

**INDUCTION OF DEFENCE RESPONSES AND  
RESISTANCE TO WHEAT LEAF RUST  
BY PLANT EXTRACTS**

by

**MARIA ELIZABETH CAWOOD**

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Department of Soil, Crop and Climate Sciences and Plant Sciences  
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South Africa

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**Promoter:**

Prof JC Pretorius

Department of Soil, Crop and Climate Sciences  
UFS

**Co-Promoter:**

Prof AJ van der Westhuizen

Department of Plant Sciences  
UFS

## **Declaration**

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I further more cede copyright of the thesis in favour of the University of the Free State.

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Date

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“ O Lord my God! when I in awesome wonder  
Consider all the works Thy hand hath made,  
I see the stars, I hear the mighty thunder,  
Thy power throughout the universe displayed:

Then sings my soul, my Saviour God to Thee,  
How great Thou art! How great Thou Art!”

Stuart Hine

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# | *Abbreviations*

**A**

A	Absorbance
<i>Avr</i>	Avirulence

**B**

BABA	DL-3-aminobutyric acid
BCIP	5-bromo-4-chloro-3-indolyl phosphate
BL	Brassinolide
BR	Brassinosteroids
BTH	benzo (1,2,3) thiadiazole-7-carbothiotic acid S-methyl ester

**C**

CC	<i>ComCat</i> <sup>®</sup>
----	----------------------------

**D**

DC	Di-electric constant
DH-JA	Dihydro jasmonic acid
DMPC	Dimethyl pyrocarbonate
DMSO	Dimethylsulfoxide
dNTP's	Deoxynucleotide triphosphates

**H**

H <sub>2</sub> O <sub>2</sub>	Hydrogen peroxide
H <sub>2</sub> SO <sub>4</sub>	Sulphuric acid
HR	Hypersensitive response
<i>hrp</i> genes	Hypersensitive response and pathogenicity genes

**I**

IgG	Immunoglobulin(s)
INA	2,6-dichloroisonicotinic acid
IR	Infected resistant
IS	Infected susceptible
IWF	Intercellular wash fluid

**J**

JA Jasmonic acid

**K**

kDa kilo Dalton(s)

**L**

LOX Lipoxygenase

**M**

MAPK Mitogen activated protein kinase

Me-JA Methyl-jasmonate

MSB Menadione sodium bisulphate

**N**

NBT Nitro blue tetrazolium

*nahG* *Nicotiana tabacum* containing the salicylate hydroxylase gene

NO Nitric oxide

*NPRI* Non-expressor of *PR1*

NMR Nuclear Magnetic Resonance

**O**O<sub>2</sub><sup>-</sup> Superoxide**P**

PAGE Polyacrylamide gel electrophoresis

PAL Phenylalanine ammonialyase

PCR Polymerase chain reaction

ppm Parts per million

PR Pathogenesis related

P-TLC Preparative Thin Layer Chromatography

**Q**

Q-TLC Qualitative thin layer chromatography

<b>R</b>	
R	Resistant uninfected
<i>R</i>	Resistance genes
Rf	Relative to frontline
ROS	Reactive oxygen species
RT	Reverse transcription
<b>S</b>	
S	Susceptible uninfected
SA	Salicylic acid
SAR	Systemic acquired resistance
SDS	Sodium dodecyl sulphate
SIR	Systemic induced resistance
SS	<i>Lupinus albus</i> seed suspension
<b>T</b>	
TMV	Tobacco mosaic virus
<b>V</b>	
v/v	Volume per volume
<b>W</b>	
w/v	Weight per volume

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# | *Chapter 1*

## INTRODUCTION AND RATIONALE

The course of evolution has determined the state of coexistence between plants, microorganisms and insects leading to interactions ranging from mutualism to antagonism. The latter understandably demands preventative measures to ensure the sustained nutritional requirements of both humans and animals and thus man has resorted to the use of chemicals in the form of pesticides, fungicides and bactericides on crop plants. The long-term effects of these conventional control techniques in contaminating the environment are of special concern together with the fact that frequent application of fungicides has resulted in fungal mutation and, subsequently, new resistant strains (Khun 1989). Fungicides also have detrimental effects on both the environment and the quality of food products, not to mention the environmentally harmful side-effects on non-target organisms. Moreover, consumer resistance towards the use of synthetic chemicals has escalated, especially in developed countries, where chemical treatment of crop plants is continuously criticized in the media by Green Revolution supporters. It is therefore not out of the ordinary that a very bleak picture is offered to the general public in terms of the sustenance of life and the environment. Hence, the urgency to reduce the catastrophic implications of agrochemicals calls for the implementation of natural product alternatives in the agricultural industry (Duke *et al.* 1995).

The use of natural plant products in agriculture is not new, but dates back to the time of Democratus (470 BC) where sprinkling of amurca, an olive residue, was recommended to control late blight disease (David 1990). In short, plant extracts have been recognized to solve agricultural problems ever since man took to farming (Pillmoor 1993).

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). For the purpose of this monograph, secondary metabolites from plants can be classified briefly into the following compounds: phenolic compounds, flavonoids, alkaloids, glycosides (cyanogenic and cardiac glycosides), saponins, anthraquinones, anthocyanins, tannins, volatile and essential oils, sulphur containing compounds and steroids (Stumpf and Conn 1981; Dey and Harborne 1989; Carte and Johnson 2001).

These natural compounds are synthesized in plants as a result of biotic and abiotic interactions (Waterman and Mole 1989; Helmut *et al.* 1994). As in the pharmacology

industry, biochemicals isolated from higher plants may contribute to the development of natural fungicides or bactericides with application potential in 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 in order to counter the problem of resistance to currently used synthetic products by bacterial and fungal pathogens.

Another related area of organic farming systems is the potential to apply natural plant extracts as plant growth regulators. 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 Environmental Protection Agency (EPA) as any substance or mixture of substances that accelerates or retards the rate of growth or maturation, or otherwise alters the behavior of plants or their produce through physiological action (Lemaux 1999). Many natural compounds contained in plant extracts, and which have an effect on the growth and development of plants, have been identified. These include compounds such as amino acids, caffeine, fatty acids, flavonoids, lactones, quinines, steroids and various sulphur containing compounds (Roberts and Hooley 1988).

A research project initiated twelve years ago by the department of Agronomy at the University of the Free State, South Africa, included the screening of more than 3000 natural plant extracts for bio-stimulatory activity in agricultural crops. From this initiative a natural product, *ComCat*<sup>®</sup>, was developed and recently commercialized by a German company, Agraforum AG. *ComCat*<sup>®</sup> has been registered by the European Union as a natural non-toxic plant strengthening agent, derived from wild plants, that enhances plant growth and yield as well as resistance towards abiotic and biotic stress factors (Agraforum 2007). Recently a seed suspension of a legume plant, referred to as SS, was shown to have an above average bio-stimulatory effect on the growth and development of different agricultural crops, very similar to that of *ComCat*<sup>®</sup> (Van der Watt 2005). From this plant a triglyceride was isolated, purified and identified as the active substance. Both *ComCat*<sup>®</sup> and SS, when applied to a crop plant as a foliar spray, are readily absorbed by all plant parts. Crops have been shown to respond to treatment by accelerating certain physiological and biochemical reactions leading to an

elevated energy status in seedlings, accelerated root growth, increased resistance towards biotic and abiotic stress factors and ultimately higher yields.

Disease resistance in plants can either be systemically acquired (SAR) or systemically induced (SIR; Schnabl *et al.* 2001) and, in recent times, there has been an elevated interest in the mechanism of especially induced resistance responses of plants towards infection by pathogens. Information on the action mechanism may be of great value both in designing new agrochemicals that stimulate plant resistance responses and in developing genetically engineered plants with enhanced disease resistance.

According to Durner *et al.* (1997), the hypersensitive response (HR) is the most efficient plant defence mechanism associated with pathogen attack. This is characterized by host cell death around the infection point and serves to restrict further spread of the invading pathogen. The HR occurs in plants in response to infection by pathogenic fungi, bacteria and viruses (Slusarenko 2000). Associated with the HR is the induction of a diverse group of defence related genes, such as the pathogenesis-related (PR) proteins, the products of which are important in destroying the pathogen (Fritig *et al.* 1990). Furthermore, a massive increase in the active oxygen species is induced. Resistance against infection with different pathogens can be expressed locally at the site of primary inoculation, but also systemically in tissues remotely located from the initial treatment. This is termed systemic acquired resistance (SAR) and manifests itself as a long-lasting resistance to the same or even unrelated pathogens (Durner *et al.* 1997; Sticher *et al.* 1997). Of special interest in the agricultural industry is the ability to manipulate crops exogenously in order to induce resistance towards diseases.

The latter prompted the underlying study where emphasis was placed on elucidating the possible induced disease resistance mechanism in wheat against leaf rust by the registered natural product *ComCat*<sup>®</sup> and the seed suspension identified by Van der Watt (2005) as well as crude extracts of *Tulbaghia violacea* (Harv.) (Nteso and Pretorius 2006a & b) and *Agapanthus africanus* (Hoffman) (Tegegne 2004).

The objectives of this study were:

1. *In vivo* induction of resistance towards pathogen infection (*Puccinia triticina*, pathotype UVPr9), in a susceptible (Thatcher) and resistant (Thatcher / *Lr15*) wheat cultivar under

the influence of two natural bio-stimulants and two extracts with anti-fungal properties under glasshouse conditions.

2. The *in vitro* activity of certain resistance-related enzymes (PR-proteins) isolated from infected and non-infected susceptible and resistant wheat, treated with the above mentioned bio-stimulants and plant extracts under glasshouse conditions.
3. The apoplastic protein profile (gel electrophoresis), making use of immunological detection of enzymes of the above mentioned crops under the influence of the bio-stimulants and plant extracts.
4. Examining the expression of pathogenesis related (*PR*) genes in wheat upon treatment with a plant extract that shows antifungal activity.
5. The isolation, purification and identification of the active compound(s) responsible for induced resistance in wheat from the plant extract that showed the highest potential.

It is anticipated that the forthcoming results could contribute to a better understanding of the mechanisms of these natural plant bio-stimulants and antimicrobial substances as ‘plant activators’ in the resistance response of wheat in general and more specific to rust.

# | *Chapter 2*

## LITERATURE REVIEW

## 2.1 Introduction

It is estimated that there are more than 250 000 different plant species on earth (Cox 1990; Cowan 1999) offering an enormous potential for the discovery of new bioactive chemical compounds with many potential uses, including their application as pharmaceuticals and agrochemicals. Widespread public concern for long-term health and environmental effects of synthetic pesticides and the value of natural products from plants for various purposes, has prompted a renewed effort to search for active compounds from plants that can be used in natural pest and disease management programmes (Ushiki *et al.* 1996; Eksteen *et al.* 2001).

Moreover, 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. According to the National Academy of Sciences (Wilson 1997), the carcinogenic risk of fungicide residues in food is more than that of insecticides and herbicides put together.

Over the past three decades intensive bioactive plant screening programs have revealed antimicrobial activity in extracts of many plants (Sato *et al.* 2000; Bohra and Purohit 2002; Pretorius *et al.* 2002a; Masoko *et al.* 2007; Eloff *et al.* 2007). By means of bioassay guided screening, a number of these natural plant compounds have been isolated (Michael 1999; Tegegne 2004; Nteso 2004). These natural compounds are usually secondary metabolites and are synthesized in plants as a result of biotic and abiotic interactions and can play a role in plant defence mechanisms against perdition by microorganisms, insects and herbivores (Cowan 1999). Most secondary metabolites are derived from just a few building blocks, namely the acetate C2-unit (polyketides), the phenylalanine/tyrosine-derived C9-unit (phenylpropanoids), the isopentenyl diphosphate C5-unit and some amino acids (Verpoorte 1998).

On the other hand, many natural compounds have an effect on growth and development of plants. These include compounds such as amino acids, caffeine, fatty acids, flavonoids, lactones, quinines, steroids and various sulphur containing compounds (Roberts and Hooley, 1988). For example extracts from *Cyperus esculentus* tubers and the foliage of immature

*C. esculentus* plants inhibited the germination of lettuce seeds significantly (Reinhardt and Bezuidenhout 2001). Aqueous extracts of *Dendrocalmus stictus* had a stimulatory effect on chlorophyll content, seed protein, nodulation and peroxidase activity in soybeans. Shoot and radicle growth of soybeans were increased by these extracts (Sadhna *et al.* 1998). An extract from sea weed with growth promoting and yield increasing properties has been commercialized under the trade name 'Kelpek' and is currently sold in many countries (Ferreira and Lourens 2002). The potential exists to apply a plant extract as foliar spray in order to stimulate growth in crop plants and hence increase yields. These plant extracts with bio-stimulatory properties could directly serve as donor plants and sources of active compounds in the production of natural plant growth regulators.

## 2.2 Plant extracts

As a result of a recent screening program in the department of Agronomy at the University of the Free State South Africa, a number of plant extracts showed above average bio-stimulatory and antimicrobial activity when applied to test plants under greenhouse and field conditions. *Lupinus albus*, *Tulbaghia violacea* and *Agapanthus africanus* have been identified as potential bio-stimulants or antimicrobial agents (Pretorius *et al.* 2002b; Tegegne 2004; Nteso 2004; Van der Watt 2005).

### 2.2.1 Bio-stimulants

In this study *ComCat*<sup>®</sup>, a new natural bio-stimulant, was used in all *in vitro* and *in vivo* tests to investigate its potential to induce defence responses and resistance to wheat leaf rust. *ComCat*<sup>®</sup> is a unique family of natural products that are based upon a combination of bio-stimulants derived from plant materials (Agraforum 2006). It is a finely ground wettable powder specially blended with a carrier to permit conventional application on seeds and growing plants. This product has demonstrated consistent plant growth enhancement and physiological efficiency in the treated plant's utilization of available nutrients.

An extract of *Lychnis viscaria* seeds, which contain the brassinosteroids 24-*epi*-castasterone and 24-*epi*-secasterone (Friebe *et al.* 1999), contributed to an increased resistance of tobacco, cucumber and tomato to viral or fungal pathogens. However, no direct antifungal effects in mycelium growth assays with *Phytophthora infestans* could be observed. Stimulation of

different pathogenesis related (PR) proteins in cucumber by *ComCat*<sup>®</sup> were reported by Roth *et al.* (2000). The “active ingredient” of *ComCat*<sup>®</sup> is a complex combination of natural biological substances including amino acids, plant proteins, mixed phytosterols (including above mentioned brassinosteroids) and flavonoids (Schnabl *et al.* 2001). The unique nature and blend of this bio-stimulant is the strength of the *ComCat*<sup>®</sup> performance.

*ComCat*<sup>®</sup> products are a diverse blend of plant materials which have been selected from specific European plant species known for their history of positive growth effects on beneficial plants (Agraforum 2006). These selected plants are grown in a controlled fashion, harvested, dried and naturally processed to produce a concentration of natural bio-stimulants which can be controlled and monitored for uniform quality and returned to nature to nurture and enhance the health of vegetables, flowers and agricultural crops.

According to the manufacturers (Agraforum 2006), *ComCat*<sup>®</sup> activates natural defence mechanisms in plants towards abiotic and biotic stress factors. The activation of the target plant by the biochemicals within *ComCat*<sup>®</sup> stimulates biosynthesis which is generally expressed by a greater production of sugars, which are building blocks for cellulose and fruiting bodies. These natural biochemicals are the transmitters of molecular signals which trigger the defence mechanisms within the plant that increase resistance to stress factors. Further claims made by the manufacturers are that treatment of crop plants with *ComCat*<sup>®</sup> promotes root development, leading to efficient nutrient uptake and yield increases.

After treatment of *Arabidopsis thaliana* plants with *ComCat*<sup>®</sup>, five genes showed induced expression due to the treatment (Barski 2002). These cDNA clones were analyzed and three showed homology to Cytochrome P450 proteins. One of the identified genes, *03WVZ04*, encodes a hypothetical protein of unknown function and was subsequently renamed to *At-HPO1*. An analysis of the promoter region of *At-HPO1* indicates a number of elements linked to light mediated induction of expression (van Zyl 2003). In a further study by van der Merwe (unpublished results), it was found that *At-HPO1* expression was activated by light, due to the presence of a light inducible element on the promoter sequence. It was also confirmed to be activated by *ComCat*<sup>®</sup> treatment, but Brassinolide, an active ingredient in *ComCat*<sup>®</sup>, had no effect on the expression of the gene. Application of a purified fraction of *ComCat*<sup>®</sup> (that did not contain any Brassinosteroids), to *Arabidopsis* plants, led to induction of *PR2* which encodes the PR-protein  $\beta$ -1,3-glucanase which is a marker for defence

induction. It thus seems that Brassinosteroids in *ComCat*<sup>®</sup> are not involved in the induction of defence responses, but may be the main force behind other processes initialized by *ComCat*<sup>®</sup>, such as growth induction (van der Merwe 2007; unpublished results).

A second bio-stimulant was isolated from a seed suspension of a legume plant, *Lupinus albus* L (van der Watt 2005). Preliminary results showed that this natural component, referred to as (SS), increased the respiration rate of monoculture yeast cells and enhanced seedling growth, especially root development, in a number of crops under laboratory conditions. Bio-stimulatory activity, in terms of its yield increasing effect, was confirmed in a number of crops under field conditions. Significant yield increases after a foliar spray treatment with SS was observed in beetroot, lettuce and carrots by increasing the yield with 9.3, 20.0 and 24.3 ton ha<sup>-1</sup> respectively. The active substance was identified by means of NMR spectroscopy and mass spectrometry as a triglyceride, glycerol trilinoleate. Although the mechanism of action is not known at this stage, it is postulated that the linoleic acid moieties of trilinoleate is either metabolized via the dihydro jasmonic acid (DH-JA) or the jasmonic acid (JA) pathways or both. This implicates an indirect effect leading to the bio-stimulatory properties of the active compound (van der Watt 2005). In 2006 this prototype product has been patented and is currently under further scrutiny.

### 2.2.2 Antimicrobial agents

Crude extracts of selected 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; Rahman *et al.* 2001; Sharma *et al.* 2001; Wang and Ng 2001; Reddy *et al.* 2002). More specifically, fungicidal properties of garlic extracts have been demonstrated against pathogens that cause damping-off diseases in plants, e.g. *Fusarium oxysporum*, *Rhizoctonia solani* and *Pythium* species (Kurucheve and Padmavathi 1997; Horberg 1998; Raja and Kurucheve 1999; Sinha and Saxena 1999; Lindsey and van Staden 2004). A number of authors have also reported on the antibacterial properties of garlic, *A sativum*, extracts (Burton 1990; Khan and Omoloso 1998; Arora *et al.* 1999; Avato *et al.* 2000; Qiao *et al.* 2001).

The antimicrobial properties of crude methanol extracts of above- and below-soil parts of *Tulbaghia violacea* (wild garlic) were determined *in vitro* by means of an agar diffusion method against six plant pathogenic bacteria and seven fungi. The growth of three out of six bacteria and six of the seven test fungi was significantly inhibited by extracts of both below-soil and aerial parts whereas only the below-soil extract inhibited the mycelial growth of *Fusarium oxysporum* significantly (Nteso and Pretorius 2006a).

The *in vivo* control of *Mycosphaerella pinodes* was conducted with a crude aerial part extract of *T. violacea* in terms of lesions that developed over a six-day period at 20°C on detached pea leaves. The extract completely suppressed lesion development by preventing spore germination of *M. pinodes* without showing phytotoxicity to pea leaves. Additionally, the control of two seed borne fungal pathogens of sorghum, covered kernel and loose smuts, was tested under field conditions. Seed treatment with the extract significantly ( $P < 0.05$ ) reduced the incidence of both sorghum loose and covered smuts diseases, compared favourably with the standard fungicide and resulted in significant yield increases compared to the untreated control (Nteso and Pretorius 2006b).

Subsequently, crude methanol extracts of *T. violacea* were purified by means of activity directed liquid-liquid extraction, column and preparative thin layer chromatography which led to the isolation of six antifungal compounds. 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 (Nteso 2004). The fifth compound that was discovered earlier by Burton and Kaye (1992), was 2,4,5,7-tetrathiaoctane.

*Agapanthus africanus* (L) Hoffm. is an evergreen plant indigenous to South Africa (van der Una 1971). Pieces of *Agapanthus* roots are traditionally used by local communities in South Africa as medicine for various disorders (van der Una 1971). According to Kaido *et al.* 1997, infusions or concoctions of *A. africanus* are used by Xhosa women during pregnancy to induce labour. It is also frequently used to treat constipation in pregnancy, as antenatal or post-natal treatment of the mother and for high blood pressure (Duncan *et al.* 1999).

Crude extracts of different *A. africanus* plant parts were screened *in vitro* against eight economically important plant pathogenic fungi, which included *Botrytis cinerea*, *Sclerotium*

*rolfsii*, *Rhizoctonia solani*, *Fusarium oxysporum*, *Botryosphaeria dothidea*, *Mycosphaerella pinodes*, *Pythium ultimum* and *Alternaria alternata* (Tegegne 2004). All of the extracts inhibited radial mycelial growth of all test fungi while the root and flower extracts as well as a combined aerial part extract showed significantly ( $P < 0.05$ ) higher *in vitro* antifungal activity than did a leaf extract. Variation in terms of the sensitivity of fungi towards the plant extracts was also observed. *P. ultimum* and to a lesser extent *F. oxysporum* and *A. alternata* showed a degree of tolerance towards all extracts. However, screening a concentration range of the combined aerial part extract confirmed that mycelial growth of even the three tolerant pathogens could be inhibited significantly at higher concentrations. In the case of six of the eight test pathogens the extract either inhibited mycelial growth significantly ( $P < 0.05$ ) than that of the different commercial fungicides or did not differ significantly from the standards (Tegegne 2004).

Crude extracts of different plant parts of *A. africanus* were also tested *in vivo* against *Mycosphaerella pinodes* infection of detached pea leaves by applying the extracts both before and after spore inoculation. Despite differences among root, flower and aerial part extracts, all extracts suppressed lesion development significantly at relatively low concentrations by preventing spore germination or mycelial growth or both, and compared favourably with the standard fungicide used as a positive control. None of the extracts had any phytotoxic effect on the pea leaves (Tegegne 2004).

Activity directed fractionation of the crude extracts by means of liquid-solid extraction revealed that most of the root antifungal activity was located in a diethyl ether fraction while an ethyl acetate fraction of the aerial parts was most active. Further purification of active compounds from these fractions by means of column and preparative thin layer chromatography lead to the isolation of a novel and highly active sapoginin, identified by means of  $^1\text{H-NMR}$  and  $^{13}\text{C-NMR}$  spectroscopy as 3-[[O- $\beta$ -D-glucopyranosyl-(1''-3')- $\alpha$ -L-rhamnosyl-(1''-2')]- $\beta$ -D-glucopyranosyloxy] agapanthegenin, from both the roots and aerial parts. Additionally, three flavonoids possessing significant fungicidal activity were isolated from the roots of *A. africanus* and identified as 5,7,4'-tri-*O*-flavanone, 5,7,3',4'-tetra-*O*-acetylflavanone and trans-4,2',4'-tri-*O*-acetylchalcone (Tegegne 2004).

### 2.3. Wheat rust

Rust fungi (Basidiomycetes of the order Uredinales) are obligate biotrophs that grow and reproduce only in living plant tissue. There are in the order of 5000 or more species of rust fungi that collectively cause disease on most crops, ornamentals and many other plants (Eckardt 2006). Wheat leaf rust caused by *Puccinia triticina* is a serious fungal disease affecting wheat. It is the most prevalent of all the wheat rust diseases, occurring in nearly all areas where wheat is grown. It has caused serious epidemics in North America, Mexico and South America. It is most destructive on winter wheat, probably because this allows the pathogen to over winter. Wheat cultivars that are susceptible to leaf rust suffer from yield reductions of between 5 to 30% or more, depending on the stage of crop development when the initial rust infection occurs (Kolmer 1996).

During infection of a host plant, rust fungi form haustoria, specialized infection structures that penetrate the plant cell wall and form invaginations in the plasma membrane that are believed to form the major sites of nutrient uptake from the host cell (Hahn and Mendgen 2001) It is also thought that signals emanating from haustoria suppress host defence responses and facilitate disease in sensitive host plants (Panstruga 2003; Vogele and Mendgen 2003) or trigger a hypersensitive response (HR) leading to disease resistance hosts (i.e, interaction between Avr factors and host *R* gene products: Heath 1997).

According to Eckardt (2006), rust fungi have extremely complex life cycles, involving up to five different spore-producing stages. The pathogen has an asexual and sexual cycle. Many rusts are heteroecious, requiring two phylogenetically distinct host plants to complete their life cycle. The rust fungi are host specific and will develop compatible or incompatible associations with their host plants in a gene-for-gene manner, depending on the presence or absence of avirulence (*Avr*) genes in the pathogen and corresponding resistance (*R*) genes in the host. Rust races that are virulent against cultivars containing resistant genes and are newly deployed in wheat can rapidly increase in frequency over large geographic areas (Kolmer 1999), thus rendering the resistance genes ineffective (Kolmer 2005). A new wheat rust epidemic is currently building in East Africa with the appearance of a highly virulent strain of *Puccinia graminis tritici*, called Ug99, which is perceived as a threat to global wheat production and has led to the establishment of a Global Rust Initiative (<http://www.globalrust.org/index.html>).

## 2.4 Perception mechanisms in plant : pathogen interactions

Plants are capable of surviving exposure to severe stress due to infection by pathogens like fungi, bacteria and viruses through perception mechanisms involving plant : pathogen interactions. A plant : pathogen interaction may be regarded as an open warfare between the two, whose weapons are proteins and low molecular mass compounds synthesized by both organisms. The outcome of this battles results in the establishment of resistance or pathogenesis (Ferreira *et al.* 2007). Penetration of plant tissue by the pathogen occurs through degradation of the plant cell walls and the plant perceives these signals, known as elicitors, resulting in signal generation and transmission to the defence genes via intracellular signaling cascades or signal transduction (Suzuki and Shinshi 1995). Furthermore, sensing of a variety of other signals, including changes in light, temperature, nutrient availability and gravity is required to enable a plant to respond appropriately. Although sensing of and response to such a signal can be restricted to a single cell, intercellular communication processes are required to coordinate growth and development of tissues and organs throughout the whole plant body (Jonak *et al.* 1994; Hirt 1997; Morris *et al.* 1997; Ichimura *et al.* 1998).

The first step in activating a defence response is to perceive the stress and then to relay the received information through complex signal transduction pathways to the genes needed to be activated. The products of these defence genes are responsible for a successful defence mechanism (Gang *et al.* 1999; Garcia-Garrido and Ocampo 2002).

According to Hucho and Buchner (1997), signal transduction is as fundamental a feature of life as metabolism or self-replication. All living cells receive information from the extracellular space and they react to it by processing and converting it into intracellular effects. For the multicellular organism this environment is supplemented and complicated by neighbouring cells, by cells far away (which send messages important for concerted growth and action) and by the matrix which holds them together and guides their growth. For all these organisms signal transduction can be defined as the complete pathway of extracellular physical or chemical/molecular signals into the cytoplasm and/or the nucleus, including its conversion into an effect. The authors concluded that two basic fundamental principles are observed in signal transduction: (i) the extracellular signal itself penetrates the plasma membrane and makes its way to the nucleus or (ii) the signal remains outside the cell and is

converted at the plasma membrane into intracellular signals which can be described as a “first messenger/receptor/second messenger” concept .

Prior to the report by Hucho and Buchner (1997), Nishida and Gotoh (1993) introduced another concept in signal transduction, namely the “cascade concept”. In essence this entails that signal transduction consists of many steps that allow for signal amplification, specific targeting, regulation and “cross-talk” (a comprehensive treatise of interactions between different signal transduction pathways). Most signals are limited to time and space and have at least one “on/off” switch.

Studies by Bhalla and Lyengar (1999), have led to the identification of many general mechanisms of signal transfer, such as regulation by protein-protein interactions, protein phosphorylation, regulation of enzymatic activity, production of second messengers and cell surface signal transduction systems. These mechanisms have been shown to occur in many pathways, including  $\text{Ca}^{2+}$  signaling pathways, tyrosine kinase pathways and other protein kinase cascades. Signaling pathways interact with each other and the final biological response is shaped by interaction between pathways. In general, persistent activation of protein kinases is the mechanism for eliciting biological effects. In biological systems, signal transmission occurs mainly through two mechanisms: (i) protein-protein interactions and enzymatic reactions such as protein phosphorylation and dephosphorylation or (ii) protein degradation or production of intracellular messengers.

For a cell to respond efficiently and rapidly to extracellular signals, all the components of a signaling cascade should be present so that the signal transduction pathway is essentially a post-translational process. More often than not, signal transduction pathways function by the transcriptional induction of genes encoding some of the signaling components themselves (Meskiene *et al.* 1998; Hirt 1999). In other reports Nishida and Gotoh (1993) as well as Yamamoto *et al.* (1998) have speculated that the reason why a response signal prompts the cell to up regulate the synthesis of components of the same signal transduction pathway may be that this up regulation represents a positive feedback mechanism to increase the availability of signaling components in the pathway. However, there is no evidence that the protein concentrations of any of the components increased (Wilkinson 1995). Although the positive feedback mechanism at the transcriptional level cannot be dismissed, reports on transcriptional up regulation of signaling factors in yeasts, animals and plants described the

function of this mechanism to switch on and/or reset a pathway (Hirt 1999). Further, a signaling component must be inactivated after activation, as a cell cannot afford to use a signaling pathway only once. Thus, the activation and inactivation mechanisms must essentially operate only transiently (Hirt 1999). Recently it was suggested that the transient transcriptional up regulation of certain genes might serve to compensate for the loss via degradation of proteins or other components involved in a signal cascade (Hirt 2000). Hence, while up regulation of genes in a signal transduction pathway may compensate for protein loss, up regulation of others serves to directly inactivate the pathway (Meskiene *et al.* 1998).

#### **2.4.1 Gene-for-gene (*avr-R*) interactions**

To date, a large amount of attention has been fixed on resistance. However, the establishment of basic pathogenicity and susceptible interactions are poorly understood. In general, when the successful colonization of a plant by a pathogen leads to disease, the plant is said to be “susceptible” and the interaction is described as “compatible” (Slusarenko 1996). According to the author, effective resistance of the plant is expressed in the “incompatible” interaction and disease fails to develop. Crute and Pink (1996) concluded that, in plant-pathogen interactions, the highly specialized form of recognition between the elicitor and the host is governed by the gene-for-gene interactions. Resistance in plants is conferred by disease resistance (*R*) genes and is the result of the gene-for-gene interactions of these genes with the corresponding pathogenic avirulence (*avr*) gene. If the plant and the pathogen carry complementary genes specifying *R*- and *avr* genes respectively, then the plant recognizes the pathogen. A report by Grant and Mansfield (1999) added that, in plants, *avr* gene expression with the matching *R* gene leads to the hypersensitive response in several interactions. The Avr proteins conveyed by the *hrp*-dependent system (genes required for both pathogenicity and the ability to cause the hypersensitive response in non-host and resistant host plants), are thus the elicitors of the hypersensitive cell death.

The perception of the elicitors by the high-affinity binding receptors on the host plasma membrane initiates an intracellular signal cascade which eventually results in the co-ordinate transcription of a large number of defence related genes and rejection of the pathogen (Lamb *et al.* 1989; Dixon *et al.* 1994). This interaction is referred to as incompatible and the plant is resistant to the disease. If either of the complimentary pairs of genes is absent or carried in a recessive form, there is neither recognition nor induction of the resistance response and the

pathogen is able to colonize the host. Such a compatible interaction is equivalent to disease susceptibility (Lawton 1997).

### 2.4.2 Signaling events

Upon bacterial infection, exposure to the intracellular space in the plant tissue induces expression of the *hrp* gene cluster in the bacterial pathogen which is required for pathogenicity and for the ability to cause the hyper sensitive response (HR) in non-host and resistance in host plants (Grant and Mansfield 1999; Vranová 2002). In plant-pathogen interactions, the signaling events begin with the elicitor binding to the receptors in the plasma membrane. This initiates a signal transduction cascade that involves the production of reactive oxygen species such as superoxide ( $O_2^-$ ) and hydrogen peroxide ( $H_2O_2$ ) catalyzed by plasma membrane-located NADPH oxidase and/or apoplastic peroxidases (Somssich and Hahlbrock 1998). The activation of certain kinases and lipases also signal the activation of genes whose products are involved in defence reactions (Viard *et al.* 1994; Tavernier *et al.* 1995; Lamb and Dixon 1997). More evidence suggests the existence of cross-talk among the induced defence mechanisms (Beckers and Spoel 2006) and that they are not controlled by independent linear signalling cascades, but components of one pathway may affect the signaling through other pathways (Maleck and Dietrich 1999).

### 2.4.3 Elicitors of defence responses

Elicitors are defined as molecules that can induce physiological or biochemical responses associated with the expression of resistance (Kogel *et al.* 1988) and can be classified as 'endogenous' if derived from hydrolytic events of the plant cell itself or as 'exogenous' if derived from the pathogen cell wall (Suzuki and Shinshi 1995). Elicitors result in a number of early physiological events, many of which are connected to signal transduction pathways (Viard *et al.* 1994; Tavernier *et al.* 1995; Lamb and Dixon 1997; Pugin *et al.* 1997; Tena and Renaudin 1998; Romeis *et al.* 1999; Nürnberger 1999; McDowell and Dangl 2000).

Many elicitors have been described including polysaccharides, oligosaccharide fragments, proteins, glycoproteins and fatty acid derivatives (Dixon and Lamb 1990). Recently, El Gueddari and Moerschbacher (2004) described the role of chitosans and partially acetylated chitosan oligomers as elicitors of disease resistance reactions in plants. Elicitors derived from

fungal plant pathogens induce defence responses normally associated with fungal infection including the action of enzymes such as chitinase, peroxidase and  $\beta$ -1,3-glucanase that have a direct inhibitory effect on fungi (Benhamou 1996; Gelli *et al.* 1997). Subsequently, a plant reacts to elicitor stimulation with a concerted biochemical defence response including changes in membrane properties, increased production of the stress hormone ethylene and transcriptional activation of the genes encoding enzymes involved in phenyl propanoid metabolism [activation of the defence gene, phenylalanine ammonia-lyase (PAL)].

Furthermore, protein phosphorylation has been shown to play a role in the perception and chemosensory transduction of elicitors in plants (Felix *et al.* 1991) as has  $\text{Ca}^{2+}$  influx and an  $\text{H}^+$  influx/ $\text{K}^+$  efflux exchange. The latter is important for activation of the oxidative burst in plant defence responses (Lamb and Dixon 1997).

