germination and seedling growth of *Amaranthus albus*, *Amaranthus viridis, Agropyron repens* (*Elymus repens*), *Ambrosia artemisiifolia, Avena fatua, Celosia cristata* [*C. argentea var. cristata*], *Chenopodium album, Chloris barbara* [*Chloris barbata*], *Cynodon dactylon, Digitaria sanguinalis, Dactyloctenium aegyptium, Digitaria ciliaris, Echinochloa crus-galli, Flaveria australasica, Parthenium hysterophorus, Portulaca oleracea, Sida spinosa, Trianthema portulacastrum,* and *Veronica persica* (Macias *et al.,* 2002). The inhibitory effects of this crop may be utilized for weed management to attain reduced herbicide usage in sustainable agricultural systems (Azania e*t al.,* 2003).

In this study ComCat® a new natural bio-stimulant registered in Germany (Agraforum) was used as a positive control to compare with a crude *T. violacea* extract. ComCat® is a unique family of natural products that are based upon a combination of bio-stimulants derived from plant materials. ComCat® products have demonstrated consistent plant growth enhancement and physiological efficiency in the treated plant's utilization of available nutrients. The products nurture and enhance the health of vegetables, flowers and agricultural crops. ComCat® is not a fertilizer substitute but,

instead, it is a biological enhancer which stimulates the plant to more properly utilize available nutrients, it activates and induces allelopathy and disease resistance in the treated plant and stimulates greater production of sugars, which are the building blocks for cellulose and fruiting bodies. The result is a more productive, healthier plant with stronger plant stalks, better flowering and greater fruit biomass (Agraforum: Germany, 2002).

2.7 Anti-infective functions of plant secondary metabolites as part of a natural defence mechanism

Plants are under constant attack from phytophagus insects, various micro-organisms and fungi. Their survival is testamentary to their ability to defend themselves chemically against these attacks by producing a myriad of secondary metabolites with anti-feedant and anti-microbial properties (McFadden, 1995; Cowan, 1999). Since most plants lack mobility (Stumpf and Conn, 1981), they must necessarily have evolved alternative strategies for survival, and among these is the synthesis of secondary compounds that serve to deter predators. Therefore, in addition to other functions, one of the most important functions of secondary metabolites in plants is antimicrobial activity against bacteria, fungi and viruses as well as acting as deterrents towards insects and predators (McFadden, 1995; Lazarides, 1998; Palmer, 1998; Minorsky, 2001). Failure of micro-organisms to colonize plants has often been attributed to the presence of these inhibitory compounds within challenged tissues.

Callow, (1983) classifies antimicrobial compounds (secondary metabolites) isolated from plants into two categories: (i) constitutive compounds, which are present in healthy plants, and (ii) induced compounds synthesized from remote precursors following infection. The term ‘constitutive’ includes compounds that are released from inactive precursors following tissue damage, for example the release of toxic hydrogen cyanide from cyanogenic glycosides. Induced compounds include the types of secondary metabolites called phytoalexins, which are low molecular weight antimicrobial compounds that are synthesized by and accumulate in plants that have

been exposed to micro-organisms. An important feature of this definition is that it restricts phytoalexins to compounds that are synthesized from remote precursors (Callow, 1983).

According to Ley (1990), since plants have evolved highly elaborate chemical defences against attack, these have provided a rich source of biologically active compounds that may be used as novel crop-protecting agents. It follows therefore that secondary metabolites with antimicrobial activity purified and isolated from plant extracts, possess the potential to be developed into natural products. At least 12 000 plant secondary metabolites have been isolated (Cowan, 1999). For the purpose of this research, plants' secondary metabolites can be classified briefly into the following compounds (Stumpf and Conn, 1981; Dey and Harborne, 1989; Carte and Johnson, 2001): Phenolic compounds, Flavonoids, Alkaloids, Glycosides (cyanogenic and cardiac glycosides), Saponins, Anthraquinones, Anthocyanins, Tannins, Volatile and essential oils, Compounds of sulphur and Steroid compounds.

Man has utilized secondary compounds for many useful purposes including their use as sources and models of industrial oils, gums, spices, saponins, waxes, resins, dyes, tannins, soaps, vegetable oils, natural rubber, pesticides, perfumes, flavours and pharmaceuticals (Carte and Johnson, 2001). In pharmacology (Cox, 1990) many drugs currently in use are derived from plant secondary compounds. The most important include atropine, bromelain, caffeine, codeine, colchicines, morphine and quinine.

In recent years research interest has turned towards isolating, purifying and identifying secondary metabolites from plants with application potential in plant disease control. In a study conducted by Hoffmann et al. (1992) a methanol extract of *Castela emoryi* was active as both a preventative and curative agent against grape downy mildew caused by *Plasmopara viticola.* An active secondary metabolite was identified as a glycoside, 15-Glucopyranosyl-glaucarubolone.

Previously young and mature leaf extracts of *Codiaeam variegatum* were assayed for anti-fungal activity (Naidu, 1988). All extracts inhibited *Alternaria alternata* and *Fusarium oxysporum in vitro,* with the young leaves being more active against *A. alternata* and the old leaves more active against *F. oxysporum.* The active secondary metabolites from the leaves extracts were identified as phenolic compounds by chromatographic analysis. Phytochemical screening of the leaves also revealed other metabolites that may be responsible for anti-fungal activity.

Parimelazhagan (2001) studied the antifungal activity of *Atalantia monophylla* leaf extract against *Magnaporthe grisea,* the causative organism of blast disease of rice, and demonstrated control of the disease up to 83% *in vitro* and growth inhibition of the pathogen as well as a check in the spread of blast disease in rice *in vivo.* The activity was attributed to high phenol (4.8 mg g^{-1}) and flavonoid (24.5 mg g^{-1}) content in the leaves. The author speculated in positive terms on the potential to apply extracts from this plant to combat blast disease of rice under field conditions.

El-Abyad *et al.* (1990) screened extracts of eleven Egyptian weeds including *Capsella*, collected from the Menoufeya district, for antibacterial activity. Alkaloids and flavonoids isolated from *Capsella* showed broad spectrum as well as the highest antibiotic potencies and had the broadest anti-microbial spectra. In another study *Alnus acuminata* nodules were extracted with either 5% NaOH or water while constituents were separated by silica gel column chromatography (Gonzalez *et al.*, 1988). The following compounds were isolated: xylose, ribose, an aromatic carboxylic acid, a fatty acid, a phenolic biarylheptanoid and a flavonoid glycoside. The flavonoid glycoside was found to inhibit the growth of *Fusarium oxysporum* and *Pythium* species.

In their study of antimicrobial properties of plant extracts, Orlikowski (2001a) used grapefruit extract (Biosept 33 SL) to control *Phytophthora* spp. Amendment of peat with grapefruit extract at a concentration of 165 µg cm^{-3} resulted in a drastic decrease of colony forming units of the pathogen *Phytophthora cryptogea* and suppression of

its development in potted gerbera (*Gerbera jamesonii*) and cypress (*Chamaecyparis lawsoniana*). About 40 µg GE cm^{-3} inhibited approximately 50% of the mycelial growth, whereas the pathogen did not develop at all in the presence of GE at a concentration of 1000 µg $cm.^{-3}$ The antifungal property of GE against *P. cryptogea* was attributed to the presence of the active ingredient 7-geranoxycoumarin in the grapefruit extract (Orlikowski, 2001a; Orlikowski *et al.,* 2001b).

Ajoene, a secondary metabolite derived from garlic (*Allium sativum*), inhibited spore germination of some fungi including *Alternaria solani* and other *Alternaria* spp., *Collectotrichum* sp., *Fusarium oxysporum* and other *Fusarium* spp. that cause serious diseases in some important crop plants in India (Singh *et al.*, 1990). Saponins from *Mimusops elengi* and *M. littoralis* seeds and crude extract of *Ammi majus* were 86-100% effective against *Phytophthora palmivora in vitro* and the saponins were 100% effective against *Colletotrichum capsici* (Johri *et al.*, 1994). Encouraging results were obtained in two years of field trials using these products for control of the pathogens on Piper beetle. No phytotoxicity was observed.

Bae *et al.* (1997) isolated an anti-fungal secondary metabolite, flavonol diglycoside, from leaves of *Phytolacca Americana L.* and identified the compound as kaempferol-3-O-beta-D-apiofwanosyl-(1<rightarrow>2)-beta-D-lucopyranoside by spectral analyses. The compound exhibited significant anti-fungal activity against *Botrytis cinerea, Botryosphaeria dothidea* and *Colletotrichum gloeosporioides (Glomerella cingulata)*.

Curir *et al.* (2003) investigated the phenol compositions of two cultivars of carnation (*Dianthus caryophyllus*) namely "Gloriana" and "Roland", which were partially and highly resistant, respectively, to *Fusarium oxysporum f. sp. Dianthi.* The aim was to determine if endogenous phenols could have an anti-fungal effect against the pathogen. Analyses were performed on healthy and *F. oxysporum*-inoculated tissues *in vitro* as well as on plants *in vivo*. Two benzoic acid derivatives, protocatechuic acid (3,4-dihydroxybenzoic acid) and vanillic acid (4-hydroxy-3-methoxybenzoic acid),

were found within healthy and inoculated tissues of both cultivars, together with the flavonol glycoside peltatoside (3-[6-O-(alpha-L-arabinopyranosyl)-beta-D-glucopyranosyl] quercetin). These molecules proved to be only slightly inhibitory towards the pathogen. 2,6-Dimethoxybenzoic acid was detected in small amounts only in the inoculated cultivar "Gloriana", while the highly resistant cultivar "Roland" showed the presence of the flavone datiscetin (3,5,7,2'-tetrahydroxyflavone). The latter compound exhibited an appreciable fungitoxic activity against *F. oxysporum f. sp. dianthi.*

Recently Jin and Sato (2003) searched for secondary metabolites in aqueous extracts from succulent young shoots of pear *Pyrus* spp. Aqueous extracts of the tissue of succulent young shoots of the pear *Pyrus* spp. exhibited strong antibacterial activity against the bacterium *Erwinia amylovora*. The substance essential for the antibacterial activity was isolated from the extract by steam distillation in vacuo and through charcoal powder column chromatography. It was identified as benzoquinone (2,5-cyclohexadiene-1,4-dione) by NMR-spectra, mass spectra and HPLC analysis. In another study the aqueous extracts from bird cherry tree (*Padus avium* [*Prunus padus*]), aspen (*Populus tremula*), and celandine (*Chelidonium majus*) effectively suppressed the germination of *Puccinia triticina* [*Puccinia recondita*] uredospores. Fungitoxic activity of the extracts was attributed to the high phenolic compound content and high peroxidase activity in the leaves of these plants (Karavaev *et al.,* 2002).

Finally, Bandara *et al.* (1988) reported that steam distillates of the leaves of *Croton aromaticus* and *C. lacciferus* and root extracts of *C. officinalis* inhibited mycelial growth of *Cladosporium cladosporioides in vitro.* Root extracts of *C. lacciferus* were moderately active while those of *C. aromaticus* were inactive. Of the six compounds isolated from root extracts showing anti-fungal activity, only 2,6-dimethoxybenzoquinone obtained from the chloroform extract of *C. lacciferus* was significantly active. Comparatively small quantities of this compound were required to

inhibit growth of the pathogens: *Botryodiplodia theobromae, Colletotrichum gloeosporioides (Glomerella cingulata)* and others.

2.8 Growth regulating functions of secondary metabolites

It has already been stated that plants contain a wealth of compounds often referred to as secondary metabolites with several functions. Many of these have the potential to have growth regulatory roles that remain to be discovered. Among these types of compounds, some have been shown to affect growth. These include phytohormones, flavonoids, carbohydrates, fatty acids, peptides, phenolic acids and alcohols. Certain phytohormones play critical roles in a number of developmental and physiological processes. In addition, phytohormones and other growth regulatory substances control a wide range of other processes and in many cases, the effects of a particular hormone depend on the action of another (Hangarter, 2001).

Phytohormones function to coordinate plant growth and development. The compounds that have been considered as plant hormones are: indole-3-acetic acid (auxin), cytokinin, gibberellin, kinetin, ethylene and abscisic acid. In addition, the following substances have been shown to have important growth regulating activities and are considered to function as phytohormones: brassinosteroids, jasmonic acid and salicylic acid (Seigler, 1998).

Auxins play a role in controlling cell elongation during photo- and gravitropism. Control of cell elongation is just one of many auxin responses. Auxin is a term that refers to compounds that, when applied to plants of plant tissues, cause growth effects that are similar to those seen with indoleacetic acid. Auxins include compounds like 2,4-D and NAA (2,4-D is a common herbicide and NAA is often included in mixtures used to stimulate root formation in cutting). Auxin-regulated responses include: induction of lateral and adventitious roots, stimulation of fruit growth, apical dominance, leaf and flower abscission and DNA synthesis. As a result auxins have found several uses in

agriculture and horticulture. The auxin 2,4-D is a commonly used herbicide against broadleaf plants. NAA and IBA are often the active ingredients in products that stimulate root formation on plant cuttings. Auxins may be used to enhance fruit production or harvesting but these effects are species specific. Auxin, along with cytokinin, is used in for culturing plant tissues for mass propagation (Hangarter, 2001).

Cytokinin controls dormancy of seeds and buds. Like auxin, cytokinin is a term that describes a class of compounds. Specifically, cytokinins are compounds that stimulate cell division or cytokinesis, although they may also do other things. Proper regulation of cell division also requires auxin, which is needed to cause DNA synthesis before a cell can divide. Cytokinin responses include: cell division (cytokinesis), organ development (shoot formation), delayed senescence and promotion of chloroplast development. Cytokinin affects nutrient sink strength of organs; it promotes both lateral bud growth and cotyledon expansion (only in certain species) and inhibition of auxin-induced elongation (Rounkova, 1985).

Cytokinins have found few uses in agriculture. They are used in plant tissue culture and they have been used to delay senescence. Possible future applications will depend on clever bio-engineering. For example, since cytokinins are important in regulating source-sink relationships, controlled production of cytokinins in fruit could potentially lead to increased nutrient mobilization into the fruit, thus, more nutritionally valuable food (Lemaux, 1999).

Therefore, plants whose extracts contain the above compounds would be a boon to crop production especially with respect to yield increase. Intensive research is necessary to understand the growth regulatory function of these secondary metabolites.

2.9 Research activities in this study

No work has been carried out previously to explore the antimicrobial and bio-stimulatory properties of the extracts of wild garlic, *Tulbaghia violacea*, well known for its medicinal uses among the indigenous people of Southern Africa. Yet, extensive research has been done on the antimicrobial and medicinal properties of domesticated garlic (*Allium sativum*) belonging to the same family (*Alliaceae*).

Therefore, due to a lack of information in the literature pertaining to the application potential of extracts from *T. violacea* or compounds therein towards the agricultural industry, emphasis was placed on this aspect in this study. Crude extracts of aerial and below soil parts were screened for antibacterial and antifungal activity against plant pathogens as well as bio-stimulatory activity (Chapter 3). The antifungal activity of crude extracts was also tested *in vivo* against *Mycosphaerella pinodes* under greenhouse conditions (Chapter 4) as well as against sorghum loose and covered smuts (Chapter 5) under field conditions. The single activity that showed the highest potential, namely the antifungal property, was chosen to follow activity directed isolation and purification of the active compounds involved. Only the compound(s) that showed the highest activity towards the chosen biological property were purified, by means of standard chromatographical methods and the molecular structure(s) elucidated by means of nuclear magnetic resonance (NMR) and mass spectroscopy (Chapter 6). An intergraded discussion on all the aspects investigated in this study is presented in Chapter 7.

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CHAPTER 3

SCREENING OF *Tulbaghia violacea* CRUDE EXTRACTS FOR ANTIMICROBIAL AND BIO-STIMULATORY ACTIVITIES

Abstract

Extracts from certain plants possess antimicrobial and bio-stimulatory properties and have the potential to be exploited as natural products in the control of plant diseases or to promote crop growth. This study was conducted to investigate the bio-stimulatory and antimicrobial properties of crude methanol extracts of *Tulbaghia violacea*. The bio-stimulatory potential of the extracts was evaluated *in vitro* by following their effect on the respiration rate of a monoculture yeast cells, the germination of Cress seeds as well as radicle and coleoptile growth of Cress seedlings. The crude aerial and below soil part extracts of *T. violacea* revealed significant bio-stimulatory activity on the respiration rate of yeast cells as well as radicle and coleoptile growth of Cress seedlings, but had no effect on the germination of the seeds. The crude extracts also showed some *in vitro* antibacterial activity against four of the seven bacteria tested but significant (P<0.05) antifungal activity against six economically important phytopathogenic fungi. The crude extracts outperformed the standard fungicide used as a positive control and completely inhibited the mycelial growth of five of the six fungi, including the highly resistant *Pythium ultimum.*

3.1 Introduction

Crude extracts of some plants belonging to the family *Alliaceae*, commonly referred to as *alliums* (*Allium cepa* and *A. sativum*), have been reported to possess antimicrobial activity and in some cases biologically active compounds have been identified (Mala *et al.,* 1998; Singh and Navi, 2000; Dwivedi and Shukla, 2000; Sharma *et al.,* 2001; Rahman *et al.,* 2001; Wang and Ng, 2001; Upadhyay *et al.,* 2001; Reddy *et al.,* 2002). Fungicidal properties of garlic extracts have been demonstrated against fungi that cause damping off diseases in plants, e.g. *Fusarium oxysporum, Rhizoctonia solani* and *Pythium species* (Kurucheve and Padmavathi, 1997; Zaman *et al.,* 1997; Horberg, 1998; Raja and Kurucheve, 1999; Sinha and Saxena, 1999; Saniewska and Zuradzka, 1999). A number of authors have also reported on the antibacterial properties of garlic (*Allium sativum*) extracts (Burton, 1990; Maidment *et al.,* 1998; Khan and Omoloso, 1998; Angulo and Gomez, 1998; Arora *et al.,* 1999; Kirubaharan *et al.,* 1999; Avato *et al.,* 2000; Qiao *et al.,* 2001).

Moreover, crude extracts of some plants applied to agricultural and horticultural crops have been reported to initiate flower and bud formation and to enhance plant growth and development (Heftman, 1975a, 1975b; Mandava, 1979). Recently, Ferreira and Lourens (2002) demonstrated the effect of a liquid seaweed extract (now trading as a natural product under the name Kelpak) on improving the yield of canola. It significantly increased canola yields when applied at the three-leaf growth stage.

Despite the above reports, relatively little information on the antimicrobial and no information on the bio-stimulatory activities of *Tulbaghia violacea* could be traced in the literature. Consequently, crude methanol extracts of different plant parts of *T. violacea* were screened for these activities. The antifungal properties of crude extracts were determined *in vitro* against six common South African plant pathogenic fungi, selected from different taxonomic groups. These included: *Botrytis cinerea* Pers.: Fr. (Hyphomycetes), *Fusarium oxysporum* Schlechtend.: Fr. (Hyphomycetes),

Sclerotium rolfsii Sacc. (Agonomycetes), *Rhizoctonia solani* Kühn (Agonomycetes), *Botryosphaeria dothidea* (Moug.: Fr.) Ces&De Not. (Loculoascomycetes) and *Pythium ultimum* Trow (Oömycetes).

In vitro screening for antibacterial properties was performed on six plant pathogenic bacteria including: *Clavibacter michiganense pv. michiganense, Pseudomonas syringae, Erwinia carotovora, Agrobacterium tumefaciens, Ralstonia solanacearum, Xanthomonas campestris pv phaseoli* and one human bacterium, *Moraxella catarrhalis.* Additionally, the bio-stimulatory activity of the methanol extracts was investigated.

3.2 Materials and Methods

3.2.1 Plant material

Whole *Tulbaghia violacea* plants were initially collected from the Blyde River Canyon Nature Reserve (BRC) South Africa. The taxonomic identification of the species was performed by a taxonomist from the National museum, Bloemfontein, South Africa. A voucher specimen was processed according to standard procedures and deposited in the herbarium of the museum. Bulk samples of the species were later collected from the Botanical Gardens, Bloemfontein (29º 07´S, 26º 11´N) between January and March 2001, 2002 and 2003.

3.2.2 Other material

Potato dextrose agar (PDA), plate count agar (PCA) and nutrient broth were purchased from Merck (Germany). All organic solvents used were from Sigma (Germany) and of the purest grade available.

3.2.3 Methods

3.2.3.1 Preparation of the plant material

Health and laboratory safety standards were followed in the handling of the unknown material as it was assumed to be dangerous. Plant material was examined beforehand for any form of infection or insect damage and such infected material was not used. Plants were divided into (a) aerial parts (stems and leaves) and (b) below soil parts (rhizome and roots). After the fresh mass of the different plant parts was determined, the plant material was dried in an oven for two weeks at 35 ºC and the dry mass determined. Subsequently, dried plant material was ground, using a Retsch SM 2000 cutting mill and the dry mass determined again. After grinding, small representative amounts of each sample were transferred into plastic Ziploc bags, sealed and stored in a freezer at -20 °C until crude extracts were prepared. Percent loss during grinding was calculated as follows:

$$\text{Percent loss at grinding} = \frac{(\text{net mass of dry sample}) - (\text{net mass of ground sample}) \times 100}{(\text{net mass of dry sample})}$$

3.2.3.2 Preparation of crude extracts

The two ground samples (aerial and below soil parts) were transferred into separate 5-litre Qorpak jars labelled and covered with 100% methanol at a ratio of two ml g^{-1} dry weight. The lids were closed firmly, sealed with parafilm to prevent leakage and placed on a roller mill for 24 hours (Fig. 3.1). Extraction was performed twice by replacing the methanol. Subsequently, each sample was filtered twice, first under vacuum through a double layer of Whatman filter paper (No. 3 and No.1) using a Buchner funnel and then by gravity through a single sheet of Whatman No.1 filter paper.

Most of the methanol was removed from the extracts by means of vacuum distillation at 35 - 40 °C using a Büchi Rotary Evaporator (Fig. 3.2).

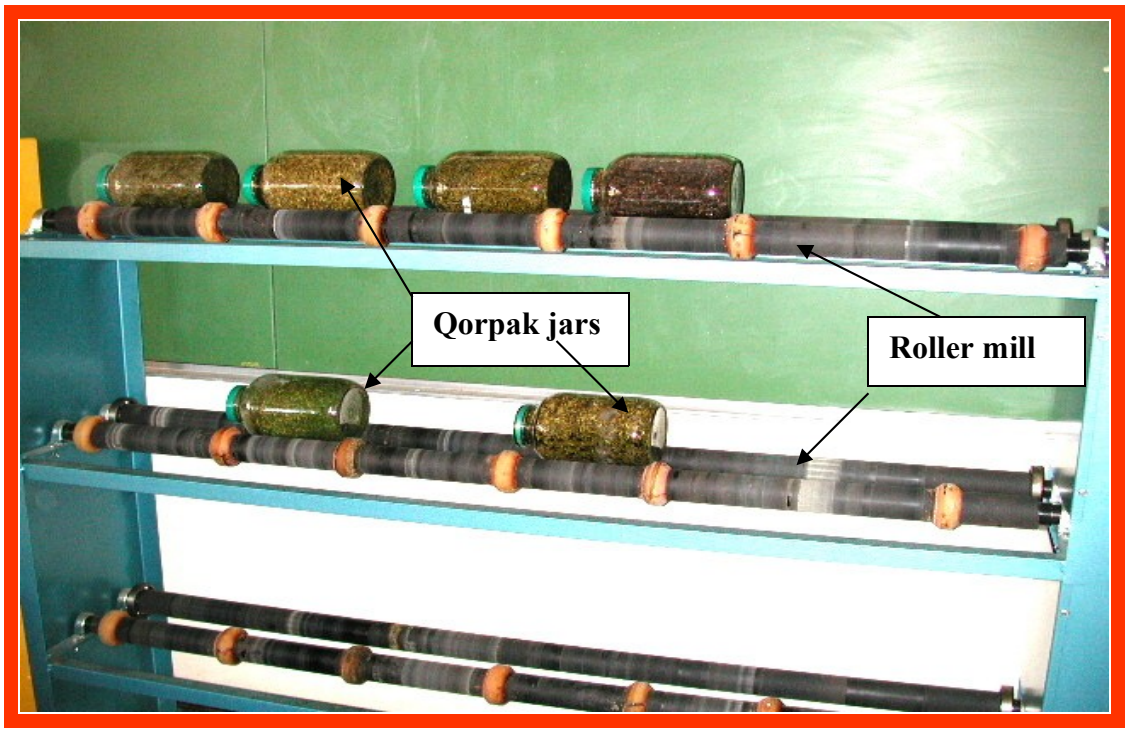

Figure 3.1: Extraction on roller mill.