Elicitors can be divided into race-specific and general elicitors. With race-specific elicitors, a resistance (*R*) gene protein interacts with a specific corresponding avirulence (*Avr*) gene product to activate a variety of signal transduction cascades that most of the time results in a hypersensitive response (HR). The HR is characterized by the death of the infected cell and can also lead to systemic acquired resistance (SAR; Nimchuk *et al.* 2003). The elicitors involved in R-gene mediated defence mechanisms are also known as race-specific elicitors. General elicitors trigger defence in host and non host plants. In the case of general elicitors, no gene-for-gene interaction takes place and a *R*-gene corresponding to the pathogen *Avr*-gene is not needed (Montesanto *et al.* 2003).

## 2.5 Resistance against disease

The course of evolution has seen the coexistence between plants, microorganisms and insects leading to interactions ranging from mutualism to antagonism. The latter has piloted plants to develop protective or defensive barriers that are induced upon contact with invaders (Schneider *et al.* 1996). When plants are infected by a nonpathogenic or an avirulent pathogen strain, elicitation of the collapse of the challenged host cells, in the hypersensitive response, ensues with an array of inducible defences in both the challenged and surrounding cells. Virulent strains however, do not elicit localized hypersensitive cell death. The induction of defence responses is often delayed and disease follows (Levine *et al.* 1994; Lamb and Dixon 1997; Bolwell 1999; McDowell and Dangl 2000).

In plants, defence mechanisms against infectious microbes involve constitutive barriers as well as reactions induced upon contact with potential pathogens (Schneider *et al.* 1996). The most common expression of resistance in the plant is the hypersensitive response (HR). The HR can be defined as “the rapid cell death in plants at the site of initial infection, with consequent colonization and death of the potential pathogen” (Grant and Mansfield 1999) and is associated with stopping the pathogen spread (Montellit *et al.* 2005). This response leads to the appearance of a restricted lesion which isolates the site of attack from the surrounding healthy tissue, and although the host cells are damaged, this cell death contributes to pathogen restraint (Levine *et al.* 1994; Lamb and Dixon 1997; Bolwell 1999; McDowell and Dangl 2000).

Three key components involved in signal transduction have, however, been implicated in HR, namely elevated cytosolic  $\text{Ca}^{2+}$ ,  $\text{Ca}^{2+}$  binding proteins [calmodulin] and protein phosphorylation [MAP kinases] (Lamb and Dixon 1997; Grant and Mansfield 1999; McDowell and Dangl 2000; Zhang and Klessig 2001). Of the earliest responses activated after host recognition are the oxidative burst and the opening of specific ion channels (Hammond-Kosack and Jones 1996). During the oxidative burst, there is a sudden increase in the generation of reactive oxygen species (ROS) (Hammond-Kosack and Jones 1996; Wojtaszek 1997; Alvarez *et al.* 1998). Reactive oxygen species ( $\text{H}_2\text{O}_2$ ,  $\text{OH}^-$  and  $\text{O}_2$ ) play a key role during defence. They can be generated by means of different mechanisms involving different enzymes such as oxalate oxidase using oxalic acid as substrate (Zhang *et al.* 1995), cell wall peroxidases, the NADPH-oxidase complex (Desikan *et al.* 1996), the xanthine oxidase complex (Montalbini 1992) and superoxide dismutase (SOD) (Liochev and Fridovich 1994; Fridovich 1995). This oxidative burst, which occurs in the cell wall, is thought to function as a signal for downstream defence responses and to participate directly in chemical reactions that strengthen the cell wall and attack pathogen surfaces, thereby limiting the progress of invasion (Cosgrove *et al.* 2000).

Another signal molecule that has been implicated in defence is nitric oxide (NO). This compound has previously been shown to serve as a key redox-active signal for the activation of various mammalian defence responses, including the inflammatory and innate immune responses (Schmidt and Walter 1994; Stamler 1994).

Other events that also happen shortly after recognition are the accumulation of salicylic acid (SA) and jasmonic acid (JA) and the activation of calcium dependant protein kinases and calmodulin by higher intracellular calcium levels (Romeis 2001). Jasmonic acid (JA) is synthesized via the octadecanoid pathway from peroxidized linolenic acid (Hamberg and Gardner 1992). Methyl-jasmonate (Me-JA), which is the volatile counterpart of JA, oxo-phytodienoic acid, the precursor Me-JA and dinor-oxo-phytodienoic acid, are all powerful cellular regulators in plant tissues (Weber *et al.* 1997). Jasmonic acid and its volatile ester methyl-jasmonate are potent inducers of proteinase inhibitors (Farmer *et al.* 1992; Ryan 1990) and of polyphenol oxidase and lipoxygenase (LOX) (Duffy and Stout 1996).

Salicylic acid plays a central role as a signal molecule being involved in both local and systemic resistance (SAR; Johal *et al.* 1995; Durner *et al.* 1997). It is not exactly known how salicylic acid induces SAR, but according to Chen *et al.* (1993), salicylic acid binds and inactivates a catalase that results in the accumulation of H<sub>2</sub>O<sub>2</sub>, which then induces the genes involved in SAR. Salicylic acid also regulates the induction of the pathogenesis-related (*PR*) genes of which many exhibit antifungal properties (Durner *et al.* 1997; Kombrinck and Somssich 1997). Inhibition of the SA signal pathway leads to susceptibility in plants towards pathogens (Delaney *et al.* 1994).

SAR is expressed systemically in other parts of the plant distant from the primary infection site, where it has long-lasting and broad-spectrum resistance to pathogens that would otherwise cause disease (Ryals *et al.* 1994).

A major feature of SAR is that resistance is expressed against pathogens that can widely differ from the initial infecting organism. Although plants do not possess immunoglobulins, the general phenomenon can be compared to immunization in animals and humans. Systemically acquired resistance is established in times ranging from several hours to several weeks, depending on the plant and the nature of the organism employed in the first inoculation. Once established, SAR may last for a relatively long time, from weeks to months, during which invasion by pathogens is hampered (Durrant and Dong 2004; Somssich 2003; Sticher *et al.* 1997). The other important feature of SAR is the expression of a set of protective genes, in particular those that encode the pathogenesis-related (*PR*) proteins (Kessmann *et al.* 1994; Datta and Muthukrishnan 1999). These *PR* proteins accumulate in the extracellular spaces between cells, the apoplast, as well as the vacuole. Examples of well-

known PR proteins involved in SAR are PR-1,  $\beta$ -1,3-glucanase (PR-2), chitinases (PR-3), PR-4 and osmotin (PR-5). Some of these PR proteins show antimicrobial activity *in vitro* in plants (Niderman *et al.* 1995; Morrissey and Osbourn 1999).  $\beta$ -1,3-glucanase and chitinase degrade the cell walls of fungi. These PR proteins accumulate in large amounts at the primary infection site, but also to a lesser extent in tissues showing SAR (Stintzi *et al.* 1993). That is why PR proteins are also sometimes known as SAR proteins. The role of PR-proteins is essential as has been shown in mutant studies with deficient plants having less or no resistance (Neuhaus 1992).

Resistance is characterized by the activation of defence mechanisms in response to pathogens and includes the HR which leads to rapid cell death, the induction of numerous low-molecular weight compounds (known as phytoalexins) with antimicrobial activity, structural barriers such as lignin as well as a range of hydrolytic enzymes, antimicrobial peptides and proteins. Furthermore, all the defence reactions are activated in the cells at or adjacent to the infection site (Schneider *et al.* 1996; Desikan *et al.* 1996; Rajasekhar *et al.* 1999).

Systemic induced resistance (SIR) is another form of resistance found in plants. Although SIR is very similar to SAR in that it involves activation of the plant's defence mechanisms leading to systemic protection, it differs from SAR in that resistance is achieved without SA accumulation while PR proteins are not associated with this resistance (Pieterse *et al.* 1996; Coventry and Dubery 2001). Through mutant studies it was discovered that SIR is dependent on elements in the JA response as well as the ethylene response. These plants with the *jar1* and *etr1* mutation do not show responsiveness to JA and ethylene respectively. On the other hand, SAR was not influenced whatsoever by the same mutations (Pieterse *et al.* 1998). Plants with the *nahG* mutation did not show a decrease in SIR, which confirms that SIR is an SA independent process.

*NPR1* is a modulator of SAR and a mutation of it results in no *PR*-gene expression or SAR. Interestingly the *npr1* mutation also affects SIR and *NPR1* is necessary for SIR to occur (Pieterse *et al.* 1998). This may indicate that although the signaling processes in SIR and SAR differ, that they still overlap. Typically SIR induces defensins like thionin that are anti-pathogenic proteins which act directly on the pathogen and mediates the resistance (Mauch-Mani and Metraux 1998). However, whichever of these pathways are followed to induce

resistance, SIR presents a natural mechanism for biological control of plant disease (Van Loon *et al.* 1998).

## 2.6 Pathogenesis-related proteins

Pathogenesis-related (PR) proteins had been defined as any protein coded for by the host plant, but induced only in pathological or related conditions (Antoniw *et al.* 1980; Cut and Klessig 1992). Accumulation of the PR proteins represents the major quantitative change in protein composition that occurs during the HR. The term pathogenesis-related (PR) protein was first used to describe the extracellular proteins that accumulate in response to tobacco mosaic virus (TMV) infection of susceptible tobacco genotypes. During plant-pathogen interactions, different PR-genes which are associated with incompatibility are induced (Bol *et al.* 1990; Bowles 1990; Linthorst 1991).

The definition of PR proteins has been broadened to include intra- and extracellular proteins that accumulate in intact plant tissue or culture cells after pathogen infection or when treated with an elicitor (Bowles 1990). Thus far PR-1 to PR-17- types have been characterized and classified from different plant species according to amino acid sequences, serological relationship and/or enzymatic or biological activity (Van Loon *et al.* 1994; Thomma *et al.* 1998; Van Loon and Van Strien 1999). Many of these PR proteins exhibit antimicrobial or antifungal activity (Thomma *et al.* 2002; Lay and Anderson 2005) and some are known to be chitinases and  $\beta$ -1,3-glucanases (Collinge *et al.* 1993; Melchers *et al.* 1994), while another group is basic cysteine-rich thionins (Bohlmann 1994).

### 2.6.1 Chitinase and $\beta$ -1,3-Glucanase

Chitinase (EC 3.2.1.14) constitute the second largest group of antifungal proteins. They catalyse the hydrolytic cleavage of the  $\beta$ -1,4-glycoside bond present in biopolymers of *N*-acetyl-D-glucosamine mainly in chitin. Chitin is a substrate found in fungal cell walls, insect exoskeletons and shells of crustaceans (Cut and Klessig 1992, Flach *et al.* 1992). Chitinases can be grouped into two categories, namely exochitinases, which act on the non-reducing ends of the chitin chain and endochitinases, which hydrolyse internal bonds (Kasprzewska 2003). It is difficult to attribute a specific role for chitinase since its substrate, chitin, does not occur in higher plants. It is believed that plants produce chitinase to protect

themselves against chitin-containing parasites (Boller, 1995). PR-3, PR-4, PR-8 and PR-11 families consist of proteins with chitinase activity (Collinge *et al.* 1993).

Chitinases have been discovered in more than 41 plant species and occur in widely different tissues including seeds (Yamagami and Funatsu 1993), leaves and stems (van Damme *et al.* 1993), roots (Majeau *et al.* 1990) and flowers (Leung 1992). Among monocotyledonous species, chitinases occur in corn, onion, rice and wheat (Ride and Barber 1990; Zhu and Lamb 1991; Verburg *et al.* 1992; Williams and Leung 1993). Like many other PR proteins, chitinase may be synthesized in both constitutive and an inducible manner (Kasprzewska 2003).

$\beta$ -1,3-Glucanase (EC 3.2.1.39) hydrolyzes  $\beta$ -1,3-glucosidic linkages in  $\beta$ -1,3-D-glucans, such as laminarin, paramylon and pachyman (Bielka *et al.* 1984).  $\beta$ -1,3-Glucans are the major cell wall constituents of common fungal pathogens, bacteria and plants (Bowles 1990). The PR-2 family consists of proteins with  $\beta$ -1,3-glucanase activity (Collinge *et al.* 1993) that is found in bacteria, fungi, higher plants and some invertebrates (Boller 1987).

Both  $\beta$ -1,3-glucanase and chitinase are constitutively expressed in different organs and tissues of higher plants and are regulated by normal developmental processes, ethylene and other plant hormones (Côtè *et al.* 1991). In addition, these enzymes are typically induced to higher levels in plants after pathogen attack, insect infestation and exposure to various biotic and abiotic elicitors (Boller *et al.* 1983; Williams and Leung 1993; Van der Westhuizen *et al.* 1998a,b).

A general characteristic of these enzymes is their complicated iso-enzymatic composition with isoforms differing in physical properties, enzyme activity, antigenicity, cellular compartmentation, tissue localization and antifungal activity encoded by a small multigene family (Linthorst 1991). At the protein level the enzymes are classified into two main groups, the acidic and basic groups. The acidic groups are extracellular whereas the basic isoforms occur in the vacuoles. Specific roles in defence against pathogens have been proposed for these two groups (Mauch and Staehelin 1989; Stintzi *et al.* 1993).

Both enzymes strongly inhibit the growth of many fungi in culture indicating that they might have a direct antifungal function (Mauch *et al.* 1988; Ji and Kuć 1996; Vannini *et al.* 1999).

In addition, they might release glucan and chitin / chitosan fragments from fungal cell walls that act as elicitors of the consequent defence response (Ham *et al.*, 1991; Okinaka *et al.* 1995; Vander *et al.* 1998). The antimicrobial potential of  $\beta$ -1,3-glucanases and chitinases has been successfully tested *in vivo* by manipulating their expression in transgenic plants (Jach *et al.* 1995). Chitinases can also display lysozyme activity and hydrolyze bacterial peptidoglycan. The chitinase / lysozyme activity ratio is variable and is characteristic of each chitinase, suggesting a dual role of these enzymes in resistance towards fungi and bacteria. In many cases, pathogenic fungi are insensitive to either chitinase or glucanase alone, but are inhibited by combinations of the two enzymes (Boller 1993; Jach *et al.* 1995).

Studies on the role of  $\beta$ -1,3-glucanase and chitinase in the resistance response of wheat (*Lr35*-bearing wheat cultivar and susceptible plants) to leaf rust infection, indicate that these enzymes were constitutively expressed, confirming a hypersensitive response of wheat to leaf rust (Anguelova *et al.* 1999; Anguelova-Merhar *et al.* 2001; 2002). Induced chitinase activity in resistant wheat leaves inoculated with *Puccinia striiformis* f. sp. *tritici*, the causal agent of yellow rust disease, suggest that chitinase may be involved in wheat defence mechanisms against yellow rust, particularly at later stages of disease development (Mohammadi *et al.* 2001).

### 2.6.2 Peroxidases

Peroxidases (EC 1.11.1.7) use  $H_2O_2$  as substrate in a range of oxidation reactions. Activation of peroxidase in response to stress is one of the key processes in the plant-cell defence responses (Dowd *et al.* 1999). It occurs after pathogen infection and treatment with elicitors (Vander *et al.* 1998), wounding (Kaothien *et al.* 2000) or changing the temperature regime (Dat *et al.* 1998). Peroxidases can be involved in the control of the level and activity of endogenous and exogenous signaling molecules in plants (Low and Merida 1996). For example, they affect the synthesis and degradation of some phytohormones, peroxides and phenols.

The involvement of peroxidases in plant defence responses against pathogens (Rasmussen *et al.* 1995; Liu and Kolattukudy 1997) and insect injury (Van der Westhuizen *et al.* 1998b) was amply reported. Phytopathogens, elicitors, various abiotic stresses and some phytohormones can induce their expression in plants (Van Loon and van Strien 1999;

Muthukrishnan *et al.* 2001). For wheat anionic peroxidases it was demonstrated that they could bind to chitins and germinating spores of the fungus *Tilletia caries* (Khairullin *et al.* 2000). This ionically-bound peroxidase appears in the cell walls of infected wheat calli demonstrating that it is involved in the reinforcement of cell walls and can hinder pathogen growth (Sharma and Singh 2002; Maksimov *et al.* 2004).

Extracellular peroxidases have been associated with several biochemical reactions including lignin and suberin synthesis (Lagrimini 1991; Polle *et al.* 1994), construction of intermolecular cross-linkages (Fry 1986; Everdeen *et al.* 1988) and NADH-dependent formation of H<sub>2</sub>O<sub>2</sub> (Pedreno *et al.* 1989).

## **2.7 Plant activators**

In addition to existing measures of disease control, knowledge on the biochemical events associated with defence responses has led to the establishment of a novel method of disease control by means of chemicals referred to as “plant activators”. Most of these chemicals induce resistance in the plant by means of systemic resistance. To date a variety of chemicals have been identified as plant activators and can be divided into three groups: natural organic, inorganic and synthetic compounds.

### **2.7.1 Natural organic compounds**

Activators that fall in this class are organic compounds that occur naturally in the plant. The most widely studied example of such a compound is salicylic acid (SA) that belongs to a diverse group of plant phenolics. Many phenolics play an essential role in the regulation of plant growth, development and interaction with other organisms (Harborne 1980). Evidence for SA being an activator of SAR is extensive and has been well documented for various species including wheat, tobacco and *Arabidopsis* (Kessmann *et al.* 1994; Bertini *et al.* 2003). Although SA has received much attention as a signaling molecule in the resistance mechanisms in plants, it is not the exclusive determining metabolite for resistance or susceptibility.

Another class of molecules that has been classified as plant activators are the jasmonates, jasmonic acid (JA) and methyl jasmonate (MeJA). Jasmonate induces local and systemic

resistance when plants are under herbivore attack. Some evidence does exist that JA also play a role in signaling during pathogenesis (Dong 1998; Pieterse *et al.* 1999).

Menadione sodium bisulphate (MSB), a vitamin K<sub>3</sub> water-soluble compound, is a novel plant defence activator in oilseed rape, which enhances local and systemic resistance to infection by *Leptosphaeria maculans*, the causal agent of stem canker (Borges *et al.* 2003). The authors reported that application of MSB to the plants does not increase *PRI* gene expression, but it does however increase expression of an ascorbate peroxidase gene. The results suggested that MSB induces resistance by increased production of reactive oxygen species (ROS) including both H<sub>2</sub>O<sub>2</sub> and O<sub>2</sub><sup>-</sup> (Borges *et al.* 2003). Menadione sodium bisulphate can be classified as a plant activator in oilseed rape because even at very high concentrations it still remains non-phytotoxic to the plant. It also acts as plant activator in banana according to tests against *Fusarium oxysporum*, the causal agent of Panama disease (Borges-Perez and Fernandez-Falcon 1996).

Ethylene is a volatile plant hormone that is derived from the amino acid methionine and is involved in various important physiological processes within the plant (Wang *et al.* 2002). It is produced by the plant in response to wounding and is also produced during infection by pathogens and treatment with elicitors. Ethylene, together with JA and Me-JA play an important role in SA-independent defence pathways (Pieterse *et al.* 1998; Wang *et al.* 2002).

Harpins are a group of bacterial proteins that can elicit a number of defence responses in plants (Keen 1999). The harpin proteins are presumed to be involved in recognition of bacteria as foreign substances in non-host plants and as virulence factors in host plants, but their precise role remains unclear (Keen 1999).

Some fatty acids have the ability to induce SAR, e.g. arachidonic, linolenic, linoleic and oleic acid that have been shown to induce SAR in potato against *Phytophthora infestans*. Some of these may well be normal elicitors that are released by the pathogen after it has infected the plant and then through HR induces SAR (Sticher *et al.* 1997).

The brassinosteroids (BR) are a group of cholesterol-like steroid molecules that occur naturally in plants in very low concentrations. They were discovered in 1979 when brassinolide (BL) was isolated by Grove and associates. Since then approximately 40

different BRs have been identified and isolated from a variety of plants. Brassinolide is the most common of all the brassinosteroids and also physiologically the most active (Bishop and Koncz 2002).

Exogenous application of BRs have a number of effects on the plant including promotion of cell elongation and division (growth), higher response to gravitropism (leaf bending), higher stress tolerance, higher rate of ethylene biosynthesis, enhancement of the differentiation of the tracheary element, proton-pump-mediated polarization of the cell membrane and retardation of abscission (Shimada *et al.* 2003; Kim *et al.* 2004). Recent micro array analysis of *Arabidopsis* plants treated with BL have shown that a vast amount of genes are up and down regulated by BL application. These genes encode cytochrome P450's, transporter proteins, ion channels, signaling proteins as well as a number of putative proteins and proteins with unknown functions (Nakamura *et al.* 2003). The important role of BRs was shown in mutant plants that are BR-deficient, which have various negative phenotypes. These negative effects include a dwarf phenotype as well as characteristic dark green leaves. It has however also been shown that application of brassinolide and some of its precursors can relieve these negative phenotypes. This suggests that BR's are essential in plants for normal growth and development (Bishop and Koncz 2002). *ComCat*<sup>®</sup>, the commercial product used in this study, contains brassinosteroids as active substance.

### **2.7.2 Inorganic compounds**

Limited knowledge is available about the induction of defence mechanisms by inorganic compounds. However, phosphate salts have been shown to induce SAR in maize, bean and cucumber through calcium sequestration (Sticher *et al.* 1997). It is believed that calcium may be the endogenous signal that triggers SAR in these plants. Further, SiO<sub>2</sub> powder applied to cucumber and tobacco increased the activity of PR-proteins such as chitinases, polyphenoloxidases,  $\beta$ -1,3-glucanase and peroxidase (Sticher *et al.* 1997).

### **2.7.3 Synthetic compounds**

The best known compounds that fall in this class of plant activators are two SA analogs namely benzo (1,2,3) thiadiazole-7-carbothioic acid S-methyl ester (BTH) and 2,6-dichloroisonicotinic acid (INA).

BTH has been commercialized as agrochemical and is supplied in a formulation commercially known as Bion<sup>®</sup> (50WG), which was developed and patented by Syngenta AG, Switzerland. It is a potent inducer of SAR in various organisms and BTH-induced resistance has been reported in tomato, bean, wheat, tobacco and *Arabidopsis* (Friedrich *et al.* 1996; Görlach *et al.* 1996; Iriti and Faoro 2003; Baysal *et al.* 2003). In all these studies a decrease in lesion formation as well as lesion size was observed. BTH also induced the resistant genes, *PR-1-4*, class III chitinase, PR-Q, basic glucanase and acidic peroxidase in tobacco (Friedrich *et al.* 1996). However, treatment with BTH did not lead to resistance in tobacco plants against *Alternaria alternata* or *Botrytis cinerea* (Friedrich *et al.* 1996).

Treatment of plants with INA induced lipoxygenase activity as well as high levels of unconjugated jasmonic acid (Schweizer *et al.* 1997). Although INA is able to induce some defence responses, it failed to be commercialized due to intolerance by some plant species.

Other known synthetic plant activators are Propanazole that protects rice against rice blast (Sekizawa and Mase 1980) and 2,2-dichloro-3,3-dimethylcyclopropane (WL 28325) that induces resistance in rice towards the pathogen *Magnaporthe grisea*, the causative agent of the disease rice blast. The latter causes the accumulation of two phytoalexins, momilactone A and B, which only occurs in rice and therefore does not have a wide applicability (Sticher *et al.* 1997). A third synthetic product, DL-3-aminobutyric acid (BABA), induces SAR in tomato, potato and tobacco protecting these plants against *Phytophthora infestans* and *Peronospora tabacina* (Sticher *et al.* 1997).

# | *Chapter 3*

## MATERIALS AND METHODS

## 3.1 Materials

### 3.1.1 Plant material

Seeds of near-isogenic wheat (*Triticum aestivum* L.) lines, resistant (RL6052 = Thatcher / *Lr15*, accession I-15, BV2005) and susceptible (Thatcher, accession I-49, BV2005) to leaf rust, were obtained from a local seed merchant and seedlings grown under controlled conditions in a glasshouse (see 3.2.3). Fresh above ground parts of *A. africanus* and *Tulbaghia violacea* were collected in Bloemfontein (29° 07' S; 26° 11' N). *Lupinus albus* seeds were purchased from SENWES, a local cooperative in Bloemfontein, South Africa.

### 3.1.2 Other materials

All the chemicals used, i.e. methanol, hexane, ethyl acetate, dichloromethane, diethyl ether, sulphuric acid, ethanol and chloroform were of the purest grade available and purchased from Merck (Germany). Aluminium thin-layer chromatography plates (Silica gel 60 F<sub>254</sub>; 20 x 20 cm<sup>2</sup>) and packing material for column chromatography (Silica gel 60; particle size 0.063 – 0.200 mm; 70 mesh ASTM) were also purchased from Merck (Germany). C<sub>18</sub> Sep-Pak<sup>®</sup> cartridges were from Waters Corporation Milford, Massachusetts, USA. *ComCat*<sup>®</sup> was purchased from Agraforum SA Pty Ltd.

## 3.2 Methods

### 3.2.1 Preparation of crude plant extracts

Fresh above ground parts of *Agapanthus africanus* and *Tulbaghia violacea* were collected, dried at 30°C and separately ground to a fine powder using a Retch SM 2000 cutting mill followed by a Freutsch<sup>®</sup> grinder (Germany). The powdered *Tulbaghia violacea* material was extracted four times with 100% methanol on a roller mill and the pooled extract vacuum dried using a Büchi<sup>®</sup> Rotavapor (Bibby Sterlin LTD, England). The moist extract was further dried overnight to a powder by means of a Savant Speed Vac<sup>®</sup> coupled to a Savant Refrigerated Vapour Trap<sup>®</sup>.

Dry *Lupinus albus* seeds (SS) were ground to a fine powder using the same equipment. This, as well as the powdered *Agapanthus africanus* material, were separately transferred to

aluminium containers and thoroughly moistened with 100% ethanol by mixing with a spatula in order to surface sterilize the material. The ethanol was allowed to evaporate in order to make the powdered material more water suspendible. The latter was repeated twice. Additionally, *ComCat*<sup>®</sup>, a commercial plant strengthening agent available in the form of a wettable powder, was also surface sterilized in the same manner.

For this study, the *T. violacea* methanol extract and the wettable powders of *ComCat*<sup>®</sup>, SS and *A. africanus* will be referred to as **plant extracts**.

### 3.2.2 *In vitro* screening for antifungal properties

A modified agar diffusion method (Rios *et al.* 1988) was used for determining the *in vitro* inhibition of radial mycelial growth of seven test fungi by *ComCat*<sup>®</sup> at 0.5 mg ml<sup>-1</sup> (Agrorum Germany), SS at 5 mg ml<sup>-1</sup> (van der Watt 2005) as well as both *Agapanthus africanus* (Tegegne 2004) and *Tulbaghia violacea* (Nteso 2004) at 1g L<sup>-1</sup>. All plant pathogenic fungi were cultured on 2% (m/v) malt agar, prepared according to the specifications of the manufacturers and autoclaved for 20 min at 121°C. Three hundred µl of a 33% (m/v) streptomycin solution was added to the basal medium to control bacterial growth. Dried and sterilized *ComCat*<sup>®</sup>, SS and *Agapanthus africanus* powder as well as *Tulbaghia violacea* crude extract were dissolved in 100 ml sterile distilled water and amended in the agar to yield the desired final concentrations. The medium was poured into 90 mm sterile plastic Petri dishes to a thickness of 2-3 mm and allowed to set. The centre of each test plate was inoculated with a 5 mm size plug of 7-10 day old cultures, for each of the pathogens separately. A plate containing only basal medium served as control.

The seven test fungi used in this study were provided by the Plant Protection Research Institute (PPRI) of the Agricultural Research Council (ARC) in South Africa and included, *Botrytis cinerea* Pers.:Fr. (PPRI 5209; Hyphomycetes), *Sclerotium rolfsii* Sacc. (PPRI 7975; Agonomycetes), *Rhizoctonia solani* Kühn (PPRI 7973; Agonomycetes), *Fusarium oxysporum* Schlechtend.:Fr. (PPRI 7972; Hyphomycetes), *Botryosphaeria dothidea* (Moug.:Fr.) . (PPRI 7974; Loculoascomycetes), *Pythium ultimum* Trow. (PPRI 6821; Oömycetes) and *Alternaria alternata* (Fr:Fr) Keissler (PPRI 6219; Deuteromycetes). Plates were incubated for 3 days at 25 ± 2°C (March *et al.* 1991) in a growth cabinet. Additionally, a plate containing a specific standard fungicide registered for each fungus was used at concentrations recommended by

the manufacturers to serve as positive control against each test organism. These included Bravo 720, CungFu<sup>®</sup> 538SC, Eria<sup>®</sup> and Metazab<sup>®</sup> 700WP. Each treatment was performed in triplicate.

### 3.2.3 Preparation of wheat seedlings under greenhouse conditions

Twenty to twenty five seedlings per pot were cultivated in a sterilised soil/peat (3:1) mixture at day and night temperatures of 25°C and 20°C. Daylight was supplemented with 120  $\mu\text{mol m}^{-2} \text{s}^{-1}$  photosynthetically active radiation provided by cool white fluorescent tubes for 14 h each day. The plants were watered daily and fertilised with 50 ml of a 3:2:1, N:P:K formulation (10 g L<sup>-1</sup>, w/v) per pot, two times per week.

### 3.2.4 Treatment of wheat seedlings with plant extracts

The different plant extracts were applied as a foliar spray to 17-day-old (three-leaf growth stage) susceptible and resistant wheat plants at the same concentrations that were tested for antifungal activity (3.2.2). Either the extract or the wettable powders were dissolved separately in 30 ml distilled water and applied to the wheat seedlings in triplicate by means of a hand spray until runoff. One set of pots was sprayed with distilled water to serve as negative control.

### 3.2.5 Inoculation of wheat seedlings with rust spores

Uredospores of *Puccinia triticina* (pathotype UVPr9) were multiplied beforehand on a susceptible cultivar and  $\pm 1 \text{ mg ml}^{-1}$  freshly harvested spores, suspended in a light mineral oil, was sprayed under high pressure onto the plants. Inoculation occurred 24 h after treatment with either the plant extract or wettable powders. Plants were left for 30 min to dry before 0 h sampling took place. The plants were left for two hours to dry at room temperature. Subsequently, the plants were incubated in a dew chamber (>96% relative humidity, 21°C) for 16 h in the dark. After a 2 h drying period at room temperature the plants were transferred to the glasshouse, randomly harvested from different pots at 24, 48, 72, 96 and 144 h after treatment and used for collection of apoplastic or intercellular wash fluid.

### 3.2.6 Collection of intercellular wash fluid (IWF) from wheat leaves

To obtain a representative sample, whole plants were randomly harvested from different pots. Leaves were cut into  $\pm 60$  mm pieces, combined and intercellular wash fluid extracted using a modified method of Jung *et al.* (1993). Leaves were rinsed in distilled water and vacuum infiltrated for 5 min with 50 mM Tris-HCl buffer, pH 7.8. The leaves were blot dried with tissue paper and centrifuged at 500 x g for 10 min at 4°C. The entire procedure was repeated and the combined IWF used for SDS-PAGE, Western blots as well as  $\beta$ -1,3-glucanase, chitinase and peroxidase activity determinations.

### 3.2.7 Enzyme activities

#### 3.2.7.1 $\beta$ -1,3-Glucanase (EC 3.2.1.39) activity

$\beta$ -1,3-glucanase activity was assayed using a modified method of Fink *et al.* (1988). The assay mixture contained 10  $\mu$ l enzyme extract, 250  $\mu$ l laminarin (2 mg ml<sup>-1</sup> water; Sigma), and 240  $\mu$ l 50 mM sodium acetate buffer, pH 4.5. After incubation at 37°C for 10 min, 500  $\mu$ l of Somogyi's reagent (Somogyi 1952) were added. The samples were then boiled at 100°C for 10 min. After cooling, 500  $\mu$ l of Nelson's reagent (Nelson 1944) were added. The absorbance of the samples was read at 540 nm against a blank prepared in the same manner, but lacking the IWF, using a Cary Spectrophotometer. The amount of glucose in the samples was estimated from a prepared glucose standard curve and specific  $\beta$ -1,3-glucanase activity was expressed as mg glucose mg<sup>-1</sup> protein min<sup>-1</sup>.  $\beta$ -1,3-Glucanase activity of the representative IWF sample was determined in triplicate.

#### 3.2.7.2 Chitinase (EC 3.2.1.14) activity

Total chitinase activity was determined with dye-labelled CM-Chitin (Loewe Biochemica GmbH, München, Germany) as substrate (Wirth and Wolf 1990). The absorbance at 550 nm was taken as a measure of enzyme activity, which was expressed as A<sub>550nm</sub> mg<sup>-1</sup> protein min<sup>-1</sup> against a blank prepared in the same manner, but lacking the IWF. All assays were performed in triplicate.

### 3.2.7.3 Peroxidase (EC 1.11.1.7) activity

Extracellular peroxidase activity in the IWF was determined spectrophotometrically, as described by Zieslin and Ben-Zaken (1991) with some modifications. The assay mixture contained 0.5% (v/v) guaiacol (Merck) in 40 mM potassium phosphate buffer, pH 5.5, 15 mM H<sub>2</sub>O<sub>2</sub> and 10 µl enzyme extract (IWF or supernatant) in a total volume of 1 ml. The increase in absorbance was measured at 470 nm for a period of 180 seconds against a blank. The specific activity was calculated using the molar extinction coefficient ( $\epsilon = 2.66 \times 10^4 \text{ mol}^{-1} \text{ cm}^{-1}$ ) of tetraguaiacol and expressed as nmol tetraguaiacol mg<sup>-1</sup> protein min<sup>-1</sup>. Peroxidase activity of the representative IWF sample was determined in triplicate.

### 3.3 Protein concentration

The protein concentration was determined according to the method of Bradford (1976), using Bio-Rad Protein Assay reagent (Richmond, CA, USA) and  $\gamma$ -globulin (Sigma, St. Louis, MO, USA) as the standard.

### 3.4 Disease rating

Seventeen day old plants were treated with *ComCat*<sup>®</sup>, SS, *T. violacea* and *A. africanus* extracts as described in 3.2.4 prior to infection. A set of plants sprayed with distilled water served as negative control. Twenty to twenty five 2<sup>nd</sup> leaves of each treatment were selected after 24 h and their upper surfaces inoculated with 2, 4, 6 and 8 mg freshly harvested spores of *Puccinia triticina*. A settling tower was used and the spores were dispersed into the settling tower as described by Negussie and Pretorius (2005). Plants were incubated in a dew chamber (>96% relative humidity, 21°C) for 16 h in the dark. After a 2 h drying period at room temperature the plants were transferred to the glasshouse.

After 7 days of infection the leaves were harvested, photographed and the area of the leaf covered by pustules was estimated using the ASSESS (Image analysis software for Plant Disease Quantification) programme (Lamari, 2002, APS Press). Data on percentage disease per leaf area from the different treatments were subjected to analysis of variance (ANOVA).