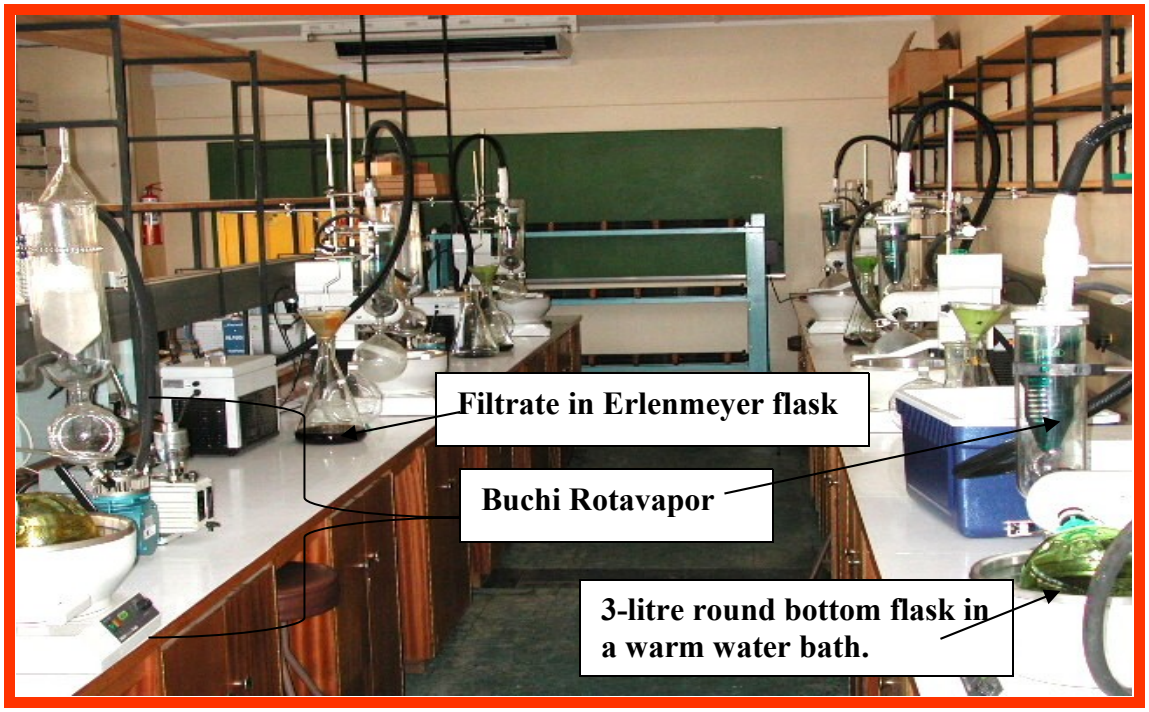

Figure 3.2: Distillation

On the following day the same procedure was followed with the re-extracted plant material. Final filtrates from the twice extracted plant material were combined and concentrated to dryness under vacuum by means of a Speedvac Concentrator at -140 °C for 24 hours (Fig. 3.3).

Figure 3.3: Vacuum drying of crude extracts.

After determining the dry matter yields, the crude aerial and below soil parts were stored separately at -20 °C for later use.

3.2.3.3 Screening crude methanol extracts of *T. violacea* for antibacterial activity

(a) Preparation of bacteria mother cultures.

Antibacterial activity was qualitatively evaluated by means of the agar plate diffusion assay technique (Rios *et al.,* 1988). Plate count agar (PCA) was used to prepare mother cultures of the following six plant pathogens and one human bacterium in advance: *Clavibacter michiganense pv. michiganense, Pseudomonas syringae pv syringae, Erwinia carotovora subsp carotovora, Agrobacterium tumefaciens, Ralstonia solanacearum, Xanthomonas campestris pv phaseoli,* all plant pathogens,

as well as *Moraxella catharrhalis (MC)* a human pathogen. Two stock cultures were prepared for each bacterium of which one was a working culture and the other a back-up culture. Cultures were stored at 5 – 15 °C, except for *Moraxella* that was stored at 4 °C.

From the bacteria mother cultures, seven nutrient agar (NA) plates were separately inoculated with the seven bacteria two days prior to the anti-bacterial bioassay. These were incubated at 25 °C, except for the *Moraxella* plate which was incubated at 35 ºC one day prior to bioassay in order to obtain pure line colonies. All cultures were checked for contamination.

After two days, the uncontaminated pure colonies of the 2-day old bacteria cultures were transferred from the agar plates into seven labelled test tubes containing sterilized distilled water, in the case of the plant pathogens, and sterilized saline water in the case of *Moraxella.* Diluted bacteria suspensions were compared to the McFarland standard in order to obtain the required concentration of 1×10^6 CFUs (colony forming units) per ml.

(b) Antibacterial bioassay

Five grams nutrient agar was dissolved in 200 ml distilled water, autoclaved at 121 °C for 20 minutes and transferred into seven Petri-dishes to cool and set. The crude extracts were assayed as aqueous suspensions in 10% (v/v) DMSO (dimethyl sulphoxide) at a concentration of 50 mg ml^{-1} by transferring a maximum of 40 µl into 5 mm holes made in the agar with a sterile cork borer (Figure 3.4). All activities were carried out in a pre-sterilized laminar flow cabinet to avoid contamination.

Each test plate was divided into five parts (Figure 3.4) and the extracts as well as the 10% (v/v) DMSO solution (positive control) transferred into the holes in the agar. Plates were left for 20 minutes to allow the extracts and DMSO to diffuse into the agar

and subsequently incubated at 25 °C (plant pathogens) or 35 °C (*Moraxella*) for three days.

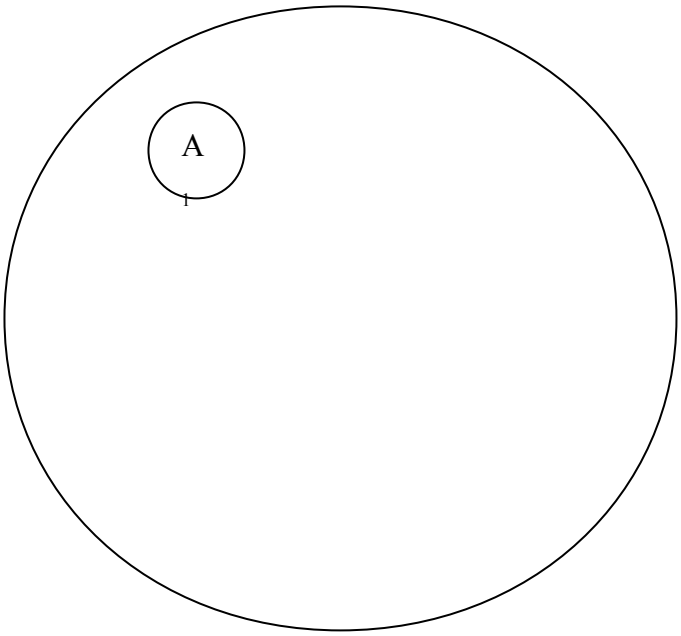

Figure 3.4: Placement of crude extracts or DMSO (10%; v/v) in holes made in nutrient agar plates. A_1 = Aerial part extract replication 1, A_2 = Aerial part extract replication 2, B_1 = Below soil part extract replication 1, B_2 = Below soil part extract replication 2 and C = 10% (v/v) DMSO.

Each plate was inoculated with the different bacterial suspensions using sterile swabs and spread evenly over the plate. After three days, the zones of inhibition were measured using a digital calliper, excluding the hole (Heisey and Gorham, 1992), and used as a qualitative indicator for antibacterial activity.

3.2.3.4 Screening crude methanol extracts of *T. violacea* for antifungal activity

A modified agar dilution method (Rios *et al.,* 1988) was used for determining the mycelial growth inhibition of the test organisms by the crude extracts.

(a) Preparation of fungus mother cultures.

Two percent malt extract agar (4g Malt extract; Difco, 2.8g Technical agar; Difco) was prepared in 200 ml distilled water and autoclaved for 20 minutes at 121 °C. Subsequently, the media was cooled in a water bath at 45 °C and 60 µl of a 33% (m/v) Streptomycin solution added to the basal medium for controlling bacterial growth. The agar medium was then transferred to Petri dishes and allowed to set. Two mother cultures for each fungus were prepared for the following six plant pathogenic fungi: *Botrytis cinerea, Fusarium oxysporum, Sclerotium rolfsii, Rhizoctonia solani, Botryosphaeria dothidea* and *Pythium ultimum.*

An inoculum of each fungus was placed face down on the agar media in separate Petri dishes (two replications) and incubated at 25 °C for 8 to 10 days. Subsequently, 12 glass culture bottles were half filled with distilled water and autoclaved for 20 minutes at 121 °C. Pieces of agar containing the organisms from a pair of Petri dishes (two replications per fungus) were then transferred to the autoclaved culture bottles, sealed with parafilm and stored at 4 °C, except for *Rhizoctonia solani* and *Pythium ultimum* that were stored at 25 °C.

(b) Bioassay

Two percent malt extract agar (MEA) was prepared as described for the preparation of mother cultures. 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} (1 g L^{-1}). The medium, also containing 33% Streptomycin, was transferred to 90 mm sterile plastic Petri dishes and left to set. The centre of each test plate was subsequently inoculated with a 5 mm size plug, for each of the pathogens separately, and incubated for three days at 25 ± 2 °C in a growth cabinet. Radial mycelial growth was determined after three days by calculating the mean of two perpendicular colony diameters on each replicate

(three replicates per organism). An inoculated plate for each pathogen, containing only the basal medium, served as control.

The means of the two measurements were used to calculate percent (%) inhibition (Fig. 3.5):

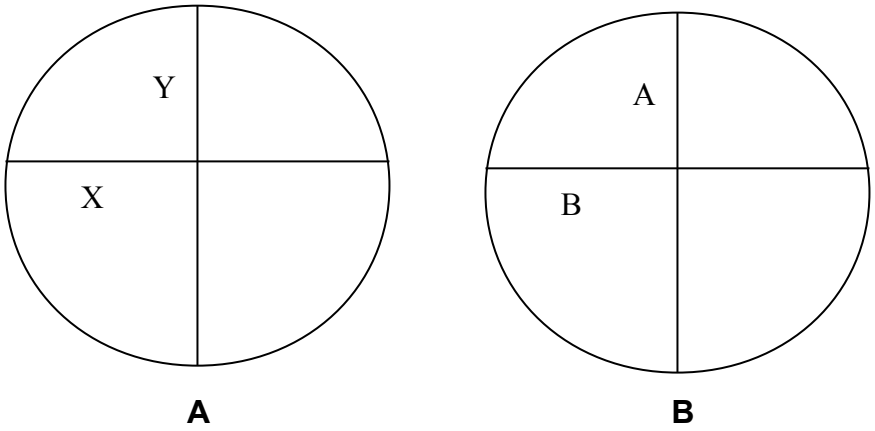

Figure 3.5: **A** = Control plate containing the basal medium only and **B** = Test plate containing an extract to determine the possible % inhibition of mycelial growth by an extract.

$$\text{MEA mean (control)} = \frac{X + Y}{2}, \qquad \text{EXTRACT mean} = \frac{A + B}{2}$$

$$\text{Percent inhibition} = \frac{(\text{MEA mean} - 5) - (\text{EXTRACT mean} - 5)}{(\text{MEA mean} - 5)} \times 100$$

Additionally, a plate containing 1µg ml $^{-1}$ Carbendazim/difenoconazole (Eria $^{®}$ - 187.5g L $^{-}$1 EC) was used as standard fungicide against each test organism separately to determine the effectiveness (expressed as % inhibition) of the extracts by comparison. Each treatment was performed in triplicate.

3.2.3.5 Screening crude methanol extracts of *T. violacea* for biostimulatory activity

Two methods were applied to determine the biostimulatory potential of the organ crude extracts of *T. violacea*:

Method 1: Manometric method to determine the effect of the crude extracts on the respiration rate of a monoculture yeast cells.

A specially constructed glass respirometer with a short bulged section (reservoir) to contain the yeast cells and a long calibrated tube, closed at the top end to collect CO_2 gas, was used to determine the effect of the crude organ extracts (aerial and below soil parts) of *T. violacea* on the respiration rate of yeast cells. Dry baker's yeast (0.8 g) was placed in the reservoir of the respirometer. Subsequently, 70 ml of each of the organ extracts previously prepared at a concentration of 0.5 mg ml^{-1} and containing 5 mg ml^{-1} glucose to serve as respiratory substrate for the yeast cells, was added to the respirometer. The apparatus was tilted sideways to release air bubbles trapped in the dry baker's yeast and placed in a water bath preheated to 29 °C. ComCat® a commercial bio-stimulant, was used as a positive control at 0.5 mg L^{-1} (optimum concentration according to the manufacturers; Agraforum, Germany) and distilled water was used as a second control. Carbon dioxide released by the yeast cells was measured in cm^3 at 30 minute intervals over a three-hour incubation period by reading the released gas volume from the calibrated tube. Tests were performed in triplicate.

Method 2: The effect of different organ extracts on the percentage germination of Cress seeds and subsequent seedling growth.

Two sheets of special germination paper (30 x 30 cm) were used to test the effect of the crude organ extracts of *T. violacea* on the germination of Cress seeds as well as the subsequent seedling growth. A line, 10 cm from the top was drawn on the one sheet and 20 Cress seeds spaced evenly on the line. A second sheet of germination paper was placed on top of the first and moistened with either 0.5 mg ml^{-1} solutions of the crude extracts, distilled water (negative control) or a 0.5 mg L^{-1} solution of ComCat® (positive control). Both sheets of paper were rolled up longitudinally and placed upright in Erlenmeyr flasks containing either the aerial or below soil part crude extracts, distilled water or the ComCat® solution and kept at 25 °C in a growing

chamber in the dark. Seed germination as well as coleoptile and root lengths were determined at 24 hours interval over a four-day incubation period. Tests were performed in triplicate.

3.2.3.6 Statistical analysis of data

Analysis of variance (ANOVA) was performed on the data, using the NCSS 2000 Statistical program, to identify differences between treatments. Tukey's mean significant difference (MSD) procedure for comparison of means (Steele and Torrie, 1980) was applied to separate means ($P<0.05$).

3.3 Results

3.3.1 Recovery of crude extract from dried plant material

After drying and grinding, 7.96% (w/w) aerial part dry material and 26.1% (w/w) below soil part dry material was obtained from the original fresh *T. violacea* material collected (Table 3.1). After extraction, 14% (w/w) dry crude material was recovered from the dried aerial part material while 6.7% (w/w) dry crude material was recovered from the dried below soil part material.

TABLE 3.1: Recovery of crude extract from dried plant material

Plant Organs	Fresh Mass(g)	Mass(g) after drying	Mass(g) after grinding	Mass(g) of methanol extract	% Recovery of crude extract from dry material
Aerial parts	16032.1	1284.8	1275.9	179.14	14
Below soil parts	16864.8	4529.0	4392.8	293.46	6.7

3.3.2 *In vitro* antibacterial activity of *T. violacea crude extracts*

Out of the six phytopathogenic bacteria used as test organisms, the growth of three plant pathogens (*C. michiganensis, R. solanacearum, X. campestris*) were significantly inhibited by both below soil and aerial part crude extracts, the results for *M. catharrhalis* are not shown (Table 3.2).

TABLE 3.2: *In vitro* growth inhibition of plant pathogenic bacteria by crude extracts of different *Tulbaghia violacea* organs.

	Mean Inhibition Zone Diameter (mm)					
	Bacteria					
Extracts	*Clavibacter michiganensis*	*Pseudomonas syringae*	*Erwinia caratovora*	*Agrobacterium tumefaciens*	*Ralstonia solanacearum*	*Xanthomonas campestris*
Aerial part	8.7 ± 1.7a	0 ± 0a	0 ± 0a	0 ± 0a	9.8 ± 1.2ab	12.2 ± 2.4ab
Below Soil part	7.3 ± 1.1a	0 ± 0a	1 ± 1.41a	0 ± 0a	15.4 ± 0.7a	18.2 ± 0.23a
DMSO Control	0 ± 0b	0 ± 0a	0 ± 0a	0 ± 0a	0 ± 0c	0 ± 0c

The other three bacteria were much more resistant to treatment with the crude extracts and were not affected by the extracts at all.

3.3.3 Antifungal activity of *T. violacea* crude extracts

The below soil part extract of *T. violacea* completely inhibited (100%) the mycelial growth of *B. cinerea, S. rolfsii* and *P. ultimum* and showed a very high inhibition (> 95%) for both *F. oxysporum* and *R. solani*. The lowest inhibition (90%) observed was

against *B. dothidea* (Figure 3.6). Mycelial growth inhibition by the aerial part extract was also very high (>95%) for *B. cinerea, S. rolfsii* and *P. ultimum* and >80% for *R. solani and B. dothidea. Fusarium oxysporum* (75% inhibition) was most resistant against treatment with the aerial part extract.

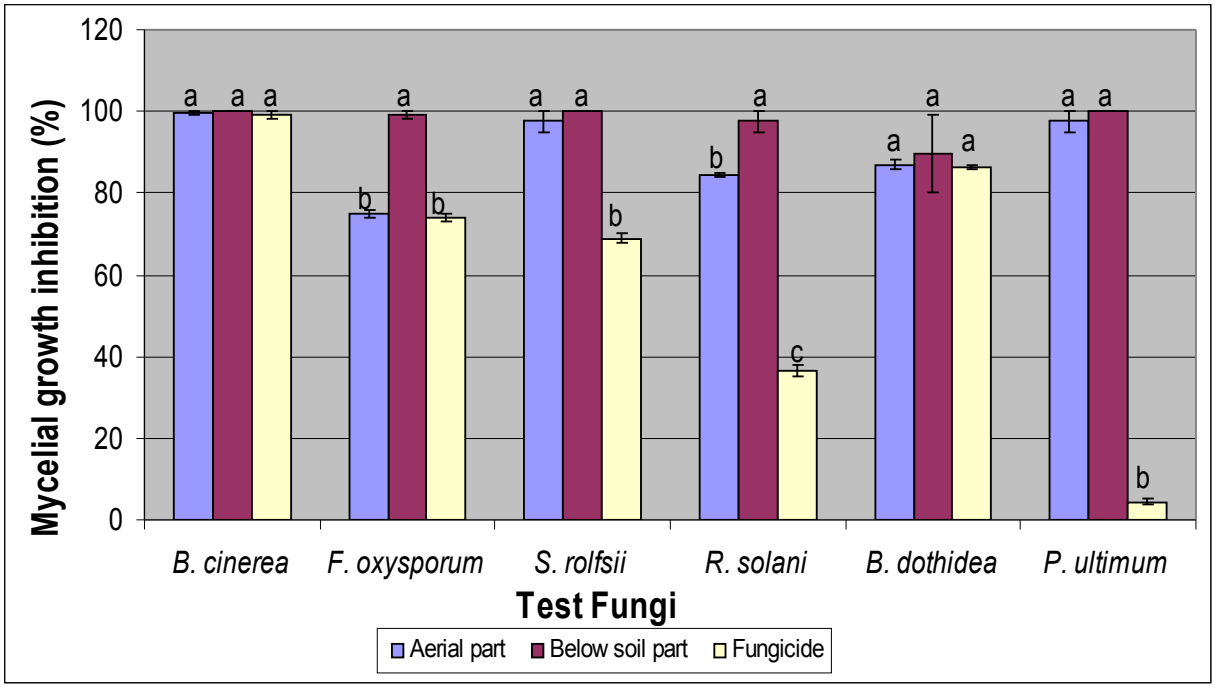

Figure 3.6: Percentage (%) *in vitro* growth inhibition of plant pathogenic fungi by fresh crude methanol extracts of *T. violacea* at a concentration of 1 g $L.^{-1}$ Bars designated with different letters for each fungus differed significantly (P<0.05) according to Tukey's Mean Significant Difference (MSD) statistical procedure.

Statistically, both the aerial and below soil part crude extracts of *T. violacea* outperformed the standard fungicide significantly (P<0.05) in terms of mycelial growth inhibition of four out of the six test fungi. However, both extracts compared favourably with the standard fungicide in terms of mycelial growth inhibition.

Figure 3.7 illustrates the antifungal activity of the same two crude extracts of *T. violacea* after storage at -20 °C for one year. Compared to the standard fungicide, a decrease in the antifungal activity of >30% was observed for both the aerial and below soil part crude extracts stored in a freezer at -20 °C for one year.

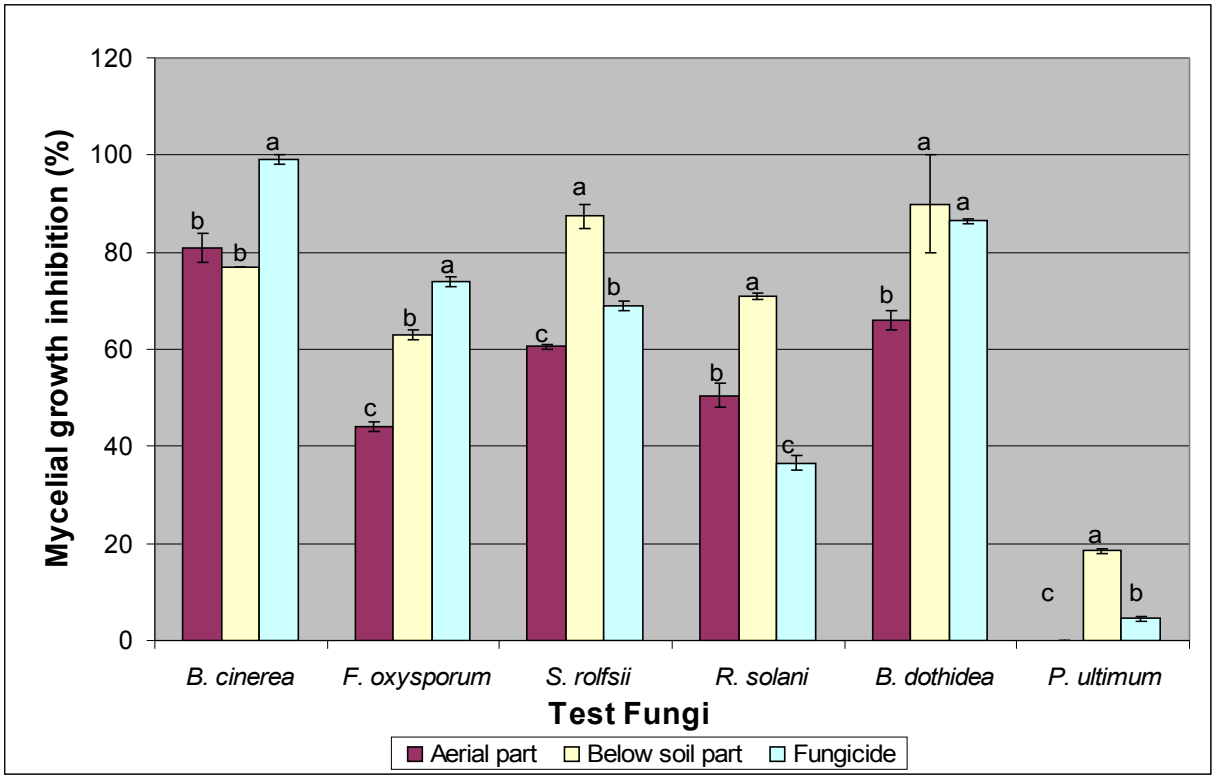

FIGURE 3.7: Percentage (%) *in vitro* growth inhibition of plant pathogenic fungi by crude methanol extracts of *T. violacea,* at a concentration of 1g $L,^{1-}$ after storage at -20 °C for one year. Bars designated with different letters for each fungus differed significantly (P<0.05) according to Tukey's Mean Significant Difference (MSD) statistical procedure.

3.3.4 Biostimulatory activity of *T. violacea* crude extracts

Both the aerial and below soil part crude extracts of *T.* violacea increased the respiration rate of a monoculture yeast cells markedly over the first 90 minutes of incubation (Fig. 3.8) compared to both the water and the ComCat® controls. However, after 120 minutes of incubation the differences in respiration rate were less pronounced as the maximum rate was probably reached. Although the commercial bio-stimulant, ComCat ® also had an increasing effect on the respiration rate of yeast cells, this was not as marked as the effect of both crude extracts of *T.* violacea.

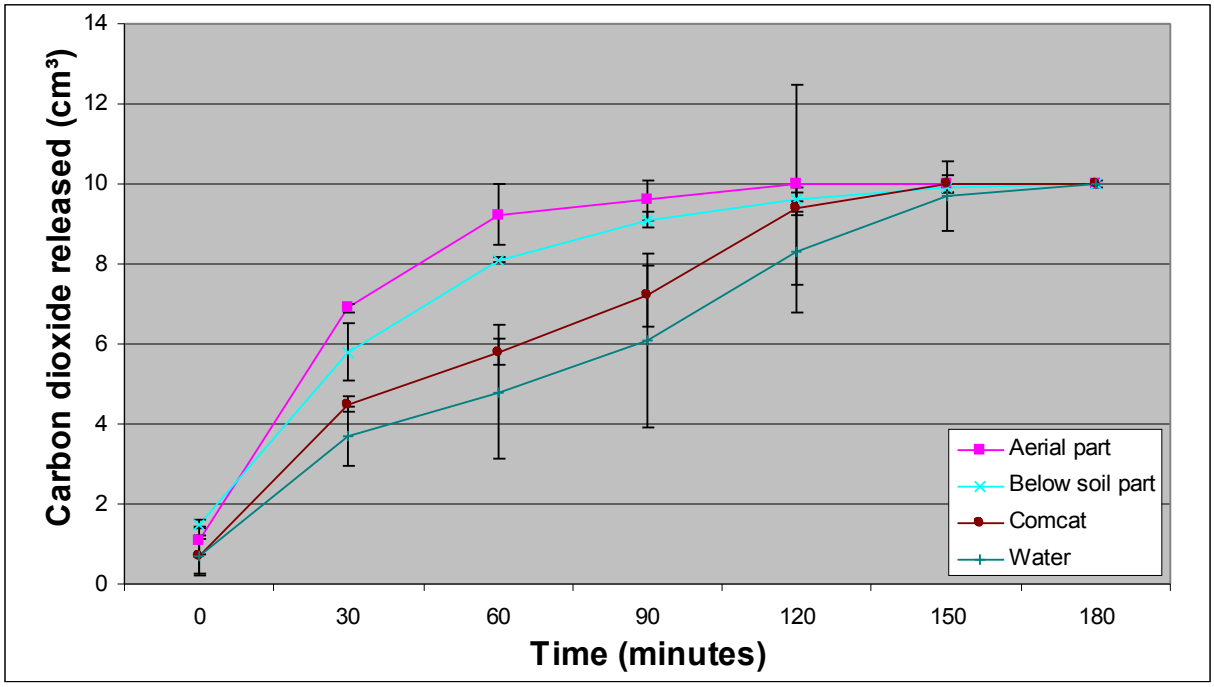

Figure 3.8: The effect of crude aerial and below soil part extracts of *T. violacea* on the respiration rate of a monoculture yeast cells at 30 minute intervals over a three-hour period. ComCat ® and distilled water served as controls.

Statistically no significant differences ($P<0.05$) in the percentage germination of Cress seeds between treated and non-treated seeds were observed (Table 3.3).

TABLE 3.3: Mean percent (%) germination of Cress seeds, treated with aerial and below soil part extracts of *T. violacea,* at 25 °C over a 96 hour incubation period. ComCat ® and distilled water served as controls.

Mean % Germination			
Aerial parts	Below soil parts	ComCat®	Water
68.33 ± 8.5a	71.67 ± 10.27a	71.67 ± 6.24a	63.33± 10.27a

However, both the aerial and below soil part crude extracts of *T. violacea* had a significant stimulatory effect on both coleoptile and root growth of Cress seedlings when compared to the water control. There was no significant difference in bio-stimulatory activity between the extracts and the commercial biostimulant ComCat.® (Fig. 3.9 and 3.10).

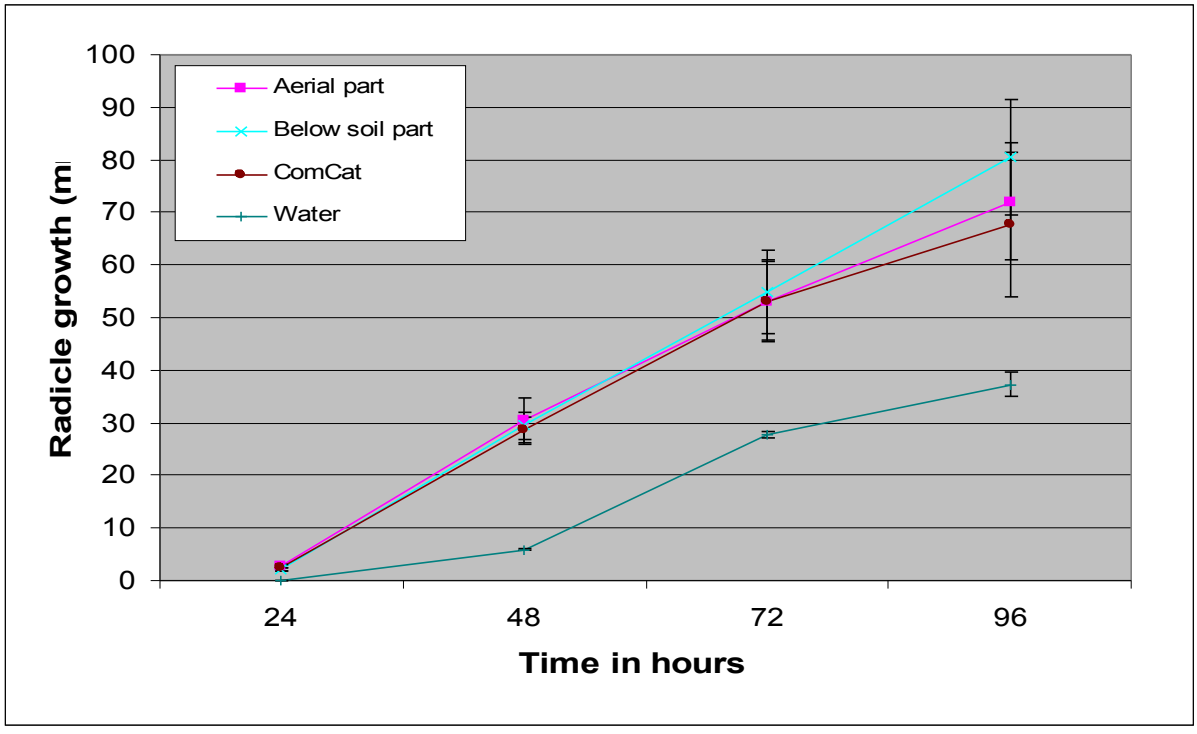

Figure 3.9: The effect of crude aerial and below soil part extracts of *T. violacea* on radicle growth of Cress seedlings at 24 hour intervals over a 96-hour incubation period. ComCat® and distilled water served as controls.

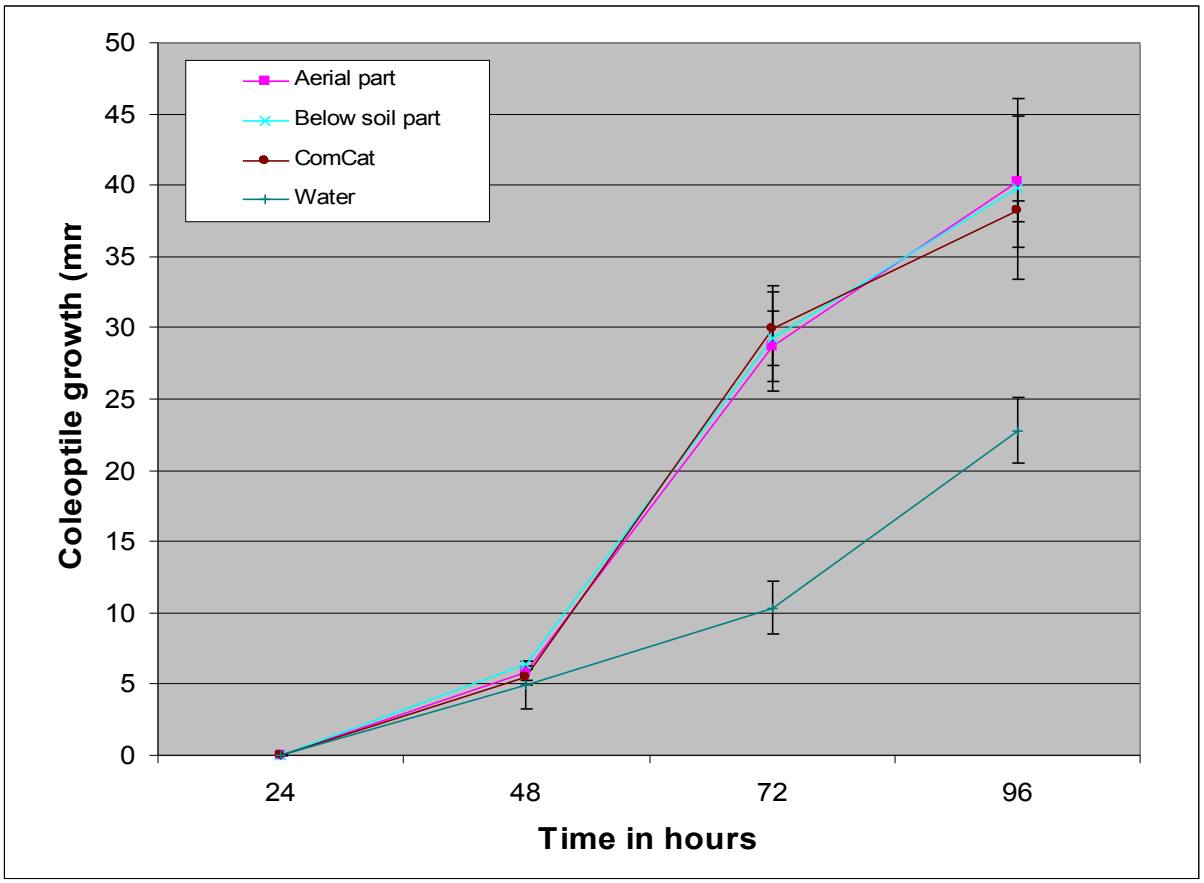

Figure 3.10: The effect of crude aerial and below soil part extracts of *T. violacea* on coleoptile growth of Cress seedlings at 24 hour intervals over a 96-hour incubation period. ComCat® and distilled water served as controls.

3.4 Discussion

Recently antibacterial activity of the extracts of garlic (*Allium* sativum) and *T. violacea* have been demonstrated against the human pathogenic bacteria *Staphylococcus aureus*, *Bacillus subtilis* and *Streptococcus pneumoniae* (Kyung and Lee, 2001; Gaidamashyili and van Staden, 2002; Dawit *et al.,* 2002). In this study both aerial and below soil part crude extracts of *T. violacea* significantly inhibited the growth of three phytopathogenic bacteria; *C. michiganensis, R. solanacearum* and *X. campestris, in vitro,* while the other three test bacteria were much more resistant and were not

affected by the extracts at all. Although Mangamma and Screeramulu (1991) and Abd *et al.* (2002) have demonstrated *in vivo* antibacterial activity of garlic (*Allium sativum*) crude extracts against plant pathogens, no indication of any reports on the antibacterial activity of wild garlic (*T. violacea*) against plant pathogenic bacteria could be traced in the literature. Nevertheless, further studies on plant bacteria were terminated and attention turned to the more effective antifungal activity of the extracts.

The *in vitro* fungitoxic effects of both the aerial and below soil part crude extracts of *T. violacea* were significantly higher than that of the synthetic fungicide. All test fungi were sensitive to the extracts emphasizing their broad spectrum potential. These results agree with the findings of Harris *et al.* (2001); Lutomski (2001) and Dhaliwal *et al.* (2002) and confirmed the 100% inhibition of the mycelial growth of *R. solani* by crude boiled and acetone extracts of *Allium sativum* reported by Kane *et al.* (2002). Many recent reports were traced in literature that confirmed the fungitoxic effect of crude extracts from domesticated garlic against plant pathogenic fungi, including *F. oxysporum, Pythium* species and *R. solani* (Zaman *et al.*, 1997; Kurucheve *et al.*, 1997; Horberg, 1998; Sinha *et al.*, 1999; Raja and Kurucheve, 1999; Saniewska *et al.*, 1999;). It has also been shown that garlic extracts compared favourably with some commercial fungicides in reducing disease intensity and increasing crop yield (Yadav *et al.,* 1998; Raja, *et al.,* 1999). In light of the fact that *T. violacea* is easy to grow and can endure neglect for many years; it is a strong candidate for large scale cultivation as a donor plant in the event that the production of a natural fungicide is considered.

The decline in the fungitoxicity of one-year old *T. violacea* extracts stored at -20 °C is environmentally desirable as indications are that it would present no residual threats under field conditions. Surely, this aspect will have to be verified in future. On the other hand, it provides a challenge for further research on the possibility to modify the active substances involved into synthetic analogues by introducing stable chemical structures to make them more robust. However, in light of the current emphasis on organic farming, the development of a natural fungicide from *T. violacea* extracts should strongly be considered. The fact that the aerial part extract was just as potent

as the below soil part extract in inhibiting the mycelial growth of a broad spectrum of plant pathogens, indicate that cultivated plants could be harvested in a non-destructable manner. Below soil parts can then remain in the soil for producing the next season's harvest in a sustainable manner.

Further, both the aerial and below soil part crude extracts of *T.* violacea and the commercial bio-stimulant ComCat® significantly increased the respiration rate of a monoculture yeast cells over the first 90 minutes and slowed down after 120 minutes of incubation. This means that both extracts and ComCat® stimulated the production of carbon dioxide more than water, i.e. increased the respiration rate making more energy available for germination and seedling growth. Consequently, a positive correlation seemed to exist between high respiration rate and seedling growth as both organ crude extracts significantly stimulated radicle and coleoptile growth of Cress seedlings over a period of 96 hours compared to the water control.

Comparable results were obtained by Neelam *et al.* (2002) who showed that lower concentrations of aqueous extracts of fresh leaves, flowers and pods of *Leucaena leucocephala* stimulated the seed germination and seedling growth of *Triticum aestivum*, while Terefa (2002) demonstrated that extracts from flowers, roots and stems of *Parthenium hysterophorus*, a weed, had a stimulatory effect on seedling growth of tef (*Eragrostis tef*) and root extracts, at a low concentration, greatly promoted root length. In this study no significant difference between the crude extracts and ComCat® in stimulating seedling growth was observed, bringing the *T. violacea* extracts on par with the commercial bio-stimulant.

Statistically ($P<0.05$), Cress seed germination showed no significant response to both plant organ extracts compared to both the ComCat® and water controls. Channal *et al.* (2002) obtained similar results when they tested the bio-stimulatory effects of fresh leaves of seven tree species on seed germination and seedling growth of pigeon pea and green gram. In pigeon pea, none of the leaf extracts showed significant effects on germination, but seedling length was increased.

Even though the literature revealed very scanty information on the bio-stimulatory effect of extracts from species belonging to the *Alliaceae* family, and none for *T. violacea*, this study revealed that the extracts of *T. violacea* indeed possess bio-stimulatory properties, especially with respect to the enhancement of seedling growth. The latter provides a challenge for full scale research on this issue in order to additionally evaluate the application potential under field conditions.

In summary, the above average broad spectrum antifungal activity of crude *T. violaceae* extracts, compared to the less convincing antibacterial activity and least documented bio-stimulatory properties, lead to the decision to explore the former property more comprehensively. Moreover, *in vitro* antifungal data alone is not sufficient to conclude that a specific plant extract either possesses the potential to be equally effective *in vivo* or the potential to be developed as a natural product with application potential under field conditions. Subsequently, this aspect was investigated under both glasshouse (Chapter 4) and field (Chapter 5) conditions.

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CHAPTER 4

IN VIVO CONTROL OF *Mycosphaerella pinodes* ON PEA (*Pisum sativum*) LEAVES BY EXTRACTS OF *Tulbaghia violacea.*

Abstract

The *in vitro* antifungal activity of crude extracts from both aerial and below soil parts of *T. violacea* against six economically important plant pathogenic fungi was previously reported (Chapter 3). A crude methanol aerial part extract of *T. violacea* was subsequently tested against *Mycosphaerella pinodes,* the cause of black spot or *Ascochyta* blight in peas (*Pisum sativum*). Fourth internode leaves were removed from four week old pea plants, placed on moist filter paper in Petri dishes and inoculated with a *M. pinodes* spore suspension both before and after treatment with a crude methanol aerial part extract of *T. violacea*. The control of *Ascochyta* blight by different concentrations of the crude extract was followed *in vivo* by leaf symptoms over a six-day period at 20 °C in a growth cabinet. The crude extract prevented *M. pinodes* spore infection of the pea leaves when the leaves were inoculated with spores both before and after treatment with the extract, confirming complete inhibition of spore germination. The crude extract showed no phytotoxic reaction on the leaves, even at the highest concentration applied.

4.1 Introduction

In vitro screening of plant extracts is an important first step in identifying plants with application potential in agriculture. However, the *in vivo* confirmation of this potential is essential in the search for plant derived preparations and for considering possible commercialization (Gorris and Smid, 1995). Therefore, after confirming the activity *in vitro* of crude *T. violacea* extracts against six phytopathogenic fungi in the previous chapter, the antifungal *in vivo* effect against *Mycosphaerella pinodes* on detached pea leaves was investigated in this chapter. This is an important step towards seeking a rationale for the isolation of the active compounds involved as well as for large-scale follow-up field trials.

Ascochyta blight of pea (*Pisum sativum*) caused by *Mycosphaerella pinodes* (Berk & Blox.) Vesterger, occurs worldwide and causes yield losses of up to 30% (Punithalingam & Holliday, 1972; Lawyer, 1984). It is a disease of economic importance and can cause serious yield reduction of peas grown for both human and animal consumption (Kraft *et al.,* 1998). It is a major constraint to field pea production (Xue, 2000) and, according to Tivoli and Lemarchand (1992), is the most destructive foliar pathogen of pea in France. The fungus infects pea seedlings as they emerge, causing girdling stem lesions. Infection of stems results in small brown streaks that later turn blue-black in colour which reduce field pea populations and increase lodging. Necrotic lesions develop on all aerial parts of the pea plant, including the pods, grown from contaminated seeds. (Ryan *et al.,* 1984; Parry, 1990; Roger *et al.,* 1999a; Béasse *et al.,* 2000; Xue, 2000).

Mycosphaerella pinodes is spread via pycnidiospores throughout the season (Banniza *et al.,* 2003). After germination of spores, the fungus grows over the plant surface before forming an aspersorium and penetrating the cuticle (Clulow *et al.,* 1991a). Symptoms may appear as early as 24 hours after infection under optimal conditions and are characterized by brown to purplish, coalescing lesions on aerial tissue (Roger *et al.,* 1999a). Non-germinated spores remain viable for up to 21 days under dry conditions (Banniza and Vandenberg, 2003). Infection and disease development are highly dependent on temperature and leaf wetness (Roger *et al.,* 1999b). During germination, and before penetration, the fungus is sensitive to dryness (Relative humidity ≤70%), resulting in reduced disease development (Banniza *et al.,* 2003).

The current control methods mainly involve the use of fungicides (Parry, 1990). There are no pea cultivars with effective field resistance to *Ascochyta* blight (Wroth, 1998; Warkentin *et al.,* 2000*;* Xue and Warkentin, 2001). Synthetic fungicides are also very expensive. According to Pretorius *et al.* (2002), compounds of plant origin are generally preferred as organic fungicides since they are regarded as environmentally

safer and hence a renewed interest in the screening of plant extracts for their application potential in agriculture. The same method used by Pretorius *et al.* (2002) to investigate the *in vivo* control of black spot (Ascochyta blight) in detached pea leaves by a crude bulb extract of *Eucomis autumnalis*, was used to assess the *in vivo* antifungal potential of *T. violaceae* crude extracts.

4.2 Materials and Methods

4.2.1 Plant materials

Whole *T. violacea* plants were initially collected from the Blyde River Canyon area in Mpumalanga, South Africa during March 2001 and the species was identified by a plant taxonomist. Later, collections of the same species were made in the Bloemfontein Botanical Gardens, Free State, South Africa. Extracts from these plants proved to be equally active and were used in follow-up studies.

4.2.2 Methods

4.2.2.1 Cultivation of pea (*Pisum sativum*) plants

One hundred *Pisum sativum* cv. Mohanderfer seeds, obtained from a local seed merchant, were sown 2 cm from the surface in 20 pots at five seeds per pot using Bainsvlei soil and applying a standard NPK fertilizer mixture. The plants were allowed to grow for four weeks in a greenhouse while maintaining the soil at field capacity. After four 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 aerial part extracts of *T. violacea* to control *Ascochyta* blight *in vivo*.

4.2.2.2 Preparation of aerial parts of crude extract of *T. violacea.*

A crude aerial part extract of *T. violacea* was prepared as previously described in Chapter 3, section 3.2.3.

4.2.2.3 Isolation of *Mycosphaerella pinodes*

Mycosphaerella pinodes 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 tissue were surface sterilized for 1 minute in 96% (v/v) ethanol, 3 minutes in a 3.5% (v/v) Sodium hypochloride 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 litre^{-1}) in 9 cm Petri dishes and incubated at 20 ± 1 ^{o}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 ^{o}C and examined under a dissecting microscope (80 x magnification). A germ tube from a pycnidiospore was severed and transferred to Coon's agar (Clulow & Lewis, 1992). 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 ^{o}C.

4.2.2.4 Preparation of a *M. pinodes* spore suspension

Oat meal agar was prepared by gently heating 30 g of oats in 1 litre distilled water for one hour, stirring frequently, and subsequently filtering through a fine sieve upon which the volume was readjusted to one litre. 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 inoculation of 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 pycnidiospores.

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 was determined by means of a haemocytometer. The pycnidiospore concentration was adjusted to 1 x 10^5 spores per ml (Nasir & Hoppe, 1997) with sterile distilled water prior to the inoculation of pea leaves.

4.2.2.5 *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 °C) (Section 4.2.2.1). Four weeks after planting, when the leaflets on the third and fourth nodes were fully expanded, three fourth node leaflets per replicate were removed from the plants, placed on Schleicher and Schull No. 595 filter paper and 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 the three leaves per Petri dish and replicated three times. Treatment of the leaves with water and a standard fungicide (Carbendazim/difenoconazole) served as controls. Petri dishes containing the treated leaflets were incubated at 20 °C in a day/night incubator programmed for a 16-hour day cycle while 2 ml sterile distilled water was added daily to keep the filter paper moistened. Six days after treatment, phytotoxicity symptoms were assessed on leaves using a six-category scale [0= symptomless; 1= < 5% necrotic flecks; 2= >5% necrotic flecks; 3= <50% of inoculated area necrotic; 4= 50-100% of inoculated area necrotic; 5= necrosis spreading beyond inoculated areas] based on stereo microscopic observations (Clulow *et al*., 1991b)].

4.2.2.6 *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 aerial part extract of *T.* violacea was followed in two ways namely, 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 the other way around. 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 °C, the optimal temperature for *M. pinodes* spore germination in a day/night incubator as illumination is necessary for spore germination (Roger & Tivoli, 1996). After incubation for six days the foliar lesions were measured and leaf damage compared to that of the controls.

4.3.2.7 Statistical analysis of data

Analysis of variance (ANOVA) was performed on the data, using the SAS statistical analysis program (SAS Institute Inc. 1999). Duncan's LSD (least significant difference) procedure for comparison of means was applied to separate means (P<0.05). Treatments differing significantly were indicated in the tables by designating different sets of letters.

4.3 Results

The *in vivo* phytotoxicity rating of the crude extract of the aerial parts of *T. violacea*, 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 4.1; Plate 4.1) and the symptomless effect of the extract was similar to that of the water and standard fungicide controls.

TABLE 4.1: Mean foliar phytotoxicity symptom rating on a six-category scale (see text) following direct inoculation of fourth node pea leaflets with different concentrations of a crude aerial part extract of *Tulbaghia violacea.*

Plant extract applied as Foliar treatment	**Extract concentration**	**Mean lesion size (mm) on pea leaf**
Aerial part extract alone	2 mg ml^{-1}	0 ± 0a
	1 mg ml^{-1}	0 ± 0a
	0.5 mg ml^{-1}	0 ± 0a
	0.25 mg ml^{-1}	0 ± 0a
Standard fungicide alone		0 ± 0a
Water control		0 ± 0a
Spore suspension only		4 ± 0.29b

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

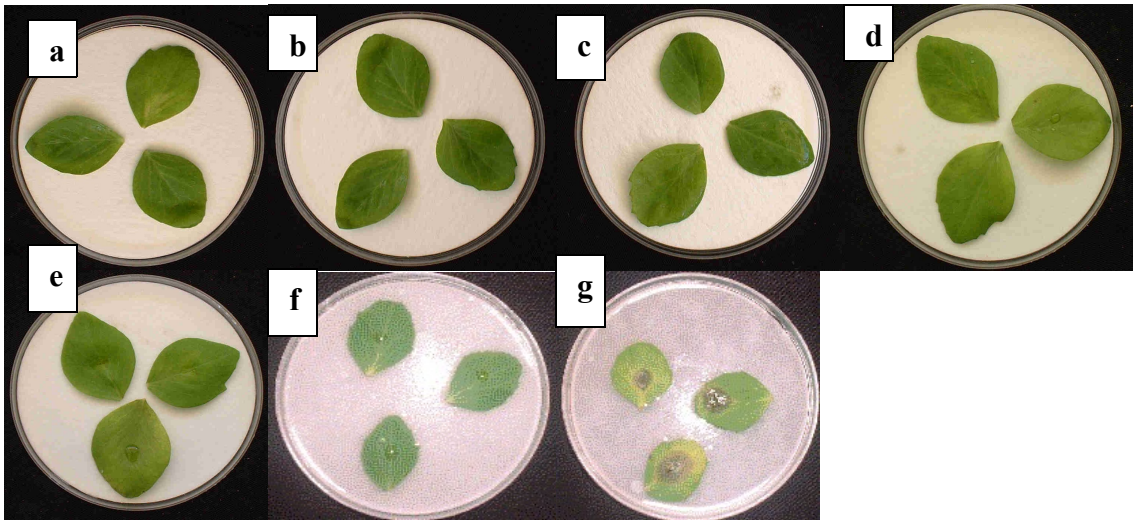

Plate 4.1: Phytotoxicity test on pea (*Pisum sativum*) leaves at different concentrations of a crude aerial part extract of *T. violacea.* Possible phytotoxic lesions caused by (a) a 0.25 mg ml^{-1} $conc^{n.}$, (b) 0.5 mg ml^{-1} $conc^{n.}$, (c) 1 mg ml^{-1} $conc^{n.}$ (d) 2 mg ml^{-1} $conc^{n.}$ (e) Standard fungicide, (f) water and (g) *Mycosphaerella pinodes* spores were recorded.