### 3.5 Spore germination

Fifteen percent bacterial agar solution (Merck) containing the different plant extracts, *ComCat*<sup>®</sup> (0.5 mg L<sup>-1</sup>), SS (5 mg L<sup>-1</sup>), *T. violacea* (1 g L<sup>-1</sup>) and *A. africanus* (1 g L<sup>-1</sup>) was used to determine the percentage germination of *Puccinia triticina* uredospores. A set of Petri dishes containing only the agar solution served as negative control. Freshly harvested spores (2 mg) were dispersed onto the Petri dishes in a settling tower as described in 3.4. Petri dishes were incubated at 20°C in the dark and after 3 h the amount of germinated spores counted. The experiment was performed in triplicate.

### 3.6 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (PAGE) was performed according to Laemmli (1970) using a 12% separating gel and a 6% stacking gel in a Mini Protein II, Bio-Rad gel system at 200 V constant voltage for 45-60 min. Wells were loaded with 30 µg protein from the IWF of susceptible and resistant, infected and uninfected plants. Low range molecular weight standards of Bio-Rad were run alongside the protein samples. Two identical gels were developed. One was stained using the Coomassie staining procedure (Zehr *et al.* 1989), while the other one was used for immunoblotting.

### 3.7 Immunoblotting (Western Blot)

Electrophoretic transfer of the polypeptides from 12% polyacrylamide gels onto nitrocellulose membranes (Hybond-C, extra membrane, Amersham Biosciences) was performed for 45 min in a semi-wet transfer unit (BioRAD) at a constant current of 350 mA in a transfer buffer. The transfer buffer contained 25 mM Tris (pH 8.3), 192 mM glycine and 20% (v/v) methanol. After the transfer, the membrane was stained in Ponceau S (Amersham Biosciences) for 2 min to verify protein transfer and destained with several changes of distilled water. All the active sites on the membrane were then blocked for 1 h at room temperature in 8% (w/v) fat-free milk powder (Elite) in TBS (10 mM Tris-HCl, pH 8.3, 1.5 M NaCl). The membranes were then incubated in the primary antibody (anti-wheat β-1,3-glucanase) diluted 1:9000 in TBS containing 4% (w/v) fat-free milk powder, for 2 h. Following extensive washing with TBST buffer [TBS, 0.1% (v/v) Tween-20], the membranes

were incubated for another 1 h at room temperature in the secondary antibody, goat-anti-rabbit IgG, that had been conjugated with alkaline phosphatase (1:9500 dilution in TBS). The membranes were then thoroughly rinsed in TBST containing 0.05% (w/v) SDS, followed by TBST and TBS. The locations of antigenic proteins were visualized by incubating the membranes in a Tris-HCl buffer containing 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and nitro blue tetrazolium (NBT) as a colour enhancer (Blake *et al.* 1984).

### 3.8 Expression of *PR* genes

#### 3.8.1 Total RNA extraction from leaves

Distilled water was treated with 0.1% (v/v) dimethyl pyrocarbonate (DMPC), left overnight and autoclaved at 121°C for 20 min. All pestles, mortars and spatulas were washed with dish wash liquid, 10% (w/v) SDS in DMPC treated water, wrapped in aluminum foil and oven-baked at 260°C for 3 h. The pestles, mortars and spatulas were finally sprayed with 100% (v/v) ethanol and set alight just before use. Plant material was ground into a fine powder in liquid nitrogen using a pestle and mortar and stored in 1.5 ml Eppendorf tubes at -80°C.

Resistant and susceptible wheat plants were treated with 1 g L<sup>-1</sup> *A. africanus* wettable powder, left for 48 h, then inoculated with *P. triticina* spore suspension and left to dry for 30 min before 0 h sampling took place (3.2.5). Controls were treated with water only. Total RNA was isolated from samples harvested at different time intervals after infection. Total RNA was extracted from approximately 0.1 g ground plant material using TRIzol Reagent (Invitrogen) according to the manufacturers specifications. The RNA was finally dissolved in 100 µl DMPC treated water. The quality and quantity of the extracted RNA was confirmed spectrophotometrically as described by Sambrook *et al.* (1989) and expressed as ng µl<sup>-1</sup>.

A 1% (w/v) agarose gel containing 0.5 µg. ml<sup>-1</sup> ethidium bromide in 0.5 x TAE [20 mM Tris hydroxymethyl aminomethane-HCl (Tris-HCl) pH 8, 0.5 mM ethylene diaminetetraacetic acid (EDTA), 0.28% (v/v) acetic acid] was used to separate the RNA. Each RNA sample (500 ng) was diluted in DMPC treated water and RNA loading buffer [0.25% (w/v) bromophenol blue, 15 % (w/v) ficoll] added in a 6:1 ratio before the samples were loaded onto the gel. Separation was done at 10 V cm<sup>-1</sup> for 30 min using 0.5 x TAE as running buffer. The RNA was visualized under UV illumination and photographed using a Bio-Rad gel documentation system.

### 3.8.2 Expression Analysis using Reverse Transcription Polymerase Chain Reaction (RT-PCR)

The RobusT II RT-PCR kit (Finnzymes) was used to amplify the differentially expressed *PR* genes in the presence of two gene specific primers (Table 3.1). Each 10 µl reaction contained 10 ng total RNA, 25 pmol of each primer, 0.2 mM dNTP's, 1.5 mM MgCl<sub>2</sub>, 1 x final dilution of the kit buffer and 0.4 µl of the RobusT II enzyme mix. The amplification regime for the reactions was the same for all the genes, except that the annealing temperatures of the primer combination differed (Table 3.1). The reaction conditions were as follows: one cycle at 48°C for 30 min and 94°C for 2 min, 30 cycles of 94°C for 30 sec, annealing temperature for 30 sec and 72°C for 2 min followed by one cycle at 72°C for 5 min. As a control, *18S rRNA* was amplified using Bovis 26 and 27 as primers (Table 3.1). After completion of the RT-PCR, 3 µl loading buffer [15% (w/v) ficoll and 0.25% (w/v) Xylene cyanol] was added to each reaction. Of each reaction, 12 µl was separated on a 1% agarose gel (3.8.1).

**Table 3.1:** Nucleotide sequences of all primers used in this study.

Primer (Gene) name	Nucleotide sequence	Annealing temperature
Bovis 26 ( <i>18S</i> ) F	5'- CAACTTTCGATGGTAGGATAG - 3'	50°C
Bovis 27 ( <i>18S</i> ) R	5'- CTCGTTAAGGGATTTAGATTG - 3'	50°C
Bovis 223 ( <i>PR2</i> ) F	5'- TCCACGGCGGTCAAGATGA - 3'	61°C
Bovis 224 ( <i>PR2</i> ) R	5'- GGTTCCTCGTTGAACATGGC - 3'	61°C
Bovis 225 ( <i>PR3</i> ) F	5'- AAGACGGCGTTGTGGTTCTG - 3'	61°C
Bovis 226 ( <i>PR3</i> ) R	5'- GTAGCGCTTGTAGAACCCGAT - 3'	61°C
Peroxidase ( <i>PR9</i> ) F	5'- ATCAGACCGTCTCCTGCG - 3'	55.4°C
Peroxidase ( <i>PR9</i> ) R	5'- GCAGCTGAGCCTGATCTG - 3'	55.4°C

### 3.8.3 PCR amplification of selected DNA fragments

After RT-PCR, the differentially expressed cDNA bands were cut and purified from the agarose gel with the Favorprep Gel/PCR Purification Kit (Favorgen Biotech) as specified by the manufacturer. The DNA was finally diluted in 50 µl water. The DNA bands were re-

amplified using the same primer combinations as described. Each 20  $\mu\text{l}$  PCR reaction contained 1  $\mu\text{l}$  of the recovered DNA, 25 pmol of each primer, 0.3 mM dNTP's, 1.5 mM  $\text{MgCl}_2$ , 1 x final dilution of the supplied buffer and 0.5  $\mu\text{l}$  KAPA *Taq*. The amplification conditions were one cycle at 94°C for 2 min and then 30 cycles of 94°C for 15 sec, annealing temperature for 30 sec and 72°C for 45 sec. An elongation step of 7 min at 72°C was finally included. After completion, 25  $\mu\text{l}$  of each sample was separated on a 1% (w/v) agarose gel (3.8.1).

#### **3.8.4 Sequencing of differentially expressed *PR* genes**

The amplified products were again cut from the gel and purified (3.8.3). The DNA fragments were then sequenced using the Big Dye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems). Each PCR sequence reaction consisted of 4  $\mu\text{l}$  DNA template, 2  $\mu\text{l}$  Ready Reaction premix, 3  $\mu\text{l}$  Big-Dye Sequencing buffer, 3.2  $\mu\text{M}$  primer (either reverse or forward primer) and water to a final volume of 20  $\mu\text{l}$ . The amplification conditions were one cycle at 96°C for 1 min, 25 cycles at 96°C for 10 sec, annealing temperature of the primer for 5 sec and 60°C for 4 min.

Purification of the sequenced products was done by adding 5  $\mu\text{l}$  of a 125 mM EDTA solution to each 20  $\mu\text{l}$  sequencing reaction, as well as 60  $\mu\text{l}$  100% (v/v) ethanol. The reactions were incubated at room temperature for 15 min, centrifuged at 13000  $g$  for 30 min and the supernatant discarded. The pellets were washed once with 60  $\mu\text{l}$  70% (v/v) ethanol, re-centrifuged for 5 min and then dried. The sequenced fragments were separated on an ABI377 Sequencer (PE Biosystems). The resulting sequences were used to do a BLAST analysis (v 2.2.13) (Altschul *et al.* 1990) with entries available at GenBank.

### **3.9 Isolation, purification and identification of substances from *Agapanthus africanus* that induce resistance in wheat against leaf rust**

#### **3.9.1 Preparation of a crude extract and subsequent activity directed liquid-liquid extraction**

Two kg of dry *A. africanus* above ground material was extracted overnight in 100% methanol (at a ratio of two ml  $g^{-1}$  dry weight) on a roller mill. The supernatant was decanted, filtered

through Whatman no. 1 and 3 filter paper and vacuum distilled at 50°C using a Büchi Rotavapor<sup>®</sup> (Bibby Sterlin LTD, England) equipped with a cooled Liebig condenser in order to remove the bulk organic solvent. This procedure was repeated three times and this fraction was referred to as the crude extract.

Subsequently, the crude extract was fractionated by means of liquid-liquid extraction (Gamoh *et al.* 1989) using hexane (DC = 2.0), diethyl ether (DC = 4.3) ethyl acetate (DC = 6.0) and dichloromethane (DC = 8.9) as solvents (1:10; v/v). Extraction was repeated more than 20 times, using fresh solvent with each step, by shaking vigorously on a mechanical shaker for 10 min. The four fractions were collected separately and vacuum distilled at 35°C using a Büchi Rotavapor<sup>®</sup>. Fractions were dried under vacuum in a Savant Speed Vac<sup>®</sup> Plus coupled to a Savant Refrigerated Vapour Trap. A qualitative thin layer chromatography (Q-TLC) profile (3.9.5) was obtained for each fraction.

### **3.9.2 Bio-assay for activity**

Each of the fractions obtained by means of liquid-liquid extraction were applied to 17 day old wheat plants as described in 3.2.4, except that the extracts were dissolved in a 5% DMSO solution. Control plants were treated with only 5% DMSO. Intercellular wash fluid was extracted after 48h (3.2.6) and induction of resistance was determined by measuring peroxidase activity in each fraction as described in 3.2.7.3.

### **3.9.3 Activity directed column chromatography fractionation**

The most active fraction obtained from the liquid-liquid extraction procedure was further fractionated by means of C<sub>18</sub> reverse phase chromatography. Seventy percent methanol, followed by 100% methanol was used as eluent. Elution was at a flow rate of 0.5 ml min<sup>-1</sup> and 0.5 ml fractions were collected. Thin layer chromatography was performed and fractions grouped according to their profile. Again, the bio-assay (3.9.2) was performed and the fraction showing the highest peroxidase activity was subjected to a second column chromatography separation step using silica gel as stationary phase.

A column (13 x 150 mm) was packed with silica gel 60 and the sample eluted with a gradient solvent system of chloroform : methanol (80:20, 60:40, 40:60 v/v), followed by 100%

methanol and finally 70% methanol. Elution was at a flow rate of  $0.5 \text{ ml min}^{-1}$  and 0.5 ml fractions were collected. Column chromatographic fractions that showed similar Q-TLC profile patterns were combined separately, dried in a Savant<sup>®</sup> SpeedVac Concentrator overnight and the bio-assay for peroxidase activity of each performed (3.9.2).

#### **3.9.4 Preparative Thin Layer Chromatography (P-TLC)**

The most active combined fractions from the silica gel column were further purified by means of  $20 \times 20 \text{ cm}^2$  preparative thin layer chromatography plates pre-coated with 1 mm Silica gel 60 F<sub>254</sub>. Thirty mg of the active fraction was dissolved in 100  $\mu\text{l}$  methanol and loaded onto the plate by streaking evenly over the baseline with the aid of a glass capillary tube. The plate was dried and developed in a saturated chamber using a chloroform : methanol : water (70:30:2) solvent system as mobile phase. A vertical piece of the TLC plate was cut off, stained with 10 % (v/v) ethanolic sulphuric acid, placed in an oven at  $100^\circ\text{C}$  for 5 minutes (Wagner and Bladt 1996) and the compounds detected. This piece of plate was compared to the other part of the TLC, individual compounds marked and the detected zones were scraped off from the plate using a spatula. The compounds were recovered from the silica by elution with methanol, followed by centrifugation for 5 min at 12000 rpm. The individual compounds were dried in a Savant<sup>®</sup> SpeedVac Concentrator and subjected to nuclear magnetic resonance (NMR) spectroscopy in order to identify them and elucidate their molecular structures. The bio-assay for peroxidase activity of each compound was performed (3.9.2).

#### **3.9.5 Qualitative Thin Layer Chromatography (Q-TLC)**

Silica gel 60 F<sub>254</sub>-aluminum backed and pre-coated plates (Mikes and Chalmers 1979) were used to obtain the profiles of the fractions during the purification process. Fifty to 70  $\mu\text{g}$  of each sample was loaded onto the plates at the baseline and compounds allowed to separate in a saturated chamber using chloroform : methanol : water (80:20:2) as mobile phase. After observation under UV-light at 254 and 356 nm, the TLC-plates were stained with a 10% (v/v) ethanolic sulphuric acid solution and developed in an oven at  $100^\circ\text{C}$  for 5 minutes. Visible organic compounds served to identify either the chemical profile of a mixture or the purity of a single compound in the case of the final purification step.

### 3.9.6 Preliminary phyto-chemical screening

Two active fractions from the silica gel column were loaded onto separate Q-TLC plates. After compounds were allowed to separate (3.9.5) each TLC plate was subjected to a different spray reagent, according to the standard methods described by Wagner and Bladt (1996), to test for different chemical groups including alkaloids, cardiac glycosides, coumarins, flavonoids, phenolic compounds, essential oils, carbon containing compounds, saponins and steroids.

### 3.9.7 Nuclear Magnetic Resonance (NMR) spectroscopy

The most bioactive compounds were submitted to nuclear magnetic resonance spectroscopy ( $^1\text{H}$  NMR). One-dimensional and two-dimensional NMR experiments were performed on a Bruker 600 MHz spectrometer at 25°C in pyridine-*d*5 and DMSO-*d*6 with tetramethylsilane ( $\text{Si}(\text{CH}_3)_4$ ; TMS) as the internal standard. Chemical shifts were reported in parts per million (ppm) on the  $\delta$ -scale and coupling constants were given in Hz.

The structure of compound X (8 mg) was confirmed by structural elucidation from a combination of NMR experiments including: HMQC (direct proton carbon correlations), HMBC (long-range proton carbon correlations, APT (carbon multiplicity: quaternary (s, C), tertiary (d, CH), secondary (t,  $\text{CH}_2$ ) or primary (q,  $\text{CH}_3$ )) and COSY (proton-proton correlations).

**Compound X:  $^1\text{H}$ -NMR (pyridine-*d*5)  $\delta$ :** 6.33 (1H, s, H-1'''), 4.97 (d,  $J = 7.8$  Hz, H-1''), 4.85 (d,  $J = 7.4$  Hz, H-1'), 4.29 (t,  $J = 9.3$  Hz, H-4''), 4.01 (t,  $J = 9.2$  Hz), 3.70 (m, H-5'''), 3.57 (t,  $J = 10.2$  Hz, H-26a), 3.49 (t,  $J = 10.2$  Hz, H-26b), 2.28 (t,  $J = 12.1$  Hz, H1 or H4), 2.08 (m, H-15), 1.96-1.940 (m, H-20), 1.940-1.903 (m, H-9), 1.39 (m, H-7), 1.78 (t,  $J = 7.9$  Hz, H-17), 1.72 (d,  $J = 6.1$  Hz, H-6''), 1.12 (d,  $J = 6.9$  Hz, H-21), 1.18 (s, H-18), 0.89 (s, H-19), 0.69 (d,  $J = 4.9$  Hz, H-27).

**Compound X:  $^{13}\text{C}$ -NMR (pyridine-*d*5)  $\delta$ :** 109.1 (C-22), 105.1 (C-1''), 102.2 (C-1'''), 100.8 (C-1'), 89.3 (C-3'), 82.9 (C-3), 81.2 (C-16), 77.8 (C-5'), 77.4 (C-5'''), 77.2 (C-2'), 75.2 (C-3'''), 74.0 (C-4''), 73.6 (C-5), 72.7 (C-3'''), 72.4 (C-2''), 72.4 (C-2'''), 70.9 (C-2), 70.0 (C-4'''),

69.6 (C-4'), 69.5 (C-5''), 66.8 (C-26), 63.1 (C-17), 62.1 (C-6'), 62.0 (C-6'''), 56.2 (C-14), 45.5 (C-9) 41.9 (C-20), 40.9 (C-13), 40.5 (C-10), 40.3 (C-4), 40.3 (C-12), 39.9 (C-1), 34.3 (C-6), 34.2 (C-8), 32.2 (C-15), 31.8 (C-23), 30.5 (C-25), 29.2 (C-24), 26.5 (C-7), 21.6 (C-11), 18.5 (C-6''), C-19 (17.3), 17.3 (C-27), 16.6 (C-18), 14.9 (C-21).

**Compound X: <sup>1</sup>H-NMR (DMSO-*d*<sub>6</sub>) δ:** 5.14 (1H, s, H-1'''), 4.34 (d, *J* = 9.4 Hz, H-1'), 4.25 (bd, H-1''), 1.07 (d, *J* = Hz, H-6''), 0.89 (d, *J* = 10.5 Hz, H-21), 0.87 (s, H-19), 0.73 (d, *J* = 5.2 Hz, H-27), 0.69 (s, H-18).

**Compound X: <sup>13</sup>C-NMR (DMSO-*d*<sub>6</sub>) δ:** 108.8 (C-22), 104.3 (C-1''), 101.1 (C-1'''), 99.6 (C-1'), 88.3 (C-3'), 81.3 (C-3), 80.7 (C-16), 76.4 (C-5'), 76.2 (C-5'''), 75.6 (C-2'), 74.0 (C-3'''), 72.9 (C-4''), 72.4 (C-5), 71.0 (C-3''), 70.9 (C-2''), 70.7 (C-2'''), 69.8 (C-2), 69.0 (C-4'''), 68.7 (C-4'), 68.5 (C-5''), 66.4 (C-26), 62.3 (C-17), 61.2 (C-6'), 60.9 (C-6'''), 55.8 (C-14), 44.9 (C-9), 41.5 (C-1), 33.9 (C-6), 33.4 (C-8), 31.8 (C-15), 31.4 (C-23), 30.2 (C-25), 29.2 (C-24), 26.1 (C-7), 21.2 (C-11), 18.5 (C-6''), C-19 (17.5), 17.3 (C-27), 16.7 (C-18), 15.1 (C-21).

### 3.10 Statistical analysis of data

Analysis of variance (ANOVA) was performed on the data, using the NCSS 2000 Statistical Programme for Windows (Hintze 1999). Tukey-Kramer's LSD (least significant difference) procedure for comparison of means (Steele and Torrie 1980; Mason *et al.* 1989) was applied to separate means (*P*<0.05). Treatments differing significantly were indicated either in figures or below tables as calculated LSD values and by using symbols for significant (\*) and insignificant (ns) differences.

# | *Chapter 4*

## RESULTS

#### 4.1 Antifungal properties of *ComCat*<sup>®</sup>, SS, *Agapanthus africanus* and *Tulbaghia violacea*.

The mycelial growth of none of the test fungi was inhibited by either *ComCat*<sup>®</sup> or SS (Table 4.1). Although the 18% mycelial growth inhibition of *B. dothidea* by *ComCat*<sup>®</sup> was highest, an inhibition percentage below 50% is not regarded as significant by any means in terms of the potential to be classified as an antifungal agent. However, both the *A. africanus* powdered material and *T. violacea* crude extract compared favourably with registered fungicides in terms of mycelial growth inhibition of all seven test fungi (Table 4.1).

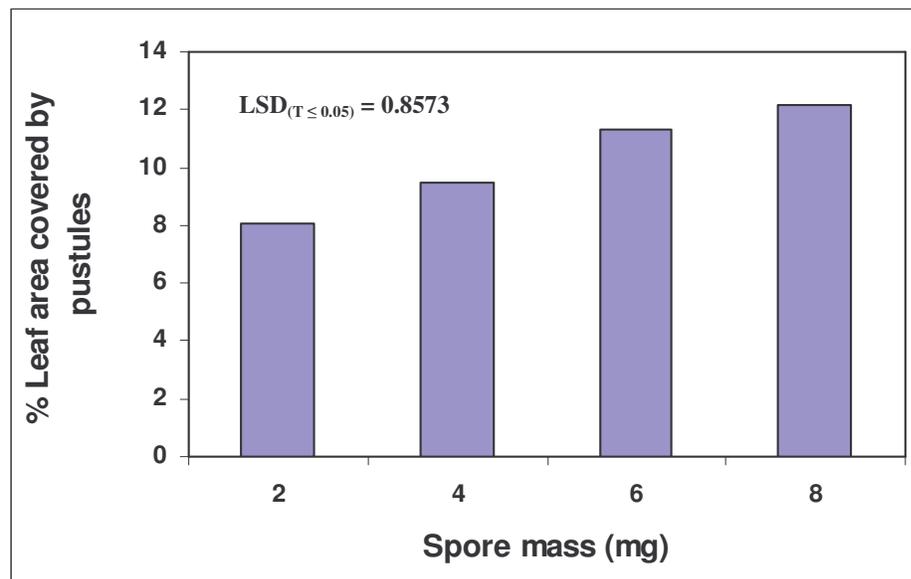
**Table 4.1:** Comparison of percentage *in vitro* mycelial growth inhibition of plant pathogenic fungi by solutions of *ComCat*<sup>®</sup> (0.5 mg ml<sup>-1</sup>), SS (5 mg ml<sup>-1</sup>), *A. africanus* (1 mg ml<sup>-1</sup>) and *T. violacea* (1 mg ml<sup>-1</sup>) to that of standard fungicides registered against the individual pathogens.

Fungus	% Mycelial growth inhibition				
	<i>ComCat</i> <sup>®</sup>	SS	<i>Agapanthus africanus</i>	<i>Tulbaghia violacea</i>	Std. Fungicide <sup>a</sup>
<i>Botrytis cinerea</i>	0.0 ± 0	0.0 ± 0	100 ± 0	100 ± 0	85.3 ± 1.8
<i>Fusarium oxysporum</i>	5.8 ± 0.5	2.7 ± 0.2	91 ± 3	75 ± 2	91.5 ± 2.2
<i>Sclerotium rolfsii</i>	3.6 ± 0.4	4.4 ± 0.5	98 ± 2	96 ± 4	90.8 ± 1.8
<i>Rhizoctonia solani</i>	0.3 ± 0.06	4.9 ± 0.6	100 ± 0	83 ± 2	81.5 ± 1.5
<i>Phytium ultimum</i>	0.0 ± 0	0.0 ± 0	82 ± 5	97 ± 5	88.0 ± 1.4
<i>Botryosphaeria dothidea</i>	18.1 ± 4.8*	0.0 ± 0	100 ± 0	85 ± 3	96.3 ± 2.3
<i>Alternaria alternata</i>	0.0 ± 0	0.0 ± 0	83 ± 3	74 ± 2	96.5 ± 2.2

<sup>a</sup> The registered standard Bravo<sup>®</sup> 720 (chlorothalonil; 3.6 µg ml<sup>-1</sup>) was used for *B. cinerea* and *R. solani*, Cungfu<sup>®</sup> 538SC (copper hydroxide; 444 µg ml<sup>-1</sup>) for *F.oxysporum* and *S. rolfsii*, Eria<sup>®</sup> (difenoconazole/carbendazim; 10.0 µg ml<sup>-1</sup>) for *B. dothidea* and Metazab<sup>®</sup> 700WP (metalaxyl and mancozeb; 1.89 µl ml<sup>-1</sup>) for *P. ultimum* and *A. alternata*.

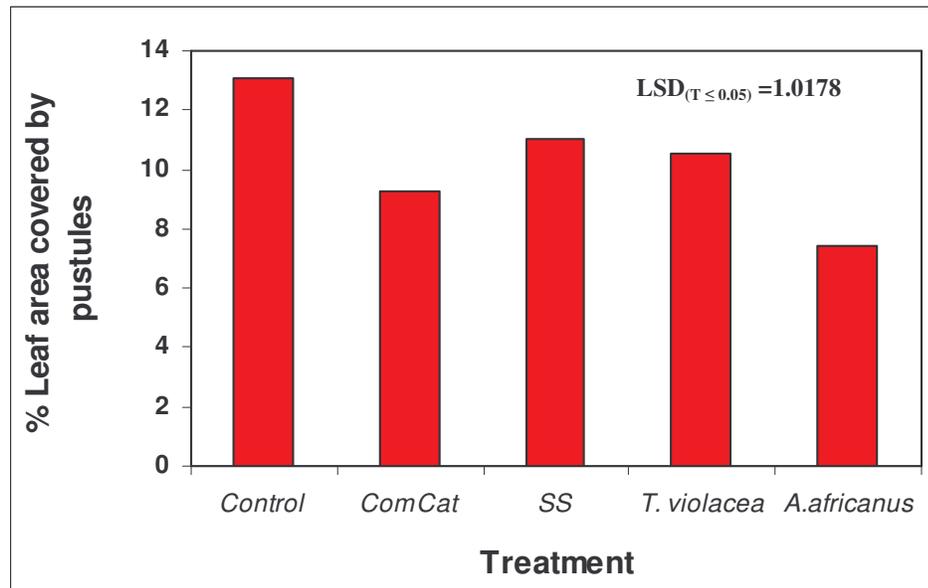
## 4.2 Disease rating

The influence of different spore masses (2, 4, 6 and 8 mg) of *P. triticina* and different plant extract treatments (*ComCat*<sup>®</sup>, SS, *A. africanus* and *T. violacea*) on disease severity in susceptible and resistant wheat cultivars was quantified. It is evident from the results that the percentage pustules formed in susceptible wheat increased significantly with increased spore concentration (Fig 4.1).



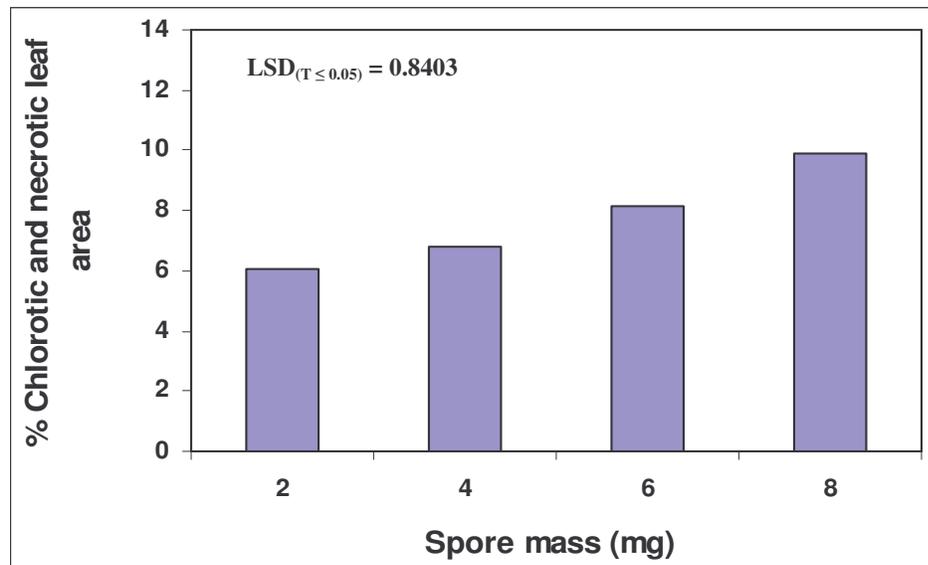
**Figure 4.1:** Effect of different spore masses of *P. triticina* on percentage leaf area covered by pustules in susceptible wheat (Thatcher).

Of the four treatments, *A. africanus* reduced the percentage pustules formed on the leaves of susceptible plants most efficiently (43%) followed by *ComCat*<sup>®</sup> (29.5%; Fig 4.2). For both treatments this reduction was statistically significant ( $P < 0.05$ ) but, albeit less efficiently, this reduction was also significant in the case of the other two treatments.

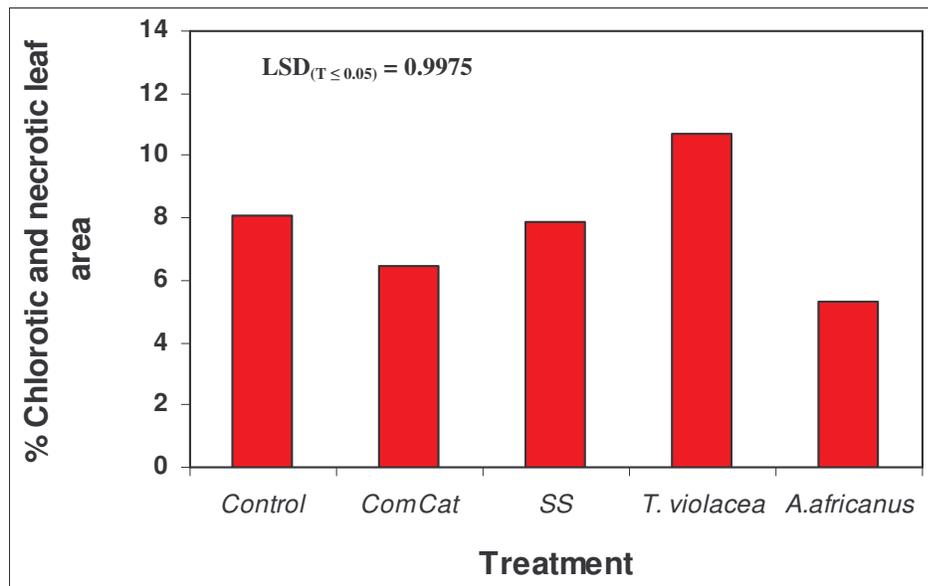


**Figure 4.2:** Effect of *ComCat*<sup>®</sup>, SS, *T. violacea* and *A. africanus* treatments on the mean percentage leaf area covered by pustules in susceptible wheat (Thatcher) calculated for all different spore masses tested.

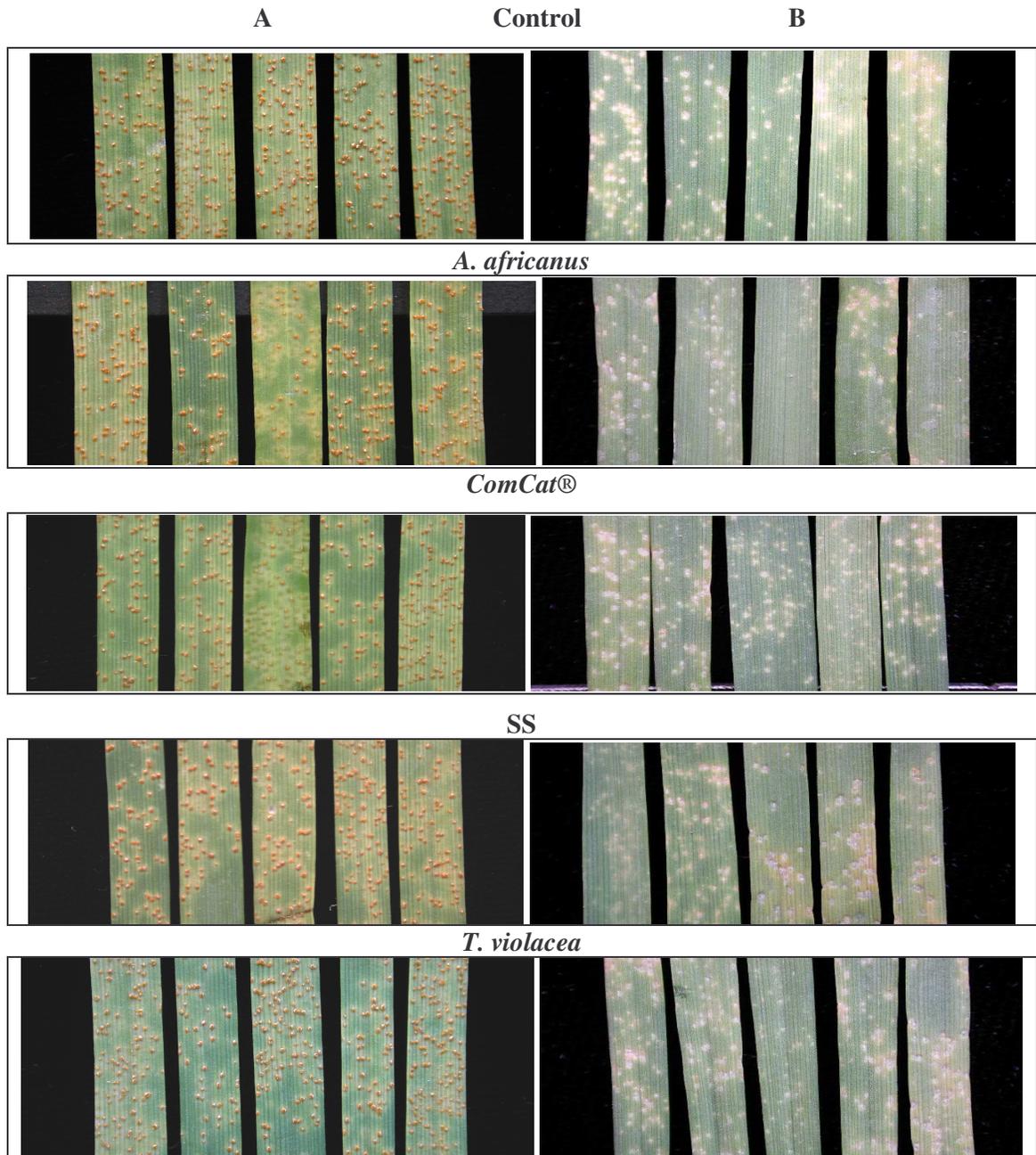
Interestingly, resistant wheat showed a tendency to increase the percentage necrotic lesions on leaves with increased spore concentration (Fig 4.3). Once again treatment with *A. africanus* reduced the percentage necrotic lesions significantly by 35%, compared to the untreated control, and *ComCat*<sup>®</sup> by 16%. (Fig 4.4). On the other hand *T. violacea* increased lesion formation significantly by 32% (Fig 4.4).