Treatment of detached pea leaves with the crude extract first, followed by spore inoculation 30 minutes later, totally suppressed lesion formation on the leaves (Table 4.2; Plate 4.2) at all concentrations tested. This was also the case for the Standard fungicide treatment before inoculation. However, when the leaves were inoculated with the spores first, followed by treatment with different concentrations of the crude extract, lesions formed on the leaves treated with the low extract concentrations of 0.25 mg ml^{-1} and 0.5 mg ml^{-1} (2.38 and 1.88 mm respectively; Table 4.2 and Plate 4.2 e and f). Spore inoculation followed by crude aerial part extract treatment at the same two low concentrations showed a significantly (P<0.05) better suppression of lesion development than spore inoculation followed by treatment with the standard fungicide (3.63 mm), but the latter significantly inhibited lesion formation when compared to the control (spore inoculation only, 9.44 mm). At concentrations of 1.0 and 2.0 mg ml^{-1} the aerial part extract of *T. violacea* totally inhibited infection of pea leaves by *M. pinodes* spores (Table 4.2 and Plate 4.2 g and h).

Table 4.2: Mean lesion size development following direct inoculation of detached fourth node pea (*Pisum sativum*) leaflets with a *Mycosphaerella pinodes* spore suspension either before or after treatment with different concentrations of an aerial part *Tulbaghia violacea* crude extract.

Treatments	*Mean lesion diameter (mm)							
	Leaves treated 30 min. before Spore inoculation				Leaves treated 30 min. after Spore inoculation			
	Concentration (mg ml^{-1} distilled water)							
	0.25	0.50	1.00	2.00	0.25	0.50	1.00	2.00
Aerial part Extract	0 ± 0a	0 ± 0a	0 ± 0a	0 ± 0a	2.38 ± 1b	1.88 ± 0.34b	0 ± 0a	0 ± 0a

+Standard Fungicide	0 ± 0a	3.63 ± 0.39c
Spore inoculation Only	9.44 ± 0.48d	9.44 ± 0.48d

*Values designated with different letters differed significantly (P<0.05) according to Duncan's least significance difference (LSD) procedure for comparison of means.
+Carbendazim / difenoconazole

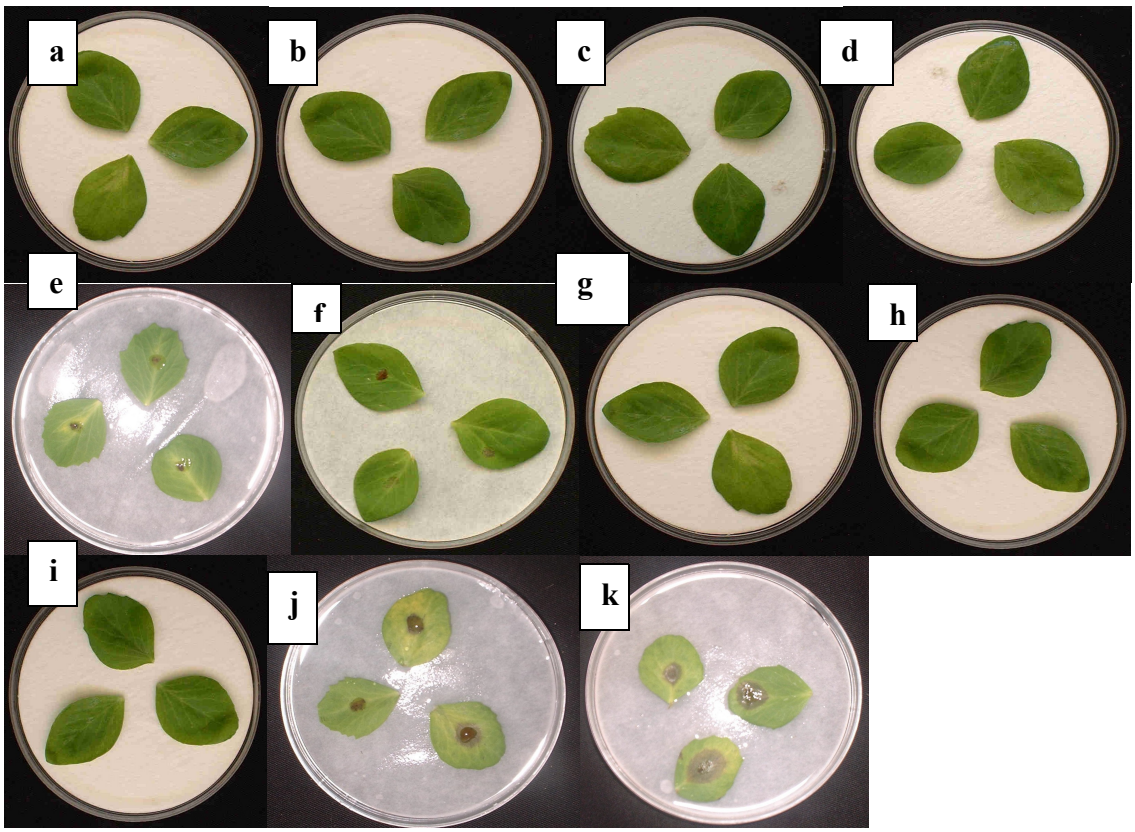

Plate 4.2: *In vivo* control of spore infection by *Mycosphaerella pinodes* in pea (*P. sativum*) leaves by different concentrations of a crude aerial part extract of *T. violacea* when pea leaves were treated with extract 30 min. before inoculation [(a) 0.25, (b) 0.5, (c) 1.0 and (d) 2.0 mg ml^{-1}] and when pea leaves were inoculated 30 min. before treatment [(e)

0.25, (f) 0.5, (g) 1.0 and (h) 2.0 mg ml^{-1}]. Control 1 = (i) Standard fungicide at 1 µg ml^{-1} applied 30 min. before inoculation, (j) Standard fungicide at 1 µg ml^{-1} applied 30 min. after inoculation and Control 2 = (k) *M. pinodes* spore inoculation only.

4.4 Discussion

None of the four concentrations (0.25, 0.5, 1.0 and 2.0 mg ml^{-1}) of a crude *T. violacea* aerial part extract was phytotoxic to pea (*Pisum sativum*) leaves. When the pea leaves were treated with the extract first and followed by spore inoculation 30 minutes later, the extract suppressed the germination of *M. pinodes* spores totally even at the lowest concentration of 0.25 mg ml^{-1}. This indicates that the crude aerial part extract of *T. violacea* shows potential to be used as a preventative measure against *Ascochyta* blight caused by *M. pinodes* on pea leaves without causing injury to the plant. According to Benner (1993), phytotoxicity is a decisive factor in evaluating plant extracts for their application potential as natural pesticides in agriculture.

Moreover, when the pea leaves were inoculated with *M. pinodes* spores 30 min. before treatment with the crude *T. violaceae* aerial part extract, the 1 mg ml^{-1} concentration totally inhibited spore germination and subsequent lesion formation indicating that the crude extract also possesses the potential to be used as a corrective measure against Ascochyta blight.

Earlier reports on the *in vivo* antifungal effects of plant extracts applied before or after inoculation of either intact plants or detached leaves correspond with the findings in this study. Mekuria *et al.* (1999) reported similar results in testing crude extracts of various *Bryophytes* species against *Phytophthora infestans* on intact tomato plants when applied before and after inoculation with the pathogen. Extracts applied 2 to 5 days before inoculation provided the best results. Amadioha (2000) reported on the same tendency of an oil extract from *Azadirachta indica* (neem) seeds to reduce infection and spreading of *Pyricularia oryzae* in rice when applied two days before inoculation. Rodriguez and Montilla (2002) demonstrated the *in vitro* and *in vivo*

antimicrobial effect of a *Citrus paradisi* seed extract (Citrex) on *F. oxysporum lycopersici* causing tomato wilt, while Kishore *et al.* (2002) reported on the *in vivo* antimicrobial activity of aqueous leaf extracts from *Lawsonia inermis* and *Datura metel* against *Mycosphaerella berkeleyi* causing late leaf spot in groundnuts (*Arachis hypogaea*).

The results obtained in this study confirmed the potential of a crude *T. violaceae* to control fungal infections of crops, at least under controlled conditions. Even the four times higher concentration that was needed to correctively inhibit *M. pinodes* spore germination, compared to the lower concentration that was needed as a preventative measure, still falls within an economically viable range of 1 g L^{-1}. Considering that an average of 400 litre fungicide solution is normally applied per hectare under agricultural field conditions, this concentration is in line with current commercially available fungicides. A minimum inhibitory concentration (MIC) of 1 g L^{-1} is also much lower than the MIC reported for a crude bulb extract of *Eucomis autumnalis* (Pretorius *et al.,* 2002).

These findings are valuable for assessing the potential application of the crude *T. violacea* aerial part extract in an integrated pest management (IPM) system in terms of minimizing crop losses caused by *Ascochyta* blight. Considering this type of data is also in line with criteria formulated by a number of authors for evaluating natural plant products for their application potential in IPM systems (Lydon *et al.,* 1989; Simmonds *et al.,*1992; Rios *et al.,*1998; Solunke *et al.,* 2001). Moreover, it seems important that further studies be carried out to explore the *in vivo* bio-stimulatory activity of the crude extracts of *T. violacea* on crops as demonstrated in Chapter 3. Stimulation of seedling growth and subsequent strengthening of a crop plant would be an added advantage possibly leading to improved yields while providing protection against Ascochyta blight.

However, in order to verify the strong indications for the *in vivo* antifungal activity of a crude *T. violaceae* extract under controlled glasshouse conditions outlined in this

chapter, this was followed up with an investigation under field conditions (Chapter 5). Two seed borne fungi, causative of loose and covered smuts in sorghum, were used as test organisms in a statistically designed field trial in Ethiopia to provide additional information on the abundance of inoculum that the extract is capable of controlling under agricultural conditions.

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CHAPTER 5

CONTROL OF SORGHUM COVERED AND LOOSE SMUTS BY AN AERIAL PART CRUDE EXTRACT OF *Tulbaghia violacea* UNDER FIELD CONDITIONS

Abstract

An aerial part crude extract of *Tulbaghia violacea* was evaluated against sorghum covered (*Sporisorium sorghi*) and loose (*Sporisorium cruentum*) kernel smuts under field conditions. The crude extract was applied at the rate of 2.0 mg ml^{-1} in lots of 90.0 g sorghum seeds by artificially inoculating separate sets of sorghum seed with smut spores at a rate of 0.5% (w/w). A standard fungicide, Thiram (65 W), was applied as a seed treatment at the rate of 0.25%/kg of sorghum seed and served as a positive control. Disease incidence observed during harvest was expressed as a percentage of infected plants. Both treatments significantly (P<0.05) reduced the incidence of both loose and covered kernel smuts and resulted in significant yield increases compared to the untreated control. Covered and loose smut incidence and yield were

significantly correlated (R^2=0.92, *P*<0.05) and (R^2=0.75, *P*=75). From these results it is envisaged that the aerial part crude extract of *T. violacea* has the potential to be developed into an environmentally friendly bio-fungicide for application in small scale farming systems where synthetic chemicals are out of reach of the average subsistence farmer.

5.1 INTRODUCTION

Sorghum (*Sorghum bicolor* L. Moench) is an important source of food in Ethiopia and serves as staple food for the majority of people (Abera *et al*., 1995). It is predominantly grown in small-scale production systems under a wide range of environmental conditions. However, production of sorghum is less than 1.0 ton ha^{-1} due to various reasons. Sorghum covered kernel (*Sporisorium sorghi* Link, G.P. Clinton) and loose kernel smuts (*Sporisorium cruenta* Kuhn, A.A. Potter) are major factors that account for low yields in Ethiopia (Abera *et al*., 1995). Both diseases occur frequently where sorghum is grown without seed treatment against these two pathogens. Consequently, contaminated sorghum seed from the previous harvest that is used for cultivating the crop in the following season is believed to be the main source of infection.

Disease incidence varies from region to region, but it is estimated to be in the range of 30 to 50 % (Martin, 1989) reducing yields by about 25 to 30%. Control of both sorghum covered smut and loose kernel smuts is commonly achieved by seed treatment with synthetic chemicals, which are beyond the reach of the majority of subsistence farmers in Ethiopia. Instead, farmers are dependent on other locally available control measures. Plant crude extracts are traditionally used as an integral component of crop protection management systems to control both insect and plant diseases under field conditions. For example, the root part of *D*. *kilimandscharicus* (local name, Bosha) is used in a slurry form to treat sorghum seeds for the control of sorghum smuts under field conditions. However, this has been practised on a small scale and only had a limited impact in the past. Experimentally, treatment of sorghum

seed with *D. kilimandscharicus, Phytolacacca dodecandra* (berries) and *Maerua subcordata* (root) material in a powder form appeared to be effective in controlling both pathogens to a certain degree, but not nearly as effective as the standard chemical, Thiram (results not published).

Information on properly developed and scientifically tested plant products for the control of both plant diseases is currently limited. Although several plant extracts with promising antifungal activity have been identified as a result of *in vitro* studies (Blaeser and Steiner, 1999; Jun Sato *et. al*., 2000; Pretorius *et. al*., 2002), their efficacy and reliability under field conditions are questionable (Benner, 1993). Subsequently, the promising antifungal activity of the aerial part crude extract of *Tulbaghia violacea* demonstrated *in vitro* (Chapter 3) and *in vivo* under glasshouse conditions (Chapter 4) was followed up with a field trial. The main aim of testing previous findings under field conditions was to evaluate the application potential of the aerial part crude extract under normal farming conditions in Ethiopia. For this purpose two seed borne pathogens of sorghum, covered and loose kernel smuts, were chosen as test organisms due to the impact they have in reducing sorghum yields in most subsistence farming systems in the country.

5.2 MATERIALS AND METHODS

5.2.1 Materials

A methanol crude extract of the aerial parts of *T. violacea* was prepared as previously described in Chapter 3 (section 3.2.3). Seed of the sorghum variety 76 T1 # 23 was obtained from the National Sorghum Improvement program. The synthetic fungicide Thiram (dimethyl thiocarbamoyl), used as a positive control, was purchased from a local merchant. Sorghum covered (*Sporisorium sorghi*) and loose kernel smuts (*Sporisorium cruentum*) spores, collected in the field during the previous year, were used to inoculate uncontaminated seed.

5.2.2 Methods

5.2.2.1 Seed treatment

Different lots of sorghum seeds were artificially inoculated separately with both covered (*Sporisorium sorghi*) and loose (*Sporisorium cruentum*) kernel smut spores at the rate of 5% (w/w) before application of seed treatments. An aerial crude extract of *T. violacea* was suspended in water at a rate of 2.0 g L^{-1}. Sorghum seed lots of 90 g each were treated with 15 ml of the crude extract by mixing thoroughly in a small plastic bag 24 h before planting. A standard synthetic seed dressing fungicide, Thiram (65 W), was applied in the same way at the rate of 0.25% (w/w) per kg seed and served as a positive control. Sorghum seeds were also artificially and separately inoculated with both loose and covered smuts spores, but were not treated with the extract or synthetic fungicide to serve as a second control.

5.2.2.2 Field trial

A field trial was conducted under irrigation at Melkassa Research Centre, Ethiopia in 2003. Plots were arranged in a randomised complete block design and treatments were replicated three times. Treated sorghum seeds were planted by hand in five rows, leaving 0.75 cm between rows, in 18.75 m^2 plots. Standard fertilizer was applied and plots were kept at field capacity by means of furrow irrigation. Disease incidence was recorded as percentage infected plants. Grain yield was determined on the whole plot.

5.3 STATISTICAL ANALYSIS OF DATA

Analysis of variance (ANOVA) was performed on the data, using the SAS (1999; SAS/IML software; Version 6; SAS Institute) program, to identify differences between treatments. Duncan's multiple range (DMR) procedure (Steele & Torrie, 1980) was applied to separate means ($P< 0.05$).

5.4 RESULTS

Treatment of sorghum seeds with an aerial part crude extract of *T. violacea* before planting completely (100%) (P<0.05) reduced the incidence of covered smut (Table 5.1) and significantly reduced loose smut incidence (Table 5.2) compared to the corresponding untreated controls, and compared favourably with the synthetic fungicide, Thiram in the control of covered kernel smut.

Table 5.1: Effect of an aerial part crude extract of *T. violacea* on the percentage covered kernel smut disease incidence in sorghum under field conditions.

Treatments	Mean plant Population	% Mean smut Incidence	Yield (ton ha^{-1})
Aerial part extract	191 ± 6a	0 ± 0a	5.0 ± 1a
Thiram	188 ± 12a	0 ± 0a	4.8 ± 0.6a
Control	174 ± 12a	9 ± 0b	2.9 ± 2.1b

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

Table 5.2: Effect of an aerial part crude extract of *T violacea* on the percentage loose kernel smut disease incidence in sorghum under field conditions.

Treatments	Mean plant Population	% Mean smut Incidence	Yield (ton ha^{-1})
Aerial part extract	175 ± 0a	14 ± 3a	4 ± 1.7a
Thiram	175 ± 0a	0 ± 0b	3.9 ± 1.3a
Control	175 ± 0a	32 ± 8c	2.5 ± 1b

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

Inoculation of pre-planted sorghum seed with covered or loose smuts spores, without also treating the seeds with either Thiram or the crude *A. violacea* extract (untreated

controls), significantly decreased the final yields (Tables 5.1 and 5.2). In the case of covered smut the yield loss was 46.7% and, in the case of loose smut, 55.2%. However, in both cases, there was no significant difference in yield between plots treated with either Thiram or the *T. violacea* crude extract. A significant difference in yield between the Thiram treated and untreated controls was also observed in both cases.

The percent covered and loose smuts incidences were negatively correlated (R^2= -0.92 and -75 respectively) with sorghum grain yield indicating the negative impact both smut diseases had on the yield.

5.5 DISCUSSION

Pre-treatment of sorghum seeds with an aerial part crude extract of *T. violacea* at a rate of 2 g L^{-1} completely prevented infection by covered kernel and significantly reduced the incidence of loose kernel smuts under field conditions. This compared favourably with the decrease in disease incidence by the standard synthetic fungicide, Thiram, and confirmed the efficacy of the crude extract. Although covered kernel smut incidence was relatively low (9%) compared to that of loose smut incidence (25%) in the untreated controls, all (100%) plants grown from inoculated seeds were totally protected against infection by covered kernel smut while the incidence of loose smut was significantly reduced following seed treatment with the aerial part crude extract of *T. violacea*. The significant differences in grain yield obtained between plants grown from treated and untreated seeds confirmed the impact that both diseases had on yield as well as the efficacy of the crude extract in controlling both diseases.

Although published accounts of the efficacy of natural plant extracts against sorghum covered and loose smuts is lacking, the results obtained in this study are in line with that of Chen *et al.* (2002) who reported on the inhibiting effect of *Chloris virgata,*

Dalbergia hupeana, Pinus massoniana, Paeonia suffruticosa and *Robinia pseudoacacia* crude extracts on spore germination of grape downy mildew (*Plasmopara viticola*). Additionally, Michele *et al.* (2002) reported on the potential of the naturally occurring phenolic acid (zosteric acid) from eelgrass (*Zostera maina* L.) to prevent spore infection of rice by *Magnaporthe grisea* as well as beans (*Phaseolus vulgaris*) by *Colletotrichum lindemuthianum*, causing anthracnose. Nwachukwu and Umechuruba (2001) also reported on the significant ($P \leq 0.05$) reduction of the incidence of seed-borne fungi and increase in seed germination and seedling emergence of African yam bean (*Sphenostylis stenocarpa*) seeds when treated with leaf extracts of some plants, including the leaf extracts of basil (*Ocimum basilicum*) and neem (*Azadirachta indica*).

In light of previous reports on the contrasting inhibiting effect that natural plant extracts with antimicrobial properties can have on seed germination (Tran and Tsuzuki, 2002) and seedling establishment (Deena *et al.,* 2003), this aspect was monitored during the field trial (results not shown). Concern with regard to these possible side effects as well as the reliability of natural plant extracts under field conditions was expressed almost a decade ago by Mathre *et al*. (1995). However, treatment of sorghum seeds with an aerial part crude extract of *T. violacea* had no apparent inhibitory effect on either seed germination or seedling growth. The latter, together with the significant *in vitro* and *in vivo* antifungal activities demonstrated in this study for an aerial part crude *T. violacea* extract against a broad spectrum of fungal pathogens, confirmed both its application potential and reliability.

In general, although the synthetic fungicide, Thiram, is very effective in controlling covered kernel and loose smuts infection in sorghum, the application of crude plant extracts seems a convenient, effective and economical alternative to the majority of subsistence farmers who cannot afford synthetic chemicals. Additionally, the risk associated with synthetic chemicals as well as consumer resistance towards its application in agriculture must be accepted as important issues when considering in depth studies on the application potential of natural products in agriculture.

Finally, crude extracts of *T. violacea* consistently showed significant *in vitro* (Chapter 3) and *in vivo* antifungal activity under glasshouse (Chapter 4) and field (Chapter 5) conditions. This supplied the rationale for the isolation, purification and identification of the active substance(s) involved (Chapter 6).

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CHAPTER 6

ISOLATION, PURIFICATION AND IDENTIFICATION OF ANTIFUNGAL COMPOUNDS FROM AERIAL AND BELOW SOIL PART EXTRACTS OF *Tulbaghia violacea*

Abstract

Crude methanol extracts of aerial and below soil parts of *T. violacea* were initially fractionated by means of activity directed liquid-liquid extraction by using organic solvents in order of increasing polarity (dielectric constants) namely, hexane, diethyl ether, ethyl acetate and dichloromethane. The semi-purified extracts were tested *in vitro* for their antifungal activity against six phytopathogenic fungi. Hexane (100%) and diethyl ether (60%) extracts from both aerial and below soil parts displayed the highest fungal inhibition against all test fungi. However, the antifungal active ingredients seemed to be distributed thinly throughout all the solvents since all exerted fungal inhibition against all the test fungi to some extent, except against *P. ultimum*. Subsequently, the active compounds were isolated and purified from the hexane fraction only using standard column and thin layer chromatographic techniques. Six aliphatic sulphur compounds were identified and their molecular

structures elucidated by means of nuclear magnetic resonance (NMR) and mass spectroscopic analysis. These included the known 2,4,5,7-tetrathiaoctane and five novel compounds, of which four from the aerial parts (2,3,5,7,8-pentathiadecane; 2,4,6-trithiaheptane; 2,4-dithiapentane; methyl thiosulphonate) and one from the below soil parts (2,4,5,6,8-pentathianonane).

6.1 Introduction

The liquid-liquid extraction technique, using organic solvents in order of increasing polarity, is commonly used for fractionating bioactive compounds from crude plant extracts (Fourneau *et al.,* 1996; Song & Oh, 1996; Amen *et al.,* 1997; Benerle & Schwab, 1997; Silva *et al.,* 1997; Volf *et al.,* 1997; Olivella *et al.,* 2001). Column and both qualitative and preparative thin layer chromatographical techniques are currently still employed for isolating and purifying active compounds from plant extracts (Froytlog *et al.* 1998; Choung, *et al.* 2000; Tang, *et al.* 2000; Jang, *et al.* 2001; Shin, *et al.* 2001; Maciejewicz, 2001; Negi and Jayaprakasha, 2001; Zhu *et al.* 2001; Khamidullina *et al.* 2002). Once active compounds are purified, nuclear magnetic resonance (NMR) and mass spectroscopy are commonly used to elucidate the chemical structures of compounds (Bonnlander and Winterhalter, 2000; Gil *et al*., 2000; Bilia *et al*., 2001; Lee *et al*., 2001; Pauli, 2001; Ratcliffe and Shacha, 2001; Takeoka *et al*., 2001).

Promising *in vitro* and *in vivo* antifungal properties of crude extracts from different plant parts of *T. violacea* against a broad spectrum of economically important plant pathogenic fungi have been confirmed in previous chapters (3, 4 and 5). This supplied the rationale for the activity directed isolation, purification and identification of the active compounds involved. As semi-purified extracts from both aerial and below soil parts of *T. violacea* showed antifungal activity, the compounds involved were purified from both and their molecular structures elucidated by means of NMR and mass spectroscopy.

6.2 Materials and Methods

6.2.1 Materials

Potato dextrose agar (PDA), plate count agar (PCA) and nutrient broth were purchased from Merck (Germany). Hexane, diethyl ether, ethyl acetate and dichloromethane used in liquid-solid extractions were of the purest form available and purchased from Merck, Germany. Sephadex LH20 used as packing material during column chromatographic purification of active compounds was purchased from Pharmacia while Silica gel packing material was from Merck. Both glass coated Silica gel F 1500/LS (1 mm) preparative (P-TLC) and Silica gel 60 F_{254}-aluminium backed and pre-coated qualitative (Q-TLC) thin layer chromatography plates were also obtained from Merck, Germany.

6.2.2 Test organisms

All micro-organisms used in this study were provided by the department of Plant Sciences, University of the Free State, Bloemfontein, South Africa. Six common South African plant fungal pathogens (the same phytopathogenic fungi used in Section 3.2.3.4 Chapter 3) were chosen to test for the fungitoxic properties of the liquid-liquid semi-purified extracts from *T. violacea* and only *Fusarium oxysporum* from the six fungi was used to test the bioactivity of the samples purified by means of column and thin layer chromatography.

6.2.3 Methods

6.2.3.1 Activity directed liquid-liquid extraction

The following organic solvents, arranged in order of increasing polarity (dielectric constants) were used for semi-purification (liquid-liquid extraction) of the crude aerial and below soil part extracts separately: Hexane (DC=2.0), Diethyl ether (DC=4.3),

Ethyl acetate (DC=6.0), Methylene chloride (DC= 8.9). The extraction was done for both aerial and below soil parts

About 63.30 g of the methanol aerial part extract and 88.24 g of the below soil part extract were each dissolved in 50 ml distilled water and mixed with 50 ml hexane (1:1 ratio). Each mixture was shaken vigorously for 20 minutes on a mechanical shaker (Fig. 6.1) and subsequently transferred to a separating funnel allowing the two liquid phases to separate. The separated upper hexane layer was transferred into a beaker while the lower crude extract layer was again mixed with fresh 50 ml hexane and shaken as before. Fractionation was repeated 20 times with fresh solvent to optimize the recovery of compounds.

Figure 6.1: Fractionation of compounds by means of liquid-liquid extraction using a mechanical shaker.

The same procedure was followed with the other organic solvents. The four separated fractions were evaporated to dryness under vacuum in a water bath at 35 °C by means of a Büchi rotavapor (Fig. 6.2).

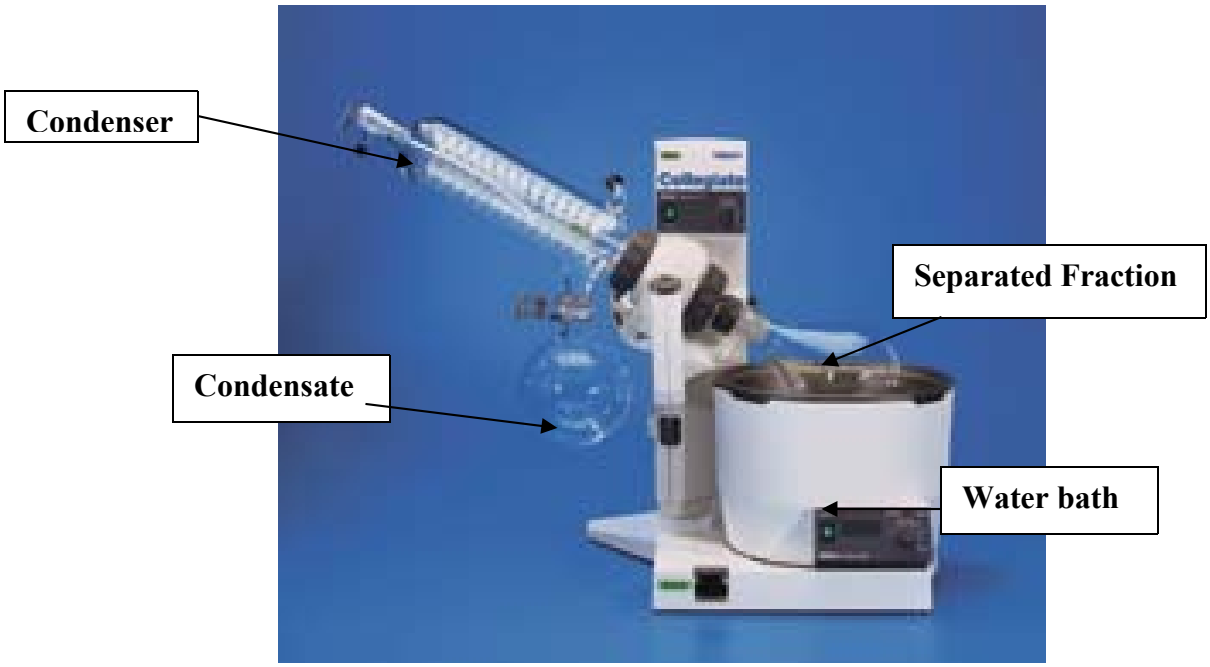


Figure 6.2: Büchi rotavapor

The mass of recovered dry material was determined for each fraction. From the dried material of each fraction, 1 mg ml $^{-1}$ stock solutions in water were prepared. In order to establish the success of the fractionation process, a qualitative thin layer chromatography (TLC) profile was obtained for each fraction on a 0.5 mm Silica Gel 60 plate using chloroform : methanol : water (80:20:10) as mobile phase (Section 6.2.3.2). To determine where the active ingredients were located, the eight extracts were screened for antifungal properties. The same procedure was used as in Section 3.2.3.4, Chapter 3.

6.2.3.2 Activity directed column chromatography fractionation (CCF)

Prior to column chromatography separation of active compounds in the semi-purified hexane extracts obtained by means of liquid-liquid extraction, using Silica Gel as stationary phase, the most effective solvent system was tested using Silica Gel TLC-plates. Solvent systems tested ranged from the least polar to the most polar and included: Hexane: Acetone (9.5:0.5 – 6:4); Hexane: Ethylacetate (8:2 – 6:4); Hexane: Acetone: Ethylacetate (6:2:2); Hexane: Acetone: Methanol (6:3:1) and Chloroform:

Methanol (9:1 - 5:5). For both the aerial and below soil part extracts the most suitable solvent system was determined as Hexane: Acetone (9.5:0.5 and 9:1 respectively).

An ordinary glass burette (1.5 m x 2 cm) was used as a separating column. After placing a small piece of cotton wool at the bottom of the column a Silica Gel 60 slurry, previously prepared using the solvent system, was slowly poured into the column and allowed to settle until a final height of 1.49 m was reached. Care was taken to avoid air bubbles from forming.

A separation funnel filled with about 1.5 litres of the solvent system was placed at the top of the column to ensure a continuous supply of the mobile phase and to maintain a 10 cm solvent column above the stationary phase. The column was equilibrated overnight before the bed volume was determined and the active semi-purified extract loaded onto the column. Twelve g of the dried active liquid-liquid extracts were dissolved in a suitable volume of the hexane: acetone solvent and gently loaded onto the column using a pipette. It was allowed to settle on the silica surface and to diffuse into the stationary phase before the mobile phase was allowed to migrate under gravitation. Compounds were eluted at a flow rate of 0.5 ml min^{-1} and 12 ml fractions were collected using a fraction collector over a period of two to two and a half months. Columns were constantly monitored to ensure that they did not run dry.

Fractions were collected until the columns were discoloured. Subsequently, the Hexane: Acetone (9.5:0.5) solvent system was replaced with a more polar Hexane: Acetone ratio of 9:1 or 8:2, 7:3, 6:4 or 5:5. Towards the end of the separation process more compounds were removed from the columns by means of 100% methanol.

6.2.3.3 Qualitative thin layer chromatography (Q-TLC).

Qualitative thin layer chromatography (TLC) was performed on 10 x 20 cm Kieselgel $60F_{254}$, 0.25 mm, aluminium plates (Merck). Development of the TLC plates in the appropriate solvent was followed by spraying with formaldehyde (40%)-sulphuric acid

(2:98) or with anisaldehyde–sulphuric acid–ethanol (5:5:90) and heated to 120 °C. A total of about 3600 test-tubes were collected for each extract during the column chromatography separation outlined above. Qualitative thin layer chromatography (Q-TLC) was performed on the compounds separated in every third test-tube fraction collected. The same solvent system used for column chromatography separation was also used for TLC-separation. The plates were developed and subsequently investigated under UV-light at 254 and 365 nm. Visible fluorescent spots were marked with a pencil before the plates were sprayed with the vanillin-sulphuric acid reagent to show all the spots.

RF-values of separate compounds as well as the TLC-profiles of the different column fractions were compared. Column fractions showing similar profiles and RF-values were combined. Combined column fractions were evaporated under reduced pressure at 40 °C in a rotary evaporator and the mass of the dried fractions determined.

6.2.3.4 Determination of the antifungal activity of combined column chromatography fractions

In order to ascertain in which column fractions most of the antifungal compounds were located, the same procedure as was outlined in Chapter 3, section 3.2.3.4, was followed using only *F. oxysporum*, relatively resistant to the aerial part extract (see Chapter 3; section 3.3.3) as test organism. Only the most active combined column chromatography fractions were considered for further purification.

6.2.3.5 Preparative thin layer chromatography (P-TLC)

Only compounds in the most active combined column chromatography fractions were further purified by means of preparative thin layer chromatography (P-TLC). Separation was carried out on 20 x 20 cm glass plates coated with 1.0 mm Kieselgel PF_{254} (Merck) which were air-dried and used without prior activation.

Approximately 250-300 mg of each active combined column fraction was dissolved in a small volume (3-5 ml) of methanol, chloroform or the mobile phase, depending on the most effective solvent. The solution was streaked onto the baselines of about 20 P-TLC plates in thin uniform bands, applying small volumes at a time and drying between applications, until about 15 mg was applied per plate. In order to separate all compounds in a combined column fraction, in this case about 20 - 25 P-TLC plates were used. Hexane:Acetone (9.5:0.5) was used as solvent system to develop the plates in glass tanks.

When the mobile phase reached the frontline, the plates were removed from the developing tanks and placed in a ventilated fume hood to dry. Subsequently, plates were examined under UV-light at 254 and 365 nm and the fluorescent bands marked with the sharp end of a spatula, before bands were scraped off using a spatula and transferred to corresponding labelled vials. Compounds were covered with a suitable volume of chloroform and eluted from the silica by vigorously shaking for 10 min before suction-filtering through a number of crucibles with labels corresponding to the labels of the silica bands. The filtrates were collected in corresponding pre-weighed flasks, placed in a ventilated fume cupboard to dry, the recovery mass determined and tested for antifungal activity.

6.2.3.6 Determination of the antifungal activity of compounds purified by means of preparative thin layer chromatography (P-TLC)

In order to ascertain which of the compounds separated by means of P-TLC were active, the same procedure as outlined in Chapter 3, section 3.2.3.4, was followed using *F. oxysporum* only as test organism. Only the most active compounds were purified further.

6.2.3.7 Final purification of isolated active compounds

Only the most active isolated compounds were again tested for purity in an original analytical thin layer chromatography (TLC) system (Mikes and Chalmers, 1979) following the same Q-TLC (see section 6.2.3.3) and P-TLC (see section 6.2.3.5) procedures with slight modifications of the mobile phases. Either chloroform:methanol:water (80:20:10 v/v) or toluene:acetone:ethyl acetate (7:2:1 v/v; Wagner and Bladt, 1996) were used as solvent systems to separate active compounds from any other compounds that might have shared the same position on the Q-TLC or P-TLC plates. After drying the plates in a stream of air, compounds were either detected under UV-light at 254 and 365 nm or the plates were stained with 5% (v/v) ethanolic H_2SO_4 or 1% (m/v) Vanillin (1 g in 100 ml H_2SO_4; Wagner and Bladt, 1996).

Non-pure compounds were again subjected to preparative TLC acidified with 1% (v/v) HCL until pure compounds were obtained. Only pure compounds that showed the highest antifungal activity were subjected to nuclear magnetic resonance (NMR) spectroscopy in order to identify them and to elucidate their molecular structures.

6.2.3.8 NMR and Mass spectroscopy

To identify the most bioactive compounds purified from the aerial and below soil plant parts and elucidate their molecular structures, isolated compounds were washed repeatedly with acetone to obtain an acceptable level of purity. Subsequently, the compounds were submitted to nuclear magnetic resonance spectroscopy (^{13}C and ^{1}H 1D and 2D NMR). NMR-spectroscopy was performed on a Bruker ADVANCE 300MHz DRX 300 spectrometer at 296K (23 ^{o}C) with tetramethylsilane ($Si(CH3)_4$; TMS) as the internal standard. The solvents used were deuteriochloroform ($CDCl_3$), or deuterioactetone [$(CD_3)_2 CO$] as indicated. Chemical shifts were reported in parts per million (ppm) on the δ-scale and coupling constants were given in Hz. The following abbreviations were used:

s	singlet
d	doublet

dd	doublet-of-doublets
m	multiplet
br	broadened
t	triplet

MS spectra and mass determinations were obtained with a Kratos MS-80 mass spectrometer in the double focus electron impact (EI) mode.

6.3 Results

6.3.1 Recovery of liquid-liquid semi-purified extracts from the crude methanol extract

Most of the compounds in both the aerial and below soil part extracts were more soluble in hexane than in the more polar solvents used. As much as 9.20 g (14.6%) of compounds from the aerial plant parts and 7.43 g (8.4%) from the below soil parts were recovered in hexane compared to the much lower recovery in the more polar solvents (Table 6.1).

Table 6.1: Recovery of compounds from *Tulbaghia violacea* by solvents in order of increasing DC-values using a liquid-liquid extraction procedure

Organic solvent	Aerial part extract		Below soil part extract	
	Mass (g)	% Recovery	Mass (g)	% Recovery
Hexane	9.20	14.6	7.43	8.4
Diethyl ether	1.57	2 .5	1.74	1.9
Ethyl acetate	0.56	0.9	1.07	1.2
Dichloromethane	0.10	0.1	0.12	0.14

6.3.2 Qualitative thin layer chromatography (Q-TLC) profiles of the liquid-liquid chromatography extractions from *T. violacea*

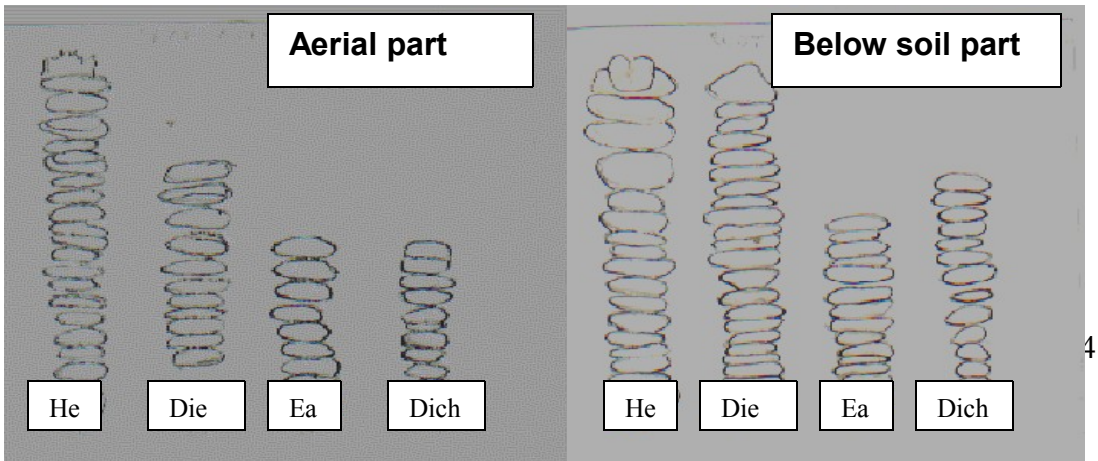


Figure 6.3: Q-TLC profiles of liquid-liquid chromatography extracts of aerial and below soil parts of *T. violacea.* (He = Hexane, Die = Diethyl ether, Ea = Ethyl acetate and Dich = Dichloromethane)

More compounds dissolved in the hexane and diethyl ether fractions than in the more polar ethyl acetate and dichloromethane fractions as illustrated by the Q-TLC profiles (Fig 6.3) and mass in grams (Table 6.1).

6.3.3 Antimicrobial properties of the liquid-liquid aerial and below soil part extracts of *T. violacea*

Although all of the liquid-liquid extractions from the aerial parts of *T. violacea* showed antifungal activity against most of the test organisms to a greater or lesser extent, that of the hexane and diethyl ether extracts was significantly ($P<0.05$) higher (Figure 6.4). Mycelial growth inhibition of all test fungi by one or both the hexane and diethyl ether extracts were higher than 80% except in the case of *P. ultimum*, well known for its resistance against fungicides, as well as *R. solani* (61%). Both these extracts compared favourably with the standard fungicide used as a positive control.

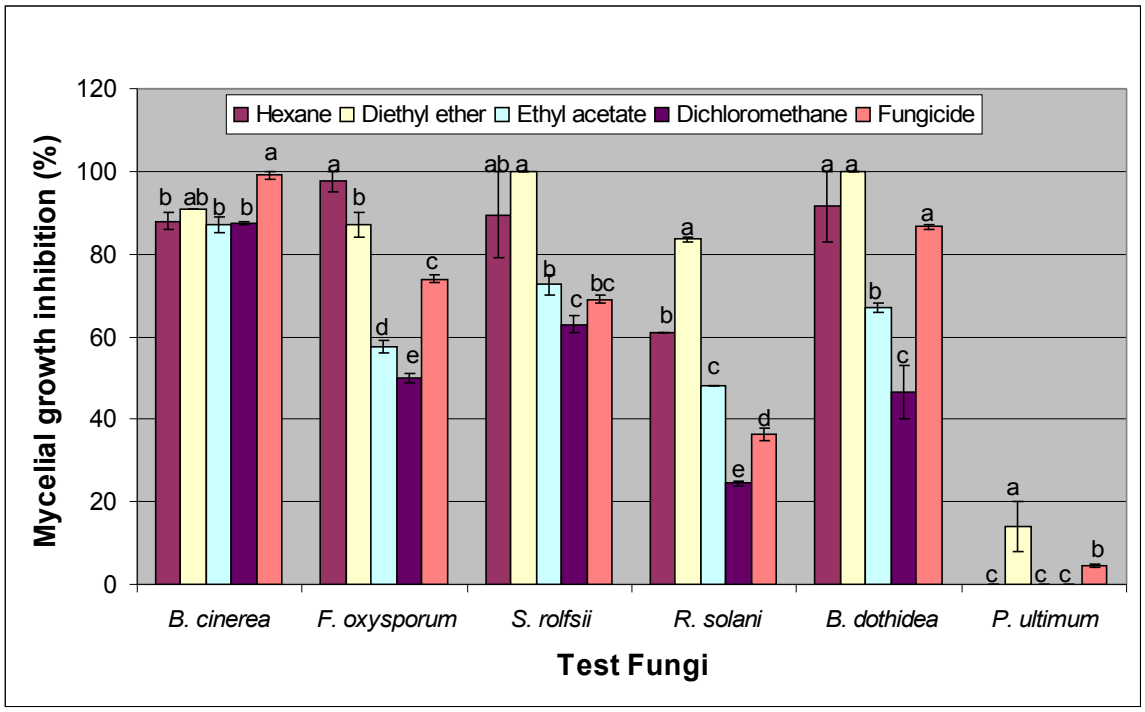


Figure 6.4: Percentage (%) *in vitro* mycelial growth inhibition of plant pathogenic fungi by semi-purified **aerial part** extracts of *T. violacea* at a concentration of 1g L^{1-}. Bars designated with different letters for each test fungus differed significantly (P<0.05) according to Tukey's Mean Difference (MSD) procedure.

Antifungal compounds in the below soil parts (Figure 6.5) of *T. violacea* showed the same tendency to accumulate in the hexane and diethyl ether fractions after liquid-liquid extraction as was the case with the aerial parts (Figure 6.4). However, in the case of *B. dothidea*, all of the extracts showed exceptionally high antifungal activity.

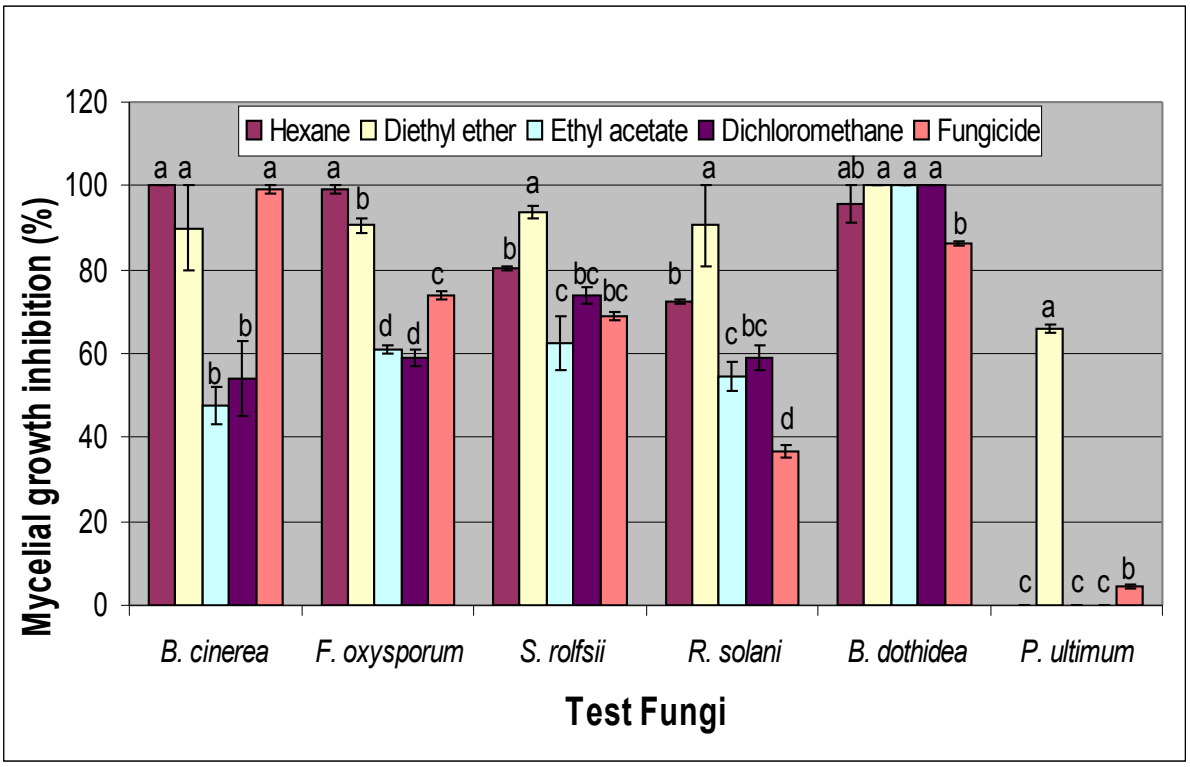


Figure 6.5: Percentage (%) *in vitro* mycelial growth inhibition of plant pathogenic fungi by semi-purified **below soil part** extracts of *T. violacea* at a concentration of 1g L^{1-}. Bars designated with different letters for each test fungus differed significantly (P<0.05) according to Tukey's Mean Difference (MSD) procedure.

6.3.4 Column chromatography fractionation (CCF) of the most active hexane liquid-liquid extracts

Only compounds in the active hexane liquid-liquid extraction were purified further by means of column chromatography in the case of both the aerial and below soil parts

of *T. violacea*. The reason for this decision was the fact that six times more compounds was retrieved from the hexane extract compared to the diethyl ether active extract in the case of the aerial parts and four times more in the case of the below soil parts.

More than 1500 column chromatography fractions were obtained from the hexane extracts for each of the aerial and below soil parts. After these were subjected to Q-TLC, those fractions with similar profiles were combined. Twenty eight combined column fractions were obtained for the aerial parts and 20 for the below soil parts in this manner. The results are shown in Table 6.2.

Only four combined column fractions (A_5, A_6, A_7 and A_8) from the aerial parts displayed above average antifungal activity (100, 95, 52.6 and 60.5%) respectively, while only two combined fractions (B_2 and B_3) from the below soil parts were active (87 and 97% respectively). The rest of the fractions failed to suppress mycelial growth of *F. oxysporum* effectively (Table 6.2). Only combined column fractions that showed more than 50% mycelial growth inhibition of the test fungus on average were purified further by means of preparative thin layer chromatography (P-TLC). The reason for taking 50% inhibition as criterion is to compensate for the possibility that active substances could act synergistically and that, on separation, activity can decline (Sakagami *et al*., 1998).

Table 6.2: Percentage (%) mycelial growth inhibition of the test organism, *Fusarium oxysporum*, by combined column chromatography fractions of aerial and below soil part extractions of *T. violacea*. (**B** = below soil parts and **A** = aerial parts).