**Figure 4.3:** Effect of different spore masses of *P. triticina* on percentage chlorotic and necrotic lesions in resistant wheat (Thatcher / *Lr* 15).



**Figure 4.4:** Effect of *ComCat*<sup>®</sup>, SS, *T. violacea* and *A. africanus* treatments on the mean percentage chlorotic and necrotic lesions in resistant wheat (Thatcher / *Lr* 15) calculated for all different spore masses tested.



**Plate 4.1:** Qualitative assesment of A) susceptible (Thatcher) and B) resistant (Thatcher / *Lr15*) wheat plants treated with *ComCat*®, SS, *T. violacea* and *A. africanus* prior to inoculation with 2 mg *P. triticina* spores.

From Plate 4.1 a clear difference in rust colony and necrotic area formation between the susceptible and resistant wheat lines is evident. The susceptible Thatcher cultivar showed

brown-red pustules on the surface of the leaves one week after infection. The resistant *Lr15* cultivar, on the other hand, mainly showed necrotic flecks, indicative of an active defence response within the plants.

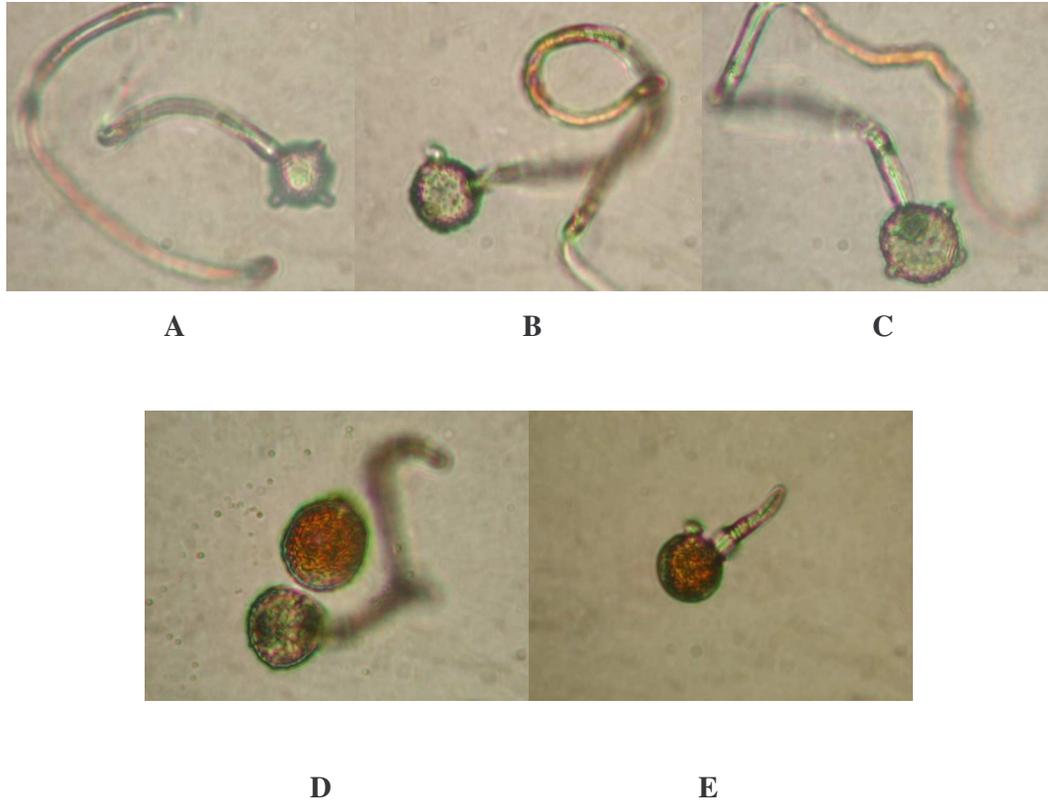
### 4.3 Spore germination

From the previous results (4.2), and compared to the other treatments as well as the untreated control, it became obvious that *A. africanus* was most efficient in inhibiting pustule and necrotic lesion formation in both susceptible and resistant wheat cultivars. The latter begged the question whether this was a result of direct inhibition of spore germination, considering previous results on the antifungal nature of *A. africanus*, or whether it could be ascribed to an indirect defence response triggered in the plant by *A. africanus*. Subsequently, the direct effect of the different plant extracts on germination of *P. triticina* spores was investigated (Chapter 3; 3.5). From the results it is clear that *A. africanus* and *T. violacea* inhibited the germination of spores dramatically (Table 4.2). When spores were incubated for another 3 hours, no significant increase in germination was detected, except in the case of *T. violacea*, where percentage germination doubled to 12% (results not shown).

**Table 4.2:** Percentage germinated spores of *P. triticina* incubated for 3 h on agar containing *ComCat*<sup>®</sup> (0.5 mg L<sup>-1</sup>), SS (5 mg L<sup>-1</sup>), *T. violacea* (1 g L<sup>-1</sup>) and *A. africanus* (1 g L<sup>-1</sup>). Data presented are means of three replicates.

<b>Treatment</b>	<b>% Germination</b>
<b>Control</b>	<b>91 ± 2.0</b>
<b><i>ComCat</i><sup>®</sup></b>	<b>88 ± 1.0</b>
<b>SS</b>	<b>94 ± 2.0</b>
<b><i>T. violacea</i></b>	<b>6 ± 1.5</b>
<b><i>A. africanus</i></b>	<b>3 ± 0.5</b>

The inhibitory effect of the four plant extracts under scrutiny on the length of germ tube development of *P. triticina* after 3 h of incubation is qualitatively illustrated in Plate 4.2. Compared to the untreated control and other treatments, *T. violacea* (Plate 4.2D) and *A. africanus* (Plate 4.2E) extracts were visibly more efficient in the post germinative inhibition of germ tube development.



**Plate 4.2:** Effect of different plant extracts, **B** = ComCat<sup>®</sup>; **C** = SS; **D** = *T. violacea* and **E** = *A. africanus* on germ tube development of *P. triticina* after 3 h incubation at 20°C in the dark. **A** = control.

#### 4.4 The effect of *ComCat*<sup>®</sup>, SS, *Agapanthus africanus* and *Tulbaghia violacea* extracts on the *in vitro* activity of different PR-proteins in wheat.

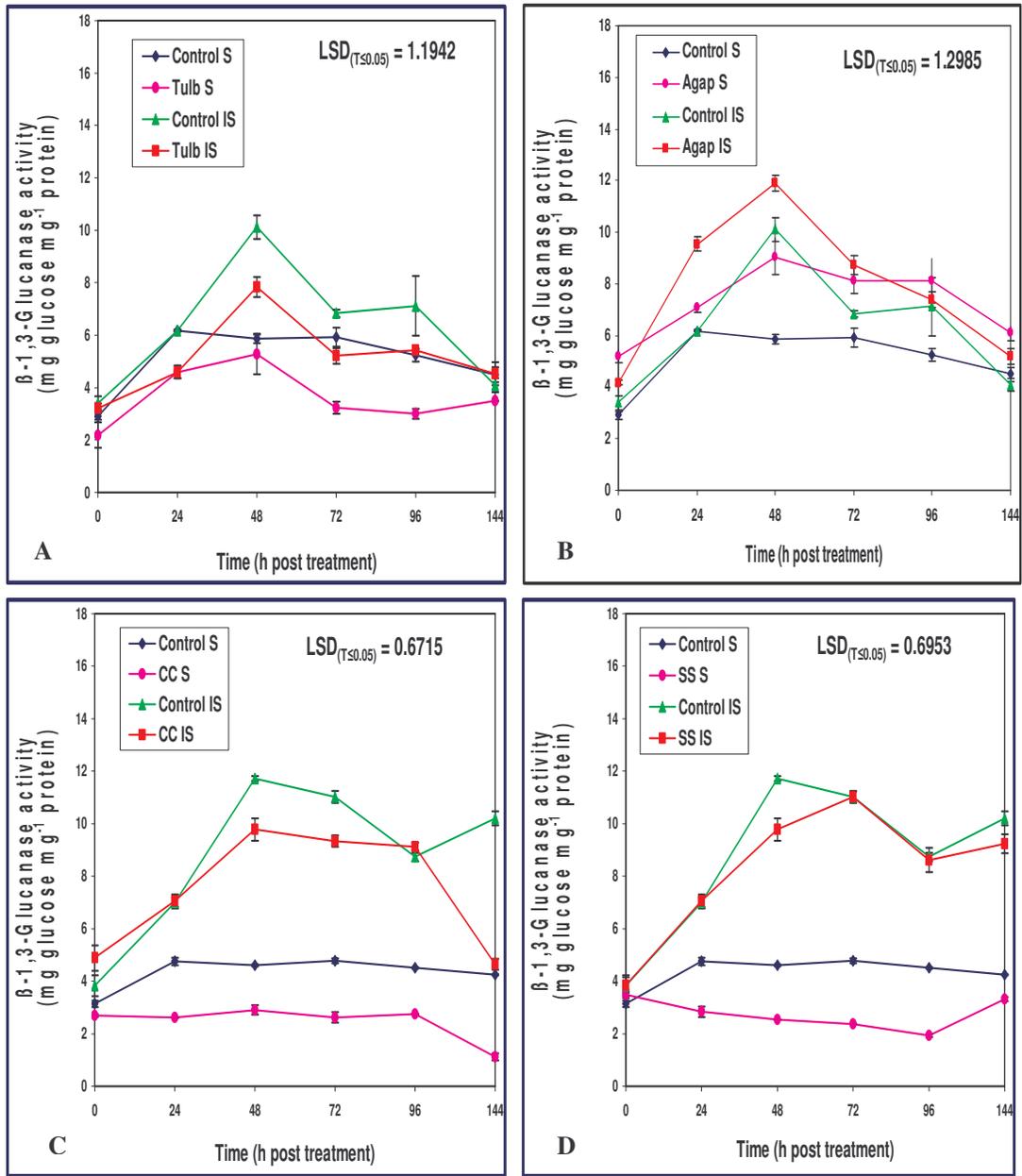
##### 4.4.1 $\beta$ -1,3-Glucanase activity

Changes in  $\beta$ -1,3-glucanase activity were measured over time in infected and uninfected susceptible (Fig 4.5) and resistant (Fig 4.6) wheat cultivars, expressing different levels of resistance to leaf rust.

###### 4.4.1.1 Susceptible wheat

In uninfected susceptible control plants (blue line), the  $\beta$ -1,3-glucanase activity was significantly lower than in the infected controls (green line; Fig. 4.5). This tendency repeated itself constantly, and with no exception, in all enzyme studies reported here. Further, the enzyme activity of the uninfected control wheat remained at a relative constant level over the entire trial period and this tendency occurred in all enzyme studies. However, compared to the uninfected susceptible control plants, treatment with *T. violacea* (Fig 4.5A), *ComCat*<sup>®</sup> (Fig 4.5C) and SS (Fig 4.5D), inhibited the  $\beta$ -1,3-glucanase activity significantly. *A. africanus* treatment on the other hand increased enzyme activity by 54% to a peak level at 48 h in the susceptible uninfected plants (Fig 4.5B).

In both the infected control and infected treated susceptible plants,  $\beta$ -1,3-glucanase activity increased dramatically within the first 48 h post inoculation (Fig 4.5). Treatment of infected susceptible plants with *ComCat*<sup>®</sup> (Fig 4.5C) and SS (Fig 4.5D) did not increase  $\beta$ -1,3-glucanase activity, while treatment with *T. violacea* tended to inhibit enzyme activity (Fig 4.5A). When infected susceptible plants were treated with *A. africanus*, an increase of more than 50% in  $\beta$ -1,3-glucanase activity occurred within 24 h (Fig 4.5B). Peak activity was reached at 48 h, where after it decreased in both the control and treated susceptible plants (Fig 4.5).

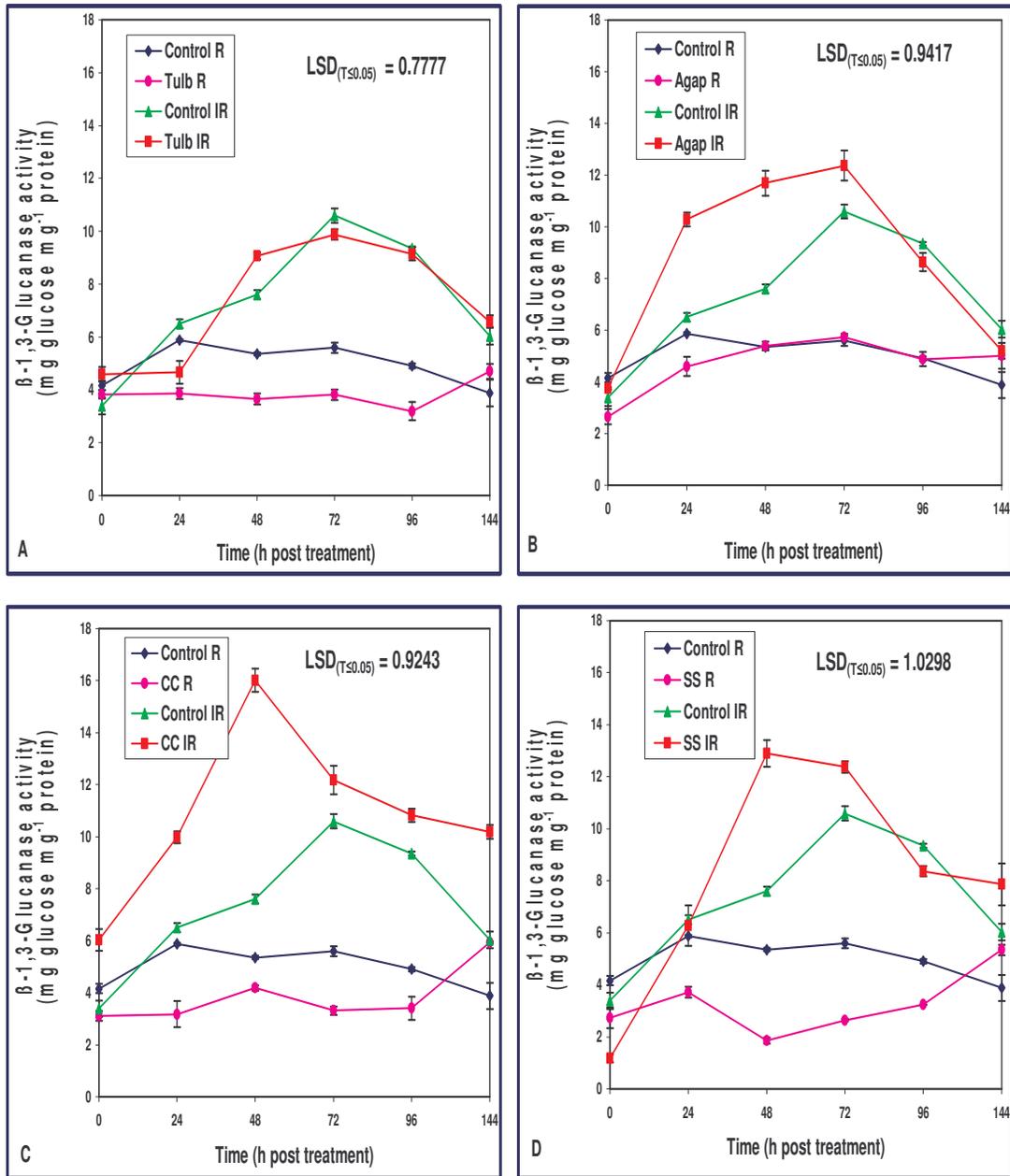


**Figure 4.5:** Effect of **A:** *T. violacea*; **B:** *A. africanus*; **C:** *ComCat*<sup>®</sup> (CC) and **D:** SS on *in vitro*  $\beta$ -1,3-glucanase activity in **susceptible** uninfected and infected wheat (Thatcher). Data presented are means  $\pm$  SD of three replicates. S = uninfected susceptible; IS = infected susceptible. LSD-values indicated in the graphs are for interactions between uninfected and infected wheat at different time intervals over a 144 h time period.

#### 4.4.1.2 Resistant wheat

$\beta$ -1,3-glucanase activity in both control and treated resistant uninfected wheat followed the same trend as in the susceptible uninfected wheat, except that inhibition of the enzyme activity by treatment with *T. violacea* (Fig 4.6A), *ComCat*<sup>®</sup> (Fig 4.6C) and SS (Fig 4.6D) was uplifted after 96 h. Treatment with *A. africanus* (Fig 4.8B) had little effect.

However, infection of the resistant plants with leaf rust resulted in an induction in  $\beta$ -1,3-glucanase activity (Fig 4.6). When resistant plants received a double signal, i.e. simultaneous inoculation with rust spores and treatment with either *A. africanus* (Fig 4.6B), *ComCat*<sup>®</sup> (Fig 4.6C) or SS (Fig 4.6D), enzyme activity increased significantly during the first 48 h by 54%, 110% and 70% respectively, compared to the control plants. *T. violacea* was the only extract that did not induce  $\beta$ -1,3-glucanase activity significantly in infected resistant plants (Fig 4.6A). After 48 h enzyme activity declined, although *A. africanus* treatment tended to exert its effect slightly longer, up to 72 h, before  $\beta$ -1,3-glucanase activity decreased (Fig 4.6B).



**Figure 4.6:** Effect of **A:** *T. violacea*; **B:** *A. africanus*; **C:** *ComCat*<sup>®</sup> (CC) and **D:** SS on *in vitro*  $\beta$ -1,3-glucanase activity in **resistant** uninfected and infected wheat (Thatcher / *Lr15*). Data presented are means  $\pm$  SD of three replicates. R = uninfected resistant; IR = infected resistant. LSD-values indicated in the graphs are for interactions between non-infected and infected wheat at different time intervals over a 144 h time period.

#### 4.4.2 Chitinase activity

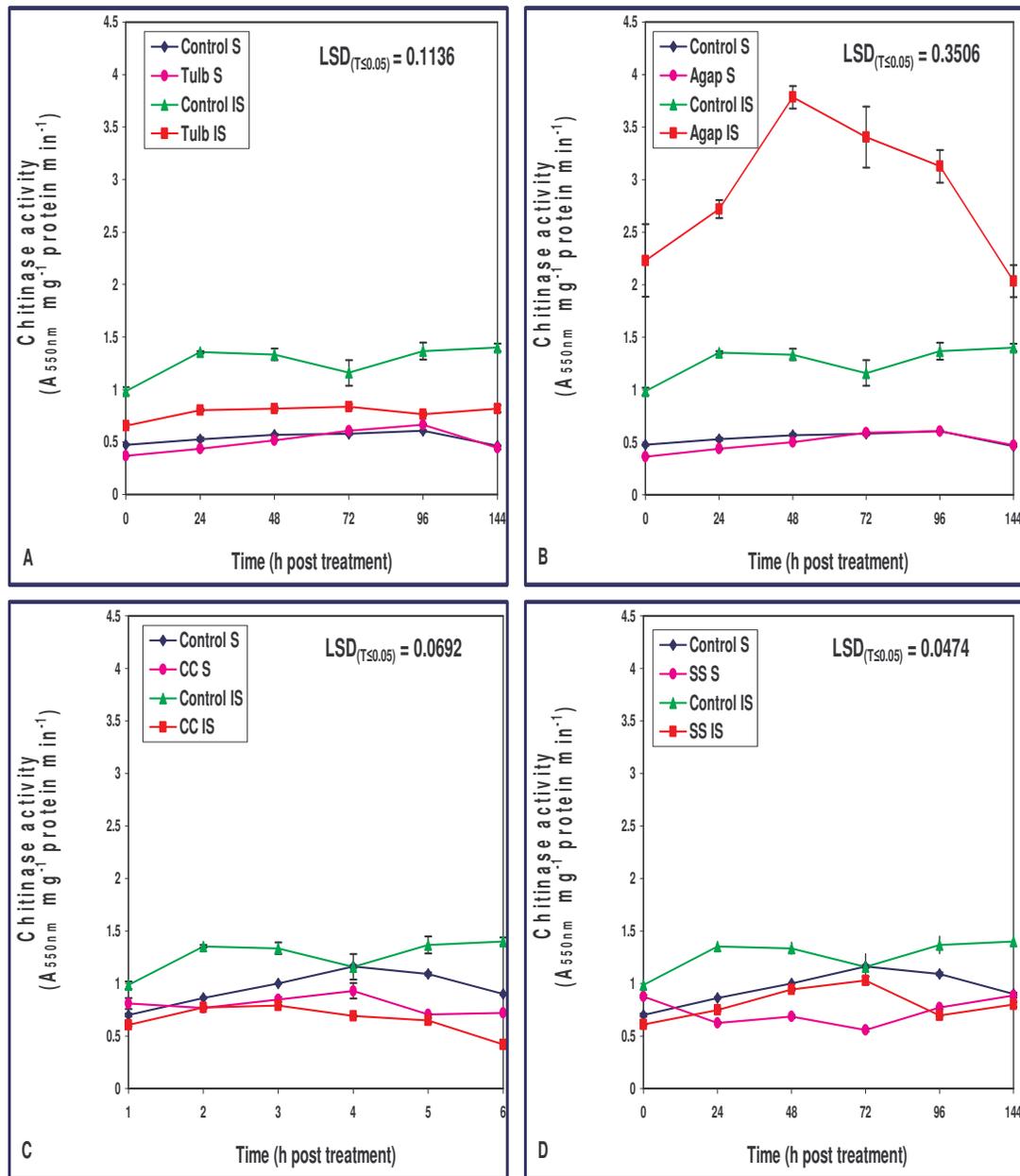
Changes in chitinase activity were measured over time in infected and uninfected susceptible (Fig 4.7) and resistant (Fig 4.8) wheat cultivars, expressing different levels of resistance to leaf rust.

##### 4.4.2.1 Susceptible wheat

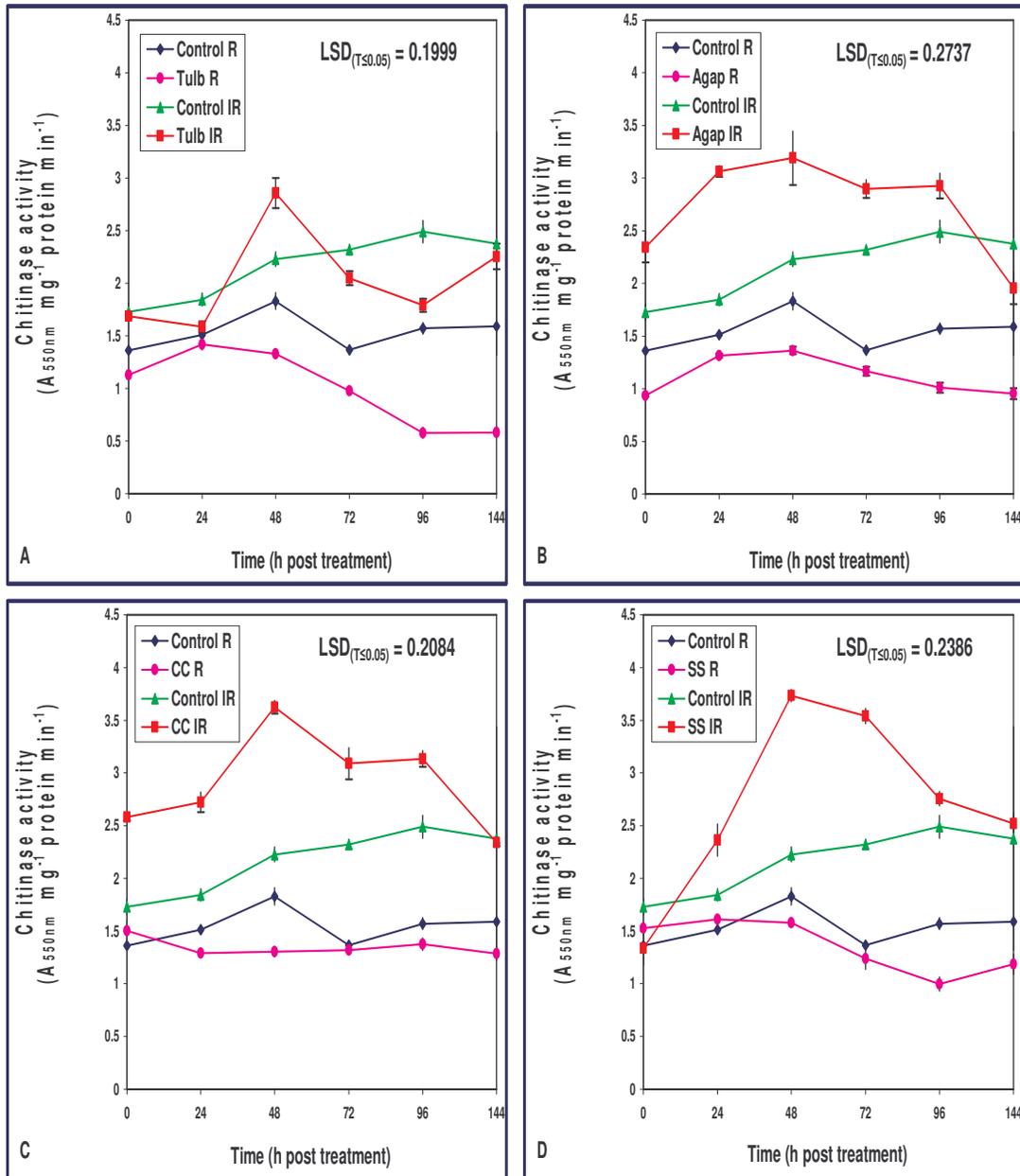
Throughout the trial, infection induced chitinase activity in the susceptible control plants (Fig 4.7). Enzyme activity was, however, inhibited by *T. violacea* (Fig 4.7 A), *ComCat*<sup>®</sup> (Fig 4.7C) and SS (Fig 4.7D) in susceptible uninfected and infected plants. In contrast, compared to the control plants, *A. africanus* was the only treatment that induced chitinase activity in susceptible infected plants, by a staggering 185% at peak activity, 48 h after inoculation (Fig 4.7B). Activity stayed high, but declined 96 h post treatment.

##### 4.4.2.2 Resistant wheat

Infection also induced chitinase activity in resistant plants (Fig 4.8). All the plant treatments inhibited enzyme activity in the uninfected resistant wheat (Fig 4.8). In contrast when the infected plants were treated with *ComCat*<sup>®</sup> (Fig 4.8C), SS (Fig 4.8D) and *A. africanus* (Fig 4.8B), where chitinase levels increased significantly within 48 h post treatment and to a much greater extent than in the uninfected plants. Except for a short burst at 48 h, treatment with *T. violacea* did not induce chitinase activity sustainably over the trial period (Fig 4.8A).



**Figure 4.7:** Effect of **A:** *T. violacea*; **B:** *A. africanus*; **C:** *ComCat*® (CC) and **D:** SS on *in vitro* chitinase activity in **susceptible** uninfected and infected wheat (Thatcher). Data presented are means  $\pm$  SD of three replicates. S = uninfected susceptible; IS = infected susceptible. LSD-values indicated in the graphs are for interactions between uninfected and infected wheat over a 144 h time interval.



**Figure 4.8:** Effect of **A:** *T. violacea*; **B:** *A. africanus*; **C:** *ComCat*® (CC) and **D:** SS on *in vitro* chitinase activity in **resistant** uninfected and infected wheat (Thatcher / *Lr15*). Data presented are means ±SD of three replicates. R = uninfected resistant; IR = infected resistant. LSD – values indicated in the graphs are for interactions between uninfected and infected wheat over a 144 h time interval.

### 4.4.3 Peroxidase activity

The peroxidase activities in response to leaf rust infection and treatment with *ComCat*<sup>®</sup>, SS, *T. violacea* and *A. africanus* extract in infected and uninfected susceptible and resistant wheat cultivars, over time, are shown in Figures 4.9 and 4.10.

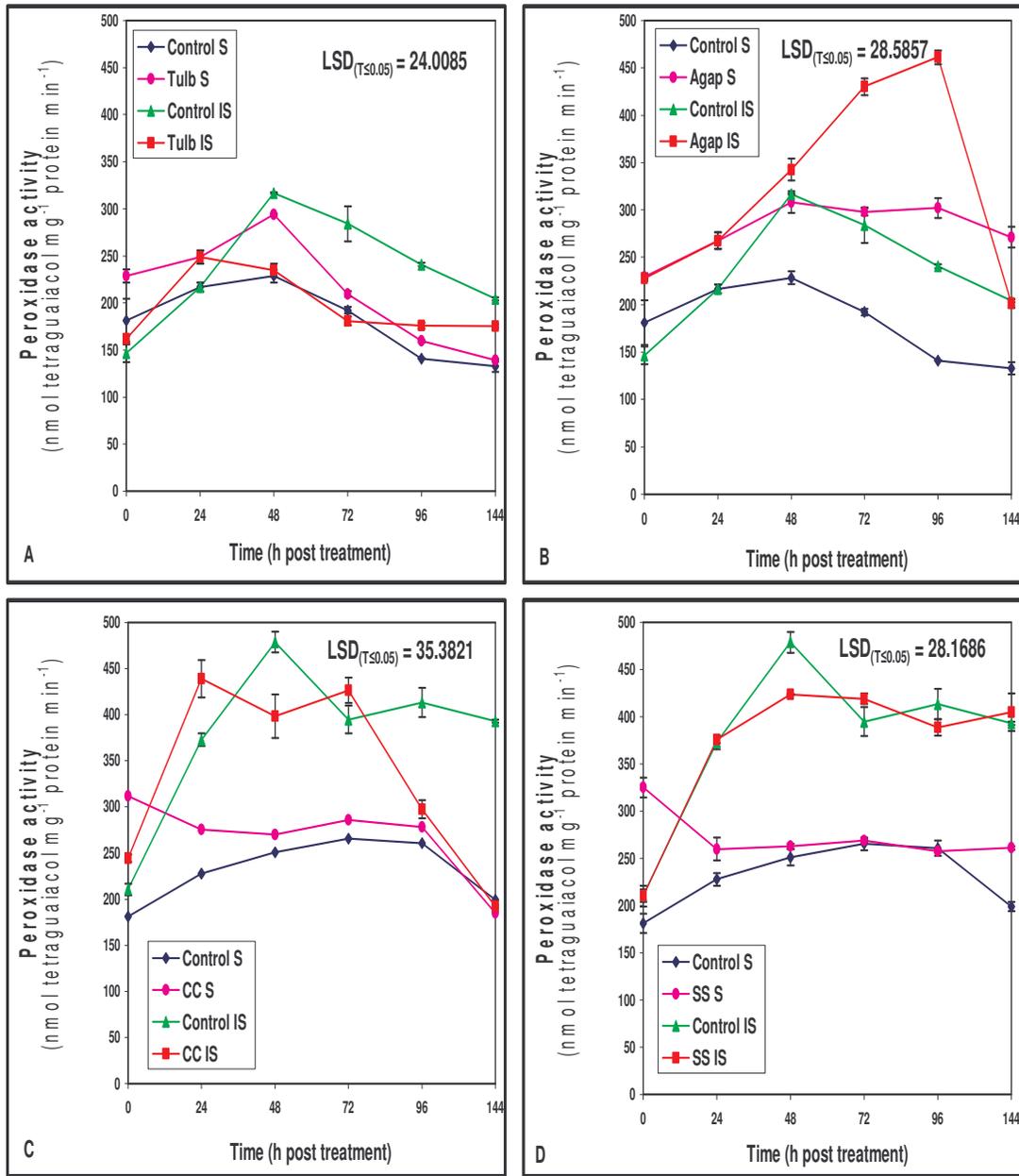
#### 4.4.3.1 Susceptible wheat

Infection of susceptible wheat with rust resulted in an induction in peroxidase activity (Fig 4.9). Treatment of uninfected susceptible wheat with *T. violacea* (Fig 4.9A), *A. africanus* (Fig 4.9B) and *ComCat*<sup>®</sup> extracts (Fig 4.9C) induced peroxidase activity significantly. No significant induction occurred during the trial period after treatment with SS extract (Fig 4.9D). The percentage increase caused by *A. africanus* was much higher than that by the other three extracts while enzyme activity also remained high over the 144 h investigation period (Fig 4.9B).

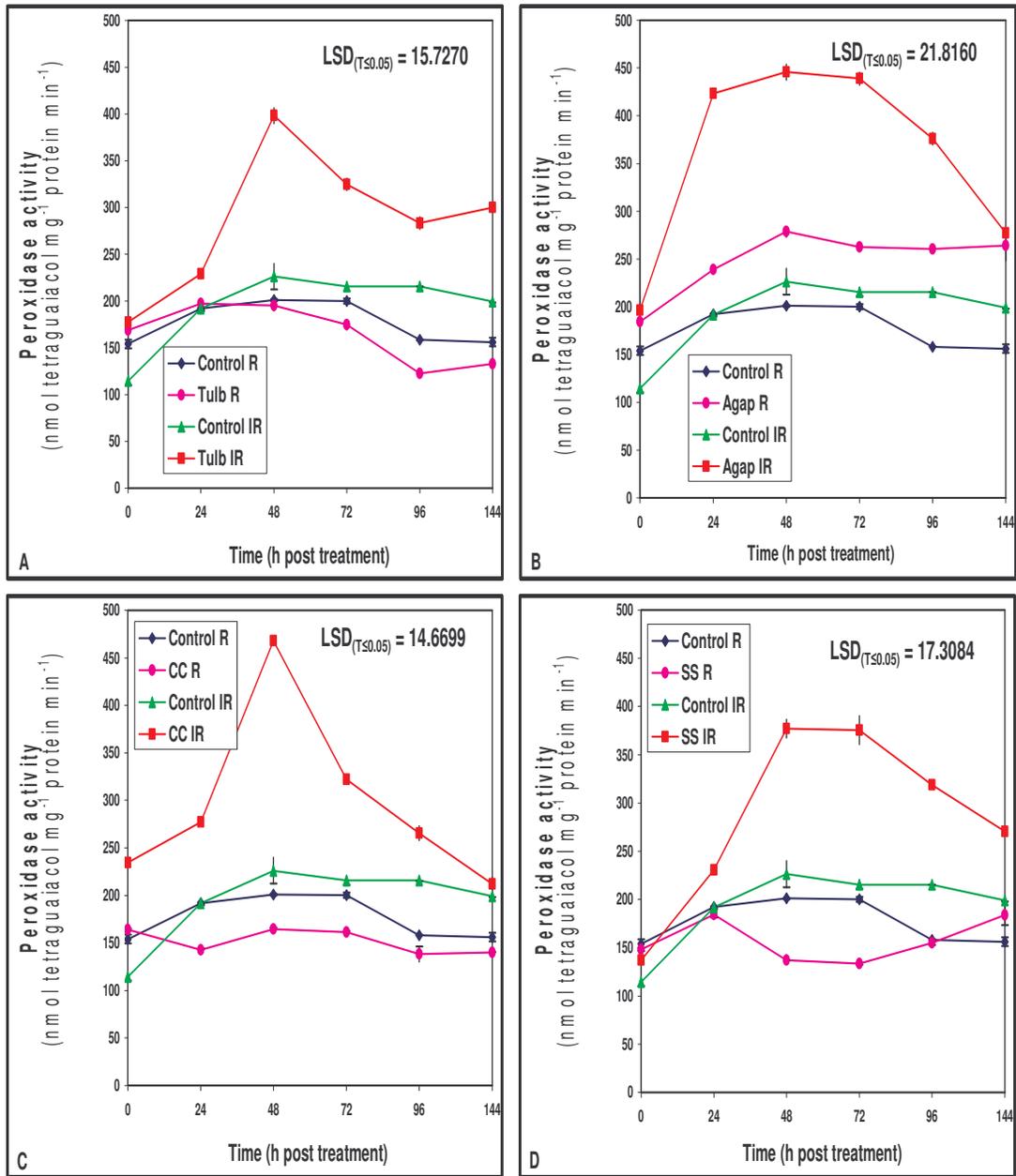
The infected plants treated with *A. africanus* responded with a rapid increase in peroxidase activity from 24 h to 96 h post treatment (Fig 4.9B). *ComCat*<sup>®</sup> (Fig 4.9C) and SS (Fig 4.9D) treatment had no significant effect on the induction of peroxidase activity in infected susceptible wheat, while *T. violacea* treatment (Fig 4.9A) tended to inhibit peroxidase activity.