Fraction number from **A**	Mass (mg) recovered	% Mycelial growth inhibition by compounds from **A**	Fraction number from **B**	Mass (mg) recovered	% Mycelial growth Inhibition by compounds from **B**
1	19.9	1.5	1	147.4	24.0
2	28.9	11.7	**B_2/2**	411.1	**87.0**
3	105.8	11.7	**B_3/3**	109.8	**97.0**
4	97.9	25.9	4	947.3	12.0
A_5/5	316.5	**100.0**	5	10.7	12.7
A_6/6	460.7	**95.0**	6	17.7	11.6
A_7/7	430.6	**52.6**	7	22.7	12.4
A_8/8	353.5	**60.5**	8	26.1	12.0
9	120.4	34.7	9	3.1	11.9
10	121.3	0.3	10	33.6	20.0
11	426.0	10.9	11	155.8	9.6
12	848.4	-10.3*	12	24.3	14.3
13	53.9	-1.4*	13	478.0	3.5
14	210.2	-3.3*	14	590.2	27.5
15	59.3	5.8	15	73.8	16.5
16	294.7	1.6	16	197.1	27.8
17	288.7	30.1	17	114.4	11.7
18	196.9	40.5	1 18	213.6	34.8
19	218.0	8.2	19	110.3	19
20	78.1	15.8	20	447.2	37.9
21-28	2201.5	-23.4*			
Fungicide		78.0			78.0
Control		0			0

*Values with negative (-) values indicate mycelial growth stimulation.

Only the six active combined column chromatography fractions shown in table 6.2 (A_5, A_6, A_7, A_8, B_2 and B_3) were subjected to further purification by means of P-TLC.

6.3.5 Activity directed preparative thin layer chromatography (P-TLC) purification of active combined column chromatography fractions

Activity directed purification of compounds in the six combined column fractions by means of P-TLC produced four compounds ($A_{5.2}$, $A_{6.1,}$ $A_{7.1}$ and $A_{7.2}$) from the aerial part fractions that showed antifungal activity higher than 50% (100, 85, 87 and 50% respectively) and two ($B_{2.2}$ and $B_{3.2}$) from the below soil part fractions (87 and 72% respectively; Table 6.3). These six compounds were identified and their chemical structures elucidated by means of nuclear magnetic resonance (NMR) spectroscopy. Interestingly, on separation, compounds in column chromatography fraction 8 (A_8) of the aerial parts, that showed 60.5% mycelial growth inhibition (Table 6.2) when together in semi-purified form, lost their antifungal activity after P-TLC purification (Table 6.3).

Table 6.3: Percentage (%) mycelial growth inhibition of *F. oxysporum* by different compounds of *T. violacea* after P-TLC separation

Aerial compd. no.	Mass (mg)	% inhibition	Below soil compd. no.	Mass (mg)	% inhibition
$A_{5.1}$	94.9	0	$B_{2.1}$	26.2	0
$A_{5.2}$	44.1	**100**	$B_{2.2}$	110.3	**87**
$A_{6.1}$	25.3	**85**	$B_{3.2}$	15.8	**72**
$A_{6.2}$	34.1	48	$B_{3.3}$	5.9	0
$A_{6.3}$	10.5	37			
$A_{7.1}$	9.3	**87**			
$A_{7.2}$	24.5	**52**			
$A_{8.1}$	7.1	0			
$A_{8.2}$	6.2	5			
$A_{8.3}$	9.1	0			

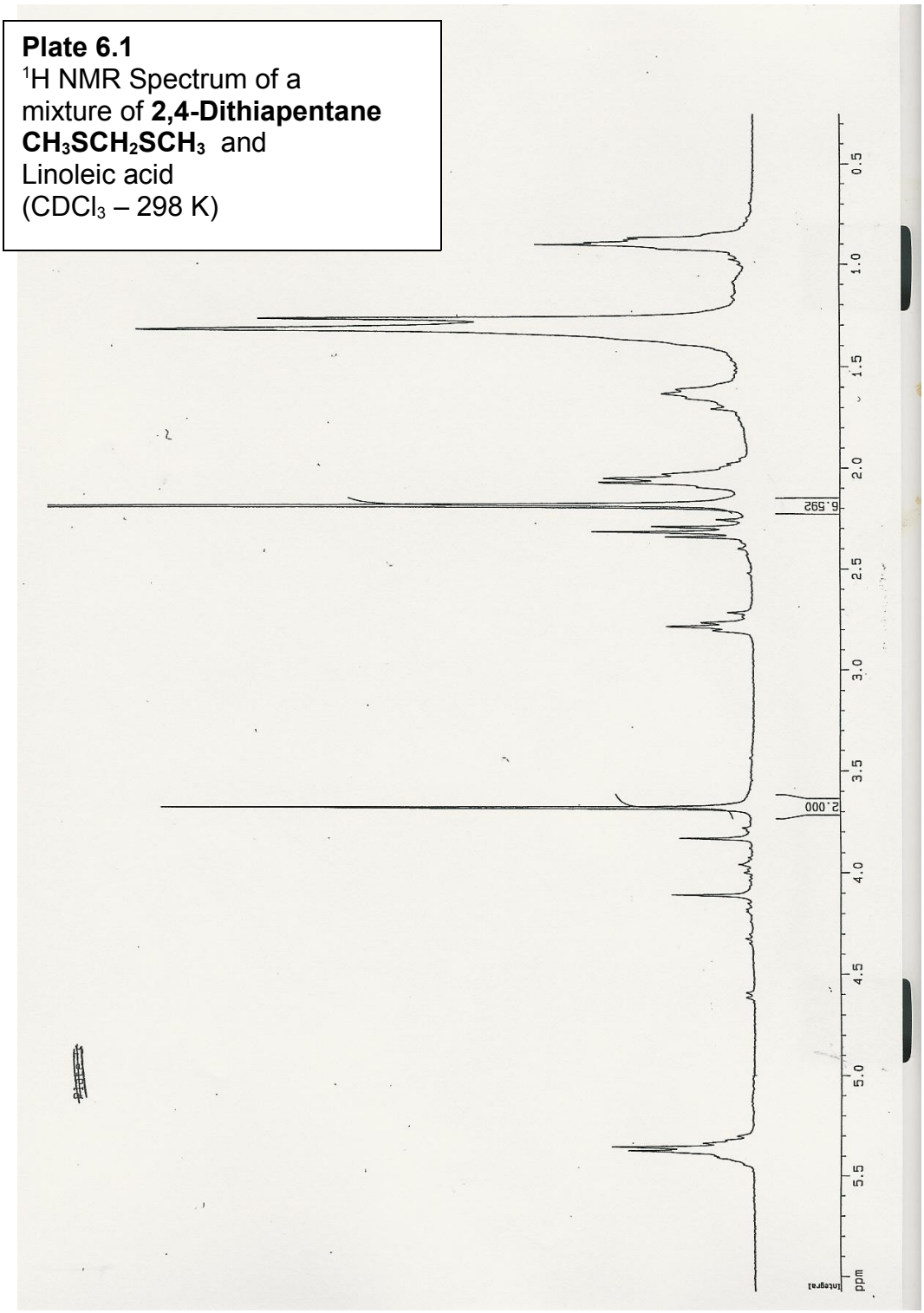
6.3.6 Identification of active compounds purified from the extracts of aerial and below soil parts of *T. violacea* by Nuclear magnetic resonance (NMR) spectroscopy and Mass spectrometry

In order to obtain an acceptable level of purity, the complex semi-purified hexane extracts of aerial and below soil parts of *T. violacea* were subjected to chromatographic fractionation which was followed by P-TLC separation. Fractionation of the hexane extract by silica column chromatography with hexane-acetone (95:5) as the eluant at a flow rate of 30 ml h^{-1}, followed by several P-TLC purifications in hexane-acetone (9.5:0.5), resulted in 6 fairly pure fractions (see section 6.2.3). Bio-assay on the fractions revealed that only two fractions from the below soil parts and four from the aerial parts showed promising results relevant to this investigation.

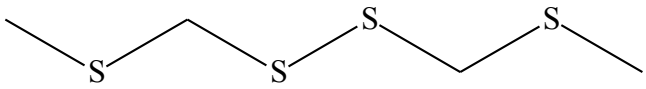
The presence of glycerol trilinoleate in all the fractions hampered separation and rendered structural elucidation difficult (see Plate 6.1). Further purification of the fractions by P-TLC afforded fairly pure compounds ($B_{2.2}$ and $B_{3.2}$ from the below soil parts and $A_{5.2,}$ $A_{6.1,}$ $A_{7.1}$ and $A_{7.2}$ from the aerial parts) whose structures were determined by standard NMR spectroscopic methods and Mass spectrometry. Mass spectra of the impure compounds could not be determined; for example compound 6. A strong garlic aroma suggested the compounds to belong to the thiol (mercaptan) class.

Absence of the aromatic and alkenoid protons (between δ 5 - 8) in the ^{1}H NMR spectra of all the compounds eliminated the aromatic character in their structures. However, simple ^{1}H NMR spectra of all the compounds displayed only singlet peaks resonating between δ 2.0 and 4.5. All the corresponding ^{13}C NMR spectra of all the compounds showed no resonance above 45 ppm, further eliminating the C=S and C=O bond characters in the structures (Kalinowski *et al.,* 1988). These findings narrowed the possibilities of the compounds to belong to either the thio or oxo aliphatic class. Further evaluation of the ^{13}C NMR spectra showed the most de-shielded signal to appear at ~45 ppm which is characteristic of the thio bond (C-S), thus eliminating the oxo bond (C-O)[1] which appears at DEPT (Distortionless enhancement by polarisation transfer) experiments of all the compounds confirmed the absence of methine protons, which eliminated the branched structures.

Plate 6.1
^{1}H NMR Spectrum of a mixture of **2,4-Dithiapentane** **$CH_3SCH_2SCH_3$** and Linoleic acid
($CDCl_3$ – 298 K)



Compound (1)



Empirical formula: $^{1}CH_3S^{3}CH_2SS^{6}CH_2S^{8}CH_3$

2,4,5,7-Tetrathiaoctane

Compound (**1**) or $B_{2.2}$ was isolated from the impure column fraction B_2 by means of P-TLC purification (Hexane:Acetone: 9.5:0.5) as a yellow oil. The ^{1}H NMR spectrum of compound (**1**) (**Plate 6.2)** showed a singlet at δ_H 4.00 (δ_C 45.2 ppm), integrating for 4 protons, and a singlet at δ_H 2.21 (δ_C 17.3 ppm), integrating for 6 protons, suggesting a thiomethylene and two thiomethyl groups. The methylene protons at δ_H 4.00 and the methyl protons at δ_H 2.11 are consistent with the presence of the CH_2-S and CH_3-S groups, respectively. Electron ionization mass spectroscopy (EIMS) of (**1**) showed the base peak at *m/z* 186, consistent with the molecular formula $C_4H_{10}S_4$, and this was in agreement with the suggested structure (**1**), a compound previously isolated by Burton and Kaye (1992).

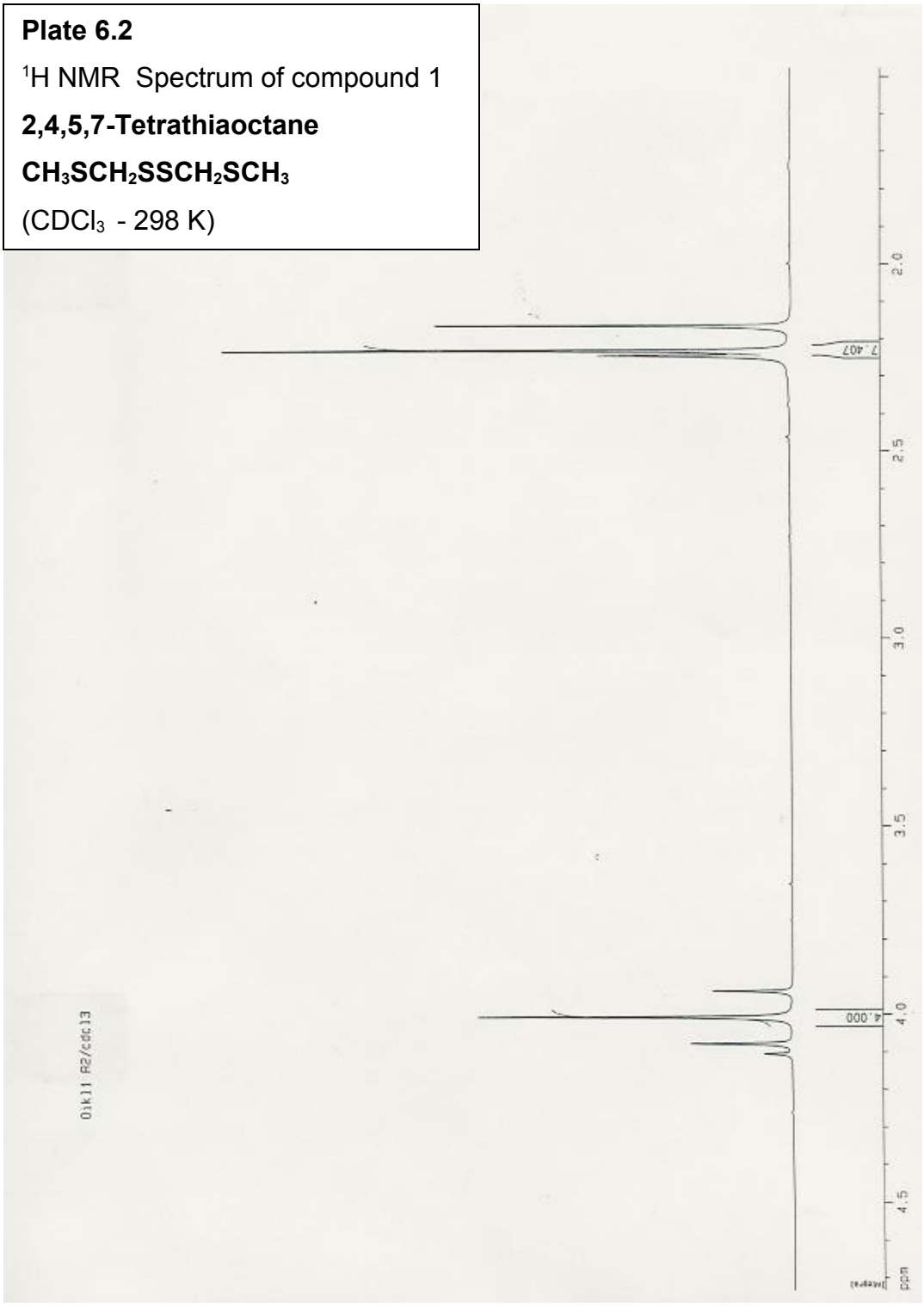
Plate 6.2

^{1}H NMR Spectrum of compound 1

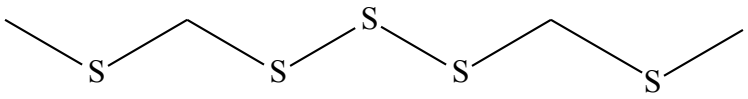
2,4,5,7-Tetrathiaoctane

$CH_3SCH_2SSCH_2SCH_3$

($CDCl_3$ - 298 K)



Compound (2)



Empirical formula: $^{1}CH_3S^{3}CH_2SSS^{7}CH_2S^{9}CH_3$

2,4,5,6,8-Pentathianonane

Following P-TLC separation (Hexane:Acetone: 9.5:0.5), compound (**2**) or $B_{3.2}$ was isolated as a yellow oil from combined column fraction B_3. In the ^{1}H NMR spectrum of compound (**2**) (**Plate 6.3)** only two singlets at δ_H 4.15 and δ_H 2.29, integrating for 2 protons and 6 protons, respectively, were observed. The ^{1}H NMR data of (**2**) was very similar to that of (**1**) suggesting a very similar structure. The intergrals of the peaks suggested the presence of two thiomethylene and two thiomethyl groups. Assignment of structure (**2**) was based on comparison of the chemical shifts of the protons in the ^{1}H NMR spectra of compounds (**1** and **2**). The more de-shielded protons in the ^{1}H NMR spectrum of (**2**) required additional sulphur atoms in the structure.

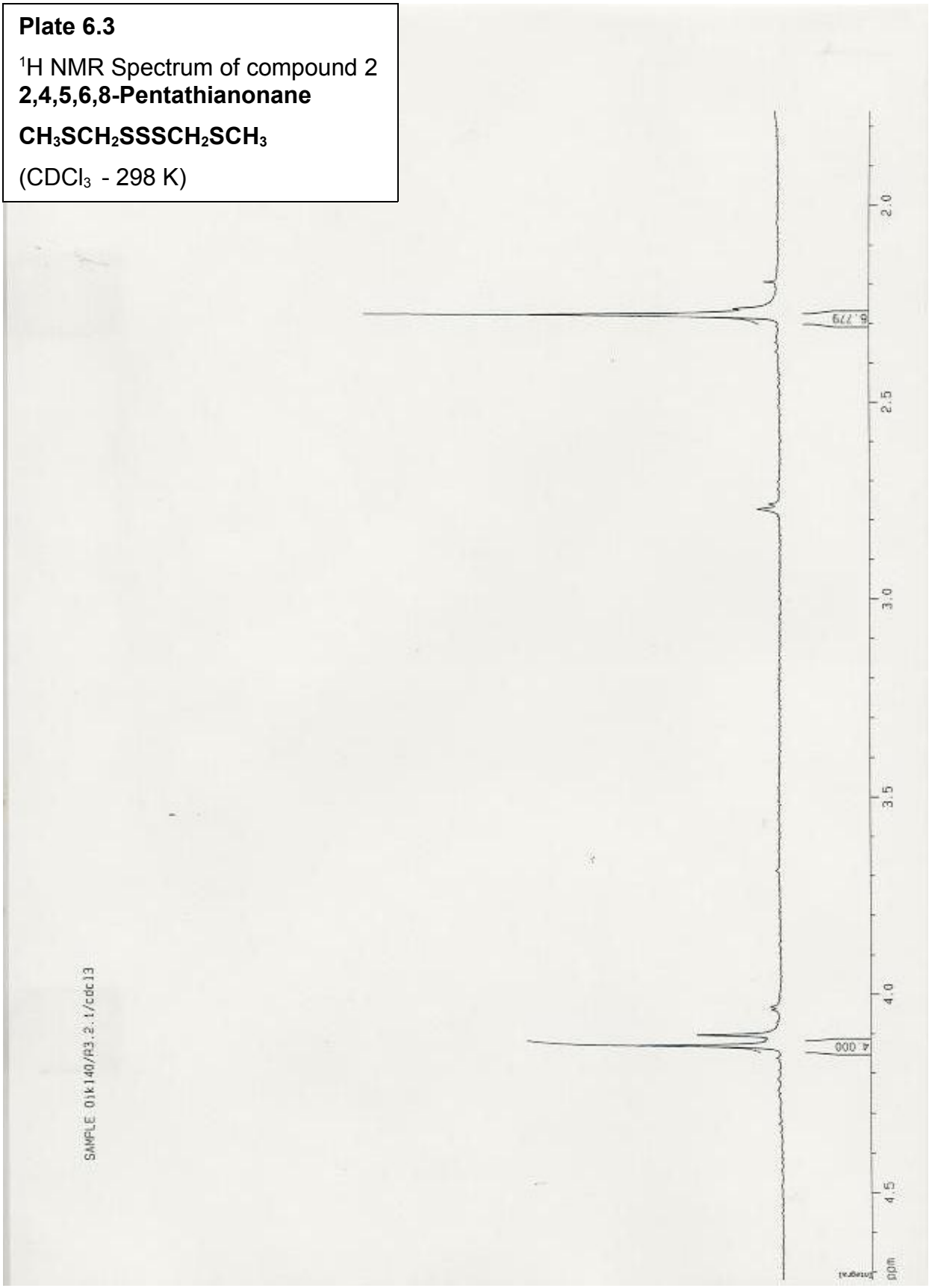
Plate 6.3

^{1}H NMR Spectrum of compound 2

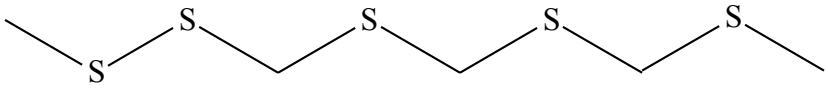
2,4,5,6,8-Pentathianonane

$CH_3SCH_2SSSCH_2SCH_3$

($CDCl_3$ - 298 K)



Compound (3)



Empirical formula: $^{1}CH_3SS^{4}CH_2S^{6}CH_2S^{8}CH_2S^{10}CH_3$

2,3,5,7,8-Pentathiadecane

Purification of fraction A_5 by P-TLC (Hexane:Acetone: 9.5:0.5) afforded compound (**3)** or $A_{5.2}$ as a yellow oil that showed similarity to compounds (**1** and **2)**, The ^{1}H NMR spectrum (**Plate 6.4a)** of compound (**3),** exhibited three methylene protons around δ_H 4.00 and two methyl protons around δ_H 2.00 consistent with the presence of CH_2-S and CH_3-S groups, respectively. Resonances of the peaks in the ^{13}C NMR spectrum (**Plate 6.4b**) was in agreement with the corresponding peaks of the ^{1}H NMR spectrum (**Plate 6.4a).** The singlets at δ_H 3.8 (δ_C 37.2 ppm), δ_H 3.95 (δ_C 40.9 ppm) and δ_H 4.11 (δ_C 45.5 ppm) were assigned to H-8, H-6, and H-4, respectively. The methyl protons were observed at δ_H 2.18 (δ_C 15.0 ppm), H-10 and 2.25 (δ_C 15.6 ppm), H-1. Electron ionization mass spectroscopy (EIMS) showed [M^+] at *m/z* 232 consistent with the molecular formula $C_5H_{12}S_5$ and this was in agreement with the suggested structure of compound (3).