#### 4.4.3.2 Resistant wheat

Peroxidase activity remained relatively constant during the trial period in uninfected control plants (Fig 4.10). As in the case of susceptible plants, infection induced enzyme activity. Of the four plant extracts, only treatment with *A. africanus* (Fig 4.10B) resulted in an increase in peroxidase activity in uninfected plants while the others had an inhibitory effect (Fig 4.10 A, C & D). All of the plant extracts induced the peroxidase activities in the infected resistant plants (Fig 4.10 A, B, C & D). Peak activities were reached after 48 h in all four cases, whereafter enzyme activities decreased, but still remained higher than that of the control during the 144 h investigation period (Fig 4.10). This suggests that sustained higher levels of peroxidase activity were maintained as a result of the treatments.

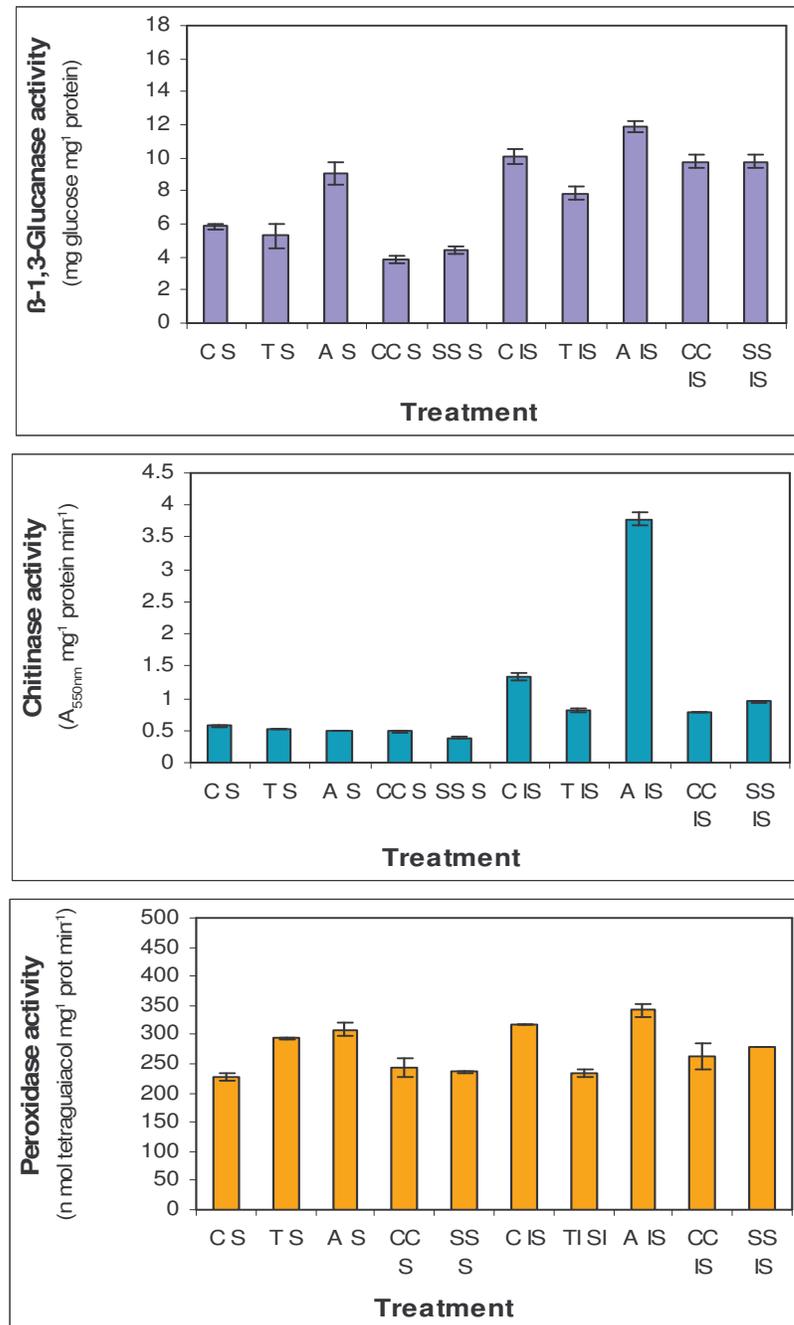


**Figure 4.9:** Effect of **A:** *T. violacea*; **B:** *A. africanus*; **C:** *ComCat*® (CC) and **D:** SS on *in vitro* peroxidase activity in **susceptible** uninfected and infected wheat (Thatcher). Data presented are means  $\pm$  SD of three replicates. S = uninfected susceptible; IS = infected susceptible. LSD-values indicated in the graphs are for interactions between uninfected and infected wheat over a 144 h time interval.

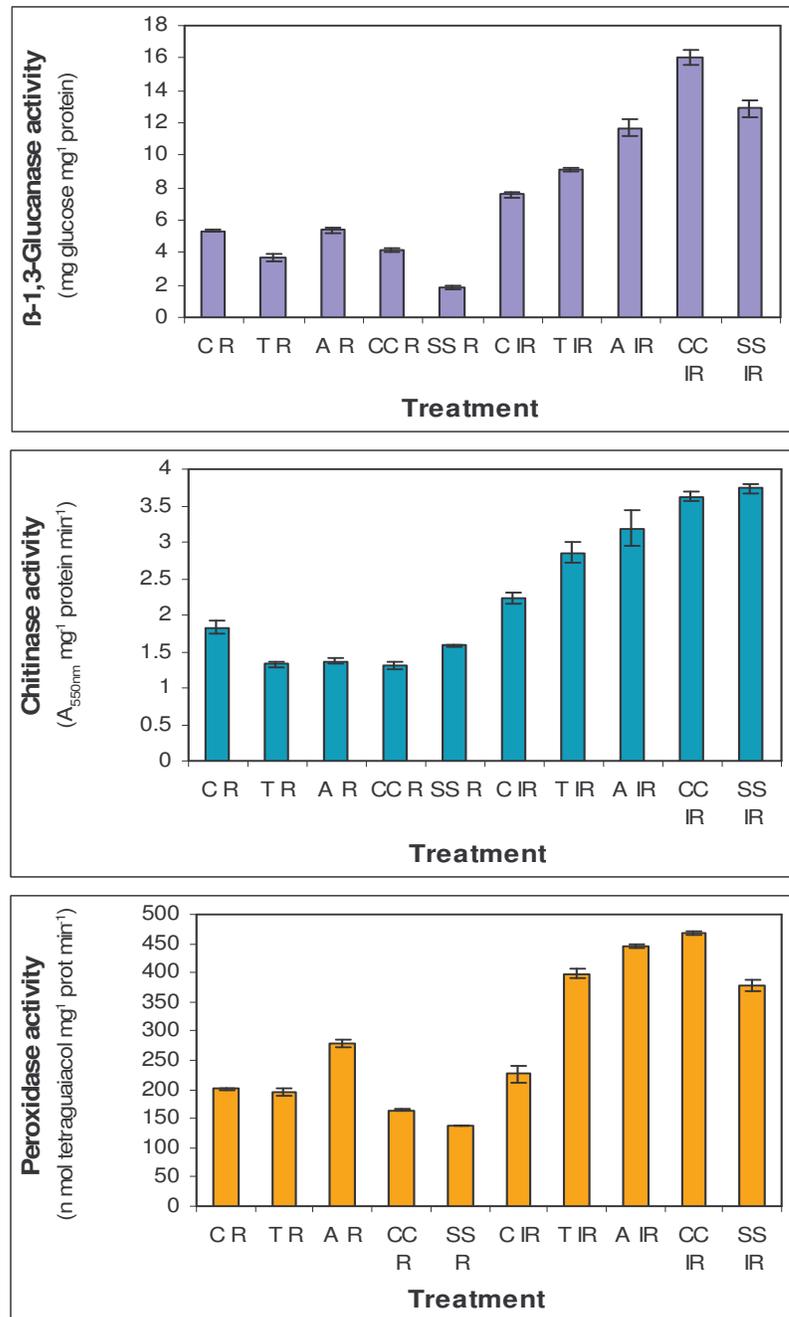


**Figure 4.10:** Effect of **A:** *T. violacea*; **B:** *A. africanus*; **C:** *ComCat*<sup>®</sup> (CC) and **D:** SS on *in vitro* peroxidase activity in **resistant** uninfected and infected wheat (Thatcher / *Lr15*). Data presented are means  $\pm$  SD of three replicates. R = uninfected resistant; IR = infected resistant. LSD-values indicated in the graphs are for interactions between uninfected and infected wheat over a 144 h time interval.

From the previous results it was persistently observed that the activities of all three enzymes studied increased most significantly ( $P < 0.05$ ) at 48 h after either infection, treatment with the extracts or both. To stress this point, comparable results between the enzyme activities are illustrated in Figures 4.11 and 4.12.



**Figure 4.11:** Enzyme activities at 48 h after treatment with *ComCat*<sup>®</sup> (CC), SS, *T. violacea* (T) and *A. africanus* (A) in uninfected (S) and infected (IS) **susceptible** (Thatcher) wheat. Data presented are means  $\pm$  SD of three replicates.



**Figure 4.12:** Enzyme activities at 48 h after treatment with *ComCat*<sup>®</sup> (CC), SS, *T. violacea* (T) and *A. africanus* (A) in uninfected (R) and infected (RI) **resistant** (Thatcher / *Lr* 15) wheat. Data presented are means  $\pm$  SD of three replicates.

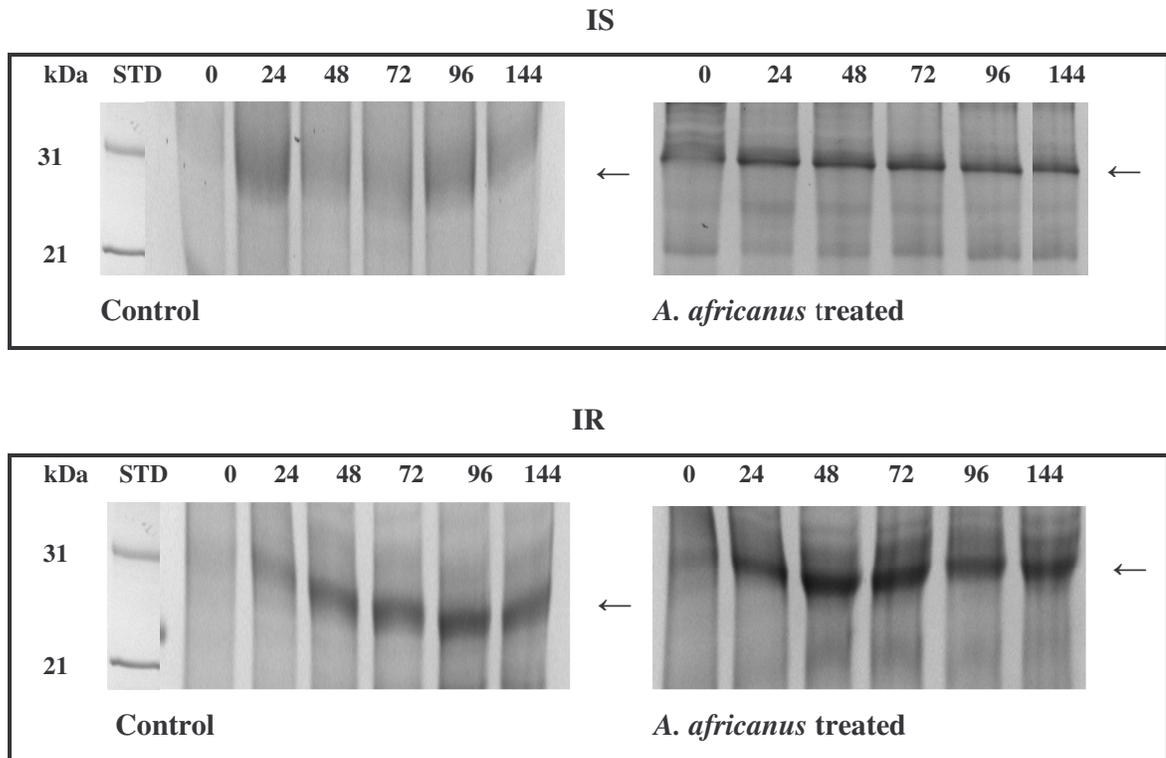
In summary, significant differences between the susceptible and resistant uninfected and infected cultivars as well as the different treatments (*T. violacea*, *A. africanus*, *ComCat*<sup>®</sup> and SS) were observed in both time and response with regard to enzyme activities. Of the four

plant extracts tested on wheat, *A. africanus* had the most significant effect on the induction of the *in vitro* activities of all three test enzymes. Therefore, it was decided to focus on the action of *A. africanus* on protein and gene expression levels in further experiments in order to establish whether the enzyme activity increases was a result of the activation of existing, or the synthesis of new enzyme protein.

#### 4.5 PR-protein expression

Since the  $\beta$ -1,3-glucanase activity was induced by *A. africanus* in both susceptible and resistant wheat cultivars, an analysis of the induction pattern of the  $\beta$ -1,3-glucanase protein by means of SDS-PAGE gel electrophoresis and Western blotting was executed. Chitinase and peroxidase activities were also induced by *A. africanus*, but it was a problem finding polyclonal antibodies from wheat, therefore only  $\beta$ -1,3-glucanase was investigated.

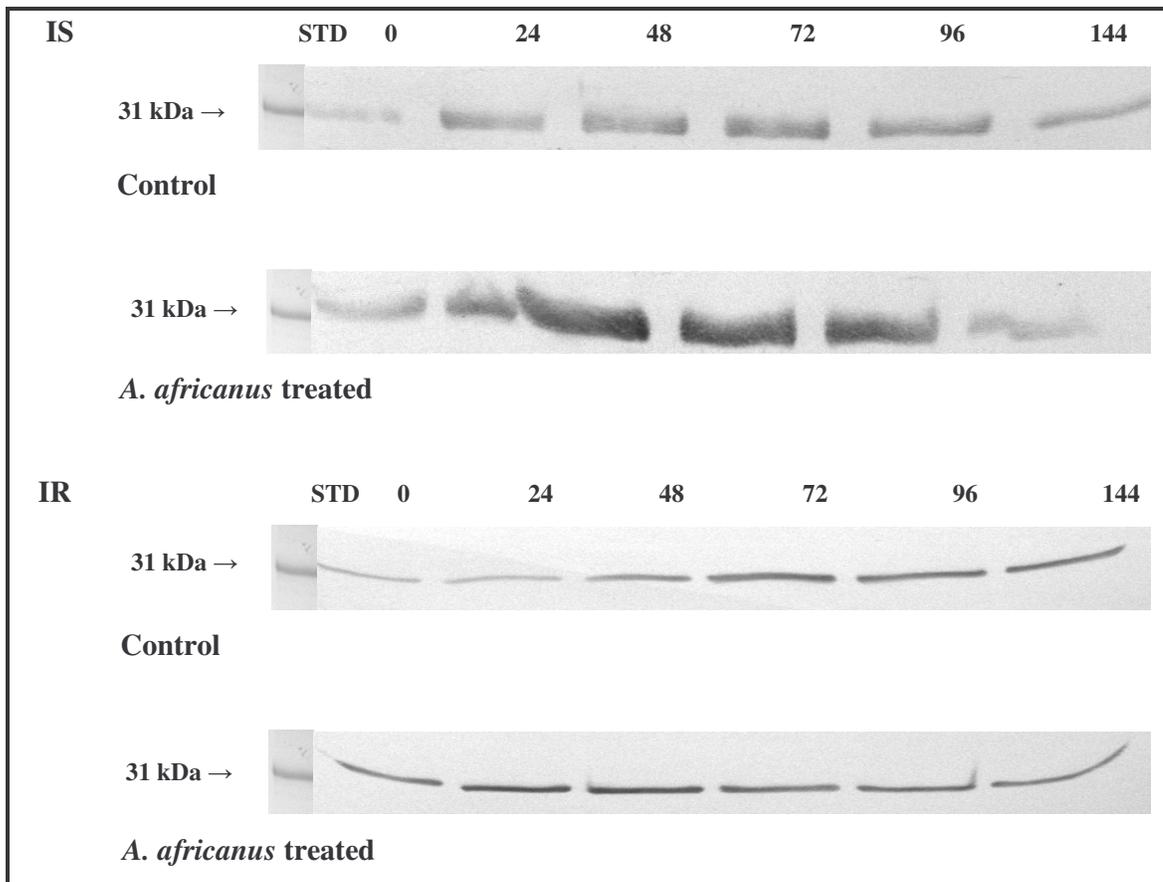
Extracellular proteins from infected susceptible and resistant wheat treated with *A. africanus* as well as a control treated with distilled water were separated on SDS-PAGE gels. The electrophoretic analysis revealed an accumulation of polypeptides in both susceptible and resistant plants treated with *A. africanus* (Plate 4.3). The  $M_r$  of the induced polypeptides was 31 kD range (Plate 4.3). These proteins did not accumulate in the infected susceptible control plants (Plate 4.3). Although these proteins were present and strongly accumulated after 48 h of infection in the resistant plants their levels were obviously much higher in the *A. africanus* treated resistant plants (Plate 4.3).



**Plate 4.3:** SDS-PAGE of apoplastic fluids from infected susceptible (IS) wheat (Thatcher) and infected resistant (IR) wheat (Thatcher / *Lr15*), over a 144 h time interval. Each lane was loaded with 30  $\mu$ g protein.

According to Western blots of the IWF of uninfected and infected susceptible and resistant leaves,  $\beta$ -1,3-glucanase antibodies cross-reacted with a polypeptide with molecular mass of ca. 31 kDa (Plate 4.4).

In accordance with visual observation, it is clear that  $\beta$ -1,3-glucanase was induced to higher levels in the susceptible and resistant plants treated with *A. africanus* extract (Plate 4.4). Although  $\beta$ -1,3-glucanase was induced especially after 24 h, in the infected control plants it was not as pronounced as in the *A. africanus* treated plants where induction already started at 0 h in the resistant and susceptible plants and increased to the highest levels at 48 h (Plate 4.4). Western blot profiles confirmed the polypeptide profiles on the SDS-PAGE gels.



**Plate 4.4:** Western blot analysis of intercellular proteins from infected susceptible (IS) (Thatcher) and infected resistant (IR) wheat (Thatcher / *Lr15*) collected at different times (h) after treatment with *A. africanus* extract. Blots were probed with antiserum against wheat  $\beta$ -1,3-glucanase (31 kDa). Each lane was loaded with 30  $\mu$ g protein.

#### 4.6 Expression of *PR* genes

The fact that the *A. africanus* extract was prominently involved in the induction of PR proteins (as previously confirmed, section 4.5), justified a study on the effect of this extract on PR gene expression. To characterize the response of infected susceptible and infected resistant wheat to treatment with the *A. africanus* extract, the expression of three PR genes, namely *PR2*, *PR3* and *PR9* were monitored using RT-PCR. In order to confirm that equal

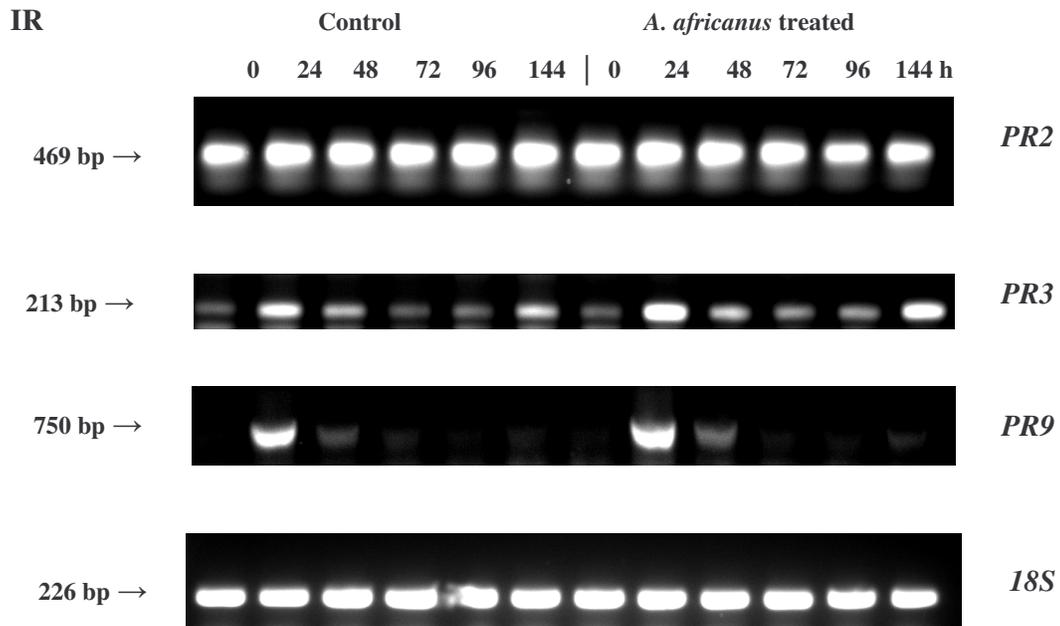
quantities RNA was used in all the RT-PCR reactions, the expression of the *18S rRNA* gene was determined. The constitutive expression of the gene in all the samples confirmed that equal quantities RNA were used for all RT-PCR reactions (Plate 4.5 & 4.6).

The expression of the different *PR* genes is presented in Plate 4.5 & 4.6. *PR2* ( $\beta$ -1,3-glucanase) was constitutively expressed in the control and *A. africanus* treated resistant plants. In the treated plants, expression of *PR2* declined after 72 h (Plate 4.5). In the susceptible control plants an induction occurred between 24 and 72 h (Plate 4.5). The expression of the *PR2* gene also showed a more prominent induced pattern in the infected susceptible plants treated with *A. africanus* extract, than in the infected resistant wheat treated with *A. africanus* extract (Plate 4.6), with highest expression between 24 and 96 h.

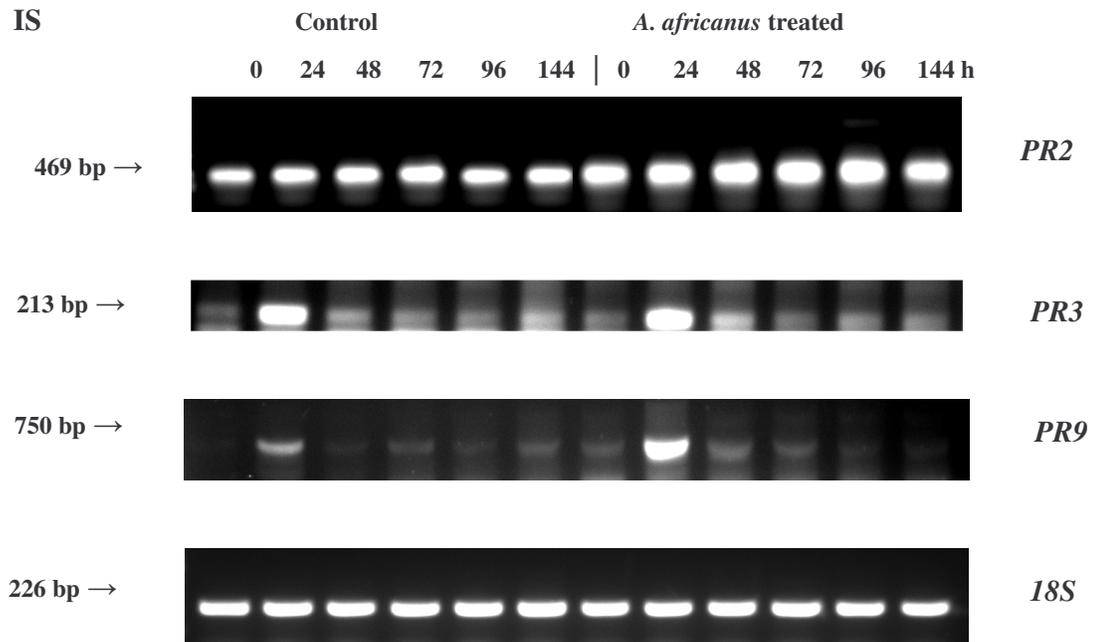
*PR3* (chitinase) and *PR9* (peroxidase) gene expression was induced in the resistant and susceptible control plants by infection with *P. triticina* after 24 h (Plate 4.5 & 4.6). It is evident that *A. africanus* treatment caused a stronger induction of gene expression in the infected plants compared to the controls. Expression of both genes declined after 48 h, but a second induction in *PR3* expression occurred at 144 h in the infected resistant plants. Again, *A. africanus* was responsible for a stronger activation (Plate 4.5).

Treatment with *A. africanus* therefore led to the induced expression of both *PR3* and *PR9* genes, where the induced expression was visually more evident in the susceptible plants than in the resistant cultivar. Induction of both genes declined after 48 h (Plate 4.6).

To summarize, the expression of all the *PR* genes tested for was substantially induced in susceptible and resistant wheat after treatment with the *A. africanus* extract (Plate 4.5 & 4.6).

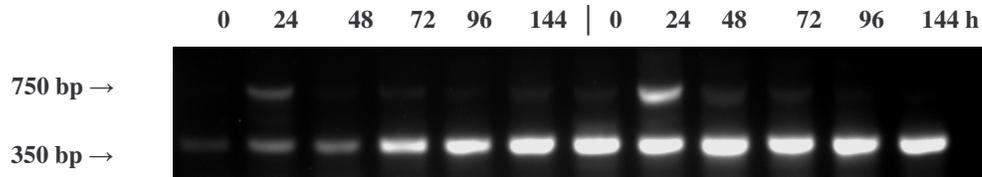


**Plate 4.5:** Expression analysis of *PR2*, *PR3*, *PR9* and *18S rRNA* in *P. triticina* infected resistant wheat (Thatcher / *Lr15*) treated with water and *A. africanus* extract, respectively. Time intervals and sizes of the amplified gene fragments are indicated.



**Plate 4.6:** Expression analysis of *PR2*, *PR3*, *PR9* and *18S rRNA* in *P. triticina* infected susceptible wheat (Thatcher) treated with water and *A. africanus* extract, respectively. Time intervals and sizes of the amplified gene fragments are indicated.

During RT-PCR of infected susceptible and resistant wheat using *PR9* specific primers, two different sized fragments were repeatedly amplified. The expression of both fragments were induced by infection (Plate 4.7). The larger fragment was approximately 750 bp in size and the smaller one approximately 350 bp. While both fragments were amplified, the smaller was amplified to much higher levels. To confirm their identity the two fragments were purified and sequenced.



**Plate 4.7:** RT-PCR amplification using *PR9* specific primers.

The larger of the two fragments had a nucleotide sequence of 750 bp. When sequenced, the 304 bp shared highest similarity with a peroxidase 5 from *Triticum monococcum* (Genbank Accession nr. AY857759.1;  $2e^{-88}$ ) (Fig 4.13).

The smaller of the two fragments was sequenced and showed 97% similarity to *Oryza sativa* clone Oss-289-384-H3 retrotransposon protein (Genbank Accession nr. EF576506.1;  $7e^{-97}$ ) (Fig 4.14).

**a**

5' -CTTGGAGGGCTTCGTGGACGGTCTCTTGGNAGAAGAGGACTTCACANATGCAAACGAGGCGGNGGCAAACA  
 NCGACCTACCACGCCATTCTTCTNCCTCTTTGACCTCGNNCAGGCCTTCNGCNACAANGGCTTCACCNNGACCG  
 ACATGGTGGCCCTCTCGGGCGCCACACCATCGGGCAGGCGCAGTGCNNGANCTTCAGGACAGANTCTATCAAC  
 GAGACCTAACATCAACTCCGGCTTCGCNACNTCGCTCAAGGCCAACTGCCCCNGCCNANCGGCTCGGCGACCGN  
 GACCTGG-3'

**b**

Top	1	CTT-GGAGGG-CTTCGTGGACGG-TCCTCTTGGNAGAAGAGGACTTCA-CANATGCAAAC	56
fragment			
<i>T. monococcum</i>	217	CTTAGGAGGGCCTTCGTGGACGGTCTCTTGGAAAGGAG-GGACTCCACCA-ATGCAAAC	274
<i>PR5</i>			
Top	57	GAGGCGGNGGCAAACANC-GACCTACCACGCCATTCTTCT-NCCTCTTTGACCTCGNNC	114
fragment			
<i>T. monococcum</i>	275	GAAGCCGTGGCGAAC-TCTGACCTACCTCCCCATTCTT-TGACCTCGTTAACCTCACCC	332
<i>PR5</i>			
Top	115	AGGCCTTCNGCNACAANGGCTTCACCNNGACCGACATGGTGGCCCTCTCGGGCGCCACACA	174
fragment			
<i>T. monococcum</i>	333	AATCCTTCGGCGACAAAGGCTTCACCGTGACCGACATGGTCGCCCTCTCGGGCGCCACACA	392
<i>PR5</i>			
Top	175	CCATCGGGCAGGCGCAGTGCNNGANCTTCAGGGACAGANTCTATCAACGAGACCTAACAT	234
fragment			
<i>T. monococcum</i>	393	CCATCGGGCAGGCGCAGTGCCTGAACTTCAGGGACAGACTCTA-CAACGAGACC-AACAT	450
<i>PR5</i>			
Top	235	CAACTCCGGCTTCGCNACNTCGCTCAAGGCCAACTGCCCCNGCCNANCGGCTC-GGCGA	293
Fragment			
<i>T. monococcum</i>	451	CAACTCCGGCTTCGCGACGTCGCTCAAGGCCAACTGCCCCAGCCGACCGGCTCCGGCGA	510
<i>PR5</i>			
Top	294	CCGNGACCTGG	304
fragment			
<i>T. monococcum</i>	511	CCGCAACCTGG	521
<i>PR5</i>			

**Figure 4.13:** Sequence analysis of the larger fragment amplified using *PR9* specific primer. Indicated are (a) the nucleotide sequence of the amplified cDNA sequence and (b) the nucleotide alignment with *PR5* (peroxidase) from *T. monococcum*. N = A, C, G or T.

**a**

5' -CGCCTTTGGCTATCTTAGGCCCTACTACCCTCGTGGACGAACCTTGC GGAGGAAACCTTGGGTTTTCGGGG  
 ATTGGATTCTACCAATGTTTTCGTTACTCAAGCCGACATTCTCGCTTCCGCTTCGTCGACCCCGCTTTCGCGG  
 TTGCTTCCTCTAAGGCGGAACGCTCCCTACCGATGCATTTTGACATCCCACAGCTTCGGCAGATCAGGCT-3'

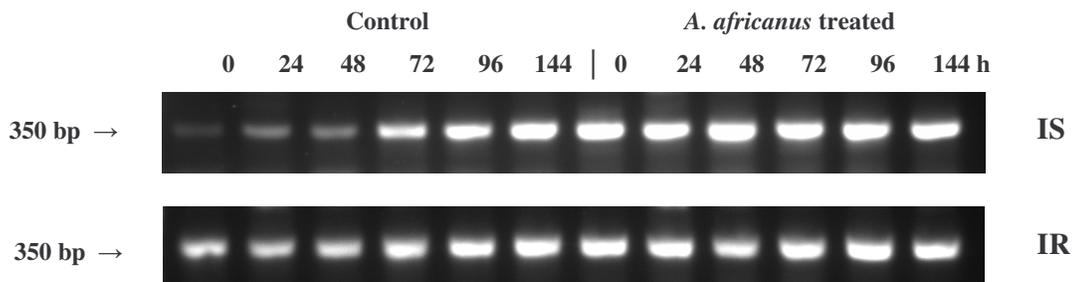
**b**

Lower fragment	1	CGCCTTT-GG-CT-ATCTTAGGCCCT-ACT-ACCCT-CGTGGACGAACCTTGC GGAGGAA	54
Retro-transposon	736	CGCCTTTCGGCCTGATCTTAGGCCCTGACTCACCTCCGTGGACGAACCTTGC GGAGGAA	677
Lower fragment	55	ACCTTGGGTTTTCGGGGCATTGGATTCTACCAATGTTTTCGTTACTCAAGCCGACATTC	114
Retro-transposon	676	ACCTTGGGTTTTCGGGGCATTGGATTCTACCAATGTTTTCGTTACTCAAGCCGACATTC	617
Lower fragment	15	TCGCTTCCGCTTCGTCGACCCCGCTTTCGCGGTTGCTTCC-TCTAAGGCGGAACGCTCC	173
Retro-transposon	616	TCGCTTCCGCTTCGTCGACCCCGCTTTCGCGGTTGCTTCCCTCTAAGGCGGAACGCTCC	557
Lower fragment	174	CCTACCGATGCATTTTGACATCCCACAGCTTCGGCAGATC	213
Retro-transposon	556	CCTACCGATGCATTTTGACATCCCACAGCTTCGGCAGATC	517

**Figure 4.14:** Sequence analysis of the lower fragment amplified using *PR9* specific primer. Indicated are (a) the nucleotide sequence of the amplified cDNA sequence and (b) the nucleotide alignment with the lower fragment.

The retrotransposon gene was differentially expressed in infected susceptible and resistant control plants (Plate 4.8). In the susceptible and resistant control plants the expression of the retrotransposon gene increased after 48 h and stayed at the same high elevated levels for the duration of the experiment. However, expression in the resistant plants was initially higher than in the susceptible plants.

If it is considered that 0 h sampling time is 48 h after treatment with the *A. africanus* extract, it is possible, based on the controls, that an induction of expression of the retrotransposon gene fragment also occurred in the treated plants, but due to the missing 48 h the induction is not evident.

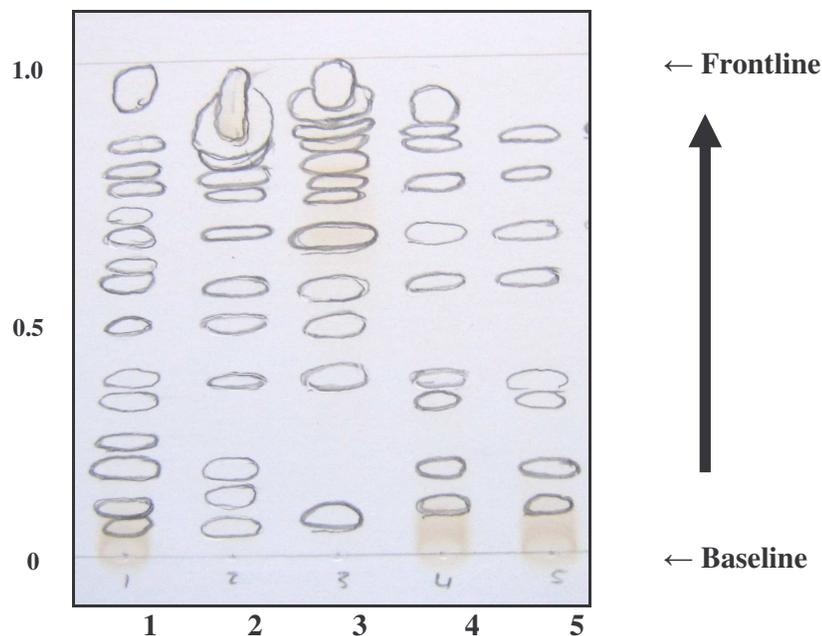


**Plate 4.8:** Expression analysis of a putative retrotransposon amplified cDNA in *P. triticina* infected wheat and in wheat treated with *A. africanus* extract 48 h prior to infection, over a 144 h period. Control plants were treated with distilled water. IS indicates the expression in infected susceptible (Thatcher) and IR in infected resistant (Thatcher / *Lr15*) wheat.

**4.7 Isolation, purification and identification of active substances from *A. africanus* that induce resistance in wheat against leaf rust.**

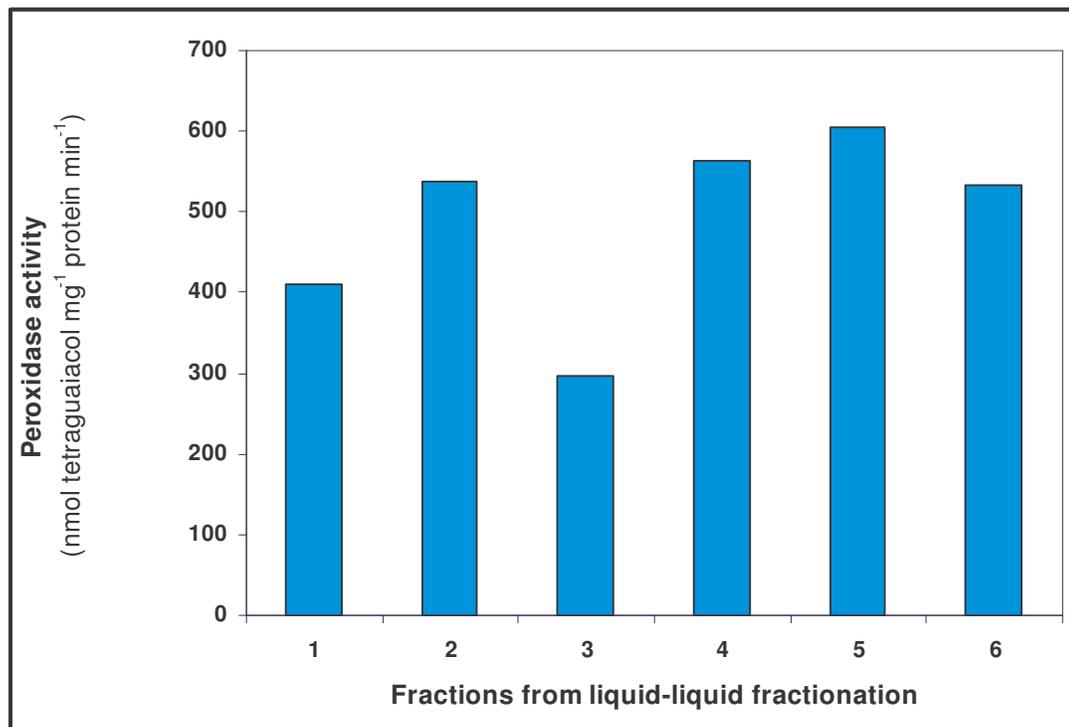
**4.7.1 A qualitative TLC profile of the liquid-liquid extraction obtained from *A. africanus* extract and the subsequent peroxidase enzyme activity of those fractions.**

Four semi-purified extracts were obtained from the crude methanol extract of *A. africanus* using organic solvents of increasing polarity. A Q-TLC profile (Plate 4.9) illustrated some similarities but also distinct differences on grounds of R<sub>f</sub>-values of compounds. Hexane (lane 2) and diethyl ether (lane 3) showed some similarities, while ethyl acetate (lane 4) and dichloromethane (lane 5) exhibited more similar patterns.



**Plate 4.9:** A qualitative TLC profile of compounds contained in an extract of *A. africanus* fractionated by means of the liquid-liquid extraction procedure of Gamoh *et al.* (1989). (1 = Methanol; 2 = Hexane; 3 = Diethyl ether; 4 = Ethyl acetate; 5 = Dichloromethane).

Peroxidase activity in wheat treated with the four semi purified extracts served as a quantitative measure of the defence reaction. Comparatively, the bio-assay revealed highest activity in the ethyl acetate extract, although higher activity was also detected in the diethyl ether and dichloromethane extracts (Fig 4.15).

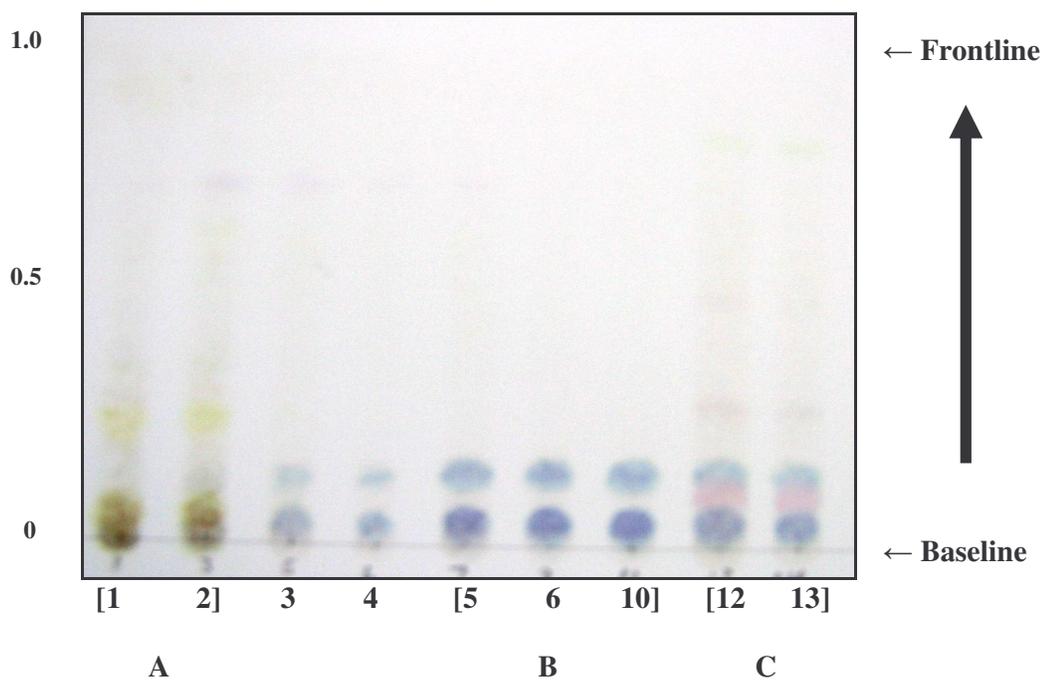


**Figure 4.15:** Intercellular peroxidase activity in wheat treated with different liquid-liquid extraction fractions of *A. africanus* (as indicated in Q-TLC, Fig 22) after 48 h. **1** = Control + DMSO; **2** = Methanol extract; **3** = Hexane; **4** = Diethyl Ether; **5** = Ethyl Acetate; **6** = Dichloromethane.

#### 4.7.2 A qualitative TLC profile of column chromatography fractions obtained from the most active ethyl acetate liquid-liquid extract and the subsequent peroxidase enzyme activity in wheat plants treated with these fractions.

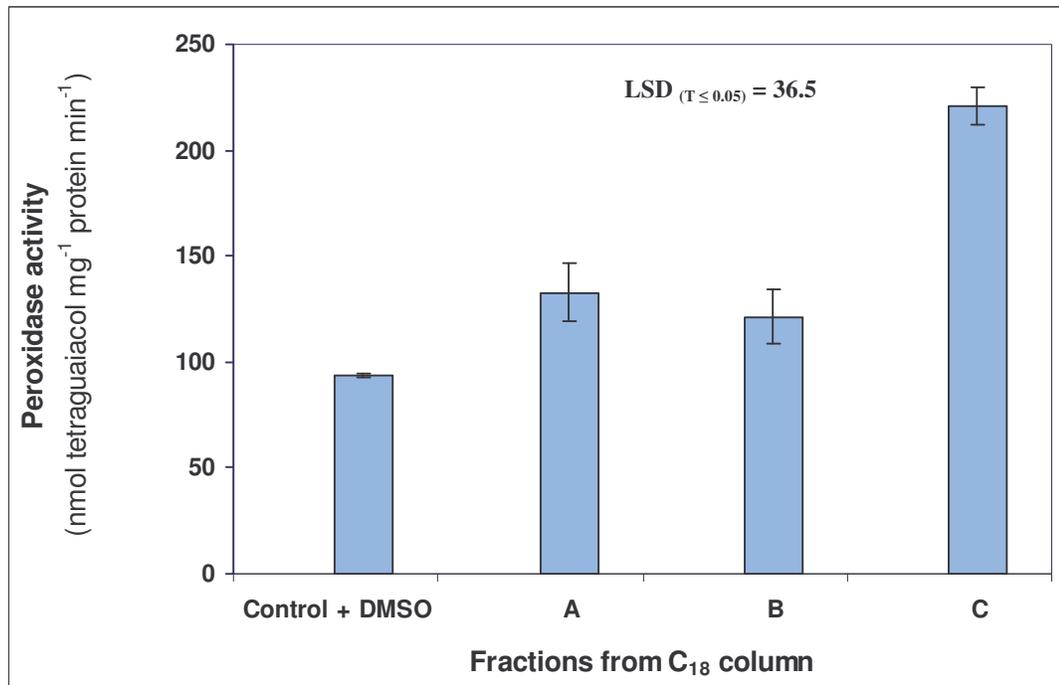
Only compounds in the active ethyl acetate fraction were purified further by means of C<sub>18</sub> reverse phase column chromatography, using 70% (v/v) methanol/water as solvent system. The Q-TLC profiles of the different fractions were determined and those with similar profiles pooled (Plate 4.10). TLC profiles of the first two fractions display yellow when sprayed with

10% ethanolic  $\text{H}_2\text{SO}_4$  and were quite different from the rest. In combined fractions 5 to 10, which eluted in the 70% methanol/water, only two prominent blue compounds were observed. After the column was eluted with 70% methanol/water, there was still some material binding to the column, therefore it was decided to wash the column with 100% methanol. A very distinct pink compound with two blue compounds above and below eluted in the 100% methanol and were detected in fractions 12 and 13 (Plate 4.10).



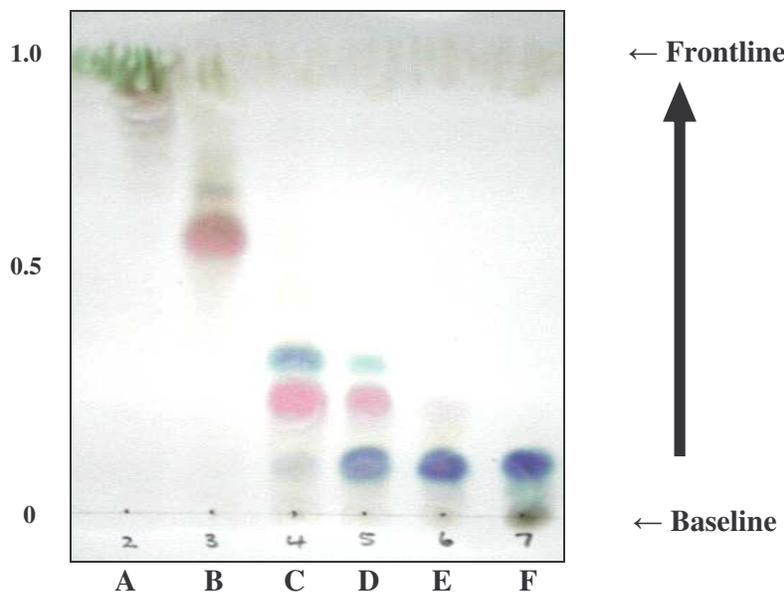
**Plate 4.10:** A qualitative TLC-profile of compounds contained in the  $\text{C}_{18}$  column chromatography fractions after fractionating the most active ethyl acetate fraction. Fractions were pooled as follows: **1 & 2 = A**; **5 -10 = B** and **12 & 13 = C**

Peroxidase activity was induced to much higher levels in wheat treated with fraction C than in any of the other fractions including the control (Fig 4.16). This indicated the presence of the active substance in fraction C.



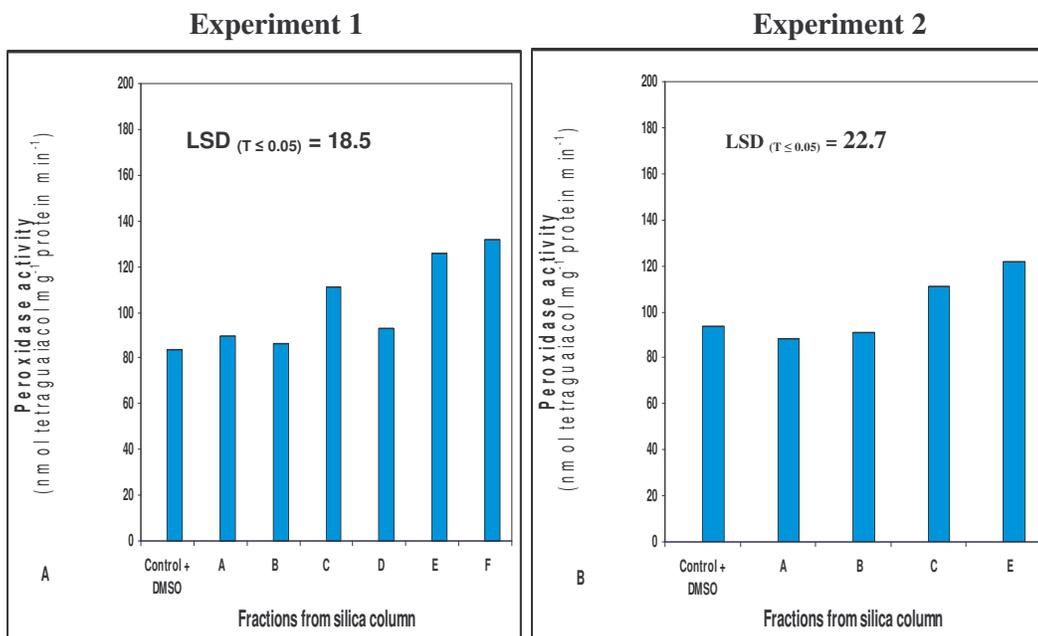
**Figure 4.16:** The effect of compounds (10 mg sprayed onto the wheat plants) in different fractions obtained from C<sub>18</sub> column chromatography (Plate 4.10), on intercellular peroxidase activity in wheat after 48h. Error bars indicate standard deviation (n = 3).

In order to further purify the active substances, compounds in fraction C were again allowed to separate on a silica gel column chromatography. A gradient solvent system was used to elute all active compounds from fraction C in a total of 60 x 0.5 ml column fractions and those with similar Q-TLC profiles were pooled in six combined fractions (Plate 4.11).



**Plate 4.11:** A qualitative TLC-profile of compounds contained in the six combined silica gel column chromatography fractions after fractionating the active fraction C obtained from C<sub>18</sub> column chromatography.

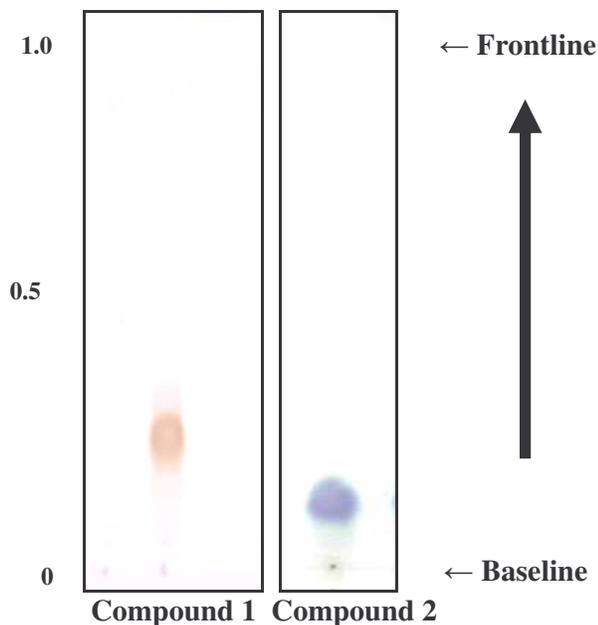
From the results obtained when peroxidase enzyme activity was measured in wheat treated with the different fractions from the silica gel column, it is evident that fractions C, E and F caused induced levels of enzyme activity after 48 h (Fig 4.17). Significant differences are indicated in Fig 4.17. The experiment was executed twice. In the first experiment 2 mg material from each fraction was sprayed onto ten plants, while in the second experiment 10 mg was used and fractions D and F were omitted from the experiment. The Q-TLC profile (Plate 4.11) strongly indicated that fraction E and F contained the same compound (blue color upon spraying with 10% ethanolic H<sub>2</sub>SO<sub>4</sub>), while the main compound in fraction C is the pink one (Plate 4.11). This indicated the presence of two possible active substances.



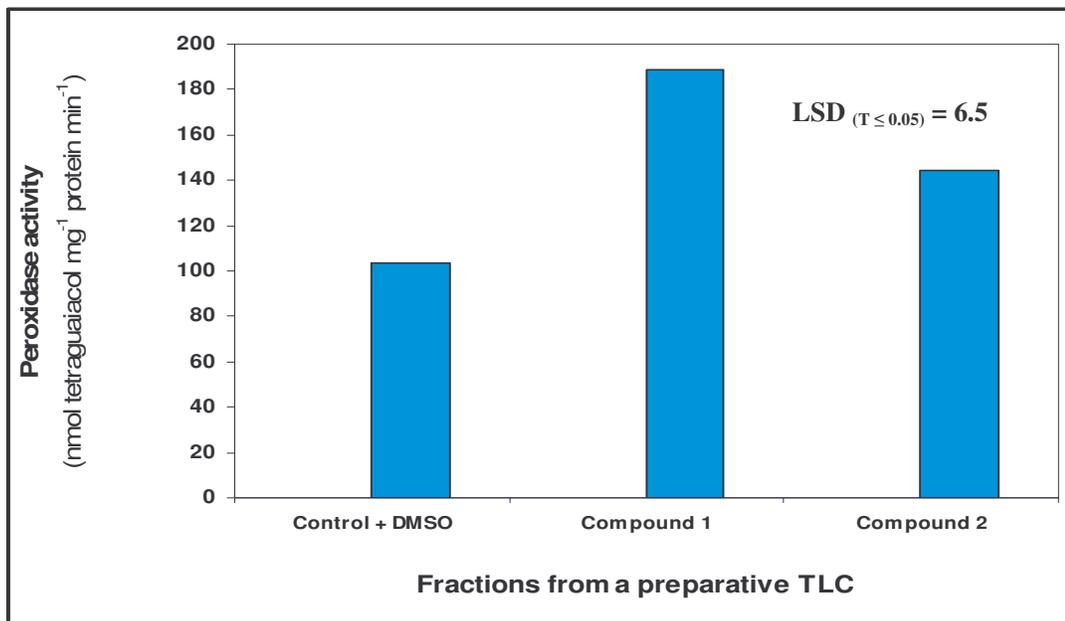
**Figure 4.17:** The effect of compounds in different fractions from silica gel column chromatography (as indicated in Plate 4.11), on intercellular peroxidase activity in wheat after 48 h. **A** = 2 mg of each fraction sprayed onto the plants, **B** = 10 mg of each fraction sprayed onto the plants

#### 4.7.3 A qualitative TLC profile of the two most active fractions purified by means of preparative TLC and the subsequent peroxidase enzyme activity in wheat plants treated with these fractions.

The two bio-active compounds were further purified using preparative thin layer chromatography and their state of purity determined by Q-TLC. Visually it appeared that both compounds were pure as only single spots were observed (Plate 4.12). The purified compounds induced peroxidase activity significantly with an increase in enzyme activity of 85% with compound 1 and 40% with compound 2 respectively when applied to wheat seedlings (Fig 4.18).



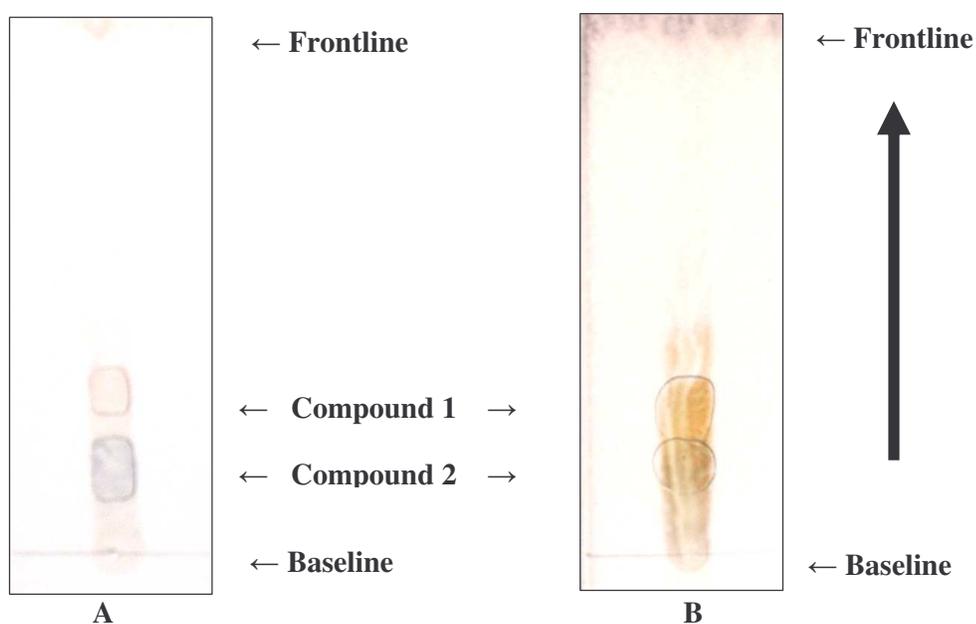
**Plate 4.12:** A qualitative TLC profile of the two compounds obtained from preparative TLC after fractionating the most active ethyl acetate extract column chromatographically.



**Figure 4.18:** The effect of two pure compounds, obtained from preparative TLC (Plate 4.12), on intercellular peroxidase activity in wheat after 48 h (2 mg of each compound was sprayed onto the plants).

#### 4.7.4 Preliminary identification of the active compounds

In order to verify the possible chemical group of the two active compounds, a fingerprinting technique (Wagner and Bladt 1996) was employed where a mixture of both compounds was spotted ( $\pm 70 \mu\text{g}$  in total) on a series of Q-TLC plates. The compounds were allowed to separate after which the plates were stained with different spray reagents. Positive results for both compounds were obtained with only two reagents, namely 15% ethanolic phosphoric acid, indicating both compounds to be of steroidal type and 1% ethanolic vanillin followed by 10% ethanolic  $\text{H}_2\text{SO}_4$ , which is indicative of essential oils (Plate 4.13).

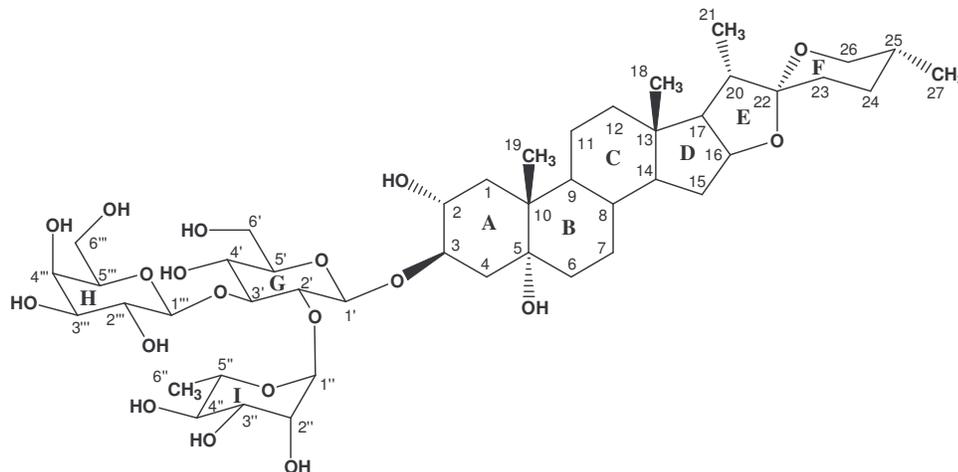


**Plate 4.13:** A qualitative TLC profile of two purified compounds, that both induced peroxidase activity in wheat, and sprayed with different reagents. **A** = 15% ethanolic phosphoric acid and **B** = 1% ethanolic vanillin /  $\text{H}_2\text{SO}_4$ .

#### 4.7.5 Identification of compounds isolated and purified from *A. africanus* that induce resistance in wheat against leaf rust, by means of Nuclear Magnetic Resonance (NMR) spectroscopy.

##### 4.7.5.1 Structural elucidation

The NMR data of compound 2 (Table 4.3) is identical to the saponin, Agapanthussaponin A, isolated from *Agapanthus inapertus* by Nakamura *et al.* (1993). Subsequently, the  $^{13}\text{C}$  NMR data (in pyridine-*d*5) obtained in this study are compared with the published data of Nakamura *et al.* (1993) in table 4.3. The  $^1\text{H}$  data of compound 2 is supplied in table 4.3. However, due to the complexity of compound 2 and overlap of  $^1\text{H}$  resonances, not all of these are distinguishable and only some of the  $^1\text{H}$  resonances and coupling constants are assigned. The  $^1\text{H}$ ,  $^{13}\text{C}$ , APT, COSY, HMQC and HMBC spectra are given in plates 4.14 to 4.20. The  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectra and data in DMSO-*d*6 of compound 2 are also included in table 4.4 and plates 4.21 and 4.22. The  $^1\text{H}$  and  $^{13}\text{C}$  NMR data (in  $\text{CDCl}_3$ ) of the peracetate are given in table 4.5.



**Figure 4.19: Compound 2: Agapanthussaponin A: (25*R*)-5 $\alpha$ -spirostane-2 $\alpha$ ,3 $\beta$ ,5 $\alpha$ -triol 3-*O*-{*O*- $\alpha$ -L-rhamnopyranosyl-(1 $\rightarrow$ 2)-*O*-[ $\beta$ -D-galactopyranosyl-(1 $\rightarrow$ 3)]-  $\beta$ -D-glucopyranoside}**

#### 4.7.5.2 Discussion of NMR of Compound 2 (Agapanthussaponin A)

Initial NMR experiments in DMSO-*d*6 indicated that the compound was a saponin with three sugar moieties. However, due to the complex structure of compound 2 its structure could not unambiguously be assigned. A consequent literature search indicated that Agapanthussaponin A had previously been assigned in pyridine-*d*6. Repetition of NMR experiments in pyridine-*d*6 gave results almost identical to the published data (Table 4.3). The NMR data for the acetylated derivative shows that all the <sup>1</sup>H resonances could be assigned.

The NMR data has the following salient features:

A total of 5 methyl signals were observed in the proton spectrum (HMQC and APT experiments confirmed this), two of which were identified as tertiary methyl groups (singlets) at  $\delta$  1.18 (s, H-19) and  $\delta$  0.87 (s, H-18). The remaining secondary methyl groups at  $\delta$  0.69 (d,  $J = 4.9$  Hz, H-27),  $\delta$  1.12 (d,  $J = 6.9$  Hz, H-21) and  $\delta$  1.72 (d,  $J = 6.1$  Hz, H-6'') were assigned to the aglycone and 6-deoxypyranose (ring I) moieties, respectively. The proton signals at  $\delta$  3.57 (dd,  $J = 10.2$  Hz, 10.2 Hz) and  $\delta$  3.49 (t,  $J = 10.2$  Hz) were identified as H-26a and H-26b.

The three anomeric protons of the glycosidic moieties were observed at  $\delta$  6.33 (s, H-1'''),  $\delta$  4.97 (d,  $J = 7.8$  Hz, H-1'') and  $\delta$  4.85 (d,  $J = 7.4$  Hz, H-1') were correlated to the respective carbons observed in the carbon spectrum C-1' at  $\delta$  100.8, C-''1 at  $\delta$  105.1 and C-1''' at  $\delta$  102.2. In the literature H-1''' and H-1'' are reported as  $\delta$  4.97 and  $\delta$  6.33 respectively. However, proton-proton correlations (COSY) were observed between the  $\delta$  1.72 (H-6'') and  $\delta$  4.97 protons and not the  $\delta$  6.33 proton.

The remaining upfield carbon signal at  $\delta$  109.1 was assigned to C-22 and was identified as a quaternary carbon from the APT spectrum. All the carbon chemical shift values were assigned by comparison to the literature and are summarized in table 4.3.

Correlation between the  $\delta$  1.72 protons and glycosidic proton at  $\delta$  4.97 confirmed the presence of the 6-deoxypyranose methyl. Long-range correlation observed between  $\delta$  4.85 (H-1) and  $\delta$  82.9 (C-3) confirmed the 3-*O*-glycosidic linkage of the first sugar unit. The C-21 methyl group was confirmed due to the HMBC correlation observed to  $\delta$  109.1 (C-22).

The good resolution of the spectra allowed for the tentative assignment of some of the methylene and methine protons that have not been reported previously. The methylene ( $\text{CH}_2$ ) protons at  $\delta$  2.28 (t,  $J = 12.1$  Hz) were identified as H-1 a/b or H-4 a/b due to long range couplings ( $\delta$  17.2 (C-19),  $\delta$  40.3 (C-4),  $\delta$  40.5 (C-10),  $\delta$  70.9 (C-2),  $\delta$  82.9 (C-3). Moreover, proton-proton correlation was observed between the H-1/H-4 proton and the 19- $\text{CH}_3$  and the H-1' protons in the COSY spectrum.

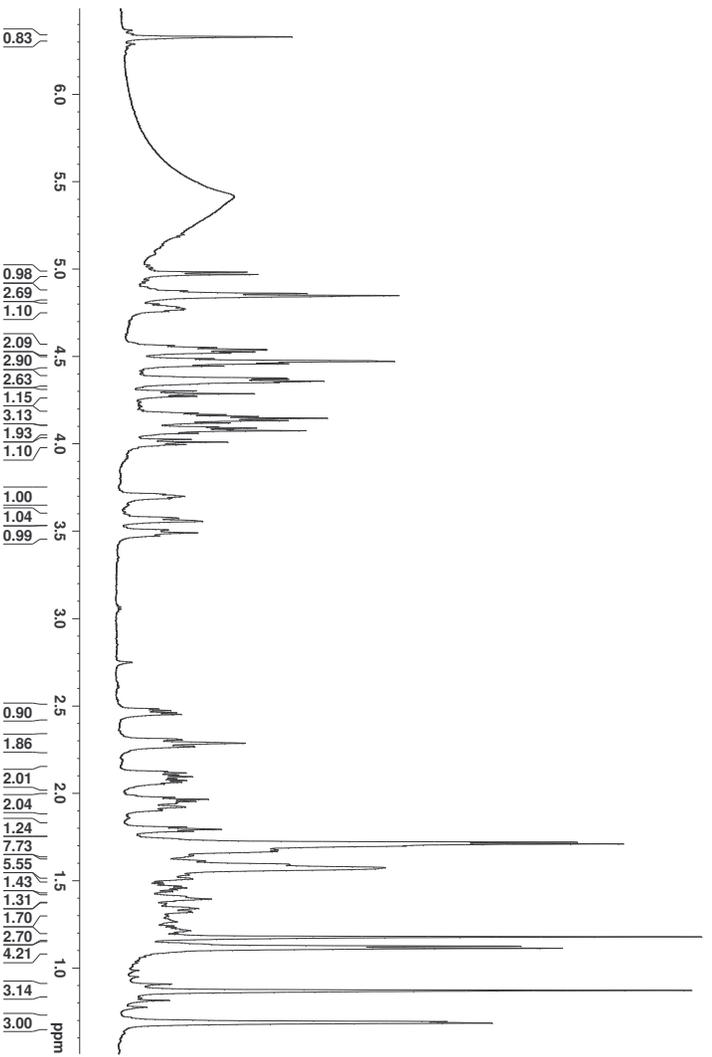
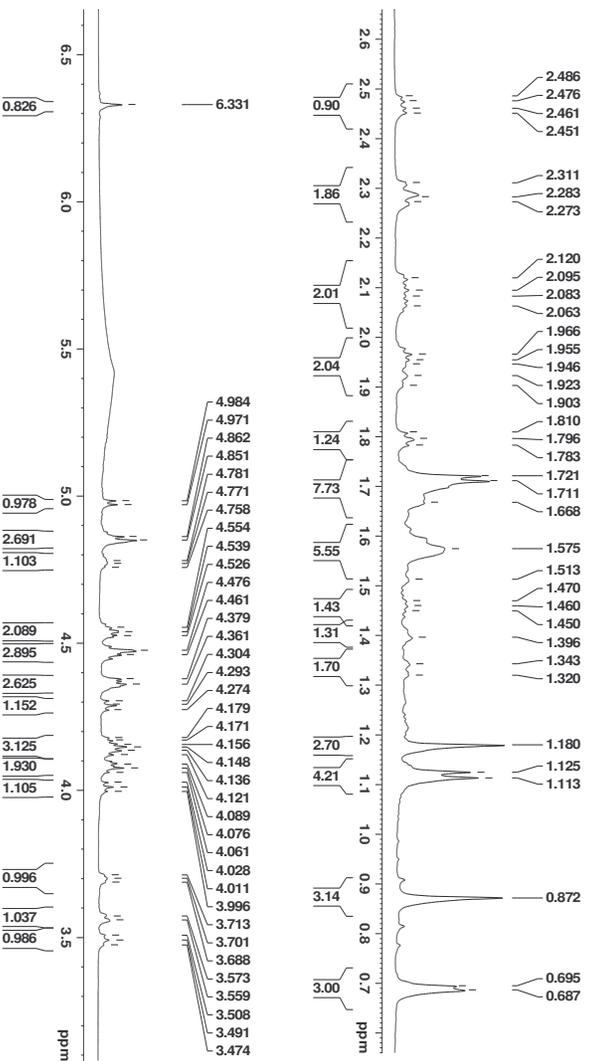
The methine (CH) H-20 and H-9 protons were assigned the respective chemical shift ranges  $\delta$  1.96-1.94 and  $\delta$  1.94-1.903, respectively (integration 2-H) due to HMBC correlations observed to the  $\delta$  14.9 (C-21) methyl carbon,  $\delta$  41.9 (C-20) and  $\delta$  63.0 (C-17) carbons. The H-15 protons were assigned the chemical shift  $\delta$  2.08 (m, 2-H), H-17:  $\delta$  1.78 (t,  $J = 7.9$  Hz) and H-7 protons  $\sim \delta$  1.39.