Plate 6.4a

^{1}H NMR Spectrum of Compound 3
2,3,5,7,8-Pentathiadecane

$CH_3SSCH_2SCH_2SCH_2SCH_3$

($CDCl_3$ - 298 K)

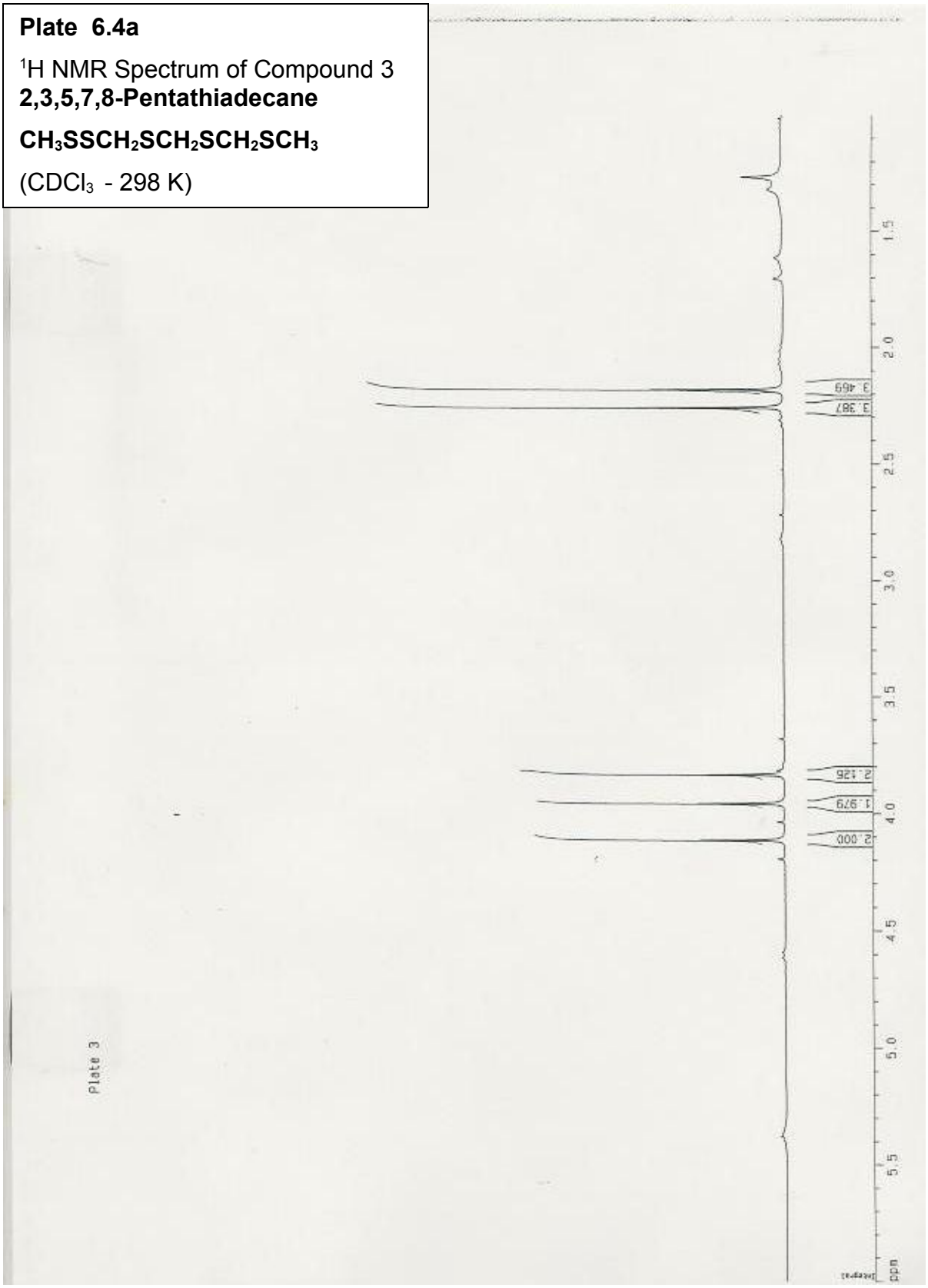
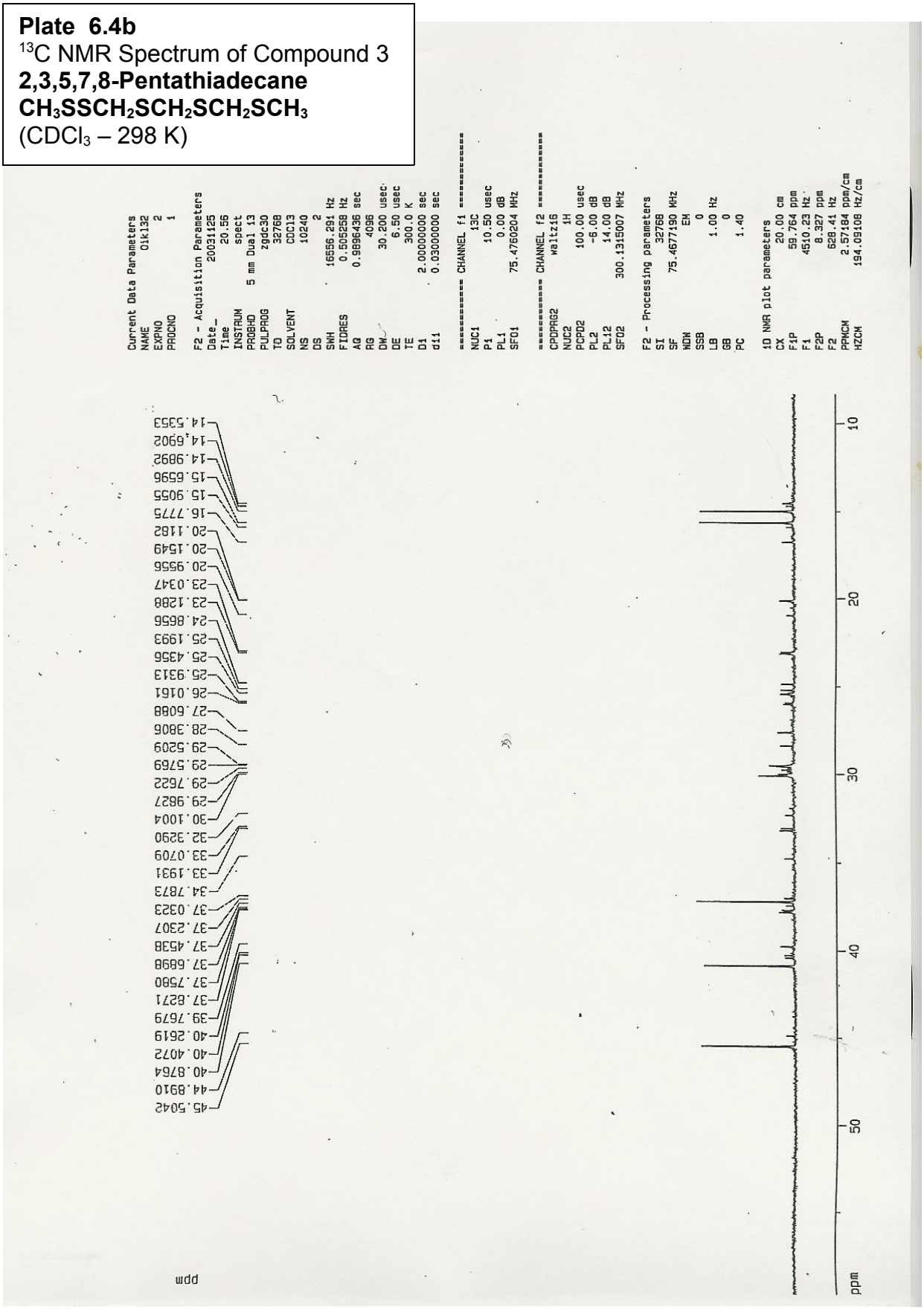
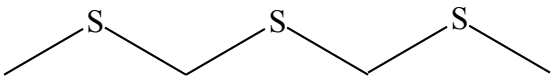


Plate 6.4b
^{13}C NMR Spectrum of Compound 3
2,3,5,7,8-Pentathiadecane
$CH_3SSCH_2SCH_2SCH_2SCH_3$
($CDCl_3$ – 298 K)



Compound (4)



Empirical formual: $^{1}CH_3S^{3}CH_2S^{5}CH_2S^{7}CH_3$

2,4,6-Trithiaheptane

Attempts to purify compound $A_{6.1}$ to yield compound **(4)** were unsuccessful. The ^{1}H NMR spectrum of compound **(4)** (**Plate 6.5a)** showed two methylene singlets and one methyl singlet corresponding to the ^{13}C NMR spectrum (**Plate 6.5b**). In comparison to the ^{1}H NMR spectrum of compound **(1)**, this indicated a mixture of compounds (4) and (1) in almost equal amounts. Furthermore it is observed that the ^{1}H NMR spectrum of compound (4), the methylene proton (δ_H 4.5) is deshielded in comparison to that in compound (1) due to an extra sulphur atom in the structure of (1). When the methylene protons (δ_H 4.00) and the methyl protons (δ_H 2.21) of compound (1) (**Plate 6.2),** identical to those in **Plate 6.5a,** were eliminated from the spectrum, the remaining protons were allocated to compound (4).

Plate 6.5a
^{1}H NMR Spectrum of Compound 4
2,4,6-Trithiaheptane
$CH_3SCH_2SCH_2SCH_3$
($CDCl_3$ - 298 K)

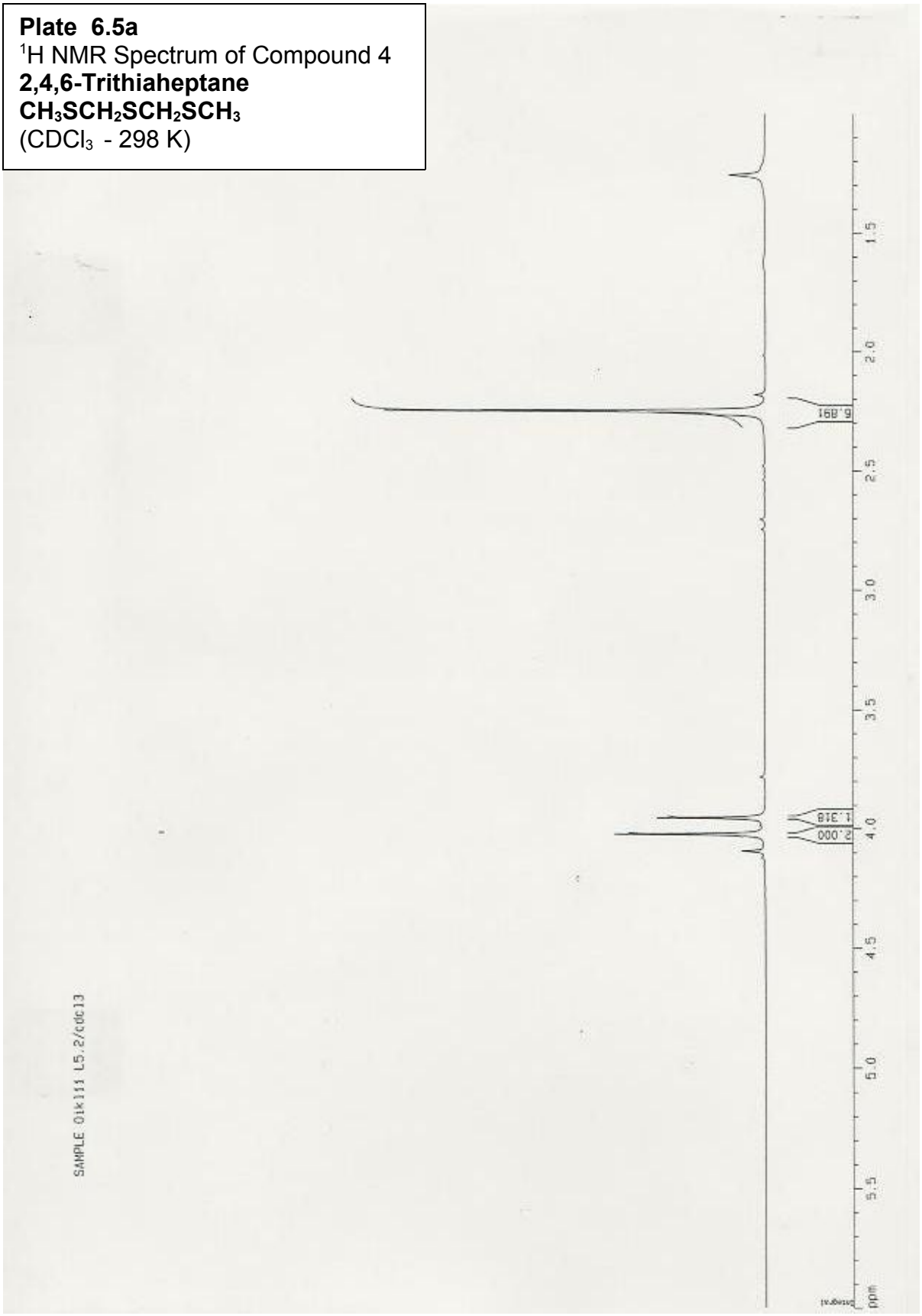
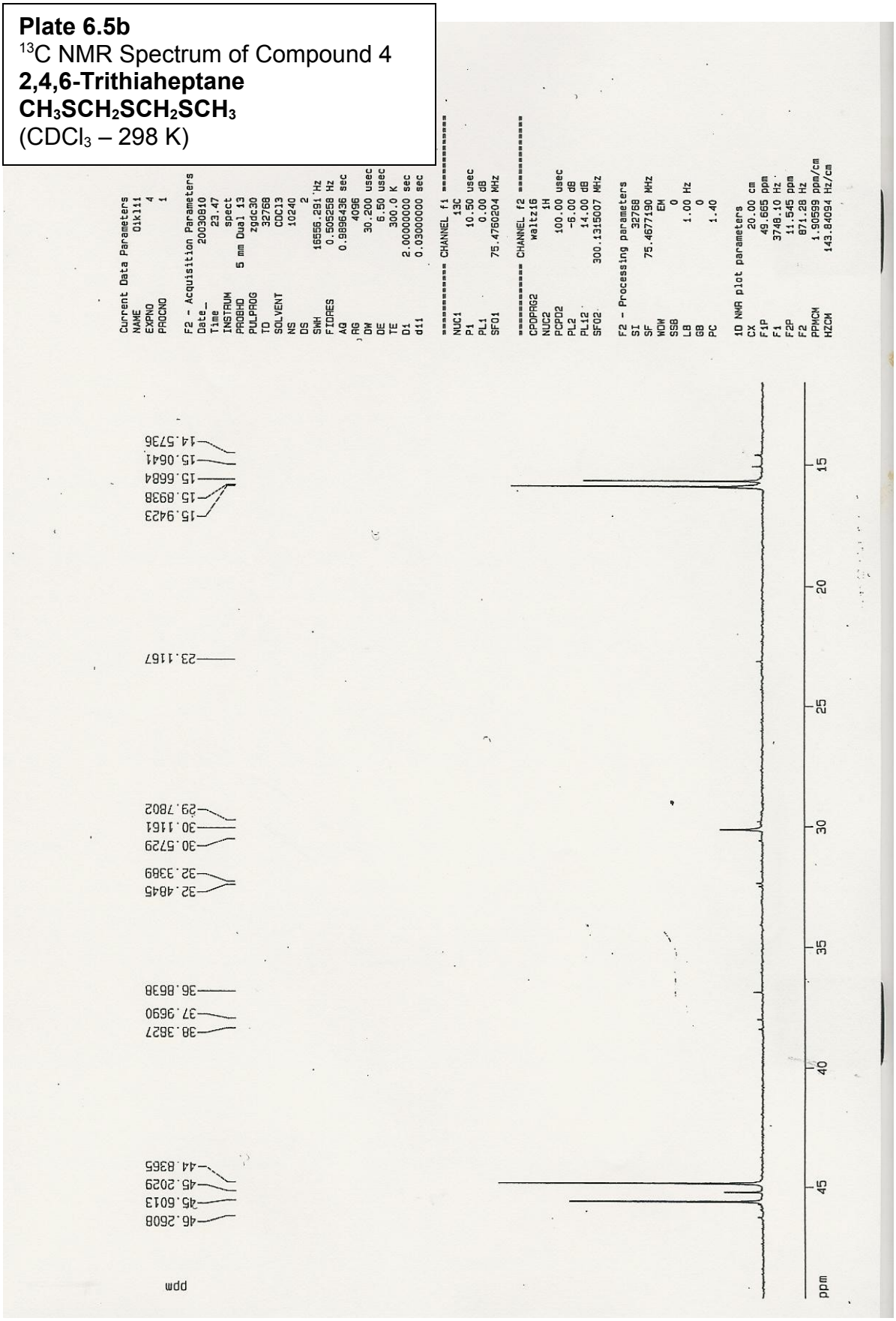
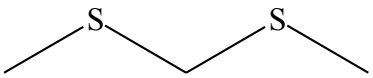


Plate 6.5b
^{13}C NMR Spectrum of Compound 4
2,4,6-Trithiaheptane
$CH_3SCH_2SCH_2SCH_3$
($CDCl_3$ – 298 K)



Compound (5)



Empirical formula: $^{1}CH_3S^{3}CH_2S^{5}CH_3$

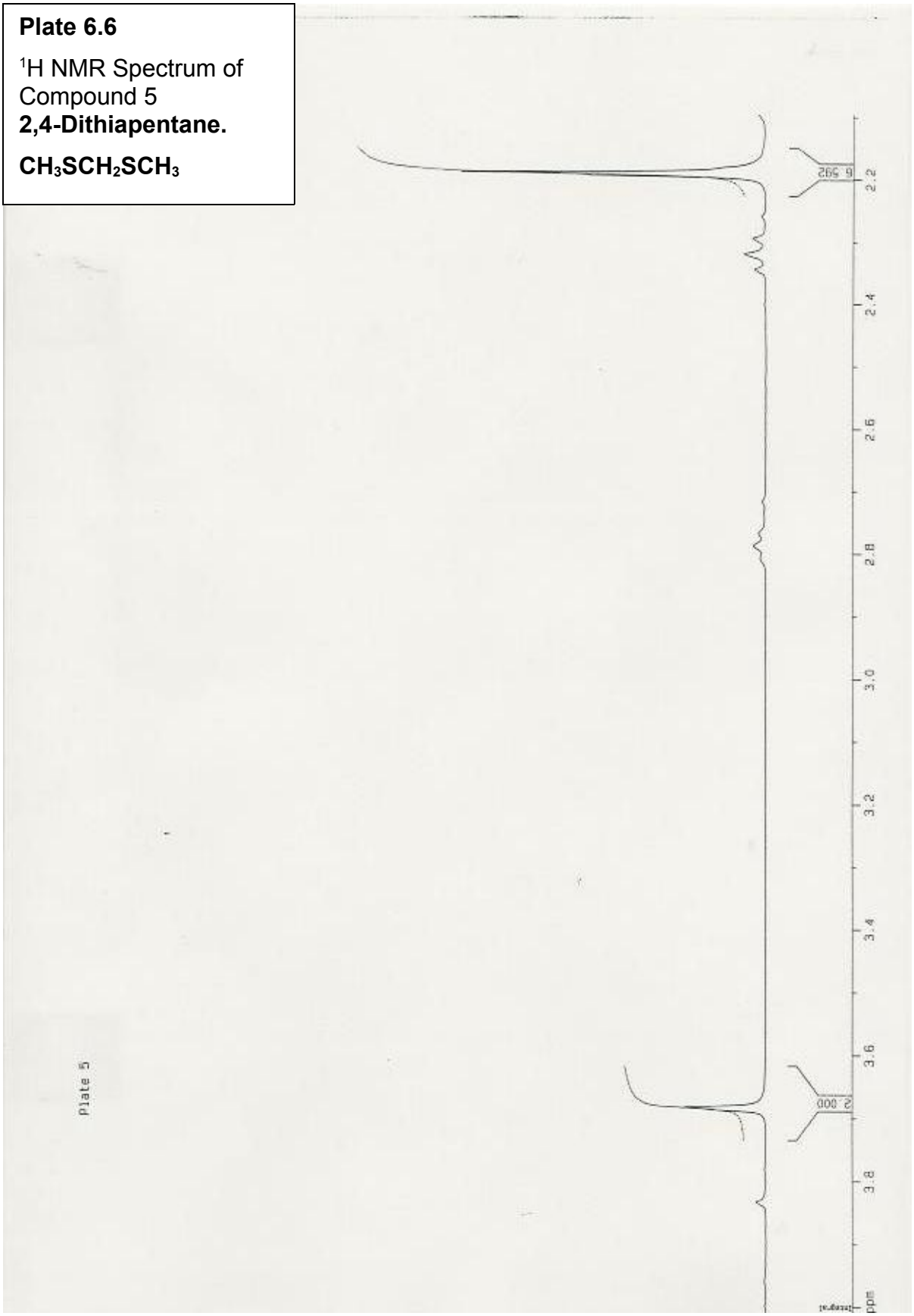
2,4-Dithiapentane.

P-TLC isolation of compound $A_{7.1}$ from fraction A_7 (Hexane:Acetone: 9.5:0.5), afforded compound (**5)** as a yellow oil. Methylene and methyl protons resonating as singlets at δ_H 3.67 and 2.18, respectively, were observed in the ^{1}H NMR spectrum (**Plate 6.6**). The less de-shielded CH_2-S methylene (δ 3.67), as compared to the CH_2-SS integrating for 2 protons, and the characteristic CH_3-S methyl protons integrating for six protons, were assigned to H-2 and H-1, respectively.

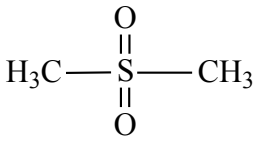
Plate 6.6

^{1}H NMR Spectrum of Compound 5
2,4-Dithiapentane.

$CH_3SCH_2SCH_3$



Compound (6)



Empirical formula: $CH_3SO_2CH_3$

Methylthiosulphonate

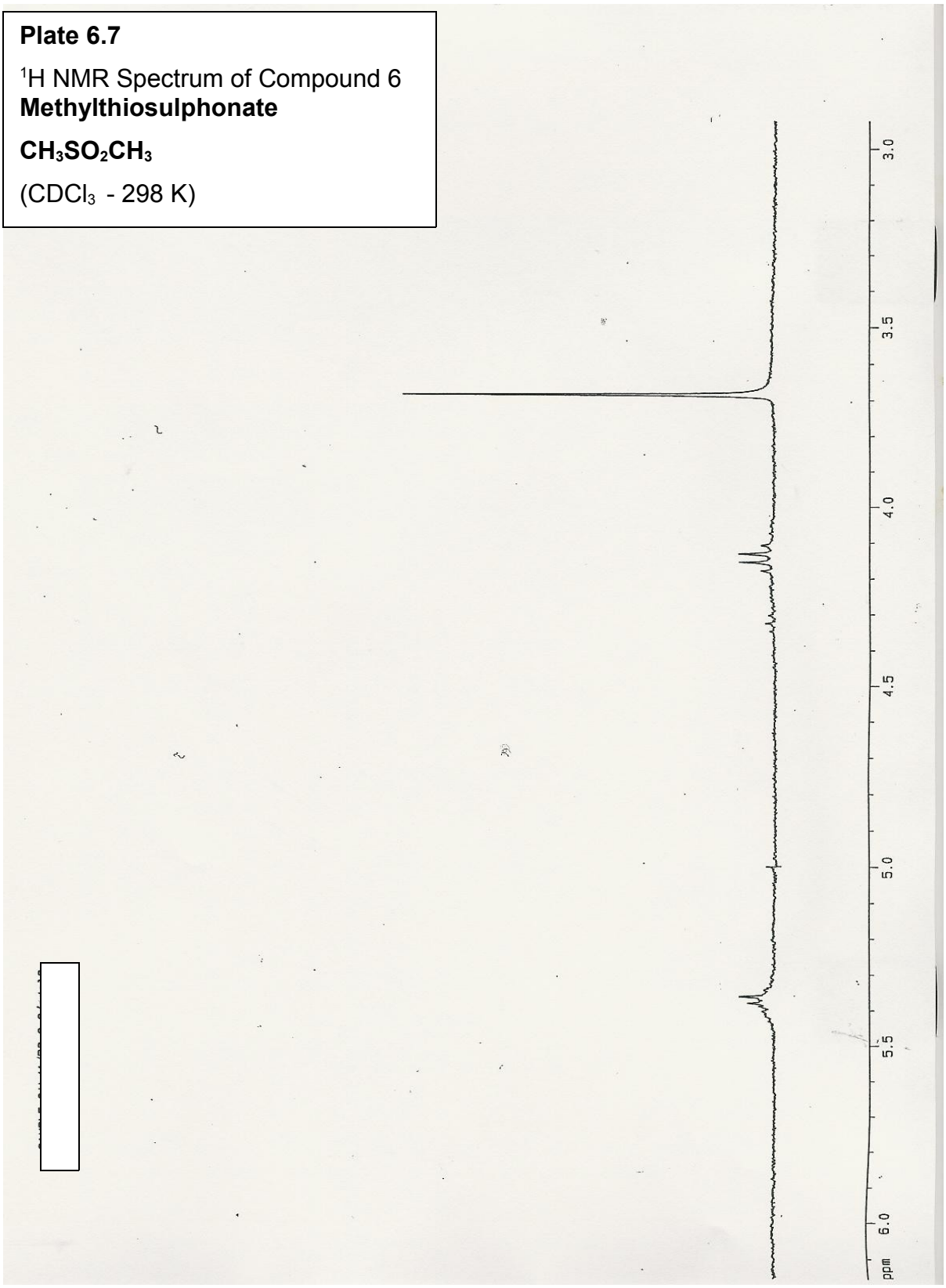
P-TLC separation (Hexane:Acetone: 9.5:0.5) of compound 6 ($A_{7.2}$) from fraction A_7 was also a yellow oil. Although the ^1H NMR spectrum of compound (**6**) (**Plate 6.7**) showed peaks which belonged to linoleic acid, a prominent singlet at δ 3.7 that was not part of those peaks observed. Owing to the impurity of the isolated compound, accurate mass could not be determined. The assignment of the tentative structure (**6**) was based entirely on the resonating position of the methyl protons in the ^1H NMR spectrum. The electronegative sulphur and oxygen atoms de-shielded the $-CH_3$ protons shifting them to chemical shift δ 3.7 as opposed to CH_3S protons which resonate around δ 2.10.

Plate 6.7

^{1}H NMR Spectrum of Compound 6
Methylthiosulphonate

$CH_3SO_2CH_3$

($CDCl_3$ - 298 K)



6.4 Discussion

According to Kubec *et al.* (2002), no systematic research on *T. violacea* has been conducted to date and only three scientific papers dealing with the chemical constituents of society garlic have been published. Jacobsen *et al*. (1968) reported the presence of a C–S lyase and three unidentified *S*-substituted cysteine sulfoxide derivatives, whereas studies of Burton (1990) and Burton and Kaye (1992) led to the isolation of 2,4,5,7-tetrathiaoctane-2,2-dioxide and 2,4,5,7-tetrathiaoctane from the leaves of *T. violacea*. Further, no previous reference to the antifungal properties of society garlic could be detected in the literature. Both these aspects supplied the rationale for this comprehensive investigation.

In this study, the liquid-liquid extraction procedure of aerial and below soil parts of *T. violaceae* showed that hexane extracted substantially more compounds than did the other organic solvents diethyl ether, ethyl acetate and dichloromethane indicating that the plant contained a large amount of rather non-polar substances (Buckley, 1999). This was confirmed by both mass recovery measurement and qualitative thin layer chromatography (Q-TLC) profiles of the liquid-liquid extracts. Although six times less compounds were recovered from the diethyl ether extract of the aerial parts and four times less from the below soil part extract compared to hexane, diethyl ether extracted the second most compounds. Much less compounds were recovered from the remaining more polar solvents, ethyl acetate and dichloromethane. This is consistent with observations made by Zidan *et al.* (2000) on isolating bioactive components from *Eucalyptys* species.

The subsequent bio-test for antifungal activity confirmed that most of the active substances were contained in the hexane and diethyl ether extracts as both showed higher activity against all six test fungi. However, albeit much lower, the ethyl acetate and dichloromethane liquid-liquid extracts also showed antifungal activity to some extent indicating that the active principles seemed to have been either thinly

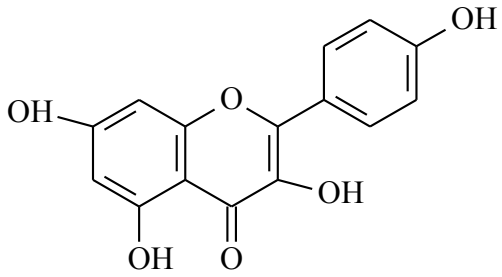
distributed between organic solvents or that other, more polar, substances were also active.