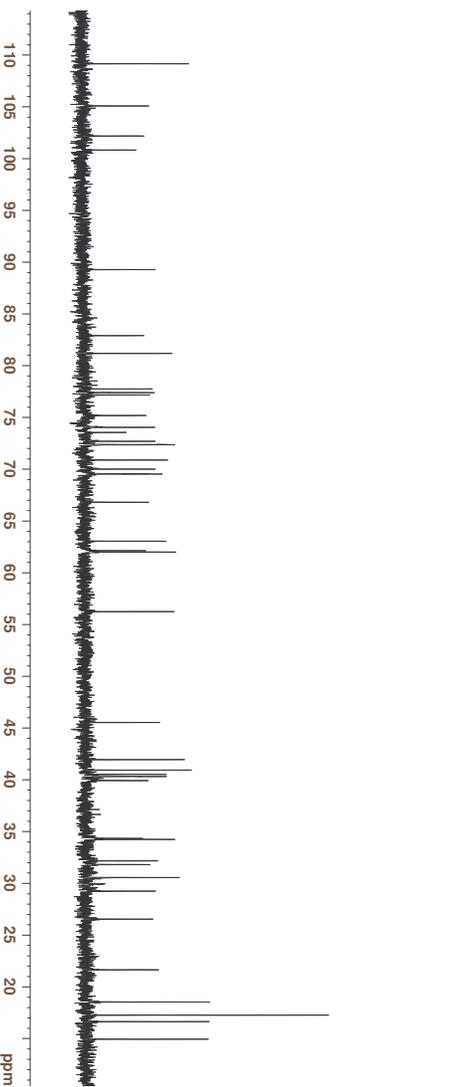
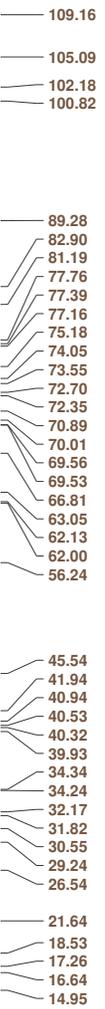
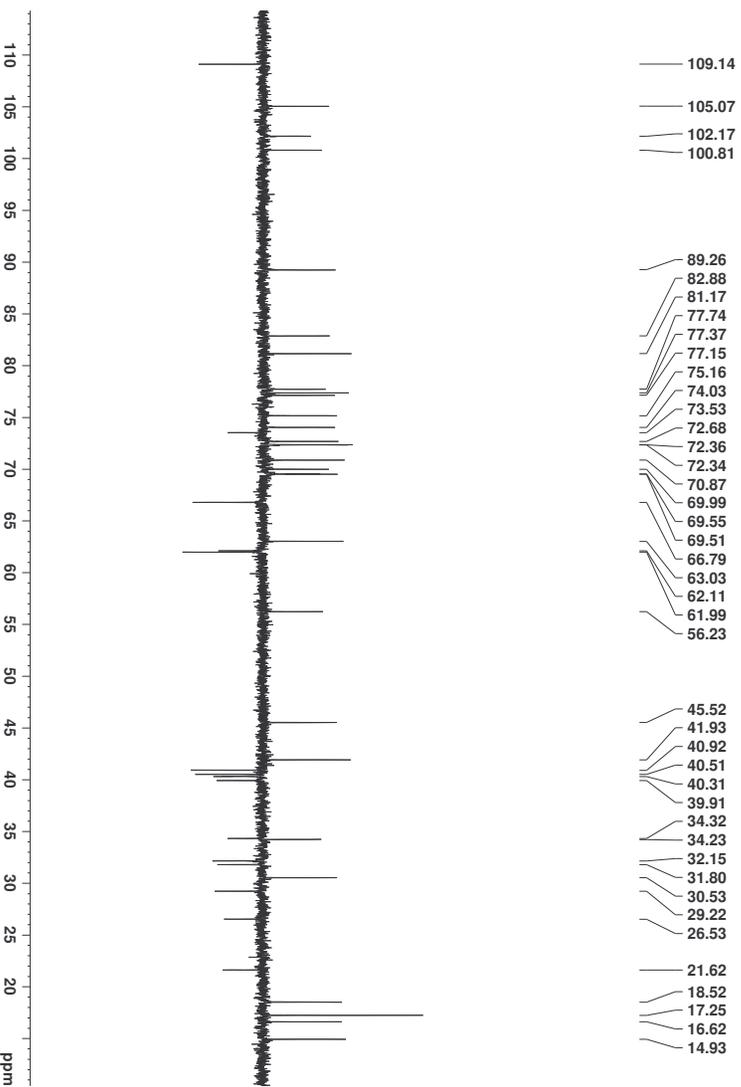
In the sugar moieties chemical shifts were assigned to the following hydroxymethine protons, H-4' ( $\delta$  4.01, t,  $J = 9.2$  Hz), H-4'' ( $\delta$  4.29, t,  $J = 9.3$  Hz) and H-5''' ( $\delta$  3.70, m). The remainder of the methylene and methine protons of the aglycone and glycosidic moieties overlapped and could not be assigned individual chemical shift values. The identified protons are summarized in table 4.3.

Several other proton-proton and proton-carbon correlations were observed in the COSY, HMQC and HMBC spectra, however due to the proximity of the peaks clear assignment of these correlations was not always possible but they still confirm the structure of the isolated saponin. All the tables and spectra given in the pages that follow:

**Table 4.3:** NMR Chemical Shift values (ppm) for Compound 2 in Pyridine-*d*5 as compared to Agapanthussaponin A data (Nakamura *et al.* 1993).

Ring	Carbon	Literature $\delta_C$	Experimental $\delta_C$	Proton	Experimental $\delta_H$		
Aglycone A-F	1	40.0	39.9 (t)	H-1/4	2.28 (t, $J = 12.1$ Hz)		
	2	71.0	70.9 (d)				
	3	82.9	82.9 (d)				
	4	40.4	40.3 (t)				
	5	73.6	73.6 (s)	H-7	1.39 (m)		
	6	34.4	34.3 (t)				
	7	26.6	26.5 (t)				
	8	34.3	34.3 (d)	H-9	1.940-1.903		
	9	45.6	45.5 (d)				
	10	40.6	40.5 (s)	H-15	2.08 (m)		
	11	21.7	21.6 (t)				
	12	40.4	40.3 (t)				
	13	40.0	40.9 (s)				
	14	56.3	56.2 (d)				
	15	32.2	32.2 (t)				
	16	81.3	81.2 (d)				
	17	63.1	63.1 (d)			H-17	1.78 (t, $J = 7.9$ Hz)
	18	16.7	16.6 (q)			H-18	0.87 (s)
	19	17.3	17.3 (q)			H-19	1.18 (s)
	20	42	41.9 (d)	H-20	1.96-1.940		
	21	15.0	14.9 (q)	H-21	1.12 (d, $J = 6.9$ Hz)		
	22	109.2	109.1 (s)	H-26 a H-26 b H-27	3.57 (t, $J = 10.2$ Hz) 3.49 (t, $J = 10.2$ Hz) 0.69 (d, $J = 4.9$ Hz)		
	23	31.9	31.8 (t)				
	24	29.3	29.2 (t)				
	25	30.6	30.5 (d)				
	26	66.9	69.5 (t)				
	27	17.3	17.3 (q)				
<b>G</b>	1'	100.8	100.8 (d)	H-1'	4.85 (d, $J = 7.4$ Hz)		
	2'	77.2	77.2 (d)				
	3'	89.3	89.3 (d)				
	4'	69.6	69.6 (d)	H-4'	4.01 (t, $J = 9.2$ Hz)		
	5'	77.8	77.8 (d)				
	6'	62.2	62.1 (t)				
<b>I</b>	1''	105.1	105.1 (d)	H-1''	4.97 (d, $J = 7.8$ Hz)		
	2''	72.4	72.4 (d)				
	3''	72.8	72.7 (d)				
	4''	74.1	74.0 (d)	H-4''	4.29 (t, $J = 9.3$ Hz)		
	5''	69.6	69.5 (d)				
	6''	18.6	18.5 (q)				
<b>H</b>	1'''	102.2	102.2 (d)	H-1'''	6.33 (s)		
	2'''	72.4	72.4 (d)				
	3'''	75.2	75.2 (d)				
	4'''	70.1	70.0 (d)	H-5'''	3.70 (m)		
	5'''	77.4	77.4 (d)				
	6'''	62.1	62.0 (d)				

Plate 4.14:  $^1\text{H-NMR}$  of Compound 2 in Pyridine- $d_5$ Plate 4.15:  $^1\text{H-NMR}$  expansion of Compound 2 in Pyridine- $d_5$

Plate 4.16: <sup>13</sup>C-NMR of Compound 2 in Pyridine-*d*<sub>5</sub>Plate 4.17: APT of Compound 2 in Pyridine-*d*<sub>5</sub>

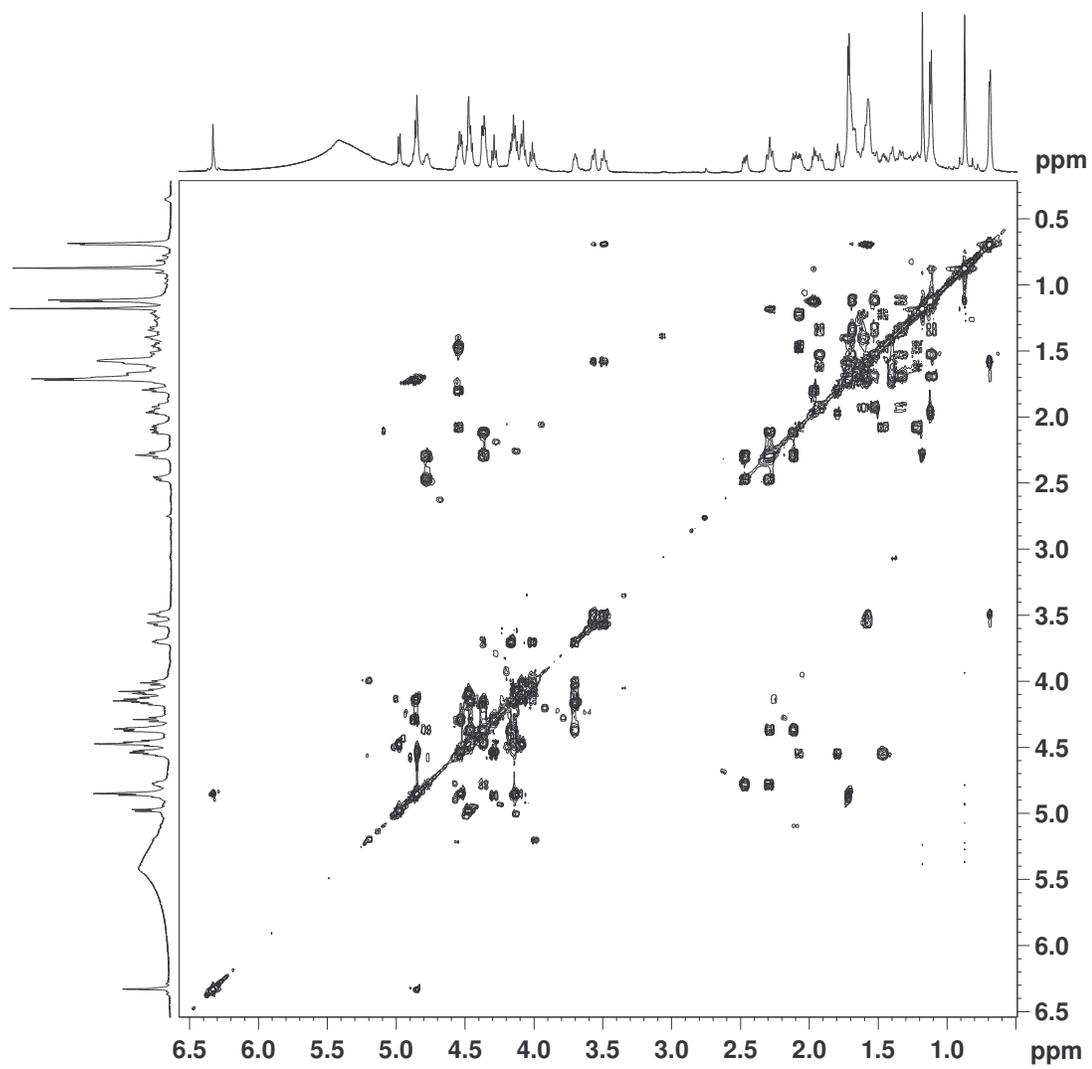


Plate 4.18: COSY spectrum of Compound 2 in Pyridine-*d*5

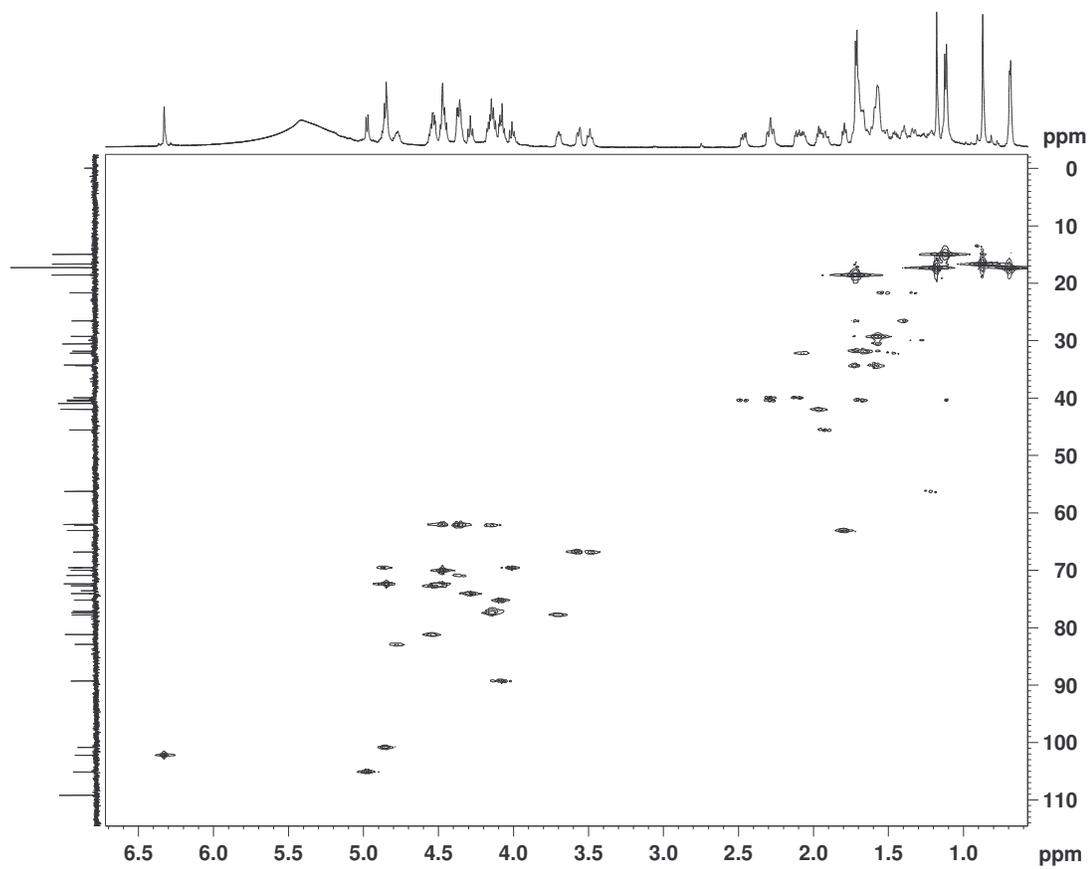


Plate 4.19: HMQC spectrum of Compound 2 in Pyridine-*d*5

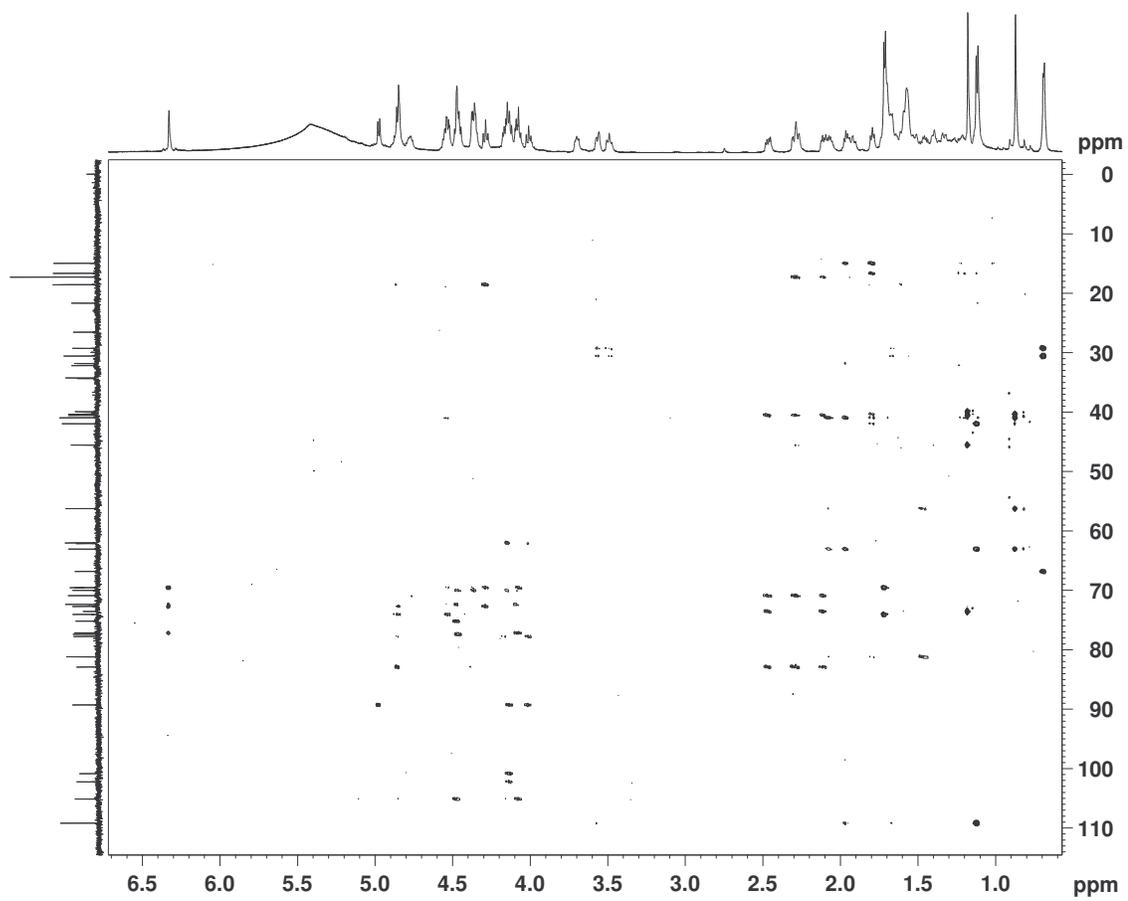
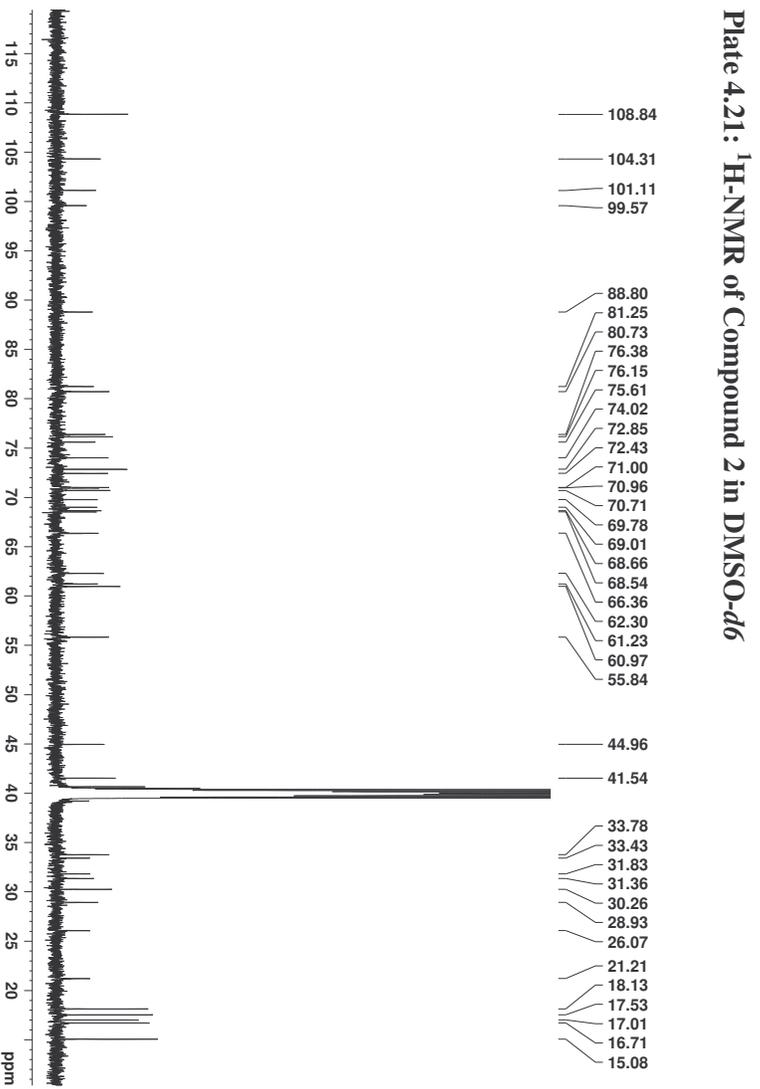
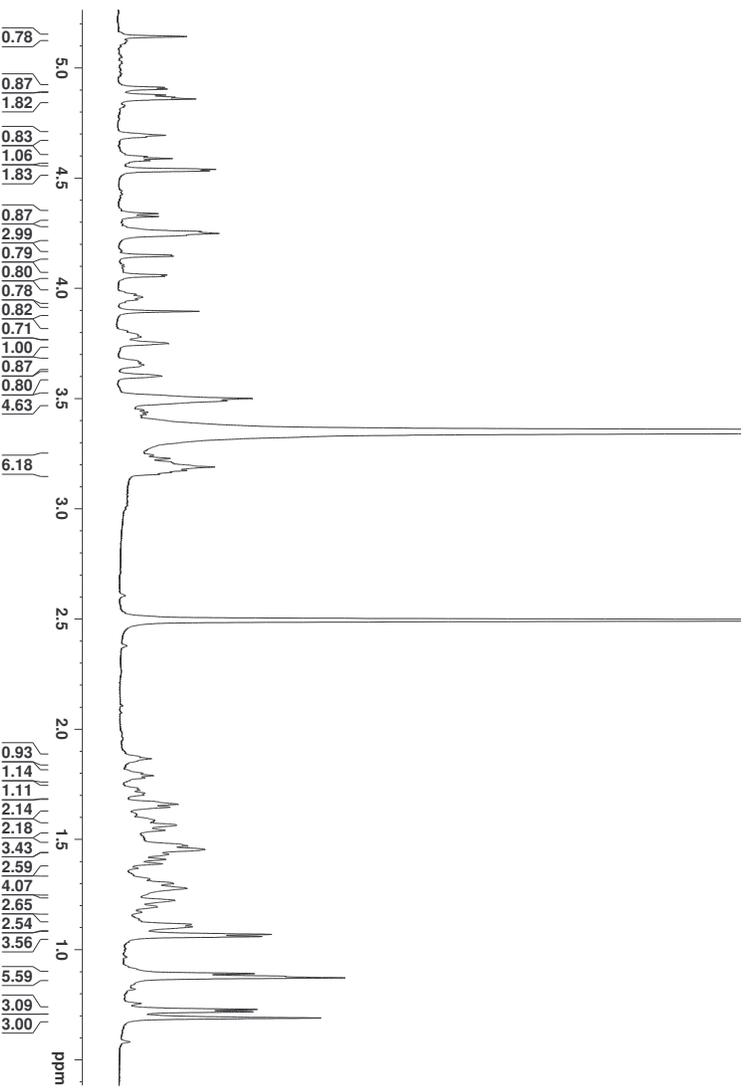


Plate 4.20: HMBC spectrum of Compound 2 in Pyridine- $d_5$

Table 4.4: Selected  $^1\text{H}$  NMR and  $^{13}\text{C}$  NMR chemical shifts of Compound 2 in DMSO-*d*<sub>6</sub>

Ring	Carbon	Experimental $\delta_{\text{C}}$	Proton	Experimental $\delta_{\text{H}}$
<b>Aglycone A-F</b>	1	41.5 (t)	H-1/4	0.69 (s) 0.87 (s) 0.89 (d, $J = 10.5$ Hz)  0.73 (d, $J = 5.2$ Hz)
	2	69.8 (d)		
	3	81.3 (d)		
	5	72.4 (s)		
	6	33.9 (t)		
	7	26.1(t)	H-7	
	8	33.4 (d)		
	9	44.9 (d)	H-9	
	11	21.2 (t)		
	14	55.8 (d)		
	15	31.8 (t)	H-15	
	16	80.7 (d)		
	17	62.3 (d)	H-17	
	18	16.7 (q)	H-18	
	19	17.5 (q)	H-19	
	21	15.1 (q)	H-21	
	22	108.8 (s)		
23	31.4 (t)			
24	29.2 (t)			
25	30.2 (d)			
26	66.4 (t)			
27	17.0 (q)	H-27		
<b>G</b>	1'	99.6 (d)	H-1'	4.34 (d, $J = 9.4$ Hz)
	2'	75.6 (d)		
	3'	88.8 (d)		
	4'	68.7 (d)		
	5'	76.4 (d)		
	6'	61.2 (t)		
<b>I</b>	1''	104.3 (d)	H-1''	4.25 (bd)
	2''	70.9(d)		
	3''	71.0 (d)		
	4''	72.9 (d)		
	5''	68.5 (d)		
	6''	18.5 (q)		
<b>H</b>	1'''	101.1 (d)	H-1'''	5.14 (s)
	2'''	70.7 (d)		
	3'''	74.0 (d)		
	4'''	69.0 (d)		
	5'''	76.2 (d)		
	6'''	60.9(d)		

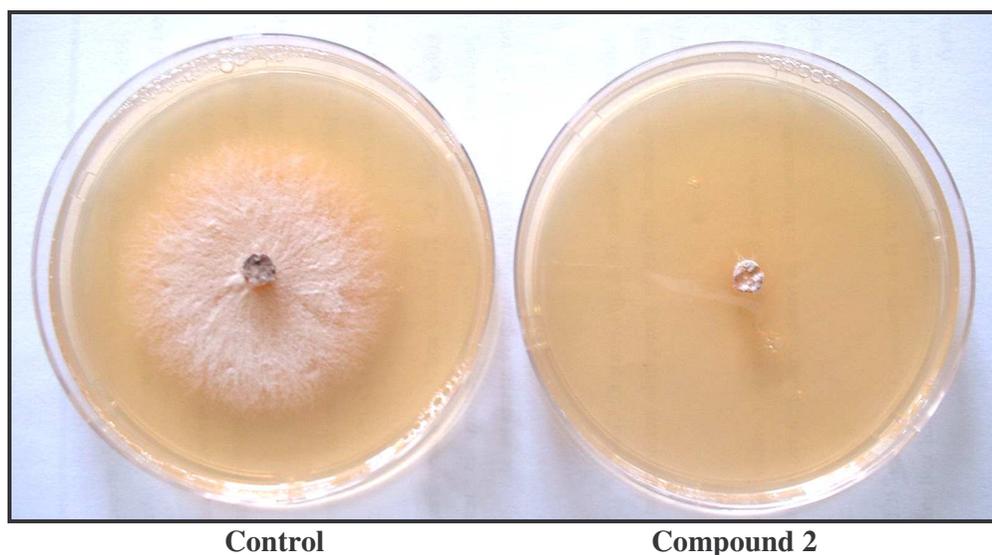


**Table 4.5: Selected  $^1\text{H}$  NMR and  $^{13}\text{C}$  NMR chemical shifts of Acetylated Agapanthussaponin A (Nakamura *et al.* 1993) in  $\text{CDCl}_3$**

Ring	Carbon	Literature $\delta_{\text{C}}$	Proton(s)	Literature $\delta_{\text{H}}$
Aglycone	2	81	2-H	4.36 (m)
	3	81	3-H	4.27 (m)
	9	17		
	10	75		
	16	77.5	16-H	4.0 (overlapped)
	18	17	18- $\text{CH}_3$	1.15 (s)
	19	17	19- $\text{CH}_3$	0.79 (s)
	21	110		
	22	14	22- $\text{CH}_3$	0.94 (d, $J = 7.0$ Hz)
	26	68.5	26- $\text{CH}_2$	3.3 (t, $J = 11.0$ Hz)
	27	16.5	27- $\text{CH}_3$	3.5 (dd, $J = 4.0, 11.0$ Hz) 0.77 (d, $J = 6.5$ Hz)
G	1'	97	1'-H	4.61 (d, $J = 8.0$ Hz)
	2'	74.9	2'-H	3.57 (dd, $J = 8.0, 9.0$ Hz)
	3'	69.5	3'-H	4.12 (dd, overlapped)
	4'	69	4'-H	4.84 (dd, $J = 9.5, 10.0$ Hz)
	5'	70	5'-H	3.80 (m)
	6'	62	6'-H	4.05 (dd, $J = 2.5, 12.5$ Hz) 4.30 (dd, $J = 5.0, 12.5$ Hz)
H	1''	100	1''-H	4.80 (d, $J = 8.0$ Hz)
	2''	70	2''-H	5.00 (dd, $J = 8.0, 10.0$ Hz)
	3''	71	3''-H	5.21 (dd, $J = 4.0, 10.0$ Hz)
	4''	67.5	4''-H	5.39 (dd, $J = 1.0, 4.0$ Hz)
	5''	69	5''-H	5.29 (dd, $J = 4.0, 9.0$ Hz)
	6''	61	6''-H	4.21 (dd, $J = 9.0, 18.0$ Hz) 4.12 (dd, overlapping)
I	1'''	94	1'''-H	5.34 (d, $J = 1.5$ Hz)
	2'''	70	2'''-H	5.28 (d, $J = 1.5, 4.0$ Hz)
	3'''	72.5	3'''-H	5.11 (dd, $J = 1.5, 10.0$ Hz)
	4'''	71	4'''-H	5.06 (t, $J = 10.0$ Hz)
	5'''	67	5'''-H	4.53 (dd, $J = 6.5, 10.0$ Hz)
	6'''	17.5	$\text{CH}_3$	1.19 (d, $J = 6.5$ Hz)

#### 4.7.6 Anti-fungal activity of compound 2, purified and identified as a saponin from *A. africanus*.

Compound 2, at a concentration of  $200 \mu\text{g ml}^{-1}$ , inhibited *in vitro* mycelial growth of *Rhizoctonia solani* by 100% after 72 h. After 7 days no mycelial back growth was observed confirming the potent antifungal activity of the purified compound identified as (25*R*)-5 $\alpha$ -spirostane-2 $\alpha$ , 3 $\beta$ ,5 $\alpha$ -triol 3-*O*-{*O*- $\alpha$ -L-rhamnopyranosyl-(1 $\rightarrow$ 2)-*O*-[ $\beta$ -D-galactopyranosyl-(1 $\rightarrow$ 3)]- $\beta$ -D-glucopyranoside}.



**Plate 4.23:** *In vitro* inhibitory effect of compound 2, purified from *Agapanthus africanus*, at a concentration of  $200 \mu\text{g ml}^{-1}$  on the mycelial growth of *Rhizoctonia solani*.

# | *Chapter 5*

DISCUSSION

Environmental damage caused by chemicals used in agriculture, especially those to control plant diseases and pests, has been a subject of global concern over the past years (Waard *et al.* 1993; Park and Seaton 1996). As a result, in many developed countries organic farming has become increasingly popular in an attempt to reduce the use of certain synthetic products that have been identified as unwanted. Of these, the banning of copper containing fungicides in Europe tops the list. As a result, extensive investigations into the potential of natural plant products, to be used as possible alternatives to synthetic chemicals that are safe for humans and the environment, followed (Daayf *et al.* 1995). For example, studies conducted over the past two decades confirmed the potential of crude extracts from several wild plants as a source of antifungal compounds that control plant fungal pathogens (Manoharachary and Gourinath 1988; Harsh 1998; Eksteen *et al.* 2001; Pretorius *et al.* 2002a & b; Nteso and Pretorius 2006a & b; Eloff *et al.* 2007). As part of a screening project, where more than 3000 crude plant extracts were tested for antimicrobial activity in wild plants at the University of the Free State, South Africa, Tegegne (2004) and Nteso (2004) found above average antifungal activity in extracts of *Agapanthus africanus* and *Tulbaghia violacea*.

Furthermore, in an attempt to improve crop yields, natural plant growth regulators have become an important component of crop production research (Gianfagna 1995). Examples include tri-iodobenzoic acid which has been registered as a yield increasing product on soybean, Dinoseb<sup>®</sup>, primarily used in maize as pre-emergence herbicide (Gianfagna 1995) and Kelpek<sup>®</sup>, a commercialized seaweed extract (Ferreira and Lourens 2002). Recently, a seed suspension of a legume plant, *Lupinus albus* (SS), was shown to have an above average bio-stimulatory effect on the growth and development of different agricultural crops (van der Watt 2005). The active substance was isolated, purified and identified as a glycerol trilinoleate, linoleic acid. Fatty acids have been shown to be involved in defence mechanisms of plants and to improve chlorophyll synthesis (Wink 2000). Edqvist and Farbos (2003) also reported on the plant growth regulatory activity of plant sterols and fatty acids. Despite these reports, very little information on the growth regulating action mechanism of either lipids or fatty acids exists. During 2006, ComCat<sup>®</sup>, a bio-stimulant and plant strengthening agent with yield increasing abilities, containing the brassinosteroids 24-epi-castasteron and 24-epi-secasteron as active compounds (Agraforum 2006), was commercialized world-wide. Since its discovery twenty years ago the mechanism of action of brassinosteroids (BRs) in promoting growth and development in plants received special attention (Marquardt and Adam 1991; Asami *et al.* 2002).

A rationale for exploring the use of plant extracts or natural products as biological pesticides or growth regulators more extensively can be found in the plant itself. Many wild plants have the potential to survive very harsh biotic and abiotic environmental stress conditions. These 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 provide a rich source of biologically active compounds that may be used as novel crop-protecting agents (Cox 1990). This initiated the incentive to pursue the use of such plants 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. Alternatively, the application potential of isolated natural compounds that act as elicitors of defence responses in plants as well as its commercial potential in the agricultural and horticultural industries has been investigated in the past (Heil and Ploss 2006).

In light of the above introductory remarks, crude extracts of *A. africanus* (Tegegne 2004) and *T. violaceae* (Nteso and Pretorius 2006a & b) containing antifungal compounds, as well as the commercial bio-stimulant *ComCat*<sup>®</sup> (Agraforum 2006) and the *L. albus* seed suspension (SS; van der Watt 2005) were included in this study. Initial screening of all four extracts for antifungal activity against seven plant fungal pathogens revealed that neither *ComCat*<sup>®</sup> nor SS showed any *in vitro* antifungal activity. On the other hand, in accordance with previous findings, crude extracts from both *T. violacea* (Nteso and Pretorius 2006a) and *A. africanus* (Tegegne 2004) showed significant *in vitro* mycelial growth inhibition of all seven plant pathogenic fungi tested.

Subsequently, the potential of these extracts to control leaf rust (*P. triticina*) in wheat was investigated. Again *ComCat*<sup>®</sup> and SS had no direct effect while the crude extracts of both *T. violacea* and *A. africanus* significantly inhibited the germination of *P. triticina* spores and prevented further germ tube development. Furthermore, a marked reduction in pustule formation occurred in susceptible plants sprayed with *A. africanus* extract 48 h prior to infection. The wheat cultivar Thatcher, with resistance conferred by the gene *Lr15* to wheat rust, showed a hypersensitive reaction (HR) when infected (McIntosh *et al.* 1995). In general, the occurrence of chlorotic and/or necrotic flecking on disease resistant plants is usually typical of a HR, resulting from the interaction between corresponding genes for resistance

and avirulence in the host and pathogen, respectively (Bowles 1990). In the case of treatment with a *T. violacea* extract, a severe hypersensitive reaction (HR) with some tissue damage was visually noticed in the infected resistant plants. However, the *A. africanus* extract reduced necrotic lesion formation in resistant plants.

This confirmed the potential of both extracts to control obligate biotrophic pathogens such as *P. triticina*. In the case of *T. violaceae*, Nteso (2004) identified six linear chain sulphur containing compounds as the active antifungal compounds contained in the plant. On the other hand, Tegegne (2004) identified four flavonoids and a single steroidal saponin purified from an *A. africanus* extract as the major antifungal active compounds responsible for both the *in vitro* and *in vivo* inhibition of the economically important plant pathogenic fungi tested. However, neither of the two authors included *P. triticina* in their studies and, out of hand, these active compounds cannot be associated with either the inhibition of spore germination or germ tube development in the case of this obligate biotrophic pathogen.

At this point a decision had to be made on the direction of the underlying study. Two possibilities were considered: (a) to isolate, purify and identify the active compounds directly involved in the inhibition of wheat leaf rust spore germination from either *T. violacea* or *A. africanus* or both and (b) to screen for possible involvement of all four plant extracts under scrutiny in the induction of a defence response in wheat against *P. triticina* infection. As information on (b) was not available at this stage, it was decided to first screen for a possible defence response in wheat on pathogen infection and treatment with the extracts before making a final decision on either (a) or (b). The effect of the extracts on the *in vitro* activities of three pathogenesis resistant (PR)-proteins,  $\beta$ -1,3-glucanase, chitinase and peroxidase was used for this purpose. The latter screening procedure included a cultivar susceptible to wheat leaf rust (Thatcher) and a resistant cultivar (Thatcher / *Lr15*; McIntosh *et al.* 1995) while the responses of both uninfected and infected wheat plants to treatment with the extracts were followed.

A rationale for this approach was that the accumulation of specific PR proteins upon infection is a well-documented defence related phenomenon (Stinzi *et al.* 1993; Somissich and Hahlbrock 1998, Montesinos 2000). The induction of some PR proteins under pathological conditions suggests, but does not prove, a role for these proteins in plant defence (van Loon 1990). However, these proteins have been generally considered as defence proteins, functioning in preventing or limiting pathogen invasion and spread, while they usually have a

poor contribution to resistance against initial infection. Nevertheless, if they are already present in a tissue, or if they have been induced in non-infected, distant tissues as a result of primary infection in the vicinity, then they confer an enhanced level of protection (Ferreira *et al.* 2007). In general, PR proteins are produced by plants during normal development or as part of an induced defence from fungal pathogens, against which they exert biocontrol. Therefore, their biosynthesis and accumulation is considered a major defence mechanism of plants against fungal pathogens (Odjakova and Hadjiivanova 2001). Not only do some of these proteins exhibit antifungal properties *in vitro*, but have they also been shown to be induced *in vivo* in a very large number of plants in response to fungal attack (Kombrink and Somssich 1997). The PR proteins encompass several different groups of structurally and functionally unrelated proteins. Seventeen classes are now considered (van Loon *et al.* 2006) of which PR-2 ( $\beta$ -1,3-glucanase), PR-3, 4, 8 and 11 (chitinases) and PR-9 (peroxidases) are quite abundant.

Results obtained in this study indicated that  $\beta$ -1,3-glucanase is present in uninfected susceptible and resistant plants at a constant relative low level. Higher levels of  $\beta$ -1,3-glucanase activity were induced by *P. triticina* infection in both susceptible and resistant wheat cultivars, reaching maximum induced levels between 48 and 72 h after inoculation. This is in agreement with several previous reports on the induction of  $\beta$ -1,3-glucanase activity by pathogens and elicitors in wheat and other plant species. Huges and Dickerson (1991) reported an eight fold selective increase in  $\beta$ -1,3-glucanase activity in elicitor treated *Phaseolus* resistant plants after 30 h.  $\beta$ -1,3-Glucanase activity in extracts of barley leaves in response to powdery mildew fungus infection was also found to be higher in resistant than in susceptible seedlings (Jutidamrongphan *et al.* 1991). Pathogen infection also induced a higher and/or earlier increase in  $\beta$ -1,3-glucanase activity in resistant plants than in susceptible plants of tomato (Joosten and de Wit 1989), tobacco (Wyatt *et al.* 1991), oats (Daugrois *et al.* 1990), muskmelon (Fink *et al.* 1990) and bean (Netzer and Kritzman 1979). More recently, Kemp *et al.* (1999) reported on the induction of  $\beta$ -1,3-glucanase activity in near isogenic lines of the wheat cultivar, Palmiet, following leaf rust infection. This implicated the role of  $\beta$ -1,3-glucanase in a general defence response, although the constitutive or induced expression of  $\beta$ -1,3-glucanase was not directly associated with the resistance genes. The involvement of  $\beta$ -1,3-glucanase in the resistance response of wheat to leaf rust was also confirmed by Anguelova *et al.* (1999) and Anguelova-Merhar *et al.* (2001) following the induction of  $\beta$ -1,3-glucanase activity within 48 h after infection.

As in the case of  $\beta$ -1,3-glucanase, chitinase activity was also induced by *P. triticina* infection in both susceptible and resistant wheat cultivars within 48 h after inoculation and activity remained constantly high. Pathogen infection resulted in a much higher chitinase activity in resistant plants than in susceptible plants. The significance of the observed induction of the apoplastic  $\beta$ -1,3-glucanase and chitinase activities is that both can hydrolyze the fungal cell walls of most higher fungi directly since glucan and chitin, the principle compounds in fungal cell walls, act as substrates for these enzymes (Honée 1999). It is also common for some proteins to display synergism. Since chitin and  $\beta$ -1,3-glucan are synthesized simultaneously in the apex of growing hyphae of filamentous fungi, the effectiveness of a hydrolase enzyme may depend on the simultaneous action of another one to hydrolyze mixed chitin-glucan fibres (Stinzi *et al.* 1993). Various authors reported on this synergistic action of  $\beta$ -1,3-glucanase and chitinase (Anguelova-Merhar *et al.* 2001; Theis and Stahl 2004). In addition, the action of these enzymes might contribute to resistance by releasing  $\beta$ -1,3-glucan and chitin-derived oligomers which are elicitors of the defence response (Klczynski *et al.* 2000; Côté and Hahn 1994). However, although fungal-glucans have been found to be potent elicitors of phytoalexin accumulation in legumes (Cosio *et al.* 1996), they have no elicitor activity in wheat (Moerschbacher *et al.* 1986). On the other hand, chitin and chitosan oligomers have been shown to be effective elicitors of defence related lignification in wheat (Moerschbacher *et al.* 1986) and are also involved as elicitors in soybean leaves (Khan *et al.* 2003). Kemp (1996) further demonstrated the *in vitro* degradation of wheat leaf rust germ tubes by a mixture of intercellular  $\beta$ -1,3-glucanase and chitinase isolated from wheat leaves. Based on these findings, and especially in accordance with that of Anguelova-Merhar *et al.* (2001), it is postulated that the role of  $\beta$ -1,3-glucanase and chitinase are confined to a direct lytic action of the enzymes on the fungal cell walls of *P. triticina*.

Besides  $\beta$ -1,3-glucanase and chitinase, peroxidases are also involved in the defence-related events that occur in the extracellular matrix during different host-pathogen interactions (Wojtaszek 1997; van der Westhuizen *et al.* 1998a,b). The peroxidase activity of the PR-9 family may act in cell wall reinforcement by catalyzing lignification, leading to enhanced resistance against multiple pathogens (Passardi *et al.* 2004). Induced increase in apoplastic peroxidase activity in infected wheat plants, observed in this study, may thus be involved in reactions that reinforce the cell walls to resist penetration by the pathogen. Peroxidases also take part in the production of active oxygen species (AOS) during plant-pathogen interactions that are associated with signaling events in resistance responses (Wojtaszek 1997).

Foliar application of the different plant extracts under scrutiny to susceptible uninfected and infected, as well as resistant uninfected wheat, revealed the inhibition of  $\beta$ -1,3-glucanase, chitinase and peroxidase activities by *ComCat*<sup>®</sup>, SS and *T. violacea*. In literature plant resistance suppressors are widely described and several strategies by pathogens to suppress the resistance reactions in plants have been identified (Jakobek *et al.* 1993; Espinosa *et al.* 2003; Bartsev *et al.* 2004). Plant pathogens cannot exist without suppression of defence responses in their hosts and are classified into non-specific and specific types. Non-specific types suppress the general metabolic pathways characteristic of all plants while specific suppressors affect only certain plant species by suppressing specific metabolic stages characteristic only of susceptible plant species. It was suggested by Ozeretskovskaya *et al.* (2001) that these suppressors act through a receptor recognition mechanism where the receptors are supposedly located on host cell membranes and plasma membranes while others are associated with mitochondrial or chloroplast membranes. Two plant resistance suppressors, 1,3- $\beta$ -1,6- $\beta$ -glucan and a pentasaccharide of xyloglucan origin, that are involved in the pathosystem of potato and are the causal agents of potato blight, were compared by the authors. The  $\beta$ -glucan caused a local and race-specific suppressor effect on the plant host defence response, while the pentasaccharide caused both local and systemic suppression of potato resistance (Ozeretskovskaya *et al.* 2001). Earlier, Moerschbacher *et al.* (1999) reported on small oligomers of galacturonic acid identified as endogenous suppressors of resistance reactions in wheat leaves. Suppression of cell death by pathogenic fungi has been documented in various plant species including barley (Huckelhoven *et al.* 2003), pea (Shiraishi *et al.* 1997) and tomato (Bouarab *et al.* 2002). In this light it is speculated that the inhibition of  $\beta$ -1,3-glucanase, chitinase and peroxidase enzyme activities by *ComCat*<sup>®</sup>, SS and *T. violacea* extracts, could possibly be as a result of an elicitor-receptor interaction that suppresses PR-protein induction and transcription of *PR* genes.

The resistance inducing effects of a *Lychnis viscaria* L. seed extract were shown by Roth *et al.* (2000) in tobacco, cucumber and tomato plants. This seed extract contains different brassinosteroides as does *ComCat*<sup>®</sup> used in this study. According to the authors,  $\beta$ -1,3-glucanase activity was more significantly induced in cucumber than chitinase and peroxidases respectively. In a preliminary study at the University of the Free State, *ComCat*<sup>®</sup> was recently shown to induce  $\beta$ -1,3-glucanase, chitinase and peroxidase activities in sunflower plants (van der Westhuizen 2005; personal communication). In another study at this university, induction of  $\beta$ -1,3-glucanase activity in sunflower by a foliar application of a

*T. violacea* extract was also demonstrated (unpublished results). This is contrary to the results obtained with wheat, an aspect that is not completely unfamiliar. Following a study on  $\beta$ -1,3-glucanase, chitinase and peroxidase activities in eighteen different plant species treated with a chemical resistance elicitor, BION, Heil and Ploss (2006) concluded that plant species differ dramatically in the presence and inducibility of resistance enzymes. Several species did not respond to the induction treatment, while enzyme activities in other species increased more than threefold after BION application. This interaction between different species and treatment could explain the differences in response of monocotyledonous wheat and dicotyledonous sunflower, cucumber and tomato plants to treatment with *ComCat*<sup>®</sup> and *T. violacea* in this study.

In contrast with the observation in the susceptible wheat cultivar, foliar treatment of the *P. triticina* infected resistant cultivar with *ComCat*<sup>®</sup>, SS and *T. violaceae* extracts induced  $\beta$ -1,3-glucanase, chitinase and peroxidase enzyme activities. This indicated that the origin of resistance and the genetic background in which the resistance gene finds itself does play a role in the level and time course of induction of pathogenesis related proteins. However, compared to the previous three extracts, treatment of both susceptible and resistant wheat cultivars with an *A. africanus* crude extract increased the *in vitro*  $\beta$ -1,3-glucanase, chitinase and peroxidase activities highly significantly in both wheat cultivars, whether uninfected or infected. This overwhelmingly distinguishes the *A. africanus* extract from the rest in terms of its potent ability to induce a defence response in wheat towards leaf rust.

Further, a unique physiological state called 'priming' can be observed following infection of plants by necrotizing attackers (Conrath *et al.* 2002; 2006). According to the authors, this primed state can also be induced by treatment of plants with various natural or synthetic compounds and is characterized by a potentiation of cellular defence responses that are induced in response to an attack by pathogens, insects or abiotic stresses. It is therefore possible that *A. africanus* - induced resistance is based on various priming mechanisms. It is suggested that the effect of *A. africanus* extract on the salicylic acid and jasmonic acid pathways be investigated. The rest of the study was concentrated on the *A. africanus* extract only.

Firstly, the induction pattern of apoplastic proteins from infected susceptible and resistant wheat treated with an *A. africanus* extract as well as a control treated with distilled water was

followed using SDS-PAGE. Clear differences between SDS-PAGE profiles of intercellular proteins from resistant and susceptible as well as untreated and treated plants were observed throughout the 144 h period after treatment with the extract. In general, resistant plants contained higher amounts of a 31 kDa protein and the protein was also present at much higher detectable levels in plants treated with the *A. africanus* extract. The molecular mass corresponded to that of  $\beta$ -1,3-glucanase, as previously described by Stinzi *et al.* (1993) and Münch-Garthoff *et al.* (1997). In an attempt to confirm the latter deduction made from the SDS-PAGE profile, a Western blot using a polyclonal antibody against  $\beta$ -1,3-glucanase from wheat, was done. This not only confirmed the identity of the 31 kDa protein to indeed be that of  $\beta$ -1,3-glucanase but also that the observed induction in the *in vitro* activity of this enzyme was the result of the synthesis of new protein and not merely activation of existing protein.

Since the apoplast is recognized as the site where many defence related compounds accumulate (Fink *et al.* 1988; van der Westhuizen and Pretorius 1996; van der Westhuizen *et al.* 1998a), it was postulated that the accumulation of these proteins in intercellular wash fluid (IWF) from plants treated with *A. africanus* extract may be associated with a compound or compounds in the extract that act as elicitors of the defence response in wheat. In order to verify this postulate it was imperative to follow the expression of *PR* genes by the extract. Subsequently, the expression of three *PR* genes, *PR2*, *PR3* and *PR9* were monitored using RT-PCR.

Analysis of the expression of these three defence related genes in time-course experiments confirmed that they were induced in resistant as well as susceptible wheat after infection. The *PR2* gene, however, was constitutively expressed at a high level in the control resistant plants confirming that it was not induced by infection in resistant plants. Although a clear cut reason for this phenomenon is rather elusive at this stage, more evidence that infection causes induction of defence related genes is available in literature. For example, Ganesh *et al.* (2006) reported that defence related genes were induced in coffee plants after infection with rust. The authors suggested that recognition of the pathogen may have occurred before penetration of the fungus into the substomatal chamber and that distinct mechanisms of resistance signaling may operate in coffee to stop pathogen infection. In other plant-rust interactions, typical host specific resistance responses were also observed concurrent with the formation of the first fungal haustoria (Heath 1997; Mould *et al.* 2003).

In this study, when resistant and susceptible wheat were treated with an extract of *A. africanus* 48 h prior to infection, a more pronounced induction of *PR2*, *PR3* and *PR9* gene expression occurred. Further, in response to treatment with the *A. africanus* extract, the expression of *PR3* in resistant wheat was induced twice at 24 h and again at 144 h. It can, therefore, be claimed that *A. africanus* is responsible for the induction of *PR* genes in wheat. A similar pattern of induction was demonstrated in *A. thaliana* and *C. arabica* in response to pathogen infection, where the expression of the *NDR1* and *CaNDR1* genes were, respectively, induced twice during the experimental time course (Century *et al.* 1997; Ganesh *et al.* 2006). The exact role of the *NDR1* protein in *A. thaliana* is still unknown, but its location in the plasma membrane suggests that it could directly interact with the pathogen. In this study, the *PR3* gene encodes for chitinase and it is well known that chitinase possess anti-fungal activity which allows it to protect the plant directly against the penetration of fungal hyphae by degrading the cell walls (Honée 1999).

During RT-PCR of infected susceptible and resistant wheat, using *PR9* specific primers, two different sized fragments were amplified and both were induced by infection. After sequencing, the larger fragment was confirmed to be peroxidase, while the smaller fragment was identified as a retrotransposon gene. Expression of the retrotransposon gene increased after 48 h in susceptible and resistant plants, but was initially stronger expressed in resistant plants. When the plants were treated with the *A. africanus* extract, it is possible that the treatment induced the expression of the retrotransposon gene, because 0 h sampling where the gene is already strongly expressed, took place 48 h after treatment with the extract. Compared to the controls this could explain the constitutive expression of the retrotransposon gene in treated susceptible and resistant plants. This phenomenon lends itself for further investigation.

Since Barbara McClintock (1950) first predicted the existence of ‘jumping genes’ in the maize genome, transposable elements have been identified in almost all plant species that have been investigated (Okamoto and Hirochika 2001). Transposons are classified into two classes based on their mechanism of transposition namely Class I: Retrotransposons and Class II: DNA transposons (Finnegan 1989). Retrotransposons are segments of RNA that can move around to different positions by a “copy and paste” mechanism. The RNA copies are then transcribed back into DNA by a reverse transcriptase which is inserted into new locations in the genome. In the process they may cause mutations or increase/decrease the

amount of DNA in the genome. The insertion of a retrotransposon in the DNA flanking a gene for pigment synthesis is thought to have produced white grapes from a black-skinned ancestor. Later the loss of that retrotransposon produced the red-skinned grape varieties cultivated today (Kimball 2007). Furthermore, retrotransposons activated under certain stress conditions, such as tissue culture and virus infection have been isolated (Hirochika 1993). Since a retrotransposon was identified in this study, the possibility exists that it is involved in the resistance mechanism of wheat against pathogens. Further research will shed light on the possible influence of the *A. africanus* extract and the expression of the retrotransposon gene.

From the results obtained thus far, it was obvious that *A. africanus* must contain an active compound(s) that act as an elicitor(s) in the mechanism of the defence reaction of wheat against leaf rust infection. Subsequently, activity directed liquid-liquid extraction, using organic solvents of increasing DC, as well as column chromatography was used to isolate and purify this/these compound(s) from a methanolic *A. africanus* crude extract. Two compounds were identified, isolated and confirmed to be active by following the *in vitro* peroxidase activity in wheat plants treated separately with combined column chromatography fractions. Purity of these two compounds was confirmed by means of qualitative TLC. In order to identify the chemical groups these two compounds belonged to, a series of staining reagents developed for each group specifically (Wagner and Bladt 1996) was used. This revealed both compounds to belong to the steroidal and essential oil types. Subsequently, the purified compounds were subjected to mass spectroscopy and nuclear magnetic resonance (NMR) spectroscopy in order to identify their true identity and structural formulas.

Identification of one of the purified compounds (compound 2) by means of  $^1\text{H-NMR}$  and  $^{13}\text{C-NMR}$  spectroscopy confirmed the compound as a steroidal saponin with a three sugar chain attached at the C3 position of ring A in the aglycone moiety. The compound was identified as (25*R*)- 5*α* spirostane-2*α*, 3*β*, 5*α*-triol 3-*O*-{*O*-*α*-L-rhamnopyranosyl-(1→2)-*O*-[*β*-D-galactopyranosyl- (1→3)]- *β*-D-glucopyranoside}. Previously, a C-3 monoglycosylated saponin, with the same aglycone, was isolated from the root system of *A. africanus* by both Stephen (1956) and Gonzalez *et al.* (1974, 1975). An identical agapanthussaponin A, with a three sugar chain, was isolated from the below soil parts of *A. inapertus* and found to exhibit inhibitory activity on cAMP phosphodiesterase (Nakamura *et al.* 1993). In 2004 a saponin (3-[*O*-*β*-D-glucopyranosyl-(1''-3')-*α*-L-rhamnosyl-(1''-2')-*β*-D-glucopyranosyloxy] agapanthegenin) with the attached three sugar chain was isolated and identified by Tegegne from both the

roots and aerial plant parts of *A. africanus*. This compound proved to be the major antifungal active compound responsible for both the *in vitro* and *in vivo* inhibition of the economically important plant pathogenic fungi tested. Singh *et al.* (2007) isolated two novel steroidal saponins from the rhizomes of *A. africanus* and one proved to be responsible for *in vivo* antifungal activity against human pathogens (Singh *et al.* 2008).

Plant saponins are widely distributed amongst plants and have a wide range of biological properties. The name 'saponin' is derived from the Latin word *sapo*, which means 'soap', because saponin molecules form soap-like foams when shaken with water (Bruneton 1995). They are glycosylated plant secondary metabolites and consist of non-polar aglycones coupled with one or more monosaccharide moieties (Oleszek 2002). Traditionally, they can be classified into two groups based on the nature of their aglycone skeleton. The first group consists of steroidal saponins, which are almost exclusively present in the monocotyledonous angiosperms, while the second more common group consists of the triterpenoid saponins and occur mainly in the dicotyledonous angiosperms (Bruneton 1995). However, based on the biosynthesis of the carbon skeletons of the aglycones, Vincken *et al.* (2007) distinguished 11 main classes of saponins.

Several reviews have been published over the last two decades, focusing on biosynthesis, isolation, structural elucidation and biological activities of saponins (Kulshrestha *et al.* 1972, Mahato *et al.* 1988, 1992, Mahato and Nandy, 1991, Mahato and Sen 1997, Connolly and Hill 2000, Sparg *et al.* 2004). Biological and pharmacological activities of saponins include haemolytic properties (Oda *et al.* 2000, Sparg *et al.* 2004), as well as antimicrobial, insecticidal and molluscicidal activities (Sparg *et al.* 2004). It is believed that the natural role of these compounds in plants is to protect against attack by potential pathogens, which would account for their antimicrobial activity (Osbourn 2003). A study on saponin-deficient mutants of a diploid oat species, *Avena strigosa*, confirmed that they are more susceptible to a variety of fungal pathogens (Papadopoulou *et al.* 1999). One of the mechanisms by which saponins display an antimicrobial activity is based on their ability to form complexes with sterols present in the membrane of micro-organisms. This causes damages in the membrane and the consequent collapse of cells (Morrissey and Osbourn 1999).

The results from this study confirmed the antifungal activity as well as the active role of this saponin, (25*R*)- 5 $\alpha$  spirostane-2 $\alpha$ , 3 $\beta$ , 5 $\alpha$ -triol 3-*O*-{*O*- $\alpha$ -L-rhamnopyranosyl-(1 $\rightarrow$ 2)-*O*-[ $\beta$ -D-

galactopyranosyl- (1→3)-  $\beta$ -D-glucopyranoside}, in inducing defence responses in wheat by induction of the PR-proteins, thus indicating another mechanism of resistance against pathogens and the possible role for saponins to act as elicitors in the defence response of plants. Unfortunately the second bio-molecule (compound 1) isolated and purified from *A. africanus* above soil parts was not recovered in sufficient amounts to identify the compound by means of NMR spectroscopy. However, it was proven that this unknown compound also acted as an elicitor leading to the induction of PR-proteins *in vitro*. A follow-up study will continue in order to recover sufficient amounts of this compound through extraction of an increased amount of dry material.

In conclusion, the initial aims stated at the beginning of the project were met, because when two natural bio-stimulants and two extracts with anti-fungal properties were tested for *in vivo* induction of resistance towards pathogen infection in wheat under glasshouse conditions, a marked reduction in pustule formation occurred in susceptible plants sprayed with the one fungicide extract (*A. africanus*). This extract was also responsible for the increase in *in vitro* activity of certain PR-proteins isolated from infected and non-infected susceptible and resistant wheat. The other extracts only increased enzyme activity in the resistant infected cultivar. SDS-gel electrophoresis and Western blotting confirmed induction of PR-proteins when treated with *A. africanus* extract. The induced expression of *PR*-genes in wheat upon treatment with *A. africanus* extract was also demonstrated. One of the active compounds which are responsible for induced resistance in wheat by treatment with *A. africanus* extract was identified as a saponin. Therefore, *A. africanus* shows great promise to be developed as a natural antifungal agent for application in the agricultural industry.

# | *Chapter 6*

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## SUMMARY

Information on the induced disease resistance mechanism in wheat against leaf rust (*Puccinia triticina*) by two natural bio-stimulants (*ComCat*<sup>®</sup> and the seed suspension of *Lupinus albus*; SS) and two extracts with antifungal activity (*Tulbaghia violacea* and *Agapanthus africanus*) may be of great value both in designing new agrochemicals that stimulate plant resistance responses and in developing genetically engineered plants with enhanced disease resistance.

The potential of these extracts to control leaf rust *in vivo* in susceptible (Thatcher) and resistant (Thatcher / *Lr15*) wheat was investigated. *ComCat*<sup>®</sup> and SS had no direct effect while the *A. africanus* extract resulted in the reduction of pustule and necrotic lesion formation in a susceptible and resistant wheat cultivar. *T. violacea* and *A. africanus* significantly inhibited the germination of *P. triticina* spores and prevented further germ tube development.

Foliar application of the different plant extracts on resistant infected wheat plants activated  $\beta$ -1,3-glucanase, chitinase and peroxidase enzyme activities. However, it was only the *A. africanus* treatment that increased the *in vitro* activities of these three apoplastic PR-proteins significantly in both susceptible and resistant wheat cultivars, whether uninfected or infected. As a result it was decided to concentrate the rest of the study on the *A. africanus* extract only.

The induction pattern of apoplastic proteins from infected susceptible and resistant wheat treated with an *A. africanus* extract as well as a control treated with distilled water was followed using SDS-PAGE. Clear differences between SDS-PAGE profiles of intercellular proteins from resistant and susceptible as well as untreated and treated plants were observed throughout the 144 h period after treatment with the extract. In general, resistant plants contained higher amounts of a 31 kDa protein and the protein was also present at much higher detectable levels in plants treated with the *A. africanus* extract. The molecular mass corresponded to that of  $\beta$ -1,3-glucanase. A Western blot using a polyclonal antibody against  $\beta$ -1,3-glucanase from wheat confirmed the identity of the 31 kDa protein to indeed be that of  $\beta$ -1,3-glucanase. This overwhelmingly excluded the *A. africanus* extract from the rest in terms of its potent ability to induce a defence response in wheat towards leaf rust.

RT-PCR was used in the analysis of the expression of the three defence related genes. Time-course experiments confirmed that they were induced in resistant as well as susceptible wheat after infection. In this study, when resistant and susceptible wheat were treated with an extract of *A. africanus* 48 h prior to infection, a more pronounced induction of *PR2*, *PR3* and *PR9* gene expression occurred. Two different sized fragments were amplified when using *PR9* specific primers and both were induced by infection and by treatment with *A. africanus* extract in susceptible and resistant wheat. After sequencing, the larger fragment was confirmed to be peroxidase, while the smaller fragment shared very high sequence similarity to a retrotransposon gene. It can, therefore, be claimed that *A. africanus* is responsible for the induction of *PR* genes and a retrotransposon gene in wheat.

From the results obtained thus far, it was obvious that *A. africanus* must contain an active compound(s) that act as an elicitor(s) in the mechanism of the defence reaction of wheat against leaf rust infection. Subsequently, activity directed isolation and purification of the active compound lead to the isolation of a saponin, identified by means of <sup>1</sup>H-NMR and <sup>13</sup>C-NMR spectroscopy as (25*R*)- 5α spirostane-2α, 3β, 5α-triol 3-*O*-{*O*-α-L-rhamnopyranosyl-(1→2)-*O*-[β-D-galactopyranosyl-(1→3)]- β-D-glucopyranoside}.

Key words: resistance, defence response, PR-proteins, bio-stimulant, anti-fungal activity, elicitor, leaf rust, wheat, *Triticum aestivum* L., saponin.

## OPSOMMING

Inligting aangaande die biochemiese weerstandsreaksiemeganisme van koring teen blaarroes (*Puccinia triticina*) geïnduseer deur twee natuurlike bio-stimulante (*ComCat*<sup>®</sup> en die saadsuspensie van *Lupinus albus*; SS), asook twee plantekstrakte met antifungale aktiwiteit (*Tulbaghia violacea* en *Agapanthus africanus*), mag van groot belang wees in die ontwikkeling van natuurlike middels asook geneties gemanipuleerde plante met verhoogde weerstand teen siektes, wat aangewend kan word in bestaans en kommersiële boerdery praktyke.

Die potensiaal van hierdie ekstrakte om roesweerstand *in vivo* in vatbare (Thatcher) en weerstandbiedende (Thatcher / *Lr15*) koring te beheer is ondersoek. *ComCat*<sup>®</sup> en SS het geen effek gehad nie, terwyl *A. africanus* 'n vermindering in roesontwikkeling in beide kultivars tot gevolg gehad het. *A. africanus* en *T. violacea* het ook die ontkieming van roesspore en ontwikkeling van infeksiehifes voorkom.

'n Blaartoediening van die verskillende ekstrakte op weerstandbiedende geïnfekteerde koringplante het gelei tot verhoogde  $\beta$ -1,3-glukanase, chitinase and peroksidase ensiemaktiwiteite. Dit was egter slegs behandeling met die *A. africanus* ekstrak wat die *in vitro* ensiemaktiwiteite van die drie apoplastiese patogeneseverwante (PR) proteïene in beide kultivars, geïnfekteer of ongeïnfekteer, betekenisvol verhoog het. As gevolg van hierdie resultate is besluit om slegs op die *A. africanus* ekstrak te konsentreer.

Deur gebruik te maak van SDS-PAGE jelektroforese is die akkumulering van apoplastiese proteïene in beide die geïnfekteerde vatbare en weerstandbiedende kultivars, sowel as die wat met met *A. africanus* ekstrak behandel is, gemonitor. Duidelike verskille in die proteïenprofile tussen die verskillende kultivars en behandeling met *A. africanus* ekstrak was waargeneem, met 'n prominente akkumulering van 'n 31 kDa grootte proteïen. Hierdie proteïen is met behulp van Westernkladanalise as  $\beta$ -1,3-glukanase bevestig en dui onteenseglik op die potensiaal van *A. africanus* om die PR- proteïene en derhalwe die weerstandsrespons van koring teen roesinfeksie te induseer.

RT-PCR was gebruik om die uiting van die drie *PR* gene te bestudeer. Roesinfeksie het tot induksie van die gene gelei, terwyl behandeling van vatbare en weerstandbiedende plante met

*A. africanus* ekstrak 'n meer prominente induksie van *PR2*, *PR3* en *PR9* gedurende die 144 uur periode tot gevolg gehad het. Twee verskillende groottes fragmente is uitgedruk by gebruik van die *PR9* spesifieke fragment en albei se uiting was aangeskakel tydens infeksie sowel as behandeling met *A. africanus* ekstrak in beide vatbare en weerstandbiedende koring. Nadat hul DNA volgorde bepaal is, is die groter fragment geïdentifiseer as peroksidase terwyl die kleiner fragment baie hoë homologie met 'n retrotransposon geen getoon het. *A. africanus* is dus verantwoordelik vir die aanskakeling en induksie van *PR* gene en 'n retrotransposon geen in koring.

Uit die resultate is dit duidelik dat *A. africanus* 'n verbinding besit wat as aktiewe bestanddeel dien om die weerstandsreaksie in koring aan te skakel. Gevolglike aktiwiteitsgerigte isolasie en suiwering van die aktiewe komponente betrokke, het aanleiding gegee tot die identifikasie van 'n saponien, (25*R*)-5 $\alpha$  spirostaan-2 $\alpha$ , 3 $\beta$ , 5 $\alpha$ -triol 3-*O*-{*O*- $\alpha$ -L-ramnopiranosiel-(1 $\rightarrow$ 2)-*O*-[ $\beta$ -D-galaktopiranosiel- (1 $\rightarrow$ 3)]-  $\beta$ -D-glukopiranosied}, met behulp van <sup>1</sup>H-KMR and <sup>13</sup>C-KMR spektroskopie.

Sleutelwoorde: weerstandsrespons, PR-proteïene, bio-stimulant, antifungale aktiwiteit, blaarroes, koring, *Triticum aestivum* L., saponien.