For some fungi (e.g. *B. cinerea, S. rolfsii* and *B. dothidea*) there was no significant difference in mycelial growth inhibition between the hexane and diethyl ether extracts of both aerial and below soil part extracts. As substantially more compounds were recovered from the hexane extract, and diethyl ether also being rather non-polar, it was speculated that the active principles contained in these two solvents were probably the same. The decision was made to purify the active substances only from the hexane extracts of both the aerial and below soil parts of *T. violaceae*. In a similar study, Murugesan *et al.* (2002) demonstrated that hexane extracts of *Acacia nilotica* seeds produced the highest antifungal activity against a wide range of plant pathogenic fungi compared to the other organic solvents while Cao *et al.* (2003) found the hexane and diethyl ether extracts of bamboo (*Phyllostachys pubescens*) to be equally active. Although the recovery of compounds from *T. violacea* in the three solvents other than hexane was rather low, it is suggested that purification of active compounds from them be attempted in future as the possibility may exist for isolating bioactive compounds different from those present in the hexane extracts.

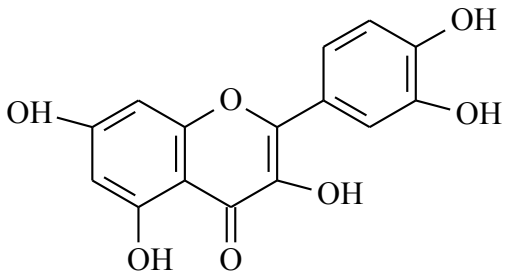
Activity directed column chromatography separation (Jang *et al.,* 2001; He *et al.,* 2002) of compounds in the hexane extracts yielded four active combined fractions from the aerial part and two from the below soil part extracts. Subsequent activity directed purification of compounds from these column fractions by means of P-TLC resulted in the isolation of six pure compounds that all showed above average antifungal activity. By means of NMR spectroscopy their chemical structures were elucidated and the compounds identified. From the below soil parts of *T. violacea* two antifungal compounds were identified as the previously described 2,4,5,7-tetrathiaoctane (Burton & Kaye, 1992) and the novel 2,4,5,6,8-pentathianonane. From the aerial parts four antifungal compounds were identified. Of these, to the best of our knowledge, only methyl thiosulphonate is known while 2,3,5,7,8-pentathiadecane, 2,4,6-trithiaheptane and 2,4-dithiapentane are most probably novel compounds.

Interestingly, both Burton (1990) and Kubec *et al.* (2002) isolated sulphoxides from water extracts of *T. violacea* but did not test these compounds for antifungal activity. In this study, probably owing to the low polarity of hexane, none was detected.

The detection of sulphur-containing compounds in *T. violacea* (wild garlic) did not come as a surprise as *Allium sativum* (garlic) has been known to be rich in sulphur compounds that give them their distinct garlic smell and pungency (Brewster, 1994). What was surprising is the fact that all of the antifungal compounds purified form the hexane extracts of aerial and below soil parts of *T. violaceae*, except for methylthiosulphonate, were rather simple aliphatic sulphur-containing alkanes. Although Burton (1990) did not test for antifungal activity, he demonstrated the presence of two sulphur compounds in the aqueous extracts of *T. violacea* namely 2,4,5,7-tetrathiaoctane-2,2-dioxide and 2,4,5,7-tetrathiaoctane. The latter is the same as compound (**1**) identified in this study. Burton (1990), in applying the qualitative TLC technique together with specific spray reagents (Wagner and Bladt, 1996), suggested the possible presence of steroidal saponins in *T. violacea*. Applying the same technique and by using commercial standards, Burton (1990) confirmed the presence of the two flavonoids kaempferol and quercetin (Burton, 1990). In a comprehensive review, Pretorius (2003) elaborated on the anti-infective properties of plant derived flavonoids against human and plant pathogens, especially plant pathogenic fungi, as well as its application potential in the pharmaceutical and agricultural industries.



Kaempferol [2-(4-hydroxyphenyl)-3,5,7-trihydroxy-1-benzopyran-4-one]

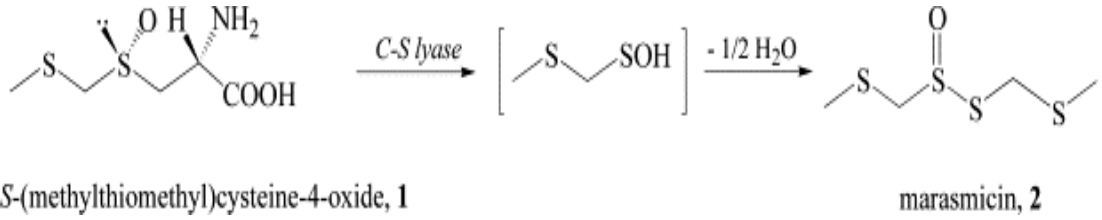


Quercetin [2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-1-benzopyran-4-one]

Other sulphur containing compounds, including *S*-methylcysteine and the *S*-ethylcysteine derivatives methiin [(S_SR_C)-*S*-methylcysteine sulfoxide] and ethiin [(S_SR_C)-*S*-ethylcysteine sulfoxide] have recently been detected in minute amounts in all plant parts of *T. violacea* (Kubec *et al.,* 2002). Methiin is a very common secondary metabolite occurring in plants of many families including *Alliaceae, Brassicaceae*, and *Leguminosae*. The other amino acid derivative, ethiin, has also only recently been found as a minor component in some *Allium* species and in members of several genera of the *Brassicaceae* family (Kubec *et al.,* 2000; Kubec *et al.,* 2001; Kubec and Musah, 2001). However, neither of these has been connected with the antifungal properties of the plant.

In extracts of both the aerial and below soil parts of *T. violacea*, marasmin (2,4,5,7-tetrathiaoctane-4-oxide) was found to be the predominant derivative accounting for nearly 99% of the *S*-alkylcysteine sulfoxide pool (Kubec *et al.*, 2002). Gmelin *et al.* (1976) were the first to propose that the enzymatic cleavage of marasmin is analogous to that of alliin (*S*-allylcysteine sulfoxide) in garlic and other alliaceous species. They suggested marasmicin to be the primary breakdown product resulting in the distinct odour. Marasmicin is very unstable and decomposes rapidly resulting in various sulphur-containing degradation products including 2,4,5,7-tetrathiaoctane-2,2-dioxide, 2,4,5,7-tetrathiaoctane-4,4-dioxide or 2,4,5,7-tetrathiaoctane-2,2,7,7-tetraoxide. Many of these compounds have been shown to possess strong antimicrobial and antifungal activity as well as anti-thrombotic properties (Takazawa *et al.* 1982; Block, 1992; Burton and Kaye, 1992; Kubota *et al*. 1994; Rapior *et al.*

1997; Kubota *et al*. 1998; Lim *et al.* 1998; Lim *et al.* 1999). The antifungal compounds 2,4-dithiapentane, 2,4,6-trithiaheptane, 2,4,5,7-tetrathiaoctane, 2,4,5,6,8-pentathianonane, 2,3,5,7,8-pentathiadecane and methylthiosulphonate identified in this study do not contain oxygen and are most probably not degradation products of marasmicin.



(R_SR_C)-*S*-(methylthiomethyl)cysteine-4-oxide, **1** marasmicin, **2**

Formation of marasmicin from marasmin [(R_SR_C)-*S*-(methylthiomethyl)cysteine-4-oxide] in *Tulbaghia violacea.*

The absence of alliin in *T. violacea,* identified as the precursor of the main volatile components detected in the breath after ingestion of real garlic (*A. sativum* L.) (Ruiz *et al.,* 1994; Cai *et al.,* 1995) may justify the trivial name of "Society garlic" linked with *T. violacea*. Apparently, the compounds generated are different from those detected in garlic breath. Furthermore, when the rhizomes or aerial parts are handled with bare hands, a strong organosulphur scent persists on the fingers for several hours. It seems reasonable to assume that the odour producing compounds are decomposition products of marasmicin, such as methylthiomethyl mercaptan as well as bis-methylthiomethyl sulfides, sulfones and thiosulfonates (Kubec *et al.*, 2002).

Finally, an aspect that needs special consideration is the fact that the methanolic crude extract of *T. violacea* showed higher broad spectrum antifungal activity against all the test fungi than did the liquid-liquid extracts and the semi-purified column chromatography fractions. Further, one of the active semi-purified column

chromatography fractions (A_8 of the aerial parts) showed high antifungal activity but on separation of the compounds contained in this fraction, the activity was lost. This complicates the isolation and identification of all active substances from plants and strongly indicates that the natural resistance of plants against biotic stress conditions largely depends on the synergistic or combined effect of different active compounds. The presence of polar saponins and flavonoids (Burton, 1990) in *T. violaceae* that are anti-infective agents (Pretorius, 2003), in combination with the presence of active sulphur containing compounds identified in this study, can explain the higher antifungal activity detected in the crude and semi-purified extracts compared to that of purified compounds.

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CHAPTER 7

GENERAL DISCUSSION AND CONCLUSIONS

Phytopathogenic fungi alone cause nearly 20% yield reduction of the major food and cash crops annually (Agrios, 2000). Since World War II, traditional agricultural practices have been replaced by the use of synthetic chemicals for the management of plant pathogens, pests and weeds. This has, without any doubt, increased crop production but with some deterioration of the environment and human health (Cutler & Cutler, 1999). Research indicates that even if one never uses pesticides, one can still be exposed to them by being a consumer of commodities that others have treated with pesticides, e.g. through food.

In addition to the target pathogen, pesticides may also kill various beneficial organisms and their toxic forms can persist in the soil (Hayes and Laws, 1991). The increasing incidence of resistance among pathogens towards synthetic chemicals is also a cause for serious concern. The above is not only of major concern to the developed countries, where consumer preferences are for organically produced foods, but also in the developing world, such as Africa, where synthetic pesticides are too expensive for subsistence farming. Because of these problems there is a need to find alternatives to synthetic pesticides.

Among the various alternatives, natural plant products that are bio-degradable and eco-friendly are receiving the attention of scientists worldwide. Such products derived from higher plants and microbes are relatively bio-efficacious, economical and environmentally safe and can be ideal candidates for use as agrochemicals (Macias *et al.,* 1997; Wilson *et al.,* 1997; Cutler, 1999). Additionally, the manufacturers of natural bio-stimulants applied in agricultural claim increased production, profit increases, cutting of operating costs and reduction of fertilizer costs with no detrimental effect to the environment (Chen *et al.,* 2003). A number of plants showing

the potential to act as donor plants for these natural products were outlined in Chapter 2.

According to Kubec *et al.* (2002), no research on the antimicrobial and bio-stimulatory properties of *Tulbaghia violacea* (wild garlic) have been conducted in the past despite it being well known for its medicinal uses among the indigenous people of Southern Africa. Yet, extensive research has been done on the antimicrobial and medicinal properties of domesticated garlic (*Allium sativum*). This supplied the rationale to screen extracts of *T. violacea* for bio-stimulatory and antimicrobial properties with the aim to evaluate the potential of developing one or more natural products for the agricultural industry.

Both the aerial and below soil part crude extracts of *T.* violacea significantly increased the respiration rate of a monoculture yeast cells and this compared favourably with that of a commercial bio-stimulant, ComCat® (Chapter 3). As an increase in the respiration rate of cells can either indicate a negative (enhanced catabolism) or a positive (enhanced anabolism) response, it was necessary to verify this aspect. A second bio-test was employed namely to test the effect of the crude extracts on Cress seed germination and subsequent seedling growth. The results indicated a positive correlation between the high respiration rate observed in monoculture yeast cells and seedling growth in terms of radicle and coleoptile growth. However, this aspect was not pursued further as preliminary bio-tests indicated that the inherent anti-microbial properties of the crude extracts were more significant in comparison. It is recommended that the bio-stimulatory potential of *T. violacea* extracts be investigated further in future.

The growth of three out of the six phytopathogenic bacteria used as test organisms was significantly inhibited *in vitro* by both aerial and below soil plant part crude extracts of *T. violacea*. However, both crude extracts showed significant *in vitro* mycelial growth inhibition of all six test fungi confirming their broad spectrum antifungal potential (Chapter 3). In all cases the crude extracts exceeded the efficacy

of the standard fungicide used as a positive control and completely inhibited the mycelial growth of the highly resistant *P. ultimum*. For this reason a further investigation on bacteria was terminated and attention turned to the comparatively more effective antifungal activity of the extracts.

In view of the significant *in vitro* antifungal activity observed, an *in vivo* investigation was conducted (Chapter 4) in order to establish whether crude extracts of *T. violacea* were able to display similar significant antifungal activity in preventing the infection of a crop by a test fungus under glasshouse conditions. The latter seemed important in view of earlier arguments (Benner, 1993) that the *in vitro* growth inhibition of micro-organisms has not always been consistent with that of *in vivo* results and that phytotoxicity might be a limiting factor in pursuing natural product development. For this purpose *Mycosphaerella pinodes*, causing Ascochyta blight in pea (*Pisum sativum*) leaves, was used as test organism.

A crude extract of aerial plant parts of *T. violacea* was tested *in vivo* against *Mycosphaerella pinodes* infection of detached pea (*Pisum sativum*) leaves by applying the extract both before and after spore inoculation. The extract completely suppressed lesion development significantly at relatively low concentrations by preventing spore germination of *M. pinodes* when applied both before and after spore inoculation. The crude extract was also not phytotoxic to pea leaves even at the highest concentration of 2.0 mg ml^{-1} indicating that the aerial part extract possesses the inherent potential to be used as either a preventative or a corrective spray on pea against Ascochyta blight caused by *M. pinodes* without causing injury to the plant.

Subsequently, in order to ascertain the *in vivo* potential of an aerial part extract of *T. violaceae* under field conditions, a field trial was conducted using covered kernel and loose smuts, two seed borne fungal pathogens of sorghum, as test organisms (Chapter 5). Seeds were inoculated with spores and treated with an aerial part crude extract before planting and the incidence of these two pathogens recorded. Pre-treatment of sorghum seeds with an aerial part crude extract of *T. violacea* at a rate of

2 g L^{-1} completely prevented infection by covered kernel smut, significantly reduced the incidence of loose smut and resulted in significant yield increases compared to the untreated control. The reduction of smuts incidence, due to treatment with the extract, was equivalent to that of the standard fungicide, Thiram, used as a positive control. Most importantly, the extract showed no observable adverse effects on sorghum seed germination. Together with the results obtained in the glasshouse trial (*M. pinodes*), this trial confirmed the inherent potential of a crude *T. violacea* aerial part extract to be applied as a natural fungicide, also under field conditions.

On the basis of this confirmation, as well as the *in vitro* results obtained earlier, it was decided to proceed with the isolation and purification (Chapter 6) of the antifungal active substances from both the aerial and below soil plant part crude extracts of *T. violacea*. Activity directed fractionation of the crude extracts by means of liquid-liquid extraction revealed that most of the antifungal activity was located in the hexane and diethyl ether fractions of both plant parts.

Further purification of active compounds from the hexane fractions by means of column and preparative thin layer chromatography led to the isolation of six antifungal compounds whose structures were elucidated by NMR spectroscopy and Mass spectrometry. These were identified as straight chain carbon sulphur-containing compounds lacking oxygen in their structures, except for compound 6 (Methyl thiosulphonate), confirming that the remaining five compounds could not have been degradation products of unstable Marasmicin (Kubec *et al.,* 2002). Of the five remaining compounds isolated from aerial and below soil part extracts of T. violacea and that revealed antifungal activity, four were identified as novel and included 2,4-dithiapentane, 2,4,6-trithiaheptane, 2,4,5,6,8-pentathianonane and 2,3,5,7,8-pentathiadecane. The sixth compound was identified as 2,4,5,7-tetrathiaoctane that was discovered earlier in *T. violacea* by Burton (1990).

Burton (1990) additionally isolated the flavonoids, Quercetin and Kaempferol from an aqueous extract of *T. violacea*. Both are known for their antifungal properties

(Weidenbörner *et al.* 1990; Harborne, 1999). As antifungal compounds from only the rather non-polar hexane fraction of *T. violacea* were isolated in this study, chances are that these more polar flavonoids might have been located in the more polar ethyl acetate or dichloro methane extracts. This might explain the antifungal activity detected in the latter two liquid-liquid extracts. In any case, this activity was much lower than what was detected in the hexane and diethyl ether extracts.

The significance of this discovery is that both *in vitro* and *in vivo* (glasshouse and field) tests confirmed the inherent antifungal activity in crude extracts of both aerial and below soil parts of *T. violacea* while no phytotoxic effects on the crops used in the *in vivo* bio-assays were observed. As both crude extracts are soluble in water, this indicates strongly that one or both possess the potential to be applied directly as natural antifungal agents in the agricultural industry. Alternatively, depending on the economics of the production process, either semi-purified extracts or purified compounds can be developed as natural fungicides. From an industrial perspective the development of at least one natural product, with broad spectrum antifungal activity, needs to be considered.

The term “natural product” implies that *T. violacea* will have to be cultivated on a large scale to act as donor plant for producing the fungicide either in crude, semi-purified or purified form. The latter further implies that *T. violacea* possess the potential to be developed as an alternative crop in the agricultural industry. Undoubtedly the economics of using the plant material directly or applying synthesized analogues of the active substances will have to be weighed against each other.

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SUMMARY

The extracts of many plants display antimicrobial properties and some show allelopathic and bio-stimulatory activities (Chapter 2). Consequently, in this study the crude extracts of *Tulbaghia violacea* (wild garlic) were screened for antimicrobial and bio-stimulatory properties (Chapter 3). The antifungal properties of crude methanol extracts were determined *in vitro* against six economically important plant pathogenic fungi. The crude extracts showed significant *in vitro* mycelial growth inhibition of all six test fungi and outperformed the standard fungicide used as a positive control (Chapter 3). *In vitro* screening for antibacterial properties performed on six plant pathogenic bacteria, showed a significant inhibition of the growth of three out of the six phytopathogenic bacteria. Both aerial and below soil part crude extracts of *T. violacea* showed significant bio-stimulatory activity in terms of seedling growth enhancement.

In view of the significant *in vitro* antifungal activity observed, an *in vivo* test was conducted (Chapter 4). A crude extract of aerial plant parts of *T. violacea* was tested against *Mycosphaerella pinodes* (causing Ascochyta blight in pea) infection of detached pea (*Pisum sativum*) leaves under glasshouse conditions. The extract completely suppressed lesion development by preventing spore germination of *M. pinodes* without showing phytotoxicity to pea leaves.

Subsequently, in order to ascertain the *in vivo* potential of an aerial part extract of *T. violacea* under field conditions, a field trial was conducted using covered kernel and loose smuts, two seed borne fungal pathogens of sorghum, as test organisms (Chapter 5). Seeds were inoculated with spores and treated with an aerial part crude extract before planting and the incidence of these two pathogens recorded. Pre-treatment of sorghum seeds with an aerial part crude extract of *T. violacea* completely prevented infection by covered kernel smut, significantly reduced the incidence of loose smut and resulted in significant yield increases compared to the untreated control. The reduction of smuts incidence, due to treatment with the extract, was equivalent to that of the standard fungicide, Thiram, used as a positive control.

Crude methanol extracts of *T. violacea* were then purified by means of activity directed liquid-liquid extraction, column and preparative thin layer chromatography (Chapter 6) which led to the isolation of six antifungal compounds whose structures were elucidated by NMR spectroscopy and Mass spectrometry. Five of these were identified as straight chain carbon sulphur-containing compounds and compound 6 as Methyl thiosulphonate. Four of these compounds were identified as novel and included 2,4-dithiapentane, 2,4,6-trithiaheptane, 2,4,5,6,8-pentathianonane and 2,3,5,7,8-pentathiadecane. The fifth compound was identified as 2,4,5,7-tetrathiaoctane that was discovered earlier.

The significance of this discovery is that the crude extracts of *T. violacea* possess the potential to be applied directly as natural antifungal agents in the agricultural industry. Alternatively, depending on the economics of the production process, either semi-purified extracts or purified compounds can be developed as natural fungicides. From an industrial perspective the development of at least one natural product, with broad spectrum antifungal activity, needs to be considered. *T. violacea* can therefore be considered as an alternative field crop in the agricultural industry to serve as a donor plant for the production of “natural products.”

OPSOMMING

Ekstrakte van ‘n verskeidenheid wilde plante besit antimikrobiese eienskappe en sommiges toon sterk allelopatiese en biostimulerende aktiwiteite (Hoofstuk 2). Gevolglik is ru-ekstrakte van *Tulbaghia violacea* (wilde knoffel) vir beide antimikrobiese eienskappe en biostimulerende aktiwiteit in hierdie studie getoets (Hoofstuk 3). Die antifungale eienskappe van ru-ekstrakte is aanvanklik *in vitro* teen ses ekonomies belangrike plant patogeniese fungi getoets. Ru-ekstrakte het betekenisvolle *in vitro* miselium groei inhibisie teen al ses fungi getoon terwyl die aktiwiteit van ‘n standaard fungisied, wat as positiewe kontrole gebruik is, oortref is (Hoofstuk 3). *In vitro* toetsing vir antibakteriese eienskappe teen ses plant patogeniese bakterieë het aangetoon dat die groei van drie betekenisvol geïnhibeer

is. Ru-ekstrakte van beide die bogrondse en ondergrondse dele van *T. violacea* het ook betekenisvolle biostimulerende aktiwiteit in terme van saailinggroei getoon.

In die lig van die betekenisvolle *in vitro* antifungale aktiwiteit wat met die ru-ekstrakte waargeneem is, is 'n *in vivo* toets gebruik om hierdie potensiaal te verifieer (Hoofstuk 4). 'n Ru-ekstrak van bogrondse dele van *T. violacea* is teen *Mycosphaerella pinodes* (oorsaak van swartvlek op ertjies) infeksie op verwyderde erjieblare (*Pisum sativum*) onder glashuis toestande getoets. Die ekstrak het letselvorming verhoed deur *M. pinodes* spoor ontkieming te inhibeer sonder enige fitotoksiese effek op die blare.

Vervolgens, ten einde die *in vivo* potensiaal van die bogrondse deel ekstrak van *T. violacea* onder veldtoestande te bevestig, is stinkbrand en losbrand, twee saaddraende fungale patogene van sorghum, as toetsorganismes gebruik (Hoofstuk 5). Sorghumsaad is vooraf apart met spore van beide patogene geïnokuleer en met die bogrondse ru-ekstrak voor uitplanting behandel terwyl die voorkoms van die twee patogene gekwantifiseer is. Voorafbehandeling van sorghum saad met die bogrondse deel ekstrak van *T. violacea* het infeksie deur stinkbrand volkome verhoed, die voorkoms van losbrand infeksie betekenisvol geïnhibeer en aanleiding gegee tot aansienlike oesopbrengsverhogings in vergelyking met die onbehandelde kontrole. Die reduksie van stink en losbrand voorkoms deur voorafbehandeling met die ru-ekstrak was net so effektief as die standard fungisied, Thiram, wat as positiewe kontrole gebruik is.

Ru metanol ekstrakte van *T. violacea* is vervolgens deur middel van aktiwiteitsgerigte vloeistof-vloeistof ekstraksie asook kolom- en preparatiewe dunlaag chromatografie (Hoofstuk 6) gefraksioneer. Laasgenoemde het gelei tot die isolasie van ses antifungale komponente waarvan die struktuurformules deur kernmagnetiese resonans (KMR) spektroskopie en Massaspektrometrie ontsyfer is. Vyf van hierdie komponente is as reguit ketting swaelbevattende alkane geïdentifiseer terwyl die sesde een as Metiel tiosulfonaat geïdentifiseer is. Vier van hierdie komponente is as raar stowwe, wat nog nie voorheen beskryf is nie, geïdentifiseer as 2,4-ditiapentaan,

2,4,6-tritiaheptaan, 2,4,5,6,8-pentatianonaan en 2,3,5,7,8-pentatiadekaan. Die vyfde komponent is as 2,4,5,7-tetratiaoktaan, wat reeds voorheen beskryf is, geïdentifiseer.

Die betekenisvolheid van hierdie ontdekking is dat die ru-ekstrak van *T. violacea* oor die potensiaal beskik om direk as natuurlike fungisied in die landboupraktyk toegepas te word. Alternatiewelik, afhangende van die ekonomiese vatbaarheid van die produksieproses, kan semi-gesuiwerde ekstrakte of gesuiwerde komponente as natuurlike produkte ontwikkel word. Vanuit ‘n industriële perspektief moet die ontwikkeling van minstens een natuurlike produk, met breëspektrum antifungale aktiwiteit, sterk oorweeg word. Oorweging moet ook geskenk word aan die moontlikheid om *T. violacea* as ‘n nuwe gewas op grootskaal te verbou om as skenkerplant vir die produksie van natuurlike produkte benut te word.